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Histochemistry of B663 Pigmentation: Ceroid-Like Pigmentation in Macrophages[1,2]

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Since its use was first reported by Browne and Hogerzeil in 1962 ([10,11]), B663, a synthesized phenazine dye, has proven to be an effective drug in the treatment of human leprosy and its use has become worldwide ([2,7,9-11,26,38,39,52,53]). The most significant and frequent side effect of this compound is an abnormal skin coloration. This can be divided into two types. One is an initial ruddiness that usually takes place within one to four weeks after the beginning of treatment ([39]) and is reported to be due to accumulation of the drug itself, mainly in the reticuloendothelial cells and fat ([5,6,16,45]). It occurs in deposits as round bodies or in crystalline form ([2,6,16,45,52,53]). The other pigmentation consists of a blackish-brown or violaceous-brown pigmentation that develops during the second and third months of treatment ([8,39,53]) and is limited mainly to lesion areas. Some investigators ([8,39]) have reported histopathologic studies indicating that the pigmentation is caused by an increase of melanin in the basal layer of the epidermis, associated with incontinence of pigment into the upper dermis ([8,39]). Others, however, have been unable to detect significant changes in the amount of melanin in such skin.

No reports have been found which attribute this pigmentation to pigments other than melanin. The present histochemical study presents evidence to the effect that part of the pigmentation is due to the deposition of a lipid pigment, a ceroid-like substance, in the cytoplasm of the macrophages (lepra cells). This substance is generally believed to be derived from lipids and to be rendered insoluble to fat solvents by oxidation ([37]).

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MATERIALS AND METHODS

Skin biopsies from three cases of lepromatous leprosy treated with B663 and four cases of untreated lepromatous leprosy together with one case of lepromatous leprosy treated with dapsone (DDS), were used for this study. The patients were all Chinese. The specimens were immediately fixed in Ridley's modified Zenker-formol solution in all instances and later were transferred to 70% alcohol, in which the tissues were kept for several weeks until they were processed. They were dehydrated with alcohols, embedded in paraffin and cut at 4 to 10 microns. In one of the B663-treated cases and all four untreated cases, the tissues were additionally fixed in 10% neutralized formalin, embedded in carbowax, polyethylene glycol (Union Carbide Corporation), which is a water-soluble wax used for lipid histochemistry. They were sectioned at 6 to 10 micron thickness in a low humidity, air-conditioned room with a temperature of approximately 22°C. Tissues from the four untreated cases were also fixed in formol-calcium solution (1% [w/v] anhydrous calcium chloride in 10% neutralized formalin), embedded in carbowax, and stained for phospholipids by Baker's acid hematin, and Klüver and Barrera's copper phthalocyanin methods.

Many different methods of staining for pigments and lipids were employed in this study. The histochemical procedures were all done according to methods detailed by Pearse ([37]), Okamoto *et al* ([35]), and Barka and Anderson ([3]). The histochemical methods employed included:

1. Unstained and methylene blue stained, 10 micron sections on both paraffin and carbowax-embedded tissues for the determination of the natural color of the pigment and its location.

2. Hematoxylin and eosin general purpose stain.

3. Ziehl-Neelsen stain for acid-fast organisms and also for acid-fast lipid pigments, lipofuscin and ceroid.

4. Bleach with 10% hydrogen peroxide.
5. Steiger's iodine-potassium iodate method for carotenoid pigment.
6. Gmelin reaction for bilirubin and hematoidin.
7. Berlin blue reaction for the iron pigment, hemosiderin.
8. Schmorl reaction for lipid pigments.
9. Chrome alum hematoxylin stain for lipid pigments.
10. Periodic acid-Schiff stain.
11. Hueck's Nile blue sulfate combined with bleaching to rule out the presence of melanin.
12. Sudan III stain on both paraffin and carbowax-embedded sections for lipids and also for lipid pigments.
13. *Fettrot* stain on both paraffin and carbowax-embedded tissues for lipids and lipid pigments together with the use of lipid extraction by fat solvents such as pyridine at 60°C for 24 hours, chloroform-methanol mixture (2:1) for 24 hours and ethanol for several days.
14. Sudan black B stain on both paraffin and carbowax-embedded tissues for lipids and lipid pigments together with the use of lipid extraction as described above.
15. Nile blue sulfate method for neutral fat and other lipids.
16. Fishler's method for fatty acids.
17. Okamoto *et al* [35] methods for cholesterol and its ester.
18. Okamoto *et al* [35] method (modified Molisch reaction) for glycolipids.
19. Okamoto *et al* [35] mercury diphenylcarbazone method for phospholipids and glycolipids.
20. Baker's acid hematin method for phospholipids.
21. Klüver and Barrera's copper phthalocyanin method for phospholipids except sphingomyelin, and probably glycolipids.

Staining for lipids on carbowax-embedded tissues was only done for one case of the B663-treated group and for all cases of the untreated group.

RESULTS

Gross changes. As has already been reported [8, 39, 53], the blackish-brown or violaceous-brown pigmentation was seen to be largely confined to areas of lepromatous infiltration (Fig. 1). Cut surfaces of the skin specimens taken from such hyperpigmented lesions showed a diffuse brown coloration

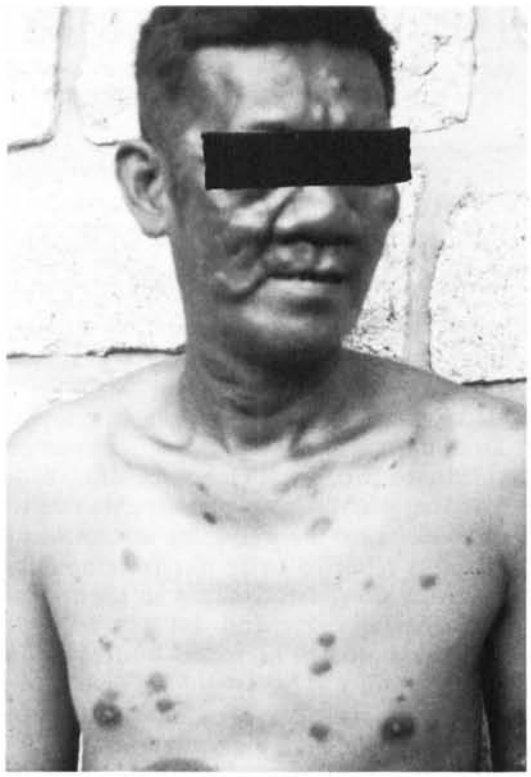

FIG. 1. Hyperpigmentation during treatment of lepromatous leprosy with B663 (HLC-1583). Pigmentation mainly limited to lepromatous lesions.

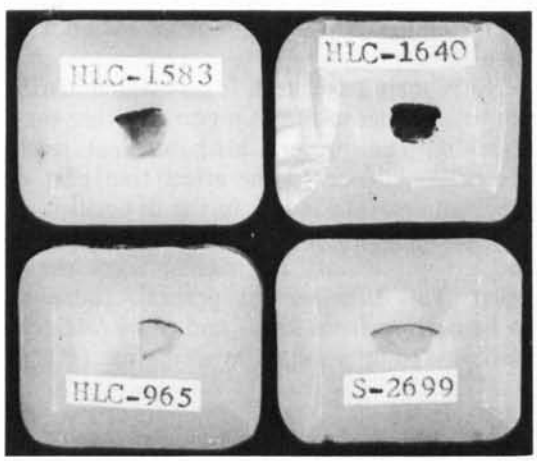

FIG. 2. Paraffin-embedded skin specimens. Diffuse pigmentation still remained after extraction by fat solvents throughout the dermis in B663-treated leprosy. Upper two specimens (HLC-1583 and -1640), B663-treated. Left lower (HLC-965), DDS-treated only. Right lower (S-2699), untreated.

throughout the dermis and the subcutaneous tissue. Such deep extension of pigmentation is not seen in melanin pigmentation except in some types of nevi such as blue nevus, Mongolian spot or Ota's nevus fusco-caeruleus ophthalomo-maxillaris, or melanoma. It is interesting that this diffuse pigmentation remained in paraffin-embedded tissues even after alcohol-xylol processing, despite pink coloration of the dehydrating alcohols by the drug (Fig. 2). This suggested that pigments other than the drug itself might still be present in the tissues and might play an important role in the pigmentation.

Pigment in the unstained state. On the unstained and methylene blue stained sections the pigment was a pale yellowish-brown and tended to form a rim around the large fat globules in the cytoplasm of the macrophages throughout the dermis and the subcutaneous tissue. The pigment was easily recognizable in thick unstained sections. There were no significant differences in characteristics of the pigment as found in paraffin and carbowax-embedded sections. This suggested that the pigment might not be soluble in ordinary fat solvents such as alcohols and/or xylol for tissue processing. Such yellowish-brown pigment was seen in all cases of the B663-treated group while the control tissues did not show such pigment in the macrophages of unstained sections. Neither the round red bodies nor the crystals of the drug, as described by some investigators, were demonstrable in the macrophages of these cases even in carbowax-embedded or frozen sections.

In only one B663 case was melanin seen to be increased in the basal layer of the epidermis. The other two cases did not demonstrate any differences in the amount of melanin as compared to the controls. Slight incontinence of pigment was present in some instances of both B663-treated and control tissues. It is concluded that melanin does not play a role in this pigmentation since there were no significant differences in amounts of melanin in the three groups and melanin was not found in the deep corium and subcutaneous tissue while the gross findings, as described above, indicate it to be there.

Histochemistry of pigment in macrophages (Table 1). The pigment present in the macrophages was not bleached out by two hours of treatment with 10% hydrogen peroxide in any of the B663-treated cases, while the melanin in the epidermis was completely bleached. However, the yellowish-brown pigment in the macrophages was almost bleached out by 24 hours.

One of the B663-treated tissues showed a slightly positive Berlin blue reaction but the other two cases were negative for iron. Gmelin reaction for bilirubin and hematoidin and Steiger's method for carotenoid pigment on carbowax-embedded tissues were negative in all three cases.

The macrophages were weakly stained by Hueck's Nile blue sulfate in combination with bleaching in all three cases, while the melanin in the basal layer of the epidermis in the same sections was not stained. On the basis of these histochemical methods, melanin, carotenoid, bilirubin, hematoidin and hemosiderin were ruled out as the responsible pigment, except for one case which had some iron in the macrophages of the lepromatous lesions.

The sections from all of the B663-treated tissues stained by the Ziehl-Neelsen method, in addition to showing a relatively small number of granular bacilli, presented brownish-red substances which tended to form a rim at the edges of the fat globules in the macrophages. These acid-fast substances were morphologically different from the mycobacteria. Acid-fast bacilli were very clearly stained purplish-red in both solid and granular forms, while in contrast these acid-fast substances were stained much paler than bacilli, were brownish-red, were seen to be foamy or diffusely present in the cytoplasm and had the tendency to rim the fat globules. In the case of DDS-treated leprosy, such acid-fast substances were not seen and only bacilli, most in a granular form, were stained densely purplish-red. Also in the untreated cases, acid-fast staining revealed a large number of bacilli, mostly in solid form, but no other acid-fast materials could be found in the macrophages. These characteristics suggested that the pigment, differing from the bacilli, might be some type of lipid pigment (Fig. 3).

The positive reaction to Sudan black B on the alcohol-dehydrated, paraffin-embedded tissues, indicated that the substance, derived from lipids probably by oxidation, became insoluble in the fat solvents used during the tissue processing (Fig. 4). Even after lipid

TABLE 1. *Histochemical characteristics of pigmented macrophages, B663-treated leprosy.*

Methods	Lipofuscin[a]	Ceroid[a]	B663-treated leprosy			Untreated leprosy	DDS-treated leprosy HLC-965
			HLC-1583	HLC-1640	HLC-1800		
Unstained	yellow-brown granular	yellow-brown rim of fat globules	yellow-brown tends to form a rim of fat globules			colorless	colorless
Acid fastness	– (occas. +)	+ (occas. –)	cytoplasm stained brown-red a few of bacilli in granular form			only bacilli stained purple-red	
Bleaching H_2O_2	+ at 48 h.	–~+	– at 2 h. + at 24 h.	– at 2 h. + at 24 h.	– at 2 h. + at 24 h.		
Berlin blue	–	– (occas. +)	–	weak +	–		
Gmelin reaction	–	–	–	–	–		
Steiger's	–	–	–	–	–		
Sudan black B on paraffin sec.	–~+	+	+	+	+		
Fat solvents	mostly insoluble	insoluble	insoluble	insoluble	insoluble		
Hueck's Nile blue & bleach.	+	–~+	weak +	weak +	weak +		
Schmorl reaction	+ (occas. –)	– (occas. +)	+	+	+		
Chrome alum hematoxylin	+	–	weak +	weak +	weak +		
PAS	–~+	–~+	weak +	weak +	weak +		

[a] Modified from Okamoto and Pearse (35, 37).

extraction by pyridine at 60°C for 24 hours, chloroform-methanol mixture (2:1) for 24 hours or alcohol for several days, the pigment still remained in the macrophages. Insolubility in the fat solvents, positive reaction to Hueck's method, and positive results for the Schmorl reaction and chrome alum hematoxylin stain also supported the thought that the pigment in the macrophages is probably one of the lipid pigments which are composed of lipofuscin or ceroid (wax-like pigment).

Histochemistry of lipids in macrophages (Table 2). For the lipid studies, carbowax-embedded tissues were available for one of the B663-treated tissues and for all four untreated cases.

Neutral fat. The tissues obtained from the B663-treated case showed bright red, large vacuoles stained with Sudan III and *Fettrot* stains in the cytoplasm of the macrophages. These were also stained red by the oxazone contained in Nile blue sulfate, while in the untreated group, the macrophages were pale orange-yellow by the Sudan III and *Fettrot* methods, and pale pink-blue or pale purplish-blue by the Nile blue sulfate stain. These color reactions indicate that the macrophages in the untreated control were stained mainly by Oxazine, true Nile blue. These differences in the staining characteristics of the two groups suggested that the macrophages in the B663-treated tissue contained more neutral fat than those in the untreated cases, which possessed more lipids, probably phospholipids as proven by other methods.

Fatty acids. Fishler's method gave a positive reaction for leprosy bacilli and also for the contents of the large globi in the untreated group, but was negative in the macrophages of the B663-treated tissues except for a small number of granular forms of bacilli in large globi.

Cholesterol and its ester. Okamoto *et al*'s sulfuric acid-acetic acid method and sulfuric acid method ([35]) were employed for this purpose and did not demonstrate a positive reaction in either the treated or untreated groups.

Glycolipids. For demonstration of glycolipids, Okamoto *et al*'s method ([35]) for the modified Molisch reaction was used and was negative in both groups.

Phospholipids. Three methods, consisting of Baker's acid hematin, Okamoto *et al*'s mercury diphenylcarbazone ([35]), and Klüver-Barrera's copper phthalocyanin, were employed for demonstration of phospholipids. The B663-treated tissue was chromated in sodium dichromate after sectioning the carbowax-embedded specimens, stained by Baker's method, and compared to those of

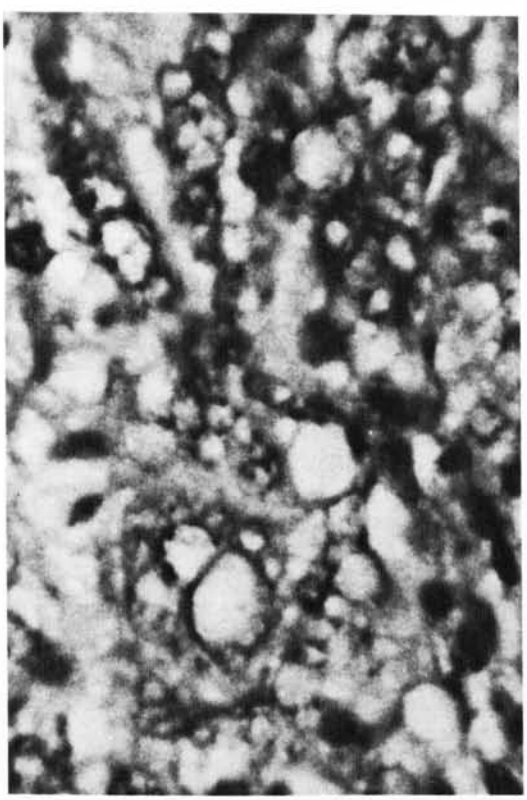

FIG. 3. Ziehl-Neelsen's stain demonstrates acid-fast substance with a tendency to form a rim of fat globules in B663-treated leprosy (HLC-1583).

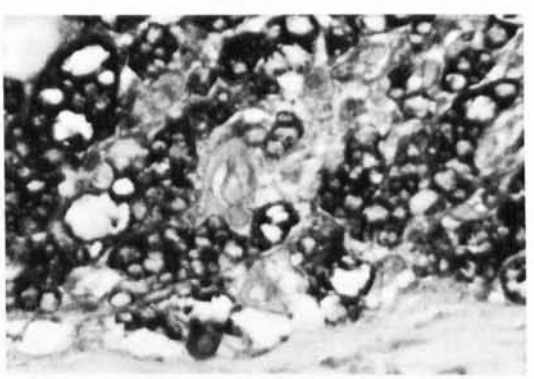

FIG. 4. Sudan black B stain on paraffin section after lipid extraction in B663-treated leprosy (HLC-1640).

the controls handled by the same procedures, since the B663-treated tissue was not fixed in formol-calcium solution. In this B663-treated tissue, only a few large globules were positive as granules, but the untreated control tissues were strongly positive in the cytoplasm of the macrophages even for the modified Baker's method. The method of Okamoto *et al* is made up of three different procedures: the "common" method for all kinds of phospholipids and cerebrosides; the "A" method for demonstration of only sphingomyelin; and the "B" method for demonstration of sphingomyelin and lecithin. Cephalin is negative to both "A" and "B" methods. The macrophages were positively stained (purplish-blue) in both groups by all three methods but the color was much paler in the B663-treated tissue than in the untreated tissue. Klüver and Barrera's method also showed the same results; the color reaction (blue) was much weaker in the B663-treated tissue than in the untreated cases.

In summary, the results of the lipid histochemical study on the macrophages were as follows: the B663-treated tissue contained more neutral fat and less phospholipids in the cytoplasm than the untreated group; both groups were negative for cholesterol

TABLE 2. *Histochemical characteristics of lipids in macrophages in human lepromatous leprosy treated with B663, comparing with untreated cases.*

Methods			B663-Treated HLC-1640	Untreated group
Neutral fat		*Fettrot*	+	+
		Sudan III[a]	bright red	pale orange-yellow partly red
		Nile blue sulf.[b]	strong red reaction weak blue reaction	pink-blue or purple-blue
Fishler's method for fatty acids			mostly –	++ (bacilli also stained)
Okamoto's method for cholesterol & ester			–	–
Okamoto's method for glycolipids			–	–
Phospholipids	Okamoto's	Common	weak +	+
		A	weak +	+
		B	weak +	+
	Baker's acid hematin		weak +	+++
	Klüver-Barrera		weak +	++

[a] Neutral fat, red. Lipoids, orange-yellow.

[b] Neutral fat and cholesterol, red (oxazone). Other lipoids, blue (oxazine, true Nile blue).

[c] "Common" method, all kinds of phospholipids and cerebrosides, positive. "A" method, only sphingomyelin, positive. Cephalin, negative. "B" method, sphingomyelin and lecithin, positive. Cephalin, negative.

and its esters, and for glycolipids (cerebrosides); Fishler's method for fatty acids was positive for leprosy bacilli as well as for globules of the macrophages, but in the B663-treated case only a small number of granular forms of the bacilli were seen in the large globi.

DISCUSSION

B663, a riminophenazine derivative of Barry's series ([4]) with the formula 2-*p*-chloranilino-5-*p*-chlorophenyl-3:5-dihydro-3-isopropyliminophenazine, has been reported to have an effect on the suppression of murine leprosy ([14]) and also of human leprosy as demonstrated by the mouse foot pad technic ([45]) and some clinical trials ([2, 7, 9–11, 26, 38, 39, 52, 53]).

The drug is practically insoluble in water but highly soluble in fat as well as fat solvents, and is reported to be deposited chiefly in the reticuloendothelial cells and fat tissue as round red bodies or in a crystalline form ([6, 16, 45]). Skin coloration has been noticed as a side effect induced by this drug. The coloration can be divided into two types: an initial redness, followed by brown pigmentation occurring after more prolonged treatment.

There are four main questions to be discussed in this study: 1) What pigment is responsible for, and does the drug itself play a major role in the late stage brown pigmentation? 2) What is the source of the pigment? 3) Is there any difference in lipids contained in lepra cells of B663-treated tissues and controls? 4) If there are any differences in lipids, what is the reason for this?

Although the initial red coloration of the skin is generally believed, and has been confirmed, to be due to deposition of the drug itself, a red dye, the histogenesis of the brown pigmentation has remained obscure. Its presence has been pointed out as one of the most significant side effects of the drug ([2, 8, 39, 52, 53]). Only two reports are available regarding its histogenesis. In 1965, Browne ([8]) described histopathologic findings of the hyperpigmented lesions of the skin, and concluded that the pigmentation was due to an increase of melanin in the epidermis with incontinence of pigment into the papillary layer and also into the reticular layer of the dermis. Later in 1967, Pettit *et al* ([39]) supported the melanin theory proposed by Browne with the results of their histopathologic study on skin biopsies. However, Browne's report ([8]) noted from personal communication that Knight and Wertlake were unable to detect significant differences in the amount of melanin in the skin specimens. In the present series of skin biopsies, only one out of three B663-treated cases revealed more melanin pigment in the basal layer of the epidermis in contrast to the controls, and incontinence of pigment could be seen in some instances of both B663-treated and untreated tissues. In addition to these findings on melanin, under simple naked eye examination the skin tissues from the B663-treated group showed intense brown pigmentation extending deeply into the subcutaneous tissue even after the tissues had been dehydrated by alcohols which dissolved the drug from the tissue (Fig. 2). These findings suggested that some pigments other than melanin and the drug itself might play an important role in the development of the pigmentation since melanin deposition does not occur as deeply in the dermis and subcutis except in some types of nevi such as blue nevus, the Mongolian spot or Ota's nevus. This concept was furthered also by the presence of a yellowish-brown pigment seen in the cytoplasm of the macrophages (lepra cells) on unstained thick sections of 10 microns, which was shown to be present in all of the B663-treated tissues but not seen in the controls. Melanin normally present in the epidermis was not stained by Hueck's method, but the pigment in the macrophages accepted the Nile blue dye in the same sections. The pigment in the macrophages also accepted other lipid stains such as Sudan black B and *Fettrot* in paraffin-embedded tissues even after lipid extraction by fat solvents (Fig. 4), in which the drug itself had been dissolved and washed out.

It is noteworthy that acid-fast substances other than leprosy bacilli were found in the macrophages. The sections stained by the Ziehl-Neelsen method showed that the non-bacillary positive material in the macrophages stained pale brownish-red in a foamy pattern and tended to form a rim about the large fat globules (Fig. 3). This acid-fast substance could be differentiated from the leprosy bacilli by the morphologic characteristics of the latter. The Schmorl reaction and chrome alum hematoxylin stain are gen-

erally believed to be positive for lipofuscin but usually negative for the ceroid pigment. In this series, the macrophages were positive or weakly positive for both reactions but in occasional instances the ceroid pigment may be positive for both methods and it is claimed by some investigators that ceroid, in contrast to lipofuscin, does not give the Schmorl reaction ([3]).

Its location, acid-fastness, lipid reaction and tendency to form a rim at the edges of the fat globules lead to the conclusion that the pigment in the macrophages is a ceroid-like substance rather than lipofuscin.

Ceroid, meaning "wax-like," was originally reported by Lillie *et al* ([30]) to be found in experimental liver cirrhosis of rats maintained on a protein deficient diet. Ceroid is a yellowish-brown, fluorescent ([40]) and acid-fast pigment, and accepts lipid dyes on both frozen and paraffin sections. The pigment, believed to be a lipid derivative, becomes insoluble in fat solvents such as pyridine, alcohols, ether, acetone and chloroform, probably by the oxidation of otherwise readily soluble unsaturated lipid ([20]), which is colorless if it has no carotenoid pigment. In addition to its presence in experimental or human liver cirrhosis with nutritional disorders such as protein or fat deficiency ([21, 30, 36, 40]), ceroid pigment is found in cases of vitamin E defiency ([18, 36, 42]) and in phagocytes in focal degenerative lesions such as necrosis, fatty degeneration or atheromas ([1, 12, 36]). The characteristics of the pigment were well described by Endicott and Lillie ([21]), and by Pappenheimer and Victor ([36]). There is no distinct border between lipofuscin and ceroid, and also no way of differentiating one clearly from another by histochemistry. Pearse ([37]) mentioned that the pigment might be called lipofuscin of ceroid type, lipofuscin of an early stage or just simply ceroid: that was a mixture of substances and a typical lipofuscin in an early stage of oxidation. Lipofuscin, in the narrow sense, is usually seen in the liver cells and heart muscle fibers as a coarse granular form, while ceroid is often observed in phagocytes, Kupffer cells and smooth muscle fibers ([36]) and has a characteristic tendency to form a rim around the fat globules.

There have been some reports of an abnormal pigmentation induced by other drugs such as Amodiaquin ([19]) and dapsone ([15, 31]). Doull reported a bluish-green pigmentation virtually confined to areas of lepromatous lesions in patients treated with high doses of Amodiaquin for long periods, and in one of nine biopsies there was observed a strong reaction for iron and in two others traces of similar pigment were found. He mentioned that the source might be local hemorrhage or increased permeability of the capillaries in lepromatous lesions. He could not, however, find any iron-positive pigment in 15 sections of untreated lepromatous leprosy. Ceroid is often present in combination with

TABLE 3. *Histochemistry of lipids in lepra cells in lepromatous leprosy.*

Authors \ Lipids		Neutral fat	Fatty acids	Cholesterol		Glyco-lipids	Phospho-lipids	Lipo-protein
				Ester	Free			
Mitsuda,	1918	−	+ bacilli	−			+ lipoid	
Ueda,	1948/49	±	−	−~+	−	−	+ lecithin	
Harada, 1955	Early	−	+				+	+
	Late	+	+				+	
Sugai,	1958	−~+	+ bacilli	−~+		−	+ lecithin	
Imaeda,	1958/60		+ saturated			+	+	+
Ghosh,	1962	±	+ bacilli	−	−	−	+	
Sakurai,	1970	−~+	+ bacilli	−	−	−	+	

iron salts at some stage ([32, 36, 37]), and in this series one of the B663-treated tissues gave an iron reaction.

Since the studies of Unna ([51]), Sakurane ([44]) and Mitsuda ([33]), it has been well known that lepra cells contain some kinds of lipids. Some attempts to analyze the lipid constituents of the lepra cells in human leprosy and to study their origin have been made by investigators utilizing histochemical methods ([17, 22, 23, 25, 34, 43, 46–50]) and by electron microscopy ([27, 28]) (Table 3). The lipids in lepra cells have generally been thought to be mainly phospholipids, particularly lecithin ([23, 43, 46–50]). Some authors have pointed out that neutral fat and cholesterol can also be seen on some occasions or in advanced stages ([22, 23, 43, 46, 48]). Fatty acids are demonstrated in leprosy bacilli by histochemical studies ([23, 43, 46]) and also in the cytoplasm of the macrophages ([43, 46]). The untreated cases of this series seem to have much phospholipid and a small amount of neutral fat in the lepra cells. In this series, all three of Okamoto's methods gave positive results in macrophages, so it may be concluded that sphingomyelin, at least, may be present since the color reaction of lecithin and cephalin, if present, may be masked by sphingomyelin. Phospholipids other than sphingomyelin may be present because of the positive reaction given by the Klüver-Barrera method which is positive when phospholipids, excluding sphingomyelin, are present. Sugai ([46]) reported one case of lepromatous leprosy which contained sphingomyelin in the lepra cells. There are some criticisms ([37, 46]) of Okamoto's method, however, with respect to its accuracy and sensitivity. In addition, lipid reaction cannot be made visible by general histochemical methods when the tissues contain less than 1.5 mg% of the lipid ([35]). Therefore, biochemical methods, such as chromatographic studies, should be attempted for more accurate and quantitative analysis of the lipids.

The results of the histochemical study of the untreated lepromatous tissue in this series are almost the same as those reported by Ghosh *et al* ([23]) but in the B663-treated leprosy the results are definitely different from those found in the untreated. It is evident that the macrophages of the B663-treated case contained much more neutral fat and less phospholipids than those of the untreated leprosy. The fat globules of the macrophages were stained red with oxazone by the Nile blue sulfate method and stained bright red by Sudan III as was the subcutaneous fat and all of the methods for phospholipids were weakly positive in the B663-treated case. Fishler's method for fatty acids, which stains leprosy bacilli greyish-black ([23, 43]), demonstrated fewer bacilli in the B663-treated case than in the untreated cases. Only a small number of granular forms could be observed in large globi by this method.

As to the origin of the ceroid in lepra cells in the B663-treated tissues, it is possible that if the action of B-663 is bactericidal, as Grumbach ([24]) reported, while sulfones have an essentially bacteriostatic effect ([13]), the ceroid-like substance may be produced through oxidation from some kinds of lipids, probably unsaturated fatty acids, resulting from the destruction of bacilli by the B663 action in the lepra cells or through their binding with the drug. The chemical components of *Mycobacterium leprae* are inadequately known because of the difficulty in its cultivation but the components of *Mycobacterium tuberculosis* are rather well known ([41, 54]). It is certain that *M. tuberculosis* contains much lipid which consists of acetone-soluble fats with free fatty acids, several kinds of waxes (wax A, B, C and D after Lederer and Asselineau), and phospholipids. Among these, mycolic acid and wax D (a macromolecular peptide-glycolipid also containing mycolic acid) are considered as important substances related to the acid-fastness of the mycobacteria ([41, 54]). The structural relationships of these substances are also thought to have some relationship to acid-fastness since the acid-fastness of *M. tuberculosis* can be destroyed by grinding the bacilli to a powder. On the other hand, the ceroid pigment is generally believed to be derived from lipids, probably unsaturated fatty acids through oxidation ([20, 37]). It is postulated that certain kinds of lipids, possibly unsaturated fatty acids, contained in leprosy bacilli may be oxidized after B663 action or may combine with the drug to yield an insoluble, yellowish-brown lipid pigment in the macrophages. As the leprosy bacilli themselves are strongly stained by Fishler's method for fatty acids, a large quantity of fatty acids may be contained in the leprosy bacilli. It is known that the carotenoid pigment is widely distributed in the microbial

world, including *M. tuberculosis* ([29, 54]). However, it is unknown whether or not the bacilli contain the ceroid pigment itself.

B663 is practically insoluble in water but highly soluble in fat or fat solvents, is absorbed through the intestinal tract mainly through the portal route and partly through the lymphatic system, and picked up by the reticuloendothelial cells ([2, 6, 16, 45]) in various organs so that the drug may circulate with fat, probably lipoproteins, in the blood. When olive oil is simultaneously administered, absorption of the compound is distinctly enhanced ([5]). This leads to a possible explanation of the fact that there is more neutral fat in the macrophages of the B663-treated tissues. As another possible mechanism, it is possible that the fatty degeneration may take place in the macrophages after destruction of the bacilli by the drug since neutral glycerides of the bacilli may be liberated and accumulate in the cells. Fukushi ([22]) reported that foam cells of leprosy tissue contained neutral fat which originated from liberated glycerides from the subcutaneous fat due to lepromatous invasion. The macrophages containing large globules of neutral fat, however, can be seen not only in the subcutis but also in the upper dermis which is far from the subcutaneous fat tissue; so accumulation of neutral fat in the macrophages may not only be due to involvement of normally present fat tissues by granulomatous infiltration.

In this series, round red bodies or crystals of the drug were not found. In two of the B663-treated group, the skin sections were embedded in paraffin. In these the drug may have been dissolved in the alcohols which became pink. In another case, the tissue was examined by frozen and carbowax-embedded sections, but no crystals were demonstrated probably because the drug was diffusely dissolved in the extensive fat globules of the macrophages.

SUMMARY

Histochemical studies were made of pigmented cutaneous lesions from three cases of lepromatous leprosy treated with B663 to determine the nature and histogenesis of the brown pigmentation which develops as a side effect of the drug. One case of DDS-treated leprosy and four cases of untreated leprosy were also investigated histochemically as controls.

The brown pigmentation of the skin is due to deposition of a ceroid-like substance in the macrophages, which is a yellowish-brown, acid-fast lipid pigment. It is insoluble in fat solvents and accepts lipid dyes even after lipid extraction by fat solvents.

The macrophages in the B663-treated leprosy contain more neutral fat and less phospholipid than the untreated lepromatous leprosy tissues.

Ceroid in the macrophages probably originated from unsaturated fatty acids of the leprosy bacilli through oxidation or their binding with the drug.

Crystals of the drug were not found in the macrophages in this series, even on the tissues embedded in carbowax or frozen sections.

RESUMEN

Se hicieron estudios histoquímicos en lesiones cutáneas pigmentadas de tres casos de lepra lepromatosa tratados con B663 para determinar la naturaleza y la histogénesis de la pigmentación café que se presenta como efecto colateral de la droga. Como controles se estudiaron un caso de lepra trado con DDS y cuatro casos de lepra sin tratar.

La pigmentación café de la piel se debe al depósito de una substancia ceroide en los macrófagos, la cual es un pigmento lipídico café-amarillento y ácido-resistente. Es insoluble en solventes para grasas y acepta colorantes para lípidos aún después de la extracción de estos con solventes para grasas.

Los macrófagos de los casos tratados con B663 contienen más grasas neutras y menos fosfolípidos que los tejidos de los pacientes lepromatosos sin tratamiento.

La substancia ceroide en los macrófagos se genera, probablemente, por la oxidación de ácidos grasos no saturados de los bacilos, o por combinación de estos ácidos con la droga.

No se encontraron cristales de la droga en los macrófagos observados, en los tejidos embebidos en carbowax, ni en los cortes hechos en congelación.

RÉSUMÉ

Des études histochimiques ont été menées sur les lésions cutanées pigmentées provenant de trois cas de lèpre lépromateuse traités par le B663. Le but de ces études était de déterminer la nature histogénèse de la pigmentation brune qui se développe comme effet secondaire de l'administration du médicament. Un cas de lèpre traité par le dapsone, et quatre cas de lèpre non traités, ont été également étudiés du point de vue histochimique, afin de servir de témoins.

La pigmentation brune de la peau est due au dépôt d'une substance cireuse dans les macrophages; il s'agit d'un pigment lipidique brun-jaunâtre, acido-résistant. Ce pigment est insoluble dans les solvants des graisses; il prend les colorants lipidiques même après extraction lipidique par les solvants des graisses.

Dans la lèpre traitée par le B663, les macrophages contiennent plus de graisses neutres et moins de phospholipides que les tissus récoltés chez des malades lépromateux non traités. Cette substance cireuse que l'on trouve dans les macrophages provient vraisemblablement des acides gras non saturés des bacilles de la lèpre, par suite de l'oxydation ou du couplage avec le médicament.

Dans cet échantillon, il n'a pas été possible de trouver des cristaux du médicament dans les macrophages, et ceci même dans les tissus enrobés dans "carbowax" ou dans les coupes congelées.

REFERENCES

1. ABELL, M. R., BEVERIDGE, J. M. R. and FISHER, J. H. Hepatic necrosis induced by dietary means. I. Structural changes occurring in the liver during the development of necrosis. Arch. Pathol. **50** (1950) 1-22.
2. ATKINSON, A. J., SHEAGREN, J. N., BARBA RUBIO, J. and KNIGHT, V. Evaluation of B663 in human leprosy. Int. J. Lepr. **35** (1967) 119-127.
3. BARKA, T. and ANDERSON, P. J. Chapter V. Lipids. Chapter VIII. Pigments. *In:* Histochemistry. Theory, Practice and Bibliography. New York: Harper & Row, 1963, pp 109-155, 183-202.
4. BARRY, V. C., BELTON, J. G., CONALTY, M. L., DENNY, J. M., EDWARD, D. W., O'SULLIVAN, J. F., TWOMEY, D. and WINDER, F. A new series of Phenazines (rimino-compounds) with high antituberculosis activity. Nature **179** (1957) 1013-1015.
5. BARRY, V. C., BUGGLE, K., BYRNE, J., CONALTY, M. L. and WINDER, F. Factors influencing the antituberculosis activity of rimino-compounds. Bull. Int. Union Tuberc. **29** (1959) 582-593.
6. BARRY, V. C. and CONALTY, M. L. The antimycobacterial activity of B663. Lepr. Rev. **36** (1965) 3-7.
7. BROWNE, S. G. "B663" possible anti-inflammatory action in lepromatous leprosy. Lepr. Rev. **36** (1965) 9-11.
8. BROWNE, S. G. Red and black pigmentation developing during treatment of leprosy with B663. Lepr. Rev. **36** (1965) 17-20.
9. BROWNE, S. G. Treatment of leprosy with B663. Appraisal of the pilot trial after three years. Lepr. Rev. **36** (1965) 13-15.
10. BROWNE, S. G. and HOGERZEIL, L. M. B663 in the treatment of leprosy. Preliminary report of a pilot trial. Lepr. Rev. **33** (1962) 6-10.
11. BROWNE, S. G. and HOGERZEIL, L. M. B663 in the treatment of leprosy. Supplementary report of the pilot trial. Lepr. Rev. **33** (1962) 182-184.
12. BURT, R. C. The incidence of acid-fast pigment (ceroid) in aortic atherosclerosis. Am. J. Pathol. **22** (1950) 135-139.
13. BUSHBY, S. R. M. Chapter XX. Chemotherapy. *In:* Leprosy in Theory and Practice, 2nd ed., R. G. Cochrane and T. F. Davey, eds. Bristol: John Wright & Sons, Ltd., 1964, pp 353-354.
14. CHANG, Y. T. Further studies on B663 in murine leprosy. Absence of resistance to isoniazid. Int. J. Lepr. **34** (1966) 1-6.
15. COCHRANE, R. G. Chapter XXI. Therapy. *In:* Leprosy in Theory and Practice, 2nd ed., R. G. Cochrane and T. F. Davey, eds., Bristol: John Wright & Sons, Ltd., 1964, pp 379-381.
16. CONALTY, M. L. and JACKSON, R. D. Uptake by reticuloendothelial cells of the riminophenazine, B663. Br. J. Exp. Pathol. **43** (1962) 650-654.
17. DAVIDSON, A. R., KOOIJ, R. and WAINWRIGHT, J. Classification of leprosy. II. The value of fat staining in classification. Int. J. Lepr. **28** (1960) 126-132.
18. DE FARIA, L. Uterine and vaginal changes in vitamin E deficiency. JAMA **131** (1946) 626.
19. DOULL, J. A. Bluish discoloration in lepromatous lesions during treatment with Amodiaquin. Int. J. Lepr. **27** (1959) 385-387.
20. ENDICOTT, K. M. Similarity of the acid-fast pigment, ceroid, and oxidized unsaturated fat. Arch. Pathol. **37** (1944) 49-53.
21. ENDICOTT, K. M. and LILLIE, R. D. Ceroid, the pigment of dietary cirrhosis of rats: its characteristics and its differentiation from hemofuscin. Am. J. Pathol. **20** (1944) 149-153.
22. FUKUSHI, K. and NARITA, M. Histopathological studies on mechanism of foam cell formation in leprosy. Lepro **29** (1960) 153-154. (In Japanese)
23. GHOSH, S., SEN GUPTA, P. C. and MUKERJEE, N. Histochemical study of lepromatous leprosy. Bull. Calcutta Sch. Trop. Med. **10** (1962) 102-105.
24. GRUMBACH, F. Activité antituberculeuse expérimentale de deux dérivés de Phénazine Pigmentée (B663 et B720) seuls et associés à d'autres antitubercleux (isoniazid et éthionamide). Ann. Inst. Pasteur **99** (1960) 567-585.
25. HARADA, K. Histochemical studies of leprosy, especially the mode of formation of lepra

cells. Lepro **24** (1955) 277-282. (In Japanese)
26. HASTINGS, R. C. and TRAUTMAN, J. R. B663 in lepromatous leprosy. Effect in *erythema nodosum leprosum*. Lepr. Rev. **34** (1968) 3-7.
27. IMAEDA, T. Electron microscopic analysis of the components of lepra cells. Int. J. Lepr. **28** (1960) 22-37.
28. IMAEDA, T. Experimental study of lepra cells by electron microscopy. Lepro **27** (1958) 351. (In Japanese)
29. JENSEN, S. L. Biosynthesis and function of carotenoid pigment in microorganisms. Ann. Rev. Microbiol. **19** (1965) 163-182.
30. LILLIÉ, R. D., DAFT, F. S. and SEBRELL, W. H. Cirrhosis of the liver in rats on a deficient diet and the effect of alcohol. Public Health Rep. **56** (1941) 1255-1258.
31. LOWE, J. Studies in sulfone therapy. Lepr. Rev. **23** (1952) 4-29.
32. MAEDA, T., TAKADA, R., NAKANO, K., HAGIWARA, T. and YAMAGATA, I. Occurrence of ceroid-like substance in rats fed tyrosin-excess diet. Trans. Soc. Pathol. Jap. **56** (1967) 70. (In Japanese)
33. MITSUDA, K. The significance of the vacuole in the Virchow's lepra cells and the distribution of lepra cells in certain organs. Tokyo Iji-shinshi (Tokyo J. Med. Affairs) **2066** (1918) 1-7. Translated into English: Int. J. Lepr. **4** (1936) 491-508.
34. OGATA, T. Some pathological studies on leprosy. Lepro **27** (1958) 303-312. (In Japanese)
35. OKAMOTO, K., UEDA, M., MAEDA, T. and MIZUTANI, A. Chapter 4. Lipids. Chapter 5. Pigments. *In:* Microscopic Histochemistry, 3rd ed., Tokyo: Kyoritsu-shuppan, 1965, vol. 3, pp 331-379, 380-419. (In Japanese)
36. PAPPENHEIMER, A. M. and VICTOR, J. "Ceroid" pigment in human tissue. Am. J. Pathol. **22** (1946) 395-413.
37. PEARSE, A. G. E. Chapter XI. Lipids, lipoprotein and proteolipids. Chapter XXIII. Pigments, and Appendices. *In:* Histochemistry. Theoretical and Applied, 2nd ed., Boston: Little, Brown & Co., 1960, pp 289-333, 632-680, 773-956.
38. PETTIT, J. H. S. and REES, R. J. W. Studies on sulfone resistance in leprosy. Treatment with a riminophenazine derivative (B663). Int. J. Lepr. **34** (1966) 391-397.
39. PETTIT, J. H. S., REES, R. J. W. and RIDLEY, D. S. Chemotherapeutic trials in leprosy. Pilot trial of a riminophenazine derivative (B663) in the treatment of lepromatous leprosy. Int. J. Lepr. **35** (1967) 25-33.
40. POPPER, H., GYORGY, P. and GOLDBLATT, H. Fluorescent material (ceroid) in experimental nutritional cirrhosis. Arch. Pathol. **37** (1944) 161-168.
41. RICH, A. R. Chapter I. The relation of the chemical constituents of the tubercle bacillus to pathogenesis. *In:* Pathogenesis of Tuberculosis, Springfield: Charles C. Thomas, 1944, pp 13-20.
42. ROBINSON, K. L. and COLY, W. E. A brown discoloration of pig fat and vitamin E deficiency. Nature **168** (1951) 997-998.
43. SAKURAI, I. and SKINSNES, O. K. Lipids in leprosy. II. Histochemistry of lipids in human leprosy. Int. J. Lepr. **38** (1970) 389-403.
44. SAKURANE, K. A supplementary report of histopathology of skin leprosy. Hifu-hinyokigakkai-zasshi (J. Dermatol. Urol.) **3** (1903) 1-19. (In Japanese)
45. SHEPARD, C. C. and CHANG, Y. T. Activity of antituberculosis drugs against *Mycobacterium leprae*. Studies with experimental infections of mouse foot pads. Int. J. Lepr. **32** (1964) 260-271.
46. SUGAI, K. Histopathological studies on human leprosy. IV. Histochemical analysis of abnormal fats in leprous lesions, especially on the fat deposition in lymph nodes. Lepro **27** (1958) 216-227. (In Japanese)
47. TANAKA, H., IZUMURA, K., ARIMORI, J., WASHIO, M., KUBO, E. and OGURO, A. Histochemical studies on tuberous leprosy lesions. IV. Phospholipids, and lipase and esterase in tuberous leprosy lesions around sebaceous glands. Lepro **27** (1958) 82-83. (In Japanese)
48. UEDA, M. On the fats of various inflammatory foci, especially leprous foci of human body and rat. Trans. Soc. Pathol. Jap. **27** (1949) 139-141. (In Japanese)
49. UEDA, M. Study on lipids in leprous foci (II). Lepro **18** (1949) 16-17. (In Japanese)
50. UEDA, M. and KUSUMOTO, Y. Study on lipids in leprous foci (II). Lepro **17** (1948) 22. (In Japanese)
51. UNNA, P. G. Die zusammensetzung des Lepra Bacillen-scheims. Monatsch. Praktische Dermatol. **26** (1898) 17-26. Cited from Ref. 41.
52. WARREN, A. G. A preliminary report on the use of B663 in the treatment of Chinese leprosy patients with chronic reaction. Lepr. Rev. **39** (1968) 61-66.
53. WILLIAMS, T. W., MOTT, P. D., WERTLAKE, P. T., BARBA RUBIO, J., ADLER, R. C., HILL, G. J., PEREZ SQUAREZ, G. and KNIGHT, V. Leprosy research at the National Institutes of Health. Experience with B663 in the treatment of leprosy. Int. J. Lepr. **33** (1965) 767-775.
54. YAMAMURA, Y. Chapter 3. Chemistry of bacillary components. Chapter 5. Biological and medical problems. *In:* Biochemistry of *Mycobacterium tuberculosis*, Tokyo: Kyoritsu-shuppan. 1955, pp 25-58, 164-165. (In Japanese)