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Electron Microscope Studies of the Antileprosy Drug B663 (Clofazimine; Lamprene)¹

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Since its discovery by Barry in 1957, the riminophenazine B663 (clofazimine; Lamprene) has been the subject of numerous publications concerning its biochemistry and pharmacology (3.4), lipid chemistry (23), experimental properties in the laboratory animal (^{4, 5, 23}), skin pigmentation (²¹), clin-ical use in leprosy (^{2, 6, 7, 8, 9, 20, 22}), action in murine leprosy (10, 11), crystallization (15), and effects on macrophages and the reticuloendothelial system (12, 16, 17).

Recently, Conalty et al (14) have published further findings on this drug in relation to the reticuloendothelial system, giving particular attention to the mechanism of its accumulation and crystallization in cells, and to the effects of this on macrophage function.

Early in 1972, observations in Oxford on tissues from the amputated leg of a leprosy patient treated for some years with B663, suggested that this drug might not penetrate so well ino peripheral nerves as it does into various other body tissues.

This was supported by light microscope findings on the histopathology of skin and peripheral nerves biopsies from patients in the Leprosy Research Unit of the Medical Research Council in Malaysia, and assessed here after some years of treatment with B663. Considering the factors of infiltrate,

anatomical structure, and acid-fast bacilli (in any form), nerve therapeutic response was often rated as good as skin, for the period of treatment, but was never better and not infrequently worse. In at least one instance, solid-staining bacilli were found in nerves several years after beginning treatment where they had apparently disappeared from the skin.

It was, therefore, decided to study by light and electron microscopy, the effects of feeding clofazimine to mice in their diet. As this work proceeded it soon became clear that a larger animal was needed for chemical analysis of drug in peripheral nerves, and that in the mouse, whole-body autoradiography would be the method of choice for following the distribution of antileprosy drugs into various body tissues.

These results and the findings on light microscopy will be published separately, and the present paper deals only with electron microscope findings as seen in cells of mouse spleen, with particular reference to 1) the position and appearance of the "mature" crystal, 2) a membrane which surrounds it, and 3) the presence of electron-dense "rods" or "bands" with which it is often closely associated.

MATERIALS AND METHODS

Parkes albino mice were fed 0.01% of powdered B663 in their diet. The animal

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whose tissues appear in Figure 2 was fed on the drug for seven months before killing, but all other animals and tissues referred to in this report were from mice fed on it continuously for ten months, and then changed to a normal diet before killing six months later. Thus, with the exception of Figure 2, all tissues shown come from animals who had not ingested any B663 for six months following a ten month period of drug loading.

Tissues taken included liver, spleen, mesenteric lymph nodes, intestine, lung, brain, spinal cord, sciatic nerves, fat, striated muscle and skin. However, the tissue reported in this study is spleen, and this was cut into 1 mm cubes or slices and fixed in a 1:1 mix-



FIG. 1. Mouse spleen. Crystal spaces are seen in the cytoplasm of macrophages in a multinucleate complex, with occasional osmiophilic bands or rods between them. X 2,400.



FIG. 2. A cluster of crystal spaces is seen in the cytoplasm of a mononuclear cell in mouse spleen. M—mitochondrion. ER—endoplasmic reticulum. CS—crystal spaces in cytoplasm of adjacent macrophage. X 10,500.



FIG. 3. Fully-formed "mature" crystal spaces (CS) in cytoplasm of a mouse spleen macrophage, with various osmiophilic bodies (OB), one of which shows 4 lighter areas in its substance (LA). X 5,200.

ture of 3% glutaraldehyde in 5% sucrose buffer and 1% paraformaldehyde, for two hours at 0.4° C. These were washed several times in sucrose buffer for 30 minutes at 0.4° C, and then treated with 1% osmium tetroxide in 5% sucrose phosphate buffer for two hours at 4° C. Dehydration was by routine graded alcohols, 10-100%, over a period of 2 hours and 20 minutes, followed by immersion in toluene, two changes each of 30 minutes. Embedding was in a toluenearaldite mixture, 1:1, overnight at room temperature; followed by araldite, in an oven at 60° C for 48 hours. Sections were cut on an LKB microtome at 500 Å, and stained with saturated uranyl acetate in 70% alcohol and Reynold's lead citrate.

RESULTS

The electron micrographs show that there is a dual phenomenon to be considered: 1) the size, shape, distribution and membranes of the electron-transparent crystal spaces, where the fully formed adult crystals have accumulated (Figs. 1-3); and 2) the presence of black "rods" or "bands," often intimately associated with or even surrounded by crystal spaces (Figs. 7-11).

When first seen over a year ago, these osmiophilic rods were thought to be an arti-

fact, and they were not photographed. Later, the possibilities of drug-induced crystalloids (19) or of physiologically normal "storage" crystalloids were considered. Normal Parkes albino mice which had not received any B663 were therefore killed and examined under EM for similar bodies, using an identical processing technic, and to these were later added various Oxford laboratory mice. Nothing whatever comparable to the osmiophilic rods was revealed. With increasing confidence in the fixation and processing, it soon became clear that in B663 mice they followed a pattern and were to be found in many areas, often lying parallel to the long axes of crystal spaces.

Although their osmiophilic properties and position in the cell were in keeping with the drug-plus-lipoprotein complex already described for B663 in mammalian tissues (^{13,} ¹⁷), their profusion in this material, and the repeated finding of long "rods" or "bands" prompted various steps to investigate the possibilities of impurity in the ingested B663, or artifact during EM processing. These results are described below.

Distribution and appearance of crystal spaces and osmiophilic rods. The choice of spleen has raised a difficulty at the outset, for the normal cytology is not always easy to interpret, particularly where immature cells are concerned. Large dense accumulations of crystals, cytoplasmic fusion and the formation of multinucleate cells (Fig. 1) all add to the difficulties. Furthermore, in some animals even after careful searching of the whole grid, deposits of drug appeared to be entirely extracellular with no clear relation to any cell membrane.

The term "crystal spaces" is used because, as processed and photographed in this study, there is no evidence of any structure within

them apart from araldite. High magnification on the electron microscope and electron diffraction have completely failed to reveal structure in these areas. Ultrathin frozen sections of unfixed and unembedded spleen, known to be full of B663 crystals, have been cut on a cryo-ultratome, and examined after freeze-drying; results of electron microscopy and X-ray microanalysis from this tissue are currently being assessed. For completely unknown reasons, attempts to embed crystal-



FIG. 4. Crystal spaces (CS) in cytoplasm of mouse spleen macrophage with surrounding membrane (M). N—nucleus. OR—osmiophilic "rod." X 20,500.



FIG. 5. Crystal spaces (CS) in cytoplasm of mouse spleen macrophage, with association osmiophilic rod (OR). N-nucleus. M-mitochondria. X 20,500.

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FIG. 6. High power view of one end of a crystal space (CS) with appearances suggesting a double outline (DO), with increased density between the layers, as compared with adjacent cytoplasm. X 60,000.



FIG. 7. Crystal spaces (CS) in cytoplasm of mouse spleen macrophage, with osmiophilic rods (OR) showing membrane surrounding them. N-nucleus. M-mitochondria. X 20,000.

laden material in water soluble durcupan or glycol methacrylate have failed, perhaps due mainly to difficulties in hardening the block and in cutting.

Crystal spaces, sometimes alone, but often associated with osmiophilic rods, have been seen in a wide variety of situations. Figure 2 shows a large cluster occupying about 25% of the revealed cytoplasmic area and, as with numerous other cells examined, having no obvious ill-effect on nucleus, membranes, remaining cytoplasm or organelles. Figure 1 shows a heavy deposit of B663, mainly involving the cytoplasm of macrophages which have merged into a multinucleated cell; close examination of the low power picture shows numerous osmiophilic rods lying between crystal spaces.

Crystal spaces and rods usually lie in mononuclear cells, this term being selected



FIG. 8. Crystal spaces (CS) with one area (CL) of concentric "lamination." N-nucleus, X 20,000.

with care since in the present study it cannot be stated that all B663 is in macrophages. Crystals have also been seen in cells indistinguishable from mouse spleen lymphocyte, and in several instances in the cytoplasm of neutrophils.

Fine spicules of the drug have been seen

in numerous micrographs, sometimes running across the main tissue without regard for cell boundaries (Figs. 9, 10). Elsewhere concentric or laminated formations have been found (Fig. 8), and occasionally deposits of the drug are closely associated with endoplasmic reticulum. Of major interest has



FIG. 9. Crystal spaces (CS) and osmiophilic rods (OR). M-mitochondria. ER-endoplasmic reticulum. N-nucleus.



Fig. 10. Crystal spaces (CS) and osmiophilic rods (OR) alternate in macrophage cytoplasm, while fine spicules (S) run across adjacent cytoplasmic membrane towards nucleus (N). X 35,000.



FIG. 11. Crystal spaces (CS) and osmiophilic rods (OR) give the impression, especially in the central complex of "streaming" one between the other. N-nucleus. X 35,000.

been the demonstration of a membrane around crystal spaces (Figs. 4-7) and osmiophilic rods (Fig. 7). This was not seen at all in our earlier mice, and may be missed completely if fixation and processing are not of high standard. It appears as a single "unit" membrane closely enveloping a space which, in shape and size, closely accords with formed crystals as seen under the light microscope. In rare instances, at high magnification (Fig. 6), some membrane areas suggested a double contour with increased electron density between the layers, as compared with adjacent cytoplasm.

In contrast to the crystal spaces, the osmiophilic rods have very definite substance. This can be seen at moderate magnification, and is abundantly confirmed at higher magnifications (Fig. 12) and by optical diffraction; aspects which will be fully described below. In some, but by no means all instances, these rods are surrounded by a membrane but elsewhere they give the impression of merging rather vaguely into the supporting cytoplasm (Fig. 11). A high percentage are aligned parallel to the long axis of crystal spaces (Figs. 9-11). Some lie in the midst of tangled, complex masses of drug deposit; others are intimately apposed to crystal spaces or alternate with them (Figs. 9, 10).

A close examination of many fields has shown osmiophilic material, associated with crystal spaces, and in a round or oval form



FIG. 12. Magnification 100,000.



FIG. 13

FIG. 14

FIGS. 12, 13 and 14. The optical diffraction pattern of the rod (a) which runs diagonally across the micrograph in Figure 12 is shown in Figure 13. Only the first order reflection is observed which corresponds to a spacing of 33 Å. The optical diffraction pattern of the rod (b) which is normal to the edge of the micrograph shown in Figure 12 is shown in Figure 14. Only the first order reflection is observed which corresponds to a spacing of 41 Å. Figure 12 magnification 100,000.

of only moderate dimensions. A striking feature of the present tissues, however, has been the profusion of this osmiophilic material in much more conspicious "rods" or "bands" generally thinner than the crystal spaces with which they are frequently aligned.

Osmiophilic rods; steps taken towards their further identification and to the elimination of artifact. As noted, the possibilities of a processing artifact or of some impurity in the B663 were considered when these rods were first seen. Steps taken to investigate these will now be described under separate headings.

An impurity in the B663 ingested by the mice. In a preliminary survey of the micrographs, we were alerted (¹³) to the possibility that other phenazines, such as B628 and B629, might be present. Although samples of the original B663 used in feeding animals in this series were no longer available, Geigy (UK) kindly supplied another sample of the micronized drug (Batch MG 98), believed to be pharmacologically very similar if not chemically identical. This moved as one spot on alumina TLC, using benzene as eluent. No other spots showed with u-v light or iodine vapor. On silica gel with benzene, the spots did not move from their origin.

Artifacts in EM processing, lack of alcohol into all parts of the tissue. This seemed unlikely, but to investigate it tissues were brought up to 100% alcohol and then left in it for one week instead of the usual two hours. There was some overall loss of definition and contrast, but absolutely no change in the character or distribution of osmiophilic rods or crystal spaces.

The influence of stains. It seemed unlikely that standard EM stain combinations could have "created" the osmophilic rod, but in view of the potential interplay between osmium, lead, uranium, B663 itself, and lipoprotein, it was decided to run various combinations of the three stains and to omit osmium from at least one series altogether. The results can be stated very simply: without osmium very little can be seen. In fact with any combination other than that described under METHODS, results are extremely poor with the one exception that if osmium is combined with lead, especially if the latter is prolonged to 30 minute staining, some osmiophilic rods can be detected, but the appearances are vastly inferior to those of the full routine.

High magnification electron microscopy and electron diffraction. Even at moderate magnifications the osmiophilic rods appeared to have internal structure, and at a magnification of 100,000 on the electron microscope crystal planes were revcaled running approximately-perpendicular to the long axis of the rod (Fig. 12). The Department of Molecular Biophysics, Oxford, very kindly examined these specimens by electron diffraction under conditions in which reflections corresponding to spacings of 10 Å or less would have been detected. No electron diffraction was found for the crystal spaces or osmiophilic rods. These results support the view that the rods are not merely aggregates of stain.

The spacings in the rods shown in Figure 12 were now submitted to examination in the optical diffractometer. For the rod marked "A" the optical diffraction pattern is shown in Figure 13 and indicates a spacing of 33 ± 2 A. For the rod marked "B" the pattern is shown in Figure 14 and indicates a spacing of 41 ± 2 A.

In the anticipation that continued efforts with water-soluble embedding media might result in the cutting of mammalian tissue with preserved crystals *in situ*, actual crystals of B663 evaporated from acetone in the laboratory, were examined by X-ray diffraction. Preliminary results suggest that "the unit cell dimensions of the crystals are considerably smaller than 33 A (as expected for a molecule of this size), and these results support the view that the rods observed in the tissue sections are most likely a crystalline array of the drug with some other molecule, possibly a lipoprotein."³

³ Report by Dr. Louise Johnson.

DISCUSSION

Although some of the electron micrographs raise considerable difficulties in interpretation, it has been thought worth presenting them in advance of other studies from the same animal tissues, because of the possibility that they may shed light on the mode of action of B663 as an antileprosy drug, and on research on macrophages, lysosomes and lipoprotein. It is important to emphasize that with the exception of the animal whose tissue appears in Figure 2, all were fed on B663 for a period of ten months at a concentration of 0.01% in the diet and then transferred to a normal diet before killing six months later. This may account for the differences between the pictures now presented and those of the only other EM studies in the literature (^{14, 17}), where animals were killed after only 14 days on the drug.

The membranes around crystal spaces and to a lesser extent around osmiophilic rods (Fig. 7) raise at least two possibilities as to their origin: first, that the drug has entered some organelle and is merely increasing in size within a preexisting membrane; and second, that the membrane has been formed by the cytoplasm in response to the presence of a growing crystal, rather as has been suggested (¹⁸) for the mycobacteria in macrophages of the mouse foot pad.

The close apposition of osmiophilic rods to crystal spaces together with the "streaming" effect already described, suggests that there may be some physical or chemical relationship between the two. In many rods, small areas of translucency have been noted, as already described (14), suggesting a transformation from the black osmiophilic form to the white fully-formed crystal space. However, in view of the factors concerning the period of ingestion of B663 and the time of killing already stressed above, it is impossible to conclude from the present material that the process is not in fact going in the other direction, and it is certainly curious that there is such a profusion of osmiophilic rods, and mainly in what seems to be longitudinal section. No final conclusion will be possible until the rods have been much more closely analyzed, but a drugplus-lipoprotein complex could be osmiophilic to the extent shown here and the physical juxtaposition with crystal spaces suggests that the two are significantly related. The reason for the variation in the spacings on optical diffraction of the osmiophilic rods (33 Å as against 41 Å) are not known, but the fact that the spacings on X-ray diffraction of the laboratory crystals were clearly different supports the view that the rods may represent a crystalline complex of the drug with some other molecule,

possibly a small lipoprotein. It is now clearly necessary to isolate osmiophilic rods from identical material and to study them by gradient centrifugation and examination in an EM pellet. Geigy Pharmaceuticals has suggested the possibility of up to 3% allied substances as impurities in batches of B663, and this is now being checked, though our investigations are against significant impurities in the batches examined so far.

The findings of groups or channels of crystals which do not relate to any obvious cell membrane, and which appear extracellular, may be due to their emergence from cells which were no longer physically capable of holding them. The mode of breakdown and excretion of B663 crystals from the body is not clearly known, but it has been suggested (²⁴) that they may go into solution and be taken up by another macrophage. Eventually, on withdrawal of the drug, they may be excreted unchanged in molecular form through the kidney and possibly the bile, though the latter route has not been investigated.

On the question of cellular entry, it has been emphasized (17) "that B663 is not taken up by macrophages in particulate form ... but rather it would appear to enter the macrophages in solution linked to a lipoprotein carrier, which is then split off, with the consequent intracellular formation of crystals of B663." The addition of the micronized drug to cell cultures is being studied and may throw light on the mode of entry as well as on the stages preceding the formation of the "mature" crystal as a residual body in a membrane-bound vacuole. B663 can be predicted to maintain some basicity in lysosomes, and it is not unreasonable to suggest (1) that it could enter the lysosomal vacuolar system, bound to a serum protein carrier, later to crystallize in secondary lysosomes.

If this can be confirmed, information of value might accrue not only on some aspects of drug pharmacology and lysosomal function, but also on the mechanism of action of B663 against the intracellular parasite which causes leprosy in man.

SUMMARY

Following the oral administration of the riminophenazine B663 (clofazimine, Lam-

prene) to mice at a concentration of 0.01%in their diet, tissues were taken for light- and electron microscopy. Findings from the latter are described as seen in cells of the spleen, where a dual phenomenon was observed. First, there were well-preserved spaces representing the fully formed crystal of drug, but dissolved out during processing; and second, osmiophilic "rods" or "bands" which had obviously retained their substance, and which showed linearity at high magnification on the electron microscope, and a lattice spacing on optical diffraction in the range $33-41 \pm 2$ Å were noted.

Drug deposits were seen in macrophages, multinucleate cells, and occasionally in neutrophil polymorphonuclear cells. Membranes have been demonstrated around crystal spaces and osmiophilic rods. The location of B663 in the cytoplasm of these cells, and the possible significance of the osmiophilic "rods" are discussed.

RESUMEN

Después de administrar a ratones rimino-fenazina B663 (clofacimina, Lamprene) por vía oral a una concentración de 0,01% en la dieta, se les tomó tejido para estudios con microscopio de luz y microscopio electrónico. Se describen los hallazgos con éste último tal como se observaron en células de bazo, donde se vió reacción. Primero, habían espacios bien conservados que representaban el cristal de la droga totalmente formado, pero disuelto durante el procesamiento y, segundo, se observaron "bastones" o "bandas" osmiofílicas que obviamente habían retenido su sustancia y que mostraban linearidad a alta magnificación en el microscopio electrónico y un espacio enrejado a una difracción óptica en el rango de 33 — 41 \pm 2 Å.

Se observaron depósitos de la droga en los macrófagos, células multinucleares y, ocasionalmente, en neutrófilos polimorfos. Se han demostrado membranas alrededor de los espacios de los cristales y de los bastones osmiofílicos. Se discute la localización del B663 en el citoplasma de estas células y el posible significado de los "bastones" osmiofílicos.

RÉSUMÉ

On a prélevé des tissus en vue d'un examen par la microscopie optique et par la microscopie électronique, à la suite de l'administration orale de riminophénazine B663 (clofazimine, Lamprène) à la souris, à une concentration de 0,01 pour cent dans la nourriture. Les observations faites au microscope électronique sont décrites telles qu'elles sont apparues dans les cellules de la rate, où un phénomène double a été observé. D'abord, on a constaté des espaces bien préservés, qui représentaient le cristal entièrement formé du médicament, qui se dissolvaient ensuite au cours des manipulations. En second lieu, on a relevé la présence de batonnets ou de bandes osmiophiles, qui avaient manifestement retenu leur substance, et qui montraient un aspect linéaire à de forts grossissements au microscope électronique, de même qu'un espacement de la diffraction optique (lattice spacing) dans la gamme $33-41 \pm 2$ Å.

Des dépots de médicaments ont été observés dans les macrophages, dans les cellules multinucléées, et à l'occasion dans les polymorphonucléaires. Des membranes ont été mises en évidence autour des espaces cristallins et des bâtonnets osmiophiles. On discute la localisation du B663 dans le cytoplasme des cellules, et la signification possible des bâtonnets osmiophiles.

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