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An Improved Embedding Method for Electron Microscopy of Lepromata[1]

Seitaro Okada, Yukiko Fukunishi, A. Mukherjee,
G. Ramu, and K. V. Desikan[2]

Epon 812 ([7]), a kind of epoxy resin, is used most widely for electron microscopic study by the ultrathin-section method in biology. It has many good points, e.g., no shrinkage in the process of polymerization, good preservation of ultrafine structures, no bubble formation, rather strong adhesive strength, and stability against the electron beam. It, however, has a weak point in the study of leprosy. It can penetrate well into the cytoplasm and leprosy bacilli in tuberculoid lesions or lesions having some bacilli which are not in foamy structures, i.e., existing freely in cytoplasm. However, in the lepromatous lesion, the penetration of Epon 812 into the foamy structure and the leprosy bacilli is not good. Therefore, Epon 812 is not polymerized uniformly in the cell, and some parts of the inside of the foamy structure and some bacilli in it are left out at the time of ultrathin-sectioning, causing a big hole in the section. Additionally, some of the bacilli which are retained in the foamy structures are homogeneously dense, their inner structures not being differentiated (Fig. 1). Vestopal W ([8]), polyester resin, and Araldite ([2]), Maraglas ([1]), and Quetol 651 ([3]), epoxy resin, are also stable in an electron beam but are not as widely used as Epon 812. They also have the same weak point as Epon 812 in the study of leprosy.

Methacrylate has very low viscosity in contrast to Epon 812, having high viscosity, and therefore it can penetrate well into tissue. On the other hand, it has the following weak points: marked shrinkage and occasional bubble formation at the time of polymerization, instability in an electron beam, its sublimation in a strong electron beam, distortion of some large molecular structures, incomplete preservation of some ultrafine structures, etc. In contrast to Epon 812, however, methacrylate can penetrate well into foamy structures and leprosy bacilli. Therefore, methacrylate is more suitable for the study of lepromatous lesions, especially leprosy bacilli, than Epon 812.

Spurr used a low viscosity epoxy resin for electron microscopic studies in botany and obtained good results ([9]). The plant cell also has a hard cell wall, and therefore the viscous resins cannot penetrate well into it. In contrast, Spurr's resin mixture includes ERL-4206, which has very low viscosity. Spurr's resin mixture can penetrate well into plant cells and yields good electron microscopic figures. Besides, it is stable against the electron beam. Therefore, this resin mixture was tried by us for the study of lepromatous lesions.

This paper deals with the examination of various resins and the embedding method for lepromata using these resins.

MATERIALS AND METHODS

The method of dehydration, the process of immersion of the specimen in the stepwise mixture of resin and ethanol or propylene oxide, and the method of incubation by stepwise rise of temperature were investigated for Epon 812. Other kinds of resin were then tried. Maraglas, Vestopal W, Araldite, styrene ([4]), glycol methacrylate ([6]), and Quetol 651 were used. Styrene was used as the mixture with n-butyl methacrylate at a ratio of 2 to 1, to which benzoyl peroxide was added to a concentration of 1%. In addition to these resins, Spurr's res-

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[2] S. Okada, M.D., Associate Professor, Leprosy Research Laboratory, Kyoto University School of Medicine, Sakyo-Ku, Kyoto 606, Japan; Y. Fukunishi, M.D., Medical Officer, National Sanatorium Oshima Seisho-en, Aji-cho, Kida-gun, Kagawa Pref. 761-02, Japan; A. Mukherjee, M.B.B.S., M.D., Research Officer; G. Ramu, M.B.B.S., M.D., Deputy Director; K. V. Desikan, M.B.B.S., M.D., Director, Central JALMA Institute for Leprosy, Taj Ganj, Agra-282 001, India.

in mixture was tried. DER 736, plasticizer, NSA, hardener, and S-1, accelerator, were mixed with ERL-4206. The standard ratio, namely ERL-4206 10.0, DER-736 6.0, NSA 26.0, and S-1 0.4, was found to be better than other ratios. For the examination of Spurr's resin mixture, four lepromata were removed from four lepromatous patients. Half of each leproma was fixed in 2.5% glutaraldehyde in M/15 phosphate buffer (pH 7.4), washed with the buffer, postfixed in 1% OsO_4 in the buffer, and dehydrated with a graded ethanol series. The other half of each leproma was fixed in 1% OsO_4 only in the same buffer. A part of each half was embedded in methacrylate, another part in Epon 812, and the remaining part in Spurr's resin mixture. Some lepromata were embedded in Quetol 651, Araldite, styrene mixed with methacrylate, and Kushida's modification of Spurr's resin mixture ([5]) also. Kushida's modification is composed of ERL-4206 23.0, Quetol 653 14.0, NSA 63.0, and S-1 0.5. With two lepromata, dimethylformamide (DMF) or dimethylsulfoxide (DMSO) was tried as substituter after dehydration with the graded ethanol series, being compared to propylene oxide (PO). The procedure for embedding was as follows:

50% ethanol	15 min
70% ethanol	15 min
90% ethanol	15 min
100% ethanol	15 min
100% ethanol	15 min
100% ethanol	15 min
PO or DMF or DMSO	20 min
PO or DMF or DMSO: Spurr's resin mixture = 3:1	30 min
PO or DMF or DMSO: Spurr's resin mixture = 2:2	30 min
PO or DMF or DMSO: Spurr's resin mixture = 1:3	30 min
Spurr's resin mixture	1 hr
Spurr's resin mixture in capsule	4–6 hr at 45°C overnight at 60°C

After the PO or DMF or DMSO is mixed with Spurr's resin mixture, the specimen and liquid was stirred by hand or with an apparatus ("Penetrator") which rotates the bottle containing the mixture and the specimen in order to accelerate the penetration of resin into the tissue.

The specimens embedded in these resins were ultrathin-sectioned, stained with uranyl acetate and lead citrate, and observed with Hitachi's H-500 type electron microscope.

RESULTS

The penetration of Epon 812 into foamy structures was improved only slightly by the different embedding methods tried. Many foamy structures were still left which were incompletely penetrated. Maraglas, Vestopal W, Araldite, and Quetol 651 also did not penetrate well into foamy structures. Holes were seen in the foamy structures, and the inner structures of the bacilli which were not left out at the time of ultrathin-sectioning were not observed well. Styrene mixed with n-butyl methacrylate could penetrate very well although some holes could be seen in a small number of foamy structures. The cell walls of leprosy bacilli were well preserved in this resin mixture while those embedded in methacrylate were frequently partially separated from the cytoplasm. The figures of bacillary cytoplasm, however, were similar to those in the specimen embedded in methacrylate, being rough and granular (Fig. 2). The section of the specimen embedded in styrene plus methacrylate was more stable against the electron beam than that embedded in methacrylate. Embedding in glycol methacrylate did not yield good figures.

When Spurr's resin mixture was used as the embedding material, the part of the polymer block without tissue was fragile, but the part having tissue could be ultrathin-sectioned easily. The sections of some specimens had a tendency to expand on the surface of the water in the knifeboat. The sections were stable against the electron beam. The contrast of the stained figures was lower than that of the section embedded in methacrylate. On the other hand, photographing was easy because membranous structures could be observed easily. Also, in the lepromata used for the trial of embedding in Spurr's resin mixture, the penetration of Epon 812 into foamy structures and leprosy bacilli was not good, and a big part of the inside of the foamy structure was left out at the time of ultrathin-sectioning, causing a big hole in the section. However, in two of four lepromata, Spurr's

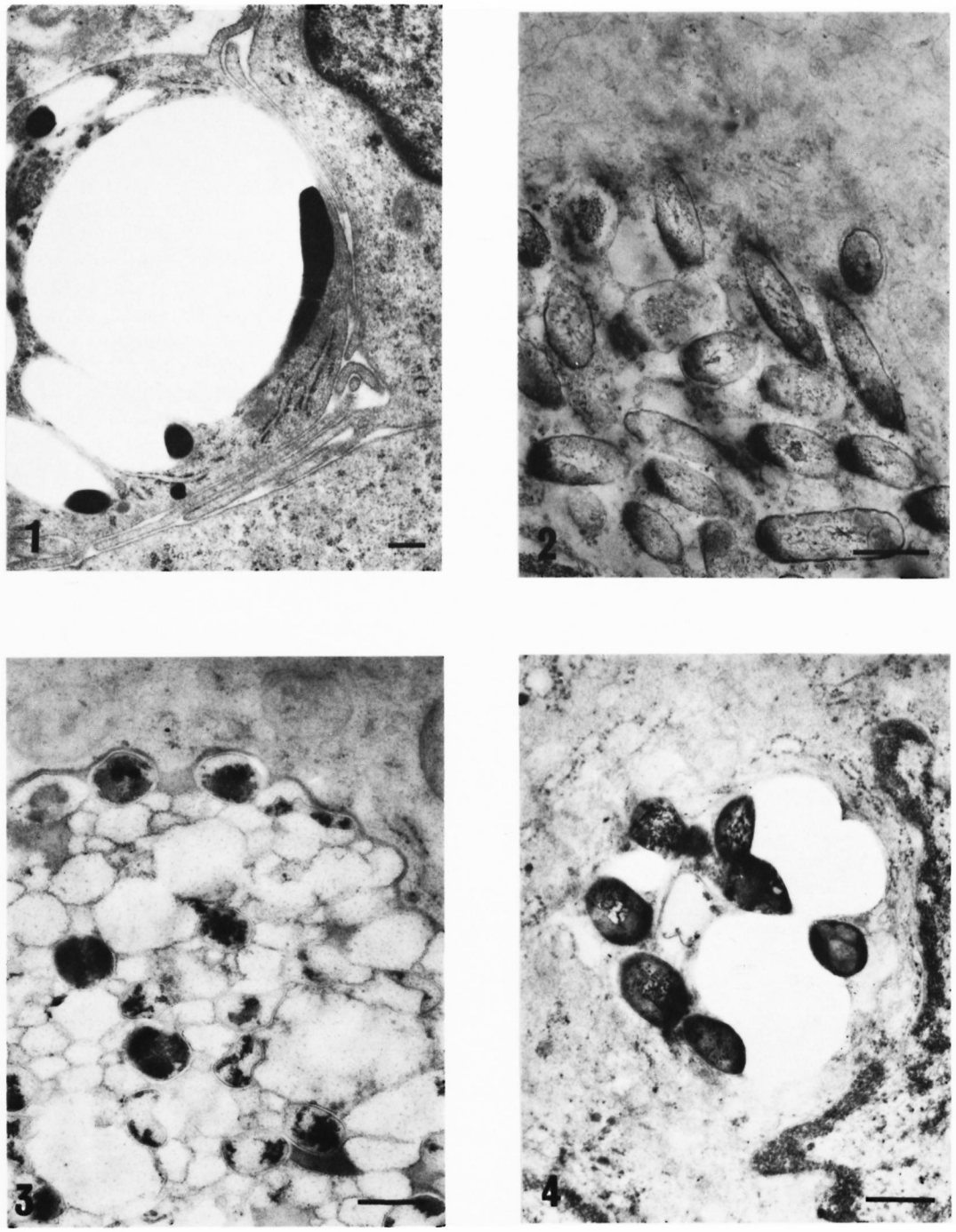

FIG. 1. Lepra cell embedded in Epon 812. Large holes are formed, and some bacilli which remain are dense, their inner structures not being differentiated. (scale bar = 0.5 μ, ×11,000)

FIG. 2. Lepra cell embedded in styrene mixed with methacrylate. This resin mixture penetrates well into foamy structures and leprosy bacilli. The cell walls of bacilli are well preserved, not being separated from the cytoplasm of the bacilli. (scale bar = 0.5 μ, ×22,000)

FIG. 3. Lepra cell embedded in Spurr's resin mixture. This resin mixture penetrates completely into foamy structures and leprosy bacilli. (scale bar = 0.5 μ, ×20,000)

FIG. 4. Lepra cell embedded in Spurr's resin mixture. In this leproma, the resin mixture penetrated incompletely, and some holes could be found in some of the foamy structures. However, even in these areas, the fine structures of leprosy bacilli are well preserved. (scale bar = 0.5 μ, ×20,000)

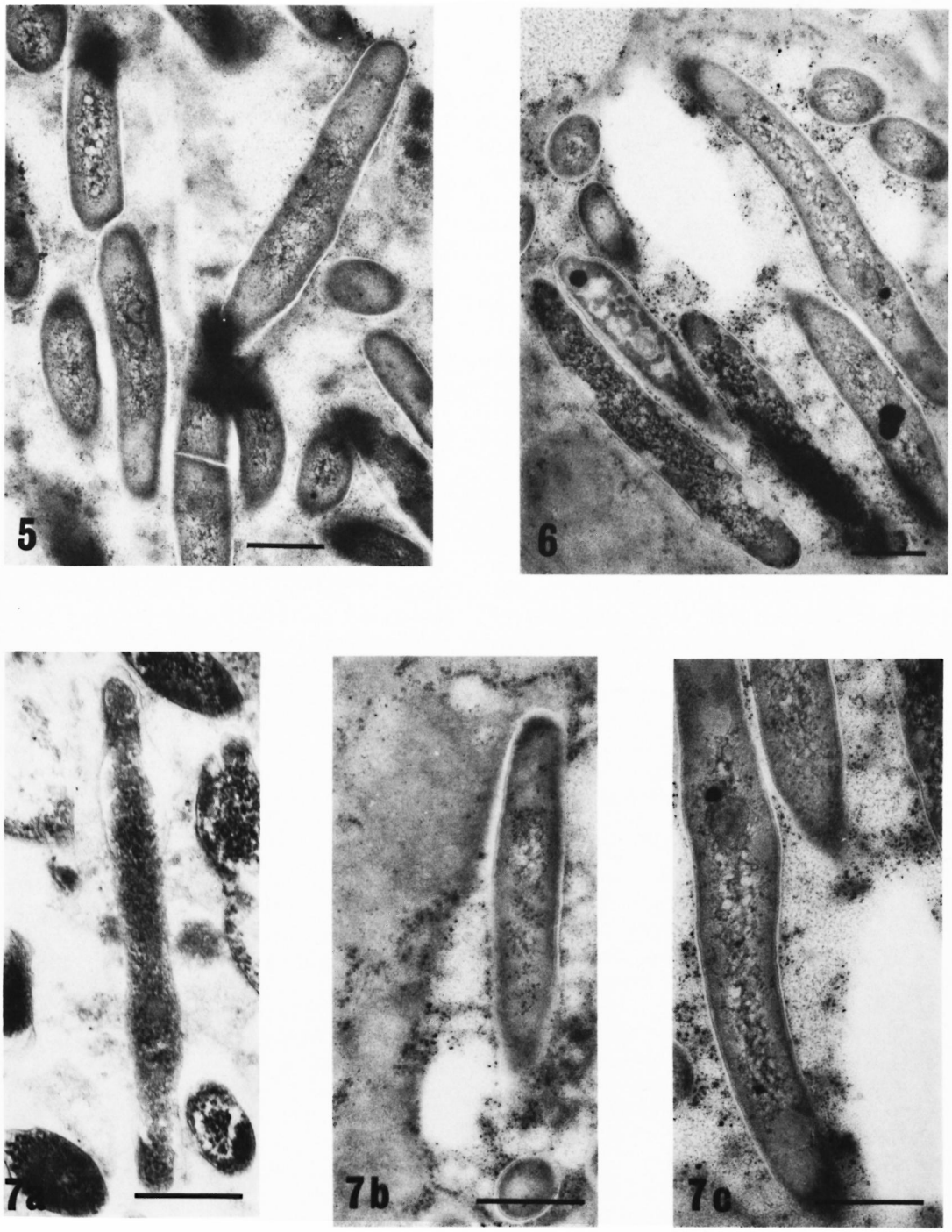

FIG. 5. Leprosy bacilli embedded in Spurr's resin mixture. For this specimen, DMF was used as substituter. The fine structure of bacilli are very well preserved. (scale bar = 0.5 μ, ×22,000)

FIG. 6. Leprosy bacilli in the same section as that shown in Fig. 5. In this picture, the degenerated bacilli can be seen in addition to the nondegenerated bacilli. The fine structures of the degenerated bacilli are also well preserved. (scale bar = 0.5 μ, ×22,000)

FIG. 7. Comparison of figures of leprosy bacilli in the same leproma among the specimen embedded in methacrylate (7a), the specimen embedded in Spurr's resin mixture for which DMSO was used as substituter (7b), and the specimen embedded in Spurr's resin mixture for which DMF was used (7c). (scale bar = 0.5 μ, ×32,000)

resin mixture penetrated completely into the foamy structures and leprosy bacilli in them, and no hole could be found in the foamy structures such as those seen in those embedded in methacrylate (Fig. 3). In the other two lepromata, small holes could be found in some of the foamy structures, but generally speaking, the penetration of this resin mixture was much better than that of Epon 812. It could penetrate well into leprosy bacilli in the foamy structure even if some holes were formed in the foamy structure. Therefore, the fine structures of leprosy bacilli were well preserved (Fig. 4). Especially in the specimens in which DMF or DMSO was used as substituter instead of PO, the fine structures of bacilli were well preserved (Figs. 5 and 6). The matrix of cytoplasm in solid bacilli was fine and homogeneous in contrast to the rough and granular appearance of the cytoplasm of bacilli embedded in methacrylate. The membranous structures were slender. The cell wall was well preserved, not being separated from the cytoplasm (Figs. 7a, 7b, and 7c). The resin mixture modified by Kushida could also penetrate well, similar to the original Spurr's resin mixture, but the figures of the cytoplasm of the bacilli were more rough than those of bacilli embedded in the original mixture.

DISCUSSION

Epon 812, Maraglas, Vestopal W, Araldite, Quetol 651, and glycol methacrylate are not suitable resins for the study of lepromata because they cannot penetrate well into foamy structures and leprosy bacilli in them. Styrene mixed with n-butyl methacrylate can penetrate into these structures very well. Additionally, the cell walls of leprosy bacilli are well preserved, not being separated from the cytoplasm. This is in contrast with the separation of the cell wall from the cytoplasm which is sometimes seen in bacilli embedded in methacrylate. The figures of cytoplasm, however, are rough and granular, being similar to those embedded in methacrylate. On the other hand, this resin mixture is stable against the electron beam, contrary to methacrylate. This resin mixture is worthy of further investigation.

Spurr's resin mixture penetrated completely into foamy structures and leprosy bacilli in two lepromata among four examined. In the other two lepromata, small holes could be found in some of the foamy structures. Further study on the resins used for the mixture and the process of dehydration and embedding is necessary to eliminate the appearance of such holes. Another good point of this resin mixture for the electron microscopic study of leprosy is the good preservation of ultrafine structures of leprosy bacilli. Especially in the specimen for which DMF or DMSO was used, the ultrafine structures of bacilli are very well preserved. In addition to these two good points, specific for the electron microscopic study of lepromata, the sections of specimens embedded in this resin mixture are stable against the electron beam. Therefore it can be said that this resin mixture has the good point of good penetration into foamy structures and leprosy bacilli, resembling methacrylate, and has the additional good points of stability against the electron beam and good preservation of ultrafine structures, resembling Epon 812.

The ultrathin sections of some lepromata embedded in this resin mixture had a tendency to expand on the surface of the water in the knifeboat. Especially the early sections by a knife had such a tendency.

It can be concluded that this resin mixture is the most suitable embedding material for lepromata at present.

SUMMARY

Spurr's resin mixture has been found to be the embedding material most suitable for electron microscopic studies of lepromata at present. Like methacrylate, it can penetrate well into foamy structures and the leprosy bacilli within foamy structures. On the other hand, like Epon 812, it is stable against the electron beam and can preserve ultrafine structures. Additionally, we have found that the use of dimethylformamide or dimethylsulfoxide instead of propylene oxide as substituter improves the preservation of the ultrafine structures of leprosy bacilli.

RESUMEN

La mezcla de resinas de Spurr ha resultado ser el material de inclusión más apropiado para el estudio de lepromas al microscopio electrónico. Como el metacrilato, puede penetrar bien dentro de las estructuras

espumosas y en los bacilos de la lepra que se encuentran dentro de estas estructuras. Por otro lado, como el Epon 812, es estable al haz de electrones y puede preservar las estructuras ultrafinas. Adicionalmente hemos encontrado que el uso de dimetilformamida o de dimetilsulfóxido en lugar del óxido de propileno mejora la preservación de las estructuras ultrafinas del bacilo de la lepra.

RÉSUMÉ

Le mélange de Spurr à base de résine s'est révélé être actuellement le matériel d'enrobement le plus approprié pour les études de microscopie électronique de la lèpre. Ce mélange, comme le méthacrylate, peut pénétrer à l'intérieur des structures spumeuses, et également à l'intérieur des bacilles de la lèpre qui se trouvent dans ces structures. Par ailleurs, de même que l'Epon 812, ce mélange est stable lorsqu'il est soumis aux faisceaux d'électrons, de telle manière que les structures ultra-fines sont préservées. De plus, nous avons observé que la dimethylformamide ou la dimethylsulfoxide peut être utilsée à la place de l'oxyde de propylène, et que ces produits améliorent la préservation des structures ultra-fines du bacille de la lèpre.

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