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Electron Microscope Study of *Erythema Nodosum Leprosum*[1]

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Many studies have been made on clinical aspects, immunology, bacteriology and treatment of *erythema nodosum leprosum* (ENL). Its pathology has been described, among others, by Murata (3), Sugai and Monobe (8), Mitsuda (2), Reiss (5), Rodriguez (6), Wolcott (9) and Sasaki (7), on the basis of light microscopy. However, electron microscopic studies have not been reported.

Recent advances in immune electron microscopy utilizing ferritin-conjugated antibody or enzyme-labeled antibody have made possible the location of antigen by electron microscopy. Ferritin is a protein including iron which is 100 Å in diameter. At the core, surrounded by protein shell, there is a square micelle 55 Å in diameter which includes iron hydroxide and iron hydroxide phosphate. The core can be differentiated as a high contrast dot under electron microscopy. Accordingly, when antibody is conjugated with ferritin as a labeling substance and reacts with antigen, the location of antigen can be observed.

MATERIALS AND METHODS

Ordinary electron microscopy. Six cases of acute ENL, lepromas of four untreated lepromatous cases, and lepromas of three cases in whom a small number of ENL sometimes appeared were excised for examination. Immediately after excision, the specimens were fixed with 1% osmium tetroxide solution adjusted to pH 7.4 with veronal acetate buffer or phosphate buffer. They were dehydrated in a graded series of ethanol and embedded in methacrylate or a mixture of styrene and methacrylate. Two specimens were embedded also in Epon 812. Ultrathin sections were made and stained with uranyl acetate and lead oxide.

Immune electron microscope by means of ferritin-conjugated antibody.

Method of isolation of leprosy bacilli from leproma. Yanagisawa *et al* (10) reported that they could not sensitize guinea pigs with an emulsion of leproma or with bacilli isolated from lepromas by Dharmendra's method (1), but they were able to sensitize with bacilli isolated by trypsin treatment. Accordingly, trypsin treatment was employed in this study. The method of isolation of murine leprosy bacilli from murine lepromata published by Nishimura *et al* (4) was adopted in this study, but modified by omission of treatment with sodium hydroxide. Thus, 5.3 mg of leprosy bacilli (dry weight) were isolated from six lepromas.

Method of immunization of rabbit against leprosy bacilli. Five milligrams of bacilli were mixed with Freund's incomplete adjuvant and injected intramuscularly into the buttocks of a rabbit. Five milligrams of bacilli were suspended in 2.5 ml of distilled water and mixed with 2.5 ml of adjuvant by use of a blender. One milliliter of this suspension was injected intramuscularly each time. A physiological saline suspension of 0.3 mg of bacilli was injected intravenously. Intramuscular injections were made two times with an interval of one week. One week later, 0.3 mg of bacilli suspended in physiological saline was injected intravenously. After that, intramuscular injections were made three times at an interval of about three weeks. Total bloodletting was done 24 days after the final injection.

Extraction of γ-globulin. G-globulin was purified by salting out through 1/3 saturation by ammonium sulphate. The γ-globulin of a patient in acute ENL was similarly prepared.

Conjugation of γ-globulin with ferritin. One step in conjugation using FNPS (p,p'-difluoro-m, m'-dinitro-diphenysulfone) was adopted in this study.

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Treatment of leproma with ferritin-conjugated antibody. The direct method was adopted. Immediately after incision, the leproma was fixed in 10% cold formalin solution adjusted to pH 7.2 with M/15 phosphate buffer for 15 minutes. Then the leproma was washed well with phosphate-buffered saline, and sectioned at about 15μ thickness in a cryostat. The sections were incubated in cold ferritin-conjugated antibody for one hour. Excessive ferritin-conjugated antibody was washed out, and then the sections were fixed with 1% osmium tetroxide solution adjusted to pH 7.2, dehydrated in a graded series of ethanol, embedded in methacrylate, ultrathin-sectioned, double-stained with uranyl acetate and lead oxide, and observed with an electron microscope. In order to ascertain that the attachment of ferritin to some place is specifically due to antigen-antibody reaction, blocking tests were made. That is, cryostat sections were treated with γ-globulin not conjugated with ferritin, and then incubated in ferritin-conjugated antibody. After that, the specimens were treated in the same manner as described.

RESULTS

Observation on ENL by electron microscopy. Some parts of the ENL lesions showed compact arrangement of neighboring lepra cells, but other parts of the same lesions showed wide intercellular spaces with polymorphonuclear leucocyte and monocyte infiltration. In those areas in which polymorphonuclear leucocyte infiltrate was found, the foamy structures of lepra bacilli were ruptured and in communication with the intercellular space. Also the cell walls of leprosy bacilli were sometimes ruptured and open to the space of foamy structures (Fig. 1). Debris from the destroyed cells was found in the intercellular space. Leprosy bacilli and cell debris were ingested by monocytes or polymorphonuclear leucocytes. Collagen fibers degenerated into an electron-dense and homogeneous substance and fused with each other. In those areas in which lepra cells were compactly gathered, the following feature could be observed. The collagen fibers were degenerating and the plasma membranes of lepra cells disappeared when adjacent to degenerating collagen fibers. These were interpreted as initial changes and termed "coincident impairment of fiber and membrane" (Fig. 2).

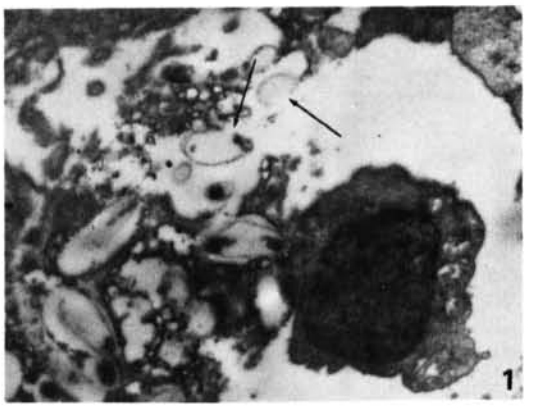

FIG. 1. A foamy structure is ruptured and open to intercellular space. Also the cell walls of leprosy bacilli are ruptured (arrow). A lymphocyte has migrated to this locale. X12,920.

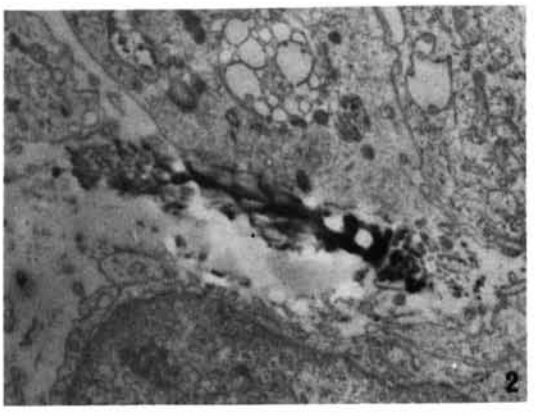

FIG. 2. The collagen fibers are degenerating, and at the part which is adjacent to degenerating collagen fibers, plasma membrane of lepra cell disappears. It is, so to speak, "coincident impairment of fiber and membrane," X18,000.

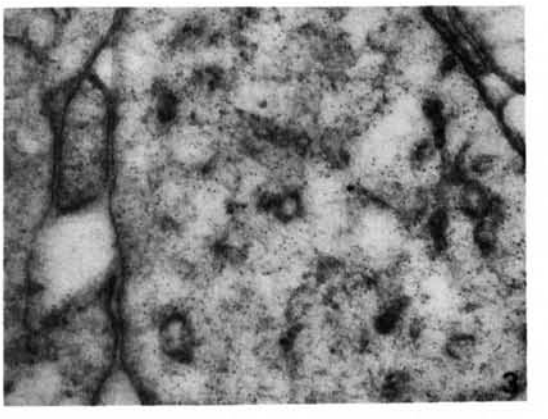

FIG. 3. The fine granules 90 Å in diameter are scattered in the cytoplasm of a lepra cell, X51,500.

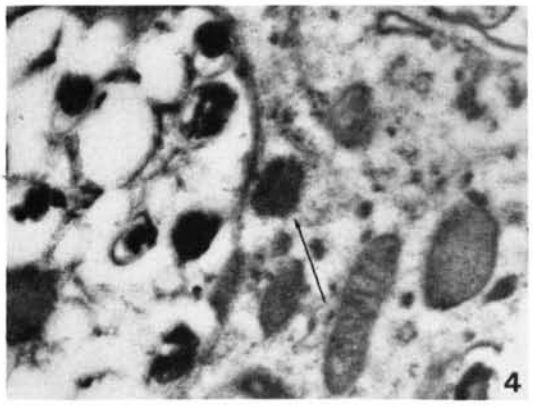

FIG. 4. Many fine granules are gathered together to form a lump adjacent to the membrane surrounding a foamy structure (arrow), X51,500.

Many fine spherical granules, 90 Å in diameter, and having high electron-density were found in the lepra cells of ENL lesions. Most such fine granules were scattered in the cytoplasm of the lepra cell (Fig. 3). In some lepra cells, many granules were gathered together to form a lump adjacent to the wall of foamy structure (Fig. 4). These granules could not always be found in the nuclear leucocytes or monocytes which infiltrated the lesion.

These fine granules were found in all of the six instances of ENL examined. On the other hand, the granules were not found in the four lepromas excised from four newly discovered and untreated lepromatous patients. In a leproma excised from three cases under treatment which sometimes had a small number of ENL, a small number of fine granules could be observed. Methacrylate embedded ENL was ultrathin-sectioned and the resin was removed from the ultrathin sections by chloroform. Next, the section was incubated in a solution of pepsin in 0.02N HCl at a concentration of 2 mg/ml for four hours at 37°C and then observed under the electron microscope. The fine granules disappeared with this treatment. Therefore, it was concluded that the granules were protein granules.

Immune electron microscope study by means of ferritin-conjugated antibody. When the leproma was treated with the antileprosy-bacilli antibody conjugated with ferritin, the ferritin-conjugated antibody attached to the cytoplasm of leprosy bacilli in the foamy structures of lepra cells. It did not attach to the cell wall of leprosy bacilli. It attached especially to ill-defined fibrous structures with low density in the cytoplasm of degenerated leprosy bacilli. The attachment was frequently observed in bacilli whose cell wall was ruptured or had partially disappeared. Because the ferritin-conjugated antibody cannot penetrate through the intact bacillary cell wall, it invades the bacillary body through a split in the wall. The ferritin-conjugated antibody attached to the foamy structure in which leprosy bacilli could not be seen. This fact means that cytoplasmic substances released from leprosy bacilli by bacteriolysis is included in the foamy material. It is thought that this substance may be included in the supernatant obtained by centrifugation of an emulsion of lepromas.

In a specimen treated by the blocking test, such attachment of ferritin to the cytoplasm of leprosy bacilli and foamy structures could not be observed. Therefore, the attachment of ferritin can be regarded as being mediated by specific antigen-antibody reaction.

When the leproma was treated with anti-leprosy-bacilli antibody conjugated with ferritin, the ferritin attached to the cytoplasm of lepra cell also. It is thought that this is due to the tissue component admixed into the leprosy bacillus fraction used for the sensitization of rabbit. The antibody against the tissue component could probably not be totally absorbed by normal skin. This fact suggests that some component different from the normal components of skin is included in the tissue of leproma.

G-globulin extracted from the serum of acute instances of ENL was conjugated with ferritin. When the leproma was treated with the ferritin-conjugated antibody, the ferritin attached to the cytoplasm of leprosy bacilli

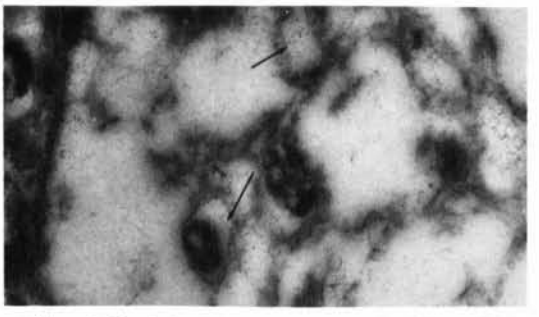

FIG. 5. γ-globulin extracted from the serum of an ENL case was conjugated with ferritin. The ferritin-conjugated antibodies attach to the cytoplasm of leprosy bacilli (arrow), X60,040.

(Fig. 5). This suggests that antileprosy-bacilli antibody is included in the serum of the ENL case.

DISCUSSION

ENL rarely occurs in cases not treated with chemotherapy and having old lepromata; most of ENL is related to chemotherapy. In many lepromatous patients, ENL appears several months to one year after the start of chemotherapy. Many degenerated bacilli are found in cases having old lepromas not treated with chemotherapy. In treated patients the degeneration of leprosy bacilli is accelerated and ENL occurs. Degenerated bacilli show condensation of dense material in some areas while others become more transparent. Leprosy bacilli which undergo such spotted condensation become swollen. It is thought that this is due to an increase in internal pressure. When leprosy bacilli are intact, the bacilli are scattered in the cytoplasm of the histiocyte. Even if intact bacilli lie in small bundles, the surrounding electron transparent zone is irregular-shaped. As bacilli degenerate, the foamy structure takes on a smooth outline due to the swelling of leprosy bacilli. As foamy structures are swollen, foamy structures adjoining each other fuse into a larger structure. If the foamy structures of two lepra cells adjoin each other, the plasma membrane disappears due to the pressure of foamy structures and fuse into a larger foamy structure. These facts suggest to us the possibility that membranes surrounding foamy structures may rupture and open into intercellular spaces. In the lesions of ENL, the membranes surrounding foamy structures are ruptured and open into intercellular spaces, and the cell walls of leprosy bacilli are also broken. This indicates that the cytoplasmic substances of leprosy bacilli are released into intercellular spaces.

The ferritin-conjugated antibody method demonstrates that the antigenicity of leprosy bacilli is localized in the cytoplasm, and the antibody against the cytoplasm of leprosy bacilli is included in the serum of ENL cases. Therefore the outflow of cytoplasmic substance having antigenicity, results in antigen-antibody reaction which leads to ENL. Such a mechanism for the development was earlier presumed by Mitsuda [2]. Mitsuda supposed from his pathologic studies that the existence of tuberculin-like substance in degenerated leprosy bacilli might play a role in the pathogenesis of ENL.

The antigen-antibody reaction causes first the "coincident impairment of fiber and membrane," and leads to degeneration of collagen fibers and the disappearance of plasma membrane. Antigen-antibody complexes also accelerate the chemotaxis of leucocytes. Leucocytes phagocytose cell debris. In this manner, the antigen-antibody reaction destroys lepra cells and causes the outflow of cytoplasmic substance of leprosy bacilli. Due to repetition of such processes, ENL appears repeatedly.

It is thought that the fine granules, 90 Å in diameter, found specifically in the lepra cells of ENL lesion have a close relation to ENL.

SUMMARY

Electron microscopic study by means of the ferritin-conjugated antibody method revealed that the antigenicity of leprosy bacilli is localized in the cytoplasm of leprosy bacilli. In the lesion of ENL, the foamy structure of the lepra cell is ruptured and opens into intercellular spaces and the cell walls of leprosy bacilli are also ruptured. This suggests that antigenic cytoplasmic substance is released from lepra cells. As antibody to the cytoplasm of leprosy bacilli is present in the serum of ENL case, the outflow of cytoplasmic substance of leprosy bacilli results in antigen-antibody reaction which leads to ENL.

RESUMEN

Un estudio hecho con el microscopio electrónico por medio del método de anticuerpos conjugados con ferritina, reveló que la antigenicidad del bacilo de lepra está localizada en el citoplasma del bacilo. En las lesiones de ENL, la estructura espumosa de la célula de lepra se rompe y se abre hacia los espacios intercelulares y, además, la pared celular de los bacilos de lepra también se rompe. Esto sugiere que las células de lepra liberan material citoplasmático antigénico. Ya que en el suero de los casos con ENL se encuentran anticuerpos contra el citoplasma de los bacilos de lepra, la salida de material citoplasmático de los bacilos de lepra produce una reacción antígeno-anticuerpo que lleva hacia el ENL.

RÉSUMÉ

Une étude menée au microscope électronique, au moyen de la méthode des anticorps conjugués a la ferritine, à révélé que l'antigénicité des ba-

cilles de la lèpre était localisée dans le cytoplasme de ces bacilles. Dans les lésions de l'*érythème noueux lépreux* (ENL) la structure spumeuse de la cellule lépreuse est disloquée et s'ouvre dans les espaces intercellulaires; les parois cellulaires des bacilles de la lèpre sont également brisées. Ceci suggère qu'une substance cytoplasmique antigénique est libérée par les cellules lépreuses. Vu qu'un anticorps contre le cytoplasme des bacilles de la lèpre est présent dans le sérum des cas d'ENL, l'irruption d'une substance cytoplasmique provenant du bacille de la lèpre entraîne une réaction antigène-anticorps qui se manifeste par l'ENL.

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