

Transmission Electron Microscopic Study of The Ultra-Structural Changes of Conjunctival Tissue in Ocular Leprosy

*Dr. Santanu sen. MS. Ph.D, Chief Consultant, Netaji Eye Hospital,
Ramchandrapur Ashram, Purulia.*

Leprosy is one of the most ancient diseases of human being known to mankind. In India, it was known even in the days of 'Vedas' (as 'Kilas') and a reasonably good descriptions of 'KUSTHA' Can be found in 'Sushruta Samhita', compiled around 600 BC and in the 'Laws of Manu' written around 1300 BC. The disease affects almost all the Cooler structures of the body. It affects skin, nerves, nose, eyes, mouth, pharynx, bones, nails, muscles, lymph nodes, kidneys and testes. Peripheral nerve involvement is a Constant feature. Ophthalmic problems arise in all types of leprosy. Kirwan (1956) stated that a lepromatous leprosy patient will develop some Ocular complication, if untreated for sufficient length of time. In fact the effect of leprosy on the eyes is perhaps the worst of all complications. "Loss of vision in normal people can partly be compensated by the sense of touch. A leprosy patient liable to develop blindness might have already lost much of the sensation in his hands or feet. For him the onset of blindness is a tragedy (Job CK et al 1991). To avoid this tragedy, early detection of ocular involvement of leprosy in pre-clinical or in suspected cases, has evident advantages. A better understanding of the evolution of such damages may enable us to evolve better methods for prevention of such damage to ocular tissues and resultant deformities due to leprosy.

The application of Transmission electron microscope in the study of histology has enabled us to study the cellular changes of any structure, either from ageing process or from any disease in great details as compared to the findings of light microscopy and/or impression cytology. The ultra-structure of this cells can be studied by this technique more elaborately with high power of magnification (X 2000 to X 100,000) and high resolution power which even the highest magnification of a light microscope (upto X 300) can not provide. The

availability of this technological advancement has opened up fascinating new-vistas for in-depth study in the field of leprosy. Which is still a priority socio-economic and Public health problem in India and other developing countries. The prevention, early detection, effective treatment and management of leprosy, is still a burning problem in this country. Studies of the cells and bacteria of the Transmission electron microscopic level thus have fascination not only for the sake of knowledge on this complex syndrome, but also for long term preventive measures. Emphasis in this study is laid on ultra structural (Cyto-skeletal) changes of conjunctiva, the most superficial (but vital) ocular structure which have to bear the brunt of many diseases, affecting the eye. No attempt is made here to provide a comprehensive, study of cell biology, covering cell dynamics, bio-physical and bio-chemical basis of the structures, or the functions of the chief sub-cellular organelles.

Plan of the Work :-

1. To study the ultra-structure of the conjunctiva of normal subjects under transmission electron microscope.
2. to study the ultra-structure of the Conjunctiva of the established leprosy patients and to compare them with the normal ones under TEM.
3. To map out the changes in the ultra-structure of the conjunctival tissue of the patients suffering from different types of leprosy.
4. To point out any change / changes specific for leprosy.
5. To search for any change / changes in the ultra structure of the conjunctival tissue which can foretell ocular affections of leprosy well beforehand in hitherto unaffected eyes in the sub-clinical stage, so that requisite measures could be taken to save the eyes from lepromatous complications.

Materials and Methods :-

Sixty six cases were finally selected for this study. Several

Address for correspondance :

*Dr Santanu Sen, 32 Amerst Street, Kolkata - 70009, Ph. 23504195
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other patients were preliminary examined and kept under observations.

The usual methods of light microscopy and electron microscopy were used. For light microscopy appropriate stains for cells, myelin and acid-fast bacilli were carried out. For transmission electron microscopy ultra-thin Glutaraldehyde fixed osmicated sections were examined. The cases were selected from the patients attending the O.P.D. of Netaji Eye Hospital, Ramchandrapur Ashram, Purulia.

The patients selected were examined thoroughly clinically. Their eyes were also examined with the help of slit lamp.

Every patient was subjected to Lepromin Test at first to detect the type of leprosy he was suffering from. Suspension of Mitsuda lepromin (lepromin A) (0.1 ml.) was used for the purpose. Although routine nasal scrapings are not recommended by experts, we had done it to confirm the diagnosis of untreated lepromatous, leprosy cases, Skin smear, taken from the dorsa of the fingers, on the glass-slide and fixed over flame, were stained with Ziehl-Neelsen Stain. A.F.B. (granular or Solid-staining) were then searched under microscope and noted accordingly. Skin-biopsy of some patients were done for correct classification and proper diagnosis. Blood samples of each patient were examined for TC, DC, ESR, Hb% and for platelet count and Prothrombin Time.

In selecting the patients for the study, we have followed the immunological classification of Ridley and Jopling as (i) Lepromatous (LL) (ii) Border line lepromatous (BL) (iii) Border line (BB) (iv) Borderline tuberculoid (BT) (v) Tuberculoid (TT).

Normal persons with age group of (i) 15 - 60 yrs. and (ii) 60 Yrs. and above - were also brought under the study. Tissues were also collected from patients suffering from non-lepromatous granulomatous diseases.

Before collecting the tissues, proper consent was taken from each of the patients and/or from his/her nearest relatives.

Collection of Tissues — After topically anaesthetising the conjunctiva with Xylocaine 4%, a small chunk of upper bulbar conjunctiva (about 2mm x 2mm) was cut and taken out of the eye very carefully. The eye was then dressed with antibiotic ointment (ophthalmic) and covered with a sterile pad. The patient was advised to apply antibiotic ointment in that eye for at least 7-10 days and to attend the clinic for regular check up.

The tissue was then fixed in 3% Glutaraldehyde + 2% Paraformaldehyde for 3 hours and then washed in 0.1 M sodium cacodylate buffer (with adjusted P_{H} 7.2 ± 0.1)

three times at 10 minutes interval each. The solutions were supplied by central drug Research Instt., Lucknow. The specimens, collected, were then Post fixed in 1% Osmium tetroxide in 0.1M cacodylate buffer at 4° C for 1-2 hours.

The specimens, so Post-fixed, were carried in Individual phials properly marked and placed in an Ice-box to Transmission electron Microscope room for further procedures.

Processing of the samples for Transmission electron microscopy - After fixation, washing in 0.1 M cacodylate buffer and post fixation in Osmium tetroxide, the samples were washed thoroughly 3-4 times in triple distilled water in order to remove excess of osmium tetroxide. Then the samples undergo the graded series of acetone and plastic mixtures for dehydration and infiltration. Acetone 30%, 50%, 70%, 90%, were used (10 mns. each) and absolute 100% acetone were used for 20 mns.

The plastic mixture consists of Epon 812, Araldite 6005 and Dibutylphthalic and a solution commercially available as Dodecyl succinic Anhydride (DDSA).

Infiltration - The dehydrated tissues were transferred in acetone plastic mixture and pure plastic mixture as follows — Acetone - plastic mixture 2:1 (one hour) — 1:1 (one hour) — 1:2 (2 hours) and then pure plastic mixture for 2 hours.

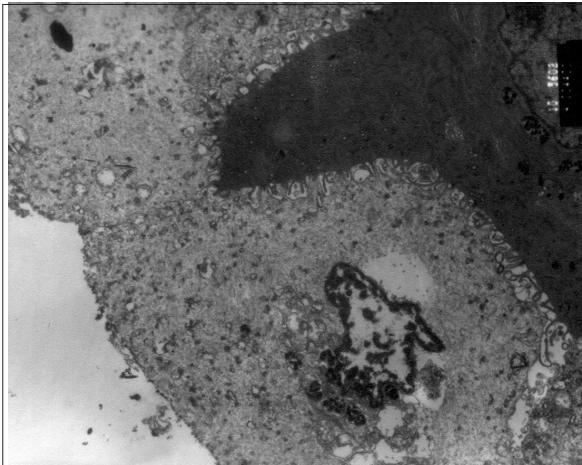
Embedding and polymerization — The tissue finally embedded individually in fresh plastic mixture in beam 00 capsules and kept in a moisture free oven at 60°C for 18-20 hours to polymerize.

Sectioning of the tissues - the blocks were carefully trimmed and initially 1 mm thick sections were cut on an LKB ultratome 8800 using glass knives prepared by cutting glass squares on an LKB knife maker. The sections were stained with 1% warm aqueous toluidine blue for identification of desired area from which ultra thin section would be made. These toluidine stained section were examined as an olympus light microscope.

For ultra thin sectioning, the selected areas of the tissues were trimmed into a smaller Pyramid and ultra-thin sections (30-50 nm) were cut on the above mentioned ultratome using non-serrated edge of the glass knife fitted with pre-cleaned metal boats with the help of nail polish / wax and filled with TOW. The ultra-thin sections were individually collected on 400 mesh copper grids pre-cleaned with 1 N HCl and water and dried with alcohol.

For light microscopy 1% toluidine blue stain and H & E stain were used. For transmission electron microscopy uranyl acetate stain and Lead Citrate stain were used.

Staining and photography of the ultrathin sections - The ultrathin sections mounted on the grids were stained for 30 minutes with 1% uranyl acetate and 5-10 minutes with lead citrate in CO₂ free atmosphere, thoroughly washed in



Conjunctiva epithelium in Tuberculoid Leprosy (TT)
Loss of microvilli (A)
D - Desmosomes
V - Vesicles
(E. M. graph X 3600)

triple distilled water, air-dried and examined under PHILLIPS EM 416 LS Electron Microscope at an accelerating voltage at 80KV.

Representative areas were photographed on on the chromatic electron microscope sheet films (KODAK, UK) under varying magnifications. These grids were stored in grid boxes for future references.

Results :-

In tuberculoid (TT) and in borderline Tuberculoid (BT) leprosy with lagophthalmos, Stratification of conjunctival epithelium with separation of superficial cell layers were found in most cases. There was



Fig. 2 Nerve Section in Conjunctive in case of Borderline Tuberculoid leprosy (BT)
(E. M. graph X 40000)

deformation and reduction of microplacae on the Surface of the Conjunctival epithelium (Fig. 1). (Next Page)

Goblet cells were seen to be fewer in number. Out of 26 Cases (17 cases of TT and 9 cases of BT) - 17 cases had Lagophthalmos, only 2 TT cases showed mild conjunctival congestion, 16 cases showed epithelial changes. Loss of microvilli was a constant feature in all of them.

7 cases of TT and BT patients having no ocular complications, but having lepromatous affections elsewhere in the body were also examined. Out of 7 cases 5 cases showed deformities and reduction of microplacae and microvilli with separation of superficial layer.

One interesting and important cell in tuberculoid leprosy was the specialised macrophage which is generally known as

epitheloid cell. It was a large cell with thin cell membrane and a large pale indented nucleus with thin uniform layer of heterochromatin. The epitheloid cells, found, were either pale or dark. The later usually contains fairly abundant RER in the cytoplasm. In most cases, two epitheloid cells were found closely apposed

to each other, while in two cases they were found to have fused together to form multinucleated "Langhan's type giant cells".

Nerve-affection is a constant feature in all types of leprosy. In BT cases, the perineurium was found to be thickened (Fig. 2). Fig. 2 shows healthy Axon (A) surrounded by a schwann cell containing Myelin debris (MB). Diffuse infiltration of the nerves by macrophages was there. The thickening of the perineurium was by the presence of increased number of collagen fibrils between many layers of perineural cells. Normal unmyelinated axons were rare. Myelinated axons were significantly reduced in number. They were replaced by inflammatory granulama.

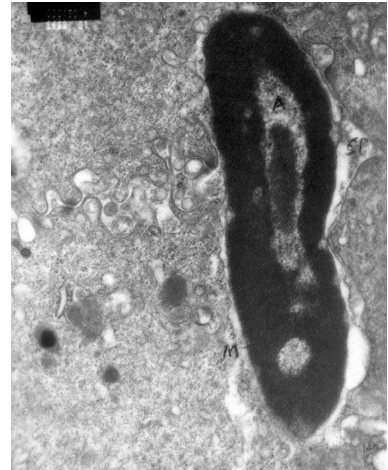


Fig. 3 A degenerated nerve fibre of Conjunctiva in a case of Tuberculoid Leprosy (TT)
(E. M. graph X 21000)

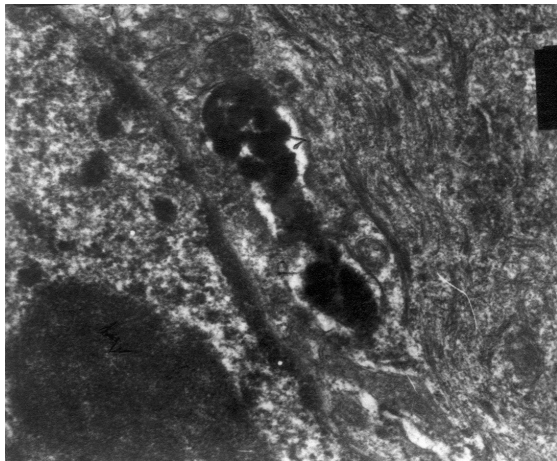


Fig. 4 A section of the Conjunctival stroma in a case of Tuberculoid Leprosy (TT)
M - A portion of mast cell
P - Pigment
V - Vesicles
(E. M. graph X 31000)

Composed of macrophages, epitheloid cels, lymphocytes and a few plasma cells. Considerable proliferation of Intra-neural collagen fibrils encircling individual nerve fibres and small clump of axon was found.

In tuberculoid leprosy, the nerve parenchyma was replaced by collagen fibrils and there was invasion cells, lymphocytes, occasional giant cells, a few macrophages. Fig. 3 shows degenerated nerve fibre with parenchyma replaced by thickened collagen fibrils. Denervated schwann process can also be seen.



Fig. 5 Conjunctival blood-vessel in a case of Tuberculoid Leprosy (TT)
(E. M. graph X 44000)

Large irregular macrophages were seen in the conjunctival stroma in tuberculoid and Borderline tuberculoid leprosy. They contain some vesicles, some of these vesicles contain pigment-granules and other substances.

Large oval or round mast cells and mast cell-granules were found in the stroma. They were numerous is

tuberculoid leprosy (Fig.4), less so in borderline tuberculoid leprosy, but only a few could be seen in lepromatous leprosy or in normal individual. Fig.4 shows two vesicles containing pigments and a part of large mast cell with mast cell granules.

Polymorpho nuclear leucocytes with lobed nucleus were found in plenty in all leprosy cases. The cytoplasm of those cells were granular, some showed vacuoles (? packets of hydrolytic enzymes).

Large Eosinophils with granular cytoplasm could also be found more in TT and in BT leprosy. An electron - dense crystalloid core surrounded by a light grannular outer layer were their constant feature.

In the blood-vessels of the conjunctiva, changes were evident in borderline tuberculoid and in tuberculoid leprosy. Out of 26 cases of BT and TT. Cases 20 cases showed some changes in the blood vessels. The basement membrane outside the endothelium was found to be proliferated into characteristic lamallae. A very large number of proliferated basement membrane layers were seen both outside the endothelial cells and outside pericytes (Fig. 5).

The extension outwards of the proliferated basement membrane and the thickness it leads to the vessel-wall could be appreciated by comparing the diameter of the lumen of the blood-vessel and that of the entire vessel-wall.

Processes of other infiltrating cells could be found amidst the basement membrane lamellae. Sometimes the pericytes had completely disappeared from the blood vessels and the number of infiltrating cells were quite large in those cases.

Fig.5 shows a conjunctival blood-vessel, probably a vein with proliferated basement membrane and pericytes. The perivascular inflammatory cells were also there. Part of a large mononuclear cell, possibly an epitheloid cell was seen just outside the basement membrane and is characterised by the presence of a clear space.

Conjunctival specimens of 4 cases of borderline laprosy were examined. Two of them had slight lagophthalmos and two had no ocular complication. Out of 4 case, 2 cases showed epithelied changes like separation of superficial layers, reduction and deformities of microplicae and microvilli. A large mast cell with mast cell granules was present within the stroma in two cases. Blood-vessels were found be normal and tubular with thickening of the basement membrane. Large vesseles were found. Inflammatory cells were present. Thickening of the perineurium around the axons were found iin two cases. Inflammatory cells were not evident around the nerve in any of the four cases.

15 Conjunctival specimens of lepromatous leprosy and 8

conjunctival specimens of borderline lepromatous leprosy cases had been examined. 7 cases of LL and 5 cases of BL were suffering from old uveitis. Some had corneal opacities following keratitis, 6 cases had lepromatous lesions elsewhere in the body, but the eyes showed no ocular affection. The rest had only mild iritis and/or corneal haze. In most of the cases, Keratinization of the epithelium with degeneration of the nuclei was evident. Reduction and deformation of microvilli was a constant feature. In cases with uveitis - masses of infiltrating cells were prominent. Blood-vessels with thickened wall, inflammatory cells, lymphocytes were also found.

Reduplication of basement membrane and thickening of vessel wall were found both in borderline lepromatous and in lepromatous leprosy. Development of Multiple pinocytic vesicles or the formation of fine vacuoles in the endothelial cells were noted. The absorptive surface of the vessel wall might have been increased by luminal protrusions of endothelial cells or filipodia arising from them. All these features have been observed in very early untreated leprosy as well as long treated lepromatous leprosy. Loosening of endothelial tight junctions were present in almost all cases of lepromatous leprosy. Pericytes were prominent.

Out of 5 conjunctival specimens of non-lepromatous granulomatous diseases 3 cases showed reduction of microvilli and microprojection of the epithelial cells, Inflammation cells within the stroma, thickening of the blood-vessels wall, but no re-duplication. 2 cases showed only separation of superficial epithelial layers, no other changes. Changes in the conjunctival nerves cells were conspicuously absent in all the cases. 8 other conjunctival specimens taken from normal eyes (control) shows no specific changes, excepting some melanocytes at basal layer and supra-basal layer of conjunctival epithelium, specially of the aged persons.

Along with systemic multidrug therapy or Dapsone monotherapy appropriate medicines (topical and systemic) were also prescribed for ocular complications in all cases. In fact, the leprosy patients who had slight or early involvement of the eyes showed mild to remarkable improvement of the clinical conditions as well as of the vision of the eyes while the patients who had gross ocular involvement and gross changes in ultra-structure of the conjunctival tissue showed little or no improvement, even after prolonged treatment.

Discussion :-

Although it is well known that electron microscopy combined with ultra-thin sectioning, has helped

enormously in understanding the inter-relationship between leprosy-bacilli and various kinds of host-tissue cells in leprosy-lesions (Hirata 1990), very little work has been done in this regards, so far ocular tissues are concerned. A few authors have demonstrated the changes in the conjunctival tissues under TEM in other diseases. Some other have done TEM studies of the cutaneous tissues and brain tissues of the patients suffering from leprosy. The reports of those studies, as documented so far in the literature are indeed most surprising. Aquavella et al (1989) showed thickened basement membrane in vessels within conjunctiva and presence of micro-fibrillar material in the extra-cellular spaces of conjunctival stroma in congenital erythropoietic porphyria. In keratoconjunctivitis sicca, Abdel Khalek and others (1978) revealed stratification of conjunctival epithelium with separation of superficial cell layer and reduction and deformation of microvilli. Almost similar changes we have found in the conjunctival epithelium, particularly in tuberculoid and in borderline tuberculoid cases, although pycnotic nuclei were not evident. Conjunctival epithelial changes, like reduction or absence of microvilli, separation of superficial layers were observed in all cases of leprosy with ocular involvement, such as lagophthalmos, uveitis etc. 60% cases of established leprosy without any ocular involvement showed similar changes. About 50% of those cases turned up for follow-up and 80% of them showed ocular involvement within a period of six months.

Golgi-apparatus were relatively decreased in number in all types of leprosy in conjunctival epithelial cells Goggel, Friend and Boruchaff (1985) also found a relative decrease in the number of Golgi-apparatus in corneal dysplasia. The Golgi-apparatus are often referred to as "packaging plant" of the cell because of its role in the formation of zymogen granules. Lesser number of Golgi-apparatus cause decreased metabolic activity of conjunctival and corneal epithelium, lesser glycoprotein synthesis and resultant dryness of the eye.

The conjunctival stroma show affection in most of the cases of leprosy. The regular pattern of the collagen fibrils was found to be lost. Imbibition of water and accumulation of infiltrating cells as a result of opening in the desmosomal tight junctions within epithelial cells might have caused those disruption within the stroma.

The vasculopathy and nerve-affection were common in all types of leprosy. Thickening of vessel wall, multi-layering and proliferation of basement membrane, Irregularly enlarged endothelial cells, accumulation of infiltrating cells, narrowing of the lumen of the vessels were common findings in the blood-vessels of conjunctiva. Dastur and others (1983) were impressed by the Glycoprotein nature

of copiously proliferated vascular basement membrane. That 'reticulin' is the substrate of vascular basement membrane has also been suggested by cervos and others (1975). This basement membrane material could have arisen from the blood itself seeping out, as suggested by Ashton (1974) in the case of retinal arteries in Diabetic Retinopathy. Other sources could be the endothelial cells or the pericytes. Whatever may be source, this basement membrane material appears to be antigenic in nature and

is capable of inviting an antibody-response. That such antibody response could have actually occurred was evidenced by the cells which had infiltrated in and around the vessels, so affected. These cells were lymphocytes, plasma cells, large mono-nuclear cells or macrophages, all of which are known to be immunologically competent in one form or other. The macrophages, moreover, is known to store antigen which

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