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Subclinical changes of oral mucosa in Hansen's disease – a histopathological and immunohistochemical study

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Abstract

Leprosy is a crippling disease with an associated social stigma. The disease is widely prevalent in India, which is one of the endemic areas for the disease. Its oral manifestations though described, is very rare. Twenty established cases of leprosy from various hospitals were taken up in the study. Thirteen were in the lepromatous variety and seven were in the tuberculoid variety. None of the cases had any oral lesions. Biopsies were taken from the buccal mucosa and histopathological sections were studied by Hematoxylin and eosin, Wade-fite and S-100 immunoperoxidase stain. The results showed positivity for *Mycobacterium leprae* in 5 cases of lepromatous leprosy by Wade fit technique. S-100 immunoperoxidase stain revealed nerve changes in the form of fragmentation in cases positive for *Mycobacterium leprae*. These findings show that even in the absence of clinically observable oral lesions, tissue changes in oral tissues do happen in some cases of leprosy and the causative organism *Mycobacterium leprae* can be demonstrated.

Keywords: Oral mucosa; *Mycobacterium leprae*; S-100; Nerve.

Introduction

Leprosy has always been a social stigma. As long as factors such as ignorance, poor living conditions, overcrowding and malnutrition exists, leprosy cannot be eradicated. Though definitive treatment is available, it is necessary to treat the disease before any irreversible permanent mutilating changes occur, so that the stigma associated with the condition may be eliminated from the society (Chacko *et al.*, 1993). To achieve this, it is necessary to institute treatment prior to the appearance of such mutilating changes. Thus, emphasis has been laid on the early diagnosis of leprosy.

Leprosy is caused by a chronic granulomatous infection of the skin and peripheral nerves with *Mycobacterium leprae*. The disease clinically presents in two immunological polar ends as tuberculoid and lepromatous and is a result of variation in the cellular immune response to the mycobacterium (Warwick *et al.*, 2004). The contribution from the dental profession to the knowledge of this disease has been limited.

This study is an attempt to analyze the oral mucosal status in leprosy patients and to visualize the oral mucosa as a diagnostic mirror of this disease entity. With limited information available on the oral mucosal changes in established cases of Hansen's disease, the present study was aimed to assess the tissue changes in clinically normal oral mucosa in such patients.

The study was carried out to evaluate the epithelial and connective tissue changes by H and E stain, the presence or absence of *Mycobacterium leprae* by Wade fit technique and the status of nerve tissue by S-100 immunoperoxidase stain.

Materials and Methods

The study consisted of twenty patients suffering from leprosy for varying periods of time and were under treatment. The patients were from different leprosy centers viz Sacred Heart Leprosy Centre, Kumbakonam (5 patients), German Leprosy Centre, Chennai (1 patient), Silver Jubilee Leprosy Centre, Chennai (2 patients) and Sri Ramachandra Medical College, Chennai (12 patients).

Clinical criteria for selection of leprosy patients

Established cases of leprosy under treatment for not more than 1 year duration. The criteria of 1 year duration of treatment was selected on the basis of the fact that the changes in the oral mucosa should be evident even up to 1 year

duration of treatment and could still demonstrate the organism (Fluery *et al.*, 1987).

The oral mucosa was clinically normal with only skin manifestations. A detailed case history highlighting their habits like smoking, tobacco chewing, alcohol consumption etc, complaints, chief clinical features, type of leprosy, duration of disease and duration of treatment were recorded. The purpose of the study was explained to the patient in his/her mother tongue and an informed consent was procured.

Clinical criteria for selection of control

One healthy person was also included in the study for the histological comparison of buccal mucosa with that of the leprosy patient. The tissue sample was obtained form the mucosal flap overlying an impacted tooth which was prophylactically removed. The control subject had no history of any systemic disease or oral habits.

Sample collection

Incisional biopsy was done under local anesthesia from an area of buccal mucosa after obtaining patients informed consent. The collected specimens were immediately fixed in 10% neutral buffered formalin and tissues were taken to the laboratory for further histopathological examination. Tissues were processed, paraffin sections 4 to 5 micron thickness were cut and routinely stained with hematoxylin and eosin and Wade fite staining. The stained sections were evaluated for the changes in the epithelium and connective tissue and for the presence or absence of *Mycobacterium leprae*.

Immunohistochemistry

Immunohistochemical analysis was performed on 3 micron paraffin embedded tissue sections on gelatin coated glass slides. After heat drying, sections were de-paraffinized in xylene and subsequently rehydrated in gradients of ethanol. Antigen retrieval to unmask antigenic sites was done using 10mM citric acid solution (pH 6) followed by a washing step with Tris buffered saline (TBS pH 7.6). Further incubations with pre-diluted ready to use primary mouse monoclonal antibody anti S-100 (Span India, Mumbai) was performed at 37^{o} C for 30 minutes. Sections were washed again and incubated with biotinylated secondary antibody (DAKO, Carpenteria, CA, USA) for 30 minutes followed by the streptavidin-biotin-peroxidase (DAKO, Carpenteria, CA, USA) for 30 minutes at room temperature. Colored reactions were developed by incubating with 3'3'–diaminobenzidine (0.05 diaminobenzidine in 0.05M Tris buffer, pH 7.6, and 0.01 H_2O_2) and subsequently counterstained with Harris hematoxylin and mounted with DPX (Dysterene Plasticizer Xylene). Positive and negative controls were included in all reactions. Positive controls were paraffin embedded sections from Schwanoma. Negative controls were performed by substituting the primary antibody with non-immune serum. Presence of brown colored reaction localized to the nucleus or cytoplasm was considered as positive reaction. The intensity of the immuno-staining for S-100 was classified as negative, weak or strong in a blinded analysis performed by three independent pathologists.

Statistical analysis

Cases were analyzed based on the clinical and pathologic parameters by SPSS statistical package. Differences in the distribution of age among groups (lepromatous and tuberculoid) were compared using students t test. Statistical analysis of associations between variables was performed by the Fisher's exact test. Comparison of median duration of treatment between positive and negative nerve change was performed using median test. Significance for all the statistical analysis was set at $p < 0.05$.

Results

The study group included a total of 20 patients, 7 patients were of the tuberculoid variety (Figure 1) and 13 patients were of the lepromatous variety (Figure 2 and 3). The majority of patients were males (17 patients), and female patients were 3 in number. The age of the patients was from 20-65 years with a mean of 41 years. Of the total 20 patients, 9 patients were positive for habits like smoking, tobacco etc., 6 in the lepromatous variety and 3 in the tuberculoid variety. The duration of treatment of the study group ranged from 2 days to 24 weeks. None of the patients in the study group showed any clinical involvement in the oral mucosa.

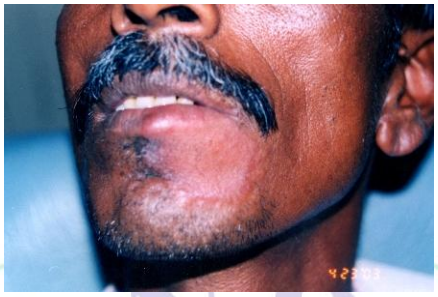

Figure 1: Tuberculoid leprosy involving the vermillion border of the lower lip, with erythema and loss of hair.

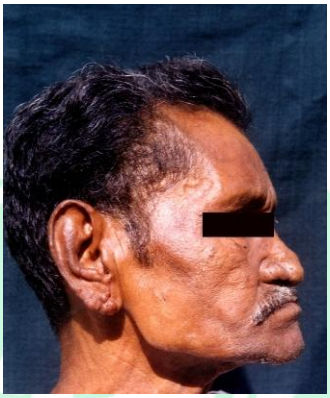

Figure 2: Infiltration of the ear lobes in lepromatous leprosy.

Figure 3: Lepromatous leprosy showing loss of eyebrows, eye lashes and depressed nasal bridge.

Wade-fite technique for *Mycobacterium leprae* demonstrated the presence of bacilli in 5 cases of lepromatous leprosy (Figure 4).

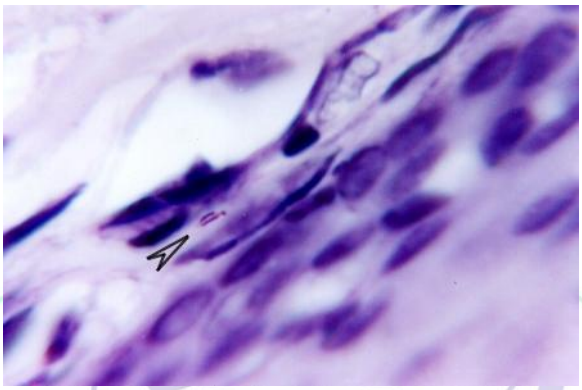

Figure 4: Wade-fite staining positive for *Mycobacterium leprae* (100x).

The hematoxylin and eosin sections were evaluated for epithelial and connective tissue changes. The epithelial changes were evaluated for the nature of keratinization and proliferation of epithelium. The connective tissue changes were assessed for inflammatory infiltrate, blood vessel status and the presence or absence of nerve tissue. The epithelium was non-keratinized in 7 cases, parakeratotic in 6 cases, and hyperplastic in 6 cases. Epithelial atrophy (Figure 5) was seen in one case of lepromatous leprosy. The connective tissue showed a chronic inflammatory infiltrate which was mild in 10 cases, moderate in 8 cases and intense in 2 cases (Figure 6). The proliferation of blood vessels was mild in 9 cases, moderate in 8 cases and intense in 3 cases. H and E sections showed presence of nerve tissue in 15 cases and no nerve tissue in the remaining 5 cases.

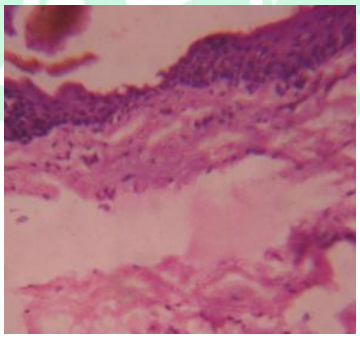

Figure 5: Atrophic epithelium in lepromatous leprosy (H and E stain, 10x).

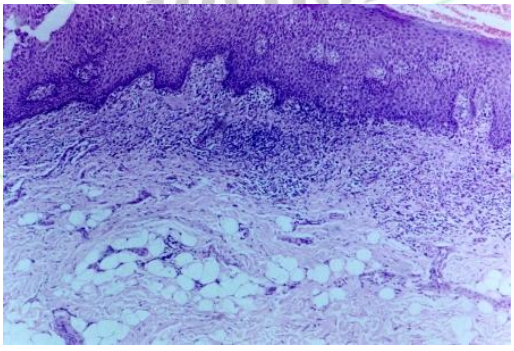

Figure 6: Inflammatory infiltrate of lymphocytes and macrophages in the connective tissue (H and E stain, 10x).

S-100 immunoperoxidase stained slides were evaluated for the presence or absence of nerve tissue and for changes in the form of fragmentation and edema of nerve tissue when present. S-100 immunoperoxidase staining showed nerve tissue in 15 cases (Figure 7) and the remaining 5 cases did not show any nerve tissue. Fragmented presentation of nerve tissue was observed in 6 cases of lepromatous variety (Figure 8) and edema like presentation was observed in one of them. Out of these, 6 cases showing an abnormality in nerve tissue, 5 were positive for *Mycobacterium leprae*. The entire five cases positive for *Mycobacterium leprae*, were under shorter duration of treatment. The solitary case with nerve change, but without *Mycobacterium leprae* was under treatment for 24 weeks which coincidentally was the longest period in the present series.

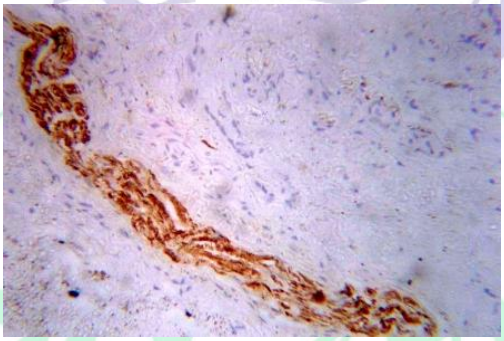

Figure 7: Normal nerve tissue as demonstrated by S-100 immuno-peroxidase staining (10x).

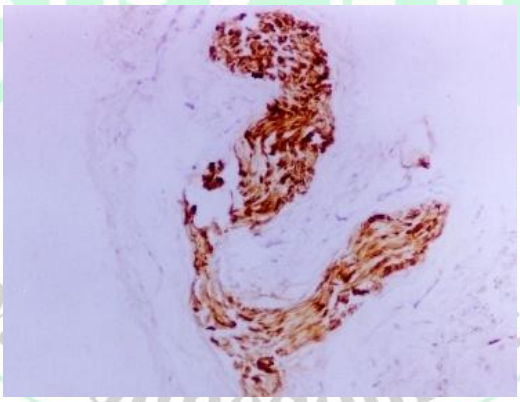

Figure 8: Fragmented nerve as demonstrated by S-100 immuno-peroxidase stain (10x).

The comparison of different characteristics between lepromatous and tuberculoid patients are depicted in Table 1, which includes age, sex, habits, epithelial changes, positivity for *Mycobacterium leprae*, duration of treatment and nerve changes. The nerve changes demonstrated a significant P-value ($P < 0.03$) in favor of lepromatous variety. The nerve changes were positive in 6 cases and negative in 3 cases of lepromatous variety. The tuberculoid variety did not show nerve tissue changes in 6 cases and one case did not show any demonstrable nerve tissue at all.

Table 1: Comparison of different characteristics between lepromatous and tuberculoid patients.

Variables	Lepromatous [n = 13]	Tuberculoid [n = 7]	P - Value
Age (Yrs)	46.8 ± 11.1	30.7± 9.2	0.004 * (Significant)
Sex : Male	11 84%	6 85.7%	1.00 + (Non-significant)
Female	215.4%	1 14.3%	
Habits : Yes	5 38.5%	4 57.1%	0.64 + (Non-significant)
No	861.5%	3 42.9%	
Epithelial change : Yes	8 61.5%	6 85.7%	0.35 + (Non-significant)
No	5 38.5%	1 14.3%	
AFB: Positive	5 38.5%	0 0%	0.11 + (Non-significant)
Negative	8 61.5%	7 100%	
Duration of the treatment Median (Range) weeks	13 (0.3 to 24)	8 (4.0 to 28.0)	0.16 ** (Non-significant)
Nerve change: Yes	966.7%	0 0%	0.03 + (Significant)
No	3 33.3%	6 100%	

* Student's independent t - test was used to calculate the P-value
+ Fisher's Exact test (2-tail) was used to calculate the P-value
** Median test was used to calculate the P-value

The age range also showed a significant P-value ($P < 0.004$) favoring the occurrence of lepromatous variety in the older age group. The Table 2 compares between different characteristics and *Mycobacterium leprae* status in lepromatous patients. The parameters compared include the duration of treatment, epithelial changes and nerve changes. Those patients with lesser duration of treatment demonstrated positivity for *Mycobacterium leprae*. The median and range for duration of treatment for AFB positive cases was 2.0 (0.3 to 12.0) and for AFB negative cases was 14.5 (8.0 to 24.0). The P-value ($P < 0.02$) for duration of treatment was significant. The nerve tissue changes were positive in all the 5 cases showing *Mycobacterium leprae*. In the AFB negative variety only one case showed positive nerve change and 3 cases did not show any nerve change. The nerve changes showed a significant P-value (0.048).

Table 2: Comparison of different characteristics between AFB +ve and AFB –ve patients in lepromatous cases only.

Variables	AFB +ve [n = 5]	AFB –ve [n = 8]	P - Value
Duration of treatment Median (Range) weeks	2.0 (0.3 to 12.0)	14.5 (8.0 to 24.0)	0.02*(Significant)
Epithelial change: Yes	4 80%	4 50%	0.56 +
No	1 20%	4 50%	(Non-significant)
Epithelial change (Excluding habit positive cases) : Yes	3 75%	2 33.3%	0.52 +
No	1 25%	4 66.7%	(Non-significant)
Nerve change : Yes	5 100%	1 25%	0.048 + (Significant)
No	0 0%	3 75%	

* Median test used to calculate the P- value
\+ Fisher's exact test (2-tail) was used to calculate the P- value

Table 3 compares the epithelial changes between tuberculoid and lepromatous variety in habit positive patients only. In the lepromatous variety 3 cases showed epithelial change and 2 cases did not. In the tuberculoid variety 3 cases showed epithelial change and 4 cases did not. These epithelial changes proved to be statistically insignificant.

Table 3: Comparison of epithelial changes between lepromatous and tuberculoid patients in habit positive patients only.

Epithelial changes	Lepromatous [n= 5]	Tuberculoid [n= 4]	P–Value*
YES	2 40%	3 42.9%	1.00 (Non-significant)
NO	3 60%	4 57.1%	

* Fisher's exact test (2-tail) was used to calculate the P-value.

Table 4 compares the median duration of treatment between positive and negative nerve changes which proved to be statistically insignificant. The median and range for nerve tissue positive cases were 2.5 and 0.3 to 28.0 weeks respectively and for those with no nerve change, the values were 12.0 and 2.0 to 28.0 weeks respectively.

Table 4: Comparison of median duration of treatment between positive and negative nerve change (lepromatous cases only).

	Nerve change		P-Value*
	YES (n= 6)	NO (n = 3)	
Duration of treatment Median (Range) weeks	2.5 (0.3 to 28.0)	12.0 (2.0 to 28.0)	0.61 (Non-significant)

* Median test was used to calculate the P-value

Discussion

Oral lesions of leprosy may be overlooked due to lack of awareness of the disease or the presence of subclinical changes in a clinically innocuous oral mucosa. With this outlook, the present study was undertaken to analyze the presence of *Mycobacterium leprae*, the causative organism, and the subsequent microscopic changes in the oral mucosa.

Intra-orally, many a times, areas of mild de-pigmentation, mucosal nodules or papules are visualized by a clinician and are not taken into serious consideration. These presentations may have the remote chances of being manifestations of leprosy. Literature supports the incidence of leprosy in the oral cavity varying from 16 to 60% (Giridhar *et al.*, 1979). The gums, hard and soft palate, uvula and tongue are the commonly reported sites (Sheskin *et al.*, 1973). The tongue generally has nodules giving it a cobble stone appearance. Fissuring of tongue has also been reported as a specific feature of leprosy (Giridhar *et al.*, 1979). Sometimes the nodules of the palate may even ulcerate resulting in palatal perforation (Sharma *et al.*, 1985). Even a simple presentation in the form of chronic periodontitis can be considered as a manifestation of leprosy in the oral cavity (Bombach *et al.*, 1987). The bone loss around the teeth is reported to be more around the maxillary central incisors (Shereef *et al.*, 1992). Intra-orally, altered taste sensation could mark an early diagnosis of leprosy. Loss of taste sensation to sweet, salt and bitter have also been reported (Sheskin *et al.*, 1973).

Extra orally, in the head and neck region, paresthesia, tingling sensation or neurological symptoms could precede leprosy. Neurological manifestations of a preclinical leprosy deserve more attention. Vague subjective symptoms preceding leprosy have been reported (Cochrane *et al.*, 1964). There is also evidence of patients with vague peripheral neurological symptoms developing clinical leprosy at a later period. Reports of cases of leprosy masquerading as trigeminal neuralgia are also available (Mishra *et al.*, 1993). Nerve abscesses of nerves involved in Hansen's disease is well documented. The clinical presentation mimics the benign swellings of the head and neck region. Nerve abscess of the great auricular nerve manifesting itself as a swelling (Desikan *et al.*, 2000), and pure neuritic leprosy manifesting itself as a swelling have been reported (Desikan *et al.*, 2001).

Hypopigmented patches in the head and neck region could be a clinical marker of leprosy. Studies show manifestations of leprosy lesions mainly in the face (Ponnighaus *et al.*, 1990). The associated cardinal signs in the form of anesthesia and loss of sensation are well documented (Bryceson *et al.*, 1990). In the face, however there is a rich innervation by the sensory nerve of the trigeminal nerve and anesthetic lesions over the face are rare. Cases of pre-senile sebaceous gland hyperplasia and yaws have mimicked facial lesions resembling leprosy (Mohamed *et al.*, 2001). Simple habit such as smoking may present itself as a de-pigmentation of the vermilion border of the lip or it may even be attributed to leprosy, where de-pigmentation is an established clinical presentation.

The present study has demonstrated the presence of *Mycobacterium leprae* in clinically uninvolved oral mucosa, only in the lepromatous variety. There are reports of granulomas in the oral cavity and presence of *Mycobacterium leprae* in palatal biopsies with no appreciable clinical involvement (Kumar *et al.*, 1988). There are reports of changes in the epithelium and connective tissue of nasal mucosa which looked relatively normal during clinical inspection (Fokken *et al.*, 1998). Truly, as a disease, leprosy not only involves the skin and nerves but almost all organs in the body. Systemic involvement is more towards the lepromatous end than those towards the tuberculoid pole (Kumar *et al.*, 2000). In this context, in geographic areas, where Hansen's disease is endemic, when oral tissue are biopsied for the diagnosis of any particular disease, the same material may be subjected to also a Wade-fite technique to detect *Mycobacterium leprae*, especially when suspected skin lesions are also present.

In the present study, histopathologically proliferative parakeratotic epithelium has been observed in some patients without any habit like smoking, tobacco or alcohol consumption. Similar hyperparakeratotic epithelium which is proliferative has been reported in patients with clinically involved oral mucosa (Scheeper *et al.*, 1993). This finding possibly suggests that epithelial proliferation may be due to a reaction to the underlying disease rather than to any oral habits. The present study showed more of proliferating epithelium and only one case of lepromatous leprosy revealed an atrophic epithelium.

The present study did not show any features of epithelial dysplasia. The epitheliums of oral lesional biopsies are reported to show dysplasia (Mukherjee *et al.*, 1979) which is again in contrast to skin lesions.

Mycobacterium leprae targeting the Schwann cells of the nerve tissue is well established. *Mycobacterium leprae* is the only known mycobacterium that invades the peripheral nerve (Shetty *et al.*, 2000). *Mycobacterium leprae* specifically bind to the G-domain, which is located at the C-terminal end of α2 chain of basal lamina of peripheral nerve (Rambukkana *et al.*, 1997). *Mycobacterium leprae*/laminin-α2 complexes bind to α/β dystroglycan complexes expressed on the Schwann cell surface (Rumbukkana *et al.*, 1998). Recently, a 21 kDa laminin binding receptor on *Mycobacterium leprae* has been identified, being a Histone–like Protein (HLP) which may function as a critical surface adhesion for the G domain of laminin-α2 (Shimoji *et al.*, 1999).

Major bacterial load was observed in both early lepromatous and tuberculoid nerve lesions suggesting that *Mycobacterium leprae* spreads only via Schwann cells within the nerve (Shetty, 1996). The present study showed positivity for *Mycobacterium leprae* in five cases of lepromatous leprosy. The presence of organism and its established affinity for nerve tissue necessitated to include the nerve status of oral mucosa by S-100 immunoperoxidase staining. Staining of nerve bundle with S-100 protein as a useful tool for the early diagnosis of leprosy has been reported (Barbosa *et al.*, 1994). There are reports of protein bands immunoreactive with leprosy sera in the western blot reacting with anti S-100 protein (Thomas *et al.*, 1990). The role of S-100 stain in the histological diagnosis of tuberculoid leprosy and other granulomatous dermatoses has been evaluated (Singh *et al.*, 1994). It has been reported that immunoperoxidase technique for detection of S-100 protein to visualize peripheral nerves represents an effective auxillary aid in the diagnosis of leprosy (Fluery *et al.*, 1987). Reports of S-100 proteins staining intensely in the lepromatous leprosy patients have been reviewed (Santos *et al.*, 1998).

Thomas *et al.* (2000) have given a classification of dermal nerve involvement in leprosy of lesional skin as intact, infiltrated and fragmented. There are reports of S-100 protein used to highlight nerve elements and to count

their number in leprosy and non-leprosy granulomas (Kahn *et al.*, 1998). S-100 protein detection seems to be a more sensitive and reliable marker for nerve damage and a useful marker for assessing nerve damage in indeterminate patients (Narayan *et al.*, 1997). The S-100 stain is considered to provide additional useful information not available by the examination of H and E stained slide alone (Jain *et al.*, 2000). The present study revealed the presence or absence of nerve tissue by Hematoxylin and Eosin staining. The further staining of the sections with S-100 immuno-peroxidase revealed the nerve tissue status. Those cases positive for *Mycobacterium leprae* invariably showed a broken or fragmented appearance of nerve tissue. Since the patients in the study group are established cases of leprosy, it may be reasonably assumed that this presentation could be fragmentation of nerve tissue secondary to *Mycobacterium leprae* infection. Similar fragmentation of nerve tissue in lesional skin biopsies by S-100 immuno-peroxidase staining has been reported by Thomas *et al.* (2000).

Recent studies have revealed the presence of phenolic glycolipid-1 (PGL-1) unique to *M. leprae* and has been shown to specifically bind to the laminin-α2 chain in vitro (Ng *et al.*, 2000). Specifically, the terminal triglyceride of this molecule has been shown to bind laminin-2. There is further evidence that this bacterial cell wall component may include de-myelination of nerve cells (Rambukkana *et al.*, 2002). Human Schwann cells can process and present *M. leprae* to *M. leprae* specific CD4+ cytotoxic T-cells and are subsequently killed during this event. Particularly during inflammatory CD4+ T-cell mediated reversal reaction, this mechanism may play an important role in causing peripheral nerve damage in leprosy (Eric *et al.*, 2000).

The study group showed an intense inflammatory infiltrate in the tuberculoid leprosy in comparison to the lepromatous leprosy. This may be attributed to the cell mediated immunity which is good in the tuberculoid leprosy in comparison to the lepromatous leprosy.

It may be hypothesized that fragmentation of nerve tissue may occur sub-clinically in the innocuous oral mucosa with *Mycobacterium leprae* positivity. No similar studies of fragmentation of nerve tissue are available in the literature for the oral tissue. This study was done to evaluate the tissue changes of clinically innocuous oral mucosa in established cases of leprosy. However, further studies in the oral mucosa are still required to confirm the present findings.

Conclusion

In conclusion, the findings of the present study show that even in the absence of clinically observable oral lesions, tissue changes in oral tissues do happen in some cases of leprosy and the causative organism *Mycobacterium leprae* can be demonstrated. The present study also supports the role of S-100 stain in revealing the nerve changes in the form of fragmentation in cases positive for *Mycobacterium leprae*.

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