



Immunohistochemical localization of mast cells and mast cell-nerve interactions in oral lichen planus

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OBJECTIVES: Mast cell mediators are likely to be involved in at least some aspects of the immunopathogenesis of oral lichen planus (OLP). The aim of this project was to map mast cell populations in OLP and identify possible sites of mast cell-nerve interactions.

MATERIALS AND METHODS: Monoclonal antibodies specific for tryptase and neurofilaments were used to identify mast cells and nerves respectively in an immunohistochemical study of OLP ($n = 25$) and normal oral buccal mucosa (NOBM) ($n = 13$) using a double-labelling protocol. Data analysis used paired t-test, multiway analysis of variance and Wilcoxon rank tests.

RESULTS: Morphometric analyses showed the greatest mast cell density in the most superficial of the three depth layers examined in OLP, an increase of 130% compared with NOBM. Mast cells associated with neurofilaments ranged from 21.9% in OLP to 10.2% in NOBM. Mean epithelial thickness was significantly lower in OLP ($P < 0.001$) but without a strong correlation with mast cell density.

CONCLUSIONS: Increased mast cell and mast cell-nerve interactions in OLP suggest both a controlling role over the lesional cell populations and a secondary role to the immune response once this becomes established.

Keywords: lichen planus; mast cells; oral mucosa

Introduction

Oral lichen planus (OLP) is a chronic inflammatory disease which is characterized histopathologically by variable epithelial thickness, basal cell destruction and a band-like infiltrate of mononuclear cells in the lamina propria. The pathogenesis of OLP is an area of active investigation, and many studies have shown a key role for cell-mediated immunity (Walsh *et al.*, 1990a). Current knowledge suggests that oral mucosal mast cells may be important in the pathogenesis of OLP, both promoting the development of the lesion and exerting immunoregulatory effects on the established lesion via release of cytokines and other mediators (Walsh *et al.*, 1995).

Several mast cell mediators, such as tumour necrosis factor- α (TNF- α) and the serine proteases, tryptase and chymase, are preformed in cytoplasmic granules (Walsh *et al.*, 1990a; Walsh *et al.*, 1991a), and can be acutely released upon degranulation, with potent effects on nearby cells. For example, mast cell tryptase is thought to be a mitogen for epithelial repair and to facilitate recruitment of granulocytes (Cairns and Walls, 1996), while mast cell chymase can function as an Interleukin-1 β convertase (Kupper *et al.*, 1990) and can damage basement membranes. Mast cell derived TNF- α activates endothelium and induces expression of adhesion molecules which bind leukocytes (Walsh *et al.*, 1991a). TNF- α can also lead to arrested growth or necrosis of epithelial cells (Philip and Epstein, 1986; Walsh *et al.*, 1990a; Sugeran *et al.*, 1996), as well as induction of cytotoxic T lymphocyte differentiation (Robinet *et al.*, 1990), and enhanced cytotoxicity mediated by monocytes and other cell types (Conkling *et al.*, 1988; Owen-Schaub *et al.*, 1988; Ostensen *et al.*, 1987).

It has been suggested that mast cell degranulation in response to release of neuropeptides is a key event in the immunopathogenesis of OLP (Walsh *et al.*, 1990a; Walsh *et al.*, 1995). Mast cells are a mobile cell population within tissues (Walsh *et al.*, 1990b; Walsh *et al.*, 1991b), and the effects of mast cell products will clearly depend on the density of mast cells in the tissues as well as their proximity to structures such as nerves, microvascular endothelium, and epithelium. While the proximity of mast cells to endothelium in OLP has been investigated previously (Walsh *et al.*, 1991b), there is little information on their spatial relationship to nerves in the same disease. Accordingly, the aim of the present study was to investigate the distribution of mast cells in OLP and their proximity to nerves, and to compare this to normal oral buccal mucosa (NOBM). For this purpose, double labelling immunohistochemistry was used to localize mast cells and nerve fibres.

Materials and methods

Biopsy specimens of OLP ($n = 25$) obtained from patients undergoing routine histological confirmation of their condition, and specimens of NOBM ($n = 13$) were all frozen archival specimens obtained during the course of previous studies (Walsh *et al.*, 1990a; Walsh *et al.*, 1995), for which institutional ethics committee approval had been obtained. None of the patients or controls were receiving medications at the time of biopsy, or within the recent past. The

diagnosis of OLP was based upon clinical and histopathological criteria in accordance with the WHO recommendations (WHO, 1978).

All specimens were bisected, with one-half fixed in neutral buffered formalin for routine histology. The other half was stored in Michel's transport medium for subsequent immunohistology. After washing three times in 150 mmol L⁻¹ phosphate buffered saline (PBS, pH 7.2), specimens were embedded in optimal cutting temperature (OCT) medium, frozen in liquid nitrogen and then stored at -70°C until sectioning. Vertically oriented 4 µm thick cryostat sections were cut and collected on poly-L-lysine coated slides. After air drying for 1 h, the sections were fixed in chloroform/acetone (50/50) at 22°C for 5 min, then wrapped in polyethylene film and stored at -20°C until used.

Sections were stained using a three layer immunoperoxidase technique. The protocol used for double labelling was that employed previously (Walsh *et al*, 1991c; Walsh *et al*, 1996). Prior to staining, slides were brought to room temperature and rehydrated in PBS for 10 min. Non-specific protein binding and endogenous peroxidase activity were blocked by incubation in PBS containing 2% swine serum, 0.1% bovine serum albumin and 0.01% sodium azide for 15 min in a humidified chamber. Excess blocking solution was drained off, and sections then incubated with the predetermined optimal dilution of the first layer antibody (monoclonal anti-tryptase, 1:200, Dako Corporation, Carpinteria, CA, USA) for 2 h at room temperature. This was followed by a poly-specific biotinylated immunoglobulin (Dako) as the secondary layer reagent and subsequently with horseradish peroxidase-conjugated streptavidin-biotin peroxidase (Dako) as the third layer reagent. Both incubations were for 15 min, with 10 min washes between each step. The peroxidase reaction was developed for 4 min using a diaminobenzidine (DAB) substrate kit with nickel (Vector Laboratories, Burlingame, CA, USA), to give a black reaction product for mast cell tryptase. The sections were then placed for 15 min in PBS and serum/albumin/azide blocking solution again applied before commencing the second labelling procedure.

Sections were then incubated with anti-neurofilament monoclonal antibody (1:200, Dako) overnight (18 h) at 4°C. The staining procedure for the primary label was then followed, with the exception that DAB alone (i.e. without nickel) was used as the peroxidase substrate. This gave a brown reaction product for neurofilaments. The sections were then counterstained for 5 s in Mayer's acid haematoxylin, dehydrated in graded alcohols, cleared in xylene and mounted.

Negative control sections were processed using an identical protocol to that described above, with replacing either the anti-tryptase or anti-neurofilament monoclonal antibodies with an irrelevant IgG₁ antibody (Dako) as a control. Single labelling was also undertaken as a positive control. Sections of normal skin obtained during previous studies (Walsh *et al*, 1996) were used as additional positive controls for mast cells and neurofilaments.

At least two frozen sections from each specimen were stained with haematoxylin and eosin for survey purposes and for the measurement of epithelial thickness.

In the evaluation of material, two representative sections of each specimen were analyzed using computer assisted morphometry. Sections were viewed on an Olympus BH microscope with an attached charge couple device (CCD) video camera and high resolution video monitor. The video image was acquired using a Macintosh LC630 computer equipped with a frame grabber, and then imported into the public domain software program NIH Image for morphometric analysis. Calibration of the system was undertaken using microscope slides with ruled grids of known dimensions.

The number of mast cells (tryptase positive cells) and the number of mast cell-nerve fiber interactions per unit area were counted using a grid with an area of 0.035 mm², and the data expressed as cells or interactions per mm². A final magnification of × 250 was used throughout for counting cells in three specific areas in each section. Level I (the superficial layer) defined as that immediately beneath the basal cell layer of the epithelium, to a constant depth of 0.22 mm. The deeper layers were immediately subjacent to the superficial layer, and represented the depths of 0.22–0.44 mm and 0.44–0.66 mm for levels II and III, respectively. This subdivision was chosen in an attempt to separate the area in immediate contact with the basal epithelium, and so most likely to contain cells directly or indirectly responsible for apoptosis and the other morphologic features of OLP, from the deeper layer of the lymphohistiocytic infiltrate and in turn the transitional area to submucosa. In a preliminary series of specimens examined, the cited depths covered these criteria. In OLP specimens, the fields selected for analysis were those which contained the most pronounced inflammatory cell infiltrations, while in NOBM, the fields selected for analysis were those showing the highest number of mast cell-nerve fiber interactions.

Cell counts were repeated three times for each area in each section. Only mast cells with clearly evident nuclei were included. Aggregations of externalized mast cell granules not obviously associated with mast cells were excluded from the analysis. For a mast cell-nerve fiber interaction to be counted, the distance between the mast cell and the neurofilament bouton had to be 50 µm or less. For intact (non-degranulated) mast cells, the minimum distance from each mast cell membrane to the membrane of the nearest nerve fiber ending was measured, while with degranulated mast cells the distance used was that from the nucleus of the mast cell to the nerve fiber endings, applying the same 50 µm limit. The percentage of mast cells showing interactions with nerve fibers was then calculated from the total number of mast cells in the same section.

To determine the epithelial thickness, specimens of OLP and NOBM were viewed under lower power (× 25) and the fields captured for morphometric analysis. The maximum thickness (perpendicular distance) from the epithelial surface to the deepest part of the basal cell layer in the epithelium was determined, and the average of four measurements per section used.

All data were collated on an individual patient basis and then group means calculated for OLP and NOBM groups. For comparison between layers and between groups, paired *t*-tests and multiway analysis of variance followed by Wilcoxon rank tests were used, respectively. The SPSS stat-

istics software package was used throughout. Non-linear (Spearman rank) correlations were assessed using the same software. A probability (*P*) value of <0.05 was accepted as an indication of statistical significance.

Results

The distributions and densities of tryptase-positive mast cells in the superficial, middle and deeper layers in OLP (expressed as cells per mm²) compared with those in NOBM are summarized in Figure 1. While mast cells were found in all three designated layers of the lamina propria in OLP, the greatest mast cell density was in the most superficial of the three layers examined. The density in level I was significantly higher than that in both levels II and III (*P* < 0.001), with an increase in the superficial level of the order of 130%. The number of mast cells was not significantly different between the two deeper layers (levels II and III). Accordingly, data from these levels were combined for subsequent analyses.

Mast cells were present in NOBM, but their density was much less than that in OLP. Using global figures for OLP (i.e. combining data for all the three layers), the mean mast cell densities (\pm s.e.m.) for NOBM and OLP were 72.2 ± 13.6 mm² vs 153.8 ± 15.4 mm², respectively (*P* < 0.001).

With regard to mast cell-nerve interactions, in OLP the mean percentage of mast cells which displayed interactions in the different layers is shown in Figure 2. Intragroup comparisons revealed that for OLP, the frequency of these interactions was highest in the most superficial layer (35.4%), with a marked decrease to the deeper layer (8.4%) (*P* < 0.001). Similarly, in NOBM the mean percentage of mast cells associated with neurofilaments also decreased from the superficial to the deeper layer (14.6% vs 7%, *P* < 0.05). Comparing OLP and NOBM, the percentage of mast cells associated with neurofilaments was twice as high in OLP

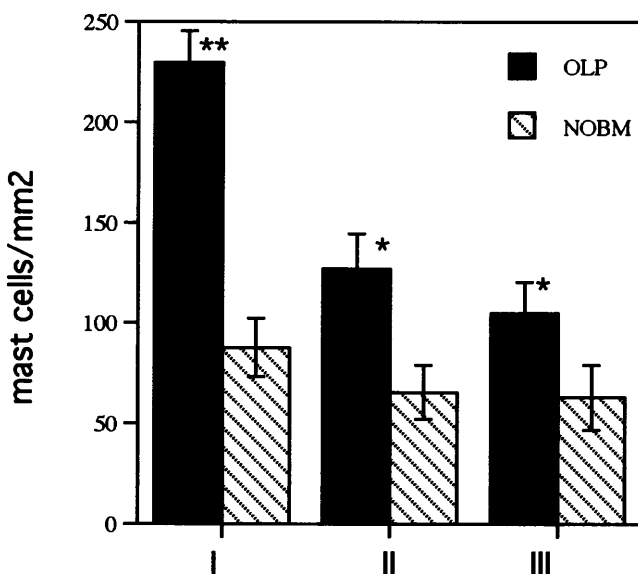


Figure 1 Density of tryptase containing mast cells in OLP (I: superficial, II: middle, III deeper levels) and the same levels in NOBM. Values are means \pm s.e.m. **P* < 0.05, ***P* < 0.001

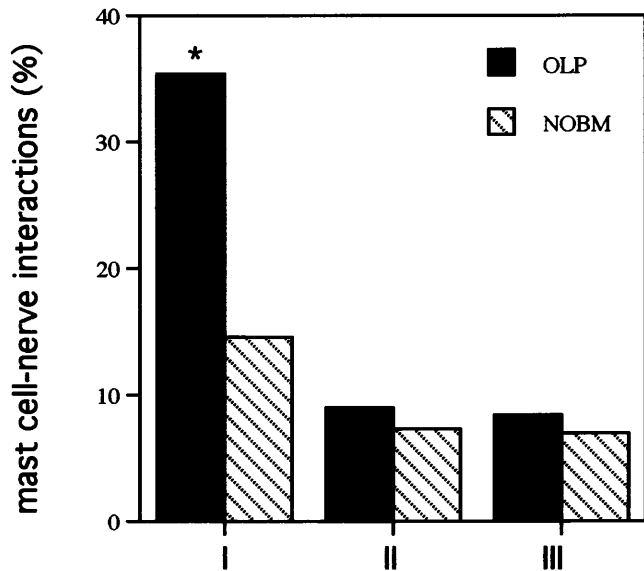


Figure 2 The percentage of mast cells closely apposed to nerves in OLP (*n* = 25) and NOBM (*n* = 13). I: superficial, II: middle, III: deeper levels. There is a significant difference (two-tailed *t*-test, **P* < 0.001) when compared with NOBM in the same layer

compared with NOBM (21.9% and 10.2%, respectively, *P* < 0.001).

Mast cells *per se* as well as mast cell-nerve interactions were most prominent in the most superficial layers, i.e. close to the epithelium, in both NOBM (Figure 3A,B) and OLP (Figure 3C–E). Numerous mast cells adjacent to nerve fibers were degranulated, and intact externalized mast cell granules could be seen (Figure 3F).

Data for mean epithelial thickness in OLP and NOBM are shown in Figure 4. The mean epithelial thickness was significantly lower in OLP than in NOBM (261 ± 15 μ m vs 512 ± 38 μ m, respectively, *P* < 0.001). However, as shown in Table 1, there was not a strong correlation between epithelial thickness and mast cell density. However, there was a moderate correlation between the overall mast cell density and the frequency of mast cell-nerve interactions in OLP.

Discussion

The results of this quantitative immunohistological study reveal a marked increase in both the density of mast cells and proportion of mast cells associated with nerves in OLP compared with NOBM. There is also evidence of a relationship between increased mast cell density in the tissues and reduced epithelial thickness. No patients or donors in the study were receiving systemic medications, nor had OLP lesions been treated with topical steroids within 6 weeks of biopsy. These are important considerations since steroid use can result in both reduced numbers of mast cells (Walsh and Murphy, 1992) as well as changes in the thickness of oral mucosal epithelium.

In the current study, spatial associations of mast cells with nerves were found in both OLP and NOBM, and these were relatively more common in the superficial layers. This pattern is consistent with previous studies in skin (Walsh

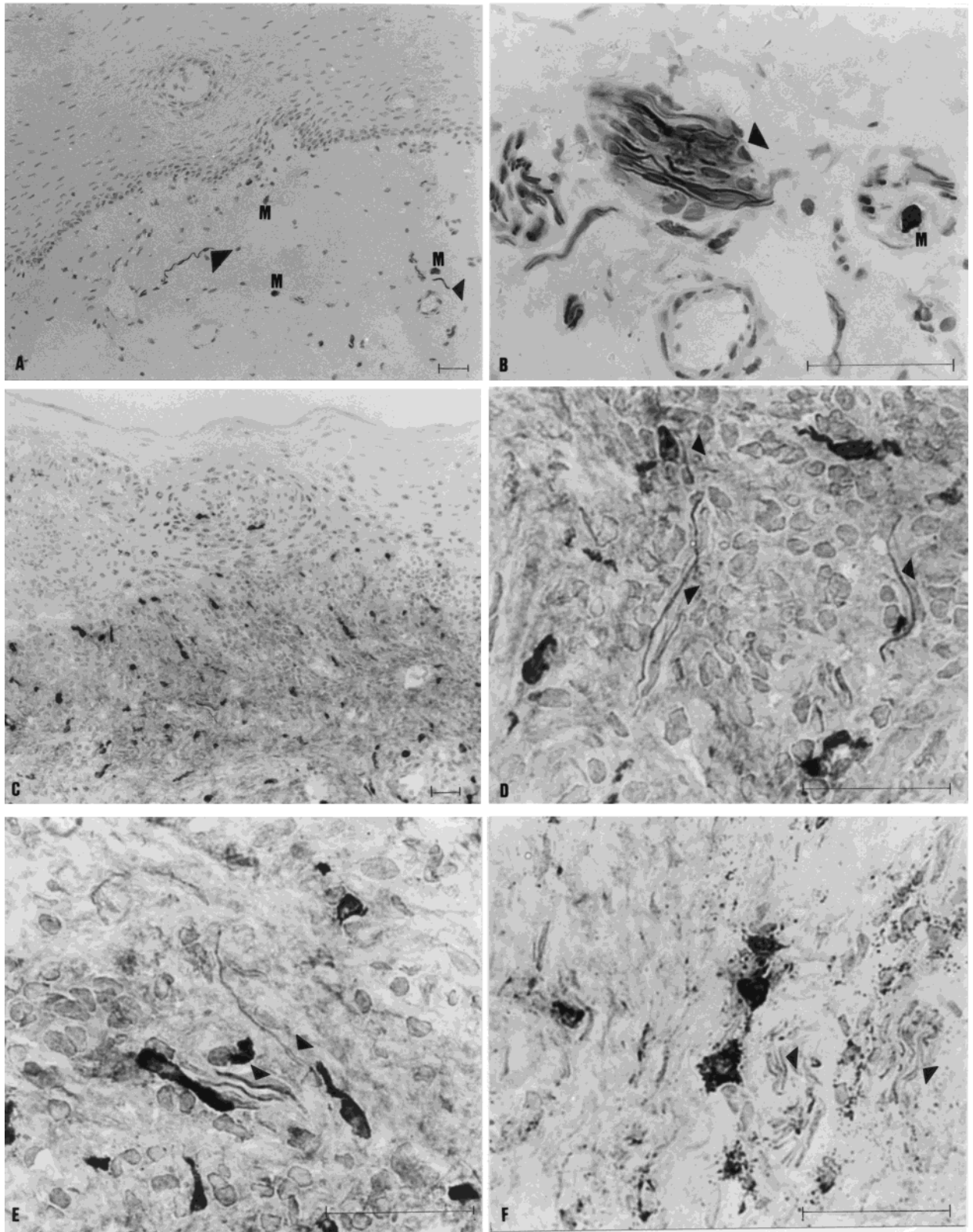


Figure 3 Mast cells and nerves in OLP and NOBM. Frozen sections were double stained for tryptase (mast cells) and neurofilaments (nerve fibers). Scale bars indicate 50 μm . (A) Low power view of normal buccal mucosa. Few mast cells (M) are present. Nerve fibers (arrowheads) can clearly be seen. Original magnification $\times 100$. (B) Higher power view of NOBM, showing nerve fibers (arrowhead) and mast cell (M). Original magnification $\times 400$. (C) Low power view of OLP showing the superficial level. The density of mast cells is increased compared with NOBM (same magnification as panel A). Original magnification $\times 100$. (D) High power view of nerves (indicated by arrowheads) and mast cells in OLP. Original magnification $\times 400$. (E) High power view of nerves (indicated by arrowheads) and mast cells in close proximity in OLP. Original magnification $\times 400$. (F) Mast cells in the deepest layer of OLP (i.e. level III). Numerous externalized granules are present in the region of the mast cells. Nerve fibers are indicated by arrowheads

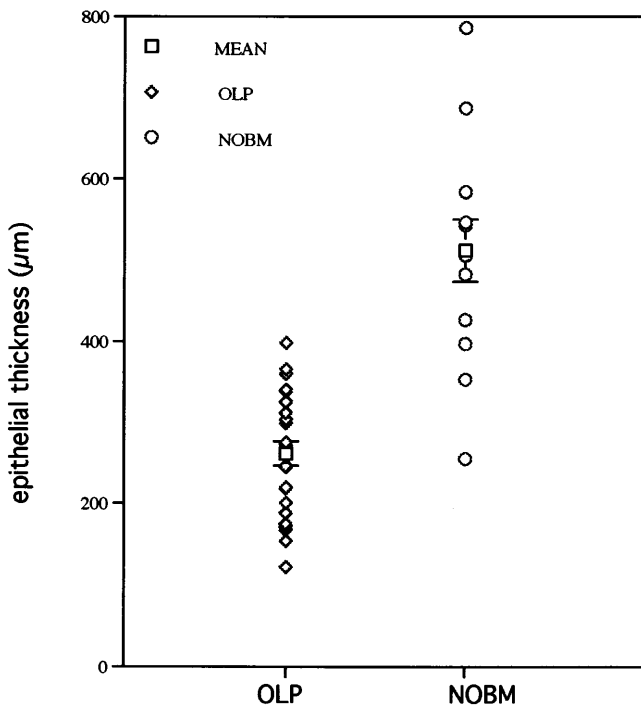


Figure 4 Epithelial thickness in OLP ($n = 25$) and NOBM ($n = 13$). Values are means \pm s.e.m. ($P < 0.001$)

Table 1 Correlations between mast cell density, with the percentage of mast cell-nerve interactions and epithelial thickness in OLP and NOBM

	OLP	NOBM
MD-MN	$r = 0.46$ at $P < 0.05$	$r = 0.48$ at $P < 0.1$
MD-ET	$r = 0.086$ at $P > 0.5$	$r = 0.085$ at $P > 0.5$

MD: mast cell density; MN: mast cell-nerve interactions; ET: epithelial thickness

et al, 1991b) as well as other sites. While a previous report (Stead *et al*, 1989) has documented that in normal human gastrointestinal mucosa, one-half to two-thirds of mast cells are associated with nerves, the proportion appears to be somewhat lower in oral tissues. This may reflect differences in the types of mast cells found in these different sites. There is a higher rate of associations with nerves for mast cells of a 'mucosal' (chymase -, tryptase +) phenotype (as in the gastrointestinal tract), and a lower rate of association with mast cells of a 'connective tissue' (chymase +, tryptase +) phenotype (as in the skin). The oral mucosa, having a mixed population, with a predominance of 'connective tissue' type mast cells (Walsh *et al*, 1990a; Walsh *et al*, 1995), would be expected to show overall an intermediate figure, and this is consistent with the present findings.

Within the oral cavity, the differences between mast cell-nerve associations in NOBM and OLP can be explained by changes in the mobility of mast cells which occur with inflammation. Prior studies have shown that the trafficking pattern of mast cells in OLP is altered compared with normal mucosa (Walsh *et al*, 1991b). The mechanisms by which mast cell precursors are recruited to putative or

established inflammatory lesions is not fully elucidated. It has been suggested that cytokines released by tissue mast cells may indeed be the trigger for the induction of vascular adhesion molecules to allow entry of mast cells to the extravascular compartment (Weiss *et al*, 1995; Sriramarao *et al*, 1996). This issue is being investigated currently by us.

In the present study, mast cell numbers overall were found to be increased in OLP, particularly in the superficial areas of the lamina propria. This is also the area with the highest number of interactions with nerves, as had been predicted (Walsh *et al*, 1990a). In this zone, essentially all mast cells were degranulated, and externalized mast cell granules were found scattered throughout the lamina propria. Key targets for mast cell products include endothelial cells, which can be induced to express adhesion molecules (Walsh *et al*, 1995), and the overlying epithelium. It has been suggested earlier that TNF released from mast cells could lead to reduced epithelial thickness in OLP (Walsh *et al*, 1990a; Sugerma *et al*, 1996), and the results of the morphometric analysis revealed that the mean epithelial thickness was reduced by approximately 50% in OLP compared with NOBM, which is consistent with this. However, a simple correlation between mast cell density and epithelial thickness could not be found, which implicates a more complex relationship involving multiple mediators and/or cell types in the control of epithelial proliferation and destruction in OLP.

The participation of mast cells in a variety of pathologic processes has been related to the nervous system, and there are numerous reports describing such modulation of immunologic responses by the nervous system. The neuropeptides substance P and calcitonin gene-related peptide stimulate mast cell degranulation and cause cytokine release *in vitro* (Cocchiara *et al*, 1995), and such mediators are involved in the profound and on-going degranulation which is a feature of OLP at both the light (Walsh *et al*, 1995) and electron microscope levels (Jontell *et al*, 1986). Mast cells appear to serve as both an 'effector' and 'responder' population in the oral cavity (Walsh *et al*, 1990a; Walsh *et al*, 1995), and as such, the mast cell-nervous system axis may contribute to the pathogenesis of OLP. The current studies support the proposal of involvement of mast cells in all phases of OLP.

With regard to NOBM, in the present study, a spatial relationship between mast cells and nerves was identified, and this was most pronounced in the superficial lamina propria. These findings are in contrast to a recent investigation, which was unable to demonstrate such close associations in normal human buccal mucosa (Ruokonen *et al*, 1993). The most likely explanation for this discrepancy is the use of different techniques to localize mast cells and neural elements. In the present study, a highly sensitive double labelling procedure using monoclonal antibodies was employed, and this allowed simultaneous co-localization of markers without significant background. Moreover, the markers employed were selected because they would allow all mast cells and nerves in the tissues to be detected, regardless of their phenotype and state of degranulation, or content of neuropeptides, respectively.

In conclusion, the present study has provided further evidence for an active role for mast cells in OLP. The finding

that both overall mast cell numbers and the number of mast cells in close apposition to nerve fibers are increased in OLP imply that mast cells may not only be modulating the immune response but also responding to it. Further studies are required to address the latter point.

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