Comparative Study of the Presence of Mast Cells in Periapical Granulomas and Periapical Cysts by Toluidine Blue and Astra Blue: Possible Role of Mast Cells in the Course of Human Periapical Lesions

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Abstract
Periapical lesions represent local immune responses to bacteria and their products emanating from the root canal system. Mast cells are recognized for their immunoregulatory properties. The aim of this study was to examine the expression of mast cells in periapical granulomas and cysts as well as to compare the specificities of toluidine blue and astra blue stains for mast cells. Thirty periapical granulomas and 30 periapical cysts were stained with the basic stains astra blue and toluidine blue. The mean mast cell count using astra blue was 37.13 ± 18.12 in granulomas and 59.5 ± 22.75 in cysts. The mean mast cell count using toluidine blue was 25.90 ± 18.45 in granulomas and 41.27 ± 26.45 in cysts. On comparing mast cell numbers, it was found that cysts contained more mast cells than granulomas. Moreover, astra blue was found to be more specific for mast cells compared to toluidine blue.

Keywords:
cyst, granuloma, immune response

Introduction
The periapical inflammatory lesion is the response of periapical tissues to microbial and chemical stimuli coming from the pulp through the root canal system. These harmful agents include viable bacteria, bacterial disintegration or metabolic products, and altered or necrotic pulpal tissues that are able to elicit an immunologic reaction. Torabinejad and Bakland (1) described periapical lesions as “areas of inflammatory response against the contents of the radicular duct system”.

Perrini and Fonzi (2) demonstrated the presence of numerous mast cells in human periapical granulomas. According to Kontiyanen et al. (3), 2% of cells in periapical granulomas and cysts are mast cells. The role of inflammatory cells present in periapical reactions has been well studied, but the role of mast cells in the development of human periapical inflammatory lesions has been less studied.

As discussed by Rodini et al. (4), mast cells control vascular tone and permeability. These cells also play a role in the immunopathology of immediate and delayed types of hypersensitivity reactions.

Mathiesen (5) reported that the selective and specific identification of mast cells is generally based on the staining properties of the sulfated mucopolysaccharides of the granules and on the activity of the trypsin-like esterase enzyme in the matrix of the granules. For the demonstration of mast cells, metachromatic staining with basic aniline dye, especially toluidine blue, has been widely employed. However, the metachromatic stains have given variable results. Not all mast cells in the mast cell population attain levels of metachromasia characteristic of sulfated compounds: the younger immature cells bind little or no dye.

Bloom and Kelly (6) have shown that the orthochromatic copper phthalocyanin dye astra blue is highly specific for the demonstration of mast cells at low pH. According to Mathiesen (5), astra blue stains
both the weakly and strongly sulfated forms of mast cell granules.

The present study has been undertaken to examine the expression of mast cells in periapical granulomas and periapical cysts to enhance the understanding of the inflammatory phenomena associated with the evolution of periapical lesions. With an increasing knowledge of the modulatory mechanisms of mast cells in periapical lesions, the clinical application of agents that block mast cell secretion will provide a new therapeutic strategy.

Materials and Methods

This histochemical study was carried out in the Department of Oral Pathology and Microbiology, Subharati Dental College, Meerut, India. The study involved 80 patients who were divided into four groups:

Group I: Periapical granulomas (30 patients).
Group II: Periapical cysts (30 patients).
Group III: Chronic tonsillitis--served as positive controls (10 patients).
Group IV: Cleft lip and palate--served as negative controls (10 patients).

Thorough clinical examination was performed for each patient. All relevant details of the history and examination were recorded. Patients were informed about the procedure beforehand and prior consent was duly obtained.

Permission was obtained from the Ear, Nose, and Throat Department, Subharati Medical College, Meerut, India to collect palatine as well as lingual tonsils removed from patients requiring tonsillectomy and from the Department of Oral and Maxillofacial Surgery, Subharati Dental College, Meerut, India to collect tissue samples from patients undergoing surgical correction of cleft lip and palate. Periapical tissue was obtained following apicectomy or extraction of involved teeth. Biopsies taken were fixed using 10% neutral buffered formalin followed by dehydration using ethyl alcohol. Paraffin blocks were made and cut with a rotary microtome. Sections were stained with hematoxylin and eosin and categorized to the particular groups. Specific staining for mast cells was done using astra blue and toluidine blue stains.

Astra blue staining

- 3-μm-thick sections were cut on a microtome and placed on adhesive-coated glass slides.
- Sections were deparaffinized using xylene and passed through decreasing grades of ethyl alcohol (100, 90, 80, 70, and 50%) for 2 s per grade, followed by washing in running tap water for 3 min.
- Sections were covered with astra blue solution (1 g of astra blue powder in 100 ml of 0.7 N HCl–pH 0.3) for 45 min, followed by differentiation in 0.7 N HCl for 1 min.
- After washing with water, counterstaining was done with 0.5% safranin for 2 s.
- Sections washed with tap water and then passed through increasing grades of alcohol for 2 s per grade.
- Clearing in xylene was performed and sections were mounted in synthetic resin.

Mast cells appeared as oval or angular cells with blue granules and red nuclei located mainly in peripheral areas. Background tissue became stained in shades of red and blue (Figs. 1 and 2).

![Fig. 1. Periapical cyst showing mast cells with blue granules and red nuclei (astra blue, 40×).](image_url)
Toluidine blue staining

- 3-μm-thick sections were cut on a microtome and placed on adhesive coated glass slides.
- Sections were deparaffinized using xylene and passed through decreasing grades of ethyl alcohol (100, 90, 80, 70, and 50%) for 2 s per grade, followed by washing in running tap water for 3 min.
- Sections were covered with toluidine blue solution (0.2 g of toluidine blue powder was dissolved in 100 ml of distilled water, pH 4.0) for 1 min followed by washing with tap water.
- Differentiation in 100% ethyl alcohol was performed for 30 s.
- Clearing in xylene was performed and sections were mounted in synthetic resin.

Mast cells appeared as oval or angular cells with purple granules and blue nuclei located mainly in the peripheral areas. Background tissue became stained in shades of blue (Figs. 3 and 4).

Counting procedure

All sections were examined under 40× magnification using an Olympus CX31 binocular light microscope. Images of 10 fields were captured and transferred to a computer where an image analysis system (Image Pro-express) was used to count mast cells using a manual tag. Selecting an icon named “spatial calibration,” 40× calibration was selected to match the magnification of the images; class and color of tags were subsequently selected. Cells were counted by clicking the appropriate tags with the computer mouse. The total number of cells counted in each image (field) was noted (Figs. 5–8). Care was taken to prevent overlapping of fields for both astra blue and toluidine blue stained sections.

Statistical analysis

The t-test was used to assess the significant difference between mast cell numbers in granulomas,
cysts, cleft lip/palate, and tonsilitis as well as the significant difference between specificity of stains (astra blue and toluidine blue) for mast cells. The chi-square test was used to test the association between the age and occurrence of granulomas and cysts. Both tests were performed at a 5% level of significance.

**Results**

*Mas cells in different groups using astra blue and toluidine blue*

Mast cells were seen in all specimens (30 granulomas, 30 cysts) using both astra blue and toluidine blue. Astra blue differentiated mast cells as oval or angular cells containing red nuclei and blue granules filling the cytoplasm and sometimes obscuring each nucleus. Following toluidine blue staining, mast cells appeared as oval or angular cells containing blue nuclei and cytoplasm filled with purple granules. Mast cells were mainly located in areas of inflammation. More mast cells were seen in peripheral areas of both periapical lesions. Both intact and degranulated mast cells were observed. Of the 30 granulomas, 16 were found in males while of the 30 cysts, 19 were found in males.

Using astra blue, the mean numbers of mast cells
in periapical granulomas were 35.44 in males and 39.07 in females, whereas the mean numbers of mast cells in periapical cysts were 56.57 in males and 64.09 in females.

Using toluidine blue, the mean numbers of mast cells in periapical granulomas were 23.62 in males and 28.5 in females, whereas the mean numbers of mast cells in periapical cysts were 33.32 in males and 55 in females.

**Comparison of granulomas and cysts with age**

The chi-square test was applied to compare the occurrence of granulomas and cysts with age. Of the 30 granulomas, 20 occurred in patients ≤30 years of age and 10 occurred in patients >30 years of age. Of the 30 cysts, 13 occurred in patients ≤30 years of age and 17 occurred in patients >30 years of age. The difference was statistically insignificant (p>0.05) (Table 1).

**Comparison of numbers of mast cells in granulomas and cysts using astra blue**

The total number of mast cells in granulomas was compared to that of cysts using astra blue stain. The unpaired t-test was applied. The mean mast cell count was 37.13±18.12 for granulomas and 59.5±22.75 for cysts. This difference was statistically significant (p<0.05) (Table 2).

**Comparison of numbers of mast cells in granulomas and cysts using toluidine blue**

The total number of mast cells in granulomas was compared to that of cysts using toluidine blue stain. The mean mast cell count was 25.90±18.45 for granulomas and 41.27±26.45 for cysts. This difference was statistically significant (p<0.05) (Table 3).

**Comparison of numbers of mast cells between cleft lip/palate samples and granulomas and cleft lip/palate samples and cysts using astra blue and toluidine blue**

The total numbers of mast cells using astra blue were 497 in cleft lip/palate samples, 1114 in granulomas, and 1779 in cysts. The total numbers of mast cells using toluidine blue were 293 in cleft lip/palate samples, 777 in granulomas, and 1238 in cysts. The mast cell count in cleft lip/palate samples was compared to the mast cell count in granulomas and cysts using both stains. The difference was statistically significant (p<0.05) (Table 4).

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Number of cases</th>
<th>( \chi^2 ) (calculated)</th>
<th>( \chi^2 ) (tabulated)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤30</td>
<td>Granulomas</td>
<td>20</td>
<td>13</td>
<td>3.29</td>
</tr>
<tr>
<td>&gt;30</td>
<td>Cysts</td>
<td>10</td>
<td>17</td>
<td></td>
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</tbody>
</table>

Table 2. Comparison of the numbers of mast cells in granulomas and cysts using astra blue

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total number of mast cells</th>
<th>Mean± S.D.</th>
<th>t (calculated)</th>
<th>t (tabulated) 58, 0.05</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulomas</td>
<td>1114</td>
<td>37.13±18.12</td>
<td>4.17</td>
<td></td>
<td>2.02</td>
</tr>
<tr>
<td>Cysts</td>
<td>1779</td>
<td>59.5±22.75</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Comparison of the numbers of mast cells in granulomas and cysts using toluidine blue

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total number of mast cells</th>
<th>Mean± S.D.</th>
<th>t (calculated)</th>
<th>t (tabulated) 58, 0.05</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulomas</td>
<td>777</td>
<td>25.90±18.45</td>
<td>2.60</td>
<td></td>
<td>2.02</td>
</tr>
<tr>
<td>Cysts</td>
<td>1238</td>
<td>41.27±26.45</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Comparison of numbers of mast cells between cleft lip/palate samples and granulomas and cleft lip/palate samples and cysts using astra blue and toluidine blue
Table 5. Comparison of the numbers of mast cells between tonsillitis samples and granulomas and tonsillitis samples and cysts using astra blue and toluidine blue

<table>
<thead>
<tr>
<th>Groups</th>
<th>t (calculated) astra blue</th>
<th>t (calculated) toluidine blue</th>
<th>t (tabulated)</th>
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</thead>
<tbody>
<tr>
<td>Granulomas</td>
<td>8.01</td>
<td>9.74</td>
<td>2.02</td>
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<tr>
<td>Cysts</td>
<td>5.62</td>
<td>8.11</td>
<td>p&lt;0.05</td>
</tr>
</tbody>
</table>

Comparison of numbers of mast cells between tonsillitis samples and granulomas and tonsillitis samples and cysts using astra blue and toluidine blue

The total numbers of mast cells using astra blue were 2814 in tonsillitis samples, 1114 in granulomas, and 1779 in cysts. The total numbers of mast cells using toluidine blue were 1980 in tonsillitis samples, 777 in granulomas, and 1238 in cysts. The mast cell count in tonsillitis was in the same range as the mast cell count in granulomas and cysts using both stains. The difference was statistically significant (p<0.05) (Table 5).

Discussion

Tani-Ishi et al. (7) discussed chronic periapical lesions of pulpal origin as the areas of inflammation in response to noxious agents in the root canal system. Kakehashi et al. (8) demonstrated the causal relationship between bacterial infection of the dental pulp and periapical lesion formation.

Johannessen et al. (9) assumed that antigens, toxins, or other noxious substances from an infected root canal or a necrotic pulp would initiate and maintain inflammation in the periapical area. Such a pathogenic role has been proposed for bacterial endotoxins. The inflammation would manifest itself as a periapical granuloma or cyst. Kontianen (3) and Farber (10) demonstrated that cells infiltrating these lesions are macrophages, neutrophils, lymphocytes, plasma cells, and mast cells.

The mast cell, with its specific membrane receptors, is positioned where potentially noxious materials are likely to enter the body. Because the mast cell is present before the entry of the noxious agent and need not be specifically recruited, it may be the sentinel cell of the local inflammatory response as discussed by Metcalfe et al. (11). The knowledge related to the role of mast cells in the pathogenesis of periapical reactions is very scanty.

The present study was conducted to compare the numbers of mast cells infiltrating periapical granulomas and periapical cysts.

Mast cells were seen in all of the lesions studied. This is in agreement with previous reports of mast cells in periapical lesions given by Stashenko et al. (12). Bhaskar (13) and Yanagisawa (14) showed equal sex distribution for periapical granulomas. In the present study, we have found only a slight difference between males (16 cases, 53.3%) and females (14 cases, 46.6%) for apical granulomas. The age of the patients with apical granulomas varied from 8 to 56 years; most were between the second and fourth decades. Our findings corresponded to those reported by Bhaskar (13) and Yanagisawa (14). Bhaskar (13) found radicular cysts about twice as common in males as females with the greatest incidence in the third decade. Our study showed similar results with cases of radicular cysts almost twice as common in males (19 cases, 63.3%) as in females (11 cases, 36.6%) with the age range of 10 to 60 years. The greatest incidence was seen in the second and third decades.

The mean number of mast cells was greater in females than males irrespective of lesion or stain. These findings agree with those previously reported by Montes et al. (15). Mast cell numbers varied from one biopsy to another. A reasonable mechanism to explain sex differences is a matter for future research. The appearance of the mast cell population depends upon the stage and severity of the disease. Although many mast cells could be present in inflamed regions, other portions within the same tissue section might contain few or no mast cells.

More mast cells were counted using astra blue both in granulomas and cysts as compared to toluidine blue (p<0.05, 0.01), which is in accordance with a study by Mathiesen (5). Mast cells were more numerous in peripheral areas in both granulomas as well as cysts. This finding was similar to that of
Rodini et al. (4). More mast cells were seen in cysts as compared to granulomas using either astra blue or toluidine blue. This finding was similar to that of Rodini et al. (4).

Another finding seen in the present study was the mast cell degranulation. Walsh et al. (16) stated that mast cell degranulation is a common feature of inflammatory lesions. Hook et al. (17) found that the administration of endotoxin in vivo caused degranulation of the mast cells. Studies of Dvorak (18) also provide indirect evidence that human mast cell degranulation is accompanied by a burst of arachidonic acid metabolite oxidation. Some previous studies, including that of Montes et al. (15), have found degranulated mast cells more frequently associated with chronic inflammation. They suggested that mast cells have an active role in these lesions.

According to Mathiesen (5), mast cells in a periapical granuloma may release histamine following a wide variety of injurious stimuli and may act on venules and small veins to increase vascular permeability. Hansen (19) emphasized that mast cells contribute to form the matrix in the connective tissue through the production of hyaluronic acid. Yanagisawa (14) indicated that mast cells promoted the growth of collagen fibers under the effect of heparin. Yanagisawa (14) also stated that mast cells release a substance thought to activate collagenase when degranulation occurs.

These observations indicate that mast cells directly or indirectly control fibrous connective tissue formation in the inflamed tissue.

According to Smith et al. (20), regarding the degranulation of mast cells, the dissociation of heparin proteoglycan may provide a mechanism for activation of the granule-associated proteases. The released enzymes degrade the components of the connective tissue capsule of odontogenic cysts. These released components diffuse into the luminal fluid and contribute to the osmotic pressure. Mast cells have also been suggested to promote collagenolytic activity, facilitate transudation of serum proteins into the luminal fluid, and also promote bone resorption and remodeling to accommodate the growing cyst. Rodini et al. (4) stated that mast cell release of prostaglandin during degranulation may have a role in bone resorption, thus promoting cyst growth.

Mast cells may have a role in the induction of specific immune responses to bacteria. A close relationship between mast cells and the immune system has been shown in studies as discussed by Silva (21). According to Farber (10), both humoral and cell-mediated immunological reactions have been suggested in the pathogenesis of periapical granulomas and cysts. Torabinejad (22) concluded that different classes of immunoglobulins have been found in human periapical lesions such as IgG and IgE. The antigen–antibody complex and IgE–mediated reactions can very well initiate the preliminary changes in the periapical tissues. Cell-mediated immunity is likely to join in the process and participate in the perpetuation and progression of periapical disease.

IgE-containing cells and mast cells were found in inflamed human dental pulp by Pulver et al. (23) and in human radicular granulomas and cysts by Pulver et al. (24). They suggested that antigen/antibody complexes and IgE–mediated reactions participate during the process of inflammation in periapical granulomas. IgE has an unusual capacity to attach itself for long periods to receptors on mast cells. The effect of this reaction is the liberation of histamine and other chemical mediators from mast cells, promoting leukocyte infiltration.

Gao et al. (25) found that dense infiltrates of lymphocytes, HLA–DR positive cells, lysozymes, and α-1 antitrypsin–positive cells were closely related to the proliferated epithelium in periapical granulomas and near the epithelial linings of the cysts. They suggested that immunological reactions may be responsible for the proliferation of the epithelium in these lesions.

Mast cells may subsequently synthesize and secrete additional mediators that are not pre–formed in their granules, including serine proteases, trypsin, chymase, pro–inflammatory cytokines such as IL–1, 3, 4, 5, 6, 8, and TNF–α. IL–4 is likely to influence inflammation in terms of progression from acute to chronic inflammation as discussed by
Montes et al. (15) and Pulver et al. (24). Montes et al. (15) demonstrated that an interaction between mast cells and T lymphocytes exist, suggesting that liberation of histamine from mast cells inhibits the T lymphocyte activity against mitogens or antigens. Dohlsten et al. (26) suggested that histamine prevents IL-2 and gamma interferon production. Frandji et al. (27) described another role of mast cells, that they were able to present antigens to immune cells. Findings of Czarnetzky and Wullenweber (28) showed that mast cells are derived from macrophage-like cells and that they are capable of phagocytosis. All of these finding suggest that mast cells could play a dual regulatory function: to inhibit T lymphocytes and to present the antigen to the immune system. These theories are sustained by the presence of numerous mast cells in inflamed areas. Their results suggest that mast cells could play an important role in the initiation, development, and persistence of the inflammatory process associated with periapical inflammatory lesions.

As discussed by Montes et al. (15), mast cells also contain tryptase and chymase, proteolytic enzymes that take part in the degradation of the extracellular matrix. Tryptase has been shown to activate matrix metalloproteinases 1 and 2, taking part in the break down of proteoglycan of the connective tissue capsule of the cyst. Heparin is also involved in bone resorption and is associated with the inhibition of collagen synthesis. IL-1, IL-6, and TNF-α secreted by mast cells have been shown to increase bone resorption, intensifying the osteoclastic activity. According to Pulver (24), chronic synthesis and release of TNF-α from mast cells may maintain leukocyte migration and promote chronicity in inflammatory lesions. Prostaglandins promote bone resorption to accommodate the growing cyst.

Clinical implications of these findings include strategies directed towards the mast cell. Based on the concept that mast cells play an important role in the chronicity of inflammation, it may be possible to use drugs therapeutically to influence mast cell secretion and thereby thwart inflammation.

**Conclusion**

Immunological reactions against bacteria and/or their products may initiate and maintain inflammation, which leads to the formation of granulomas and cysts. The study has demonstrated greater number of mast cells by astra blue as compared to toluidine blue, which is routinely used. Based on these findings, astra blue can be considered to be more specific for mast cells as compared to toluidine blue. Because astra blue is simple to use, it can be routinely used for the demonstration of mast cells. The mean number of mast cells was found to be greater in females than males. The reason for this difference needs to be further investigated. The present study has also shown a greater number of mast cells in cysts than in granulomas. The presence of degranulated cells suggests the active role of these cells in the pathogenesis of these lesions.

The multiple interactions among mast cells and other cells of the immune system provide a basis for therapies for targeting mast cell responses. It may be possible to develop novel approaches that influence the release of pro-inflammatory molecules or neuropeptides to ameliorate mast cell-driven inflammation.

**References**

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