*International Journal of Paediatric Dentistry* 2006; **16:** 2–9

# Immunocytochemical investigation of immune cells within human primary and permanent tooth pulp

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**Summary.** *Aim.* The aim of this study was to determine whether there are any differences in the number and distribution of immune cells within human primary and permanent tooth pulp, both in health and disease.

*Design.* The research took the form of a quantitative immunocytochemical study.

One hundred and twenty-four mandibular first permanent molars and second primary molars were obtained from children requiring dental extractions under general anaesthesia. Following exodontia, 10-μm-thick frozen pulp sections were processed for indirect immunofluorescence. Triple-labelling regimes were employed using combinations of the following: (1) protein gene product 9·5, a general neuronal marker; (2) leucocyte common antigen (LCA); and (3) *Ulex europaeus* I lectin, a marker of vascular endothelium. Image analysis was then used to determine the percentage area of immunostaining for LCA.

*Results.* Leucocytes were significantly more abundant in the pulp horn and mid-coronal region of intact and carious primary teeth, as compared to permanent teeth ($P < 0{\cdot}05$, ANOVA). Both dentitions demonstrated the presence of well-localized inflammatory cell infiltrates and marked aborization of pulpal nerves in areas of dense leucocyte accumulation.

*Conclusions.* Primary and permanent tooth pulps appear to have a similar potential to mount inflammatory responses to gross caries The management of the compromised primary tooth pulp needs to be reappraised in the light of these findings.

## Introduction

The body's white blood cells (leucocytes) play an essential part in host defence mechanisms against microbial or foreign-body invasion [1]. They may be involved in nonspecific inflammatory responses as well as cell-mediated or humoral immune reactions. A number of different leucocyte subpopulations exist, but there are essentially two main groups: granuloctyes (polymorphonuclear neutrophils, eosinophils and basophils) and mononuclear cells (lymphocytes and monocytes). Lymphocytes are further classified as B cells, T cells or natural killer cells. Each cell population has specific functions and tends to be associated with different stages of the inflammatory process.

Immunocytochemical identification of the different leucocyte subsets relies on their individual expression of specific cell-surface proteins, known as cluster designation (CD) antigens [2]. The leucocyte common antigen (LCA), also known as CD45, is a cell-surface glycoprotein complex that is selectively expressed on all heamatopoetic cells, excluding mature erythroid cells. Thus, the use of an antibody to LCA is generally considered to be a universal marker for all leucocyte cell types [3,4].

A number of histological investigations have described the presence of inflammatory cells within the pulp of carious primary and permanent teeth [5–9]. The initial caries-evoked inflammatory infiltrate principally includes mononuclear cell types including lymphocytes, macrophages and plasma cells. With caries progression and resultant pulpal suppuration, there is a marked increase in neutrophils.

However, it is important to appreciate that immune cell reactions do not occur independently, but interrelate closely with vascular and neural changes. Fundamental to the overall inflammatory process is the initial adhesion of leucocytes to vascular endothelial cells, which must occur prior to

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leucocyte extravasation into the surrounding tissues [10]. Functional interactions also exist between the neural and immune systems, since these two systems are able to communicate bi-directionally via a number of shared signals and receptors [11,12].

To date, histological investigations of caries-induced pulpal changes have been largely limited to the permanent dentition, and there is little understanding of how the primary tooth pulp responds to this disease. Indeed, the commonly held belief that inflammatory reactions are more excessive and poorly localized within the primary tooth pulp appears to be founded on limited subjective observation. Therefore, the overall aim of this study was to investigate the presence of leucocytes within human tooth pulp using an immunocytochemical approach. The specific objectives were to:

- compare the presence of leucocytes in intact and carious primary and permanent teeth;
- explore the anatomical relationships between leucocytes, blood vessels and intradental nerve fibres in both dentitions; and
- determine whether there was any correlation between leucocyte accumulation and reported pain history.

## Materials and methods

The experimental material comprised 62 mandibular second primary molars and 62 first permanent molars, which were obtained from children requiring dental extractions under general anaesthesia. Primary teeth with evidence of root resorption were excluded from the study in view of the known degenerative neural changes which accompany this biological process [13]. Immediately prior to surgery, the child and accompanying guardian were interviewed to ascertain a simple pain history. A positive pain history was recorded either when the child personally reported that she or he had experienced some spontaneous toothache during the previous 48 h or when the parent stated that the child had suffered sleep loss attributed to dental pain over the past two nights. The pain history was thus considered consistent with that of irreversible pulpitis. The South Sheffield Research Ethics Committee, Sheffield, UK, granted ethical approval for the study.

Immediately following simple forceps extraction, a groove was cut on the buccal aspect of each crown and the tooth was split longitudinally. The mesial half of the tooth was retained and placed in fixative (4% paraformaldehyde and 0·2% picric acid in 0·1-M phosphate buffer, pH 7·4) for 24 h at 4 °C. The coronal pulp was carefully removed from the pulp chamber and placed in phosphate-buffered saline (PBS). The degree of occlusal caries in each tooth was then assessed visually under a dissection microscope at × 20 magnification. Each half of the tooth was categorized as intact (no colour change within dentine, but with possible staining confined to enamel), moderately carious (colour changes did not extend beyond half the dentine thickness), or grossly carious (colour changes extended beyond half the dentinal thickness).

The coronal pulps were left in PBS for 24 h at 4 °C before being placed in 0·1-M PBS containing 30% sucrose solution for cryoprotection (5 h at 4 °C). The pulp tissue was then embedded in Tissue-Tek OCT compound (Bayer Diagnostics, Basingstoke, UK) and three, 10-μm longitudinal sections (200 μm apart) were cut from each tooth pulp and collected on poly D-lysine-coated glass slides. Slides were left for 60 min at room temperature to air-dry prior to long-term storage at −70 °C.

### *Immunocytochemistry and lectin histochemistry*

Immunostaining was performed using an indirect immunofluorescence method [14]. Slides were removed as required from storage and were left to air-dry at room temperature for 60 min. Slides were then washed in PBS containing 0·2% Triton X-100 (PBST) (2 × 10 min). To reduce nonspecific background staining and to increase the permeability of cell membranes to antibodies, sections were first incubated in PBST containing 10% normal goat serum (Vector Laboratories, Peterborough, UK) for 30 min at room temperature. Following this, sections were triple-labelled using a mixture of:

**1** a monoclonal antibody to protein gene product 9·5 (PGP 9·5), which is a general neuronal marker (rabbit antihuman PGP 9·5, dilution 1:1000, Ultraclone, Isle of White, UK);

**2** a monoclonal antibody to LCA (mouse antihuman LCA, dilution 1:100, Dako, Buckinghamshire, UK); and

**3** biotinylated *Ulex europaeus* agglutinin I lectin (UEIL) (dilution 20 μg $mL^{-1}$, Vector Laboratories); this lectin, derived from the gorse plant, is specific for α-L-fucose-containing compounds and is considered an excellent marker of human vascular endothelium [15].

The antisera and UEIL were diluted in PBST containing 5% normal goat serum, and sections were incubated for 24 h at 4 °C.

Slides were then washed again in PBS (2 × 10 min) before incubation for a further 90 min at room temperature, with a mixture of fluorescent secondary antibodies. Goat antirabbit IgG conjugated to fluorescein isothiocyanate (dilution 1:20, Vector Laboratories), horse antimouse IgG conjugated to Texas red (dilution 1:100; Vector Laboratories) and 7-amino-4-methyl-3-acetic acid-conjugated streptavidin (dilution 1:25, Vector Laboratories) were employed to visualize PGP 9·5, LCA and UEIL labelling, respectively. The fluorescent labels were diluted in PBST containing 2% normal goat serum. Slides were finally washed again in PBS (2 × 10 min), and sections were carefully dried and mounted in Vectashield (Vector Laboratories).

Immunohistochemical controls for PGP 9·5 and LCA were performed by incubating sections with the nonimmune serum and secondary antibody only. The specificity of the lectin reaction was tested by inhibiting lectin binding. This was accomplished by preincubating the lectin conjugate with 0·2-M α-L-fucose (Vector Laboratories) dissolved in PBS containing 0·2% PBST for 60 min at room temperature prior to applying this mixture to tissue sections. No positive labelling was seen in any of the controls.

### *Analysis of labelling*

Sections were viewed using a Zeiss axioplan fluorescent microscope and all analyses were performed blind. Three different fields were subject to quantitative analysis: the mesio-buccal pulp horn, the buccal subodontoblastic region and the mid-coronal pulp region (containing large neurovascular bundles). Each field was viewed with the × 20 objective and represented 0·22 $mm^2$ of pulp tissue.

The method used to quantify labelling has been described previously [16–18]. Essentially, computer-assisted image analysis software (Image-Pro Plus, Version 3·0; Media Cybernetics, Silver Spring, MD, USA) was used to create a digital image from the microscopic image. The percentage area of staining (PAS) for LCA-labelled tissue was then automatically determined within each field of analysis.

### *Statistical analysis*

A two-way analysis of variance (ANOVA) was employed to test for statistically significant differences for PAS LCA according to the two independent variables: dentition type (primary/permanent) and the degree of caries (none/moderate/gross). Where appropriate, this was followed by Tukey's test for multiple pairwise comparisons of mean values in order to determine whether there were any significant differences between specific subgroups. An independent *t*-test was used to determine whether there was any significant difference in PAS LCA between grossly carious painful and grossly carious asymptomatic samples from either dentition. All statistical analyses were performed on logarithmically transformed ($Log_{10}$) data, but the data are presented graphically in their raw form. The significance levels were set at $P < 0{\cdot}05$.

## Results

A total of 62 primary tooth pulps were analysed, comprising 22 intact, 20 moderately carious and 20 grossly carious samples. Of the grossly carious samples, 10 had reportedly been painful. The mean age (± SD) of patients from whom a primary tooth was obtained was 4·9 ± 0·88 years (range = 3·3–7·0 years). In addition, 62 permanent tooth pulps were also analysed, comprising 19 intact, 22 moderately carious and 21 grossly carious samples (10 of which were painful). The mean age (± SD) of patients from whom a permanent tooth was obtained was 9·7 ± 2·02 years (range = 6·1–14·1 years).

### *Anatomical observations*

Labelling for PGP 9·5, LCA and UEIL provided excellent visualization of the overall pulpal innervation, immune cell population and vascular system, respectively. Detailed observations for pulpal innervation and vasculature have been described previously [16,19], and thus, this paper principally focuses on findings relating to immune cells.

Essentially, two different LCA-labelled cell types were recognizable by their distinctive morphology. The most abundant was an intensely labelled round cell, which was seen throughout the coronal pulp (Fig. 1a). The diameter of this cell was found to be in the region of 6–7 μm and the overall surface area was approximately 50 $\mu m^2$. The second cell type demonstrated paler immunolabelling and was generally localized at the pulp periphery. This cell had an irregular outline with small dendritic extensions and had an estimated surface area of 150–175 μm (Fig. 1b).

In the intact primary tooth pulp, a moderate number of round LCA-immunoreactive (LCA-ir) cells were seen scattered throughout the coronal pulp. However, small, dense clusters of both round and irregular cells were also occasionally seen, particularly in the pulp horn regions (Fig. 1c). In contrast, only a sparse number of round LCA-ir cells were evident within the pulps of intact permanent teeth (Fig. 1d).

There was an obvious increase in the number of both round and irregular LCA-ir cells within carious primary and permanent pulp sections, although round cells were by far the most predominant subpopulation. In some grossly carious samples, an intense accumulation of LCA-ir cells could be seen within the pulp horn region (Fig. 1e). The vast majority of LCA-ir cells were seen to be extravascular, although round LCA-ir cells were occasionally seen within blood vessels.

Close anatomical relationships were observed between PGP 9·5-immunoreactive (PGP 9·5-ir) structures and LCA-ir cells. First, in carious samples showing an increased number of leucocytes, these cells were sometimes localized along a nerve trunk. Also, small, beaded PGP-ir nerve fibres were occasionally seen in close proximity to round LCA-ir cells, and sometimes, the nerve fibres appeared to give off a small branch towards these cells. An interesting anatomical association between neural tissue and leucocytes was observed in areas of dense leucocyte accumulations. In these regions, PGP-ir nerve fibres appeared to form a neural 'barrier' between the LCA-ir cells and the relatively normal pulp tissue (Fig. 1f).

*Quantitative analysis*

It can be seen in Fig. 2 that mean PAS for LCA was significantly greater in primary teeth than in permanent teeth in the pulp horn and mid-coronal region ($P \leq 0{\cdot}005$, ANOVA). However, further pairwise comparisons did not reveal any significant inter-dentition differences for any specific caries subgroup ($P > 0{\cdot}05$, Tukey's test).

Caries had a highly significant effect on mean PAS for LCA in all three fields of analysis, with an overall increase in immunolabelling for LCA with caries progression ($P = 0{\cdot}001$, ANOVA). Further pairwise comparisons revealed that grossly carious permanent samples demonstrated a significantly greater PAS for LCA than corresponding intact or moderately carious samples in all fields of analysis ($P < 0{\cdot}05$, ANOVA)

*Prevalence of leucocytes in relation to pain history*

The mean (± SD, range) PAS for LCA within grossly carious teeth according to the reported pain history is presented in Table 1. It can be seen that, for both dentitions, there was no significant difference in leucocyte accumulation within any field between samples which were reportedly painful or asymptomatic ($P > 0{\cdot}05$, independent sample *t*-test on log-transformed data).

## Discussion

The presence and distribution of inflammatory cells are important parameters in the histopathological

**Table 1.** Mean (± SD, range) percentage area of staining for leucocyte common antigen according to the reported pain history.

| Field and dentition | Pain history | | *P*-value‡ |
|---|---|---|---|
| | Asymptomatic* | Painful† | |
| Pulp horn: | | | |
| primary | 1·92 (± 2·42, 0·10–7·46) | 2·73 (± 4·82, 0·05–15·56) | 0·950 |
| permanent | 1·40 (± 2·78, 0·05–8·53) | 1·63 (± 1·80, 0·10–5·87) | 0·187 |
| Subodontoblastic plexus: | | | |
| primary | 0·26 (± 0·31, 0·03–0·88) | 0·20 (± 0·21, 0·04–0·76) | 0·364 |
| permanent | 1·79 (± 0·82, 0·42–3·24) | 1·21 (± 2·72, 0·02–8·34) | 0·507 |
| Mid-coronal region: | | | |
| primary | 1·38 (± 2·45, 0·09–7·69) | 0·41 (± 0·62, 0·04–1·84) | 0·121 |
| permanent | 0·31 (± 0·51, 0·06–1·77) | 0·52 (± 0·48, 0·13–1·47) | 0·078 |

*$n$ = 10, 11 (primary teeth, permanent teeth).
†$n$ = 10, 10 (primary teeth, permanent teeth).
‡$P$ = two-tail significance, independent sample *t*-test on log-transformed data.

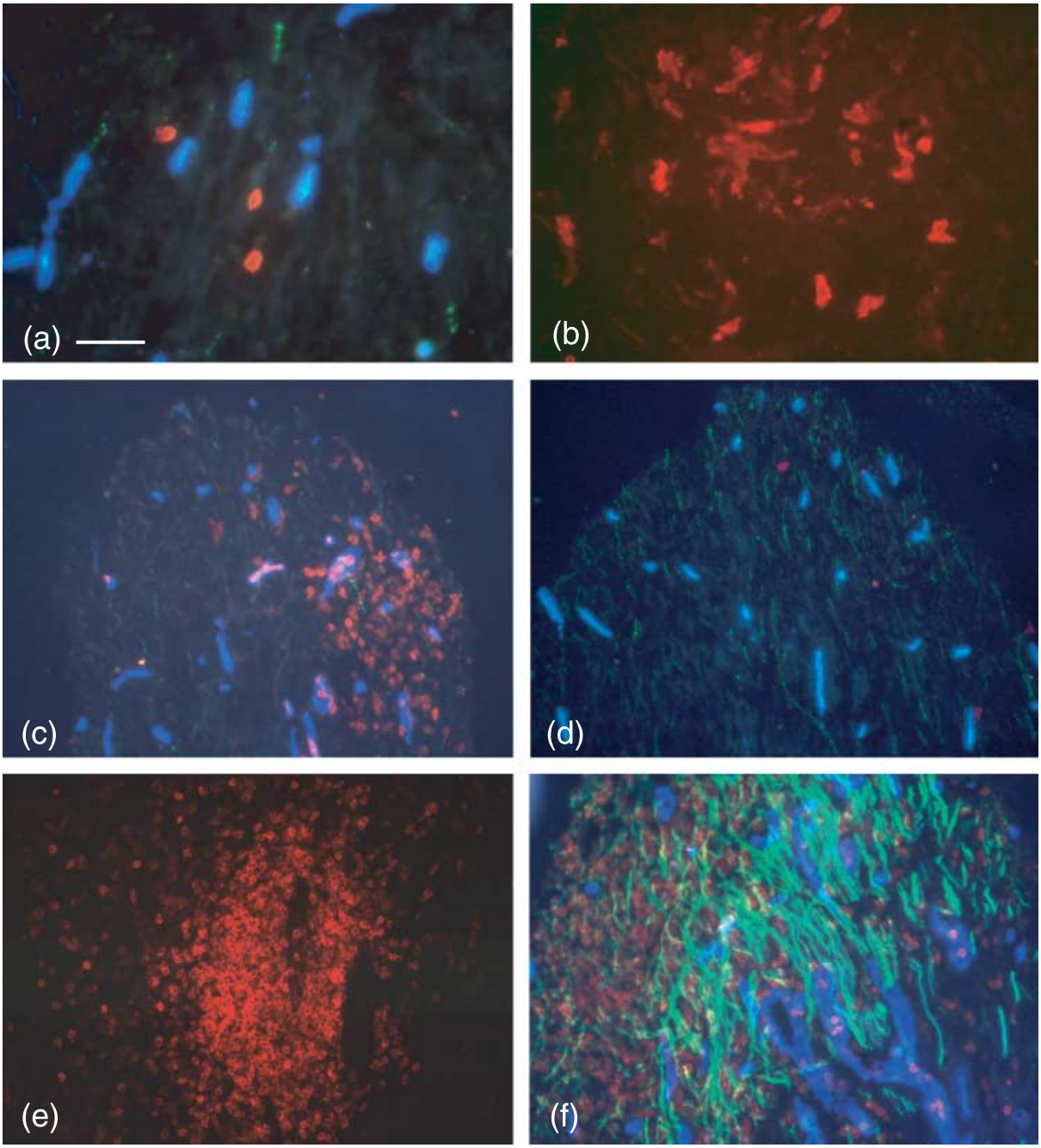


**Fig. 1.** Photomicrographs demonstrating differences in labelling for leucocyte common antigen (LCA) in the coronal pulp of intact and carious primary and permanent teeth: (a) labelling for *Ulex Europaeus* agglutinin I lectin (UEIL) (blue) and LCA (red) in the pulp horn of an intact primary molar showing round LCA-immunoreactive (LCA-ir) cells; (b) labelling for LCA to show larger irregularly shaped cells in the pulp horn region; (c) labelling for UEIL and LCA in the pulp horn region of an intact primary tooth showing a moderate accumulation of immune cells; (d) labelling for UEIL and LCA in the pulp horn region of an intact permanent tooth showing a single immune cell; (e) labelling for LCA in the pulp horn region of a carious primary molar showing a dense accumulation of immune cells; and (f) labelling for protein gene product 9·5 (green), UEIL and LCA in the pulp of a grossly carious primary tooth showing the presence of a neural 'barrier' between areas of dense LCA-ir and relatively normal tissue. [Scale bar = 30 μm (a, b), 60 μm (c, d, e, f).]

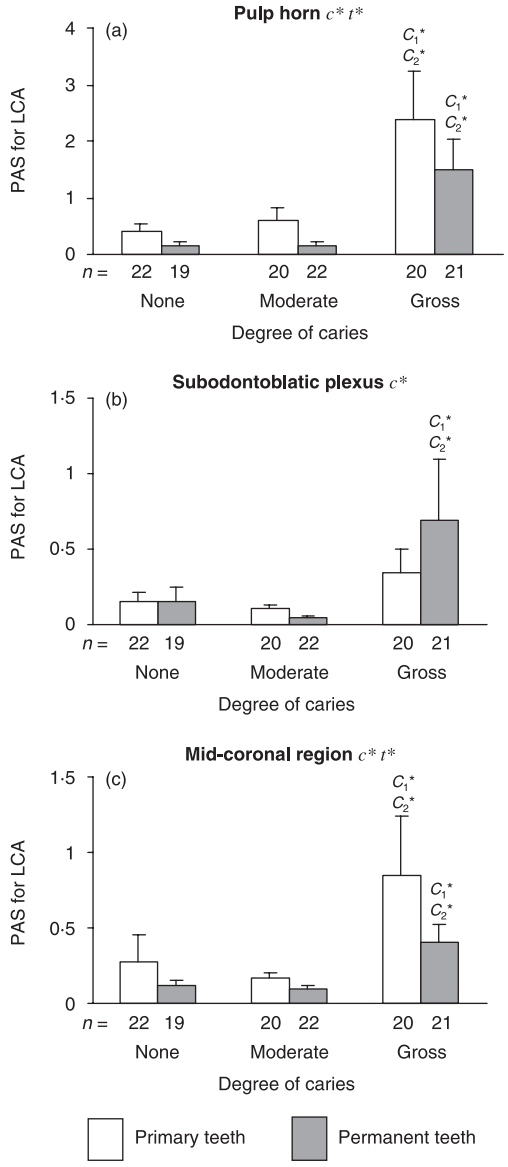


**Fig. 2.** Bar charts showing the mean (± SEM) percentage area of staining (PAS) for leucocyte common antigen (LCA)-labelled tissue for primary and permanent teeth according to the degree of caries: (a) pulp horn; (b) subodontoblatic plexus; and (c) mid-coronal region. Key: ($c^*$) significant difference in mean PAS for LCA according to the degree of caries; ($t^*$) significant difference between the primary and permanent teeth ($P < 0{\cdot}05$, ANOVA); ($c_1^*$) significantly different from intact samples in the same dentition; and ($c_2^*$) significantly different from moderately carious samples in the same dentition ($P < 0{\cdot}05$, Tukey's test).

assessment of pulpal inflammation [20,21]. This study sought to identify the total leucocyte population within intact and carious tooth pulps by employing antiserum to the general leucocyte marker, LCA. Immunolabelling for this antigen permitted good visualization of pulpal leucocytes and revealed the presence of two different subpopulations, distinguishable by their distinct morphologies. The predominant cell type was a small, round cell that demonstrated morphological characteristics consistent with those described for T lymphocytes [22]. The morphology, prevalence and distribution of the irregularly shaped cells would seem to be comparable with those ascribed to macrophages [22,23].

This study has shown that, with the exception of the subodontoblastic region, the primary tooth pulp contains a greater number of immune cells than the permanent tooth pulp. This finding may be attributed to a number of biological factors. First, previous studies have reported the presence of numerous chronic inflammatory cells within the pulps of primary teeth which are undergoing physiological resorption [24]. Although, there was no macroscopic evidence of root resorption affecting this sample, it is possible that subtle biochemical changes were already occurring. In addition, the calcified tissues of primary teeth may be less resistant to the influx of antigenic substances in comparison to their successors. Thus, early immune cell reactions may take place in the primary tooth pulp even in the absence of caries.

Therefore, it is possible, by virtue of the greater number of resident leucocytes present in primary tooth pulp, that inflammatory cell responses may be more rapidly mounted in the primary dentition. However, anatomical observations did not support the theory that primary teeth are unable to localize inflammatory reactions. Carious samples from both dentitions clearly demonstrated evidence of localized inflammatory cell infiltrates which were predominantly restricted to the pulp horn regions.

Both dentitions showed an overall significant increase in pulpal leucocytes with the progression of caries. Immune cell proliferation and migration are fundamental to the body's defence mechanisms and have been well described in the tooth pulp following caries [25,26]. However, there was no significant difference in leucocyte accumulation between asymptomatic and reportedly painful grossly carious samples. This was not an unexpected finding since a number of previous studies have also failed to establish any correlation between patient symptoms and the histopathological status of the pulp [27,28]. Indeed, extensive pulpal invasion by inflammatory cells has frequently been seen in the

absence of any symptoms in both primary and permanent teeth [29,30].

Over the past decade, there has been increasing interest in immune cell and neural interactions, particularly in relation to neurogenic inflammation. The observations made in this study appear to support the existence of close anatomical neural and leucocyte relationships within the dental pulp. The most notable finding was the presence of neural 'barriers' between dense leucocyte accumulations and tissue that was relatively free of LCA-ir cells. A similar spatial relationship has been identified between peptidergic nerve fibres and 'zones of inflammation' in animal models of pulpal inflammation [31].

Several regulatory mechanisms have been proposed to explain this phenomenon. Byers and her colleagues speculated that proliferating fibroblasts, contained within the outer inflammatory zone, exert stimulatory effects on neural tissue via the release of various regulatory factors [32]. Other investigators have suggested that nerve growth factor, synthesized by activated lymphocytes, may play an important role in stimulating axonal growth responses during inflammation [33]. Neuropeptides, which are biologically active proteins transmitted and released by certain nerve subpopulations, are also known to have important immune cell interactions. Substance P (SP), for example, is known to mediate a number of pro-inflammatory responses. It acts as a chemoattractant for mononuclear and polymorphonuclear leucocytes and is able to stimulate human T lymphocyte proliferation via receptor-mediated mechanisms [34]. Previous work has established that there is a significant increase in SP expression in carious teeth [18], and thus, this neuropeptide may play an important role in immune cell regulation and proliferation within the carious tooth pulp.

Clinical management of the compromised primary and permanent tooth pulp is fundamentally different. Traditionally, the cariously exposed, asymptomatic, primary tooth pulp has been subject to a pulpotomy technique whereby the vital coronal pulp is removed and the remaining radicular pulp is fixed using formocresol [35,36]. However, it is interesting to note that indirect pulp treatment has recently gained more recognition as a successful alternative to the formocresol pulpotomy [37,38]. In contrast, every effort is made to maintain the vitality of the compromised permanent tooth pulp with the use of much more biologically compatible pulp-capping techniques. The rationale for adopting such different approaches for the inflamed primary and permanent tooth pulp does not appear to have a sound biological basis. It was evident from this study that both dentitions are able to mount a localized inflammatory response, and thus, should have the same regenerative potential for pulpal healing. However, it would appear that inflammatory cell infiltrates are greater in the primary coronal pulp, and thus, successful outcomes may be more dependent on instigating appropriate treatment at the earliest possible stage.

**What this paper adds**

- The study compares the presence of immune cells within the pulps of intact and carious human primary and permanent teeth.
- The findings are quantitative and novel and thus help to give further insight into the defence and healing potential of carious primary teeth.

**Why this paper is relevant to paediatric dentists**

- It is important that clinicians have a good understanding of pulp biology and pathophysiology, and thus appreciate the potential for healing and repair in both permanent and primary teeth.
- The development of new and more biologically compatible pulp therapy treatments for the primary dentition should be based on a sound understanding of pulp defence mechanisms.

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