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Characterization of antigen-presenting cells in human apical periodontitis lesions by flow cytometry and immunocytochemistry

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Abstract

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Aim To analyse phenotypic characteristics of antigen-presenting cells (APC), isolated from human periapical lesions by flow cytometry and immunocytochemistry.

Methodology Sixteen periapical lesions were digested for 15 min with 0.05% collagenase. Mononuclear cells, separated from other inflammatory cells by density centrifugation, were processed for flow cytometry and/or immunocytochemistry. Single and double immunostainings were performed using monoclonal antibodies specific for human CD45, CD3, CD19, CD14, HLA-DR, CD1a, CD83 and CD123.

Results Antigen-presenting cells (HLA-DR^+ cells) represented 32.9 ± 17.8% of total mononuclear cells. Amongst them, B cells (HLA-DR^+ $CD19^+$) were the predominant APC population, followed by activated macrophages (HLA-DR^+ $CD14^+$), dendritic cells (DC) (HLA-DR^+ $CD14^-$ $CD19^-$ $CD3^-$) and activated T cells (HLA-DR^+ $CD3^+$). Based on the predominance of T cells ($CD3^+$) or B cells and plasma cells ($CD19^+$ and $CD19^{lo}$, respectively) amongst mononuclear cell infiltrates, lesions were divided into T- and B-types. The percentage of DC in T-type lesions (27.1 ± 6.8% of total HLA-DR^+ cells) was higher, compared with B-type lesions (10.3 ± 5.2%) ($P < 0.01$). Within the DC population, the percentages of CD1a (Langerhans cell type) and CD123 (probably plasmacytoid DC type) did not differ significantly between the groups ($P > 0.05$). However, the percentage of mature DC ($CD83^+$) was significantly higher in T-type periapical lesions ($P < 0.05$).

Conclusion Flow cytometry and immunocytochemistry are suitable methods for phenotypic analysis of APC after their isolation from human periapical lesions. APC, that were phenotypically heterogeneous, constituted a significant component of infiltrating cells. Lesions with the predominance of T cells were characterized by a higher proportion of mature DC (HLA-DR^+CD83^+ cells) than lesions with predominance of B cells/plasma cells.

Keywords: antigen-presenting cells, flow cytometry, immunocytochemistry, periapical lesions, phenotype.

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Introduction

Periapical lesions develop in response to chronic irritation of periapical tissue by microbes and their products, usually from an infected root canal. Microbial antigens may stimulate both nonspecific inflammatory reactions and specific immune responses in the periradicular tissue. As a consequence of these processes and the inability of host defense mechanisms to eradicate antigens, periapical lesions such as apical abscesses, granulomas and periapical cysts may be formed, with the aim of restricting microbial invasion (Nair 1997).

Periapical granuloma is a chronic periapical periodontitis consisting of granulomatous tissue with

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infiltrating cells, fibroblasts, small blood vessels and fibrous capsule (Stashenko 1990, Nair 2004). Serial sectioning has shown that many granulomas are epithelialized (Nair *et al.* 1996). Proliferating epithelium forms an irregular epithelial mass in which vascular and infiltrated connective tissue is enclosed. Some chronic periapical lesions develop into cysts. More than half of cystic lesions are true apical cysts containing cavities completely enclosed in epithelial lining. The rest of them are apical pocket cysts with epithelium-lined cavities that open to the root canals (Simon 1980, Nair *et al.* 1996, Nair 2003). In spite of numerous experimental and clinical studies, specific-inducing factors, participating cells and growth mediators associated with maintenance and resolution of periapical lesions are not understood completely (Marton & Kiss 2000, Rodini *et al.* 2004).

Most of the existing data on infiltrating cells within periapical lesions have been derived from immunohistological studies (Stern *et al.* 1981, Cymerman *et al.* 1984, Torabinejad & Kettering 1985, Babal *et al.* 1987, Barkhordar & Desouza 1988, Gao *et al.* 1988, Kopp & Schwarting 1989, Lukić *et al.* 1990, Piattelli *et al.* 1991, Rodini *et al.* 2001, Liapatas *et al.* 2003, Rodini *et al.* 2004). It has been demonstrated that lymphocytes, plasma cells, macrophages and neutrophil granulocytes represent the predominant cells. Other cells, such as mast cells, eosinophils, dendritic cells (DC) and natural killer cells comprise minor, but functionally important cell populations (Kettering & Torabinejad 1993, Marton & Kiss 2000). Few studies on the characterization of inflammatory cells from periapical lesions in suspension have been reported (Stern *et al.* 1981, 1982, Kontiainen *et al.* 1986, Schmitt *et al.* 1989, Marton & Kiss 1993, Yamamoto *et al.* 1997, Fernando *et al.* 2000). Results reported on the number, ratio and distribution of inflammatory cells in periapical lesions have differed and may reflect the histological heterogeneity of different stages in the development and progression of chronic inflammation.

Antigen-presenting cells (APC) such as DC, macrophages and B cells play an important role in activation and function of T cells. Whilst macrophages and B cells activate effector T cells locally, DC are the only APC responsible for activation of naive T cells in regional lymphoid tissues. Published data relating to DC in periapical lesions are limited. Kopp & Schwarting (1989) studied APC (HLA-DR$^+$ cells) in periapical lesions by immunohistochemistry and found a higher number of HLA-DR$^+$ cells in periradicular cysts than in granulomas. Similar cells have been identified within the epithelial wall of different odontogenic cysts, including periradicular cysts, together with T and B cells (Matthews & Browne 1987, Gao *et al.* 1988). Macrophages and DC (CD11$^+$ cells) were found to be more abundant in symptomatic periapical lesions (Matsuo *et al.* 1992). Of the DC in periapical lesions, only Langerhans cells (CD1a$^+$) were identified, mostly in the epithelium (Contos *et al.* 1987, Akhlaghi & Dourov 1995, Suzuki *et al.* 2001). An increase in the number of OX-6$^+$ cells (DC and macrophages) was observed in induced periapical lesions in rat molars (Okiji *et al.* 1994), but it is not known whether they originate from circulating precursors or from dental pulp DC (Jontell *et al.* 1998).

The aim of this study was to assess the phenotypic characteristics of APC, including DC, within the mononuclear cell population isolated from human periapical lesions, as well as to correlate the proportion of DC, DC subsets, T- and B cells with clinical characteristics of the lesions.

Materials and methods

Tissue

Sixteen periapical lesions of pulpal origin were collected with informed consent from patients at the time of tooth extraction ($n = 10$) or apical surgery ($n = 6$). The mean age of patients was 34 years, with a range of 17–60 years. All patients were without systemic diseases, had radiographic evidence of periapical lesions, including periapical alveolar bone loss and had not been treated with antibiotics at least 1 month prior to surgery. Six patients had moderate swelling with or without moderate pain. Periapical lesions of these patients ($n = 6$) were termed symptomatic lesions. Lesions from the remaining patients ($n = 10$) were asymptomatic. The teeth of eight patients (five symptomatic and three asymptomatic) had been root filled. Tooth indices for the symptomatic lesions were 15, 21, 22, 34, 34, 45 and 12, 15 ,21, 22, 24, 24, 32, 32, 34 45 for the asymptomatic lesions. After extraction, the tissue was immediately placed into transport medium consisting of RPMI 1640 medium (Sigma, Munich, Germany) and antibiotics/antifungals.

Preparation of total and mononuclear inflammatory cells

Periapical tissue was placed in a Petri dish containing 1 mL of RPMI medium and minced with a scalpel into 2–3 mm pieces. The tissue was then digested for 15 min

with 0.05% collagenase type IV (Sigma) and 0.02% DNAse (Sigma) in 10 mL of RPMI medium at +37 °C. Soft tissue was then pressed through a stainless steel mesh with a syringe plunger, filtered through nylon gauze to remove tissue fragments and resuspended in 10 mL of fresh RPMI medium containing 1 mmol L^{-1} EDTA (Sigma). Cells were washed twice by centrifugation in RPMI/0.5 mmol L^{-1} EDTA medium at room temperature (400 *g*, 7 min) and counted. Cell viability, as determined by Trypan Blue Dye (Sigma) exclusion, was usually between 90% and 95%. The cell suspension of total inflammatory cells (4 mL) was layered on to a density gradient Lymphoprep (Nycomed, Oslo, Norway) and centrifuged at 800 *g* for 20 min. Mononuclear cells were collected from the interphase, washed twice in RPMI medium containing 2% heat-inactivated foetal calf serum (FCS) (ICN, Costa Mesa, CA, USA) and counted. Viability was usually >97%. From each sample, cytospins were prepared using a cytocentrifuge (MPW-350; MPW Med. Instruments, Warsaw, Poland) on poly-L-lysine-coated glass slides. Cytospins were stained with May Grünwald-Giemsa (Merck, Darmstat, Germany). Cytospins from seven samples were further processed for immunocytochemistry. Mononuclear cells from nine periapical lesions (four symptomatic and five asymptomatic) were processed for flow cytometry.

Monoclonal antibodies

For immunofluorescent staining, the following monoclonal antibodies (mAbs) were used: CD45, CD3, CD19, CD14 and CD83 mAbs conjugated with fluorescein isothyocyanate (FITC) and HLA-DR mAb conjugated with biotin or phycoerythrin (PE) were obtained from Serotec (Oxford, UK). CD1a-FITC mAb was from Caltag Laboratory (Burlingame, CA, USA) whereas CD123-FITC mAb was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). CD45, CD3, CD19 and CD14 unconjugated mAbs were from DAKO (Glostrup, Denmark), whereas CD123 unconjugated mAb was from R & D Systems (Minneapolis, MN, USA). Rabbit antimouse immunoglobulin (Ig) unconjungated, FITC- and peroxidase-conjugated antibodies, as well as alkaline phosphatase antialkaline phosphatase (APAAP) complex, were purchased from DAKO. Streptavidin-alkaline phosphatase was from Serotec.

Immunocytochemistry

Cytospins were fixed with 2% pararosaniline (Sigma) for 2 min at room temperature, washed with phosphate-buffered saline (PBS) (Sigma) for 10 min and incubated with 20% rabbit serum diluted in Tris buffered saline (TBS) (Sigma), PH 7.6 and 0.05% Tween 20 (Sigma) for 20 min. After washing in TBS/0.5% bovine serum albumin (BSA) (Sigma)/0.05% Tween 20, slides were incubated with unconjugated mAbs for 60 min at room temperature in a humidified slide chamber. Cytospins were incubated with rabbit antimouse Ig containing 10% human AB serum, previously inactivated at +56 °C for 45 min, followed by APAAP solution. After each incubation step, slides were washed with TBS/BSA/Tween 20 solution for 10 min. AP reaction was visualized using Fast Red (DAKO) as substrate. Finally, slides were lightly counterstained with haematoxyllin, mounted in Keiser gel and examined by light microscopy.

For double immunofluorescent stains, cytospins, that were fixed and pre-incubated with rabbit serum as described, were incubated with unconjugated CD14, CD19, CD3 or a mixture of these three mAbs, followed by rabbit antimouse Ig conjugated with peroxidase. Revelation of the enzymatic reaction was performed using 0.05% diaminobenzidine (Sigma) and 0.3% H_20_2 (Sigma). After washing in TBS, slides were incubated with normal mouse serum to block unsaturated-binding sites at the rabbit Ig and then overlayered with HLA-DR biotinylated mAb, followed by streptavidin-AP. The reaction was developed using the substrate for AP as described above. For each experiment, adequate controls were performed using a mouse mAb reactive with rat CD4 (Serotec), noncross-reactive with human antigens. All controls gave negative results.

Cytospins were analysed by light microscopy. At least 500 cells were counted on each slide. The percentages of single- or double-positive cells, depending on the staining procedure, were based on total cell counts.

Flow cytometry

Mononuclear cells were resuspended in PBS containing 2% FCS and 0.1% sodium azide and aliquoted into tubes ($1–2 \times 10^5$ cells/tube in 100 μL). For single staining, cells were incubated with appropriate dilutions of FITC-conjugated mAbs (CD3, CD19 and CD14) or HLA-DR PE-conjugated mAb for 45 min at +4 °C. For double staining, cells were incubated with either FITC-conjuagted CD19, CD14, CD3 or their combination, together with HLA-DR PE-conjuagted mAb or FITC-conjugated CD1a, CD83 and CD123 mAbs together with HLA-DR PE-conjuagted mAb. Negative controls were relevant isotype control mAbs

conjugated with FITC and PE (Serotec). After washing twice in PBS/sodium azide, cells were fixed with 1% paraformaldehyde and analysed on an EPICS XL-MCL flow cytometry (Coulter, Krefeld, Germany) using the SYSTEM^{TM} II Software (Coulter). At least 5000 cells were analysed per sample. Results are given as percentages of positive cells in the whole cell population. For double staining analysis, APC cells were gated on HLA-DR$^+$ cells. Percentages of DC subsets are given as proportions of HLA-DR$^+$ cells and total values of DC (HLA-DR$^+$/CD19$^-$CD14$^-$CD3$^-$), previously determined in double labelling experiments as described.

Statistical analysis

Statistical analysis was performed with the nonparametric Mann–Whitney test. Values of $P < 0.05$ were considered statistically significant.

Results

Phenotypic characteristics of mononuclear cells isolated from periapical lesions

Mononuclear cells were isolated from 16 human periapical lesions. The number of isolated cells varied both in symptomatic and asymptomatic lesions and ranged between 1.9×10^5 and 2.8×10^6 per lesion. Cells isolated from nine lesions (four symptomatic and five asymptomatic) were studied by flow cytometry. Seven samples containing lower number of cells (two symptomatic and five asymptomatic lesions) were processed for immunocytochemistry, whereas three samples of asymptomatic lesions were examined using both flow cytometry and immunocytochemistry.

Initially, it was determined whether both methods give comparable results, in the light of other reports (Fernando *et al.* 2000), suggesting that collagenase digestion and vortexing of periodontal tissue during cell isolation could affect the expression of cell surface molecules. As presented in Table 1, only the CD45 expression as detected by flow cytometry was slightly decreased, compared with immunocytochemistry. Consequently, in the subsequent analysis, with the exception of CD45, the results of flow cytometry and immunocytochemistry were pooled. Results for all samples, given in Table 2, show that most cells (81.4 ± 5.7%) were CD45$^+$. Negative cells were small plasma cells, apoptotic cells and fibroblast-like cells. The majority of cells were lymphocytes. T cells (CD3$^+$) constituted 34.3 ± 15.1%, , whereas B cells and plasma cells (CD19$^+$ and CD19^{lo}), respectively, comprised 26.7 ± 15.4% of analysed cells. The percentage of CD14$^+$ macrophages was 16.9 ± 5.8%, whereas the proportion of total APC (HLA-DR$^+$) was 32.9 ± 17.8%. No statistically significant differences were observed between symptomatic and asymptomatic lesions (Table 2).

Table 1 Comparative analysis of phenotypic characteristics of mononuclear cells isolated from periapical lesions studied by flow cytometry and immunocytochemistry

	Periapical lesions					
	1		2		3	
Markers	IC	FC	IC	FC	IC	FC
CD45	86.2	79.1	78.0	70.4	90.1	84.3
CD3	30.2	28.1	22.0	21.3	37.0	39.2
CD19	19.0	17.3	32.4	28.6	18.1	18.4
CD14	16.1	18.2	24.1	22.3	23.6	25.0
HLA-DR	28.1	30.0	36.1	33.4	34.1	35.0

Mononuclear cells were isolated from three different periapical lesions and processed for flow cytometry (FC) and immunocytochemistry (IC) as described in Materials and methods. Cytospins were stained using an alkaline phosphatase antialkaline phosphatase (APAAP) method, whereas cells in suspensions were stained using monoclonal antibodies directly conjugated with fluorescein isothyocyanate (FITC). The mean percentages of positive cells within total population are presented, based on calculation of 500 cells on each cytospin or 5000 cells studied by flow cytometry.

As HLA-DR is expressed on B cells, macrophages, DC and certain activated T cells, a double staining was undertaken to assess the relative contribution of each of these cell in the HLA-DR$^+$ APC population. As seen in Table 2, (average values in all lesions studied), HLA-DR$^+$ macrophages expressing CD14 comprised 8.8 ± 5.9%, and HLA-DR$^+$ CD19$^+$ B cells 18.1 ± 6.8% of total mononuclear cells. Activated T cells (HLA-DR$^+$ CD3$^+$) were a minor population (0.4 ± 0.2% cells). The rest of HLA-DR$^+$ cells, negative for CD19, CD14 and CD3 (3.9 ± 1.8%) are likely DC. The majority of these cells exhibited typical morphology of DC (the presence of dendritic protrusions and processes), but some of them were monocyte-like and plasma cell-like in appearance (Fig. 1). No differences were found in APC subsets between symptomatic and asymptomatic lesions (Table 2).

Characterization of APC in T- and B-type periapical lesions

Based on the predominance of T- or B cells/plasma cells amongst mononuclear cells, periapical lesions

Table 2 Phenotypic characteristics of mononuclear cells isolated from periapical lesions

Markers	% positive cells		
	Total lesions	Symptomatic lesions	Asymptomatic lesions
CD45*	81.4 ± 5.7	82.3 ± 3.6	80.3 ± 6.2
CD3	34.3 ± 15.1	30.1 ± 13.2	36.2 ± 14.1
CD19	26.7 ± 15.4	29.2 ± 14.0	24.4 ± 16.1
CD14	16.9 ± 5.8	15.0 ± 6.2	18.1 ± 4.4
HLA-DR	32.9 ± 17.8	31.0 ± 14.2	34.2 ± 18.0
HLA-DR$^+$/CD14$^+$	8.8 ± 5.9	9.1 ± 4.8	8.3 ± 5.0
HLA-DR$^+$/CD19$^+$	18.1 ± 6.8	19.9 ± 6.0	17.2 ± 7.1
HLA-DR$^+$/CD3$^+$	0.4 ± 0.2	0.4 ± 0.1	0.3 ± 0.2
HLA-DR$^+$/CD14$^-$ CD19$^-$ CD3$^-$	3.9 ± 1.8	3.7 ± 2.0	4.1 ± 1.7

Mononuclear cells were isolated from periapical lesions and processed either for flow cytometry (FC), immunocytochemistry (IC) or both methods as described in Materials and Methods. Cytospins were stained using single (APAAP method) or double immunoenzymatic labelling. Cells in suspension were stained using mAbs directly conjugated with FITC or FITC and phycoerythrin. Values represent mean percentages of positive cells ± SD within total population ($n = 16$), symptomatic lesions ($n = 6$), or asymptomatic lesions ($n = 10$) based on the counting 500 cells on each cytospin or 5000 cells studied by flow cytometry. The differences between asymptomatic and symptomatic lesions were not statistically significant ($P > 0.05$). * = results are from IC studies. Other results are collected and pooled together from FC and IC studies.

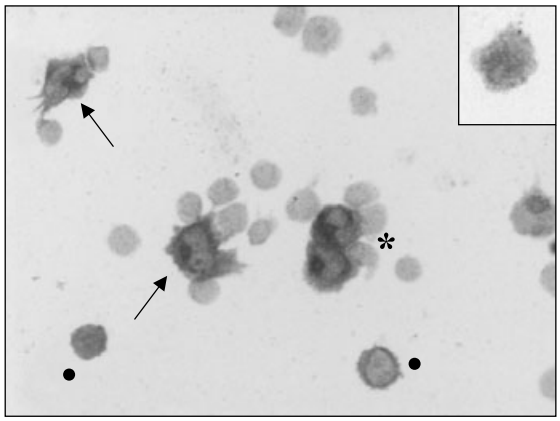

Figure 1 A representative cytospin of a T-type periapical lesion stained with anti-HLA-DR monoclonal antibodies (mAb). Note: dendritic cells (DC)-like positive cells (arrows) macrophage-like cells (asterix), lymphocyte-like cells (black points) and a plasmacytoid DC-like cell (insert). Cells with similar morphology, as presented in the insert, are also CD123$^+$. Magnifications: ×520; insert: ×640.

were divided into T- ($n = 9$) and B-type ($n = 7$) (Figs 2 and 3). The proportion of HLA-DR$^+$ APC did not significantly differ between groups (38.4 ± 18.2% in T type; 30.1 ± 10.2% in B type). However, the contribution of different subsets of APC varied. As presented in Fig. 4, higher percentage of DC in the T-group (27.1 ± 6.8% of total HLA-DR$^+$ cells) vs. 10.3 ± 5.2% in the B-group ($P < 0.01$) was the most notable difference.

Characterization of DC subsets in periapical lesions

Phenotypic characteristics of DC in T- and B-type periapical lesions were analysed based on the expression of CD1a, CD123 and CD83. Results are shown in Figs 5 and 6. The percentages of CD1a (Langerhans-type DC) and CD123 (plasmacytoid type DC) did not significantly differ between groups. In contrast, a higher proportion of mature (CD83$^+$) DC was present in T-type than in B-type lesions ($P < 0.05$).

Discussion

In this study, APC subsets were analysed by immunocytochemistry and flow cytometry after *ex vivo* isolation of inflammatory cells from periapical lesions. Up to now, these methods have not been applied for studying APC in periapical or periodontal lesions. The advantage of using immunohistochemistry is that it enables assessment of the localization of APC and their relationship with other cells and tissue components within the microenvironment; however, for cell quantification a large number of sections is needed. *Ex vivo* study of APC has other advantages as it enables their quantitative phenotypic and functional characterization. Consequently, the best way to study APC in

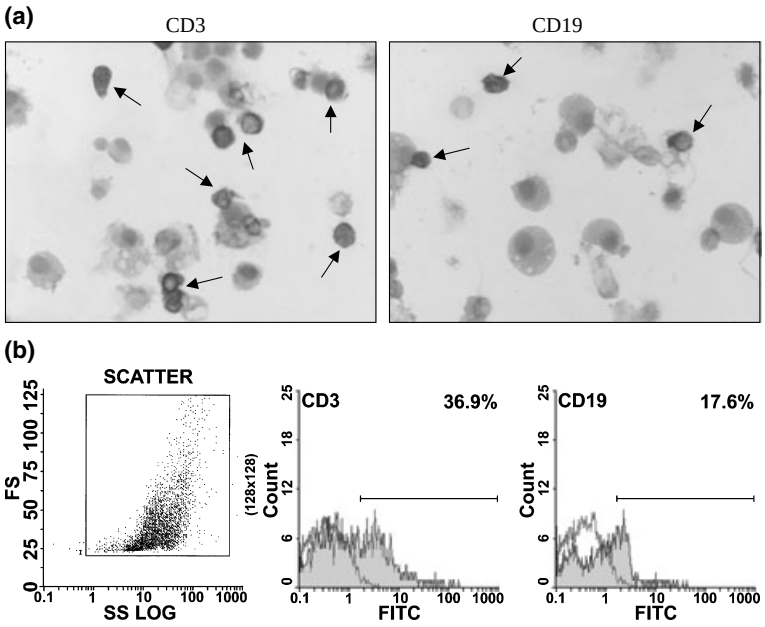

Figure 2 Representative examples of a T-type periapical lesion studied by immunocytochemistry (a) and flow cytometry (b). Mononuclear cells were stained with anti-CD3 and anti-CD19 mAbs as described in Materials and methods. (a) Positive cells detected by the alkaline phosphatase antialkaline phosphatase method can be recognized by dark staining (arrows). Magnifications: ×520; (b) Grey histograms: specific fluorescence, marked by horizontal bars; white histograms: negative (isotype) controls.

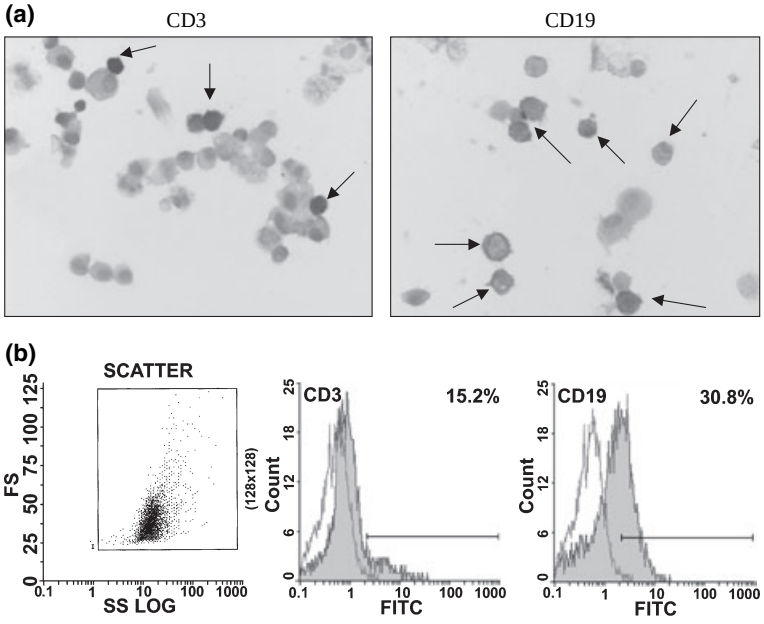

Figure 3 Representative examples of a B-type periapical lesion studied by immunocytochemistry (a) and flow cytometry (b). Mononuclear cells were stained with anti-CD3 and -CD19 mAbs as described in Materials and methods. (a) Positive cells detected by the APAAP method can be recognized by dark staining (arrows). Magnifications: ×520; (b) Grey histograms: specific fluorescence, marked by horizontal bars; white histograms: negative (isotype) controls.

periapical lesions is to combine both methods. However, in the present study, it was not possible to use the same tissue sample for *in situ* staining and *in vitro* isolation of cells, because a large number of cells is required for flow cytometry.

Initially, a protocol for isolation of inflammatory cells was established based on recent data published by Fernando *et al.* (2000), who concluded that flow cytometry was not an acceptable method for analysing leucocytes in periapical lesions, because prolonged enzymatic digestion and vortexing of tissue reduced the expression of cell surface markers. To overcome this problem, a four times lower concentration of collagenase, very mild vortexing and considerably shorter time for tissue digestion were employed. This procedure, which was previously established for isolation of DC from lymphoid organs (Vasilijić *et al.* 2003), enables recovery of up to 95% of inflammatory cells from

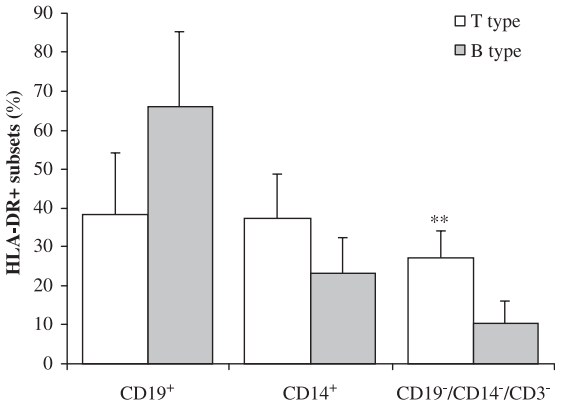

Figure 4 Analysis of different antigen-presenting cells (APC) (HLA-DR$^+$) subsets *in vitro* within isolated mononuclear cells from periapical lesions. Mononuclear cells were isolated from periapical lesions and processed for immunocytochemistry and flow cytometry using double staining as described in Materials and methods. APC were identified as total HLA-DR$^+$ cells. B cells were identified as HLA-DR$^+$CD19$^+$ cells, macrophages as HLA-DR$^+$CD14$^+$cells and DC as HLA-DR$^+$CD3$^-$CD14$^-$CD19$^-$. The proportion of APC subsets was calculated based on the total number of HLA-DR$^+$ cells being 100%. Values represent mean percentages ± SD ($n = 9$, T-type lesions; $n = 7$, B-type lesions). $^{**}P < 0.01$ compared with B-type lesions.

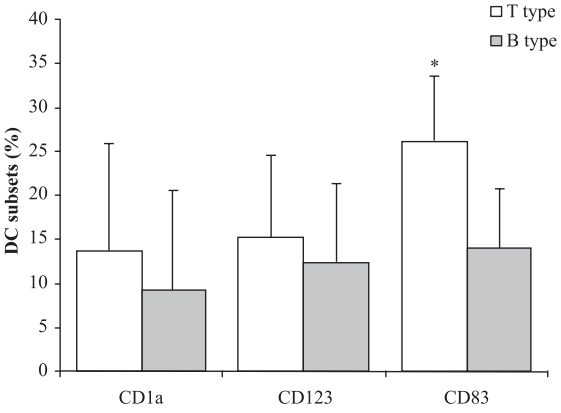

Figure 5 Characterization of DC subsets from periapical lesions. Mononuclear cells isolated from periapical lesions were double stained with anti-HLA-DR-PE mAb together with a cocktail of CD3-, CD14- and CD19 fluorescein isothyocyanate (FITC)-labelled mAbs or with anti-HLA-DR-PE mAb in combination with mAbs reactive with DC subsets (CD1a, CD83 and CD123) as described in Materials and methods. The percentages of total DC (HLA-DR$^+$/CD19$^-$CD14$^-$CD3$^-$) and DC subsets (HLA-DR$^+$CD1a$^+$), HLA-DR$^+$CD83$^+$, HLA-DR$^+$CD123$^+$ were calculated. The percentages of DC subsets were determined using total DC as 100%. Values represent mean percentages ± SD ($n = 9$, T-type lesions; $n = 7$, B-type lesions). $^*P < 0.05$ compared with B-type lesions.

periapical lesions and preservation of cell surface molecules. Only the expression of CD45 (a pan leucocyte antigen) was slightly decreased as studied by flow cytometry, compared with immunostaining of cytospins. For other markers, both methods gave comparable results.

The present study focused on general phenotypic characterization of mononuclear cells isolated from the lesions. The majority of cells were lymphocytes and plasma cells, followed by macrophages. DC represented a minor cell subset. In nine lesions, T cells predominated and in seven lesions B cells and plasma cells outnumbered T cells. Certain cells that had a typical plasma-cell morphology were both CD19$^-$ and CD45$^-$, which may be relevant when analysing B cells and their effectors. Based on the above results, it is possible to divide lesions into predominantly T- or B-cell types.

Different T/B cell ratios have been observed in periapical lesions. Tani *et al.* (1992) reported that the T/B cell ratio in radicular cysts was significantly higher than in radicular granulomas, the opposite was found for macrophages. Others (Akamine *et al.* 1994) suggested that the initiation and progression of lesions was characterized by a predominance of T cells, whereas the humoral immune response mediated by B cells was associated with an advanced stage of lesion development and healing processes.

The aim of this study was characterization of APC (HLA-DR$^+$cells). HLA-DR$^+$ cells consisted of activated macrophages (HLA-DR$^+$ CD14$^+$), B cells (HLA-DR$^+$CD19$^+$) activated T cells (HLA-DR$^+$CD3$^+$) and DC (HLA-DR$^+$ CD14$^-$ CD19$^-$ CD3$^-$). APC at the site of inflammation activate effector and memory T cells and promote both cellular and humoral immune responses (Cutler & Jotwani 2004). Amongst APC, DC are uniquely capable of initiating an adaptive immune response by priming naive T cells in peripheral lymphoid organs to undergo clonal expansion and differentiation into effector cells (Banchereau *et al.* 2000).

Periodontal tissue contains two type of immature DC: Langerhans-type DC (CD1a$^+$) and interstitial type DC (CD1a$^-$). Both types of DC develop from myeloid blood DC precursors after their migration into periodontal tissue (Cutler & Jotwani 2004). It was noted that CD1a$^+$ DC are less frequent than CD1a$^-$. As CD1a$^+$DC are predominately localized within epithelium, especially in radicular cysts (Suzuki *et al.* 2001), the results at present study suggest that the epithelium was not a dominant component in the periapical lesions. The CD1a$^-$ DC population probably belongs to

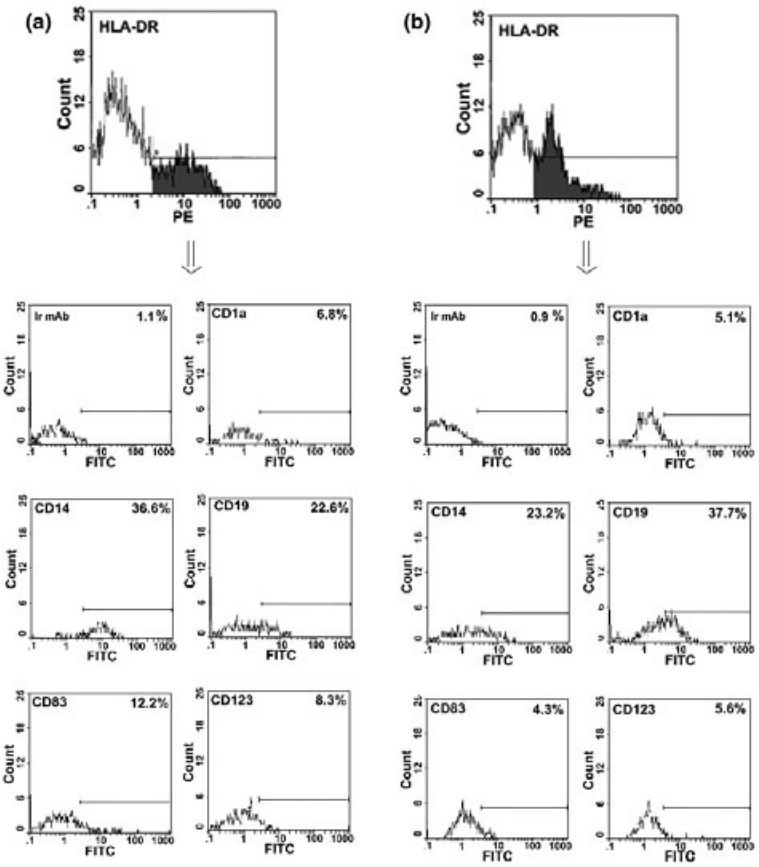

Figure 6 Flow cytometric characterization of HLA-DR$^+$ subsets within mononuclear cell populations of T- and B-type periapical lesions. Cells of a T-type (a) and a B-type (b) periapical lesion were double stained in suspensions, as described in Materials and methods using anti-HLA-DR-PE mAb together with various FITC-labelled mAbs: (irrelevant mAb, CD1a, CD14, CD19, CD83 and CD123). FITC-labelled APC subsets were analysed within PE-labelled HLA-DR$^+$ cells (marked in black). Results are presented as histograms of fluorescence and the percentages of positive cells within HLA-DR$^+$ cells (representing 100%). Horizontal bars represent non-specific staining cut-offs using an isotype control mAb (ir mAb).

both resident and inflammatory DC, localized outside the epithelium.

In apical periodontitis, both CD1a$^+$ and CD1a$^-$ immature DC may capture and process microbial antigens within inflamed tissue and then migrate to the draining lymph node, where, as mature DC, they activate naive T cells and trigger specific T-cell immune responses. This causes clonal expansion of naive T cells and their differentiation into effector and memory T cells. Such T cells migrate to the site of inflammation where, upon reactivation with local APC, they perform different effector functions. In addition, CD1a$^-$ interstitial DC, but not CD1a$^+$ DC, activate naive B cells (Palucka *et al.* 2002). In chronic inflammation, such as periapical periodontitis, a number of DC are retained at the site and undergone local maturation, manifested by the expression of CD83 molecule. Many proinflammatory mediators such as IL-1β, TNF-α, IL-6 and prostaglandin E_2 that are found in periapical lesions (Nair 2004) are well known DC maturation stimuli (Banchereau *et al.* 2000). It was noted that about 10–30% of DC isolated from periapical lesions express CD83, which has not been reported previously. A higher proportion of both total DC and mature CD83$^+$ DC, as observed in T-type lesions, is in accordance with data published for other chronic inflammatory lesions and in line with the recognized association of DC with T cells. In marginal periodontitis lesions, CD83$^+$ DC were also identified predominantly in the CD4$^+$ lymphoid-rich lamina propria (Jotwani *et al.* 2001).

Mature DC in T-type periapical lesions may be responsible for accumulation of T cells, as was previously proposed for DC in marginal periodontitis (Jotwani *et al.* 2001). It is possible that, by production of IL-12, these cells could stimulate the Th1 immune response that is involved in progression of chronic inflammation. However, certain DC subsets may acquire tolerogenic properties at the site of chronic inflammation and down-regulate the inflammatory response through mechanisms involving IL-10 and TGF-β (Vasilijić *et al.* 2005). Indeed, it has been reported previously that a significant increase in TGF-β levels occurs in periapical lesions (Lukić 2000).

A novel finding in this study is the presence of $CD123^+$ DC in periapical lesions. The proportion of these cells varied between samples, but no significant differences were observed in T- vs. B-type lesions. It is postulated that most HLA-DR^+CD123^+ cells are plasmacytoid DC according to their morphology and the phenotype, as CD123 (IL-3R) is the major marker of plasmocytoid DC (Colonna *et al.* 2004). Plasmacytoid DC are present in blood, lymphoid organs and in some lesions of autoimmune diseases such as lupus erythematosus and psoriasis vulgaris (Ronnblom *et al.* 2003, Colonna *et al.* 2004). A low level of CD123 was expressed also in indoleamine 2.3-dioxygenase-positive DC of myeloid origin. These cells are involved in the induction of tolerance and suppression of the immune response (Munn *et al.* 2002, Mellor & Munn 2004). Therefore, the exact nature of $CD123^+$ DC and their functions in periapical lesions remain to be elucidated.

Macrophages comprised 15–30% of mononuclear cells in periapical lesions and 40–70% of them were activated (HLA-DR^+CD14^+) cells. Stern *et al.* (1982) demonstrated that macrophages constituted 30% of total inflammatory cells from human periapical granulomas. Other authors reported equal distribution of $CD68^+$ macrophages in periapical granulomas and periapical cysts that were mainly localized in the inner portion of the lesions (Rodini *et al.* 2001). The presence of macrophages is extremely important for protective responses in periapical lesions, as well as for the development and perpetuation of inflammatory reactions (Ma *et al.* 2003). Based on the results of this study, it can be postulated that, in lesions with a higher number of activated macrophages that function as APC, the Th1 immune response may be stronger. This hypothesis is based on the fact that IFN-γ, a dominant Th1 cell cytokine, is the most potent activator of macrophages (Watford *et al.* 2003).

Conclusion

This study has shown that immunocytochemistry and flow cytometry are suitable for characterization of APC and other leucocytes after their isolation from periapical lesions. The phenotypic heterogeneity of total APC and DC might reflect the complexity of mechanisms involved in the development of different periradicular diseases. Future studies of periapical lesions, classified according to the aetiology or their clinical presentation, including functional characterization of DC subsets, will significantly improve our knowledge of these important immune cells.

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