

The localisation of ED1 antibody in the resorbing hard tissues of the periodontium

C.W. Dreyer

BDS, MDS, FRACDS

Senior Lecturer, Orthodontics

A.M. Pierce

BDS, MDS, OdontDr, FRACDS,
FFOP(RCPA)

Senior Lecturer, Oral Pathology
Division of Health Sciences
School of Dentistry
The University of Adelaide
Australia

A dental hard tissue resorptive model was used to determine the periodontal ligament (PDL) distribution of lysosomal membrane antibody ED1 to cells of the macrophage-phagocyte lineage. Immunolabel was identified in mononuclear cells around inflammatory sites in the PDL, while multinuclear cells were labelled in resorption bays present in both bone and dentine. As repair of the tissues occurred, the label became less obvious. The presence of strong ED1 label in alveolar bone marrow provided evidence supporting the haemopoietic origin of the similarly labelled PDL cells. Also, evidence confirming the current theory that multinucleated resorptive cells differentiate along a monocyte-macrophage pathway was provided. It was concluded that ED1 is a positive PDL marker for mononuclear and multinuclear cells involved in the inflammatory and resorptive processes. Aust Orthod J 2001; 17: 35–40

Introduction

The osteoclast is recognised as the principal cell involved in bone resorption.¹ The regulation of its generation, differentiation and activity is a complex interplay between calcium-regulating hormones, growth factors, cytokines and regulatory molecules, and receptors that act through osteoblast/stromal cell mediators on haemopoietic stem cells.^{2, 3} Although the histological identification and localisation of resorptive cells has often relied on their multinuclear appearance at the light microscopic level,⁴ and their characteristic ultrastructural features of cellular polarisation, ruffled borders and clear cytoplasmic zones,⁵ their precursor cells have been difficult to identify. To reveal clast cells and their precursors, reactivity with tartrate-resistant acid phosphatase,⁶ neutral red vital staining⁷ and succinic dehydrogenase activity⁸ have all been employed with limited success because of technical difficulties and uncertain specificity. Hattersley and Chambers⁹ reported that multinuclearity and tartrate-resistant acid phosphatase (TRAP) reactivity as morphologic and enzyme histochemical markers, respectively, were unreliable as indicators of osteoclast activity in tissue culture. The amount of induced experimental bone resorption quantitatively exceeded the number of multinuclear cells present, thereby confusing the identity and the nature of the resorptive elements.⁹

Cell identification problems have recently been enhanced by the use of immunological techniques¹⁰ utilising antibodies prepared against tissue or cellular antigens. Athanasou¹¹ identified a number of antibodies that were useful in revealing antigens on several structural protein components of active human osteoclasts in routinely processed formalin-fixed paraffin-embedded tissues. As the origin of multinucleated resorptive cells is reported

Legend for figures

- AB: Alveolar bone
 Ank: Ankylosis
 D: Dentine
 Inf: Inflammation
 M: Bone marrow
 Od: Odontoclast
 Oc: Osteoclast
 PDL: Periodontal ligament
 RR: Repairing resorption

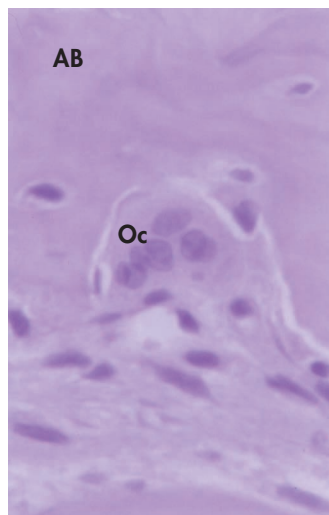


Figure 1. Photomicrograph of an osteoclast in a resorption lacuna within inter-radicular alveolar bone. H&E, original magnification x 150.

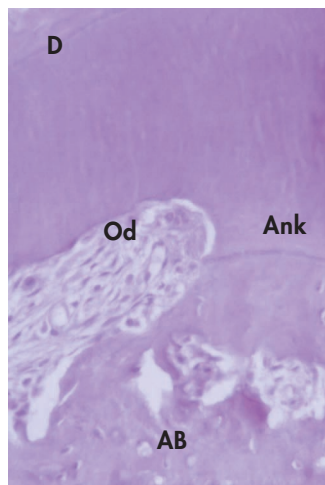


Figure 2. Photomicrograph of a dental resorption lacuna containing an odontoclast adjacent to an area of inter-radicular ankylosis. H&E, original magnification x 100.

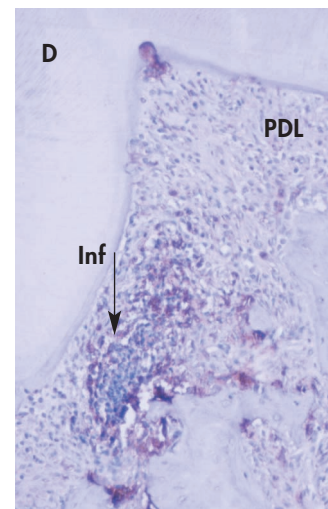


Figure 3. Photomicrograph of periodontal ligament revealing brown staining ED1 activity associated with inflammation (arrow). H&E, magnification x 50.

to be from a monocyte-phagocyte lineage,¹² antigens derived from the macrophage cell line may also be useful indicators of resorptive cell activity. Although a number of macrophage immunolabels were assessed by Athanasou,¹¹ not all were capable of labelling resorptive cells. However, Jager *et al.*¹³ reported that ED1 (a monoclonal antibody against macrophage lysosomal membranes) was a useful lysosomal marker for cells of the mononuclear phagocyte lineage and it was, therefore, considered that ED1 might be valuable in labelling resorptive cells in the periodontal ligament.

Aim

The aim of this study was to determine the periodontal ligament distribution of ED1.¹⁴ It was expected that the detection of ED1 would provide information on the differentiation pathway of monocytes/macrophages and osteoclasts, and, further, that it would reveal possible mechanisms for the control of resorptive cells.

Materials and methods

Root resorption was induced in a homogeneous population of thirty-two, eight-week old, anaesthetised, male Sprague-Dawley rats by the continuous application of dry ice to the crown of the upper right first molar for periods ranging from 10 to 20 minutes.¹⁵ The upper left first molar was left unfrozen and served as a tissue

control. Animals were sacrificed 7 days after the experimental procedure by carotid perfusion of 20 mL of Zamboni's fixative¹⁶ containing paraformaldehyde and picric acid. Maxillae were dissected and placed in the same fixative for 24 hours. Excess picric acid was removed using 70 per cent alcohol washes before specimens were decalcified in 10 per cent ethylenediaminetetra-acetic acid (EDTA) at 4°C. Decalcification was determined radiographically, following which tissues were dehydrated in graded alcohols and embedded in paraffin before being processed for immunological labelling utilising the avidin-biotin peroxidase complex (ABC) technique. In order to block endogenous peroxidase activity, 5µm cut serial sections on silane-coated glass slides were dewaxed and placed in a solution of 0.5 per cent H₂O₂ in methanol. Trypsin digestion of sections for 3 minutes at 37°C was used as an antigen-unmasking procedure, prior to incubation in normal horse serum for 30 minutes. After washing, the slides were incubated overnight with ED1 mouse anti-rat primary antibody at a concentration of 1 mg/mL diluted 1:50 in phosphate-buffered saline. Successive incubations in biotinylated linking or secondary anti-mouse antibody (30 min) and streptavidin peroxidase (1 hour) occurred prior to the application of diaminobenzidine (DAB) as a peroxidase substrate. The slides were washed between each incubation, before a light counterstaining with Mayer's haematoxylin and mounting in aquatex. Negative immuno-

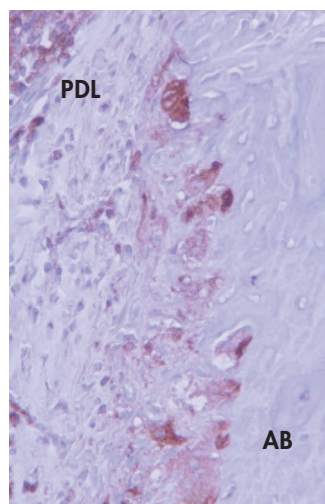


Figure 4. Photomicrograph of resorptive alveolar bone surface showing immunolabelling of resorptive cells. H&E, original magnification x 100.

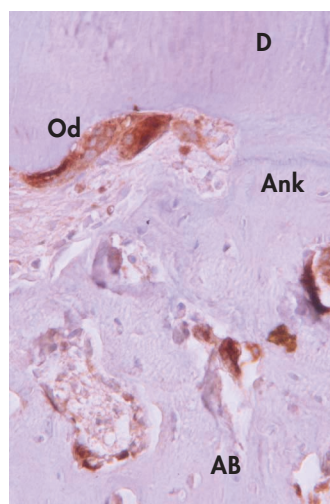


Figure 5. Photomicrograph of immunolabelled odontoclast adjacent to ankyrotic area. Compare Figure 2. Haematoxylin, original magnified x 100.

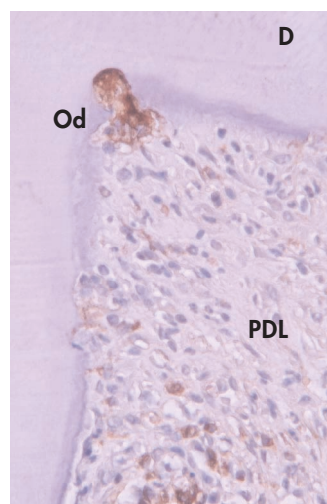


Figure 6. Photomicrograph of immunolabelled odontoclast in resorption lacuna. Haematoxylin, original magnification x 100.

logical controls were provided by three slides in each staining run to which primary antibody (ED1) had not been added. Positive controls were provided by sections of rat spleen.

Results

The freezing of rat molars produced pulpal necrosis, as well as hyalinisation and disruption of the periodontal ligament. Hard tissue resorption was evident and localised to the cervical and interradicular areas of the tooth root. Hard tissue resorption was also seen in the alveolar crestal and interradicular bone. Large multinucleated cells were seen in resorptive lacunae (Figure 1). In six animals, ankylosis was observed in the interradicular areas (Figure 2) alongside areas of active tooth resorption and odontoclastic activity. Large numbers of brown staining ED1-immunopositive cells were distributed throughout the periodontal ligament, but showed more intense activity and staining in areas of tissue inflammation (Figure 3). These areas were seen in the cervical regions of the periodontal ligament and corresponded to areas of greatest resorbing activity. Strong immunolabelling was evident along bone resorptive surfaces (Figure 4) where large clastic cell attack occurred. A positive reaction to ED1 was present in tooth resorptive bays (Figures 5 and 6). Label was detected in smaller mononuclear cells throughout the PDL, and in large multinucleate cells in the adjacent alveolar

bone marrow spaces (Figure 7). The latter reaction became less evident as repair of the dental tissues occurred (Figure 8).

Histological examination of sections from the untreated contralateral first molars revealed ED1 label in multinuclear cells associated with resorption lacunae in bone and tooth. Negative immunological control sections in which primary antibody was omitted remained unlabelled while spleen sections labelled positively around, but not in, germinal lymphopoietic centres (Figure 9).

Discussion

Cryotherapy has many applications but essentially it has the capability of permitting tissue preservation for reuse after thawing, in addition to causing localised tissue necrosis.¹⁷ Cellular destruction arises by means of irreversible thermal alterations in structure and function as a result of a cessation of molecular movement.¹⁷ In the present study, the freezing of the molar for the purpose of generating hard tissue resorption initially caused disruption of the soft tissues in the surrounding areas. This was demonstrated by the dramatic changes seen in the pulp chamber of the thermally traumatised teeth, and involved sterile necrosis of the cellular elements with obliteration of the odontoblastic layer.

Legend for figures

- AB: Alveolar bone
 Ank: Ankylosis
 D: Dentine
 Inf: Inflammation
 M: Bone marrow
 Od: Odontoclast
 Oc: Osteoclast
 PDL: Periodontal ligament
 RR: Repairing resorption

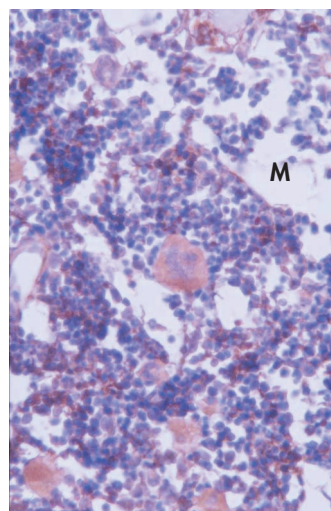


Figure 7. Photomicrograph of alveolar bone marrow showing immunolabelling of multi-nuclear cells. Haematoxylin, original magnified x 100.

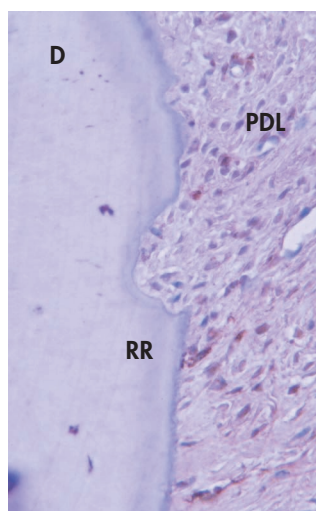


Figure 8. Photomicrograph of repairing dental resorption with no labelling of cells. Haematoxylin, original magnification x 100.

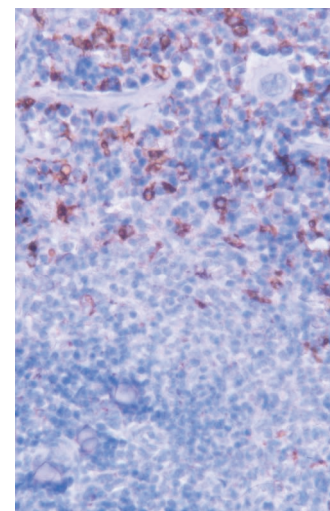


Figure 9. Photomicrograph of spleen tissue reacting positively to immunolabel. Haematoxylin, original magnification x 100.

Wessellink *et al.*,¹⁸ Tal and Stahl¹⁹ and Dreyer *et al.*¹⁵ examined the effects of low temperatures applied to periodontal structures and, subsequently, described the thermal injuries and resorption of adjacent root surfaces. The application of a cold stimulus caused periodontal cell death and bared root surfaces to clastic cell attack followed by repopulation of the PDL with fibroblasts and macrophages. The technique of tooth freezing *in situ* by the application of dry ice, as demonstrated in the present study, is an effective method for inducing PDL damage that facilitates investigation of the sterile inflammatory and hard tissue resorptive processes.¹⁵

ED1 has been identified as one of a family of monoclonal antibodies that recognises different specific antigens on lysosomal membranes of mononuclear phagocytic cells.¹⁴ Damoiseaux *et al.*¹⁴ reported that the immunolabel was highly specific for macrophages rather than for other cell types containing lysosomes and, as such, the ED1 antibody was useful for the identification of cells from the monocyte/macrophage lineage. However, Mighell *et al.*¹⁰ and McNicol and Richmond²⁰ indicated the importance of the preservation of tissue antigenic properties throughout cell differentiation and more particularly during the histological processing of tissues. McNicol and Richmond²⁰ found that heavy fixation or the

affects of heat on tissues due to paraffin embedding could remove or alter antigenic sites. Dijkstra *et al.*²¹ therefore recommended the cryosectioning of unfixed tissues to preserve antigens, but considered the resulting preservation of frozen tissue morphology poor. Jäger *et al.*¹³ suggested that formalin-fixed paraffin-embedded tissues may be suitable for immunolabelling. However, Stirling²² reported that the formalin cross-linking fixation process often rendered the antigenic sites unavailable for antibody reaction, unless unmasking procedures were performed. Unmasked tissues failed to label in the present study, whereas formalin-fixed paraffin-embedded rat tissues reacted positively with ED1 antibody following antigen retrieval using trypsin digestion. This suggests that the ED1 antibody could be successfully used with routine histological tissue preparation protocols provided that suitable antigen preservation and unmasking procedures are followed. Moreover, Stirling²² indicated that complete confidence in the results of immunolabelling relied heavily on the use of positive and negative tissue section controls. The omission of primary antibody and the subsequent lack of labelling in negative control sections of rat periodontium in the present study, plus the strong staining of positive control sections of rat spleen, provided assurance as to the veracity of the tissue preparation and labelling techniques.

The pattern of ED1 label indicated that macrophage-related cells were widely distributed throughout the thermally insulted periodontium and surrounding alveolar bone: mononuclear cells were located within the PDL, while larger, positively-staining multinuclear cells were found adjacent to hard tissue surfaces undergoing resorption. In addition, the intense labelling of cells in the marrow spaces strongly suggested a haemopoietic association between these and the large hard tissue-related cells in resorptive zones. It is likely that the clastic cells resorbing bone and tooth originate from the same source. Proof that osteoclasts and odontoclasts are the same cells has always resided in their similar morphological appearances²³ and functional attributes,⁵ as well as their positive tartrate-resistant acid phosphatase staining.⁶ The positive ED1 staining in the periodontal tissues indicates that haemopoietic cells, osteoclasts and odontoclasts all possess the ED1 antigen, thereby accentuating the strong, positive link between these cell types and further supporting the theory that they emanate from the same differentiation pathway.

Vandevska-Radunovic *et al.*²⁴ evaluated the number and distribution of immunocompetent cells in the normal and orthodontically affected periodontium. Their examination of serial cryostat sections to which a variety of monoclonal antibodies directed against macrophages, dendritic cells and other cell members of the immune system had been applied, revealed that experimental tooth movement attracted cells of the mononuclear phagocyte system, rather than lymphocytes, granulocytes and other immunological cell types. An additional study by Jäger *et al.*,¹³ utilising ED1 on the periodontium of orthodontically moved teeth, revealed a positive reaction to the ED1 antibody on both test and control specimens, with macrophages, osteoclasts and odontoclasts all identified in the stressed PDL.

By using ED1 immunohistochemical techniques, Akamine *et al.*²⁵ examined the pathogenesis of rat periapical lesions and described the localisation of macrophages and plasma cells around areas of infection. The same workers reported that macrophages have a close relationship with bone destruction. This relationship was also reported by Sminia and Dijkstra²⁶ following an examination of mononuclear phagocytes in embryonic rat bone. Furthermore, Sabokbar *et al.*²⁷ showed that hard tissue resorption was directly mediated by inflammatory cells. The release of osteolytic cytokines by heavy macrophage response to bio-mechanical wear has been identified as a prime

cause of hip replacement failure.²⁷ The ED1-labelling of mononuclear cells responding to cold thermal necrosis of the cervical PDL implicates macrophages and provides further evidence of their intimate association in the processes of bone resorption and remodelling or, at least, in the associated inflammatory mechanisms.

Although ED1 identified mononuclear inflammatory cells in the present study, Beelen *et al.*²⁸ showed that there are cytochemically-distinct macrophage populations related to the state of the induced inflammation. These same workers²⁹ reported further that, although ED1 is a general rat-macrophage marker, other ED labels exist for specific types of macrophages related to other tissues. Since ED1 is a lysosomal antibody, it is possible that specific macrophages, with varying functions depending on their lysosomal activity and content, are attracted to the resorptive site.

Stutzmann *et al.*³⁰ suggested that marrow-derived giant macrophage polykaryons migrate to nearby hard tissue surfaces to initiate resorption. Alternatively, Sabokbar *et al.*²⁷ suggested that the large number of immunopositive mononuclear cells identified within the periodontal ligament might continue to differentiate into large multinuclear clastic cells for the same purpose. Although both mechanisms for clast cell derivation within the periodontal ligament are possible, highly specific differentiation labels would be required in order to provide conclusive evidence. Nevertheless, the periodontal ligament of the rat molar provides a useful model whereby the origin and differentiation pathways of hard tissue resorptive cells of monocyte/macrophage origin might immuno-histochemically be further investigated.

Conclusions

1. ED1 positively labels mononuclear and multinuclear cellular elements of the PDL.
2. Mononuclear-cell labelling further implicates macrophages in the process of bone and dentin resorption and in the derivation of clast cells.
3. The periodontal ligament and adjacent bone provides an effective model for the investigation of the cellular origin of resorptive cells.

This project was supported by the Australian Society of Orthodontists' Foundation for Research and Education, for which the authors are grateful.

Address for correspondence and reprints:

Dr C. W. Dreyer
Division of Health Sciences
School of Dentistry
The University of Adelaide
South Australia 5005
Australia
Email: craig.dreyer@adelaide.edu.au

References

1. Vänänen K. Osteoclast function: Biology and mechanisms. In: Bilezikian JP, Raisz LG, Rodan GA, eds. *The Biology of Bone*. San Diego: Academic Press, 1996; 103–13.
2. Suda T, Takahashi N, Martin TJ. Modulation of osteoclast differentiation. *Endocr Rev* 1992; 13: 66–80.
3. Greenfield EM, Bi Y, Miyauchi A. Regulation of osteoclast activity. *Life Sciences* 1999; 65: 1087–102.
4. Chambers TJ. The pathobiology of the osteoclast. *J Clin Path* 1985; 38: 241–52.
5. Lucht U. Osteoclasts and their relationship to bone as studied by electron microscopy. *Z Zellforsch Mikrosk Anat* 1972; 135: 211–28.
6. Andersson GN, Marks SC Jr. Tartrate-resistant acid ATPase as a cytological marker for osteoclasts. *J Histochem Cytochem* 1989; 37: 115–17.
7. Barnicot NA. The supravital staining of osteoclasts by neutral red: their distribution on the parietal bone of normal growing mice, and a comparison with mutants grey-lethal and hydrocephalus-3. *Proc Roy Soc B* 1947; 134: 467–84.
8. Burstone MS. Histochemical demonstration of succinic dehydrogenase activity in osteoclasts. *Nature* 1960; 185: 866.
9. Hattersley G, Chambers TJ. Generation of osteoclastic function in mouse bone marrow cultures: multinuclearity and tartrate-resistant acid phosphatase are unreliable markers for osteoclast differentiation. *Endocrinology* 1989; 124: 1689–96.
10. Mighell AJ, Hume WJ, Robinson PA. An overview of the complexities and subtleties of immunohistochemistry. *Oral Diseases* 1998; 4: 217–33.
11. Athanasou NA. Cellular biology of bone-resorbing cells. *J Bone Jt Surg* 1996; 78A: 1096–112.
12. Suda T, Udagawa N, Takahashi N. Cells of bone: osteoclast generation. In: Bilezikian JP, Raisz LG, Rodan GA, eds. *The Biology of Bone*. San Diego: Academic Press, 1996; 87–102.
13. Jäger A, Radlanski RJ, Götz W. Demonstration of cells of the monocyte phagocyte lineage in the periodontium following experimental tooth movement in the rat. *Histochemistry* 1993; 100: 161–6.
14. Damoiseaux JG, Dopp EA, Calame W et al. Rat macrophage lysosomal membrane antigen recognized by monoclonal antibody ED1. *Immunology* 1994; 83: 140–7.
15. Dreyer CW, Pierce AM, Lindskog S. Hypothermic insult to the periodontium: a model for the study of aseptic tooth resorption. *Endod Dent Traumatol* 2000; 16: 9–15.
16. Zamboni L, De Martino C. Buffered picric acid: formaldehyde: a new, rapid fixative for electron microscopy. *J Cell Biol* 1967; 35:148A.
17. Sherman JK. Freeze/thaw induced structural changes in cells. *J Cryosurgery* 1969; 2: 189–205.
18. Wesselink PR, Beertsen W, Everts V. Resorption of the mouse incisor after the application of cold to the periodontal attachment apparatus. *Calcif Tiss Int* 1986; 39: 11–21.
19. Tal H, Stahl SS. Healing following devitalization of sites within the periodontal ligament by ultra-low temperatures. *J Periodontol* 1986; 57: 735–41.
20. McNicol AM, Richmond JA. Optimizing immunohistochemistry: antigen retrieval and signal amplification. *Histopathology* 1998; 32: 97–103.
21. Dijkstra CD, Dopp EA, Joling P, Kraal G. The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2 and ED3. *Immunology* 1985; 54: 589–99.
22. Stirling JK. Controls for immunogold labelling. *J Histochem Cytochem* 1993; 41:1869–70.
23. Jones S, Boyde A. The resorption of dentine and cementum in vivo and in vitro. In: Z. Davidovitch, ed. *The Biological Mechanisms of Tooth Eruption and Root Resorption*. Birmingham: EBSCO Media, 1988; 335–54.
24. Vandeyska-Radunovic V, Hals Kvinnslund I, Kvinnslund S, Jonsson R. Immunocompetent cells in rat periodontal ligament incident to experimental tooth movement. *Eur J Oral Sci* 1997; 105: 36–44.
25. Akamine A, Hashigushi I, Toriya Y, Maeda K. Immunohistochemical examination on the localization of macrophages and plasma cells in induced rat periapical lesions. *Endod Dent Traumatol* 1994; 10: 121–8.
26. Sminia T, Dijkstra CD. The origin of osteoclasts: an immunohistochemical study on macrophages and osteoclasts in embryonic rat bone. *Calcif Tissue Int* 1986; 39: 263–6.
27. Sabokbar A, Fujikawa Y, Neale S, Murray DW, Athanasou N. Human arthroplasty-derived macrophages differentiate into osteoclastic bone resorbing cells. *Ann Rheum Dis* 1997; 56: 414–20.
28. Beelen RHJ, Walker WS. Dynamics of cytochemically distinct subpopulations of macrophages in elicited rat peritoneal exudates. *Cell Immunol* 1983; 82: 246–57.
29. Beelen RHJ, Eestermans IL, Dopp EA, Dijkstra CD. Monoclonal antibodies ED1, ED2 and ED3 against rat macrophages: expression of recognized antigens in different stages of differentiation. *Transplantation Proceedings* 1987; 19: 3166–70.
30. Stutzmann J, Petrovic A, Shaye R. Extrinsic origin of bone-resorbing cells in orthodontic tooth movement. *J Dent Res* 1980; 59: 440.