Histochemical and Immunohistochemical Studies on the Immunocompetent Cells in the Periodontal Ligament of Rat Incisors and Molars

Ichiro KAWAHARA

Department of Second Oral Anatomy, Niigata University School of Dentistry, Niigata, Japan

Summary

The distribution of antigen presenting cells such as dendritic cells and macrophages in the periodontal ligament of rat incisors and molars was surveyed by histochemical and immunohistochemical methods. The antigen presenting cells are known to display MHC-class II molecules on their cell surfaces that are recognized by OX6-monoclonal antibody. Many OX6 immunopositive cells were localized in the periodontal ligament of rat incisors and molars showing region-specific distributions under a physiological condition. In the incisor, the antigen presenting cells were shown throughout the lingual periodontal ligament, located preferentially in the bone-related zone and shear zone. In contrast to the distribution of immunopositive macrophages that showed very intense ACPase activity and round or oval profiles associated with sinusoidal blood vessels, immunopositive dendritic cells without significant ACPase activity were located throughout the tooth-related zone as well. In the periodontal ligament of rat molars that are undergoing physiological distal migration associated with alveolar bone resorption, the OX6 immunopositive dendritic cells and the immunonegative cells of the osteoclastic lineage showed a clear-cut segregated localization when viewed in the transverse sections of the molar tooth ligaments. Immunostaining with an ED1-monoclonal antibody that recognizes various subpopulations of monocyte system cells showed even spatial distribution of the cells of monocyte origin including osteoclastic cells, dendritic cells and macrophages in the periodontal ligament of rat molar.

These data are the first to display the abundance and unique distribution of dendritic cells and macrophages expressing MHC-class II molecules in the periodontal ligament of rat. A possibility is suggested that the periodontal ligament of rat can be utilized as an exquisite model tissue whereby the origin and differentiation pathways of the antigen presenting cells of monocyte origin can be examined further *in vivo*.

Arch. Histol. Cytol., Vol. 55, No. 2 (1992)
p. 211-217

Histochemical and Immunohistochemical Demonstration of Macrophages and Dendritic Cells in the Lingual Periodontal Ligament of Rat Incisors

Ichiro KAWAHARA, Yoshiro TAKANO, Osamu SATO, Takeyasu MAEDA and Koichi KANNARI

Department of Oral Anatomy, Niigata University School of Dentistry, Niigata, Japan

Received April 24, 1992

Summary. **The distribution of macrophages in the lingual periodontal ligament of rat incisors was surveyed by histochemical and immunohistochemical methods. Numerous macrophages showing intense ACPase reactions were located primarily in the shear zone of the periodontal ligament. Immunostaining with an ED1-monoclonal antibody that recognizes various subpopulations of macrophages revealed plentiful positive cells showing flamelike profiles throughout the periodontal ligament, in addition to regular macrophages associated with sinusoidal blood vessels. A similar distribution of flamelike cells expressing Ia antigens was demonstrable with immunostaining using an OX6-monoclonal antibody. A consecutive staining of sections for ACPase histochemistry followed by immunoreactions for Ia antigens revealed the presence of two types of the flamelike cells in the periodontal ligament: one with and the other without distinct ACPase activity, corresponding to the macrophage and the dendritic cell, respectively. Either type of flamelike cells was located in the bone-related and shear zones, whereas only dendritic cells without ACPase activity were restricted to the tooth-related zone. OX6-immunonegative cells showing ACPase reactions were also found in the periphery of the sinusoidal blood vessels.**

Our data are the first to demonstrate the abundance of macrophages and dendritic cells expressing Ia antigens throughout the lingual periodontal ligament of rat incisors. In addition to regular macrophages, an exclusive localization of macrophages with flamelike extensions has been demonstrated in the bone-related and shear zones of the ligament. The region-specific arrangement of macrophages and dendritic cells with various histochemical and immunological features suggests that the periodontal ligament of rat incisor is a useful model for analyzing the process of differentiation of antigen-presenting cells.

Dendritic cells have been identified in lymphoid tissues as cells with extensive cytoplasmic projections showing flamelike profiles; they have little or no endocytic activity (STEINMAN et al., 1986). They are known to have I-region associated (Ia) antigens and play an important role in the immune system as tissue antigen-presenting cells (STEINMAN et al., 1986). Recent immunohistochemical study on the dental tissues has revealed the presence of a large population of dendritic cells in the dental pulp, serving towards tissue protection under physiological as well as pathological conditions (KAWASHIMA, 1990).

Macrophages, a kind of antigen-presenting cells in the immune system, have been suggested in recent studies to cooperate with dendritic cells for antigen-presentation (KAPSENBERG et al., 1986). In gingival connective tissues constantly exposed to antigenic stimuli through sulcular epithelium, the role of the macrophage has been investigated in relation to the pathology of periodontal diseases (SEYMOUR et al., 1988; MATSUKI et al., 1991). In the periodontal ligament continuous with gingival tissues, however, little attention has been focused upon macrophages and, hence, neither the precise location nor their role has been established in periodontal biology.

The present study was therefore undertaken to disclose the distribution of macrophages in the periodontal ligament and their relationship with dendritic cells using histochemical as well as immunohistochemical markers for macrophages and/or dendritic cells. The central one-third of the lingual periodontal ligament of the rat incisor was chosen for observations because the influence of exogenous antigenic stimuli could then be disregarded.

MATERIALS AND METHODS

Adult male rats of the Wistar strain weighing (260 ± 5 g) were anesthetized by an intraperitoneal injection of

chloral hydrate (400 mg/kg body wt) and perfused either with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) or a mixture of 4% paraformaldehyde and 0.25% glutaraldehyde in the same buffer for 20 min. Some of the animals were perfused with 3% paraformaldehyde and 2.5% glutaraldehyde mixture in 0.06 M cacodylate buffer supplemented with 0.05% $CaCl_2$. Upper jaws were dissected and processed for decalcification with neutral 5% EDTA at 4°C. Following decalcification, the tissues were immersed in a 30% sucrose solution at 4°C overnight, and frozen in liquid nitrogen. Longitudinal cryosections of the upper incisors with adherent periodontal tissues, 20 μm in thickness, were made in a cryostat.

ACPase histochemistry

For histochemcal demonstration of acid phosphatase (ACPase) activity, the azo-dye method (BURSTONE, 1961) and lead salt method (GOMORI, 1952) were used with slight modifications. The incubation medium for the azo-dye method comprised 0.01% naphthol AS-MX phosphate (Na salt) and 0.06% fast red violet LB salt in 0.1 M acetate buffer (pH 5.2). The modified Gomori's medium comprised 0.12% sodium-glycerophosphate, 0.1% lead nitrate, and 2% dextrose in 0.05 M acetate buffer (pH 5.2). In both methods, the cryosections were incubated for 30–45 min at 37°C. The sections incubated in the lead salt medium were treated with diluted ammonium sulfide for light microscopic identification of reaction sites. An incubation with the medium containing 10 mM NaF or with a substrate-free medium completely eliminated the reactions in both experiments.

Immunohistochemistry

Free floating sections were processed for immunohistochemistry by use of ED1- and OX6-monoclonal antibodies (Serotec, Oxford, England), which could recognize most of the monocyte-macrophage system cells (DIJKSTRA et al., 1985) and Ia antigens (MCMASTER and WILLIAMS, 1979), respectively. A solution of 0.01 M phosphate-buffered saline (PBS, pH 7.4) was used to dilute the antibody and to rinse the sections. After blocking endogenous peroxidase with absolute methanol containing 0.3% H_2O_2, the sections were incubated for 48 h at 4°C with the primary antibodies, diluted to 1 : 500 (ED1), and 1 : 5000 (OX6). The sites of antigen-antibody reaction were made visible by an avidin-biotin peroxidase complex (ABC) method (HSU et al., 1981), using a commercially available ABC kit (Vector Lab. Inc., Burlingame, USA).

Some of the sections were sequentially stained for ACPase histochemistry with the azo-dye method followed by immunostaining for Ia antigens with an OX6-monoclonal antibody.

RESULTS

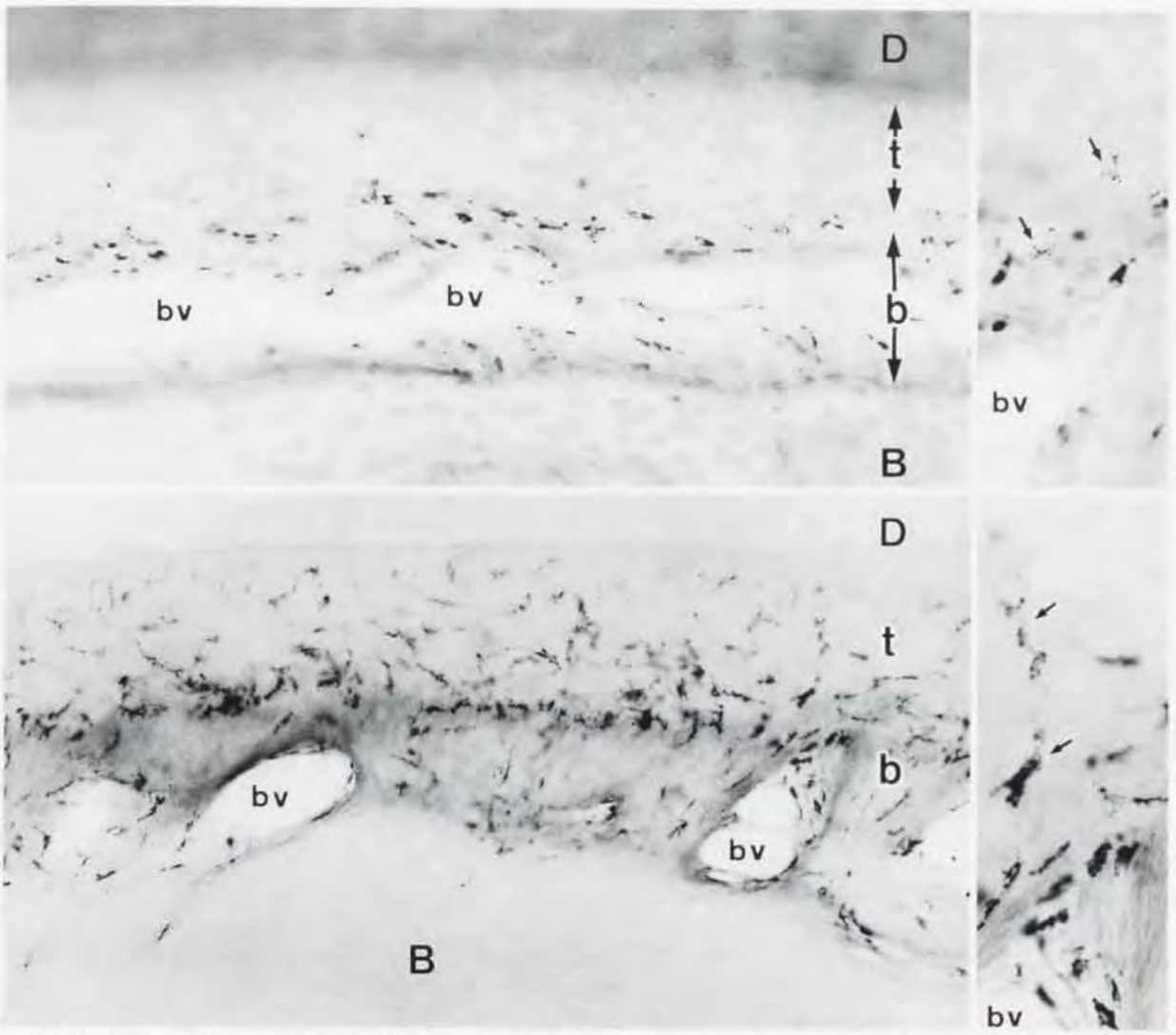
ACPase activities

Intense reactions for ACPase were localized in macrophages located in the bone-related and shear zones of the lingual periodontal ligament. The reactive cells appeared to be concentrated along the shear zone, the border region between the tooth-related and bone-related zones (Fig. 1a). They were also seen in the periphery of the sinusoidal blood vessels in the bone-related zone. Enzymatic reactions were observed to be confined in the large sized lysosomal structures in the individual cells (Fig. 1b). Only a few cells in the tooth-related zone showed similar enzymatic reactions. Fibroblasts did not show any ACPase reactions as large granular structure but as fine fibrous structures; they could be easily distinguished from macrophages under the light microscope.

Immunohistochemistry

Immunostaining with an ED1-monoclonal antibody revealed distinct reactions in numerous cells possessing flamelike extensions throughout the periodontal ligament. A number of oval or spindle-shaped macrophages showing the ED1-immunoreactivity were also recognized, primarily associated with sinusoidal blood vessels in the bone-related zone of the ligament. Immunostaining with an OX6-monoclonal antibody that recognizes Ia antigens, a class of surface antigens encoded for the major histocompatibility complex (THOMAS and SHEVACH, 1978), revealed a similar distribution of reactive cells showing flamelike profiles as seen with an ED1-antibody. The immunoreactive cells with flamelike extensions to either ED1- or OX6-antibody were most highly concentrated in the shear zone of the lingual periodontal ligament (Figs. 2–4).

A double staining both with ACPase histochemistry and OX6-immunohistochemstry (Fig. 5a), allowed a clear-cut distinction between the two types of flamelike cells expressing Ia antigens: one with and the other without significant ACPase activity (Fig. 5b,c). The latter type of cell was distributed throughout the periodontal ligament. The OX6-positive flamelike cells showing intense ACPase activity were relatively small in number and were located primarily in the bone-related and shear zones where those



1a 1b

2a 2b

Fig. 1 a. ACPase reactions (lead salt method) in the lingual periodontal ligament of the upper incisor. Note the exclusive localization of reactive cells in the bone-related zone (*b*) of the ligament. ×250. **b.** Regular macrophage-like cells and irregular-shaped cells with relatively weak reactions (*arrows*) in the bone-related zone. *B* alveolar bone, *bv* blood vessel, *D* dentin, *t* tooth-related zone. ×290

Fig. 2 a. Immunoreactions to an ED1-monoclonal antibody in the lingual periodontal ligament of the upper incisor. Note the abundance of reactive cells showing irregular or flamelike profiles throughout the ligament. ×250. **b.** A closer view of immunoreactive flamelike cells (*arrows*). Regular macrophages are primarily located in the periphery of the blood vessels (*bv*). *B* alveolar bone, *b* bone-related zone, *D* dentin, *t* tooth-related zone. ×290

without ACPase activity were also located.

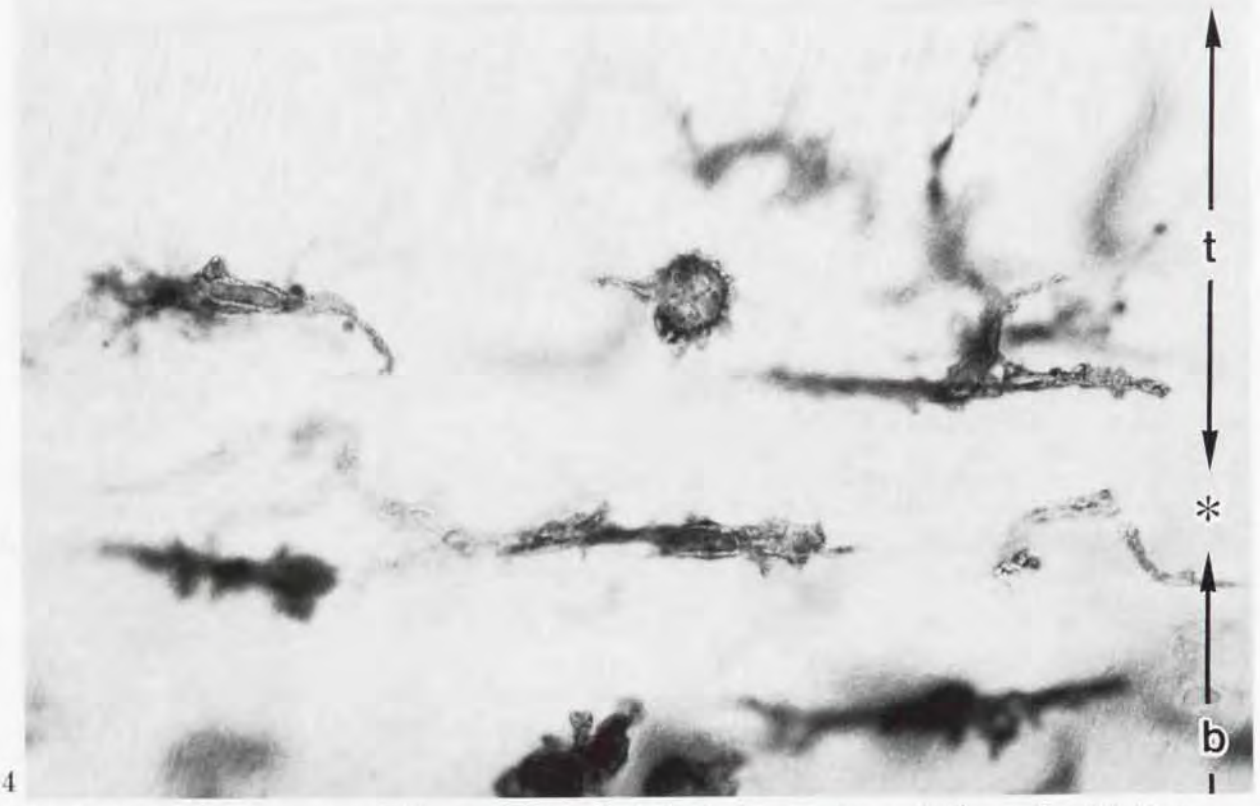
Two types of oval or spindle-shaped macrophages (ACPase positive) could further be distinguished by the presence or absence of OX6-immunoreactivity. These types of cells were mostly located in the periphery of the sinusoidal blood vessels.

The histochemical and immunohistochemical characterizations of these cells demonstrated in this study are summarized in Table 1.

DISCUSSION

Our data indicated the presence of numerous macro-

3



4

Fig. 3. Immunoreactions to an OX6-monoclonal antibody in the same region as in Figures 1 and 2. Most immunoreactive cells show typical flamelike profiles and are crowded at the border region between tooth-related (*t*) and bone-related zones (*b*). *B* alveolar bone, *bv* blood vessel, *D* dentin. ×330

Fig. 4. A close-up of flamelike cells in the ligament. *b* Bone-related zone, *t* tooth-related zone, * shear zone. ×635

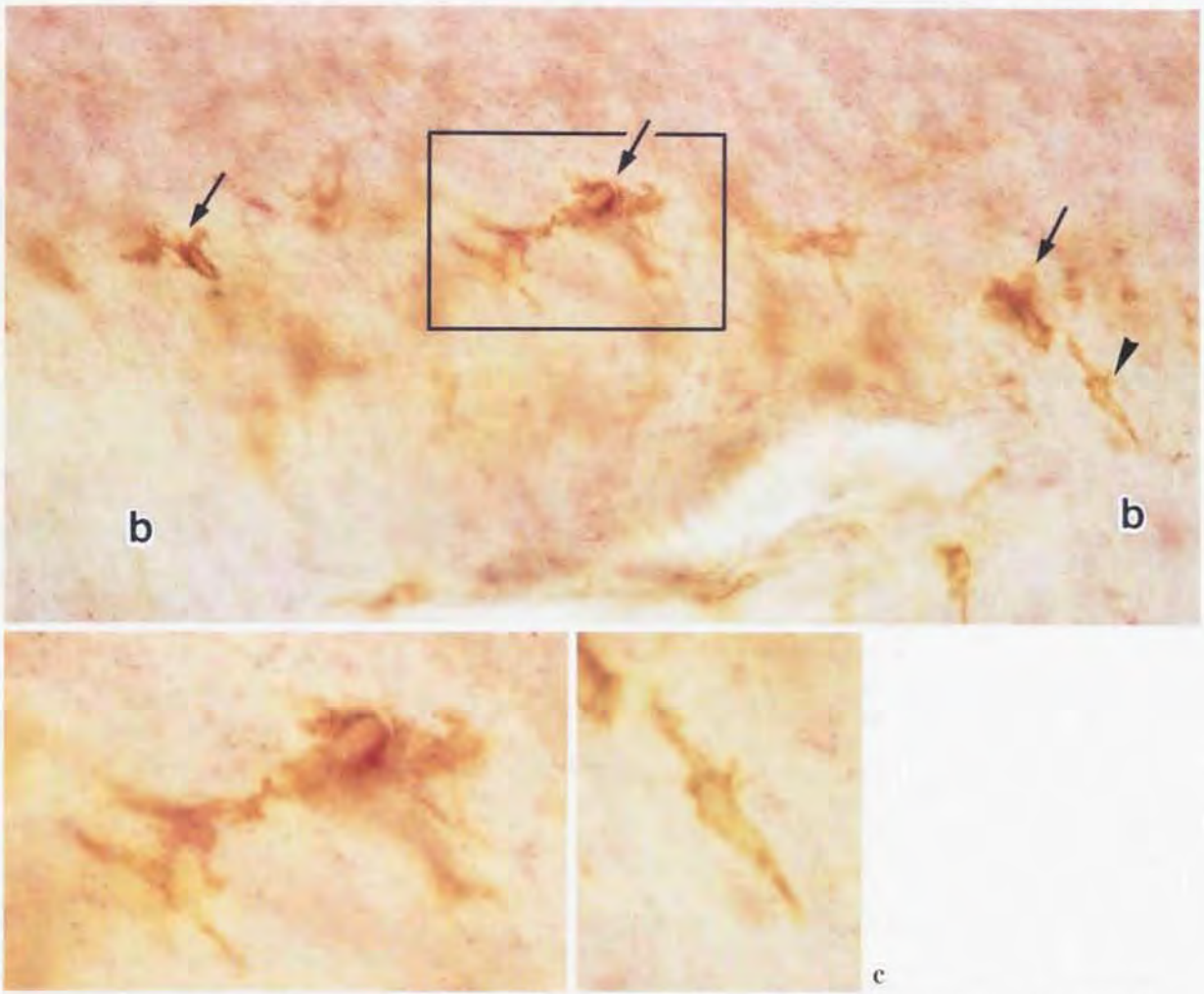


Fig. 5 a. A double staining for histochemical ACPase activity and OX6-immunoreactivity. A distinction between immunoreactive cells with (*arrows*) and without significant ACPase reactions (*arrowhead*) can be made. *b* Bone-related zone. ×460. **b.** Higher magnification of boxed area shown in Fig. 5a. An immunoreactive cell showing intense ACPase reaction. ×910. **c.** Closer view of the dedritic cell (*arrowhead* in Fig. 5a). An immunoreactive cell lacking ACPase reaction. ×910

Table 1. Histochemical and immunohistochemical characterization of macrophages and dendritic cells in the periodontal ligament of rat incisor.

	Macrophage		Dendritic cell
	Regular-shaped	Flamelike	
ED1	+	+	+
OX6	+	−	+
ACPase	+	+	−

phages and dendritic cells in the lingual periodontal ligament of rat incisors, and further could distinguish two types of macrophages with respect to the presence or absence of extensive flamelike cytoplasmic extensions.

Dendritic cells are irregular-shaped cells which were initially identified *in vitro* in the glass and plastic adherent population of cells from the mouse spleen (for review, STEINMAN and NUSSENZWEIG, 1980). Dendritic cells are clearly distinguishable from lymphocytes due to their lack of surface Ig, thy-1 and brain antigens, and also by differences in the properties of surface polysaccharides (STEINMAN et al., 1979). Dendritic cells can be also distinguished from

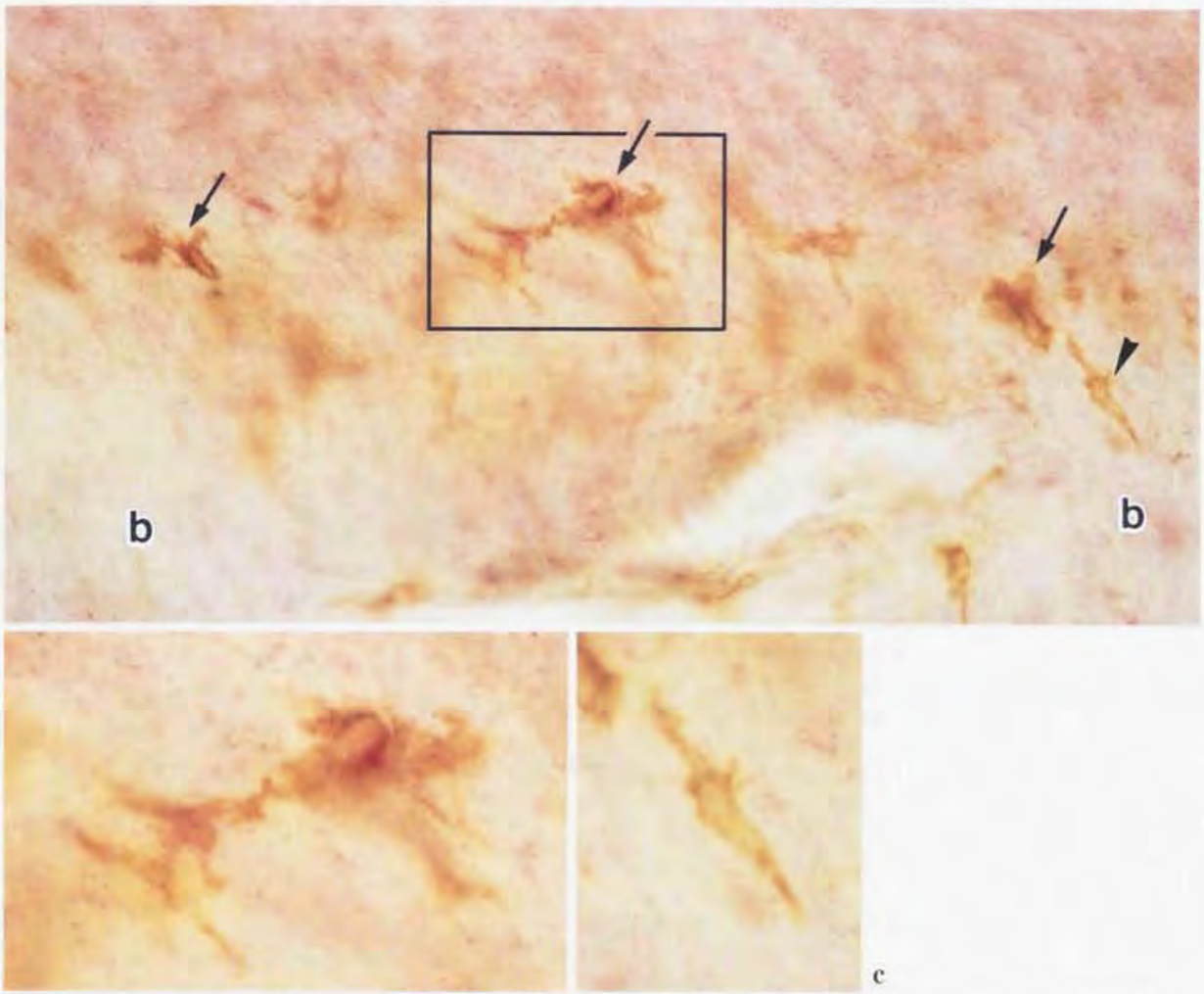


Fig. 5 a. A double staining for histochemical ACPase activity and OX6-immunoreactivity. A distinction between immunoreactive cells with (*arrows*) and without significant ACPase reactions (*arrowhead*) can be made. *b* Bone-related zone. ×460. **b.** Higher magnification of boxed area shown in Fig. 5a. An immunoreactive cell showing intense ACPase reaction. ×910. **c.** Closer view of the dedritic cell (*arrowhead* in Fig. 5a). An immunoreactive cell lacking ACPase reaction. ×910

Table 1. Histochemical and immunohistochemical characterization of macrophages and dendritic cells in the periodontal ligament of rat incisor.

	Macrophage		Dendritic cell
	Regular-shaped	Flamelike	
ED1	+	+	+
OX6	+	−	+
ACPase	+	+	−

phages and dendritic cells in the lingual periodontal ligament of rat incisors, and further could distinguish two types of macrophages with respect to the presence or absence of extensive flamelike cytoplasmic extensions.

Dendritic cells are irregular-shaped cells which were initially identified *in vitro* in the glass and plastic adherent population of cells from the mouse spleen (for review, STEINMAN and NUSSENZWEIG, 1980). Dendritic cells are clearly distinguishable from lymphocytes due to their lack of surface Ig, thy-1 and brain antigens, and also by differences in the properties of surface polysaccharides (STEINMAN et al., 1979). Dendritic cells can be also distinguished from

macrophages, since they show little or no endocytic activity *in vivo* and *in vitro* (STEINMAN and NUSSENZWEIG, 1980) and, hence, display low ACPase activity (STEINMAN et al., 1986) in contrast to macrophages that have a phagocytic activity and intense ACPase activity in lysosomal cytoplasmic vesicles.

Tissue macrophages generally appear oval or spindle in profile under the light microscope *in vivo*. As shown in Figure 1, however, histochemical reactions for ACPase alone do not allow the precise determination of the profiles of the reactive cells. Since an ED1-monoclonal antibody recognizes a monocyte-macrophage lineage as well as dendritic cells (DIJKSTRA et al., 1985) and since a large number of cells immunoreactive to ED1, including those located in the shear zone, have all displayed flamelike profiles in this study, it seems reasonable to infer the presence of macrophages with flamelike cytoplasmic extensions in the periodontal ligament.

It is well known that either dendritic cells or macrophages express I-region associated (Ia) antigens, and that dendritic cells lack ACPase activity, a marker enzyme for lysosomes rich in macrophages. The present double staining with ACPase histochemistry and Ia-immunohistochemistry serves a distinction between the two types of cells, both showing flamelike profiles. Current data indicate that, at least, some of the cells with flamelike extensions in the bone-related and shear zones of periodontal ligament of rat incisors can be regarded as macrophages from histochemical as well as immunohistochemical criteria.

The double staining of sections further allowed a distinction between the regular (oval or spindle) shaped macrophages located at the peripheral regions of sinusoidal blood vessels in the bone-related zone: one with and the other without Ia-immunoreactivity. Macrophages that lack Ia antigens may be of the monocyte-macrophage lineage having, directly or indirectly something to do with the osteoclasts that also lack Ia antigens.

Regarding the location of macrophages in the periodontal ligament, it is interesting to note that those without flamelike extensions (regular macrophages) are principally located in the periphery of blood vessels, whereas flamelike macrophages are distributed throughout the bone-related and shear zones of the ligament. Since macrophages have been thought to be differentiated from monocytes in the connective tissue (VAN FURTH et al., 1972), it is tempting to speculate that monocytes derived from local blood vessels may initially differentiate into regular macrophages in the periphery of blood vessels, and further into flamelike macrophages in the bone-related zone of the periodontal ligament. Some of the flamelike macrophages might migrate toward the shear zone where they differentiate into Ia-antigen-presenting flamelike cells (ACPase reactive flamelike cells). It should be noted that the tooth-related zone of the periodontal ligament is an avascular zone (BEERTSEN et al., 1974) where only dendritic cells (flamelike cells without ACPase activity) are located. It is assumed, therefore, that the dendritic cells in the periodontal ligament of rat incisors might be the derivative of the flamelike macrophages that obtained Ia antigens instead of a loss of ACPase by undefined mechanisms.

The correlation and functional significance of these cells shown in this study require future clarification.

Acknowledgments. The authors express their thanks to Mr. M. HOSHINO and K. TAKEUCHI for their technical assistance.

REFERENCES

BEERTSEN, W., V. EVERTS and A. VAN DEN HOOFF: Fine structure of fibroblasts in the periodontal ligament of the rat incisor and their possible role in tooth eruption. Arch. Oral Biol. 19: 1087–1098 (1974).

BURSTONE, M. S.: Histochemical demonstration of acid phosphatases in frozen sections with naphthol AS-phosphates. J. Histochem. Cytochem. 9: 146–153 (1961).

DIJKSTRA C. D., E. A. JOPP, P. JOLING and G. KARAAL: The heterogeneity of mononuclear phagocytes in lymphoid organs: Distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2 and ED3. Immunology 54: 598–599 (1985).

GOMORI, G.: Microscopic histochemistry. Chicago Univ. Press, Chicago, 1952.

HSU, S.-M., L. RAINE and H. FANGER: Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabeled antibody (PAP) procedures. J. Histochem. Cytochem. 29: 577–580 (1981).

KAPSENBERG, M. L., M. B. M. TEUNISSEN, F. E. M. STIEKEMA and H. G. KEIZER: Antigen-presenting cell function of dendritic cells and macrophages in proliferative T cell responses to soluble and particulate antigens. Europ. J. Immunol. 16: 345–350 (1986).

KAWASHIMA, N.: Immunohistochemical demonstration of various immunocomponent cells in developing rat periapical lesions (In Japanese). Jap. J. Conserv. Dent. 33: 1659–1677 (1990).

MATSUKI, Y., T. YAMAMOTO and K. HARA: Interleukin-1 mRNA-expressing macrophages in human chronically inflamed gingival tissues. Amer. J. Pathol. 138: 1299–1305 (1991).

McMaster, E. R. and A. F. Williams: Identification of Ia glycoproteins in rat thymus and purification from rat spleen. Europ. J. Immunol. 9: 426–433 (1979).

Seymour, G. J., E. Gemmell, L. J. Walsh and R. N. Powell: Immunohistological analysis of experimental gingivitis in humans. Clin. Exp. Immunol. 71: 132–137 (1988).

Steinman, R. M. and C. Nussenzweig: Dendritic cells: features and functions. Immunol. Rev. 53: 127–147 (1980).

Steinman, R. M., G. Kaplan, M. Witmer and Z. A. Cohn: Identification of a novel cell type in peripheral lymphoid organs of mice. V. Purification of spleen dendritic cells, maintenance *in vitro*, and new surface markers of dendritic cells. J. Exp. Med. 149: 1–7 (1979).

Steinman, R. M., W. C. Van Voorhis and D. M. Spalding: Dendritic cells. In: (ed. by) D. M. Weir et al.: Handbook of experimental immunology. Blackwell Scientific Publications, Oxford, 1986 (Chap. 49, p. 1–9).

Thomas, D. W. and E. M. Shevach: Nature of the antigenic complex recognized by T lymphocytes. VI. The effect of anti-TNP antibody on T cell responses to TNP-conjugated macrophages. J. Immunol. 121: 1145–1151 (1978).

Van Furth, R., Z. A. Cohn, J. G. Hirsch, W. G. Spector and H. L. Langevoot: The mononuclear phagocyte system: A new classification of macrophages, monocytes, and their precursor cells. Bull. World Health Organ. 46: 845–852 (1972).

Prof. Yoshiro Takano
Department of Oral Anatomy
Niigata University School of Dentistry
2-5274 Gakkocho-dori
Niigata, 951 Japan

高野 吉郎
951 新潟市学校町通2-5274
新潟大学歯学部
口腔解剖学第二教室

McMaster, E. R. and A. F. Williams: Identification of Ia glycoproteins in rat thymus and purification from rat spleen. Europ. J. Immunol. 9: 426–433 (1979).

Seymour, G. J., E. Gemmell, L. J. Walsh and R. N. Powell: Immunohistological analysis of experimental gingivitis in humans. Clin. Exp. Immunol. 71: 132–137 (1988).

Steinman, R. M. and C. Nussenzweig: Dendritic cells: features and functions. Immunol. Rev. 53: 127–147 (1980).

Steinman, R. M., G. Kaplan, M. Witmer and Z. A. Cohn: Identification of a novel cell type in peripheral lymphoid organs of mice. V. Purification of spleen dendritic cells, maintenance *in vitro*, and new surface markers of dendritic cells. J. Exp. Med. 149: 1–7 (1979).

Steinman, R. M., W. C. Van Voorhis and D. M. Spalding: Dendritic cells. In: (ed. by) D. M. Weir et al.: Handbook of experimental immunology. Blackwell Scientific Publications, Oxford, 1986 (Chap. 49, p. 1–9).

Thomas, D. W. and E. M. Shevach: Nature of the antigenic complex recognized by T lymphocytes. VI. The effect of anti-TNP antibody on T cell responses to TNP-conjugated macrophages. J. Immunol. 121: 1145–1151 (1978).

Van Furth, R., Z. A. Cohn, J. G. Hirsch, W. G. Spector and H. L. Langevoot: The mononuclear phagocyte system: A new classification of macrophages, monocytes, and their precursor cells. Bull. World Health Organ. 46: 845–852 (1972).

Prof. Yoshiro Takano
Department of Oral Anatomy
Niigata University School of Dentistry
2-5274 Gakkocho-dori
Niigata, 951 Japan

高野 吉郎
951 新潟市学校町通2-5274
新潟大学歯学部
口腔解剖学第二教室

Printed in Niigata, Japan by Daiichi Printing Co. Ltd.

Arch. Histol. Cytol. (1995) in press.

Segregated Localization of Immunocompetent Cells and Osteoclasts in the Periodontal Ligament of Rat Molar Tooth

Ichiro KAWAHARA and Yoshiro TAKANO

Running title: Antigen Presenting Cells in Rat Molar Periodontal Ligament

Address all correspondence to: Ichiro KAWAHARA, Department of Oral Anatomy, Niigata University School of Dentistry, Gakkotyo 2–5274, Niigata 951, Japan

Summary

A spatial distribution of dendritic cells, macrophages, and their respective precursor cells in the periodontal ligament of rat molars was examined by means of ACPase enzyme histochemistry and immunohistochemistry. Intense reactions for ACPase were localized in both the multinucleated- and mononucleated cells of the periodontal ligament located exclusively in the potions of physiological bone resorption due to physiological migration of the molar teeth. Immunohistochemical staining with OX6-monoclonal antigen that recognizes antigen-presenting cells such as dendritic cells and macrophages revealed localization of immunopositive cells predominantly in the potions of the periodontal ligament that showed only trace reactions for ACPase. On the other hand, a large number of ED1-immunopositive cells, comprising a broad spectrum of cells of the monocyte origin including dendritic cells and osteoclasts, displayed almost even distribution throughout the periodontal ligament. After all, our current study is the first to show a clear-cut *in vivo* morphological evidence that the cells of the bone-resorbing, osteoclastic cell lineage and those of the non-bone resorbing, macrophagic and/or dendritic cell lineages are exclusively localize in roughly the distal and proximal regions of the periodontal ligament of rat molars, respectively. An advantage of the use of rat molar periodontal ligament as an *in vivo* model system for pursuing differentiation pathways of cells of the monocyte lineage, particularly of the osteoclastic cells is proposed.

INTRODUCTION

The macrophages have been known as the antigen-presenting cells that have a phagocytic activity and characteristically well-developed lysosomal systems showing intense ACPase activities. In recent years the presence of dendritic cell is well acknowledged in various tissues, which have no significant ACPase activity and have antigen-presenting capability mach stronger than that of the macrophages such as sensitization of MHC-restricted T cells (VAN FURTH, 1986; STEINMAN, 1986). The precise origin and function of dendritic cells have been sought (INABA, 1992 and 1993).

Unlike in ordinary rooted teeth of limited growth, the periodontal ligament of continuously erupting rodent incisors undergoes constant remodeling and hence is recognized as the physiological stable tissue free from infection and foreign antigens. In previous studies, we reported on the presence of a large number of dendritic cells in the periodontal ligament of rat incisors and demonstrated their region-specific localization different from that of macrophages and elaborated on their differentiation pathways (KAWAHARA and TAKANO 1992 and 1995).

The rat molar teeth are of the limited growth and known to migrate toward the distal direction constantly through the life (VIGNERY and BARON, 1980; ROBINSON and SCHNEIDER, 1992; LASFARGUES and SAFFAR, 1993). Accordingly in the periodontal ligament, the ligament fibers and alveolar bone undergo dynamic remodeling in accordance with distal migration of molar teeth (VIGNERY and BARON, 1980; ROBINSON and SCHNEIDER, 1992; LASFARGUES and SAFFAR, 1993). Since biological significance of dendritic cells in the periodontal ligament of rat incisors is obscure, the present study examines the periodontal ligament of rat molars to define whether or not the dendritic cells are ubiquitous in the periodontal ligament. The results obtained confirmed presence of dendritic cells and further revealed a distinct habitat segregation between the dendritic cells and

osteoclastic cells in the molar ligament both are of the monocyte lineage.

MATERIALS AND METHODS

Adult male rats of the Wistar strain weighing 260±10g were anesthetized by an intraperitoneal injection of chloral hydrate (400mg/Kg body wt.) and perfused via aorta either with 4% paraformaldehyde in 0.1M phosphate buffer (pH7.4) or a mixture of 4% paraformaldehyde and 0.25% glutaraldehyde in the same buffer for 20 min. Upper jaws were dissected and processed for decalcification with neutral 5% EDTA at 4°C for 30 days. Following decalcification, the tissues were immersed in a 30% sucrose solution at 4°C overnight, and frozen in liquid nitrogen. Cross or longitudinal sections of the root of the upper molars with the surrounding alveolar bone, 20μ in thickness, were made in a cryostat (Coldtome CM-41 SAKURA corp. Japan).

Immunohistochemistry

Free floating sections were processed for immunohistochemistry by use of ED1 monoclonal antibody (DIJKSTRA et al., 1985), and OX6 monoclonal antibody (Mc MASTER and WILLIAMS, 1979), which could recognize most of the cells of monocyte-macrophage linage and MHC-class II molecules, respectively. A solution of 0.01M phosphate-buffered saline (PBS pH7.4) was used to dilute the antibody and to rinse the sections. After blocking endogenous peroxidase with absolute methanol containing 0.3% H_2O_2, the sections were incubated for 24h at 4°C with the primary antibodies, diluted to 1:500 (ED1), and 1:5000 (OX6). The sites of antigen-antibody reaction were made visible by an avidin-biotin peroxidase complex (ABC) method (HSU et al., 1981), using a commercially available ABC kit (Vector Lab. Inc., Burlingame, USA).

Double staining of OX6 immunohistochemistry and ACPase enzyme histochemistry by Azo dye method.

First by, free floating sections were processed for immunohistochemistry by use of OX6 as described above except that the DAB treatment was omitted. The sections immunostained for OX6 were then reacted for ACPase activity reactions by the Azo-dye method (BURSTONE, 1961; MAEDA et al., 1993). Briefly, the sections were incubation for 10 min in a medium consisting of 0.7% fast red violet LB salt (Siguma, St. Louis, USA), 0.8% naphthol AS-MX phosphate (Sigma, St. Louis, USA), and 0.1M acetate buffer(ph 5.3), at room temperature. An incubation with the medium containing 10mM NaF or with a substrate-free medium completely abolished the ACPase reaction in both experiments. After completion of histochemical staining for ACPase activity, the OX6-immunoreaction site was visualized by incubating in DAB medium.

Distribution density of immunopositive cells in the periodontal ligament

Differences in distribution density of the OX6-immunopositive cells in the periodontal ligament between the ACPase reactive area and non-reactive area were examined on the cross sections of the central 1/3 of the upper second molar roots. On the photoprints, the periodontal space of each root was divide evenly into 36 units by drawing straight lines extending radially from the center of the dental pulp as shown in Figure 5. The mean cell density per unit area of the periodontal ligament was calculated independently in the intensely ACPase positive portion and ACPase weak portion of the ligament, and the values compared between the two. Since the unit surface area of the examined periodontal ligament differed among the specimens, a paired T-test between the values of the immuno-positive cell density from the ACPase positive- and ACPase weak portions in the respective roots was

executed. The ACPase positive- and ACPase weak portions of the periodontal ligament in each examined root were distinguished precisely by staining the adjacent cryosections for ACPase by the Azo dye method. The roots observed were randomly selected from those of the upper second molars and no specific type of root was chosen. Twenty-eight cut surfaces of the roots were selected for counting OX6-immunopositive cells, and thirteen cut surfaces for ED1-immunopositive cells.

RESULTS

ACPase activity in the periodontal ligament

Intense reactions for ACPase were localized in cells of the periodontal ligament located more or less in the distal portions of the periodontal space toward which a physiological migration of molar is known to take place (Fig 1). Very intense ACPase reactions appeared in the osteoclasts on the bone surface. Among the cut surfaces of the roots of upper second molars, the portions of the alveolar bone surface, enriched with intensely ACPase reactive osteoclasts and undergoing bone resorption, were not necessary restricted to the distal side and differed variably among the individual roots. A number of resorption cavities were occasionally seen on the distal root surface extending through the cementum to the dentin matrix (Fig. 1–b).

In the ACPase reactive areas of the ligament, there were some small and round cells showing intense ACPase reactions equal to the that of the osteoclasts (Fig. 1–b). Moreover fibroblasts in this area also showed some ACPase activity, whereas the ACPase reaction was almost negative in the rest of the periodontal ligament where the osteoclasts were lacking (Fig.1–b).

OX6–immunopositve cells and ACPase reaction

Presence of a large population of OX6–immunopositive cells in the periodontal ligament of rat molars was confirmed (Fig. 2). In longitudinal sections molar roots, OX6–immunopositive cells showed fairly even distribution from cervical to the apical portion of the periodontal ligament (Fig. 2–a). As examined in the cross sections of the molar roots, however, an apparent heterogeneity in distribution of OX6–immunopositive cells became evident in the periodontal ligament (Fig. 2–b). In cross sections, the OX6–immunopositive cells were shown to be much smaller in number in the areas where a large number of multinucleated osteoclasts, which were immunonegative to OX6, were located. Many of the OX6–immunopositive

cells had large cytoplasmic projections and displayed dendritic profiles, while some showed spindle or ovoid contours.

A double staining of sections for OX6-immunohistochemistry and ACPase enzyme histochemistry clearly indicated that the predominant population of OX6-immunopositive cells were located in the ACPase weak or negative portion of the periodontal ligament. There were only a small number of immunoreactive cells in the intensively ACPase reactive area of the ligament where a bone resorption by the osteoclasts appeared to be in progress (Figs. 3-a,b.).

The eccentric distribution pattern of the OX6-immunopositive cells in the periodontal ligament of the doubly stained specimens was similar as that in the specimens simply stained for OX6 alone and, hence, should not be considered as an artifact (compare Fig. 2-b and Fig. 3-a).

ED1-immunopositve cells

A large number of ED1-immunopositive cells, being recognized as a broad spectrum of cells of the monocyte origin appeared and showed almost equal distribution in the periodontal ligament (Fig 4-a). Large sized osteoclasts were easily recognized due to particularly intense ED1-immunoreactions. ED1-immunopositive mononuclear cells in the osteoclast-rich area mostly had rich cytoplasm and hardly showed typical dendritic profiles (Fig 4-c), whereas those in the osteoclast-free areas showed relatively slim cell bodies and displayed rather weaker and somewhat granular immunoreactions in the cytoplasm(Fig 4-b).

OX6- and ED1-immunopositive cell densities in the osteoclast-rich area and osteoclast-free area

On the cross sections of the molar roots, OX6- and ED1-immunopositve cell densities in both the osteoclast-rich area and osteoclast-free area of the

periodontal ligament were respectively computed and statistical analysis of the difference examined. As shown in Table 1 and Figure 6, there was a significant difference between the values from ACPase reactive area and ACPase weak area of the periodontal ligament on both the OX6- and ED1-immunopositive cell densities. OX6-immunopositive cells showed a distinct difference in cell density between the two areas, being combinant in the ACPase wear area. ED1-immunopositive cells that optically appeared to have equal distribution in the periodontal ligament also showed slight, but significant difference in cell density between the two areas. But in the case of ED1-immunopositive cells, the density was higher in the ACPase reactive, osteoclast-rich area.

DISCUSSION

Difference in distribution of antigen presenting cells in incisor- and molar periodontal ligament

In previous studies we could demonstrate presence of a large number of immunocompetent cells, the dendritic cells in particular, in the lingual periodontal ligament of rat incisors and compared their spatial distribution, morphological as well as immunohistochemical features with those of the macrophages known to have an antigen presenting capability as the dendritic cells (VAN FURTH,1986; STEINMAN, 1986). At least in the lingual aspect of the periodontal ligament of rat incisors where we had examined, the distribution pattern and morphological profiles of the cells, immunoreactive respectively to OX6- and ED1-monoclonal antibodies, were basically similar. Accordingly, the cells immunopositive to either of the two types of the antibodies appeared, if not all, to belong to the same group of antigen presenting cells, though a double immunostaining with OX6- and ED1-antibodies was not successfully attempted (VAN FURTH,1986; STEINMAN, 1986).

In the present study, an abundance of dendritic cells was also confirmed in the periodontal ligament of rat molar teeth and indicated that the OX6-immunopositive dendritic cells being the cellular constituent common to the intact periodontal ligament. However, when viewed in the cross cut sections of the molar roots, the spatial distribution of the dendritic cells in the periodontal ligament was eccentric and was different from that of the ED1-immunopositive cells that showed almost even distribution throughout the ligament. As shown in Figure 4-c, the ED1 monoclonal antibody recognizes osteoclasts and its precursor cells. It is therefore possible to safely conclude that the difference between the distribution patterns of the OX6- and ED1-immunopositive cells in the molar periodontal ligament are due in part to the exclusive localization of the cells of the osteoclastic lineage in the distal

aspects of the periodontal ligament that show immunoreactivity to ED1 but not to OX6, and also to the scarceness of other types of OX6–immunopositive cells in such areas.

Since the rat molar teeth are known to undergo physiological distal migration throughout life, it can be expressed that the OX6–immunopositive dendritic cells and immunopositive macrophages are preferentially localized in the proximal, bone forming aspects of the periodontal ligament, whereas they are sparse in the distal, bone resorbing aspects of the periodontal ligament in rat molars. Thus our current observation is the first to provide morphological evidence showing a clear–cut habitat segregation between the bone–resorbing osteoclastic cell lineage and non–bone resorbing macrophagic and/or dendritic cell lineage occurring *in vivo*.

Differentiation pathways of dendritic cells and osteoclasts

It is generally accepted that both the OX6– and ED1–immunopositive cells originate from the hematopoietic stem cells in the bone marrow. Some of the marrow cells have been shown to leave marrow early to the peripheral connective tissue via blood stream where they proliferate and differentiate to become residential macrophages such as Kupffer cells in the liver or other types of antigen presenting cells under the influence of local factors, while most of the macrophages and dendritic cells derive from further differentiated monocytes arriving via blood stream (NAITO, 1993).

As regards the origin of the osteoclasts, a possibility remains that they differentiate secondarily in the peripheral tissues from monocytes as is the case for the macrophages. However, a large body of experimental evidence has suggested that the differentiation pathway of the cells of the osteoclast lineage is determined prior to the differentiation of monocytes has started (SCOTT,1967a and b; RIFKIN et al., 1967b; HORTON et al., 1984; EJIRI and OZAWA, 1984; SCHEVEN, 1986A; MARKS and POPOFF, 1988;

PETER and GROOTH, 1992; SUDA et al., 1992). Nevertheless, most of the above data are of the *in vitro* experiments and, hence, a clear-cut distinction between the macrophagic and osteoclastic precursor cells in the peripheral connective tissue *in vivo* can not be made before the latter type of cells further differentiate and acquire phenotypical expression typical of bone resorbing cells; large number of mitochondria and relatively small amounts of rough-surfaced endoplasmic reticulum, and cytoplasmic vesicles showing intense ACPase activity (EJIRI, 1983; EJIRI and OZAWA, 1984).

A morphological marker whereby a discrimination between the two types of the precursor cells either of the bone resorbing, osteoclastic cell lineage and of the non-bone resorbing, macrophagic and/or dendritic cell lineage *in vivo* can be madehas thus been awaited.

Molar periodontal ligament as a model system

As already mentioned, our current study is the first to show a clear cut *in vivo* morphological evidence that the cells of the bone-resorptive, osteoclastic lineage and those of the non-bone resorptive, macrophagic and/or dendritic cell lineages are respectively localized in roughly the distal and proximal regions of the periodontal ligament of rat molars. To our knowledge, the periodontal ligament of rat molars is so far the only tissue where an apparent habitat segregation between the bone resorptive- and non-bone resorptive cells of the monocyte origin has been identified.

Although no conclusive explanation has yet been given to be able to explain why dendritic cell population is so small in the portion of the periodontal ligament where the cells showing intense ACPase activities are prevalent, it is important to note that such a condition is maintained and lasts throughout life in the molar periodontal ligament due to physiological distal migration of molar teeth (VIGNERY and BARON, 1980; ROBINSON and

SCHNEIDER, 1992; LASFARGUES and SAFFAR, 1993). It is speculated that if an orthodontic force is being applied to rat molars so that the direction of tooth movement is changed from physiological one to the opposite direction, a drastic shift of the sites of bone resorption and formation should be induced rather rapidly. In this context, the periodontal ligament of rat molars is expected to become an exquisite model tissue whereby the origin and differentiation pathways of the bone resorptive- and non-bone resorptive cells of monocyte origin can be examined further *in vivo*.

Our preliminary ultracytochemical observations have in fact shown undifferentiated mononuclear wondering cells displaying osteoclast-like intensely ACPase reactive tubulovesicular structures in the cytoplasm to be localized exclusively in the distal aspects of the periodontal ligament, and their absence in the proximal ligament of rat molars (unpublished data). Histocytochemical and immunohistochemical observations of molar periodontal ligament under an orthodontic force are currently in progress in our laboratory by means of light- and electron microscopy.

In conclusion, a clear-cut habitat segregation between the cells of the bone resorptive cell lineage and non-bone resorptive, macrophagic and/or dendritic cell lineage has been confirmed in rat molar periodontal ligament by immunohistochemistry and enzyme histochemistry. An advantage of the use of rat molar periodontal ligament as a model system for pursuing differentiation pathways of cells of the monocyte lineage is proposed.

Acknowledgments.

The authors express their thanks to Mr. M. HOSHINO and K. TAKEUCHI for their technical assistance.

References

BURSTONE, M. S.:
Histochemical demonstration of acid phosphatases in frozen sections with naphthol AS–phosphates.
J. Histochem. Cytochem. 9: 146–153 (1961).

DIJKSTRA, C. D., E. A. JOPP P. JOLING and G. KARAAL:
The heterogeneity of monoclonal phagocytes in lymphoid organs: Distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2 and ED3. Immunology 54: 598–599(1985).

EJIRI, S.:
The preosteoclast and its cytodifferentiation into the osteoclast: ultrastructural and histochemical studies of rat fetal parietal bone. Ach. histol. Jap., 46: 533–557 (1983).

EJIRI, S. and H. OZAWA:
Identification and characterization of the preosteoclast and its cytodifferentiation into the osteoclast: Ultrastructural and histochemical studies of rat fetal parietal bone.
In: Endocrine Control of Bone and Calcium Metabolism. D.V.Cohn, T.Fujita, J.T.Potts, Jr and R.V.Talmage, eds. Elsevier, Amsterdam, pp90–93 (1984).

HORTON, J. E., E. F. RIMMER, D. LEWIS, J. A. S. PRINGLE, K. FULLER and T. J. CHAMBERS:
Cell surface characterization of the human osteoclast: Phenotype relationship to other bone marrow–derived cell types.
J. Pathol. 144: 281–294 (1984).

HSU, S. -M., L. RAINE and H. FANGER:
Use of avidin–biotin–peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabeled antibody (PAP) procedures.
J. Histochem. Cytochem. 29: 577–580 (1981).

INABA, K.:
The dendritic cell system. Medical Immunol.vol.25 No1 (1993) 13–22.(In Japanese)

INABA, K., RALPH M.STEINMAN, MARGIT WITMER PACK, HIKEKI AYA, MUNEO INABA, TETUO SUDO, STEPHAEN WOLPE and GEROLD SCHULER:
Identification of Proliferating Dendritic cell Precursors in Mouse Blood. J. Exp. Med. Vol175, May (1992) 1157–1167.

KAWAHARA, I., Y. TAKANO, O. SATO, T. MAEDA and K. KANNARI:
Histochemical and Immunohistochemical Demonstration of Macrophages and Dendritic cells in the Lingual Periodontal Ligament of Rat Incisors.
Arch. Histol. Cytol. 55: 211–217 (1992).

KAWAHARA, I. and Y. TAKANO:
Immnuohistochemical characterization of MHC class II antigen presenting cells in the periodontal ligament of rat incisors.
Connect. Tissue Res. Vol. 31, No. 3, pp1–10 (1995).

LASFARGUES, J. J. and J. L. SFFAR:
Inhibition of Prostanoid Synthesis Depresses Alveolar Bone Resorption but

Enhances Root Resorption in the Rat.
Anat. Rec. 237: 458–465 (1993).

MARKS, C. Sandy JR., and STEVEN N. POPOFF:
Bone cell Biology: The Regulation of Development, Structure, and Function in the Skeleton.
Am. J. Anat. 183: 1–44 (1988).

MAEDA, T., O. SATO, I. KAWAHARA and Y. TAKANO:
Histochemical demonstration of acid phosphatase activity in terminal shwann cells associated with ruffini endings in the periodontal ligament of rat incisors.
Arch. Oral Biol. 38: 611–617 (1993).

McMASTER, E.R. and A. F. WILLIAMS:
Identification of Ia glycoproteins in rat thymus and purification from rat spleen.
Europ. J.Immunol. 9: 426–433(1979).

NAITO, MAKOTO:
Macrophage Heterogeneity in Development and Differentiation.
Arch. Histol. Cytol. 56: 331–351 (1993).

NIJIWEIDE J. PETER and RICARDO DE GROOTH:
Ontogeny of the Osteoclast.
Biology and Physiology of the Osteoclast.
Edited by Barry R.Rifkin and Carol V.Gay, CRC press 1992 (Chap.4, 81–104).

RIFKIN, B. R., J. S. BRAND, J. E. CUSHING, J. J. COLEMAN and F. SANAVI:
Fine structure of fetal rat calavarum: Provision identification of pre–osteoclasts.
Calcif. Tissue Int. 31: 21–28 (1980).

ROBINSON, A. JOHN and BERNARD J. SCHNEIDER:
Histological evolution of the effect of transsepital fibre resection on the rate of physiological migration of rat molar teeth. Arch. Oral Biol. 37: 371–375 (1992).

SCHEVEN, B. A. A., E. M. M. KAWILARANG–DE HAAS, A. –M. WASSENAAR and P. J. NIJIWEID:
Differentiation kinetics of osteoclasts in the periosteum of embryonic bones in vivo and in vitro.
Anat. Rec. 214: 418–423 (1986a).

SCOTT, B. L:
The occurrence of specific cytoplasmic granules in the osteoclast.
J. Ultrastruct. Res. 19: 417–431 (1967a).

SCOTTO, B. L:
Thymidine–3H electron microscope radioautography of osteogenic cells in the fetal rat.
J. Cell Biol. 35: 115–126 (1967b).

STEINMAN, R.M., W.C.VAN VOORHIS and D.M.SPALDING:
Dendritic cells.
Handbook of experimental immunology vol.2 Cellular Immunology 4th edition. edited by D.M.Weir, M.D.Frcp, Blachwell Scientific Publications, Oxford 1986 (Chap.49, 1–9)

SUDA, T., TAKAHASHI N., and MARTIN J.:
Modulation of osteoclast differentiation.
Endocrino. Rev. 13: 66–80 (1992).

VAN FURTH, R.:
Overview: the mononuclear phagocyte system.
Handbook of experimental immunology vol.2 Cellular Immunology 4th edition. edited by D.M.Weir, M.D.Frcp, Blackwell Scientific Publications, Oxford 1986 (Chap.42, 1–5)

VIGNERY, AGNES and ROLAND BARON:
Dynamic Histomorphometry of Alveolar Bone Remodeling in the Adult Rat.
Anat. Rec. 196: 191–200 (1980).

FIGURES

All the pictures are aligned so that the right hand side indicates distal direction and top of the pictures, buccal direction.

Fig. 1: ACPase reactions (Azo dye method) in the periodontal ligament of upper second molars (cross section).
a) Note a region-specific localization of ACPase reactive cells in the ligament of each root (*). b) Enlarged view of the disto-buccal root, showing intense ACPase reactions of the osteoclasts (single arrows) and mononuclear cells around the root (arrow heads). Double arrows indicate resorption cavities on the root surface.
A, alveolar bone. p, periodontal ligament. d, dentin. pu, dental pulp. v, blood vessels. bar, 0.5mm.

Fig. 2: Immunoreactions to an OX6-monoclonal antibody in the periodontal ligament of upper second molar. a) Sagittal section through an upper second molar root, showing many immunopositive cells in the periodontal ligament. b) Cross section of distal roots of upper second molar showing eccentric distribution of immunopositive cells. *, low cell density area. d, dentin. pu, dental pulp. A, alveolar bone. bar, 0.2mm.

Fig. 3: A double staining for ACPase histochemical reactions(red) and OX6 immuno-reactivity (black). a) A habitat segregation between OX6-immunopositive cells and intensely ACPase reactive cells in the periodontal ligament is displayed.
b) Higher magnification of the disto-lingual root.
A, alveolar bone. d, dentin. pu, pulp. *, ACPase reactive area. bar, 0.5mm.

Fig. 4: Immunoreactions to an ED1-monoclonal antibody in the periodontal

ligament of upper second molar (cross-cut section). a) In each root, small immunoreactive cells are located almost evenly throughout the periodontal ligament. Large osteoclasts (arrows) display most intense immunoreactions. bar: 0.5mm.
b) Enlarged view of the immunoreactive cells in the proximal portion of the periodontal ligament. The ED1-immunoreactive cells have large cytoplasmic projections and show spindle-or dendritic profiles. Some cells show granular immunoreactions in the cytoplasm. bar: 0.1mm.
c) Immunoreactive cells in the distal resorption of the periodontal ligament where large osteoclasts (arrows) show intense immunoreactions. Besides osteoclasts, round or oval shaped small cells also show stronger immunoreactions compared with those in the proximal region of the ligament. bar: 0.1mm.
v, blood vessels.

Fig. 5: Schematic diagram showing the areas for statistical analysis of immunopositive cells. A cross-cut sectional image of a rat molar root.

Fig. 6: A diagrammatic representation of OX6- or ED1-immunopositive cell densities in the ACPase-reactive and ACPase-weak or non reactive regions of rat molar periodontal ligament.

The values in the ACPase-reactive regions represent density of immunopositive cells in regions undergoing bone resorption, whereas those in ACPase weak or non reactive regions, those undergoing bone formation. See text for the detail.

Table 1: Mean of density and statistical analysis of OX6- or ED1-immunopositive cells in a unit area of the periodontal ligament from ACPase-reactive and ACPase-weak areas as illustrated in Figure 6.

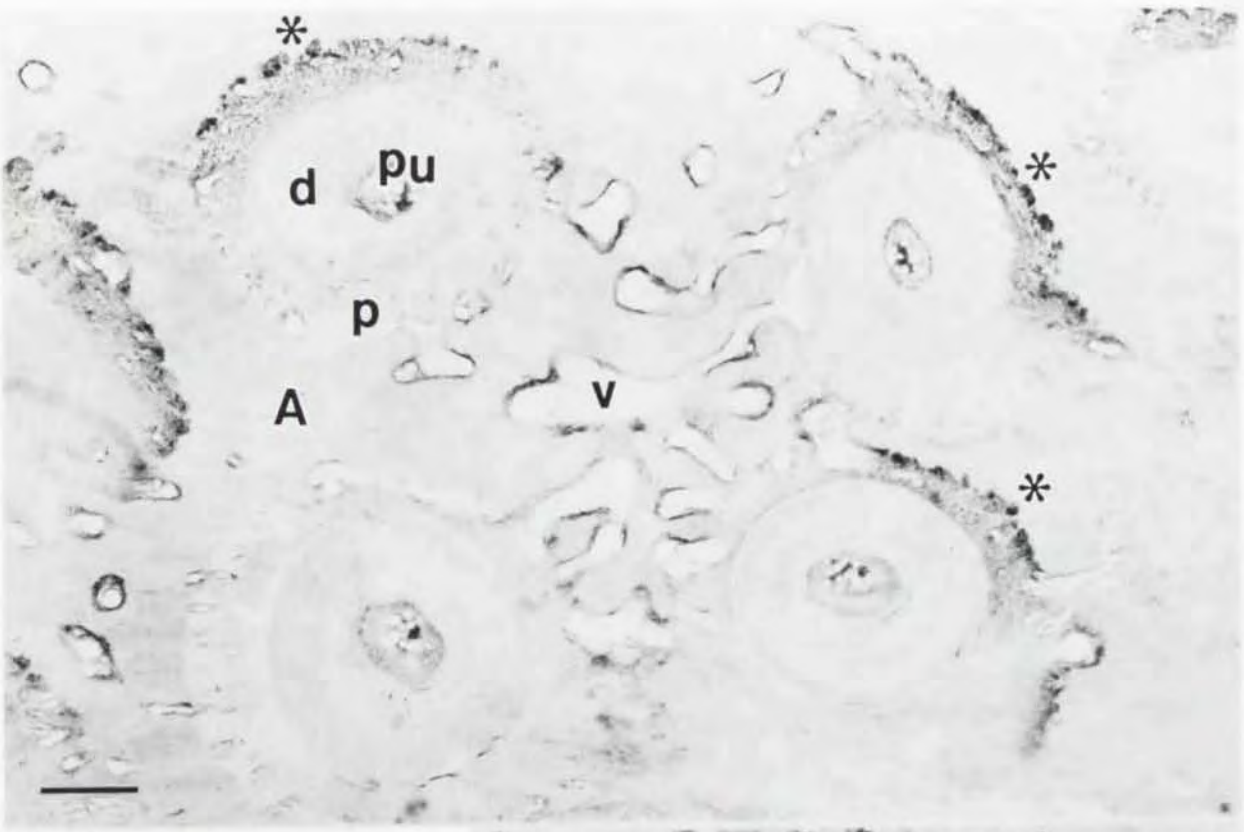
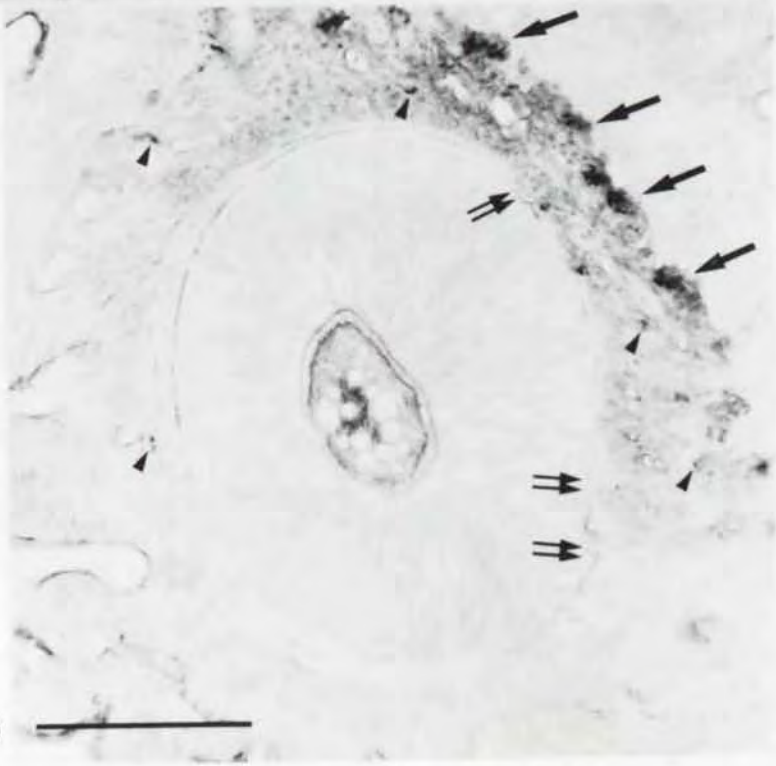


Fig. 1. **a**



b

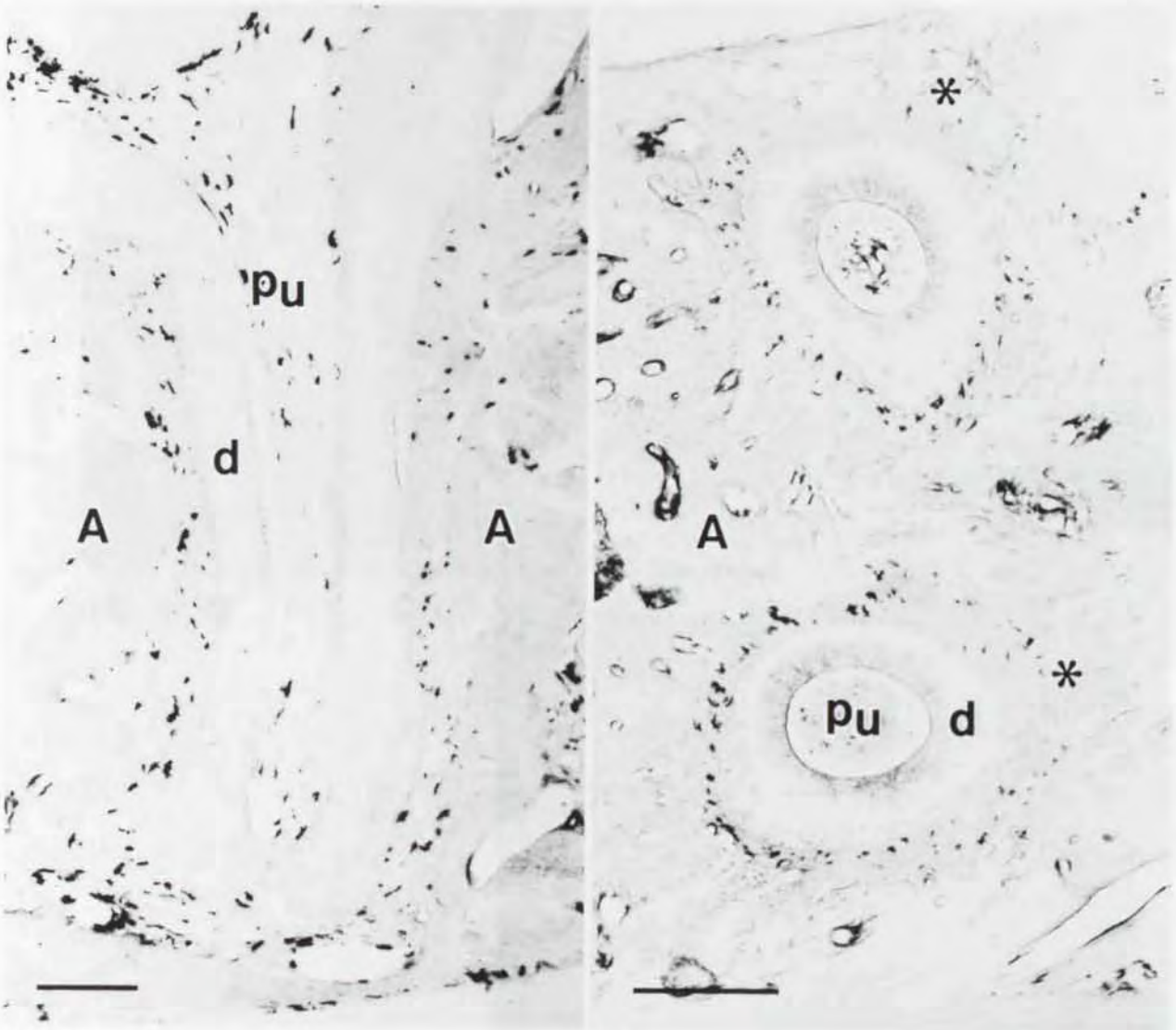


Fig. 2. a b

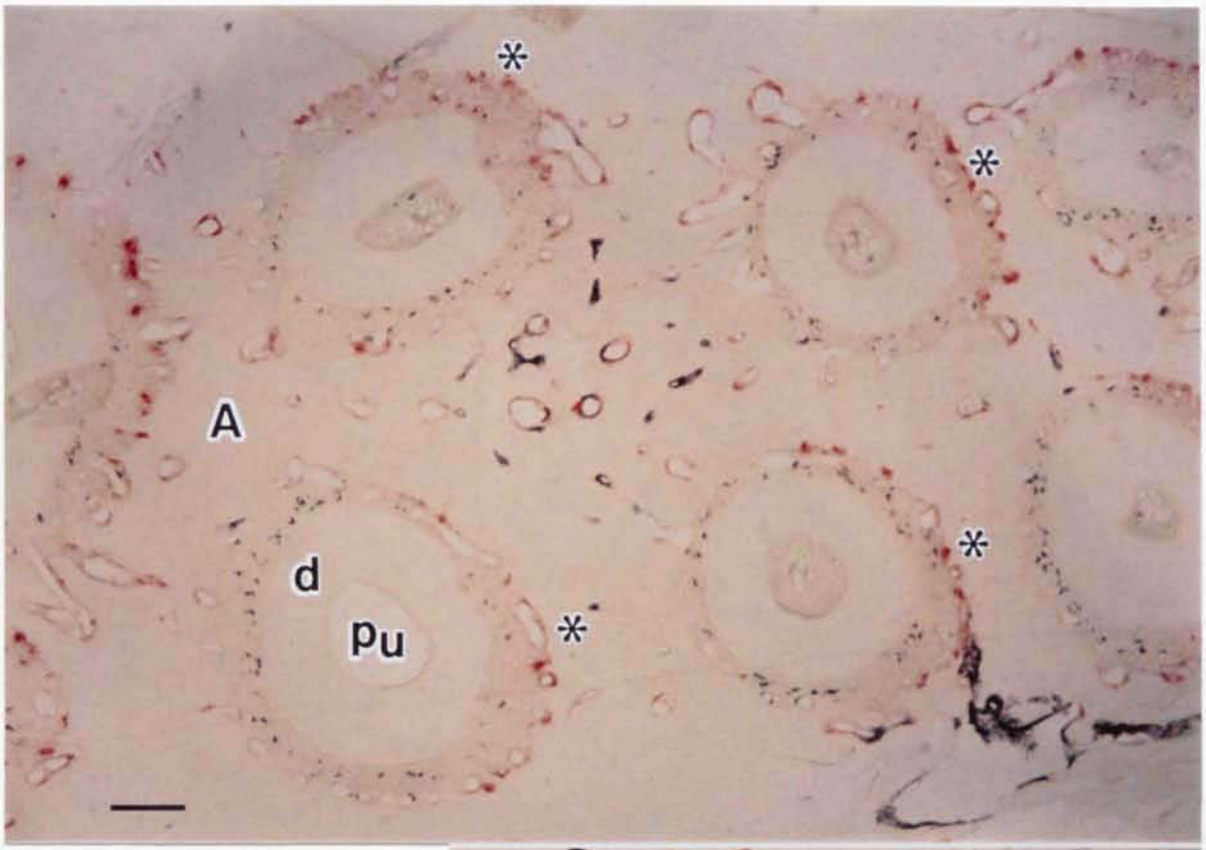
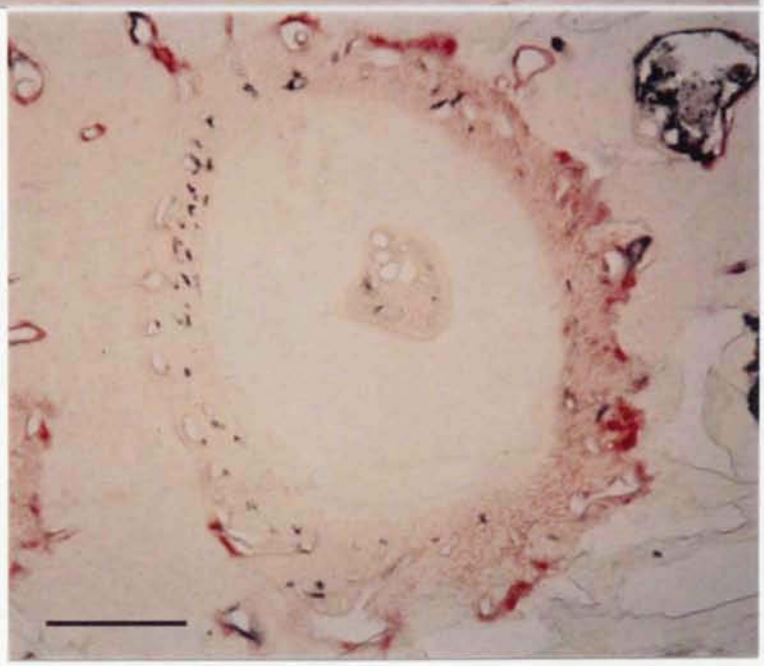


Fig. 3. **a**



b

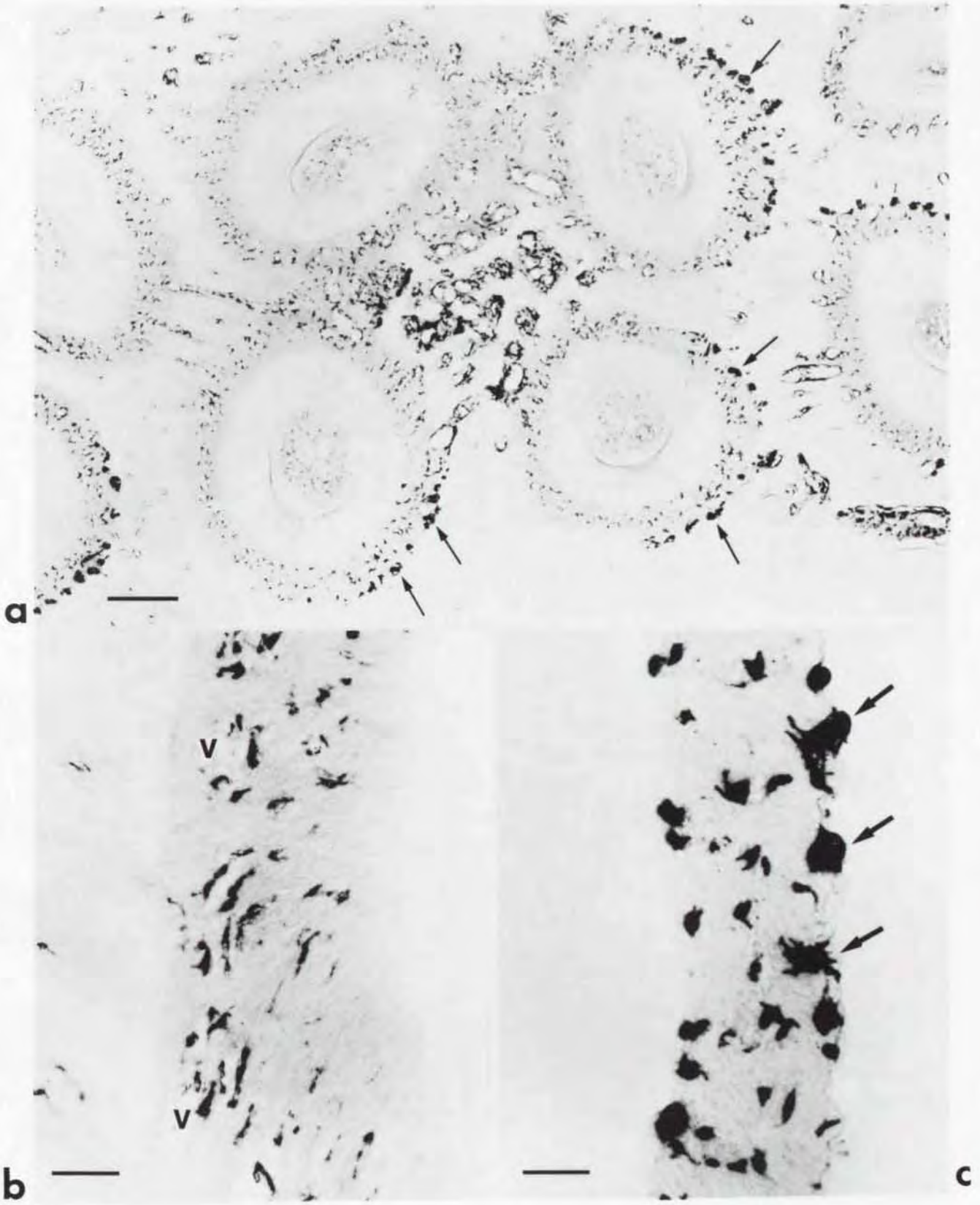


Fig. 4.

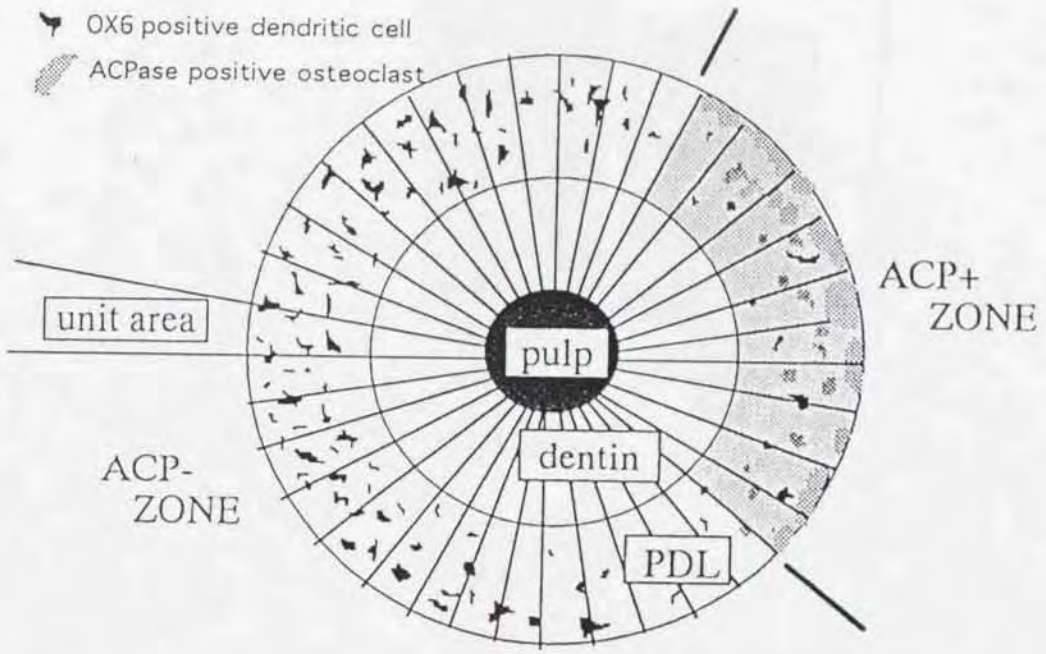

OX6 positive dendritic cell
ACPase positive osteoclast
unit area
ACP+
ZONE
pulp
dentin
ACP-
ZONE
PDL

Fig. 5.

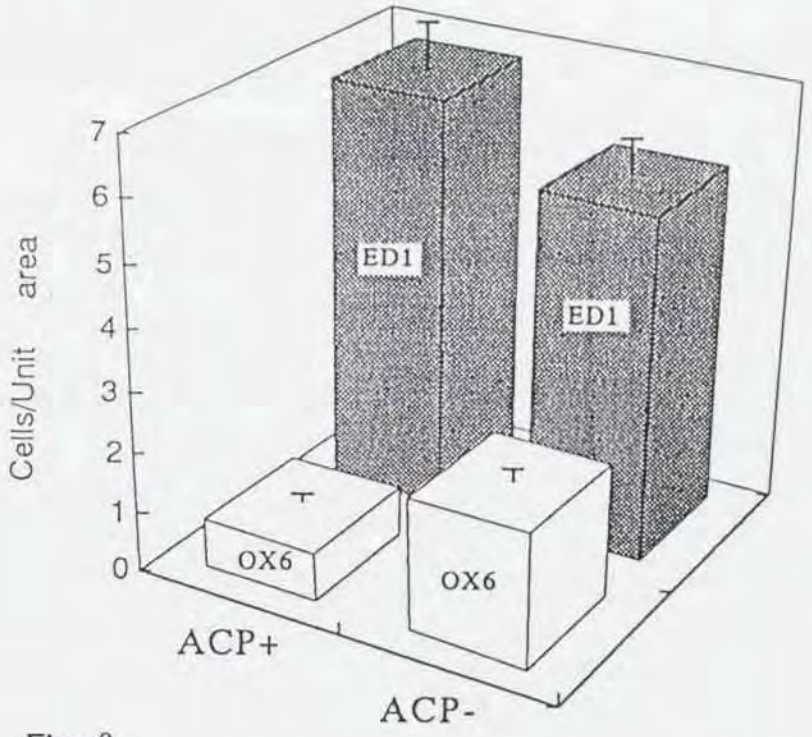


Fig. 6.

		MEAN	PAIRD T-TEST		
			MEAN OF DIFFERENCE	SD OF DIFFERENCE	SIGNIFICANT
OX6 n=28	ACPase+	0.81429±0.15238	-1.41429	0.597968	P<0.001
	ACPase−	2.22857±0.291			
ED1 n=13	ACPase+	6.76154±1.71422	1.11538	1.06915	P<0.01
	ACPase−	5.64615±1.19769			

Table 1.