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Production of monoclonal antibodies against mouse molar papilla cells

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ABSTRACT. To develop markers for the analysis of the molecular mechanisms of dental papilla cells differentiation, 10 monoclonal antibodies were produced against trypsin-isolated mouse molar dental papilla cells. These antibodies identify matrix components, cell membrane associated antigens and intracellular-constituents. Changes of the staining patterns were correlated with a typological hierarchy of dental papilla cells and with terminal differentiation of odontoblasts.

KEY WORDS: *monoclonal antibodies, immunofluorescence, dental papilla cells, differentiation*

Introduction

Histological, cytological and functional organization determine the identity of odontoblasts. It is not known whether the presumptive dental papilla is composed of distinct odontoblast and pulp-cell progenitors or if all the dental papilla cells are potential odontoblasts but only some of them will overtly differentiate according to a specific spatial distribution of epigenetic signals. Furthermore the possible typological hierarchy of maturing dental papilla cells is not well known (see Ruch, 1987 for review). A monoclonal approach might provide information concerning the lineage potential of these cells. In a previous paper we provided data concerning five monoclonals directed against native mouse molar papillae (Zidan and Ruch, 1987). In this paper we summarize preliminary observations concerning 10 monoclonals generated by immunization of one rat with trypsin-isolated mouse molar papilla cells.

Results

Hybridoma cells obtained from 10 of 170 wells produced antibodies reacting with dental tissues with good staining intensity. Table 1 summarizes the initial immunoglobulin isotypes and the selected monoclonals. The cloning led mainly to selection of IgM's. Cloning of MC22-3 by repeated limiting dilution and finally using a micropipette to isolate small colonies did not allow the separation of the initial immunoglobulins.

The main data of indirect immunofluorescence are summarized in Table 2. According to the staining patterns extracellular components, plasma membrane associated epitopes and intracellular antigens were recognized.

Extracellular components

-Antibodies MC22-3B stained the dental papilla including the preodontoblast-layer. The odontoblast layer, the enamel organ predentin-dentin and enamel were negative. The staining of day-14 head sections revealed strong reactivity of bone and some mesenchyme around the oral epithelium. Fluorescence was observed in the mesenchyme located at the lingual side of the dental bud (Fig. 1).

-Monoclonals MC22-9F and MC22-9I, stained fibrilar structures of the dental papilla. Intense fluorescence was detected at the secretory pole of odontoblasts. The enamel organ was not stained. In day-14 head sections antigen defined by these monoclonals was present in the brain, retina and dermis. The mesenchyme around the dental bud showed very faint staining (Fig. 2).

-Monoclonal MC22-45D reacted with an enamel component (Fig.3).

Plasma membrane associated antigens

-Monoclonal MC22-14A reacted intensely with dental papilla cells. Antigen of MC22-14A concentrated at the secretory pole of odontoblasts. The preameloblasts-ameloblasts were negative whilst the cells of the *stratum intermedium* and the *stellate reticulum* were stained.

The staining of day-14 and -16 head sections revealed intense fluorescence at the epithelial-mesenchymal junctions. The retina, the brain, bone and dermis were also stained. Permeabilized and not permeabilized dental cells of primary culture were stained (Fig. 4).

-Monoclonals MC22-33D and 33F stained with changing intensity most of the examined tissues. Non permeabilized and permeabilized dental cells were stained. The antigen defined by these antibodies was enriched in odontoblasts (Fig. 5).

-Monoclonal MC22-37Y reacted more or less uniformly

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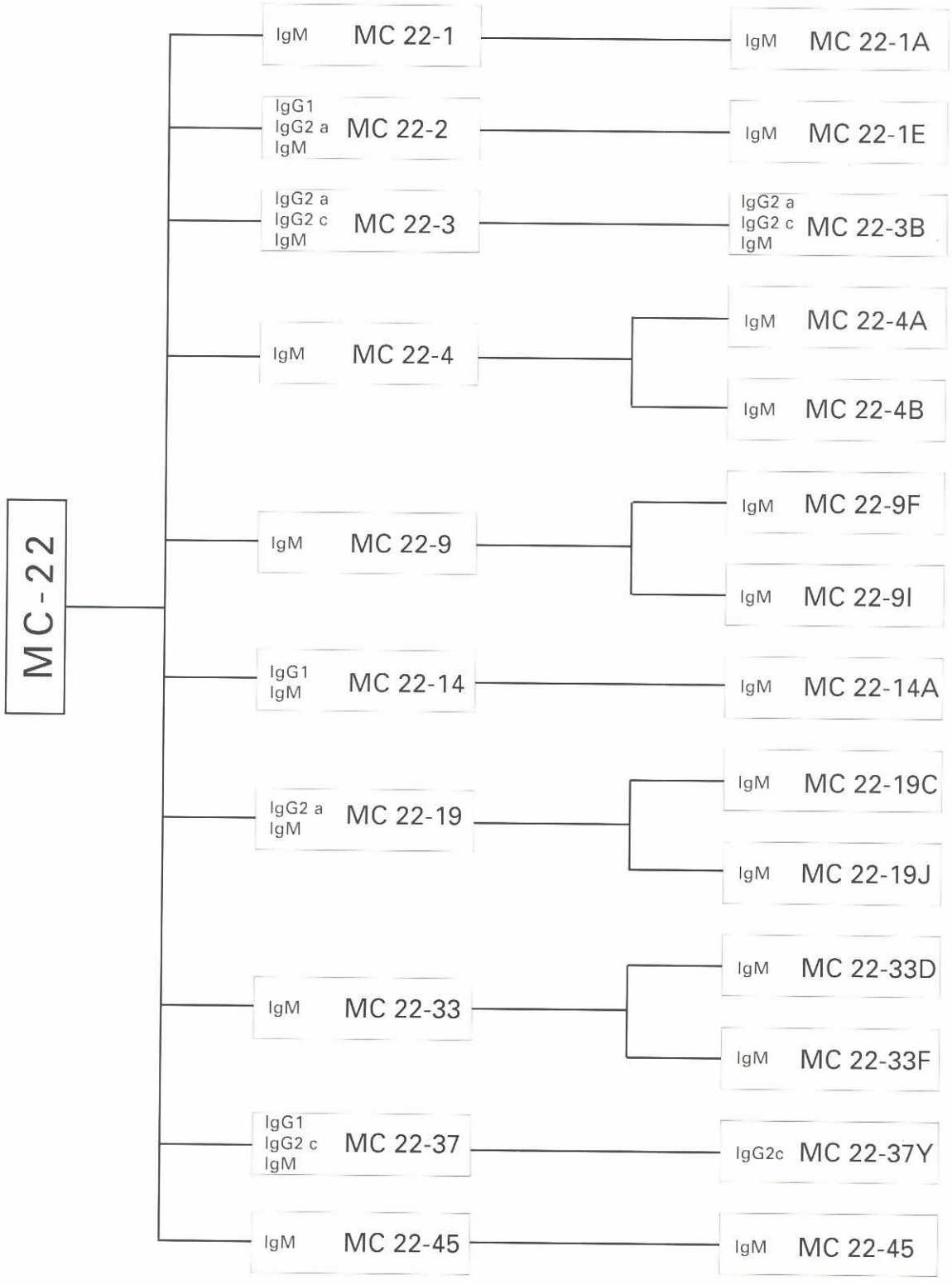
TABLE 1

FILIATION OF THE PRODUCED MONOCLONALS



with most of the stained tissues. However, bone did not react. Non permeabilized and permeabilized dental cells were stained. (Fig. 6).

Intracellular components

-Monoclonal MC22-2E stained mainly epithelial cells. The inner and outer dental epithelia, the *stratum intermedium* and the *stellate reticulum* reacted with this antibody. The identified antigen concentrated at the secretory pole of the ameloblasts. The day-14 head-sections revealed staining of the dental bud, the oral epithelium and the epidermis. Permeabilized dental epithelial cells were stained (Fig. 7).

-Monoclonal MC22-4A and 4B are bound to the whole enamel organ. Cells of the *stratum intermedium* and *stellate reticulum* reacted very intensely. Staining of day-14 and -16 head sections demonstrated staining of the dental bud, the oral epithelium, the epidermis and dermis and cartilage. The reactivity of dental mesenchyme decreased during odontogenesis. Permeabilized dental epithelial cells were stained (Fig. 8).

-Monoclonals MC22-19C and 19J reacted intensely with the odontoblasts and weakly with dental papilla cells and the enamel organ. The day-14 and -16 head sections revealed positive reactions with the dental bud and the enamel organ. The oral epithelium, the epidermis, cartilage, brain and retina were stained.

Permeabilized dental epitelial cells showed positive reactions (Fig. 9).

Extracellular and intracellular components

-Monoclonal MC22-1A stained both extracellular enamel and the ameloblasts. Preameloblasts, cells of the outer dental epithelium, also reacted.

The dental bud, the oral epithelium, epidermis and cartilage of day-14 head sections were also stained. Permeabilized dental epithelial cells were positive (Fig. 10).

Discussion

The dental papilla cells control the histo-morphogenesis of the enamel organ and are capable of differentiating into odontoblasts secreting predentin-dentin matrix (for review see Ruch, 1987). Immunohistochemistry using antibodies directed against defined components, i.e. matrix molecules, cell membrane associated antigens and cytoskeleton components has provided some interesting data: the distribution patterns of 1) tenascin (Thesleff *et al.*, 1987), 2) EGF binding sites (Partanen and Thesleff, 1987), 3) cell surface proteoglycan (Thesleff *et al.*, 1988), 4) cell adhesion molecules (Thesleff, III International Tooth Morphogenesis and Differentiation Workshop, Aland, 1986), 5) chondroitin sulfates (Mark and Ruch, manuscript submitted) and 6) TGB (Heine *et al.*, 1987) have been correlated with morphogenetic aspects of odontogenesis.

The pattern of distribution of 1) fibronectin (Lesot *et al.*, 1981), 2) cell surface antigen 165 kd (Lesot et al., 1988) and 3) cytoskeleton components (Lesot *et al.*, 1982; Kubler *et al.*, 1988) have been correlated with cytodifferentiation of odontoblasts.

Immunohistochemistry provides a sensitive approach for the localization of molecules *in situ*. However even if highly purified antigen preparations are used the antibody population obtained is directed at best against numerous antigenic sites and at worst also against contaminants. The monoclonal approach leading to the detection of specific epitopes might improve our knowledge of the molecular basis of dental papilla cells potencies. A previous immunization using intact mouse molar dental papillae as immunogen (Zidan and Ruch, 1987) allowed the generation of 5 distinct monoclonals directed against matrix components and intracellular antigens. These antigens were not tooth-specific. Two of the epitopes disappeared during terminal differentiation of odontoblasts. Vainio *et al.* (1988) injected fixed and sonicated mouse dental papillae and generated 6 antibodies recognizing intracellular components. One of them was enriched in secretory odontoblasts and ameloblasts. These antigens were not tooth specific.

The immunization with trypsin-isolated dental papilla cells allowed the generation of 10 distinct, new monoclonals. Only one of them - MC22-45D - is tooth specific and recognizes an enamel constituent. Preliminary western-blots indicate that the defined antigen is an amelogenin. MC22-45D reacted with a number of bands in enamel extracts with molecular weight material in the range of 13-22 Kd. Furthermore, to see whether MC22-45D could affect odontogenesis, day-15 molars containing only preodontoblasts and preameloblasts were cultured for 8 days in control medium or in the presence of MC22-45D hybridoma cells. In the presence of MC22-45D the odontoblasts did not differentiate. Hybridomas producing unrelated antibodies had no effect.

Two further antibodies (MC22-3B, MC22-9F and 9I) interact with extracellular constituents. The staining patterns of MC22-3B are different from the fluorescence produced using polyclonal antibodies directed against collagen type I, type III, fibronectin and tenascin (Lesot *et al.*, 1978, 1981; Thesleff *et al.*, 1979, 1987; Andujar *et al.*, 1988; Magloire *et al.* 1988). MC22-9 might recognize fibronectin. MC22-3B binds asymmetrically to the presumptive dental papilla. Later during odontogenesis the staining increased; however, the odontoblast layer has lost reactivity. On the other hand, MC22-9F and 9I identify an epitope which is enriched at the secretory pole of odontoblast.

The monoclonals MC22-14A, MC22-33D (and 33F) and MC22-37Y identify plasma membrane associated antigens which might be involved in cell-matrix and/or cell-cell interactions: preliminary data indicate that these antibodies affect the morphology of cultured cells. The anti-

TABLE 2

SUMMARY OF IMMUNOFLUORESCENT STAINING PATTERNS

Distribution		Extracellular			Plasma Membrane			Intracellular			Extra- and intra-celular
Tissues \ Monoclonals		MC 22-38	MC22-9J (91)	MC22-450	MC22-14A	MC22-330 (33F)	MC22 37Y	MC22-2E	MC22-4A (48)	MC22-19J (19C)	MC22-1A
TOOTH SECTIONS	DP	+++	++	-	+++	+	+	(+)	-	+-	-
	PO	+	++	-	++	+	+	-	-	++	-
	O	-	++(2)	-	+++(3)	+++	-	-	-	++	-
	IDE	-	-	-	-	+	++	++	++	+	+
	ODE	-	-	-	-	+-	++	+	-	+	+
	SI	-	-	-	++	+	+	++	+++	+	-
	SR	-	-	-	++	++	-	++	++	+-	-
	P.Mes	-	-	-	-	-	+	+-	-	-	-
	Dentin	-	-	-	-	-	-	-	-	-	-
	Enamel	-	-	+++	-	-	-	-	-	-	++
HEAD SECTIONS	D.Bud.	-	-	-	-	+	+	++	+	++	+
	D. Mes.	+-(1)	+	-	+-(4)	+	+	+	+-+-(5)	+-	-
	Oral EP.	-	-	-	-	-	+	++	++	++	+
	Epiderm.	-	-	-	-	+	+-	++	+	+	+
	Derma.	-	-	-	+	+	+-	+-	+	+-	-
	Epi/mes.J	-	-	-	++	-	-	-	-	-	-
	Carti.	+-	-	-	-	+	++	-	+	++	++
	Bone	++	-	-	++	+++	-	-	-	-	-
	Tongue	+	-	-	+	+	-	+-	-	-	-
	Brain	-	++	-	++	-	++	-	+	+	-
	Retina	-	+	-	++	+-	++	-	-	+	-
CELL CULTURES	EP Perm.	-	-	-	++	+++	++	++	++	++	+
	EP NP	-	-	-	++	+++	+	-	-	-	-
	Mes. Perm	-	-	-	++	++	++	-	-	+	-
	Mes. NP	-	-	-	+	++	+	-	-	-	-

- : fluorescence was absent
+- : fluorescence was faint
+, ++, +++ : increasing intensity of fluorescence

DP: dental papilla; PO: preodontoblasts; O: odontoblasts; IDE: inner dental epithelium; ODE: outer dental epitelium; SI: *stratum intermedium*; SR: *stellate reticulum*; P. Mes.: peridental mesenchyme; D. BUD. : dental bud; D. Mes.: dental mesenchyme; Oral Ep.: oral epithelium; EP/Mes.J: epithelial-mesenchymal junction; carti.: cartilage; EP Perm.: permeabilized epithelial dental cells; EP NP: non permeabilized epithelial dental cells; Mes. Perm,: permeabilized mesenchymal dental cells; Mes. NP : non permeabilized mesenchymal dental cells.

1) only the lingual mesenchyme was stained.
2) intense staining at the secretory pole.
3) intense staining of the secretory pole
4) increasing staining during odontogenesis
5) decreasing staining during odontogenesis

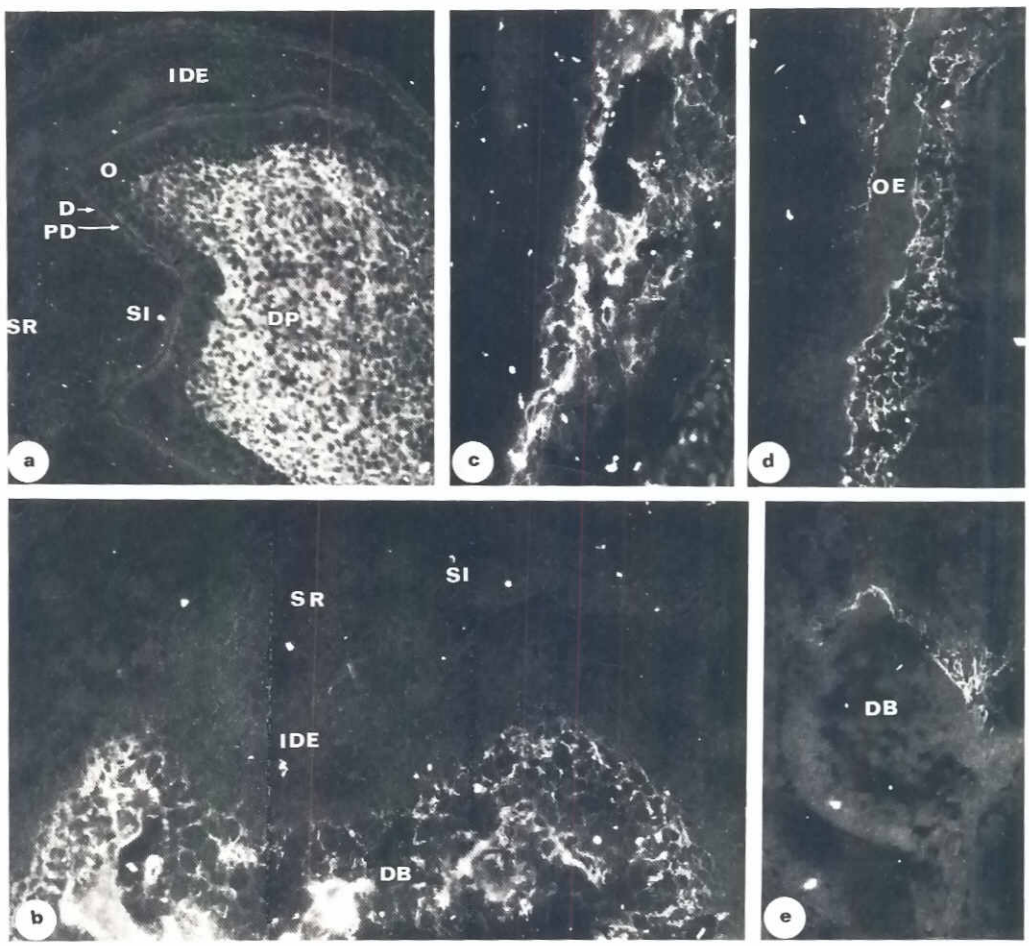


Fig. 1. Immunohistologic staining with antibodies MC22-3B. ***a*** *and* ***b*** *show respectively reactivity of dental papilla (DP) of 2 day old and day-16 embryonic mouse molars. The odontoblasts (O) and the enamel organ (EO) are negative. IDE: inner dental epithelium; SI:* stratum intermedium*; SR:* stellate reticulum; *PD: predentin; D: dentin.* ***c, d, e.*** *day-14 head sections: bone (****c****) and some mesenchyme adjacent to the oral epithelium (OE) are stained (****d****). The mesenchyme on the lingual side of the dental bud (DB) shows fluorescence (****e****).*

gens recognized by MC22-33 and MC22-14 are enriched in functional odontoblasts, whereas epitope identified by MC22-37 is not. Pera *et al.* (1988) produced monoclonals to cytostructural antigens of embryonal carcinoma cells. One of them, GCTM-2, led to very similar cell surface staining to that of MC22-33. GCTM-2 identifies the core protein of a membrane associated keratan sulfate proteoglycan.

Three monoclonals (MC22-2E; MC22-4A (4B); MC22-19J (19C)) interact with intracellular components. MC22-2E probably identifies a cytokeratin polypeptide, which demonstrates an apical accumulation in ameloblasts. Lesot *et al.* (1982) have shown such an accumulation using antibodies directed against epidermal prekeratin. MC22-4 stains an intracellular component which disappears or becomes masked during odontogenesis. The antigen defined by MC22-19 is significantly enriched in functional odontoblasts.

Finally, MC22-1A stains enamel and intracellular component present in epithelial cells and cartilage. Recently it has been shown that an enamel protein of 68 Kd shares common antigenic determinants with keratins (Lesot *et al.*, 1988).

All together these preliminary data strongly suggest the existence of a typological hierarchy of the dental papilla cell lineages: six of the present antibodies (MC22-3B; -9; -33; -14; -4 and -19) react with epitopes demonstrating changing patterns. Metabolic alterations exist during dental papilla cell "maturation" and particularly during terminal differentiation of odontoblast. Furthermore these data argue against the existence of tooth specific "morphogens". In order to generate tissue specific information, the rearranging of ubiquitous components might play an important role (Ruch, 1987).

Further investigations will include the analysis of biological effects of all these antibodies on cultured teeth and dental cells and the identification of the specific-antigens.

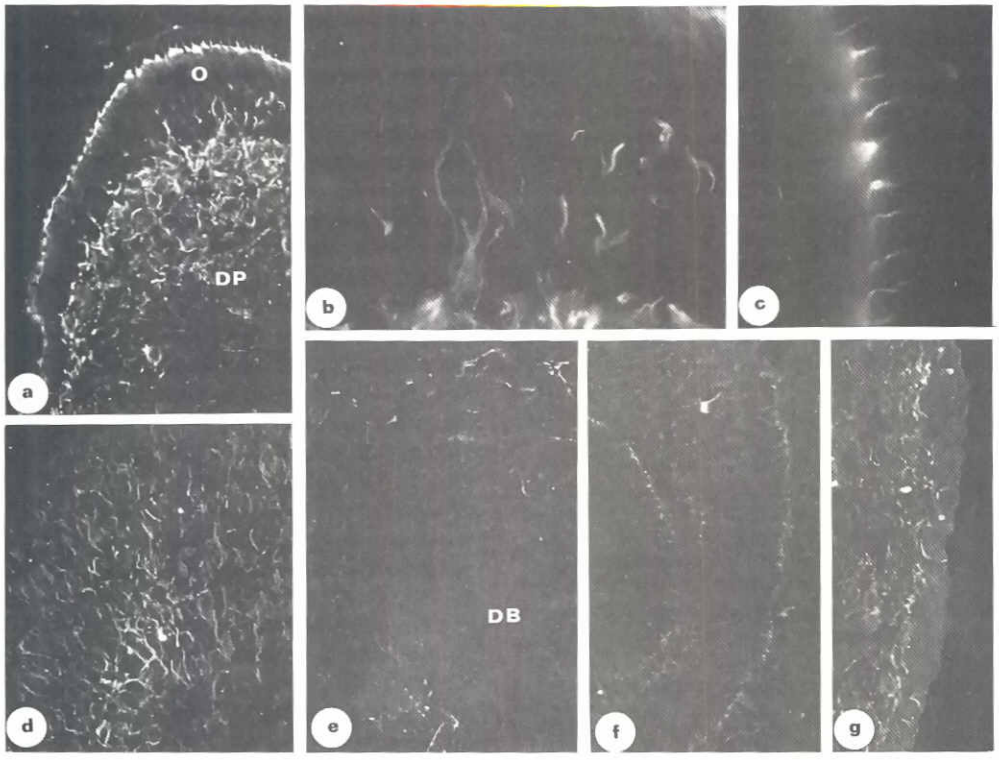


Fig. 2. Immunohistologic staining of tooth (a, b, c) and day-14 head sections (d, e, f, g) with monoclonal MC22-9I. ***a.*** *The dental papilla (DP) is stained. Antigen concentrates to the secretory pole of odontoblasts (O).* ***b.*** *Higher magnification of the dental papilla illustrating the staining of fibrilar material.* ***c.*** *The staining of odontoblast cell processes might be an artifact.* ***d.*** *The brain is positive.* ***e.*** *The dental bud (DB) is not stained whilst the mesenchyme is faintly positive.* ***f.*** *The retina is positive.* ***g.*** *The dermis shows fibrilar structures.*

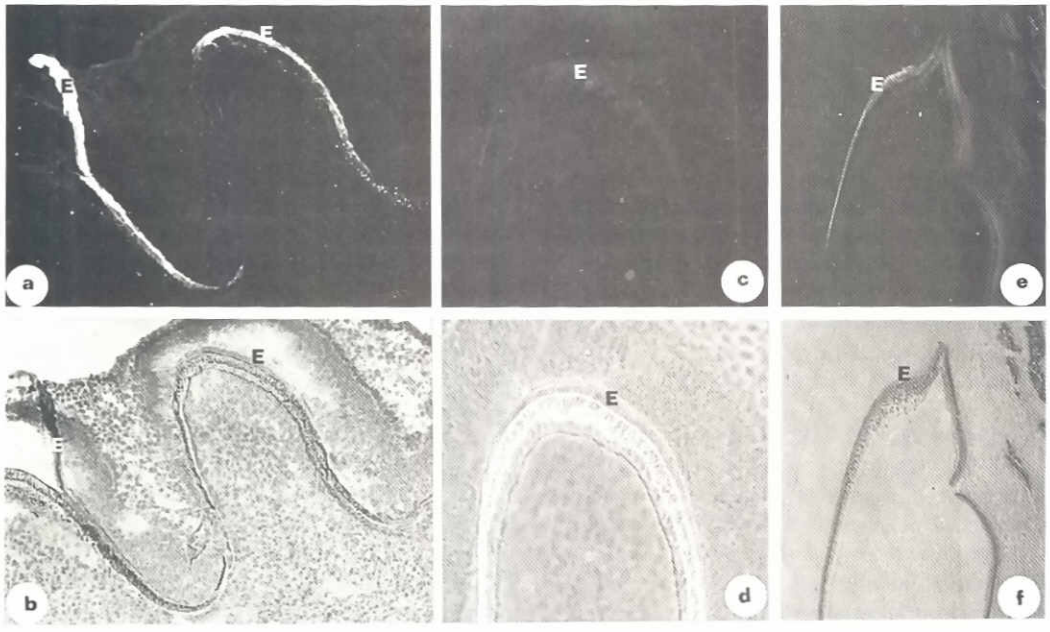


Fig. 3. Immunohistochemical staining (a, c, e) and phase contrast pictures (b, d, f) of 2 day old molars and incisors with monoclonal MC22-45D. ***a.*** *The enamel (E) reacted intensely.* ***b.*** *Corresponding phase contrast.* ***c.*** *Control section which has been incubated with PBS instead of primary antibody: the enamel (E) is negative.* ***d.*** *corresponding phase contrast.* ***e.*** *The incisor demonstrates positive reaction of the labial enamel (E).f. Corresponding phase contrast.*

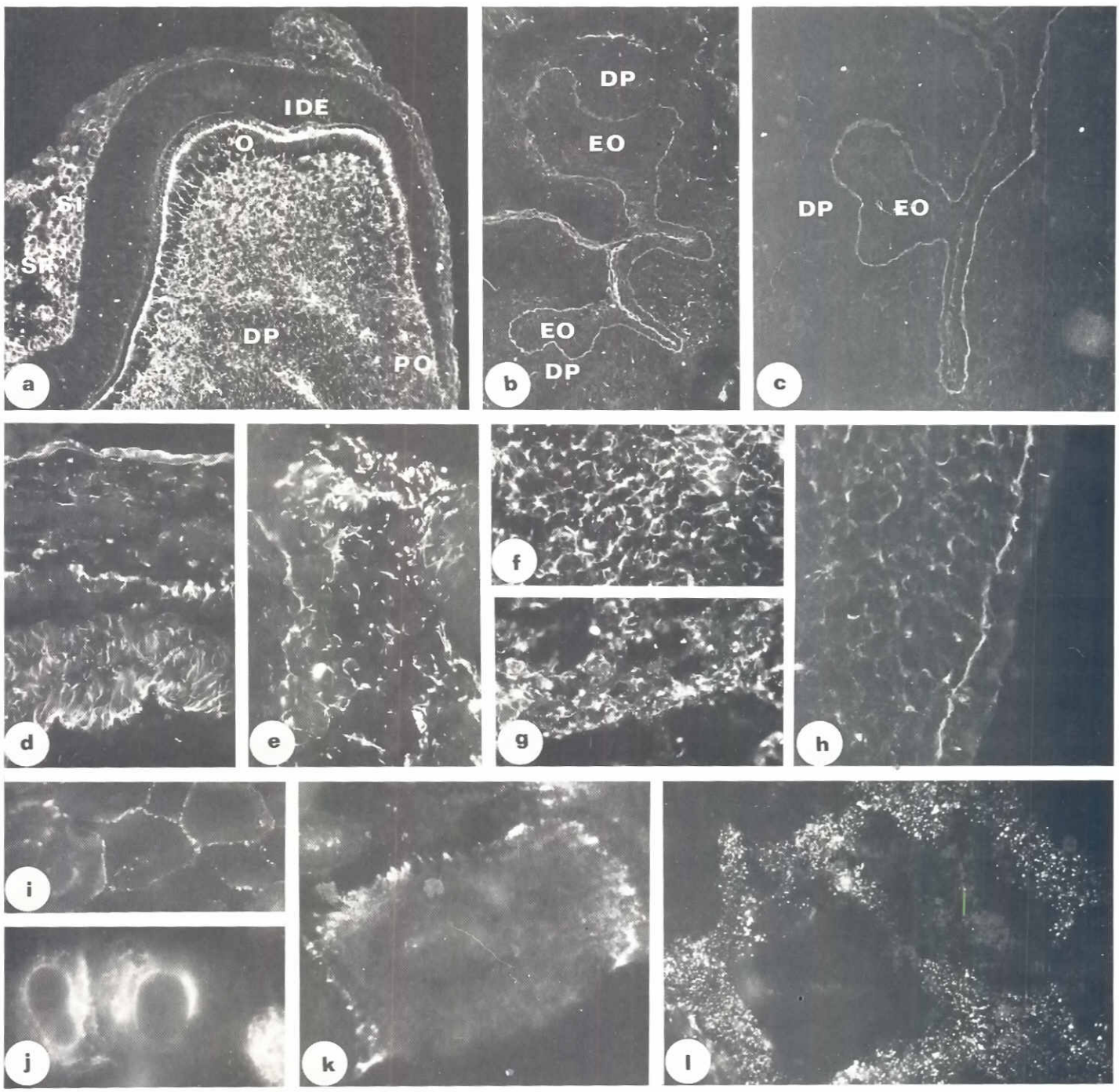


Fig. 4. Immunohistologic staining of tooth (a), day-16 head (b), and day-14 head sections (c, d, e, f, g, h) and dental cells (i, j, k, l) with monoclonal MC22-14A. ***a.*** *The dental papilla cells (DP) including preodontoblasts (PO) are stained. Antigen defined by MC22-14A is enriched at the secretory pole of odontoblasts (O). The inner dental epithelium (IDE)is negative. Cells of the* stratum intermedium *(SI) and the* stellate reticulum *(SR) are fluorescent.* ***b, c.*** *Day-16 and -14 tooth germs demonstrate strong fluorescence of the epithelial-mesenchymal junction. The cells of the enamel organ (EO) and the dental papilla (DP) are faintly stained.* ***d, e.*** *The retina (d) and the optic nerve (e) are positive.* ***f, g.*** *Brain cells (f) and bone cells (g) are stained h. The epidermal-dermal junction and derma are positive.i, k. Permeabilized dental epithelial cells showing accumulation of the antigen at the periphery of the cells.l. Non permeabilized dental epithelial cells showing granular staining.* ***j.*** *Positive permeabilized dental papilla cells.*

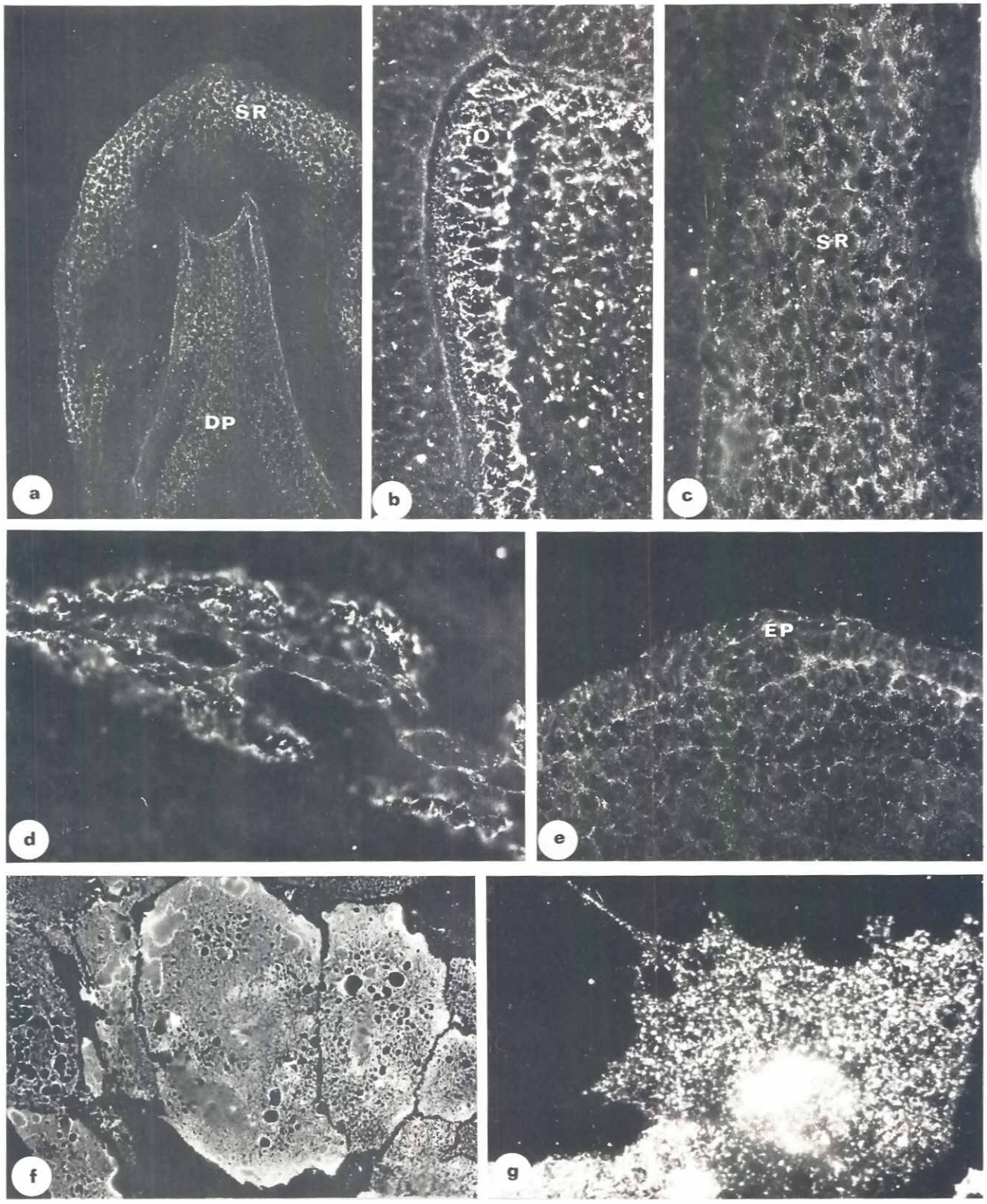


Fig. 5. Immunohistologic staining of tooth (a, b, c), and day-14 head sections (d, e) and dental cells (f, g) with monoclonal MC22-33D. ***a****. Dental papilla cells (DP), and cells of the* stellate reticulum *(SR) react positively.* ***b****,* ***c****. Higher magnifications illustrating the intense staining of odontoblasts (O) and the cells of the* stellate reticulum *(SR).* ***d****,* ***e****. Bone cells (d) epidermal (EP) and dermal cells (e) are positive.* ***f****,* ***g****. Staining patterns of non permeabilized (f) and permeabilized (g) dental epithelial cells.*

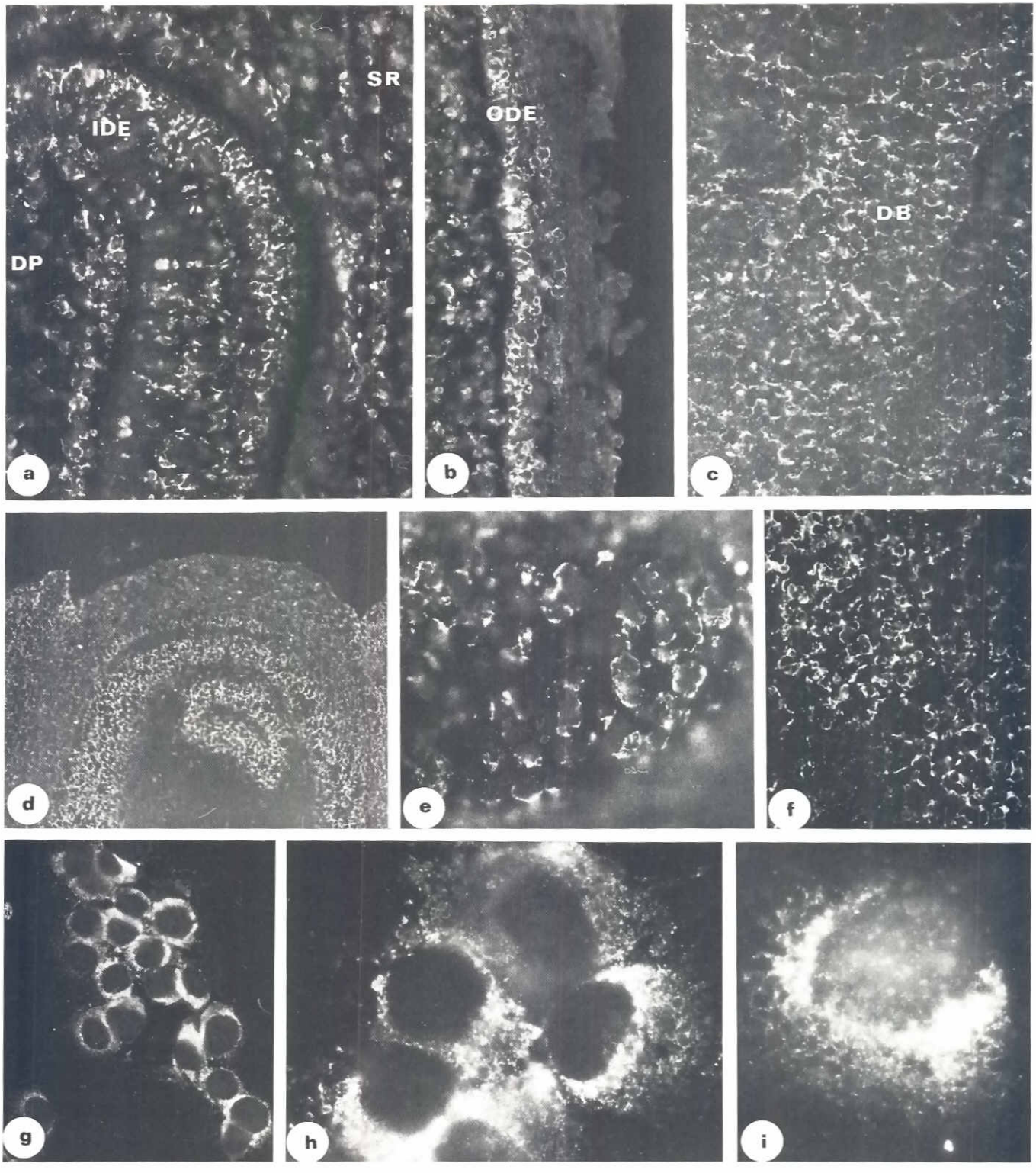


Fig. 6. Immunohistologic staining of tooth (a, b) and day-14 head sections (c, d, e, f) and dental cells (g, h, i) with monoclonal MC22-37Y. ***a****. Dental papilla cells (DP), inner dental epithelium (IDE) and cells of the* stellate reticulum *(SR) are positive.* ***b****. Staining of cells of the outer dental epithelium (ODE).* ***c****. The cells of the dental bud (DB) and adjacent mesenchyme react with MC22-37Y.* ***d****,* ***e****,* ***f****. Positive reactions in the eye (d), cartilage (e) and brain (f).* ***g****,* ***h****,* ***i****. Perinuclear positive reaction of permeabilized dental papilla cells (g, h) and permeabilized dental epithelial cell (i).*

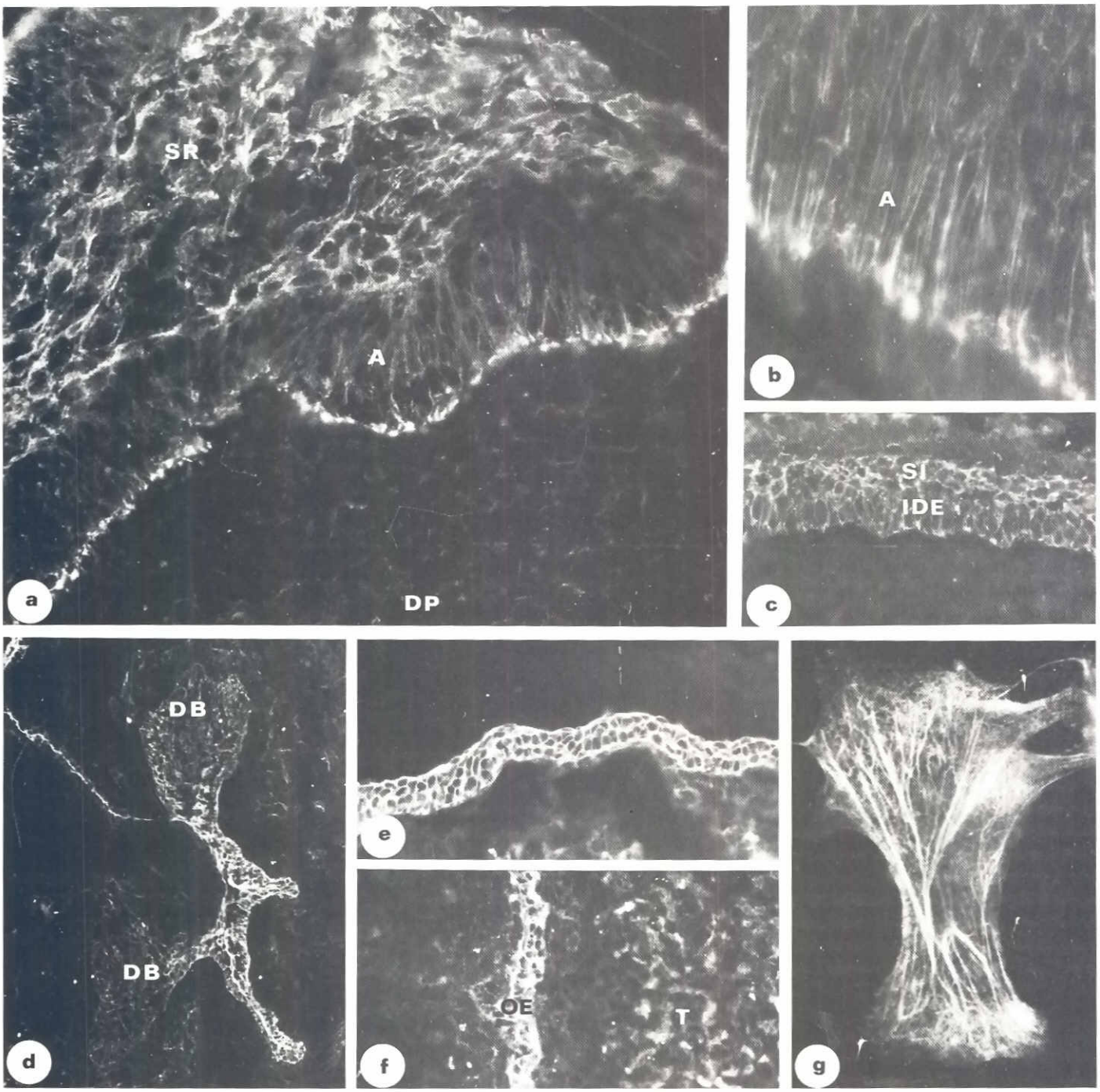


Fig. 7. Immunohistologic staining of tooth (a, b, c) and day-14 head sections (d, e, f) and dental epithelial cell (g) with monoclonal MC22-2E. ***a****. The cells of the enamel organ are stained. Ameloblasts (A) demonstrate accumulation of the antigen at the secretory pole. SR: stellate reticulum; DP: dental papilla.* ***b****. Higher magnification of ameloblasts (A) revealing intracellular fibers.* ***c****. Preameloblasts (IDE) and cells of the stratum intermedium (SI) are positive.* ***d****,* ***e****,* ***f****. MC22-2E reacts with the dental buds (DB) (d) the epidermis (e) the oral epithelium (OE) and the tongue(T) (f).* ***g****. The stained permeabilized dental epithelial cells reveal a cytoskeleton component.*

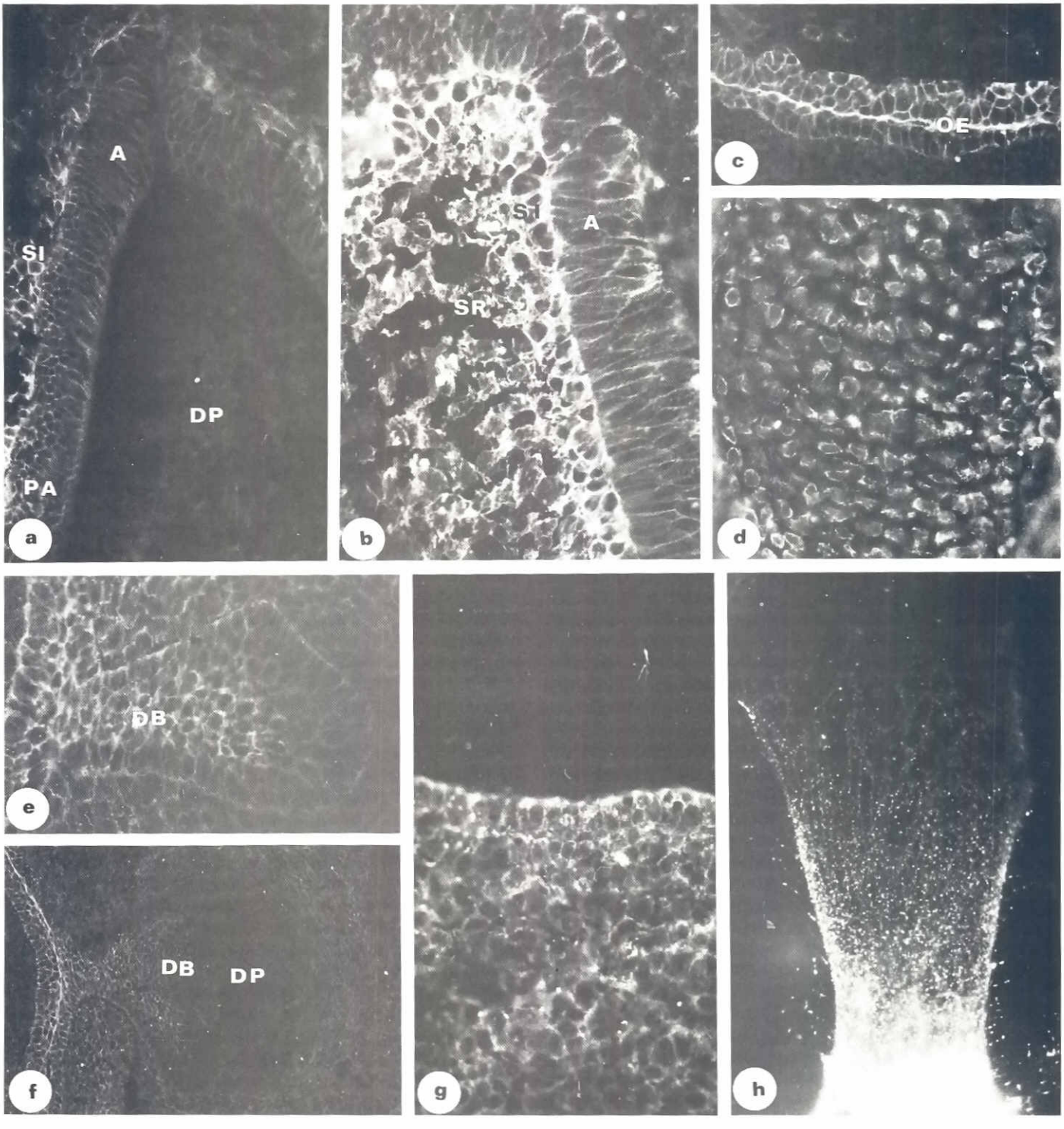


Fig. 8. Immunohistological staining of tooth (a, b), day-16 (f) and day-14 head sections (c, d, e, g) and epithelial dental cell (h) with monoclonal MC22-4A. ***a, b****. Preameloblasts (PA), ameloblasts (A) and cells of the* stratum intermedium *(SI) and* stellate reticulum *(SR) react intensely. The dental papilla (DP) is negative.* ***c, d, g****. Cells of the oral epithelium (OE) (c), chondrocytes (d) and epidermal-dermal cells (g) are positive.* ***e****. The day-14 head section reveals staining of the dental bud (DB) and adjacent mesenchymal cells.* ***f****. The day-16 bell stage demonstrates absence of reactivity of dental papilla cells (DP).* ***h****. The permeabilized epithelial dental cell shows a fibrogranular staining pattern.*

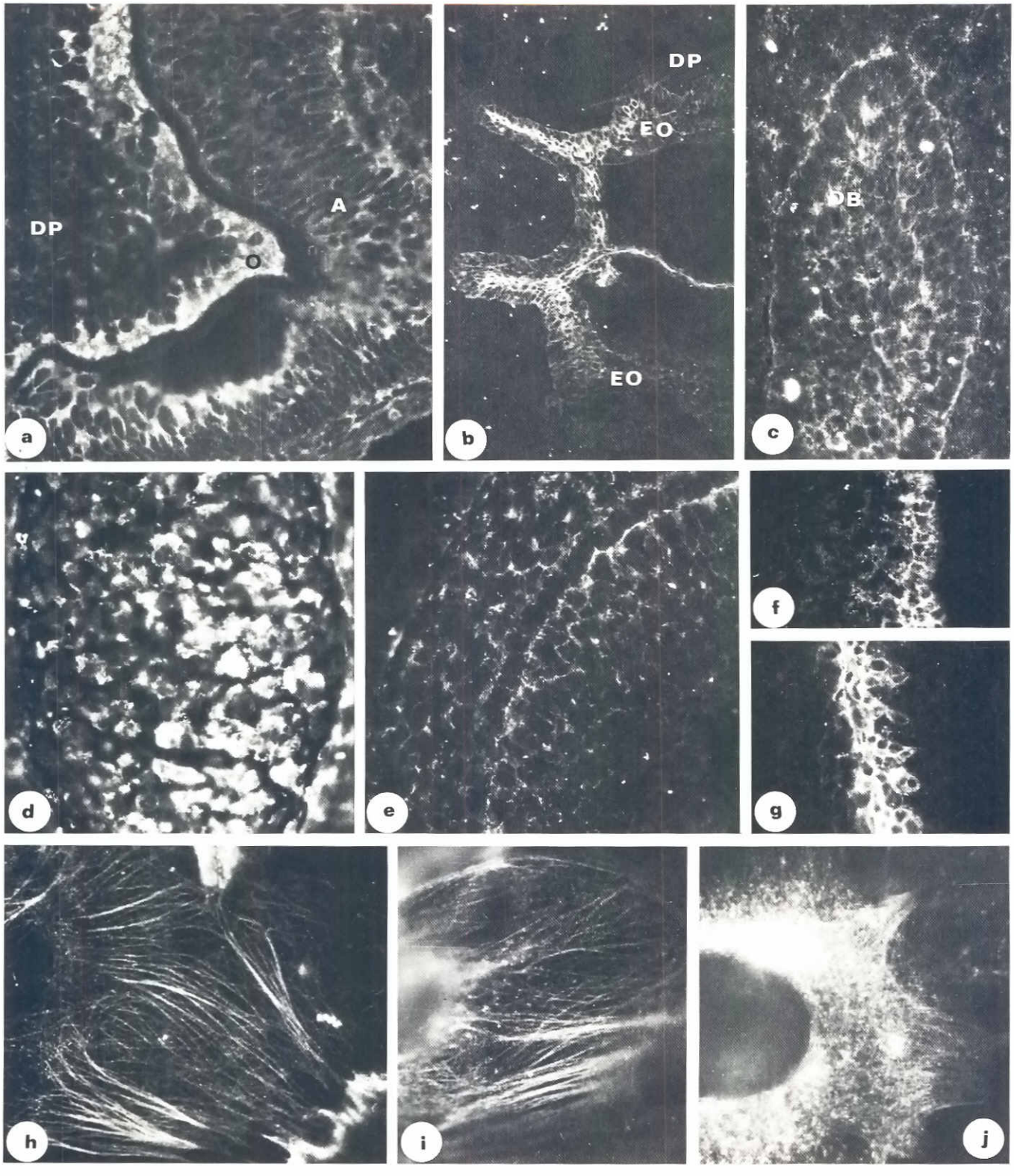


Fig. 9. Immunohistochemical staining of tooth (a), day-16 (b) and day-14 head sections (c, d, e, f, g) and permeabilized dental cells (h, i, j) with monoclonal MC22-19J. ***a****. This antibody reacts intensely with odontoblasts (O). PD: dental papilla; A: ameloblasts.****b****. The enamel organs (EO) of day-16 molars are stained whilst the dental papillae (DP) are negative.* ***c****. The dental bud (DB) is positive - the adjacent mesenchyme is faintly stained.* ***d****,* ***e****,* ***f****,* ***g****. Positive reactions of chondrocytes (d), the retina (e), the epidermis (f) and the oral epithelium (g).* ***h****. Permeabilized dental epithelial cell demonstrating staining of a cytoskeleton component.* ***i, j****. Two aspects of staining of permeabilized dental papilla cells.*

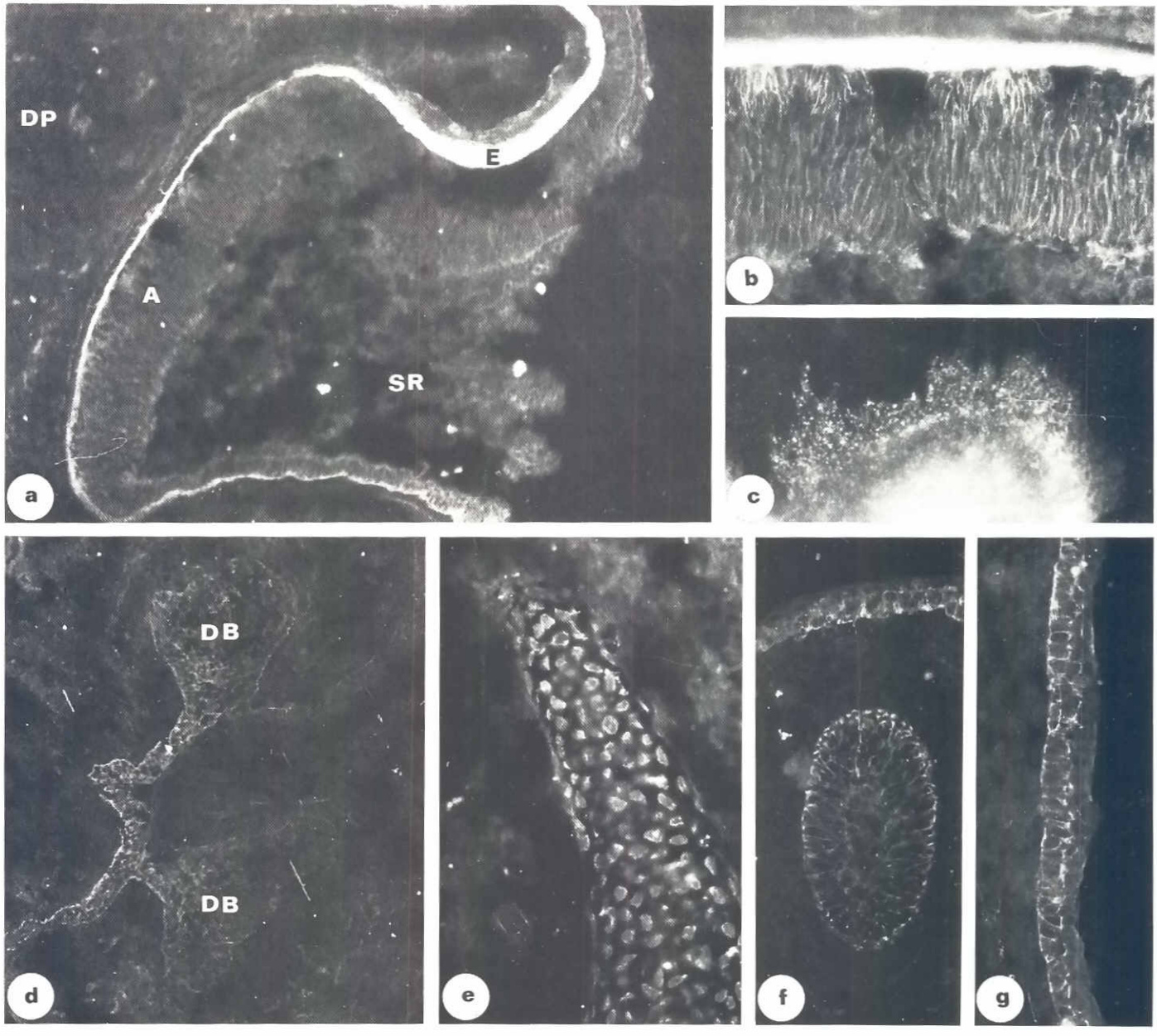


Fig. 10. Immunohistochemical staining of tooth (a, b) , and day-14 head sections (d, e, f, g) and dental epithelial cell (c) with monoclonal MC22-1A. ***a****. The enamel (E) and basal pole of ameloblasts (A) demonstrate strong fluorescence. DP: dental papilla; SR:* stellate *reticulum.* ***b****. Higher magnification of ameloblasts showing positive fibrilar constituents.* ***c****. Granular staining pattern of a permeabilized dental epithelial cell.* ***d, e, f, g****. The cells of the dental buds (DB) - d, chondrocytes - e, the lens - f, and epidermis (g) show positive reactions.*

Materials and Methods

Preparation of immunogen

Mandibular first molars were dissected from 2-day old, laboratory raised Swiss mice. The teeth were incubated at 4°C in 1% trypsin (Difco 1:250) in phosphate buffered saline (PBS) for 2 h. The dental papillae were then mechanically isolated (Osman and Ruch, 1981). The isolated dental papillae were incubated at 37° for 30 min in 10mM EDTA in Ca^{++} and Mg^{++} free PBS containing 0.5% trypsin (Difco 1:250). After trypsin inhibition with fetal calf serum (Gibco) the isolated cells were collected by sieving (20μ mesh) and after 3 washes in PBS the cells were allowed to regenerate in RPMI-1640 (Gibco) at 37°C for 30 min. This preparation of immunogen did not allow the complete exclusion of the contamination of dental papilla cells with dentin-enamel components and peridental mesenchyme.

Immunization

One Fisher rat (12 weeks old) was immunized at 2 week intervals by intraperitoneal injection of 2x $1x10^7$ and $1.5x10^7$ cells in

PBS, emulsified in complete or incomplete (last-injection) Freund's adjuvant (1:1).

Production of hybridomas and screening

3 days after the last injection the rat splenotytes were fused with the mouse myeloma cell line NS_1 according to Kohler and Milstein (1975). Spleen cells and NS_1 cells were washed briefly in RPMI-1640, combined in a 1:1 ratio and centrifuged. One milliliter of polyethyleneglycol-PEG-1500 at 37°C was gently dropped to the cell pellet and then diluted with RPMI-1640. Cells were centrifuged and resuspended in RPMI-1640 supplemented with 15% fetal calf serum. The cell suspension was seeded onto 96 well plates (NUNC). After 24 h incubation at 37°C in humidified 5% CO_2 in air the hybrid selection was started by replacing one half of the supranatant with the same medium containing 13.6µg/ml hypoxanthine, 0.67 µg/ml aminoptherine and 3.88µg/ml thymidine. Growing hybridoma cultures were screened for antibody production by indirect immunofluorescence using frozen sections of 2-day old mouse first lower molars. Positive hybridomas were cloned twice under conditions of limiting dilution.

Antibody-isotype analysis

The immunoglobulin subclasses were determined by Ouchterlony double diffusion against specific antisera directed against rat IgG subclasses, IgA and IgM (Serotec, Paris). Some culture supernatants were concentrated tenfold with 50% ammonium sulfate.

Indirect immunofluorescence

Frozen sections of 2-day old first mouse mandibular molars and frozen sections of 14-16-day old mouse embryos (vaginal plug = day 0) were used. Furthermore primary cultures of trypsin-isolated enamel organs and dental papillae (day-18 mouse first madibular molars) were performed according to Lesot *et al.* (1984). Briefly, fragments of dental papillae and enamel organs were cultured on glass coverslips in RPMI-1640 supplemented with 15% fetal calf serum. Penicillin (50µg/ml) and streptomycin (50µg/ml) were added to the culture medium. The culture dishes were incubated in a humidified incubator under 5% CO_2/95% air atmosphere. The medium was changed every two days.

The sections and the cells were used unfixed. Half of the cell cultures were used after permeabilization for 2 min in Triton X-100 (0.5% in PBS). For indirect immunofluorescence staining the sections and cells were first incubated with hybridoma culture supernatant (1:1) at 37°C for 30 min and washed in PBS (3x10 min), and treated with fluorescein isothiocyanate (FITC)-conjugated antibodies against rat immunoglobulins (Cappel). Controls were incubated with PBS or with normal rat serum. The FITC-labeled samples were examined with a Leitz fluorescence microscope.

Acknowledgments

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