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Localization and synthesis of type III collagen and fibronectin in human reparative dentine

Immunoperoxidase and immunogold staining

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Summary. The injury of dental pulp tissue, following caries, is accompanied by the deposit of a typical hard scar tissue known as reparative dentine which should be regarded as the mineralization of a new organic matrix. Highly purified antibodies were used in combination with immunoperoxidase or immunogold technique at the ultrastructural level to reveal the distribution and synthesis of types I and III collagen and fibronectin elaborated by typical matrix-forming cells in the new tissue.

Specific immunoperoxidase labelling, on demineralized teeth, clearly demonstrated that type I collagen represents the main type of collagen (88%). It is associated with bundles of fine striated fibrils of type III collagen and in close vicinity with fibronectin and constituted, at least, the new organic matrix of reparative dentine.

Immunogold staining gave precise localization mainly over Golgi apparatus for the 3 components, thus suggesting that the cells concerned should not be considered as new odontoblasts but rather as pulpal cells in the process of differentiation participating in the formation of new dentine. Moreover, these events are very similar to those observed during wound healing in other tissues.

Introduction

Pulp and dentine are the two major components of the tooth and should not be regarded as separate tissues. The former is a soft connective tissue whereas the latter represents the calcified organic matrix composed of collagen type I along with some type I trimer (Munsksgaard et al. 1978; Munksgaard and Moe 1980; Magloire et al. 1983; Butler 1984; Linde 1985), proteoglycans (Linde 1973; Jones and Leaver 1974; Nygren et al. 1976), phosphoproteins (Dimuzio and Veis 1978; Linde et al. 1981) and Gla-containing proteins (Dimuzio et al. 1983; Butler 1984; Linde 1985).

The predentine matrix differs from that of dentine and other soft connective tissues because it does not contain any phosphoproteins, Gla-containing proteins, type III collagen or fibronectin (Linde 1985). At the ultrastructural level, predentinal collagen fibrils form a fine irregular network and increase in size toward the dentine-predentine junction. The cellular components involved in the formation of dentine are the odontoblasts, post-mitotic and highly specialized cells, the differentiation of which is mediated by a continuous epithelially derived basement membrane (Thesleff and Hurmerinta 1981; Ruch et al. 1982; Ruch 1985). This inductive component, then disappears, the ameloblasts secrete enamel and later also disappear when the crown of the tooth is completely achieved. In contrast, circumpulpal dentinogenesis continues throughout the life and cover the entire pulpal surface.

When a tooth becomes carious, or when the dentine is cut following the operative procedures of clinical dentistry (Massler 1967), the pulp tissue responds to irritation of the odontoblasts by laying down reparative or irritative dentine subjacent to the involved tubules (Trowbridge 1981; Bergenholtz 1981). This healing process of the pulp should be regarded as scar formation resulting in the mineralization of a typical organic matrix.

The reparative dentine differs from primary dentine in structure and the quality of the predentine is also changed, more amorphous, less tubular but mainly composed of collagen fibres (Schröder and Sundström 1974; Sela et al. 1981). It is likely that most if not all of the original odontoblasts, at least in the restricted area of the injured tissue, are dead. The new matrix components might therefore be elaborated by odontoblast-like cells which could differentiate from other pulpal cells exhibiting more closely fibroblastic appearance rather than the columnar shape of odontoblasts (Harrop and Mackay 1968; Sveen and Hawes 1968; Cotton 1968; Skogedal and Mjör 1979; Wennberg et al. 1982).

So the aim of this paper was to identifiy collagens and fibronectin as a marker of cell differentiation in the organic matrix of human reparative dentine using specific antibodies and immunoperoxidase labelling for light and electron microscopy. In addition, the immunogold method was used to give more precise labelling, particularly at the intracellular level. Preliminary reports have been described previously (Magloire et al. 1985).

Materials and methods

Preparation and control of antibodies. Human type I and III collagens were extracted from skin and placenta respectively according to the routine procedures. Types-specific antibodies raised in rab-

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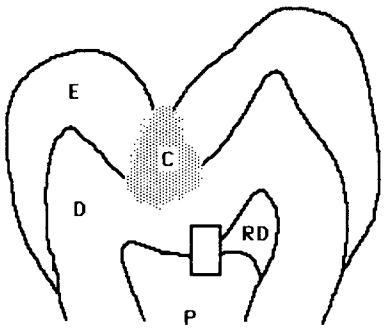


Fig. 1. Schematic drawing of the deposition of reparative dentine (*RD*) following the effect of carious process (*C*). The frame correspond to the investigated area. *P*: pulp tissue

bits were isolated by affinity chromatography on the corresponding collagen type. The specificity and cross reactivity of the anti-collagen antibodies were determined by immunoblotting and by radioimmunoassay as described previously (Magloire et al. 1984; Magloire et al. 1986). Antibodies against human fibronectin were raised in rabbits according to Linck et al. (1983).

Morphology. For morphological studies by light and electron microscopy 4 carious teeth in active phase (Fig. 1) were extracted, the crown separated from the roots and immediately placed in 2% glutaraldehyde-0.1 *M* cacodylate solution for 2 h at room temperature. After washing in 0.2 *M* cacodylate-sucrose buffer (pH 7.4), the specimens were progressively demineralized in a series of dilutions of HNO_3 (from 10% to 2%) for one week at room temperature. The teeth were then washed again, post-fixed in 1% OsO_4, dehydrated and embedded in Epon 812. Sem-thin or ultrathin sections were cut and contrasted respectively with toluidine blue or uranyl acetate and lead citrate.

Immunostaining for electron microscopy. For immunoperoxidase staining, 22 carious human third molars, selected as described above, were immediately fixed in 4% paraformaldehyde-cacodylate (0.1 *M*)-sucrose (5%) solution (pH 7.4) for 24 h at 4° C as described previously (Magloire et al. 1982). After washing in cacodylate buffer (0.1 *M*) for 24 h, the samples were progressively demineralized in HNO_3 (from 10% to 5%) for one week at room temperature. After washing in cacodylate buffer overnight, the samples were rinsed in phosphate buffer (0.1 *M*)-glycerol (20%) solution, embedded in tissue teck II medium and quickly frozen in isopenthane cooled in liquid nitrogen. Cryostat sections (15 microns) were treated for 90 min at 20° C with hyaluronidase (bovine testes, Sigma Chemical Co.) in phosphate buffer (150 IU/ml). Following incubation in enzyme, sections were rinsed 3 times with phosphate buffer (0.1 *M*) and the endogenous peroxidase were abolished using H_2O_2 (1%) in phosphate buffer for one hour at room temperature. After washing in phosphate buffer with 1% bovine serum albumin for 90 min at room temperature, the sections were incubated overnight at 4° C in affinity-purified antibodies against type I, III or fibronectin prepared as described above. After washing sections were incubated with sheep anti-rabbit-IgG peroxidase conjugate diluted 1:50 (ref. 75011, Institut Pasteur, France) and subjected to the D.A.B. (3-3'diaminobenzidine) procedure according to Graham and Karnovsky (1966). The sections were then osmicated, dehydrated in ethanol and embedded in Epon 812. The semi-thin sections were cut and briefly contrasted with toluidine blue but ultra-thin sections were observed without further contrast. For light microscopical examinations of immunostained tissue, cryostat sections (15 microns) subjected to the same immunoperoxidase procedure were mounted in Epon 812 on glass slides and observed under the light microscope. Control samples were incubated with sheep anti-rabbit-IgG-peroxidase conjugate or non immune rabbit serum instead of the primary anti-serum while the other steps were unchanged.

For immunogold staining, ten teeth exhibiting active carious lesions and suceptible to develop reparative dentine were washed in PBS and carefully sectioned with a sawing machine equipped with a diamond disk (Isomet, Buehler, USA) into sections about 100 microns thick.

During sectioning, PBS was permanently dropped onto the tissue. These sections were further fixed overnight in a solution containing 4% paraformaldehyde, 0.1 *M* sodium cacodylate and 5% sucrose. After washing (5 h), the slices were demineralized in 2% ascorbic acid-0.3 *M* sodium chloride solution for one week (fresh medium being renewed every day) at 4° C according to Dietrich and Fontaine (1975). After rinsing, samples were embedded in glycol methacrylate according to Leduc and Bernhard (1967). Thin sections mounted on nickel grids having a formvar film, were processed for immunocytochemistry. The immunogold staining method (IGS) for the detection of antigenic sites was used according to De Mey et al. (1981). Briefly, the thin sections were first floated on a drop of distilled water for 10 min and further on a drop of tris (0.02 *M*)-bovine serum albumin (0.1%) buffer for 15 min. Next, the sections were placed on a drop of the described antibodies for 30 min. The washed grids were then transferred onto a drop of non immune goat serum (1/50) for a further 30 min, washed again and incubated with IGS EM grade reagents (1/20,; granules size: 15 nm; Janssen Pharmaceutica, Belgium). After incubation, the sections were thoroughly washed with tris buffer, rinsed with distilled water, dried for 2 days and then post-fixed with 2% OsO_4 for 15 min. Finally, the sections were routinely contrasted with uranyl acetate and lead citrate and dried again for 2 days before examination under the electron microscope (Hitachi 12A). In controls, sections were exposed to non immune rabbit serum prior to immunogold staining or were incubated with the IGS EM reagents alone.

Quantitative evaluation of type I and type III collagen fibrils (Weibel 1979). For evaluation of the percentage of type I (coarse) and type III (fine) striated collagen fibrils, approximately 100 ultrathin sections were systematically surveyed in an electron microscope.

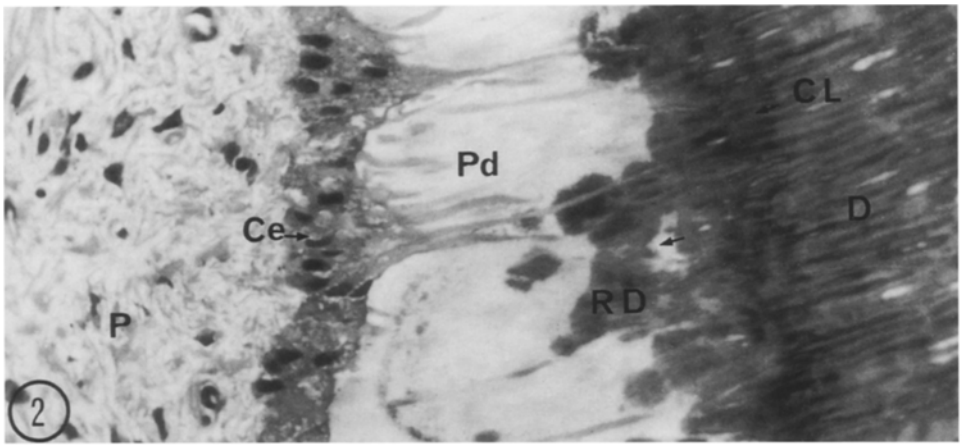
Every area showing the presence of both fibrils was photographed at ×12000. From a pool of 30 electron micrographs selected, the number of coarse (type I collagen) and fine (type III collagen) striated fibrils was recorded with a manual laboratory counter. These data were expressed as mean plus or minus standard deviation of the mean (SD).

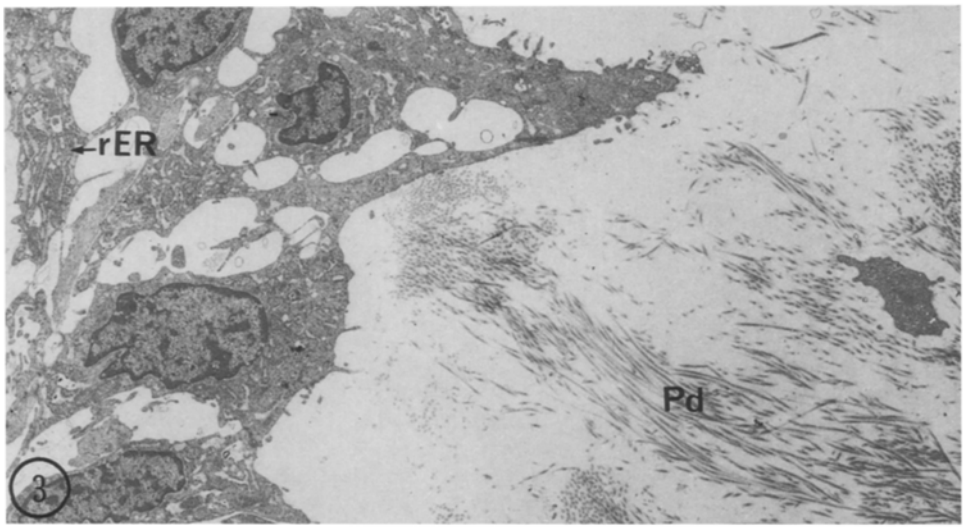
Results

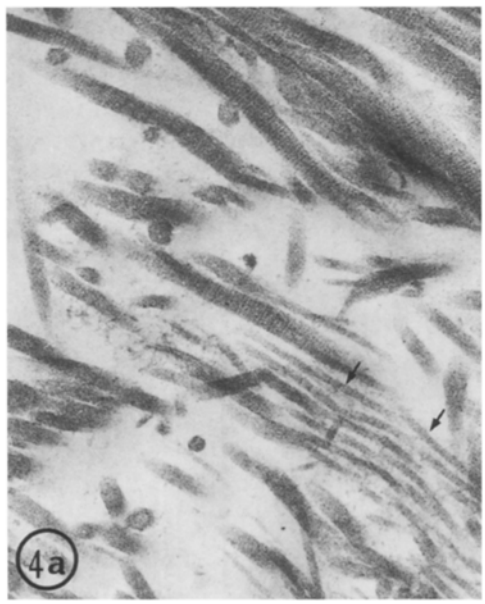
Morphological observations

Examination of semi-thin sections of the 26 carious teeth (standard or immunoperoxidase specimens) demonstrated the presence of a barrier of reparative dentine in 14 only cases. Reparative dentine was easily recognizable on Fig. 2 by its irregular arrangment, a reduced number of dentinal tubules and spherical foci of calcification coalescing to a more homogeneous mineralized zone. Most of this barrier of newly-formed material was lined by typical polygonal or spindle shaped cells (Figs. 2 and 3). At the ultrastructural level, they exhibited well developed rough endoplasmic reticulum, electron dense cytoplasm and cellular extensions. Adjacent to these cells, the organic matrix was mainly composed of patches of packed collagen fibrils which appeared, at a higher magnification (Fig. 4), composed of thick fibrils (around 100 nm in diameter) associated with bundles of more slender parallel ones (around 25 nm in diameter).

Thick longitudinal sections (15 microns) of demineralized teeth exposed to anti-type I collagen antibodies (Fig. 5) showed an intense staining of the new predentine barrier as well as underlying cells. The pulp tissue stained diffusely







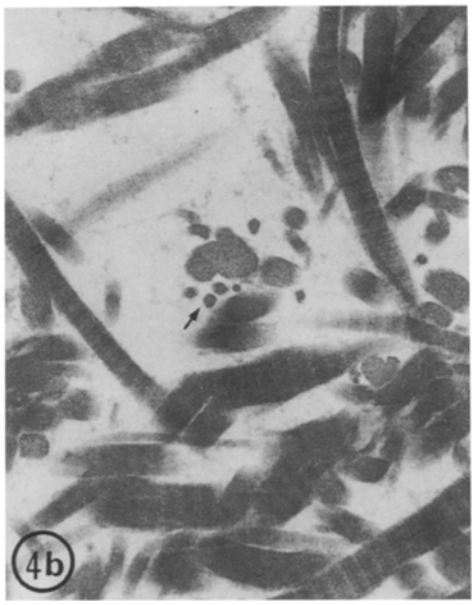


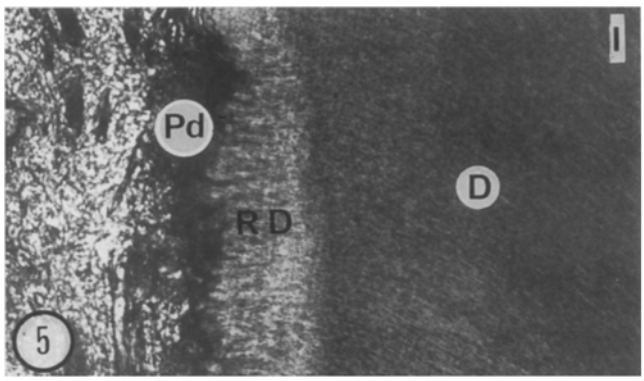
Fig. 2. Semi-thin section of the formation of reparative dentin (*RD*) subjacent to a carious lesion. The scar tissue (*RD*) is limited by a so-called calciotraumatic line (*CL*) from the sound dentin (*D*) and by a barrier of typical matrix-forming cells (*Ce*) with cytoplasmic extensions and organic matrix (*Pd*). Note the entrapment of uncalcified matrix (*arrow*). *P*: pulp tissue. *Pd*: predentine. Toluidine blue. Original magnification ×1000

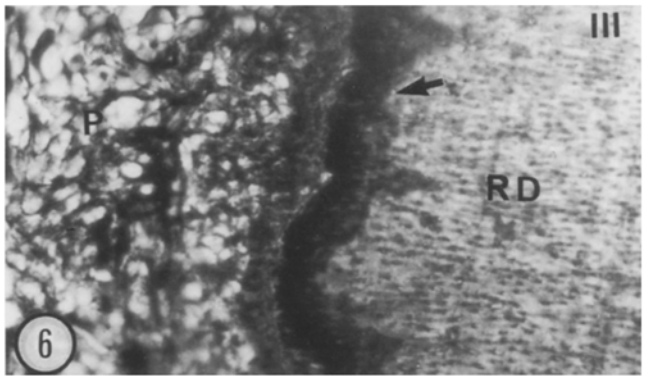
Fig. 3. Electron micrograph of predentine (*Pd*) of reparative dentine made up of newly collagen fibrils lined by differentiating cells containing an abundance of profiles of rough endoplasmic reticulum (rER). Original magnification ×3350

Fig. 4a and b. Higher magnification of the organic matrix showing the thick, densely packed and irregularly arranged collagen fibrils. Note the presence of bundles of more slender ones (*arrows*), around 25 nm diameter, in longitudinal (**a**) or cross (**b**) sections. Original magnification (**a**) ×35000, (**b**) ×38000

and moderately, excepted immediately adjacent to the blood vessel walls. Reparative dentine itself exhibited varying degrees of labelling.

The more intense was associated with sound dentinal tubules. Type III collagen (Fig. 6) was also abundant in the matrix of reparative dentine and an equivalent distribution was found for anti-fibronectin antibodies (Fig. 7). This staining extended to the cell layer as well as the dentinal tubules of the demineralized tissue. On control semi-thin sections (Fig. 8) subjected to non immune rabbit serum, no labelling was observed in the predentine border and the subjacent cell layer.





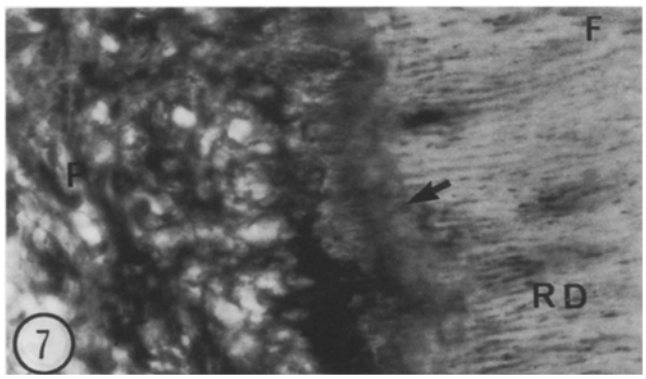


Fig. 5. Thick longitudinal sections (15 microns) of demineralized tissue exposed to anti-type I collagen antibodies as revealed by immunoperoxidase procedure. A strong staining is associated with sound dentine (*D*) as well as predentine and underlying cells (*Pd*). Reparative dentine is less reactive (*RD*). *P*: pulp tissue. Original magnification × 200

Fig. 6. Thick longitudinal section of demineralized tissue exposed to antitype III collagen antibodies. The predentine border (*arrow*) and the the subjacent cell layer are intensely labeled. *RD*: reparative dentine. *P*: pulp tissue. Original magnification × 625

Fig. 7. Thick longitudinal section of demineralized tissue exposed to anti-fibronectin antibodies. The staining is evident in the predentine (*arrow*)-cell barrier. *RD*: reparative dentine. *P*: pulp tissue. Original magnification × 625

Electron microscopic observations on immunostaining

At the ultrastructural level, the coarse fibrils constituting the new matrix were peripherally outlined by peroxidase deposits, following type I collagen detection (Fig. 9). Gold particles gave also intense labelling over the same fibrils (Fig. 10). Reaction product was mainly present intracellularly over Golgi apparatus distributed on the supra nuclear zone (Fig. 12a) whereas rough endoplasmic reticulum or dense granules were sometimes labelled. The presence of type III collagen as well as fibronectin was revealed by immunoperoxidase deposits associated with fine striated fibrils (around 25 nm diameter) grouped in bundles (Figs. 13a and 14a). After faint contrast with uranyl acetate (Figs. 13b and 14b), the distribution of peroxidase along these fine fibrils showed some regularity while the thicker fibrils were clearly devoided of staining. In addition, fibronectin was also detected as dot-like material scattered throughout the new organic matrix. Immunogold staining revealed the same fibrils (Figs. 13c and 14c) but without periodicity of labelling. At the intracytoplasmic level, gold particles were present over the saccules of the Golgi apparatus (Figs. 12b and 13d) in both cases (fibronectin and type III collagen).

Control experiments (data not shown) gave negligible peroxidase staining and only a few randomly distributed gold particles (Fig. 11) when using immunoperoxidase or immunogold labelling respectively.

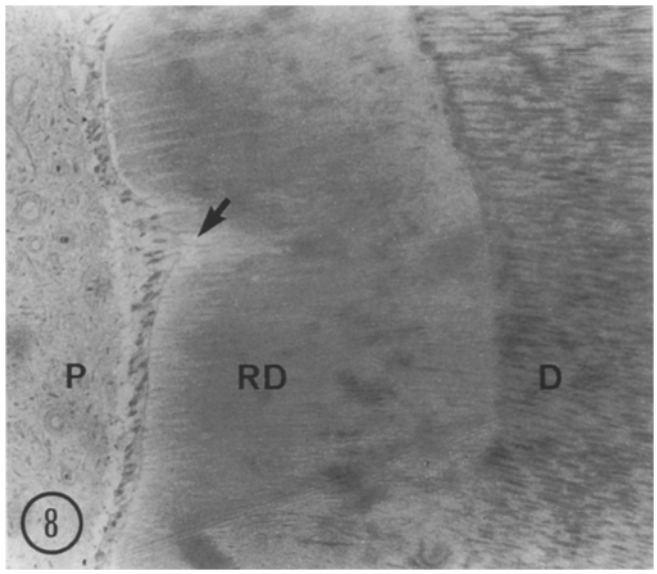


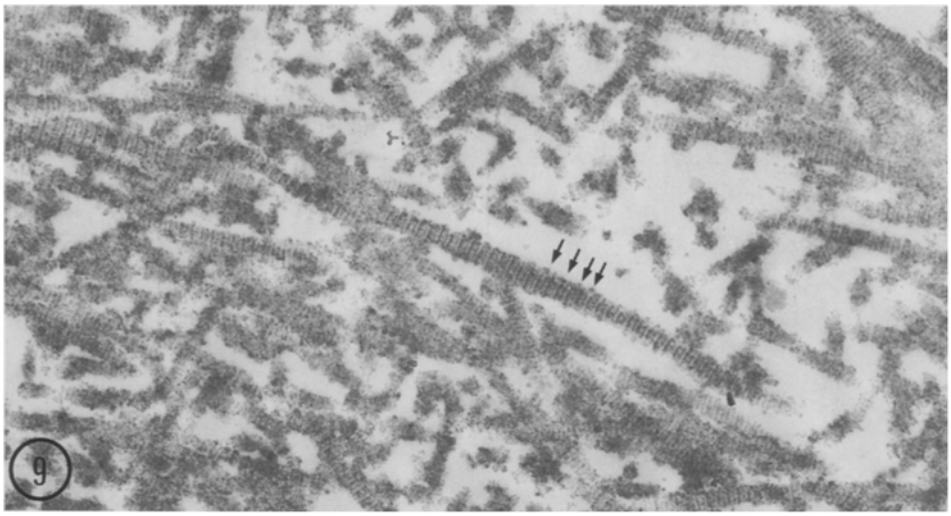
Fig. 8. Control semi-thin section treated with non immune rabbit serum followed by immunoperoxydase sequence and contrasted with toluidine blue. Note the absence of labelling (*arrow*) in the predentine border of the reparative dentine (*RD*) as well as the underlying cells. *P*: pulp tissue. *D*: dentine. Original magnification × 500

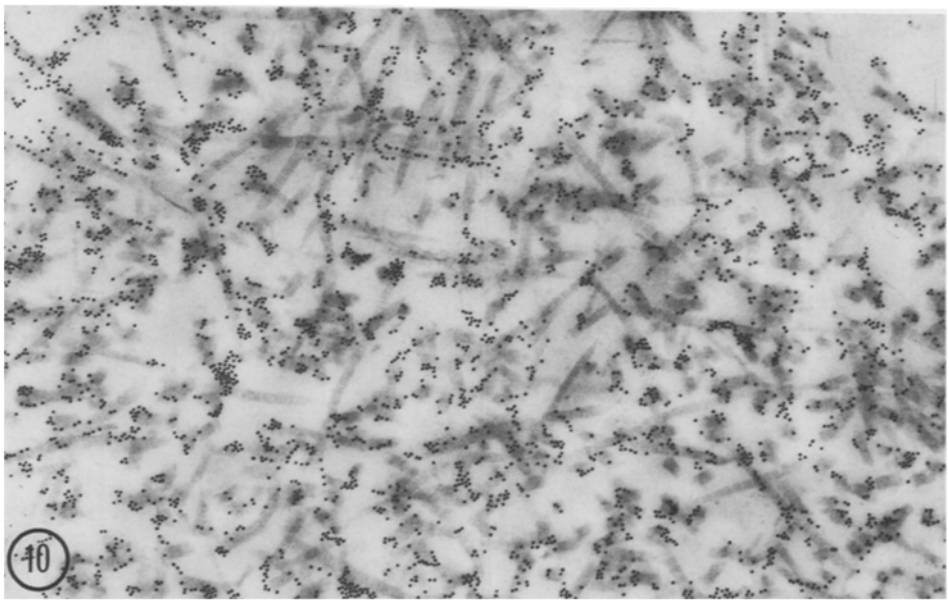
Quantitative evaluation

The quantitative evaluation (Table 1) shows that type I collagen fibrils represent around 88% of total collagen found in reparative dentine.

Discussion

They are many reports concerning the morphological aspects of reparative dentine formation under carious lesions (see Baume's review 1980). The observations support the view that the healing processes of the dental pulp tissue, in response to bacterial irritation result in the elaboration of a mineralized scar tissue. Our findings yielded no new information at the standard light and electron microscope





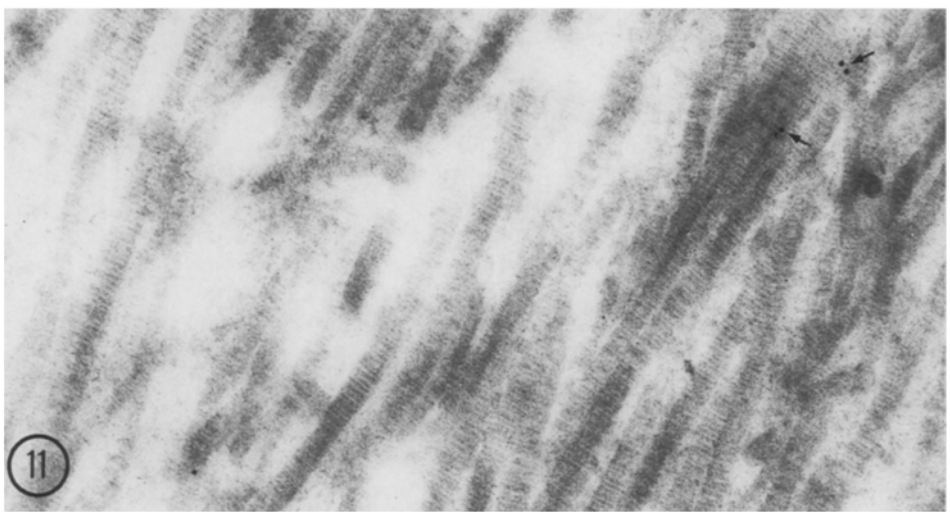


Fig. 9. Electron microscope localization of type I collagen as revealed by immunoperoxidase procedure. Coarse collagen fibrils, peripherally emphasized by peroxidase deposits (*arrows*) correspond to the matrix of reparative dentine. Original magnification ×33000

Fig. 10. Thin section of the new predentine of reparative dentine labelled for type I collagen with human anti type I collagen antibodies and the immunogold staining. Tissues were contrasted with uranyl acetate and lead citrate. Gold particles can be seen over thick collagen fibrils present in the extracellular matrix. Original magnification ×18000

Fig. 11. Control section of collagen matrix exposed to non immune rabbit serum prior to immunogold staining. Note the very few, randomly distributed gold particles over the tissue (*arrows*). Original magnification ×40000

level (see Yamamura's review 1985) but clearly demonstrated the presence of type I and III collagen as well as fibronectin in the matrix of reparative dentine. The poorly calcified aspect of this tissue (Trowbridge 1981), the entrapment of uncalcified matrix giving a typical Swiss-cheese appearance, might possibly be correlated with the presence of type III collagen (around 12%) and fibronectin. Predentine of sound tooth was shown to be mainly composed of type I collagen (see Linde's review 1985) consisting of coarse fibrils whereas definite amounts of type III collagen

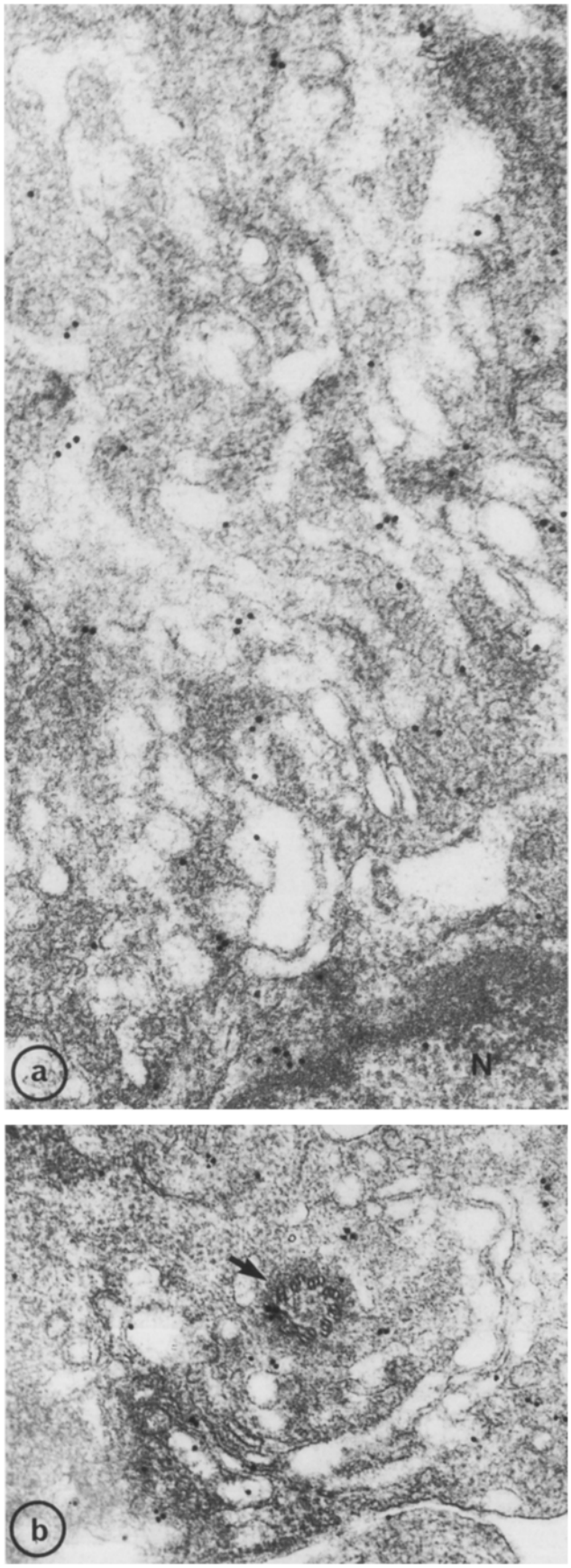


Fig. 12a and b. Golgi areas of matrix forming cells. **a** Underlying cell of new predentine barrier labelled for type I collagen. Gold particles are detected over the saccules of the well developed Golgi apparatus. *N*: nucleus. Original magnification × 60000. **b** Underlying cell of new predentine barrier labelled for fibronectin. Gold particles are scattered over the Golgi saccules in vicinity with a cilium (*arrow*) in transverse section. Original magnification × 47300

were detected in affected dentine from patients with osteogenesis imperfecta (Sauk et al. 1980) Moreover, previous investigations on other tissues (Holund et al. 1982; Williams et al. 1984) strongly suggested that type III collagen is the predominant form of collagen synthesized in the earliest phase of tissue repair, concomitantly with fibronectin (Grinnell et al. 1981; Kischer and Hendrix 1983).

These two components then decrease while type I collagen increases. A similar sequence of events might occur during the recovery of wounded dentine but it is possible that the low level of constant inflammation due to continuous bacterial micro-irritation (dental caries are considered as a chronic disease which may take months or even years to develop) could be responsible for unceasing stimulation of type III collagen or fibronectin synthesis. This last point is supported by the immunogold detection of these antigens over Golgi apparatus, rough endoplasmic reticulum or secretory granules of the matrix-forming cells.

In addition, our results confirm that fibronectin has a stronger affinity for type III collagen than other collagen types (Grinnell 1984; Fleischmajer and Timpl 1984; Ruoslahti et al. 1985) with a typical immunoperoxidase localization in a periodic pattern in close vicinity to these fine cross-striated fibrils. At the ultrastructural level, the immunogold stainings are similar to those obtained with anti-collagen antibodies in skeletal muscle by Stephens et al. (1982) or Geerts et al. (1986) on normal rat liver. The absence of periodicity, in contrast with the immunoperoxidase technique presented in this paper, is correlated to the fact that the gold technique is a post-embedding technique and consequently only the antigenic sites located at the surface of the section are exposed as described previously by Bendayan (1984). Nevertheless, the use of immunoperoxidase method failed to reveal intracellular activities whereas immunogold succeeded. In these conditions, at least for mineralized tissues, both methods must be used in order to obtain maximal revelation of the different antigenic sites.

Finally, it is clear that the new organic matrix of reparative dentine following dental pulp healing under carious lesions, is laid down by cells which cannot be considered as new functional differentiated odontoblasts. The original odontoblasts are injured and autoradiographic studies (Sveen and Hawes 1968; Yamamura 1985) brought evidence for their replacement by underlying pulp cells in process of differentiation, also supported in this paper by the well developed rough endoplasmic reticulum and synthesis of both type I, III collagens and fibronectin. This is in agreement with recent biochemical data (Karjalainen and Söderling 1984). Indeed, it is commonly assumed that odontoblasts elaborate type I procollagen only (Cournil et al. 1979; Lesot and Ruch 1979; Ruch 1985) and this fonctional aspect is related to the presence of a typical basement membrane, the structure of which does not appear during dentine repair. Thus, the possible detection of basement membrane components in the wounded tissue as well as the evaluation of level of differentiation of the concerned cells through Gla-containing proteins or phosphoproteins (Finkerlman and Butler 1985; Gorter de Vries et al. 1986)

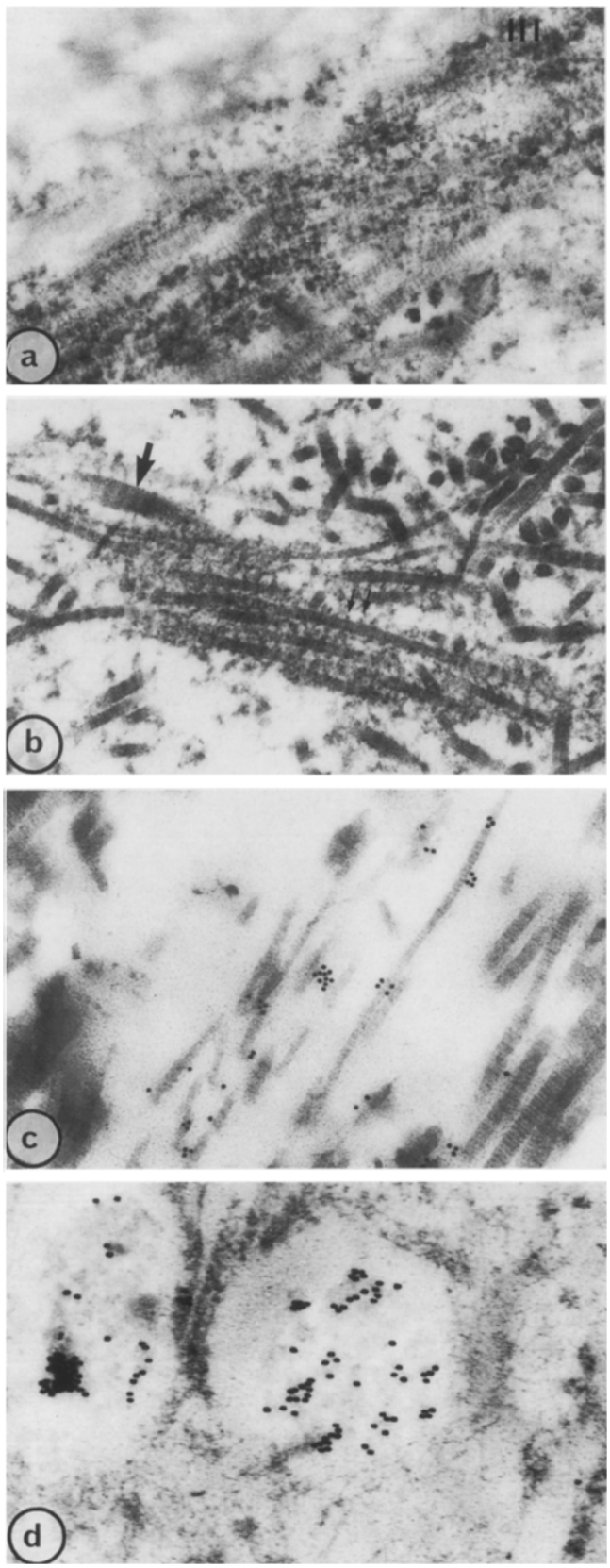


Fig. 13 a and b. Immunoperoxidase staining patterns of human organic matrix of reparative dentin with antibodies against type III collagen. Thin collagenous fibrils (25 nm) are heavily stained with antibodies (*arrows*). Original magnification (**a**) ×38000. **b** A faint contrast with uranyl acetate reveals coarse type I collagen fibrils without peroxidase deposits (*large arrow*). Original magnification

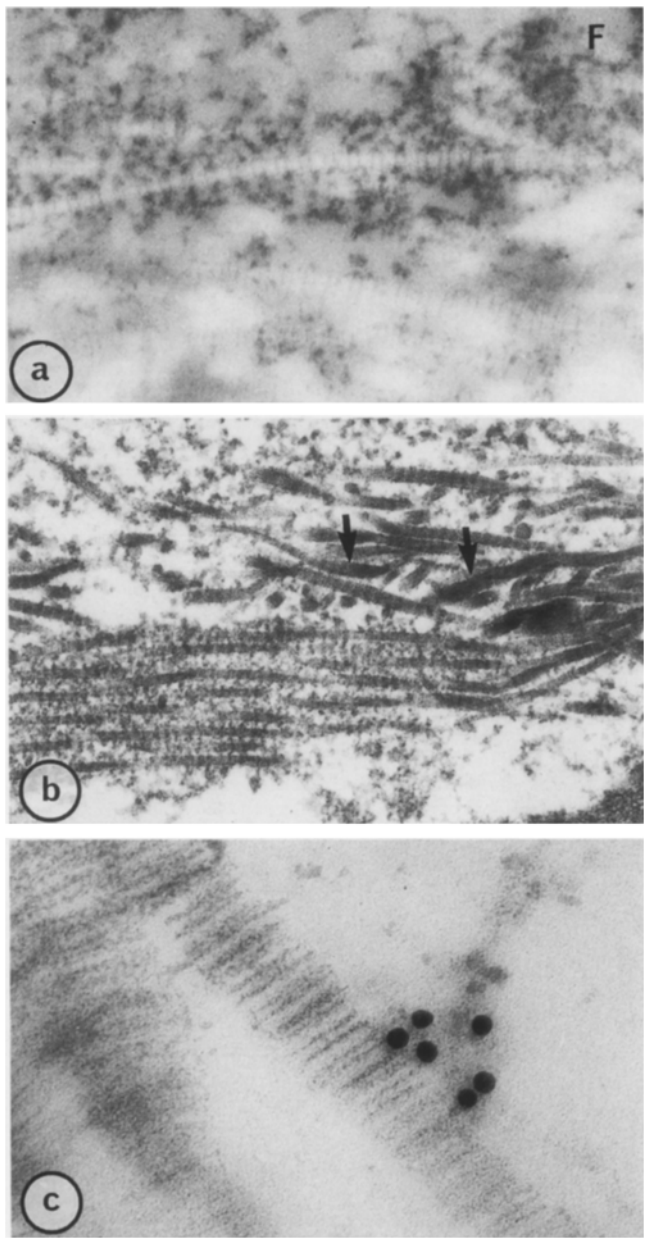


Fig. 14 a and b. Immunoperoxidase staining patterns of human organic matrix of reparative dentine with antibodies against fibronectin. Thin collagenous fibrils (25 nm) are heavily stained, indicating that fibronectin is closely associated with type III collagen fibrils. Original magnification (**a**) ×72000. **b** As in Fig. 13, a faint contrast with uranyl acetate reveals coarse type I collagen fibrils without peroxidase deposits (*arrows*). Original magnification ×24000. **c** Gold particles appear to be associated with material in close vicinity to fine fibrils after incubation with human anti-rabbit fibronectin antibodies. Original magnification ×146000

(**b**) ×45000. **c** type III collagen fibrils are similarly detected with immunogold staining. Original magnification ×36000. **d** The underlying cells of new predentine barrier regularly exhibit intracellular gold particles in colse association with Golgi saccules. Original magnification ×42000

Table 1. Mean numbers (±SD) of type I and type III collagen fibrils recorded in reparative dentine (per μm^2)

Coarse fibrils [type I collagen]	fine striated fibrils [type III collagen]
8.6±2.7	1.03±0.4

might lead us to precise the dental basement membrane requirement for odontoblast differentiation.

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