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Light Microscopic Detection of Sugar Residues in Rabbit Embryo Teeth With Lectin-Horseradish Peroxidase Conjugates

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ABSTRACT We investigated the binding of five HRP-conjugated lectins to rabbit tooth germs at the cap and late bell stages of development. The results revealed some changes in the glycosylation patterns of the glycoconjugates. Sugar residues, such as α-D-mannose, methyl-D-glucose, N-acetylglucosamine, β-D-galactosamine, D-galactose, and sialic acid, were detectable in some components of the tooth germs. The most conspicuous developmental change was increased binding of Con A and WGA. These lectins showed, at the cap stage, moderate binding to the (pre)-ameloblasts and (pre)-odontoblasts. With further development to the late bell stage, but prior to the achievement of well-defined morphological-functional characteristics, the odontoblasts and ameloblasts displayed considerable amounts of α-D-mannose, α-D-glucose as well as β-D-acetylglucosamine and sialic acid. Appropriate control studies confirmed the specificity of the binding of the lectins. Two lectins (DBA and PNA) with known specificity for N-acetylgalactosamine groups were bound by the basement membranes in tooth germs at the cap stage. A third lectin (RCA) with the same specificity did not produce any detectable staining in the same material. Further studies must be planned to determine the specific functions and significance of lectin-HRP-binding glycoconjugates in odontogenesis.

Odontogenesis may be considered a sequence of biochemical and physical interactions between dental epithelium and mesenchyme. Currently available information indicates that fibronectin, type III collagen, tenascin, basement membrane, syndecan, growth factors and their receptors, homeobox genes and protooncogene transcriptors, retinoic acid receptors, protein modifications, carbohydrate moieties, etc., seem to play important roles in the developing tooth (Lemus et al., '87, '94, '95; Thesleff et al., '91; Hu et al., '92; Mark et al., '92; Sasano et al., '92; Zeichner-David et al., '92; Bloch-Zupan et al., '94; Ruch, '95). Although important progress has been made toward the elucidation of numerous developmental events involved in odontogenesis, the molecular signals and processes underlying these events remain unknown.

In recent years a number of studies have been performed to reveal the specific types, distributions, and significance of glycoconjugates in various developing systems (e.g., Kokiler and Barondes, '77; Rutherford and Cook, '84; Currie et al., '89; Fazel et al., '89; Griffith and Sanders, '91; Varki, '93). It is generally perceived that changes in the complement of cell surface carbohydrate determinants accompany, and may influence, many morphogenetic events during early embryonic development. The structural diversity possible among the many oligosaccharide chains of surface glycoconjugates renders them likely candidates as modulators of cell-cell interactions, cellular movements, and differentiation. Lectins that bind to specific sugar residues have been used as potent probes for the demonstration of carbohydrates associated with the surfaces of cells as well as with their cytoplasmic organelles.

The process of morphogenesis of the tooth may be considered a good model to use in the study of the distribution and significance of sugar residues during cellular differentiation (Gheri et al., '92; Sasano et al., '92; Lemus et al., '94). In the present study, the appearance of, and changes in, specific sugar residues during differentiation of rabbit tooth germs

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were investigated using lectins conjugated to horseradish peroxidase.

MATERIALS AND METHODS

Two gravid females of the species *Oryctolagus cuniculus* were anesthetized with ether and sacrificed. Nine embryos ranging from ~15–16 days of gestation were collected. Mandibular arches were isolated and fixed in Carnoy's fixative. The tissues were dehydrated through graded alcohols and xylene and embedded in paraffin blocks in specific orientations. Paraffin-embedded sections were serially cut at a thickness of 5 μm.

Lectin histochemistry

After hydration, sections were treated with 0.3% hydrogen peroxide for 10 min to inhibit endogenous peroxidase, rinsed in distilled water, and washed with 1% bovine serum albumin (BSA) in 0.1 M phosphate-buffered saline PBS (pH 7.2). The sections were then incubated for 30 min at room temperature in a series of HRP-conjugated lectins (Con A *Canavalia ensiformis,* WGA *Triticum vulgaris,* PNA *Arachis hipogea,* DBA *Dolichos biflorus,* and RCA *Ricinus communis*). Each lectin was dissolved in 0.1 M PBS (pH 7.2) containing 0.1 M NaCl, 0.1 mM CaCl2, 0.1 mM MgCl2, and 0.1 mM MnCl2. The sections were then rinsed three times in PBS and incubated for 10 minutes at room temperature in PBS (pH 7.0) containing 3,3'-diaminobenzidine (DAB) (25 mg/dl) and 0.003% hydrogen peroxide. The specimens were rinsed in distilled water, dehydrated using graded ethanol solutions, cleared in xylene, and mounted in Permount. The optimal concentration of each lectin (Sigma Chemical Co., St. Louis, MO), which allowed maximum staining with minimum background, was as follows: Con A 50 μg/ml, WGA 20 μg/ml, PNA 20 μg/ml, DBA 20 μg/ml, and RCA 120 20 μg/ml. The sugar-binding specificity of each lectin is shown in Table 1.

TABLE 1. Lectin characteristics[1]

Lectin (common name) acronym	Carbohydrate binding specificity	Inhibitory sugar[2]
Canavalia ensiformes (Jack bean) ConA	-D-Man > -D-Glc	Methyl- -Man
Triticum vulgaris (Wheat germ) WGA	(β-D-GlcNAc)n Sialic acid	D-GLcNAc Sialidase
Arachis hypogea (Peanut) PNA	D-Gal(β1–3)-D-GalNAc	—
Dolichos biflorus (Horse gram) DBA	-D-GalNAc	GalNac
Ricinus communis (Castor bea) RCA 120	β-D-Gal	—

[1]D-Mannose = mannose; α-D-Glc = Methyl-D-glucose; Methyl-α-Man = Methyl-α-D-mannopyranoside; GLcNAc = N-acetylglucosamine; GalNAc = N-acetylgalactosamine; GalNAc = N-acetylgalactosamine; β = D-Gal = Gal = galactose.
[2]Sugar not listed failed to inhibit.

Control experiments

Controls for lectin staining included exposure to HRP and substrate medium without lectin and oxidation with 1% periodic acid for 10 min prior to lectin staining. As a control to confirm the sugar specificity of each of the lectins, adjacent sections were incubated for 2 hours at room temperature in a mixture of lectin-HRP conjugate (20 μg/ml) and appropriate inhibitory sugars (0.1 M) before they were exposed to DAB. Methyl-α-D-mannopyranoside (methyl-α-man) and methyl-α-D-glucoside were used as inhibitory sugars for Con A; N-acetylglucosamine (D-GLcNAc) was used as the inhibitory sugar for WGA; and N-acetylgalactosamine (GalNAc) was used for DBA. Under these conditions, either decreased staining or inhibition of staining was considered evidence of specific binding of the lectin to the carbohydrate moiety in question.

In some experiments, N-acetyl-neuraminic acid (sialic acid) was removed by pretreating the sections for 18 hours at 37°C in a 0.25 M sodium acetate buffer (pH 5.5) containing 0.1 unit/ml sialidase (neuraminidase Type X from *Clostridium perfringens,* Sigma Chemical Co., St. Louis, MO), 5.0 mM CaCl2, and 154 mM NaCl, prior to staining with lectin-HRP conjugates. Controls exposed to the sialidase buffer without enzyme were also prepared. The sections were examined by light and phase contrast microscopy.

RESULTS

The principal findings of this study are summarized in Tables 2 and 3, for tooth germs in the cap stage and late bell stage respectively. For each lectin, binding was sought in each of the localities shown in the tables.

Dental cap stage

Each dental cap develops into a concave enamel organ displaying differentiation of

TABLE 2. Lectin reactivity of cells of rabbit tooth germs at cap stage: Summary of lectin binding[1]

	PO[2]	BM[3]	PA[4]
ConA	±	−	+
WGA	−	±	±
PNA	−	+	±
DBA	−	±	−
RCA120	−	−	−

[1]Evaluation of binding. Signs indicate staining intensity on a subjectively estimated scale: −, no binding; ±, traces; +, ++, binding to heavy binding.
[2](pre)-odontoblasts.
[3]Basement membrane.
[4](pre)-ameloblasts.

the inner and outer dental epithelia. The stratum intermedium and the stellate reticulum also differentiate progressively (Fig. 1). Lectin distributions at this stage are summarized in Figure 2. The surfaces of all of the cells showed a diffuse positivity with Con A. The reaction was most marked in the (pre)-ameloblasts of the inner dental epithelium, whereas no staining was detected in the stratum intermedium and stellate reticulum (Fig. 2). After treatment with methyl- -D- mannopyranoside (inhibitory sugar for Con A), all of the positive sites described above showed decreased staining (Fig. 3).

Treatment of (pre)-odontoblasts and (pre)-ameloblasts with WGA-HRP produced diffuse cytoplasmic staining, whereas the cell surfaces and the basement membrane were strongly stained (Fig. 4). Following exposure to neuraminidase and D-GlcNac, slight decreases in staining were observed (Figs. 5 and 6, respectively).

PNA-HRP was bound by the basement membrane more avidly than it was by (pre)-ameloblasts and (pre)-odontoblasts (Figs. 7, 8). Treatment with DBA-HRP produced slight, diffuse staining of the basement membrane and cytoplasm of the cells (Fig. 9). In contrast, RCA 120-HRP showed marked binding to the oral epithelial surface, but it did not bind to any region of the tooth germs (Fig. 10).

TABLE 3. Lectin reactivity of cells of rabbit tooth germs at late bell stage: Summary of lectin binding[1]

	PPC[2]	O[3]	A[4]
Con A	−	++	++
WGA	−	++	±
DBA	−	±	−

[1]See fn. 1, Table 2.
[2]Presumptive pulp-cell progenitors.
[3]Odontoblasts.
[4]Ameloblasts.

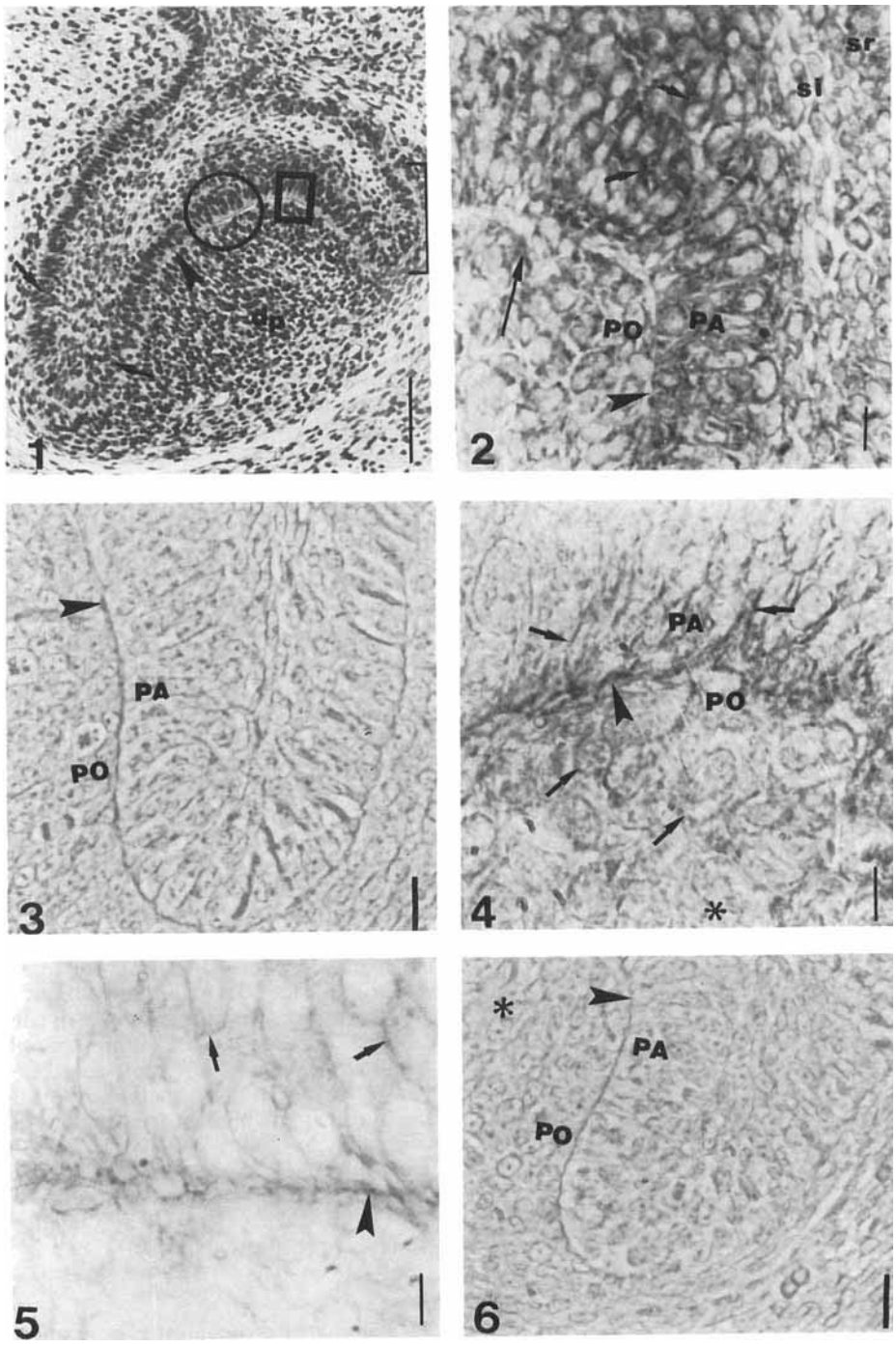
Late bell stage

At this stage, the tooth-specific crown morphology appears progressively. The dental papilla delimited by the cervical loop (junction between the inner and outer dental epithelia) consists of two regions: one containing odontoblasts and the other containing presumptive pulp-cell progenitor cells (Fig. 11). During the bell stage, the odontoblasts and ameloblasts gradually become functional (i.e., the odontoblasts assume an epithelial arrangement, polarize, and secrete predentin, whereas the ameloblasts withdraw from the cell cycle, polarize progressively, and gradually lose their basement membrane). The exact mechanism involved in the degradation of the basement membrane and the effects of this resorption per se on ameloblasts differentiation are not known. At the late bell stage, the odontoblasts reacted strongly with Con A-HRP, whereas the presumptive pulp-cell progenitor cells lacked detectable staining (Fig. 12). Higher magnification showed an intense granular positivity with Con-HRP in the cytoplasm as well as at the apical surfaces of the odontoblasts, whereas reactive (pre)-ameloblasts in the cervical loop were not stained (Figs. 13, 14). Exposure of sections to Con A-HRP conjugate containing methyl-α-D-mannopyranoside produced an appreciable decrease in staining (Fig. 15).

WGA-HRP, which is specific for N-acetyl-D-glucosamine and sialic acid, produced the most intense overall staining during the late bell stage (Fig. 16). At higher magnification, intense staining of the cytoplasm of the odontoblasts was apparent; some cytoplasmic granules were seen, and a moderate reaction was observed in the ameloblasts (Figs. 17, 18). Following sialidase treatment, the reactivity of ConA and WGA was depressed in both the ameloblasts and the odontoblasts (Fig. 19). When sections of dental tissues were stained with DBA-HRP, all dental components were stained diffusely (Fig. 20).

DISCUSSION

Carbohydrate-containing macromolecules of the cell surface are thought to play significant roles in many developmental phenomena, including cell-cell interactions and differentiation (Lis and Sharon, '86; Reuter et al.,



Figures 1–6

'82; Takata and Hirano, '83; Takahashi, '88; Gheri et al., '92; Varki, '93; Lemus et al., '94). The developing tooth is an excellent system with which to study the mechanisms governing organogenesis, particularly since it is well known that morphogenesis as well as cellular differentiation in the tooth germ are under the control of reciprocal interactions between epithelial and mesenchymal tissues (Koch, '67; Kollar and Baird, '70; Ruch, '87; Thesleff et al., '91; Ruch, '95).

In the present investigation of the lectin binding patterns of rabbit tooth germs at cap and late bell stages, the major change was increased binding of Con A and WGA as too development progressed. At the cap stage of development, these lectins showed a moderate binding to (pre)-ameloblasts and (pre)-odontoblasts. With further development to the late bell stage, the odontoblasts and ameloblasts displayed progressively grater binding of lectin specific for α-D-man, α-D-Glc as well as β-D-GlcNAc and sialic acid.

The high lectin-binding capacity of the odontoblasts and ameloblasts could be due to the presence of glycoproteins and/or proteoglycans secreted by these cells. It appears likely that the reactive glycoconjugates localized in areas thought to contain Golgi complexes are destined for incorporation into the cell surface. Ultrastructural localization of Con A binding sites on the surfaces of odontoblasts of developing mouse molars revealed an apical accumulation of these receptors during polarization of the cells (Meyer et al., '81, cited by Ruch, '87). In an immunocytochemical study, Thesleff et al., ('91) demonstrated that fibronectin and tenascin are present at the early bell stage in mouse molar tooth germs. Other studies revealed the incorporation of 3H-fucose into glycoproteins during early developmental stages in the chick heart (Manasek, '76; Hay and Markwald, '81), but the precise histological distribution and origin of the fucosylated glycoproteins have not been established (Fazel et al., '89).

Positive staining of (β-D-GLcNAc)n and sialic acid sugar residues was present in the Golgi region, and to a lesser extent in the cytoplasm of ameloblasts during the periods considered. Reactivity was also associated with the apical surfaces of the odontoblasts. However, following sialidase digestion, all of the positive sites showed decreased staining. Glycoconjugates containing sialic acid and fucose have been of particular interest in developmental studies since these sugars invariably occupy terminal positions on oligosaccharide side chains (Bennett et al., '74). The terminal position of sialic acid makes it a potential candidate as a receptor for many developmentally regulated recognition molecules, such as endogenous lectins, glycosyltransferases, or exoglycosidases on the surfaces of similar or different embryological cell types (Reuter et al., '82; Fazel et al., '89). Recently, Lemus et al. ('94) demonstrated that glycoconjugates recognized by Con A and WGA are also present during lizard odontogenesis.

Some of the most striking examples of binding by N-acetylgalactosamine were ob-

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Fig. 1. *Oryctolagus cuniculus.* Tooth at the cap stage from rabbit embryo showing initiation of histogenesis in enamel organ. The area enclosed by the circle is shown in Figure 4. The area encompassed by the rectangle is shown in Figure 2, and the area indicated by the bracket (cervical loop) is shown in Figures 3 and 6. Arrows point to inner and outer dental epithelia. Arrowhead points to basement membrane. dp, dental papilla. Scale bar = 76 μm.

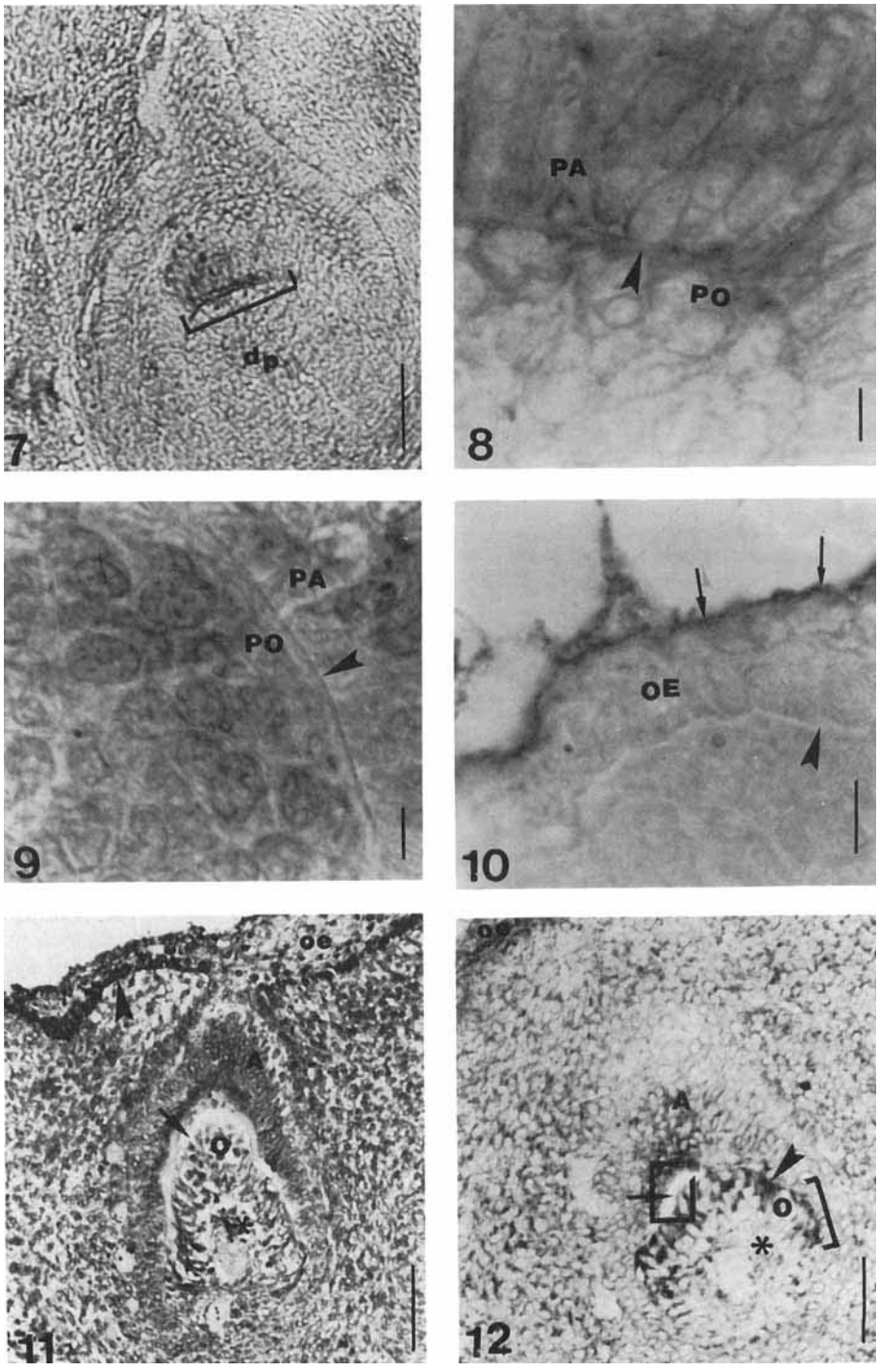
Fig. 2. *Oryctolagus cuniculus.* Con A-HRP. The surfaces of the cells show a diffuse positivity. The reaction is most marked in the (pre)-ameloblasts (short arrows) and in marked contrast to the unstained stratum intermedium (si) and stellate reticulum (sr). A moderate reaction on the dental papilla is visible (long arrow). Arrowhead points to basement membrane. PO, (pre)-odontoblasts; PA, (pre)-ameloblasts. Scale bar = 10 μm.

Fig. 3. *Oryctolagus cuniculus.* Section through the cervical loop of dental cap from rabbit embryo exposed to Con A-HRP in the presence of methyl-α-D-mannopyranoside (methyl-α-man). The Con A-HRP binding is completely inhibited. PO, (pre)-odontoblasts; PA, (pre)-ameloblasts. Arrowhead points to basement membrane. Phase contrast microscopy. Scale bar = 20 μm.

Fig. 4. *Oryctolagus cuniculus.* WGA-HRP. The outer dental epithelium and dental papilla show moderate staining. The basement membrane (arrowhead) and the surfaces of the dental cells (arrows) display the greatest amount of staining. Asterisk marks presumptive pulp-cell progenitors. PO, (pre)-odontoblasts; PA, (pre)-ameloblasts. Scale bar = 6 μm.

Fig. 5. *Oryctolagus cuniculus.* Sialidase-WGA-HRP. Following sialidase treatment, all the positive sites shown in Figure 4 show decreased staining. Arrows point to (pre)-ameloblast surfaces, and arrowhead points to basement membrane. Scale bar = 6 μm.

Fig. 6. *Oryctolagus cuniculus.* WGA-HRP in the presence of D-GLcNac. The WGA-HRP stain is completely inhibited. Arrowhead points to basement membrane. Asterisk marks presumptive pulp-cell progenitors; PA, (pre)-ameloblasts; PO, (pre)-odontoblasts. Phase contrast microscopy. Scale bar = 20 μm.



Figures 7–12

served in developing chick embryos treated with DBA, PNA, and RCA (Damjanov, '87; cited by Griffith and Sanders, '91). In rabbit tooth germs at the cap stage, PNA and DBA showed an affinity for the basement membrane. PNA, specific for the sequence Gal(β1-3)-GalNAc, has been associated previously with the early phases of differentiation (Zimmerman and Thies, '84; Slack, '85; Stern et al., '86; Aulthouse and Solursh, '87; Watt et al., '89). It appears to be capable of recognizing oligosaccharides of significance to embryonic induction at certain critical times in development.

Observations made by Bagnall and Sanders ('89) and by Griffith and Sanders ('91) indicate that PNA-binding, although not exclusively extracellular, is associated mostly with the extracellular matrix. One of the components of the extracellular matrix of the dental papilla is hyaluronic acid. This glycosaminoglycan is a component of the basement membrane (Kollar, '83), which also binds PNA, suggesting that at dental cap stage at least, the binding possibly might be attributable to molecules associated with hyaluronic acid, but not to hyaluronic acid itself. In contrast, some authors suggest that oligosaccharides recognized by PNA may be functionally related to molecules that contribute to root formation and cementogenesis during mouse molar development (Sasano et al., '92).

With regard to RCA, this lectin has been localized in the basement membrane during gastrulation and neurulation of chick development, and its principal site of binding is known to be the interstitial bodies attached to the lamina densa (Griffith and Sanders, '91). The avidity of these bodies for RCA might be due to their abundant content of fibronectin, particularly since this molecule has the appropriate oligoasaccharide content and can be quantitatively extracted using an RCA column (Carter and Hakomori, '79; Takasaki et al., '79; Fakuda et al., '82; Takamoto et al., '89, cited by Griffith and Sanders, '91). Perhaps the lack of binding of RCA to the basement membrane of the rabbit tooth germ at the cap stage might be due to early resorption of the interstitial bodies. Eventually the complete resorption of the basement membrane allows heterotopic cell contacts between (pre)-ameloblasts and odontoblasts as well as an interaction between (pre)-ameloblasts and predentin.

In the present work, the presence of sugar residues: α-D-mannose, methyl-α-D-glucose, N-acetylglucosamine, sialic acid, and acetylgalactosamine has been confirmed in (pre)-ameloblasts, (pre)-odontoblasts, and the basement membrane of rabbit tooth germs at the cap stage. At the late bell stage, the increase in surface-related and cytoplasmic binding sites for Con A and WGA might play important roles during the formation of dentin and enamel. Finally, further studies must be planned to determine the specific identities of these lectin-HRP-binding glycoconjugates, and to elucidate the roles played by these glycoconjugates in the complex processes of odontogenesis.

Fig. 7. *Oryctolagus cuniculus.* PNA-HRP. The (pre)-odontoblasts and (pre)-ameloblasts are diffusely positive for the lectin, while a moderate reaction is observable in the basement membrane. dp, dental papilla. Scale bar = 70 μm.

Fig. 8. Higher magnification of the area indicated by the bracket in Figure 7. The basement membrane (arrowhead) shows moderate staining. PO, (pre)-odontoblasts; PA, (pre)-ameloblasts. Scale bar = 6 μm.

Fig. 9. *Oryctolagus cuniculus.* DBA-HRP. The basement membrane is stained (arrowhead). PO, (pre)-odontoblasts; PA, (pre)-ameloblasts. Scale bar = 6 μm.

Fig. 10. *Oryctolagus cuniculus.* RCA-HRP. Affinity for this lectin is restricted to only the apical border (arrows) of the oral epithelium (OE). Arrowhead points to basement membrane. Scale bar = 10 μm.

Fig. 11. *Oryctolagus cuniculus.* Tooth at late bell stage of embryo ranging from ~15–16 days of gestation. O, odontoblasts; asterisk, presumptive pulp-cell progenitors. A, ameloblasts; oe, oral epithelium. Arrow points to dentin, and arrowhead points to basement membrane. Hematoxylin-eosin staining. Scale bar = 74 μm.

Fig. 12. *Oryctolagus cuniculus.* Tooth at late bell stage, 14th–15th day of gestation. Odontoblasts (O) display strong reaction with Con A-HRP (arrowhead). Reactive presumptive pulp-cell progenitors are not stained (asterisk). The reactivity is also observable at the apical surfaces of the ameloblasts (A). Arrow points to dentin. The areas enclosed by the rectangle and marked by the bracket are shown in Figures 13 and 14. Scale bar = 74 μm.

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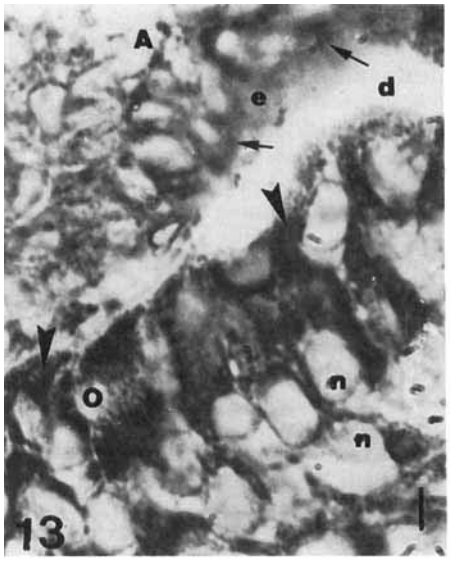

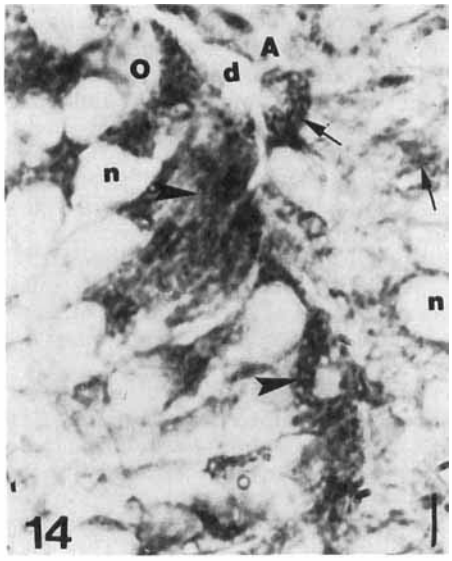

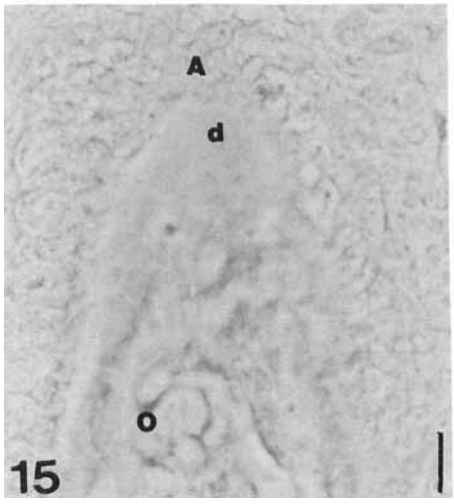

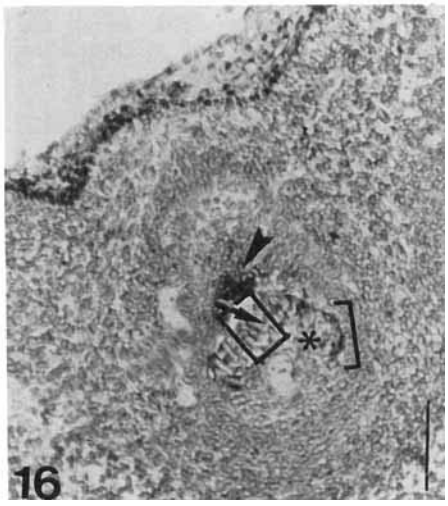

Fig. 13. Con A-HRP. Higher magnification of the area enclosed by the rectangle in Figure 12. Intense positivity is shown in the cytoplasm as well as at the apical surfaces (arrowheads) of the odontoblasts (O). Reactivity is also observable at the apical surface and in the supranuclear areas of the ameloblast (A). Arrow points to enamel. d, dentin; e, enamel; n, nucleus. Scale bar = 6 μm.

Fig. 14. Con A-HRP. Higher magnification of the area (cervical loop) indicated by the bracket in Figure 12. Intense granular positivity is shown in the supranuclear cytoplasm of the odontoblasts (O). Reactive (pre)-ameloblast cells are also observable (arrows). Arrowhead points to intense positivity to the lectin. A, ameloblasts; d, dentin; n, nucleus. Scale bar = 6 μm.

Fig. 15. *Oryctolagus cuniculus*. Tooth at late bell stage stained with ConA-HRP in presence of 0.1 M methyl-α-D-mannopyranoside. Con A stain is completely inhibited. A, ameloblasts; d, dentin; O, odontoblasts. Phase contrast microscopy. Scale bar = 20 μm.

Fig. 16. *Oryctolagus cuniculus*. Tooth at late bell stage of embryo ranging from approximately 15 to 16 days of gestation. Odontoblasts display strong reaction with WGA-HRP (arrow). Reactivity is also observable at the apical surface of the ameloblasts (arrowhead). Reactive presumptive pulp-cell progenitors are not detectable (asterisk). The areas enclosed in the rectangle and marked by the bracket are showed in Figures 17 and 18. Scale bar = 74 μm.

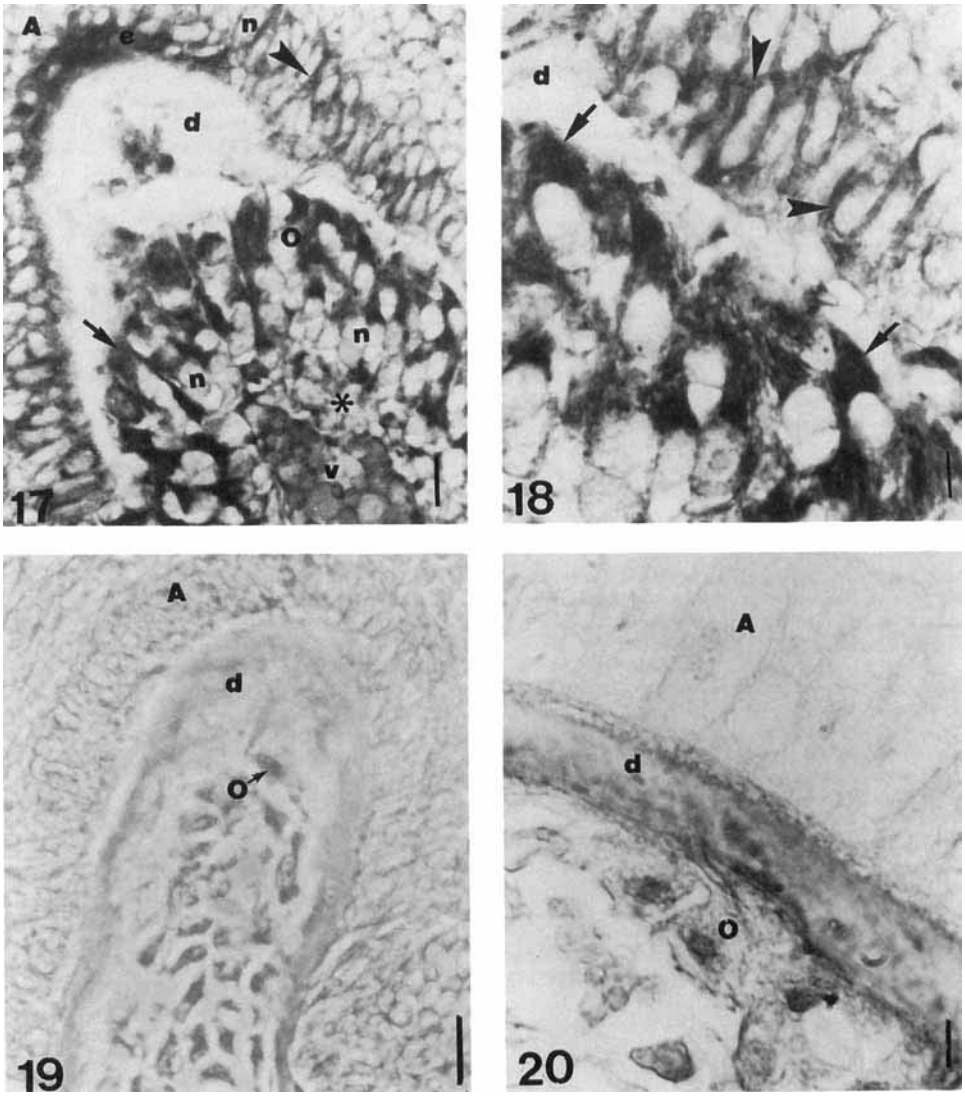

Fig. 17. *Oryctolagus cuniculus.* WGA-HRP. Higher magnification of the area encompassed by the rectangle in Figure 16. Apical surfaces of odontoblasts are stained strongly, while a moderate reaction is observable in the ameloblasts (arrowhead). Arrow points to apical surface of odontoblast stained strongly. A, ameloblasts; e, enamel; d, dentin; O, odontoblasts; asterisk, presumptive pulp-cell progenitors; v, blood vessel; n, nucleus. Scale bar = 10 μm.

Fig. 18. *Oryctolagus cuniculus.* WGA-HRP. Higher magnification of the area indicated by the bracket in Figure 16. The cytoplasm of odontoblasts is strongly stained (arrows). Moderate intensity is observable in the ameloblasts (arrowheads). d, dentin. Scale bar = 6 μm.

Fig. 19. *Oryctolagus cuniculus.* WGA-HRP after treatment with sialidase. All positive sites described in Figures 17 and 18 are completely inhibited. A, ameloblasts; d; dentin; O, odontoblasts. Phase contrast microscopy. Scale bar = 20 μm.

Fig. 20. *Oryctolagus cuniculus.* DBA-HRP. The lectin has reacted diffusely with all tooth germ components. A, ameloblasts; d, dentin; O, odontoblasts. Scale bar = 6 μm.

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