JOURNAL OF MORPHOLOGY 231:175–184 (1997)

Detection of Sugar Residues in Rabbit Embryo Teeth With Lectin-Horseradish Peroxidase Conjugate: II. A Light Microscopal Study

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ABSTRACT The cellular distribution and changes of sugar residues during tooth development in embryos of the rabbit *Oryctolagus cuniculus* were investigated by using horseradish peroxidase-conjugated lectins (lectin-HRP). The lectins SBA, ECA, and LTA show no binding to any region of the dental cap and bell stages, whereas BS-1 and UEA-1 bind to dental cells at both stages. Appropriate control studies confirmed the specificity of the binding of the lectins. At cap stage, the lectins BS-1 and UEA-1 show moderate binding to the (pre)-ameloblast and (pre)-odontoblast cells. These results suggest that the acetylgalactosamine and α-L-fucose residues present in (pre)-ameloblasts and (pre)-odontoblasts, respectively, are common to determined but relatively undifferentiated cells capable of forming matrices of hard tissues. Since the odontoblast and ameloblast express dentin and enamel, respectively, it can be speculated that the abundance of these residues in these cells might be associated with the maintenance of the capacity of the cells to produce such matrices. At the bell stage, the odontoblasts display considerable amounts of α-L-fucose, whereas α-L-fucose is poorly localized in ameloblasts. However, ameloblasts contain significant quantities of N-acetylgalactosamine, whereas only a diffuse positivity for this carbohydrate is apparent in odontoblasts. The marked changes of the glycosylation pattern of these glycoconjugates might indicate that they play a role during the cell-to-cell interaction and might also be involved in the odontoblastic and ameloblastic functional activity. Such a possibility is entirely speculative until specific in vitro experiments are conducted. J. Morphol. *231*:175–184, 1997.

Cell-surface macromolecules containing carbohydrate components play an important role in inducing cellular differentiation, cell-to-cell recognition, interaction, and pinocytosis during embryonic development (e.g., Kokiler and Barondes, '77; Currie et al., '84; Rutherford and Cook, '84; Fazel et al., '89; Griffith and Sanders, '91; Varki, '93). Lectins that bind to specific sugar residues have been used as histochemical reagents for the demonstration of carbohydrates associated with the surfaces of cells as well as with their cytoplasmic organelles.

Some studies have been performed on the characterization, distribution, and significance of glycoconjugates in the teeth of embryonic and adult animals (Meyer et al., '81; Sasano et al., '92; Lemus et al., '94). Recently, we investigated the binding of five HRP-conjugated lectins (Con A, WGA, PNA, DBA, and RCA 120) 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, α-D-glucose, N-acetylglucosamine, β-D-galactosamine, sialic acid, and acetylgalactosamine were detectable in some components of the tooth germs (Lemus et al., '96).

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Contact grant sponsor: DTI, Universidad de Chile; Contact grant number: B-3191-9644.

TABLE 1. Lectin characteristics[1]

Lectin (common name) acronym	Carbohydrate binding specificity	Inhibitory sugar
Bandeiraea simplicifolia BS 1	αGalNac > αGal	GalNac; D-Gal β-lactose
Glycine max (soybean) SBA	α/β-GalNAc > α/β-Gal	D-GalNAc
Erythrina cristagalli (Coral tree) ECA	Galβ1-4GlcNAc	GalNAc; D-Gal; β-lactose
Ulex europaeus (Gorse seed) UEA 1	α-L-Fuc	β-L-Fuc
Lotus tetragonolobus purpureas (Asparagus pea) LTA	α-L-Fuc	β-L-Fuc

[1]GalNAc = N-acetylgalactosamine; GLcNAc = N-acetylglucosamine; Gal = galactose; Fuc = fucose.

In the present study, we used another panel of lectins-HRP to characterize cell-surface changes in other specific sugar residues during differentiation of rabbit tooth germs.

MATERIALS AND METHODS

Two gravid females of the species *Oryctolagus cuniculus* were anesthetized with ether and sacrificed. Seven 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, cleared in 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 (BS-1 *Bandeiraea simplicifolia*, SBA *Glycine max*), ECA *Erithrina cristagally*), UEA-1 *Ulex europaeus*, LTA and *Lotus tetragonolobus purpureas*). 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 MnCl2. The

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

	PO[2]	BM[3]	PA[4]	BE[5]	PDM[6]	BVW[7]
BS-1	±	±	±	++	±	++
SBA	−	−	−	−	−	−
ECA	−	−	−	−	−	−
UEA-1	+	−	+	++	−	+
LTA	−	−	−	−	−	−

[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.
[5]Buccal epithelium.
[6]Peridental mesenchyme (dental sac).
[7]Blood vessel wall.

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]
BS-1	−	+	+++
UEA	+	+++	+
SBA	−	−	−

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

Fig. 1. *Oryctolagus cuniculus*. Tooth at the cap stage from rabbit embryo showing initiation of histogenesis in enamel organ. The line depicts a longitudinal section through the lateral portion of the dental cap magnified in Figure 2. Arrow points to outer dental epithelium. Arrowhead points to (pre)-ameloblasts. Hematoxylin-eosin staining. Scale bar = 260 µm.

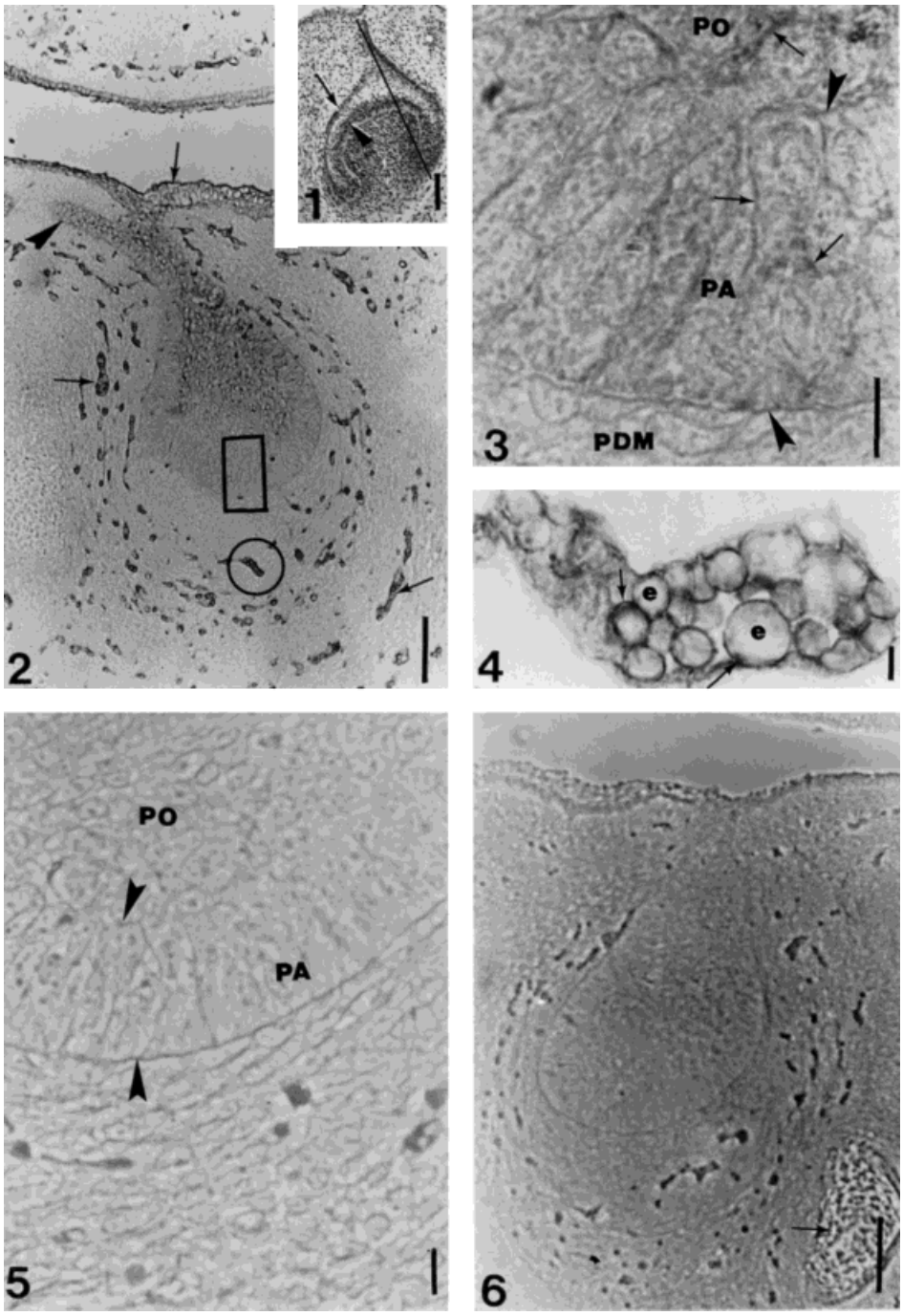
Fig. 2. *Oryctolagus cuniculus*. BS 1-HRP. The surface of the buccal epithelium and walls of blood vessels are positive for the lectin (arrows). Arrowhead shows dental lamina of replacement tooth. Scale bar = 260 µm.

Fig. 3. *Oryctolagus cuniculus*. Higher magnification of the area enclosed by the rectangle in Figure 2, showing a moderate reaction in the cytoplasm as well as in the cell membrane of the (pre)-odontoblast and (pre)-ameloblast cells (arrows). Diffuse reactivity is also observable at the basement membrane and peridental mesenchyme. Arrowheads point to basement membrane; PO, (pre)-odontoblasts; PA, (pre)-ameloblasts; PDM, peridental mesenchyme. Scale bar = 7 µm.

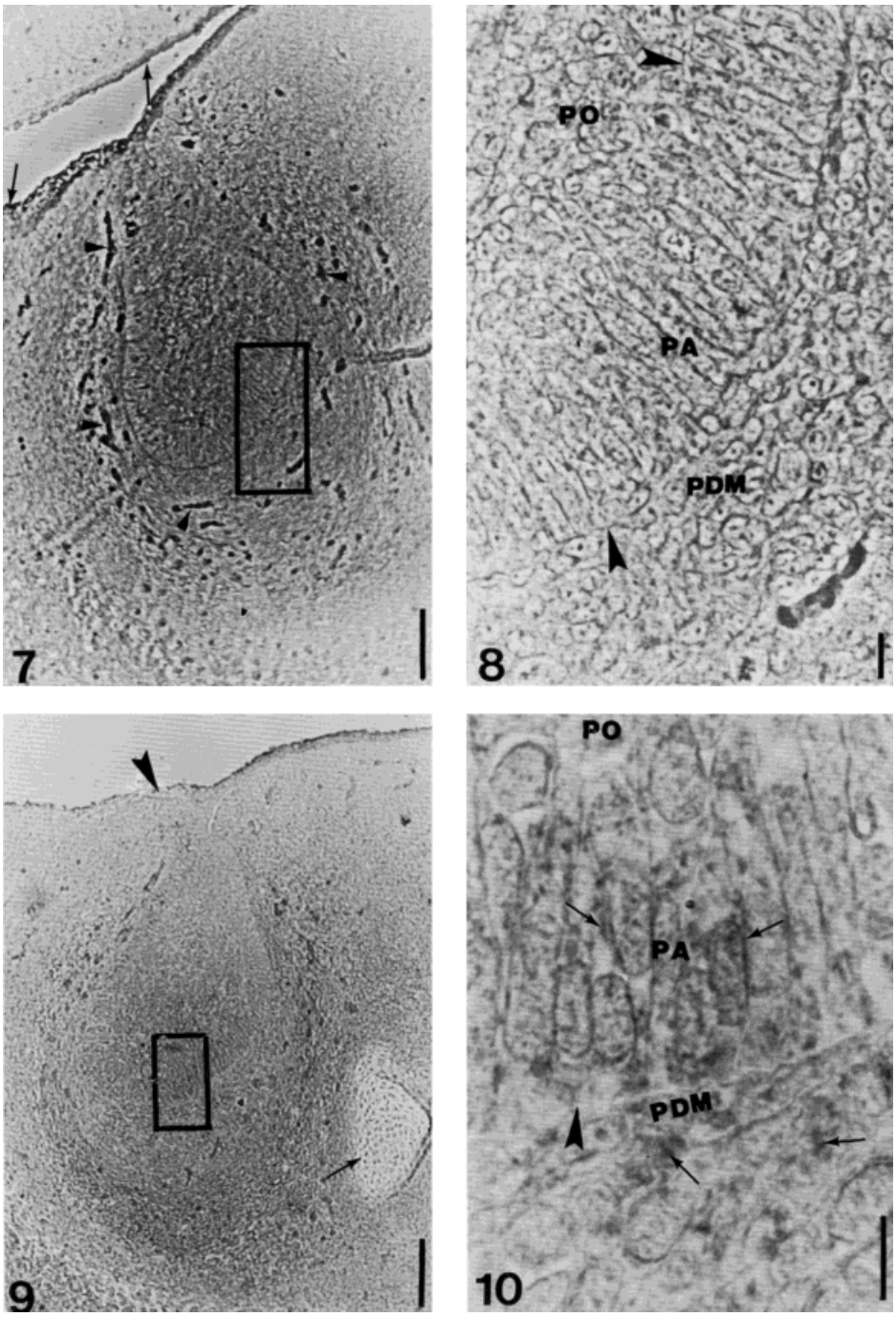
Fig. 4. *Oryctolagus cuniculus*. Higher magnification of the area enclosed by the circle in Figure 2. Affinity for BS 1-HRP is restricted to only the plasmalemma of erythrocytes and vessel walls (arrows). e, erythrocytes. Scale bar = 5 µm.

Fig. 5. *Oryctolagus cuniculus*. BS 1-HRP in the presence of N-acetylgalactosamine (GalNAc). All the positives sites shown in Figure 3 show decreased staining. PO, (pre)-odontoblasts; PA, (pre)-ameloblasts. Arrowheads point to basement membrane. Scale bar = 8 µm.

Fig. 6. *Oryctolagus cuniculus*. SBA-HRP. Affinity for this lectin is not observable. Arrow points to Meckel's cartilage. Phase contrast microscopy. Scale bar = 260 µm.



Figures 1–6



Figures 7–10

sections were then rinsed three times in PBS and incubated for 10 min 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 gradedethanol solutions, cleared in xylene, and mounted in 1% poly-L-lysine solution (Sigma Chemical Co., St. Louis, MO). The optimal concentration of each lectin (Sigma), which allowed maximum staining with minimum background, was as follows: BS-1 20 µg/ml, SBA 20 µg/ml, ECA-1 25 µg/ml, UEA I 25 µg/ml and, LTA 25 µg/ml. The sugar-binding specificity of each lectin is shown in Table 1.

Control experiments

To confirm the binding specificity of a lectin for a particular sugar, 0.1 M of an appropriate competing sugar (Table 1) was added to the solution of each lectin and allowed to react for 2 hr at room temperature prior to use. N-acetylgalactosamine (GalNAc) was used as an inhibitory sugar for BS-1, SBA, and ECA, and fucose (α-L-Fuc) was used for UEA-1 and LTA. 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.

RESULTS

The principal findings of this study are summarized in Tables 2 and 3 for the dental cap and late bell stages, respectively.

Dental cap stage

The buccal epithelium invaginates at its undersurface, encompassing an aggregation of mesenchymal cells. During this stage of development, the mesenchymal cells diverge into two different pathways: the dental papilla cells and the presumptive pulp-cell progenitors. The whole tooth germ is surrounded by the dental sac, which consists of condensed mesenchymal cells (Fig. 1) BS 1-HRP has a major affinity for terminal α-D-galactosyl residues with a secondary affinity for terminal N-acetyl-α-D-galactosaminyl residues. This lectin is bound avidly by the surface of the buccal epithelium and walls of blood vessels (see Figs. 2, 4). Higher magnification shows traces of staining of the surface and cytoplasm of the (pre)-ameloblasts and (pre)-odontoblasts, as well as the basement membrane and peridental mesenchyme (Fig. 3). When the sections are treated with N-acetylgalactosamine (inhibitory sugar for BS-1), all of the positive sites described above show decreased staining (Fig. 5).

SBA-HRP has an affinity for N-acetyl-D-galactosamine, and the lectin ECA binds to sugar sequences found in the poly-N-lactosamine series. Neither of these lectins reacts at any site of the dental germs at either the cap or bell stages (see Figs. 6, 7, 8).

UEA 1-HRP binding sites (affinity for α-L-fucose) are detected specially on the surface of the (pre)-ameloblasts and in areas of the cytoplasm of mesenchymal dental sac cells surrounding the tooth germ (peridental mesenchyme). The basement membrane is negative (Figs. 9, 10). After treatment with α-L-fucose (inhibitory sugar for UEA 1), all of the positive sites described above show decreased staining (Fig. 11). When sections of dental tissues are treated stained with LTA-HRP, which is specific for α-L-fucosyl residues, staining is not observed in any of the dental components (Fig. 12).

Dental bell stage

During this stage, the form of the tooth crown is established, and odontoblasts and ameloblasts differentiate and start to secrete dentin and enamel matrices, respectively.

The staining with BS 1-HRP is very intense in the cytoplasm as well as on the

Fig. 7. *Oryctolagus cuniculus*. ECA-HRP. Specific for N-acetyl-D-galactosamine, N-acetyl-lactosamine, lactose and D-galactose, this lectin did not bind at all in the dental cap stage. Arrows point to buccal epithelium. Arrowheads mark blood vessels. Phase contrast microscopy. Scale bar = 260 µm.

Fig. 8. Higher magnification of the area enclosed by the rectangle in Figure 7. PO, (pre)-odontoblasts; PA, (pre)-ameloblasts, PDM, peridental mesenchyme. Arrowheads point to basement membrane. Phase contrast microscopy. Scale bar = 10 µm.

Fig. 9. *Oryctolagus cuniculus*. UEA-HRP. A strong reaction is shown by the surface of the buccal epithelium, whereas a moderate reaction is observable in the mesenchyme around the dental germ as well as in Meckel's cartilage (arrow). Arrowhead points to buccal epithelium. Scale bar = 260 µm.

Fig. 10. Higher magnification of the area enclosed by the rectangle in Figure 9. Diffuse positivity is observable in the (pre)-odontoblast cells. Arrows point to some positive staining seen in the intercellular spaces between the (pre)-ameloblast and peridental mesenchyme cells. The basement membrane is negative (arrowhead). PO, (pre)-odontoblasts; (pre)-ameloblasts; PDM, peridental mesenchyme. Scale bar = 7 µm.

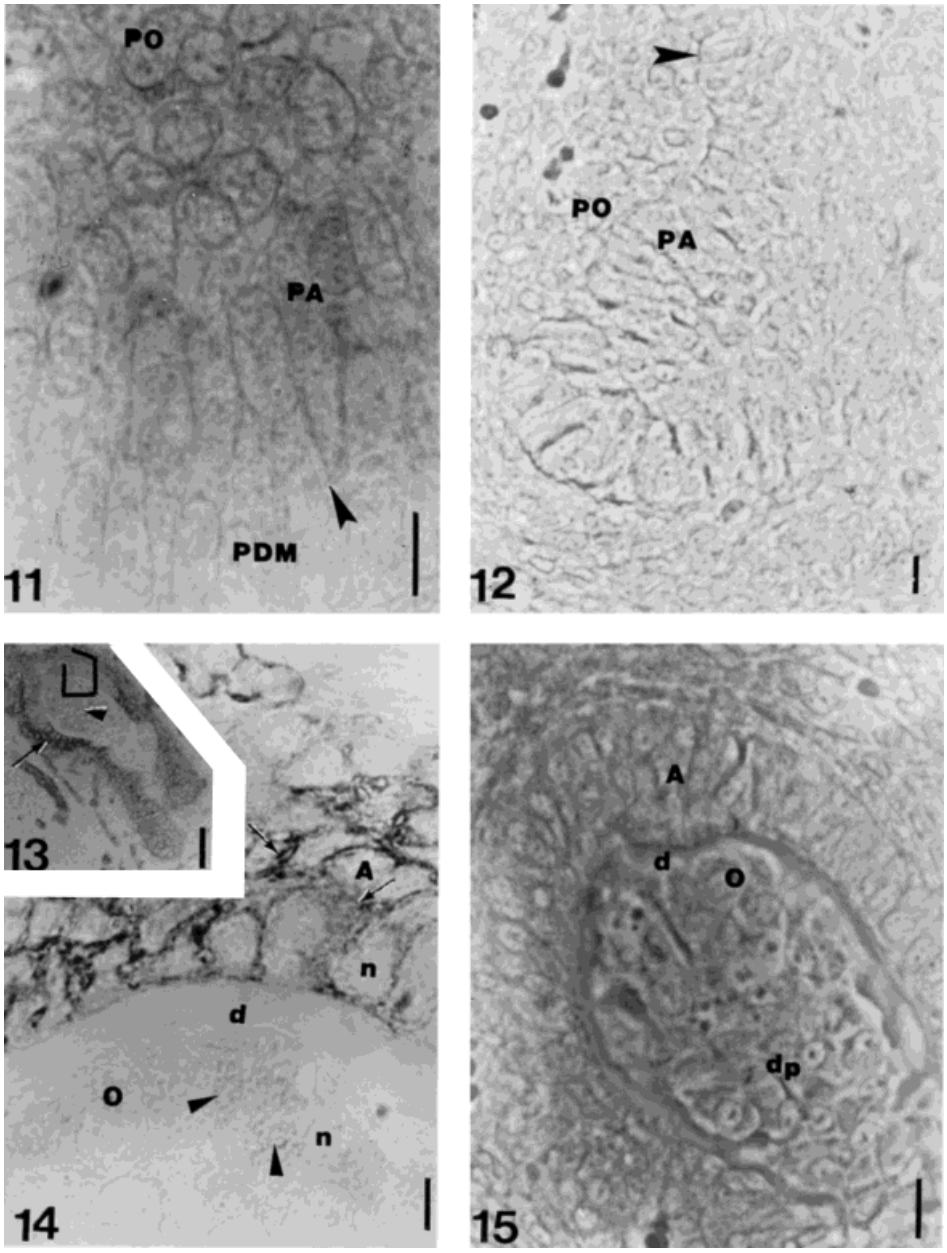


Fig. 11. *Oryctolagus cuniculus.* Tooth at cap stage stained with UEA-HRP in presence of 0.1 M alpha-L-fucose. UEA stain is partially inhibited. PO, (pre)-odontoblasts; PA, (pre)-ameloblasts; PDM, peridental mesenchyme. Arrowhead points to basement membrane. Scale bar = 7 µm.

Fig. 12. *Oryctolagus cuniculus.* LTA-HRP. All dental tissues lack staining. PO, (pre)-odontoblasts; PA, (pre)-ameloblasts. Arrowhead points to basement membrane. Phase contrast microscopy. Scale bar = 8 µm.

Fig. 13. *Oryctolagus cuniculus.* BS I-HRP. Tooth at late bell stage, 14–15th day of gestation. Arrow points to ameloblast cells. Arrowhead marks to dental pulp. Scale bar = 500 µm.

Fig. 14. Higher magnification of the area encompassed in Figure 13. Strong positive granular material is observable in the supranuclear cytoplasm of the functional ameloblasts with BS 1-HRP (arrows). The odontoblasts are diffusely positive for the lectin (arrowheads) whereas the dentin is negative. A, ameloblasts; d, dentin; O, odontoblasts; n, nucleous. Scale bar = 6 µm.

Fig. 15. *Oryctolagus cuniculus.* BS 1-HRP in the presence of N-acetylgalactosamine (GalNAc). All the positive sites shown in Figure 14 show decreased staining. A, ameloblasts; O, odontoblasts; d, dentin; dp, dental pulp. Scale bar = 8 µm.

surface of the ameloblast cells. The odontoblast cells show diffuse cytoplasmic positivity, which is more intense in the supranuclear region. The dentin is negative (Figs. 13, 14). Exposure of sections to BS 1-HRP conjugate containing α-L-fucose produce an appreciable decrease in staining (Fig. 15).

After treatment with UEA-HRP, the odontoblasts show a strong cytoplasmic affinity, whereas the ameloblast cells show a diffuse granular cytoplasmic positivity (Figs. 16, 17). Following α-L-fucose treatment, a slight decrease in stainability is seen in the odontoblast cells, whereas staining is abolished in the ameloblast cells (Fig. 18).

With SBA-HRP, binding is absent at all sites of the dental germ (Fig. 19).

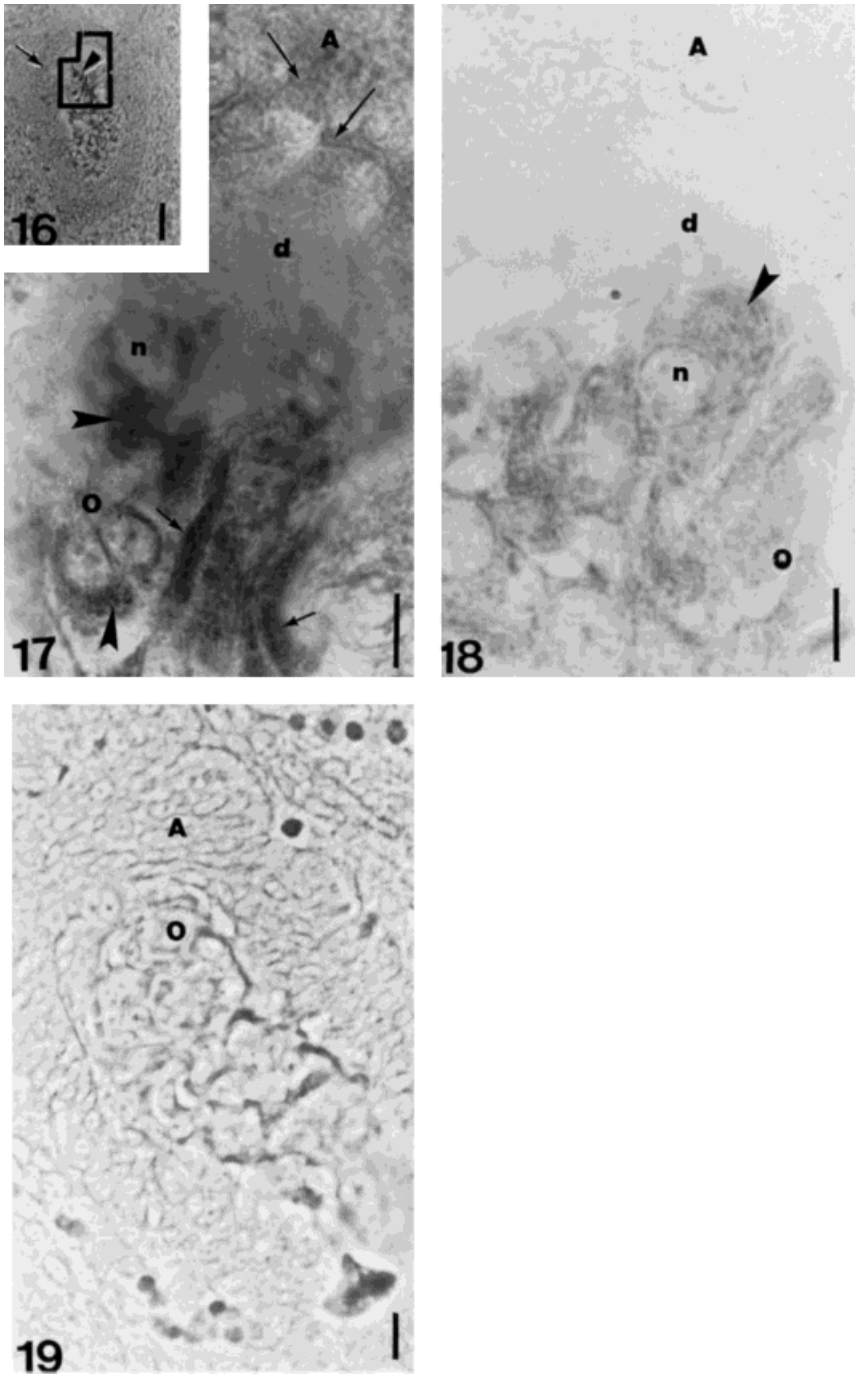
DISCUSSION

Cell surface glycoconjugates, and in particular their constituent carbohydrate moieties, are known to play an important role in many critical events during embryogenesis.

In this work we investigated the binding patterns of lectins to embryonic dental tissues in an effort to establish a relationship between morphogenesis and stage-specific changes in sugar residues during odontogenesis. Of the different lectins used, three show no binding to any region of the dental cap and late bell stages: SBA, ECA, and LTA. The remaining lectins, BS-1 and UEA-1, bind to dental cells at both stages. The appropriate control studies confirm the specific binding of the lectins. The affinity for BS-1 and the lack of positivity with SBA, although nominally both lectins recognize the same sugar residue N-acetyl-galactosamine, suggest the presence of the anomer β-N-acetyl-galactosamine (Schulte et al., '85). In the case of ECA, the absence of Galβ1-4GLcNAc in the cells during rabbit odontogenesis might represent a step in the evolution of the dental cells.

At the cap stage, a moderate staining for N-acetylgalactosamine and α-L-fucose residues is observed in (pre)-ameloblasts and (pre)-odontoblasts. The moderate expression of N-acetyl-D-galactosamine- and fucose-containing glycoconjugates at the surface of (pre)-ameloblast and (pre)-odontoblast cells probably is related to early differentiation of these cells. However, it remains possible that low levels of these glycoconjugates, not detected by these lectin methods, may play specific roles in development. With further development to the bell stage, the cytoplasm and surface of the odontoblasts display considerable amounts of fucose, whereas fucose is poorly localized in ameloblast cells. Conversely, considerable amounts of N-acetyl-D-galactosamine are present in the cytoplasm and surface of the ameloblasts, but only a diffuse positivity in the odontoblast cells is apparent. The marked changes of the glycosylation pattern of glycoconjugates such as N-acetyl-D-galactosamine in ameloblasts, and α-L-fucose in odontoblasts might indicate that they play a role during the secretion of N-acetylgalactosylated and fucosylated glycoconjugates during cell-to-cell interaction and also might be involved in regulating the beginning of the odontoblastic and ameloblastic functional activity. In fact, since all cells are covered with a dense coating of sugars, it has long been predicted that oligosaccharides must be critical determinants of cell-cell interactions.

The tooth is a good example of an organ that undergoes morphogenesis as a result of epithelial-mesenchymal interactions and in which dental cells, e.g., odontoblasts and ameloblasts, differentiate as a response to tissue interactions. These interactions initiate a series of biochemical changes that lead to the synthesis of dentin by the odontoblasts and of enamel by the ameloblasts (reviewed by Kollar, '83; Thesleff et al., '95; Zeichner et al., '95). However, there are relatively few examples in which the roles of oligosaccharides in cell-cell interactions have been clearly defined. Perhaps the best documented example is that of the selectin family of receptor proteins that mediates the adhesion of leukocytes to endothelial cells (L-selectin), the recognition of leukocytes by stimulated or wounded endothelium (E-selectin), and the interactions of activated platelets or endothelium with leukocytes (P-selectin). In each case, the minimal carbohydrate ligands involved in recognition appear to be sialylated fucosylated sugar chains, such as sialyl Lewis (Foxall et al., '92). At the cap stage, N-acetyl-D-galactosamine is present in the basement membrane between the (pre)-ameloblast and (pre)-odontoblast cells. The presence of this sugar residue is also abundant in the cell surface of the oral epithelium and in the plasmalemma of erythrocytes and the vessel walls. The dental basement membrane is believed to be significant for the alignment and differentiation of the dental papilla cells into odontoblasts (Thesleff and Hurmerinta, '81; Ruch, '85).



Figures 16–19

The staining of the basement membrane by BS-1 has been known for some time (Peters and Goldstein, '79). Arguments can be made for laminin as the molecule responsible for the basement membrane staining of BS-1, since laminin can be affinity-purified withthis lectin (Knibbs et al., '89) and contains appropriate galactosyl residues (Li Vecchi et al., '89). Other glycoproteins such as tenascin and fibronectin are involved in the interaction between the basement membrane and the mesenchymal cell surface that leads to the polarization and differentiation of odontoblasts (Thesleff et al., '87; Chiquet-Ehrismann et al., '88). Glycoconjugates containing fucose and sialic acid have been of particular interest in developmental studies since these sugars invariably occupy terminal positions on oligosaccharide side chains (Bennet et al., '74). The terminal position of fucose makes it a potential candidate as a receptor for many developmentally regulated recognition molecules, such as endogenous lectins, glycosyltransferases, or exoglycosidases on the surface of similar or different embryological cell types (Fazel et al., '89). Recently, Lemus et al. ('96) demonstrated that glycoconjugates recognized by WGA and Con A are present during rabbit odontogenesis. The most conspicuous developmental changes involve increased binding of WGA and Con A. With further development to the bell stage, the odontoblasts and ameloblasts display considerable amounts of sialic acid, as well as acetyl-glucosamine, α-D-mannose, and α-D-glucose. Glycoconjugates recognized by WGA and Con A are also present during lizard odontogenesis (Lemus et al., '94). The increases in surface-related and cytoplasmic binding sites for WGA and Con A during rabbit and lizard odontogenesis might play important roles during the formation of dentin and enamel (Lemus et al., '96).

The present study clearly shows that of the two fucose-specific lectins tested, only the lectin from *Ulex europaeus* (UEA 1) recognizes glycoconjugates in some dental tissues. Although each of these lectins has a nominal binding specificity for α-L-fucose, their preferences as to linkage and underlying sugar chain structure are known to be different. In fact, UEA 1 and LTA are thought to show the highest affinity for fucose linked (α 1–2) to galactose or other more complex difucosyl structures (Pereira and Kabat, '74; Allen and Johnson, '77; Pereira et al., '78). It has been shown that LTA does not bind to fucose linked (α 1–6) to N-acetyl-D-glucosamine (GlcNAc), and it has a very poor affinity for the fucose (α 1–3) GlcNAc linkage (Susz and Dawson, '79). The widespread binding in different tissues and cell types and the broad binding specificity of UEA 1 further suggest that this lectin is recognizing a heterogenous population of fucosylated macromolecules. Similar results have been observed in other developing systems. For example, the affinity for UEA 1 and the lack of staining by LTA in human fetal olfactory epithelium indicate the presence of a terminal α-L-fucose bound via β1.2-linkage to penultimate D-galactose-(β1-4)-N-acetyl-D-glucosamine residues and not difucosylated oligosaccharides (Foster et al., '91).

Finally, the results suggest that acetyl galactosamine and α-L-fucose residues present in (pre)-ameloblast and (pre)-odontoblast cells are common to determined but relatively undifferentiated cells capable of forming the matrices of hard tissues. Since the odontoblast and ameloblast express dentin and enamel, respectively, it can be speculated that the abundance of these residues in these cells is associated with the maintenence of their capacities to produce such matrices. Such a possibility is entirely speculative until specific in vitro experiments are conducted (Lemus, '95).

Fig. 16. *Oryctolagus cuniculus*. UEA-HRP. Tooth at late bell stage. Arrow points to ameloblast cells. Arrowhead marks dental pulp. Scale bar = 600 μm.

Fig. 17. Higher magnification of the area encompassed in Figure 16. Diffuse positivity is observable in the intercellular spaces between the ameloblast cells (long arrows), and dentin. The cytoplasm of the odontoblast cells (arrowheads), and the cells of the dental pulp (short arrows) show an appreciable binding to BS 1. O, odontoblasts; n, nucleus. Scale bar = 10 μm.

Fig. 18. *Oryctolagus cuniculus*. BS 1-HRP in the presence of N-acetylgalactosamine. The positive sites described in the ameloblasts and dentin shown in Figure 17 are completely inhibited, with the exception of the cytoplasm of the odontoblasts which shows a decrease in staining (arrow). A, ameloblasts; d, dentin; O, odontoblast; n, nucleus. Scale bar = 10 μm.

Fig. 19. *Oryctolagus cuniculus*. SBA-HRP. Affinity for this lectin is not observable. A, ameloblasts; O, odontoblasts. Phase contrast microscopy. Scale bar = 8 μm.

ACKNOWLEDGMENTS

This investigation was supported in part by grant B-3191-9644 from the DTI, Univer-

sidad de Chile. The authors thank two anonymous reviewers for their valuable comments on the article.

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