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Expression of the alpha 6 beta 4 integrin in tooth development

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EXPRESSION OF THE $\alpha_6\beta_4$ INTEGRIN IN TOOTH DEVELOPMENT

by

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THESIS

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ABSTRACT

EXPRESSION OF THE $\alpha_6\beta_4$ INTEGRIN
IN TOOTH DEVELOPMENT
by
GORDON D. DOUGLASS

Integrins are major cell membrane receptors implicated in many physiologic activities. One member of the integrin family of molecules, $\alpha_6\beta_4$, has been specifically identified with epithelial tissues. Dental enamel is the result of the calcification of a matrix secreted by ameloblasts. Since ameloblasts are epithelially derived cells, an investigation of the expression of integrins could enhance our understanding of cell-matrix interactions.

This study uses immunohistochemical techniques on cryostat sections to evaluate the presence and expression of the $\alpha_6\beta_4$ member of the integrin family in the developing teeth of the mouse. Four stages of development are used: 16 and 18 day fetuses and one day and three day old neonatal mice.

Results of this study confirm the expression of integrins in developing teeth and suggest a temporal expression of the $\alpha_6\beta_4$ integrin that is consistent with the role of this integrin as a cell-membrane receptor and it's association with stable cell contacts. Results also demonstrate a separation of basement membrane components into an ameloblastic layer and an odontoblastic layer within the developing enamel organ, with a specificity of $\alpha_6\beta_4$ for the ameloblastic layer.

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DEVELOPMENT OF THE LOWER DENTITION IN MICE:

Embryologic development of the lower dentition in mice begins at approximately the eleventh day of gestation. At this time the oral epithelium begins to thicken in three areas and invade the deeper mesenchymal tissues. Two of these ingrowths are bilateral and give rise to two dental laminae (one on each side of the arch) from which the molar tooth buds originate. The third ingrowth is anterior and gives rise to two incisor tooth buds, as well as, a lip furrow band that will eventually separate within itself to form the facial vestibule (Hay, 1961).

Each epithelial tooth bud develops into a "bell" shape with the concavity of the bell filled with mesenchymal tissue called a dental papilla. The layer of mesenchymal cells which line the bell further differentiate into the precursor cells of the dentine, the odontoblasts (Fig. 1). Differentiation of the odontoblasts seems to be a signal for the further differentiation of the apposed epithelial cells into ameloblasts (Fig. 1) (Gorter de Vries et al., 1986; Thesleff et al., 1981; Slavkin and Bringas, 1976). Each fully developed tooth thus appears to be derived from two embryologic tissue types. This perception has been

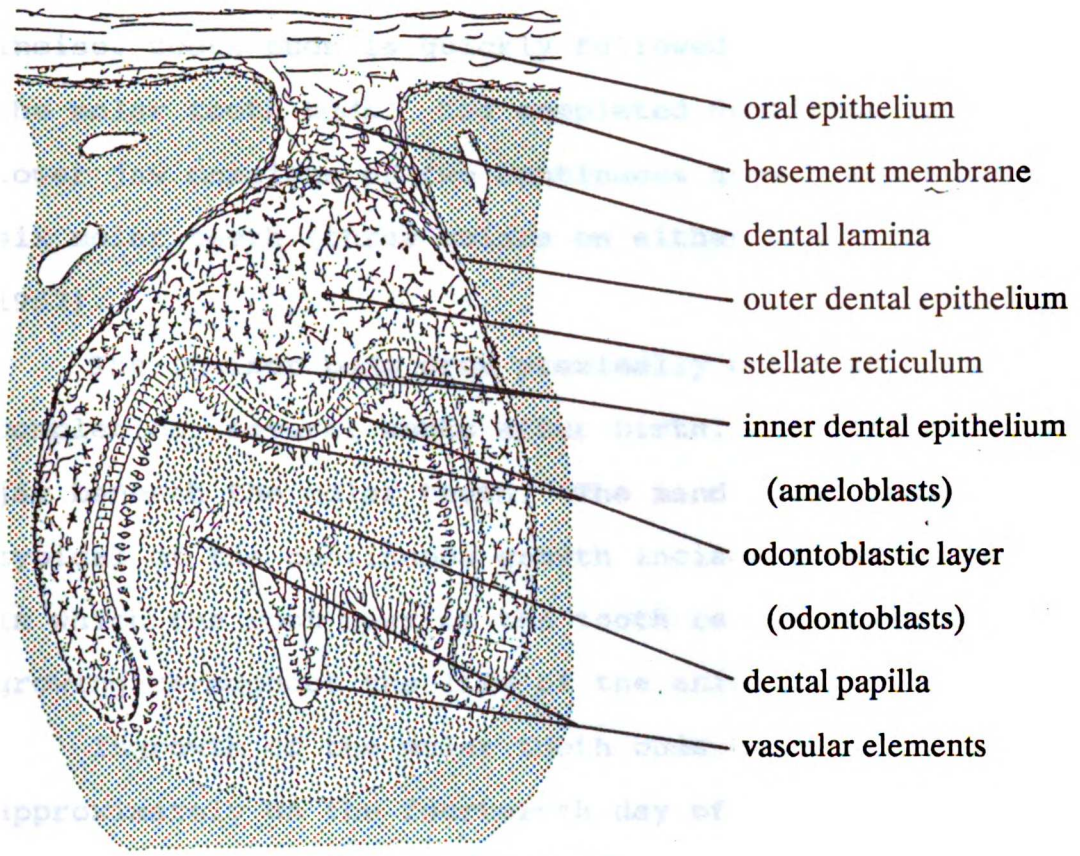

Figure 1

Diagrammatic representation of a tooth bud at the bell stage of development

blurred by evidence that the differentiating mesenchymal cells are proto-ectodermally derived cells (ectomesenchyme) that arise from the neural crests of the neurectoderm (Thesleff et al., 1990; Smith and Hall, 1990).

Development of all the teeth does not take place at one time but rather occurs over a period of time beginning with the incisor teeth. The ingrowth of the

incisor tooth buds is quickly followed by an ingrowth of the molar tooth buds. The completed dentition in the lower jaw consists of two continuous growth incisors and six molar teeth (three molars on either side) (Cohn, 1957).

The incisor buds grow proximally until at completion, several weeks after birth, they lie along the jaw beneath the molar teeth. The mandibular incisor is typical of the continuous growth incisor found in rodents in which the root apex of the tooth remains open and growing throughout the life of the animal.

Ingrowth of the molar tooth buds begins approximately on the fourteenth day of gestation, about one day later than the incisors. The first molars precede the second molars by three to four days but each develops in a similar fashion as a bell-shaped invasion off of the dental lamina into the mesenchyme (Fig. 2). The molar teeth grow to have completed roots and are not continuously growing as are the incisors

Differentiation of mesenchymal cells into odontoblasts and the secretion of predentin occurs prior to the beginning of amelogenesis in molars and incisors (Slavkin and Bringas, 1976; Thesleff et al., 1981; Gorter de Vries et al., 1986). As in the human dentition, amelogenesis is limited to the inner dental epithelium (IDE) (Fig. 1). Interestingly, the development of the

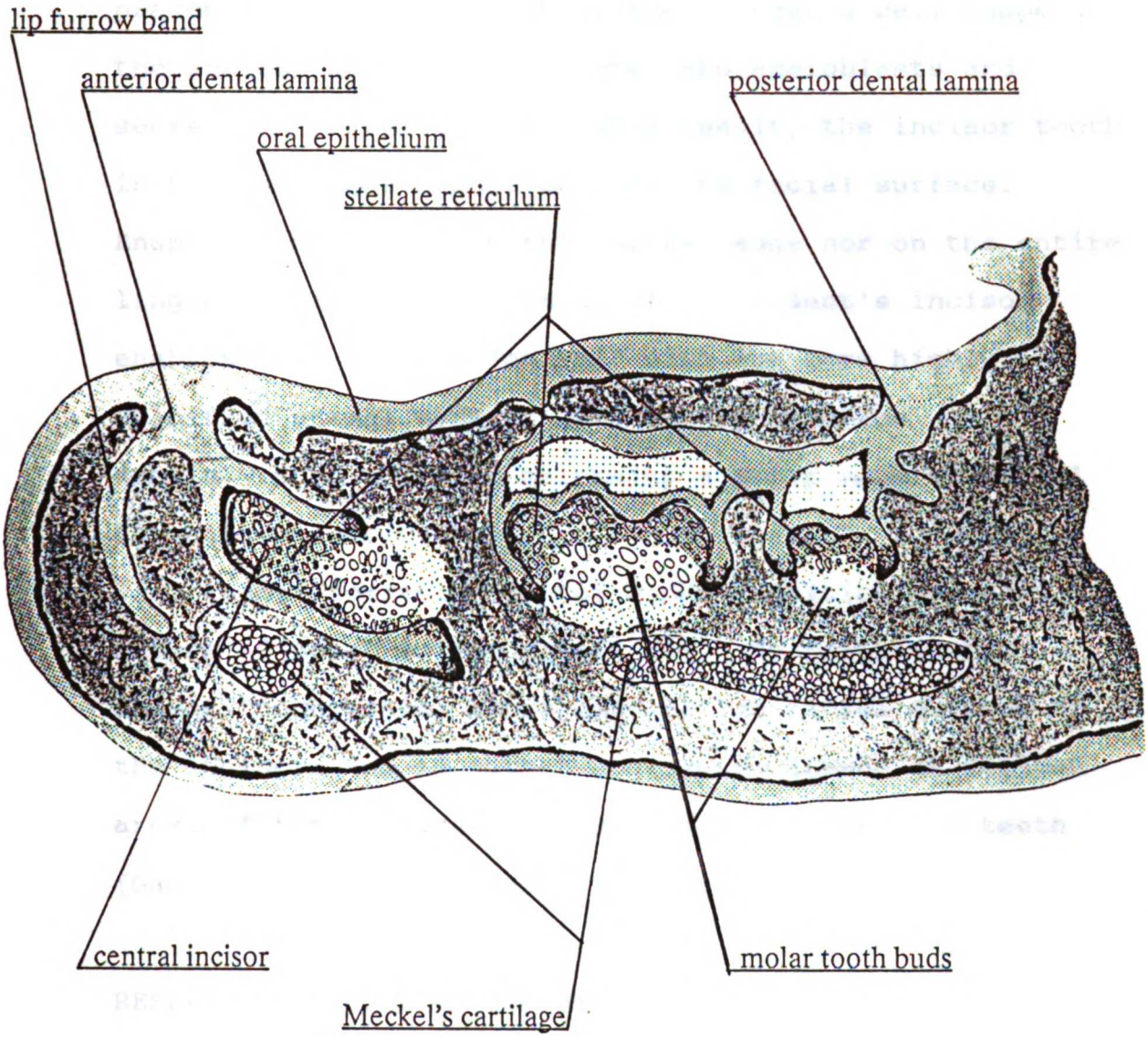

Figure 2

A diagrammatic, composite representation of a mouse mandible, based upon several time points from fetus to newborn, to illustrate the anatomical relationships of the developing mandible and its associated teeth.

incisor parallels the molar teeth to the bell stage but only those epithelially derived cells positioned on the predestined facial aspect of the elongated bell shape of the incisor bud, differentiate into ameloblasts and secrete an enamel matrix. As a result, the incisor tooth in the adult has enamel only on its facial surface. Enamel is not found on the incisal edge nor on the entire lingual surface. The design of the rodent's incisor enables its use as a "chisel" with the more highly calcified enamel wearing more slowly than the dentin. Such a design maintains a sharp edge of enamel for cutting efficiency.

Enamel formation in the human dentition normally occurs adjacent to the entire IDE. By contrast the rodent tooth enamel forms only on the facial surface of the incisors and is either reduced or absent in defined areas of the occlusal surfaces of the molariform teeth (Gaunt , 1956; Cohn, 1957; Snead et al., 1988).

RESEARCH IN TOOTH DEVELOPMENT:

Hay (1961) described in detail the development of the mouse incisors and molars but since development of the mouse molar teeth is more analogous to human tooth development, most of the papers cited in this work refer to molar development. Developing teeth offer a unique opportunity to study organogenesis because the spectrum

of development is broad and tissues at a given age can display a wide range of developmental stages.

Intercellular and cell-matrix communication during embryogenesis is essential for the orderly development of the organism. In the development of the teeth it has been hypothesized that the extracellular matrix (ECM) present at the epithelio-mesenchymal interface of developing tooth germs mediates differentiation of odontoblasts and ameloblasts (Slavkin and Bringas, 1976; Lesot, H. et al., 1985). Studies have shown that a separation of the two developing cell types interferes with the formation of predentin and further differentiation of the ameloblasts (Koch, 1967). Reith (1970), Slavkin and Bringas (1976), Katchburian and Burgess (1983), and Sawada et al. (1990) found evidence of a disappearance of the basement membrane (BM) between the odontoblasts and the ameloblasts indicating cell-cell interaction. This suggests that direct odontoblast-ameloblast cell interaction may be essential for the induction of enamel proteins.

Research has also been directed toward the changing expression of specific molecules associated with ameloblasts, odontoblasts and the ECM during tooth development (Lesot et al., 1981; Thesleff et al., 1981; Laurie et al., 1982; Thesleff et al., 1988; Lesot et al., 1988). Lesot et al. (1981) used immunofluorescence to

determine the localization of collagens, fibronectin and laminin in the developing *molar* teeth of eighteen day mouse fetuses. They concluded that collagen types I, III, IV, laminin and fibronectin were associated with the dental epithelio-mesenchymal junction but that type III collagen could no longer be detected in the BM of terminally differentiated odontoblasts. They further stated that type IV collagen and laminin were specifically localized to the BM and that fibronectin within the dental papilla was less intense at the tip of the developing principal cusps with fluorescence increasing toward the central part of the papilla.

Thesleff et al. (1981) used mandibular *first molars* of 16 and 18 day fetuses, and 1, 2, and 3 day neonatal mice to show type IV collagen, laminin, and heparan sulfate proteoglycans to be evenly distributed along the BM prior to differentiation of the mesenchymal and epithelial cells of the tooth germ. They also detected fibronectin in the BM of the IDE and suggested it was more intense there than in the BM associated with the oral epithelium or the outer dental epithelium (ODE). It was suggested that as tooth development progressed the BM of the IDE was degraded and, as a result, staining for type IV collagen, laminin and the BM proteoglycan was lost.

Laurie et al. (1982) looked at developing rat *incisor* teeth and found positive immunostaining of the enamel

organ "2 mm from the tooth's posterior extremity" for type IV collagen laminin and heparan sulfate proteoglycan in the basal lamina. They did not report detecting a gradient of staining. It is likely that the area used as a standard was within the continuous growing area of the root and thus would tend to confirm the findings of Thesleff et al. (1981) and Lesot, et al. (1981) regarding the presence of these molecules in the BM.

Later work by Thesleff et al. (1988) correlated the expression of the cell surface proteoglycan, syndecan, with *molar* tooth development in the 11 to 19 day embryos of mice. Results indicated an intense immunofluorescent staining of the oral epithelium. With ingrowth of the tooth bud and differentiation of the mesenchyme, staining was progressively lost in the developing enamel epithelium while more intense staining occurred in the dental papilla. It was speculated that syndecan might function as a cell adhesion molecule during certain developmental stages.

Snead et al. (1988) used *in situ* hybridization to demonstrate a temporal and spatially restricted pattern for the expression of the amelogenin gene during mouse molar development. Results indicated that amelogenesis is coordinated between left and right molars and initiated in specific areas on the slopes of the cusps before spreading to adjacent ameloblasts. A question

left to be answered is how cells (ameloblasts) that are members of a continuous sheet of epithelial cells, linked by gap junctions and without a BM can have a variable transcription of gene expression leaving enamel free areas.

Lesot et al. (1988) used a monoclonal antibody (MAb) to a non-integrin cell membrane protein (165 Kd) to show an association of the protein with intracellular microfilaments in cultured "dental mesenchymal cells" from fifteen day mouse embryos. It was suggested that the, as yet, uncharacterized protein was involved in transmembrane signal transduction.

Obviously there are many questions left to be answered. Most of these seem to revolve around the question of intercellular and cell-ECM communication. This question has been made more interesting by the suggestion that the BM is degraded as a part of the terminal differentiation of ameloblasts (Reith, 1970; Slavkin and Bringas, 1976; Katchburian and Burgess, 1983 and Sawada et al., 1990). Seeking an answer has been made more difficult by the plethora of techniques (fixed, frozen, paraffin, epoxy resins, etc.), the variety and ages of the animals reported on (rat, mouse, monkey, swine, human etc.), the teeth chosen (molar or continuous growth incisor of the rodent) and the spatial variations in matrix production of the IDE (Gaunt, W., 1956;

Cohn, S., 1957; Snead et al., 1988).

The discovery of a family of cell surface molecules, some members of which have been shown to be associated with the BM, may shed some light on the subject.

INTEGRINS: CELL ADHESION RECEPTOR MOLECULES:

Studies on cell membrane constituents in the 1970's and early 80's demonstrated the ability to identify and manipulate cell-cell and cell-substrate interactions (Wylie et al., 1979; Damsky et al., 1981). In 1981 a glycoprotein complex called IIbIIIa was detected in platelet membranes (Jennings and Phillips, 1982). Since then a growing family of cell membrane molecules known as integrins have been described (Hynes, 1987; Ginsberg et al., 1988). Integrins have been implicated in many important physiologic activities including embryologic development, hemostasis, thrombosis, wound healing, immune and non-immune defense mechanisms and oncogenic transformations (Hynes, 1987; Rouslahti and Giancotti, 1989; Hemler, M. 1990).

Integrins are non-covalently linked heterodimers which were originally divided into three subfamilies: the VLA protein family, the Leu-CAM family and the cytoadhesins (Hogervorst, F. et al., 1990). The subfamilies were characterized by a common β subunit that could associate with one of several α subunits. These

original family delineations have been blurred by the discovery of at least seven different β subunits and that some α subunits can associate with more than one β subunit (Albelda and Buck, 1990).

INTEGRINS SPECIFIC TO EPITHELIAL CELLS

Recently a unique β subunit was identified that associates with a previously described α_6 subunit. It has been accepted that this heterodimer is a fourth subfamily of integrins and should be called "$\alpha_E\beta_4$" (Kajiji et al., 1989). The label α_E was suggested because the heterodimer was thought to be limited to epithelial cells. Immunohistochemical analysis however, showed that β_4 is also expressed weakly on Schwann cells (Hogervorst et al., 1990). Since the α_6 subunit had been previously described and associated with a common β_1 subunit (Sonnenberg et al., 1988), and three other β subunits had been identified (Kajiji et al., 1989), it seemed logical that the name for this unique member of the integrin family should be designated $\alpha_6\beta_4$.

The α_6 subunit to date has only been found in association with either the β_1 or the β_4 subunit. It has been suggested that the $\alpha_6\beta_1$ integrin is a specific receptor for the E8 fragment of the A chain of laminin (Sonnenberg et al., 1990; Hall et al., 1990; Sorokin et al., 1990) and that antibodies to the α_6 subunit inhibit

development of kidney epithelium in mice (Sorokin et al., 1990) . Korhonen et al. (1990) found the $\alpha_6\beta_1$ integrin to be associated with developing and adult human kidney tubular epithelial cells but found the $\alpha_6\beta_4$ integrin to be absent in the adult kidney and isolated to the fetal kidney collecting ducts. Carter et al. (1990) suggests that β_1 subunit integrins are associated with dynamic focal adhesions associated with the spreading and migrating of cells while the $\alpha_6\beta_4$ integrin is associated with stable non-motile cells.

$\alpha_6\beta_4$ is a unique integrin in that it is expressed strongly only in epithelial cells. It has been identified and characterized in mouse mammary tumor cells (Sonnenberg et al., 1988), a variety of other diverse malignant carcinomas (Falcioni et al., 1988; Kennel et al., 1989) and in embryogenesis (Korhonen et al., 1990 and Stepp et al., 1990). Its uniqueness has been corroborated by its lack of reactivity with various antisera to the three previously identified integrin β chains (Kajiji et al., 1989).

Cloning and sequence analysis of the β_4 subunit has identified even more unique features. The extracellular part is largely homologous to other β subunits but the intracellular domain is considerably larger than all other identified β subunits (205 Kd vs. 90-130Kd) (Hogervorst et al., 1990). It has been suggested that

one of the functions of the exceptionally large cytoplasmic domain is to enable direct interaction with the cytoskeleton without the involvement of the linker proteins: talin, vinculin and α-actinin. Evidence indicates this may be true (Hogervorst et al., 1990).

Carter et al. (1990), Stepp et al. (1990), and Sonnenberg et al. (1991) suggested that the $\alpha_6\beta_4$ integrin is associated with hemidesmosomes, and Jones et al. (1991) demonstrated that antibodies to $\alpha_6\beta_4$ interfere with the assembly of hemidesmosomes in cultured cells. Evidence seems to indicate that $\alpha_6\beta_4$ is an integral part of hemidesmosomes and that the integrin may play a major role in interactions with intermediate filaments within the cytoplasm.

The $\alpha_6\beta_4$ integrin does not bind to the E8 fragment of laminin (Sonnenberg et al., 1990), however, recent work by Carter et al (1991) characterizes a newly identified glycoprotein, "epiligrin", present in the BM, and offers this as a ligand for a super adhesion complex of $\alpha_3\beta_1$ and $\alpha_6\beta_4$. The actual ligand for $\alpha_6\beta_4$ still remains to be identified.

If hemidesmosomes are the anchoring structures between cells and the ECM, then one could expect a decreased number in migrating cells. Stepp et al., (1990) found that the $\alpha_6\beta_4$ integrin localized to hemidesmosomes and used the development of the rabbit

cornea as a model to follow their development in stratified squamous epithelium. This work indicated a gradient of hemidesmosome formation consistent with migrating cells, starting at the eyelid margin and spreading along the conjunctiva to finally form in the cornea at 20 days of fetal development.

INTEGRIN EXPRESSION IN DEVELOPING TEETH

Hormia et al. (1990) surveyed adult human gingivae for integrin localization and demonstrated expression of α_6 in the basal cell layers and along the basal lamina of the epithelium. They also found β_4 to be localized to the basal lamina. There has been no published research, however, on the expression of integrins in the developing dentition. Given the findings of others on the expression of integrins in developing tissues (Stepp, et al. 1990; Kehonen et al. 1990 and Sorokin et al. 1990) one would expect the $\alpha_6\beta_1$ and the $\alpha_6\beta_4$ integrins to be significantly represented in odontogenesis. Are these integrins expressed in developing teeth and if so, does their distribution change during the ingrowth of the tooth bud, the formation of the bell stage and the degradation of the basement membrane of the IDE during the maturation of the ameloblasts?

The purpose of this project was to investigate the expression of the α_6 and the β_4 integrin subunits in

developing molar teeth of the lower jaw of the mouse. Evidence of the α_6 subunit in tissue was taken to indicate an expression of either $\alpha_6\beta_1$ or $\alpha_6\beta_4$ since α_6 is not known to associate with any other β subunit. Evidence of β_4 colocalization with the α_6 integrin in tissue was taken to indicate the possible existence of hemidesmosomes. Confirmation of the existence of the β_1 subunit was not possible due to the unavailability of a β_1 MAb that cross reacts with mouse antigen.

MATERIALS AND METHODS:

PREPARATION OF TISSUES:

Fetuses dissected from pregnant female Swiss Webster mice at days 16 and 18 gestation and neonatal mice of the ICR strain at 1 day, and 3 days of age were used. Fragility of tissues required whole head preparations of embryos while mandibles were dissected from the neonatal animals and sectioned in half using a dissecting scope at 20x. No attempt to remove the overlying epithelium and connective tissue was made. Specimens were immediately embedded in O.C.T. compound (Miles Laboratories, Naperville, IL), quick frozen in liquid nitrogen and stored at -80°C.

Tissue sections were made on a cryostat (Bright Instrument Company Limited, Huntington, England).

Various cryostat cutting speeds, temperatures, and section thicknesses were tried. The most acceptable specimens were achieved with slow cutting speeds at -20°C and sections of 10μm. Sections were immediately placed on uncoated glass slides and allowed to air dry. Because of the relative size of the tissue samples to the actual tooth buds, periodic sections were quick stained with toluidine blue for referencing.

At a later date, prior to further processing, all frozen sections were evaluated using phase contrast microscopy to determine that artifacts did not obscure the tissues of interest. This was especially a problem with the 3 day mandibles because of early enamel and dentine calcification and the inability to section these without distorting the specimen. Tissue sections were stored at -80°C in black, slide storage boxes until further processed.

PRIMARY ANTIBODIES:

Rat derived MAbs from a rat/mouse hybridoma recognizing the β_4 subunit (346-11A) was a gift from S. J. Kennel (Oak Ridge National Laboratory, Oak Ridge, Tennessee). Rat derived MAb for the α_6 subunit (GoH3) was acquired from A. Sonnenberg (The Netherlands Cancer Institute, Amsterdam, The Netherlands). This antibody generally stained tissues well but seemed to stain

embryonic tissues inconsistently. To supplement staining for embryonic tissues, the anti-human MAb to α_6, J1B5 (C. Damsky, University of California, San Francisco), proved to cross react well. Anti-rabbit polyclonal antibodies (PAb) to the fibronectin receptor (FNR), β_1, from E. Rouslahti (Cancer Research Center, La Jolla, CA) and PAb to the FNR., β_1, from Telios (San Diego, CA 92121) were tried but these did not show staining of the tissues tested.

Rabbit derived PAb to type IV collagen was acquired from H. Furthmayr (Department of Pathology, Stanford University, Palo Alto, CA) and rabbit derived PAb to laminin was purchased from Gibco Laboratories (Life Technologies Inc. Grand Island, N.Y.). The use of laminin and collagen type IV PAbs enabled the localization of the apparent basement membrane since these two antigens have been shown to be co-localized in that structure in dental tissues (Thesleff, et al., 1981; Lesot, et al., 1981; Laurie, et al., 1982).

A negative control (no primary antibodies) was used with each experiment. Control tissue sections were kept moist with D-PBS with 1% normal goat serum and Na-azide (Appendix A). Concentrations and times of applications used for all antibodies are listed in Table I.

SECONDARY ANTIBODIES:

Antibodies and suppliers of stains used for immunofluorescent techniques are listed in Table I. One and three day neonatal tissues displayed considerable autofluorescence. To overcome this problem, the results of immunofluorescent techniques on neonatal tissues were confirmed with peroxidase conjugated immunoglobulin G techniques. Concentrations and times of application used are listed in Table I.

IMMUNOFLUORESCENT STAINING:

Tissue sections to be stained were removed from storage and placed flat under a hood in open air to dehydrate for 30 minutes. Tissues were fixed with freshly diluted 0.1% formaldehyde (see Appendix A for Media, Buffers and substrates) for 3 minutes and then washed five times with DME H-16 culture medium to quench the fixation. The culture medium was immediately rinsed away with five washes using modified D-PBS (Appendix A).

Primary antibodies were placed over the tissues using 25μl of each antibody for each tissue section. Antibodies were left on the tissues according to the times listed in Table 1 and then rinsed off five times with D-PBS modified as noted previously. Secondary antibodies were placed over the tissues using 25μl of each antibody for each tissue section according to the

times listed in Table 1 and then rinsed off five times with modified D-PBS.

Excess D-PBS was removed and the moist tissues covered with Fluoromount (Fisher Scientific, Orangeburg, New York) and coverslip. To maintain the coverslip in position a small amount of fingernail polish was used at three points to tack the coverslip to the slide surface. Tissue sections processed for immunofluorescence were kept in darkness until evaluation in order to avoid quenching of the secondary antibody fluorescence.

IMMUNOPEROXIDASE STAINING:

Tissue sections to be stained for the immunoperoxidase technique were removed from storage and allowed to dehydrate for 30 min. under a hood. Sections were fixed with 0.1% formaldehyde for 30 min. and then rinsed five times with D-PBS modified as previously noted but without Na-azide. Other than maintaining azide free D-PBS, the placement of primary and secondary antibodies was the same for peroxidase and fluorescence techniques.

Secondary antibodies used for immunoperoxidase techniques were either goat anti-rat for the anti α_6 and β_4 primary antibodies, or goat anti-rabbit for the PAbs (Gibco, Sigma Chemical Company, St. Louis, MO.) After the peroxidase conjugated secondary antibodies were rinsed off, the sections were treated with

diaminobenzidine (DAB)(Appendix A). The time of treatment with DAB was based upon the development of an adequate depth of staining. This was determined by visual evaluation of the amount of enzymatic precipitation on the section using a magnifying hand lens.

When an adequate depth of staining was achieved, the DAB was immediately washed off with five rinses in distilled water and dehydrated with 70% and then absolute ethyl alcohol. Sections were allowed to air dry prior to the placement of Permount (Fisher Scientific, Fairlawn, New Jersey) and coverslip.

MICROSCOPY AND PHOTOGRAPHY:

A Nikon microscope (Labophot with Episcopic-Fluorescence Attachment EF-D) with attached 35mm camera was used to view the slide sections. Sections were viewed and photographed under oil immersion at 40x or more. Photographs of fluorescent tissues were taken on Kodak ASA 400 T-MAX film and developed using Accufine developer (Accufine Inc., Chicago Il 60611) to push the ASA rating to 800. Immunoperoxidase slides were photographed using the same equipment and developing technique with Kodak ASA 100 T-MAX film pushed to ASA 200 with the Accufine developer.

Exposed film was placed in a developing canister in

total darkness. The canister was filled with developer and allowed to sit with only slight agitation for 4.5 minutes. Developing was stopped with a water wash and the canister then filled with Kodak Rapid Fixer for 3 minutes. The fixer was replaced with Hypo clearing solution for 1 to 2 minutes, rinsed with running water for 5 minutes and soaked for 30 seconds in standing water to which Kodak Photoflo was added. The processed film was then allowed to air dry. Black and white prints were made using AGFA P 1-4 paper.

RESULTS:

SIXTEEN DAY EMBRYO:

Development of the first molar tooth at sixteen days of gestation had progressed to the early bud stage. No evidence of second molar development was seen in the sections studied. There was no polarization of eloblasts nor any evidence of predentin matrix production by the odontoblasts. Staining with antibodies to laminin, collagen type IV, α_6, and β_4 revealed continuous staining around the entire tooth bud (Fig. 3). Vascular elements (VE) within the mesenchyme stained prominently for laminin and collagen IV. There was no staining of VE's for the β_4 subunit and staining for the α_6 subunit was inconsistent in VE's (Fig. 3).

FIGURE 3

Immunofluorescent stained tissue sections of the whole head at 20X showing just the area of the developing molar bud at 16 days of gestation. A): staining for collagen IV showing both the epithelial basement membrane of the maxillary arch (small arrowheads) and the tooth bud of the mandibular first molar (large arrowheads). Vascular elements (VE) stain prominently and one area of the basement membrane has been sectioned tangentially (T). The apposed maxillary and mandibular epithelial surfaces have been marked with a dotted line. B): staining for laminin of a similar cross section, showing the maxillary basement membrane (small arrowheads) and the mandibular tooth bud (large arrowheads). VE's stain prominently. C): staining for α_6 showing positive staining of the maxillary basement membrane (small arrowheads) and the mandibular tooth bud (large arrowheads). Note the absence of staining of VE's. D): staining for β_4 showing positive staining of the maxillary basement membrane (small arrowheads) and the mandibular tooth bud (large arrowheads). E and F): double staining of sections at 60X for laminin (E) and for β_4 (F) illustrate the colocalization of these two molecules in the basement membrane (arrows).

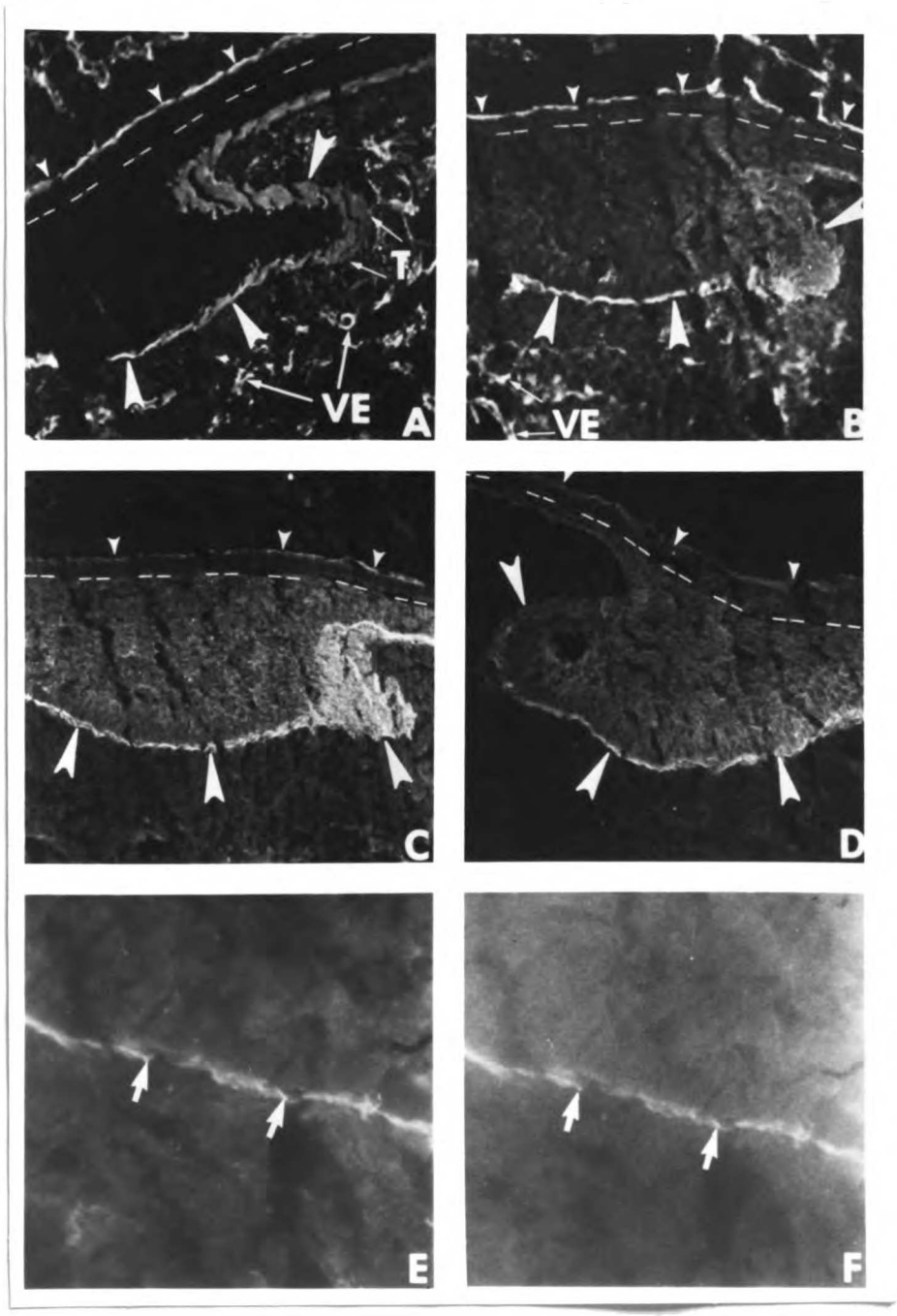

FIGURE 3

EIGHTEEN DAY EMBRYO:

By the eighteenth day of gestation the mandibular first molar tooth bud had developed to the bell stage while the second molar was now at an early bell stage. Staining of the BM at the epithelial lining of the oral cavity was positive for laminin, collagen IV, α_6, and β_4 (Fig.4). Positive staining for the α_6 subunit, laminin and type IV collagen extended around the entire tooth bud (Fig. 4 A,B,C). Staining for the β_4 subunit as compared to the α_6 subunit, however, became less intense at the interface of the presumptive IDE and the mesenchyme (Fig. 4 B,D,E,F).

Intercellular staining of the epithelially derived cells with antibodies to α_6 and β_4 was more evident in specimens beginning at the eighteenth day of development (Fig. 4 B,D,E,F). VE's stained strongly for collagen IV, laminin, and α_6 (Fig. 4 A,B,C,E) at the 18 day time-point, but only weakly for β_4 (Fig. 4 D) or not at all (Fig. 4 E).

ONE DAY NEONATAL MOUSE:

Staining for laminin and type IV collagen in the one day old mouse molar was less intense along the ODE but strongly positive in the BM of the enamel organ. A single line of staining was found along the root sheath but this separated into two distinct apparent BM's as the

FIGURE 4

Double immunofluorescent staining of an 18 day fetal first molar at 20X for collagen IV (A) and α_6 (B) and for laminin (C) and β_4 (D), and successive sections of a second molar from another fetus of the same age, stained for α_6 (E) and β_4 (F). A): staining for collagen IV using FITC conjugated secondary antibodies, is positive at the epithelial surface basement membrane (small arrowheads). Staining at the outer dental epithelium (ODE) and the inner dental epithelium (IDE) is positive. B): staining of the same section as (A) for α_6 using rhodamine conjugated secondary antibodies is similar in pattern to the staining for collagen IV. Note the evidence of staining of VE's. C): staining for laminin using FITC conjugated secondary antibodies is similar in pattern to the staining for collagen IV shown in (A). D): staining for the β_4 subunit in the same section as (C) using rhodamine conjugated secondary antibodies is positive at the oral epithelial basement membrane (OE) as well as at the ODE. Staining at the IDE is more diffuse at the basement membrane than for the ODE. Note the intercellular staining in the oral epithelium as well as in the ameloblasts of the stellate reticulum (SR). Staining for α_6 is more distinct at the IDE basement membrane in (B) than is seen for β_4 in (D). E and F show a comparison of consecutive sections of a second molar tooth bud stained for α_6 (E) and for β_4 (F). Note the more diffuse IDE and the intercellular staining of the stellate reticulum (SR) in F.

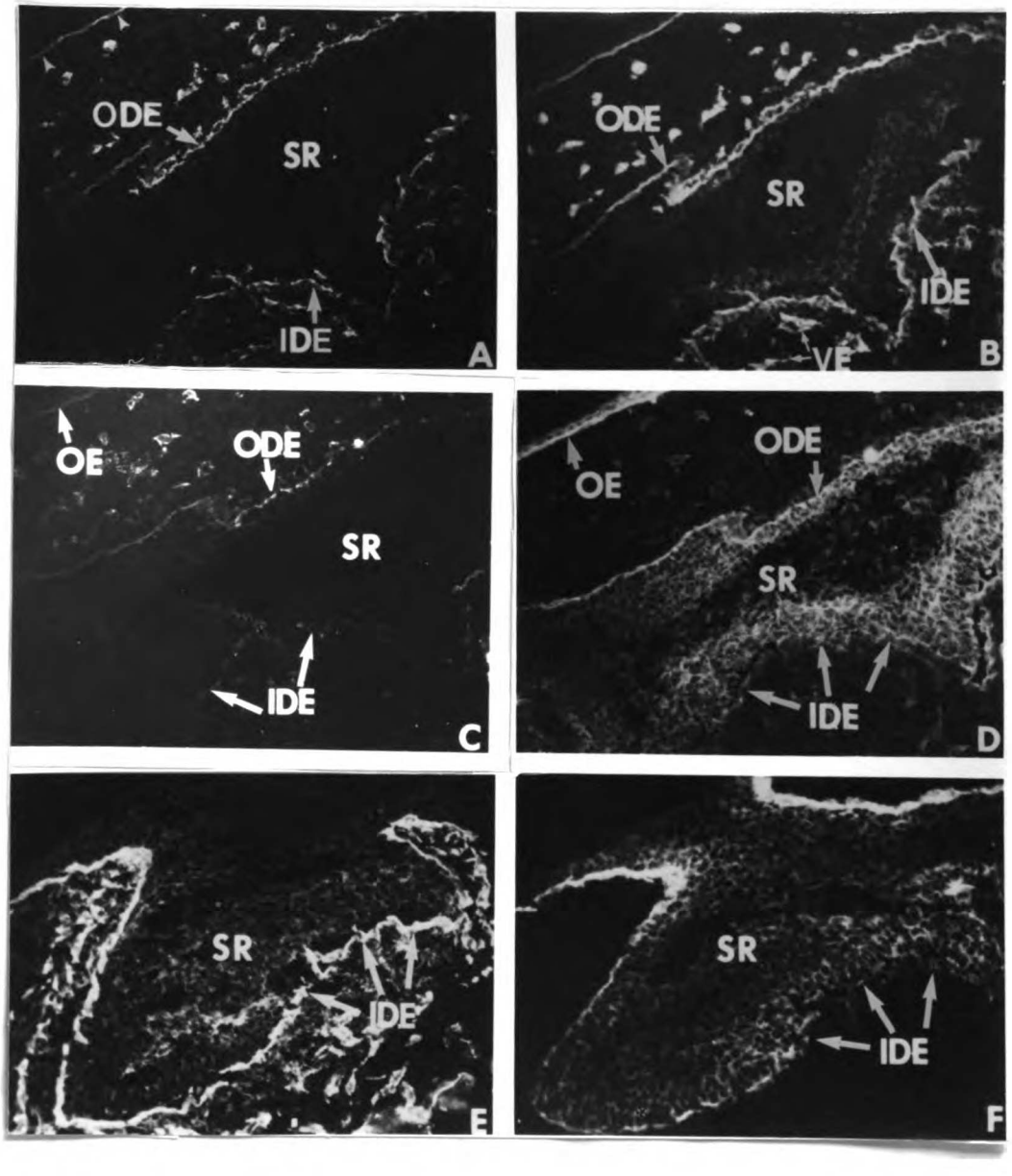
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FIGURE 4

tip of the cusp was approached (Fig. 5 A,B).

In contrast to the staining for laminin and collagen IV, the α_6 and β_4 antibodies did not indicate a double BM but instead were limited to the BM of the ameloblasts (Fig. 5 C,D).

Significant autofluorescence of the developing dentin and enamel was a concern and tended to obscure results (not shown). For this reason, parallel staining with immunoperoxidase was used to support the research findings. Results of these studies confirmed the results found with immuno-fluorescence (Figs. 6 A-D and 7 A-D).

FIGURE 5

Tissue sections of a 1 day neonatal mouse tooth buds stained using immunofluorescence. A): At 10X, staining for collagen IV is positive at the apparent basement membrane of the inner dental epithelium (IDE) and along the root sheath (RS). At the cusp tip the staining divides (arrowheads). B): a cusp tip, at 40X, stained for laminin, with staining of an apparent ameloblastic basement membrane (ABM), and an odontoblastic basement membrane (OBM). C): staining for α_6 at the cusp tip is positive for the ABM but not at the OBM. D): staining of a typical root sheath for β_4, shows positive staining at the ABM (arrows) with a decreasing intensity toward the tip of the sheath ((RS) and along the outer dental epithelium (arrowheads).

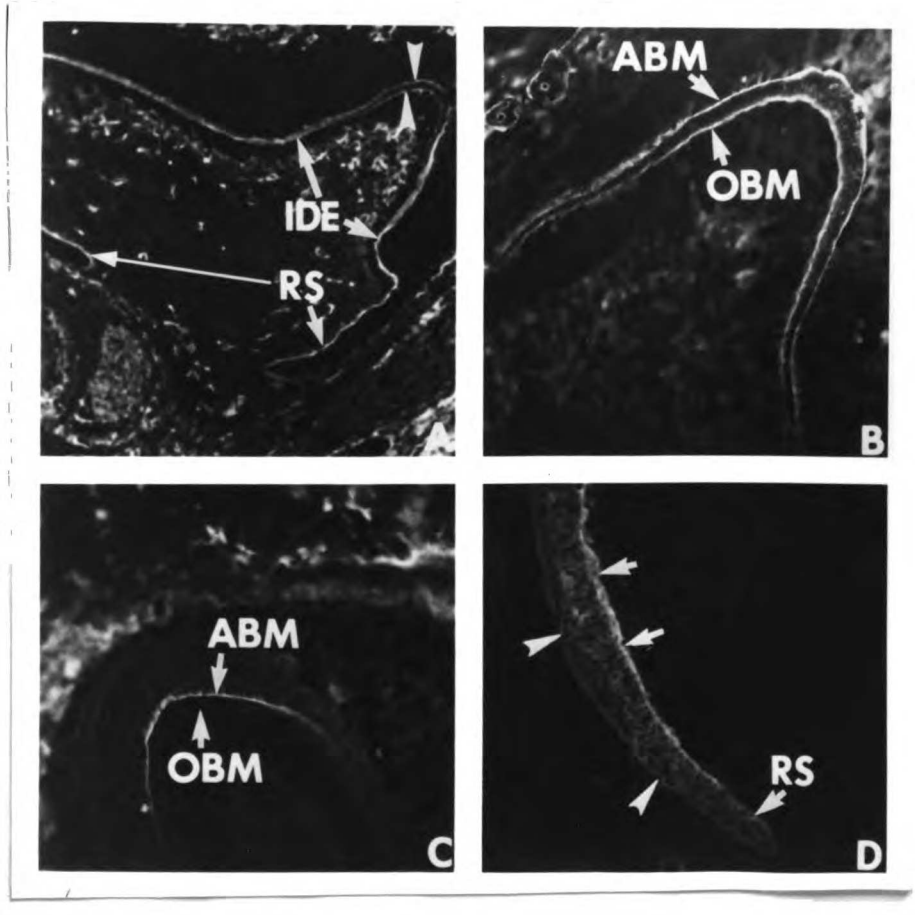

FIGURE 5

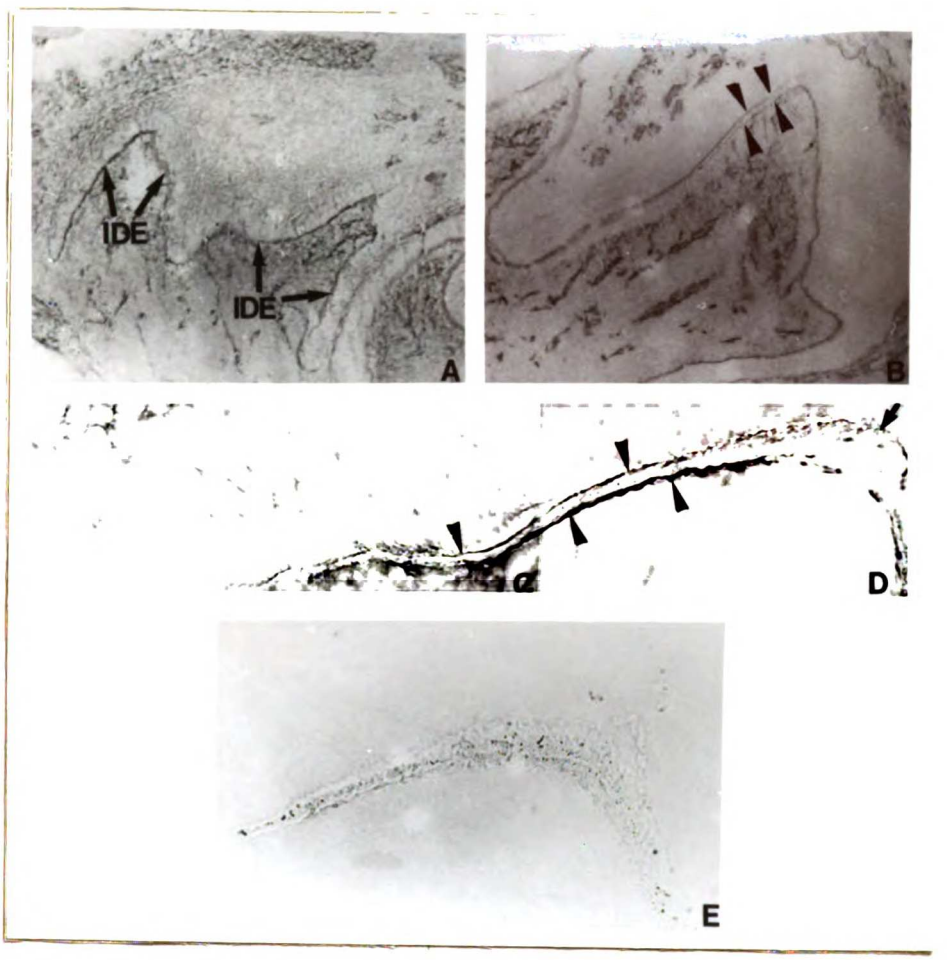

FIGURE 6

Immunoperoxidase stained sections of a 1 day neonatal mouse tooth bud. A): first molar at, 10X, stained for collagen IV with more intense staining at the inner enamel epithelium (IDE). B): similar first molar section from another specimen and stained for laminin, at 20X, showing the separation of the basement membrane (arrowheads). C) and D): is a composite of a representative cuspal area stained for laminin, at 40X, showing the separation of the basement membrane components (arrowheads). The arrow indicates the cusp tip where the laminin membrane component appears to be decreasing. E): a typical control specimen (no primary antibodies).

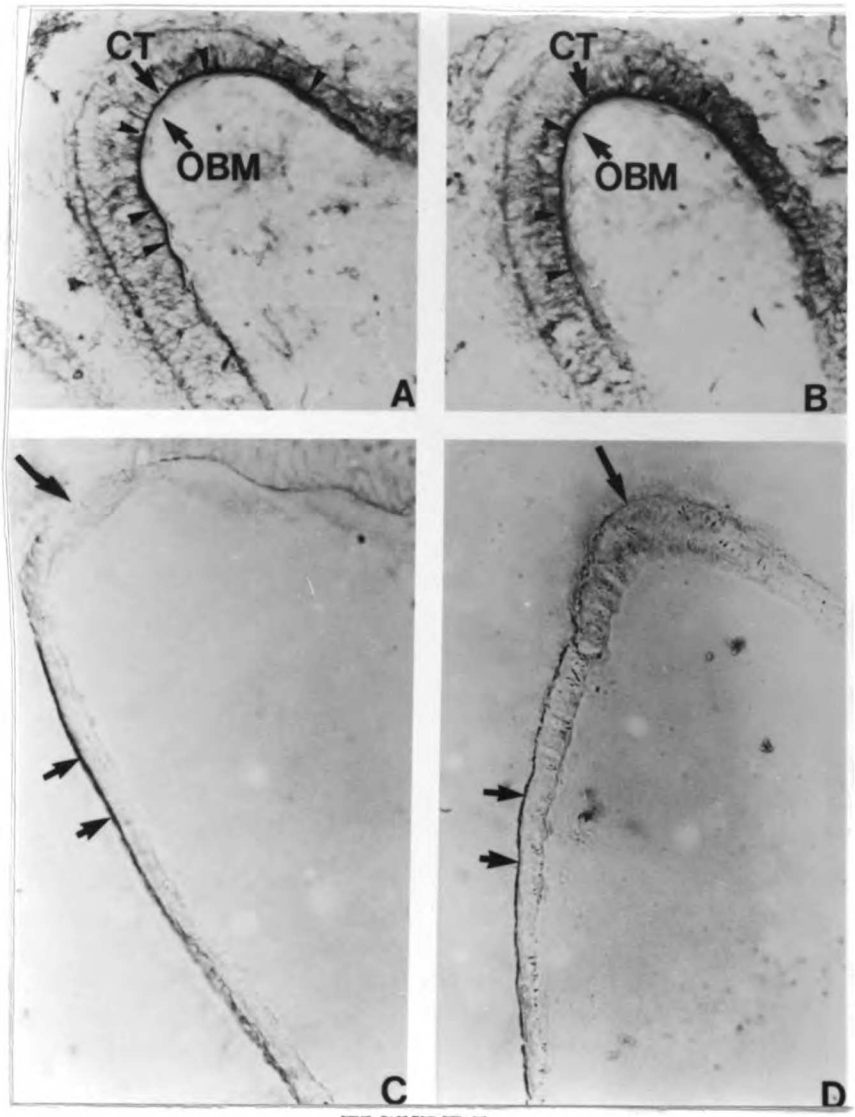

FIGURE 7

Sections from a 1 day neonatal tooth bud stained for α_6 and β_4 using immunoperoxidase techniques. A): cusp tip, at 40X, stained for α_6 with increased intensity toward the cusp tip (CT) and an absence of staining of the odontoblastic basement membrane (OBM). B): a similar specimen to (A) stained for β_4. The similarity in these two sections is typical of the staining patterns for α_6 and β_4, showing an increased intensity of stain at the cusp tip (arrows) and diminishing toward the proximal contours. C): the cusp tip stained for α_6 (original at 40X) showing the intensity of stain near the cusp tip (small arrows) with a decreased staining at the cusp tip (large arrow). D): a cusp tip stained for β_4, displaying a similar pattern to (C) with a similar loss of staining at the cusp tip (large arrow).

THREE DAY NEONATAL MOUSE:

Immunofluorescent and peroxidase staining of tissues in the three day neonatal mouse indicated a similar pattern of distribution to those of the one day neonatal mouse. The staining of collagen IV and laminin continued to be less intense at the ODE than along the IDE. The single line of staining along the IDE still divided in two as the cusp was approached but this was less frequently found than in the one day neonate (Figs. 8 C and 9 D). Nearer the cusp tip, staining for laminin and collagen IV became less distinct (Fig. 9 A,B).

As in the one day neonate, antibody staining for the α_6 and β_4 subunits indicated a more intense and well defined expression at the BM of the ameloblasts near the cusp tips than along the root sheath (Fig. 8 A,B). In contrast to the one day neonate, the intensity of stain for α_6 and β_4 more frequently decreased in some areas at the cusp tip (Fig. 8 A,B).

THE DENTAL LAMINA AND OUTER ENAMEL EPITHELIUM

Staining for α_6 and β_4 in the basement membrane of the oral epithelium and the dental lamina was positive but with a decreasing intensity approaching the ODE (Fig. 10), beginning at the 1 day old mouse. The intensity of stain began to increase again as the IDE became established (Figs. 5 D and 8 A).

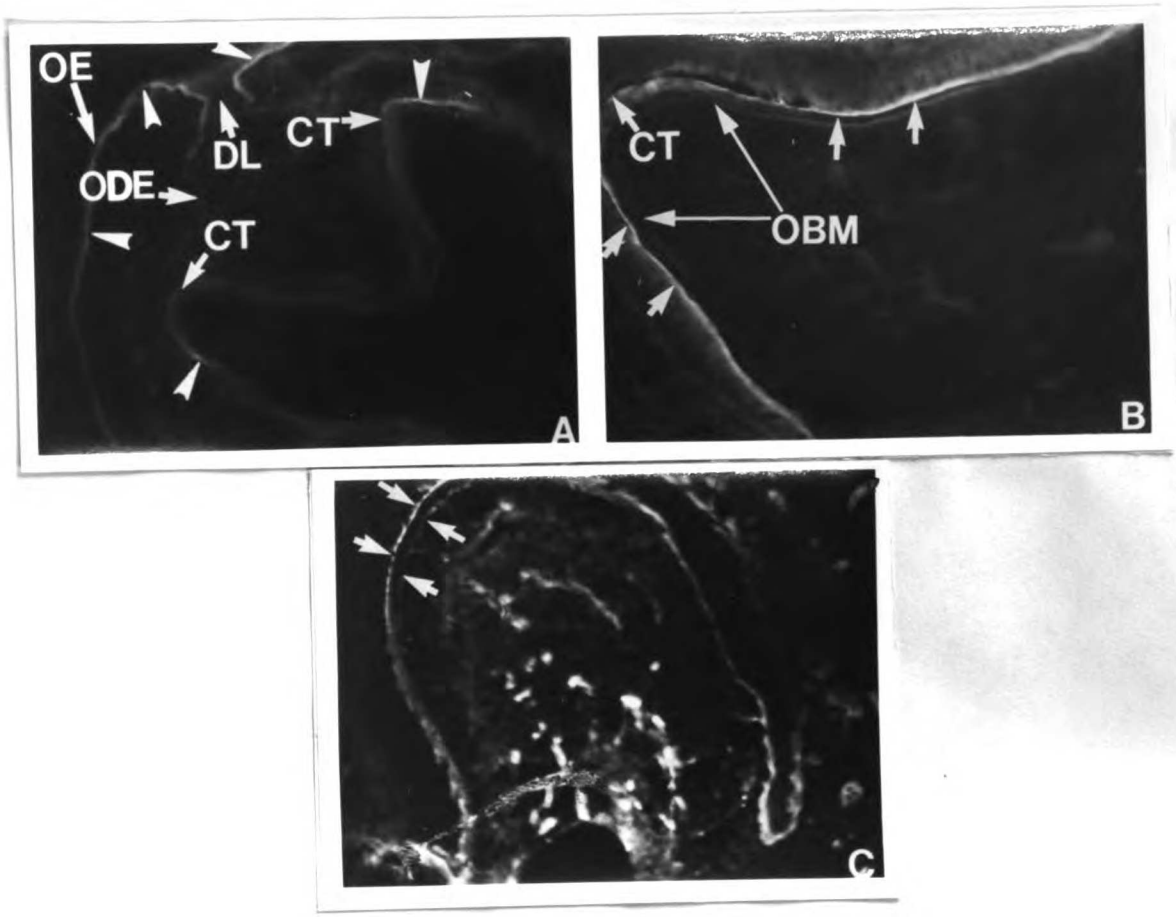

FIGURE 8

Tissue sections of 3 day old mouse tooth buds stained using immunofluorescence. A): first molar tooth bud and dental lamina, at 10X, stained for β_4 (arrowheads) showing a nearly equal intensity of stain at the base of the oral epithelium (OE) and near the cusp tip (CT). Staining of the dental lamina (DL) is prominent but deminishes in the outer dental epithelium (ODE). B): a section of a cusp tip, at 40X, stained for α_6 with intense staining on the slopes of the cusp (arrows) and a decreased intensity at the cusp tip (CT). There is no staining of an odontoblastic basement membrane (OBM). C): a section, at 20X, stained for laminin showing the typical division of the basement membrane as previously seen (arrows).

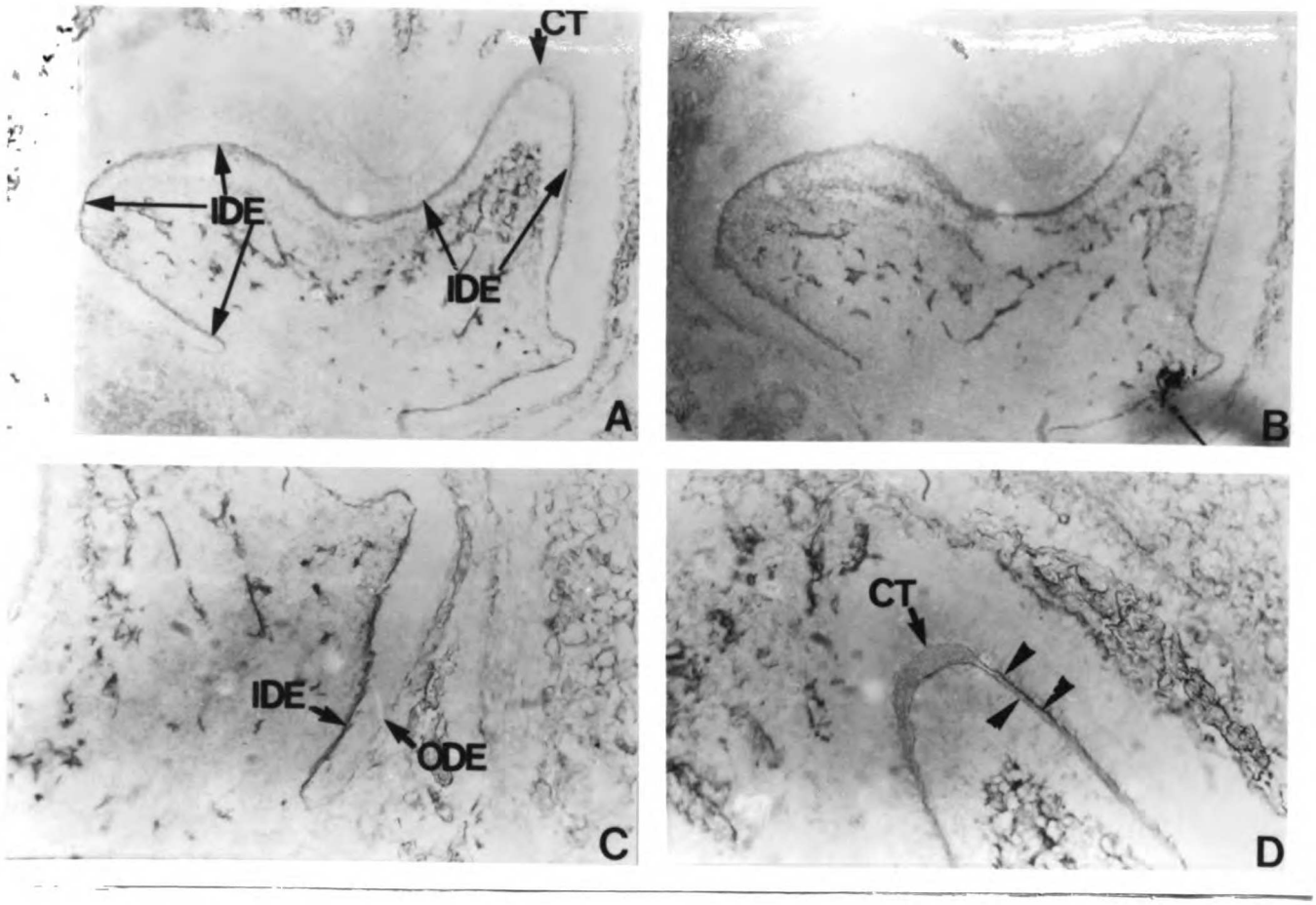

FIGURE 9

Sections of 3 day old mouse tooth buds stained with immunoperoxidase techniques. A): section of a first molar, at 10X, stained for collagen IV. Note the increased intensity of staining of the inner dental epithelial basement membrane (IDE) and the decreased intensity at the cusp tip (CT). B): an adjacent section to (A) stained for laminin. Note the pattern similarities to the staining for collagen IV in (A). C): a section of the root sheath stained for collagen IV, at 20X, with intense staining along the IDE but decreased intensity along the outer enamel epithelium (ODE). D): a section of a cusp tip, at 40X, stained for collagen IV showing a typical division of the basement membrane (arrowheads) and a loss of staining at the cusp tip at base of the ameloblasts (CT).

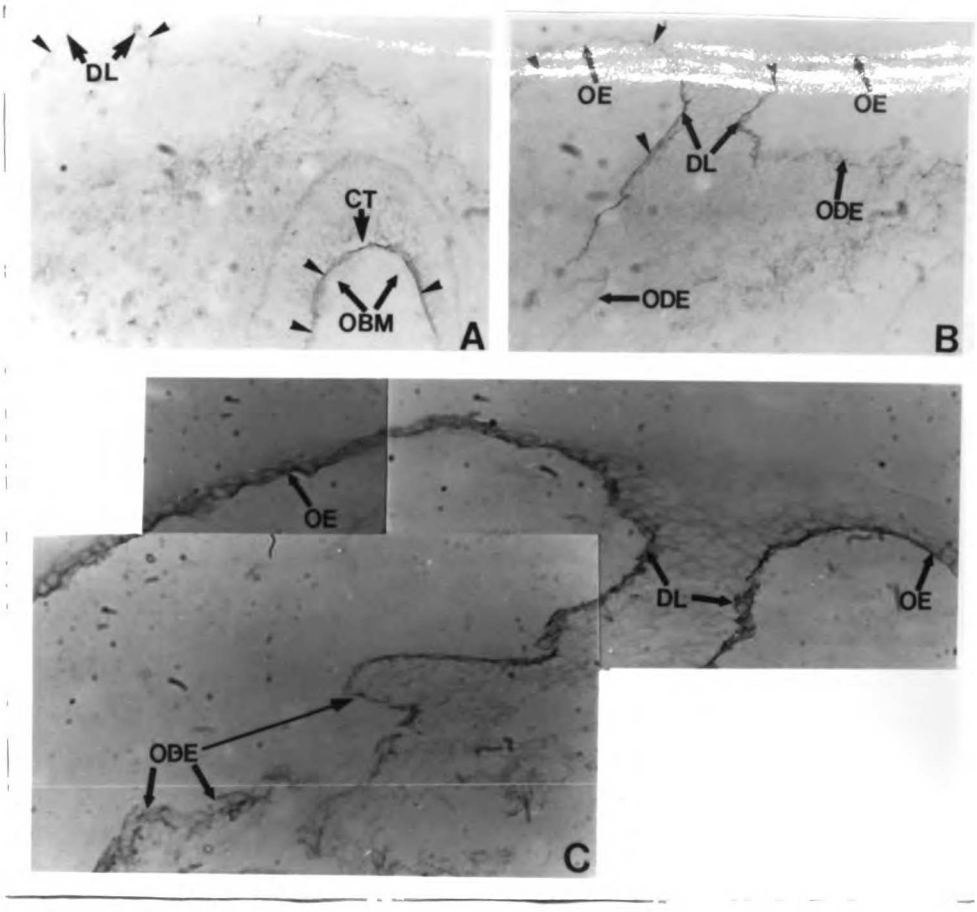

FIGURE 10

Sections of 3 day old mouse tooth buds and dental lamina, stained for α_6 and β_4 using immunoperoxidase techniques. A): Section of a first molar cusp tip (CT), at 20X, and dental lamina (DL), showing positive staining for α_6 of the DL and an apparent ameloblastic basement membrane (arrowheads), but no staining of an odontoblastic basement membrane (OBM). Note the increased intensity of stain for α_6 at the CT. B): The DL, at 20X, stained for β_4 (arrowheads) showing positive staining at the base of the oral epithelium (OE) and DL with decreased staining of the outer dental epithelium (ODE). C): a composite of three photos (originals at 40X) to illustrate the base of the oral epithelium (OE) and the dental lamina (DL) stained for α_6. Note the decrease in intensity in the ODE.

DISCUSSION:

EXPRESSION OF THE $\alpha_6\beta_4$ INTEGRIN IN DEVELOPING TEETH:

Expression of the α_6 and β_4 integrin subunits in developing teeth of the mouse has been shown. Considering the many other studies showing expression of integrins in developing and adult tissues, this is not a surprise. Adhesion of epithelial cells to each other and to a common structural element such as the basement membrane is essential for the integrity of mature tissues. Adhesion flexibility on the other hand is essential to the changes that must occur to enable tissue modifications in wound healing, tumorogenesis and embryogenesis. Focal adhesion plaques in keratinocyte cell cultures have been described as relatively unstable but dynamic and associated with cell migration. These have been associated with the β_1 subunit in association with several α subunits (Carter et al., 1990).

The stable anchoring contact (SAC) has been related to the $\alpha_6\beta_4$ integrin in keratinocytes (Carter et al., 1990). SACs apparently form only in nonmigrating cells and have been likened to hemidesmosomes of skin (Carter et al., 1990). Stepp et al. (1990), Sonnenberg et al. (1991), and Jones et al. (1991) established that the $\alpha_6\beta_4$ integrin is a component of hemidesmosomes.

This study has shown the integrin subunits α_6 and

β_4, to be coincident and continuous around the tooth bud at the early stages of invagination in the 16 day embryo. As development progressed to the 18 day embryo, there was a more diffuse staining adjacent to the presumptive IDE for the β_4 subunit, as compared to α_6. The possibility that this represents non-complexed β_4 subunits as a result of the disassembly of hemidesmosomes will be discussed later. The staining patterns found in the developing teeth, however, support the contention that $\alpha_6\beta_4$ is not strongly expressed in migrating cells, but once the cells are morphologically positioned, the intensity of staining is increased again.

A model proposed for the staining patterns found, is based upon accepting the hypothesis that only the basal cells of the epithelium are capable of division. This would then suggest that the tooth bud begins when a small, limited number of basal cells within the dental lamina, are induced to divide at a more rapid rate. Rapid cell division would result in a "ballooning" of the basal cell layer and then a "blossoming" of cells from the original basal layer into the mesenchyme. The growing blossom could be expected to displace the adjacent basal cells and their basement membrane components in a fashion similar to Fig. 4 F.

The induction of the basal cells may include a reduction in the assembly of hemidesmosomes. The altered

staining patterns in the 16 day embryo, as seen with the staining for β_4 in Fig. 4 D, and F at the IDE, support this concept. In addition, the constancy of the staining for BM components and the α_6 and β_4 subunits at the base of the oral epithelium and the dental lamina (Figs. 8 A and 10 A,B,C), strongly suggests a difference in cell-ECM interactions between these cells and the cells of the dental organ. It appears as if tooth bud cells deeper than the dental lamina have shut down their assembly of hemidesmosomes at this early stage in order to migrate.

The decreased staining for BM components found in the ODE of the 1 and 3 day neonate, suggests two possible scenarios. First, these cells, having been derived from the originally induced basal cells, may not be producing a BM nor assembling hemidesmosomes. Second, the decreased staining may represent a deterioration of an ODE that is no longer needed (rather like the wake of a ship at sea) now that the tooth bud is in proper morphological position. The functional consequences of the altered staining pattern for β_4 remains unanswered.

Hay (1961), Reith (1970) and Ruch (1984) have suggested that mature ameloblasts no longer undergo cell division. If cell division stops in mature ameloblasts and these cells are at the cusp tips, then cells peripheral to them will continue to divide and descend

into the mesenchyme. The morphologic development of the tooth, and the ameloblasts' loss of an ability to divide, seems to correlate nicely with the staining patterns observed for the $\alpha_6\beta_4$ subunits.

The expression of the α_6 and β_4 subunits appears to be strong in the BM of the oral epithelium, shut down during the descent of the progenitor cells into the mesenchyme, and then re-expressed for a transitory period during terminal differentiation of the ameloblasts. One can imagine the expression of $\alpha_6\beta_4$ in ameloblasts beginning at the cusp tip and moving proximally as a wave, shutting down after the wave passes and the enamel matrix is secreted.

It is interesting to speculate on the effect antibodies to $\alpha_6\beta_4$ might have on tooth development. From the model outlined, one could predict that tooth form would not be severely affected but that enamel production would be restricted or absent. It would be of further interest to investigate a possible correlation between the clinical condition known as amelogenesis imperfecta and a perturbation of the $\alpha_6\beta_4$ integrin in ameloblasts.

THE BASEMENT MEMBRANE IN TOOTH DEVELOPMENT:

Many investigators have suggested the disappearance of the BM at the time of ameloblast differentiation (Reith, 1970; Slavkin and Bringas, 1976; Thesleff et al.,

1981; Katchburian and Burgess, 1983 and Sawada et al., 1990). This study supports that hypothesis.

Even at the cusp tip, however, depending upon where tissue sections were taken, the results of staining were different in the same aged animal. A variation in the staining patterns illustrates the problem of studies in rapidly developing animal models and, specifically, the problem of enamel free areas in the rodent dentition. The BM components appeared to be lost when enamel matrix was laid down but their apparent retention in some sections could have been in areas that were to be enamel free. Human ameloblast differentiation and enamel matrix secretion is more symmetrical and there are normally no enamel-free areas on the crowns of the teeth. These differing, species-specific patterns of development must be recognized when interpreting the literature and attempting to draw parallels between human and animal models.

Thesleff et al. (1979) and Thesleff et al. (1981) are the only investigations found in the literature to have followed temporal changes in BM components in developing teeth. Both of these studies used paraffinized sectioning techniques. Several studies have alluded to the fragility of integrins (Kennel et al. 1990; Costantini et al. 1990 and Hogervorst et al. 1990). Since this study was done entirely on frozen tissue

sections to try to preserve antigen sites, some findings may differ from those of previous studies.

The existence of a BM in the developing tooth has been described prior to ameloblast differentiation but no investigator has described a division of the BM components as shown in this study. Careful evaluation of photographs in the work by Thesleff et al. (1981) indicates a similar division in staining for the BM proteoglycan in the neonatal mouse at one, two and three days (Thesleff et al., 1981, Figs. 5, 6 and 7) but no mention is made of this in the text. Similarly, work by Cournil et al. (1979) seems to show a division of BM components, but no mention is made in the text.

The morphologic separation of collagen IV and laminin into two apparent BMs in the early stages of cuspal development suggests that the "BM" of the ameloblasts is different from that of the odontoblasts. This possibility is further supported by the limitation of staining for the α_6 and β_4 subunits to the interface of the ameloblasts with the predentin.

The use of MAb's for indirect visualization of antigens, is based upon the specificity of the antibody to the protein of interest. The technique is useful for determining the presence of specific proteins but it does not determine the quality nor functional capacity of the antigen. Sawada et al. (1990) demonstrated at the

ultrastructural level, evidence of an uptake of the BM by differentiating ameloblasts. They also demonstrated remnants of type IV collagen remaining in coated pits and deep invaginations of differentiating ameloblasts after the basement membrane was no longer discernible. The work of Sawada et al. would suggest that the staining seen in this study is of remnants of BM components and not of a structurally defined BM.

Although previous evidence suggests a loss of the BM with terminal differentiation of the ameloblasts, this does not explain the increased intensity of staining nor the specificity of staining for α_6 and β_4 at the interface of the ameloblasts and the predentin. If these subunits are normally complexed in a hemidesmosomal unit and associated with a BM, it does not make sense for their staining to be enhanced during differentiation of the ameloblasts when the BM is supposedly being degraded.

This study supports the concept that $\alpha_6\beta_4$ acts as a mediator of intermediate filament-cell surface interactions (Jones et al., 1991). It is possible that $\alpha_6\beta_4$ expression increases during differentiation of ameloblasts because of the increased need for cell-substrate interaction during this period and not as a function of a need for SAC's. Once differentiation is completed, it is degraded. It has been shown that the integrin does not have to be complexed in hemidesmosomes

(Jones et al. 1991).

Ultrastructural studies of dentinogenesis and amelogenesis by Slavkin and Bringas (1976), Laurie et al. (1982), and Gorter de Vries et al. (1986) do not describe the presence of hemidesmosomes between ameloblasts and the ECM. Slavkin and Bringas, however, described a "homogeneous, electron dense, material" filling the contact zones between preameloblasts and mesenchymal cell processes, when these areas were stained with tannic acid-glutaraldehyde. Since binding of integrins to their ligands is a cation-dependent process (Albelda and Buck, 1990), perhaps the Slavkin and Bringas finding is the result of a tannic acid affinity for the cations of the $\alpha_6\beta_4$ integrin.

It has been suggested that the unique cytoplasmic domain of the β_4 subunit plays an important role in cell-matrix interactions. Staining of the IDE, and the increased intensity of staining near the cusp tips as differentiation of the ameloblasts proceeded, is consistent with the high level of inter and extracellular interaction one would expect at this stage of differentiation. Multiple forms of the β_4 subunit have been identified (Hemler et al., 1989; Tamura et al., 1990). Perhaps the β_4 subunit found in the epithelium is different from that used in differentiating ameloblasts. Further work at the ultrastructural level will be

required to answer the question of $\alpha_6\beta_4$ expression and its relationship to BM components during differentiation of the ameloblasts. It will also be interesting to see if antibodies to epiligrin (Carter et al., 1991) can further characterize a difference in the two apparent BM's found.

Although evidence from this study supports the concept that the α_6 and β_4 subunits are associated with the basement membrane components and involved in cell-matrix interactions, the observation that staining occurred intercellularly within epithelially derived tissues was also described. Intercellular staining has been shown for α_6, where it associates with the β_1 subunit to form cell-cell contacts (Sonnenberg et al. 1991), but to date $\alpha_6\beta_1$ has not been detected in keratinocytes in cell culture (Sonnenberg et al., 1990; Carter et al. 1991). Intercellular (suprabasal cell) staining for α_6 in kerato-epithelial tissues, however, has been described by Hormia et al. (1990) and Carter et al. (1990). Why this discrepancy exists is not known.

Intercellular (suprabasal cell) staining of β_4 has also been described by Carter et al (1990). Although Sonnenberg et al. (1988) suggests that free β_4 is not translocated to the cell surface, Sonnenberg et al. (1990) describes free β_4 found on the surfaces of certain *in vitro* cell lines. They suggest that the free β_4 subunit

was originally part of the $\alpha_6\beta_4$ complex. Intercellular staining for α_6 and β_4 in this study could indicate the presence of non-complexed and perhaps non-functional β_4 subunits remaining in the cell membrane. Considering the rather rapid rate at which the teeth are developing (a matter of hours between some stages), cellular clearing of uncomplexed, non-functional subunits could overlap several stages of development and result in the staining of non-functional proteins.

Other possibilities for the unexplained intercellular staining of the α_6 and β_4 subunits, could be the existence of other, as yet unidentified, integrin subunits with which these subunits secondarily complex. It is also possible that a variety of the $\alpha_6\beta_4$ integrin complex, itself, is used by the cell as an intercellular receptor in some instances. Further investigation will be necessary to determine answers to these questions.

CONCLUSION:

This study has established the expression of the α_6 and the β_4 integrin subunits in developing teeth. It demonstrated that this expression is stage-related during the differentiation of the ameloblasts. The basement membrane components, collagen IV and laminin, have been

described as separating into two layers, one at the interface between the ameloblasts and the predentin and the other between the predentin and the odontoblasts. While both interfaces stained for collagen IV and laminin, one was unique in that it was associated with the ameloblasts and stained specifically for the α_6 and β_4 subunits. A correlation between the degradation of the BM and the role $\alpha_6\beta_4$ plays in differentiation of ameloblasts remains to be examined at the ultrastructural level.

Evidence from this study supports the concept that the α_6 and β_4 subunits are associated with basement membrane components but suggests that the $\alpha_6\beta_4$ integrin may be used by ameloblasts as receptors while not associated with hemidesmosomes. There is also the suggestion that both subunits may stain intercellulary as a result of the existence of non-functional integrin subunits, or of some, as yet unidentified, integrin complex.

There was no evidence shown that would suggest a retention of basement membrane components after differentiation of the ameloblasts. In order to test for detectable remnants of $\alpha_6\beta_4$ at the dentino-enamel junction in fully developed teeth an addendum experiment was run.

ADDENDUM:

Four, freshly extracted, soft tissue impacted, third molar teeth from an eighteen year old female patient in the University of California San Francisco, Oral Surgery Clinic, were obtained. These teeth were immediately immersed in liquid nitrogen and then shattered with a hammer to obtain fresh cross sections of the mature dentino-enamel junction.

Frozen sections were fixed in the manner described for the mouse tissues in this study and stained according to the protocol used for the peroxidase technique, except primary antibodies used were GoH3, anti-α_6, (Sonnenberg) at 1:100 dilution and 439-9B, anti-human, β_4 (Kennel) at 1:100 dilution for 1 hour.

Six sections were examined and none showed any evidence of an immunoprecipitate at the dentino-enamel junction. These results help to confirm that the basement membrane of the ameloblasts is degraded as a function of the maturation of the dentino-enamel junction.

BIBLIOGRAPHY:

ALBELDA, S. and BUCK, C. (1990): Integrins and Other Cell Adhesion Molecules, FASEB J 4:2868-2880.

CARTER, W.; KAUR, P.; GIL, S.; GAHR, P. and WAYNER, E. (1990): Distinct Functions for Integrins $\alpha_3\beta_1$ in Focal Adhesions and $\alpha_6\beta_4$/Bullous Pemphigoid Antigen in a New Stable Anchoring Contact (SAC) of Keratinocytes: Relation to Hemidesmosomes, J Cell Biol 111:3141-3154.

CARTER, W.; RYAN, M. and GAHR, P. (1991): Epiligrin, A New Cell Adhesion Ligand for Integrin $\alpha_3\beta_1$ in Epithelial Basement Membranes, Cell 65:599-610.

COHN, S. (1957): Development of The Molar Teeth in The Albino Mouse, Am J Anat 101:295-317.

COSTANTINI, R.; FALCIONI, R.; BATTISTA, P.; ZUPI, G.; KENNEL, S.; COLASANTE, A.; VENTURO, I.; CURCIO, C. AND SACCHI, A. (1990): Integrin ($\alpha_6\beta_4$) Expression in Human Lung Cancer as Monitored by Specific Monoclonal Antibodies, Cancer Research 50:6107-6112.

COURNIL, I.; LEBLOND, C.; POMPONIO, J.; HAND, A.; SEDERLOF, L. and MARTIN, G. (1979): Immunohistochemical Localization of Procollagens I. Light Microscospic Distribution of Procollagen I, III, and IV Antigenicity in the Rat Incisor Tooth by the Indirect Peroxidase-Anti-peroxidase Method, J Histochem Cytochem 27: 1059-1069.

DAMSKY, C.; KNUDSEN, K.; DORIO, R. and BUCK, C. (1981): Manipulation of Cell-Cell and Cell-Substratum Interactions in Mouse Mammary Tumor Epithelial Cells Using Broad Spectrum Antisera, J Cell Biol 89:173-184.

FALCIONI, R.; SACCHI, A.; RESAU, J. and KENNEL, S. (1988): Monoclonal Antibody to Human Carcinoma-associated Protein Complex: Quantitation in Normal and Tumor Tissue, Cancer Research 48:816-821.

GAUNT, W. (1956): The Development of Enamel and Dentine on The Molars of The Mouse, with An Account of The Enamel-Free Areas, Acta Anat 28:111-134.

GINSBERG, M.; LOFTUS, J. AND PLOW, E. (1988): Cytoadhesins, Integrins, and Platelets, Thrombosis and Haemostasis 59:1-6.

GORTER DE VRIES, I.; AMELOOT, P.; COOMANS, D. and WISSE, E. (1986): An Ultrastructural Study of Dentinogenesis and Amelogenesis in Rat Molar Tooth Germs Cultured *in vitro*, Cell Tissue Res 246:623-634.

HALL, D.; REICHARDT, L.; CROWLEY, E.; HOLLEY, B.; MOEZZI, H.; SONNENBERG, A. and DAMSKY, C. (1990): The $\alpha_1\beta_1$ and $\alpha_6\beta_1$ Integrin Heterodimers Mediate Cell Attachment to Distinct Sites on Laminin, J Cell Biol 110:2175-2184.

HAY, M. (1961): The Development *in vivo* and *in vitro* of the Lower Incisor and Molars of the Mouse, Arch Oral Biol 3:86-109.

HEMLER, M.; CROUSE, C. and SONNENBERG, A. (1989): Association of the VLA α_6 Subunit with a Novel Protein, J Biol Chem 264:6529-6535.

HEMLER, M. (1990): VLA Proteins in the Integrin Family: Structures, Functions and Their Role on Leukocytes, Annu Rev Immunol 8:365-400.

HOGERVORST, F.; KUIKMAN, I.; VON DEM BORNE, A. and SONNENBERG (1990): Cloning and Sequence Analysis of Beta-4 cDNA: An Integrin Subunit That Contains a Unique 118 kd Cytoplasmic Domain, EMBO J 9:765-770.

HORMIA, M.; YLÄNNE, J. and VIRTANEN, (1990): Expression of Integrins in Human Gingiva, J Dent Res 69:1817-1823.

HYNES, R. O. (1987): Integrins: A Family of Cell Surface Receptors, Cell 48:549-554.

JENNINGS, L. and PHILLIPS, D. (1982): Purification of Glycoproteins IIb and III from Human Platelet Plasma Membranes and Characterization of a Calcium-dependent glycoprotein IIb-III Complex, J Biol Chem 257:10458-10463.

JONES, J.; KURPAKUS, M.; COOPER, H. and QUARANTA, V. (1991): A Function for the Integrin $\alpha_6\beta_4$ in the Hemidesmosome, Cell Reg 2:427-438

KAJIJI, S.; TAMURA, and QUARANTA, V. (1989): A Novel Integrin ($\alpha_E\beta_4$) from Human Epithelial Cells Suggests a Fourth Family of Integrin Adhesion receptors, EMBO J 8:73-680.

KATCHBURIAN, E. and BURGESS, M. (1983): Lysosomes and Removal of the Basal Lamina of Ameloblasts in Early Stages of Odontogenesis, Cell Biol Inter Rep 7:407-415.

KENNEL, S.; FOOTE, L.; FALCIONI, R.; SONNENBERG, A.; STRINGER, C.; CROUSE, C., and HEMLER, M. (1989): Analysis of the Tumor-associated Antigen TSP-180, Biol Chem 264:15515-15521.

KENNEL, S.; EPLER, R.; LANKFORD, T.; FOOTE, L.; DICKAS, V.; CONAMUCIO, M.; CAVALIERIE, R.; COSIMELLI, M.; VENTURO, I.; FALCIONI, R. and SACCHI, A. (1990): Second Generation Monoclonal Antibodies to the Human Integrin $\alpha_6\beta_4$, Hybridoma 9:243-255.

KOCH, W. (1967): *In vitro* Differentiation of Tooth Rudiments of Embryonic Mice, J Exp Zool 165:155-170.

KORHONEN, M.; YLÄNNE, J. LAITINEN, L., and VIRTAINEN (1990): The α_1-α_6 Subunits of Integrins Are Characteristically Expressed in Distinct Segments of Developing and Adult Human Nephron, J Cell Biol 111:1245-1254.

LAURIE, G.; LEBLOND, C. and MARTIN, G. (1982): Localization of Type IV Collagen, Laminin, Heparan Sulfate Proteoglycan, and Fibronectin to the Basal Lamina of Basement Membranes, J Cell Biol 95:340-344.

LESOT, H.; OSMAN, M. and RUCH, J. (1981): Immunofluorescent Localization of Collagens, Fibronectin, and Laminin during Terminal Differentiation of Odontoblasts, Dev Biol 82:371-381.

LESOT, H.; KARCHER-DJURICIC, V.; MARK, M.; MEYER, J. and RUCH, J. (1985): Dental Cell Interaction with Extracellular-Matrix Constituents: Type-I Collagen and Fibronectin, Differentiation 29:176-181.

LESOT, H.; KARCHER-DJURICIC, V.; KUBLER, M. and RUCH, J. (1988): Membrane-Cytoskeleton Interactions: Inhibition of Odontoblast Differentiation by a Monoclonal Antibody Directed Against a Membrane Protein, Differentiation 37:62-72.

RUCH, J. (1984): Tooth Morphogenesis and Differentiation. In: Dentin and Dentinogenesis, Vol. 1, A. Linde, Ed., Boca Raton, Florida: CRC Press, pp. 49-75.

REITH, E. (1970): The Stages of Amelogenesis as Observed in Molar Teeth of Young Rats, J Ultrastructure Res 30:111-151.

ROUSLAHTI, E. and GIANCOTTI, F. (1989): Integrins and Tumor Cell Dissemination, Cancer Cells 1:119-126.

SAWADA, T.; YAMAMOTO, T.; YANAGISAWA, T.; TAKUMA, S.; HASEGAWA, H. and WATANABE, K. (1990): Evidence of Uptake of Basement Membrane by Differentiating Ameloblasts in the Rat Incisor Enamel Organ, J Dent Res 69:1508-1511.

SLAVKIN, H. and BRINGAS, P., JR. (1976): Epithelial-Mesenchyme Interactions during Odontogenesis, Dev Biol 50:428-442.

SMITH, M. and HALL, B/ (1990): Development and Evolutionary Origins of Vertebrate Skeletogenic and Odontogenic Tissues, Biol Rev 65:277-373.

SNEAD, M.; LUO, W.; LAU, E. and SLAVKIN, H. (1988): Spatial-and Temporal-restricted Pattern for Amelogenin Gene Expression During Mouse Molar Tooth Organogenesis, Development 104:77-85.

SONNENBERG, A.; HOGERVORST, F.; OSTEROP, A. and VELTMAN, F. (1988): Identification and Characterization of a Novel Antigen Complex on Mouse Mammary Tumor Cells using a Monoclonal Antibody against Platelet Glycoprotein Ic, J Biol Chem 263:14030-1438.

SONNENBERG, A.; LINDERS, C.; DAAMS, J. and KENNEL, S. (1990): The $\alpha_6\beta_1$ (VLA-6) and $\alpha_6\beta_4$ Protein Complexes: Tissue Distribution and Biochemical Properties, J Cell Sci 96:207-217.

SONNENBERG, A.; CALAFAT, J.; JANSSEN, H.; DAAMS, H.; van der RAAIJ-HELMER, L.; FALCIONI R.; KENNEL, S.; APLIN, J.; BAKER, J.; LOIZIDOU M. and GARROD, D. (1991): Integrin $\alpha_6\beta_4$ Complex is Located in Hemidesmosomes, Suggesting a Major Role in Epidermal Cell-Basement Membrane Adhesion, J Cell Biol 113:907-917.

SOROKIN, L.; SONNENBERG, M.; AUMAILLEY, M.; TIMPL, R. and EKBLOM, P. (1990): Recognition of the Laminin E8 Cell-Binding Site by an Integrin Possessing the α_6 Subunit is Essential for Epithelial Polarization in Developing Kidney Tubules, J Cell Biol 111:1265-1273.

STEPP, M.; SPURR-MICHAUD, S.; TISDALE, A. and ELWELL, J. (1990): $\alpha_6\beta_4$ Integrin Heterodimer is a Component of Hemidesmosomes, Proc Natl Acad Sci USA 87:8970-8974.

TAMURA, R.; ROZZO, C.; STARR, L.; CHAMBERS, J.; REICHARDT, L.; COOPER, H. AND QUARANTA, V. (1990):

Epithelial Integrin $\alpha_6\beta_4$: Complete Primary Structure of α_6 and variant Forms of β_4, J Cell Biol 111:1593-1604.

THESLEFF, I.; BARRACH, H.; FOIDART, J.; VAHERI, A.; PRATT, R. and MARTIN, G. (1981): Changes in the Distribution of Type IV Collagen, Laminin, Proteoglycan, and Fibronectin during Mouse Tooth Development, Dev Biol 81:182-192.

THESLEFF, I.; JALKANEN, M.; VAINIO, S. and BERNFIELD, M. (1988): Cell Surface Proteoglycan Expresseion Correlates with Epithelial-Mesenchymal Interaction during Tooth Morphogenesis, Dev Biol 129:565-572.

THESLEFF, I.; VAAHTOKARI, A. and VAINIO, S. (1990): Molecular Changes during Determination and Differentiation of the Dental Mesenchymal Cell Lineage, J Biol Buccale 18:179-188.

WYLIE, D.; DAMSKY, C. and BUCK, C. (1979): Studies on the Function of Cell Surface Glycoproteins. I. Use of Antisera to Surface Membranes in the Identification of Membrane Components Relative to Cell Substrate Adhesion, J Cell Biol 80:385-402

TABLE I

ANTIBODY	CONCENTRATION	TIME OF APPLICATION
PRIMARY ANTIBODIES:		
Anti-α_6 (GoH3)	1:100	30 Min.
Anti-α_6 (J1B5)	supernatant (as supplied)	30 Min.
Anti-β_4 (346-11A)	1:300	30 Min.
Anti-laminin	1:200	30 Min.
Anti-collagen IV	1:100	30 Min.
Anti-amelogenin	1:200	30 Min.
SECONDARY ANTIBODIES:		
Rhodamine goat anti-rabbit (Boehringer Mannheim Biochemicals, Indianapolis IN 46250)	1:500	30 Min.
Rhodamine goat anti-rat (Organon Teknika Corp. West Chester PA 19380)	1:500	30 Min.
FITC goat anti-rabbit (Boehringer Mannheim Biochemicals, Indianapolis IN 46250)	1:25	30 Min.
Peroxidase conjugate Anti-rabbit (Sigma Chemical Co. P.O. Box 14508 St. Louis MO 63178)	1:500	30 Min.
Peroxidase conjugate Anti-rat (Sigma Chemical Co. P.O. Box 14508 St. Louis MO 63178)	1:500	30 Min.

APPENDIX A

MEDIA, BUFFERS AND SUBSTRATES

ITEM:	USED FOR:
O.C.T. Compound (Miles Laboratories, Naperville, IL)	Used to embed tissues for quick freezing
0.1% formaldehyde	Fixing of tissues
DME culture medium	Quenching of fixative
D-PBS with 1% normal goat serum with Na-azide (50 μl of 10% Na-azide per 100 ml)	Rinsing of tissues for immunofluorescence and diluting agent for fluorescent technique antibodies
D-PBS with 1% normal goat serum without Na-azide	Rinsing of tissues for peroxidase technique and diluting agent for peroxidase technique antibodies
Diaminobenzidine (DAB) substrate (prepared by dissolving 6 mg. of DAB in 10 ml of 0.05M Tris to which was added 0.1 ml of 3% peroxide)	Used as substrate for peroxidase technique
Fluoromount (Fisher Scientific, Orangeburg, New York)	Used for mounting of tissues for fluorescence technique
Permount (Fisher Scientific, Fair Lawn, New Jersey)	Used for mounting of tissues for peroxidase technique