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Enamel organ proteins as targets for antibodies in celiac disease: implications for oral health

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Enamel defects in permanent and deciduous teeth may be oral manifestations of celiac disease. Sometimes they are the only sign that points to this underdiagnosed autoimmune pathology. However, the etiology of these specific enamel defects remains unknown. Based on previously reported cross-reactivity of antibodies to gliadin with the enamel proteins, amelogenin and ameloblastin, we analyzed (using immunohistochemistry) the ability of anti-gliadin IgG, produced during untreated disease, to recognize enamel organ structures. We used swine germ teeth as a tissue model because they are highly homologous to human teeth in terms of proteins and development biology. Strong staining of the enamel matrix and of the layer of ameloblasts was observed with serum samples from women with celiac disease; high IgG reactivity was found against both gliadin peptides and enamel matrix protein extract, but there was no IgG reactivity against tissue antigens. In line with these findings, the gamma globulin fraction from gliadin-immunized BALB/c mice showed a similar staining pattern to that of amelogenin-specific staining. These results strongly suggest a pathological role for antibodies to gliadin in enamel defect dentition for both deciduous and permanent teeth, considering that IgG can be transported through the placenta during fetal tooth development.

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Links between celiac disease (CD) and developmental defects of enamel (DDEs) have been reported since the 1990s (1). More recently, several studies have confirmed that patients with CD have a significantly higher occurrence of DDE than do otherwise healthy people (2–5). It is generally accepted that specific DDEs may be the only clinical manifestations of CD; therefore, clinicians look for them as signs of CD during screening (6).

A wide range of DDEs have been described in patients with CD, including discolorations, changes in opacity, and the presence of pits and horizontal grooves, ranging from hypomineralized enamel areas (Grade I) to evident structural defects (Grades II–IV), according to the Aine classification (7). Several authors report that Grade I and Grade II lesions are more prevalent (7–10). Reports of DDEs state involvement of both permanent and deciduous teeth, with permanent incisors and first-molar teeth being most commonly affected (2, 3, 8, 11, 12).

Developmental defects of enamel specifically related to CD are characterized by symmetrical and chronological distribution, and involvement of the same type of tooth in all four quadrants, in contrast to DDEs observed in a wide range of other unrelated conditions (such as premature birth, or after infections or exposure to chemicals) (13).

Dental enamel is a highly mineralized tissue without any metabolic activity after its formation. Therefore, DDEs are the result of disturbances of the amelogenesis process that take place from the intrauterine period to adolescence, depending on which tooth group is involved.

Dental enamel is the hardest mineralized tissue in the body, made of highly structured crystal prisms of calcium hydroxyapatite. The growth and organization of enamel prisms is controlled by proteins secreted by ameloblasts, the specialized epithelial-like cells of the transient enamel organ (14, 15). Amelogenin is the predominant matrix protein in developing enamel and plays a major role in the biomineralization and structural organization of these crystals (16).

The amelogenesis process can be divided into various consecutive stages, the secretory and maturation phases being the most readily distinguished. During the secretory stage, the ameloblasts deposit the matrix protein and the initial mineral phase is completed (17). Amelogenin self-assembles into nanospheres that serve as a scaffold for the oriented elongation of calcium hydrox-

yapatite crystals until the tooth's thickness is reached (18). Mineralization becomes complete during the maturation stage, when the production of amelogenin declines and crystals grow (19).

Disruption of amelogenesis at different stages can cause several DDE phenotypes. The disorders that occur during the secretory stage can result in an altered architecture of teeth (hypoplasia), whereas inadequate maturation of crystals is associated with hypomineralized teeth (20). Any stage of amelogenesis could be compromised, depending on the specific pattern of DDE linked to CD, but the mechanisms and triggers behind them are still unknown. Hypocalcemia has been regarded as the main factor, but the results are inconclusive (21–23).

The origin of DDE in CD is poorly studied and has not yet been elucidated. We previously described the presence of common peptide motifs among amelogenin, ameloblastin, and gliadin sequences. Cross-reactivity with amelogenin was demonstrated for serum samples with high anti-gliadin titers, obtained from patients with CD, which specifically recognized amelogenin in a crude extract of swine-matrix-derived enamel protein (24).

Antibody-mediated immune reactions in the transitory enamel organ during tooth development may cause disturbances in the enamel structure. Therefore, we studied the reactivity of serum samples from patients with CD against enamel organ tissue. This study may provide useful insight into the mechanisms behind DDEs observed in patients with CD and eventually in other autoimmune diseases.

Material and methods

Serum samples from patients with CD and from healthy donors were used with the informed consent of all parties, according to the provisions of the local Ethics Committee (Facultad de Medicina, Universidad de la República, Uruguay).

Human serum samples

We studied a group of 21 women with CD (27–63 yr of age, mean age = 34.5 yr). The diagnosis of all patients was confirmed by the presence of a lesion in the small intestine (Type >2) according to the Marsh–Oberhuber classification (25). Serum samples were selected for this study on the basis of strong reactivity to a preparation of enamel-derived matrix protein, previously reported by our group (24). Serum samples from eight age-matched healthy women (21–55 yr of age, mean age = 33.4 yr) were used as controls for immunostaining assays.

Tissue-transglutaminase-specific antibodies (anti-tTG Igs) were analyzed using Quanta Lite h-tTG IgG or IgA ELISAs (Inova, San Diego, CA, USA). Anti-gliadin reactivity was measured with deamidated synthetic peptides as target antigens (Quanta Lite DGP screen; Inova) and the reactivity against actin was detected using Quanta Lite Actin ELISA (Inova). Serum samples from healthy blood donors with negative results in these immunoassays were used as controls. In these ELISAs, the cut-off values (20 units) for each antigen were defined by the manufacturer based on a panel of healthy individuals.

Animals and tissue preparation

The mandibles of two full-term newborn pigs (*Sus scrofa domestica*), killed no more than 72 h after birth, were obtained. The heads were separated at Departamento de Neonatología, Hospital de Clínicas, Montevideo immediately after death, in accordance with the approved practices of the Ethics Committee for Animal Research [Comisión Honoraria de Experimentación Animal (CHEA), Universidad de la República]. The lower mandibles were removed, and germ tissue pieces of the molar teeth were dissected under a microscope and immediately immersed for 24 h in a neutral 4% paraformaldehyde solution. The samples were decalcified with neutralized 10% EDTA (Sigma-Aldrich, St Louis, MO, USA) for 3 d at 4°C, then the tissue pieces were dehydrated and soaked in increasing concentrations of ethanol (from 70% to 100%) and kept in ethanol before embedding in paraffin.

Anti-gliadin Igs produced in mice

Mice were obtained from DILAVE (Veterinary Laboratory Division, Uruguay) and housed at the Animal Facility of Instituto de Higiene (Montevideo, Uruguay). Animal experiments were performed in compliance with the provisions of CHEA.

Six adult BALB/c mice were inoculated intraperitoneally, twice (2 wk apart), with 50 μg of gliadin from wheat (Sigma-Aldrich) emulsified in incomplete Freund's adjuvant; the mice were bled just before immunization (pre-immune serum samples) and 15 d after the last inoculation. Serum samples were collected and kept at −20°C until required for use.

The presence of anti-gliadin Igs was determined by ELISA using a peptide extract (GLDp), obtained by sequential digestion of gliadin by peptic-tryptic treatment, as previously described (24). Briefly, microplates (Greiner, Frickenhausen, Germany) were coated with GLDp at 10 μg ml^{-1} and subsequently blocked with 1% BSA (Sigma-Aldrich) in PBS (1 h, 37°C). Then, the plates were incubated, for 1 h at 37°C, with appropriate serial dilutions of sera made in PBS-Tween 20 (0.05%)/1% BSA. After washing, a goat anti-mouse IgG–horseradish peroxidase (HRP) conjugate (Sigma-Aldrich) was added and the plates were incubated for 1 h at 37°C. Finally, the color reaction was developed with 0.005% (v/v) hydrogen peroxide (H_2O_2) containing 0.1 mg ml^{-1} of tetramethylbenzidine substrate solution (Sigma-Aldrich). The absorbance was read at 450 nm after stopping the reaction with 0.5 M sulfuric acid (H_2SO_4).

The gamma globulin fraction was obtained by precipitation with ammonium sulfate (40% saturation), and the protein content was measured using the bicinchoninic acid assay (Pierce, Rockford, IL, USA).

Immunostaining of tooth germ sections

Sagittal, 4-μm-thick sections were obtained from molar tooth germs. The slides were subsequently dewaxed, rehydrated with PBS, and then epitopes were unmasked by boiling for 20 min at pH 6.0 in 10 mM sodium citrate.

Sections were pretreated with normal goat serum (to decrease background staining) and then incubated overnight at 4°C with suitable dilutions in PBS containing 1% BSA of mouse serum (1:100), mouse gamma globulin fraction (1:200), or human serum (1:50). Simultaneously, a 1:100 dilution of a rabbit anti-amelogenin polyclonal antibody (FL-191; Santa Cruz, Santa Cruz, CA, USA) was used as a positive control. After washing three times with PBS, the endogenous peroxidase activity was quenched by incubation with 3% H_2O_2 in PBS for 30 min. Sections were then incubated for 1 h at room temperature with the following biotin-labeled goat antibodies: anti-mouse (Thermo Fisher Scientific, Waltham, MA, USA), anti-human (1:200), or anti-rabbit (1:300) (Sigma-Aldrich), respectively. Finally, an HRP–streptavidin conjugate (1:500) (Sigma-Aldrich) was added for 45 min.

The reaction was developed by incubation, for 15 min, in tris-buffered saline containing 10 mg ml^{-1} of 3,3′-diamobenzidine tetrahydrochloride (Sigma-Aldrich) and 0.03% H_2O_2. Slides were counterstained with Mayer's hematoxylin and mounted.

For all assays, control incubations were carried out using identical procedures, except for the addition of primary antibody.

Results

We previously hypothesized that antibodies could mediate enamel defects in patients with CD based on the observation that antibodies generated during the untreated disease cross-react with amelogenin, the main component of crude extracts of enamel matrix proteins (24). Here, we studied if the amelogenin contained in secretory/transition ameloblasts and immature enamel is recognized by antibodies from patients with CD.

In view of the high interspecies homology of amelogenin sequences and the similarities between human and porcine enamel development biology (26), we used sections of first-molar tooth germs isolated from newborn pigs as the test tissue for immunostaining (Fig. 1). As depicted in Fig. 1B, amelogenin was detected in the enamel matrix and inside the layer of ameloblasts when the slides were incubated with a reference antibody. It is worth noting that the amelogenin protein shows a typical distribution pattern in the enamel matrix, with the highest expression at the mineralization front as well as some expression at the dentin–enamel junction.

We analyzed the serological reactivity profiles of a panel of serum samples from women with untreated CD, which had previously been shown to react to a crude extract of enamel matrix protein and gliadin extract (GLDp) (24). The reactivity against gliadin was further studied using deamidated gliadin peptides (DGP) as highly specific targets. Fifteen out of 21 serum samples exhibited reactivity against DGP (patients 1–15, Table 1).

All serum samples were positive for anti-tTG IgA and two also expressed specific IgG, tTG being the most specific target of CD (27). Interestingly, expression of this ubiquitous protein has been reported in the enamel organ (28). Moreover, other autoantibodies are also linked to CD. Actin is one major conserved tissue protein that is frequently recognized by antibodies in patients with untreated CD (29). Actin was also reported to be present in the commercial enamel protein extract (30) and this could account for the reactivity of serum samples that were negative for GDP antibodies (patients 16–19, Table 1).

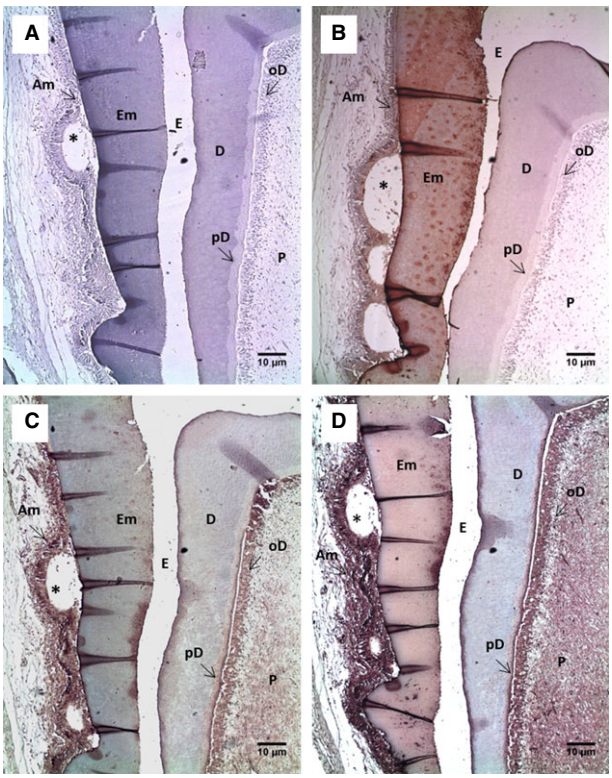

Fig. 1. Immunohistochemical analysis of swine molar germ using human serum samples. (A) Negative control (PBS instead of serum followed by secondary biotinylated antibody and streptavidin–horseradish peroxidase conjugate). (B) Anti-amelogenin reference antibody (FL-191, 1:100 dilution; Santa Cruz Biotechnology). (C) Representative serum control (1:50 dilution). (D) Representative serum from a patient with celiac disease (CD) (1:50 dilution). Scale bar = 10 μm. Am, ameloblasts; D, dentin; E, space occupied by decalcified enamel; Em, enamel matrix; pD, predentin; oD, odontoblasts; P, dental pulp. *Artifacts attributable to tissue processing for histological techniques.

Based on the serological reactivity profile found in the ELISA analysis, we selected serum samples lacking specific IgG against both highly conserved proteins in order to discriminate recognition of enamel proteins in the tooth germ tissue more accurately (patients 1–8, Table 1). Strong staining of enamel matrix and ameloblasts was observed with serum samples from five patients with CD, the staining pattern being similar to that for the reference antibody against amelogenin (a representative example is shown in Fig. 1D), and a weak signal was obtained with three serum samples. In contrast, no significant staining was observed for normal serum samples (a representative example is shown in Fig. 1C).

To confirm the ability of in-vivo-generated anti-gliadin Igs to recognize enamel proteins in the tooth germ, we immunized BALB/c mice, a strain known to

Table 1

Serological profile of patients with celiac disease

Patient	IgG reactivity (units)		
	DGP	Actin	tTG
1	239	ND	ND
2	183	ND	ND
3	141	ND	ND
4	106	ND	ND
5	96	ND	ND
6	67	ND	ND
7	37	ND	ND
8	28	ND	ND
9	157	20	22
10	75	26	139
11	267	23	ND
12	133	38	ND
13	132	26	ND
14	82	34	ND
15	68	23	ND
16	ND	24	ND
17	ND	25	ND
18	ND	79	ND
19	ND	25	ND
20	ND	ND	ND
21	ND	ND	ND

The levels of specific antibodies obtained for each antigen tested are expressed in the units provided by each commercial kit, according to the manufacturer's instructions (Quanta Lite ELISA; Inova). DGP, deamidated gliadin peptides; ND, not detected (the antibody levels in the serum sample were below the cut-off value of 20 units); tTG, tissue transglutaminase.

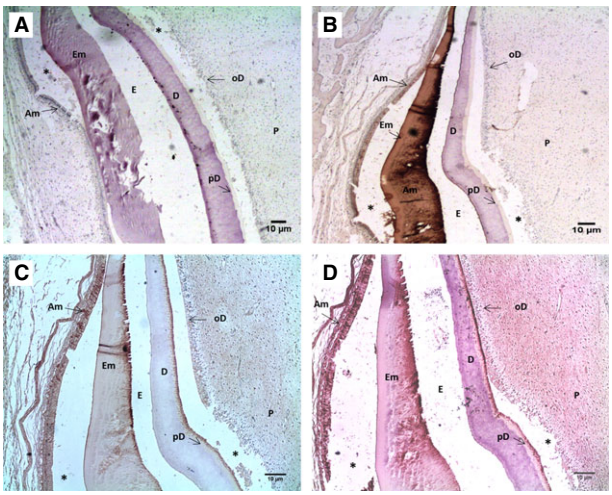

Fig. 2. Immunohistochemical analysis of swine molar germ using mice serum samples. (A) Negative control (PBS instead of serum followed by secondary biotinylated antibody and streptavidin–horseradish peroxidase conjugate). (B) Anti-amelogenin reference antibody (FL-191, 1:100 dilution; Santa Cruz Biotechnology). (C) Pre-immune mouse serum (1:100 dilution). (D) Gamma globulin fraction from gliadin-immunized mice (4.2 mg ml^{-1}; 1:200 dilution). Scale bar = 10 μm. Am, ameloblasts; D, dentin; E, space occupied by decalcified enamel; Em, enamel matrix; oD, odontoblasts; P, dental pulp; pD, predentin. *Artifacts attributable to tissue processing for histological techniques.

be resistant to autoimmunity, with commercial wheat gliadin. The reactivity of serum from these mice against GLDp was verified by ELISA in samples obtained 2 wk after the second inoculation; all serum samples diluted 1:2,000 exhibited a 10-fold increase in absorbance with respect to pre-immune serum samples. These serum samples were pooled together and the corresponding pre-immune sera were pooled separately for use in immunohistochemistry assays.

We found that antibodies induced by gliadin immunization (Fig. 2D) immunolocalize to structures in the swine enamel organ similar to those recognized by the reference anti-amelogenin Ig (Fig. 2B). On the other hand, no significant reactivity was observed with serum from pre-immune mice (Fig. 2C).

Discussion

Developmental enamel defects are commonly described in clinical practice and may be defined as aberrations in the quality or quantity of enamel that are caused by disruption and/or damage of the enamel organ (13).

Interestingly, as enamel does not have any remodeling activity, these observed defects may serve as a record of the insults to the enamel organ throughout its development. As a result of the chronology of the development of human teeth (from intrauterine stages to late adolescence), the occurrence of a transient condition disturbing the enamel organ physiology may become evident months or years later. Determining which groups of teeth are affected, and what types of defect are present, can contribute to predicting the onset of CD, which frequently remains underdiagnosed for years. This is especially relevant because the presence of DDE may be the only clinical manifestation of CD. In contrast, if CD starts after teeth have completely developed, no changes on dental enamel are expected, as confirmed in adult patients with CD (31).

Based on a theoretical study of the sequence motifs of swine enamel matrix proteins and in vitro immunoassays (24), we previously hypothesized that defects could be attributed to immune mechanisms mediated by anti-gliadin cross-reactive antibodies. In this study we proposed to analyze this topic further using a swine tooth-germ tissue as a tissue model.

Pigs are generally considered to be a suitable animal model for dental biomedical research because of the high similarity of tooth morphology and the presence of deciduous teeth and all other tooth types (i.e. canine, premolars, etc.) (32). Furthermore, high homology of several swine bioactive molecules with their human counterparts allows the use of swine- derived enamel matrix extracts in regenerative medicine (33) and enables the use of swine tissue in immunochemical studies with human antibodies.

We showed that antibodies in sera from patients with CD recognize tissue components in the enamel organ. The staining pattern of these antibodies was similar to those of antibodies generated in gliadin-immunized

mice and to an amelogenin-specific reference antibody. The strongest signal was observed in immature enamel, where most amelogenin is intact. In mature enamel, however, where amelogenin has gradually been cleaved, the weaker signal may be attributed to both the native protein and some of the derived peptides.

Infiltration of serum proteins through the ameloblast layer into the enamel has been previously described, which can be explained by the permeability of the ameloblast layer and the proximity of fenestrated capillaries in the transition/mature stages (34). In particular, serum albumin was reported to diffuse into porous enamel, and passage of other proteins has also been suggested (35). In this context, in vivo infiltration of antibodies could occur under basal conditions and could be enhanced by local inflammation. In line with this, DDEs in premature newborns were associated with local trauma caused by tracheal intubation, suggesting that even in the absence of autoantibodies, an autoimmune response against enamel proteins could take place and mediate the formation of DDEs (36).

Taking all the above into consideration, we speculate that an in vivo reaction in patients with CD is feasible given the high homology of enamel proteins from both species (37) and the infiltration of serum proteins into the enamel organ under physiological and inflammatory conditions.

Both deciduous and permanent teeth have been reported to be affected in children with CD. Consequently, it is possible that both endogenous and maternal antibodies could participate in the initial DDE, according to our hypothesis. Celiac disease is still underdiagnosed (38) and affects women twice as frequently as it affects men; therefore, we focused our study on IgG from women with CD. This isotype can access fetal tissues when high levels of circulating antibodies are present during pregnancy, coinciding with development of teeth during fetal life.

Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) is another autoimmune clinical condition that has recently been suspected to be an etiology for DDE. Similarly to CD, the pattern of enamel disorders may differ from one individual to another. Unlike CD, permanent teeth are almost exclusively affected. In line with our findings, it was suggested that autoantibodies may attack ameloblasts and other cell types, or interact with proteins that are secreted into the extracellular matrix (39), although the target has not yet been characterized.

In CD, the autoimmune episode can be associated with a high titer of serum autoantibodies that eventually enter the enamel organ. This could provide a reasonable explanation of why adhesion to a gluten-free diet leads to normalization of tooth development: circulating autoantibodies that cause DDEs simply decrease or disappear entirely (40).

Amelogenin expression, typically attributed exclusively to ameloblasts, has recently been described in other cell types (41, 42). It has been proposed that, in addition to its main role in enamel mineralization, amelogenin is involved in depositing other hard tissues during development and regeneration, acting as a signaling molecule (43). In this context, alterations of the amelogenin function could have consequences beyond enamel formation, and involvement of other immune components cannot be ruled out.

To our knowledge, no other study has evaluated immunological involvement in DDE formation in patients with CD. Here, we offer conclusive evidence supporting a pathological role of anti-gliadin Igs in the etiology of DDE. This insight could be very valuable to clinicians and aid the diagnosis of CD through exhaustive examination of the appearance of enamel, particularly in asymptomatic children who have a genetic predisposition to CD.

Future identification of the specific peptide motifs on enamel proteins that serve as antigens for autoantibodies will help to define preventive strategies to avoid enamel defects on permanent teeth in children with CD.

Whether antibodies that gain access to the tooth germ in vivo could cause transient changes in enamel development remains to be verified. Amelogenin acts as a scaffold protein for calcium hydroxyapatite crystal formation; hence, it is possible that antibodies interacting with fully formed amelogenin nanospheres or growing nanospheres could interfere with biomineralization. Experimental approaches to elucidate this are underway in our laboratory.

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Conflicts of interest – The authors have no conflicts of interest.

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