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Polymorphonuclears in oral mucosa of celiac children not gluten free diet

Polimorfo-nucleares en mucosa oral de niños celíacos sin dieta libre de gluten

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Abstract

Our work group first described the presence of Polymorphonuclears (PMNs) in the oral mucosa smears of children patients with CD and not comply a gluten-free diet. The objective of this work was to associate the PMN in oral mucosa smears of celiac children and factors as recurrent aphthous stomatitis (RAS) or other typical signs and symptoms. To established two groups: a) celiac children *(CD)* (n=27); b) non celiac children *(NCD)* (n=24) were not affected by gastrointestinal pathology or other diseases related to CD and negative for IgA AGA, IgG AGA, total IgA, and IgA EMA. Both groups included, female/male, 4-12 years old with similar sociodemographic, cultural, and odonto-medical features. It recorder the irritable behavior, RAS and pick-up smears of non-keratinized zones of oral cavity. The association between PMN and RAS in celiac children was evaluated by logistic model and bivariate relations by Fisher test. For all test the statistical significance was set $p<0.05$. RAS were observed in a significantly higher percentage ($p=0.0007$) in CD children (55.6%) compared to NCD (8.3%), while PMNs were only observed in CD children (44.4%; $p=0.0002$). Also, 70.4% of children with CD exhibited anxiety state in comparison to 12.5% of NCD children ($p=0.0001$). A strongly significant association was observed between the presence of RAS and its lesions and PMNs in smears of CD. Our results showed an association between PMNs and RAS in CD children, probably due to anxiety and/or stress states in children having CD. It could be said that in CD patients not complying with a gluten free diet, PMNs and other specific markers could be used as markers of a presumptive diagnosis; also, in patients with a confirmed diagnosis, they could signal the fulfillment of a diet together with other signs and symptoms.

KEYWORDS: celiac disease, leukocytes, recurrent aphthous stomatitis

Resumen

Nuestro grupo de trabajo describió por primera vez la presencia de polimorfonucleares (PMN) en los frotis de mucosa oral en pacientes pediátricos con Enfermedad Celíaca (EC) y que no cumplían con una dieta libre de gluten. El objetivo de este trabajo fue asociar los PMN presentes en frotis de mucosa oral de niños celíacos con factores como la estomatitis aftosa recurrente (EAR) u otros signos y síntomas típicos de EC. Se establecieron dos grupos: a) niños celíacos (EC) (n=27); b) niños no celíacos (NEC) (n=24), estos últimos que presentaran patología gastrointestinal u otras enfermedades relacionadas con EC y negativos para IgA AGA, IgG AGA, IgA total e IgA EMA. Ambos grupos incluyeron, niños y niñas, de 4 a 12 años con características socio-demográficas, culturales y odonto-médicas similares. Se registró el comportamiento irritable, EAR y muestras de zonas no queratinizadas de la cavidad oral. La asociación entre PMN y EAR en niños celíacos se evaluó mediante modelo logístico y relaciones bivariadas, prueba de Fisher. Para todas las pruebas, la significación estadística se estableció $p<0,05$. Se observaron EAR en un porcentaje significativamente mayor ($p=0,0007$) en niños con EC (55,6%) en comparación con NEC (8,3%), mientras que los PMN solo se observaron en niños con EC (44,4%; $p = 0,0002$). Además, el 70.4% de los niños con EC exhibieron estado de ansiedad en comparación con el 12.5% de los niños con NEC ($p=0.0001$). Se observó una asociación fuertemente significativa entre la presencia de EAR y los PMN en frotis de EC. Nuestros resultados mostraron una asociación entre PMN y EAR en niños con EC, probablemente debido a estados de ansiedad y / o estrés en niños con EC. Podría decirse que en pacientes con EC que no cumplen con una dieta libre de gluten, los PMN y otros marcadores específicos podrían ser útiles como biomarcadores de un diagnóstico presuntivo; Además, en pacientes con un diagnóstico confirmado, podrían indicar, junto con otros signos y síntomas típicos de EC, el cumplimiento de la dieta libre de gluten.

PALABRAS CLAVE: enfermedad celíaca, leucocitos, estomatitis aftosa recurrente

Introduction

Celiac disease (CD) is a complex non transmissible disease affecting the small bowel, and it is caused by the activation of immune cells response when exposed to prolamines and glutenins of wheat, barley, rye, and (not often) oats, in a genetically predisposed person. This kind of pathology frequently produces alterations of the oral ecosystem[1].

In some CD patients, the symptoms are obvious to recognize, but others may be present slight symptoms or become manifest as long-standing complications of untreated pathology [2]; thus the study of new simply markers allowing to establish presumptive and/or monitoring diagnoses in time, mainly within risk groups, by means of non-bloody methodologies, would enhance therapeutics and, consequently, these patients' quality of life. In this context, the study of components of the oral ecosystem is of great interest for predicting CD for its accessibility and easy collection.

As regards the oral cavity, it is known that the majority of patients with celiac disease showed enamel alterations and the presence of Recurrent Aphthous Stomatitis (RAS) among others oral features. In previous own research (cross-sectional and follow-up studies), a high percentage of child patients that reported RAS and showed a significant presence of polymorphonuclear neutrophils (PMNs) in oral smears was observed, compared to child patients without CD[1,3].

Several factors, including genetic predisposition, immunologic disturbances, viral and bacterial infections, food allergies, vitamin and microelement deficiencies, systemic diseases, hormonal imbalance, mechanical injuries, and stress, have been suggested to cause RAS or to be associated with it [4-6].

Pathogenesis of RAS involves an immune-complex response, such as cell infiltration into the epithelium during the initial stages of the lesion and then ulceration with a fibrous membrane covering the sore, which is penetrated mainly by neutrophils, lymphocytes and plasma cells[7,8].

However, the etiology of RAS remains controversial: one accepted hypothesis proposes the existence of a possible genetic predisposition of some patients. In those patients, the presence of local and/or systemic factors causes alterations of oral mucosal cells, leading to the uncontrolled activity of lymphocytes, monocytes and neutrophils [9]. On the other hand, authors as Pastore et al. 2008[10] suggest that exposing the oral mucosa to gluten in patients with CD could stimulate lymphocyte activity, so they state that the oral mucosa and salivary glands are effectors' areas of the mucosal immune system and when it is gluten-sensitized, lymphocytes present in CD-patients' saliva associate with antibodies in the oral mucosa of these patients[10].

Previously exposed background leads to the following questions: a) is celiac disease not gluten-free treatment associated *per se* to the presence of PMNs in smears of oral mucosa?; b) does celiac disease produce anxiety behaviors in patients which relate to the occurrence of RAS, in which PMNs play a role?; c) does celiac disease produce RAS, in which PMNs play a role?

Thus, the objective of the present work was to assess whether there exists a relationship between the presence of PMNs in oral mucosa smears and the presence of RAS, or celiac disease, or both.

Materials and methods

Children both genders (n=51) aged 4-12 years, who attended the Gastroenterology Service at the ''Santísima Trinidad'' Children's Hospital, Córdoba, Argentina and the Department of Paediatric Dentistry at the School of Dentistry of the National University of Córdoba, Argentina were assessed. Children were assigned to two groups of cases: children with CD (n=27) and non-celiac children (NCD) (n=24). All children included in the study had a similar socio-economic background, measured through social features of families, father's occupation, level of education, source of income, type of housing and characteristics of the geographical area.

CD children were diagnosed by a physician specializing in Gastroenterology, according to the European Society of Paediatric Gastroenterology and Nutrition (ESPGAN) criteria, amended in 1990[11], and the World Gastroenterology Organisation, in 2015 by means of intestinal biopsy as grade III or IV, considering the small intestine as normal when the villous height to crypt depth ratio is approximately 4:1 to 5:1[12].

NCD children were not affected by gastrointestinal pathology or other diseases that may be related to CD and their routine serological examinations at the hospital showed negative IgA AGA, IgG AGA, total IgA and IgA EMA.
Individuals who at the time of the study were taking any type of medication acting on the immune system were excluded from the study, as was anyone who had another immunological disease and/or serious periodontal disease. This study was approved by the Research and Ethics Committee, Ministry of Health of the province of Córdoba, Argentina (Protocol no. 1379; http://www.cba.gov.ar/listado-de-investigaciones-registradas/), and it followed the guidelines of the Declarations of Nüremberg, Helsinki and Tokyo of the World Medical Association. Informed consent forms were signed by parents or guardians of all child patients with or without CD.
Signs and symptoms of children, mainly of features such as frequent negative emotional state characterized by feelings of worry and nervousness, and accompanied by specific somatic, cognitive, and anxious behavior, were collected on a single clinical record through reports of parents[13].

Clinical-odontological exam

It was conducted by Mina S., through routine instrumental exploration, artificial lighting, after cleaning the teeth with a toothbrush and drying them. The lips, cheeks, soft and hard palates, tongue, floor of the mouth, and gums were inspected and assessed. Recurrent Aphthous Stomatitis (RAS) was recorded by clinical examination or by parent report according to features such as recurrent, small, round, or ovoid ulcers with circumscribed margins, erythematous halos, and yellow or grey floors (Scully and Porter 2008).
The oral hygiene was assessed according to the simplified Greene and Vermillion index (1964)[14] and the degree of gingival inflammation by the gingival health index proposed by Löe and Sillnes (1963)[15].

Oral Mucosa Smears

Oral mucosa smears were taken by brushing (Cytobrush) from the non-keratinized zones of cheek and/or lips of celiac and control children without clinical trauma of oral mucosa. Smears were prepared and observed following own protocols [3].

Microbiological study on saliva

The levels of *Streptococcus mutans*, and *Lactobacillus spp*. were determined according to the protocol described by Mina et al, 2008[1]. Scores of bacteria were reported in CFU/mL of saliva (colony forming units per mL of saliva): *High Activity*; $>10^4$ CFU/mL, *Medium Activity*; 10^3- 10^4 CFU/mL; *Low Activity*; $<10^3$ CFU/mL.

Statistical Analysis

Quantitative data were described by median values, and qualitative data were expressed as percentages. The association between PMNs and explain variables was analyzed by a logistic model and the bivariate association by Fisher test. The Whitney U test was performed to evaluate significant differences between averages of age (years) groups by gender. For all test, the critical level was set at $p<0.05$ for establishing significant differences. Data analysis was performed with the R 2.15.3 software (www.r-project.org).

Results

Percentages of boys and girls were similar among CD patients and NCD patients (Table 1); same as the average age of CD patients (median male age=7.36±3.29 years; median female age=7.50±3.90 years) and NCD children (median male age=8.00±3.77 years; median female age=8.36±2.62 years) (p-value female=0.1760; p-value male=0.7768).
RAS lesions were observed in a significantly higher percentage (p=0.0007) in CD children (55.6%) compared to NCD (8.3%) (Table 1), while PMNs were only observed in oral smears of CD children (44.4%; p=0.0002) (Table 1).
Also, 70.4% of parents of children with CD described anxiety state in comparison to 12.5% of NCD children (p=0.0001) (Table 1).

Table 1. Demographic, oral mucosa and behavioral features measured. *RF*: relative frequencies; *AF*: absolute frequencies. *Gen*: gender; *OM*: oral mucosa; *BS*: behavior state; *Anx*: anxiety. ***Bold***: Bivariate association statistical significance set at p≤0.05 between celiac/non celiac and other variables. *Yate's correction for the categorical data that is made when the number of cases in any class is small and there is one degree of freedom.

		Celiac AF (RF%) (n=27)	Non celiac AF (RF%) (n=24)	Fisher's p-value	OR crude	LB 95 %	UB 95%
Gen.	Fem.	16 (59.3%)	14 (58.3%)	0.9465	1.04	0.35	3.1
	Male	11 (40.7%)	10 (41.7%)				
OM	RAS	15 (55.6%)	2 (8.3%)	**0.0007**	12.5	2.76	56.57
	PMN	12 (44.4%)	0 (0%)	**0.0006**(*)			
BS	Anx and Stress	19 (70.4%)	3 (12.5%)	**0.0001**	16.6	4.15	66.58

Possible relationships among the variables considered as explanatory of the presence of PMNs in oral smears are shown in Fig. 1. All variables in the graph are showed significant association (by simple logistic regression), but it was observed a strongest significant association between the presence of RAS/CD (P=0.0004; OR=13.75; CI [3.06, 61.88], and RAS/PMNs in smears of CD (P=0.0001; OR=16.63; CI [4.15, 66.58] (Fig. 1).

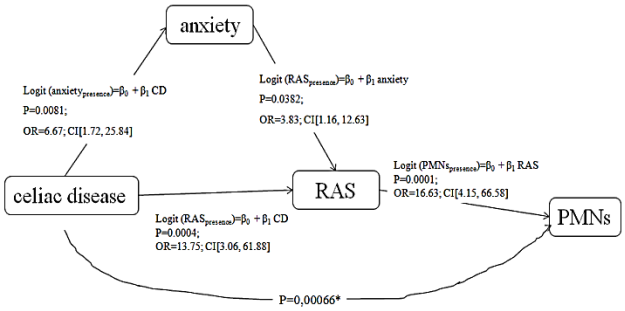

Figure 1. Graph model of possible relationships among the presence of PMNs in oral smears and other explanation variables. Arrows indicated a direction of association and the numbers correspond to Odd Ratios (OR) and its respective 95% confidence intervals (CI). *p-value is estimated by Yates's correction; *PMNs*: polymorphonuclear cells; *RAS*: recurrent aphthous stomatitis

The assessment of oral health conditions showed that the population exhibited middle-low bacterial activity (*Streptococcos mutans*; 70% CD and 90% NCD ≤ 10^4; p-value=0.1138; *Lactobacillus spp.*; 77% CD and 87% NCD ≤ 10^4; p-value=0.4349), good oral hygiene (CD= 66.39%; NCD=65.21%; p-value=0.9304) and low-mild gingivitis (CD=99%; NCD=100%; p-value=0.9435), in control patients as well as in CD patients.

Discussion

Our work group first described the presence of PMNs in the oral mucosa smears of patients with CD who did not comply with a gluten-free diet. In general, the presence of PMNs in smears from subjects has been related to infectious processes and/or other systemic diseases involving the immune system[17,18].

In this study, a high percentage of child patients with CD have presented or reported RAS. RAS is the most common ulcerative lesion of the oral mucosa, and it affects around 20% of the general population. In agreement with our observations, several authors described RAS in similar percentages in child and adult patients with CD[19-21]. Although the majority of patients did not present this lesion at the time of dental exam, in many occasions their parents had informed that children had presence of RAS. The percentage of children with RAS is reliable because Baccaglini et al. 2013[22] proved the diagnosis accuracy based on anamnesis.

Studies have proved that oral microbiota composition may regulate oral immunity and, consequently, have an influence on presence of RAS in oral cavity[5]. Nevertheless the population in this study showed middle-low bacterial activity, good oral hygiene and low-mild gingivitis, in both groups (control and CD). It suggests that the presence of RAS is due to non-infectious factors. On the other hand, up to date the etiology of RAS still remains uncertain. Diverse factors, including genetic predisposition, immunologic disturbances, viral and bacterial infections, food allergies, vitamin and microelement deficiencies, systemic diseases, hormonal imbalance, mechanical injuries, and psychological stress, have been suggested to produce or to be associated with RAS[4,5,14]. Recent studies have demonstrated that during RAS onset, an abnormal apoptosis of oral epithelial cells is

observed, which can progressively lead to necrosis later and a passive release of products such as proinflammatory cytokines leading to acute inflammation[23]. Moreover, it is known that in the pre-ulcerative phase of RAS formation, there appears a mononuclear infiltrate with a large amount of granulate lymphocytes and $CD4^+$ helpers. It can also be observed in polymorphonuclear cells [24,25].

In this research, it has been noted that CD children showed a strong association between the presence of RAS and CD, and the presence of PMNs in smears and RAS. As mentioned above the presence of RAS in CD children is a fact already described in previous research [1,26,27]. It is known that CD is a pathology affecting nutrient absorption, mainly because intestinal villi's extension is altered, which can lead to anemia[28]. Akintoye and Greenberg (2014)[29] have demonstrated that RAS patients suffer from anemia and/or iron, calcium, and B and C vitamin deficiency; and other studies have described that CD patients habitually have nutritional deficiencies of iron, vitamin D, folate, vitamin B12, vitamin B6, and zinc [2,30]. And the researches have shown that some neurologic disorders have been association with mal absorption of vitamin B12, folate, copper, and vitamin D[31].

Besides, we observed that 70% of CD children exhibited behavior states as anxiety. In agreement with our results, other researchers have confirmed that these psychological states, like anxiety and stress, are related to the presence of RAS[19]. In the somatic level, there exists evidence proving that psychological stress alters immunologic activity thus leading to an increase in leukocytes in inflammatory areas, like the ones observed in RAS lesions[32,33]. Furthermore, experimental studies conducted in mice have shown that high anxiety levels inhibit humoral and cellular parts of the immune system, and a decrease in lymphocytes, and IgG A and E concentration can be observed, although not a change in the number of granulocytes, monocytes and natural killer cells[34].

In summary, our results showed that the presence of PMNs in oral smears of CD children is associated with RAS and CD condition; it could be probably anxiety and/or stress states or both together in children having CD. Figure 2 shows the hypothetical biological pathway leading to the presence of PMNs in the oral mucosa of children with CD.

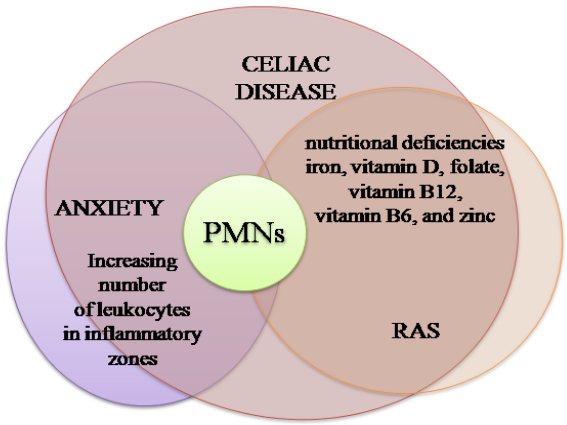

Figure 2. Possible biological relationships between celiac disease and PMNs in celiac children's smears. *PMNs*: polymorphonuclear cells; *RAS*: recurrent aphthous stomatitis.

It could be said that in CD patients not complying with a gluten-free diet, PMNs and other specific signs/symptoms of CD could be used as markers of a presumptive diagnosis; also, in patients with a confirmed diagnosis, they could signal the fulfillment of a gluten-free diet.

Since oral mucosa proves an accessible organ for clinical and histological diagnosis, it could be highly valuable for monitoring patients at risk. However, conditions under which the oral mucosa morphology must be considered as a CD marker need to be determined, so it is important to carry further research in order to clarify the changes in said morphology in relation to CD.

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