IMMUNOGLOBULIN A, G AND M LEVELS IN SALIVA IN CHILDREN BETWEEN 3 – 12 YEARS OF AGE, HEALTHY AND WITH GINGIVITIS

Mario R. Romero[1], Marta L Lozano[2], Carolina Posada[1], Paola A Rueda[1], Nelly S. Roa[3], Adriana Rodríguez[3]

[1] Craniofacial Department, School of Dentistry, Pontificia Universidad Javeriana,Colombia.

[2] Dental Department, School of Dentistry, Pontificia Universidad Javeriana, Colombia.

[3] Center for Dental Research, School of Dentistry, Pontificia Universidad Javeriana, Colombia.

ABSTRACT

The aim of this study was to measure the level of immunoglobulin A, G and M in saliva of 3- to 12-year-old children, both healthy and diagnosed with gingivitis. Methods: A sample of 177 children was selected, of whom 24 were healthy and 153 were diagnosed with gingivitis according to Loe´s index. Samples of saliva were taken and the ELISA test was applied to obtain the immunoglobulin concentrations expressed in µg/ml. A relationship was established between the immunoglobulin levels, the disease (gingival index) and Loe´s bacterial plaque index.

IgG levels were higher in healthy children. In the group with gingivitis, 95.8% of the children had incipient gingivitis with a low average index of bacterial plaque (1.33). A direct correlation was found between age and gingival index, while an inverse correlation was found between age and bacterial plaque index. The analysis of the behavior of immunoglobulin according to age showed that age was only significantly correlated to IgA levels. The analysis comparing immunoglobulin levels and clinical parameters for gingivitis showed a direct correlation between gingival index and IgM. The gingival index was found that to increase with the age of the subject, even though bacterial plaque decreases. It was also found that age is a better predictor of IgA level than gingival index and bacterial plaque index are; and that gingival index is a better predictor of IgM level than age and bacterial plaque index are.

Keywords: Immunoglobulin A, Immunoglobulin G, Immunoglobulin M, saliva, gingivitis, child.

NIVELES DE INMUNOGLOBULINA A, G Y M EN NIÑOS DE 3 – 12 AÑOS SANOS Y CON GINGIVITIS

RESUMEN

El objetivo de este estudio fue cuantificar los niveles de inmunoglobulinas A, G y M en saliva de niños entre 3 – 12 años sanos y con gingivitis. Métodos: la muestra fue de 177 niños distribuidos en dos grupos: 24 sanos y 153 con diagnóstico de gingivitis según el índice de Loe a quienes se les tomaron muestras de saliva y por medio de la prueba de ELISA se obtuvieron las concentraciones de las inmunoglobulinas expresadas en µg/ml. Resultados: Se encontró que en la saliva de los niños sanos los niveles de IgG son significativamente mayores que en los niños con gingivitis. El grupo gingivitis estuvo conformado por un 95.8% de niños con diagnóstico de gingivitis incipiente que presentó un promedio bajo de índice de placa bacteriana. Al hacer análisis de correlación entre las variables estudiadas, se encontró una correlación directa entre la edad y el índice gingival, una correlación inversa entre la edad y el índice de placa bacteriana, correlación directa entre los niveles de IgA y la edad y correlación directa entre el índice gingival y la IgM. Conclusiones: Se encontró que en la medida en que el individuo crece aumenta el índice gingival, aunque se presenta menor cantidad de placa bacteriana. También se concluyó que la edad es mejor predictor de los niveles de IgA que el índice gingival y el índice de placa bacteriana y que el índice gingival es mejor predictor de los niveles de IgM que la edad y el índice de placa bacteriana.

Palabras clave: Inmunoglobulina A, inmunoglobulina G, inmunoglobulina M, saliva, gingivitis, niños.

Introduction

Periodontal disease in children has often been described as limited to marginal gingivitis, because advanced forms are typical of adults. Evidence of gingival inflammation in young children is lower than in older children with similar amounts of bacterial plaque[1]. The differences in the clinical manifestation of periodontal disease as age increases are attributed

to functional changes of the periodontal structures, the presence and maturation of different microflora, and the maturity of the immune system[2,3].

Mooder and Wondimu report that tissue inflammation due to the accumulation of bacterial plaque begins in early childhood and reflects the host's bacterial challenge[4].

This manifestation of the inflammatory reaction depends on the interaction between local and systemic factors, including the individual's immune response. In children, the inflammatory cell infiltrate of gums with gingivitis especially compromises T-lymphocytes and a humoral response with activation of B-lymphocytes, which synthesize antibodies such as IgA, IgG and IgM. The immune system in preschoolers is immature, and these serum immunoglobulins attain their highest levels towards puberty[5].

The serum levels of antibodies against microorganisms involved in periodontal disease increase gradually from childhood to adulthood. To date, studies of immunoglobulins and their increase against periodontal pathogens in children with gingivitis have been performed on serum samples.

The base level of immunoglobulin in total saliva allows an assumption to be made regarding the host's degree of susceptibility when responding to aggression, because the immunoglobulin sub-type found in a fluid takes part in the effector functions of neutralization, complement activation and phagocytosis. The protective effects of humoral immunity are mediated by the specific functions of each immunoglobulin, thus, IgA neutralizes antigens and macromolecules in general that penetrate through the mucosa, inhibiting sensitization to foreign macromolecules and protect againts infectious diseases such as periodontal disease; IgG has classically been defined as being responsible for secondary immune response and memory, and IgM is the most important in the primary response against the large majority of microorganisms. IgA and IgM can be actively transported through the secretor epithelium, while IgG plays a small part in the immune defense of mucosas, due to the absence of receptors in the secretory epithelial cell enabling its transcytosis. However, IgG can be found in whole saliva, therefore its role in the immune response in the oral cavity should not be dismissed[6].

Given the relevance of evaluating the role of antibodies in gingival disease in children, the aim of this study was to measure levels of immunoglobulins A, G and M in saliva, and to establish the differences between children with and without gingival disease in different age groups, in addition to determining the relationship with the clinical parameters gingival index and bacterial plaque.

MATERIALS AND METHODS

A transversal, analytical, observational study was performed, with a quantitative focus.

Selection of Population and Sample

We selected 177 boys and girls aged 3 to 12 years, of whom 24 were healthy and 153 had gingivitis, diagnosed according to Loe's index[7], from a population of children belonging to Social Welfare Homes and socio-economic levels 1 and 2, with similar living conditions and oral hygiene habits. The following criteria were used in the selection:

Inclusion criteria

Systemically healthy children.

No dental/maxillofacial anomalies or alterations in dental development.

Not undergoing antibiotic therapy or taking any medication at the time or during the 3 months prior to sample collection.

No orthodontic appliances.

No teeth about to exfoliate, with pathological mobility, in eruption or with purulent exudate.

No dental treatment received within 3 months prior to taking the samples.

Exclusion Criteria

Children unable to collect the amount of saliva needed for quantifying immunoglobulins.

Clinical Parameters

Gingivitis was diagnosed according to the gingival index and Loe's bacterial plaque index[7] on 6 teeth selected for the study: 16 or 55, 21 or 61, 24 or 54, 36 or 75, 41 or 81 and 44 or 84.

Saliva Samples

The samples were taken by spontaneous salivation and transported on ice. In the laboratory, saliva samples were centrifuged at 15000 rpm for 15 minutes at 4°C. The supernatant was collected and stored at – 20°C until the ELISA test was performed.

Quantification of saliva IgA, IgG and IgM by ELISA

Polystyrene microtiter plates with 96 wells were sensitized with 100 µl of each previously titered monoclonal antibodies (anti-IgA ref A-7032 1/500, anti-IgG ref I-6135 1/1000 and anti-IgM ref I-6385 1/2500 from Sigma Chemical Co.) diluted in carbonate buffer pH 9.6 for 3 hours at 37°C and overnight at 4°C. After 3 washes with PBS-tween 20 (0.05%), the free sites on the solid phase were blocked with a solution of 100 µl of 1% skim milk in PBS, for 1 hour at 37°C in a moist chamber. The washes were repeated between each step under the same conditions. 100 of µl the saliva sample were placed in solid phase, diluted in PBS, after standardization (IgA 1/10, IgG 1/40 and concentrated IgM). For the detection of the antigen-antibody reaction, 100 µl of each conjugate diluted in PBS were added, after titering (anti-IgA with alkaline phosphatase ref A-3063 1/2500, anti-IgG with alkaline phosphatase ref A-9544 1/5000 and anti IgM with alkaline phosphatase ref A-9794 1/2500 from Sigma Chemical Co.).

To obtain the immunoglobulin concentrations in the saliva samples, known concentration patterns for each immunoglobulin were included in the procedure (human IgA ref 2636, human IgG ref l-4506 and human IgM ref l-8260, Sigma Chemical Co.). The absorbance values recorded were used to produce a calibration curve by plotting concentration on the X-axis and absorbance on the Y-axis. The absorbance values obtained for each saliva sample were extrapolated and the concentration corresponded to the cut on the X-axis, expressed in µg/ml.

Statistical Analysis

To process the information, a data base was prepared in Excel format and exported to the SPSS software version 10.0, which preformed the relevant calculations. The analysis initially developed the description of the sample with measures of central tendency and dispersion for quantitative variables (means – standard deviation). In addition, homogeneity of variance and normal behavior were evaluated for the use of parametric models with inferentials.

The comparative analysis was processed from the 95% confidence intervals for means and significant differences were evaluated as from their limits. In addition, single classification factor analysis of variance was used, taking $p \leq 0.05$ as significance criterion.

Finally, after evaluating the statistical assumptions of homoscedasticty and normality, a multivariate analysis was processed from the Pearson's correlation model, from which the correlation matrix was constructed and linear regression equations were modeled.

Parametric tests were used for this analysis because the sample does not fall within the range of a small sample and these tests are robust enough to handle data that have some parametric variation.

RESULTS

This study quantified levels of IgA, IgG and IgM in saliva from children aged 3 to 12 years with gingivitis, in order to determine whether these levels vary among different age groups and if they are related to the clinical parameters gingival index and bacterial plaque.

Description of the sample

This study considered 24 healthy children and 153 children diagnosed with gingivitis according to Loe's gingival index. Mean age was 6 years ± 2.4 for the healthy group and 8 years ± 2.9 for the group with gingivitis. It was found that healthy children were significantly younger than children with gingivitis.

The clinical parameters show that the group of children with gingivitis was composed mainly of children diagnosed with incipient gingivitis (95.8%), while only 4.2% had moderate gingivitis. No patient was found to have severe gingivitis.

To determine the relationship between immunoglobulin levels and disease, the clinical parameters gingival index and Loe's bacterial plaque index were used. Mean gingival index was 0.45, ranging from 0.04 to 1.42. Mean plaque index was 1.33, ranging from 0.17 to 2.58, i.e. the group had low bacterial plaque index (Table 1).

Immunoglobulin levels

The analysis of the whole group showed heterogeneity in the behavior of immunoglobulin concentrations. When all three immunoglobulins were compared, IgA was found in the highest proportion, followed by IgG and IgM, in that order. IgA and IgM were present in the saliva of all children, but IgG was absent from some of them (Table 1). This might be explained by the high biological variability in the humoral immune response in each individual.

Table 1. Indicators for the study variables in the two groups.

	Bacterial plaque	Gingival index	IgA μg/ml		IgG μg/ml		IgM μg/ml	
	Gingivitis	*Gingivitis*	*Healthy*	*Gingivitis*	*Healthy*	*Gingivitis*	*Healthy*	*Gingivitis*
Mean	1.33	0.45	81.0	69.84	14.8	7.98	0.2	0.22
Standard deviations	0.51	0.28	53.4	41.7	11.1	10.43	8.8	0.17
Minimun	0.17	0.04	13.8	8.1	0.076	0	0.014	0.14
Maximum	2.58	1.42	191.3	213.6	34.8	49.9	0.4	1.45

The analysis of confidence intervals showed that healthy children have significantly higher values of saliva IgG than children with gingivitis ($p<0.05$). IgA and IgM levels in saliva do not differ between groups.

Correlation analysis

In order to evaluate the multivariate behavior of the immunoglobulins as a function of gingival and bacterial plaque indices, a correlation matrix was prepared using Pearson's correlation coefficient with a significance level of 0.05, for the group of children with gingivitis exclusively.

The analysis of the relationship between age and clinical parameters showed a direct correlation between age and gingival index ($p<0.05$) and an inverse (negative) correlation between age and bacterial plaque index ($p<0.05$). These findings suggest that as the child grows, the gingival index increases although there is less bacterial plaque. Nevertheless, the analysis showed a direct correlation between bacterial plaque index and gingival index ($p<0.05$).

Analysis of immunoglobulin behaviour in relation to age showed a statistically significant correlation only with IgA levels, i.e., older children had higher levels of IgA. IgG and IgM showed no statistically significant relationship with age.

Analysis of the relationship between immunoglobulin levels and the clinical parameters of gingivitis showed that there was a direct correlation between gingival index and IgM ($p<0.05$), but that gingival index was not related to IgG or IgA. No significant correlation was found between plaque index and the levels of any of three immunoglobulins studied.

Considering that higher IgA levels were found at older ages, that IgM was positively correlated to gingival index and that gingival index was higher in older children, a linear regression analysis was performed based on Pearson's model, to evaluate how useful the variables age and gingival index are as predictors of IgA and IgM levels.

Analysis of the results between gingival index and immunoglobulins, with statistical control of the effect of age, showed that gingival index behaves as a significant predictor of IgM, but does not predict IgA or IgG; i.e., it fit a significant regression model of gingival index and IgM.

Similarly, when gingival index was controlled, it was found that age acts as a significant predictor of IgA, fitting the regression model for IgA, but not for IgM or IgG.

In general, this analysis establishes that age is a better predictor of IgA levels than gingival index and bacterial plaque are, and that the gingival index is a better predictor of IgM levels than age and bacterial plaque index are.

Analysis by age groups

The total 153 children were divided into 3 age groups: 3- to 6-year-olds (preschoolers), 7- to 9-year-olds (school age) and 10- to 12-year-olds (preteens). A descriptive analysis according to age group showed that 32% of the sample belonged to the 3- to 6-year-old group, 28.8% to the 7- to 9-year-old group and 39.2% to the 10- to 12-year-old group.

Analysis of the mean values for gingival index showed that it is lower in 3- to 6-year-olds than in 10- to 12-year-olds ($p<0.05$), but similar in 3- to 6-year-olds and 7- to 9-year-olds, suggesting that gingival index is higher in older children. These results determine two groups for gingival index: 3- to 9-year-olds and 10- to 12-year olds (Table 2).

Bacterial plaque index was found to be higher in younger children (3- to 6-year olds) ($p<0.05$) than in 7- to 9-year-olds and 10- to 12-year-olds, in

Table 2. Mean values for clinical parameters and immunoglobulin levels in children with gingivitis, by age group.

Age (years)	Bacterial plaque	Gingival index	IgA μg/ml	IgG μg/ml	IgM μg/ml
3 - 6	1.61	0.35	57.81	8.61	0.24
7 - 9	1.08	0.45	81.27	7.82	0.2
10 - 12	1.26	0.52	70.52	7.46	0.22

which it behaves similarly. Two age groups are therefore also established by bacterial plaque index: 3- to 6-year-olds and 7- to 12-year-olds (Table 2).

Comparison of the mean values for immunoglobulins according to age group showed that IgA levels in 3- to 6-year-olds were significantly lower than in 7- to 9-year-olds ($p<0.05$); although there was no significant difference with 10- to 12-year-olds (Table 2). No significant difference was found in IgG and IgM levels among age groups. However, it is interesting to note that IgG levels were lower in older age groups, even though the difference was not statistically significant (Table 2). The behavior of IgM was opposite to that of IgA, with highest levels in 3- to 6-year olds, declining in 7- to 9-year-olds, and increasing again in 10- to 12-year-olds, although not reaching levels as high as in 3- to 6-year-olds (Table 2).

DISCUSSION

The lack of studies seeking to further the knowledge of clinical, microbiological and immunological parameters of periodontal disease in children has hindered the development of better diagnostic, prevention and early treatment alternatives for children[8]. One of the interesting aspects of the development of gingival disease in children is that the severity of the clinical response to similar triggering parameters such as bacterial plaque is lower than in adults. This might be due to the behaviour of the immune response maturing as the individual faces antigenic challenge, until it attains a maximum during adulthood. Thus, this study was designed to establish the relationship between age and total levels of saliva antibodies IgA, IgM and IgG in subjects affected by gingival disease.

It is clear from direct observations and different studies that the inflammatory gingival response is lower in children than in adults when confronted by a similar antigenic challenge such as quantity of bacterial plaque. This leads to the statement that prevalence and severity of gingival disease increase gradually from childhood to adulthood[2]. These findings, which reflect current consensus on epidemiology of gingival disease in the pediatric population, have been confirmed by this study, which has shown how the gingival index increases with age, although the bacterial challenge represented by the bacterial plaque index is higher in younger children.

The local antigen challenge triggers a humoral immune response that can be detected in saliva. The influence of the local response is given by secretory IgA, which becomes the predominant antibody in saliva, while the influence of the systemic response is given by serum IgA (monomeric), IgG and IgM, which reach the saliva through the gingival fluid[6]. Almost 74% of the blood antibodies are IgG, 15% are IgA and 11% are IgM[9]. The mean concentrations found in this study correspond to 0.091% and 0.014% of the reference values for serum IgG and IgM for this age group. Consistently with this theoretical aspect, this study found that the predominant immunoglobulin in saliva is la IgA, followed by IgG and, as expected, considering the relationship with serum levels, a lower level of IgM.

Regarding levels of immunoglobulins in saliva, there is no report in the literature on the values for IgG and IgM. For IgA, Nagao et al. performed a study on systemically healthy Brazilian children, without considering their periodontal state, and reported that the range of IgA concentration is 17.1 to 93.1 μg/ml[10]. The range observed in our study was different, as it was 10.95 to 202.5 μg/ml, indicating variable behaviour of these immune proteins, not only among individuals in a population, but also among different populations.

The comparative analysis of the three isotypes of saliva antibodies in healthy children and those with gingivitis showed that there is no statistically significant difference for IgA and IgM, although the average was lower for the diseased group. IgG levels were found to be significantly greater in healthy subjects than in subjects with gingivitis. The significant reduction in IgG levels in children with gingivitis may be interpreted by means of two hypotheses, which could be discussed: the reduction could be either a consequence or a cause of the disease.

If the reduction is considered to be a consequence, then the antibodies produced are consumed in the gingival tissue due to the effector response of IgG against the periodontal pathogens, contributing to the inflamed tissue characteristic, which is a fundamental parameter in the clinical diagnosis of gingivitis. This means that a lower proportion of antibodies reaches the oral cavity than in healthy subjects, in whom these antibodies do not face a bacterial challenge from pathogenic microorganisms. Although it is true that periodontal pathogens may be found in healthy children, they are found in lower amounts than in children with gingivitis.

However, if the reduction is analyzed as a cause of the disease, the lower amount of antibodies may predispose the subject to developing gingivitis, because it has lower capacity for defense against microorganisms that colonize the gingival tissue; this would lead to gingivitis being considered as a manifestation of immunodeficiency, which does not agree with clinical findings in these patients. Moreover, considering reports in the literature that show that specific antibody levels (for the different periodontal pathogenic bacteria) are higher in inflamed tissue[11,12], it may be assumed that the consequence argument is more valid for explaining the results found in this study. It could be suggested that IgG plays a relevant part in the physiopathogenesis of gingivitis in children. However, when the behaviour of la IgG is analyzed within the diseased group, no significant correlation is found between these levels and a disease indicator such as gingival index, although there is a gradual decline in IgG as gingival index increases.

The literature reports that saliva IgA against microorganisms of the oral and intestinal microbiota increases with age regardless of disease parameters[13]. The results of our study confirm that IgA levels definitely increase with age in response to the constant microbial challenge in the oral cavity, without showing relationship with gingival disease.

Studies seeking a relationship between gingival disease, age and antibody types IgG and IgM have been performed on serum and against specific microorganisms. The results have been controversial. Some authors suggest that the levels of antibodies against microorganisms involved in periodontal disease seem to increase gradually from childhood to adulthood[14-17] and with the severity of the disease[18], while others conclude that age has a greater influence than severity of the disease does on the levels of antibodies, mainly for IgM values[1].

In contrast to these reports, the results of this study showed that saliva IgG levels were not related to age or gingival index, while saliva IgM behaves independently of age, although it increases proportionally to the gingival index. Nevertheless, it is important to highlight that the immunoglobulin concentrations attained in the oral cavity do not necessarily reflect serum levels. No report was found in the literature on total saliva immunoglobulins in patients with gingivitis.

The meaning of the direct relationship between IgM levels and gingival index is not clear; it may be interpreted as the response to activation of IgM production as a result of the antigenic challenge leading to tissue inflammation or that the increase in exudate as a result of the inflammation generates more IgM in saliva. Considering that gingival disease in children behaves as an acute, transitory biphasic phenomenon[19], IgM levels might be higher due to their role in the primary response. However, it is important to highlight that the results vary according to the microorganism studied. Morinushi reports an inverse relationship between levels of serum anti-*P gingivalis* antibodies and gingival inflammation in children aged 3 to 18 years, while for *A. actinomycetemcomitans* this relationship is direct only in children over 12 years old[20].

The analysis of age groups shows that the positive relationship found between IgA levels and age differs between 3- to 6-year-olds and 7- to 12-year olds. As from 6 years of age, there are changes caused by the beginning of the mixed dentition stage, when permanent teeth emerge. This leads to a change in the crevicular environment, facilitating colonization by microorganisms associated to periodontal disease, producing an increase in the antigenic challenge, which is reflected by higher IgA levels. In addition, it is important to consider that hormonal changes beginning in the pre-adolescent stage also have an influence on the changes in the subgingival microbiota observed as age increases[1].

The main conclusions drawn from this study are: saliva IgG levels are higher in healthy children than in those with gingival disease; as the subject grows, the gingival index increases, although there is less bacterial plaque; high heterogeneity was found among immunoglobulin concentration values; IgA levels are higher at older ages; IgM levels are higher at when the gingival index is higher.

CORRESPONDENCE
Dr. Mario R. Romero
Departamento Sistema Craneofacial,
Facultad de Odontología, Pontificia Universidad Javeriana.
Carrera 7 No. 40-62, edificio 26, piso 3.
Bogotá, Colombia.
E-mail: romero.mario@javeriana.edu.co

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