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ORIGINAL ARTICLE

Levels of salivary IgA in patients with minor recurrent aphthous stomatitis: a matched case–control study

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Abstract
Objectives Recurrent aphthous stomatitis (RAS) is the most common oral mucosal disease. Despite plenty of studies on aetiopathogenesis of RAS, a definite cause is not clear. The objective of this study was to determine the potential changes of salivary IgA and salivary flow rate in patients affected with minor form of RAS.
Materials and methods Levels of salivary IgA in 33 patients with acute RAS (minor form) and 33 matched healthy controls were determined using enzyme-linked immunosorbent assay. Resting salivary flow rates were determined too. Both measurements, levels of salivary IgA and resting salivary flow rate, were performed again for each RAS patient in remission phase.
Results Levels of salivary IgA were significantly increased in acute phase of RAS [median (interquartile range)—124.94 μg/mL (106.22–136.31)] in comparison with the levels in healthy controls [88.92 μg/mL (76.85–93.91; $P<0.001$)] and with the levels in remission phase [102.4 μg/mL (84.6–120.16; $P=0.01$)]. Even in the disease-free period (remission phase), levels of salivary IgA remained significantly higher in comparison with the levels in healthy controls ($P=0.01$). Salivary flow rates, on the other side, were not influenced by the disease state (RAS vs. healthy), phase (acute vs. remission) or even gender (males vs. females).
Conclusion Marked increase of salivary IgA in acute and remission phases of the minor RAS may suggest a potential role for this immunoglobulin in pathogenesis of the disease.
Clinical relevance Salivary IgA may be an important aetiological agent in the pathogenesis of RAS, and hence, its immunomodulation may help prevent the disease.

Keywords Salivary IgA · Minor ulcer · Recurrent aphthous stomatitis · Acute phase · Remission phase

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Introduction

Recurrent aphthous stomatitis (RAS) is the most common oral mucosal disease. It is characterized by recurring painful ulcers of the mouth that are round or ovoid covered by greyish-white fibrin pseudomembranes and surrounded by inflammatory halos [1]. On the basis of ulcer size and number, RAS is classified as minor, major and herpetiform [2]. The cumulative prevalence of RAS varies from 5 to 66 % of the population, depending on the group examined [2, 3]. However; up to 80 % of the affected patients present with the minor form of RAS [2, 4]. Despite its high prevalence rate and plenty of studies, the aetiology of RAS is not entirely clear.

Many studies suggested different aetiological and predisposing factors and aetiopathogenesis mechanisms. These studies were critically reviewed elsewhere [1, 2, 4–6]. Among these mechanisms, cell-mediated [7–9] and humoral immune responses [10, 11] were reported. The latter comprises circulating and secretory immunoglobulins. Secretory IgA plays a pivotal role in mucosal immunity [12, 13]. Salivary IgA alters the motility and adhesiveness properties of microorganisms and thus increases oral mucosal resistance to infections [14]. Apart from studies which investigated the potential association of serum IgA with RAS; few

studies investigated the potential role of salivary IgA [10, 11, 15–17]. These studies recruited small samples, and many of them [10, 11, 15, 16] included heterogeneous patients (affected by minor and major forms of RAS). Furthermore, these studies applied less sensitive detection methods than enzyme-linked immunosorbent assay (ELISA). Hence they reported conflicting results. The objective of the current study was to determine the potential changes in salivary IgA, along with salivary flow rate, in patients affected with the minor form of RAS.

Materials and methods

Subjects

The present matched case–control study was conducted during 2009/2010 academic year in the Oral Medicine Department, Faculty of Dentistry, Damascus University, Syria. It is approved by the faculty board and the scientific committee. Prior to investigation, each participant was informed about the study aims and protocol and an informed consent was obtained accordingly. The sample size was calculated to detect a difference of more than 15 % between the mean values of levels of salivary IgA of the cases and controls. The standard deviation in each group was considered as 20 % of the mean value. The power and the significance values were set at 85 % and <0.05, respectively. The resulted sample size was 30 subjects in each group. The cases group consisted of 33 patients affected with the minor form of RAS which developed within the last 72 h of their attendance. Their clinical presentations and medical histories were reviewed to confirm the criteria proposed by Natah et al. [2] for diagnosing the minor form of RAS. Patients were excluded if they had any systemic disease or used any medication 1 month prior to their attendance. Data regarding size, number and site of the ulcer(s) were recorded through clinical examination, while the age, the potential predisposing factors and the annual recurrence rate were obtained through questioning the patient. For each patient, a healthy subject (matched for age, sex and smoking) was recruited, yielding a control group of 33 healthy subjects. They were either dental students or dental patients attending the faculty for conservative treatment. All control subjects had never been affected by RAS, as they reported. No attempt was made to match the cases and the controls regarding oral health status; however, they had somewhat comparable oral health.

Saliva collection

For each RAS patient, two salivary samples were obtained. The first was within 72 h of ulcer development while the second was after quiescence; i.e. a complete remission of the ulcer(s) (mostly 10–14 days after attack). For each healthy control, one saliva sample was obtained. Salivary collection was between 9 a.m. and 1 p.m. All participants were instructed not to eat, drink (except water) or smoke 1 h before saliva collection. Passive drooling technique was used to collect the total spontaneous (non-stimulated) saliva. After mouth washing by distilled water, the participant instructed to swallow and then tilt his/her head anteriorly and let the saliva to drool over the lower lip through an open-ended glass cylinder into a centrifugable polypropylene tube (Vissal®; France). This lasted for 5 min during which the participant was supervised not to swallow or move his/her tongue or lip. The tubes were sealed and immediately weighted (Electronic Balance BEB43, BOECO; Germany). The salivary samples were then immediately stored at −20 °C freezer (DS-208, DAIREI; Japan) until required. The salivary flow rate was determined in millilitres per minute.

Determination of IgA

Solid-phase enzyme-linked immunosorbent assay (IgA SALIVA; DiaMetra, Italy) was used to determine the levels of salivary IgA. Briefly, upon thawing at room temperature, the saliva sample was centrifuged at 3,000 rpm for 15 min (EBA20, Hettich; Germany). The supernatant was diluted by IgA assay buffer (HEPES buffer 25 mM, pH 7.4; BSA 0.5 g/L) in two subsequent stages (1/20 and 1/50) yielding 1:1,000 diluted sample. After each dilution, the sample was shaked (Orbital Shaker OS-20, BOECO; Germany) for 5 min at room temperature. Two 25-μL samples of the diluted supernatant were added separately into two wells of the microplate where the antibody anti-IgA adsorbed. Similarly, five samples (25 μL each) of the supplied IgA saliva standards were added into five wells. One well was left blank as negative control. The microplate was incubated at 20–28 °C for 1 h and then automatically washed (Autostrip washer ELx50, Bio-TEK instruments, INC.; USA) three times using NaCl 45 g/L and Tween 55 g/L wash solution. For each well, 100 μL of the diluted conjugate (antibody anti-IgA conjugated with horseradish peroxidase) was added with further incubation for 1 h and then washing as indicated. One hundred microliters of TBM substrate (H_2O_2-TMB 0.26 g/L) was then added to each well with further incubation for 15 min in the dark. Finally, 100 μL of stop solution (sulphuric acid 0.15 mol/L) was added to each well and the microplate was shaked gently. Absorbance at 450 nm was read against blank using microplate reader (Universal Microplate Reader, ELx-800, Bio-TEK instruments, Inc., USA). Based on the known concentrations of the standards samples, the concentrations of the salivary IgA were calculated and presented as micrograms per millilitre.

Table 1 Demographic and clinical characteristics of the recurrent aphthous stomatitis (RAS) patients

Variable	RAS patients	
	n	%
Gender		
Male	21	63.6
Female	12	36.4
Smoking		
Yes	3	9.1
No	30	90.9
No. of ulcers		
One	21	63.6
Two	10	30.3
>2	2	6.1
Recurrence rate (per year)		
Up to 4 episodes	7	21.2
5–6 episodes	5	15.2
7–8 episodes	7	21.2
9–10 episodes	3	9.1
11–12 episodes	5	15.2
>12 episodes	6	18.2
Ulcer site[a]		
Buccal mucosa	15	28.3
Lower lip	25	47.2
Flour of the mouth	7	13.2
Tongue	5	9.1
Soft palate	1	1.9

[a] Some patients presented with more than one ulcer

Statistics

The qualitative variables were presented as frequencies and proportions. The quantitative variables were checked for normal distribution using Kolmogorov–Smirnov test. As they were not normally distributed, they were presented as medians and interquartile ranges. The differences, between and within groups, were analysed using nonparametric tests, i.e. Mann–Whitney *U* and Wilcoxon signed rank tests, respectively. A significance value of less than 0.05 was considered. All statistical analyses were performed with SPSS software version 19.

Results

Of the included RAS patients, 21 (63.6 %) were males. Three males (9.1 %) were smokers. The median age of RAS patients was 22 years (range 19–41 years). Ages of male and female patients were comparable. The most affected oral site was the lower lip (25 ulcers, 47.2 %) followed by the buccal mucosa (15 ulcers, 28.3). The patients were distributed evenly regarding the annual recurrence rate. Seven patients (21.2 %) reported up to four episodes/year, while six patients (18.2 %) reported more than one episode a month. Table 1 summarizes the demographic and clinical characteristics of the patients.

Levels of salivary IgA in RAS patients in acute phase were increased significantly in comparison with the values in healthy controls. Although these levels were decreased significantly in remission phase, they remained significantly higher than that of healthy controls (Table 2).

When considered separately, male and female RAS patients revealed significantly higher levels of salivary IgA in acute phase when compared with the values of healthy control counterparts and their values in remission phase. Even in the remission phase, levels of salivary IgA in the affected males and females remained higher than the values in healthy control males and females respectively; the differences were statistically insignificant however (Table 3).

Levels of salivary IgA in RAS patients were not found to be gender related. Contrarily, levels of salivary IgA in healthy males were significantly higher in comparison with the values of healthy females (Table 4). On the other hand, salivary flow rate was not found to be influenced by the health state (healthy vs. RAS), RAS phase (acute vs. remission) or gender (males vs. females; Tables 2 and 3).

In acute phase of RAS, levels of salivary IgA were inversely but insignificantly correlated with the number of ulcers and with recurrence rate/year (Pearson correlation=−0.134 and −0.03, P=0.458 and 0.869, respectively). Contrarily, in remission phase, these parameters were proportionally but insignificantly correlated (Pearson correlation=0.008 and 0.236, P=0.963 and 0.078, respectively).

Table 2 Salivary IgA concentrations and salivary flow rates in recurrent aphthous stomatitis (RAS) patients and healthy controls

Group/subgroup	No. of subjects	Salivary IgA (μg/mL), median (IQR)	Salivary flow rate (mL/min), median (IQR)
Healthy controls	33	88.92 (76.85–93.91)	0.54 (0.41–0.80)
Acute RAS	33	124.94 (106.22–136.31)*, **	0.48 (0.33–0.82)
Remission RAS	33	102.4 (84.6–120.16)***	0.57 (0.47–0.60)

IQR interquartile range

P<0.001 vs. healthy controls; **P=0.01 vs. remission; ***P=0.01 vs. healthy controls

Table 3 Salivary IgA concentrations and salivary flow rates in recurrent aphthous stomatitis (RAS) and healthy control males and females separately

Gender	Subgroups	No. of subjects	Salivary IgA (μg/mL), median (IQR)	Salivary flow rate (mL/min), median (IQR)
Males	Healthy controls	21	92.34 (86.06–95.12)	0.56 (0.42–0.82)
	Acute RAS	21	125.92 (106.22–132.53)*, **	0.55 (0.35–0.89)
	Remission RAS	21	105.04 (85.6–119.58)	0.57 (0.39–0.81)
Females	Healthy controls	12	70.9 (50.82–92)	0.49 (0.28–0.75)
	Acute RAS	12	121 (102.24–128.93)*, ***	0.42 (0.30–0.65)
	Remission RAS	12	98.4 (67.95–129.6)	0.57 (0.48–0.59)

IQR interquartile range

*$P<0.000$ vs. healthy controls; **$P=0.001$ vs. remission; ***$P=0.021$ vs. remission

Discussion

Many issues should be considered while critically discussing studies on a potential association of an aetiological factor with RAS. Among these are: the diagnostic criteria which applied, the clinical forms of RAS and sensitivity of the detection methods. Experts have suggested reserving the term RAS for recurrent ulcers confined to the mouth and seen in the absence of systemic disease [1, 4, 6]. To avoid enrolling patients with intraoral aphthous-like ulcer(s) associated with several systemic diseases or conditions [1], the medical and family histories of RAS patients in the present study were reviewed in details along with application of the diagnostic criteria proposed by Natah et al. [2]. Moreover, and unlike the previous studies [10, 11, 16], including patients with the minor form of RAS only makes the study sample more homogenous, and reduces the potential biases. Furthermore, the method used for salivary IgA detection, ELISA, is more sensitive than nephelometry and radial immunodiffusion methods used by Martinez Kde et al. [11], and Sistig et al. [10], Saluja et al. [17] and Brozovic et al. [16], respectively.

Irrespective of the gender, levels of salivary IgA are significantly increased in acute phase of RAS in comparison with the values in healthy controls and with the values in remission phase (Tables 2 and 3). This is in agreement with many studies [10, 11, 16, 17], but it contradicts others [15, 18, 19]. This could reflect an important role for salivary IgA in pathogenesis of RAS. Four mechanisms could be proposed: firstly, the presence of a threshold level of salivary IgA above which direct destruction of keratinocytes may commence; secondly, indirect destruction through IgA immune complex-mediated mechanism, although this type of uncontrolled immune response occurs in the circulation [20]; thirdly, these IgA antibodies are autoantibodies against oral mucosal cell antigens, or they may be produced against a foreign antigen that is immunologically cross-reactive with a component of oral self tissues [21, 22] and fourthly, these elevated levels might be a normal local immune response (innate or adaptive) to neutralize the aetiological factor(s) that has/have already caused epithelial destruction. The last mechanism is more appropriate in context of the normal functioning immune system [20]. The principle mechanism of protective immunity against antigens in mucosal lumen is antibody-mediated neutralization which is dominated by IgA [12, 14].

Interestingly, levels of salivary IgA in remission phase remained significantly higher in comparison with the levels in healthy controls (Table 2). Previous studies did not report such a result [15, 18, 19]. However, Sistig et al. [10] and Saluja et al. [17] took this one step further as they found increased levels of IgA-2 subclass in remission phase, in contrast to IgA-1 subclass which returned to levels comparable to the levels in healthy controls. They related their findings to the chronic antigenic stimulation because of the high concentration of the specific microorganism that could be involved in the pathogenesis of RAS [10, 17].

In line with that, the immune response during an active disease process might end up with collateral tissue damage; this might be the cause of acute RAS. Such a response leads to development of a specific (adaptive) immune protection in

Table 4 Salivary IgA concentrations in recurrent aphthous stomatitis (RAS) and healthy control by gender

Group/subgroup	Salivary IgA (μg/mL), median (IQR)		*P* value
	Males ($n=21$ each)	Females ($n=12$ each)	
Healthy controls	92.34 (86.06–95.12)	70.9 (50.82–92)	0.024
Acute RAS	125.92 (106.22–132.53)	121 (102.24–128.93)	NS
Remission RAS	105.04 (85.6–119.58)	98.4 (67.95–129.6)	NS

NS not significant, *IQR* interquartile range

remission phase (healthy state) against future attacks [20]. It is not the case in RAS however; the increased levels of salivary IgA in remission phase appears to be a determinant for the recurrence. Previous studies revealed that low levels, but not high levels, of salivary IgA were associated with development of dental caries [23], gingivitis [24] and periodontitis [25, 26]. Therefore, with elevated levels of local secretory immunity, RAS patients should be less vulnerable to recurring attacks. This holds true unless multifactorial aetiopathogenesis, defective quality of the secretory immunity and/or antigenic variation of the causative microbe are implicated. On the other hand, some investigators classified RAS as an autoimmune disease mediated by salivary IgA autoantibody [21, 27].

Healthy males revealed significantly higher levels of salivary IgA than healthy females did (Table 4). In addition to the role of the gender difference in body mass index, levels of many biological parameters are gender related; e.g. haemoglobin. Less number of females included in the present study could not be overlooked, however. Regarding RAS patients, levels of salivary IgA in males, in acute or remission phase, were higher than levels in females; the differences were statistically insignificant.

Measuring resting salivary flow is preferred over the stimulated one. Salivary flow at rest reflects the function of saliva most of the time. Moreover, it is more reliable to represent the physiology of oral secretions without the interference of external factors. Moreover, Hagewald et al. [26] found that with stimulation, salivary parameters were diluted and measured lower concentrations than they were in resting. Overall, quantity and quality of saliva together determine its biological functions [28]. In our study, neither the health status (affected vs. healthy), RAS phase (acute vs. remission) nor the gender affected the levels of salivary flow rate. This is in agreement with previous studies [10, 11, 15, 29].

In conclusion, the quality, but not the quantity, of saliva is altered in RAS patients. Marked increase of salivary IgA in acute and remission phases of RAS may suggest an important role for this immunoglobulin in pathogenesis of the disease. Whatever the involved mechanisms are, protective or, indirectly or directly, destructive, they are hypotheses for future studies.

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Conflict of interest The authors declare that they have no conflict of interest.

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