ORIGINAL ARTICLE

Detection of salivary interleukin-2 in recurrent aphthous stomatitis

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Received: 04-07-2014
Accepted: 30-12-2014

ABSTRACT

Objective: The present study was undertaken to estimate and compare salivary interleukin-2 (IL-2) levels in patients with recurrent aphthous stomatitis, among healthy controls and their variation with age and sex. **Study Design:** Saliva was collected from 60 patients within the age range of 16-60 years which included 30 patients (17 Females and 13 Males) with recurrent aphthous stomatitis and healthy control group consisted of 30 participants (18 Females and 12 Males). IL-2 estimation was done in both the groups using enzyme linked immunosorbent assay (ELISA). Statistical analysis of the data was done using Independent '*t*' test. **Results:** The results showed increased salivary IL-2 levels in patients with recurrent aphthous stomatitis compared to the healthy controls. The IL-2 levels were also increased in patients with the age group of 16-30 years compared to other age groups. Similar increase of IL-2 was also seen in female patients. **Conclusion:** Age related and sex related alterations of IL-2 in recurrent aphthous stomatitis patients were observed.

Key words: Enzyme linked immunosorbent assay, interleukin-2, recurrent aphthous stomatitis

INTRODUCTION

Recurrent aphthous stomatitiis (RAS), an unfortunate common disease is characterized by the development of painful, recurring solitary or multiple ulcerations of the oral mucosa.[1] The term "aphthous" originated with Hippocrates as far back as 460-370 BC in reference to disorders of the mouth.[2] Cooke classified the lesions of RAS into three groups and Lehner characterized them as minor aphthous ulcers, major aphthous ulcers and herpetiform ulcers.[3]

Molecularly defined cytokines are called interleukins, implying that they mediate communications between leukocytes.[4] Interleukins are biologically active glycoproteins derived primarily from activated lymphocytes and macrophages.[5] interleukin-2(IL-2) is a 15kDa glycoprotein originally known as T cell growth factor (TCGF). It is secreted mainly by activated T helper cells. It plays a critical role in regulating both cellular and humoral chronic inflammatory responses. Binding of IL-2 to the IL-2 receptor on T lymphocytes leads to cell proliferation and increased lymphokine secretion.[6]

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Quick Response Code:

Website: www.jomfp.in

DOI: 10.4103/0973-029X.151313

The pathogenesis of RAS involves cell-mediated responses, involving T cells and tumor necrosis factor (TNF)-α production by these and other leucocytes. TNF-α induces inflammation by its effect on endothelial cell adhesion and neutrophil chemotaxis.[7] Cytokines of type-1 includes interleukins such as IL-2, IL-12 and interferons such as interferon (IFN)-γ and TNF-α which are pro-inflammatory cytokines that induce cell-mediated immunity.[8] During inflammation, IL-2 stimulates secretion of pro-inflammatory cytokines such as IL-1, TNF-α and TNF-β. An increased local expression of Th1 genes and systemic production of cytokines, such as IL-2, TNF-a and IL-6 were observed in RAS patients.[9] This study has been undertaken to detect the levels of IL-2 in a inflammatory condition such as RAS.

MATERIALS AND METHODS

Thirty patients with RAS were included in the study. Thirty age-matched healthy volunteers were selected as a control group. Selection of cases was done based on the patients' history and a thorough clinical examination. Inclusion criteria included patients in the age group of 16-60 years with the

history and/or clinical presentation of RAS (active lesion in ulcerative phase). Exclusion criteria excluded subjects with other inflammatory oral lesions and systemic diseases. Informed consent of the patient was obtained and medical, dental and social histories were collected.

The RAS group consisted of 13 males and 17 females. The patients age ranged from 16-60 years of age. They were divided into three groups as 16-30 years (Group-A), 31-45 years (Group-B) and 46-60 years (Group-C). The control group consisted of 12 males and 18 females.

All the patients presented with active lesions of RAS in ulcerative phase. The subjects were asked to rinse their mouth. Saliva was collected from each participant in sitting position. Each person was asked to expectorate 10 ml of whole unstimulated saliva in a sterile tube. The saliva was stored at 80°C and analysis was made. For determination of salivary IL-2, enzyme linked immunosorbent assay (ELISA) was performed and the results were expressed in pg/mL. Statistical analysis of the data was done using independent *t* test.

ELISA procedure

The microtiter plate provided in this ELISA kit had been pre-coated with an antibody specific to IL-2. Standards or samples were then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for IL-2. Next, Avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated. Then a 3,3',5,5'-Tetramethyl benzidine (TMB) substrate solution was added to each well. Wells that contain IL-2, biotin conjugated antibody and enzyme-conjugated Avidin exhibited a change in color. The enzyme-substrate reaction was terminated by the addition of a stop solution and the color change was measured by ELISA microplate reader at a wavelength of 450 nm.

The substance to be determined in our study was IL-2 and hence known concentrations of IL-2 solutions were prepared for evaluation. IL-2 in varying concentrations were 4000 pg/ml, 2000 pg/ml, 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml and 62.5 pg/ml.

Statistical analysis

The data were analysed using the version 14.00 of statistical package of social sciences (SPSS) software. The value of biochemical parameters were expressed as mean + standard deviation (SD). The levels of significance were determined by employing Independent't' test. Statistical significance was defined at $P < 0.001$.

RESULTS

There was a significant increase in IL-2 in RAS patients compared to controls [Table 1 and Figure 1].

Average values of IL-2 concentration were significantly higher in the group of patients with RAS: Mean 30.23 + 3.46 pg/mL compared to the controls: Mean 11.91 + 1.7 pg/mL ($P < 0.001$).

Age distribution in RAS patients Group A (16-30 years) were affected more of about 63.3% compared to Group B (31-45 years) and Group C (46-60 years) which is of 20% and 16.7% respectively [Table 2 and Figure 2].

Gender distribution in RAS group shows females were more affected compared to the males [Table 3and Figure 3].

DISCUSSION

RAS (aphthae; canker sore) is a common condition which is characterized by multiple recurrent small, round or ovoid ulcers with circumscribed margins, erythematous haloes and yellow or gray floors appearing first in childhood or adolescence.[10] It is a common disorder affecting 5% to 66% of examined adult patient groups.[11]

In RAS, mostly a cell mediated immune response mechanism is involved and results in generation of T-cells and TNF-α by other leucocytes (macrophages and mast cells). The TNF-α cytokine, a major inflammatory mediator, induces initiation of the inflammatory process by its effect on endothelial cell adhesion and has a chemotactic effect on neutrophils. Studies have shown that RAS can be prevented by treatment with substances that prevent the synthesis of endogenous TNF-α such as thalidomide and pentoxifylline.[12]

Table 1: Comparison of interleukin-2 in saliva pg/mL in healthy controls and recurrent aphthous stomatitis patients

Group	*n*	Mean	Standard deviation	*t*-value	*P* value
Healthy controls	30	11.91	1.70	26.06	0.000
Recurrent aphthous stomatitis patients	30	30.23	3.46		

S: Significant at $P<0.001$

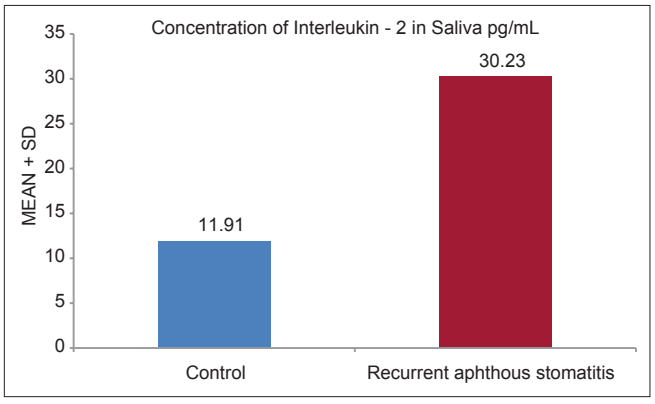

Figure 1: Comparison of interleukin-2 levels in patients with recurrent aphthous stomatitis and healthy controls. Data shows the mean expressed in pg per milliliter of saliva

As TNF-α may be implicated in pathogenesis of RAS, it might be expected that anti-TNF-α agents like pentoxifylline and thalidomide could be beneficial especially in case of patients with frequent or severe RAS who require systemic immunosuppressive therapy. Pentoxifylline (400mg three times daily) significantly reduced the number of RAS for up to 9 months after 1 month of therapy (Pizarro *et al*., 1995, 1996; Wahba-Yahav 1995). Thalidomide acts via its action upon Th_1/Th_2 immune response and/or its angiogenic properties. (Porter and Jorge, 2002). But due to its adverse effects of teratogenicity it is avoided during pregnancy.[12] A markedly increased plasma level of IL-2 was recorded in the active stage of RAS. Natural killer (NK) cells activated by IL-2 may play a role in the process of this disease. An increased activity of these cells was noted in active lesions, diminishing during periods of remission.[12]

The host defences against infection vary in different oral micro-environments or domains represented by the oral mucosa, salivary glands, saliva and the gingival crevice. This has laid the focus on detection of IL-2 in saliva and its significance to assess the possible role in etiology and progression of the disease**.** In this study, saliva was used for the estimation of IL-2 level as it is readily available, easier to collect and the procedure is non-invasive.

Table 2: Age distribution in recurrent aphthous stomatitis group

Age groups	Recurrent aphthous stomatitis	
	Frequency (*n*)	Percentage
A (16-30 years)	19	63.3
B (31-45 years)	6	20.0
C (46-60 years)	5	16.7

Table 3: Gender distribution in recurrent aphthous stomatitis group

Group	Recurrent aphthous stomatitis	
	Frequency (*n*)	Percentage
Males	13	43.3
Females	17	56.7

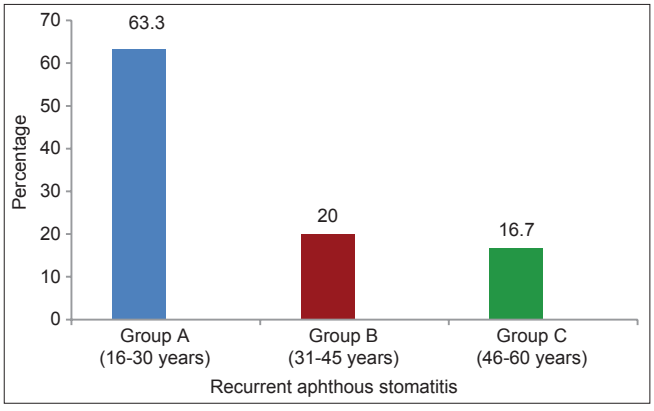

Figure 2: Age distribution in recurrent aphthous stomatitis study groups

Our findings can be correlated with the research conducted by Stephen R. Porter *et al*.,[11] who stated that the recurrent aphthous ulceration usually commences in the second decade and there may be female predominance in some adult and child patient groups. Reports exist regarding association of hormonal changes in women and RAS. Studies state association of oral ulceration with onset of menstruation or in the luteal phase of the menstrual cycle.[13] This could be a reason for female predominance in the present study.

The values of salivary IL-2 levels among the RAS group correlated with the values of the study group (burning mouth syndrome) in the study conducted by Daria Simcic *et al*.,[14] The author showed that an increase in IL-2 concentration offers a possible explanation for the causative mechanism of burning mouth syndrome through immunological reactions during inflammation where the concentrations of the cytokine IL-2 were increased. This implies that RAS can be due to changes caused by inflammatory mediators in the oral mucosa. Patients with RAS may be liable to uncontrolled or excessive release of locally active inflammatory mediators, like IL-2, IFN-γ[12] perhaps in response to the etiological factors.

RAS is a common oral mucosal disease with altered humoral and cellular immunities.[15] Specific antigens are capable of activating clonal population of resting T cells when presented by macrophages displaying the appropriate major histocompatibility antigens. This activation is initiated by antigen binding to specific receptors present on the surface of resting T cells. Antigen binding in the presence of macrophage-derived interleukin-1 (IL-1), then triggers *de novo* synthesis and secretion of IL-2 or T-cell growth factor and the transient expression of high and low affinity IL-2 receptors. The subsequent interaction of IL-2 with its high affinity membrane receptors stimulates cellular proliferation

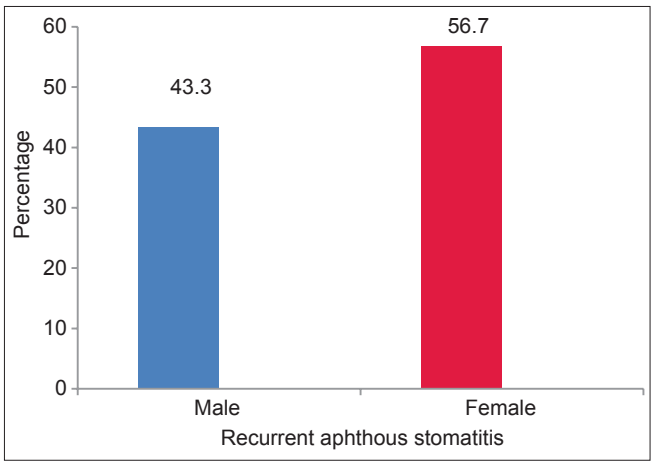

Figure 3: Gender distribution in recurrent aphthous stomatitis study groups

resulting in the expansion of T-cell population,[16,17] potentiates B-cell growth, enhances NK-cell and monocyte activation.[18] TNF-α is a pro-inflammatory cytokine[19] secreted by activated monocytes that causes activation of cytotoxic T-cells and neutrophils; necrosis of the epithelium and finally the development of an aphthous lesion.[20] This explains the reason why there is an increased IL-2 levels in patients with RAS in comparison with the healthy control group in the present study.

Increased IL-2 expression in RAS offers a possible explanation for causative mechanism and its reactions during inflammation. However, whether RAS is mainly due to the release of IL-2 or a result of combined effect of other cytokines is still unknown. It is a well known fact that RAS is a multifactorial disorder, the most realistic approach is to analyze other substances which are immunologically active which could serve as a useful target for assessing the causes and the quality of therapy. Since some drugs like pentoxifylline are implicated in the treatment of RAS and in preventing the endogenous synthesis of TNF-α (a cytokine), it needs to be emphasized that other cytokines have to be identified in preventing the disease process.

CONCLUSION

The present study shows an increase in IL-2 in RAS patients compared to control group. Age and gender wise distribution in RAS shows patients in Group A (16-30 years) were affected more than other groups with female predilection. Therefore an age related and sex related alterations of IL-2 were observed in RAS patients.

The important role of this study was to educate the patient regarding the nature of this condition and especially the fact that RAS can be controlled by treatment. Early diagnosis and identification of precipitating factor which might have caused the lesion could prevent the recurrences of RAS. As IL-2 has a synergistic effect on activation of other cytokines, treatment of RAS using drugs specific to block IL-2 could be a new approach to prevent recurrences.

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How to cite this article: Kalpana R, Thubashini M, Sundharam BS. Detection of salivary interleukin-2 in recurrent aphthous stomatitis. J Oral Maxillofac Pathol 2014;18:361-4.

Source of Support: Nil. **Conflict of Interest:** None declared.