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— SHORT COMMUNICATION —

Evaluation of cellular immunity in recurrent aphthous stomatitis (RAS)

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The purpose of the current study was to evaluate fluctuations of T lymphocyte subsets (CD3+ CD4+ and CD3+CD8+), as well as B lymphocyte (CD3–, CD19+) and NK cells (CD3-CD16/56+) and NKT cell (CD3+CD16/56) subsets in the peripheral blood of patients with Recurrent Aphthous Stomatitis (RAS). In addition, the ratio of CD3+CD4+/CD3+CD8+ (TH/TC) cells was calculated. Thirty cases with RAS (23 females and 7 males), mean age 48.5 ± 14.8 years, were examined both during active and non-active phase of the disease. Thirty healthy individuals, age- and sex-matched were used as controls. Flow cytometry was used for the detection and counting of all six types of lymphocytes. The findings showed a statistically significant decrease of the CD3+CD4+/CD3+CD8+ (TH/TC) ratio ($p < 0.05$). No differences were observed in CD3+, CD3+CD4+, CD3-CD16/56+ (NK), CD3+CD16/56+ (NKT), CD3+CD8+ and CD3– CD19+ (B) subsets, when compared to control group or during the elation phase of the disease. Our findings suggest an association between a cellular type immunoregulation disorder and RAS immunopathogenesis. Variation in lymphocyte subsets between RAS and controls in addition to variations recorded between the active and non-active phase of the disease in the same group, delineate the distinctive immunological profile of RAS.

Key words: cellular immunity, RAS, T lymphocyte subsets, immunopathogenesis, immunoregulation, oral ulceration.

INTRODUCTION

Recurrent aphthous stomatitis (RAS) is one of the most frequent oral mucosal diseases (Lehner, 1978; Kleinman *et al*., 1991; Zunt, 2003; Scully, 2006).

The most common form is minor RAS which accounts for more than 80% of RAS. The former comprises of shallow ulcers covered by a white-grey pseudomembrane and surrounded by a red inflammatory halo (Lehner, 1977; Albanidou-Farmaki, 2000; Porter & Scully, 2005).

The precise etiology of the disease remains elusive despite the intensive research efforts (Carrozzo *et al*., 1995). A great emphasis has been given to the immunological changes mainly during the course of the disease (Landesberg *et al*., 1990; Scully & Porter, 2008).

Immunological changes have been observed at the systemic and local level and were related to cellular and humoral immunity or to the autoimmune nature of the disease (Kayavis *et al*., 1987a, b; Albani-

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dou-Farmaki *et al*., 1991; Petersen & Hornsleth, 1993; Regezi *et al*., 1993; Kayavis *et al*., 1995; Vicente *et al*., 1996; Buño *et al*., 1998; Koridze *et al*., 2007)

The issue of participation of cell mediated immunity at the systemic level during the active and non-active stage of the disease is conflicting in the literature. For this reason we undertook this study to investigate the presence of T lymphocyte subsets, B lymphocytes, natural killer cells and NKT cell (CD3+, CD3+CD4+, CD3+CD8+, CD3-CD19+, CD3-CD 16/56+ and CD3+CD16/56+) and the ratio of CD3+ CD4+/CD3+CD8+ (TH/TC) cells in the peripheral blood of patients with RAS.

MATERIALS AND METHODS

The first group consisted of 30 adults (23 females and 7 males) with RAS, who were selected from the clinic of Oral Medicine. Their age ranged from 22 to 63 years (48.5 ± 14.8 years). They fulfilled the diagnostic criteria for RAS and underwent a complete clinical and laboratory examination. Individuals suffering from any kind of oral disease with erosions or ulcerations and diseases that could affect their immune response, namely infections, immunological diseases or immunosupression were excluded from the study. The patients group were evaluated during the active phase (group a) and the non-active phase of the disease (group b). Thirty healthy individuals (16 females and 14 males, 21-60 years old) were used as control group (group c). All the participants gave informed consent and the whole study was performed according to Helsinki II Declaration.

Peripheral blood lymphocyte subsets (PBLS) were measured by flow cytometry, using a two color cytometer (FACSCAN, Becton Dickinson) and a direct immunofluorescence double staining method. Each lymphocyte subset was determined by its individual immunophenotype, after immunostaining with the appropriate monoclonal antibodies (moAbs, Bectoy Dickinson), conjugated either to fluorescein isothiocyanate (FITC) or to phycoerythrin (PE), as follows: T cells (CD3+ cells) after staining with anti-CD3-FITC moAb, B cells (CD19+ cells) after staining with anti-CD19-PE moAb, NK cells (CD16/56+cells) after staining with anti-CD16/56-PE moAb, T-helper cells (CD3+CD4+ cells) after staining with anti-CD3-FITC and anti-CD4-PE moAbs, T-cytotoxic cells (CD3+ CD8+ cells) after staining with anti-CD3-FITC and anti-CD8-PE moAbs, and non-MHC restricted T cells or NKT cells (CD3+CD16/56+ cells) after staining with anti-CD3-FITC and anti-CD16/56-PE moAbs. After the determination of the percentage of each subset to the total lymphocyte population, the ratio between T-helper and T-cytotoxic cells (TH/TC or CD3+CD4+/CD3+CD8+) was also calculated (Shapiro, 2003).

Statistical evaluation of our findings was performed using the unpaired Student's t-test.

RESULTS

The percentage of the CD3+ lymphocytes during the active phase in the patients group was 54.6 ± 9.6%, while in the non-active phase was 57.8 ± 9.9%. In the control group the recorded value was 66.55 ± 3.6% (Table 1).

The percentage of the CD3+CD4+ cells during the active phase was 30.2 ± 7.7%, while in the non-active phase 33.72 ± 7.28%. In the control group the percentage of the CD3+CD4+ cells was 35.05 ± 2.54% (Table 1).

The percentage of the CD3+CD8+ and CD3-CD19+ lymphocytes during the active period was 25.17 ± 5.34% and 18.63 ± 8.88, respectively; for the non-active period, the values for the same variables were 23.38 ± 5.34% and 17.6 ± 8.86%, respectively. CD3+CD8+ and CD3-CD19+ subsets in the control group were found to be 20.4 ± 3.2% and 16.83 ± 2.46%, respectively (Table 1).

TABLE 1. The percentage of T-lymphocytes of patients in i) active phase of RAS and ii) non-active phase of RAS and healthy individuals (control). Values in bold are significantly different ($p < 0.05$)

	CD3+ (T-cells) %	CD3+CD4+ (T-helper) %	CD3+CD8+ (T-cytotoxic) %	CD3-CD19+ (B cells) %	CD3-CD16/56+ NK %	CD3+CD16/56+ NKT %	CD3+CD4+/ CD3+CD8+ TH/TC
Active phase	54.60±9.60	30.20±7.70	25.17±5.34	18.63±8.88	12.00±5.80	9.00±3.90	**1.300±0.50**
Non-active phase	57.80±9.90	33.72±7.28	23.38±5.34	17.60±8.86	15.57±8.89	9.60±4.10	**1.550±0.55**
Controls	66.55±3.60	35.05±2.54	20.40±3.20	16.83±2.46	17.90±3.02	10.70±4.90	1.595±1.25

NK cells (CD3-CD16/56+ cells) and NKT cells (CD3+CD16/56+ cells) during the active period were 12.0 ± 5.8% and 9.0 ± 3.9%, respectively; the mean percentages of NK and NKT cells during the non-active phase were 15.57 ± 8.89% and 9.6 ± 4.1%. In the control group, the same subsets were found to be 17.9 ± 3.02% and 10.7 ± 4.9%, respectively.

It sould be noted that Student's t-test showed no statistical differences among the above mentioned groups and variables ($p > 0.05$, Table 1).

CD3+CD4+/CD3+CD8+ (TH/TC) ratio differ significantly ($p < 0.05$) between active (1.3 ± 0.5%) and non-active phase (1.55 ± 0.55%), while in the control group was 1.595 ± 1.25.

DISCUSSION

The pathogenesis and etiology of RAS is associated with disorders of the immunity mechanisms, which are already known to be implicated in the disease. This can be concluded from the large quantity of existing research data, although the significance of predisposing or other factors should not be ignored (Scully & Porter, 2008).

Based on the research findings to date, the contribution of certain immunological disorders to the pathogenesis of the disease is well documented, without taking into account the possible differences between the active phase of the disease and/or the findings of the depression phase in the same patients (Koridze *et al*., 2007; Scully & Porter, 2008). Moreover, the role of variances of certain cell subpopulations in correlation with the clinical expression of the disease (size of ulceration, depression period in association with elation periods, etc.) has not been clarified. An association between the activity as well as the intensity of the lesions and the numerous disorders (percentage ratio) of CD3+CD4+ and CD3+CD8+ cells, both in minor and major oral ulceration has been detected (Bachtiar *et al*., 1998).

In another study, during the elation phase of the disease, there was a significant increase of the percentage ratio of CD3+ and CD4+ as well as that of CD4+ IL-2R+ and CD4+IL-2R+ cells compared to those of healthy individuals. The elations of the disease were accompanied by a significant increase in CD3+CD4+ as well as in the CD3+ CD4+/CD3+ CD8+ ratio, and by a significant increase in CD3+ CD8+ cells of the group of patients with parallel increase in IL-2 serum levels (Sun *et al*., 2000).

In the group with RAS, a non statistically significant decrease in the percentage of Tαβ lymphocytes should be considered to have a special dynamic and that they may represent an important controlling factor in the immunopathogenesis of aphthous ulceration.

According to our results, an immunoregulation disorder has been confirmed, particularly in the cellular part. A statistically significant decrease in the TH/TC ratio occurred in the active phase of the disease compared to depression phase ($p < 0.05$). This decrease appears to result from both the decrease in the numerator (absolute number and percentage ratio of CD3+CD4+ cells).

Comparing the lymphocyte numbers between men and women we did not find any statistical significant differences (data not shown). It should be noted that the role of gender and of specific hormone conditions or endocrinopathies in patients, has not been studied to date. Furthermore, there was a non statistically significant decrease in the ratio of CD3+ and CD3+ CD4+ lymphocytes during the elation phase of the disease; this finding suggests a movement of the above cell subpopulations towards the peripheral zone, as a result of potential virus infection or autoimmunity procedure.

Based on the above, the necessity of combined studies using the latest molecular immunological techniques both *in vivo* and *in vitro*, in strictly selected groups of RAS, is obvious in order to clearly define the pathogenesis of the disease.

In conclusion, the findings of our study revealed a statistically significant decrease of the TH/TC ratio, as well as a non statistically significant decrease during the elation phase of the disease, of CD3+, CD3+ CD4+, CD3-CD16/56+(NK), and CD3+CD16/56+ (NKT), cells and increase of CD3+CD8+ and CD3-CD19+ (B) lymphocytes, which may suggest a characteristic profile of the immunoregulation disorder.

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