Detection, Enumeration and Characterization of T Helper Cells Secreting Type 1 and Type 2 Cytokines in Patients with Recurrent Aphthous Stomatitis

ELENI ALBANIDOU-FARMAKI, ANASTASIOS K. MARKOPOULOS, FILANTHI KALOGERAKOU and DEMETRIOS Z. ANTONIADES

Department of Oral Medicine & Maxillofacial Pathology, School of Dentistry, Aristotle University of Thessaloniki, Thessaloniki, Greece

ALBANIDOU-FARMAKI, E., MARKOPOULOS, A.K., KALOGERAKOU, F. and ANTONIADES, D.Z. Detection, Enumeration and Characterization of T Helper Cells Secreting Type 1 and Type 2 Cytokines in Patients with Recurrent Aphthous Stomatitis. Tohoku J. Exp. Med., 2007, 212 (2), 101-105 —— One of the factors involved in the pathogenesis of recurrent aphthous stomatitis (RAS) is a cell-mediated immune response in which several cytokines seem to play a major role. The aim of this study was to detect, enumerate and characterize T helper cells which are secreting type 1 cytokines (interleukin [IL]-2, IL-12, interferon [IFN]-γ, and tumor necrosis factor [TNF]-α) and type 2 cytokines (IL-4, IL-5, IL-6, and IL-10) in the peripheral blood of patients with RAS. Thirty-two patients in the active phase of RAS (14 men and 18 women) and 40 healthy individuals participated in the study. T helper (T) cells were detected and characterized using Elispot assay. T cells secreting IL-2, IL-12 or IFN-γ were increased in patients with RAS compared with the controls (p < 0.05, p < 0.001 and p < 0.001, respectively). T cells secreting TNF-α in RAS patients and controls were not statistically different (p > 0.05). T cells secreting IL-10 were increased in patients with RAS compared with the controls (p < 0.05). T cells secreting IL-4 were decreased in patients with RAS compared with the controls (p < 0.001). No statistical difference was observed between T cells secreting IL-5 or IL-6 in patients with RAS and controls. Our findings suggest that the increased numbers of T cells secreting type 1 cytokines may influence the immune response against RAS. Whether this action is of etiological importance or epigenetic phenomenon is a question that needs to be answered. ——

imunopathogenesis of recurrent aphthous stomatitis; Type 1 and 2 T helper cells; peripheral blood mononuclear cells

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Recurrent aphthous stomatitis (RAS) is one of the most common oral ulcerative diseases. Although this condition has been described by Hippocrates in 360 B.C. there is still no proven etiology for RAS. Precipitating factors include trauma, stress, chemical irritants, hormones, certain foods and heredity. Emotional and physical stress has been implicated in the pathogenesis of RAS. Deficiencies in iron, folate, and vitamin B-12 have been noted in relation with this disease.
(Albanidou-Farmaki et al. 1988; Scully et al. 2003).

The pathogenesis of RAS also involves an immune-complex mediated vasculitis. Autoantibodies against the oral mucosal membranes have been suggested due to the histopathological features. A lymphocytic cell infiltrate in the epithelium in the initial stages of the disease is followed by a localized papular swelling due to keratinocyte vacuolation surrounded by a reactive erythematous halo representing vasculitis. The painful papule then ulcerates and a fibrinous membrane covers the ulcer, which is infiltrated mainly by neutrophils, lymphocytes and plasma cells. Finally, there is healing with epithelial regeneration (Natah et al. 2000).

Cytokines are the major mediators of immune response against microorganisms, tumors and self-antigens. They are a major focus in the study of the pathogenesis of immune responses. Cytokines are produced by a wide variety of cell types including type 1 and type 2 T helper cells. Generally, two types of cytokines are secreted from T helper cells; Type 1 (interleukin [IL]-2, IL-12, interferon [IFN]-γ, and tumor necrosis factor [TNF]-α) which are considered pro-inflammatory cytokines that induce cell-mediated immunity and Type 2 (IL-4, IL-5, IL-6, IL-10, and IL-13) which are also anti-inflammatory cytokines that promote humoral immunity and tolerance. The cytokine profile is crucial in the determination of immune activation and tolerance (Mackay and Rosen 1992).

The aim of this study was to detect, enumerate and characterize T helper cells which are secreting type 1 and 2 cytokines (IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IFN-γ, and TNF-α) in the peripheral blood of patients with RAS.

### MATERIALS AND METHODS

#### Patients and samples

Thirty two patients with minor RAS, in the active phase of the disease (14 men and 18 women, mean age 35.4 ± 12.03 years, range 19-56 years) who did not have any other systemic or inflammatory diseases and 40 healthy individuals, matched for age and sex (Table 1) were selected for this study. All patients with RAS and controls were recruited from the clinic of Oral Medicine. All patients were non-smokers and manifested minor aphthous lesions every month during the last year prior to the study. Patients did not receive any treatment and their lesions were healed spontaneously. The present study was conducted in compliance with the relevant regulations and especially according to Helsinki declaration.

#### Isolation and culture of PBMC

Peripheral blood was taken from all patients and controls. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood with phosphate buffer saline (PBS, 1:1) and after centrifugation on Ficoll-Hypaque separating solution (Biochrom AG, Berlin, Germany). After three washings with PBS, the viability of PBMC determined by trypan blue exclusion was 97%.

#### Enzyme-linked immunospot (ELISPOT) assay

The Elispot plates are 96-well Microtiter plates with nitrocellulose membrane filters in each well.

**Step 1:** The plates were coated aseptically with anti-IFN-γ, anti-IL-2, anti-IL-4, anti-IL-5, anti-IL-6, anti-IL-10, anti-IL-12 or anti-TNFα antibody diluted in PBS pH 7.4 at a concentration of 5 μg/ml (100 μl per well) and were incubated overnight at room temperature.

<table>
<thead>
<tr>
<th>TABLE 1. Gender and ages of patients and controls.</th>
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<tbody>
<tr>
<td># of patients</td>
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<tr>
<td># of patients</td>
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<td># of controls</td>
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Step 2: The plates were washed three times with PBS pH 7.4 and they were blocked with 200 μl of RPMI 1,640 + 10% fetal calf serum (FCS).

Step 3: Suspensions of viable PBMCs, at a range of concentrations of between 1 × 10^5 – 5 × 10^4 per well were prepared in another wells overnight at 37°C in 5% CO₂ incubator and 90% humidity.

Step 4: PBMCs suspensions were added to the wells blocked with RPMI and were incubated in 5% CO₂ at 37°C for 1 hr. During this period antigen-specific responding cells secrete the cytokine.

Step 5: The plates were washed with PBS-T20 containing 1% FCS.

Step 6: The plates were then incubated with either biotinylated anti-mouse IFN-γ, anti-IL-2, anti-IL-4, anti-IL-5, anti-IL-6, anti-IL-10, anti-IL-12, or anti-TNF-α antibody at a concentration of 5 μg /ml (room temperature overnight) and they were then washed three times with PBS-T20.

Step 7: Avidin-peroxidase (Sigma-Aldrich Corporation, St. Louis., MO, USA) at 1:1,000 dilution in PBS-T20 1% FCS was added to the wells and was incubated for 2 hrs at room temperature.

After incubations spots appeared on all responding cells. The spots were developed by adding the substrate 3-amino-9-ethylcarbazole plus hydrogen peroxide and were counted under a stereoscopic microscope. The numbers of spot-forming cells were expressed per 10^6 cells.

**Statistical analysis**

Student’s t-test was performed to assess the significance of differences between the means of type 1 and type 2 T helper cells in patients and control group.

**RESULTS**

The numbers of type 1 T helper cells secreting IL-2, IL-12, and IFN-γ were increased in the peripheral blood of RAS patients compared with the healthy controls (p < 0.05, p < 0.001 and p < 0.001, respectively). No statistical difference was observed in the number of TNF-α secreting cells between RAS patients and controls (p > 0.05) (Table 2).

The number of type 2 T helper cells secreting IL-10 was also increased in patients with RAS (p < 0.05). (Table 3). The number of IL-4 secreting cells in patients with RAS was decreased compared with the controls (p < 0.001), while no statistical differences were noted in the numbers of IL-5 and IL-6 secreting cells between patients with RAS and controls (p > 0.05 for each) (Table 3).

**DISCUSSION**

Our immune system represents the essential defense line against foreign intruders. This is achieved with its capability to recognize the mali-

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**Table 2.** Numbers of T helper cells secreting Type 1 cytokines in patients with RAS and control group.

<table>
<thead>
<tr>
<th></th>
<th># of Type 1 T helper cells in patients with RAS</th>
<th># of Type 1 T helper cells in controls</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ secreting cells</td>
<td>128.5 ± 13</td>
<td>59.3 ± 14</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>IL-2 secreting cells</td>
<td>98.6 ± 7.3</td>
<td>49.7 ± 5.7</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>IL-12 secreting cells</td>
<td>200.2 ± 2.9</td>
<td>80.9 ± 1.3</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>TNF-α secreting cells</td>
<td>184 ± 16</td>
<td>177.6 ± 16</td>
<td>p &gt; 0.05</td>
</tr>
</tbody>
</table>

**Table 3.** Numbers of T helper cells secreting type 2 cytokines in patients with RAS and control group.

<table>
<thead>
<tr>
<th></th>
<th># of Type 2 T helper cells in patients with RAS</th>
<th># of Type 2 T helper cells in controls</th>
<th>p</th>
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</thead>
<tbody>
<tr>
<td>IL-4 secreting cells</td>
<td>50.81 ± 0.88</td>
<td>120.1 ± 1.6</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>IL-5 secreting cells</td>
<td>60.63 ± 0.88</td>
<td>80.82 ± 0.99</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>IL-6 secreting cells</td>
<td>84.1 ± 11</td>
<td>80.4 ± 8.5</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>IL-10 secreting cells</td>
<td>297 ± 41</td>
<td>193 ± 29</td>
<td>p &lt; 0.05</td>
</tr>
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</table>
cious antigens and to prevent their catastrophic action as well as the autodestructive immune response. Given that there is no difference between the foreign body and the autoantigen, some authors suggested that the cells of the immune system react to the antigens only by the presence of cytokines (Hasan et al. 2002).

In the present investigation we studied the parameters of immune reaction that may be involved in the pathogenesis of RAS. We detected, enumerated and characterized type 1 and type 2 T helper cells and we indirectly studied their capacity for secretion of certain cytokines (IFN-γ, IL-2, IL-12, TNF-α, IL-4, IL-5, IL-6, and IL-10). We believe that the determination of the capacity of type 1 and type 2 T helper cells for cytokine production is a more reliable method compared with the measurement of cytokines in serum. Our results revealed statistically increased numbers of Type 1 T helper cells (IL-2, IL-12, IFN-γ secreting cells) compared with the controls (p < 0.05, p < 0.001 and p < 0.001, respectively). Similar findings have been reported in other studies. Buno et al. (1998) using the RT-PCR method, reported that the serum levels of IFN-γ, TNF-α, IL-2 and IL-4 were increased in patients with RAS, while the levels of IL-10 were proportional with the healthy controls. Similarly, Borra et al. (2004) using DNA analysis have shown that the type 1 cytokines expression in patients with RAS is statistically increased compared with the controls.

Despite the pathogenetic importance that has been reported for TNF-α in the literature, our results did not reveal any statistical difference in TNF-α between RAS patients and controls (p > 0.05). TNF-α has been proposed to induce inflammation by its effect on endothelial cell adhesion and neutrophil chemotaxis (Natah et al. 2000). Increased serum levels of TNF-α from stimulated PBMC in RAS patients have been reported in several studies (Taylor et al. 1992; Sun et al. 2003, 2004).

In contrast, the number of certain Type 2 T helper cells, such as IL-4 producing cells which antagonize the production of type 2 cytokines was found to be statistically decreased compared with the controls (p < 0.05). No statistical difference was observed in the numbers of IL-5 and IL-6 secreting cells between RAS patients and controls (p > 0.05).

All these findings indicate a predominance of proinflammatory cytokines in lesional level and peripheral blood of RAS patients. This may be significant for the pathogenesis of RAS.

The nature of immune response and the profile of cytokines in RAS, indicate a type of immune response mediated by Type 1 cytokines. The increased expression of proinflammatory cytokines (TNF-α, IFN-γ and IL-2) from PBMC cells and the inadequate immunosuppressive mechanisms in RAS, may lead to excessive immune response to oral antigens. This fact may cause the clinical appearance of RAS. The increased expression of TNF-α, IFN-γ and IL-6 in RAS may also lead to the maturation of the oral epithelial Langerhans cells and to the subsequent activation of T cells. The tissue destruction is explained by the fact that RAS may potentially activate the cytotoxic cells which may lead to the appearance of lesions in local level.

Conclusively our results showed an increase of T cells secreting type 1 cytokines. Considering the diverse and important role of T helper cells in the immune system, we suggest that these cells may influence the immune response against RAS. Whether this action is of etiological importance or reactive to the ulcerative damage of the oral mucosa is a question that needs to be answered.

References


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Type 1 and 2 T Helper Cells in RAS

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