Elevated Levels of Interferon Gamma, Tumor Necrosis Factor α, Interleukins 2, 4, and 5, but Not Interleukin 10, Are Present in Recurrent Aphthous Stomatitis

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Objective: To investigate our hypothesis that recurrent aphthous stomatitis (RAS), an inflammatory disease of the oral mucosa, is the result of an abnormal oral mucosal cytokine cascade leading to an enhanced cell-mediated immune response directed toward focal areas of the oral mucosa.

Design: Prospective nonrandomized case-control study.

Setting: Academic referral center

Patients: For part 1, 21 patients with RAS and 7 control patients; for part 2, 6 patients with RAS and 6 control patients.

Intervention: For study part 1, lesional and clinically normal oral mucosal biopsy specimens were obtained during an acute episode (within 72 hours of onset of ulcer) from 21 patients with RAS. Normal oral mucosal biopsy specimens were obtained from 7 healthy individuals, who served as controls. In study part 2, oral mucosal biopsy specimens were obtained from 6 RAS and 6 control patients at 24 and 48 hours after surgical trauma to those sites.

Main Outcome Measures: Detection of the following messenger RNA (mRNA) types by use of semiquantitative reverse transcriptase polymerase chain reaction. For part 1, interleukins (IL) 2, 4, 5, and 10, interferon gamma, and tumor necrosis factor α were measured. For study part 2, IL-10 and interferon gamma were measured.

Results: In part 1, elevated levels of IL-2, interferon gamma, and tumor necrosis factor α mRNAs were detected in RAS lesions, consistent with a cell-mediated immune response. The IL-10 mRNA was not increased in RAS lesions. In addition, lower resting levels of IL-10 mRNA were detected in the clinically normal mucosa from patients with RAS, as compared with levels seen in the healthy controls. In part 2, at both 24 and 48 hours following trauma to the oral mucosa, the levels of mucosal IL-10 mRNA remained lower in patients with RAS than those observed in healthy controls, while interferon gamma mRNA levels were higher.

Conclusion: Failure to suppress the inflammatory reaction initiated by trauma or other external stimuli, likely involving a functional deficiency of IL-10 in the oral mucosa, appears to be important in the pathogenesis of RAS.

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SUBJECTS, MATERIALS, AND METHODS

SUBJECTS AND SPECIMENS

Informed consent was obtained from all the study participants after the nature of the procedure was fully explained.

For study part 1, lesional and clinically normal mucosal biopsy specimens from 21 patients with RAS were obtained during an acute episode, generally fewer than 3 days from the onset of the lesion. Biopsy specimens of normal oral mucosa from 7 healthy individuals with no history of oral disease served as controls. In all cases, biopsy specimens were taken from either the buccal or labial mucosae, as opposed to the gingivae, tongue, or palate.

In study part 2, 6 RAS and 6 normal control patients participated. Trauma was induced by placement of a surgical suture in 2 sites of the labial mucosa in each patient (time 0). A baseline biopsy specimen of clinically normal mucosa was also obtained at that time. Patients returned 24 hours later, and a biopsy specimen was obtained from 1 of the suture sites. Patients returned again, 48 hours after trauma, and the last biopsy specimen was obtained from the second suture site.

The biopsy specimens were immediately placed in immunofluorescence transport medium, composed of N-ethylmaleimide, sodium phosphate dibasic, ammonium sulfate, and thimerosal (Zeus Scientific Inc, Branchburg, NJ), treated with 0.1% diethyl pyrocarbonate, a ribonuclease inhibitor (Sigma Chemical Co, St Louis, Mo), and reserved for RNA isolation.

To serve as a positive control, peripheral blood specimens were obtained from healthy subjects. Peripheral blood mononuclear cells were isolated via the Ficoll-Hypaque (Pharmacia, Piscataway, NJ) phase separation technique and washed in RPMI 1640 medium (Roswell Park Memorial Institute 1640 medium, Life Technologies Inc, Gibco BRL, Grand Island, NY). The peripheral blood mononuclear cells were stimulated with either concanavalin A or phytohemagglutinin A (Sigma Chemical Co) for 24 hours at 37°C; then cells were centrifuged. The pellet was reserved for the RNA extraction.

RNA EXTRACTION

Each mucosal tissue specimen was mounted in a compound embedding medium (Tissue-Tek OCT, Fisher Scientific, Pittsburgh, Pa) and cut into 10-µm sections with a cryostat. Mucosal tissue sections were homogenized with a phenol–guanidine isothiocyanate monophase solution (TRIzol, Life Technologies Inc). The mRNA was isolated using chloroform and isopropyl alcohol, and the product was quantified with the RNA-DNA calculator (Pharmacia Gene Quant, Pharmacia, Piscataway, NJ) at an absorbance of 260 nm. The peripheral blood mononuclear cell pellets were processed in a similar manner.

Table 1. Oral Mucosal Cytokine Messenger RNA Levels in Patients With Recurrent Aphthous Stomatitis (RAS) and in Control Patients

<table>
<thead>
<tr>
<th>Patient Characteristic</th>
<th>Mean (SEM) Oral Mucosal Cytokine Messenger RNA Levels</th>
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<tbody>
<tr>
<td></td>
<td>IFN-γ</td>
</tr>
<tr>
<td>Patients with RAS, lesional (n = 21)</td>
<td>1.54 (0.23)</td>
</tr>
<tr>
<td>Patients with RAS, nonlesional (n = 21)</td>
<td>0.60 (0.13)</td>
</tr>
<tr>
<td>Control patients (n = 7)</td>
<td>0.21 (0.04)</td>
</tr>
</tbody>
</table>

*IFN-γ indicates interferon gamma; IL, interleukin; TNF-α, tumor necrosis factor α; and NA, data not applicable.
†Compared with biopsy specimens from control patients.
‡Compared with nonlesional biopsy specimens from patients with RAS.

It is now appreciated that dysfunction of the cutaneous cytokine network is involved in the pathogenesis of many inflammatory skin diseases. The roles played by CD4+ T-cell subsets (helper T cells T H1 and T H2) and their specific cytokine products have recently been described.10-13 The T H1 cells secrete interleukin 2 (IL-2), tumor necrosis factor α (TNF-α), and interferon gamma (IFN-γ), and they are the principal effectors of cell-mediated immunity.12 The T H2 cells produce interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 10 (IL-10), and interleukin 13 (IL-13). Immunoglobulin E antibody production is stimulated by IL-4, while IL-5 is an eosinophil-activating factor. Macrophage functions are inhibited by IL-4, IL-10, and IL-13. The T H2 products are
REVERSE TRANSCRIPTION AND POLYMERASE CHAIN REACTION

A uniform amount of mRNA from each specimen was used in the preamplification system (Gibco BRL, Grand Island, NY). To serve as a control, an additional sample of mRNA from each specimen was preamplified without reverse transcriptase in the reaction mixture.

An equal quantity of complementary DNA from each specimen was combined with cytokine-specific and control primers (IL-2, IL-4, IL-5, IL-10, TNF-α, IFN-γ, and β-actin) and amplified for 30 cycles in the DNA thermal cycler (Perkin Elmer Cetus, Norwalk, Conn). The cytokine-specific primer sets span introns, so that amplified product originating from mRNA could be distinguished from any amplification of contaminating DNA, based on size of the product.

Amplified products were identified via gel electrophoresis, using a 2% agarose gel (Sigma Chemical Co) immersed in a solution with 0.5-µg/mL ethidium bromide, and run on a DNA subcell apparatus (Bio-Rad Laboratories, Hercules, Calif) at 150 V. A positive control specimen was included on each gel.

SOUTHERN BLOT ANALYSIS

A blotting system (Turboblotter, Schleicher & Schuell, Keene, NH) was used to transfer the amplified DNA products from the gel onto positively charged nylon membranes (Nytran, Schleicher & Schuell, Keene, NH). The cytokine and β-actin DNA products and a digoxigenin tail-labeled DNA marker were hybridized with digoxigenin-labeled probes. Detection of hybridized products was performed according to a system (Genius System, Boehringer Mannheim, Indianapolis, Ind), which uses an antidigoxigenin alkaline phosphatase conjugate and the substrate (CDP-Star, Boehringer Mannheim). The products were exposed to autoradiograph film.

DENSITOMETRY

National Institutes of Health Image 1.59 software was used to semiquantitate the polymerase chain reaction products as described above. For each blot, mRNA values were equalized to the positive control (peripheral blood mononuclear cells stimulated by concanavalin A or phytohemagglutinin A), which had been hybridized and detected under identical conditions. Then, for each specimen, the amplified cytokine product was normalized to the β-actin product from that same specimen. The means of all the control, lesional, and nonlesional values were calculated for each cytokine.

STATISTICAL ANALYSIS

A Student t test statistical analysis (2-sample test assuming unequal variances and paired 2-sample test) was done to determine significance.

<table>
<thead>
<tr>
<th></th>
<th>IL-5</th>
<th>P†</th>
<th>P‡</th>
<th>IL-10</th>
<th>P†</th>
<th>P‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.44 (0.14)</td>
<td>&lt;.01</td>
<td>&lt;.04</td>
<td>1.67 (0.30)</td>
<td>&lt;.34</td>
<td>&lt;1.00</td>
</tr>
<tr>
<td></td>
<td>0.14 (0.02)</td>
<td>&lt;.003</td>
<td>NA</td>
<td>1.29 (0.22)</td>
<td>&lt;.82</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>0.05 (0.01)</td>
<td>NA</td>
<td>NA</td>
<td>1.28 (0.15)</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Elevated levels of IL-2, IFN-α, and TNF-α mRNA (T_h1 subtype) were detected in lesional mucosa of patients with RAS compared with mucosa from healthy controls (Table 1 and Figure 1). These values were statistically significant. Similarly, elevated values of IL-2, IFN-γ, and TNF-α mRNA were detected in lesional mucosa of patients with RAS compared with nonlesional mucosa from patients with RAS. However, only IFN-γ was elevated to a significant level. Interleukin 4 and IL-5 (T_h2 subtype) mRNA levels were elevated in lesional mucosa (Table 1 and Figure 1) as compared with nonlesional mucosa from patients with RAS and mucosa from healthy controls. These differences were statistically significant. Significantly increased levels of IFN-γ, TNF-α, IL-4, and IL-5 mRNA were also detected in the normal mucosa of patients with RAS, compared with those seen in the non-RAS controls (Table 1 and Figure 1).

No significant increase in IL-10 mRNA level was noted in RAS lesions and, in the clinically normal mucosa from patients with RAS, the levels of IL-10 mRNA were lower than those seen in the healthy controls. In the biopsy specimen of traumatized mucosa, IL-10 mRNA levels were consistently lower, and IFN-γ mRNA levels higher, in the patients with RAS compared with normal controls at both times examined (Table 2 and Figure 2), although these differences were not statistically significant. All of the patients with RAS developed ulcers at the sites of trauma, compared with the con-
trol patients in whom minimal inflammation was noted at the sites of suture placement.

The healthy oral mucosa responds to trauma or other external factors by initiating a series of homeostatic repair mechanisms involving a complex interaction between the cellular constituents of the mucosal tissue and the immune system. These repair mechanisms lead to healing and prompt return of normal mucosal function. In individuals with RAS, however, trauma to the oral mucosa instead leads to tissue damage and ulceration. This isomorphic phenomenon has been previously described and was clearly demonstrated in the second part of the present study where the patients with RAS uniformly developed clinically evident aphthae within 24 hours following the suture-induced mucosal trauma. Interestingly, the ulcer that developed assumed the shape of the inciting trauma, as if the damaged keratinocytes were specifically targeted for destruction (Figure 3). This was in contrast to the non-RAS controls, whose mucosal trauma sites quickly healed. The exact sequence of events in the isomorphic phenomenon is not known, but current evidence supports the concept that T-cell activation, together with an increased sensitivity of keratinocytes to locally derived cytokines, forms the basic underlying mechanism. Proinflammatory cytokines within the mucosa, such as TNF-α, may initiate or up-regulate the inflammatory response, while the absence of other mediators, such as IL-10, may result in a failure to down-regulate an inflammatory reaction.

Table 2. Oral Mucosal Cytokine Messenger RNA Levels in Patients With Recurrent Aphthous Stomatitis (RAS) and in Control Patients After Surgical Trauma

<table>
<thead>
<tr>
<th>Messenger RNA Type</th>
<th>Baseline</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interferon gamma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients with RAS</td>
<td>0.66 (0.42)</td>
<td>2.04 (0.67)</td>
<td>2.04 (0.56)</td>
</tr>
<tr>
<td>Control patients</td>
<td>0.56 (0.28)†</td>
<td>1.12 (0.40)</td>
<td>1.08 (0.71)</td>
</tr>
<tr>
<td>Interleukin 10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients with RAS</td>
<td>1.44 (0.28)†</td>
<td>2.40 (0.41)</td>
<td>2.42 (0.47)</td>
</tr>
<tr>
<td>Control patients</td>
<td>1.91 (0.72)†</td>
<td>3.01 (0.76)</td>
<td>2.83 (0.64)</td>
</tr>
</tbody>
</table>

* Data are given as mean (SEM).
† For these groups, n = 5. For all other groups, n = 6.
The present study was designed to identify the pattern of mucosal cytokine mRNA expression in RAS. The elevated levels of IL-2, IFN-γ, and TNF-α mRNA detected in RAS lesions are consistent with a cell-mediated immune response, while the elevated levels of IL-4 and IL-5 mRNA suggest that the immune response is not exclusively of the Th1 or Th2 subtype. In addition, there appears to be an intrinsic abnormality of cytokine mRNA expression in the oral mucosa of patients with RAS, demonstrated by the elevated levels of IFN-γ, TNF-α, IL-4, and IL-5 mRNA in the “normal” mucosa of a patient with RAS compared with levels seen in the non-RAS controls. Furthermore, there are higher resting levels of the proinflammatory cytokines, IL-2 and TNF-α, in lesional and nonlesional mucosa of patients with RAS compared with controls. Interleukin 10 was the only cytokine examined that was not detected in increased levels in lesions of patients with RAS, or in the healthy mucosa from patients with RAS. Human IL-10, also known as cytokine synthesis inhibitory factor, is produced by both Th1 and Th2 CD4+ T cells and inhibits their antigen-specific proliferation and cytokine production. Cutaneous basal and squamous cell carcinomas produce IL-10, which may provide a mechanism for evading the local T-cell–mediated immune response. Interleukin-10–knockout mice develop a chronic enterocolitis because of an aberrant immune response to normal enteric antigens, and low levels of IL-10 have been shown in humans with inflammatory bowel disease. Blocking endogenous IL-10 in mice accelerates skin allograft rejection. The ability of human dermal microvascular endothelial cells to present antigen to CD4+ T cells was stimulated by IFN-γ, but inhibited by IL-10, although the latter effect was not associated with a down-regulation of class II major histocompatibility complex expression. Given the natural history of RAS to diminish with advancing age, it is also of interest that whole blood assays revealed greater IL-10 production in the elderly compared with younger controls. In the present study, lower levels of IL-10 mRNA and higher levels of IFN-γ mRNA were detected following trauma to the mucosa from patients with RAS, compared with control patients. Although these differences were not statistically significant, this trend was seen at each time examined. Since most of the patients with RAS already had clinically evident ulcers by the first point chosen for study (24 hours), it is possible that examination sooner after trauma may have yielded more striking differences.

In conclusion, elevated mRNA levels of proinflammatory cytokines were detected in clinically normal mucosa of patients with RAS as well as in the active RAS ulcers, while mRNA levels of the anti-inflammatory cytokine IL-10 were not elevated in acute RAS lesions and were decreased in both clinically normal mucosa of patients with RAS and mucosa of patient with RAS following trauma. This cytokine imbalance favoring an active cell-mediated immune process may fail to suppress the inflammatory reaction initiated by trauma or other external stimuli, resulting in the mucosal damage clinically evident as an aphthous ulcer. Further investigation of the mechanisms underlying this dysregulation of the normal cytokine network in RAS will lead to a better understanding of this common and sometimes debilitating disorder.

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