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Levamisole can modulate the serum tumor necrosis factor-α level in patients with recurrent aphthous ulcerations

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BACKGROUND: Recurrent aphthous ulcerations (RAU) are common oral inflammatory lesions. Tumor necrosis factor (TNF)-α is an important inflammatory mediator and a critical cytokine for adequate host defense. Our previous studies have shown that 14–43% and 59–63% of patients in the ulcerative stage of major, minor or herpetiform RAU have significantly higher than normal serum levels of interleukin (IL)-6 and IL-8, respectively. In this study, we examined whether RAU patients in the ulcerative stage had a significantly higher than normal serum level of TNF-α and assessed whether treatment with levamisole can modulate serum TNF-α levels in RAU patients.

METHODS: This study used a solid phase, two-site sequential chemiluminescent immunometric assay to determine the baseline serum levels of TNF-α in 146 patients with RAU, nine patients with traumatic ulcers (TU), and 54 normal control subjects. Fifty-five RAU patients with serum TNF-α levels higher than 5.0 pg/ml were treated with levamisole for 0.5–4 months and their serum TNF-α levels were measured after treatment.

RESULTS: We found that 29% (42 of 146) RAU patients as well as 39% (24 of 61) major type, 20% (14 of 69) minor type, and 25% (four of 16) herpetiform type RAU patients had a serum level of TNF-α greater than the upper normal limit of 7.4 pg/ml. The mean serum level of TNF-α in patients with RAU (9.1 ± 1.0 pg/ml, $P < 0.001$), major type RAU (11.6 ± 1.9 pg/ml, $P < 0.001$), minor type RAU (6.9 ± 0.9 pg/ml, $P < 0.005$), or herpetiform type RAU (9.6 ± 2.7 pg/ml, $P < 0.001$) was higher than that (3.8 ± 0.2 pg/ml) in normal control subjects. The mean serum TNF-α level was significantly higher in patients with major type RAU than in patients with minor type RAU ($P < 0.05$) and was significantly higher in major type RAU patients in the exacerbation stage than in the post-exacerbation stage ($P < 0.05$). In 55 RAU patients with serum TNF-α levels higher than 5.0 pg/ml, treatment with levamisole for a period of 0.5–4 months could significantly reduce the serum TNF-α level from 16.4 ± 1.9 to 5.8 ± 0.6 pg/ml ($P < 0.001$).

CONCLUSIONS: We conclude that a significantly higher than normal serum level of TNF-α can be detected in 20–39% of patients in the ulcerative stage of major, minor or herpetiform RAU. The serum TNF-α level may be associated with the severity and the stage of RAU. Levamisole can modulate serum TNF-α levels in RAU patients.

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Introduction

Recurrent aphthous ulcerations (RAU) are common inflammatory lesions characterized by recurrent and painful ulcerations of the oral non-keratinized mucosa. Previous investigations have demonstrated alterations of serum immunoglobulins (1), circulating immune complexes (2), T-lymphocytes subsets (3–7), and natural killer (NK) cell activity (8) in patients with RAU. Immunohistochemical studies have shown an increased number of CD4+ lymphocytes in the pre-ulcerative oral aphthous lesions and a predominance of CD8+ lymphocytes in the ulcerative oral aphthous lesions (4). Quantitative analysis has shown that the inflammatory infiltrate in active RAU lesion consists of 30–60% of CD4+ cells, 10–30% of CD8+ cells, 5–12% of B cells, 5–35% of macrophages, 2–5% of mast cells, and a low number of monocytes (9). Many activated T cells and very few activated B cells are found in both local active RAU lesions (10) and peripheral lymphocytes (11). The results of previous studies on peripheral blood mononuclear cells (PBMC) and tissue infiltrated mononuclear cells (TIMC) favored the role of cell-mediated cytotoxicity in the immunopathogenesis of RAU (3–7, 9–12).

Tumor necrosis factor (TNF)-α is an important inflammatory mediator and a critical cytokine for

adequate host defense. A variety of cell types including macrophages, CD4+ T cells, mast cells, and NK cells have been shown to produce TNF-α after stimulation with lipopolysaccharides (13), some viruses (14, 15) or parasites (16). Elevated levels of TNF-α mRNA have been detected in oral aphthous lesion (17). TNF-α immunoreactivity has been detected in macrophages and lymphocytes within the mononuclear inflammatory infiltrates of the oral aphthous lesion. TNF-α is also seen in mast cells and vascular endothelial cells in connective tissue lateral to the inflammatory infiltrates (18). Significantly greater amounts of TNF-α are released from unstimulated monocyte-enriched and monocyte-depleted leukocyte fractions in active RAU compared with those from healthy control donors (19). TNF-α has multiple stimulatory activities on activated T cells, including increasing the proliferation in response to antigen, increasing interleukin (IL)-2 receptor expression and the response to IL-2 stimulus, and inducing IL-1 production. Both TNF-α and IL-1 induce expression of intercellular adhesion molecule 1, a cell adhesion molecule involved in binding T cells to antigen-presenting cells. These TNF-α effects on T cells should upregulate T-cell activation (20). Moreover, TNF-α can induce the secretion of IL-6 and IL-8 by several cell types. IL-6 can promote T-cell growth and differentiation as well as cytotoxic T-cell differentiation (21). IL-8 can attract more T cells, including cytotoxic T cells, to the aphthous lesion (22). Because RAU is a T-cell-mediated disease, TNF-α may be involved in the pathogenesis of RAU.

Levamisole is an effective immunodulating agent that can restore the normal phagocytic activity of macrophages and neutrophils, modulate T-cell-mediated immunity, and potentiate the activity of human interferon (IFN) and IL-2 (23–26). Our previous studies have shown that levamisole has modulating effects on both cell-mediated and humoral immunity in RAU patients (27). Levamisole can also modulate the serum IL-6 and IL-8 levels in RAU patients (21, 22). Therefore, both IL-6 and IL-8 are useful serum markers in evaluating therapeutic effects of levamisole on RAU patients. In addition, serum IL-8 level is a more sensitive marker than serum IL-6 level in monitoring the disease activity of RAU (22). In this study, we measured the serum TNF-α levels in 146 RAU patients in the active stage of disease at baseline to clarify whether the serum TNF-α level was elevated in RAU patients. For comparison, we also measured the serum TNF-α levels in nine patients with traumatic ulcers (TU) and in 54 normal control subjects. In addition, we followed the serum TNF-α levels serially in 55 RAU patients both during and after treatment with levamisole to see whether levamisole could modulate the serum TNF-α levels in RAU patients.

Materials and methods

The study group consisted of 146 RAU patients (66 men and 80 women, mean age 39.4 years, range: 10–79). All the RAU patients had at least one episode of oral ulcerations per month during the preceding years. The development of RAU was divided into two stages. (i) Exacerbation stage: from the onset of oral mucosal ulceration to the day of maximum ulcer pain and (ii) post-exacerbation stage: from the day of maximum ulcer pain to the complete healing of the oral ulceration. The severity of RAU was subdivided into major, minor, and herpetiform types as described previously (21). The disease and normal control groups consisted of nine patients with TU and 54 healthy subjects without any oral mucosal disease, respectively. All the patients and control subjects were diagnosed and treated in the dental department of National Taiwan University Hospital. None of them had taken any prescription medication at least 3 months before entering the study. The age and sex distributions of the subjects in both the study and control groups are summarized in Table 1.

Fifty-five RAU patients with the serum TNF-α levels above 5.0 pg/ml were treated by levamisole. Levamisole was administered at a dose of 50 mg twice per day for patients with 30–50 kg of body weight, or at a dose of 50 mg three times per day for patients with 50–70 kg of body weight for 3 consecutive days at the beginning of each 2-week interval. Compliance was monitored by asking the patients to record the time at which each drug was taken. Before the start of therapy, data on all cases were recorded according to a set protocol, and the patients were examined by the same dentist at each visit. Patients were monitored once or twice a month for the recording of changes of clinical parameters including frequency, duration, and number of oral aphthous ulcerations.

Blood samples were withdrawn from the RAU patients in the active (ulcerative) stage (from the onset to the complete healing of the oral mucosal ulceration) before treatment and from the disease and normal control subjects. To assess whether the serum level of TNF-α in patients with RAU could be modulated by

Table 1 Age and sex distributions of 146 patients with RAU, nine patients with TU, and 54 NC subjects

	Age (years)														
	10–19		*20–29*		*30–39*		*40–49*		*50–59*		*60–69*		*70–79*		
	M	*F*	*M*	*F*	*M*	*F*	*M*	*F*	*M*	*F*	*M*	*F*	*M*	*F*	*Total*
RAU	8	4	10	17	14	21	19	19	7	11	6	4	2	4	146
TU	0	0	0	1	2	3	0	2	0	1	0	0	0	0	9
NC	4	0	4	5	6	8	5	10	2	6	3	1	0	0	54

M, male; F, female; RAU, recurrent aphthous ulceration; TU, traumatic ulcer; NC, normal control.

Table 2 Baseline serum levels of TNF-α in the patients with RAU, TU, and in healthy control subjects

	Number of subjects	*Serum levels of TNF-α (pg/ml)*		
		Range	*Mean ± SEM*	*Level >7.4 pg/ml, n (%)*
RAU*	146	1.0–74.3	9.1 ± 1.0**	42 (29)
TU*	9	1.0–6.8	4.1 ± 0.5	0 (0)
Normal control subjects*	54	1.0–8.5	3.8 ± 0.2	1 (2)

*Comparison among three groups by ANOVA ($P < 0.05$).
**Comparison between patients and normal controls ($P < 0.05$).
TNF, tumor necrosis factor; RAU, recurrent aphthous ulceration; TU, traumatic ulcer.

treatment with levamisole, one or up to four serial blood samples were obtained after treatment until the patients' serum TNF-α levels returned to normal.

The TNF-α concentration in serum samples was determined using Immulite TNF-α assay (Diagnostic Products Corporation, Los Angeles, CA, USA). Immulite TNF-α was a solid phase, two-site sequential chemiluminescent immunometric assay. The solid phase, a polystyrene bead enclosed within an Immulite Test Unit, was coated with monoclonal murine antibody specific for TNF-α. The patient sample and a protein/buffer matrix were simultaneously introduced into the Test Unit, and incubated for approximately 30 min at 37°C with intermittent agitation. During this time, TNF-α in the patient sample bound to the monoclonal antibody-coated beads. Unbound serum was then removed by a centrifugal wash. An alkaline phosphatase-labeled polyclonal rabbit anti-TNF-α antibody was introduced, and the Test Unit was incubated for another 30-min cycle. The unbound enzyme conjugate was removed by a centrifugal wash. Substrate was then added, and the Test Unit was incubated for a further 10 min. The chemiluminescent substrate, a phosphate ester of adamantly dioxetane, underwent hydrolysis in the presence of alkaline phosphatase to yield an unstable intermediate. The continuous production of this intermediate resulted in the sustained emission of light, thus improving precision by providing a window for multiple readings. The bound complex – and thus also the photon output, as measured by the luminometer – was proportional to the concentration of TNF-α in the sample. The TNF-α concentration was calculated from the standard curve. The sensitivity of this assay for TNF-α is 1.0 pg/ml.

Analysis of variance and multiple comparison were used to test whether the mean baseline serum TNF-α levels were different among RAU patients, TU patients, and normal control subjects. The serum TNF-α level and clinical parameters (frequency, duration, and number of oral ulcers) at baseline and after treatment with levamisole were compared to each other using a paired *t*-test. The result was considered to be significant if the *P*-value was <0.05.

Results

The baseline serum levels of TNF-α in the patients with RAU or TU and in healthy control subjects were measured and compared between groups (Table 2). In 54 normal control subjects, the mean serum TNF-α level was 3.8 ± 0.2 pg/ml. A value of 7.4 pg/ml (equal to approximate mean normal level + 2 SD) was adopted as the upper limit of the normal range of TNF-α. About 98% of the normal control subjects and the patients with TU had a serum level of TNF-α within the normal limit of 7.4 pg/ml. However, 29% (42 of 146) RAU patients had a serum level of TNF-α >7.4 pg/ml. A significant difference in the mean baseline serum level of TNF-α was found among three groups tested ($P < 0.05$). Furthermore, the mean serum level of TNF-α in RAU patients (9.1 ± 1.0 pg/ml, $P < 0.05$) was significantly higher than that in normal control subjects (3.8 ± 0.2 pg/ml; Table 2).

When 146 RAU patients were further divided into subgroups according to the disease types (major, minor, and herpetiform) and disease stages (exacerbation and post-exacerbation), we found that 39% (24 of 61) major type, 20% (14 of 69) minor type, and 25% (four of 16) herpetiform type RAU patients had a serum level of TNF-α >7.4 pg/ml (Table 3). A significant difference in the mean baseline serum TNF-α level was found among three types of RAU patients and normal control subjects ($P < 0.001$). The mean serum level of TNF-α in major type (11.6 ± 1.9 pg/ml, $P < 0.001$), in minor type (6.9 ± 0.9 pg/ml, $P < 0.005$), or in herpetiform type RAU patients (9.6 ± 2.7 pg/ml, $P < 0.001$) was greater than that in normal control subjects. In addition, a higher mean serum TNF-α level was also found in major type RAU patients in both the exacerbation (16.0 ± 3.6 pg/ml, $P < 0.005$) and post-exacerbation stages (8.4 ± 1.8 pg/ml, $P < 0.005$), in minor type RAU patients in both the exacerbation (8.6 ± 2.3 pg/ml, $P < 0.005$) and post-exacerbation stages (5.9 ± 0.7 pg/ml, $P < 0.005$), and in herpetiform type RAU patients in both the exacerbation (10.7 ± 3.7 pg/ml, $P < 0.001$) and post-exacerbation stages (8.1 ± 4.1 pg/ml, $P < 0.01$) than in normal control subjects. Furthermore, the mean serum TNF-α level was significantly higher in patients with major type RAU than in patients with minor type RAU ($P < 0.05$) and was significantly higher in major type RAU patients in the exacerbation stage than in the post-exacerbation stage ($P < 0.05$; Table 3).

We monitored the serum TNF-α level in RAU patients before and after treatment and found that levamisole treatment for a period of 0.5–4 months could

Table 3 Baseline serum levels of TNF-α in the 146 patients with subgroups of RAUs and in healthy control subjects

	Number of subjects	*Serum levels of TNF-α (pg/ml)*		
		Range	*Mean ± SEM*	*Level >7.4 pg/ml, n (%)*
Major type RAU[f]	61	1.0–74.3	11.6 ± 1.9[a, d]	24 (39)
Exacerbation stage	26	1.0–74.3	16.0 ± 3.6[b, e]	13 (50)
Post-exacerbation stage	35	1.0–49.2	8.4 ± 1.8[b]	11 (31)
Minor type RAU[f]	69	1.0–49.7	6.9 ± 0.9[b]	14 (20)
Exacerbation stage	25	1.0–49.7	8.6 ± 2.3[b]	5 (20)
Post-exacerbation stage	44	1.0–21.2	5.9 ± 0.7[b]	9 (20)
Herpetiform type RAU[f]	16	1.0–36.1	9.6 ± 2.7[a]	4 (25)
Exacerbation stage	9	1.0–36.1	10.7 ± 3.7[a]	3 (33)
Post-exacerbation stage	7	1.0–32.6	8.1 ± 4.1[c]	1 (14)
Normal control subjects[f]	54	1.0–8.5	3.8 ± 0.2	1 (2)

Comparison between RAU patients and normal controls: [a]$P < 0.001$, [b]$P < 0.005$, and [c]$P < 0.01$.
[d]The mean serum TNF-α level was significantly higher in patients with major type RAU than in patients with minor type RAU with $P < 0.05$.
[e]The mean serum TNF-α level was significantly higher in major type RAU patients in the exacerbation stage than in the post-exacerbation stage with $P < 0.05$.
[f]Comparison among four groups by ANOVA ($P < 0.001$).
TNF, tumor necrosis factor; RAU, recurrent aphthous ulceration.

Table 4 The serum levels of TNF-α before and after treatment with levamisole in RAU patients with the serum TNF-α levels above 5.0 pg/ml

Treatment	*RAU patients (number and type of patients)*	*Duration of treatment (months)*		*Serum levels of TNF-α (pg/ml; mean ± SEM)*		
		Range	*Mean ± SD*	*At baseline*	*After treatment*	*Difference*
Levamisole	28 major type	0.5–3.0	1.2 ± 0.8	19.0 ± 3.0	5.9 ± 0.9[a]	13.1 ± 2.9
Levamisole	22 minor type	0.5–4.0	1.1 ± 0.9	13.2 ± 2.4	6.1 ± 0.9[b]	7.1 ± 2.4
Levamisole	5 herpetiform type	0.5–1.0	0.6 ± 0.2	15.7 ± 5.9	4.1 ± 0.8	11.6 ± 6.3
Total	55 patients	0.5–4.0	1.1 ± 0.8	16.4 ± 1.9	5.8 ± 0.6[a]	10.6 ± 1.8

Significant difference in serum level of TNF-α between patients at baseline and patients after treatment with [a]$P < 0.001$ and [b]$P < 0.01$ by paired *t*-test.
TNF, tumor necrosis factor; RAU, recurrent aphthous ulcerations.

Table 5 Comparison of clinical parameters in 28 major type, 22 minor type, and five herpetiform type RAU patients before and after treatment with levamisole for 1 or 2 months

Duration of treatment (months)	*Clinical parameters (mean ± SD)*					
	Frequency of oral ulcers (times/month)		*Duration of oral ulcers (days)*		*Number of oral ulcers*	
	At baseline	*After treatment*	*At baseline*	*After treatment*	*At baseline*	*After treatment*
1	2.6 ± 0.8	1.3 ± 0.8[a]	17.7 ± 4.8	12.1 ± 4.1[a]	5.2 ± 3.7	3.1 ± 2.4[a]
2		1.0 ± 0.7[a]		9.9 ± 2.9[a]		2.5 ± 1.5[a]

Significant difference in the mean frequency, duration or number of oral ulcers between patients at baseline and patients after treatment with [a]$P < 0.001$ by paired *t*-test.

reduce the serum TNF-α concentration from 16.4 ± 1.9 to 5.8 ± 0.6 pg/ml ($P < 0.001$) in 55 RAU patients with the serum TNF-α level above 5.0 pg/ml (Table 4). A significant reduction of the serum TNF-α level from 19.0 ± 3.0 to 5.9 ± 0.9 pg/ml was observed after treatment with levamisole in 28 major type RAU patients for a period of 0.5–3 months ($P < 0.001$). Treatment with levamisole for a period of 0.5–4 months could also significantly reduce the serum TNF-α level from 13.2 ± 2.4 to 6.1 ± 0.9 pg/ml in 22 minor type RAU patients ($P < 0.01$). Although treatment with levamisole for a period of 0.5–1 month could reduce the serum TNF-α level from 15.7 ± 5.9 to 4.1 ± 0.8 pg/ml in five herpetiform type RAU patients, the difference was not significant ($P > 0.05$). The serum TNF-α levels of the treated RAU patients declined during the remission stage and elevated during the exacerbation stage (data not shown).

Comparison of clinical parameters in 28 major type, 22 minor type, and five herpetiform type RAU patients before and after treatment with levamisole for 1 or 2 months is shown in Table 5. We found that RAU

patients treated with levamisole only for 1 or 2 months showed a significant reduction in the frequency, duration, and number of the oral aphthous ulcerations ($P < 0.001$). These findings suggest that the drop in TNF-α levels in RAU patients is accompanied by a significant clinical improvement.

Discussion

This study showed that the mean serum TNF-α levels in patients with RAU including major, minor, and herpetiform types of RAU were significantly higher than that in normal control subjects. In 55 RAU patients with the serum TNF-α levels above 5.0 pg/ml, the relatively high-serum TNF-α levels could be significantly reduced to the normal level after treatment with levamisole for 0.5–4.0 months. This finding indicates levamisole can modulate the serum TNF-α levels in RAU patients. In this study, the mean serum TNF-α level was significantly higher in patients with major type RAU than in patients with minor type RAU and was significantly higher in major type RAU patients in the exacerbation stage than in the post-exacerbation stage. Furthermore, the serum TNF-α levels of the treated RAU patients declined during the remission stage and elevated during the exacerbation stage. These findings suggest that the serum TNF-α level may be associated with the severity and the stage of RAU.

The significant increase in serum TNF-α level in RAU patients in the active stage compared with the normal controls could be due to local and systemic production of TNF-α by many cell types. A variety of cell types including macrophages, CD4+ T cells, mast cells, and NK cells have been shown to produce TNF-α after stimulation with lipopolysaccharides (13), some viruses (14, 15), or parasites (16). Previous studies have shown the presence of mature tissue macrophages, recently recruited monocytes, CD4+ T cells, activated T cells, and mast cells in oral aphthous lesions (4, 9, 10). NK cells and activated T cells are also found in the peripheral blood of RAU patients (8, 11). Elevated levels of TNF-α mRNA have been detected in oral aphthous lesion (17). TNF-α has been immunolocalized in macrophages, lymphocytes, mast cells, and vascular endothelial cells in local oral aphthous tissues (18). Increased production of TNF-α by peripheral blood leukocytes is found in active RAU patients (19). Taking these findings together, we suggest that macrophages, lymphocytes, mast cells, and vascular endothelial cells in RAU lesional oral mucosa may be the local cellular sources of TNF-α, and peripheral blood monocytes, lymphocytes, NK cells, and other leukocytes as well as endothelial cells may be the systemic cellular sources of TNF-α.

The IL-1 and granulocyte-macrophage colony-stimulating factor (GM-CSF) augment TNF-α production by monocytes (20). IFN-γ, GM-CSF, and TNF-α itself can promote TNF-α synthesis and/or release from activated macrophages. TNF-α, in turn, induces IL-1 production by macrophages, IFN-γ production by fibroblasts and T lymphocytes, and GM-CSF production by several cell types (20, 28). Because the production of TNF-α is controlled by such a reciprocally stimulatory network of cytokines through cooperation of many cell types, we suggest that the significant increase in the serum TNF-α level in RAU patients in the active stage compared with the normal controls could be due to local and systemic production of TNF-α by many cell types through a complicated autocrine and paracrine cytokine stimulation mechanisms.

The reasons why treatment with levamisole can result in a significant improvement in symptoms and signs of RAU and can reduce the serum TNF-α level in RAU patients are still not clear. The results of previous studies on PBMC and TIMC favored that RAU may be a T-cell-mediated disease (3–7, 9–12). Levamisole has been found to immunomodule T-cell-mediated immunity (24–26). Our previous studies have shown that levamisole can reduce the abnormally high-serum IL-6 and IL-8 levels in RAU patients to the normal level. In addition, levamisole also has modulating effects on both cell-mediated and humoral immunities in the patients with RAU (27). Normalization of the decreased CD4+/CD8+ cell ratio and of the increased serum levels of immunoglobulin (Ig)A and IgM has been found in RAU patients after levamisole treatment (27). The reversion of aberrant cellular and humoral immunities after levamisole therapy may explain why RAU patients experience marked symptom improvement (27). Furthermore, healing or disappearance of RAU lesions after drug therapy may give rise to a reduction of the number of altered macrophages, lymphocytes, mast cells, and vascular endothelial cells in RAU lesional oral mucosa, which in turn resulting in a decrease in the production of TNF-α because these cells can secrete TNF-α in the local lesional tissues. In addition, normalization of the cellular immunity after drug therapy may also reduce the number of altered peripheral blood monocytes, lymphocytes, NK cells, and other leukocytes as well as endothelial cells that are capable of producing TNF-α systemically, or may reduce the secretion of cytokines such as IL-1, GM-CSF, IFN-γ, and TNF-α itself that are found to induce the production of TNF-α by monocytes and activated macrophages (20). Healing of local RAU lesions and normalization of the local and systemic cellular immunity may explain why treatment with levamisole can decrease the serum TNF-α level in RAU patients.

To conclude, a significantly higher than normal serum level of TNF-α can be detected in 20–39% of patients in the ulcerative stage of major, minor or herpetiform RAU. The serum TNF-α level may be associated with the severity and the stage of RAU. Levamisole can modulate serum TNF-α levels in RAU patients.

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