Immunomodulation by LongoVital in patients with recurrent aphthous ulceration


LongoVital (LV) (DK, Reg. No. 5178/75) is a herbal based tablet enriched with recommended doses of vitamins. Peripheral lymphocyte subsets: T-helper/CD4 (OKT4+) and T-suppressor/cytotoxic/CD8 (OKT8+) were studied quantitatively in 31 otherwise healthy patients with minor recurrent aphthous ulceration (RAU) during 6 months' daily LV intake in a double-blind, randomized, crossover 1-year study. Fourteen had had LV during the first 6 months (GrA) and 17 LV during the latter 6 months (GrB). OKT4+ percentages increased significantly during the LV period in both groups (P<0.05). OKT8+ percentages increased in both groups, however, only significantly in GrA (P<0.05). It is concluded that LV acts as an immunostimulator in patients with RAU and that the increase in T-lymphocyte subsets may account for the previously reported benefit of LV in RAU prevention.

Key words: aphthae; herbs; immunomodulation; LongoVital; mouth; diseases; natural killer cells; oral; T-lymphocytes; ulceration; vitamins.

Studies on peripheral blood have indicated the existence of a general immunoregulatory imbalance in patients with recurrent aphthous ulceration (RAU). Irrespective of disease stage, CD8+ (T-suppressor/cytotoxic cells) lymphocyte counts have been found elevated (1-3), CD4+ (T-helper cells) numbers depressed (1, 2), and the ratio CD4+ :CD8+ lowered (1-3) as compared with non-RAU healthy subjects. Furthermore, a significantly decreased number of cells bearing pan T-cell marker (CD3+) has been reported in the patients compared with controls (1).

Levamisole stimulates T-cell-medi ated immune functions and is so far the only systemic immunoregulatory drug which has been studied in the prevention of RAU in controlled trials. The drug has occasionally been reported to be beneficial in preventing RAU (4-6) but adverse effects are quite common (4-6), and consequently levamisole is not a feasible treatment.

In a double-blind, placebo-controlled study we have shown that daily intake of LongoVital (LV) for 6 months significantly reduces RAU recurrence rate in the absence of unwanted effects (7). LV (DK, Reg. No. 5178/75) is a tablet based on dried and ground herbs from pumpkin seeds, arnica flowers, rosemary leaves, paprika and milfoil flower supplemented with the nationally recommended doses of vitamins. The purpose of the present study was to investigate changes in immunologic parameters in RAU patients during 6 months' daily intake of LV.

Material and methods

The population consisted of 31 patients with minor RAU (19F, 12 M), mean age 36.5 yr (18-67). The average number of recurrences the previous year was 12.4 (3-30), and the mean duration of RAU experience was 19.5 yr (3-41). Apart from one female suffering from myxedema, systemic diseases as well as extraoral manifestations along with RAU were absent. Objectively, all patients were in physical as well as psychologic good health and oral diseases apart from RAU were not diagnosed. Eleven women had a regular medicine intake, 7 used contraceptives, 3 postmenopausal hormones and 1 thyroid hormones. The patients participated in a previously described double-blind study where the effect of LV in the prevention of RAU was investigated (7). The present study includes the 2 patients who had had no recurrences during the study period.

The protocol was approved by the local ethics committee, and informed consent to participate was obtained according to The Declaration of Helsinki II. The investigation was designed as a double-blind, randomized 1-yr crossover study where patients daily took 3 tablets of LV or placebo together with breakfast for 6 months. A wash-out period was omitted as the study design took into account a potential carry-over effect of 60 days. Peripheral blood samples were obtained at the beginning of the study (Day 0), after 2 months (Day 60), 6 months (Day 180), 8 months (Day 240) and finally after 12 months (Day 360) in order to determine differential white blood cell counts, serum levels of immunoglobulins A, G, M, and E as well as percentages of T-helper cells (CD4+), T-suppressor/cytotoxic cells (CD8+) and natural killer (NK) cells (CD45 RA, Leu-18+). All samples were collected between 8:00 and 11:00 AM, and to further control for circadian periodicity, the five samples of each patient were obtained within the same hour.

Differential counts and serum immunoglobulins were determined by routine methods. Lymphocytes were isolated from blood samples after 30 min centrifugation on Lymphoprep at 20°C. After washing three times with RPMI-1640, 10⁶ lymphocytes were labelled with 10 μl, 1:20 diluted mouse monoclonal antibodies OKT4, OKT8 (Ortho-Mune, or
Table 1. Differential white blood cell counts and immunoglobulin levels (medians) during daily intake of LongoVital (LV) and placebo in double-blind, cross-over, 1-yr trial on 31 patients with minor RAU.

<table>
<thead>
<tr>
<th></th>
<th>LV in the first period</th>
<th>Placebo</th>
<th>LV in the second period</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=14</td>
<td></td>
<td>n=17</td>
<td></td>
</tr>
<tr>
<td>Normal range</td>
<td>0  60  180  240  360</td>
<td></td>
<td>0  60  180  240  360</td>
<td></td>
</tr>
<tr>
<td>Leukocytes (10^9/L)</td>
<td>3.0–9.0</td>
<td></td>
<td>4.3  4.8  4.2  4.4  4.3</td>
<td></td>
</tr>
<tr>
<td>Neutrophils (10^9/L)</td>
<td>1.8–7.4</td>
<td></td>
<td>2.7  2.8  2.4  2.8  2.6</td>
<td></td>
</tr>
<tr>
<td>Eosinophils (10^9/L)</td>
<td>&lt;0.45</td>
<td></td>
<td>0.12  0.15  0.08  0.13  0.15</td>
<td></td>
</tr>
<tr>
<td>Basophils (10^9/L)</td>
<td>&lt;0.20</td>
<td></td>
<td>0.04  0.02  0.03  0.03  0.04</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes (10^9/L)</td>
<td>0.7–4.8</td>
<td></td>
<td>1.7  1.7  1.4  1.6  1.4</td>
<td></td>
</tr>
<tr>
<td>Monocytes (10^9/L)</td>
<td>&lt;0.80</td>
<td></td>
<td>0.19  0.20  0.26  0.27  0.21</td>
<td></td>
</tr>
<tr>
<td>Total mononuclear cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(lymphocytes+monocytes)</td>
<td>0.7–5.6</td>
<td></td>
<td>1.8  1.8  1.6  1.7  1.9</td>
<td></td>
</tr>
<tr>
<td>IgA (mmol/L)</td>
<td>3.8–21</td>
<td></td>
<td>14.0  15.0  15.0  16.0  15.0</td>
<td></td>
</tr>
<tr>
<td>IgG (mmol/L)</td>
<td>36–89</td>
<td></td>
<td>83  84  89  89  89</td>
<td></td>
</tr>
<tr>
<td>IgM (mmol/L)</td>
<td>0.3–3.7</td>
<td></td>
<td>1.4  1.6  1.6  1.5  1.4</td>
<td></td>
</tr>
<tr>
<td>IgE (KIU/L)</td>
<td>&lt;150</td>
<td></td>
<td>29  27  29  27  37</td>
<td></td>
</tr>
</tbody>
</table>

1Day 0, 60, 180 values significantly different, P<0.01, Friedman
2Day 180, 240, 360 values significantly different, P<0.01, Friedman
3Day 0, 60, 180 values significantly different, P<0.05, Friedman

Leu-18 (Becton Dickinson) for 30 min at 4°C. After renewed washing, the cells were incubated for 30 min with 100 μL 1:30 diluted FITC-conjugated rabbit anti-mouse-Ig (anti-kappa and anti-lambda) (Dakopatts Copenhagen, Denmark). Finally, the washed cells were stored in 150 μL 1% formalin at 4°C and quantified in a fluorescence activated cell sorter (FACS IV, Becton Dickinson). Lymphocyte subsets were determined as percentages of the total number of mononuclear cells (lymphocyte plus monocyte counts).

Statistical analysis – The Friedman test was applied for the statistical analysis of variance and P-values below 0.05 were considered significant.

Results
Before breaking the trial code, data were analyzed for the whole population disregarding the order of treatment. After the code was broken, it appeared that 14 patients had taken LV during the first 6 months (Day 0–180) (GrA), and 17 LV during the second period (Day 180–360) (GrB). Figures on the various parameters in GrA indicated a sustained effect of LV lasting throughout the entire 6 months on placebo. Data analysis disregarding treatment order was therefore considered inappropriate as differentiation between carry-over effect and any possible effect of time would be impossible. Hence we decided to perform statistical analyses on GrA and GrB individually.

Differential white blood cell counts – Numbers of leucocytes, neutrophils, eosinophils and basophils did not change significantly during the study period in any of the treatment groups (Table 1). In both groups, the mean lymphocyte counts were statistically significantly different during the first 6 months with an average decrease of the mean on 18% in GrA (Day 0/180:1.7/1.4; P<0.01) and 14% in GrB (Day 0/180:2.1/1.8; P<0.05) from Day 0 to Day 180. No significant differences were demonstrated during the last 6 months in any of the groups. In GrA, the monocyte count increased over the period from Day 0 to Day 180 with an average of the mean on 78% (Day 0/180:0.18/0.32; P<0.01). The total number of mononuclear cells (lymphocytes plus monocytes) did, however, not change significantly during any of the intervals in neither GrA nor GrB.

Immunoglobulin levels – No significant changes in plasma IgA, IgG and IgE levels were demonstrated in any of the groups during the study period (Table 1). In GrA, the mean IgM level was significantly different during the 6 months after discontinuation of LV (P<0.01) with an average decrease on 13% from Day 180 to Day 360 (Day 180–360) (GrB).
Table 2. Analysis of variance (Friedman) of lymphocyte subset percentages during daily intake of LongoVital (LV) and placebo in double-blind, cross-over, 1 yr trial on 31 patients with minor RAU.

<table>
<thead>
<tr>
<th></th>
<th>LV during day 0-180</th>
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<th>LV during 180-360</th>
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<tbody>
<tr>
<td></td>
<td>0 60 180 240 360</td>
<td>n=14</td>
<td>0 60 180 240 360</td>
<td>n=17</td>
</tr>
<tr>
<td>OKT4</td>
<td>* (t) NS</td>
<td></td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>OKT8</td>
<td>* (t) NS</td>
<td></td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>4/8</td>
<td>NS ** (t)</td>
<td></td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Leu-18</td>
<td>NS NS ** (t)</td>
<td></td>
<td>**** (t)</td>
<td>* (t)</td>
</tr>
</tbody>
</table>

NS not significant; *P<0.05; **P<0.01; ****P<0.0001; (t) (t) direction of the shift.

180/360 = 1.81.6). In GrB, the IgM level decreased with an average of the mean on 15% during the 6 months' LV period (Day 180/360=2.0/1.7; P<0.01).

OKT4+ – In GrA, the OKT4+ mean count was statistically different during the 6 months on LV (P<0.05) with an average increase of 23% from Day 0 (x̄ = 20.7) to Day 180 (x̄ = 25.5) (Fig. 1, Table 2). No significant changes appeared during the study period even if a further increase on an average of 8% took place from Day 180 to Day 360 (x̄ = 27.6).

In GrB, no significant changes in OKT4+ percentages appeared over the placebo period. During the 6 months on LV, however, the mean was significantly different (P<0.05) and an average increase on 29% had taken place from Day 180 (x̄ = 21.3) to Day 360 (x̄ = 27.3).

OKT8+ – In GrA, the mean of OKT8+ counts were significantly different over the 6 months on LV (P<0.05) with an average increase on 31% from Day 0 (x̄ = 11.4) to Day 180 (x̄ = 16.6) (Fig. 2, Table 2).

No significant changes took place during the following 6 months on placebo even if the increase declined slightly from Day 180 to Day 360 (x̄ = 14.6). In GrB, no significant changes in the OKT8+ counts were demonstrated during the placebo period. On LV, OKT8+ figures increased with an average of 42% from Day 180 (x̄ = 11.9) to Day 360 (x̄ = 16.9). However, the values on Day 180, 240 and 360 were not significantly different.

Ratio OKT4+:OKT8+ – The OKT4+:OKT8 ratio remained statistically unchanged during the LV period in both groups, but in GrA, the ratio was significantly different during the 6 months after discontinuation of LV (P<0.01) with an average increase on 18% from Day 180 (x̄ = 2.7) to Day 360 (x̄ = 2.0) (Fig. 3, Table 2).

Leu-18+ – In GrA, no significant changes in Leu-18+ counts took place during the entire study period (Fig. 4, Table 2). In GrB, Leu-18+ values were significantly different (P<0.0001) during the 6 months on placebo with an average decrease on 76% from Day 0 (x̄ = 3.6) to Day 180 (x̄ = 2.0). On LV, Leu-18+ counts were also significantly different (P<0.05), but in this period an increase on 110% appeared from Day 180 to Day 360 (x̄ = 4.2).

Discussion

The previously reported improvement of RAU due to the intake of LV for 6 months (7) must at least partly be ascribed to the hereby demonstrated increase of OKT4+ and OKT8+ counts as pan lymphocytopenia (decreased OKT3+ counts) (1) and decreased OKT4+ counts has been demonstrated in RAU patients as compared with non-RAU subjects (1, 2). It seems, however, somewhat surprising that OKT8+ counts increased in the present study considering that increased OKT8+ percentages might be a characteristic feature of RAU patients (1–3). Meanwhile, the number of circulating lymphocytes might not be related to the functional state of the cells, and the effect of LV on T-lymphocyte responsiveness was not evaluated in the present study.

Reduced in vitro T-lymphocyte responses to standard mitogens (phytohemagglutinin (PHA) and concanavalin A (Con A)) has occasionally been reported in RAU patients (8, 9). Levamisole is an immune modulator which restores the number of circulating T-lymphocytes and enhances their in vitro response to PHA and Con A (10). One of both functions might account for the reported positive effect of levamisole in preventing RAU (4–6). The increase in OKT4+ and OKT8+ counts implies a contemporary decline of B-lymphocyte numbers which is somewhat supported by the decrease of serum IgM during LV intake in GrB, and over the 6 months after discontinuation of LV in GrA. The decline of IgM levels along with the observed clinical improvement of RAU might support the occasionally reported increased IgM levels in the patients (11).

In both treatment groups, the OKT4:OKT8 ratio remained unchanged dur-
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Fig. 3. OKT4:OKT8 ratios in peripheral blood during daily intake of LongoVital (LV) and placebo in double-blind, cross-over, 1-yr trial on 31 minor RAU patients. Horizontal bars indicate the medians and vertical bars one standard deviation.

Fig. 4. Percentages of Leu-18+ cells in peripheral blood during daily intake of LongoVital (LV) and placebo in double-blind, cross-over, 1-yr trial on 31 minor RAU patients. Horizontal bars indicate the medians and vertical bars one standard deviation.

The increase in the OKT4+ subpopulation seems more pronounced and lasting longer than the increase in the OKT8+ subpopulation. Leu-18 detects a complex of peripheral lymphocyte subsets, including “virgin” T-lymphocytes but mainly NK cells (12). Investigations employing different test systems have substantiated a high susceptibility of NK cell function to the state of the mind (13–17). Meanwhile, the influence of psychologic interventions on the circulatory numbers has not been studied, but the considerable decline in Leu-18+ counts in the present study during the first 6 months in GrB might very well reflect an interesting psychologic effect of the placebo treatment. LV seems to induce an augmentation in Leu-18+ counts as figures increased on LV in GrB. Leu-18+ figures remained unchanged during the entire study period in GrA, and if applying the results from GrB, the unchanged Leu-18+ counts in GrA might be ascribed reverse reactions of LV and the psychologic placebo effect.

It is concluded from the present study that LV acts as an immunostimulator in patients with RAU. The stimulation implies enhancement of T-helper, T-suppressor/cytotoxic and possibly also NK cell percentages in peripheral blood. The increase of T-lymphocyte counts due to LV intake may account for the contemporary clinical benefit of LV in RAU as low T-lymphocyte percentages seem to be a characteristic feature of the disease. Investigations on the effect of LV in other diseases with immunoregulatory imbalances seems to be a challenge for the future.

Acknowledgements — We thank Ms. Jette Pedersen, Bartholin Institutet, Kommunehospital, Copenhagen, for valuable technical assistance.

References


5. MILLER MF, SILVERT ME, LASTER LL, GREEN P, SHIP II. Effect of levamisole on