NATIONAL INSTITUTE OF CHEMICAL SAFETY

REPORT

The effect of d-Lenolate® on the immune parameters of healthy volunteers

Budapest
2011
Report
The effect of d-Lenolate® on the immune parameters of healthy volunteers

Antecedents

KAQUN HUNGÁRIA Ltd. (2144 Kerepes, Szabadság út 102), as Client has contracted the National Institute of Chemical Safety/NICS (1096 Budapest, Nagyvárad tér 2.) as contractor in contract no. GOKBI-90/2011 to test the immune effects of d-Lenolate® in healthy volunteers at the Department of Cytogenetics and Immunology of NICS. d-Lenolate (Olive Leaf Extract) is a dietary supplement patented by East Park™ Research, Inc. d-Lenolate formulation is prepared on a patented extraction process of selected olive leaves that contain Oleuropein. Each capsule contains 500 mg olive leaf (Olea Europaea) extract. In our study we examined the effect of 21 days of d-Lenolate® treatment on the immune parameters of healthy volunteers. The end points measured were: qualitative and quantitative blood counts, the ratio of lymphocyte populations, lymphocyte activation and the oxidative burst of neutrophil granulocytes. The measurements were carried out on the first day before the start of the treatment (0 point) and on the 8th, 15th and 21st days.

The theoretical basis of immunology tests

Immune-toxicology examines the damaging/modifying effects caused by exposure at the workplace, environment or therapy on the immune system. Its task is to detect and assess the modifying factors affecting the immune system especially from the aspect of their effect on human health. An immune response may be elicited when the immune system is the passive target of a chemical agent or when the chemical, as an antigen, triggers a specific response. In consequence of the complexity of the immune system the chemical agents have a broad target of attack. They can affect the development, maturation, division, differentiation and function of cells, or modify the regulation of the immune system.

The immunology tests were carried out on peripheral blood samples. White blood cells have an important role in the defence mechanisms of the body. Blood contains an average of 9x10^9 / l white blood cells, but 4-10 x 10^7 / l is also within the normal range. There are 3 main types of leukocytes: lymphocytes, monocytes and granulocytes. 20-45 % of leukocytes of a healthy person are lymphocytes, 2-9 % are monocytes and 50-75 % are granulocytes. Normally the lymphocyte count is in the range of 1.5-3.5 x 10^9 / l, and their importance lies in mediating the adaptive immune response. They are relatively small cells, their round shaped nucleus fills the cytoplasm almost completely. Lymphocytes are classified into 3 main groups: T and B lymphocytes and NK-cells. During the adaptive immune response cytotoxic T (Tc) cells are generated which are able to destroy the pathogens directly (cellular immune response), and B lymphocytes, which produce antibodies (humoral immune reaction). The presence of helper T lymphocytes (Th) is essential for the division and differentiation of T and B cells. NK cells kill virus infected or cancerous cells. Monocytes make up about 2-9 % of the white blood cells (1-8 x 10^8 /l), their nucleus is large, kidney or bean shaped. They originate from the bone marrow, they then enter the circulation where they spend about 72 hours, and then pass through the blood vessel wall and change into tissue macrophages. Their activation is initiated by lymphokines secreted by T lymphocytes, and as a result they become able to phagocytose foreign matter such as bacteria, and to release a number of inflammatory mediators.
The nucleus of granulocytes becomes lobed as they mature. Another characteristic feature is the presence of large quantities of granules in the cytoplasm – the biologically active material stored within them has a very important role in the development of inflammatory and allergic reactions. The **neutrophil**, **basophil** and **eosinophil granulocytes** can be distinguished on the basis of their histological staining properties. Most of the granulocytes are **neutrophils** (3-6 x 10<sup>9</sup>/l). Since their half life in the circulation is short, (generally ~6 hours), they are produced in large quantities every day. They are the basis of cellular protection against infection, and can enter the tissues in large quantities. In the course of bacterial or fungal infection the neutrophil granulocytes phagocytose and destroy the pathogens. The intracellular killing of pathogens is achieved by oxygen-independent enzymes (lysosomal elastase, lysozyme) and oxygen-dependent enzymatic systems (principally NADPH-oxidase). The activated phagocytic cells produce antimicrobial reactive radicals, so called reactive oxygen intermediates (ROI) in a reaction named oxidative burst.

A number of molecules, "markers" appear on the surface of lymphocytes and with their help the lymphocyte populations can be distinguished from each other. These markers have been classified into groups, and each marker has been given a CD (Cluster of Differentiation) number. The basic lymphocyte populations (T, B, NK cells) can be defined with cell markers: **T lymphocytes** express CD3 (CD3+ cells), **helper T cells** also express CD4 (CD4+/CD3+ cells), **cytotoxic T cells** express CD8 besides CD3 (CD8+/CD3+ cells). Immature T cells express both the CD4, and the CD8 molecules (CD4+/CD8+ cells). **B lymphocytes** can be characterized by the CD19 cell surface antigen (CD19+cells). **NK cells** have CD56 surface molecules, but do not express CD3, therefore they are characterized as CD56+/CD3- cells. CD25 (IL-2 receptor) and CD71 (transferrin receptor) surface antigens cannot be detected on resting lymphocytes, they are expressed when the lymphocytes are activated (e.g. by an antigen). Therefore these surface molecules can be used to detect the activation of lymphocytes.

**Immunotoxic materials** can affect different immune parameters; therefore we have adjusted our measurements to characterize different parameters. This is important, because the change in one parameter or another is not suitable to characterize the general condition of the immune system, conclusions can only be drawn from changes in the data pattern. We characterized the immune status of the studied subjects by measuring characteristics of white blood cells gained from peripheral blood. Qualitative and quantitative blood count was determined, and immune phenotyping was used to determine lymphocyte subpopulations and the CD25 (IL-2R) and CD71 (transferrin receptor) activation antigens expressed on lymphocytes with the aid of monoclonal antibodies produced against cell surface molecules. Innate immunity was characterized with the help of a functional test: **the killing capacity of white blood cells** was determined by measuring the production of reactive oxygen intermediates (ROI) of granulocytes.
Test procedure

Selection of healthy volunteers
The selection of 30 healthy volunteers (15 women, 15 men) was carried out by KAQUN HUNGÁRIA Kft. Exclusion criteria in this study were: smoking, acute or chronic illness, infection, the use of any kind of drugs or dietary supplements, because these could affect immune parameters.

The participants were informed about the purpose and the course of the study, and they signed a Declaration of Agreement confirming that they had received information about the study and that their participation was voluntary.

Duration of the study and the procedure:
The examined persons participated in a 21 day d-Lenolate treatment which consisted of taking 2 capsules 3 times a day. The measurements were done on the first day, before treatment (0-point), then on days 8, 15, and 21.

Methods:

Blood sampling: Blood sampling at the site: day 1 before the treatment, (0-point), then on days 8, 15, and 21 after the first 2 capsules of d-Lenolate, during the same part of the day. The blood samples were taken from the cubital vein of the examined persons in sitting position, under sterile conditions with venipuncture. Standard 3 ml sterile vacuum blood sampling tubes containing anti-coagulant were used for blood sampling. One 3 ml tube with EDTA anti-coagulant for determining the qualitative and quantitative blood count, one 3 ml tube with heparin for the immunology tests. The blood samples were given unique identifiers marked on the blood sampling tubes.

The following tests were carried out on the blood samples:

1) Qualitative and quantitative blood count
The qualitative and quantitative blood count was carried out with an automated analyser in the blood sampling laboratory of the Hungarian Institute of Occupational Health (Bp. IX. Nagyvárad tér 2.).

Determined parameters:
- WBC leukocyte count
- abs LY, abs MO, abs NEUTR, abs EO: the absolute number of lymphocytes, monocytes, neutrophil- and eosinophil granulocytes
- LY %, MO %, NEUTR %, EO %, BA %: percentile distribution of lymphocytes, monocytes, neutrophil- eosinophil- and basophil granulocytes
- RBC red blood cell count
- Hb concentration of hemoglobin in the blood
- HTK hematocrit
- MCV mean cell volume
- MCHC mean corpuscular hemoglobin concentration
- RDW-CV red blood cell distribution width
- MCH mean cell hemoglobin
- Thrombocyte count
2) Determination of qualitative immune parameters (immune phenotyping)

The subpopulations and activation of circulating lymphocytes were determined by immune phenotyping, using flow cytometry. Heparinised whole blood was used for the measurement. The surface markers of peripheral lymphocytes were measured with fluorescent labelled monoclonal antibodies in a flow cytometer. The surface antigens examined were: CD3 (T-cell receptor), CD4 and CD8 (T-cell co-receptors), CD19 (B-cell co-receptor), CD25 (interleukin-2 receptor), CD45 (protein-tirosine-phosphatase, pan leukocyte marker), CD56 (neural cell adhesion molecule, NK-cell marker), CD71 (transferrin receptor). Using 3 and 4 colour staining the following antibody combinations were used: (1) CD25-FITC / CD8-PE / CD3-PerCP / CD4-APC; (2) CD56-FITC / CD3-PerCP / CD45-APC; and (3) CD71-FITC / CD3-PerCP / CD19-APC. Standard forward and side scatter gating combined with CD45 was used to separate leukocyte populations and to set the lymphocyte gate. The lymphocyte subpopulations of the donors (T lymphocyte, helper T, cytotoxic T, B lymphocyte and NK-cell) were determined with the aid of cell markers. CD25 and CD71 surface antigens were used to determine the activation of lymphocytes.

Determined parameters:

- **Ly, Mo, Neu, Eos**: percentage of lymphocytes, monocytes, neutrophil- and eosinophil granulocytes
- **Total T, T helper, T cytotoxic, Immature T, B cell, NK-cell**: percentage of T lymphocytes, cytotoxic and helper T lymphocytes, immature T lymphocytes, B lymphocytes and NK-cells within lymphocytes
- **Th/Tc**: The ratio of helper and cytotoxic T lymphocytes
- **Activated T**: percentage of CD25 (IL-2 receptor) activation antigen carrying T cells within the T cells
- **Activated Th**: percentage of CD25 activation antigen molecule carrying helper T cells within the helper T cells
- **Activated Tc**: percentage of CD25 activation antigen expressing cytotoxic T lymphocytes within the cytotoxic T lymphocytes
- **CD71 positive T**: percentage of CD71 (transferrin receptor) molecule carrying T cells within the T cells
- **CD71 positive B**: percentage of CD71 (transferrin receptor) molecule carrying B cells within the B cells

3) Determination of functional immune parameters

**Measurement of killing capacity of neutrophil granulocytes (reactive oxygen intermediate measurement)**

The production of reactive oxygen intermediates (ROI) which is directly proportional with the killing potential of white blood cells was measured with the aid of Bursttest (Phagoburst®) kit. Neutrophil granulocytes respond to activation by producing reactive oxygen intermediates, which oxidize the fluorogenic substrate. The quantity of oxidized substrate is proportional to the production of reactive oxygen radicals. Heparinized whole blood was used, and the measurement was carried out on a flow cytometer. We measured the quantity of oxidized substrate in the control and the stimulated samples, and determined the percentage of ROI producing cells. The activation stimuli: 1) fMLP chemotactic peptide (weak stimulus). 2) E. coli opsonized with antibody, which stimulates through the Fc receptors that recognize the
constant part of the antibody (particulate stimulus) 3) PMA (phorbol-myristil-acetate), which transports signals through protein kinase C (strong stimulus)

**Determined parameters:**

**Production of reactive oxygen intermediates (ROI)**
- Control, fMLP, E. coli, PMA: ROI production in unstimulated samples, and samples stimulated with fMLP, E. coli, and PMA

**Percent of ROI producing cells**
- Control, fMLP, E. coli, PMA: Percent of ROI producing cells in unstimulated samples, and samples stimulated with fMLP, E. coli, and PMA

**Statistical analysis:**

Student’s paired-t test was used for the group level statistical evaluation of the results, the level of significance was set at p<0.05.

**Results and conclusions**

One person (L114) did not show up at the last measurement.

1) **Qualitative and quantitative blood count**

The group results of qualitative and quantitative blood counts are shown in *Table 1.*, the individual results in *Table 2.* The absolute numbers of leukocytes at the group level and individual level showed little changes, and individually remained within the normal range in most cases. Biologically significant change was not observed in the qualitative and quantitative blood count either at group or individual level, except for two cases. The subjects coded L16/46/76/106 and L21/51/81/111 had a highly increased leukocyte count (caused by the increase in neutrophil count) at the time of the last measurement, compared to the previous week.

2) **Determination of qualitative immune parameters (immune phenotyping)**

The measurements carried out with the flow cytometer produced very similar results to those carried out with the automated analyser regarding the percentile distribution of lymphocytes, monocytes, neutrophil- and eosinophil granulocytes. This can be considered as the internal control of the measurements.

**Results at the group level**

The group averages of immune parameters are shown in *Table 3.* The percentage of activated (CD25+) T lymphocytes increased statistically significantly from the first week compared to the 0 point, and the same was observed in the case of activated (CD25+) helper T cells. The percentage of activated (CD25+) cytotoxic T cells increased significantly on the first and the third week compared to the initial value. The percentage of transferrin receptor positive (CD71+) T and B lymphocytes also increased at the first and third week of treatment.
compared to the 0 point. We found no gender differences: the same results could be observed for the whole group, and also for men and women.

In the course of the treatment the ratio of leukocytes changes statistically, but the changes are so small that probably no physiological importance can be attached to them. No significant changes were observed in the ratio of lymphocyte subpopulations (total T cells, helper T cells, immature T cells and B lymphocytes).

**Individual results**

Figure 1. shows the changes in T and B lymphocyte activation after 3 weeks treatment with d-Lenolate at the individual level. The activation of T lymphocytes increases after 3 weeks treatment compared to the initial values: the ratio of CD25+ T cells increases in 89.7 %, is unchanged in 6.9 %, and decreases in 3.4 % of the subjects compared to initial values. The same tendency can be found in the case of helper T lymphocytes: the ratio of activated (CD25+) helper T cells increases in 96.6 %, and decreases in only 3.4 % of the subjects at the end of treatment compared to initial values. The ratio of CD25 positive cytotoxic T cells increases in more than half of the investigated subjects (55.2 %) remains unchanged in 41.4 and decreases in 3.4 % compared to the 0 point. The ratio of CD71+ T cells increases in half of the subjects, and stays unchanged in the remaining half. The activation of B lymphocytes also increases, the ratio of CD71+ B increases in 75 % of the subjects and remains unchanged in 25%.

Fig. 1. Activation of lymphocytes after 3 weeks treatment with D-lenolate®

<table>
<thead>
<tr>
<th></th>
<th>CD25+/CD3+</th>
<th>CD25+/CD4+</th>
<th>CD25+/CD8+</th>
<th>CD71+/CD3+</th>
<th>CD71+/CD19+</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>Unchanged</td>
<td>Decrease</td>
<td>Increase</td>
<td>Unchanged</td>
<td>Decrease</td>
</tr>
<tr>
<td>CD25+/CD3+ Activated T cell</td>
<td>69.3</td>
<td>3.4</td>
<td>25.0</td>
<td>89.7</td>
<td>6.9</td>
</tr>
<tr>
<td>CD25+/CD4+ Activated Th cell</td>
<td>96.6</td>
<td>3.4</td>
<td>0.0</td>
<td>96.6</td>
<td>25.0</td>
</tr>
<tr>
<td>CD25+/CD8+ Activated Tc cell</td>
<td>55.2</td>
<td>3.4</td>
<td>41.4</td>
<td>55.2</td>
<td>3.4</td>
</tr>
<tr>
<td>CD71+/CD3+ CD71+ T cell</td>
<td>50.0</td>
<td>0.0</td>
<td>50.0</td>
<td>50.0</td>
<td>0.0</td>
</tr>
<tr>
<td>CD71+/CD19+ CD71+ B cell</td>
<td>75.0</td>
<td>0.0</td>
<td>25.0</td>
<td>75.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

The individual results of immune parameters are shown in Table 4. Individually both increased and decreased white blood cell and lymphocyte percentages could be observed during the three weeks of the study, the changes are usually minimal, and the values remained in the normal range in most cases. In the two subjects mentioned in the Qualitative and quantitative blood count section (L16/46/76/106 and L21/51/81/111) the percent of lymphocytes decreased at the last measurement, but this was caused by the increase in neutrophil count, as stated above, and not by a drastic decrease in the number of lymphocytes.
3) Determination of functional immune parameters

Measurement of killing capacity of neutrophil granulocytes (reactive oxygen intermediate measurement)

Results at the group level

The group averages for the production of reactive oxygen intermediates (ROI) of neutrophil granulocytes are shown in Table 5. The ROI production of neutrophil granulocytes increased significantly in both the control and the stimulated samples (fMLP, E. coli, PMA) in the whole group and in men and women from the first week of the treatment. Moreover, ROI production increases with every week in every sample. The percentage of ROI producing cells does not change significantly by the treatment with D-lenolate.

Individual results

Figure 2. shows the individual changes in ROI production of neutrophils after 3 weeks treatment with d-Lenolate. In the case of fMLP 96.6 % of the subjects showed an increase in ROI production while 3.4 % showed no change. In the case of E. coli and PMA stimulation 100% of subjects showed increased ROI production after three weeks of treatment compared to initial values.

![Figure 2. ROI production after 3 weeks treatment with D-lenolate®](image)

The individual results for the production of reactive oxygen intermediates (ROI) of neutrophil granulocytes are shown in Table 6. Similarly to group results, the individual results show a weekly rise.
Summary

In our study we examined the effect of 21 days of d-Lenolate® treatment on the immune parameters of healthy volunteers. The end points measured were: qualitative and quantitative blood counts, the ratio of lymphocyte populations, lymphocyte activation and the oxidative burst of neutrophil granulocytes. The measurements were carried out on the first day before the start of the treatment (0 point) and on the 8th, 15th and 21st days. Student’s paired-t test was used for the group level statistical evaluation of the results, the level of significance was set at p<0.05.

A non-specific activation of T lymphocytes (indicated by the increase in the expression of the CD25 and CD71 cell surface antigens) could be detected, presumably caused by the D-Lenolate treatment, indicating the increased activity of the immune response.

The increase of the production of reactive oxygen intermediates both at group level and at individual level shows the intensification of the killing potential of neutrophil granulocytes.

No biologically significant changes were observed in the qualitative and quantitative blood count either at group level or individual level during the 21 days of D-Lenolate treatment.

The changes in the ratio of lymphocyte subpopulations are so small that it they probably do not have a physiological relevance.

10th of June 2011

Dr. Anna Biró
head of department

Dr. Gyula Sebestyén
advisor
associate professor