Influence of Peroral Application of a Herbal Immunomodulator on the Antibody Production of Peyer’s Patches Cells

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Summary

The influence of the peroral administration of the macromolecular components of a herbal immunomodulator isolated from an aqueous-ethanolic extract of the mixed herbal drugs \textit{Thujasummitate}, \textit{Baptisia tinctoriae} radix, \textit{Echinacea purpureae} radix and \textit{Echinacea pallidae} radix on the function of Peyer’s patches cells was investigated in mice.

Peyer’s patches cells isolated from mice which had received oral administration of the macromolecular fraction of the plant extract developed a significantly enhanced plaque-forming cell (PFC) response to sheep red blood cells after incubation with these cells in the presence of lipopolysaccharide or the extract fraction for 7 days in vitro. These results show that after oral administration of the herbal immunomodulator, the immunologically active macromolecules can contact the cells of the gut-associated lymphoid tissue and modulate the mucosal immune response.

Zusammenfassung

Einfluß der peroralen Applikation eines pflanzlichen Immunmodulators auf die Antikörperproduktion von Peyer’schen Plaques-Zellen


Die Ergebnisse zeigen, daß nach peroraler Applikation des pflanzlichen Immunmodulators seine immunologisch wirksamen Makromoleküle mit den Zellen des darmsozialisierten Immunsystems in Kontakt treten und eine Modulation ihrer Immunität induzieren.

Key words

- \textit{Baptisia} spp.
- \textit{Echinacea} spp.
- Immunomodulator, herbal,
peroral application
- Peyer’s patches
- \textit{Thuja} spp.

1. Introduction

Echinacea purpurea (L.) Moench, Echinacea pallida (Nutt.) Nutt., Baptisia tinctoria L. und Thuja occidentalis L. belong to the most important medicinal plants used as immunomodulators. Extracts of them or expressed sap of the aerial parts of Echinacea species are mainly used to overcome a susceptibility to infections due to a temporary immunodeficiency, for the therapy of acute and chronic respiratory tract infections and as adjuvant to antibiotic therapy in severe bacterial infections [1-3].

The positive effects of preparations from these medicinal plants on different immune parameters have been shown in many pharmacological studies. Among other things a stimulation of phagocytosis activity, an enhanced cytokine production of macrophages, a stimulation of the proliferation of murine spleen cells and an increase of antibody production could be proven [4-9]. Almost all of these experiments were performed in vitro using isolated immune cells. In case of in vivo investigations test fractions were mostly applied i.v. or i.p.

We could show in previous publications, however, that also peroral application, as it is commonly used in human therapy, had positive effects on the antibody production and on the cytokine production of spleen cells and peritoneal macrophages and provided a protection against influenza A virus infection in Balb/c mice [10-13].

The points of attack and the mechanisms of action of orally applied extracts, however, are still unclear. It is assumed that the active constituents in these herbal immunomodulators are macromolecules (polysaccharides and/or glycoproteins) [14, 7-9], which do not act systemically but exert their effects by a direct contact with the cells of the mucosa-associated lymphoid tissue (MALT) [15]. The experimental evidence for this theory is still missing.

To verify this hypothesis we tested in the experiments described here, the influence of the peroral application of the macromolecular constituents of these plants on the MALT with special focus on the Peyer’s patches (PP). PP appear as white areas in the submucosa of the small intestine and are relatively easy to isolate. They are the main components of the gut-associated lymphoid tissue (GALT) that together with the bronchial-associated tissue forms the MALT. The epithelium overlying the lymphoid follicles of the PP contains specialised pinocytic cells (called M cells) which are able to engulf high molecular weight molecules which have entered the intestinal tract.

In our experiments we applied the retentate (cut-off 10,000 Dalton) of an aqueous-ethanolic extract of a mixture of Thujae summitiae, Baptisiae tinctoriae radix, Echinaceae purpureae radix and Echinaceae pallidae radix to mice by gavage. After 15 h the antigen-specific antibody-production of isolated PP cells was investigated by means of an in vivo-in vitro variant of the plaque-forming cell assay (PFC assay) [16].

The combination of drugs was used for the experiments because its efficacy has been proven clinically and it had been shown in earlier investigations that the extract of this drug mixture exerts stronger and more reliable effects than extracts of the single drugs [10].

2. Materials and methods

2.1. Manufacturing of the extract

Thujae occidentalis herba (Alfred Galke, Göttelborn, Germany), Baptisiae tinctoriae radix (Paul Miggenreburg, Hamburg, Germany), Echinaceae purpureae radix (Dieter Müller, Staufenberg, Germany) and Echinaceae pallidae radix (Berghof-Kräuter, Heilbronn, Germany) were checked for identity and purity using the specifications of the quality control department of Schaper & Brümmel, Salzgitter (Germany). A mixture of the drugs was percolated exhaustively with 30 % EtOH identical to the manufacturing specification for Esberitox® N (Schaper & Brümmel). The extract was characterised by TLC and HPLC [11]. For the preparation of the retentate fraction, the extract was filtrated through a deep-bed filter, dialysed at room temperature against demineralised water in the dialysis device (SP 20, Amicon, Eschborn, Germany) via an S10Y10 cartridge (cut-off: 10,000 Dalton, Amicon) and freeze-dried. The amount of polysaccharides and glycoproteins in the retentate was quantified by means of a specific ELISA [29].

2.2. Experimental animals

The tests were carried out with male NMRI mice weighing approx. 30 g (Charles River, Sulzfeld, Germany). The animals were housed under standard conditions with food and water ad libitum. The keeping and handling of the animals corresponded to the principles of laboratory animals care under consideration of EU guidelines (Official Journal EU L1358 (68/609/EWG)) [17].

2.3. Treatment

Ten NMRI mice were distributed randomly on two groups of 5 animals each. 10 mg of the retentate was dissolved in phys. NaCl, sterile-filtered and with the same volume of preheated fat matrix (Miglyol-Harrfett, Scherzer, Germany) a suspension was prepared. For the controls a corresponding suspension was prepared with physiological saline. 0.5 ml of the respective suspensions were applied by gavage to the corresponding control and verum animals.

2.4. Preparation of the Peyer’s patches

15 h after the application, the animals were sacrificed by CO₂ inhalation. The small intestine of each animal was dissected under aseptic conditions. The Peyer’s patches (PP), visible as whitish areas on the small intestine, were cut off with fine scissors and washed with 8 ml MEM (Minimal Essential Medium) as per Eagle with Earle’s salts and L-glutamine, pH 7.2, modified with penicillin (100 U/ml) and streptomycin sulphate, (100 μg/ml), 5 % fetal cell serum (FCS), 1 x non-essential amino acids and 1 mmol/L sodium pyruvate. The PP were then put onto a sieve and with the tender die of a 5 ml syringe they were pressed through the sieve (0.18 mm, Witegro, Altenhain/Taunus, Germany) into a Petri dish with cell culture medium.
The cell suspension thus obtained was centrifuged (1,000 rpm, 10 min., 4°C) and washed twice. After the last washing step the cells were suspended in 7.5 ml cell culture medium. In order to have enough cells for the adjacent PFC assay the PP cells from the 5 animals of each group were pooled and the cell density was adjusted to 1 x 10^6 cells/ml.

2.5. PFC assay

400 µl of the PP cell suspensions and 100 µl of a suspension of sheep red blood cells (SRBC), adjusted to 1 x 10^6 cells/ml, were pipetted into the cavities of a 24-well test plate. In duplicate each 100 µl cell culture medium, 100 µl LPS solution (lipopolysaccharide E. coli 0128: B12, concentrations: 30, 60, 90 µg/ml) or 100 µl of the retentate fraction (concentrations: 1,000–125 µg/ml) were added. The cultures were incubated for 7 days at 37°C and 5% CO2 with fresh cell culture medium (100 µl) added daily. The cells from the individual cavities were then harvested, the cells from the respective double test batches were pooled, centrifuged (1,000 rpm, 4°C, 10 min), resuspended in 1 ml buffered salt solution (BSS T + II) and counted.

Narrow glass tubes were preheated at 43°C in a water bath and filled with 0.5 ml agarose solution (0.5%). Each 70 µl of a 10% suspension of Protein A-labelled SRBC [18] and 100 µl of the cultured cells were added (double test batch). After thorough mixing the content of every glass tube was poured onto slides (coated with 0.1% agarose), and distributed homogeneously. After drying of the layer, the slides were covered with 1 ml anti-mouse-Ig-developer serum (1:100, Dako, Hamburg, Germany) and incubated in a moist chamber for 1 h at 37°C.

Then the slides were coated with 1 ml of the diluted guinea pig complement solution (1:7.5, Behring, Liederbach, Germany). After incubation for 3 h at 37°C the plaques were counted and the number of the plaques/10^6 PP cells was calculated.

2.6. Evaluation and statistics

Four independent tests were carried out. The number of antibody-producing cells (number of plaques (PFC)/10^6 PP cells) was set into relation to the respective control and computed as a stimulation index (SI). From the SI values of the individual test batches the mean values and standard deviations were calculated. Comparison between the values of the verum animals and the controls were performed using the Student's t-test with a limit of significance at p = 0.05.

3. Results

In Table 1 the data (raw data and SI calculations) of the four independently performed tests are summarised. Fig. 1 shows the SI mean values and standard deviations and the result of the statistical evaluation.

Obviously, without additional stimulation in vitro, no significant differences in the number of plaque-forming cells between treated and control animals are to be seen. However, very impressive differences between the two groups are evident, if there is an additional stimulation with LPS or the retentate fraction in the in vitro part of the assay.

While the PP cells isolated from the control animals could indeed be stimulated to an amplified production of antibody-producing cells through the further incubation with LPS (SI = 3.43 with LPS 90 µg/ml), PP cells stemming from the animals treated with the immunomodulator, however, reacted significantly more strongly to the additional stimulation. The number of antibody-producing cells in the PP cultures increased after stimulation with LPS 90 µg/ml by a factor of 10.45, which means three-times more than in the cultures with the PP cells of the control mice.

After additional stimulation with the retentate fraction the PP cells from the control animals produced more plaque-forming cells in vitro than unstimulated cells, while the stimulation indices achieved with the PP cells from the treated animals were again significantly higher. For example a stimulation index of 6.79 was calculated after additional stimulation with the retentate fraction (1,000 µg/ml) being 3 times higher than the corresponding stimulation index obtained with the PP cells from the control animals (SI = 2.12).
Table 1: Influence of the peroral application of a herbal immunomodulator on the PFC-response of Peyer’s patches cells in mice.

<table>
<thead>
<tr>
<th>Group in vivo</th>
<th>Test substances (µg/ml) In vivo</th>
<th>PFC/10^6 PP cells</th>
<th>Test 1</th>
<th>Test 2</th>
<th>Test 3</th>
<th>Test</th>
<th>Mean value SI</th>
<th>Standard deviation SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>medium control</td>
<td>200</td>
<td>1.00</td>
<td>193</td>
<td>1.00</td>
<td>111</td>
<td>1.00</td>
<td>132</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>738</td>
<td>3.70</td>
<td>461</td>
<td>2.39</td>
<td>245</td>
<td>2.21</td>
<td>235</td>
</tr>
<tr>
<td></td>
<td>retentate fraction</td>
<td>554</td>
<td>2.77</td>
<td>304</td>
<td>1.50</td>
<td>101</td>
<td>1.63</td>
<td>248</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>542</td>
<td>2.71</td>
<td>332</td>
<td>1.72</td>
<td>217</td>
<td>1.95</td>
<td>277</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>474</td>
<td>2.37</td>
<td>286</td>
<td>1.48</td>
<td>177</td>
<td>1.59</td>
<td>269</td>
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<tr>
<td></td>
<td>250</td>
<td>442</td>
<td>2.21</td>
<td>221</td>
<td>1.15</td>
<td>157</td>
<td>1.41</td>
<td>239</td>
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<tr>
<td></td>
<td>125</td>
<td>348</td>
<td>1.74</td>
<td>225</td>
<td>1.17</td>
<td>147</td>
<td>1.32</td>
<td>223</td>
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</table>

Verum

<table>
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<tr>
<th>Group in vivo</th>
<th>Test substances (µg/ml) In vivo</th>
<th>PFC/10^6 PP cells</th>
<th>Test 1</th>
<th>Test 2</th>
<th>Test 3</th>
<th>Test</th>
<th>Mean value SI</th>
<th>Standard deviation SI</th>
</tr>
</thead>
<tbody>
<tr>
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<td>205</td>
<td>1.03</td>
<td>277</td>
<td>1.44</td>
<td>246</td>
<td>2.22</td>
<td>226</td>
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<td>90</td>
<td>1615</td>
<td>8.08</td>
<td>1024</td>
<td>5.31</td>
<td>1660</td>
<td>14.77</td>
<td>1800</td>
<td>13.64</td>
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<td>60</td>
<td>1544</td>
<td>6.72</td>
<td>721</td>
<td>3.74</td>
<td>889</td>
<td>6.01</td>
<td>1066</td>
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<td>892</td>
<td>4.46</td>
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<td>3.46</td>
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<td>6.23</td>
<td>678</td>
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</tr>
<tr>
<td>125</td>
<td>569</td>
<td>2.85</td>
<td>556</td>
<td>2.88</td>
<td>569</td>
<td>5.31</td>
<td>620</td>
<td>4.70</td>
</tr>
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</table>

SI (stimulation index) = PFC/10^6 PP cells test batch / PFC/10^6 PP cells control

15 h after application of 10 mg of the immunomodulator by gavage the Peyer's patches cells were isolated and incubated in vitro with SRBC and cell medium or LPS or the retentate fraction for 7 days. Then the number of antibody-producing cells in the cultures was determined by means of the PFC assay. Verum: PP cells from mice treated with 10 mg of the immunomodulator; Control: PP cells from mice treated with NaCl. PFC = plaque-forming cells; PP cells = cells isolated from the Peyer's patches.

4. Discussion

The test results show that the peroral application of a macromolecular fraction of the extract of a mixture of *Thujae summitates*, *Baptitae tinctoriae* radix, *Echinaee purpureae* radix and *Echinacea pallidae* radix induces an activation of PP cells. The immune response in the Peyer’s patches was increased through the administration of the immunomodulator. PP cells from the treated animals developed considerably more antibody-producing cells after stimulation with SRBC and LPS than the PP cells from the untreated control animals, suggesting that the active compounds of the herbal immunomodulator can contact the cells of the GALT and are able to stimulate them. So for the first time it was possible to verify experimentally for a clinically-applied herbal immunomodulator that its effects are exerted by such a route of activation, and that the macromolecular constituents of the extract are responsible for its efficacy, or at least play an essential role.

With the aid of the same test model for SSG, too, a β-1,3-D-glucan from the ascomycete *Sclerotinia sclerotiorum*, a modulation of the mucosal immune response was experimentally proven. Additionally the peroral administration of the glucan led to an increase in the activity of peritoneal macrophages of NK (natural killer) cells in the spleen and an induction of the cytokine release. It was accepted, due to its high molecular mass, that the glucan is incorporated by M cells via endocytosis and reaches the Peyer’s plaques from there [19–21].

A prerequisite for this route of activation is that the macromolecular constituents of the herbal immunomodulator reach the Peyer’s patches after peroral application. It could be shown that after peroral administration glycoproteins with a high molecular mass (M, > 10,000 Dalton) were found in the Peyer’s plaques, while the substances with a low molecular mass (M, < 10,000 D) were found in the duodenum [22].

Also the peroral efficacy of PSK (Krestin), a protein-bound polysaccharide from the basidiomycete *Coriolus versicolor*, was justified with absorption through the lymphoid cells of the GALT and a stimulation of the immunoresponsive cells in the Peyer's plaques [23, 24]. With the aid of an antisemur, radioactively labeled Krestin could be located in the blood serum as soon as 1 h after p.o. administration – obviously unchanged [25, 26], which was also explained with a pinocytosis under participation of the GALT cells. By means of tests with microparticles and “microspheres” it was possible to show that M cells can transport particles of 100 nm up to 10 µm diameters [27, 28].

Remarkable is the fact that the activation of the PP cells was only to be seen when, by addition of LPS, an endotoxin from the cell wall of gram-negative bacteria, a microbial attack was simulated. Only in this case, the anti-SRBC-antibody response of the activated PP cells was strongly enhanced. This increase, however, could also be induced by additional stimulation with the extract retentate, using substantially higher dosages. The retentate itself was not contaminated by LPS as we could show in previous investigations [10].

It can be derived from the results that the cells – in the sense of a "priming" through an adjuvant – are put on the alert through the immunomodulator. The reaction itself, however, is started only in the case of a definite microbial attack. It may be assumed that the alert...
mode maintains for some time because the immunomodu-
lar was applied a considerable time before the anti-
genic stimulation of the PP cells. This is very useful for a
medicinal preparation which is intended for the de-
ference and fight against infections. Only in the case of a
real microbial attack is the immune system fully activ-
ated.

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