Introduction

Radiation pneumonitis (RP) is defined as an exudative inflammation which occurs as a result of ionizing radiation (5) and it is considered as an alveolitis resulting from irradiation of the lungs with a single dose of 7 Gy or more (2). It involves changes in the irradiated areas only (29).

Apoptosis (programmed cell death) is important for cellular homeostasis in a variety of tissues. The mechanisms regulating apoptosis are complex and involve the interaction of nuclear and cytoplasmic proteins. Recently, the sensitivity of tissues to apoptosis induced by a variety of external stimuli has been closely linked to the intracellular concentrations of a family of cell-death regulators, which include bcl-2 (14). High levels of bcl-2 protect against apoptosis through inhibition of caspases activation, the key enzymes of apoptosis (7). Guinee et al. (15) and Bardales et al. (1) have shown that apoptosis is demonstrable in type II pneumocytes in DAD and not in normal lung tissues. In this regard, the action of bcl-2 in DAD is potentially analogous to its action in lymphomas and carcinomas: the prevention of apoptosis (19). Fukuk et al. (8) first described apoptosis in irritated lung tissue by the DNA terminal transferase nick-end labeling method. This method revealed time and dose-dependent apoptosis mainly in endothelial and mesothelial cells at 1 week after irradiation.

Pentoxifylline (PTX) ameliorates radiation-induced histological changes in irritated lungs (25). Funk et al. (9) observed the in vitro influence of cytokine production (27) and PTX-therapy causes diminished inflammation development.

Summary: We measured number of bcl-2, apoptotic, neutrophil, and surfactant apoprotein D (SP-D) positive cells in irradiated rat lungs during different time points after the sublethal whole-thorax irradiation of rats. We also investigated the influence of pentoxifylline (PTX) therapy on these markers. Wistar rats were given 15 Gy thoracic irradiation and PTX (35 mg/kg) twice a week. Animals were examined histologically and immuno histochemically at intervals from 1-12 weeks. In non-treated rats compared with treated rats, bcl-2 expression was significantly inhibited from 4 weeks after irradiation. A higher apoptosis presence in non-treated rats from 4 weeks was found and apoptosis development in PTX-treated animals was delayed and started 8 weeks after irradiation. Similar differences were measured during neutrophil granulocytes examination. Neutrophil penetration in non-treated rats was found 5 weeks after irradiation in contrast to the RP onset of PTX-treated animals 8 weeks after irradiation. The number of SP-D positive cells in non-treated rats observed until 5 weeks after irradiation was higher than in the control group. PTX-treated animals expressed higher number of SP-D positive cells during the whole experiment than the control group. We suggest that apoptosis is linked to neutrophil granulocyte actions during the RP onset and that PTX-therapy causes diminished inflammation development.

Key words: Irradiation; Lung; Pentoxifylline; Apoptosis; Bcl-2

Abbreviations: RP - radiation pneumonitis; ARDS - adult respiratory distress syndrome; DAD - diffuse alveolar damage (DAD); TNF - tumor necrosis factor; IL - interleukin; S.E.M - standard error of mean. PTX - pentoxifylline; PBS - phosphate buffered saline; cAMP - cyclic adenosine monophosphate; ATP - adenosine triphosphate; DXM - dexamethasone; IFN - interferon; SP-D - surfactant apoprotein D.
ly confirmed inhibition of TNF-α and other pro-inflammatory cytokines during PTX therapy. If the hypothesis of RP development via the overexpressed pro-inflammatory cytokine cascade (TNF-α, IL-1) and others is correct (26), then the therapeutic period influencing the expression of cytokines starts immediately after irradiation because an increased production of pro-inflammatory cytokines was found immediately after irradiation.

In this study, we sought to test the effect of PTX administered from day 1 to day 84 (12 weeks) after irradiation and to study the link with bcl-2 expression. We investigated apoptosis by the caspase cleavage product of cytokeratin 18 as well as the number of neutrophils and surfactant apoprotein D positive cells in the irradiated lungs by computer image analysis.

Material and Methods

Male Wistar rats (Konarowice, Czech Republic) aged 8-12 weeks and weighing 150-200 g were given local thoracic irradiation using a 60Co unit (Chisotron Chirana) at a dose rate of 1.0 Gy/min, target distance 1 m. The animals were slightly anaesthetised before irradiation using a mixture of one volume of Rompet (Spofa Company, Prague), 3 volumes of Narkamon (Lečiva Company, Prague) and 12 volumes of physiological saline. This solution was injected intratracheally at 10 ml/kg. Local thoracic irradiation was performed in a jig in which a 10 cm thick layer of lead reduced the dose to other parts of the body to around 2-3% of the lung dose.

Three groups of rats were used: Group S was given physiological saline only. Group PTX 35 was given pentoxifylline (Pentilin, Krka, 100 mg/kg) and ampicillin (Anapec, 200 mg/kg) intraperitoneally immediately after irradiation and for 14 days. Group C consisted of 6 non-irradiated rats. In each irradiated group (Group S and PTX 35) included 7 irradiated subgroups by 6 animals in each subgroup. The first drug administration was given 2 h after irradiation and the last at two days before the animals were sacrificed.

Table 1: Average number of bcl-2 positive cells ± SEM in the lungs.

<table>
<thead>
<tr>
<th>Group</th>
<th>1 week</th>
<th>2 weeks</th>
<th>3 weeks</th>
<th>4 weeks</th>
<th>5 weeks</th>
<th>6 weeks</th>
<th>7 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group S</td>
<td>13.5±7.2</td>
<td>18.6±5.36</td>
<td>14.7±8.1</td>
<td>10.7±8.64</td>
<td>15.3±0.72</td>
<td>10.8±1.52</td>
<td>17.4±2.88</td>
</tr>
<tr>
<td>Group PTX 35</td>
<td>11.9±2.20</td>
<td>17.0±6.32</td>
<td>19.5±8.34</td>
<td>29.2±5.20</td>
<td>34.0±8.52</td>
<td>29.5±4.01</td>
<td>31.8±2.24</td>
</tr>
<tr>
<td>Group C</td>
<td>27.7±4.52</td>
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</tr>
</tbody>
</table>

Probability of value differences between Group S and Group PTX 35 in the same time interval: p<0.05 - a; p<0.01 – b; p<0.001 - c.

Table 2: Average number of apoptotic cells ± SEM in the lungs.

<table>
<thead>
<tr>
<th>Group</th>
<th>1 week</th>
<th>2 weeks</th>
<th>3 weeks</th>
<th>4 weeks</th>
<th>5 weeks</th>
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<th>7 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group S</td>
<td>0.18±0.12</td>
<td>1.6±0.88</td>
<td>0.2±0.10</td>
<td>4.25±2.04</td>
<td>1.05±2.68</td>
<td>10.25±3.08</td>
<td>8.4±1.92</td>
</tr>
<tr>
<td>Group PTX 35</td>
<td>3.38±1.08</td>
<td>1.6±1.03</td>
<td>0.2±0.16</td>
<td>0.08±0.12</td>
<td>0.02±0.04</td>
<td>2.67±1.12</td>
<td>3.8±1.28</td>
</tr>
<tr>
<td>Group C</td>
<td>0.35±0.12</td>
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</tr>
</tbody>
</table>

Probability of value differences to Group C 12 weeks after irradiation: p<0.05 - 1; p<0.01 - 2; p<0.001 - 3.

Table 3: Average number of neutrophils in the lungs ± 2 x S.E.M.

<table>
<thead>
<tr>
<th>Group</th>
<th>1 week</th>
<th>2 weeks</th>
<th>3 weeks</th>
<th>4 weeks</th>
<th>5 weeks</th>
<th>6 weeks</th>
<th>7 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group S</td>
<td>0.09±0.05</td>
<td>0.04±0.04</td>
<td>0.1±0.07</td>
<td>0.07±0.07</td>
<td>1.24±0.34</td>
<td>3.80±0.64</td>
<td>5.9±0.56</td>
</tr>
<tr>
<td>Group PTX 35</td>
<td>0.14±0.06</td>
<td>0.21±0.07</td>
<td>0.1±0.06</td>
<td>0.08±0.04</td>
<td>0.16±0.06</td>
<td>1.96±0.27</td>
<td>3.8±0.74</td>
</tr>
<tr>
<td>Group C</td>
<td>0.46±0.11</td>
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</tr>
</tbody>
</table>

Probability of value differences to Group C: p<0.05 - 1; p<0.01 - 2; p<0.001 - 3.

The Table 4: Average number of SP-D positive cells in the lungs ± 2 x S.E.M.

<table>
<thead>
<tr>
<th>Group</th>
<th>1 week</th>
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<th>5 weeks</th>
<th>6 weeks</th>
<th>7 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group S</td>
<td>9.02±1.00</td>
<td>10.4±0.97</td>
<td>10.55±0.93</td>
<td>8.48±1.32</td>
<td>7.95±0.76</td>
<td>7.3±0.87</td>
<td>6.9±0.65</td>
</tr>
<tr>
<td>Group PTX 35</td>
<td>12.40±0.92</td>
<td>9.5±0.87</td>
<td>14.3±1.04</td>
<td>12.3±1.06</td>
<td>7.8±0.96</td>
<td>9.5±0.83</td>
<td>9.0±1.20</td>
</tr>
<tr>
<td>Group C</td>
<td>6.38±0.89</td>
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</tr>
</tbody>
</table>

Probability of value differences between Group S and Group PTX 35 in the same time interval: p<0.05 - 1; p<0.01 – b; p<0.001 - c.

The bcl-2 expression measurement

Significantly higher values of Group C compared with Group S were measured 12 weeks after irradiation. The effect of PTX therapy was expressed by a higher number of positive staining cells beyond 4 weeks than in Group S.

Apopotic cell measurement

Significantly higher values of this marker of Group C compared with Group S were measured 12 weeks after irradiation. In Group PTX 35, the effect of therapy expressed...
The following bcl-2 positive cells were found: type-II pneumocytes, fibroblasts, fibrocytes, endothelial cells and with 10% neutral buffered formalin, embedded into paraffin and 4 mm thick tissue sections were cut, stained with chlo-roacetic esterase to detect neutrophil granulocytes and Gramm’s staining for bacterial infection.

Immunohistochemical examinations for bcl-2, direct detection of apoptotic cells, and surfactant apoprotein D positive cells were performed with a standard immunoperoxidase technique. After blocking endogenous peroxidase activity for 20 min, tissue sections were incubated with in- dividual polyclonal antibodies (anti-bcl-2 rabbit antibody, Santa Cruz, CA, USA) diluted 1:300 in phosphate-buffered saline (PBS, pH 7.2) for 24 h at 4°C, and then washed three times in PBS. The slides for bcl-2 detection were then incu-bated with a horseradish peroxidase-coupled anti-rabbit an-tibody (Santa Cruz, CA, USA) for 45 minutes at 37°C. Excess antibodies were washed off with PBS. Finally, a 0.05% 3,3-diaminobenzidine tetrahydrochloride chromo-gen solution (Sigma, Prague, Czech Republic) in PBS contain- ing 0.02% hydrogen peroxide was added for 10 min to visualise the antigen-antibody complex in situ.

A M30 CytoDeath kit (Roche Diagnostics, Mannheim, Germany) was employed for the detection of apoptotic cells. The staining procedure was as for bcl-2, whereas in-cubation with the primary antibody was for 1 hour at room temperature and incubation with the anti-mouse secondary horseradish peroxidase-coupled antibody for 30 minutes at room temperature.

Surfactant apoprotein D was detected as with M30 Cyto-death, whereas samples were put in citrate saline (pH 6.0) and twice heated in a microwave (750W) for a period of 5 minutes. Samples were then incubated with the mouse mo-noclonal anti-surfactant apoprotein D antibody (clone VI F11, from Prof. Michael Kasper, Technische Universität, Dresden, Germany) diluted 1:10 in PBS for 1 h at room temperature.

As negative controls were used samples stained without primary antibodies.

Measurement of bcl-2, M 30, and SP-D positive cell numbers

Immunohistochemical samples were evaluated using an IMT-2 light microscope (Olympus Company, Prague) and computer image analysis (Image Pro, Media Cybernetics, MD, USA). Ten randomly selected viewing fields with a size of 10744.32 µm² without bronchi and large vessels from each sample were evaluated at a 600 fold original magnification.

Data Processing

The Mann-Whitney test was used for statistical analysis, giving a mean ± 2 x SEM.

Results

The following bcl-2 positive cells were found: type-II pneumocytes, fibroblasts, fibrocytes, endothelial cells and

<table>
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</tr>
<tr>
<td>Group S</td>
</tr>
<tr>
<td>Group PTX 35</td>
</tr>
<tr>
<td>Group C</td>
</tr>
</tbody>
</table>

Probability of value differences to Group C: 12 weeks after irradiation: p<0.005 - a; p<0.001 - b; p<0.0001 - c.

Probability of value differences between Group S and Group PTX 35 in the same time interval: p<0.005 - c; p<0.01 - b; p<0.001 - a.

Probability of value differences to Group C 12 weeks after irradiation: p<0.005 - a; p<0.01 - b; p<0.001 - c.

Probability of value differences between Group S and Group PTX 35 in the same time interval: p<0.005 - c; p<0.01 - b; p<0.001 - a.

Tab. 2: Average number of apoptotic cells ± 2 x SEM in the lungs.

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<tr>
<td>Group S</td>
<td>0.18±0.12</td>
<td>1.64±0.88</td>
<td>0.12±1.06</td>
<td>2.25±2.04</td>
<td>17.05±2.68</td>
<td>10.25±3.08</td>
<td>8.43±1.92</td>
</tr>
<tr>
<td>Group PTX 35</td>
<td>3.38±1.08</td>
<td>3.67±1.03</td>
<td>0.2±1.16</td>
<td>0.08±0.12</td>
<td>0.02±0.04</td>
<td>2.67±1.12</td>
<td>3.83±1.28</td>
</tr>
<tr>
<td>Group C</td>
<td>0.55±0.28</td>
<td>0.58±0.32</td>
<td>0.39±0.28</td>
<td>0.49±0.43</td>
<td>0.31±0.31</td>
<td>0.47±0.26</td>
<td>0.46±0.21</td>
</tr>
</tbody>
</table>

Probability of value differences to Group C 12 weeks after irradiation: p<0.005 - 1; p<0.01 - 2; p<0.001 - 3.

Probability of value differences between Group S and Group PTX 35 in the same time interval: p<0.005 - 1; p<0.01 - 2; p<0.001 - 3.

Tab. 3: Average number of neutrophils in the lungs ± 2 x S.E.M.

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<td>0.09±0.05</td>
<td>0.02±0.04</td>
<td>0.1±0.07</td>
<td>0.0±1.02</td>
<td>1.24±0.34</td>
<td>4.83±0.40</td>
<td>5.82±0.56</td>
</tr>
<tr>
<td>Group PTX 35</td>
<td>0.1±0.06</td>
<td>0.2±0.07</td>
<td>0.1±0.06</td>
<td>0.08±1.00</td>
<td>0.16±0.06</td>
<td>1.96±0.27</td>
<td>3.41±0.45</td>
</tr>
<tr>
<td>Group C</td>
<td>0.4±0.06</td>
<td>0.4±0.06</td>
<td>0.2±0.06</td>
<td>0.1±0.06</td>
<td>0.3±0.06</td>
<td>0.4±0.06</td>
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</tbody>
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Probability of value differences to Group C: p<0.005 - 2; p<0.01 - 2; p<0.001 - 3.

Probability of value differences between Group S and Group PTX 35 in the same time interval: p<0.005 - 2; p<0.01 - 2; p<0.001 - 3.

Tab. 4: Average number of SP-D positive cells in the lungs ± 2 x S.E.M.

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</tr>
</thead>
<tbody>
<tr>
<td>Group S</td>
<td>9.01±1.00</td>
<td>16.48±0.90</td>
<td>10.05±2.99</td>
<td>8.48±1.32</td>
<td>7.55±0.74</td>
<td>6.94±0.96</td>
<td>6.84±0.89</td>
</tr>
<tr>
<td>Group PTX 35</td>
<td>12.40±0.92</td>
<td>9.55±0.83</td>
<td>14.32±1.40</td>
<td>12.3±1.65</td>
<td>8.75±0.96</td>
<td>9.3±0.83</td>
<td>9.1±0.20</td>
</tr>
<tr>
<td>Group C</td>
<td>6.38±0.89</td>
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</tbody>
</table>

Probability of value differences to Group C: p<0.005 - 2; p<0.01 - 2; p<0.001 - 3.

Probability of value differences between Group S and Group PTX 35 in the same time interval: p<0.005 - 2; p<0.01 - 2; p<0.001 - 3.

The bcl-2 expression measurement

Significantly higher values of Group C compared with Group S were measured 12 weeks after irradiation. The effect of PTX therapy was expressed by a higher number of positive staining cells beyond 4 weeks than in Group S.

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The number of SP-D positive cells

Until 5 weeks after irradiation, a significantly higher number of SP-D positive cells were measured in Group S than in Group C. In the lungs of PTX-treated rats during the whole experiment, a higher number of SP-D immunoreactive cells were detected than in Group C. The effect of PTX therapy was observed in all time intervals, except 2 and 5 weeks after sublethal irradiation.

Discussion

Many of the known etiologies of diffuse alveolar damage, including radiation, may cause DNA damage and thereby induce apoptosis (13,17). In animal models, DAD is associated with the generation of free radicals, which include the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical (OH•), and singlet oxygen (O_2^*) (6,23). These free radicals have been shown to induce apoptosis, presumably through damage to DNA (32). Korsmeyer et al. (20) postulated that DNA damage and p53-dependent apoptosis may contribute to the pathogenesis and/or evolution of this disorder.

In our study, at 1 week after irradiation of the physiological saline–administered animals, an inhibition of bcl-2 followed by the presence of the apoptotic cells in the next time interval were seen. We assume that the presence of the apoptotic cells early after irradiation has a relationship to the action of the radiation-induced free radicals. Apoptotic cells increased significantly beyond 4 weeks after irradiation, one week before production of the neutrophils. On the other hand, the relationship between inflammation and apoptotic phenomena might be bilateral. Apoptotic cells and bodies are primarily removed by non-professional phagocytes, e.g. type II pneumocytes in the lungs. However, if they are not readily removed by phagocytes, they undergo “secondary necrosis”. During the secondary necrosis, the lysosomal enzymes reach in the extracellular space and cause further destruction of the internal components including the plasma membrane. Once the plasma membrane loses its integrity, the cell cytoskeleton, releasing hydrolytic enzymes into the extracellular space and guarantying an inflammatory response with possible tissue damage (12).

The number of neutrophil granulocytes


The beneficial effects of repeated applications of PTX, DXM and their combination to sublethally irradiated mice were noted (25). PTX diminishes and/or delays the neutrophil granulocyte penetration through the vascular wall in the lungs and the interstitial edema intensity during the RP onset (24,25). In addition, it has been found in the Paragauta-affected tissue culture model formed by isolated pulmonary cells (28) that PTX reduces the production of oxygen radicals and scavenges free radicals.

The observed apoptosis inhibition during the RP onset phase in PTX-treated rats may have been caused via PTX-induced TNF-α inhibition and subsequently a lower inflammatory response in irradiated lungs. Moreover, PTX therapy was observed in all time intervals, except 2 and 5 weeks after sublethal irradiation.

Further investigation of the early molecular postradiation pulmonary changes with cell identification will be the objective of our work.

Conclusion

The reduced and delayed expression of apoptosis in irradiated lungs caused by PTX might be used as an effective tool for inhibition of radiation-induced changes of lungs. The inflammatory process and apoptosis in irradiated lungs are closely-related phenomena with a possible bilateral influence.

Acknowledgements

We would like to thank Dr. Michael Kasper for his rat reactive anti-SP-D antibody and we thank Mrs. Sarka Prčihová and Hana Burzkova for their skillful assistance. This study has been supported by the grants 60020398127, 0203100602, and FJ MSM 11110003 grants.

References

The number of SP-D positive cells

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In our study, at 1 week after irradiation of the physiological saline-administered animals, an inhibition of Bcl-2 followed by the presence of the apoptotic cells in the next time interval were seen. We assume that the presence of the apoptotic cells early after irradiation has a relationship to the action of the radiation-induced free radicals. Apoptotic cells increased significantly beyond 4 weeks after irradiation, one week before presentation of the neutrophils. On the other hand, the relationship between inflammation and apoptotic phenomena might be bilateral. Apoptotic cells and bodies are primarily removed by non-professional phagocytes, e.g. type II pneumocytes in the lungs. However, if they are not readily removed by phagocytes, they undergo “secondary necrosis”. During the secondary necrosis, the lysosomes rupture and the cytoplasmic hydroxides enter the cytoplasm causing further destruction of the intracellular components including the plasma membrane. Once the plasma membrane loses integrity, the cell lyses, releasing hydroxides into the extracellular space and guaranteeing an inflammatory response with possible tissue damage (12).

At present, PTX is considered to be a preparation with various immunomodulatory effects which were found in vitro in pharmacological concentrations as low as 10 µg/ml (9) and 20 µg/kg/day per os in the rat model (27). The main effect of PTX in immune reactions is a decrease in the production of TNFα (9,22) at the protein and as well as at the mRNA level (3). Because of this effect, we used pentoxifylline for the treatment of post-irradiation apoptotic changes. Ward et al. (31) reported that PTX had only a small beneficial effect on a radiation-induced lung injury in a rat by some indices such as lung wet weight and protein content, but not by hydroxyproline content, the activity of the lung angiotensin converting enzyme and the plasminogen activator. From their results it follows that the PTX effect is not only through the pulmonary endothelial function, but also via other mechanisms.

The beneficial effects of repeated applications of PTX, DXM and their combination to sublethally irradiated mice were noted (25). PTX diminishes and/or delays the neutrophil granulocyte penetrates through the vascular wall in the lungs and the interstitial edema intensity during the RP onset (24,25). In addition, it has been found in the PPARα-affecting tissue culture model formed by isolated pulmonary cells (28) that PTX reduces the production of oxygen radicals and scavenges free radicals. The observed apoptosis inhibition during the RP onset phase in PTX-treated rats may have been caused via PTX-induced TNFα inhibition and subsequently a lower inflammatory response in irradiated lungs. Moreover, TNFα is known to induce a P-53 dependent apoptotic pathway.

PTX-mediated overproduction of OSM and IL-1β (3) and subsequent expression of p-38 MAPK may stimulate surafactant secretion (11). Our results of surfactant apoprotein D positive cells show that PTX facilitates surfactant production in our model.

We suggested that apoptotic cells in our model is linked to inflammation onset after thoracic irradiation. Moreover, PTX therapy would cause a delayed and dose-dependent radiation-induced inflammatory response in the lungs.

Further investigation of the early molecular postradiation pulmonary changes with cell identifications will be the objective of our work.

Conclusion

The reduced and delayed expression of apoptosis in irradiated lungs caused by PTX might be used as an effective tool for inhibition of radiation-induced changes of lungs. The inflammatory process and apoptosis in irradiated lungs are closely related phenomena with a possible bilaterar influence.

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References

Introduction

Continuing search for etiological factors of dyslexia motivated also a lot of recent studies using various methods (PET, functional MRI, electrophysiology of the CNS, psychophysical examination) to verify the hypothesis of the visual magnocellular pathway involvement (5). There are quite new findings supporting the theories about a delayed signal transmission within the magnocellular pathway and/or a decreased activation of the visual association cortex – particularly of the medio-temporal (V5) area which is reported to be specialised for motion processing (4,13). However, some other results (7,14) are not in agreement with the opinion that quite large part of the dyslexic people has a deficit in visual functions. Since knowledge of the basis of dyslexia is crucial for strategy of its rehabilitation, we tried to enlarge our previous study (10) of motion related visual evoked potentials (VEPs) specifically testing the magnocellular pathway (1,9) in dyslexic children. Simultaneously, we have tested whether the suspected visual deficit in dyslexics is attributable to a delay in magnocellular system maturation (according to some reports – e.g. by Barnard et al. (2)) and also if it is possible to influence the function of this system with the use of various light wavelengths (reported e.g. by Williams et al. (16) or Solan (11)).

Methods and subjects

Three groups of subjects were used for electrophysiological testing of visual function. We have examined 10 dyslexics (mean age 14.0 ± 1.1 years) from the group of 20 children who have been tested already 4 years ago (10) – group No. 1 in Tab. 1. The second group consisted from 25 dyslexic children (mean age 10.0 ± 1.9 years) randomly selected in the 2nd and 3rd classes of a specialised school for dyslexics. In the third group 7 control subjects (normal readers of the mean age 13.7 ± 1.1 years) from the previous study (10) were repeatedly examined after 4 years interval. All subjects had visual acuity 6/6 or better (with correction if needed).

The same set of VEPs examinations was done in each subject. Transient pattern-reversal visual evoked potentials (P-VEPs) were acquired with high contrast (96%) square-wave black and white checkerboard (element size 40'). Two variants of motion-onset VEPs (M-VEPs) were used. The first one - linear motion (random order of fundamental directions, velocity 10 deg/s) of low contrast (10%) isolated checks (40' check size and 120' check-to-check distances) had grey, green, blue, yellow and pink modifications (equivalent wavelengths to recommendations by Wilkins et al. (15)). Second motion stimulus consisted of low contrast (10%) grey concentric frames with increasing size and motion (131).