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CANNABINOID RECEPTORS EXPRESSION IN BONE MARROW TREPHINE BIOPSY OF CHRONIC LYMPHOCYTIC LEUKAEMIA PATIENTS TREATED WITH PURINE ANALOGUES

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Background: **Cannabinoid receptors CB1 and CB2 are part the endocannabinoid system that plays an important role in the process of proliferation and apoptosis of different neoplastic cells. B-cell chronic lymphocytic leukaemia is one of the diseases in which these processes are altered.** ***Aim:*** **The aim of our study was the assessment of cannabinoid receptor expression on the B-lymphocytes in bone marrow trephine biopsy from leukaemic patients at diagnosis and after purine analogue treatment.** ***Methods:*** **The biopsy was taken routinely and standard immunohistochemical staining procedure for paraffin embedded sections was applied. The cannabinoid receptors were detected using specific primary polyclonal antibody anti-CB1 and anti-CB2. Additionally, an existence of cannabinoid receptors was confirmed by flow cytometry.** ***Results*****: The results showed that the expression of CB1 receptor on the surface of neoplastic cells was lower than that of CB2 (17.0 ± 3.1% and 92.1 ± 1.7% respectively, $p < 0.001$). Nine of the patients responded to applied treatment with a reduction in leukaemic infiltration (77.2 ± 6.9% to 30.2 ± 6.5%, $p = 0.007$) and CB1 receptor expression (24.4 ± 4.8% to 8.6 ± 2.9%, $p = 0.01$), but there was no change in CB2 expression (91.7 ± 2.7% vs 90.9 ± 2.8%, $p = 0.69$). Four patients without remission expressed even greater number of the receptors. In all of the cases both cannabinoid receptor types antibodies gave positive reaction. Furthermore, the existence of cannabinoid receptors on neoplastic lymphocytes was confirmed by flow cytometry.** ***Conclusion:*** **The study provides original evidence for the existence of cannabinoid receptors on B-lymphocytes in chronic lymphocytic leukaemia patients. The receptors are thought to be a new structure that can modify the course of the disease and may be considered as a new target in leukaemia treatment.**

Key Words: **chronic lymphocytic leukaemia, endocannabinoid system, purine analogues, cannabinoid receptor, trephine biopsy.**

Marijuana derived from *cannabis sativa (Moraceae*) has been one of the oldest psychoactive and healing substances widely used by men for many centuries. In contemporary medicine these substances are still applied in such complications like nausea related to chemotherapy, and used as an appetite stimulator in AIDS patients. Moreover, cannabinoid receptor antagonists are currently used in obesity treatment [1, 2].

Studies on the rational use of cannabinoids have grown up recently and to a great extent have been related to the discovery of two cannabinoid receptors, CB1 and CB2. CB1 receptors are expressed predominantly in the brain, whereas CB2 receptors are found primarily in the cells of the immune system [6, 7, 12, 14]. Furthermore, endogenous ligands for these receptors have also been discovered and called endocannabinoids [4]. The physiological interactions of this system in the field of immunology and carcinogenesis remain unclear.

B-cell chronic lymphocytic leukaemia (CLL) is a disease characterised primarily by defects in programmed cell death regulation [15]. CLL is still considered an incurable disease. So far great efforts have been undertaken to improve the results of CLL treatment and finally eradicate this disease.

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Abbreviations used: BMT – bone marrow trephine biopsy; CLL – chronic lymphocytic leukaemia; FC – flow cytometry; IHC – immunohistochemistry; MFI – mean fluorescence intensity.

Our former study showed an existence of significant differences in cannabinoid receptors expression between CLL patients and healthy individuals. A higher percentage of B-lymphocytes expressing cannabinoid receptors and their higher fluorescence intensity assessed by flow cytometry (FC) in the CLL patient group suggest that the cannabinoid system may take part in CLL development [16].

The aim of our study was the assessment of cannabinoid receptor expression on the lymphocytes B in bone marrow trephine biopsy (BMT) derived from patients with diagnosed chronic lymphocytic leukaemia. The influence of purine nucleoside analogues treatment (fludarabine or 2-chlorodeoxyadenosine) on the cannabinoid receptors expression was evaluated.

MATERIALS AND METHODS

The studied specimens were obtained from 18 previously untreated CLL patients and 13 of the patients after the treatment with purine nucleoside analogues containing regimens (fludarabine, n = 7, 2-chlorodeoxyadenosine, n = 6). The study was approved by the local Ethic Committee and all evaluated subjects signed the consent for the study. The individuals, females and males aged 35 to 72, were in II–IV clinical stage of the disease according to Rai staging system. BMT was performed in every patient as initial examination prior to chemotherapy and after six courses of treatment. Bone marrow tissue was taken routinely from the posterior iliac spine using the Jamshidi type needle. The sample was fixed in the so called Oxford solution (100 ml of 40% formaldehyde solution,

900 ml of destilated water, 8.7 g NaCl, 20 ml of acetic acid glacial) for 48 h. After fixing, the representative trephine specimens were put into paraffin bars. 5 μm sections were cut using microtom. In the BMT, morphology was analysed on standard haematoxylin and eosin stained sections. The origin of neoplastic cells forming infiltration was confirmed by coupling with antiCD20 monoclonal antibody (DAKO, catalog No M0755). For identification of other leukocytes we used MAC 387 monoclonal antibody (DAKO, catalog No M0747). The cannabinoid receptors were detected using specific primary polyclonal CB1 antibody (CB1, catalog No PA1-745, Affinity BioReagents; Golden, Co, USA) and CB2 antibody (CB2 receptor polyclonal antibody, catalog No. 101550, Cayman Chemical, Ann Arbor, USA) in dilution 1 : 50 and 1 : 100, respectively. All sections required microwave heating for receptor retrieval. Primary antibodies were visualised by the biotin-streptavidin-peroxidase method using LSAB + HRPkit with DAB (3,3-diaminobenzidine). The positive reaction was seen as a brown colour. For negative control the sections underwent the above procedure without primary antibodies. Positive control for CB1 and CB2 antibodies was performed using lymph node slices obtained from T cell lymphoma patient. The intensity of immunohistochemical (IHC) staining was defined as negative, weak staining, moderate staining and strong staining.

The percentage of lymphoid cells with the positive reactions was assessed using morphometric analyser set MicroImage (OLYMPUS) with Analysis DP12 program.

Flow cytometry assessment of aberrant CLL phenotype and cannabinoid receptor expression were performed in all patients at the time of diagnosis (n = 18). Additionally, bone marrow FC assessment was performed in 3 cases before the treatment. To confirm a specific phenotype commercially available monoclonal antibodies (Becton Dickinson) were used. CB1 and CB2 human cannabinoid receptors polyclonal antibody (affinity pure) were obtained from Cayman Chemical. All the other reagents were obtained from Coulter. For flow cytometric analysis 1×10^5–1×10^6 peripheral blood cells (n = 18) or bone marrow (n = 3) were incubated with 10 μl of anty-CB1 or anty-CB2 antybodies for 30 min, in the dark, at 4 °C. Then, 10 μl of monoclonal antibodies IgG1-FITC and CD19-PE were added and samples were incubated for 20 min, in the dark, at 4 °C. Then samples were lysed, fixed and stabilized using Immuno-Prep (Coulter procedure) and estimated by flow cytometry (Epics XL, Coulter). Percentage and mean fluorescence intensity (MFI) were assessed.

All results are presented as mean ± SEM. Distribution of variables in every study group was evaluated via the Shapiro-Wilk test and the differences between study groups (with nonparametric distribution) were assessed with the use of Wilcoxon's test.

RESULTS

The results showed that the neoplastic infiltration at the time of diagnosis was very extensive and amounted to 81.9 ± 1.3%. Both types of cannabinoid receptors were expressed on the surface of neoplastic cells infiltrating bone marrow. The expression of CB1 receptor was significantly lower than that of CB2 (17.0 ± 3.1% and 92.1 ± 1.7% respectively, $p < 0.001$). In all the cases an existence of cannabinoid receptors was confirmed by FC, and we observed very high percentages of both CB1/CD19 and CB2/CD19 positive cells in all patients (82.67 ± 8.6% and 86.66 ± 9.6 respectively, $p = 0.13$). However, MFI for CB1 receptor was statistically significantly lower than CB2 (2.84 ± 0.7% and 6.53 ± 2.2 respectively, $p = 0.0001$). No differences in CLL phenotype and cannabinoid receptor expression were observed depending on the source of blood cells (peripheral blood or bone marrow).

Nine of the patients responded to the applied treatment with a statistically significant reduction in leukaemic infiltration (77.2 ± 6.9% to 30.2 ± 6.5%, $p = 0.007$) and the reduction of CB1 receptor expression (24.4 ± 4.8% to 8.6 ± 2.9%, $p = 0.01$), but there was no change in CB2 expression (91.7 ± 2.7% vs 90.9 ± 2.8%, $p = 0.69$).

Four patients without remission presented no changes in cannabinoid receptors expression after treatment.

In all of the investigated cases the antibodies against both cannabinoid receptor types gave positive immunohistochemical reaction. A diversity in intensity of the reaction was observed. At the diagnosis the intensity of CB1 and CB2 receptors was mainly weak and strong, respectively. In spite of a decrease in neoplastic infiltration of the responders the treatment did not affect the pattern of antibody-staining intensity. The same phenomenon was observed in non responders.

The immunohistochemical staining of the selected trephine specimens is presented on the Figures below (Fig. 1–5). An example of FC cannabinoid receptor analysis is shown in Fig. 6.

DISCUSSION

Normal cells of the immune system express high levels of CB2 receptors. Those receptors play an important role in the B-cells differentiation [3, 17]. However, up to date, there has been insufficient data concerning the presence of cannabinoid receptors on the transformed immune cells in CLL patients. This study confirms the existence of both types of cannabinoid receptors on the neoplastic lymphocyte surface in the CLL patients.

Previous studies of Galiegue et al. [5] using RT-PCR method showed that CB2 receptor is abundant in human blood cells with the expression level 10–100-fold higher than that of CB1. Further observation of McKalip et al. [13] indicated the presence of CB2 receptors in human leukaemia and lymphoma cell lines [5, 13]. However, they did not detect the presence of CB1 receptors. Other authors also reported a high density of

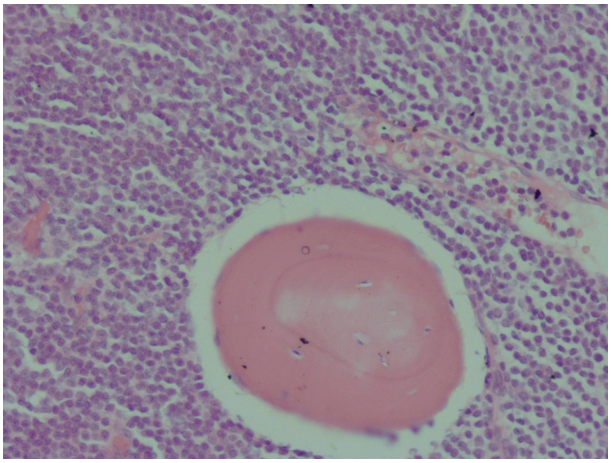

Fig. 1. Bone marrow trephine biopsy section. Diffuse infiltration in CLL. Bone trabecula and sinus in centre. Paraffin — embedded, H & E, x 240

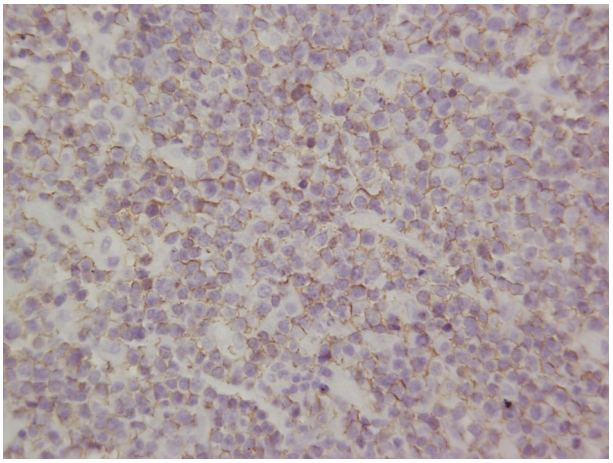

Fig. 2. Bone marrow trephine biopsy section, CLL. Lymphoid cells with positive staining for CB1 antibody — before treatment. Immunohistochemical staining (CB1, x 480)

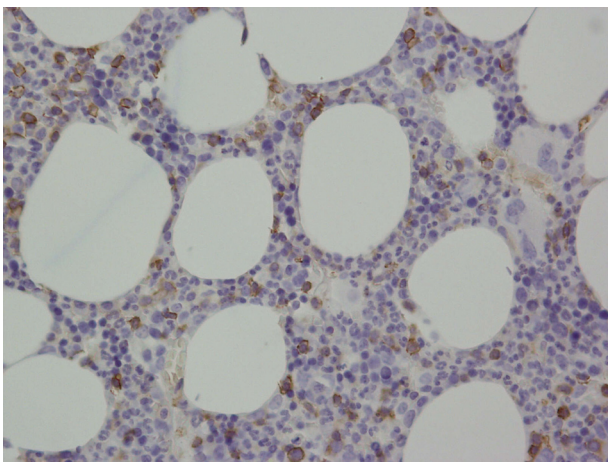

Fig. 3. Bone marrow trephine biopsy section, CLL. Lymphoid cells with positive staining for CB1 antibody — after treatment. Strong positive staining of single cells. Immunohistochemical staining (CB1, x 480)

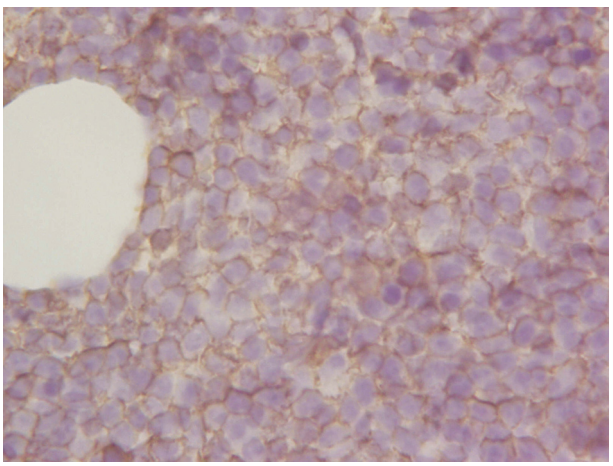

Fig. 4. Bone marrow trephine biopsy section. Diffuse infiltration in CLL. The majority of cells with characteristic expression of CB2 receptors (moderate staining) - before treatment. Immunohistochemical staining (CB2, x 1200)

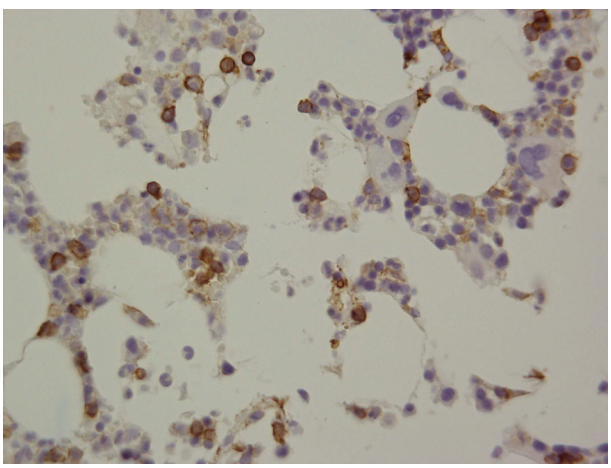

Fig. 5. Bone marrow trephine biopsy section, CLL. Bone marrow hypoplasia. Some cells with positive staining for CB2 antibody. Immunohistochemical staining (CB2, x 240)

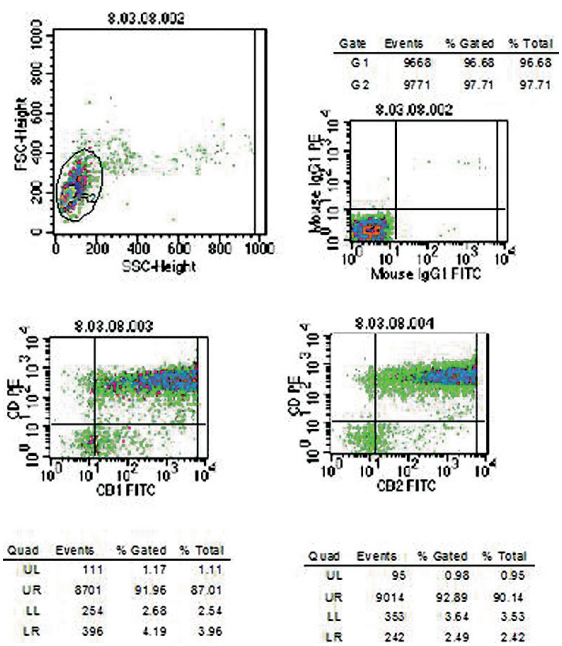

Fig. 6. An example of flow cytometry. Study presents co-expression of both cannabinoid receptors with CD19+ on lymphocytes in peripheral blood of CLL patients. The majority of B-lymphocytes expressed cannabinoid receptors

CB2 receptors by flow cytometry in the B-lymphocytes subpopulation [11]. Interestingly, our study proved that leukaemic cells display a relatively high expression of CB1 receptors. However, similarly to observations made by Galiegue et al. [5], this expression was lower than that of CB2. It is worth mentioning that, according to one report by Islam et al [10], neoplastic cells in mantle cell lymphoma present a very high expression of CB1 receptors. Our findings indirectly confirm the very high density of CB2 receptors through the intensity of the immunohistochemical reaction/staining. In the case of CB1 receptors this intensity was much lower. In fact, we found in FC analysis equal percentage of lymphocytes that present CB1 and CB2 receptors on their surface. However, the MFI that may express density of the receptor was statistically higher for CB2. As we know the FC may be more specific in assessment of particular molecules when their density is low [18]. So, difference between IHC and FC assessment of cannabinoid CB1 receptor may reflect its relative low density on the cell surface.

Other peripheral blood mononuclear cells may express cannabinoid receptors on their surface [3, 17]. To make sure that we analysed lymphocytes the IHC included CD20(+) and CD3(–) staining on serial sections and flow cytometry analysis of neoplastic cells in peripheral blood (all cases) and bone marrow (3 cases) were performed.

Up to date there has been no evidence of the influence of chemotherapy on the cannabinoid receptors expression in the CLL patients. The reduction of the leukaemic bone marrow infiltration after purine analogues treatment did not affect the CB2 expression. In contrast, we noted a significant reduction in the expression of CB1 receptors after the chemotherapy.

In some of the patients who did not respond to the treatment, no changes in CB1 receptor expression were observed. Micro-array analysis of gene expression profile of CLL patients performed by Haslinger et al. [8] showed that CB1 receptor is differently expressed (upregulated) within the group with 11q22–23 deletion. In our study, we did not perform analysis of cytogenetic changes that could reveal whether the individuals that did not respond to the treatment and presented no changes in CB1 expression belong to the group of patients with poor prognosis due to the deletion.

There are two splice variants of CB1 receptor discovered in human tissues. The application of the polyclonal antibody was not helpful to differentiate between the variants. The CB 1 antibody recognised the C terminal amino acid fragment of the cannabinoid CB1 human receptor. The production of Cb2 antibodies has been based on a sequence between the N-terminus and the first transmembrane domain of the receptor [9]. The existence of distinct conserved sequence of amino acids between CB1 and CB2 receptors in these regions excludes the possibility of cross-reactions of the antibodies. The applied indirect immunohistochemical technique did not allow for performing simultaneous assessment of both cannabinoid receptors.

In conclusion, our study provides original evidence for the existence of cannabinoid receptors on lymphocytes B in CLL patients. CB2 receptor expressed in great number on the neoplastic cells may be considered a new particular surface structure modifying the course of the disease. The presence of CB2 receptor on the neoplastic lymphocytes after chemotherapy may be considered as a new target in the treatment of residual disease. Elucidating and understanding the role of endocnnabinoid system in leukaemia genesis could give us an additional possibility in fighting against chronic lymphocytic leukaemia.

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ЭКСПРЕССИЯ РЕЦЕПТОРОВ КАННАБИНОИДОВ В ТРЕПАНОБИОПТАТАХ КОСТНОГО МОЗГА БОЛЬНЫХ ХРОНИЧЕСКИМ ЛИМФОЛЕЙКОЗОМ ПОСЛЕ ЛЕЧЕНИЯ ПУРИНОВЫМИ АНАЛОГАМИ

Обоснование: **рецепторы каннабиноидов CB1 и CB2 являются частью системы эндоканнабиноидов, которая играет важную роль в процессах пролиферации и апоптоза различных неопластических клеток. Одним из заболеваний, при которых происходит нарушение этих процессов, является В-клеточный хронический лимфлейкоз.** ***Цель:*** **оценка экспрессии рецепторов каннабиноидов на В-лимфоцитах в трепанобиоптатах у больных лейкозом до и после проведения лечения с использованием пуриновых аналогов.** ***Методы:*** **биоптаты получили рутинными методами; иммуногистохимическое исследование депарафинизированных срезов проводили по стандартной процедуре. Рецепторы каннабиноидов определяли с использованием специфических моноклональных антител анти-CB1 и анти-CB2. Кроме того, наличие рецепторов подтверждено при помощи проточной цитофлуориметрии.** ***Результаты*****: показано, что экспрессия рецептора CB1 на поверхности опухолевых клеток ниже, чем экспрессия CB2 (17,0 ± 3,1% и 92,1 ± 1,7% соответственно,** $p < 0{,}001$**). После проведенного лечения у 9 пациентов отмечалось уменьшение лейкозного инфильтрата (77,2 ± 6,9% до 30,2 ± 6,5%,** $p = 0{,}007$**) и снижение экспрессии рецептора CB1 (24,4 ± 4,8% до 8,6 ± 2,9%,** $p = 0{,}01$**), однако различий в экспрессии CB2 не отмечали (91,7 ± 2,7% против 90,9 ± 2,8%,** $p = 0{,}69$**). У 4 пациентов, у которых не удалось достичь ремиссии, определяли даже повышение экспрессии рецепторов. Во всех случаях маркировки антителами к обоим типам рецепторов каннабиноидов отмечали положительную реакцию. Более того, присутствие рецепторов каннабиноидов на злокачественных клетках подтверждали при помощи проточной цитометрии.** ***Выводы*****: в ходе исследования показано изначальное наличие рецепторов каннабиноидов на В-лимфоцитах у больных хроническим лимфолейкозом. Указанные рецепторы могут быть новой структурой, которая может быть модифицирована в течение болезни, и могут считаться новой мишенью при лечении больных лейкозом.**

Ключевые слова: **хронический лимфолейкоз, система эндоканнабиоидов, пуриновые аналоги, рецепторы каннабиоидов, трепанобиопсия.**