Type and intensity of tumor-infiltrating lymphocytes (TILs) in close proximity to the primary tumor are prognostically significant in postoperative patients. High intensity of TILs is considered to be a prognostically beneficial factor. The research included 66 postoperative colorectal cancer patients. The control group comprised 20 colon segments. Monoclonal antibodies LCA, CD3, CD4, CD5, CD8, CD20, CD23 and CD138 were used to differentiate between T and B lymphocytes. Types of cells in the infiltrate were defined. We found greater numbers of T and B lymphocytes located in close proximity to the cancerous tissue when compared to the control group. T lymphocyte intensity in the inflammatory infiltrations was directly correlated with the size of resected tumors, presence of regional lymphatic node metastases and histological grade of malignancy. Lymphocytic infiltrations of greater intensity located in close proximity to the primary tumor were found in subjects with less advanced colorectal cancer.

The research presented here proves direct dependence between the immune system and colorectal cancer. The presence of lymphocytes in the inflammatory infiltrations located in close proximity to the cancerous tissue has been proved to be prognostically beneficial. The obtained results support the application of immunotherapy in colorectal cancer treatment.

**Key words:** colorectal cancer, immune response, T and B lymphocytes.
be the most beneficial prognostically, whereas tumors presented in the following order are characterized by an increasingly worse prognosis compared to adenocarcinomas: gelatinous carcinoma, signet ring cell carcinoma, microcellular carcinoma, undifferentiated carcinoma [1].

The intensity of inflammatory infiltration in close proximity to the tumor tissue is significant in the post-surgery prognosis in subjects with colorectal cancer. The classification suggested by Jass in 1987, apart from differentiating between the invasive margin, limitation of growth into the bowel wall and the number of lymph nodes with metastasis, also takes into consideration the conspicuous peritumoral lymphocytic infiltrate, which is commonly known to constitute a prognostically beneficial factor in colorectal cancer cases.

The immune system in cancer subjects has been researched by numerous scientists around the world. Its close correlation with the development of malignant tumors has been defined. An increased number of peritumoral lymphocytes has been found to improve the survival rates in cases of various malignant tumors. In addition, the effector cells of the immune system – T and B lymphocytes, as well as NK cells – produce anticancer cytokines, kill atypical cells by means of apoptosis and destroy tumor antigens by means of antibody-dependent cell-mediated cytotoxicity (ADCC).

However, there are numerous factors which encourage cancer development. Some of them derive from tumor cells, while others result from disorders of the immune system. Tumor cells avoid the immune system response by regulating the number of major histocompatibility complex class I molecules (MHC I) on their surface and by producing numerous biologically active immunosuppressive agents.

The correlation between the immune system and biology of malignant tumors encourages oncologists to search for new alternative methods of cancer immunotherapy.

**Material and methods**

The research was conducted on a group of 66 postoperative colorectal cancer subjects, aged 46-82 years, at the Medical University of Białystok Clinical Hospital. Subjects with distant metastases, low tumor grade (G1) and the histological type of mucinous adenocarcinoma were excluded from the study, since the low incidence level did not allow us to compose a statistically significant group.

Tumor grading (G) was applied to define moderately differentiated tumors (G2) in 34 patients (52%) and poorly differentiated tumors (G3) in 32 subjects (48%). The TNM staging system size measuring mandatory parameters were used to select two groups of subjects: (1) pT1 + pT2 (35 cases) and (2) pT3 + pT4 (31 cases). Two age ranges were taken into account: ≤ 65 years (29 subjects – 44%) and > 65 years (37 subjects – 56%). The study included 30 female (45%) and 36 male subjects (55%). In 27 (41%) cases tumors were located in the colon, in 39 (59%) cases in the rectum. Lymph node metastases were found in 32 subjects (48%) by means of histopathological analysis. Correlations between age, gender of the subjects and the following anatomical and clinical features were investigated: size of the primary tumor (pT), presence of lymphatic node metastases (pN), histological grade of malignancy (G) and presence of inflammatory infiltration. Invasion into healthy tissue was analyzed by Dukes’ classification. All the examined tumors were classified as Dukes’ A and Dukes’ B.

The control group was made up of 20 colon segments resected from the proximal part of the ascending colon at the minimum distance of 10 cm from the primary tumor, which had been analyzed histopathologically to eliminate the presence of cancerous infiltration.

The histological type of tumor, histological grade (G) and pathologic stage (pTNM) were analyzed in the Department of Pathomorphology at the Medical University of Białystok. Tissue samples were formalin-fixed, embedded in paraffin blocks and further analyzed immunohistochemically in the Department of Human Anatomy at the Medical University of Białystok. Paraffin blocks were then cut on a microtome into 6 μm paraffin sections, which were stained with hematoxylin and eosin prior to basic histological analysis. Tissue sections were deparaffinized by hot incubation and then consecutively bathed and incubated in decreasing solutions of xylene and ethyl alcohol.

The immunohistochemical (IHC) method was applied to identify lymphatic cells. Primary monoclonal antibodies (Dako) were employed to detect T and B lymphocytes: CDA diluted 1 : 50 for lymphoid cells, CD5 diluted 1 : 50 for T lymphocytes, CD4 diluted 1 : 40 for T helper lymphocytes, CD5 diluted 1 : 50 for T lymphocytes, CD8 diluted 1 : 100 for T cytotoxic and T suppressor lymphocytes, CD20 diluted 1 : 50 for B lymphocytes, CD23 diluted 1 : 100 for T lymphocytes, CD138 diluted 1 : 50 for mature B lymphocytes. The incubation period for all the antibodies was 30 minutes. The LSAB + System-HRP was used as a detection kit and so was 2,3’-diaminobenzidine chromogen solution.

A light microscope at 480× magnification was used to analyze IHC reactions. The numbers of lymphocytes in 1 mm² of tissue samples were counted, evaluating four visual fields of the peritumoral area. The average number of particular lymphocytic cells was calculated.
The number of cells was counted by means of the Olympus morphometric program MicroImage InCD UDF Packed Writing Software for Windows. Numbers of particular lymphocyte types present in the inflammatory infiltrations were calculated. The results were analyzed statistically by means of Statistica 7.1 software using Student’s t-test for dependent and independent samples, as well as the Mann-Whitney test. Selection of the test to be applied was relevant to the distribution within groups.

Subjects were divided into 8 groups based on the following histopathological and clinical features:
- IA-G2, T1+2, N0,– (9 subjects),
- IB-G2, T1+4, N0,– (8 subjects),
- IC-G2, T1+2, N+,– (9 subjects),
- ID-G2, T1+4, N+,– (8 subjects),
- IIA-G3, T1+2, N0,– (9 subjects),
- IIB-G3, T1+2, N0,– (9 subjects),
- IIC-G3, T1+2, N+,– (8 subjects),
- IID-G3, T1+4, N+,– (7 subjects).

Correlations between the selected anatomical features, clinical features and the presence of inflammatory infiltration, as well as the types of cells in the infiltrate, were defined. Calculations were considered statistically significant at p < 0.05.

Results

Tables I and II present the average numbers of particular lymphocyte types in patient groups I and II, respectively.

Analysis of the lymphocytes present in the control group proved presence of singular lymphocytic cells, mainly CD3+ and CD20+ lymphocytes, which were located within the physiological lymph follicles. Correlations between all the analyzed features and the tumor tissue proved to be statistically significant in each case, as there were only a few lymphocytic cells present.

As far as group I is concerned, statistically significant correlations were established with regard to the numbers of particular types of lymphocytes. Statistically significant correlations were found in: CD3+ lymphocytes in group IA – 128 and in group ID – 42.8 and in group IIA – 42.8 and in group IIC – 13.8 (p = 0.002). Statistically significant correlations were also found in cases characterized by an outstanding tumor size in group IIA – 42.8 and in group IIB – 13.2 (p = 0.002). Also, statistically significant correlations were revealed in subjects with advanced cancer: in group IIA – 42.8 and in group IID – 12.6 (p = 0.002) (Fig. 5).

Correlations proved to be statistically significant with regard to the histological grade (G2, G3). Statistically significant relationships were found in CD4+ lymphocytes in group IA – 128 and in group IIA – 67.2 (p = 0.033); CD5+ lymphocytes in group IA – 65.8, in group IIA – 42.8 (p = 0.027), in group IC – 47.2 and in group IIC – 15.2 (p = 0.005). It was similarly the case in LCA+ cells in group IA – 393 and in group IIA – 274 (p = 0.015) (Figs. 4B and 6).

Discussion

The immune system participates in numerous body reactions to bacterial and viral infections, autoimmune diseases and allergic events. It also exerts an impact on the process of cancer development and its prognosis. While stimulation of the immune system results in tumor regression, its suppression creates preferential circumstances for cancer development [2].

Higher average numbers of LCA+, CD3+, CD8+, CD4+ and CD5+ cells were detected in the analyzed samples compared to the control group. The average numbers of CD20+, CD23+ and CD138+ cells detected in the studied cases of colorectal cancer were significantly lower compared to the average numbers of LCA+, CD3+, CD8+, CD4+ and CD5+ cells. The results of our research showed a preponderance of the cellular type of reactions over hormonal ones in close proximity to the colorectal tumor. In T lymphocytes the greatest number of cells was found to be associated with CD3+ expression, rather than with CD8+, CD4+ or CD5+. Our findings proved a preponderance of cytotoxic CD8+ lymphocytes over helper CD4+ lymphocytes in the cellular-type reactions within the analyzed cases of colorectal cancer.

An increased preponderance of CD3+ and CD8+ lymphocytes over CD4+ cells was observed by Rich-
Pawel Laskowski, Beata Klim, Karol Ostrowski et al.

Ards et al. and Dolcetti et al. [3, 4]. Michael-Robinson et al. reported a similar preponderance of CD8+ lymphocytes, which constituted 75% of the cells in the inflammatory infiltration located in close proximity to the colorectal tumor, over CD4+ lymphocytes which made up only 25% of the infiltrate. They also established a positive correlation between the intensity of inflammatory infiltration and the intensity of apoptotic processes [5]. Koch et al. did not find any statistically significant correlations between the numbers of CD4+ and CD8+ lymphocytes. However, their research showed intense infiltration in close proximity to the primary tumor compared to the healthy mucous tissue of the colon, the percentages of CD4+ and CD8+ lymphocytes being similar [6].

Our research results proves the preponderance of CD8+ lymphocytes. CD8+ lymphocytes produce anticancer cytokines called tumor necrosis factors (TNF). Tumor necrosis factors belong to a group of proteins involved in numerous kinds of proliferation,
including TNF-related apoptosis inducing ligand (TRAIL). TRAIL induces apoptotic processes in the majority of neoplastic types, and this feature is employed in cancer immunotherapy [7]. Apoptosis of cancer cells is also triggered by TNF through binding with its TNFR1 and TNFR2 receptors. T lymphocytes carry TNF cytokines on their surface, and Fas ligand (FasL), by activating the Fas receptor (FasR), induces apoptosis. Moreover, CD8+ lymphocytes contain granzymes and perforin, which insert themselves into the target cell’s plasma membrane, forming pores, and then damage the DNA of cancerous cells [4].

Funada et al. observed a correlation between the presence of CD8+ T lymphocytes in inflammatory infiltrations and increased colorectal cancer mortality [8]. They proved the existence of a positive correlation between CD8+ lymphocytes and deeper growth into the bowel wall, as well as increased neoangiogenesis.
In our research we observed increased expression of CD4+ helper lymphocytes. Functions of helper lymphocytes are vast and cover stimulation of the effector cells of both the congenital and developed immune response.

CD4+ lymphocytes produce many cytokines, which makes them an important part of the cancer defense mechanism. Interleukin-21 (IL-21), produced by helper lymphocytes, increases cancer survival rates and NK cell activation, as well as the expression of NK cell receptors and their cytotoxicity. Similarly, IL-21 accelerates the maturation of NK cells and increases anticancer functions in NK cells and cytotoxic CD8+ lymphocytes. Moreover, IL-21 increases the proliferation of cytotoxic T lymphocytes, whereas interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), IL-1 and IL-15 increase the abilities of antigen-presenting cells (APC) to present anti-

Fig. 1. Differences in the average number of CD3+, CD4+, CD23+, LCA+ cells in subjects without (NO) and with (N+) lymph node metastases

Fig. 2. Differences in the average number of CD5 lymphocytes in subjects with tumors limited to the muscle tissue (T1,2) and subjects with tumors perforating the bowel wall (T3,4)

Fig. 3. Differences in the average number of CD4 and CD5 lymphocytes in subjects without lymph node metastases and a smaller tumor size (T1,2 NO) and subjects with lymph node metastases and a greater tumor size (T3,4 N+)

Fig. 4. Immunohistochemical reaction in subject: A) T2 NO, anti-CD4 monoclonal antibody is used, magnification 240×; B) with G2, anti-CD5 monoclonal antibody is used, magnification 480×
Locally inflammatory response in colorectal cancer

gens, including cancer CD8+ lymphocytes. Furthermore, CD4+ lymphocytes are able to kill cancer cells by means of apoptosis [9]. McMillan et al., having observed postoperative colorectal cancer subjects for two years, proved anticancer functions of helper lymphocytes [10].

Unfortunately, cytokines produced by helper lymphocytes are able to accelerate cancer development. TNF-α and IL-1 stimulate neoangiogenesis, whereas TGF-β decelerates the proliferation of antigen-specific T lymphocytes. IL-4 and IL-10, produced by CD4+ lymphocytes, decelerate the responsive production of IFN-γ by CD4+ lymphocytes, whereas IL-6 promotes the proliferation of tumor cells and triggers suppression of the immune system. German scientists proved higher concentrations of IL-6 and IL-10 in the plasma of subjects with advanced colorectal cancer compared to a control group composed of healthy volunteers. The higher concentrations of IL-6 and IL-10 in plasma positively correlated with cancer progression [11].

Our research clearly proved that the functioning of helper T lymphocytes is not fully relevant to deceleration of cancer development. There exist numerous interactions, as well as positive and negative reactions between the cytokines produced by helper lymphocytes. We were not able to define which cytokines dominated cancer or anticancer.

We detected CD20+ and CD138+ expression mainly within the lymph follicles and in close proximity to blood vessels. The average numbers of CD20+ and CD138+ cells were low compared to CD3+, CD8+, CD4+, CD5+ lymphocytes and LCA+ cells. However, Nascimbeni et al., while analyzing the morphology of lymph follicles and B lymphocytes in colorectal cancer, did not observe any differences in the numbers of CD20+ cells in their subjects [12].

The hormonal response undoubtedly affects cancer regression. Maxwell-Armstrong et al., Buckley et al. and Habal et al. all reported the decelerating influence of hormonal response on cancer development. Production of specific antibodies and IL-4 appears to reduce tumor mass [13, 14, 15]. Cellular cytotoxicity, which depends on ADCC antibodies, is another mechanism which damages atypical cells. B lymphocytes produce specific antibodies which, when bound to NK cells, initiate cytotoxic mechanisms and production, which results in killing cancer cells. Cytotoxic and NK cell production are stimulated to a significant degree by IL-2, which is produced by B lymphocytes.

Research conducted on animal models proved that B lymphocytes decelerate the anticancer response of CD8+ lymphocytes. B lymphocytes compete with APC cells for antigens released by tumor cells, blocking antigen presentation to CD8+ lymphocytes. Moreover, B lymphocytes are known to activate suppressive lymphocytes.

We observed no correlation between infiltration intensity and age or gender of our subjects, like numerous previous studies [8]. Moreover, age does not influence the survival rates in subjects with colorectal cancer. The lack of correlation between infiltration intensity and age or gender suggests that these features do not influence the survival rates.

We established a correlation between the tumor size and intensity of inflammatory infiltration in subjects with colorectal cancer. We found a decreased number of cells within the inflammatory infiltration in subjects with more advanced disease (T3+4). Statistically significant differences were found in CD4+ and CD5+ cells. Similar results were reported in plentiful previous studies which showed a negative

![Fig. 5. Differences in the average number of CD5 lymphocytes in subjects without lymph node metastases and tumors limited to the muscle tissue (T1,2 NO) and subjects with lymph node metastases and tumors perforating the bowel wall (T3,4 N+)](image)

![Fig. 6. Differences in the average number of CD4+, CD5+, LCA+ cells in subjects with G2 and G3 tumors](image)
correlation between the intensity of inflammatory infiltration, tumor size and survival rates [16].

The histological grading of malignancy was proved to influence the average number of cells in the inflammatory infiltration. Subjects with a higher grade had a decreased number of lymphatic cells. Statistically significant differences were found in the numbers of LCA+, CD4+ and CD5+ cells. Similar results were reported by Di Georgio and Lackner, who proved a correlation between the intensity of inflammatory infiltration, grading and survival rates in subjects with colorectal cancer [17, 18]. Our results established a correlation between the numbers of T lymphocytes and grading in subjects with colorectal cancer.

We found a correlation between the intensity of inflammatory infiltration and the presence of lymph node metastases. Statistically significant differences were found in the numbers of CD3+, CD4+, CD5+ and CD23+ cells. Studies by Ropponen et al., George et al., Pages et al. and Nagtegaal et al. all showed a decreased number of lymph node metastases and cancer recurrences in subjects with intensive lymphocytic infiltration [19, 20, 21, 22]. Dillman et al. proved that in cases of colorectal cancer, kidney cancer and melanoma subjects with distant metastases who received high dosages of IL-2 the survival rates were twice as good as in subjects treated conventionally [23]. Application of high dosages of IL-2 resulted in an increased number of CD8+ cells in the inflammatory infiltration in close proximity to the primary tumor and distant metastases. Our results suggest a hypothesis that the presence of T lymphocytes in the inflammatory infiltration in close proximity to the primary tumor decreases the number of lymph node metastases.

When assessing the efficiency of the immune response to malignant cancer cells, the following fact should be considered: effector mechanisms of the immune system are decelerated by cancer cells. Tumor cells possess decreased expression of MHC I connected with a mutation of genes which encode β2-microglobulin, which results in the loss of antigen features. It is estimated that around 16-50% of MHC I are subject to negative regulation in cancer cells. A correlation was found between MHC I downregulation, higher grading, reduced histologic differentiation and worse survival rates in cases of ovarian, nipple, lung and colorectal cancers. These facts undoubtedly suggest that suppression of the immune system and its mechanism greatly influence cancer prognosis.

Cancer cells are able to produce biologically active substances which influence tumor progression. Vascular endothelial growth factor (VEGF), produced by tumor cells, accelerates neoangiogenesis and decelerates APC to present genes, similarly to IL-10 produced by cancer cells. Moreover, cancer cells are able to produce pro-cancer cytokines.

TGF-β suppresses B, T and NK cells and promotes tumor growth by dissolving the extracellular matrix and accelerating neoangiogenesis. IL-23, also produced by tumor cells, promotes suppressive lymphocytes and simultaneously decelerates the functioning and proliferation of cytotoxic lymphocytes.

Glycoprotein, produced by atypical cells, described as MUC-1, exhibits pro-cancer features. MUC-1 disables T lymphocytes to present antigens [24]. Cancer cell also release tumor-associated antigens (TAA). CA19.9, which belongs to the TAA group, causes energy of cells capable of apoptosis. The immunosuppressive influence of CA19.9 on digestive tract cancer was proved by Li et al. in their in vitro research [25].

The immunology of colorectal cancer is extremely complex and depends on mutual interactions between features which disable and promote tumor growth. It should be emphasized that tumor growth promoting and disabling substances are produced by both the effector cells of the immune system and tumor cells.

Conclusions

Our research results prove that the immune system, heavily dependent upon the environment to function properly, is closely correlated with the development of colorectal cancer and its prognosis. The presence of lymphocytes in the inflammatory infiltrate in close proximity to the primary tumor is prognostically beneficial. There are correspondences between the intensity of T lymphocytes in the inflammatory infiltrate and the size of resected tumors, the presence of regional lymph node metastases and grading (CD4+ lymphocytes, CD5+ lymphocytes and LCA+ cells in G2-G3/T1+2). In our study, there was no correlation between the humoral immune response cells and the anatomical and clinical features we took into consideration. Our research established a correlation between the immune system and colorectal cancer. It should be emphasized that we revealed dominance of T lymphocytes in the cellular response to colorectal cancer.

Analyzing interactions between colorectal cancer and anticancer properties of all the effector cells in the immune system, we must also take the immunosuppressive properties of tumors into consideration. In addition, the effect of biologically active substances, including numerous cytokines, produced by both the immune system cells and colorectal cancer cells, should also be considered.

The authors declare no conflict of interest.


