Immunity profile in breast cancer patients

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Abstract: Objectives: Despite the multifactorial pathogenesis of malignant transformation, it is assumed that deficiency in some immune mechanisms plays a considerable role in its development. Background: Chronically activated immune cells exert tumour-promoting effects directly by influencing the proliferation and survival of neoplastic cells, as well as by indirect modulation of neoplastic microenvironments in favour of tumour progression.

Patients and methods: We refer to results of two separate investigations that aim to monitor the immune functions in patients with breast cancer. In the first investigation, we compare the picture of basic cellular immunity profile of patients in early stage of breast cancer with those suffering from advanced disease; in the second one, we compare the production of Th1-cytokines in patients in different stages of breast cancer and atopic healthy controls.

Results: We recognized that the totals of T-lymphocytes and T-helpers were lower and the expression of HLADR on T-lymphocytes were higher in patients with advanced disease; the expression of IL-2 and IFN-γ by T-lymphocytes was decreased in metastatic breast cancer patients, however IL-2 production was increased in patients in early stage of disease.

Conclusion: We conclude that the role of immune system in cancer development is ambivalent as it may be not only protective, but also harmful (Tab. 1, Fig. 3, Ref. 22). Full Text (Free, PDF) www.bmj.sk.

Key words: breast cancer, immunity profile.

The immune system comprises many immune cell types and mediators that interact among themselves and with non-immune cells. These interactions compose a dynamic network that protects against foreign pathogens along with maintaining tolerance towards harmless foreign antigens and self-antigens. Based on antigen specificity and timing of activation, the immune system is composed of two distinct compartments – adaptive and innate (non-adaptive). Innate immune cells, such as dendritic cells (DCs), natural killer (NK) cells, macrophages, neutrophils, basophils, eosinophils and mast cells form the first line of defence against foreign pathogens. The ability of rapid response to tissue injury (with no previous experience and independently of antigen specificity) is a unique feature of innate immune cells distinguishing them from adaptive immune cells. The activation of innate immunity leads to activation of more sophisticated specific adaptive immune responses.

Activated innate immune cells possess an enormous capacity to produce a myriad of cytokines, chemokines and pro-angiogenic mediators, metalloproteinases, reactive oxygen radicals, enzymes such as cyclooxygenase-2, histamine and many other bioactive mediators. These innate immune cells are the key modulators of cell survival (regulating cell proliferation and death) (1). Despite intensive research, pathogenesis of malignant transformation is not fully understood. The programmed cell death is one of crucial components of health as well as of malignant cell transformation. We know that cancer is a disease that originates from mutant DNA sequences leading to deregulation of tissue homeostasis and violation of cell survival and/or cell death. Cancers are not merely masses of mutant cells, but they are composed of multiple cell types, such as fibroblasts, epithelial cells, cells forming blood and lymphatic vasculature, mesenchymal cells as well as innate and adaptive immune cells. The role of immune cells in cancer tissue is controversial because at this stage of tumor genesis they are incapable of damaging the cancer development.

From the immunological viewpoint, the cancer development largely depends on the ability of mutant cells to hijack and exploit the normal physiological processes of the host. Each stage of cancer development is exceedingly susceptible to regulation by immune cells (1). We can assume that full activation of adaptive immune cells in response to the tumour might result in eradication of malignant cells around pre-malignant tissues. However, in the environment of a growing tumour, the balance between...
innate and adaptive immunity and tumour-cell growth is often violated in favour of cancer progression.

**Immune system in health and disease**

When tissue homeostasis is perturbed, sentinel macrophages and mast cells immediately release cytokines, chemokines, matrix proteases, reactive oxygen radicals and bioactive mediators such as histamine; all of them induce mobilization and infiltration of additional immune cells into the damaged tissue. Macrophages and mast cells also activate the vascular and fibroblast responses in order to initiate the local tissue repair. DCs take up foreign antigens and thereafter migrate to lymphoid organs where they present their antigens to adaptive immune cells. NK interact with DCs in two manners; some NK-cell subsets eliminate immature DCs, others promote DC maturation. Reciprocally, DCs can regulate the activation of NK cells (2).

The induction of efficient primary adaptive immune responses requires direct interactions with mature antigen-presenting cells and a pro-inflammatory cytokine milieu. Adaptive immune cells, such as B-lymphocytes, CD4+ helper T lymphocytes and CD8+ cytotoxic T lymphocytes (CTLs) distinguish themselves from innate leukocytes by the expression of diverse antigen-specific receptors. The latter receptors allow a flexible and broader repertoire of responses than innate immune cells, which express germline-encoded receptors. Individual B and T lymphocytes are antigenically committed to a specific unique antigen.

In addition to the elimination of invading pathogens, immune cells are crucially involved in normalizing the cell-proliferation and cell-death pathways to enable tissue repair. Inflammation should resolve, and normally the tissue homeostasis recovers. When inadequate inflammation persists, many morbid processes occur. The immune system is thereby involved in maintaining tissue homeostasis as well as in the pathogenesis of many chronic diseases, such as arthritis, heart disease, Alzheimer disease and cancer. The destructive cycles initiated within tissues by failure to appropriately “switch on” and/or “switch off” distinct arms of the immune system can result in tissue remodelling and/or to tissue destruction, protein and DNA alterations due to oxidative stress, and, under some circumstances, increased risk of cancer development (1).

**Associations between immunity and cancer**

Why do tumour cells escape the immune-surveillance mechanisms? Neoplastic microenvironments can favour the chronic pro-tumorigenic inflammatory state rather than anti-tumour immune responses (3). Some clinical data indicate that the immune profile of patients with malignant tumours is distinct from healthy individuals. Functional activity of T lymphocytes from malignant patients is impaired, and chronically activated myeloid suppressor cells and regulatory T cells are found in the circulation as well as in lymphoid organs and neoplastic tissues (4–6). These changes can lead to disabling the tumour-killing cytotoxic lymphocytes (CTL CD8+).

Population-based studies reveal that individuals who are prone to chronic inflammatory diseases are at increased risk of cancer development (3). It is estimated that over 15% of human cancers are caused by infectious conditions, which indirectly promote carcinogenesis through the induction of chronic inflammatory states (7). It was believed that leukocytic infiltrates in and around malignant tissue represented an attempt of the organism to eradicate neoplastic cells. However, this was confirmed only in some cases: extensive infiltration of NK cells in some carcinomas is associated with a favourable prognosis (8, 9), but malignant tissues containing other innate-immune cell types (e.g. macrophages in human breast carcinoma, mast cells in human lung adenocarcinoma and melanoma) tend to be associated with poor clinical prognosis (10–13).

Now it is clear that polymorphisms in genes that encode “modified inflammation” exist in individuals with chronic inflammatory disorders who are at increased risk of cancer. Hence, genetic polymorphisms in genes that encode crucial cytokines, proteases and signalling proteins have been identified as aetiological factors in several chronic inflammatory disorders (3). These findings indicate that therapeutics that are aimed at normalizing the immune balance might be efficacious in prevention of carcinogenesis.

How do chronically activated innate immune cells participate in cancer development? Which mechanisms and which inflammatory-cell-derived mediators are involved in human malignancies? Despite the fact that many of these questions remain unanswered, some experimental models elucidate particular mechanisms by which innate immune cells regulate cancer processes. Mechanisms by which innate immune cells (macrophages, mast cells and granulocytes) contribute to cancer can function either directly or indirectly. Direct mechanisms imply induction of DNA damage by generating free radicals and by paracrine regulation of intracellular pathways; indirect mechanisms involve cytokines, chemokines and matrix metalloproteinases, promotion of angiogenesis and tissue remodelling by the production of growth-factors, cyclooxygenase-2 upregulation and suppression of antitumour adaptive immune responses allowing the tumour escape from immune surveillance. Adaptive immune cells modulate cancer development ambivalently: they can inhibit the tumour growth directly by antitumour cytotoxic-T-cell activity and by cytokine-mediated lysis of tumour cells. However, they can also contribute to tumour growth through the activity of regulatory T cells that suppress the antitumour T-cell responses, and by humoral immune responses that increase chronic inflammation in the tumour microenvironment (1).
infiltration of pre-malignant tissue reduces the angiogenesis and limits the tumour development (16). The tumour microenvironment is rich in cytokines, chemokines and pro-angiogenic mediators derived from immune cells (e.g. tumour necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), and interleukins 1 (IL-1) and 6 (IL-6) (3). VEGF is a fundamental chemokine that increases the angiogenesis and enhances the tumour development (14). Similarly, TNF-α, a key cytokine of acute inflammation, mediates the cancer development. Mice deficient for TNF-α or TNF-α receptors have a reduced susceptibility to chemically induced skin cancers and develop fewer experimental metastases (17).

Myeloid suppressor cells are known to induce T-lymphocyte dysfunction by direct cell-cell contact and by production of immunosuppressive mediators that actively inhibit the antitumour adaptive immunity (6, 18). It is also known that malignant tissues attract regulatory T cells that suppress the effector functions of cytotoxic T cells (19). Experiments have revealed that depletion of regulatory T cells (using monoclonal antibodies anti-CD25) enhances antitumour T-cell responses and induce the regression of experimental tumours. The presence of regulatory T cells (in patients with ovarian cancer) is correlated with reduced survival (4).

Tumors express antigenic peptides that can become the targets of a tumor-specific T-cell response. It is assumed that the role of adaptive immunity in carcinogenesis is – in general – protective. This is supported by epidemiological studies of cancer incidence in immune-suppressed individuals (e.g. subjects with AIDS, patients on immunosuppressive therapy). Their risk for viral-associated cancers (e.g. Kaposi sarcoma, non-Hodgkin lymphoma, HPV-associated squamous carcinoma) is elevated significantly. Among other malignancies, the relative risk (RR) varies between individual cancers. Surprisingly, the RR for the most common non-viral-associated solid tumours of epithelial origin in immune-suppressed patients is decreased (breast, prostate and bladder cancer have an RR <1.0) (1).

The occurrence of autoantibodies in the serum of cancer patients is sufficiently documented. The early presence of autoantibodies in the serum correlates with unfavourable prognosis (20). It is not clear whether this correlation indicates that individuals with tumours in progress have a higher antigen load and therefore trigger greater antibody production, or the presence of antibodies predisposes patients to the development of more advanced cancers. However, these data outline that B-lymphocytes are also involved in human cancer development.

Our data

In our outpatient department we are often consulted about the “immunological status” of patients with malignant disease. Our experiences show that the so-called immunity profile (differential count of leukocyte subtypes; expression of basal CD markers on leukocytes; total level of immunoglobulins IgA, IgG, IgM; level of C3 and C4 components of complement system; level of C-reactive protein and prealbumin) of patients with malignant disease is very variable and not specific. Actual levels/values of a particular parameter vary in dependence on the disease stage, therapy (chemotherapy, radiotherapy); actual patient’s stage and health (not only according to the malignant disease, but also in connection with his/her other diseases, concomitant infection), age, etc.

With the exception of hematological malignancies (leukemia, lymphoma), we have not seen any special dependence on the type of tumour, moreover – with exception of terminal stage of disease – we have not observed any great dependence on the disease stage. This observation, namely that there was a small difference between immunity parameters of patients with initial disease and those of patients in advanced stage, was the motive behind our study. We have compared the immunity profiles from patients with newly diagnosed early stage of breast carcinoma (carcinoma in situ) with those from patients with advanced disease.

The aim of the second part of our work was to evaluate the production of IL-2 and IFN-γ in breast cancer patients. T-lymphocytes (CD3+) produce several types of cytokines. We investigated Th1-type cytokines – interleukin-2 (IL-2) and interferon-gamma (IFN-γ), i.e. the cytokines that are responsible for cell-mediated inflammatory reaction, delayed-type hypersensitivity and tissue injury in infections and autoimmune disease. The cancer development (especially in advanced stage with metastases) leads to the impairment of T-lymphocyte-mediated immunity, including the downregulation of Th1-lymphocyte function since the production of IL-2 and IFN-γ is systemically or locally inhibited in cancer patients.

Methods

We assigned patients from our outpatient department of clinical immunology dispatched by oncologists to immunological consultation because of breast cancer in any stage. No selection was done; we examined the immunity profiles of all patients in chronological order. The only exclusion criterion was acute infectious disease. The cohort was divided into two groups, namely the patients in the earliest stage of disease – so-called carcinoma in situ – and patients with advanced disease with metastases; each group comprises 30 patients. Informed consent was obtained from all subjects or their guardians prior to blood collection. Venous blood samples were collected directly into Vacutainer containing EDTA-anticoagulant. The expression of CD markers was detected on FACS-Canto flow cytometer (Becton Dickinson).

Our observation was focused on two arrays of parameters obtained from these two patient groups. In both groups we observed the expression (%) of positive cells) of CD markers and absolute count of cells as follows: CD3 (T-lymphocytes), CD4 (helper T-lymphocytes), CD8 (cytotoxic T-lymphocytes), CD19 (B-lymphocytes), CD16+56 (natural killers) as well as the expression of activating marker HLADR on CD3-positive cells.

Our aim was to assess whether the two cohorts of patients with breast cancer in different disease stages differ in these cellular immunological parameters significantly or not. To fulfil this
target we used the variance data of monitored parameters and their average values as characteristics. By using the minimal and maximal values of monitored parameters (in each group of patients) we ranked their diffuse scattering. We estimated the average value as an interval of incidence on the significance level of 95%. In such case the average μ occurs in interval (x̄ – 1.96 · σ/√n) till (x̄ + 1.96 · σ/√n); x̄ is median of spotted parameter, x̄ = (x1 + x2 + … + xn) / n; n represents total number of members in group, in both cases n = s 1, 2, ..., 30, and δ is standard deviation of evaluated file.

Results are depicted graphically in Figures 1 and 2 by means of box graphs. Lower and upper sides of each box represent lower and upper interval bounds in which the average of evaluated parameter occurs; vectors on boxes represent minimal (lower abcissa) and maximal (upper abcissa) values which occurred in each evaluated parameter. In each figure, we classified each parameter side by side from both groups of patients. Ranks of parameters from two evaluated groups differ significantly when their boxes do not overlap (on the other hand – parameters do not differ significantly, when they interlap or overlap). The lengths of lower and upper abcissas characterize the index of dispersion – too long abcissas signalize the occurrence of distant (and probably not accurate) values.

In the second part of our investigation, venous blood samples were collected (directly into Vacutainer containing sodium heparin anticoagulant) from 28 breast cancer patients, of which 20 were in early stage of breast carcinoma and 8 patients had metastatic breast carcinoma. The control group comprised 16 atopic subjects (allergic rhinitis without other health impairment) from our allergy outpatient department. Blood samples were stored at room temperature (22 °C) in the dark. Informed consent was obtained from all subjects or their guardians prior to blood collection.

**Cell culture and activation of T-lymphocytes**

Whole blood (500 μL) was diluted 1:1 with RPMI 1640 in 12x75 mm fluorescence-activated cell sorting tubes and activated with phorbol 12-myristate 13-acetate (PMA; 2.5 ng/mL, Sigma Chemical Co., St Louis, MO) and ionomycin (1 Ag/mL, Sigma Chemical). These cultures were incubated for 4 hours at 37 °C and 5 % CO₂ in the presence of brefeldin A (10 μg/mL, Sigma Chemical), a transport inhibitor that prevents cytokine release from cells. Samples incubated with brefeldin A alone served as nonstimulated controls.

**Fluorescent labelling and flow cytometry of intracellular cytokines**

Activated or non-stimulated blood (100 μL) was pipetted directly into a 12x75-mm polystyrene tube containing 20 μL of monoclonal antibodies for T-lymphocytes surface antigen CD3 (CD3-PerCP, Becton Dickinson, San Diego, CA) and incubated at room temperature in the dark for 15 minutes. Then 3 mL of 1×FACS lysing solution (Becton Dickinson) was added to lyse red cells (and fix white cells) and incubated at room temperature in the dark for 10 minutes. After centrifugation at 500-x g for 5
minutes, the supernatant was aspirated and 500 μL of 1xFACS permeabilizing solution (Becton Dickinson) was added into the pellet and incubated for 10 minutes at room temperature in the dark. After washing with 3 mL buffer (1% bovine serum albumin, 0.1% NaN₃, 1xPBS), cytokine-specific antibodies (IFN-gamma-FITC, IL-2-FITC Becton Dickinson) were added to the cells and incubated for 45 minutes at room temperature in the dark. After one final wash, cells were resuspended in 1% paraformaldehyde and stored at 4 °C until flow cytometry analysis. Cells were acquired using a Beckman-Coulter EPICS ALTRA flow cytometer equipped by Expo 32 program for analysis. A minimum of 5,000 CD3+ cells was counted from each sample.

Isotype control, fluorochrome-equivalent

IgG1-FITC and IgG2b-FITC isotype controls (Becton Dickinson) were used to detect non-specific bindings. Compensation for dual-fluorochrome spectral overlap was made using cells individually stained with FITC-only and PerCP-only antibodies.

Results and discussion

Part 1

Figures 1 and 2 imply that in most of evaluated basic cellular immunological parameters we do not observe any statistically significant difference between patients with early stage and those with advanced breast cancer. The only exceptions are the absolute counts of T-lymphocytes (CD3+ cells) and helper T lymphocytes (CD3+4+ cells) and the expression of HLADR on CD3+-positive cells. Total T-lymphocytes and T-helpers are both lover, and the expression of HLADR on T-lymphocytes is higher in patients with advanced disease. In the total count of CD3+8+ cells (cytotoxic T-lymphocytes) we can see some tendency to lower values in patients with advanced disease, however this trend is not statistically significant.

Part 2

In the control group (n = 16, healthy atopics), the mean ± SD for CD3+/IL-2+ cells was 23.8 ± 9.5 %, with a range of 6.2–33 %. The upper limit of the normal range was defined as the mean + 1 SD (33.3 %) and the lower limit as the mean - 1 SD (14.3 %) of CD3+/IL-2+ cells. The mean ± SD for CD3+/IFN-γ+ cells was 31.2 ± 12.8 %, with a range of 9.6–49.5 %. The upper limit of the normal range was defined as the mean + 1 SD (44 %) and the lower limit as the mean - 1 SD (18.4 %) of CD3+/IFN-γ+ cells.

In the non-metastatic breast cancer group (n = 20), the mean ± SD for CD3+/IL-2+ cells was 30.6 ± 19.8 %, with a range of 3.3–62.7 %. The mean ± SD for CD3+/IFN-γ+ cells was 24.9 ± 12.9 %, with a range of 7.9–51.2 %. In the metastatic breast cancer group (n = 8), the mean ± SD for CD3+/IL-2+ cells was 13.6 ± 12.1 %, with a range of 0.3–34.8 %. The mean ± SD for CD3+/IFN-γ+ cells was 20.4 ± 22.8 %, with a range of 3.5–67.6 %.

We observed an increase in the number of patients with expression of IL-2 on T-lymphocytes below lower limit depending...
on the stage of disease. Similar results were seen in expression of IFN-γ on T-lymphocytes. In comparison with the control group, we observed a higher expression of IL-2 on T-lymphocytes in more non-metastatic breast-cancer patients (Fig. 3, Tab. 1).

Discussion

Plenty of experimental work has been done in the field of immunology in cancer; however, literature is destitute of basic immune parameters used in clinical practice in malignant patients. We can find many articles dealing with basic research, but nothing applicable in praxis. Our results show that changes in basic cellular parameters (expression of CD markers CD3, CD4, CD8, CD19, CD16+56) are mainly not significant. Lower counts of CD3+ / CD3+4+ cells in advanced disease are balanced by their higher activation rate. The latter fact is shown in higher expression of HLA-DR on CD3+ cells.

Our results show that the reactivity of T-cells in cancer is ambivalent. We have observed a decrease in expression of IL-2 and IFN-γ on T-lymphocytes only in metastatic breast cancer patients. Paradoxically, the expression of IL-2 in patients with non-metastatic breast cancer was higher than in the control group. These results document the ambivalent role of immunity in cancer. In the early stage of cancer, the immunity is stimulated but it cannot stop the development of cancer (and metastases), on the contrary, it can even support it. On the other hand, the immune functions in patients suffering from advanced stages of disease are more or less decreased. However, in such cases it is difficult to distinguish what is induced by cancer and what is a consequence of chemo-/radio- therapy.

Conclusion and perspective

According to available experimental and clinical data, we can conclude that the immune system plays a crucial role in cancer occurrence, development and prognosis. However, the role of immune cells and mediators is ambivalent, sometimes preventive and/or protective, and on the other hand in many cases harmful. Clinicians (immunologists) are often consulted to evaluate the patient’s immune status in order to help in the treatment of malignancy. Unfortunately though, the information obtained from routinely evaluated immunological profiles is too obscure to be used in the management of supportive therapy. Data from experimental animal studies scoring different immunological parameters and their application in the treatment strategy are not applicable in general; moreover, we must consider the possible stimulatory effect of immunotherapy on survival and propagation of cancer cells.

Our data support the fact that one-shot evaluation of essential leukocyte CD markers (CD3, CD4, CD8, CD19 and CD16+56 expression) is of little significance. Such examination may help to determine the global status of patient, but it becomes meaningful only when the monitoring is focused on the dynamics of immunity profile. In global, the changes are rather minor and not specific. In our two groups (patients with initial stage of breast carcinoma and patients with progressive disease with metastases), the only statistically significant differences were those in absolute counts of CD3+4+ cells (lesser in progressive disease) and the expression of HLA-DR on CD3+ cells (higher in progressive disease).

The core of critical question that should be resolved is whether the therapeutic manipulation of the immune system in patients with malignant disease is helpful. Because of the known ambivalent role of immunity in cancer development, we cannot exclude also the enhancement of cancer-promoting activity. For example, the induction of antitumor humoral immune responses might be beneficial in patients with established cancer; however, the activation of humoral immune responses in patients who are predisposed to cancer development or in patients with latent or pre-malignant disease might enhance the neoplastic programming of tissue rather than eradicating it. With reference to deVisser and co-workers, in these latter patients, it might be beneficial to monitor their parameters of B-cell activation and/or humoral immunity, as this might open a therapeutic window for anti-B-lymphocyte-based therapies or for modalities aimed at neutralizing the tumour-promoting properties of innate immune cells (1). However, according to our knowledge, such therapeutic strategies are not available in clinical praxis.

Table 1. Number of patients (%) out of limit with IL-2 and IFN-γ expression on T-lymphocytes in control group, non-metastatic breast cancer patients and metastatic breast cancer patients

<table>
<thead>
<tr>
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<th>IL-2 %</th>
<th>IFN-γ %</th>
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<tr>
<td></td>
<td>&lt;14,3%</td>
<td>&gt;33,3%</td>
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<tr>
<td>Control group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=16)</td>
<td>18,75 %</td>
<td>12,5 %</td>
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<tr>
<td>Non-metastatic</td>
<td></td>
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<tr>
<td>Breast-cancer</td>
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<tr>
<td>patients (n=20)</td>
<td>15 %</td>
<td>40 %</td>
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<tr>
<td>Metastatic</td>
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<tr>
<td>Breast-cancer</td>
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<tr>
<td>patients (n=8)</td>
<td>57,1 %</td>
<td>0 %</td>
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Hrubisko M et al. Immunity profile in breast cancer patients
Nevertheless, it seems reasonable to consider immunomodulating strategies targeting the cancer-promoting properties of both innate and adaptive immune-cell populations in order to stimulate antitumor properties of immunity without enhancing the inflammatory response and potentially the pro-cancer activity. One of such possibilities is to use the so-called transfer factors (TF) (21). In Slovak and Czech Republics, TFs made from human and pig leukocytes are available but in most other European countries and in USA, such therapy is unavailable. Our clinical experience with TF in malignant patients is very good. Another possibility lies in immunomodulation with bacterial remedies (lysates, proteoglycans and ribosomal preparation). In our clinical experiments with different bacterial immunomodulators, we have seen anti-inflammatory activity rather than pro-inflammatory one (22, 23). Immunomodulating therapies definitely help patients treated with chemotherapy and/or radiotherapy to alleviate the harmful side effects of these cytotoxic therapies.

The goal of future research should be to identify concrete immunological parameters that can help the clinicians to determine the actual status of a malignant patient. It should aim to positively influence the therapeutic strategy, establish effective and safe combinations of immunomodulating and cytotoxic therapies, and thus to improve the quality-of-life and significant survival extension for patients with cancer.

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References


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