REVIEW

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Cancer immunotherapy in mice and humans: personal observations*

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Abstract

An experiment involving active immunotherapy of leukemia in mice and its extension to human patients with chronic lymphocytic leukemia is described. First, anticancer activation of the immune system, how it is achieved, and observations of the central blockade of the immune system in leukemia are discussed. Clinical results of antileukemia immune activation are then presented, along with a description of new attempts to remove the immune checkpoint blockade. Personal observations are presented on possible further steps to achieving more effective active immunotherapy of cancer. *(Folia Histochemica et Cytobiologica 2018, Vol. 56, No. 1, 1–10)*

Key words: apoptosis; L1210 leukemia cells; DBA/2 mice; thymus; human B-CLL; vaccination with apoptotic CLL lymphocytes; clinical trial

Introduction

Immune responses to mutation events within cells leading to cancer or microbial pathogens are organized by the interaction between cells of the innate and adaptive parts of the immune systems. One aspect of the adaptive immune system is its capacity to remember previous encounters with the same antigen. This could serve as a basis for constructing anticancer vaccines. The main theoretical basis of this therapy is the fact that almost every cell of the 10^{16} cells in the human body is monitored by the immune system, and virally infected or mutated cells are recognized and removed. The hypothesis suggests that further activation of the immune system would result in more effective removal of mutated cancer cells expressing tumor antigens (now called neoantigens) that are not present in the normal cells. They are recognized by

T cells differentiated in the thymus against the tumor neoantigens. Vaccination with tumor antigens present within cancer cells should increase the activation of actually present anti-tumor T-cells and lead to the activation of new ones, as the collection of tumor antigens changes during development of the tumor.

An experiment on an animal model was performed on DBA/2 mice treated with leukemia L1210 cells. The leukemia cells were cultured *in vitro*, essentially as described in the National Cancer Institute Program [1], and after implanting in the healthy animals, an acute leukemia process developed. It was assumed that the leukemia cells were syngeneic with DBA/2 mice, so the L1210 cell implantation was not an allogenic transplant. That was first tested by comparing MHC class I of the DBA/2 mice lymphocytes with L1210 cells [2], confirming the syngeneic character of the leukemia cells (Table 1). In the model, activation of the mouse immune system should target the L1210 leukemia cells.

The immune system may theoretically be activated by implanting a large quantity of leukemia cell antigens. The genetic instability of cancer cells is well known [3, 4], and so the best collection of L1210

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Table 1. Leukemia L1210 cells are syngeneic with the lymph node cells of DBA/2 mice. Result of the reaction with various antibodies against H-2 alloantigens [2]

Antibody	Cytotoxic titer	
	L1210 cells	**Lymph-node cells***
anti H-2K^a	> 80 (positive)	> 160
anti H-2D^d	> 160 (positive)	> 160
anti H-2K^b	0 (negative)	> 160 (negative control)
anti H-2D^b	0 (negative)	> 160 (negative control)

*Lymphocytes taken from a strain of mouse of specified haplotype.

Table 2. Survival of mice with implanted malignant L1210 cells (10^3 per animal) one week earlier vaccinated with apoptotic or dead L1210 cells, compared to unvaccinated control animals [12]

Number of cells	No. of animals	MST days	Range, days
Control (-L1210CY)	58	20	18–22
L1210 dead, 10^5	10	19	17–21
L1210CY 10^3	10	18	14–22
L1210CY 10^4	10	32	20–51
L1210CY 10^5	10	66*	32–135

MST — Median Survival Time. *Statistically significant difference compared to unvaccinated control, $p < 0.05$.

antigens would be from within the leukemia cells themselves. This hypothesis suggested induction of T cell responses to the mouse leukemia cells antigens now called neoantigens which are absent in the normal cells [5]. Theoretically, a tumor surgically removed from a patient would constitute the best source of tumor antigens. However, the malignant antigenic tumor cells cannot be directly applied to induce anticancer immune responses. Moreover, dead, necrotic cells cannot be used to induce anticancer responses, because they are immediately recognized and removed by local inflammatory processes. Another argument against use of necrotic cells is that they release K^+ ions, which has been found to suppress the activity of the immune system's T cells [6]. Therefore, in the proposed animal model, leukemia antigens were applied in the form of leukemia cells with induced apoptosis. The induction of apoptosis in cells blocks their proliferation; however, the commencement of the apoptotic process is delayed in many cells. The cell apoptotic process begins several hours, or even some days, after induction [7–9]; however, the apoptotic process itself takes only about 2 h to proceed. A cell in apoptosis will be recognized by surrounding cells and immediately removed by phagocytosis. It appears that leukemia cells with induced apoptosis are a long-term antigen stimulating system that functions within days, without inducing an inflammatory response [10, 11].

Anticancer activation of the immune system

The first experimental test of the antigen properties of the system was in DBA/2 mice vaccinated with various doses of apoptosis-induced L1210 leukemia cells. It was expected that implantation of apoptotic

cells would delay leukemia growth in the vaccinated mice. Apoptosis in L1210 cells was induced *in vitro* by treatment with cyclophosphamide or mafosfamide (a derivative of cyclophosphamide) (L1210CY or L1210Maf cells). The *in vitro* treatment of cells with cyclophosphamide or mafosfamide blocked the mitotic cycle within the cells, which were then used for vaccinating the mice. One week later, the mice were implanted with 10^3 malignant L1210 leukemia cells in the logarithmic phase of growth [12]. Mice vaccinated with 10^5 L1210CY cells before the implantation of malignant L1210 cells survive the malignant leukemia significantly longer than mice not vaccinated, or vaccinated with dead leukemia cells (Table 2) [12]. This result suggests that the apoptotic L1210 cells are an effective antileukemia antigen. The effect was immunologically specific, as it did not prolong the survival of mice with malignant L-1 sarcoma cells.

In the next step of the experiment, we proceeded into the treatment of mice with already growing malignant leukemia [13]. The mice were intraperitoneally inoculated with 10 malignant L1210 leukemia cells, and their survival of the leukemia was observed. We found that *i.p.* inoculated L1210 cells induced the death of mice in an average of 12.5 days (median survival time; MST), while *i.p.* injection of 10^6 or 10^7 apoptotic L1210Maf cells at days 0, 3, 6, 9, and 12 from start of the leukemia led to an MST of 13.5 days (Table 3). The vaccination was therefore ineffective over a period shorter than the full immunological response against the fast-growing leukemia. In order to prolong the survival of leukemic mice inoculated with malignant L1210 cells, chemotherapy with cyclophosphamide (100 mg/kg) was administered *i.p.* at day 8, which resulted in a prolongation of the survival period to 19 days. When the mice treated with chemotherapy were vaccinated *i.p.* with 10^7 apoptotic L1210Maf cells at 0, 3, 6, 9, and

Table 3. Chemoimmunotherapy of L1210 leukemia in mice model [13]

Group of mice	Leukemia	CY	BRM	L1210 Maf Cells	MST (range) (Survivors/total)
1	+	–	–	–	12.5 (12.2–12.8)
					(0/54)
2	+	–	–	10^6 *i.p.*	13.5 (12.3–14.8)
					(0/10)
3	+	–	–	10^7 *i.p.*	12.5 (11.6–13.4)
					(0/10)
4	+	+	–	–	19.0 (18.5–19.5)[a]
					(3/56)
5	+	+	–	10^6 *i.p.*	27.0 (22.6–32.3)[b]
					(6/20)[b]
6	+	+	–	10^7 *i.p.*	25.0 (23.5–26.6)[b]
					(17/60)[c]
7	+	+	–	10^7 *i.p.* + *s.c.*	> 100
					(16/30)[de]
8	+	+	+	10^7 *i.p.* + *s.c.*	> 100
					(6/10)[b]

Leukemia: each mouse received 10 malignant L1210 cells *i.p.* at day 0. CY: 100 mg/kg of cyclophosphamide was injected *i.p.* eight days after L1210 leukemia challenge. BMR: BCG (1 mg/mouse = 3 × 10⁶ viable bacilli) were injected *i.p.* on days 0, +3, +6, +9, +12 after L1210 leukemia challenge. L1210 Maf: the cells were injected on days 0, +3, +6, +9, +12 after L1210 leukemia challenge. [a]p < 0.05 as compared with group 1; [b]p < 0.05 as compared with group 4; [c]p < 0.01 as compared with group 4; [d]p < 0.001 as compared with group 4; [e]p < 0.05 as compared with group 6.

12 days (as described before), the MST was 25 days (28.3% mice survival, 17 of 60). These leukemic mice lived for 100 days or more from the time of inoculation with malignant cells. A 100-day survival period of mice can be taken as recovery of animals from malignant leukemia; most mice were observed for 2 years. The success rate could be increased to 53% (16 of 30) by vaccination of the leukemia-inoculated mice with 10⁷ apoptotic L1210Maf cells given intraperitoneally and subcutaneously, and additional BCG (1 mg/mice) inoculation further increased the rate to 60% (6 of 10) of the leukemia-recovered animals. Thus, the experiment demonstrated the possibility of successful treatment of acute leukemia in an animal model by activation of the immune system [13] and as shown by further tests, even when cells were resistant to the cytoplasmic agents [14]. It can be suggested that, using an appropriate vaccination and additional cyclophosphamide chemotherapy, the number of antileukemia lymphocyte clones could be significantly increased to effectively attack the malignant leukemia cells in the mice.

Leukemia blockade of the immune system

The observation that only some of the vaccinated leukemic mice recovered suggests that the positive outcome was blocked by some mechanism in the nonrecovered animals. We thus decided to check the status of the thymus in the leukemic mice, in cooperation with HJ Seidel of the Department of Clinical Physiology and Occupational Medicine, University of Ulm, Germany. The plan was to test thymocyte differentiation *in vivo* in DBA/2 mice with L1210 leukemia. Within the first 3–6 days after *i.p.* implantation of 10⁶ L1210 cells, we found profound changes in thymocyte subpopulations. Microscope observations clearly showed that leukemia significantly altered the differentiation of T cells, centrally blocking the immunological response to malignant cells [15]. Shortly thereafter, we repeated the thymocyte test in leukemic animals and examined changes in thymocyte subpopulations by flow cytometry; the results of the observations agreed well with the microscopic observations (Table 4) [16].

Table 4. Subpopulations of thymocytes in leukemia-bearing mice or normal mice at different days after *i.p.* inoculation of 10^6 L1210 malignant cells [15, 16]

Mice	Subpopulation of thymocytes, percentage*			
	L3T4⁺Lyt 2⁺	**L3T4⁺Lyt 2⁻**	**L3T4⁻Lyt 2⁺**	**L3T4⁻Lyt 2⁻**
Normal, n = 7	78.0 ± 3.8	10.7 ± 2.0	5.0 ± 1.0	6.6 ± 1.8
L1210, 2 d., n = 2	73.0 ± 2.8	14.0 ± 1.4	7.0 ± 0	5.5 ± 0.7
L1210, 3 d., n = 5	71.6 ± 5.0	13.8 ± 1.9[b]	6.8 ± 0.4[a]	7.8 ± 2.8
L1210, 5 d., n = 4	62.0 ± 14.9[b]	19.3 ± 7.2	9.3 ± 2.1[a]	9.5 ± 5.4
L1210, 6 d., n = 6	48.3 ± 20.0[b]	25.2 ± 9.0	11.2 ± 5.3[b]	15.8 ± 7.8[b]

*Mean values ± SD; n = number of animals in the group, difference as compared with normal mice; [a]significant at $p < 0.01$; [b]significant at $p < 0.05$.

In summary, we determined the conditions necessary to activate the immune system using leukemia apoptotic cells, in order to achieve effective recovery from leukemia in mice. These were (i) small mass (number) of leukemia cells, and (ii) activation of the immune system of mice with apoptotic leukemia cells containing neoantigens.

Immune monitoring of cells in the human body

Human lymphocytes regularly monitor the amino acid sequence of cytoplasmic and nuclear proteins within cells. The knowledge of these mechanisms began with experiments on the presence of proteinases within normal cells. The presentation of intracellular protein structure to cells of the immune system is associated with their degradation to peptides. These peptides are then associated with major histocompatibility complex (MHC) class I molecules, and the peptides of this complex are presented to lymphocytes on the surface of each cell (Fig. 1). The peptides are usually 8-mer to 12-mer peptides, produced within the proteasome [17–19]. Peptide aldehydes that inhibit major peptidase activities of the 20S and 26S proteasomes are shown to reduce the degradation of protein and ubiquitinated protein substrates. These compounds inhibit the degradation of abnormal (and also normal) proteins in intact cells, and may be used to test the importance of the proteasome in antigen presentation. Blockage of proteasomal activity within cells leads to apoptosis.

In our experiment, the proteasomal protease active within leukemia L1210 cells was tested by its inhibition using specific chymotrypsin inhibitor. Treatment of L1210 cells with inhibitor induced apoptosis (Fig. 2) [20]. The proteases are thus referred to as chymotrypsin-like (ChTr) proteases; they were present and active in the proteasomes of L1210 cells. Similar ChTr proteases have been observed in the other cancer cells,
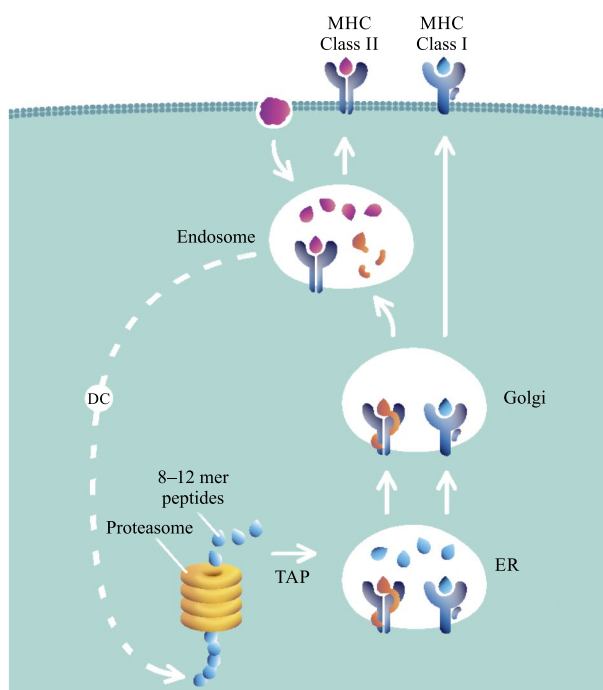

Figure 1. Schematic presentation of the mechanism of protein antigen processing in the proteasome and endosome and peptide presentation with MHCs in most cells in the human body. The proteasome-processed protein fragments are transported to the endoplasmic reticulum and bound to MHC class I, while endosome processed proteins are bound to MHC class II. Protein fragments with MHC are transported to the surface of cell. However, proteins from apoptotic cells or their fragments, phagocytosed by the dendritic cells (DC), are released from the endosome to cytoplasm, where they are processed in the proteasome and bound to MHC class I before being presented on the cell surface. (- - -→) pathway of antigens in the dendritic cell. TAP — transport associated processing; ER — endoplasmic reticulum.

as well as in the normal cells. The ChTr protease of the type present in proteasomes was isolated and purified, and its properties in normal mast cells have
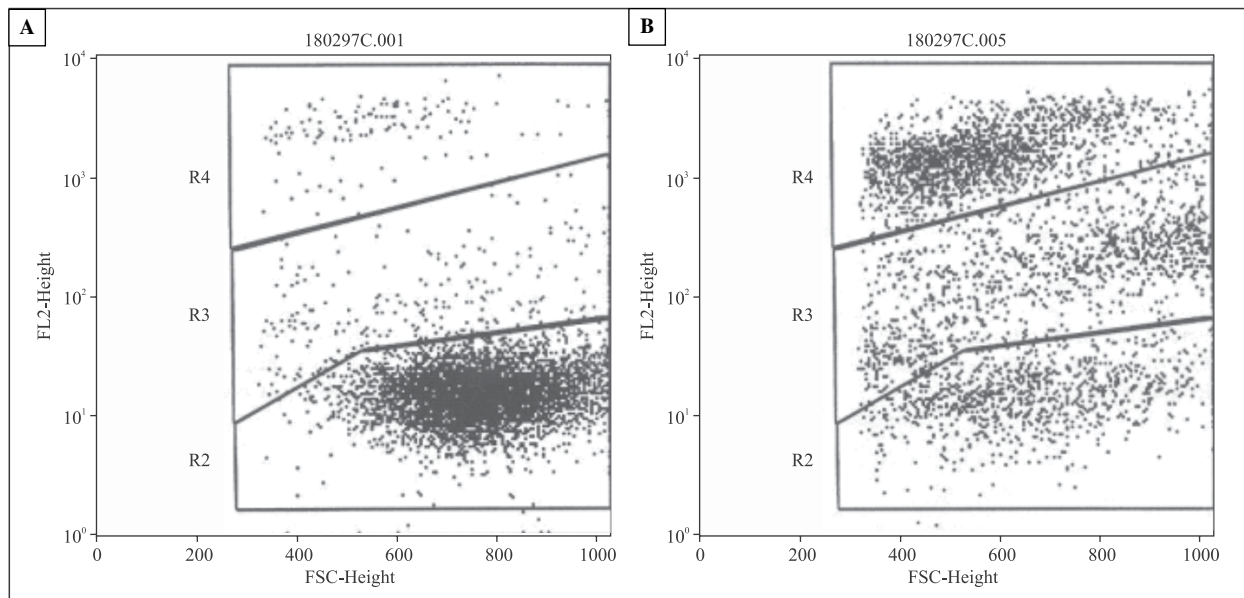
Figure 2. Inhibition of chymotrypsin-like protease in L1210 leukemia cells induces apoptosis of cells. Cells analyzed by the propidium iodide (PI) method in a control sample (**A**) and in the sample incubated for 24 h in the presence of 500 nM PSI (chymotrypsin inhibitor) (**B**). Plot axes: FSC *vs.* FL2 (PI on *y*-axis). R2 living cells, R3 apoptotic cells, R4 necrotic cells [20].

been described [21]. It is well known that the 26S proteasome is a multimeric complex consisting of a centrally located 20S core catalytic complex flanked by 19S regulatory subunits. The 20S complex contains β rings with three active enzymatic sites of distinct substrate specificities: chymotrypsin-like on the $\beta 5$ subunit, trypsin-like on the $\beta 2$ subunit, and caspase-like on the $\beta 1$ subunit [22].

Within the immune system, there is an active specialized type of cells called myeloid dendritic cells (mDCs), which have some additional functions in the presentation of peptide antigens. These mDCs have the additional property that their endocytotic membrane is capable of transporting part of the extracellular endocytosed proteins to the cytoplasm [23, 24], where they are processed by proteasomes of the phagocytosing cell. The phagocytosed extracellular protein peptides are then transported to the endoplasmic reticulum and presented to T lymphocytes with MHC class I on their cell surface. In this way, the exogenous antigens access the cytosol of mDC and are processed for presentation via the same pathway as described for conventional MHC class I restricted cytosolic protein antigens.

mDCs differentiate following phagocytosis of apoptotic cells and translocate to the lymph nodes [25, 26], where there are the optimal conditions for the presentation of cancer/leukemia antigens to naïve T cells constantly flowing (translocating) in the lymph. It has been described that mDCs remain and function within the lymph nodes over 2–3 weeks. This mean of presenting of mDCs is referred to as the crosspresentation of antigens, because with MHC class I not only are own-cell proteins presented, but so are the phagocytosed proteins of the apoptotic leukemia cells. We therefore suggest that the described recovery from leukemia of the mice treated with apoptotic L1210 leukemia cells was possible due to this process of crosspresentation of antigens in the phagocytosed cells by mDCs.

Clinical attempts to activate antileukemia immunity

The experiment in mice, with its conclusion that it is possible to activate the immune system to cure leukemia, was the basis for performing similar clinical tests in patients. Human chronic lymphocytic leukemia (B-CLL) is a hematologic neoplasm characterized by accumulation of a subpopulation of B-lymphocytes $CD19^+CD5^+$. The disease typically occurs over the age of 50 [27]. The clinical course of B-CLL is highly variable. When we first discussed the clinical experiment, the therapy of patients with the stable disease was limited to watching and waiting, coupled with treatment of disease-related symptoms: progressive marrow failure (anemia, thrombocytopenia), autoimmune complications, massive splenomegaly, lymphadenopathy, and infectious complications. Very precise prognostic diagnosis based on cytometric
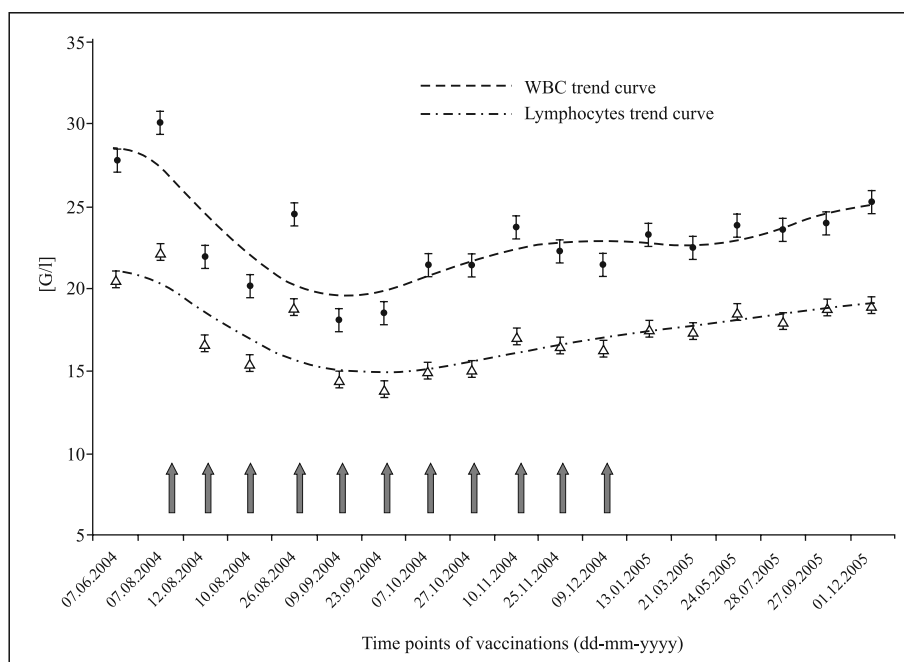
Figure 3. Leukocyte (WBC) and lymphocyte trend curves of a patient with B-CLL treated with irradiated leukemia cell vaccines from the time of initial diagnosis during immunotherapy to twelve months after the end of vaccination (patient from hematological improvement group) [37]. Arrows — time of vaccine injections.

analysis was required [28, 29]. Chemotherapy and passive immunotherapy with monoclonal antibodies were also possible treatments. About 15 years ago, it was established that rituximab, a chimeric anti-CD20 monoclonal antibody, is effective when administered with chemotherapy. It was shown that rituximab significantly improves the outcome of both untreated and previously treated B-CLL patients [30–33]. Some other antibodies, such as ofatumumab, are also advised clinically [34].

The slow progress of B-CLL in some patients provides time for active immunotherapy. We therefore proposed to follow the treatment described earlier. Patients' consent was obtained, as was the agreement of the Ethical Commission at the School of Medicine, Lublin. It was assumed that B-CLL antigens could be crosspresented by mDCs. Preliminary results were presented in 2003 [35]. The clinical test was based on the use of the patient's CLL autologous apoptotic B-cells as a vaccine. The cells contained actual leukemia neoantigens of mutated proteins, with contents likely differing in different patients. We first performed tests *in vitro*, using gamma-irradiation doses to induce apoptosis in peripheral blood mononuclear cells (MNC) isolated from patients with B-CLL. The optimal radiation dose for inducing leukemia cells apoptosis *in vitro* was found to be 1000 Gy for 1 hour [8]. In different irradiated MNCs, the apoptosis process started hours or even days later, as

shown by *in vitro* tests, which may be evidence of the variability of leukemia cells. The irradiated cells were given *s.c.* in the arm region near the lymph nodes. As irradiated cells begun to undergo the apoptotic process within few days, the procedure was repeated once a week, 11–12 times, to keep the leukemia antigen constantly exposed to the patient's immune system for a prolonged time. The procedure did not induce significant adverse effects, except for some small redness of the skin at the injection site, and sometimes a temperature rise of 1°C on the following day. Long-term observation of patients over a number of years did not disclose any adverse problems related to the vaccination [36, 37].

One positive outcome for patients during the vaccination, and for some 2–3 months longer, was a lower level of leukocytosis and lymphocytosis than they started with (Fig. 3). However, some patients (7 of 17) did not respond to the treatment, for which we have not explanation (Fig. 4). The most important difference, compared to the results of the animal *in vivo* experiment, was that there was no cure for patients with B-CLL. This could be expected as, during the long period of B-CLL development, the general mass of leukemia cells becomes large and the patient's immune system is constantly paralyzed. In our mice, L1210 leukemia was treated within a short time, and the leukemia cell mass (the number of L1210 cells) remained low.
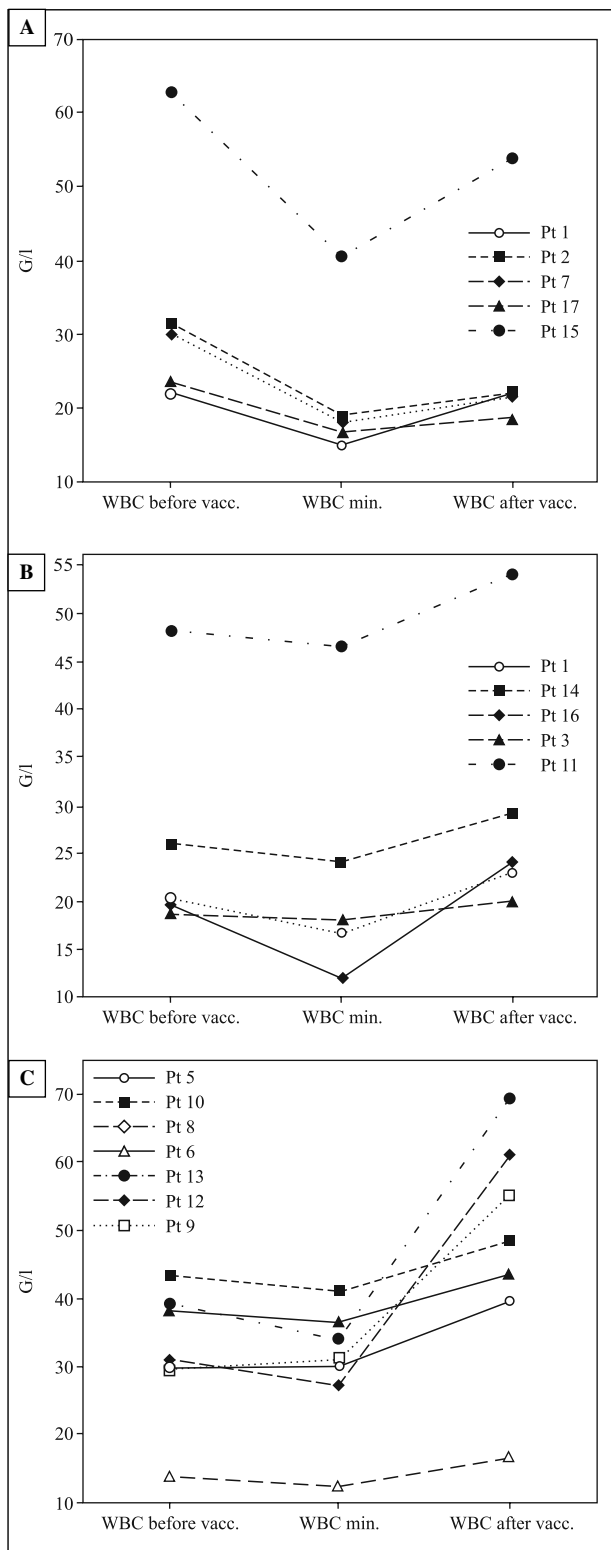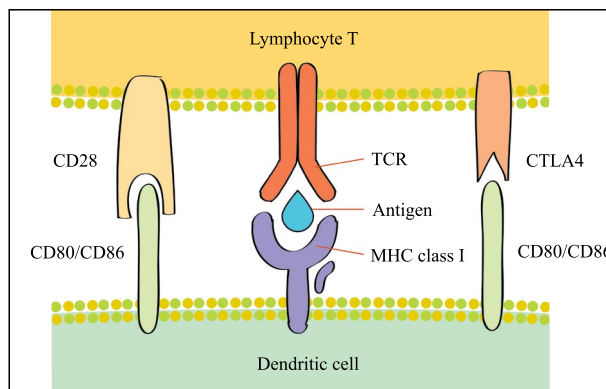
Figure 5. Schematic presentation of the activation of T lymphocytes through recognition of the antigen presented with MHC class I and costimulation of the activated T cell necessary for exerting a cytotoxic effect on the target cell. There are two processes necessary for effector function of the T cell: recognition of the presented peptide antigen, and costimulation by interaction between CD80CD86 and CD28 on the lymphocyte. After some time, the checkpoint blocking protein CTLA4T appears on the T cell surface. Interaction of CTLA4 on the T cell with CD80CD86, however, blocks effector function. Moreover, CTLA4 has higher affinity to the CD80/CD86 than to CD28.

Figure 4. Summary of the vaccination results of 17 patients: (**A**) five responded with lower WBC (0.29), (**B**) five stabilized the increase of WBC (0.29) and (**C**) seven did not respond (0.41) [37].

The following conclusions can be drawn from the clinical experiment: applied vaccination treatment cannot cure patient with B-cell chronic lymphocytic leukemia; however, in about two out of three patients, a delay in disease progression was observed for some period. These can be beneficial for the patient, as the well-known hematological problems (anemia, thrombocytopenia) are blocked for some time. New clones of T lymphocytes directed against leukemia cells enlarge, and preserve the activation markers for a long time, as has been shown with cytometric analysis of peripheral blood leukocytes of vaccinated patients. However, the activated T cells are cytotoxic to the recognized leukemia cells only for a short period of time (Fig. 5), as their costimulation is then blocked by the expression of checkpoint-blocking proteins such as CTLA4 (cytotoxic T-lymphocyte associated protein 4) or PD-1 (programmed cell death-1). Also, as part of the immune response in vaccinated patients, T_{reg} cell clones are also rapidly enlarged and activated. The blocking of the T cell responses is probably the natural, physiological process that prevents long-term allergic activation. The vaccination did not lead to any undesired processes in patients, even after observation of some of them for 14 years.

Anticancer immune activation:
More recent attempts

The observation of patients with different cancer types now suggests some additional general conclusions. Immunological therapy in patients with cancer is rather of the passive type, involving the application of monoclonal antibodies. The chemotherapeutic pathway is intended to decrease the mass and lower the number of cancer cells, as well to block some known cellular regulatory processes. The active immunotherapy described here, based as it is on the activation of the immune T cells, involves recognizing the neoproteins of the mutated cancer cells. This should lead to better recognition of altered, mutated cells, allowing them to be more efficiently removed. This works for a period of time, but is then blocked by the expression of checkpoint blockers on activated lymphocytes [38–41]. In B-CLL, it can thus be proposed that complex associated active immunotherapy be used in conjunction with passive immunotherapy (rituximab), followed by antibodies that block the checkpoint blockers — *e.g.*, directed at CTLA4 (ipilimumab) or PD-1 and its ligands [42, 43]. Promising clinical attempts to block the checkpoint blockers are now underway, so the application of complex associated active immunotherapy may soon appear as a promising therapeutic approach.

Long-term observations of both healthy individuals and B-CLL patients suggest that an important role is played in cancer immunity by the T lymphocyte subpopulations discussed above ($CD3^+CD4^+$ and $CD3^+CD8^+$) recognizing infected or mutated cytoplasmic proteins within the cells. Mammalian T cells monitor the proteome of most cells; however, the lymphocyte subpopulation called NKT cells recognize another group of compounds in cells (lipids and glycolipids) and appear to be very important for the normal functioning and regulation of the immune system [44–46]. The activation of NKT cells is probably also important for cancer immunity. A subpopulation of these cells, iNKT cells, recognizes glycolipids presented by the nonpolymorphic CD1d antigen-presenting molecule [47]. These iNKT cells are capable of rapidly producing type Th1, Th2, and Th17 cytokines upon activation, as they store ready-to-use cytokine mRNA. This shortens the route and time needed to cytokine production and release. The constant renewal, exchange, and activation of NKT cells may be maintained, as noted by several years of personal observations. These are induced by proper nutritional composition, containing lipids such as unsaturated fatty acids, omega-3, omega-6 lipids, and cod liver oil [46–50].

A system of vaccination, similar to ours, with autologous tumor cells for active specific immunotherapy, has also been presented by the other authors [51–54]. It seems that passive and active immunotherapy, supported by the additional activation of NKT cells, may help in preventing cancer in healthy persons, as well as curing for cancer patients.

In conclusion, the induction of apoptosis blocks cell proliferation. However, the apoptotic process, which takes about 2 h to complete once begun, is delayed by hours or days (4–5 days in irradiated B-CLL cells). Thus, the patient's leukemia cells form heterogenous population, as apoptosis starts at varius times after their induction in different cells. Vaccination with the actual cancer antigens of apoptosis-induced cells induces activation of several anticancer T cell clones. However, it was observed in B-CLL vaccinated patients that the response is blocked after 2–4 months or more, which probably results from the physiological checkpoint regulation of immune system function; this is undesirable in cancer treatment. The anticancer vaccination should thus be followed by passive immunotherapy by treatment that prevents immune checkpoint blocking. Recently, anti-PD-1 monoclonal antibodies, nivolumab (fully human IgG4 antibody), and pembrolizumab (humanized IgG4-*k* antibody) have been approved for advanced kidney cancer [55, 56] and metastatic non-small-cell lung cancer (NSCLC), whose tumors express programmed death-ligand 1 (PD-L1) [54]. The treatment of B-CLL patients with anti-checkpoint blocker antibodies was unknown in 2003–2008 when we performed our study of apoptotic leukemia cell treatment in CLL patients. Since then, technological advances have led to the approval of immune checkpoint blocking monoclonal antibodies for the treatment of malignant lymphomas and B-CLL [57–60].

Acknowledgments

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