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RESEARCH

Evaluation of Significance of Lymphocyte Subpopulations and Non-specific Serologic Markers in B-cell Non-Hodgkin's Lymphoma Patients

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Abstract The use of rituximab brought attention to the hosts' immune system and to the microenvironment in non-Hodgkin's lymphoma cases. Our aim was to identify prognostic factors that can be measured easily to indicate the current state of the patient's immune status and possible reaction against malignant cells. In the retrospective analysis (2000–2008), 66 patients diagnosed with B-cell non-Hodgkin's lymphomas were enrolled (40 women, 26 men; mean age: 51 years). White blood cells, lymphocytes, CD3 +; CD4 +; CD8+T-cells, immunoglobulin types A; G; M, anti-cardiolipin antibody isotypes A; G; M; and levels of beta-2-microglobulin were measured before the initiation of the first cycle of chemotherapy, during and after 4-weeks treatment. As for CD 3+ T-lymphocytes, the absolute CD 3+ T – lymphocyte numbers were higher before (0.78×10^9/L) versus during (0.27×10^9/L) treatment, and increased percentages were detected in pre- (66.57 %) and post-treatment (75.32 %). Absolute numbers of CD 8+ T-lymphocyte levels showed reduction before (0.26×10^9/L) versus during (0.10×10^9/L) therapy, but were elevated after (0.28×10^9/L) treatment, while increased percentage before (21.99 %) versus after (29.85 %), and during (24.56 %) versus after (29.85 %) therapy were seen. Average white blood cell numbers were increased before (9.71×10^9/L) versus during (12.07×10^9/L) treatment, while decreased numbers could be observed, after (5.47×10^9/L) treatment. IgA levels were decreased before (2.51 g/L) versus after (1.63 g/L) therapy. IgG levels were higher before (12.25 g/L) vs. after (8.64 g/L) treatment. IgM levels were decreased before (1.76 g/L) and after (0.83 g/L) as well as before (1.76 g/L) versus during (0.73 g/L) treatment. Anti-cardiolipin antibody type A level were decreased before (2.76 U/ml) versus after (2.49 U/ml) treatment. Decreased level of beta-2-microglobulin could be observed before (2.91 mg/L) versus post (2.28 mg/L) chemotherapy. Findings may provide better insight into the effects of immuno-chemotherapy on the hosts' immune system.

Keywords Lymphocyte subpopulations · Non-specific serologic markers · Non-Hodgkin's lymphoma · Immuno-chemotherapy · Host immune system

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Introduction

One of the largest heterogeneous group of lymphoproliferative disorders in oncohematology are non-Hodgkin lymphomas (NHL). In 2010, an estimated 65,540 new cases of NHL were diagnosed in the US [1]. During the past 40 years, the field of oncohematology has greatly developed. Parallel with

the advances in classification methods and diagnostic options, the management of non-Hodgkin's lymphomas has also been significantly changed from radiotherapy to modern polychemotherapy with the application of monoclonal antibodies and the approach of treatment for lymphoma patients has also changed [2, 3].

The development of R-CHOP (rituximab combined chemotherapy: cyclophosphamide, hydroxydaunorubicin, vincristine, prednisolone) combination was the first significant advancement, which has a dramatic positive impact on event-free survival and overall survival of the patients [4, 5]. Successful administration of specific anti-tumor immunotherapies redirected the attention to the hosts' immune system during the treatment of the disease [6, 7]. The understanding of background processes in the immune system of the patients, antitumor immunity and the relation between the microenvironment and the tumor cells all affect prognosis and have become more and more important for both researchers and physicians.

A wide repertoire of specific cell subpopulations constitute the tumor microenvironment in each lymphoproliferative syndrome, which could be an important diagnostic, prognostic and therapeutic factor [8]. Recent evidence supports a significant influence of surrounding lymphoid infiltrates, reactive microenvironment, immune cell subsets, and especially the presence of non-malignant T-cells in the microenvironment of the tumor pre-, during, and post-treatment. The cell subpopulations could not only be responsible for histological morphology and immune phenotype, but it is also hypothesized that they have clinical importance in certain lymphoproliferative syndromes and therefore, in the outcome of the disease [7, 9, 10].

Our aim was to identify prognostic factors that can be measured easily in everyday medical practice to indicate the current state of the patients' immune system and to find a possible connection with prognosis. During our research, our long-term goal was to detect a measurable parameter that can indicate the immune systems' condition and reaction against malignant cells. The prognostic value of these parameters would help us set up a therapy that is tailored not only to the disease, but to the immune system of the patients as well. In our retrospective analysis, we measured subpopulations of lymphocytes and non-specific serologic markers in peripheral blood samples diagnosed with B-cell non-Hodgkin lymphomas. Levels of white blood cells; absolute lymphocyte counts; lymphocyte percentages; total immunoglobulin types A, G, M; anti-cardiolipin antibody isotypes A, G, M; percentages and counts of CD 3+, CD 4+, CD 8+ T-cells along with the levels of beta-2-microglobulin (B2MG) were measured, pre- (before the initiation of the first cycle of chemotherapy), during (after the third cycle of chemotherapy) and post- (4 weeks after the administration of chemotherapy) treatment. The investigation and follow up of these specific parameters is an important issue, which should not be neglected due to the fact that the applied therapies have effects on B-cells. Immunoglobulins play an important role in immune defense activities such as antibody-dependent cell-mediated cytotoxicity (ADCC), or Fas-mediated apoptosis. Cardiolipin, as an autoantibody, is part of the autoreactive immune mechanisms, thus it may let us investigate the degree of autoimmunity which could relate to the presence of peripheral immune tolerance. The role of the immune tolerance is suggested to be important, particularly in the peripheries because it can be assumed that it may help the initial survival of the tumor cells.

Patients and Methods

From September 2000 to April 2008, 66 patients who were treated at our institute for B-cell non-Hodgkin lymphoma were randomly selected. Patients with autoimmune diseases and prior arterial or venous thromboembolic disturbances were excluded.

The study was approved by the IRB (Institutional Revision Board) as only routine blood sampling was performed, therefore, no informed consent was required. Sufficient follow up data were only available from the selected patients, and data were reported in these cases.

There were 40 female and 26 male patients, the mean age at diagnosis was 51 years [range: 19–80 years]. The median follow up time was 32 months [range: 10–76 months]. Patients' distribution according histological subtype, Ann Arbor stage, Eastern Cooperative Oncology Group (ECOG) categorization, and international prognostic index (IPI) distributed as shown in Table 1.

All cases were examined by 3 color flow cytometry and had no detectable circulating lymphoma cells in peripheral blood. Routine staging consisted of bone marrow biopsy with histology and aspirate for flow cytometry, CT scans of neck-chest, abdomen and pelvic regions with additional ultrasound of peripheral lymph nodes. PET-CT was only used to confirm residual tumor masses in all cases, and was used in every case for staging as well since March 2007. Complete remission (CR) was stated only when no active tumor was found, as described by the Cheson criteria [11]. Standard treatment regimens were 8× R-CHOP 21 for DLBCL and follicular NHL, 6–8× CHOP 21 for mediastinal B-cell lymphoma (MBCL) and mucosa associated lymphoid tissue (MALT), 6–8× CVP (cyclophosphamide, vincristine and prednisone) or CHOP for small lymphocytic lymphoma. In case of bulky disease at presentation, involved field irradiation (30–36 Gy) was performed after chemotherapy. Patients not receiving rituximab for first line (due to insurance coverage) did receive rituximab for second line regimens if needed. Granulocyte colony-stimulating factor (G-CSF) support was used when needed, and for allpatients above age 65. The concentration of anti-cardiolipin antibodies, beta-2-microglobulin and

Table 1 Patients' distribution according to histological subtype, Ann Arbor stage, ECOG categorization, and international prognostic index

Histological subtypes	No. of patients
DLBCL	31
FL	16
MBCL	10
SLL	9
Ann Arbor stage	
Stage I	3
Stage II	18
Stage III	14
Stage IV	31
ECOG categorization	
ECOG 0	31
ECOG 1	22
ECOG 2	5
ECOG 3	5
ECOG 4	3
International prognostic index	
IPI 0	4
IPI 1	21
IPI 2	22
IPI 3	10
IPI 4	8
IPI 5	1
B symptoms	24
Bulky disease	19

Our distribution of lymphoma subtypes matched the one concluded by the The Leukemia & Lymphoma Society in 2008. Abbreviations: *DLBCL* Diffuse Large B-Cell Lymphoma, *FL* follicular NHL, *MBCL* Mediastinal B-cell lymphoma, *SLL* Small lymphocytic lymphoma

immonoglobulin subtypes were detected in the Regional Immune Monitoring Laboratory of the University of Debrecen, Medical Health and Science Centre. The level of anti-cardiolipin antibodies were measure by standard ELISA technology (Orgentec Diagnostik GmbH, Mainz, Germany). The levels of beta-2-microglobulin and immunoglobulin subtypes were measured by nephelomerty. Routine blood analysis was done as part of the treatment protocol by automated blood counter. All blood count samples were drawn at the same time with heparinized samples. Flow cytometry analysis was done using the following reagents: Dako MultiMix™ CD4/FITC, CD8/RPE, CD3/ RPE-Cy5 (Dako Denmark A/S), BD Simultest CD3/FITC, CD16+56/PE (Becton-Dickinson, NJ, USA), and CD19/ PC5 (Immunotech, Marseille, France). Heparin anti-coagulated whole blood samples were freshly drawn from patients and stained with monoclonal antibodies according to the recommended protocol. Samples were washed, and red cells were lysed, and were fixed with 1 % paraformaldehyde solution. Measurements were performed on Coulter XL4 flow cytometer (Coulter, Hialeah, FL, USA) using the supplied EPICS software, and from June 2006 on Beckman-Coulter CYTOMICS FC500 flow cytometer (Beckman-Coulter, Fullerton, CA, USA) using the supplied CXP software. Gates and analytic regions were set by isotype control antibodies simultaneously stained with samples. Absolute numbers were calculated using routine whole blood count results multiplied by the flow cytometry percentages.

Our data was collected with Microsoft Office Excel and statistical analysis was performed by Statsoft Statistica v 9.1 and SPSS 17 software. Our dataset was not normally distributed, thus suitable statistical analysis had to be chosen. Our measurements were performed pre- (before the initiation of the first cycle of chemotherapy), during (after the third cycle of chemotherapy) and post- (4 weeks after the administration of chemotherapy) treatment, comparing pre- and during treatment; pre- and post-treatment; during and post-treatment period results. Considering the statistical characteristics and the claim of comparability of our results, we chose a robust test, the Mann–Whitney U (or Mann–Whitney-Wilcoxon) non-parametric test as a non-two-sample test. Statistical significance was stated when $p<0.05$.

Results

Our results concluded that the number of T-lymphocytes changed as follows: mean CD 3+ T-lymphocyte percentages compared before (66.57 %), during (72.60 %), and after (75.32 %) treatment showed significant increased pre-treatment versus (vs.) after chemotherapy treatment CD 3+ T-lymphocytes cell percentages ($p=0.030$). The absolute CD 3+ T –lymphocyte numbers showed significant differences, before (0.78×10^9/L) versus during (0.27×10^9/L) ($p=0.030$), and before (0.78×10^9/L) versus after (0.67×10^9/L) ($p=0.080$) treatment.

The mean CD 4+ T-lymphocyte percentages were the following: before (41.91 %), during (43.79 %), and after (39.80 %) treatment, without any significant differences between compared periods. Absolute numbers of CD 4+ T-cells also did not show any specific or significant changes, before (0.49×10^9/L), during (0.16×10^9/L), or after (0.36×10^9/L) treatment.

CD 8+ T- lymphocyte percentages showed a significant increased level before (21.99 %) versus after (29.85 %) ($p=0.000$), and significant increased level during (24.56 %) versus after (29.85 %) ($p=0.004$) chemotherapy. Absolute numbers of CD 8+ T-lymphocytes showed significant differences before (0.26×10^9/L) versus during (0.10×10^9/L) ($p=0.040$), and before (0.26×10^9/L) versus after (0.28×10^9/L) ($p=0.030$) treatment. The changes of CD 8+ T- lymphocytes, and related CD 19+ B-cells could be important, as it may be related to production of immunoglobulin levels and autoantibodies.

Total lymphocyte percentages did not changed significantly, before (20.90 %) during (22.41 %) after (22.83 %) ($p=0.300$) treatment. Mean absolute lymphocyte numbers showed significant decrease, before (2.96×10^9/L) versus during (2.41×10^9/L) ($p=0.080$), and before (2.96×10^9/L) versus after (0.71×10^9/L) ($p=0.000$) treatment.

The average white blood cell numbers were significantly increased before (9.71×10^9/L) versus during (12.07×10^9/L) (p=0.015) treatment, which level were influenced by G-CSF support, used necessarily during the chemotherapy. Decreased numbers could be observed, before (9.71×10^9/L) vs. after (5.47×10^9/L) chemotherapy (p=0.000) and during (12.07×10^9/L) vs. after (5.47×10^9/L) (p=0.030) treatment.

Average IgA levels showed significantly decreased level before (2.51 g/L) versus after (1.63 g/L) (p=0.050) therapy. Mean IgG levels were significantly different before (12.25 g/L) vs. after (8.64 g/L) treatment (p=0.002) and during (11.03 g/L) vs. after (8.64 g/L) treatment (p=0.100). IgM levels significantly decreased before (1.76 g/L) and after (0.83 g/L) (p=0.040) and before (1.76 g/L) vs. during (0.73 g/L) (p=0.050) treatment. Despite the observed changes in the immunoglobulin levels we did not observe higher occurrence of infectious diseases.

Anti-cardiolipin antibody types A showed significantly decreased level before (2.76 U/ml) versus after (2.49 U/ml) (p=0.020) treatment. Anti-cardiolipin antibody types G; M did not show any significant changes. Mean levels of anti-cardiolipin IgG were lower during (5.58 U/ml) and after (5.94 U/ml) treatment compared before treatment (6.42 U/ml) mean levels, but not essentially. Increasing but not essentially tendency could be observed in mean anti-cardiolipin IgM antibody levels comparing the mean levels of before (7.26 U/ml), during (7.53 U/ml) and after (10.38 U/ml) treatment. Significantly decreased level of beta-2-microglobulin could be observed in the view of pre (2.91 mg/L) versus post (2.28 mg/L) (p=0.002) chemotherapy. As a summary, mean and median values of these indicators before, during and after treatment are shown in Table 2. The p-values of Mann–Whitney are shown in Table 3.

Discussion

Prognostic markers are essential for physicians to predict patients' response and to choose adequate therapy. The tests performed in our study are easily reproducible and widely available in all hospitals. International Prognostic Index (IPI) and its variations and newer factors, such as beta-2-microglobulin could also have an important role, but recent data on their role is still inconsistent. Nowadays, it is supposed that the microenvironment reflecting both the cells surrounding malignant cells as well as certain cell subtypes infiltrating the tumor, and the immune response may be an additional predictor in response to therapy. To characterize the immune system, several approaches were used. We measured anti-cardiolipin antibody isotypes A; G; M; beta-2-microglobulin and non-malignant lymphoid cells, like CD 3+, CD 4+, CD 8+ T cells, and levels of peripheral blood lymphocytes and white blood cells to characterize the functional capabilities of patients' immune system [7, 12–16].

During the last 3 years, several reports highlighted the importance of pretreatment absolute lymphocyte numbers

Table 2 Mean and median values of the observed indicators pre-during-post treatment

	Pre-treatment Before the initiation of the first cycle of chemotherapy		During- treatment After the third cycle of chemotherapy		Post treatment 4 weeks after the administration of chemotherapy	
	Mean	Median	Mean	Median	Mean	Median
CD 3+T-lymphocyte (%)	66.57	70.00	72.60	74.00	75.32	76.35
Abs CD 3 (x10^9/L)	0.78	0.73	0.27	0.78	0.67	0.70
CD 4+T-lymphocyte (%)	41.91	42.45	43.79	44.00	39.80	39.90
Abs CD 4 (x10^9/L)	0.49	0.45	0.16	0.42	0.36	0.35
CD 8+T-lymphocyte (%)	21.99	21.00	24.56	24.00	29.85	30.10
Abs CD 8 (x10^9/L)	0.26	0.21	0.10	0.27	0.28	0.24
WBC (x10^9/L)	9.71	7.80	12.07	6.19	5.47	5.45
Ly %	20.90	19.00	22.41	20.70	22.83	21.95
AbsLy (x10^9/L)	2.96	1.19	2.41	0.96	0.71	0.80
IgA (g/L)	2.51	2.18	2.09	2.14	1.63	1.41
IgG (g/L)	12.25	10.94	11.03	11.02	8.64	8.59
IgM (g/L)	1.76	1.12	0.73	0.62	0.83	0.56
aCLIgA (U/ml)	2.76	2.30	2.89	1.75	2.49	1.60
aCLIgG (U/ml)	6.42	5.15	5.58	3.60	5.94	4.60
aCLIgM (U/ml)	7.26	3.50	7.53	3.30	10.38	3.75
B2MG (mg/L)	2.91	2.40	2.10	2.05	2.28	2.10

Abbreviations: *B2MG* Beta-2-microglobulin, *IgA* Immunoglobulin A, *IgG* Immunglobulin G, *IgM* Immunglobulin M, *aCLIgA* anti-cardiolipin antibody types A, *aCLIgG* anti-cardiolipin antibody types G, *aCLIgM* anti-cardiolipin antibody types M, *Abs CD3* absolute CD 3+ T lymphocytes, *Abs CD4* absolute CD 4+ T lymphocytes, *Abs CD8* absolute CD 8+ T lymphocytes, *WBC* white blood cell, *Ly %* lymphocyte percentages, *AbsLy* absolute lymphocyte

Table 3 Introduction of p-values in the comparison of indicators pre, during and post-treatment by Mann–Whitney analyses

	Pre-During treatment	During-Post treatment	Pre-Post treatment
CD3+ T-lymphocyte	0.296	0.262	**0.030****
Abs CD3	**0.030****	0.650	**0.080***
CD4+ T-lymphocyte	0.336	0.293	0.846
Abs CD4	0.670	0.910	0.700
CD8+ T-lymphocyte	**0.070***	**0.004*****	**0.000*****
Abs CD8	**0.040****	0.344	**0.030****
WBC	**0.015****	**0.030****	**0.000*****
Ly%	0.295	0.990	0.300
AbsLy	**0.080***	0.690	**0.000*****
IgA	0.530	0.697	**0.050****
IgG	1.000	0.100*	**0.002*****
IgM	**0.050****	0.057*	**0.040****
aCLIgA	0.236	0.337	**0.020****
aCLIgG	0.299	0.820	0.288
aCLIgM	0.420	0.616	0.740
B2MG	0.160	0.500	**0.002*****

Significant differences at 10 % are marked by *, at 5 % by ** and at 1 % by ***

Abbreviations: *B2MG* Beta-2-microglobulin, *IgA* Immunoglobulin A, *IgG* Immunoglobulin G, *IgM* Immunoglobulin M, *aCLIgA* anti-cardiolipin antibody types A, *aCLIgG* anti-cardiolipin antibody types G, *aCLIgM* anti-cardiolipin antibody types M, *Abs CD3* absolute CD 3+ T lymphocytes, *Abs CD4* absolute CD 4+ T lymphocytes, *Abs CD8* absolute CD 8+ T lymphocytes, *WBC* white blood cell, *Ly %* lymphocyte percentages, *AbsLy* absolute lymphocyte

and beta-2-microglobulin [14, 15, 17]. As the lymphocyte subpopulations are constantly changing and consist of various major cell types, it is essential to subcategorize them. A more precise predictive factor might be identified by distinguishing between the various lymphocytes by the means of flow cytometry and the routine technique [18–20]. Czuczman et al. have identified CD 3+ T-cells to be the most important lymphocyte population in follicular lymphoma patients [21]. They highlighted that CD 3+ T-cells, LDH level, and beta-2-microglobulin are stronger predictors than the conventional prognostic factors when rituximab is administered beside standard chemotherapy. Subdividing T-cells into CD 4 and CD 8 groups, only CD 8+ T-cells corresponded significantly with overall survival [22]. Patients with low CD 8+ T-cell numbers (less than 0.2 g/L) had a significantly lower overall survival, and it is consistent with our results in the low risk IPI group showing better therapeutic response [18]. These findings evoked our interest in CD 8+ T-cells. Constituting the majority of effector immune cells, their absence may result in reduced cell mediated killing and apoptosis induction through cell–cell binding. This finding is partially supported by the promising results found in in vitro expanded and primed autologous LAK-cell therapy in malignancies [23, 24]. Altered cytokine profile in patients might be another possible explanation for this finding, and it may result in unfavorable T-helper 2 shift and the suppression of T-helper 1 like effector mechanisms and CD 8+ cell numbers [23]. Due to our results, IgG and IgM levels in peripheral blood were significantly higher in the group of patients with unsatisfactory response to therapy. We suppose that cytokines, such as TNFα, IL-1α, IL-1β, IL-6, and IL-8, suppressing the immune system could be responsible for insufficient response to therapy in the long-term. Due to these results, it may be suggested that altered immunoglobulin levels in peripheral blood could be associated with an advanced state and poorer prognosis of the disease. Melbye's observations about IgE and other specific antibodies also support our hypothesis [25]. Nevertheless, some reports have revealed that higher ENA (extractable nuclear antigen) autoantibody levels might be associated with better prognosis of the disease [26]. This can reflect diminished peripheral and central immune tolerance and a more pronounced anti-tumor immune response.

Our research is an overview of the clinical importance of lymphocyte subtypes, white blood cells and autoimmunitity in malignant B-cell originated entities. Despite of the conclusive results in our research, we have to emphasize that patients would greatly benefit from further prospective analysis in each B-cell lymphoma subtypes. The evaluation of these markers separately in B-cell lymphomas could be of remarkable use in everyday clinical practice.

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