PhD THESES

CLINICAL AND EXPERIMENTAL INVESTIGATIONS IN NON-HODGKIN'S LYMPHOMAS

DR. LÁSZLÓ VÁRÓCZY

Tutor: Dr. Árpád Illés MD, PhD

Supervisor: Prof. Dr. Zoltán Nemes MD, DSci

3rd Department of Medicine, Institute for Medicine, Medical and Health Science Centre,

University of Debrecen

2005

INTRODUCTION

Non-Hodgkin's lymphomas (NHLs) as tumours of the immune system are considered to be malignant lymphoproliferative disorders. Pathological lymphocytes at different maturity stages grow up in the central and / or peripheral lymphoid organs, changing the normal structure of the lymph nodes. These cell clones can also appear in extranodal localizations. The heterogeneity of malignant lymphomas has posed demands to create their classifications. The development of immunohistochemical and molecular biological methods facilitated the birth of the latest WHO-classification which provides unambiguous histological diagnosis, therapeutical and prognostical relevances for clinicians.

The number of diagnosed NHL cases increases worldwide: nowadays they represent 4 % of all malignant tumours. In Hungary the incidence is 15-20/100.000 inhabitants annually. Factors that may promote the development of NHLs: infective agents, immunocompromised stages and autoimmune disorders. Pathogens that play an important role in lymphomagenesis are the Epstein-Barr virus, human T-cell lymphotropic virus and Helicobacter pylori in MALT-lymphomas of the stomach. Since the early 1990s several studies have been published about hepatitis viruses as risk factors for malignant lymphomas. Hepatitis C (HCV) and hepatitis G (HGV) viruses were found to be lymphotropic, that means that they repilicate themselves in lymphocytes as well. Their genomes and their protein products can be detected in lymphoid cell lines. Most of the evidences reflect HCV that is able to infect all peripheral and bone marrow mononuclear cells both in vitro and in vivo. HCV infection is present in 70 % of some rare NHL subtypes (e.g. primary hepatosplenic lymphoma). In case of hepatitis infections, chronic antigene stimuli result first polyclonal then oligo- and monoclonal lymphocyte growth,

which may lead to malignant transformation of these cells. In hereditary and acquired immunodeficient states, disturbances of T-cell immunoregulation occur, facilitating uncontrolled B-cell proliferation. Defects of cytokine production and some genetical changes result monoclonal rearrangement of immunoglobulin genes during the maturation of lymphocytes. This explains why primary and secondary immunodeficiencies often associate with malignant lymphomas. As for autoimmune diseases, there are several factors that promote the development of NHL: chronic inflammation, hyperreactivity of the immune system and immunosuppressive therapy. These autoimmunity-associated NHLs are mainly low grade, extranodal MALT-lymphomas. In Sjögren's syndrome, lymphoid cell infiltration can be detected in the salivary glands and the risk for the development of malignant lymphoma is 6-7 times higher than in the rest of the population. NHLs often develop in patients with Hashimoto's thyroiditis, celiac disease and autoimmune lymphoproliferative syndrome.

The most common symptom in NHL is painless enlargement of the lymph nodes. To get a diagnosis, the histopathological examination has got a primary role whereas it is unambigouos to use immunohistochemical and molecular biological methods. On the other hand, there are several other diseases that show up with lymphadenomegaly which can easily be misinterpreted as malignant lymphomas. This is why good cooperation is needed between clinicians and pathologists. In the treatment of NHLs, novel methods like monoclonal autoantibodies, radioimmunotherapy and active immunotherapy have been recently introduced besides „traditional" chemo- and radiotherapy. Thanks to these therapies, dramatical improvement can be detected in surivival rates. To achieve complete remission, it is also important, how the patient's immune system defends against malignant cell clones. All known effector mechanisms play a role in antitumour immunity: B-cells

and immunoglobulins the produce, CD4+ and CD8+ T-cells and elements of the innate immunity (macropages, NK-cells, granulocytes, complements).

AIMS

My clinical and experimental investigations with non-Hodgkin's lymphoma patients aimed the following:

1. Determining the associated systemic and organ-specific autoimmune diseases among our patients. Finding the similarities in the pathogenesis of NHLs and autoimmune diseases.
2. Reporting on the immunodefeiciency-associated lymphoma in connection with a patient suffering from common variable immunodeficiency. Discussing a patient with chronic lymphocytic leukemia as an example for secondary tumours in NHL.
3. Characterizing BALT-lymphoma, a rare extranodal B-cell marginal zone disorder. Working up the cases with BALT-lymphoma at our department and presenting the changes of the immune system that may play a role in the pathogenesis.
4. Presenting Castleman's disease, Rosai-Dorfman disease and Kikuchi's disease as rare diseases with lymphadenomegaly that can be misinterpreted as malignant lymphomas.
5. Investigating the frequency of hepatitis virus (HCV, HGV and HBV) infections in our NHL patients, comparing to the healthy population and to the results of other studies. Examining the possible role of these viruses in the pathogenesis of malignant lymphomas.
6. T-cell immunity is a main element of anti-tumour defence. My aim was to investigate how the ratio of CD3+/HLA-DR+ and CD3+/CD69+ activated T-cells change in NHL patients' blood sample during the treatment and if these cells have any prognostic value in lymphomas.

PATIENTS AND METHODS

1. Patients

I analyzed the data of those NHL patients who have been treated and are taken care at the 3rd Department of Medicine, University of Debrecen. Collecting data, I used written and electronic documentation: patients's files, patients' reports and the Medsolution health informatical system. I could process the data of 612 patients until the end of 2004.

The diagnostics of non-Hodgkin's lymphoma have been provided by histopathological examinations of tissue samples, which have been performed at the Department of Pathology, University of Debrecen in most of the cases. Determining the histological subtypes, we followed formerly the Kiel's then the WHO's classification. We used the Ann Arbor's principles by staging. We followed the actual clinical principles by the treatment of the patients. In each study, the number and main characteristics (mean age, sex, histological subtypes) were stated.

2. Methods

a, Analyzing hepatitis infections

The method of nested polymerase chain reaction (PCR) was used to determine HCV and HGV infections in the blood of the patients. Enzyme linked immunosorbent assay (ELISA) was performed to determine the ratio of hepatitis B surface antigen (HbsAg) carriers. Our results were compared to the ratio of hepatitis virus infections in Hungarian blood donors.

b, Investigation of activated T-cells in blood samples

There were three blood samples taken from each patient: one right before the start of treatment, one after three cycles and the last one two months after the last cytostatic infusion. 100 μl of heparinized whole blood samples were incubated with antibodies against CD3 and HLA-DR (Sigma, ST. Louis, MO, USA) conjugated with fluorescent stain in dark at room temperature for thirty minutes. After the lysis of red blood cells, white blood cells were fixed with paraformaldehyde. The assessment was performed on EPICS XL-4 (Coulter, Hialeah, FL, USA) and Becton Dickinson FACS Calibur (Mountain View, CA, USA) flow cytometers, where lymphocytes were separated from monocytes and granulocytes. We gave the percentage of cells giving positive signals after counting 5000 lymphocytes

c, Statistics

The chi-square test was used for all statistical analysis. Probability levels (p) <0.05 were considered significant.

RESULTS AND NEW CONCLUSIONS

1. Association of systemic and organ-specific autoimmune diseases in NHL patients

I reviewed 421 NHL patients' charts, 230 males, 191 females, mean age was 54.1 years at the time of diagnosis. They were treated in the period of 1980-2002, the mean follow-up was 6.7 years. 32 (7.6 %) of them had any kind of autoimmune diseases. 26 (81 %) of them were women, 6 (19 %) of them were men. Mean age was 48.3 years (20-77). The most frequent autoimmune disease was Sjögren's syndrome with 11 cases, the other cases were autoimmune skin diseases (5), thyroiditis (4), polymyositis (2), scleroderma (2), rheumatoid arthritis (2), vasculitis (1), undifferentiated collagenosis (1), myasthenia gravis (1), autoimmune hepatitis (1), Addison's disease (1) and autoimmune hemolytic anaemia (1). Taking the priority of appearance of the disease's type, 10 patients (31%) were initially diagnosed with lymphoma and 22 (69%) had been already diagnosed autoimmune disease at the time of lymphoma occurrance. The mean follow-up with lymphoma was 4.5 years at the first group and it was 11.2 years with autoimmunity at the second group at the time of diagnosis of the second disease.

The prevalence of autoimmune diseases is 4-5 % in the whole population, while I found a frequency of 7.6 % among NHL patients. It was interesting that autoimmune symptoms developed first in 70 % of 32 patients, which supports the theory that regulational disorders of the immune system promote the development of malignant lymphomas. In a study with large number of patients like ours, Jonsson et al examined the prevalence of associated autoimmune diseases in 380 patients with lymphoproliferative disorders. They found an overall frequency of 12.8 % and 8.5 % by NHL patients. My

results highlighted that malignant lymphomas often associate with autoimmune diseases and vice versa, so this fact must be strongly considered in patients' care.

2a. Association of angiocentric lymphomatoid granulomatosis and common variable immunodeficiency

Examining immunodeficiency-associated lymphoma, I processed the case of a male patient who was born in 1949. In 1996 he was treated because of recurrent upper respiratory infections, and severe hypogammaglobulinaemia with extremely low IgA, IgM and IgG levels was detected. In 1998 he was admitted to our hospital because of fever, fatigue, night sweating and weight loss. Chest X-ray examination showed multiple round opacities bilaterally in his lungs and this finding was confirmed by CT as well. Thoracoscopy and lung biopsy were made. Histologic examination of the removed lung tissue revealed grade 3 angiocentric lymphomatoid granulomatosis, a rare subtype of NHL. Six cycles of CHOP polychemotherapy was administerred, which resulted complete remission in half a year. Since then his lymphoma has been in remission, but we are constantly detecting his severe hypogammaglobulinaemia which is due to common variable immunodeficiency (CVID). Since 2000 the patient has been on regular intravenous immunoglobulin substitution which helps preventing and treating his common respiratory infections.

Former studies found the prevalence of NHLs 8 % in patients with CVID. This way it is not surprising that angiocentric lymphomatoid granulomatosis, a rare disorder mimicking granulomatous inflammation developed in the lung of our patient. We could achieve complete remission by administerring CHOP chemotherapy, and so far there has

been no relapse of lymphoma, probably thanks to the regular intravenous immunoglobulin substitution.

b2. Association of malignant diseases to chronic lymphocytic leukemia

Non-Hodgkin's lymphomas that affect the bone marrow result constant immunodeficient states which may lead to the development of secondary malignancies. I examined this in connection with the case of an elderly male patient who was born in 1923. We diagnosed Rai 1 stage B-cell chronic lymphocytic leukaemia (B-CLL) by him in 1998. Six months later, purple-coloured skin lesions appeared on his lower extremities which were equal to Kaposi's sarcoma. In 2001 a prostate biopsy was performed because of increased level of PSA, the histological diagnosis was adenocarcinoma. This way the patient had three types of malignant diseases at the same time and the following therapies were administerred: CLL – chlorambucile, Kaposi's sarcoma – electron radiotherapy, prostate tumour – anti-androgene and LHRH-antagonist drugs. Recently, the patient is in good condition, his B-CLL is in partial remission.

Secondary malignant diseases appear in 11 % of all CLL patients. The most common associated tumours are Kaposi's sarcoma, melanoma and lung cancer. The association of prostate cancer in our case can be attributed rather to the patient's age and not to the immunodeficient state.

3. The clinical features of pulmonary BALT-lymphoma and immunological changes that promote its development

Among 617 NHL patients, we diagnosed pulmonary BALT-lymphoma in four cases. The patients were three elderly ladies and a middle-aged man. They all went through routine chest X-rays and the results presumed that they had maligant tumours in their lungs. Thoracosurgical operations (lung resections) were performed by two of them, the other two patients went through percutaneous lung biopsies. The histological examinations revealed BALT-type non-Hodgkin's lymphoma in all cases. None of them had B-symptoms. In their former histories, immunological disorders (Sjögren's syndrome and myasthenia gravis) and pulmonary tuberculosis could be found. One patient achieved complete remission after six cycles of CVP polychemoterapy, but the other three showed unsatisfactory therapeutical response, so the treatment had to be continued with more agressive chemotherapy regimens (CHOP, FC) and irradiation. We lost one of the elderly female patients because of cardiac insufficiency.

The bronchial mucosa-associated lymphoid tissue is usually not present in healthy adults, it develops in the airways of patients with chronic or recurrent respiratory infections, in chronic bronchitis of smokers and in chronic hypersensitive pneumonitis. Chronic antigenic stimuli result hyperplasia of the BALT and the increase of lymphoid cells can turn to monoclonal and lymphoma may develop. Looking at the patients' histories, three of them sufferred from chronic inflammatory – autoimmune or infectious – disease. I declared that BALT-lymphoma shows the features of low-grade NHLs: slow progression anf frequent relapses. By treatment, non-agressive chemotherapeutical regimens must be considered firstly, providing a good qualiy of life and avoiding side effects.

4. Rare lymphadenopathies that can be misinterpreted as malignant lymphoma

At our outpatient department for hematology we found three patients suffering from rare lymphadenopathies of probable immunological origin that caused difficulties in diagnostics. They were a young female with Rosai-Dorfman disease, a middle-aged male with multicentric Castleman's disease and a middle-aged female with Kikuchi's disease. By all of them, lymph node biopsy had to be performed several times to get the correct histopathological result. The female patients had enlarged lymph nodes only in single localizations, they were in good general condition. The male patient showed up with generalized lymphadenomegaly, his disease affected the cervical, axillar and inguinofemoral regions and appeared in his tonsillas causing difficulties in swallowing. CVP polychemotherapy and interferon-alpha were administerred without any improvement in his condition. In the end, involved field irradiation resulted regression of all enlarged lymph nodes.

All three diseases are difficult to distinguish from NHLs both in their clinical and histological appearance. Patients showing up with lymphadenomegaly must be examined in onco-hematological centres, which supposes good cooperation between clinicians and hemato-pathologists. Rosai-Dorman disease, Kikuchi's disease and multicentric Castleman's disease call for agressive treatment (chemotherapy, irradiation) only in cases of enormously enlarged lymph nodes causing compression symptoms.

5. Hepatitis virus infections in non-Hodgkin's lymphoma patients

From all the patients treated for NHL at our hospital, 80 (41 females, 39 males, mean age 55.7 years) were randomly selected for the study. None of the patients suffered from chronic liver disease or HIV infection. Out of these 80 patients, HCV was detected in 8 cases (10 %), HGV in 8 cases (10 %) and HCV-HGV coinfection was shown in 1 patient (1.25 %) by nested PCR. HbsAg positivity was confirmed in only 1 case. The frequencies were compared to the data of healthy blood donors. We found that rate of HCV infection was significantly higher in lymphoma patients, on the other hand, we did not find any significant alteration as regards to the prevalence of HGV infection. A total of 19 patients received blood transfusion, 5 were HGV-carriers and 1 was HCV-carrier.

My investigations are the first in Hungary that present hepatitis B, C and G infections among NHL patients. Assuming data from several countries, the rate of HCV infection is approximately 20 % in all lymphoma patients. In our patients, I found a frequency of 10 % which is lower than is other studies, however, it is significantly higher than in healthy blood donors. The rate of HGV infection was also lower in my study than in other countries. Our results seem to support the hypothesis that hepatitis virus infections – especially HCV - must be considered as risk factors for the development of malignant lymphomas. On the other hand, it is strongly recommended to test hepatitis virus infections among lymphoma patients in order to avoid late complications like cirrhosis or liver insufficiency.

6. Investigation of activated T-cells in NHL patients

There were forty-three people (20 females, 23 males, mean age 52.4 years) included to the study from among the non-Hodgkin's lymphoma patients between 1999 and 2002. As the result of combination polychemotherapy, all patients reached complete or good partial remission. Those people, whose disease seemed chemoresistant or did not react well to the treatment and those who sufferred from any infectious diseases or had leukemia, were excluded from the investigation. We used fifty-two healthy individuals as the control group, they had the same distribution in sex and age as the patients. In the end, we separated those patients who relapsed within one year after the polychemotherapy from the ones who stayed in constant remission. First we compared lymphoma patients to healthy control population. We found that the level of CD3+/HLA-DR+ activated T-cells is significantly higher in the blood of NHL patients right before the treatment than in healthy controls (10.63 % vs. 2.07 %, $p<0.001$) There was no significant difference found in the case of CD3+/CD69+ lymphocytes (1.257 % vs. 0.96%). After this, we compared the ratio of activated T-cells before and during polychemotherapy. This time only the number of CD3+/HLA-DR+ cells increased (10.63 % vs. 16.94 %, $p=0.005$), but not the CD3+/CD69+ cells. The ratio of activated T-cells during treatment did not differ significantly from the value measured after reaching remission. Following the patients for one year after polychemotherapy, approximately half of them (twenty-two people) stayed in remission, the rest twenty-one relapsed. Comparing the levels of CD3+/HLA-DR+ cells in these two groups, we found a really significant difference as there were much more activated lymphocytes in the post-treatment blood samples of patients with worse prognosis (20.62 % vs. 9.55 %, $p<0.001$). We could not show any relation in the case of CD3+/CD69+ cells.

The significance and novelty of these experiments are that we made a follow-up of the ratio of CD3+/HLA-DR+ and CD3+/CD69+ activated T-lymphoyctes in the peripheral blood samples of NHL patients in the period of chemotherapy. We found the ratio of CD3+/HLADR+ T-cells significantly higher in the blood of untreated lymphoma patients than in healthy control population, which can be explained with the fact that the immune system really recognizes tumour cells and tries to defend against them. Chemotherapy kills plenty of malignant cells, so several hidden intracellular antigenes will be set free and become approachable for the immune system, which results T-cell activation. When the third blood samples were taken from the patients after therapy, we supposed and proved by imaging and laboratory analysis that all of them had reached remission of their diseases. Later it turned out that almost half of them, twenty-two people relapsed. In this group the ratio of CD3+/HLADR+ activated T-cells was significantly higher and this showed the „stand-by" state of the immune system. Examinations with more cases and longer follow-up will decide whether the investigation of activated T-cells is a proper method to determine the immune defence and prognosis of lymphoma patients.

PUBLICATION

A. Articles that directly grounded the PhD theses:

1. Váróczy L, Gergely L, Zeher M, Szegedi Gy, Illés Á: Malignant lymphoma-associated autoimmune diseases - a descriptive epidemiological study. Rheumat Intern 2002; 22: 233-237.

   IF: 1,00, citation: 9

2. Váróczy L, Gergely L, Szakáll Sz, Illés Á: Angiocentric lymphomatoid granulomatosis and severe hypogammaglobulinaemia. Haematologia 2002; 32: 535-541.

   IF: 0,293

3. Váróczy L, Miltényi Zs, Keresztes K, Gergely L, Remenyik É, Illés Á: Association of malignant diseases to chronic lymphocytic leukemia – case report. Magy Belorv Arch 2003; 56: 127-130. (In Hungarian)

4. Váróczy L, Gergely L, Illés Á: Diagnostics and treatment of pulmonary BALT-lymphoma - a report on four cases. Ann Hematol 2003; 82. 363-366.

   IF: 1,241, citation: 1

5. Váróczy L, Gergely L, Miltényi Zs, Illés Á: Treatment and follow-up of pulmonary BALT-lymphoma. Magy Belorv Arch 2004; 57: 83-87. (In Hungarian)

6. Váróczy L, Gergely L, Aleksza M, Miltényi Zs, Illés Á: Investigation of activated T-cells by non-Hodgkin's lymphoma patients. Magy Immunol 2004; 3: 35-39. (In Hungarian)

7. Váróczy L, Krenács L, Gergely L, Bassam A, Illés Á: Some rare diseases causing lymphadenomegaly. Hemat Transzf 2004; 37: 248-256. (In Hungarian)

8. Váróczy L, Gergely L, Miltényi Zs, Aleksza M, Illés Á: Can CD3+/HLA-DR+ activated T-cells predict the prognosis of non-Hodgkin's lymphoma patients? Immunol Letters 2005; 97: 155-157.

   IF: 1,71

9. Váróczy L, Keresztes K, Gergely L, Illés K, Takács M, Horányi M, Miltényi Zs, Simon Zs, Illés Á: The prevalence of hepatitis virus infections and autoimmune phenomena in non-Hodgkin's lymphoma patients. Magy Belorv Arch 2005; accepted for publication (in Hungarian).

10. Váróczy L, Keresztes K, Gergely L, Takács M, Horányi M, Miltényi Zs, Simon Zs, Illés Á: Malignant lymphoma-associated hepatitis virus infections in East-Hungary. Int J Haematol, in submission

11. Váróczy L, Illés Á, Gergely L, Bassam A, Krenács L: Uncommon lymphadenopathies can be misinterpreted as malignant lymphoma. Acta Hematol, in submission.

B. Other articles

12. Váróczy L, Szegedi Gy: The role of hemopoietic stem cell transplantation in the treatment of autoimmune diseases. Orv Hetil 2000; 141: 595-600. (In Hungarian)

13. Illés Á, Keresztes K, Miltényi Zs, Váróczy L, Olvasztó S, Redl P, Gergely L, Dankó K: Unusual and late complication following treatment for Hodgkin's disease. Magy Belorv Arch 2002; 55: 105-109. (In Hungarian)

14. Miltényi Zs, Keresztes K, Váróczy L, Illés Á: Renal and ureter complications for the treatment of Hodgkin's disease. Magy Onkol 2002; 46: 351-355. (In Hungarian)

15. Miltényi Zs, Keresztes K, Lakos G, Váróczy L, Miltényi L, Illés Á: Is the treatment of the Hodgkin's disease detrimental to the parathyroid gland? Magy Belorv Arch 2002; 55: 135-138. (In Hungarian)

16. Illés Á, Bíró E, Miltényi Zs, Keresztes K, Váróczy L, András Cs, Sipka S, Bakó Gy: Hypothyreoidism and thyroiditis after therapy for Hodgkin's disease. Acta Haematol 2002; 109: 11-17.

IF: 1,252, citation: 2

17. Gergely L, Váróczy L, Vadász Gy, Remenyik É, Illés Á: Successful treatment of B-CLL associated severe paraneoplastic pemphigus with cyclosporine A. Acta Haematol 2003; 109: 202-205.

IF: 1,874, citation: 1

18. Miltényi Zs, Garai I, Édes I, Galajda Z, Tóth L, Keresztes K, Váróczy L, Illés Á: Early coronaria sclerosis in Hodgkin's disease. Magy Belorv Arch 2003; 56: 157-162. (In Hungarian)

19. Miltényi Zs, Keresztes K, Lakos G, Váróczy L, Miltényi L, Illés Á: Is the treatment of Hodgkin's disease detrimental to the parathyroid gland? Acta Haematol 2004; 112: 148-151.

IF: 1,874

20. Gergely L, Illés Á, Nagy Zs, Adamkovich N, Rejtő L, Szerafin L, Ujj Gy, Váróczy L, Radványi G, Borbényi Z, Varga Gy, Udvardy M: Results of combination immuno-chemotherapy in the treatment of diffuse large B-cell lymphoma. Magy Belorv Arch 2004; 57: 26-30 (In Hungarian)

21. Miltényi Zs, Keresztes K, Váróczy L, Mekkel G, Illés Á: Serious rhabdomyolysis in coeliac disease. Magy Belorv Arch 2004; 57: 44-47 (in Hungarian)

22. Simon Zs, Vadász Gy, Csípő I, Váróczy L, Illés Á: The biclonal type of myeloma multiplex: a case report . Magy Belorv Arch 2004; 57: 93-95.

23. Gergely L, Aleksza M, Váróczy L, Ponyi A, Sipka S, Illés Á: Intracellular IL-4 / IFN-gamma producing peripheral T lymphocyte subsets in B-cell non-Hodgkin's lymphoma patients. Eur J Haematol 2004; 72: 336-341.
IF: 1,714

24. Keresztes K, Aleksza M, Baráth S, Miltényi Zs, Váróczy L, Gergely L, Sipka S, Illés Á: Helicobacter pylori and Hodgkin's disease. Hemat Transzf 2004; 37: 265-271.

25. Gergely L, Dankó A, Csípő I, Váróczy L, Sipka S, Zeher M, Illés Á: Antibodies against extractable nuclear antigen (ENA) in non-Hodgkin lymphoma patients may be a non-specific marker of apoptosis. J Scan Immunol, in press
IF: 1,942

SCIENTOMETRY

Articles in extenso published or accepted for publication: 23

In English:	9
In Hungarian:	14
First author:	10
Impact factor:	12,9
Citation index (independent):	13