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ОТ РЕДАКТОРА

Журнал «Иммунология гемопоэза» — «Hæmatopoïesis Immunology» приобретает все большие черты периодического издания. В настоящем (№ 2/2006) номере мы учли интересы наших зарубежных коллег и приводим английскую версию трех статей, которые были опубликованы в №1/2006 год только на русском языке.

Кроме того, вы найдете в этом томе статью (дается на русском языке) по оценке субпопуляций мобилизованных стволовых клеток у больных с последствиями тяжелой спинномозговой травмы. Расширенная английская версия опубликована в «Journal of Biological Regulators and Homeostatic Agents», 2006, V. 20; № 1–2. В статье приводятся данные о выраженной пропорции $CD45^-$ клеток среди мобилизованных $CD34^+$ позитивных клеток периферической крови, обосновываются возможности исследования активации рецепторного комплекса *gp 130* на этих клетках.

С 2007 года мы выходим на полномасштабную двуязычную версию журнала. В № 1 будут представлены статьи по иммуноморфологической диагностике MALT-лимфом на материале гастробиопсий; иммуноглобулин-секретирующим периферическим В-клеточным лимфомам; роли иммуноморфологических исследований опухолевого субстрата при лимфоме Ходжкина.

Надеемся, что эти публикации вызовут у читателя большой интерес.

Главный редактор журнала «Иммунология гемопоэза»
доктор медицинских наук, профессор Н.Н. Тупицын
заведующий лабораторией иммунологии гемопоэза
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Настоящий номер журнала «Иммунология гемопоэза» — «Haematopoiesis Immunology» издан при финансовой поддержке Клиники восстановительной интервенционной неврологии и терапии «НейроВита», Москва, Россия

EDITORIAL

The journal Hæmopoïesis Immunology is increasingly acquiring features of a periodical publication. To meet the interests of our foreign colleagues we are publishing in this issue (№ 2/2006) English versions of three presentations from issue 1/2006 that were in Russian only. The volume also contains a description of a study of mobilized stem cell subpopulations in patients with severe spine cord trauma. This paper is in Russian because its extended English version is published in Journal of Biological Regulators and Homeostatic Agents, 2006, V. 20, № 1–2. The presentation provides evidence of the presence of a marked proportion of $CD45^-$ cells among mobilized $CD34^+$ peripheral blood cells and provides a rationale for study of *gp 130* receptor activation on these cells.

Since 2007 we are launching the journal as a fully double-language periodical. The first issue will contain presentations on immunomorphologic diagnosis of MALT-lymphomas by gastric biopsy, immunoglobulin-secreting peripheral B-cell lymphomas, the role of immunomorphologic study of tumor substrate in Hodgkin's lymphoma.

Hope these publications will be of interest for our readers.

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N. A. Kupryshina, M. A. Frenkel, N. N. Tupitsyn

IMMUNOPHENOTYPING AND MORPHOCYTOCHEMICAL CHARACTERIZATION OF ACUTE MYELOID LEUKEMIAS WITH EXPRESSION OF STEM CELL ANTIGEN CD34

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Abstract

Morphocytochemical and immunophenotypic features of CD34+ blast cell cases were studied in 81 patients with newly diagnosed acute myeloid leukemia (AML). AML M0 FAB-subtype was found in 10 (12.3%), AML M1 in 18 (22.2%), AML M2 in 18 (22.2%), AML M3 in 5 (6.2%), AML M4 in 17 (21.0%), AML M5 in 8 (9.9%), AML M6 in 3 (3.7%) patients. There were also AML M7 and mixed linear subtypes found. Stem cell antigen CD34 (>10%) was discovered in 61/81 (76.5%) patients. The CD34+ type was defined in 100% of AML M0, 66.7% of AML M1, 94.4% of AML M2, 20.0% of AML M3, 78.6% of AML M4 and 50.0% of AML M5 cases. Features specific of CD34+ versus CD34 cases were: by morphology, a less prominent granularity ($p=0.0001$) and monocytoid-shape nuclei ($p=0.001$); by cytochemistry, a lower myeloperoxidase (MPO) activity in M0 — M3 cases ($p=0.003$) and inhibitable α-naphthyl acetate esterase (ANAE) activity in M4 — M5 cases ($p=0.006$) as well as sudan black positivity ($p=0.005$) and diffuse PAS positivity ($p=0.0001$); by immunophenotyping, higher levels of HLA-DR ($p=0.0001$) and CD38 ($p=0.0001$). Proportion of CD34+ blasts was positively related with CD13+ ($p=0.01$) and negatively related with CD33+ blasts ($p=0.0001$). Leukemic stem cells ($CD34+CD38^-$) were seen in 23.4% of AML cases. Dysgranulopoiesis, dyserythropoiesis and dysmegakaryopoiesis were defined in 58 (71.6), 55 (67.9%) and 56 (69.1%) cases respectively. Granulocytic dysplasia was detected 2-fold more frequently in CD34+ AML as compared to CD34 AML. Moreover, CD34 expression was associated with the count of pseudo Pelger-Huet anomaly granulocytes ($p=0.002$).

Key words:
Acute myeloid leukemia (AML), stem cell antigen CD34, myeloperoxidase (MPO).

List of abbreviations:
ALL — Acute lymphoblastic leukemia
AML — Acute myeloid leukemia
ANAE — Nonspecific esterase (α-naphthyl acetate esterase)
BM — Bone marrow
HSC — Hemopoietic stem cell

LSC — Leukemic stem cell
MDS — Myelodysplastic syndrome
MKC — Megakaryocyte
MPO — Myeloperoxidase
MRD — Minimal residual disease

Introduction

The diagnosis of acute myeloid leukemia (AML) is historically based on comparison of morphological, cytochemical and immunophenotyping features. Blasts of granulocytic leukemia look similar to normal granulocytes by granularity and myeloperoxidase (MPO) activity. Monoblastic leukemia cells are similar to normal cells of the same lineage by morphology, nucleus shape and nonspecific esterase activity. Morphocytochemical signs are a basis for AML subtyping by degree of blast differentiation (M0-M3, M5a, M5b etc). If blasts have no clear morphocytochemical characteristics, it is immunophenotyping that plays the main role in the diagnosis to confirm myeloid nature of the leukemia in question and degree of blast maturity. Immunophenotyping is the most important in erythroblastic (M6), megakaryoblastic (M7) and minimally differentiated (M0) AML.

It should be noted that morphological and cytochemical features help to identify the most mature blast fraction with clear-cut hemopoietic lineage characteristics, however identification of blast lineage provides no information about the level of malignant transformation or leukemic clone structure.

Given the high intensity of today AML polychemotherapy regimens, most conventional clinical, hematologic and morphocytochemical (FAB subtype) signs fail to play important role in the prognosis of AML (I.G. Markina, N.N. Tupitsyn, L.Yu. Andreeva et al 2002). That is why new molecular criteria for the diagnosis and prognosis of AML are required.

The current WHO, 2001 classification is based on conventional morphocytochemical and immunophenotyping characteristics of blasts together with molecular and cytogenetic signs such as the presence of repeating chromosomal aberrations or specific changes (dysplasia) in cells of residual myelopoiesis.

There is much debate about potential utility of characteristics of the least mature AML fraction in the diagnosis. Modern tests identify $CD34^+$ early precursors and provide detailed immunophenotyping and functional analysis. Promising prospects of stem cell fraction study in leukemic clones are based on the following provisions:

1. In AML the $CD34^+$ blast fraction contains leukemic stem cells. There are reports (H. T. Hassan, A. Zander, 1996; R.T. Costello, F. Mallet ,B. Gaugler et al., 2000) demonstrating that overall efficacy of treatment may depend upon their proportion in the leukemic clone and degree of eradication as a result of therapy.
2. The $CD34^+$ fraction in the leukemic clone is significantly often associated with aberrant immunophenotype of malignant cells and expression of linearly unrelated antigens, which may be used in the monitoring of minimal residual disease (MRD) (A. Macedo, A. Orfao, M. Gonzales et al., 1995; D. M. Bahia, M. Yamamoto, M. L. Chauffaile et al., 2001).

3. Many reports demonstrate association of blast resistance with the presence of $CD34^+$ leukemic cells, that may account for poor results of the treatment in such cases (O. Legrand, S. Zompi, J. Y. Perrot et al., 2004).
4. High-dose chemotherapy with autologous hemopoietic stem cell (HSC) grafting is a common treatment in AML. This requires clear-cut characterization of $CD34^+$ stem cells to differentiate normal from leukemic ones (J. L. Harousseau, J. Y. Cahn, Pignon et al., 1997; P. A. Casileth, D. P. Harrington, F. R. Appelbaum et al., 1998).

Discovery of a specific stem cell marker, antigen CD34 in the mid 1980's allowed characterization of the earliest blast fraction.

There are different opinions about the effect of CD34-positivity on prognosis: some authors report of $CD34^+$ cases having a poor prognosis (S. J. Wells, R. A. Bray, L. L. Stempora et al., 1996; H. T. Hassan, A. Zander, 1996) or CD34-positivity to be inversely related to complete response rate (E. Solary, R. O. Casasnovas, L. Campos et al., 1992), while others deny any relationship between CD34-positivity and prognosis (S. Ciolli, F. Leoni, R. Caporale et al., 1993). Both $CD34^+$ blast proportion in the leukemic clone and degree of antigen expression play a role in the prognosis (G. M. Rigolin, F. Lanza, L. Ferrari et al., 1995).

There is a vast literature on the poor prognostic role of CD34 expression in AML (D. Raspadori, F. Lauris, M. A. Ventura et al., 1997; R. Costello, F. Malet, H. Chambost et al., 1999), which is also confirmed by studies conducted at the N. N. Blokhin CRC (I. G. Markina, N. N. Tupitsyn, L. Yu. Andreeva, 2002; O. Yu. Baranova, M. A. Volkova, M. A. Frenkel, 2005).

It should be noted that study potential of the least mature blast component in AML is limited, however new markers have recently appeared in addition to CD34 such as AC 133, CD117 (in comparison with other common myeloid markers), as well as non-linearly restricted antigens such as HLA-DR, CD71, CD38 and others.

Study of the earliest CD34-expressing blast fraction in AML seems therefore promising.

Materials and methods

The study group consisted of 81 patients with AML. All the patients had newly diagnosed AML, received no previous chemoradiotherapy and were free from myelodysplasia syndrome (MDS). The patients were examined at the Laboratory of Haematopoiesis Immunology (head Professor N. N. Tupitsyn.) N. N. Blokhin CRC RAMS (director Academician, Professor M. I. Davydov) during 2003 to 2005.

There were 47 (58.0%) females and 34 (42.0%) males in the study group. Mean age of males and females with AML was 46.1±3.1 and 48.6±2.6 years respectively.

Morphological, cytochemical study and immunophenotyping were performed on bone marrow (BM) aspiration biopsies. BM, 0.5–1 ml was harvested to Vakutainer tubes with dry EDTA. BM smears were prepared for morphologic and cytochemical studies, the remaining cells were used for immunophenotyping.

BM characteristics were assessed by a procedure described by A.I.Vorobyev (2002). BM cellularity was assessed using a Goryaev chamber, myelokaryocyte count was presented as a number of cells $\times 10^9$/l. Megakaryocyte (MKC) count was determined in a Goryaev

chamber ($\times 10^9$/l) and in BM smears during morphologic study, as well as in nonspecific esterase-stained smears.

Myelogram was counted by two independent morphologists (250 cells each) on Pappenheim-stained BM smears. Blastogram including blast size, nucleus-cytoplasm ratio, ratio of cells with regular and irregular nuclear shape, count of blasts with granularity and Auer rods, count of blasts with cytoplasm vacuolization and basophily was obtained for all cases.

Cytochemical study included activity measurement of myeloperoxidase (MPO), α-naphthyl-acetate esterase (ANAE) alone and with NaF inhibition, lipid identification in a reaction with sudan black B, diffuse or granular PAS-positive substance. The number of blasts with positive cytochemical test was presented as percentage.

AML was diagnosed by WHO (2001) classification in all cases, proportion of blasts was above 20% in all cases. AML subtype was defined by FAB (1991) criteria (J. M. Bennet, D. Catovsky, M. T. Daniel et al., 1991) basing on blast morphocytochemical and immunophenotyping characteristics.

Distribution of cases by FAB subtypes is shown in table 1.

Table 1.

Distribution of AML cases by FAB types

Types	Patients	
	№	%
M0 (poorly differentiated, myeloblastic)	10	12.3
M1 (myeloblastic without maturation)	18	22.2
M2 (myeloblastic with maturation)	18	22.2
M3 (promyelocytic)	5	6.2
M4 (myelomonoblastic)	14	17.3
$M4_{eos}$ (myelomonoblastic with eosinophilia)	3	3.7
M5a (monoblastic without maturation) M5b (monoblastic with maturation)	5/8 3/8	9.9
M6 (erythroblastic)	3	3.7
M7 (megakaryoblastic)	1	1.2
Mixed lineage	1	1.2
Total	81	100

Immunophenotyping was performed by direct immunofluorescence using triple fluorescent labeling. The monoclonal antibody panel included antibodies to common leukocytic antigen CD45, stem cell antigen CD34, non-linearly restricted antigens CD38 and HLA-DR, antigens of myeloid (CD13 and CD33), monocytic (CD14 and CD64), erythroid (glycophorin A — GlyA), megakaryocytic (CD61) differentiation lineages, common acute lymphoblastic leukemia (ALL) antigen CD10, B-, T-lineage antigens and NK-cell antigen CD56. A test was considered positive, if the respective marker was present on more than 10% of leukemic cells for CD34 and on more than 20% blasts for the remaining antigens. The blast study panel is shown in table 2.

Table 2.

Blast study panel in AML

Characteristics	Sign analyzed	No. of patients examined (%)
Morphology	1. % of blasts	81 (100)
	2. Blastogram	81 (100)
Cytochemistry	1. Peroxidase	80 (98.8)
	2. Lipids	51 (64.2)
	3. PAS-positive substance	80 (98.8)
	4. α-Naphthyl-acetate esterase with NaF inhibition	80 (98.8)
Immunophenotyping	1. Early antigen CD34	81 (100)
	2. Common leukocytic antigen CD45	81 (100)
	3. Non-linearly restricted antigens	
	CD38	76 (93.8)
	HLA-DR	81 (100)
	4. Myeloid CD13	81 (100)
	CD33	81 (100)
	5. Monocytic CD64	55 (67.9)
	CD14	19 (23.5)
	6. Common ALL antigen CD10	79 (97.5)
	7. B-lineage CD19	81 (100)
	CD20	76 (93.8)
	CD23	72 (88.9)
	8. T-lineage CD7	81 (100)
	CD3	76 (93.8)
	CD5	76 (93.8)
	CD4	72 (88.9)
	CD8	72 (88.9)
	9. NK-cell CD56	70 (86.4)

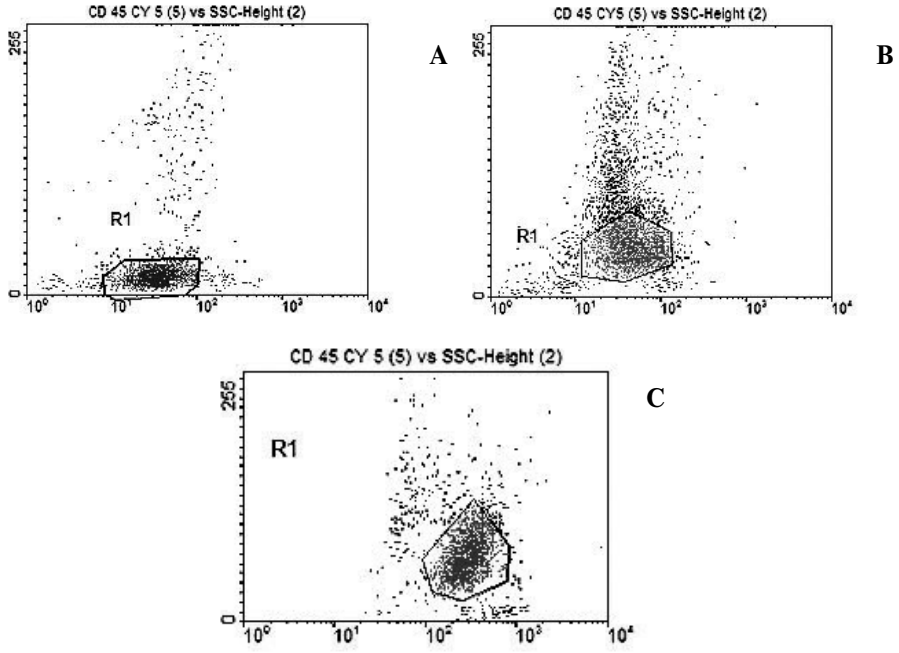
Immunophenotyping findings were analyzed by flow cytofluorimetry using a FACScan (Becton Dickinson, USA) instrument. At least 10,000 events were collected.

Blast gate was determined by light scattering and exrpession of common leukocyte antigen CD45 (N.N.Tupitsyn.,Z.G.Kadagidze,N.N.Shatinina et al. Immunodiagnosis of human haemoblastoses 2003). Blasts demonstrate a weaker CD45 expression as compared to lymphocytes and lower light scattering characteristics as compared to mature/maturating granulocytes.

The method is schematically presented in pic. 1.

Features of residual myelopoiesis elements were assessed in parallel with blast cells. Frequency of dysplasia signs in granulocytic, erythroid and megakaryocytic elements was analyzed. Dyshemopoiesis assessment was performed by two independent cytologists.

Granulocyte, erythroid and MKC dysplasias were assessed by WHO (2001) criteria. More than 50% cells of each hemopoietic lineage had to show dysplasia signs. One hundred cells of granulocytic and erythroid lineages are normally counted, however in cytopenic cases dysplasia is diagnosed by counting at least 25 granulocytes or erythroid cells and 5 MKC (or by the presence of 3 dysplastic MKC). One-, two-, or three-lineage dysplasia is defined basing on the number of dysplastic cell lineages diagnosed. At the same

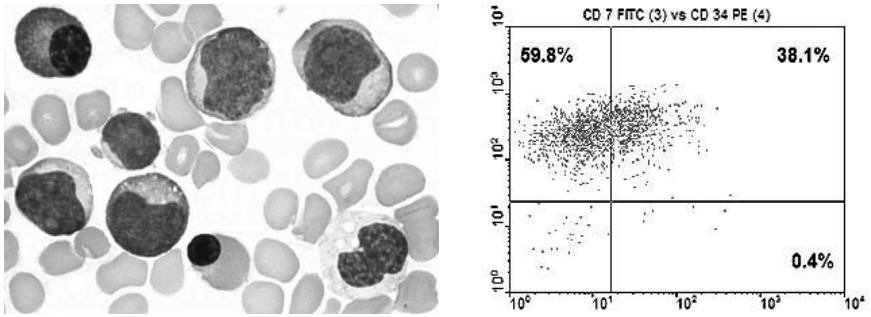


Pic. 1. Blast gate identification by light scattering and expression of common leukocytic antigen CD45:

A. — Blast gate in M0 AML;

B. — Blast gate in M2 AML;

C. — Blast gate in M5 AML



Pic. 2. Bone marrow profile and CD34 expression on blasts in M0 AML:

A. — Blasts in M0 AML;

B. — CD7 FITC-CD34 PE

time, the WHO classification specifies an individual AML subtype with multilinear dysplasia as defined by changes in 2 or 3 myelopoiesis lineages.

We counted partial contents of normal and dysplastic cells in each (granulocyte, erythroid and MKC) cell population.

When counting dysplastic neutrophils (granulocytogram) we specified hypogranular and pseudo Pelger-Huet anomaly neutrophils and MPO activity in neutrophils. Dysplastic erythroid cells were defined as cells with megaloblastoid features and dysplasia signs. If extended erythroid population was detected, BM smears were stained for siderophilic granules. The diagnosis of MKC dysplasia involved identification of mononuclear cells and MKC microforms in Pappenheim- and nonspecific esterase-stained BM smears. MKC contain nonspecific esterase resistant to NaF inhibition and are clearly seen by light microscopy. This technique allows identification of micro- or mononuclear MKC that are hardly seen after morphologic staining (table 3).

Table 3.

Morphological and cytochemical characteristics of myelopoiesis in AML patients

Myelopoiesis lineage	Characteristics	No. of patients assessed/Total No. of patients (%)
Granulocytes	1. Morphology - normal - with pseudo Pelger-Huet anomaly - hypogranular 2. MPO activity	58/81 (71.6) 58/81 (71.6)
Erythroids	1. Morphology - normal - megaloblastoid - dysplastic 2. Ring sideroblasts	55/81 (67.9) 2/81 (2.5)
Megakaryocytes	Morphology - normal - mononuclear - microforms	56/81 (69.1)
Two-lineage dysplasia	-	56/81 (69.1)
Three-lineage dysplasia	-	40/81 (49.4)

Assessment of dysgranulopoiesis, dyserythropoiesis and dysmegakaryopoiesis was made in 58 (71.6), 55 (67.9%) and 56 (69.1%) cases respectively.

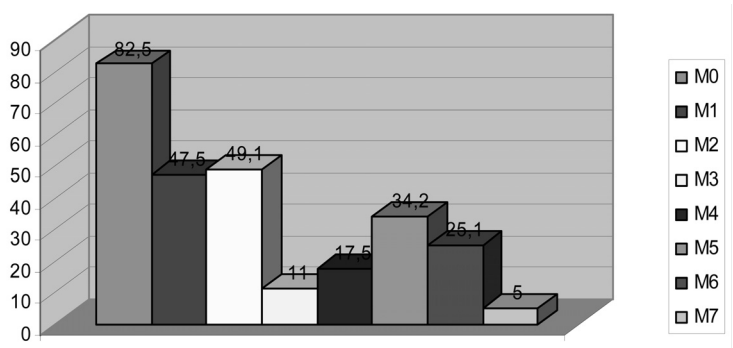
Cytometry findings were analyzed using PC WinMDI.

Statistical analysis of data involved correlation analysis and comparison of means. Significance of parametric signs was assessed by Student's test. Comparison of nonparametric data was made by means in contingency tables (chi-squared test). Statistical analysis was performed using SPSS 10.0 for Windows.

Results

Stem cell antigen CD34 exspression (>10% cells) was found in 62 of 81 (76.5%) AML patients. Distribution of antigen expression frequencies with respect to FAB-subtypes was as follows: 100% in M0, 66.7% in M1, 94.4% in M2, 20.0% in M3, 78.6% in M4, 100% in M4eos, 50% in M5. CD34 was present on cells from all 3 patients with M6 subtype and the patient with mixed lineage leukemia, and was absent in M7 (1 case).

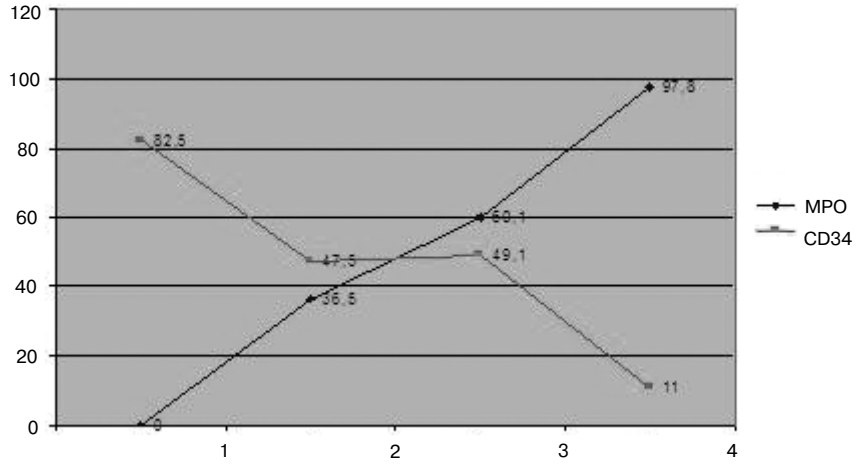
High levels of the antigen expression (>50%) were found on blasts with minimal differentiation only (pic. 2). A greater than 50% CD34 expression was discovered in all M0 cases, with an average level of $CD34^+$ cells 82.5%. In all other AML subtypes (M1–M6) mean number of $CD34^+$ cells was not greater than 50% (11.0 to 49.1%) and was significantly lower ($p<0.01$) (pic. 3). These results show that expression of the stem cell antigen on early precursors significantly differs by percentage from that on more differentiated cells.



Pic. 3. CD34 expression in FAB subtypes

Correlation analysis between CD34 expression and morphocytochemical characteristics of blasts discovered some regularities. CD34 expression was associated significantly with low granularity rate (r= -0.51, p=0.000), number of sudan-positive (r= -0.38, p=0.005) and PAS-positive blasts (r= -0.43, p=0.000), i.e. CD34 cell proportion was inversely related to percent of cells with morphocytochemical parameters characteristic of myeloblast differentiation. There was no relationship between CD34 expression and MPO in the entire AML patient group (p=0.08), however these parameters were inversely and significantly related in M0-M4 subtypes (p=0.02) in which MPO expression was a diagnostic marker (pic. 4). The statistical significance increased for granulocytic (M1–M3) leukemia subtypes (p=0.003).

We performed a similar study to assess relationship between CD34 expression and characteristic features of monoblasts. CD34 cell percentage was inversely related to the number of blasts with irregular (monocytoid) nuclei (r= -0.36, p=0.001) and nonspecific esterase activity (r= -0.30, p=0.006).



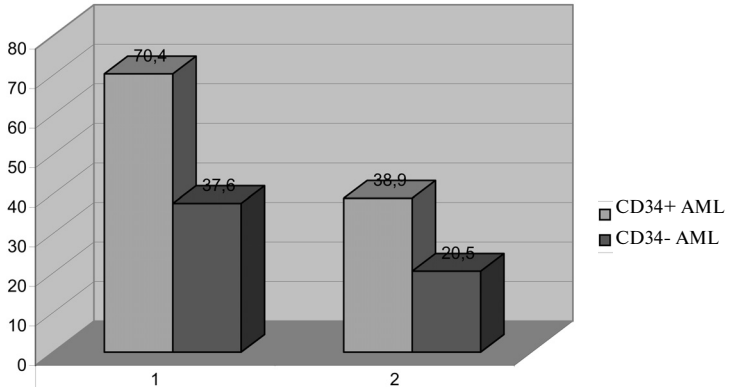
Pic. 4. MPO activity and CD34 expression in M0–M3 AML:
1. — M0 AML; 2. — M1 AML; 3. — M2 AML; 4. — M3 AML

These findings demonstrate that high levels of the CD34 expression (%% of blasts) are characteristic of the least mature precursors of M0 and decrease with blasts differentiation as determined by morphocytochemistry.

MPO activity is not found in normal $CD34^+$ HSC population. This enzyme appears in cells with granulocytic commitment. Of much interest are cases with high levels of CD34 expression and MPO activity in blasts. In our study high levels of CD34 expression (>50%) together with high number of MPO+ blasts (>50%) were found in 9 (11.1%) AML patients including 2 with M1, 4 with M2 and 3 with M4. The total of MPO+ and $CD34^+$ blasts was greater than 100%. This means that some $CD34^+$ blasts also contained the specific granulocytic enzyme MPO. These findings suggest that asynchronous maturation of leukemic cells with remaining CD34 expression on MPO+ differentiated blasts may be detectable. Such specific characterization of blasts may be useful in monitoring of residual disease.

Our findings demonstrate a great variability of relationship between blast antigen profile and enzyme status in AML. In early AML (M0 and M1) high levels of stem cell antigen CD34 are found together with the absence or low levels of the specific enzyme (MPO) which makes unlikely their simultaneous presence on the same cell. Coexpression of CD34 and MPO characteristic of aberrant phenotype may be seen in rare cases (about 10%) of differentiated disease.

CD34+ blasts had specific immunophenotyping characteristics. CD34 expression was correlated with other markers of early stages of granulocytic differentiation such as CD38 (r= 0.46, p=0.000) and HLA-DR (r= 0.52, p=0.000) which might be evidence of very early myeloid precursors transformation in AML cases (pic. 5).



Pic. 5. HLA-DR and CD38 expression on blasts with respect to CD34 expression in AML:
1. — HLA-DR expression parameters;
2. — CD38 expression parameters

Leukemic clones have a fraction of early, so called leukemic stem cells (LSC). As reported in the literature, cells with CD34+CD38$^-$ phenotype are the earliest in the population of normal and leukemic stem cells. These cells demonstrate increased expression of multiple drug resistance gene and decreased expression of apoptosis mediator antigens.

Analysis of occurrence of this fraction was a separate objective of the study.

As seen in table 4, CD34$^+$CD38$^-$ hemopoietic precursor fraction was found in almost all AML subtypes (in 23.4% of the patients). Subtypes M3, M6 and M7 were an exception and had no such a blast fraction. Primitive (CD34$^+$CD38$^-$) stem leukemic elements may be discovered in most FAB-subtypes of AML in about a quarter of cases. It seems reasonable to include such patients first of all in the group for monitoring MRD since it is this fraction that can resist to drug therapy and may account for treatment failure in AML.

Expression of HLA-DR molecule associated with early stages of blast granulocytic differentiation was found in most cases (72.2 to 100%) in all AML types except M3 in our study. However, mean intensity of its expression was significantly greater in the earliest M0 type (84.4±3.0% of positive cells) as compared with granulocytic leukemia types (51.3±8.3% and 56.5±4.2% respectively, $p<0.01$). HLA-DR was expressed in a vast majority (87.5–100%) of monoblastic (M4-M5) AML types, the difference in mean numbers of positive cells in comparison with M0 (77.1±5.1 and 78.9±10.8% respectively) was not significant. One of 5 cases with M3 type was HLA-DR-positive.

AML blasts with myeloid commitment are characterized by expression of pan-myeloid antigens CD33 and CD13. Analysis of myeloid antigen expression with respect to the presence or absence of stem cell antigen CD34 revealed some regularities. For instance, CD34 expression demonstrated a positive correlation with CD13 expression and a negative relationship with CD33 expression ($p=0.01$ and $p=0.000$, respectively).

Analysis of myeloid antigen expression separately in CD34$^+$ and CD34$^-$ patients showed

Table 4.

Frequency of early blast fraction with CD34+CD38 phenotype with respect to FAB-subtypes

Type	Patients	
	No.	%
M0	3/10	33.3
M1	4/18	22.2
M2	6/17	35.3
M4	4/16	25.0
M5	1/7	14.3
Total	18/81	23.4

both myeloid antigens to be present in most cases (table 5).

CD34$^+$CD33$^+$CD13$^+$ blast phenotype was most common in all FAB-subtypes except M3.

Expression of CD13 alone was characteristic of CD34$^+$ AML. Cells with CD34$^+$CD13$^+$CD33$^-$ phenotype appear at early stages of leukemic myeloid cell differentiation. Such cells were typical of M0 cases and found in a quarter of M1 ones. It should be emphasized that this blast subtype is considered the earliest one by morphocytochemical characteristics of myeloid differentiation (no granularity, low MPO activity, low lipid content).

In opposite, expression of myeloid antigen CD33 alone was more typical for CD34 cases mainly with M5 AML. The earliest M0 subtype was characterized by much higher mean numbers of CD13$^+$ (84.0±4.1%) blasts and much lower numbers of CD33$^+$ (35.3±9.3%) ones than in other AML subtypes (35.8 to 68.1% and 65.3 to 82.9% respectively, $p<0.05$).

CD64 expression was inversely correlated with CD34 ($p<0.001$). Monoblastic leukemia types (M4–M5) with high activity of inhibitable ANAE demonstrated no expression of specific antigen CD64 in 19% (4/12) of cases. All the CD64$^-$ cases had high expression of CD34 as

Table 5.

Myeloid precursor subpopulations in AML types

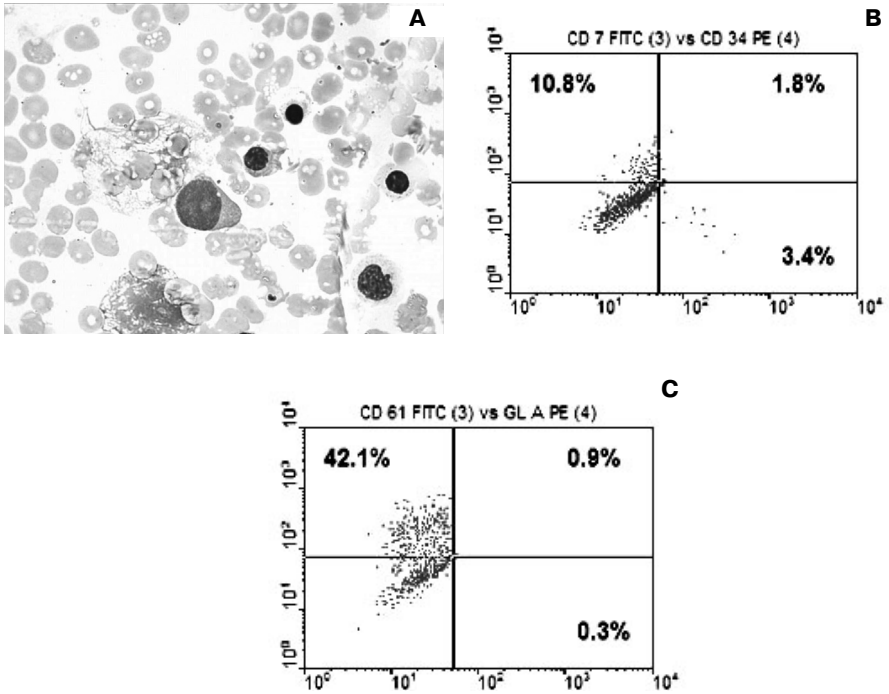
Blast subpopulation	M0 n=10	M1 n=18	M2 n=18	M3 n=5	M4 n=17	M5 n=8	Total* n=76
CD34$^+$/33$^+$/13$^+$	6/10 (60	6/12 (50)	16/17 (94.1)	1/1 (100)	12/14 (85.7)	2/4 (50)	43/58 (74.2)
CD34$^+$/33$^-$/13$^+$	4/10 (40)	4/12 (33.3)	0	0	2/14 (14.3)	0	10/58 (17.2)
CD34$^+$/33$^+$/13$^-$	0	2/12 (16.7)	1/17 (5.9)	0	0	2/4 (50)	5/58 (8.6)
CD34$^-$/33$^+$/13$^+$	0	6/6 (100)	1/1 (100)	3/4 (75)	3/3 (100)	1/4 (25)	14/18 (77.8)
CD34$^-$/33$^-$/13$^+$	0	0	0	1/4 (25)	0	0	1/18 (5.6)
CD34$^-$/33$^+$/13$^-$	0	0	0	0	0	3/4 (75)	3/18 (16.6)

* No. of patients having the sign/No. of patients assessed (%)

compared to other monoblastic leukemias with lower CD34 expression (93.8% versus 28.6%).

Acute erythromyelosis (M6) and acute megakaryoblastic leukemia were diagnosed in 3 and 1 cases respectively. By immunology, blasts in M6 AML were characterized by low CD34 expression (17.2 to 39.7%) and the presence of a specific molecule Gly A (pic. 6). In M7 AML blasts were classified as $CD34^-$ with expression of specific megakaryoblast antigen CD61 (pic. 7).

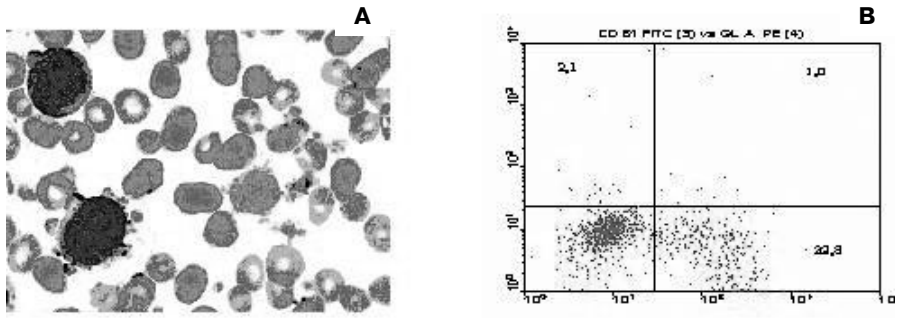
Blasts in acute leukemia with mixed differentiation lineages were characterized by



Pic. 6. Bone marrow picture and immunophenotyping findings in M6 AML:
A. — Bone marrow profile;
B. — CD7 FITC-CD34 PE;
C. — CD61 FITC-Gly A PE

high CD34 expression and the presence of markers of different lineages such as CD33, CD13 and B-lymphoid ones CD10, CD19 (pic. 8).

Leukemic blasts in AML have a characteristic disorder in antigenic profile on surface membranes. Aberrant immunophenotype demonstrates expression of myeloid and lymphoid antigens, the latter correlating with immaturity of clone generation elements and



Pic. 7. Bone marrow picture and immunophenotyping findings in M6 AML:
A. — Bone marrow profile;
B. — CD61 FITC-Gly A PE

early differentiation stages. Frequency of aberrant expression of "lymphoid" antigens in our study is demonstrated in table 6.

Expression of CD7, typically present at early stages of cell differentiation, was found in 17 of 81 (21%) patients. There was no CD7 expression in M3, M5b, M6 and M7 types. There was a high, significant and direct correlation between CD7 and stem cell CD34 expressions ($r=0.31$, $p=0.005$) that might characterize early precursors in the leukemic clone in most AML types.

The most frequent expression of B-lymphoid antigen CD19 in M2 AML, a rather mature acute myeloleukemia by morphocytochemistry, may seem to be a contradiction. However CD34 was expressed almost in all M2 cases and tended to correlate with lymphoid marker CD19.

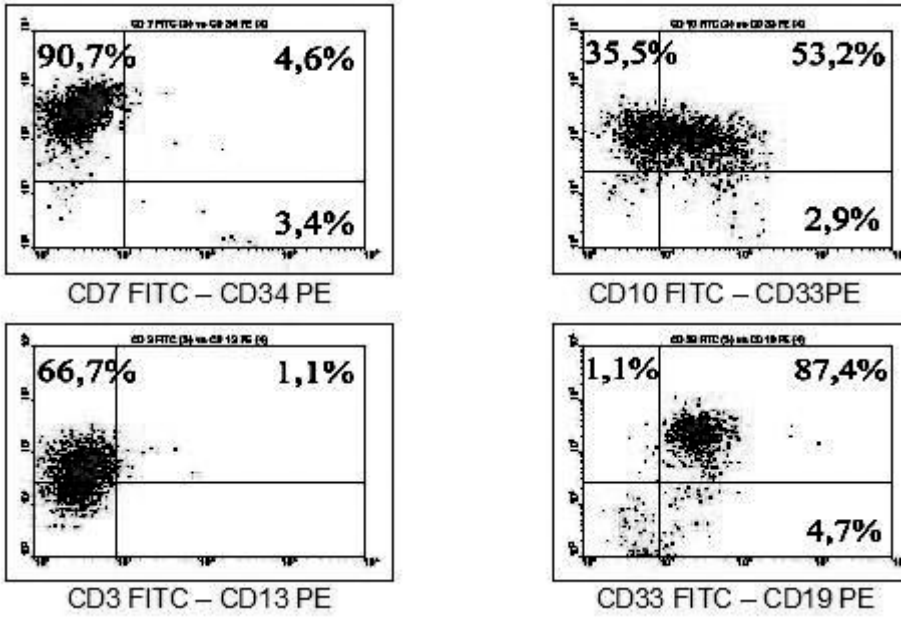
Aberrant expression of CD56, CD4, CD5 and CD10 demonstrated in our study may be

Table 6.

Frequency of "lymphoid" associated antigens in AML types

Antigen	M0	M1	M2	M3	M4	M5	M6	M7	Total*
CD7	3/10 (30)	4/14 (28.6)	3/18 (16.7)	0	4/17 (23.5)	2/8 (25)	0	1	17/81 (21)
CD5	0	1/18 (5.6)	1/18 (5.6)	0	1/16 (6.3)	0	0	0	3/77 (3.9)
CD4	0	2/18 (11.1)	2/16 (12.5)	0	3/16 (18.8)	3/7 (42.9)	0	0	10/72 (13.9)
CD10	0	0	0	0	0	1/8 (12.5)	0	0	1/79 (1.3)
CD19	0	0	4/18 (22.2)	0	1/17 5.9)	1/8 (12.5)	0	0	6/80 (7.5)
CD56	0	5/17 (29.4)	4/18 (22.2)	0	3/14 (21.4)	3/5 (60)	0	0	15/71 (21.1)

* No. of positive cases/No. of patients assessed (%)



Pic. 8. CD34, CD33, CD13, CD10, CD19, CD7 and CD3 expression in acute mixed lineage leukemia

used in monitoring of MRD during remission as well as for differentiation between leukemic and normal precursors during cell mobilization for autologous grafting.

Aberrant expression may be a combination of antigens of different myeloid lineages. For instance, there were the following molecules expressed on myeloblasts of various degree of differentiation: monocytic antigen CD64 in 35.5% (11/31), erythroid antigen GlyA in 9.1% (4/14), MKC antigen CD61 in 8.5% (3/34) of cases. This may be explained by asynchronous appearance of antigenic and enzymatic characteristics of blasts. Such observations are of much importance in the diagnostic practice and prove reasonable the use of various approaches to blast assessment in AML.

Assessment of multilineage dysplasia of residual myelopoiesis elements has recently started to play an important role in characterization of AML. Granulocyte and MKC dysplasias were the most common types as detected in about half of the 56 patients examined (44.8% and 51.8% respectively). Erythroid dysplasia was found in isolated cases (8.9%). Multilineage dysplasia (23.2%) was represented as dysplasia of two lineages, mainly of granulocytes and MKC. There were no three-lineage dysplasias in our patient group.

Analysis of occurrence of different dysplasia types with respect to CD34 expression on blasts demonstrated that granulocyte dysplasia was found about two-fold more frequently (55.6% versus 30.8%, $p<0.1$) in $CD34^+$ cases, while frequency of MKC, erythroid and

two-lineage dysplasias showed no relation with expression of this antigen.

Some parameters of neutrophil changes were analyzed to clarify granulocyte dysplasia characteristics, such as size, the presence of granularity and pseudo Pelger-Huet anomaly.

Pseudo Pelger-Huet anomaly was found in half of the cases. The number of neutrophils with pseudo Pelger-Huet anomaly was significantly higher in $CD34^+$ than in $CD34^-$ blasts (87.1% versus 12.9%, $p=0.002$).

Erythroid dysplasia was seen only in $CD34^-$ blasts, though there were too few cases in our study to make definite conclusions about this finding.

As a whole, our assessment of relationship between dysplasia and blast maturity or CD34-positivity suggests certain significant associations. This may be another sign of relationship between CD34 expression on blasts and dysplasia of recognizable myelopoiesis elements.

Therefore, results of our study demonstrate that CD34 expression on blasts in AML is a characteristic of less mature leukemia forms as assessed by morphocytochemistry and immunology and is associated with myelodysplasia manifestations in residual blasts.

Conclusions

1. Blasts in AML may be divided into two groups with respect to the presence of membrane stem cell antigen CD34, i.e. less mature ($CD34^+$) and more mature ($CD34^-$) types by most morphocytochemical and immunophenotypical characteristics.

2. $CD34^+$ AML are characterized by significantly lower content of morphologically differentiated myeloblasts with granularity ($p=0.000$) and monoblasts with typical monocytoid nuclei ($p=0.001$) as compared with CD34 disease.

3. Characteristic cytochemical features of $CD34^+$ AML include low activity of MPO in M0–M3 AML ($p=0.003$) and of inhibitable ANAE in M4-M5 ($p=0.006$) as well as decreased content of lipids ($p=0.005$) and diffuse PAS-substance ($p=0.000$).

4. By immunophenotyping, $CD34^+$ AML differ from $CD34^-$ ones by higher degree of expression of early granulocyte differentiation markers such as HLA-DR ($p=0.000$) and CD38 ($p=0.000$).

5. About a quarter (23.4%) of AML cases have blasts with stem leukemic cell phenotype $CD34^+CD38^-$. This fraction is the largest in M0 (33.3%) and M2 (35.3%), is absent in M3 and is found with an intermediate frequency (14.3 to 25.0%) in other AML types.

6. CD34 expression is related directly to CD13 expression ($p=0.01$) and inversely to CD33 one ($p=0.000$). Basing on this relationship we defined a new AML immunosubtype $CD34^+CD13^+CD33^-$ typical of immature myeloid leukemias M0 and M1.

7. As compared to $CD34^-$ AML, $CD34^+$ type is characterized by aberrant blast immunophenotype with

— coexpression of lymphoid antigens CD7 and CD19

— coexpression of erythroid and MKC markers in granulocytic and monoblastic leukemias

— absence of Fc RI (CD64) in monoblastic types

— coexpression of CD34 and MPO in 11.1% of AML.

These characteristics may be useful in monitoring of MRD.

8. CD34-positive AML is characterized by granulocyte dysplasia which is encountered almost two-fold more frequently than in CD34-negative disease. There is a significant direct relationship between CD34+ blast proportion and the presence of neutrophils with pseudo Pelger-Huet anomaly (p=0.002).

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THE ROLE OF IMMUNOPHENOTYPING IN THE DIAGNOSIS OF BONE-MARROW INVOLVEMENT IN PATIENTS WITH PERIPHERAL B-CELL NON-HODGKIN'S LYMPHOMA

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Abstract

Bone marrow is a frequent site of involvement in mature B-cell non-Hodgkin's lymphomas. Bone marrow specimens from 58 patients with various B-cell non-Hodgkin's lymphoma types were analyzed to study specific features of the bone marrow lesions, to identify their specific histology and immunomorphology patterns, and possible differences from lesions in other sites involved. The diagnosis was made according to WHO (2001) classification in all cases. The selected group included diffused large-cell B-cell lymphoma (20/35%), all types of marginal zone lymphoma (10/17%), follicular lymphoma (7/12%), B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma (6/10%), mantle cell lymphoma (6/10%), Burkitt's lymphoma (4/7%), plasmacytic types (2/3.5%), lymphoplasmacytic lymphoma (2/3.5%), mediastinal large-cell B-lymphoma (1/2%). Analysis involved comparison of cytology and flow cytometry findings by aspiration biopsies, and morphology and immynohistochemistry data from bone marrow trephine biopsies. Immunophenotyping by flow cytometry ensures sensitive detection of bone marrow involvement especially in CD5+ B-cell non-Hodgkin's lymphomas, while paraffin immunohistochemical immunophenotyping provides preservation of architecture, identification of histologically distinct lesions in patients with B-cell non-Hodgkin's lymphoma and differentiation of the latter from reactive lymphoid infiltration. We did not see any immunological differences in antigen expression on tumor cells between bone marrow and extramedullary B-cell non-Hodgkin's lymphoma sites. Basing on the analysis results we developed a diagnosis algorithm to study bone marrow immunomorphology in each B-cell non-Hodgkin's lymphoma type.

Key words:

Non-Hodgkin's lymphoma, bone marrow, aspiration biopsy, trephine biopsy, flow cytometry, immunohistochemistry.

List of abbreviations

WHO — World Health Organization

NHL — non-Hodgkin's lymphoma
B-NHL — B-cell non-Hodgkin's lymphoma
BM — bone marrow
AM — adhesion molecules
McAb — monoclonal antibodies
FC — flow cytometry
IHC — Immunohistochemistry
B-CLL/CLL — B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma.
MCL — mantle cell lymphoma
MZL — marginal zone lymphoma
LPL — lymphoplazmacytic lymphoma
FC — follicular lymphoma
DLBCL — Diffuse Large B-cell Lymphoma
MLCBL — Mediastinal Large -cell B- Lymphoma
MALT — mucosa- associated lymphoid tissue lymphoma
CD — cluster differentiation

All types of mature B-cell non-Hodgkin's lymphomas (B-NHL) demonstrate affinity to bone marrow (BM) with hemopoietic tissue being the most frequent site of extranodal involvement (a so called 'homing-effect'). Detection of BM involvement in lymphoma cases plays a very important role in the clinical practice for the following reasons.

1. Accurate primary disease staging to ensure adequate treatment strategy, first of all as concerns small-cell B-cell lymphomas. BM involvement, for instance in follicular lymphoma, is associated with stage IV disease and requires systemic chemotherapy in opposite to a combined chemoradiotherapy approach indicated in early stage disease. Patients with stage I-II mucosa-associated lymphoid tissue lymphomas (MALT-lymphomas) and Helicobacter pylori-positive gastric lesions may benefit from antihelicobacter eradication therapy. While polychemotherapy is the only treatment modality used in advanced disease with BM involvement (S. N. Malek 2003).
2. BM findings may be useful in follow-up of patients achieving complete response. This diagnostic approach is of a special importance now, when novel chemotherapies and therapeutic monoclonal antibodies are used successfully to achieve complete response in some B-NHL, e.g. in B-cell small lymphocytic lymphoma/chronic lymphocytic leukemia (B-CLL/SLL) (F. Bosch, A. Ferrer, A. Lopez-Guillermo et al., 2002). Of importance, degree of response to induction polychemotherapy determines intensity and number of consolidation cycles in B-NHL.
3. Lymphoma-affected BM may be the only source for diagnostic sampling in cases with primary bone/bone marrow involvement or with inaccessible primary extramedullary lesions (isolated lesions in retroperitoneal or mediastinal lymph nodes, spleen), as well as due to patient' s poor performance status; pan- or thrombocytopenia.

Histological signs for differential diagnosis such as disease size, shape, location as well as cytological features of lymphoma cells as found by standard staining of BM specimens

play an important role in the diagnosis though fail to ensure accurate diagnosis (D.S. Osmanov , 2004). For instance, paratrabecular and diffuse lymphoma infiltration types are considered markers of lymphoma while focal BM lesions may be very similar to reactive disease, and detection of minimal interstitial or intrasinusoidal lymphoma cell settlement is always very difficult even for high-skilled investigators (B. J. Bain, 2001; C. F. Feller and J. Deibold, 2004).

Immunological and molecular biological tests are currently applied to improve sensitivity in detecting minimal BM lesions in B-NHL (E.V. Chigrinova., 2005). Immunophenotyping of lymphoma cells by BM aspiration biopsy detects neoplastic cells even in samples with a small number of lymphocytes/prolymphocytes in BM basing on immunophenotype features, lineage and differentiation of cells from the primary tumor (G. K. Valet, H.G. Hoffkes 1997). Flow cytofluorimetry (FC) has the advantage of identifying coexpression of several molecules (2 or 3 or more) on a single cell (N.N. Tupitsyn.,Z.G. Kadagidze.,N.N. Shatinina. et al 2003).

Immunohistochemistry (IHC) by trephine biopsy is another immunological approach to study BM lesions in patients with B-NHL (E.V.Chigrinova, 2004). This method comprises identification of tumor cell lineage and evaluation of relationship between immunophenotypes of the BM and extramedullary lesions, as well as allows assessment of this diagnostically significant tumor cell settlement in its relationship with bone and hemopoietic tissues (B. J. Bain, 2001; C. F. Feller and J. Deibold, 2004). Sensitivity assessment in immunological identification of BM involvement in B-NHL by comparison of immunophenotyping (FC and IHC) and standard (cytology and histology) findings is useful for improvement in B-NHL staging accuracy and classification according to the current WHO system (2001) (N. Harris, E.S. Jaffe, J. Diebold et al., WHO, 2001). The most accurate technique for staging and immunophenotyping of lymphoma types will then ensure optimal choice of treatment strategy both in primary and recurrent disease in every individual case of B-NHL.

Purpose

The purpose of this study was to assess the role of immunophenotyping in the diagnosis of BM involvement in different morphological types of peripheral B-NHL.

Materials and methods

This study was performed in 58 patients with different B-cell peripheral non-Hodgkin's lymphoma (NHL) types managed outpatiently at the Hematology Department of the clinic or inpatiently at the Department of Chemotherapy for Hematology Malignancy, Institute of Clinical Oncology, N. N. Blokhin Cancer Research Center.

The patients' age at diagnosis ranged from 16 to 82 years, median 54 years. Male to female ratio was 0.87. Case histories were collected basing on careful analysis of medical records and interviews with patients. All patients had standard clinical diagnostic tests at baseline, during therapy and when assessing treatment response taking into account B-NHL specific dissemination. Disease stage was therefore determined basing on a comprehensive assessment and patients' histories. Patient distribution with respect to principal site

involved is demonstrated in table 1. Proportional site involvement in cases with extranodal primary tumors is given in table 2.

Table 1.

Case distribution with respect to prevalent site involvement

Tumor site	No. of patients	Percentage
Nodal primary	34	58.6
Extranodal primary	24	41.4

Table 2.

Prevalent site involvement in extranodal primary B-NHL

Site	No. of patients	Percentage
GI	6	25
Spleen	3	13
Bones/bone marrow	5	21
Soft tissues	3	13
Liver+spleen	2	8
Ovaries	2	8
Waldeyer's throat ring	1	4
Breast	1	4
Mediastinum	1	4

The diagnosis of B-NHL (WHO, 2001) was based on complex immunomorphology findings by patients' biopsies. When possible the primary diagnosis was made by surgical biopsy of extramedullary lesions. Fixation and paraffin embedding procedures were in compliance with standard protocols adopted at the Human Tumor Pathology Department, N. N. Blokhin Cancer Research Center. Histology analysis was made on sections from paraffin blocks using hematoxylin-eosin staining and additionally on Brachet- and pycrofuchsin-stained sections. In a vast majority of cases tumor tissue immunophenotyping was made on fresh-frozen sections from biopsy samples (acetone fixation) at the Hemopoiesis Immunology Laboratory, Clinical Immunology Department, Institute of Clinical Oncology, N. N. Blokhin Cancer Research Center. Enzyme immunostaining (IHC) was used at the Human Tumor Pathology Department if fresh biopsy samples were not available or as an extra test.

Sixteen patients had no or inaccessible solid extramedullary lesions. Serous exudation (ascitic fluid) was studied as a sample of primary extramedullary lesions by cytofluorimetry in one case. The sample was centrifuged and cell smears were made for the study. Another sample from an extramedullary lesion (lymph node) was taken by fine-needle

biopsy to be studied by immunocytology. Leishman's staining was used in both cases. All cytology testing was done at the Clinical Cytology Laboratory.

The standard monoclonal antibody (MAB) panel used at the Hemopoiesis Immunology Laboratory, Clinical Immunology Department, Institute of Clinical Oncology, N. N. Blokhin Cancer Research Center, to specify NHL diagnosis is shown in table 3.

Table 4 presents distribution of B-NHL types with respect to the WHO (2001) classes.

Table 3.

Monoclonal antibody panel for lymphoma immunodiagnosis

Marker type	Immunofluorescence, frozen sections	Flow cytofluorimetry	Immunohistochemistry, paraffin sections
Nonlinearly restricted	CD45, CD23, CD10, CD30, CD38, HLA-DR	=IFR	CD45, CD23, CD10*, CD30, HLA-DR
B-lineage	CD19, CD20, CD21, CD138, κ, λ, Ig	=IFR	CD20, CD21, CD45RA, CD79α, CD138, κ, λ, Ig
T/NK-lineage	CD45RO, CD3, CD56, CD5, CD7, CD4, CD8	=IFR	CD45RO, CD3, CD5, CD4, CD8
Myelomonocytic	CD15, CD68, CD163	CD13, CD33, CD64	CD15, CD68, CD163
Additional	Bcl-2, Bcl-6, cyclin D1, Ki-67	Bcl-2, Ki-67	Bcl-2, Bcl-6, cyclin D1, Ki-67
Precursor	CD34, TdT	=IFR	=IFR
* the Hemopoiesis Immunology Laboratory did not have this antibody for fixed samples at the time of the study			

Table 4.

Non-Hodgkin's lymphoma types by WHO (2001) classification

WHO (2001) type	No. of patient	%
DLBCL	20	35
Marginal zone lymphoma*	10	17
Follicular lymphoma	7	12
Small lymphocyte lymphoma/B-CLL	6	10
Mantle cell lymphoma	6	10
Burkitt's lymphoma	4	7
Plasma cell NHL	2	3.5
Lymphoplasmacytic lymphoma	2	3.5
Primary mediastinal B-cell lymphoma	1	2
Total	58	100
* The term includes all marginal zone lymphoma subtypes assessed in this study		

The study was based on detailed morphology investigation and immunophenotyping of BM from patients with B-NHL. Cytology analysis (myelogram), FC by aspiration biopsies, histology study and IHC by trephine biopsies were used (table 5). Of note, BM was the only disease site in 5 cases.

Table 5.

Methods of bone marrow study in non-Hodgkin's lymphoma

Method	No. of patients	%
Cytology (myelogram)	58	100
Trephine biopsy, histology	51	88
Aspiration biopsy, flow cytometry	31	53
Trephine biopsy, immunohistochemistry	40	69

BM sampling was made by puncture of the body and handle of the sternum, and of posterior spine of huckle-bones. Cytology study of puncture samples was made at the Hemopoiesis Immunology Laboratory on smears stained by Romanovsky-Giemsa after fixation in 96% methanol.

Immunophenotyping of BM aspiration biopsies was made by FC using a FACScan (Becton Dickinson) sorter with two- or three-color labeling and direct conjugates of monoclonal antibodies with fluorescein isothiocyanate (FITC), phycoerythrin (PE) and peridinine-chlorophyll (PerCP) fluorochromes. Table 6 demonstrates frequency of morphological verification and immunophenotyping confirmation of the diagnosis of B-NHL by FC on aspiration biopsies.

BM trephine biopsy was also made in posterior superior spine of huckle-bones. The trephine biopsy samples were routinely fixed in Carnoy solution, decalcificated in nitric acid-based solution and paraffin embedded. Histology study was done on hematoxylin-eosin- and pyrofuchsin-stained sections from paraffin blocks.

Immunophenotyping of BM trephine biopsies was performed by a combination of immunofluorescent assay and enzyme immunoassay on de-embedded sections at the Hemopoiesis Immunology Laboratory. The MAB panel routinely used in the NHL diagnosis basing on BM trephine biopsy was demonstrated above in table 7.

Therefore all patients underwent a comprehensive BM assessment that helped to select a priority technique with respect to the clinical situation and B-NHL subtype.

Results and discussion

Owing to specific biological features of B-NHL, lymphoma cells demonstrate affinity to BM (homing-effect) thus making hemopoietic tissue a principal site of disease dissemination. This is a key stage of lymphoid tumor advance that influences both the disease course and patient's performance status.

Table 6.

Frequency of detection of bone marrow involvement in NHL by cytology and flow cytofluorimetry

Method	Frequency of involvement	
	No. of bone marrow aspiration biopsies assessed	Percentage of positive tests
Cytology	58	64
Flow cytofluorimetry	31	84

Table 7.

Frequency of detection of bone marrow involvement in B-NHL by histology and immunohistochemistry

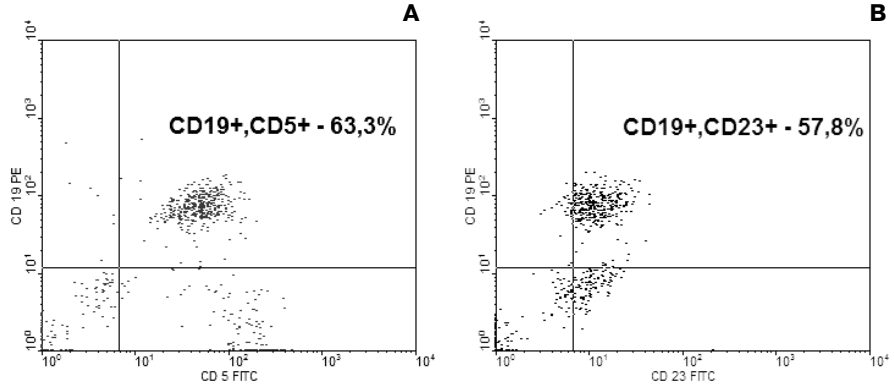
Method	Frequency of involvement	
	No. of bone marrow aspiration biopsies assessed	Percentage of positive tests
Histology	51	94
Immunohistochemistry	40	62.5

Each clinical entity of B-peripheral NHL specified in the WHO system, last revision (2001) identifies a degree of block of normal B-lineage differentiation leading to immortalization of a specific stage together with all molecular biological features of lymphoid cells of the given «age» (N. Harris, E.S. Jaffe, J. Diebold et al., WHO, 2001). In other words, each peripheral B-NHL subtype in parallel with a common dissemination vector has peculiarities of its own including the risk of leukemization at early disease stage and the mode of extramedullary advance.

In an attempt to optimize the diagnosis of BM involvement in B-NHL we believed it necessary to develop personified diagnostic algorithms for every clinical entity basing on selection of an optimal combination of immunomorphologic tests on two principal BM sample types, i.e. aspiration and trephine biopsies.

B-CLL/SLL

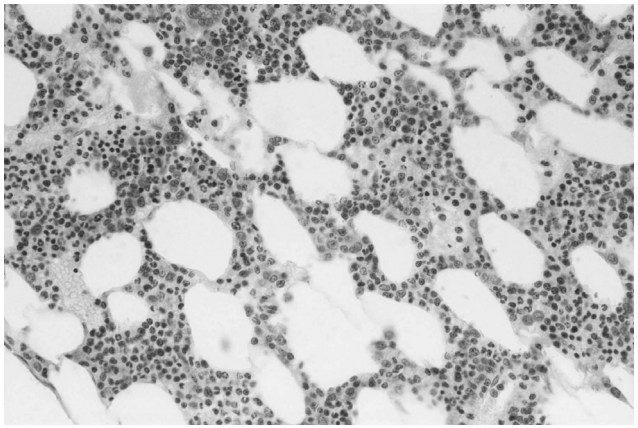
All 6 cases with B-CLL/SLL had a marked pathologic lymphocytosis by BM aspiration biopsy. The specific appearance of tumor cells in association with a known immunomorphology of the extramedullary lesion suggested BM involvement with high probability already at the level of standard cytology, notwithstanding low levels of neoplastic lymphocytes (25.8%). Immunophenotyping by FC confirmed the disease specific character. Detection of diagnostic co-expression of CD5 and CD23 on surface of $CD19^+$ lymphocytes and quantification of CD20 expression (pic. 1) (N. Harris, E.S. Jaffe, J. Diebold et al.,WHO, 2001) were the most important findings. Morphologic study of trephine biopsies in 2 of 6 cases did not add any diagnostic information: the classical appearance of neoplastic lymphocytes and typical dispersed intramedullary dissemination between normal hemopoiesis elements (interstitial type) confirmed the results of the complex aspiration biopsy study (pic. 2).



Pic. 1. CD5 and CD23 coexpression on B-lymphocytes ($CD19^+$)

None of the cases required IHC of trephine biopsy. Besides, difficulty of using diagnostic antibodies CD5 and CD23 in paraffin-embedded tissue and impossibility to assess their coexpression in the lymphoid component rendered FC the method of absolute priority in cases under study.

The question arises in these circumstances, whether BM trephine biopsy is at all needed in primary diagnosis of B-CLL/SLL, if BM aspiration biopsy demonstrates a marked



Pic. 2. Morphology of bone marrow trephine biopsy in B-CLL/SLL. Interstitial lymphoma growth, Hematoxylin-eosin ×250. Patient B., 66 years old

pathologic lymphocytosis (B. J. Bain, 2001). Our findings are evidence against morphologic (histologic) study of BM in classical B-CLL/SLL cases provided complex immunomorphologic study of aspiration biopsy by FC is conducted.

Mantle-cell lymphoma (MCL)

This entity, like B-CLL/SLL, belongs to lymphomas with a high-risk leukemization at early-stage disease. Some foreign authors report of a higher than 90% rate of BM involvement at diagnosis (P. L. Cohen, P.J. Kurtin, K.A. Donovan et al., 1998). ALL 6 MCL cases in our study presented with a high level of leukemic substrate with at least 50% of pathological lymphocytes in each myelogram.

The marked pathologic lymphocytosis in BM aspiration biopsies rendered FC useful in characterization of disease immunophenotype in all cases. Quantification of CD20 expression on lymphoma cells by FC together with identification of T-cell antigen, CD5, on clonal B-lymphocytes were important investigation findings (pic. 3). Expression levels of pan-B-cell marker, CD20, are a good diagnostic bench-mark to further differentiate MCL from B-CLL/SLL and a useful alternative to identification of FMC7 expression (W. Hubl, J. Iturraspe and R.C. Braylan 1998).

As found by trephine biopsy study, lymphoma demonstrated a tendency to settle in intertrabecular hemopoietic tissue and to generate foci with a confluent tendency, the character of lymphoma intramedullary dissemination was similar in all 6 cases (table 8).

We strongly recommend performing standard morphology study of trephine biopsy in all cases because MCL frequently transforms into a more aggressive blastoid type. This phenomenon cannot be detected by FC because it is not accompanied by changes in profile of immunological marker expression and noticeable transformations are seen in cell morphological appearance only (C. H. Dunphy, S. E. Wheaton, S. L. Perkins, 1997). We present detailed description of two such cases (pic. 4).

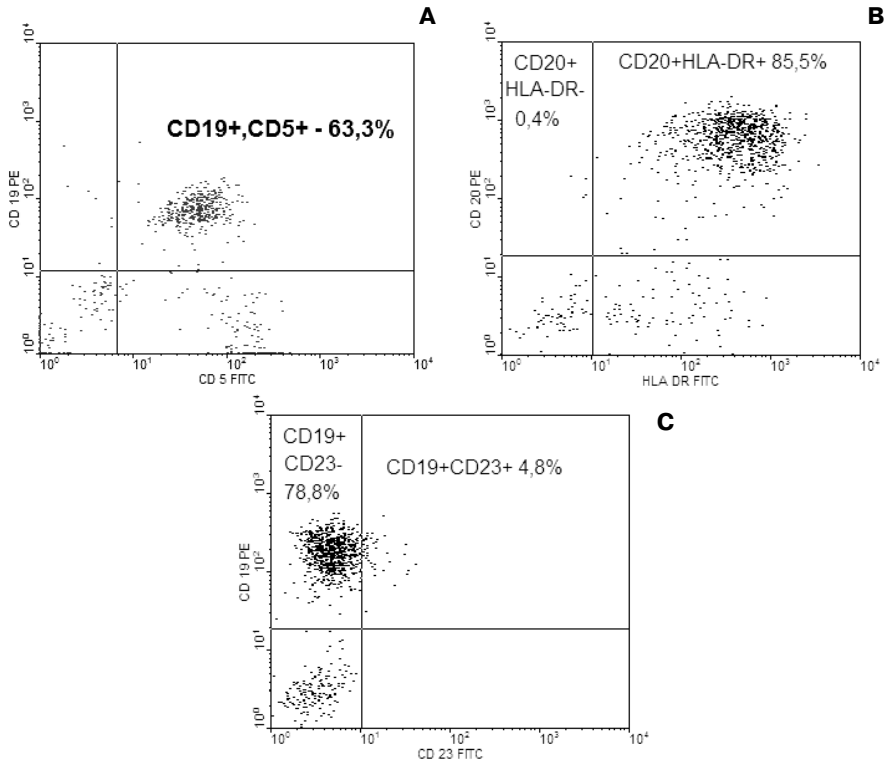
Thus, FC may be considered the optimal immunophenotyping test in MCL; however, unlike in B-CLL/SLL, even a comprehensive immunomorphologic study of aspiration biopsy is not sufficient and should be supplemented with standard morphologic study of trephine biopsy to rule out transformation into blastoid type.

Table 8.

Levels of relative lymphocytosis in bone marrow aspiration biopsies with respect to histological type of involvement by trephine biopsy in mantle cell lymphoma

Patient initial/ gender/age, years	Percentage of lymphoid elements in bone marrow aspiration biopsy/cell type	Lesion histology by bone marrow trephine biopsy/cell type
B./f/61	lymphocytes 72% prolymphocytes* 1.6%	The study was not performed
V./f/62	lymphocytes 68% prolymphocytes* 5%	The study was not performed
K./m/43	lymphocytes 32% prolymphocytes* 15%	Diffuse/lymphocytic
K./m/47	lymphocytes 50.8% prolymphocytes* 4.8%	Focal-diffuse/lymphocytic
E./m/82	prolymphocytes* 53% blasts 4.3%	Diffuse/blastic
M./f/56	lymphocytes 10.8%	Focal-diffuse/lymphocytic

* the term 'prolymphocyte' refers to cells of a larger size as compared to general lymphoma population. These cells were also characterized by an irregular "scalloped" nuclear outline and a finer chromatin structure with a single or several visible nucleoli. In the modern literature including the WHO (2001) classification this term refers to certain cell types encountered in various proportions in B-CLL/SLL and also composing substrate of B-cell prolymphocytic leukemia. MCL is characterized by pleomorphic cell substrate except for rather monomorphic blastoid type.



Pic. 3. CD5 expression on B-lymphocytes (CD23$^-$). Bright CD20 expression. Patient E., 82 years old

Marginal zone lymphoma (MZL) of the spleen

Our findings are in a complete agreement with reports of foreign authors that this peripheral B-NHL type demonstrates high-degree homing-effect to bone marrow (J. Dierlamm, S. Pittaluga, I. Wlodarska et al., 1996). Marked pathological lymphocytosis was found in 5 of 6 aspiration biopsies at primary diagnosis (table 9). Since lymphoma

extramedullary lesions were inaccessible, all diagnostic procedures required by the WHO (2001) classification were restricted to BM lesion. Careful case histories were very useful in these cases: evidence of splenomegaly with echo-changes in all cases and the presence of 'villous' lymphocytes helped to define lymphoma subtype.

Table 9.

Levels of relative lymphocytosis in bone marrow aspiration biopsies with respect to lesion histology by trephine biopsy in marginal zone lymphoma

Patient initial/ gender/age, years	Percentage of lymphoid elements in bone marrow aspiration biopsy/cell type	Lesion histology by bone marrow trephine biopsy/location (immunomorphology)
A./f/57	lymphocytes 85%	Diffuse/interstitial/intrasinusoidal*
G./m/54	lymphocytes 60%	Nodular/intertrabecular/intrasinusoidal*
L./f/57	lymphocytes 80%	Diffuse/intrasinusoidal*
O./m/58	lymphocytes 55%	Diffuse/interstitial/intrasinusoidal*
R./f/47	lymphocytes 54.6%	Nodular/intertrabecular/intrasinusoidal*
C./f/54	blasts 5.8% lymphocytes 18%	Nodular/intertrabecular

* this lesion type was detectable only by immunohistochemistry
** pathological lymphoid infiltration was not discovered by light microscopy in patient L. Bone marrow involvement could be detected only by immunohistochemistry of trephine biopsy

FC of aspiration biopsies discovered a $CD5^-$ and $CD103^-$ (in cases with villous lymphocytes) B-immunosubtype.

Thus, FC ruled out other peripheral B-NHL subtypes such as B-CLL/SLL, MCL and hairy-cell leukemia though failed to differentiate the disease from FL ($CD10^-$ cases).

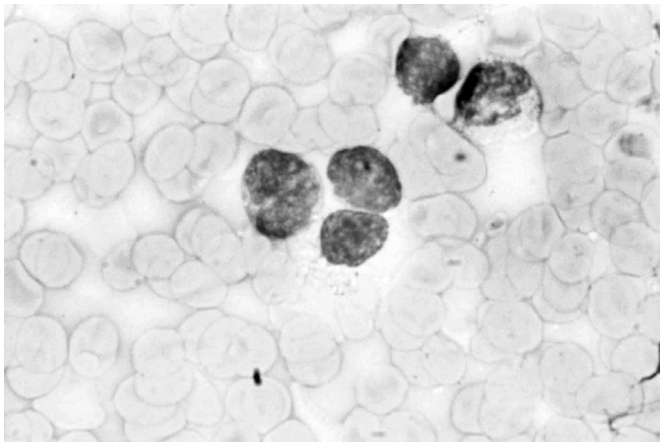
Study of trephine biopsies divided all cases equally into two main histological subtypes, i.e. focal lesions with marked zones and intertrabecular location, and intrasinusoidal lesions with lymphoid elements located inside dilated BM microvessels, both being also found in combination (pic. 5). As already mentioned, pathologic nodules demonstrated, beside a clear-cut outline, marked zonal structure very similar to that of reactive lymphoid follicles.

Thus, there were two indications for IHC of BM trephine biopsy, i.e. to differentiate from FL and to rule out reactive disease. By IHC the foci had monomorphous B-cell composition therefore excluding reactive disease. The presence of B-cell conglomerations ($CD20^+$, $CD79\alpha^+$) in sinusoids helped both to identify pathognomic type of infiltration and to detect BM lesion not found by light microscopy (pic. 6, see cover, page 2).

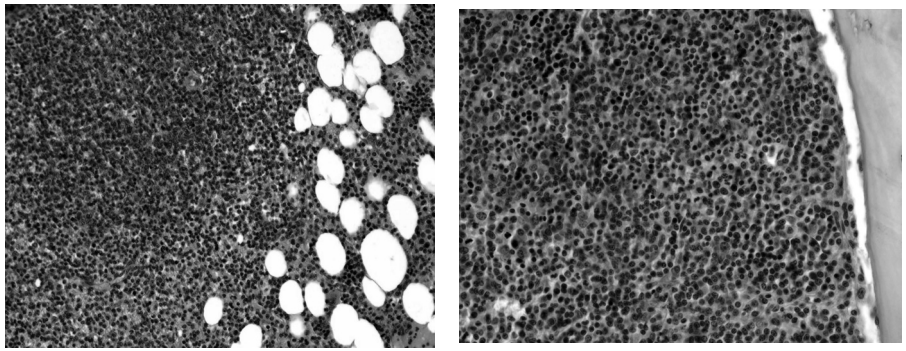
Of much help was the detection of follicular dendritic cells in nodular central portions — an MZL characteristic sign (pic. 7, see cover, page 2).

The absence of paratrabecular lesion or typical centrocytoid-centroblastic morphology in combination with a trend to formation of lymphoid sludge in microvessels helped to differentiate the disease from FL (R. Henrique, R. Achten, B. Maes et al., 1999).

We may therefore conclude that even immunologically and cytologically "colorless" lymphoma such as MZL of the spleen demonstrates specific immunomorphologic features indicative of BM involvement, and IHC of BM trephine biopsy (if splenectomy is impossible) is a determinant diagnostic test (V. Costes, E. Duchayne, J.Taib et al., 2002). This



Pic. 4. Substrate of discordant MCL. Aspiration biopsy blast component. Romanovsky-Giemsa staining, ×1000. Patient E., 82 years old



Pic. 5. Morphologic profile of bone marrow trephine biopsy in marginal zone lymphoma of the spleen. Right: diffuse intrasinusoidal disease. Hematoxylin-eosin staining ×400. Patient O., 58 years old. Left: focal intertrabecular disease. Hematoxylin-eosin staining, ×200. Patient C., 54 years old.

test is beyond comparison in differential diagnosis from FL ($CD10^-$) and reactive disease, as well as in accurate staging (discovery of intrasinusoidal infiltration undetectable by light microscopy).

Follicular lymphoma

Authors report a 40-60% frequency of FL leukemization. BM involvement was present in 4 of 7 cases in our study. As known, FL leukemization manifests itself by rare presence of pathologic lymphocytosis in BM aspiration biopsy and specific intramedullary tumor growth in close contact with trabecules (paratrabecular type). Only 3 BM aspiration biopsies from FL patients in our study had seemingly neoplastic cells, the cells being less than 5% of the total myelokaryocyte count. This minimal concentration of cells with tumor-like morphology (centrocytoid type) did not allowed FC to be used in immunophenotyping. In the third case (27.4% of lymphoid cells) FC discovered preponderance of T-cell population with no clone markers on B-cells, i.e. ruled out the presence of lymphoid lesion.

Study of trephine biopsies identified a small-cell lymphoid infiltration in all cases (table 10). Cytological peculiarities of lymphoid cells, e.g. small size in combination with dense chromatin structure in angular nuclei, were a strong evidence in favor of FL, the pathognomic paratrabecular disease location was found in 4 of 7 cases including 2 cases with lymphoid clusters also located between trabecules (pic. 8).

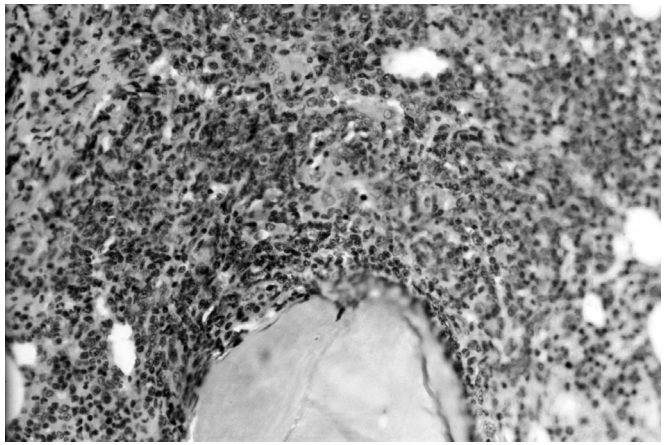
There was no paratrabecular lymphoid foci (all were seen between trabecules) in 3 cases.

The diagnosis of FL in all cases (except one) was based on primary immunomorphologic study of extramedullary substrate. As seen in table 10, information about the baseline lymphoma immunomorphologic type in the case with small-cell centrocytoid BM infiltration made the morphologist define the case as BM invasion of small-cell lymphoma, supposedly FL.

Table 10.

Immunohistochemistry findings in trephine biopsies from patients with follicular lymphoma

Patient initial/ gender/age, years	Lesion histology type/location	Histological diagnosis	Cell composition	Immunological diagnosis
V./f/57	Focal/ intertrabecular	Small cell lymphoma	T- and B-cells B without atypia; 2:1 ratio	Reactive disease
M./f/58	Focal/inter- and paratrabecular	Small cell lymphoma	B- monomorphous; centrocytoid-centroblastic	Follicular lymphoma
S./f/71	Focal/ paratrabecular	Follicular lymphoma (?)	T- and B-cells B without atypia; 2:1 ratio	Limit of method: Reactive disease (?) Minimal disease (?)
T./f/47	Focal/inter- and paratrabecular	Small cell lymphoma	T- monomorphous; isolated B without atypia	Reactive disease
H./f/58	Focal/ intertrabecular	Small cell lymphoma	B- monomorphous; centrocytoid-centroblastic	Follicular lymphoma
G./m/	Focal/inter- and paratrabecular	Small cell lymphoma	B- monomorphous; centrocytoid-centroblastic	Follicular lymphoma
B./m/52	Focal/ intertrabecular	Small cell lymphoma	B- monomorphous; centrocytoid-centroblastic	Follicular lymphoma



Pic. 8. Morphological profile of bone marrow trephine biopsy in follicular lymphoma. Paratrabecular growth. Hematoxylin-eosin staining, ×400. Patient M., 58 years old

The primary purpose of IHC was to differentiate the case from reactive disease. This problem can be solved immunologically by ratio of T and B lymphoid cells which normally is shifted towards a greater T-component. We used the following, rather simple practical recommendations for peripheral small-cell B-NHL developed by other investigators as a diagnostic basis:

A considerable rise in B-lymphocyte ($CD20^+$, $CD79\alpha^+$) count in BM with a trend to clustering and changing appearance is a clear sign of B-NHL;

Monomorphous T-cell composition of lymphoid infiltration in the patient with extramedullary B-NHL is a reactive disease;

Composition of lymphoid foci (the presence of T- and B-cells) with mandatory considerable preponderance of T-cells ($CD3^+$, $CD45RO^+$) is evidence of a reactive (benign) rather than malignant disease.

In one case of FL the foci were monomorphous and were composed of T-cells; the disease was therefore considered reactive. In another two cases lymphoid clusters including those located paratrabecularly consisted of B- and T-cells with prevalence of the latter. B-lymphocytes (CD20+, CD79 +) were free from cytological centrocytoid features, were small and had round nuclei. Immunomorphologic study discovered no evidence of typical lymphoma and the disease was defined as reactive. Evaluation of B-cell clonality (IgVh genes) in such cases can provide more objective information. In the remaining 4 cases the lymphoid component consisted mainly (90%) of B-cells with centrocytoid morphology with some B-cells having centroblastic morphology, therefore these cases were defined as FL.

So, the morphologic diagnosis of BM involvement in FL was not confirmed immunomorphologically in 43% of cases, and immunomorphologic study played the principal role to rule out BM involvement and to establish correct disease stage. Summarizing our findings concerning the diagnosis of BM involvement in FL, it should be emphasized that immunomorphologic study of both aspiration and trephine biopsies (the latter being of more value) is an optimal combination of diagnostic investigations in all clinical entities. Lymphoma leukemization cannot be identified basing on standard morphologic study of trephine biopsy alone even, if the typical centrocytoid appearance and/or paratrabecular location of lymphoid cells are found. IHC of trephine biopsy is a mandatory requirement even in cases with negative immunological test of BM aspiration biopsy by FC. Although the latter is recommended in FL with minimal percentage of lymphoid elements of suspicious morphology or with a relative increase in mature lymphocytes more than 15% of all myelokaryocytes.

Marginal zone lymphoma Mucosa-associated lymphoid tissue type (MALT-lymphoma)

This entity is worldwide attributed to peripheral B-cell NHL with low homing effect to BM. (C. Montalban, J.M. Castrillo, V. Abraira et al., 1995; J. Dierlamm, S. Pittaluga, I. Wlodarska et al., 1996). The few cases in our study may be considered an indirect evidence in favor of this opinion. All these cases presented with a classical lymphoma site (stomach) and a proved association with Helicobacter pylori.

BM lymphoid infiltration was discovered only by trephine biopsy with no qualitative or quantitative changes in myelogram. Leukemization of small-cell lymphoma was an interim morphologic diagnosis in all cases as based on immunomorphology of the primary substrate, thus making the study of BM involvement the determinant phase of disease staging (table 11).

The discovery of BM involvement would have defined stage IV disease which in turn would have required a significant intensification of treatment with systemic polychemotherapy instead of Helicobacter pylori eradication therapy (S. N. Malek, 2003).

Table 11.

Immunohistochemistry findings in trephine biopsies from patients with MALT-lymphoma

Patient initial/ gender/age, years	Lesion histology type/ location	Histological diagnosis	Cell composition by immunohistochemistry	Immunological diagnosis
G./m/36	Focal/inter- and paratrabecular	Small cell lymphoma Follicular lymphoma (?)	T-monomorphous; isolated B without atypia	Reactive disease
I./f/31	Focal/ intertrabecular	Small cell lymphoma (?)	T-monomorphous; isolated B without atypia	Reactive disease
T./m/52	Focal/not determined*	Small cell lymphoma	T- and B-cells B without atypia; 2:1 ratio	Limit of method: Reactive disease (?) Minimal disease (?)

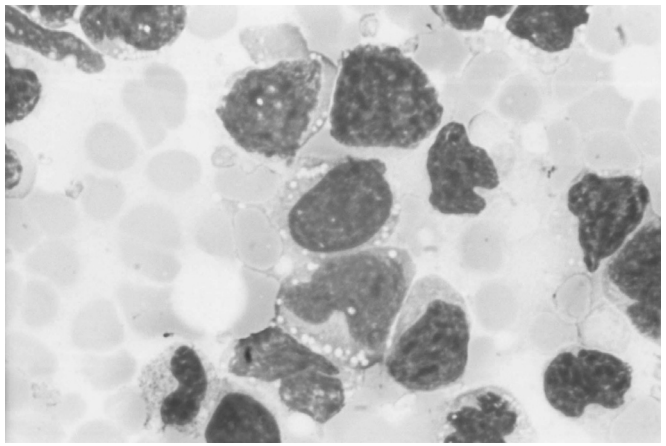
* disease location could not be determined due to material deformation during harvesting

As seen in table 11, the lesions consisted of both T- and B-cells in 2 cases and of T-cells only in the third case. This meant that basing on the diagnostic algorithm mentioned above there was no strong evidence in favor BM involvement (pic. 9, see cover, page 2).

The conclusion may be therefore made that discovery of any lymphoid infiltration by BM trephine biopsy in patients with MALT-type lymphoma is an absolute indication for IHC to determine disease stage and to choose treatment strategy.

Diffuse large B-cell lymphoma (DLBCL)

Of much interest was that patients with the clinical entity having a risk of leukemization less than 30% by many authors were a majority in our study (K. Foucar ,W. Robert, 1982; M. G. Conlan, M. Bast, J.O. Armitage et al., 1990). We should like to emphasize that it is this peripheral B-NHL type that has given most interesting findings. All patients with this disease type divided into two groups with respect to verification of BM lesion, i.e. those with or without indications for IHC of BM trephine biopsy. The group without indications of IHC consisted of 6 (30%) of 20 patients. In these cases DLBCL substrate was found in both trephine and aspiration biopsies (pic. 10).

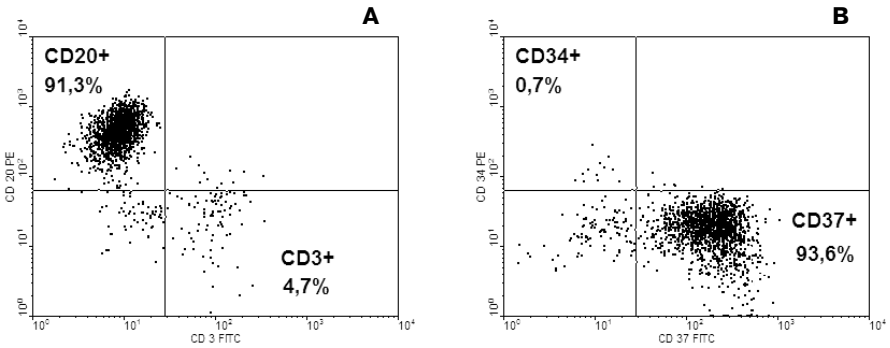


Pic. 10. DLBCL substrate. Aspiration biopsy. Romanovsky-Giemsa staining, ×1000.

Comparison of the findings demonstrated that in parallel with a rather low (not more than 20%) level of pathologic blasts in aspiration biopsies, trephine biopsies showed subtotal neoplastic replacement of normal hemopoietic tissue. Basing on our own findings and reports in the literature this phenomenon could be explained by biological characteristics of lymphoma that demonstrated multifocal, sarcoma-like growth rather than interstitial, cellular dissemination (B. J. Bain, 2001; C. F. Feller and J. Diebold, 2004). The presence

of intact, normal hemopoiesis islands might proof this hypothesis (pic. 11, see cover, page 2).

Clear pathological BM blastosis discovered in 6 cases allowed the use of FC that proved tumor cells to belong to peripheral stages of B-cell differentiation (CD20+, CD19+, $CD37^+$, $CD34^-$). These immunological characteristics of tumor cells in aspiration biopsies in combination with characteristic histological pattern of trephine biopsies were evidence in favor of DLBCL leukemization (pic. 12).



Pic. 12. Expression of B-lineage differentiation peripheral stage molecules CD20 and CD37 on tumor blasts with no expression of precursor cell marker CD34. Patient V., 71 years old

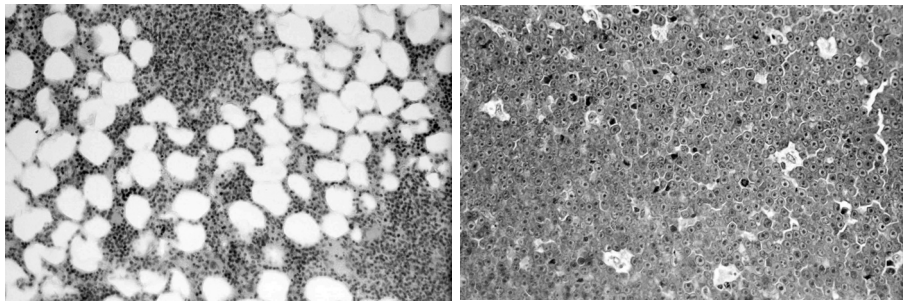
IHC of trephine biopsy made in some cases failed to provide additional information to FC findings or was even less informative due to a narrower antibody panel for fixed material.

In the remaining 14 DLBCL cases BM infiltration suspected for BM involvement was found in trephine biopsies only, cytological characteristics of lymphoid cells in 10 cases demonstrated histologic discordance with extramedullary substrate (pic. 13).

BM lymphoid substrate had blast morphology in 2 cases. It was IHC of trephine biopsies that allowed the diagnosis in accordance with the WHO classification, because biopsy of extramedullary component was not possible by technical reasons in 1 case and the second one was a rare example of BM/bone lymphoma location. Immunologic discovery of blasts belonging to peripheral stages of B-lineage differentiation ($CD20^+$, $CD3^-$) in combination with a characteristic centroblastic/immunoblastic cell appearance and local diffuse (multifocal) growth were a basis for the diagnosis of DLBCL leukemization in both cases.

We consider analysis of 10 cases with small-cell and mixed lymphoid infiltration a key event in the whole study.

There are few reports on histologic discordance in large B-cell lymphoma in the literature. There are three main reasons for the discordance defined by foreign colleagues (M. Klemer, M. Spitzer 2003), as follows:



Pic. 13. Histologic discordance of extramedullary and bone marrow substrates of DLBCL. Right: lymph node. Hematoxylin-eosin staining, ×200. Patient K., 49 years old. Left: BM small lymphoid cell nodular lesions

1. Transformation into a more aggressive type (large-cell NHL) most typical of B-NHL (Richter's syndrome) and manifestation of neoplastic progression.
2. Reactive disease. In opposite to extramedullary large B-cell lymphoma small-cell infiltrations in BM are represented by T cells with different proportions of CD4+/CD8+ population.
3. True biclonality. Simultaneous existence of two lymphoproliferative diseases with different immunomorphologic and molecular biologic clonal features.

IHC of trephine biopsies in 9 DLBCL cases was determinant for the diagnosis (table 12). In all cases with lymphocytic lesion the small-cell lymphoid component consisted of T-cells only ($CD3^+$, $CD45RO^+$).

Cases with mixed (centrocytoid/centroblast-like) composition of the lesion looked as FL by standard microscopic staining, their paratrabecular location discovered in two cases added to the similarity. IHC demonstrated that all small-cell components expressed T-cell markers, while B-cell antigens (CD20, CD79α) were seen only on large blasts with marked nuclear pleomorphism and high mitotic activity. The discrete location of the latter between dense T-components resembled the T-cell rich DLBCL type, though in fact was nothing else but a unique morphologic reflection of T-cell immunity response to starting DLBCL invasion into BM (pic. 14, see cover, page 4).

Of importance is the fact that all cases (except one) with primary extranodal DLBCL location were included in the group of histologic discordance or with T-cell reaction phenomenon as defined by IHC. Most cases with primary nodal location of extramedullary DLBCL substrate typically demonstrated minimal levels of T-cells in BM and rapid intramedullary growth and appearance of pathological blasts in aspiration biopsies. No doubt that our findings are just a prologue for further profound study of mechanisms of antitumor activity or tolerance of the immune system.

So, speaking of the principal purpose of our study, optimal combination of immunologic tests to determine nature of lymphoid infiltration in DLBCL may be established only after comprehensive morphologic study of both aspiration and trephine biopsies of BM.

Table 12.

Comparison of morphology and immunohistochemistry of bone marrow trephine biopsies in DLBCL patients

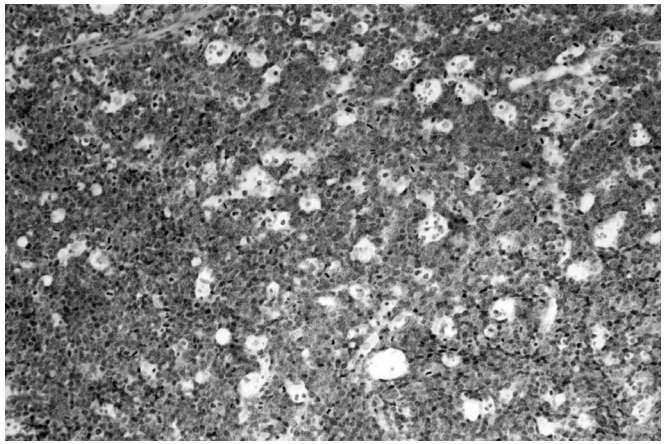
Patient initial/ gender/age, years	Lesion histology type/location	Cell composition	Histological diagnosis	Cell composition by immunohistochemistry	Immunological diagnosis
B./m/69	Focal/ intertrabecular	Lymphocytic	Small cell lymphoma	T-monomorphous; isolated B without atypia	Reactive disease
B./m/ unknown	Focal/ intertrabecular	Lymphocytic	Small cell lymphoma (?)	T-monomorphous; isolated B without atypia	Reactive disease
K./f/49	Focal/ intertrabecular	Lymphocytic	Discordance/ Small cell lymphoma (?) Reactive disease (?)	T-monomorphous; isolated B without atypia	Reactive disease
P./f/51	Focal/ intertrabecular	Lymphocytic	Discordance/ Small cell lymphoma (?) Reactive disease (?)	T-monomorphous; isolated B without atypia	Reactive disease
R./f/ unknown	Focal/ intertrabecular	Lymphocytic	Small cell lymphoma	T-monomorphous; isolated B without atypia	Reactive disease
S./m/56	Focal/ intertrabecular	Lymphocytic	Discordance/ Small cell lymphoma (?) Reactive disease (?)	T-monomorphous; isolated B without atypia	Reactive disease
B./m/43	Focal/ inter- and paratrabecular	Mixed	Discordance/ Small cell lymphoma (?) Reactive disease (?)	T prevail, B are large, with atypia, nuclear pleomorphism, discrete	B-large cell
K./m/60	Focal/ intertrabecular	Mixed	Discordance/ Small cell lymphoma (?) Reactive disease (?)	T prevail, B are large with atypia and nuclear pleomorphism, discrete	B-large cell
S./f/53	Focal/ inter- and paratrabecular	Mixed	Discordance/ Small cell lymphoma (?) Reactive disease (?)	T prevail, B are large with atypia and nuclear pleomorphism, discrete	B-large cell

In case of clear-cut blastosis in BM aspiration biopsy, FC may be recommended, which should be obligatory supplemented with standard study of trephine biopsy, while discovery of characteristics histologic pattern of DLBCL makes IHC unnecessary. Discovery of a lymphoid infiltration that differs by morphology from the principal extramedullary DLBCL substrate is an absolute indication for IHC of BM trephine biopsy.

Burkitt's lymphoma

BM involvement was found in 50% of cases in this study with leukemization starting at early-stage disease in 2 of 4 cases.

The BM component both in aspiration and trephine biopsies was characterized by typical features for this entity such as L3 morphology of blast component in aspiration biopsy and characteristic 'starry sky' morphology in trephine biopsy (pic. 15) (B. J. Bain, 2001; C. F. Feller and J. Diebold, 2004).

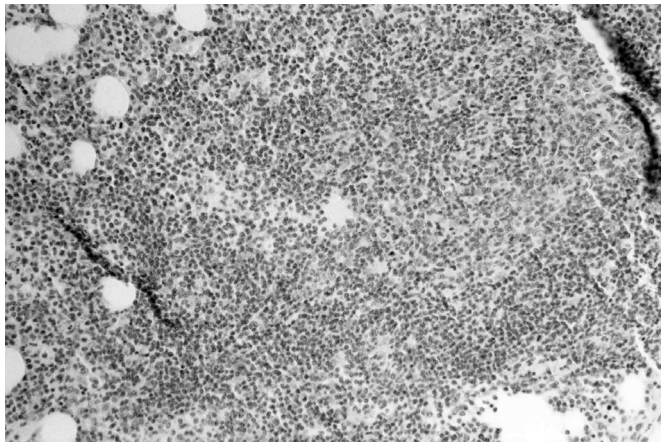


Pic. 15. Morphologic pattern 'starry sky' of Burkitt's lymphoma in bone marrow trephine biopsy. Hematoxylin-eosin staining, ×250. Patient B., 16 years old

Comprehensive study of aspiration biopsies by FC discovered classical Burkitt's lymphoma immunophenotype, i.e. expression of molecules of B-cell peripheral-stage differentiation in combination with CD10 expression (it is understood that proliferative activity, i.e. Ki-67 level, and the presence of translocation t(8;14) would have been assessed in the absence of L3 morphology). Morphologic study of trephine biopsy was of secondary value and did not require verification by morphologic immunophenotyping of extramedullary component. There were no changes in BM aspiration biopsies, while morphological study of trephine biopsies discovered small-cell focal lymphoid infiltration with formation of a single nodal structure having clear-cut outline and zones in one case, and multiple interstitial intertrabecular clusters without clear-cut contours in the other (pic. 16).

Discordance of lymphoid infiltrations was indication for IHC of trephine biopsies. The lesions had a mixed composition and consisted of T- and B-cells with a considerable T-lineage prevalence in both cases. By morphology, B-cells looked like small lymphocytes and were absolutely different from Burkitt's lymphoma substrate. So, BM involvement was ruled out in both cases (pic. 17, see cover, page 4).

Summarizing the above-said, leukemization manifests itself as rapid intramedullary disease dissemination due to high proliferation potential of Burkitt's lymphoma cells and generation of leukemia-like pattern of BM aspiration biopsy. Blast cytologic specificity (L3) in



Pic. 16. Morphologic pattern of small-cell lymphoid infiltration in bone marrow trephine biopsy from a Burkitt's lymphoma patient. Hematoxylin-eosin staining, ×200. Patient K., 28 years old

combination with characteristic immunologic profile including expression of B-cell peripheral-stage differentiation markers make comprehensive study of aspiration biopsies from lymphoma patients sufficient both for detection of BM involvement and direct lymphoma classification by the WHO (2001) system. In view of the above-said the question arises whether it is reasonable to perform IHC study of BM trephine biopsy or even standard morphologic study of BM after leukemization of Burkitt's lymphoma is established (B. J. Bain, 2001).

The situation is quite opposite for cases with histologic discordance: only comprehensive immunologic study of both aspiration and trephine biopsies of BM can discover true nature of BM lymphoid infiltration atypical for Burkitt's lymphoma, pic. 16

Nonstandard diagnostic situations

Nonstandard diagnostic situations include rare examples of lymphoma leukemization with BM involvement considered in the world literature as anecdotal or occurring at a less than 5% rate. It is very important to describe in detail characteristic features of their leukemization to be further used as a model for diagnostic algorithms.

This type of peripheral B-NHL was in our study represented by cases of nodal MZL and primary B-cell mediastinal lymphoma with BM involvement.

In conclusion, IHC of BM in peripheral non-Hodgkin's lymphoma is a useful diagnostic test to establish lymphoma subtype and stage. IHC can give maximal information only if data of comprehensive study of all tumor substrates were available in every individual clinical case.

Conclusions

1. Immunophenotyping of BM lymphoid cells (FC, IHC) is a method of choice in the diagnosis and staging of non-Hodgkin's lymphoma by BM involvement, because there are no pathognomic cytologic or histologic signs of neoplastic BM involvement for most lymphoma types specified by the WHO (2001) classification.
2. B-cell chronic lymphatic leukemia or small-lymphocytic lymphoma demonstrate characteristic interstitial BM invasion by neoplastic B-lymphocytes coexpressing CD5 and CD23 antigens. The diagnosis (or BM involvement) is established by immunocytometry of BM aspiration biopsy while IHC of trephine biopsy is not reasonable.
3. Generalization of mantle-cell lymphoma is accompanied by focal (rarer focal-diffuse) intertrabecular (rarer together with paratrabecular) infiltration of BM. Diagnostic immunophenotype of neoplastic B-lymphocytes ($CD5^+$ $CD23^-$) is established by BM puncture, and IHC is not needed. Morphologic study of BM trephine biopsy is indicated to detect transformation into a blastoid type.
4. BM involvement in FL is focal, with paratrabecular, intertrabecular or mixed location. Comprehensive immunomorphologic study of BM trephine and aspiration biopsies is mandatory in all cases to differentiate the lesion from marginal zone lymphoma of the spleen and reactive disease.
5. Marginal zone lymphoma of the spleen is characterized by intrasinusoidal, follicle-like and diffuse interstitial BM lesion. Intrasinusoidal and follicle-like intertrabecular growth of tumor cells in BM is specific of the lymphoma type in question and established by IHC only basing on confirmation of B-cell nature of tumor elements and detection of CD21+CD23+ follicular dendritic cells.
6. BM involvement in diffuse large B-cell lymphoma is to a high degree associated with tumor primary site: diffuse localized blast growth is characteristic mainly of primary nodal tumors, while morphologic discordance with generation of T-cell foci in BM is more specific of extranodal primary disease. IHC of trephine biopsy is needed in the absence of lymphoma cells (blasts) in BM aspiration biopsy or if morphologic evidence of discordance is present.
7. Burkitt's lymphoma is characterized by high frequency of BM involvement of acute leukemia type (cytological L3 type). BM trephine biopsy sections present with isolated macrophages among diffusely growing blasts (a 'starry sky' pattern similar to the primary tumor). Peripheral immunophenotype of B-blast with high proliferative activity is established by BM puncture biopsy. IHC of trephine biopsy is needed, if atypical lymphoid infiltration is discovered.
8. BM from patients with MALT-lymphoma of the stomach diagnosed immunomorphologically may have small-cell lymphoid infiltration (intertrabecular or paratrabecular) looking like lymphoma lesion at the morphology level. These infiltrations are mainly composed of T-cells (reactive type) in most cases as discovered by IHC of BM trephine biopsy.
9. In cases with plasmacytic or lymphoplasmacytic tumors BM may be the only extranodal site of lymphoma accessible for histological study. Immunologic verification of the diagnosis is based on establishing monoclonality (κ/λ) with confirmation of plasmacytic differentiation (CD138).

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FROM THEORY TO PRACTICE: MUC-1 IS A NEW TARGET FOR CANCER THERAPY

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Abstract

We studied MUC-1 expression using monoclonal antibodies ICO-25 and LU-BCRU-G7 on breast cancer cells and assessed anti-MUC-1 monoclonal antibody potential in the treatment of cancer. IHC discovered ICO-25 epitope on breast cancer cells in 94% of all cases (including 56.3% of monomorphic and 37.7% of mosaic reactivity cases). MUC-1 expression correlated with CEA ($p<0.05$) and did not correlate with levels of lymphocyte or macrophage infiltration, or breast cancer stage. Overall and disease-free survival rates were decreasing with increase in the antigen expression. ICO-25 detected lymph node involvement 9.7% more frequently than conventional histologic tests. Antigen Galβ1-3GlcNAc (Le^c — a MUC-1 determinant, LU-BCRU-G7 monoclonal antibody) was studied in early breast cancer. The antigen was present in 56.8% of cases, was significantly correlated with CD71 and tumor size grater than 3 cm. Le^c expression was associated with poorer survival of patients with early breast cancer (the difference was significant for grade III tumors). LU-BCRU-G7+ breast cancer tended to metastasize to lungs. Galβ1-3GlcNAc-binding B-lymphocytes were found in peripheral blood from 85% (18/21) of breast cancer patients and 75% (6/8) of normal donors. An ICO-25-derived drug Imuteran was studied in phase I trial in 10 patients. Single and total dose escalation was performed by a modified Fibonacci scale. Allergic reactions were the main adverse events. A regimen of Imuteran administration at 35 mg (700% of the starting dose) by i.v. drip once weekly was recommended for stage II clinical study. By now 20 enrolled patients have received 26 treatment cycles, response was assessed in 16 cases. There were no complete or partial responses to treatment. Disease stabilization for 11.8+4.3 weeks (median 10 weeks) was detected in 37.5%, progressive disease was reported in 62.5% of cases. Treatment was discontinued in 15% (3/20) of patients due to toxicity (allergic reactions in 2 and intestinal paresis in 1 cases).

Key words:
MUC-1, immunohistochemistry, monoclonal antibodies, Imuteran, clinical trial.

List of abbreviations
MAB — Monoclonal antibodies
BC — Breast cancer
OS — Overall survival
DFS — Disease-free survival
IHC — Immunohistochemical analysis

There was a true break-through in cancer chemotherapy at the borderline of the second and third millenniums owing to development and clinical use of targeted therapies, i.e. agents with antitumor activity against specific molecules. Now experimental knowledge about a huge number of tumor-associated antigens is of much importance for development of new targeted therapies rather than for diagnostic or prognostic purpose only. New drugs may be derived from monoclonal antibodies (MAB) to various determinants, antisense oligonucleotides, small molecules such as signal transduction inhibitors etc.

Epithelial mucins are a large family of tumor-associated antigens. Mucins are large, extensively glycosilated macromolecules that are expressed and secreted by various epithelium types. By physiological function, they are protection antigens and physiological lubricants with their expression mainly increasing and/or changing in cancer.

There are several mucin families identified so far. MUC-1 (CD227) is of the most clinical significance both as a tumor marker in general and as a breast cancer (BC) marker in particular [19]. This is a complex of glycoproteins that includes a polypeptide body with multiple oligosaccharide side chains containing O-compounds with serin and treonin residues. As the mature MUC-1 molecule is anchored to cell surface by a transmembrane domain, the greater portion of the molecule is expressed extracellularly as an elongated structure with a much greater extension from cell membrane as compared to other macromolecules expressed on cell surface. The huge region of MUC-1 protein body contains a variable number of highly constant repeated sequences of chains or units each consisting of 20 amino acids.

MUC-1 physiological functions are not yet quite clear and are debated, though there is no doubt about its most valuable property as a marker of many human cancer types. MUC-1 expression is increased on malignant cells. The molecule loses its extended orientation with a tendency to polarize on the cell apex. Its glycosilation (with side chains in O-compounds with serin and treonin residues) is changed and impaired due to neoplastic transformation. Incomplete or aberrant glycosilation in neoplastic cells leads to shortening or less marked branching of oligosaccharide chains, accumulation of previous structures and increase in or de novo exposure of protein body regions. MUC-1 loses its surface location and comes into circulation (CA 15,3) as a marker of tumor occurrence and, if detected repeatedly, of disease recurrence, progression or response to treatment.

There are a large number of MAB to different MUC-1 epitopes available by now [10; 14]. Most of them recognize epitopes inside the protein body of the molecule [22; 23], while just a few of them (mainly of recent development) react with epitopes including carbohydrate determinants [20]. Of these MAB, LU-BCRU-G7 to terminal disaccharide Galβ1-3GlcNAc seems most interesting and promising. This MAB has a unique property of being absent on cells of normal breast tissue or epithelium in benign hyperplasia.

Data concerning MUC-1 expression in BC are equivocal because different MAB are targeted at different epitopes. Besides phenotyping of the primary tumor, MAB to MUC-1 are widely used in BC to monitor disease recurrence and response to chemotherapy [18; 21], to detect regional micrometastases [8; 17]. MUC-1 derivatives are reported to induce HLA-restricted cytotoxic T-lymphocyte reaction to BC cell lines leading to lysis of target cells [12]. A number of studies are currently investigating potential use of MUC-1-expressing autologous and allogeneic tumor cells and synthesized polypeptide sequences in vaccination of BC patients [13; 15].

Among the great number of antibodies to different MUC-1 epitopes there is an ICO-25 MAB developed by Russian scientists A. Yu. Baryshnikov and R. I. Yakubovskaya. The determinant ICO-25 is frequently found on cells of epithelial tumors including BC, cancer of the ovary, uterine tube, stomach, colon and other sites. These MAB are widely used in this country in study of BC and some other tumors [4; 6].

Immunohistochemical study (IHC) of BC demonstrated the specific reactivity with ICO-25 being preserved after trypsin and lipase treatment of sections and to disappear after neuroaminidase treatment which was evidence of the carbohydrate nature of the determinant [9]. In the normal non-lactating breast ICO-25 reacted with apical membranes of acinuses and ducts only. In non-proliferative and proliferative mastopathies with grade I-II epithelial dysplasia the antigen was found on apical membranes of epithelial cells. Among multiple layers of proliferating ductal epithelium the reactivity was detected only in cells immediately lining the lumen.

ICO-25 reacted with both the primary tumor and metastatic cells in regional lymph nodes and bone marrow [1; 2]. Frequency of regional lymph node involvement was higher by this test as compared to standard pathologic study by 9.7% for BC and 27.2% for gastric cancer, the presence of morphologically undetectable micrometastases in gastric cancer correlated with disease progression during the follow-up period [8]. In bone marrow ICO-25 can detect one cancer cell per million hemopoietic cells, the presence of tumor cells in sternal puncture biopsy from BC patients correlated with a higher frequency of bone metastases [3].

IHC results provided a rationale for development of an original Russian drug Imuteran on the basis of ICO-25 in a joint project of the N. N. Blokhin CRC RAMS and the P. A. Herzen Moscow Oncology Institute. Experiments on nude mice with xenografts of human colonic carcinoma demonstrated ICO-25 to markedly inhibit tumor growth, by toxicology study Imuteran was associated with low risk of acute or chronic toxicity [5; 7; 16]. These data were a basis for study of Imuteran in a clinical setting.

This paper describes study of MUC-1 expression (determinants ICO-25 and LU-BCRU-G7) on BC cells as well as results of phase I-II clinical trials of Imuteran (as derived from ICO-25) in patients with advanced epithelial cancer.

MUC-1 expression on BC cells

Determinant ICO-25.4

Immunofluorescence reaction (IFR) with ICO-25 to MUC-1 was studied on frozen histological tumor sections from 183 patients with BC (including 138 ductal carcinomas, 15 lobular carcinomas, 15 mixed lubular-ductal carcinomas, 5 medullary carcinomas, 5 tubular carcinomas, 1 papillary carcinoma and 1 cancer with metaplasia). Table 1 presents results of this study.

Table 1.

MUC-1 expression on breast cancer cells

Tumor histology	Reaction type (No. of patients, %)			Total No. of patients
	monomorphous	mosaic	negative	
Ductal carcinoma	76–55.1%	51–37.0%	11–8.0%	138
Lobular carcinoma	8–52.3%	7–46.7%	-	15
Rare types	19–63.3%	11–36.7%	-	30
Whole group	103–56.3%	69–37.7%	11–6.0%	183

The ICO-25 determinant was found on tumor cells from 94.0% (172/183) of patients. The expression was monomorphous (more than 70% of antigen-positive tumor cells) in 56.3% (103/183), mosaic (10 to 70% of antigen-positive tumor cells) in 37.7% (69/183) of cases, and 6.0% (11/183) tumors were antigen-negative.

The antigen was located in a diffuse manner both in cytoplasm and on tumor cell membrane with a very bright fluorescence by IFR. Cell staining intensity in each of the tumors with mosaic antigen distribution varied from fully negative to very intensive, the cells having bright, weak or no staining both as homogeneous areas or (more frequently) near each other. In cases with areas of preserved breast tissue MUC-1 was found on apical areas of ductal epithelium cell membranes (unlike cancer cells, the normal epithelium demonstrated reactivity on cell membranes only).

MUC-1 expression analysis (ICO-25 determinant) in different BC histotypes demonstrated all the 11 antigen-negative cases to be ductal carcinomas. It should also be noted that all the 5 medullary carcinomas had this marker on a majority of cells.

Analysis of MUC-1 expression in relation to other BC cell antigens (CEA, HLA-DR, CD71, CD29) demonstrated MUC-1 to be positively related with CEA (χ^2=14.152; p=0.007): all 100% tumors with monomorphous CEA expression also had monomorphous MUC-1 expression. Monomorphous expression of MUC-1 was encountered more frequently in the CEA-mosaic than the CEA-negative group (68.4% vs 49.3% respectively). The only MUC-1-negative case was CEA-negative too. Statistical analysis showed MUC-1 and CEA being of equal value. Of note, CEA expression and MUC-1 cytoplasmatic location are not characteristic of normal BC epithelium. The presence of CEA-positive and MUC-1-positive phenotypes may be interrelated and may be evidence of malignant transformation with specific anaplasia and dedifferentiation.

MUC-1 expression had no effect on levels of tumor infiltration by various immunocompetent cell populations including CD45 (all lymphocytes); CD7, CD5, CD4, CD8 (T-cells and their populations); CD19, CD37 (B-cells), CD38 (plasmatic cells) and CD163 (macrophages).

MUC-1 expression showed no relationship with disease advance as defined by tumor size (T), presence of positive regional lymph nodes (N) and disease stage.

Study of disease-free survival (DFS) of BC patients with respect to MUC-1 expression on tumor cells demonstrated that DFS was decreasing with increase in the marker expression (the differences were not statistically significant, $p>0.05$) (table 2).

Table 2.

Disease-free survival in breast cancer patients with respect to MUC-1 expression on tumor cells

MUC-1 expression (No. of patients)	Disease-free survival		
	5 years, % (mean±SD)	10 years, % (mean±SD)	median
None (8)	70.0±18.2	—	not reached
Mosaic (59)	63.5±6.4	56.0±7.5	not reached
Monomorphous (82)	51.1±5.8	41.6±6.8	72 months

A similar trend was discovered for overall survival (OS): the 5-year OS rates for cases with antigen-negative, mosaic and monomorphous reactivity were 75.0±21.7%, 74.8±5.9% and 67.1±5.4% respectively ($p>0.05$).

We used ICO-25 to study frozen sections of regional lymph nodes from 72 BC patients. Standard morphology study discovered regional lymph node involvement in 45.8% (33/72) of cases. The use of ICO-25 increased metastasis detection by 9.7% as compared to histologic study to reach 55.6 (40/72) of cases. IHC also discovered metastases in 7 cases with negative histologic tests. Our findings prove reasonable the use of this additional test in node-negative cases.

Terminal disaccharide Galβ1-3GlcNAc (Le^c-a MUC-1 determinant, LU-BCRU-G7)

Reactivity with MAB LU-BCRU-G7 to Le^c was studied on de-embedded tumor sections from 88 early BC (T1-2N0M0) patients. There were 59 ductal, 7 lobular, 13 mixed, 5 medullary carcinomas and 4 cancers of other types. Terminal disaccharide Galβ1-3GlcNAc (Le^c) was found on malignant cells in 56.8% (50/88) of cases including 21.6% (19/88) with monomorphous and 35.2% (31/88) with mosaic reactivity patterns. 43.2% (38/88) of tumors were antigen-negative.

LU-BCRU-G7 expression demonstrated no relationship with tumor histology or grade, though increased with decrease in cancer cell differentiation: the antigen was discovered in 50% (8/16) of well differentiated, 57.1% (12/21) of moderately differentiated and 61.5% (8/13) of poorly differentiated cases ($p>0.05$).

Le^c detection frequency was significantly related with the presence of transferrin receptor CD71 on cancer cells ($p=0.026$) and not related with major histocompatibility class I and II antigens, adhesion molecule CD29, or tumor infiltration by $CD45^+$ leukocytes, $CD7^+$ T-lymphocytes and $CD163^+$ macrophages.

Interestingly, ICO-25 and LU-BCRU-G7 reacted differently with the same tumors. As known, the framework of the intermediate region carbohydrate chains in O-bound glycoproteins including MUC-1 contains a type 1 carbohydrate chain connecting the intermediate and the core regions and consisting exactly of Galβ1-3GlcNAc. This region is normally «covered» by the carbohydrate chain peripheral region. As a result of glycosilation impairment in cancer the Galβ1-3GlcNAc becomes a terminal disaccharide easily accessible for antibodies. Thus, two carbohydrate determinants in the same glycoprotein MUC-1 may demonstrate absolutely different expression on cancer cells.

Since all our patients were node-negative we studied relationship of Galβ1-3GlcNAc (Le^c) with tumor size only. The disaccharide was expressed significantly more frequently on tumors greater than 3 cm as compared to smaller than 3 cm tumors (85% [17/20] vs 48.4% [33/68] respectively, $p=0.004$). OS and DFS rates were somewhat higher in Le^c cases (table 3), the differences being not statistically significant.

Table 3.

Disease-free (DFS) and overall (OS) survival in breast cancer patients with respect to Galβ1-3GlcNAc (Lec) expression on tumor cells

Galβ1-3GlcNAc expression (No. of patients)	DFS		OS	
	5 years, % (mean±SD)	median	5 years, % (mean±SD)	median
No (38)	79.0±6.0	not reached	81.1±6.4	not reached
Yes (50)	65.7±6.8	not reached	79.6±5.8	not reached

In grade III tumors ($n=30$) Le^c expression correlated with significant decrease in survival of early BC patients ($p=0.05$).

Additional analysis of metastasis in cases with disease progression after 6 months following surgery demonstrated that lung was the most common site of involvement in Galβ1-3GlcNAc-positive cases: the antigen expression was found in 87.5% (7/8) of cases with lung metastases versus 53.2% (41/77) of patients free from lung metastasis ($p=0.047$).

B-lymphocyte subpopulation analysis in BC patients: Le^c-specific B-cells

Over the recent years there were increasing data about the role of so called "natural" humoral immunity due to polyreactive pentameric IgM produced by $CD5^+$ B-lymphocytes in the antitumor defense in BC [11]. We studied B-lymphocyte subpopulations in blood from 21 BC patients and 8 healthy donors involving assessment of B-lymphocyte ability to specifically bind to disaccharide Galβ1-3GlcNAc (Le^c) with fluorescent label in fluorochromic probe Le^c-PAA-flu (further referred to as Le^c). A fluorochrome-labeled lactosamine, a disaccharide stucturally similar to Lec (Galβ1-3GlcNAc), was used as control against Le^c. Samples with fluorescent labels were kindly supplied by Professor N. V. Bovin (Institute of Biological Chemistry RAS, Moscow).

Le^c-binding B-cells were found in 85% (18/21) of BC patients and 75% (6/8) of donors. Average levels of Le^c-binding B-lymphocytes were 6.0% (0.73 to 32.0%) and 5.4% (1.5 to

16.0%) respectively, the differences being not significant. B-cells with marked Le^c-binding and intensive fluorescence were seen in 78% (14/18) of positive BC cases (0.6 to 3.9%, median 0.9%).

We also analyzed Le^c binding with $CD5^+$ B-lymphocytes that play an important role in antibody response to carbohydrate antigens and produce so called natural IgM antibodies. $Le^{c+}CD5^+$ B-cells were assessed in 12 of 21 patients. In all cases receptors to Le^{c+} were expressed on both $CD5^+$ and $CD5^-$ B-lymphocytes. $CD5^+$ B-cells were about 50% of B-lymphocytes with high affinity to Le^{c+}. We are currently studying Le^c-specific antibodies in sera from BC patients.

Results of phase I-II clinical trials of a new Russian drug Imuteran (MAB ICO-25)

The use of biological agents or biotherapies is a new promising approach in cancer therapy. Application of MAB to various tumor-associated antigens is the most important field. These antibodies alone or in immunoconjugates, immunoliposomes etc. can directly attack tumor cells and destroy them through immunity effector cells.

As mentioned above, high specificity of ICO-25, MUC-1 low expression in normal tissues and high concentration in cancer cells as well as in vivo antitumor activity and low toxicity were a rationale for development of a drug Imuteran.

Pharmaceutical formulation: sterile solution of murine monoclonal antibody ICO-25 at 5 mg/ml; 5 ml (25 mg) vials.

Results of phase I clinical trial of Imuteran

Phase I clinical trial of Imuteran as an anticancer therapy was performed at the N. N. Blokhin CRC RAMS on approval of the RF HM Pharmacology Committee.

Ten patients with advanced cancer were enrolled. The diagnosis was verified morphologically and all conventional specific treatments proved ineffective in all cases. Table 4 shows distribution of the patients by disease clinical entities.

Table 4.

Patient distribution by clinical entities (phase I)

Diagnosis	No. of patients
Breast cancer	6
Metastases from unknown primary site	2
Ovarian cancer	1
Cancer of the body of the womb	1
Total	10

Objectives of phase I clinical trial are: to define single and maximal tolerated doses, dosing regimen and dose-limiting toxicity. Adverse events were reported by the WHO criteria. Imuteran phase I study was conducted with escalation of the single and total doses of Imuteran by a modified Fibonacci scale (dose escalation scheme is shown in table 5). The starting dose was 5 mg.

Imuteran was dissolved in 250 ml of isotonic NaCl solution and administered by intravenous drip at 5 ml/min on days 1, 8, 15 and 22 (once weekly, 4 doses in a total).

Table 5.

Imuteran dose escalation by modified Fibonacci scale

% of Dose	Imuteran single dose, mg	No. of administrations	Imuteran course dose, mg	No. of patients
100%	5	4	20	1
350%	17.50	4	70	2
525%	26.25	4	105	1
700%	35.00	4	140	3
925%	46.25	1	185 46.25	1 2

In phase I study 10 patients received 10 cycles of Imuteran therapy (a total of 34 doses). Eight patients completed treatment, in another two patients the treatment was discontinued due to toxicity after the first dose of Imuteran of 925% of the initial dose (46.25 mg). The following adverse events were reported:

- chills during infusion, immediately or at several hours after infusion completion in 60% (6/10) of patients with 32.4% (11/34) of administrations; 3 patients had chills during the first infusion at 17.5 mg (1 case) and 46.22 mg (2 cases), and another 3 developed chills during repeated administrations at 26.25 mg (1 case), 35 mg (1 case) and 46.25 mg (1 case);
- nausea and one vomiting episode in 20% (2/10) of patients with 5.9% (2/34) of administrations;
- urticaria and angioedema in combination with chills and increase in blood pressure in 1 patient (10%) with bone metastases during the first Imuteran infusion at 46.25 mg (treatment discontinued);
- fall in blood pressure, orthostatic collapse in 2 patients (20%) with 11.8% (4/34) of administrations;
- rise in blood pressure in 2 (20%) patients with 8.8% (3/34) of administrations.

Thus, the treatment was discontinued in 2 of 10 (20%) patients after the first Imuteran administration at 46.25 mg (925%) of starting dose) due to adverse events.

Imuteran did not affect basic parameters of peripheral blood (hemoglobin, leukocytes, platelets), clotting system (protrombin time, fibrinogen, RKMP ethanol test, plasma heparin tolerance) and biochemistry (glucose, creatinine, bilirubin, total protein, transaminases, alkaline phosphatase).

The dose escalation program was accomplished fully in the phase I trial. Imuteran at 46.25 mg (925% of the starting dose) induced adverse events requiring treatment discontinuation in 2 of 3 patients. Most common adverse events reported during Imuteran therapy were hypersensitivity reactions (chills, collapse, urticaria, angioedema). The following treatment regimen was recommended for phase II clinical trial: Imuteran 35 mg (700% of the starting dose) to be administered once weekly, 4 doses in the total. Premedication with dexamethasone 20 mg i.m., dimedrol 1% 3.0 i/m at 30 min before Imuteran infusion was recommended to prevent hypersensitivity reactions.

Interim results of phase II clinical trial of Imuteran

In Imuteran phase II clinical trial program, Imuteran treatment was given to 20 patients with advanced cancer (table 6) in whom all previous conventional specific treatments (including 3 to 5 chemotherapy regimens) proved ineffective.

Table 6.

Patient distribution by clinical entities (phase II)

Diagnosis	No. of patients
Breast cancer	6
Colon cancer	5
Ovarian cancer	6
Uterine tube cancer	2
Germ-cell cancer of the ovary	1
Total	20

Involvement of the liver was found in 45% (9/20), lymph nodes in 35% (7/20), bones in 20 (4/20), lungs in 20% (4/20, 1 in combination with pleuritis), peritoneum in 30% (6/20), soft tissues in 10% (2/20), small pelvis in 20% (4/20) of the cases.

At the first stage of phase II trial 7 patients received Imuteran 35 mg by i.v. drip once weekly to a total of 4 administrations with a 3-week interval to follow. Further treatment was given on a weekly basis without intervals till the scheduled assessment.

A total of 26 cycles (23 complete, 3 incomplete) were given: 16 patients received 1 cycle each, 3 patients received 2 cycles each and another 1 received 4 cycles of Imuteran therapy.

85% (17/20) of patients receiving a total of 23/26 (88.5%) cycles presented with no morbidity.

Two patients developed allergic reactions as a bronchospasm in combination with allergic dermatitis and a bronchospasm in combination with angioedema during the 2nd and 3rd infusions of cycle 1. One patient had intestinal paresis imitating intestinal obstruction during cycle 1 (received surgical treatment at the place of residence). Thus, nonaccessible toxicity requiring treatment discontinuation was reported in 15% (3/20) of patients (efficacy was not assessed).

One patient presented with germ-cell cancer as determined by repeated histology study and was excluded from efficacy analysis because nonepithelial tumors do not express MUC-1.

Therefore, Imuteran efficacy was assessed in 16 patients, the results are given in table 7.

There were no complete or partial responses to treatment.

Six of 16 (37.5% including 1 case with minimal response) demonstrated stable disease for a mean period of 11.8±4.3 weeks, median 10 weeks. Disease stabilization was reported in 2 of 4 BC patients (stabilization duration 15 weeks and 9 weeks), 2 of 5 colon cancer patients (stabilization duration 9 weeks and 9 weeks) and 2 of 2 patients with uterine tube cancer (stabilization duration 19 weeks and 11 weeks). Of note that all patients with stable disease after Imuteran treatment presented with disease progression after 3 to 5 chemotherapy regimens when starting the study drug.

Table 7.

Response to Imuteran (phase II clinical trial)

Diagnosis	No. of patients/ No. of patients evaluated	No. of cycles/ No. of cycles completed	Response (16 patients evaluated)
Breast cancer	6 (4)	7 (5)	**2/4 stable disease 15 and 9 wks** 2/4 progressive disease 2 not evaluated (treatment discontinued due to allergic reactions)
Colon cancer	5 (5)	6 (6)	**2/5 stable disease 9 and 9 wks** 3/5 progressive disease
Ovarian cancer	6 (5)	6 (5)	5/5 progressive disease 1 not evaluated (treatment discontinued due to intestinal paresis)
Uterine tube cancer	2 (2)	6 96)	**2/2 stable disease 19 and 11 wks**
Total	19 (16)	25 (22)	**6/16 (37.5%) stable disease** 11.8±4.3 wks (median 10 wks) 10/16 (62.5%) progressive disease

Disease progression was reported in 62% (10/16) of cases.

Imuteran effects on hematology, blood biochemistry and clotting ability were not reported in phase II clinical trial either.

Summarizing interim results of Imuteran phase II clinical trial, Imuteran can stop disease progression as demonstrated by disease stabilization in one third of patients with advanced epithelial cancer types such as cancer of the uterine tube, breast and colon. In spite of premedication some patients (10–15%) developed severe hypersensitivity reactions due to Imuteran being derived from native murine MAB, which hampers its application in clinical trials. Development of humanized MAB inducing minimal immune response may be reasonable to avoid or considerably reduce these effects. A research in this field is currently conducted by Academician of RAS G. P. Georgiev and colleagues.

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СУБПОПУЛЯЦИИ МОБИЛИЗОВАННЫХ СТВОЛОВЫХ (CD34$^+$) КЛЕТОК КРОВИ БОЛЬНЫХ С ПОСЛЕДСТВИЯМИ ТЯЖЕЛОЙ ТРАВМЫ СПИННОГО МОЗГА*

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Резюме

Мобилизованные стволовые клетки (CD34$^+$) больных позднего периода травматической болезни спинного мозга гетерогенны по экспрессии CD45, CD38, мономорфных детерминант HLA-DR, эпитопов *gp130*. У большинства пациентов обнаруживается фракция CD34$^+$ клеток, на которых отсутствует или представлен с низкой плотностью общелейкоцитарный антиген CD45. Процент HLA-DR-CD38- клеток лишь у 2 пациентов была >15% в пределах CD34$^+$ клеток. Экспрессия *gp130* — общей трансдуцерной молекулы цитокинов семейства интерлейкина-6 - на CD34$^+$ клетках имела место во всех случаях, у 26% больных — в активированной форме (сочетание эпитопов C7$^+$A1$^-$).

Ключевые слова:
спинномозговая травма, CD34, CD45, эпитопы gp130

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IMMUNOPHENOTYPIC PECULIARITIES OF MOBILIZED STEM (CD34$^+$) CELLS IN BLOOD FROM PATIENTS WITH SEVERE SPINAL CORD INJURY

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Abstract

Immunophenotype of mobilized stem blood cells (CD34$^+$) was studied in 29 patients with late post-traumatic spinal lesions. The CD34$^+$ cells demonstrated different levels of expression of CD45, CD38, monomorphic determinants HLA-DR and *gp130* epitopes. Most patients presented with a CD34$^+$ cell fraction with no or low expression of common leukocytic antigen CD45. Only 2 patients had >15% of HLA-DR-CD38$^-$ cells in the CD34$^+$ fraction. A common transducer molecule of interleukin-6 family cytokines *gp130* was expressed on stem (CD34$^+$) cells in all the cases, 26% of the patients had an activated *gp130* phenotype, i.e. a combination of C7$^+$ and A1$^-$ epitopes.

Key words:
spinal cord injury, CD34, CD45, *gp130* epitopes.

Работа выполнена в рамках отраслевой программы РАМН «Новые клеточные технологии — медицине». В период с 2003 по 2005 гг. интратекальное введение мобилизованных аутологичных стволовых клеток (МАСК) проведено 78 пациентам с травматической болезнью спинного мозга, в 61% случаев отмечен положительный клинический эффект в виде появления или улучшения двигательной активности в паретичных конечностях, улучшения функции тазовых органов, а также частичного, мозаичного улучшения чувствительности. Клинические данные подтверждены результатами комплексного уродинамического исследования и электромиографии.

Трансплантация МАСК — это новое направление в неврологии, и механизм восстановления (улучшения) иннервации интратекально вводимыми стволовыми клетками не известен. Трансплантация аутологичных стволовых мобилизованных гемопоэтических клеток наиболее часто применяется в онкологии и онкогематологии. Суть метода заключается во введении пациенту для восстановления уничтоженного химиотерапией гемопоэза обогащенной CD34$^+$ клетками фракции мононуклеаров крови, выделенной лейкаферезом. Подобный «заместительный» механизм действия, то есть генерация нейронов из CD34$^+$ стволовых клеток при их интратекальном введении больным спинальной травмой, представляется маловероятным, хотя и не исключен полностью.

Необходимым условием применяемой нами лечебной процедуры являлась мобилизация стволовых (CD34$^+$) клеток в периферическое кровяное русло (этот факт был подтвержден у всех больных) под действием колониестимулирующих факторов. По этой причине, не исключая, в принципе, роли CD34$^-$ фракции интратекально трансплантируемых клеток, мы в рамках данной работы, в первую очередь, сосредоточились на детальном изучении CD34$^-$позитивных стволовых клетках, т.е. МАСК.

Определенный ключ к разгадке проблемы может дать углубленная характеристика качественного состава CD34+ МАСК с позиций возможной негемопоэтической коммитированности этих клеток. Для ответа на этот вопрос нами проведено исследование экспрессии на CD34$^+$ МАСК молекул CD45 и коэкспрессии HLA-DR и CD38. Другим аспектом работы явилось изучение на CD34$^+$ клетках экспрессии и активации рецептора *gp130* — трансдуцерной молекулы цитокинов семейства интерлейкина-6 (ИЛ-6). В гемопоэтических и негемопоэтических стволовых клетках *gp130* является корецептором, необходимым для осуществления пролиферативного и дифференцировочного действия следующих цитокинов: ИЛ-6, ЦНТФ (цилиарный нейротрофный фактор), ЛИФ (лейкоз-ингибирующий фактор), ОМ (онкостатин М), КТ-1 (кардиотрофин-1) и ИЛ-11 [1; 2; 5]. Активация *gp130* под действием того или иного цитокина может быть оценена на основании эпитопной структуры *gp130* [6].

Методика исследования

В исследование включены 29 пациентов позднего периода травматической болезни спинного мозга (от 3 до 10 лет после травмы). Возраст больных — 17–65 лет (медиана — 30 лет, лишь 4 пациента старше 50 лет); мужчины — 24, женщины — 5.

Мобилизация и сбор аутологичных стволовых клеток крови осуществлялись по стандартной методике, рекомендованной и утвержденной Европейским обществом трансплантации костного мозга. В качестве колониестимулирующего фактора применен нейпоген.

Стволовые клетки и их субпопуляции в лейкоконцентрате определяли методом прямой иммунофлуоресценции с последующей проточной цитометрией. Использовали моноклональные антитела (МКА) к CD34 HPCA-2, меченные фикоэритрином /PE/ (Beckton Dickinson, США), контролем служил мышиный IgG1-PE. В исследованиях субпопуляций стволовых клеток использовали конъюгаты МКА к CD34 с PE/Cy5 и FITC.

Экспрессия молекулы CD45 изучена на мобилизованных стволовых CD34$^+$ клетках у всех 29 пациентов. Использовали МКА к CD45, напрямую меченные FITC (DakoCytomation, Дания). Определение экспрессии молекул HLA-DR (мономорфные детерминанты) и CD38 на мобилизованных стволовых клетках проводили с использованием напрямую меченых антител - HLA-DR-PE и CD38-FITC (Becton Dickinson, США).

МКА к трансдуцерному рецептору *gp130* предоставлены J. Brochier (INSERM, Франция). Использовали антитела к 2 эпитопам молекулы: A1 в сайте димеризации и C7 в другом функционально активном домене. Конъюгаты этих антител с PE/Cy5 (для A1) и фикоэритрином (для C7) были получены совместно с R. Jones (HMDS, Leeds, UK).

Анализ данных проводили на проточном цитометре FACScan (Becton Dikinson, США). Субпопуляции стволовых клеток изучали в гейте $CD34^+$ клеток с низкими (близкими к таковым для лимфоидных клеток) характеристиками светорассеяния лазерного луча (Low SSC).

Результаты исследования

Антиген CD45 экспрессирован на клетках крови за исключением эритроцитов - незрелых предшественниках, лимфоцитах, гранулоцитах, моноцитах. По мере созревания клеток крови экспрессия CD45 на них возрастает. Стволовые гемопоэтические клетки характеризуются различными уровнями экспрессии CD45 — от незначительной до выраженной. Наличие CD45 на стволовых гемопоэтических клетках крови и костного мозга положено в основу одного из наиболее известных протоколов определения этих клеток — ISHAGE [3]. У 21 из 29 больных с травматической болезнью спинного мозга среди $CD34^+$ клеток выявлялась отчетливая популяция клеток с крайне слабой экспрессией (ниже уровня на гранулоцитах) или отсутствием CD45. У 5 больных (17%) процент $CD45^-$ клеток среди $CD34^+$ клеток был небольшим (5–18,9%), у 10 (34%) — умеренным (24–49%) и у 6 (21%) — высоким (62-98%). В 8 случаях (27%) CD45-негативная фракция $CD34^+$ клеток отсутствовала. На рис. 1 приведен пример слабой экспрессии и полного отсутствия CD45 на 98% $CD34^+$ клеток, отчетливо видна $CD45^-$ фракция.

Экспрессия CD34 характерна не только для гемопоэтических, но и для стромальных предшественников, которые составляют небольшой процент в пределах фракции $CD34^+CD38^-HLA\text{-}DR^-$ [7]. Для более детального изучения этого вопроса нами проведен анализ экспрессии CD38 и HLA-DR на мобилизованных $CD34^+$ клетках крови 12 больных с травматической болезнью спинного мозга. Пропорция $CD38^-HLA\text{-}DR^-$ клеток среди $CD34^+$ клеток в большинстве случаев была незначительной (менее 9% у 10 больных) и лишь у 2 больных составила 16% и 23% (рис. 2). В обоих случаях с выраженной пропорцией HLA-DR-CD38- стволовых ($CD34^+$) клеток большинство $CD34^+$ клеток не имело на мембране CD45. В приведенном примере CD45-негативная фракция составила 98%.

Особое внимание нами было уделено оценке экспрессии и активации на мобилизованных стволовых клетках трансдуцерного рецептора цитокинов семейства интерлейкина-6 — молекуле *gp130*. Ранее подобных исследований в России и за рубежом не проводилось. У 15 больных с травматической болезнью спинного мозга изучены 2 эпитопа молекулы — функциональный эпитоп C7, участвующий в передаче сигнала лейкоз-ингибирующего фактора (ЛИФ) и онкостатина M (ОМ), а также функциональный эпитоп A1, вовлеченный в димеризацию *gp130* под действием любого цитокина семейства ИЛ-6 [1]. Различные уровни экспрессии *gp130* на $CD34^+$ клетках имели место у всех пациентов: в 13 случаях эпитоп C7 присутствовал на всех $CD34^+$ клетках, в 9 из них отмечена коэкспрессия эпитопа A1, что соответствует не активированному состоянию *gp130* [6]. У 4 больных мобилизованные стволовые клетки мономорфно экспрессировали эпитоп C7, а эпитоп A1 отсутствовал или присутствовал лишь на части клеток. Подобная эпитопная структура характерна для активации молекулы *gp130* под действием ИЛ-6, ИЛ-11,

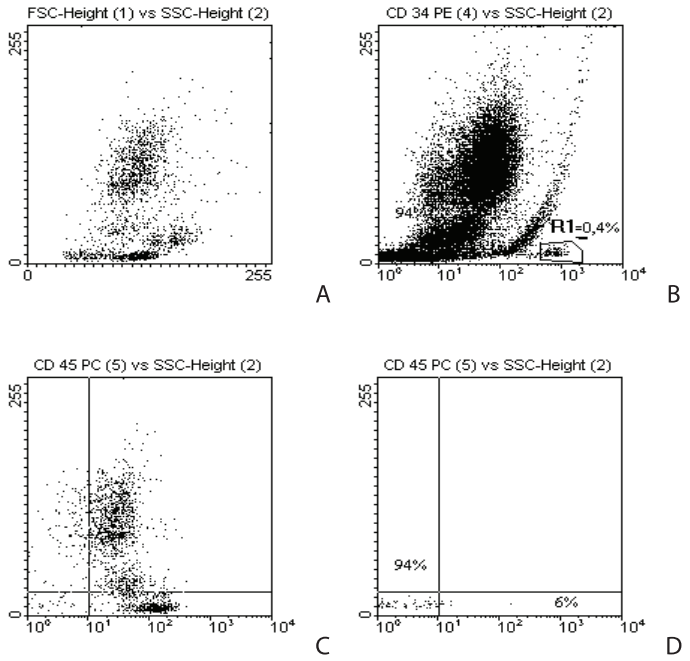

Рис. 1. Мобилизованные стволовые ($CD34^+$) клетки крови, не имеющие на мембране антигена CD45:

A — характеристики рассеяния света лазерного луча клетками цитаферезного продукта. По оси X — прямое светорассеяние (FSC) в обычных единицах (канал детектора); по оси Y (на рисунках 1A, 1B, 1C, 1D) — боковое светорассеяние (SSC);

B — стволовые клетки ($CD34^+$) в гейте R1 составляют 0.4% от общей клеточной популяции. По оси X (на рисунках 1B, 1C, 1D) — интенсивность флуоресцентного сигнала в обычных единицах (lg канала флуоресценции) по PE/FL2/;

C — пороговое значение уровня экспрессии CD45 (PerCP — FL3), определенное на основании наименьшего позитивного значения для гранулоцитов.

D — лишь 6% клеток гейта R1 (стволовые $CD34^+$ клетки) экспрессируют CD45, остальные — CD45-негативны. $CD45^-$ фракция содержит как абсолютно CD45-негативные клетки, так и клетки слабопозитивные по CD45. На диаграммах A и C представлено 2,000 событий, на диаграмме B — 50,000 и на диаграмме D — 200 событий (0.4% от 50,000, гейт R1)

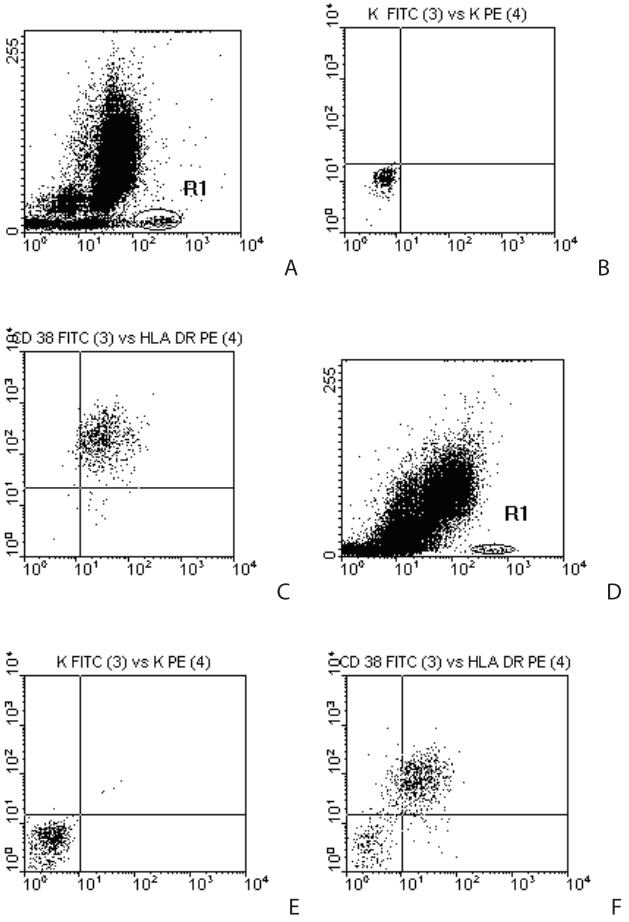

Рис. 2. Экспрессия HLA-DR и CD38 на мобилизованных стволовых ($CD34^+$) клетках крови:

A, B, C — коэкспрессия молекул. C, D, E, F — фракция HLA-DR CD38- среди $CD34^+$ клеток.

A — стволовые ($CD34^+$) клетки составляют 0.44% от общего числа клеток (гейт R1);

B — контрольное окрашивание в гейте $CD34^+$ (FL1 vs FL2);

C — 95% $CD34^+$ клеток коэкспрессируют CD38 и HLA-DR на мембране. CD45-негативные стволовые клетки ($CD34^+$) составили в этом случае 65%;

D — гейт стволовых клеток;

E — контрольное окрашивание;

F — 23% CD34+ клеток не экспрессируют HLA-DR and CD38 (HLA-DR CD38). 98% стволовых (CD34+) клеток не экспрессируют CD45 в этом случае.

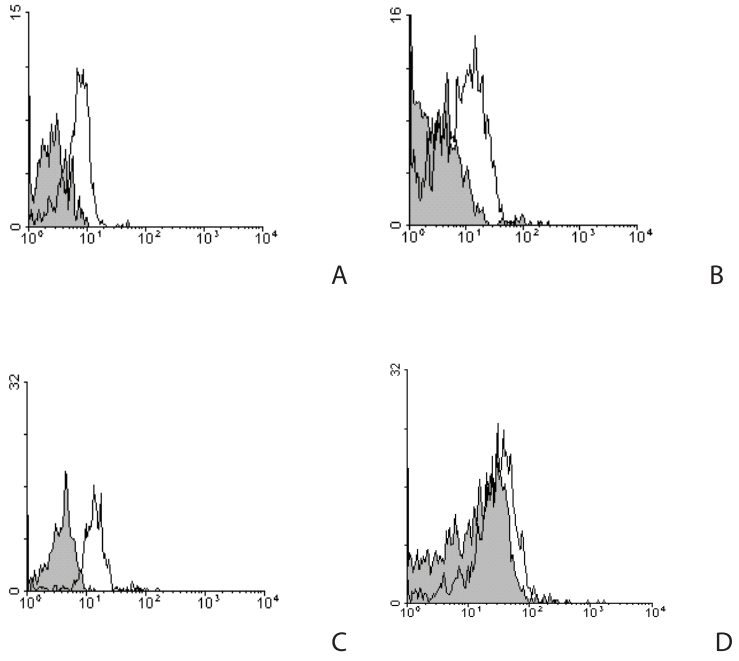

Рис. 3. Мембранная экспрессия эпитопов gp130 на мобилизованных стволовых клетках крови больных (гейт $CD34^+$ клеток):

По оси X — интенсивность флуоресцентного сигнала (канал флуоресценции), по оси Y — количество клеток. Заштрихованный пик — контрольное окрашивание, незаштрихованный пик — окраска на эпитопы C7 (A, C) и A1 (B, D). A, B — клетки пациента K.: gp130 не активирован (оба эпитопа присутствуют на мембране). C, D — клетки пациента B.: молекула gp130 находится в активированном состоянии (присутствует эпитоп C7, эпитоп A1 практически отсутствует).

кардиотрофина 1 и цилиарного нейротрофного фактора [6]. У 2 больных наблюдалась экспрессия эпитопа A1 при практически полном отсутствии эпитопа C7. В гематологических системах (клетки гемобластозов, стволовые гемопоэтические клетки) такое сочетание эпитопов мембранного *gp130* не описано, и теоретически это может соответствовать начальным этапам сигналинга ЛИФ и ОМ, еще не приведшим к гетеродимеризации *gp130*. Различные варианты экспрессии *gp130* представлены на рис. 3.

Полученные нами данные свидетельствуют о гетерогенности мобилизованных стволовых ($CD34^+$) клеток крови больных с травматической болезнью спинного мозга по экспрессии общелейкоцитарного антигена CD45. У большинства пациентов в популяции МАСК присутствовала отчетливая фракция CD45-негативных

клеток ($CD34^+CD45^-$). Согласно данным литературы, именно эти клетки, так же как и $CD38^-$HLA-DR- клетки потенциально могут дифференцироваться в негемопоэтическом направлении [7]. На сегодняшний день нет методов прямого подтверждения нейрональной или глиальной коммитированности мобилизованных стволовых клеток. Не исключена возможность приобретения CD45-негативными МАСК негемопоэтической направленности дифференцировки в условиях специфического микроокружения при интратекальном введении; для $CD45^+CD34^+$ клеток подобный ход развития событий, согласно современным представлениям, полностью исключен.

Наиболее перспективным, на наш взгляд, в проверке гипотезы «заместительной» роли МАСК представляется исследование возможности индукции нейрональной или глиальной дифференцировки стволовых клеток прямым бесцитокиновым воздействием на *gp130*. Ранее нами подобный механизм был установлен для гемопоэтических стволовых клеток [2; 4], однако на нейрональных предшественниках не изучался. Представленные в работе данные свидетельствуют об экспрессии *gp130* на МАСК ($CD34^+$) как в неактивированном состоянии (эпитопы $A1^+C7^+$), так и в активированном ($A1^-C7^+$). Более того, в 2 случаях отмечено практически полное отсутствие эпитопа С7, что, теоретически, может иметь место на начальных этапах сигналинга ОМ и ЛИФ.

Нами не ставилось задачи оценить эффективность лечения последствий травмы с помощью МАСК в зависимости от наличия в их популяции CD45-негативных клеток и от активационного статуса *gp130*. Положительные клинические эффекты отмечены и у больных с отсутствием CD45-негативных МАСК. Это говорит о возможности терапевтического эффекта не только $CD34^+CD45^-$ МАСК, но и вводимых одновременно с ними CD34-негативных или $CD34^+CD45^+$ клеток. На сегодняшний день «заместительный» механизм действия МАСК (упрощенно говоря, генерация из них нейронов) представляется наиболее уязвимым и трудно доказуемым. Представляется очевидным, что важную роль в восстановлении или улучшении иннервации тканей под действием МАСК могут играть процессы индукции роста нервных отростков, их миелинизации, ангиогенеза, а также целый ряд других факторов.

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