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Immunophenotyping of Non-Hodgkin's Lymphoma

Lack of Correlation Between Immunophenotype and Cell Morphology

HENK-JAN SCHUURMAN, PhD, JOOP VAN BAARLEN, MD, WIM HUPPES, MD, BERT W. LAM, LEO F. VERDONCK, MD, and JAN A. M. VAN UNNIK, MD

From the Division of Immunopathology, Department of Internal Medicine, Hematology and Institute for Pathology, University Hospital, Utrecht, The Netherlands

The establishment of Clusters of Differentiation for T- and B-lymphoid cells during International Workshops on Human Leukocyte Differentiation Antigens prompted the authors to evaluate the immunophenotypes in 160 cases of non-Hodgkin's lymphoma (NHL). In this group, 130 were of B-lymphocyte lineage (117 by monotypic immunoglobulin expression), and 30 of T-cell lineage. In the B-NHL series the expression of immunoglobulin isotypes, B-cell maturation/differentiation antigens of CD9, CD10, CD19–24, CD37, and CD38 (OKT10), HLA-DR and peanut agglutinin binding showed no significant relationship with histopathologic diagnosis as defined by the Kiel classification. Of the T-cell markers, CD5, CD6, and CD7 showed lineage promiscuity by their presence on some B-NHL. Conversely, the authors grouped the cases according to phenotypes (either CD antigens or immunoglobulin isotypes) which occur in distinct stages of (physiologic) B-cell maturation/differentiation. Eighty-six of the 130 cases could be fitted according to CD phenotype expression. This approach did not yield a significant relationship between phenotype and individual histopathologic categories either. The staging by CD phenotype and by immunoglobulin isotype yielded different results in this respect. Most B-NHL had an intermediate stage of B-cell maturation/differentiation. In the T-NHL series most cases showed a phenotype (CD1-CD8, CD38, TdT, and peanut agglutinin binding capacity) compatible with mature T-lymphocyte characteristics. The exceptions were lymphoblastic convoluted lymphomas, which exhibited an immature immunophenotype. It is concluded that NHL in distinct histopathologic categories are heterogeneous in immunologic phenotypes, and that the immunophenotype of lymphoma cells has no evident association with that of their presumed counterparts in physiologic cell maturation/differentiation. (Am J Pathol 1987, 129:140–151)

THE DIAGNOSIS of non-Hodgkin's lymphoma (NHL) is made by conventional histopathology, mostly on sections of formalin-fixed, paraffin-embedded tissue. A number of classification schemes have been proposed, mainly based on cell morphology and growth pattern (summarized in the Working Formulation[1]). These schemes presuppose the resemblance of pathologic cells to their physiologic counterparts. This is well illustrated by the Kiel classification[2]; for instance, the characterizations "centrocytic" and "centroblastic" indicate a morphology resembling that of cells in the normal lymph node follicle center.

Conventional histopathology and cytology are currently being extended by adopting other histologic techniques such as electron microscopy, enzyme histochemistry, and immunohistochemistry.[3,4] The immunohistochemical assessment of immunoglobulin light- and heavy-chain expression has proven its value in the identification of NHL of B-lymphocyte lineage. The monoclonal antibody technology has provided an increasing battery of reagents to identify leukocyte subpopulations in cell suspensions and on tissue sections. During international workshops[5–7] antibodies have been compared and grouped. This has resulted in the establishment of so-called Clusters of Differen-

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Address reprint requests to H.-J. Schuurman, PhD, Division of Immunopathology, Department of Internal Medicine, University Hospital, 101 Catharijnesingel, 3511 GV Utrecht, The Netherlands.

tiation (CD). For some of the corresponding antigens, the association with cell function has been shown (in particular for CDs of the T-lymphocyte series), as well as associations between expression of CD antigens and maturation/differentiation stage of the cell.[5-7]

The immunophenotyping of tumors of hematopoietic origin serves in the first place to distinguish these neoplasms from others such as carcinomas[8,9] and to distinguish neoplasms from reactive processes.[3,4] Furthermore, it serves in determining the leukocyte subset lineage of the neoplasm,[10,14] and finally in assessing the maturation/differentiation stage of the malignant cell population.[13-21] This has been extensively studied for leukemias.[17,18,22,23] For NHL, especially those of B-lymphocyte lineage, the establishment of CDs has supplied the evaluation of the immunophenotype with dimensions exceeding that of the immunoglobulin (Ig) isotype expression. Studies on this subject, including extensive series of NHL,[19] are fragmentary with respect to the application of monoclonal anti-B-lymphocyte reagents. We evaluated the diagnostic relevance of immunotyping in a large group of NHLs. Apart from Ig isotype expression, the maturation/differentiation stage was evaluated with the use of commercially available reagents in the CD series.

Materials and Methods

Specimens

The study included 160 NHLs presented for histopathologic diagnosis to the Institute for Pathology. The routine analysis included histopathology and cytology, which was followed by frozen-tissue-section analysis in immuno- and enzyme-histochemistry and electron microscopy. The final diagnosis included the results of all histologic assessments. In case of doubt about the histopathologic diagnosis of lymphoma versus carcinoma (22 cases), immunohistochemistry with anti-leukocyte antibody was performed (T29/33, Hybritech, San Diego, Calif[8]; DAKO-LC, Dakopatts, Copenhagen, Denmark[9]); cases with no staining were excluded from further study (exclusion was confirmed by electron microscopy). The immunophenotyping was performed with the use of the reagents listed in Table 1. All cases could be classified into either T- (n = 30) or B- (n = 130) cell lineage. For B-cell NHL the analysis included all reagents to B cells and the assessment of peanut agglutinin binding capacity (PNA). To exclude T-lymphocyte characteristics, anti-T reagents in CD2, CD5, CD6, and CD7 were applied. The analysis of T-cell NHL included all reagents to T cells, and it involved the assessment of PNA and terminal deoxynucleotidyl transferase (TdT). To exclude B-lymphocyte characteristics, immunoglobulin (Ig) light and heavy chains, CD20, and CD22 applied. In the B-NHL series 93 specimens were lymph nodes: the other ones included 16 tonsils and 3 skin specimens. In the T-NHL series 23 specimens were lymph nodes and 7 were from other sites (including 1 spleen and 3 skin specimens). The histologic diagnosis of B-cell NHL was done according to the Kiel classification,[2] which presupposes a close resemblance to cells in normal lymphoid cell maturation/differentiation. Table 2 summarizes the diagnoses; in individual categories the classification following the Working Formulation[1] is also listed. According to the histologic diagnosis, 86 of the B-NHLs were of low-grade malignancy and 44 of high-grade malignancy. This distribution among the various histopathologic categories is similar to that of a population-based registry of NHL in the western part of the Netherlands.[36]

There is no suitable classification scheme for T-cell lymphomas. We used a scheme based on cell size (small, medium, large, and lymphoblastic) and morphologic variability of the nuclei (mono- or pleiomorphic, convoluted), similar to that described by Stansfeld (see Table 4).[37] For instance, T-NHL, small-sized monomorphic, resembles chronic lymphocytic leukemia; T-NHL, large-sized monomorphic, resembles B-NHL, immunoblastic (K. Lennert, personal communication, 1986). In the present series, there were 1 case of lymphocytic lymphoma and 4 cases of small-sized monomorphic lymphoma. Because these two histopathologic categories have much in common with regard to cell morphology, the data are presented for the combined categories.

Immunohistochemistry

Frozen tissue sections 4–6 μ in thickness were air-dried and fixed in acetone for 10 minutes at room temperature. Immunoperoxidase with monoclonal antibodies was performed by a two-step procedure, using horseradish peroxidase-conjugated rabbit Ig to mouse Ig (Dakopatts). Staining was performed with 3-amino-9-ethylcarbazole (Aldrich Chemical Co., Milwaukee, WI) and H_2O_2 in 0.1 M acetate buffer, pH 4.6.[38] Sections were embedded in an aqueous suspension of gelatin (18% wt/vol), glycerin (50% vol/vol) and phenol (1% wt/vol). Ig expression was analyzed by two-color immunofluorescence, using a first incubation with tetramethyl rhodamin isothiocyanate (TRITC)-conjugated anti-κ or anti-λ, and a second one with fluorescein isothiocyanate (FITC)-conjugated antibody to λ or κ light chains or μ, γ, α, δ, or ϵ heavy chains.[39] J-chain was assessed by direct immu-

Table 1—List of Reagents Used*

Reagent	CD	MW	Specificity	Immunohistochemistry of lymphoid tissues	References
To B cells					
Immunoglobulin†			B cells, plasma cells	B cells in GC (IgM,IgG,IgA) and mantle (IgM,IgD): monotypic κ or λ in B-NHL	24
BA-2‡	CD9	gp24	Late pre-B cells	Faint staining in GC, minority of cells in thymus cortex	25–27
BA-3‡ (J5)	CD10	gp100	Late B-precursor, CALLA	Faint staining in GC, minority of cells in thymus cortex	24–27
Anti-Leu-12§ (B4)	CD19	gp95	Pan-B, specific	B cells in GC and mantle, FDC	28–31
B1‖	CD20	p35	Pan-B, specific	B cells in GC and mantle, FDC	28–31
BL 13¶ (B2)	CD21	gp140	B-restricted, specific	B cells in GC and mantle (faint), FDC	28–31
To 15** (anti-Leu-14)	CD22	gp135	B-restricted, specific	B cells in GC and mantle	24,28–31
Tü 1††	CD23	p45	B-restricted, activated B, specific	Some B cells in GC (strong) and mantle (faint), subset FDC	7,24,28,30–32
BA-1‡	CD24	p45,55,65	Pan-B associated	B cells in GC and mantle, myeloid cells	31
BL 14 (IOB 1)¶	CD37	gp40–45	Pan-B, specific	B cells in GC and mantle	7,28,29,31
OKT10‡‡	CD38	gp45	Immature cells, activated cells, plasma cells	Lymphocytes in thymus cortex, cells in GC (weak), plasma cells	25,33
Anti-HLA-DR§		p29–34	Pan-B, activated T	B cells in GC and mantle, stromal cells	
To T cells					
OKT6‡‡ (NA1/34)	CD1	gp45,12	Corticothymocytes	Lymphocytes in thymus cortex, Langerhans cells, interdigitating cells in paracortex	25,33
Anti-Leu-5§ (OKT11)	CD2	gp50	All T cells with sheep erythrocyte receptor	Nearly all T cells in thymus and peripheral lymphoid tissue	25,33
OKT3‡‡ (anti-Leu-4)	CD3	gp19–29	Mature T cells	T cells in thymus medulla and peripheral lymphoid tissue	25,33
Anti-Leu-3§ (OKT4)	CD4	gp55	T-helper-inducer	Lymphocytes in thymus cortex, about ⅔ of T cells in peripheral lymphoid tissue	25,33
Anti-Leu-1§	CD5	gp56–62	Pan-T, subset B	Lymphocytes in thymus cortex (faint), majority of T cells in thymus medulla and peripheral lymphoid tissue	25,33
Tü 33††	CD6	gp120	Mature T, subset B	T cells in thymus medulla and peripheral lymphoid tissue	24
WT1§§ (3A1)	CD7	gp41	Pan-T	Nearly all T cells in thymus and peripheral lymphoid tissue	25,33
Anti-Leu-2§ (OKT8)	CD8	gp32–33	T-cytotox-suppressor	Lymphocytes in thymus cortex, about ⅓ of T cells in peripheral lymphoid tissue	25,33
34/28 HLK‖‖			HLA Class I, in T-cell lineage on mature cells	Majority of T cells in thymus medulla and peripheral lymphoid organs, stromal cells	33
Anti-Leu-8§			Subset of mature T cells, T-inducer-suppressor	T cells in thymus medulla and peripheral lymphoid tissue	25,34
Other					
Peanut agglutinin (PNA)¶¶			Corticothymocytes, plasma cells	Lymphocytes in thymus cortex, cells in GC plasma cells	35
Terminal deoxynucleotidyl transferase (TdT)***			Immature lymphoid cells	Lymphocytes in thymus cortex	25,33

*Other antibodies in individual CDs are shown within brackets in the Reagent column.

Abbreviations used: CD, Cluster of Differentiation; MW, molecular weight; CALLA, common acute lymphoblastic leukemia antigen; GC, germinal center; FDC, follicular dendritic cell. CD37 and CD38 have been proposed during the Third International Workshop on Human Leukocyte Differentiation Antigens, Oxford, England, September 1986,[7] and have to be approved by WHO/IUIS.

†Fluorescein isothiocyanate-conjugated anti-κ, anti-λ, anti-IgD, and anti-IgE from Behringwerke, Marburg/Lahn, FRG; anti-IgM from Dakopatts, Copenhagen, Denmark; anti-IgG and anti-IgA from Kallestad, Austin, Texas; tetramethyl rhodamine isothiocyanate-conjugated anti-κ and anti-λ from Dakopatts, anti-J-chain from Nordic Immunological Laboratories, Tilburg, The Netherlands.

‡Hybritech, San Diego, California.

§Becton Dickinson, Mountain View, California.

‖Coulter Immunology, Hialeah, Florida.

¶Serotec, Blackthorn, Bicester, Oxon, UK.

**Dakopatts.

††Biotest Diagnostics, Dreieich, FRG.

‡‡Ortho Diagnostic Systems, Raritan, New Jersey.

§§Dr. W. J. M. Tax, Department of Nephrology, St. Radboud Hospital, Nijmegen, The Netherlands.

‖‖Sera-Lab, Crawley Down, Sussex, UK.

¶¶Fluorescein isothiocyanate-conjugated, Vector, Burlingame, California.

***Bethesda Research Laboratories, Gaithersburg, Maryland.

Table 2—Diagnosis of 130 NHLs of B-Lymphocyte Lineage

n	Kiel Classification	n	Working formulation
FCC-derived (n = 80)			
25	Centrocytic/centroblastic follicular (low grade)	17	Follicular, predominantly small cleaved cell (B)
		4	Follicular, mixed, small cleaved and large cell (C)
		4	Follicular, predominantly large cell (D)
13	Centrocytic/centroblastic diffuse (low-grade)	7	Diffuse, mixed, small and large cell (F)
		6	Diffuse, large cell, cleaved (G)
17	Centrocytic (low-grade)	11	Diffuse, small cleaved cell (E)
		1	Diffuse, mixed, small and large cell (F)
		5	Diffuse, large cell, cleaved (G)
25	Centroblastic (high-grade)	25	Diffuse, large cell, noncleaved (G)
Non-FCC-derived (n = 15)			
5	Lymphocytic (low-grade)	5	Small lymphocytic (A)
8	Lymphoblastic (high-grade)	8	Small noncleaved cell (J)
2	Plasmacytoma (low-grade)	2	Miscellaneous, extramedullary plasmacytoma
Other (n = 35)			
24	Immunocytoma (low-grade)		
18	Lymphoplasmacytoid	18	Small lymphocytic (A)
6	Polymorphic	6	Diffuse, mixed, small and large cell (F)
11	Immunoblastic (high-grade)	11	Large cell, immunoblastic (H)

nofluorescence using TRITC-conjugated rabbit anti-human J-chain,[40] and PNA by immunofluorescence using FITC-conjugated PNA.[35] TdT was assessed by indirect immunofluorescence using rabbit anti-TdT in the first incubation, and FITC-conjugated goat anti-rabbit antibody (Nordic Immunological Laboratories, Tilburg, The Netherlands) in the second one. Sections were embedded in PBS supplemented with 10% (vol/vol) glycerin and read with the use of a fluorescence microscope (Laborlux 12, Leitz, with HBO 100 epi-illumination and I2 or K2 (FITC) and N2 (TRITC) filter combinations, 25/0.60 or 50/1.00 water immersion objectives, 12.5× oculars).

In reading the results and in presenting the data, we are only concerned with the staining of tumor cells. The Ig expression on B-NHL described here is only monotypic Ig light-chain expression, either cytoplasmic or membranous. The results of immunoperoxidase or immunofluorescence were scored as negative (−), either faintly positive or positive on part of the malignant population (±), or positive (+).

Statistical Analysis

The nonparametric chi-square test was applied. To assess the correlation, the contingency coefficient C was calculated.

Results

B-Lymphoid NHL

The expression of individual markers in individual histologic categories is presented in Table 3. Ig (light chains), CD19, CD20, CD22, CD37, and HLA-DR showed the highest prevalence. The highest proportion of Ig heavy chains was by IgM. None of the cases expressed IgE. The expression of Ig was clearly membranous in lymphocytic lymphomas. In lymphomas derived from follicle center cells (FCCs) and in immunoblastic and lymphoblastic lymphomas, it was membranous/cytoplasmic instead. A clear cytoplasmic staining was observed in plasmacytomas. Of the T-cell markers, none of the cases expressed CD2. A number of cases exhibited expression of CD5, CD6, and CD7.

In the comparison of immunologic phenotype to histopathologic diagnosis, we followed two approaches. First, we considered the phenotype expression of lymphomas grouped according to histopathologic classification. None of the markers studied was restricted to any individual histopathologic category. The three major groups FCC-derived, non-follicle center-cell-derived, and other lymphomas) differed significantly in prevalence of IgD, CD20, CD37, CD5, and CD6 (Table 3, P values < 0.05 or less). The prevalence of IgD and CD5 was lowest in FCC-derived NHL, and that of CD20 and CD37 in NHL of non-FCC origin. For the FCC-derived lymphomas, significant differences were found for CD21, CD23, and PNA. These markers were most prevalent in CBCC follicular lymphomas ($P < 0.01$). For non-FCC-derived NHL, CD23 was significantly more prevalent in lymphocytic than in lymphoblastic lymphomas or plasmacytomas ($P < 0.01$). The proportions of IgD, CD21, CD23, PNA, and CD5 were significantly higher in low-grade malignancies than in high-grade malignancies (Figure 1).

Table 3—Marker Expression on 130 NHLs of B-Lymphocyte Lineage*

Histopathologic diagnosis	n	Immunoglobulin κ or λ n(%)	κ/λ ratio	M n(%)	D n(%)	G n(%)	A n(%)	J-chain n(%)	CD9 n(%)	CD10 n(%)	CD19 n(%)
FCC-derived											
CBCC follicular	25	25(100)	1.08	13(52)	2(8)	12(48)	1(0)	8(32)	22(88)	19(76)	23(92)
CBCC diffuse	13	13(100)	0.86	5(38)	0(0)	8(62)	1(8)	1(8)	7(54)	7(54)	9(69)
CC	17	14(82)	1.00	10(59)	3(18)	3(18)	1(6)	5(29)	7(41)	13(76)	12(71)
CB	25	22(88)	2.67	14(56)	0(0)	12(48)	3(12)	8(32)	11(44)	16(64)	24(96)
Total	80	74(93)	1.31	42(53)	5(6)	35(44)	6(8)	22(28)	47(59)	55(69)	68(85)
Non-FCC origin											
Lymphocytic	5	5(100)	0.67	5(100)	2(40)	0(0)	0(0)	4(80)	2(40)	2(40)	5(100)
Lymphoblastic	8	5(63)	0.67	4(50)	1(13)	1(13)	1(13)	0(0)	7(88)	6(75)	8(100)
Plasmacytoma	2	2(100)	†	0(0)	0(0)	2(100)	0(0)	1(50)	1(50)	1(50)	0(0)
Total	15	12(80)	1.00	9(60)	3(20)	3(20)	1(7)	5(33)	10(67)	9(60)	13(87)
Other											
Immunocytoma	24	21(88)	1.45	19(79)	9(38)	4(17)	3(13)	9(38)	14(58)	17(71)	16(67)
Immunoblastic	11	10(91)	4.00	5(45)	0(0)	8(73)	0(0)	1(9)	7(64)	9(82)	8(73)
Total	35	31(89)	1.91	24(69)	9(26)	12(34)	3(9)	10(29)	21(60)	26(74)	24(69)
Total	130	117(90)	1.39	75(58)	17(13)	50(38)	10(8)	37(28)	78(60)	90(69)	105(81)

*Abbreviations used: CBCC, centroblastic-centrocytic; CC, centrocytic; CB, centroblastic.
†Both plasmacytoma cases were cytoplasmic κ-positive.

In the second approach, lymphomas were grouped according to phenotypic expression. The International Workshops on Human Leukocyte Differentiation Antigens[5,6] established the restricted occurrence of CD9, CD10, CD21, CD22, and CD23 during B-lymphocyte differentiation. A model of B-cell differentiation has been proposed in which the stages correspond to different CD phenotype expression. These stages are listed in the heading in Figure 2. Stages 1–5 represent the stages from pre-B-cell to resting B-cell, based on positivity for CD9, CD10, and CD22. Stages 4–6 represent the resting B-cell stage, based on the presence of CD21. CD23 occurs late in B-cell differentiation and is considered to be an activation marker (Stage 7). Of the 130 B-NHL cases, 86 could be fitted to either one or more of the 7 stages. In fitting the cases to the model, we interpreted faint positivity or positivity for an individual CD on only part of the tumor either negative or positive. Forty-four cases had an immunologic phenotype that was not compatible with any of the 7 stages; 25 of these cases were positive for both CD21 and CD23.

Of the 86 fitted NHL, most were in Stage 4 (n = 25), 5 (n = 14), or 6 (n = 22). With regard to the composition of histopathologic categories and the malignancy grade, there were no significant differ-

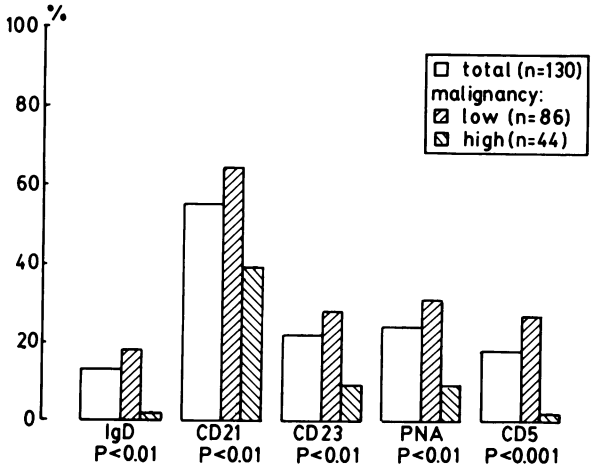

Figure 1—Prevalence of some markers in B-lymphoid NHL grouped according to malignancy grade. Only data with statistical significance between groups are presented.

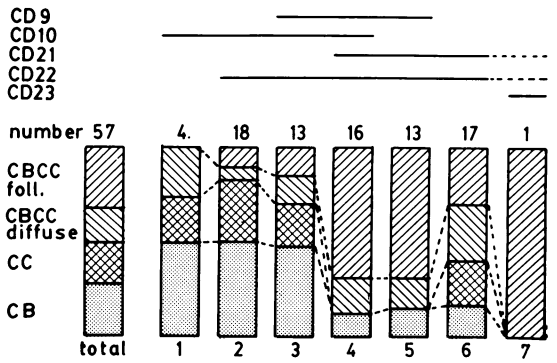

Figure 2—Staging of B-lymphoid NHL according to CD phenotype. Data shown are for the group of lymphomas derived from FCCs. Fifty-seven of the 80 lymphomas in this group fitted to one or more of the stages depicted. The sum of the number of cases in the various stages (82) exceeds the total number of lymphomas classified (57), because 18 cases could be categorized in two or three stages when faint staining or staining of only part of the cells was considered either negative or positive. The distinct stages differed in composition of histopathologic category of the lymphomas grouped ($P < 0.02$).

CD20 n(%)	CD21 n(%)	CD22 n(%)	CD23 n(%)	CD24 n(%)	CD37 n(%)	CD38 n(%)	HLA-DR n(%)	PNA n(%)	CD5 n(%)	CD6 n(%)	CD7 n(%)
25(100)	25(100)	25(100)	9(36)	20(80)	23(92)	15(60)	22(88)	16(64)	2(8)	1(4)	6(24)
13(100)	7(54)	12(92)	0(0)	7(54)	12(92)	4(31)	13(100)	4(31)	2(15)	2(15)	4(31)
16(94)	8(47)	16(94)	3(18)	13(76)	17(100)	10(59)	16(94)	2(12)	4(24)	2(12)	4(24)
25(100)	5(20)	25(100)	0(0)	20(80)	23(92)	9(36)	19(76)	2(8)	1(4)	1(4)	2(8)
79(99)	45(56)	78(98)	12(15)	60(75)	75(94)	38(48)	70(88)	24(30)	9(11)	6(8)	16(20)
3(60)	4(80)	3(60)	4(80)	5(100)	3(60)	0(0)	5(100)	0(0)	4(80)	4(80)	1(20)
6(75)	5(63)	8(100)	0(0)	7(88)	6(75)	6(75)	7(88)	1(13)	0(0)	0(0)	2(25)
0(0)	0(0)	0(0)	0(0)	0(0)	1(50)	2(100)	1(50)	2(100)	0(0)	0(0)	0(0)
9(60)	9(60)	11(73)	4(27)	12(80)	10(67)	8(53)	13(87)	3(20)	4(27)	4(27)	3(20)
21(88)	11(46)	20(83)	8(33)	18(75)	21(88)	8(33)	24(100)	3(13)	11(46)	2(8)	10(42)
9(82)	7(64)	10(91)	4(36)	6(55)	11(100)	3(27)	8(73)	1(9)	0(0)	0(0)	4(36)
30(86)	18(51)	30(86)	12(34)	24(69)	32(91)	11(31)	32(91)	4(11)	11(31)	2(6)	14(40)
118(91)	72(55)	119(92)	28(22)	96(74)	117(90)	57(44)	115(88)	31(24)	24(18)	12(9)	33(25)

ences between NHL in the 7 stages (data not shown). One exception is formed by immunoblastic lymphomas, which exhibited the highest prevalence in Stage 1 (25%) and Stage 7 (50%, $P < 0.05$). Another exception is formed by individual histologic categories in FCC-derived NHLs, which were unevenly distributed among the stages (Figure 2). In Stages 1–3 the contribution of CB lymphomas was highest, and in Stages 4, 5, and 7 that of (follicular) CBCC lymphomas was highest ($P < 0.02$). The proportion of follicular CBCC lymphomas was highest in Stage 5 ($P < 0.001$). We subsequently analyzed the occurrence of markers other than those defining individual stages. Significant differences were observed for IgM, IgG, J-chain, and PNA (Figure 3).

Before the establishment of CD for the B-lymphocyte lineage, stages in B-cell differentiation were defined by Ig isotype expression on the membrane or in the cytoplasm of the cell. The first stage in Ig expression is the development of cytoplasmic μ-chain positivity. During subsequent physiological B-cell differentiation, the appearance of IgM molecules on the cell surface is followed by IgD expression (small resting B-cell stage). Then IgG or IgA is expressed, and gradually IgD and IgM disappear. These stages in B-cell differentiation are shown in the heading in Figure 4.

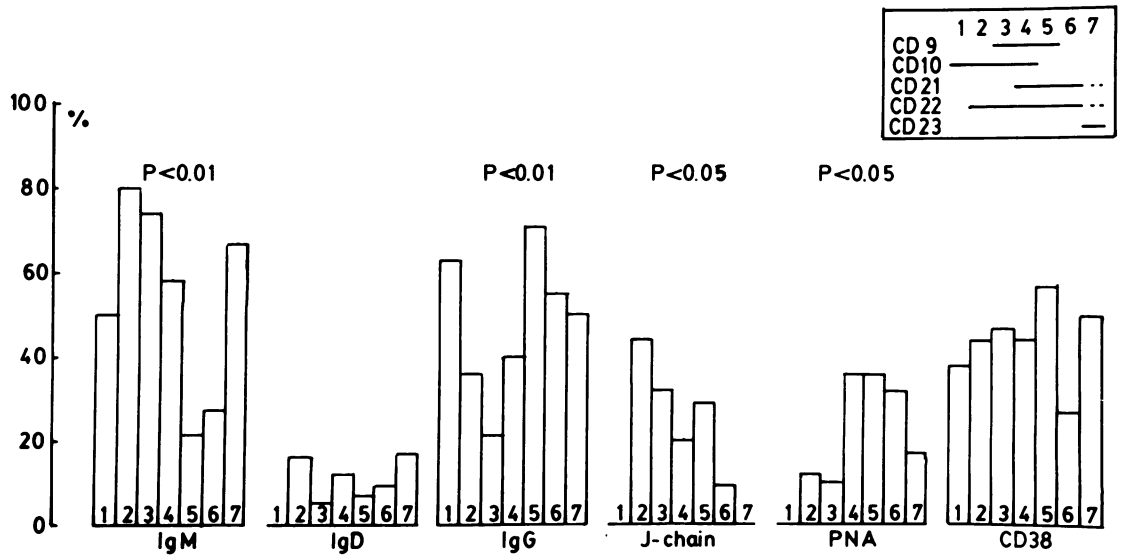

Figure 3—Expression of some markers in B-lymphoid NHL grouped according to CD phenotype. Statistical significance is indicated.

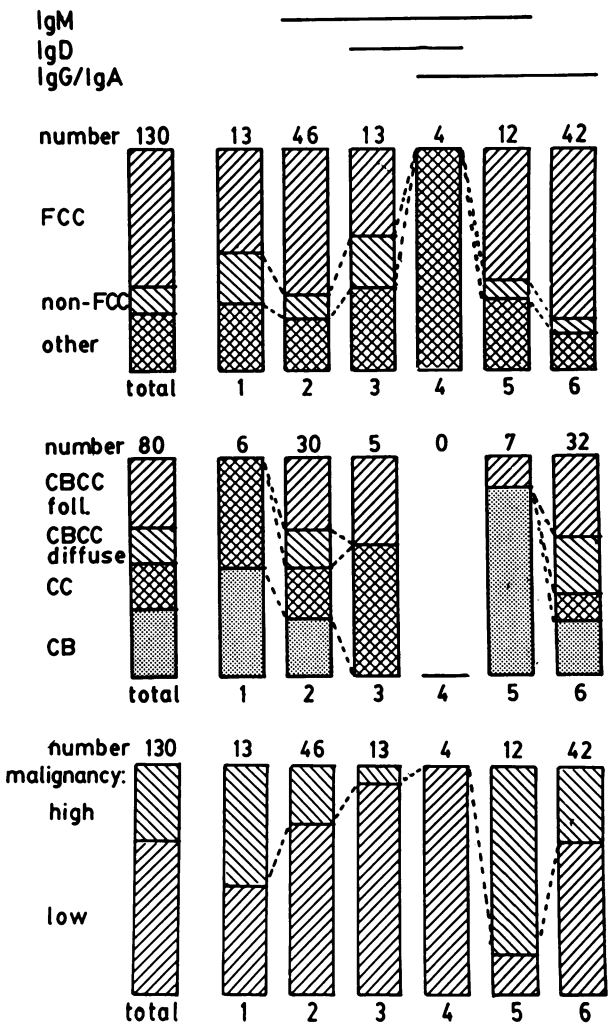

Figure 4—Staging of B-lymphoid NHL according to Ig isotype expression. For individual stages, the composition of lymphomas grouped by FCC origin of the lymphoma (**upper panel**, $P < 0.02$), within the group of lymphomas derived from FCCs (**middle panel**, $P < 0.01$), and grouped by malignancy grade (**lower panel**, $P < 0.001$) are depicted.

Stage 2 (only IgM expression) is placed before the small-resting Stage 3 (IgM and IgD expression). This is not entirely correct, because expression of solely IgM occurs either before or after Stage 3. All B-NHLs were classified in one of the six categories. The main groups were B-NHL with only IgM expression (Stage 2, 46 cases) and B-NHL with either IgG or IgA expression (Stage 6, 42 cases). There were significant differences in histopathologic classification between lymphomas in specific stages (Figure 4). In addition, differences were observed for CB lymphomas ($P < 0.05$) and immunocytomas ($P < 0.001$). None of the CB lymphomas expressed IgD (Stages 3 and 4), whereas the highest proportions of immunocytomas were found in stages with IgD expression (Stage 3 (38%) and 4 (100%)). We subsequently analyzed non-Ig marker expression in each of the stages (Figure 5). Significant differences were found for CD21, CD38, and CD5. Noteworthy is the presence of J-chain in Ig-negative lymphomas (Stage 1, 38%).

T-Lineage NHL

Table 4 presents the expression of cell markers in categories of histopathologic diagnosis. None of the cases exhibited the B-lymphocyte markers Ig, CD20, and CD22, with one exception: CD22 was present in one case of lymphoblastic convoluted lymphoma. The pan-T reagents CD2, CD5, and CD7 produced staining in the vast majority of cases. Positivity for CD3, CD6, and HLA Class I is considered indicative of a mature T-lymphocyte stage. This positivity was observed in high proportions. CD1, PNA, and TdT, which are markers for immature T lymphocytes, were present in low percentages. These markers did occur on lymphoblastic convoluted lymphomas, on 1 case of lymphocytic lymphoma (CD1 and TdT), and 1 case of medium-sized monomorphic lymphoma (CD1). Most T-NHL were positive for CD4, whereas the prevalence of CD8 was low. Coexpression of these two markers, indicative of immature T-cell characteristics, was observed in 3 cases: 1 lymphocytic and 2 medium-sized monomorphic lymphomas. Two of these 3 cases showed expression of CD1, and 1 was PNA-positive.

Leu-8 was observed in 14 of the cases, all but 1 being CD4-positive as well. One of the positive lym-

Table 4—Marker Expression on 30 NHL of T-Lymphocyte Lineage

Histopathologic diagnosis	n	CD1 n(%)	CD2 n(%)	CD3 n(%)	CD4 n(%)	CD5 n(%)	CD6 n(%)
Lymphocytic							
Small-sized, monomorphic	5	1(20)	5(100)	3(60)	5(100)	5(100)	4(80)
Medium-sized, monomorphic	6	1(17)	3(50)	6(100)	5(83)	6(100)	2(33)
Medium-sized, pleiomorphic	5	0(0)	4(80)	5(100)	5(100)	5(100)	4(80)
Large-sized, monomorphic	4	0(0)	4(100)	3(75)	4(100)	3(75)	3(75)
Large-sized, pleiomorphic	4	0(0)	2(50)	2(50)	3(75)	2(50)	3(75)
Lymphoblastic, convoluted	5	3(60)	4(80)	4(80)	4(80)	5(100)	2(40)
Lymphoblastic	1	0	1	1	1	1	1
Total	30	5(17)	23(77)	24(80)	27(90)	27(90)	19(63)

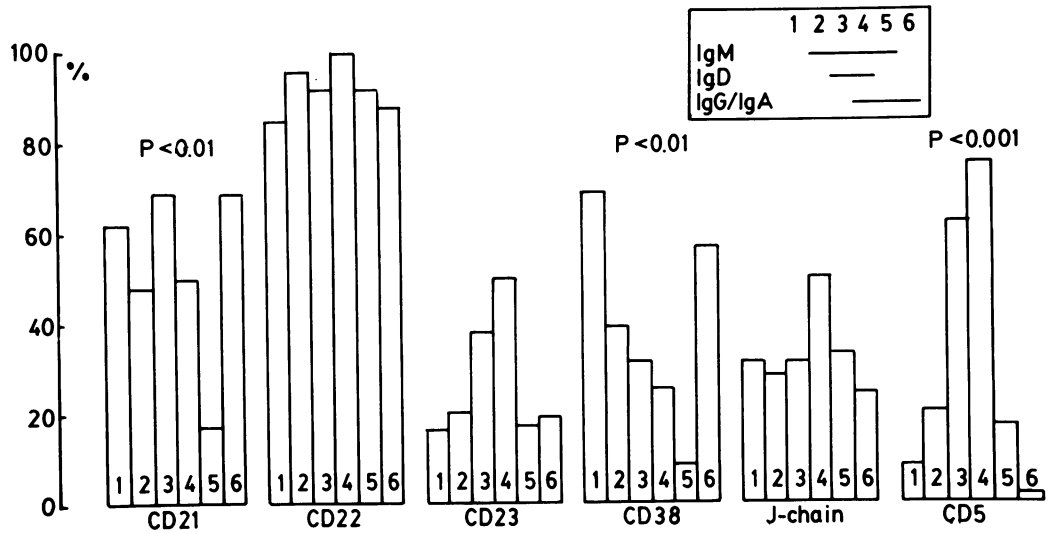

Figure 5—Expression of some non-Ig markers in B-lymphoid NHL grouped according to Ig isotype expression. Statistical significance is indicated.

phomas (medium-sized monomorphic) was also positive for CD1, CD3, CD4, CD8, and HLA-class I, but negative for CD6.

We analyzed the immunologic phenotype in T-NHLs, which were positive for differentiation/maturation markers CD1, CD3, CD6, HLA-Class I, and CD38 (Table 5). CD1 and TdT were significantly correlated (C = 0.56). There was no significant correlation between CD1 and CD3, but an inverse correlation was observed for CD1 and CD6 (C = − 0.34). CD1-positive NHLs revealed a significantly higher prevalence of CD8. Remarkably, none of the CD1-positive cases exhibited PNA binding. Markers for immature T cells and mature T cells for the group of CD38-positive lymphomas showed the same prevalence as in the total group of T-NHLs, except for the presence of CD3 in all cases.

Discussion

Use of the antibodies described in the papers of the International Workshops on Human Leukocyte Differentiation Antigens[5-7] has been reported in a limited number of studies on lymphoma typing. The positivity of almost all B-lymphoid NHL for pan-B reagents in CD19, CD20, CD22, CD24, and CD37 and the lower proportions of CD21- and CD23-positive cases are confirmed in the present study (Table 3).[7,28-31] Even before the establishment of CD in the B-cell lineage, anti-B-cell reagents had been applied in typing of B-lymphocyte malignancies. Stein et al[19,24] found CD22 (To15) expression on almost all B-NHL. In a number of studies, CD20 (B1) has been found on B-NHL irrespective of histopathologic diagnosis.[13-16] Concordant with our findings, lower proportions of CD21-positive cases have been reported for diffuse large-cell lymphomas[14] and follicular lymphomas.[16]

The primary concern of the present study is the correlation between immunologic phenotype and histopathologic classification (Kiel classification) on the one hand, and on the other, the maturation/differentiation stages of the malignant cell identified by phenotype. For B-lymphoid NHL two approaches were followed: first, we analyzed individual markers in

CD7 n(%)	CD8 n(%)	HLA Class I n(%)	Leu-8 n(%)	PNA n(%)	CD38 n(%)	TdT n(%)
5(100)	1(20)	4(80)	3(60)	0(0)	3(60)	1(20)
6(100)	2(33)	5(83)	4(67)	1(17)	3(50)	0(0)
4(80)	0(0)	3(60)	3(60)	0(0)	3(60)	0(0)
3(75)	0(0)	3(75)	0(0)	0(0)	2(50)	0(0)
3(75)	1(25)	4(100)	2(50)	0(0)	2(50)	0(0)
4(80)	1(20)	3(60)	2(40)	1(20)	1(20)	4(80)
1	0	1	0	0	0	1
26(87)	5(17)	23(77)	14(47)	2(7)	14(47)	6(20)

Table 5—Markers on T-NHL With CD1, CD3, CD6, HLA Class I, or CD38 Expression

	CD1 (5) n(%)	CD3 (24) n(%)	CD6 (19) n(%)	HLA Class I (23) n(%)	CD38 (14) n(%)	Total (30) n(%)
CD3					14(100)†	24(80)
CD6	1(20)*					19(63)
CD8	3(60)*					5(17)
Leu-8					10(71)*	14(47)
PNA				0(0)*		2(7)
TdT	4(80)†					6(20)

Only data with statistical significance compared with total are shown.
*$P < 0.05$.
†$P < 0.01$.

lymphomas grouped by histopathologic categories (Table 3, Figure 1); and second, we analyzed the histopathologic diagnosis in NHL grouped according to phenotype (Figures 2–5).

On the basis of findings in the first approach, we conclude that even for markers with a restricted occurrence, marker expression is not related to the histopathologic category of the NHL. Yet some of the features we found do merit discussion:

IgD, CD20, CD37, CD5 and CD6 discriminated with respect to the follicle-center-cell origin. The distribution of CD20 and CD37, which occur on all B cells but are absent on B-progenitor cells,[6,7] is about the same. In the total group of B-NHL, the correlation of positivity for these markers was significant ($P < 0.001$, $C = 0.36$). The expression of IgD and CD5 were also significantly correlated ($P < 0.001$, $C = 0.43$). This is illustrated in Figure 5, which shows CD5 expression to be most prevalent in IgD-positive NHL. The highest percentages of CD5- or IgD-positive NHL were found in lymphocytic lymphomas and immunocytomas (Table 3).

Within the group of FCC-derived NHL, the lowest prevalence of CD21, CD23, and PNA was found in CB lymphomas. This weak presence contributes to the low percentage of high-grade malignancies positive for these markers (Figure 1), because CB lymphomas are of high-grade malignancy and the other FCC-derived NHL are of low-grade malignancy. This aspect aside, a correlation was found between the occurrence of CD21 and CD23 ($P < 0.001$, $C = 0.32$), and between CD21 and PNA ($P < 0.01$, $C = 0.28$). The co-expression of CD21 and CD23 is discordant with the model of B-cell maturation/differentiation (Figure 2), which explains why a number of B-NHL did not fit this model (25 cases). In secondary follicles of normal lymph nodes, CD21 and PNA preferentially stain germinal center cells (Table 1). Therefore, higher proportions of positive cases are expected for FCC-derived NHL. This was not observed in the present study, either for the markers specified or for other antigens (CD9, CD10, and CD38) which preferentially occur in germinal centers. The immunoglobulin isotype, such as the absence of IgD (Table 3, Figure 4), shows a better correlation with the follicle-center-cell origin of the tumor. Within the group FCC-derived tumors, follicular CBCC lymphomas demonstrated the closest resemblance in terms of marker expression to normal germinal center cells (CD21, CD23 in Table 3, lack of IgD in Figure 4).

On the basis of findings in the second approach, we extend the conclusion made above. Furthermore, because the Kiel classification scheme we used assumes a close resemblance to lymphocytes in normal lymphoid organs, we conclude that there is no relation of immunologic phenotype between malignant lymphoma B cells and their presumed physiologic counterpart. Nonetheless, some aspects of these findings merit discussion:

CB and CC lymphomas had a more immature CD phenotype than (follicular) CBCC lymphomas (Figure 2). This was less evident in staging according to Ig isotype expression (Figure 4): high proportions of CB lymphomas were present in Ig-negative lymphomas and in stages lacking IgD expression. On the basis of phenotyping, Stein et al[19,24] have proposed the presence of two types of centrocytes: one occurs in CBCC follicular lymphomas and expresses IgM or IgG, CD10, and CD23; the other occurs in CC lymphomas and expresses IgM (not IgG), CD5, and CD6. Our data do not unequivocally confirm this suggestion. We found CC lymphomas mainly in the group of IgG-negative NHLs derived from FCCs (Figure 4). However, CD23 positivity was found in comparable proportions in CBCC follicular and CC lymphomas, whereas it was absent in the other FCC-derived NHL (Table 3). With regard to staging by Ig isotype expression, we found J-chain in B-NHL irrespective of Ig isotype expression, even in Ig-negative lymphomas. This finding has been reported previously.[42]

The grouping by CD phenotype (Figure 2) and by Ig isotype (Figure 4) yielded quite different results. This is illustrated by the analysis of markers not included in the definition of individual stages (Figures 3 and 5). There were significant differences for IgM and IgG expression in stages defined by CD phenotype, and for CD21 in stages according to Ig isotype expression, but the patterns found do not allow an unequivocal interpretation. It is noted that the expression of IgM alone can occur either before or after the B-cell stage when both IgM and IgD are expressed. Thus, the order of Stages 2 and 3 in Figure 4 is not entirely correct, which may partly explain the different results of staging according to CD phenotype or Ig isotype.

Other groups have followed approaches similar to ours. On the basis of expression of IgM, IgG, IgD, CD10, CD23, and CD5, Stein et al[19,24] have proposed the presence of seven immunophenotypes in B-NHL. We can confirm their observation that IgM and IgD expression is preferentially found in lymphocytic lymphomas and immunocytomas (data not shown) and that IgG (IgA) expression is restricted to NHL of FCC origin (Figure 4).

Some suggestions may be offered to explain the absence of a relation in B-NHL between the immunologic phenotype and the histopathologic category, and hence with the presumed physiologic counterpart. First, B-lymphoid lymphoma is generally considered to be a malignancy occurring in a small area during physiologic maturation/differentiation. This does not permit a fine substaging by immunologic phenotype. Most of the lymphomas had a CD phenotype compatible with intermediate stages of B-cell differentiation (out of 86 fitted cases, 25 were in Stage 4, 14 in Stage 5 and 22 in Stage 6 in Figure 2). Second, the model of immunologic phenotype at different stages of normal maturation/differentiation may not be correct. Based on cell morphology, a large part of B-NHL are of follicle-center origin, whereas models of B-cell differentiation are mainly concerned with the pathway leading to plasma cells, rather than the germinal-center differentiation pathway. The expression of CD9, CD10 and CD38 on germinal-center cells illustrates this phenomenon. Except for CD38, which occurs on plasma cells, the reexpression of these markers after their appearance on immature cells is not considered in most models of B-cell differentiation.[5,6] Third, some error may have ensued from a change in marker expression during the genesis of the malignancy.

The presence of T-lymphocyte markers on B-lymphoid lymphomas illustrates the validity of the suggestions made. For CD5 this lineage promiscuity is well established.[15,19,24,41] CD6 exhibited a similar expression in B-NHL of follicle-center and non-follicle-center origin (Table 3); the positivity of these two markers was significantly correlated ($P < 0.01$, $C = 0.33$). The presence of CD7 on B-NHL has not been reported thus far. Apparently, in the series of pan-T markers investigated, CD2 is the most reliable marker to exclude T-lymphocyte characteristics of suspected B-cell lymphomas.

For the series of T-NHL investigated, an association between histologic diagnosis and immunophenotype was only apparent for lymphoblastic convoluted lymphomas. The presence of CD1 and TdT in this category indicates an immature phenotype, which concurs with data from the literature.[17,18] Immature phenotype characteristics were also found in a lymphocytic and a medium-sized monomorphic lymphoma. All other T-NHLs had the phenotype of mature T cells. An inverse correlation between markers for mature cells (CD3, CD6, and HLA Class I) and markers for immature cells was expected[25,33] but was found only for CD1 and CD6 and PNA and HLA Class I (Table 5). It is unfruitful to assess the maturation/differentiation stage of malignant cells in T-NHL by analysis of CD38. Although some of the CD38-positive cases did express CD1 and TdT, most of the CD38-positive cases expressed CD6 and other markers for mature cells. In normal mature T cells, Leu-8 expression is found both within the CD4-subset and within the CD8-subset.[34] In the present study Leu-8 occurred on only some CD4-positive cases and on only one CD8-positive case, which was also CD4-positive and had an immature immunophenotype. The presence of Leu-8 on an immature T-NHL was not expected, because Leu-8 does not occur on immature thymus cortex cells (Table 1).

We offer some final remarks on the usefulness of immunologic phenotyping of NHL. To analyze the maturation or differentiation stage of malignant lymphoid cells in NHL, complete immunophenotyping seems unwarranted. Groups of NHL with similar cell morphology are heterogeneous in immunophenotype, and this heterogeneity occurs in all histopathologic categories. The histopathologic diagnosis of NHL is of prognostic value.[1,2,43] Whether this also applies to the immunophenotype remains open for investigation. For this purpose, other markers associated with proliferation may be more suitable than the cell-lineage markers and the markers associated with maturation/differentiation used in the present study. Some examples are the transferrin receptors[44] and the antibody Ki67 directed to a nuclear antigen in proliferating cells.[45] This aspect aside, immunophenotyping is of value in the assessment of cell lineage and the distinction between neoplastic and reactive pro-

cesses.[3,4] In the present series, all lymphoma cases could be classified into the B- or T-cell lineage. For B-lymphoid characteristics, the assessment of CD antigens of the B-cell series is of limited value, because 117 out of 130 B-lymphoid lymphomas did express monotypic immunoglobulin (Table 3).

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