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Immunoglobulin and T cell receptor gene rearrangements and in situ immunophenotyping in lymphoproliferative disorders*

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Summary. We investigated for rearrangements of the immunoglobulin (Ig) heavy and light chain genes and of the T cell receptor γ (TCRγ) and β(TCRβ) genes 45 biopsy samples from a variety of lymphoproliferative disorders. They were diagnosed histopathologically and immunophenotypically as non-Hodgkin's lymphomas (NHLs) of the B cell type (19 cases), NHLs of the T cell type (3 cases), NHLs of "undetermined" cell type (3 cases), atypical lymphoid proliferation (1 case) and AIDS-related lymphadenopathies with florid polyclonal follicular hyperplasia (19 cases). A monoclonal proliferation of B cells was shown by DNA analysis in all 19 B cell NHLs. In two immunohistologically determined T cell NHLs (both diagnosed as mycosis fungoides) the cells had rearrangements of TCRβ gene, whereas in the third case (lymphoblastic NHL) the cells had rearrangements of Ig heavy chain and TCRγ and TCRβ genes. None of the B cell NHLs exhibited TCRγ and TCRβ gene rearrangement bands. All the "undetermined" cell NHLs demonstrated rearrangements of Ig heavy chain gene associated with the germ line TCRγ and TCRβ genes; in two cases light chain gene rearrangements were also found. The atypical lymphoid proliferation, in which the differential diagnosis was between a reactive or malignant process, and two out of 19 cases of florid polyclonal follicular hyperplasia showed a clonal B cell population by DNA analysis. This study indicates that there was a strong correlation between the rearrangements of specific genes and the immunophenotype of the NHL; moreover, DNA analysis of tissue biopsy specimens from phenotypically "undetermined" cell NHLs and from equivocal lymphoid proliferation using Ig and TCR gene probes yelded an answer in the cases analyzed. The significance of clonal B cell expansions found in two AIDS-related lymphadenopathies should be interpreted with caution.

Key words: DNA analysis – Gene rearrangements – Molecular hybridization – Immunohistology – Histopathology – Malignant lymphoma – AIDS

Introduction

The accurate diagnosis of malignant lymphoma has become increasingly dependent upon immunohistochemical techniques (Norton and Isaacson 1987; Picker et al. 1987; Sheibani and Winberg 1987) used in conjunction with good histopathological studies (Wright 1987). However, immunohistochemistry has not solved all the problems of lymphoma identification (Asou et al. 1987b; Gonzalez-Crussi et al. 1987; Neri et al. 1987; O'Connor et al. 1987; Wright 1987): there are biopsy specimens which show a malignant lymphoid neoplasm but in which the cell lineage cannot be determined by immunohisto-cytological studies (Asou et al. 1987b; Knowles et al. 1986; O'Connor et al. 1987). Moreover, in some lymphoproliferative disorders it is difficult to distinguish between a reactive and a malignant process (Neri et al. 1987; O'Connor et al. 1987).

Clonal expansion in a lymphoproliferative disorder can now be demonstrated by the presence of immunoglobulin (Ig) and/or T cell receptor (TCR) gene rearrangements using DNA probes

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and Southern blot analysis (Arnold et al. 1983; Bertness et al. 1985; Cleary et al. 1984; Flug et al. 1985; Waldmann et al. 1985; Williams et al. 1987). Although the presence of clonality does not equate with malignancy (Weiss et al. 1986), the practical value of immunogenotypic analysis in resolving many of the diagnostic problems that arise in the assessment of lymphoid tissue biopsy specimens has been shown (Knowles et al. 1986; Neri et al. 1987; O'Connor et al. 1987; Smith et al. 1988).

In the last year, we evaluated for rearrangements of the Ig heavy – and light-chain genes, and of the TCRγ-chain (TCRγ) and TCRβ-chain (TCRβ) genes a variety of histopathologically and immunophenotypically characterized lymphoproliferative disorders. In this paper we report our findings, using gene rearrangement studies, to investigate a series of 45 biopsy specimens showing reactive and malignant lymphoid disorders, some of which had given rise to difficulty after histological analysis and immunophenotyping.

Material and methods

The biopsy specimens selected for study included 25 cases in which a pathological diagnosis of non-Hodgkin's lymphoma (NHL) was established after examination of routinely stained slides and immunohistologically labelled sections, and 19 lymph node samples with florid reactive follicular hyperplasia obtained from human immunodeficiency virus (HIV) positive patients with AIDS – related lymphadenopathy. The immunohistological findings of the majority of these latter have been previously reported by some of us (Carbone et al. 1985). The NHL cases were classified according to a modification (Nathwani 1979) of the Rappaport original classification and to the categories of the Working Formulation (WF) of NHLs for clinical usage (1982). The classification used (WF) was also related to the Kiel classification (Gerard-Marchant et al. 1974; Lennert et al. 1975).

A biopsy specimen in which it was not possible to differentiate definitely between a reactive or neoplastic lymphoproliferative process was also selected for study.

Samples with banal reactive lymphadenopathies, overt chronic lymphocytic leukaemia diagnosed by hematological criteria, AIDS – related lymphomas, and Hodgkin's disease were excluded from the present study.

Representative fresh samples were collected during standard diagnostic procedures and were obtained from lymph nodes (38 cases), skin (two cases), breast, maxillary sinus, lung, stomach and small bowel in one case each.

After submitting necessary tissue for histopathological studies, the remaining tissue was snap frozen in liquid nitrogen and stored at $-80°$ C.

The expression of surface immunoglobulin (sIg), common acute lymphoblastic leukemia antigen (CALLA-CD10) and B and T-cell-associated differentiation antigens (Leu12-CD19, LEU14-CD22, OKB7-CD21, BA1-CD24, BA2-CD9, HLA-DR, Leu4-CD3, Leu2a-CD8, Leu3a-CD4, LEU1-CD5, OKT6-CD1, OKT10-CD38, Leu7, Leu8, LeuM1-CD15) was determined as described (Carbone et al. 1987; Carbone et al. 1988).

Genomic DNAs were extracted from frozen tissues as described by Ceccherini-Nelli et al. (1987). Ten or twenty μg of DNA were digested with the appropriate restriction enzyme for analysis of gene rearrangements. Size-fractionation of DNA fragments, Southern blotting, molecular hybridization and autoradiography were carried out as described by Ceccherini-Nelli et al. (1987). The probes used are the following: Ig heavy chain: 2.5 Kb EcoRi fragment specific for Ig-JH region (Flanagan and Rabbitts 1982); Ig K light chain: 0.6 Kb SacI fragment specific for the C_K region (Bentley and Rabbitts 1983); Igλ light chain: 8 Kb EcoRi fragment specific for the Cλ region (Rabbitts and Forster 1983); TCRγ: 0.7 Kb HindIII – EcoRI fragment containing the Joining (Jγ_1) region (Lefranc and Rabbitts 1985); TCRβ: 0.6 Kb HindIII – EcoRI fragment specific for the Cβ region (Sims et al. 1984).

Plasmids were grown and purified by standard methods. Specific fragments were purified by the low melting agarose procedure as described (Ceccherini-Nelli 1987). Probes were 32P labeled by the multiprime DNA labeling system (Amersham Int. Buckinghamshire, U.K.) at specific activity 10^9 cpm/μg.

A rearrangement was defined as having occurred if, after digestion with restriction endonucleases, a new band was detected on hybridization. All rearrangements were determined using at least two different restriction enzymes.

The technique is sensitive and reliable enough to detect a clonal proliferation when it represents about the 5% of the cellular population.

Results

Table 1 summarizes the results of histopathology, immunophenotyping and DNA analysis of the overall series.

Twenty-five cases were diagnosed histologically as non-Hodgkin's lymphomas (NHLs). They were grouped according to the WF for clinical usage (1982) as follows: Group A (small lymphocytic, 8 cases), Group B (follicular predominantly small cleaved cell, 2 cases); Group D (follicular predominantly large cell, 1 case); Group E (diffuse small cleaved cell, 2 cases); Group F (diffuse mixed, small and large cell, 1 case); Group G (diffuse large cell; 2 cases); Group H (large cell, immunoblastic, 2 cases); Group I (lymphoblastic, 1 case); Group J (small non cleaved cell, 3 cases). Two cases were diagnosed as cutaneous mycosis fungoides, whereas one case diagnosed as intermediately differentiated lymphocytic lymphoma was not classified according to the WF. After immunohistological staining 19 of 25 cases were diagnosed as B cell NHLs since the cells expressed HLA-DR, Leu12-CD19, Leu14-CD22 antigens, sIg, and a single Ig light chain isotype, K or λ. Three cases including both mycosis fungoides were diagnosed as T cell NHLs. In particular in one case (n° 20 – Fig. 1) more than 90% of cells reacted with Leu4-CD3, Leu3a-CD4, Leu2a-CD8 and anti CALLA-CD10 monoclonal antibodies.

The other 3 cases (case n° 21, 22, 23) which could not be assessed unequivocally as being of

Table 1. Correlation of histopathological, immunophenotypic and genotypic characteristics in non-Hodgkin's lymphoma (NHL) and other lymphoproliferative disorders

Patient N°[a]	Histologic diagnosis[b]	Immunophenotype	Genotype[c]				
			IgH	IgK	Igλ	TcRγ	TcRβ
1	NPDL-NHL/B/CB-CC Follicular	B-Cell neoplasm	R	R	G	G	G
2	DM-NHL/F/CB-CC Diffuse	B-Cell neoplasm	R	R	G	G	G
3	DU-Burkitt's-NHL/J/Lymphoblastic	B-Cell neoplasm	R	ND	R	G	G
4	DU-non Burkitt's-NHL/J/Lymphoblastic	B-Cell neoplasm	R	G	R	G	G
5	DH-NHL/G/Centroblastic	B-Cell neoplasm	R	G	R	G	G
6	WDL-NHL/A/Lymphocytic	B-Cell neoplasm	R	G	R	G	G
7	WDL-NHL/A/Lymphocytic	B-Cell neoplasm	R	R	G	G	G
8	WDL-NHL/A/Lymphocytic	B-Cell neoplasm	R	G	R	G	G
9	WDL-NHL/A/Lymphocytic	B-Cell neoplasm	R	R	G	G	G
10	DPDL-NHL/E/Centrocytic	B-Cell neoplasm	R	G	G	G	G
11	DU-Burkitt's-NHL/J/Lymphoblastic	B-Cell neoplasm	R	G	R	G	G
12	Intermediately Lymphocytic NHL	B-Cell neoplasm	R	R	G	G	G
13	DPDL-NHL/E/Centrocytic	B-Cell neoplasm	R	G	R	G	G
14	WDL-NHL/A/Lymphocytic	B-Cell neoplasm	R	R	G	G	G
15	NPDL-NHL/B/CB-CC Follicular	B-Cell neoplasm	R	G	R	G	G
16	WDL-NHL/A/Lymphocytic	B-Cell neoplasm	R	G	R	G	G
17	NH-NHL/D/CB-CC (large) Follicular	B-Cell neoplasm	R	R	G	G	G
18	WDL-NHL/A/Lymphocytic	B-Cell neoplasm	R	R	G	G	G
19	WDL-NHL/A/Lymphocytic	B-Cell neoplasm	R	G	G	G	G
20	Lymphoblastic-NHL/I/Lymphobl. Convol.	T-Cell Neoplasm	R	G	G	R	R
21	DH-NHL/G/Centroblastic	Undetermined	R	G	G	G	G
22	DH-NHL/H/Immunoblastic	Undetermined	R	R	G	G	G
23	DH-NHL/H/Immunoblastic	Undetermined	R	R	G	G	G
24	Mycosis Fungoides	T-Cell Neoplasm	G	ND	G	ND	R
25	Mycosis Fungoides	T-Cell Neoplasm	G	ND	G	ND	R
26	Atypical Lymphoid Proliferation	Polycl. Disorder	R	G	G	G	G
27	Florid Reactive Hyperplasia	Polycl. Disorder	R	ND	G	G	G
28	Florid Reactive Hyperplasia	Polycl. Disorder	R	G	G	G	G
29 to 45	Florid Reactive Hyperplasia	Polycl. Disorder	G	G	G	G	G

[a] Patients n. 27 to 45 are human immunodeficiency virus (HIV) positive patients with AIDS related lymphadenopathy

[b] The NHL cases are classified according to a modification of the Rappaport classification and to the categories (from A to J) of the Working Formulation (WF) of NHLs for clinical usage. WDL: Well Differentiated Lymphocytic; NPDL: Nodular Poorly Differentiated Lymphocytic; NH: Nodular Histiocytic; DPDL: Diffuse Poorly Differentiated Lymphocytic; DM: Diffuse Mixed; DH: Diffuse Histiocytic; DU: Diffuse Undifferentiated; Case 12 is not classificable according to the WF. The classification used (WF) is also related to the Kiel classification. CB: centroblastic; CC: centrocytic (small)

[c] G: germinal configuration; R: rearranged; ND: not done. In the patients n. 29 to 45 analysis for IgK chain gene was not done in three cases (35, 36, 37); for Igλ chain gene it was not done in three cases (35, 39, 40); for TcRγ gene it was not done in one case (35) and for TcRβ gene in three cases (39, 44, 45)

either B or T cell origin were diagnosed as "undetermined" cell NHLs (Fig. 2). In 2 cases (case n° 21, 22) of the latter group, the majority of cells were B cells (Leu 14^+-CD22, Leu 12^+-CD19) but staining for K and λ light chains had shown no clear pattern of light chain restriction. In case number 23, the antigenic determinants recognized by anti-Leu4-CD3, and Leu1-CD5 were negative. Pan B cell antigens (Leu12-CD19, Leu14-CD22) and heavy and light chains were not detectable by frozen section immunohistochemical studies.

A clonal B cell population was shown by DNA analysis in all 19 B cell NHLs (see Table 1). In the mycosis fungoides cases (n° 24, 25) the cells had rearrangements of TCRβ gene associated with the germ line Ig chain genes tested, whereas in the remaining immunohistologically determined T-cell NHL the cells had rearrangements of Ig heavy chain and TCRγ and TCRβ genes (Figs. 3 and 4). Thus clonality of these cells was demonstrated, but cell lineage was not unequivocally determined. All the immunohistologically "undetermined" cell NHLs demonstrated rearrangements of Ig heavy chain gene associated with the germ line TCRγ and TCRβ genes (Figs. 3 and 4). In two cases light chain gene rearrangements were also found (Fig. 4). Therefore these cases could be determined as B cell NHLs.

In case 26 it was very difficult to differentiate a reactive from a malignant process. An incisional

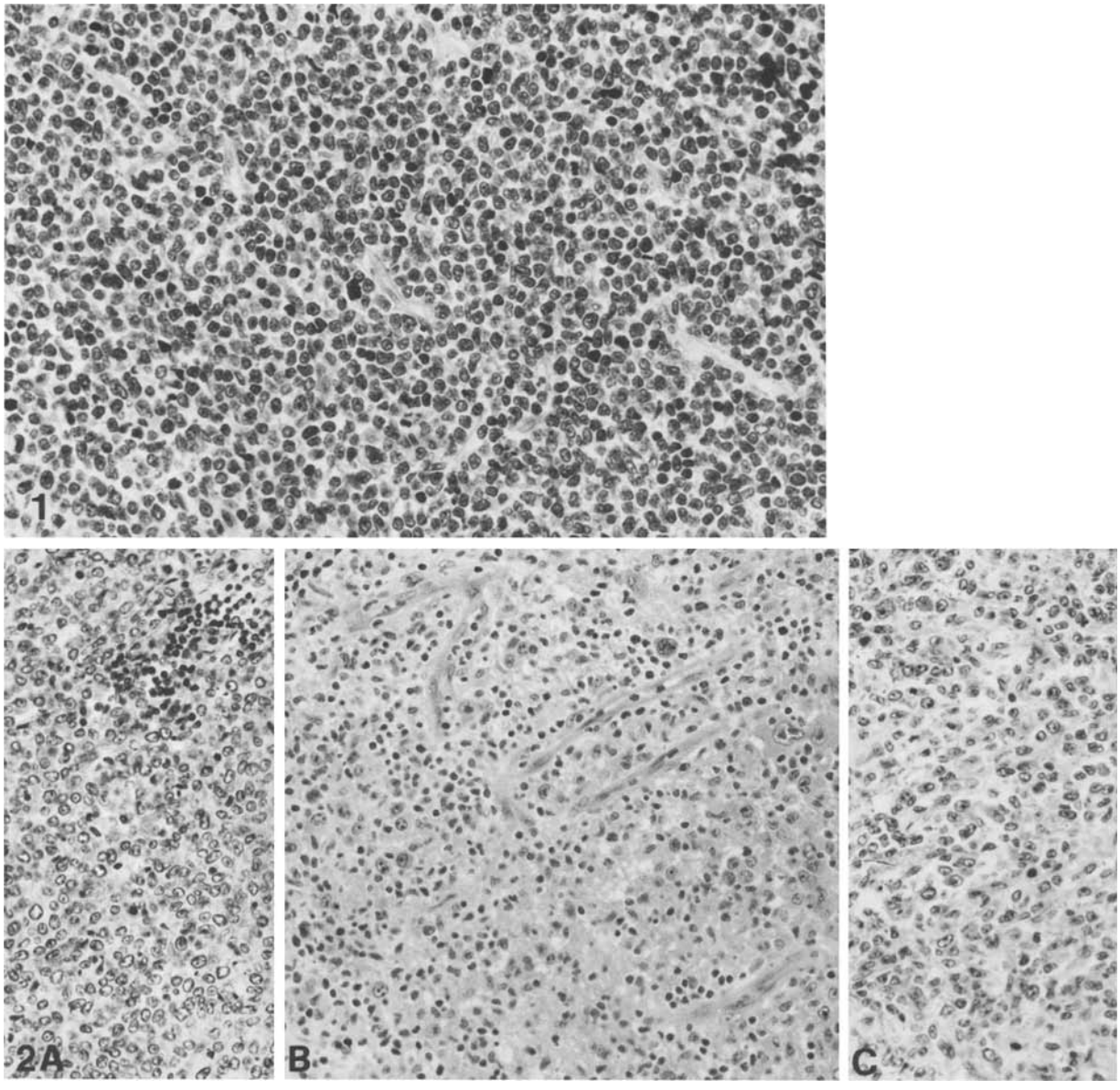

Fig. 1. Case n° 20 (lymph node). The immunohistologically determined T-cell lymphoma was classified as lymphoblastic lymphoma; it consists of an uniform population of small lymphoid cells with scanty or indistinct cytoplasm and nuclear convolutions. (Haematoxylin and eosin, ×400)

Fig. 2 A–C. Cases n° 21 (lymph node-**A**), 22 (lung-**B**), 23 (breast-**C**). The immunohistologically "undetermined" cell lymphomas consist of a diffuse growth of large lymphoid cells; they are of the large non cleaved cell type (**A**) or the immunoblastic cell type (**B–C**) with a polymorphous cell component in case 22 (**B**). (Haematoxylin and eosin, ×250)

biopsy of a submandibular lymph node was examined. Histologically, the lymph node fragment showed partial structural effacement by a pleomorphic cell population with lymphocytes, lymphoplasmacytoid cells and immunoblasts; numerous histiocytes, macrophages and epithelioid cells were also present (Fig. 5). Residual lymphoid follicles were noted. Mitotic figures were rare, capillary vessels were abundant. Immunohistology on frozen sections demonstrated a low number of polyclonal B cells associated with T cell hyperplasia with equal number of Leu3a-CD4 and Leu2a-CD8 positive cells. OKT10-CD38 positive cells were focally increased, whereas OKT6-CD1 and LeuM1-CD15 positive cells were absent. DNA analysis showed rearrangements of Ig heavy chain gene, suggesting the presence of B cell NHL (Fig. 4). The patient, a 13 year old girl, was admit-

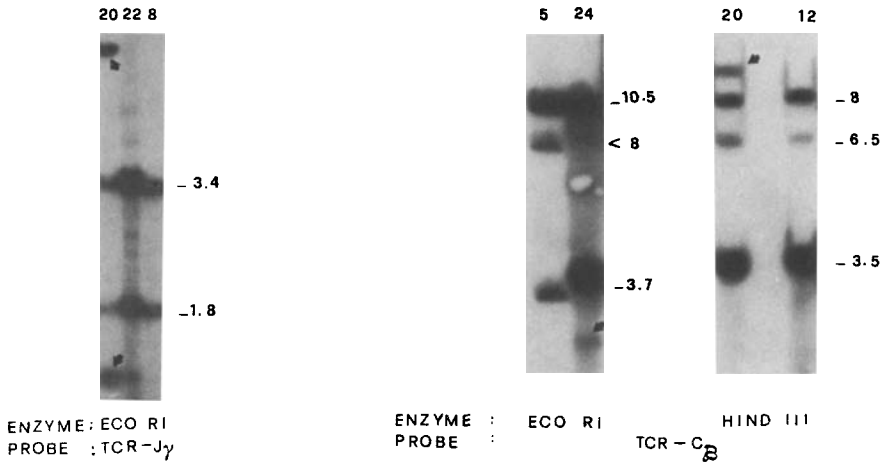

Fig. 3. Southern blot of genomic DNA from non-Hodgkin's lymphomas of the T cell (n° 20 and n° 24) and B cell (n° 22, 8, 5, 12) type hybridized with TCR-Jγ and TCR-Cβ. DNAs were digested with the reported enzymes. Germ line fragments are indicated in Kilobase units. *Arrows* indicate rearranged fragments. An EcoRI site (marked with an < in figure), partly resistant to normal digestion, gives rise to an 8.0 Kilobase fragment, which hybridizes to TCR-Cβ probe (Furley et al. 1987)

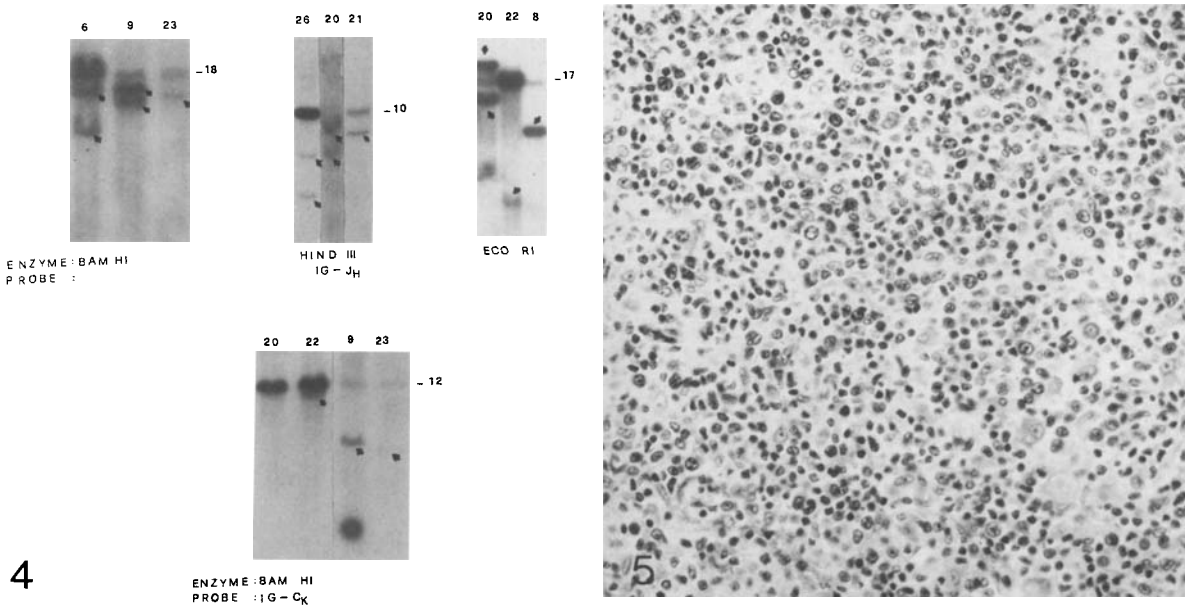

Fig. 4. Southern blot of genomic DNA from non Hodgkin's lymphomas and atypical lymphoid proliferation (n° 26) hybridized with heavy (Ig-JH) and light (Ig-C_k) chain immunoglobulins. The lane numbers indicate cases reported in Table 1. DNAs were digested with the reported enzymes. Germ line fragments are indicated in Kilobase units. *Arrows* indicate rearranged fragments

Fig. 5. Case N° 26 (lymph node). The micrograph illustrates a representative histological area of the lymph node in which several "blast" cells are admixed with numerous smaller lymphoid cells and some histiocytes. (Haematoxylin and eosin, ×250)

ted to our center because of a left submandibular lymphadenopathy. This had been present for five months in her parent's opinion. Past history was unremarkable and results of the physical examination were completely normal with the exception of the lymphadenopathy. The lymph node measured about 10 cm. Chest X-ray and laboratory data including serological tests were normal. After incisional lymph node biopsy a cycle of corticosteroid therapy was administered with a reduction in size of the lymphadenopathy. Fifteen months after initial investigation physical examination showed a persistence of the lymphadenopathy. The lymph node measured 5 × 3 cm.

Nineteen lymph node specimens obtained from HIV positive patients with persistent generalized

lymphadenopathy were diagnosed histologically as nonmalignant lymphadenopathies. All cases showed exuberant follicular hyperplasia involving both the cortex and the medulla, associated with a striking proliferation of capillary vessels in the interfollicular areas. The follicles were round or oval and often contained a group of small vessels. A prominent and diffuse plasmacytosis was almost invariably present. By immunohistological staining, the B cell population proved to be polyclonal in all cases. No germinal center showed positive staining restricted to a single heavy or light Ig chain. Immunohistological analysis also showed a thinning of the mantle zones and abnormalities of T cell population. These abnormalities included increased numbers of Leu2a-CD8 positive cells in follicles and paracortical regions, with a concurrent decrease in paracortical Leu 3a-CD4 positive cells. DNA analysis demonstrated rearrangements of Ig heavy chain gene in two samples (case n° 27, 28), but no Ig light chain rearranged genes were detected. None of the remaining 17 cases exhibited clonal Ig or TCRγ and TCRβ gene rearrangement bands.

Both patients, in which DNA analysis showed a clonal B cell proliferation, were intravenous drug abusers. They remained apparently free from progression to lymphoma or AIDS after 35 and 36 months respectively. No therapy was administered.

Discussion

The application of molecular hybridization as a complementary method to conventional histopathological and immunohistological studies has recently been shown to be useful in the diagnosis and characterization of various lymphoproliferative disorders that are difficult to evaluate histologically (Arnold et al. 1983; Bertness et al. 1985; Cleary et al. 1984; Flug et al. 1985; O'Connor et al. 1987; Waldmann et al. 1985). In particular, this approach is helpful in distinguishing B and T cell NHLs among lymphoid tumours that fail to show the definitive immunological markers for either B or T cell differentiation (Bertness et al. 1985; Cleary et al. 1984; O'Connor et al. 1987). Warnke et al. (1980) have indicated that these tumours may represent up to 25% of large cell NHLs or 10–15% of all NHLs. Moreover, because the DNA hybridization technique is an extremely sensitive test for the presence of a minor clonal population of lymphocytes (Cleary et al. 1984), the clonality of lymphoproliferative disorders can be established with this technique even in the absence of detectable immunological markers. We have analyzed rearrangements of the Ig heavy and light chain genes and of TCRγ, TCRβ genes, and surface phenotypes in a series of 45 lymphoproliferative disorders.

Rearrangements of the Ig heavy chain gene were present in DNA from each of 19 immunophenotypically diagnosed B cell NHLs. Seventeen of these tumours also had rearrangements of the Ig light chain genes. None of the 19 B cell NHL cases exhibited TCRγ and TCRβ gene rearrangement bands. There was thus a strong correlation between the rearrangements of specific genes and the immunophenotype of the tumour.

In the case (n° 20) in which rearrangements of Ig heavy chain gene were found with the presence of the TCRγ and TCRβ gene rearrangements, the neoplastic cells immunohistologically expressed only T cell associated antigens. The occurrence of TCRβ gene rearrangements with Ig heavy chain gene rearrangements in T cell tumours has been reported (Williams et al. 1987). Sheibani et al. (1987) have reported 3 NHL cases that were immunologically classified as having a T cell phenotype and in which, in addition to rearrangements of the TCRβ gene, a rearrangement of an Ig light chain gene was detected. This finding was interpreted as indicative of an unusual lineage infedelity or, alternatively of T cell lymphomas that additionally contain monoclonal expansion of B cells (Sheibani et al. 1987). However, Griesser et al. (1986) have found rearrangement of TCRβ in two of seven B-cell NHLs; in all seven cases there was rearrangement of both the heavy chain and K-light chain genes. The observation that the intensity of the rearranged Ig and TCRβ bands were roughly equal supported the view that both rearrangements co-existed in a single clone of cells.

The precise function of TCRγ gene, a third gene that rearranges in T cells, remains uncertain. Recently, Davey et al. (1986) by examining the spectrum of leukaemias of the T-cell series have provided evidence for a hierarchy of rearrangement of TCR genes in T-lymphocyte ontogeny, and supported the view that TCRγ gene is rearranged first, followed by the TCRβ gene. This is followed by TCRα gene activation. TCRγ gene rearrangements have been found in immature B cells and are not always found in T cells showing TCRβ gene rearrangements, but it is not detected in non lymphoid cells (Asou et al. 1987a). Asou et al. (1987a) observed TCRγ gene rearrangements in 19 of 21 T cell neoplasms; in 14 of 21 immature B cell leukemias, including 4 out of 5 cases with

rearrangements of both Ig heavy chain and TCRβ genes. Finally, analogously to the results of the present study none of 14 B cell neoplasms showed TCRγ gene rearrangements.

As concerns to the three cases of NHL of immunohistologically "undetermined" cell origin, this study indicates that DNA analysis yelded an answer in all cases. All these cases were diagnosed histologically as large cell NHLs analogously to the majority of the phenotypically "undetermined" cell lymphomas investigated by others (Asou et al. 1987b; O'Connor et al. 1987) by using DNA hybridization.

In addition to the NHL cases we studied DNA from a lymph node specimen showing atypical lymphoid proliferation. The difficulty in diagnosis seems to have been caused by the presence of large numbers of reactive T cells. The genotypic suggestion of lymphoma was in keeping with the clinical course of the disease revealing a persistence of the lymphadenopathy after 15 months. A longer follow-up, however, is necessary to permit a firm conclusion to be drawn.

Finally, we investigated the clonality of the B or T lymphoid populations in 19 lymph node samples obtained from HIV positive patients with persistent generalized lymphadenopathy. HIV infection may be associated with a spectrum of lymphoproliferative disorders ranging from persistent generalized lymphadenopathy, an apparently benign polyclonal lymphoid hyperplasia (Carbone et al. 1985; Turner et al. 1987) with several, although non-specific, histological patterns (O'Murchadha et al. 1987), to B cell NHLs, that is to say a malignant, presumably monoclonal B cell proliferation (Pelicci et al. 1986). None of our cases contained a clonal B cell expansion by immunophenotype analysis. In contrast, DNA analysis detected rearrangements of Ig heavy chain gene in two cases. Previous data have indicated that multiple clonal B expansions were present in a significant percentage ($\simeq$20%) of AIDS-related non malignant lymphoproliferative disorders (Pelicci et al. 1986). The hypothesis that these disorders would represent a predisposing condition to malignant lymphomas has been suggested (Lippman et al. 1988; Pelicci et al. 1986).

Clonal populations are also demonstrable in the so called morphologically benign monoclonal small lymphocytic proliferations of the gastrointestinal tract (Burke et al. 1987), lung and orbit (Neri et al. 1987) which have an uncertain malignant potential. The biological meaning of these clonal B cell expansions is not clearly understood, at present. The alternative, that they represent small benign clonal populations, should also be considered (Cossman et al. 1988).

At present, this finding should be interpreted with caution; the clinical and pathological follow up of large series of such patients will provide an answer to the question.

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