Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 7942–7946, October 1986
Medical Sciences

Immunoglobulin and T-cell receptor β-chain gene rearrangement analysis of Hodgkin's disease: Implications for lineage determination and differential diagnosis

(Reed–Sternberg cell/T-cell receptor genes/non-Hodgkin's lymphoma/immunoglobulin genes)

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Communicated by Gertrude Henle, July 7, 1986

ABSTRACT **The lineage and clonality of Hodgkin's disease (HD) were investigated by analyzing the organization of the immunoglobulin and T-cell receptor β-chain (T_β) gene loci in 18 cases of HD, and for comparison, in a panel of 103 cases of B- and T-cell non-Hodgkin's lymphomas (NHLs) and lymphoid leukemias (LLs). Sizable clonal B- or T-cell populations, representing ≥10% of the pathologic sample, were readily detectable by immunogenotypic analysis in all 103 NHLs and LLs but not in any of the 18 cases of HD. However, extremely minor clonal populations (≤1%) were detectable in 3 of 18 cases of HD. We demonstrated that these minor clonal populations do not correspond to Reed–Sternberg (RS) cells since clonal immunoglobulin or T_β gene rearrangements are not detectable in cases of HD containing >25% RS cells. The number of RS cells present in these samples appeared to correlate directly with the pattern of gene rearrangements characteristic of polyclonal T cells. These studies demonstrate (*i*) that Southern blot hybridization analysis for clonal immunoglobulin and T_β gene rearrangements represents an accurate, objective tool in the differential diagnosis between HD and NHL; (*ii*) that HD is predominantly composed of polyclonal B and T cells; (*iii*) that minor clonal B- or T-cell populations unrelated to RS cells occasionally can be found in HD; and (*iv*) that RS cells do not represent clonal B- or T-cell expansions. Finally, our data preliminarily suggest that RS cells may represent polyclonal T-cell populations.**

Lymphoid neoplasms are broadly divided by clinical, morphologic, and immunophenotypic criteria into two major categories: (*i*) non-Hodgkin's lymphoma (NHL) and lymphoid leukemia (LL) and (*ii*) Hodgkin's disease (HD) (1). Extensive investigations have suggested that nearly all NHLs and LLs represent clonal expansions of B or T lymphocytes (2). In contrast, the lineage derivation and the clonal origin of HD have remained controversial since the origin and nature of the Reed–Sternberg (RS) cell, the diagnostic cell of HD, remains unknown. Morphologic and immunophenotypic studies have left suggestions that RS cells may derive from B or T lymphocytes, monocytes, dendritic cells, or an as yet undefined cell population (3–8), unresolved. Failure to resolve this issue has contributed to the controversy concerning the pathogenesis of HD and whether it represents a clonal malignant proliferation.

In addition to pathogenetic relevance, the determination of the lineage and clonality of HD has critical diagnostic implications. Distinguishing between HD and NHL is extremely important since clinical staging and evaluation, choice of therapeutic regimen, clinical course, and eventual outcome differ significantly (1). This differential diagnosis is based on established histopathologic criteria (1). However, instances arise in which the histopathologic differential is difficult and an accurate diagnosis cannot be made (9). Immunophenotypic analysis has been extensively used as an adjunct to the histopathologic diagnosis and classification of lymphoid neoplasia (2). However, cases of HD often have increased numbers of T cells, predominantly of the $T3^+T4^+$ or occasionally of the $T3^+T8^+$ subset (10), similar to the majority of cases of peripheral T-cell lymphoma (2). Moreover, the presence of benign lymphoid cells within a B- or T-cell NHL may erroneously suggest a polyclonal lymphoid cell proliferation, which in the face of confusing histopathology may result in the misdiagnosis of NHL as HD. Finally, the LeuM1 antigen, recently proposed as a useful immunodiagnostic marker of HD (11, 12), is often expressed by the large pleomorphic RS-like cells of peripheral and cutaneous T-cell lymphoma (13, 14). Therefore, immunophenotypic analysis sometimes may be of limited value in the differential diagnosis of HD.

The antigen recognition molecules of B and T cells, immunoglobulin and T-cell receptor(s), respectively, are encoded by genetic loci that undergo somatic recombinations (rearrangements) to become functionally active in mature lymphocytes (15, 16). Clonal immunoglobulin and T-cell receptor β-chain (T_β) gene rearrangements are useful genetic markers of the lineage and clonality of B and T cells, respectively. They have been successfully used to determine the lineage and the clonality of a variety of NHLs and LLs (17–24), but not HD.

We investigated the organization of the immunoglobulin and the T_β gene loci in 18 well-documented cases of HD to probe the origin and the nature of the lymphoid and the RS cells of HD. We similarly analyzed a heterogeneous collection of 103 B- and T-cell NHLs and LLs and 2 lymphoid neoplasms of uncertain histogenesis to determine the utility of immunogenotypic analysis in the differential diagnosis between HD and NHL and LL.

METHODS

Pathologic Samples. Representative samples of lymph nodes, peripheral blood, and bone marrow were collected during standard diagnostic procedures. The diagnosis and classification of each lymphoid neoplasm were established by conventional clinical, histochemical, and histopathologic

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Abbreviations: NHL, non-Hodgkin's lymphoma; LL, lymphoid leukemia; HD, Hodgkin's disease; RS cells, Reed–Sternberg cells; H chain, immunoglobulin heavy chain; J_H, joining region H chain; C region, constant region of immunoglobulin; kb, kilobase(s).

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criteria. Lineage assignment was determined by immunophenotypic analysis.

Immunophenotypic Analysis. The expression of surface immunoglobulin, cytoplasmic μ heavy chains, sheep erythrocyte rosettes, common acute lymphoblastic leukemia antigen, terminal deoxynucleotidyl transferase, and B- and T-cell-associated differentiation antigens (HLA-DR, OKT3, OKT4, OKT6, OKT8, OKT9, OKT10, OKT11, OKB1, OKB2, OKB4, OKB7, BA-1, BA-2, B1, B2, B4, Leu1, Leu12, Leu14, LeuM1, BL1, BL2, BL3, BL4, and BL7) were determined as described (2, 25, 26).

DNA Analysis. DNA was extracted from each pathologic sample by standard techniques (27). DNA (10–15 μg) was digested with the appropriate restriction endonuclease and analyzed for immunoglobulin and T_β gene rearrangements according to Southern (28). Hybridization was performed in 50% formamide/3× standard saline citrate (SSC; 1× SSC = 0.15 M NaCl/0.015 M sodium citrate) at 37°C for 16 hr using 5–10 μg of DNA probe per ml. Filters were washed in 0.2× SSC/0.5% $NaDodSO_4$, pH 7.0, at 60°C for 2 hr (29). DNA clones were ^{32}P-labeled by nick-translation (30) or by random priming (31) for use as probes. The ranges of sensitivities obtained by these DNA labeling methods were 2–5 × 10^8 dpm/μg and 0.8–3 × 10^9 dpm/μg, respectively. Autoradiographic exposure time and efficiency of detection of hybridization signals varied accordingly. Clonal populations representing 5–10% and 0.5–1% of the total DNA are detectable using probes labeled by nick-translation and random priming, respectively, using these hybridization and washing conditions.

The T_β gene locus was analyzed by hybridization of *Eco*RI-, *Bam*HI- and *Hin*dIII-digested DNA to a T_β probe (courtesy of T. Mak, Ontario Cancer Institute) that hybridizes to both constant (C) region alleles ($C_\beta 1$ and $C_\beta 2$) (19, 32). The immunoglobulin heavy (H) chain and the κ and λ light-chain gene loci were studied by hybridization of *Hin*dIII- and *Eco*RI-digested DNA to a joining region heavy chain (J_H) specific probe, by hybridization of *Bam*HI-digested DNA to a C-region κ-specific (C_κ) probe, and by hybridization of *Eco*RI- and *Hin*dIII-digested DNA to a C-region λ-specific (C_λ) probe, respectively (courtesy of S. Korsmeyer, National Institutes of Health) (22).

RESULTS

Immunoglobulin and T_β Gene Rearrangement Analysis of NHLs, LLs, and HD. First, we comparatively investigated the organization of the immunoglobulin and the T_β gene loci in NHL, LL, and HD. We and others had already demonstrated that B- and T-cell NHLs and LLs exhibit clonal immunoglobulin and T_β gene rearrangements, respectively (17–24). However, we extended these studies by investigating an additional expanded collection of 48 B-cell and 55 T-cell NHLs and LLs, which we carefully selected to be representative of the entire diverse clinical, morphologic, and immunophenotypic spectrum of non-Hodgkin's lymphoid neoplasia (Table 1). Each of the 48 B-cell neoplasms exhibited clonal H-chain gene rearrangements upon hybridization of *Eco*RI- or *Hin*dIII-digested DNA to a J_H-specific probe. Each of the 55 T-cell neoplasms displayed the distinctive *Eco*RI 12.0-kilobase (kb) DNA fragment deletion characteristic of the T-cell lineage (19) and a unique set of clonal T_β gene rearrangements, indicative of a monoclonal T-cell proliferation, upon hybridization of *Eco*RI- or *Bam*HI-digested DNA to a T_β probe. Eight of the 48 B-cell and 3 of the 55 T-cell neoplasms (11%) were bigenotypic (Fig. 1). Thus, all 103 NHLs and LLs exhibited clonal rearrangements of the immunoglobulin or the T_β genes, or occasionally both; no cases of NHL or LL displayed solely the germline H chain or the T_β gene configuration. In addition, the clonal rearrange-

Table 1. Immunoglobulin H-chain and T-cell receptor T_β gene rearrangements in B- and T-cell-derived NHL neoplasms

Diagnosis	Cases	T_β, R/ H chain, G	T_β, G/ H chain, R	T_β, R/ H chain, R	T_β, G/ H chain, G
			T-cell neoplasms		
ALL	9	7	0	2	0
LBL	5	5	0	0	0
PLL	5	5	0	0	0
CLL	6	5	0	1	0
CTCL	8	8	0	0	0
SS	8	8	0	0	0
PTCL	14	14	0	0	0
			B-cell neoplasms		
ALL	12	0	8	4	0
CLL	8	0	7	1	0
NHL	26	0	23	3	0
MM	2	0	2	0	0

ALL, acute lymphoblastic leukemia; SS, Sezary syndrome; LBL, lymphoblastic lymphoma; PTCL, peripheral T-cell lymphoma; PLL, prolymphocytic leukemia; NHL, non-Hodgkin's lymphoma; CLL, chronic lymphocytic leukemia; MM, multiple myeloma; CTCL, cutaneous T-cell lymphoma; G, germline; R, rearranged.

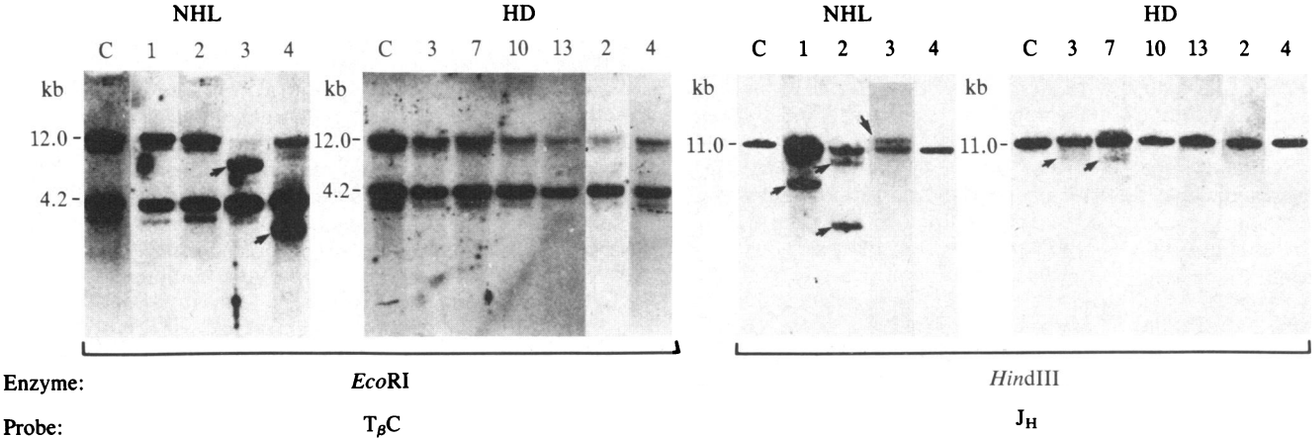

FIG. 1. Southern blot hybridization analysis of H-chain and T_β genes in DNA extracted from NHLs and from HD. Lanes C (human fibroblast DNA) show the germline restriction enzyme pattern of the T_β and H-chain genes after digestion with *Eco*RI or *Hin*dIII and hybridization to a T_β or J_H probe, respectively, as indicated in the two NHL panels. Lanes 1–4 show the organization of the T_β and the H-chain loci of two B-cell NHLs (lanes 1 and 2) and two T-cell NHLs (lanes 3 and 4). The number above each lane in the HD panels corresponds to the patient number in Table 2. Rearrangement bands are indicated by arrows.

Table 2. Clinical, histopathologic, immunophenotypic, and genotypic characteristics of 15 cases of HD and two unclassified lymphoid neoplasms

Patient	Age, yr	Sex	Clinical stage	Histo-pathology	Immunophenotype										Immuno-genotype	
					Ia	B1	SIg	K/λ	E/T11	LEU1	T3	T4	T8	T4/T8	H chain	T_β
1	28	M	IA	HD, LP	29	23	21	1.6	66	60	56	19	52	0.4	G	G
2	41	F	IA	HD, LP	2	ND	1	ND	92	91	90	90	10	9.0	G	G
3*	29	M	IA	HD, LP	50	36	32	1.8	40	45	61	51	7	7.3	G	G
4	58	M	IIA	HD, NS	63	ND	16	1.7	77	76	77	63	12	5.3	G	G
5	16	F	IIA	HD, NS	48	39	32	1.6	50	52	51	38	14	2.7	G	G
6	26	M	IIA	HD, NS	37	35	34	1.9	53	ND	60	55	11	5.0	G	G
7*	12	M	IIA	ND, NS	48	ND	40	2.6	ND	53	45	38	17	2.2	G	G
8	36	F	IIA	HD, NS	19	ND	16	1.7	66	88	88	72	16	4.5	G	G
9	24	M	IIA	HD, NS	60	ND	29	2.0	44	63	58	60	12	5.0	G	G
10	52	M	IIB	HD, NS	13	ND	10	1.0	80	ND	78	59	18	3.3	G	G
11	25	F	IIIB	HD, NS	56	54	22	1.7	ND	40	39	25	17	1.7	G	G
12	22	M	IIIA	HD, MC	8	4	2	1.0	82	80	82	43	43	1.0	G	G
13	57	M	IIIA	HD, MC	63	ND	20	1.2	68	86	87	75	7	10.7	G	G
14*	68	M	IIIB	HD, MC	35	25	21	1.6	ND	58	62	54	9	6.0	G	G
15	23	M	IIIB	HD, MC	17	ND	16	1.3	69	ND	77	62	18	3.4	G	G
16	25	M	I	Unc	33	24	24	2.4	60	55	59	40	19	2.1	R	G
17	74	M	IV	Unc	33	ND	17	8.5	ND	ND	60	38	17	2.2	R	G

LP, lymphocyte predominance; NS, nodular sclerosis; Unc, unclassified; MC, mixed cellularity; ND, not done; SIg, surface Ig; G, germline; R, rearranged.
*Minor clonal populations (<1%) detected.

ment bands in all 103 cases were of high intensity and easily identified, consistent with the high percentage of clonal tumor cells in each sample.

Next, we studied 15 patients with clinically and morphologically typical HD (Table 2, patients 1–15). These cases were subclassified as lymphocyte predominance, mixed cellularity, and nodular sclerosis in 3, 4, and 8 instances, respectively. None of these cases contained a clonal B-cell expansion by immunophenotypic analysis. Seven cases exhibited an increase in the total proportion of T cells and 9 cases had an increase in the $T3^+T4^+$ or the $T3^+T8^+$ T-cell subsets beyond that usually seen in benign and hyperplastic lymph nodes, but similar to that described in HD (10).

We investigated the organization of the immunoglobulin and the T_β gene loci in each of these 15 cases of HD by Southern blot hybridization analysis of DNA extracted from cryopreserved blocks of solid tissue and/or isolated cells in suspension under the same experimental conditions used in our analysis of NHLs and LLs. None of the 15 cases exhibited clonal immunoglobulin or T_β gene rearrangement bands upon hybridization of *Eco*RI- and *Hin*dIII-digested DNA to J_H, C_κ, and C_λ probes and *Eco*RI-, *Hin*dIII-, and *Bam*HI-digested DNA to a T_β probe. However, using sensitive experimental conditions involving high specific activity probes and long autoradiographic exposure times, barely detectable clonal immunoglobulin and/or T_β gene rearrangement bands appeared in 3 of the 15 cases of HD (Fig. 1). The extremely low intensity of these hybridization signals suggests the presence of clonal populations representing ≈1% or less of the total cell population present in each of these 3 pathologic samples. In addition, several cases displayed a variable decrease in the intensity of the *Eco*RI 12.0-kb T_β band. The intensity of this band appeared to decrease in direct proportion to the increase in the number of T cells in the sample (Table 2 and Fig. 1). We had previously demonstrated that this pattern, in the absence of rearrangement bands, represents a genetic marker of polyclonal T cells (19).

These results indicate that pathologic samples of non-Hodgkin's lymphoid neoplasia and HD clearly differ with respect to the frequency and abundance of clonal B- or T-cell populations. Sizable monoclonal lymphoid populations (>10%) are detectable in all NHLs and LLs but are absent from HD. Minor clonal B- and/or T-cell populations (≤1%) are detectable in a small minority of cases of HD. We conclude that the identification of sizable clonal B- or T-cell populations by immunogenotypic analysis represents a useful diagnostic adjunct for distinguishing non-Hodgkin's lymphoid neoplasia from HD in cases in which the differential diagnosis cannot be determined by histopathologic and/or immunophenotypic criteria.

Immunoglobulin and T_β Gene Rearrangement Analysis of RS Cell-Rich HD. RS cells are only present in small numbers in most cases of HD (1). Therefore, our inability to detect sizable clonal B- or T-cell populations in samples of classical HD does not exclude the possibility that RS cells represent clonal B- or T-cell expansions. Such minor cell populations may merely fall below the threshold of sensitivity of our technique. This argument is supported by our identification of weak hybridization signals in a small number of cases of HD.

Therefore, we directly addressed the issue of the lineage and clonality of RS cells by investigating the organization of the immunoglobulin and the T_β gene loci in three patients with lymphocyte-depleted, RS-cell-rich HD. These three samples contained clusters and sheets of RS and RS-cell variants, which constituted from 25% to >50% of the total cell population (Fig. 2), considerably above the threshold of detectability of clonal gene rearrangements by our methods. Immunohistologic analysis demonstrated that each patient had a small polyclonal B-cell population, a mixed T-cell population, and variable numbers of LeuM1 antigen-positive large cells, an immunophenotypic profile consistent with HD.

We analyzed the organization of the immunoglobulin and the T_β genes in these three patients by Southern blot hybridization analysis of DNA extracted from cryopreserved blocks of solid tissue. This approach avoided RS cell loss, which might occur during cell isolation procedures. None of the three patients displayed clonal immunoglobulin or T_β gene rearrangements upon hybridization of *Eco*RI-, *Hin*dIII-, or *Bam*HI-digested DNA to J_H and T_β probes, respectively (Fig. 3). In the same experiment, we added an internal control, which clearly shows that the sensitivity of our assay is at least 5%. Thus, any clonal gene rearrangements that existed in these RS cell populations would have been readily

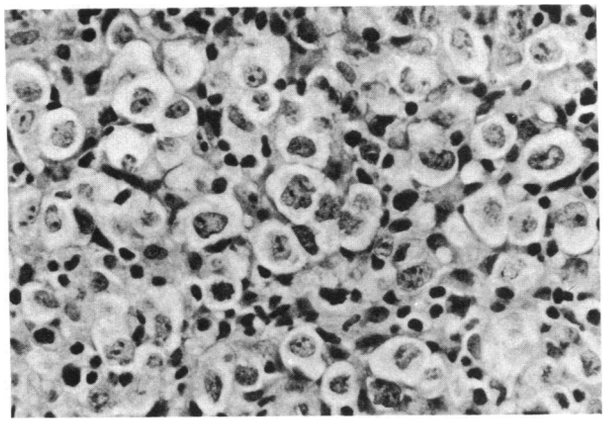

FIG. 2. The biopsy samples from three patients displayed the histopathology of lymphocyte-depleted RS-cell-rich HD. Each biopsy sample contained areas almost entirely composed of atypical mononuclear cells, including sheets of lacunar cells, RS cells, and RS cell variants. (×390.)

detected. These results provide direct evidence that RS cells and their morphologic variants do not represent clonal B- or T-cell populations and indirect evidence that the minor clonal populations that are detected in occasional cases of HD do not represent RS cells.

Additional preliminary information concerning the lineage of RS cells may be deduced from the T_β gene rearrangement patterns in these three cases of RS-cell-rich HD. We have already mentioned that the intensity of the *Eco*RI 12.0-kb T_β band, the disappearance of which represents a marker of the T-cell lineage (19), approximately correlated with the percentage of $T3^+$ (T) cells present in each HD sample. Experiments involving the dilution of polyclonal T-cell DNA with T_β germline DNA indicate that this band disappears when >80% of the sample DNA is from T cells. The three RS-cell-rich cases of HD display the pattern consistent with a very high percentage of T cells (Fig. 3). In particular, one patient with >50% RS cells (Fig. 2) almost entirely lacked the *Eco*RI 12.0-kb T_β band, indicating that at least 80% of the cells in this sample are polyclonal T cells. Therefore, a simple arithmetic calculation allows the conclusion that the RS cells must account for a substantial portion of the total T-cell population in this sample, which is contributing to the disappearance of this band. These data preliminarily suggest

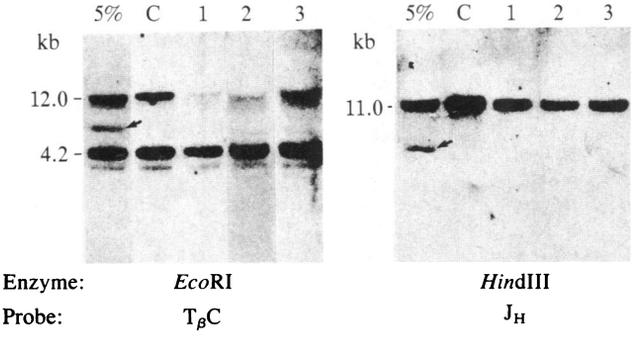

FIG. 3. Southern blot hybridization analysis of H-chain and T_β genes in three cases of HD containing a high proportion of RS cells and RS cell variants. Lanes: C, human fibroblast DNA; 1–3, DNA from lymphocyte-depleted RS-cell-rich HD. Case 2 corresponds to DNA isolated from a portion of the pathologic sample of HD illustrated in Fig. 2. Lane 5%, DNAs extracted from T- and B-cell NHLs were diluted to 5% with human fibroblast DNA, digested with *Eco*RI or *Hin*dIII restriction enzymes, and hybridized to $T_\beta C$ or J_H probes, respectively, as indicated. Rearrangement bands are indicated by arrows.

that RS cells may represent a polyclonal T-cell or T-cell-related population that contains unrearranged T_β genes.

DISCUSSION

A large number of morphologic and immunophenotypic studies have contributed to the characterization of HD (3–8, 10–12, 33–35). However, these studies have not determined the lineage and the clonality of the lymphoid cells and the RS cells of HD. We addressed these questions directly by investigating the organization of the immunoglobulin and T_β gene loci of a panel of cases representative of each clinical and morphologic subtype of HD. This approach allows the unequivocal identification of polyclonal T cells and of clonal B- and T-cell populations, even when the latter represent as little as 1% of a pathologic sample. The results of our studies bear important implications for the differential diagnosis between HD and non-Hodgkin's lymphoid neoplasia, and the lineage derivation and the clonal origin of the major pathogenetic component of HD, the RS cell.

First, our studies demonstrate that immunogenotypic analysis is an accurate and objective tool in the differential diagnosis between non-Hodgkin's lymphoid neoplasia and HD. This approach has considerable diagnostic value in all instances in which this differential arises since our results were comparable for all subcategories of NHL and HD. All cases of non-Hodgkin's lymphoid neoplasia display easily detectable clonal immunoglobulin and/or T_β gene rearrangements corresponding to clonal B- or T-cell populations, which always represent ≥10%, and usually >50%, of the total cells in the pathologic sample. In contrast, only occasional cases of HD contain minor clonal populations representing <1% of the total cells in the pathologic sample. The minimal size of these minor clonal populations and the consequent substantial sensitivity of the assay required for their detection virtually eliminate any possibility of mistaking them for a major clonal population typical of NHL. Thus, the identification of a clonal population ≥10%—i.e., clearly visible by classic Southern blot hybridization analysis—within a pathologic sample appears to represent a definitive and unequivocal marker for NHL or LL and eliminates HD as a diagnostic consideration. The detection of extremely minor clonal populations requires further examination to distinguish between HD and a NHL clone, which is poorly represented in the pathologic sample.

We preliminarily tested the utility of this immunogenotypic approach by analyzing two lymphoid neoplasms in which the histopathologic differential between NHL and HD was difficult (Table 2, patients 16 and 17). Both patients predominantly had T cells and contained only a small proportion of B cells, similar to the cell marker profile commonly seen in HD (10). A monoclonal B-cell proliferation could not be convincingly demonstrated by immunophenotypic analysis in either case because of the small numbers of B cells, although κ light-chain-positive B cells clearly predominated in one instance (patient 17). We retrospectively investigated the organization of the immunoglobulin and the T_β gene loci and found prominent high intensity clonal immunoglobulin gene rearrangement bands, characteristic of a monoclonal B-cell proliferation, in each case. These results strongly suggest that NHL and not HD is the correct diagnosis in these two cases. These results further illustrate the utility of immunogenotypic analysis in the accurate diagnosis of NHL and its differential from HD, although prospective studies of additional similar cases are necessary.

The lymphoid cells of HD vary considerably in quantity and in cytomorphologic and immunophenotypic composition from case to case (1, 10). Our results clearly indicate that these diverse lymphoid populations, including the sizable expansions of $T4^+$ or $T8^+$ T cells present in some cases of

HD, are predominantly polyclonal. Preliminary observations suggest that the minor clonal populations demonstrable in a small number of cases of HD may not have pathogenetic significance. Their only occasional presence, their apparent indifferent B- or T-cell-lineage derivation, and their extremely small size strongly suggest a reactive and not a malignant nature. The possibility that they represent small benign clonal populations is not unprecedented, since similar clones are detected by immunogenotypic analysis of other histologically complex, but apparently benign, lymphoid proliferations such as toxoplasmic lymphadenitis and acquired immunodeficiency syndrome (AIDS)-associated lymphadenopathy (36).

Finally, our studies address the critical and long-standing issues of the lineage derivation and clonality of the RS cell and its morphologic variants. Our data provide partial resolution by demonstrating that RS cells do not represent clonal B- or T-cell populations. These results leave open the possibility that RS cells represent polyclonal lymphoid cells or, alternatively, nonlymphoid cells, the clonality of which cannot be determined at the present time. In this context, our studies, based on T_β gene rearrangement analysis, provide the provocative but preliminary suggestion that RS cells may represent polyclonal T cells or a T-cell-related population. A postulated relationship between RS cells and polyclonal activated T cells should not be surprising in view of the results of several recent studies that demonstrate that RS cells express a variety of antigens associated with T-cell activation, such as HLA-DR, LeuM1, and Tac (interleukin 2 receptor), which are absent from resting peripheral T cells (11, 12, 33, 34). Interestingly, monoclonal antibodies KI-1 and HeFi-I, prepared by immunizing with the HD cell line L428 and which preferentially react with RS cells (35, 37), also react with some activated and neoplastic T cells but not with mature peripheral lymphoid tissue (37, 38).

We thank Dr. T. Mak and S. Korsmeyer for the T-cell receptor β chain and immunoglobulin DNA probes, and Angela Ubriaco and Laurie McFall for excellent technical assistance. These studies were partially supported by National Institutes of Health Grants EYO6337 (D.M.K.), CA37165 (R.D.-F.), CA37295 (R.D.-F.), CA26422 (C.D.W.), and CA33572 (C.D.W.); the New York State AIDS Institute (D.M.K.); The Bernard and Frances Laterman Project Chai Philanthropic Trust (D.M.K.); and the James H. Harless Research Fund (C.D.W.). A.N. is supported by a Fellowship from the Associazione Italiana Ricerca Sul Cancro. R.D.-F. is a Scholar of the Leukemia Society of America.

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