TRANSLOCATION t(14;18) IN B CELL LYMPHOMAS
AS A CAUSE FOR DEFECTIVE IMMUNOGLOBULIN PRODUCTION

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B cell non-Hodgkin's lymphomas (NHL) represent malignant counterparts of the various stages of the physiological B cell development. Many immunophenotypic and functional properties are retained in the malignant situation. Thus, follicle center cell (FCC)-derived lymphomas have an immunophenotype of mature B cells with, among other antigens, weak or absent expression of CD10 and strong expression of CD19, CD20, CD22, HLA-DR, and surface Igs. In 10–30% of the cases, however, the cells fail to express detectable Ig proteins on the membrane or in the cytoplasm (1, 2). It is not known why Ig are not expressed in these mature B cells. Besides at the gene level, a block in the Ig synthesis could be located at any step between gene and product: Ig gene transcription, processing of the primary transcript, translation of mature mRNA, or assembly of the final protein product. At the gene level, Ig synthesis requires a functional Ig gene rearrangement on one of the alleles. Nonfunctional rearrangements can be produced by association with pseudo genes, of which three are interspersed between the JH genes, and out-of-phase rearrangement of VH and JH genes. Gene transcription may also be disturbed by stop codons, nonsense codons, or mutations in promoter regions or splice sites created by the recombinational processes.

A reciprocal translocation t(14;18)(q32;q21) is found in ~85% of follicular lymphomas. In this translocation, the bcl-2 gene on chromosome 18 is joined to one of the JH genes by similar mechanisms as involved in normal VDJH joining (3). The resulting bcl-2/Igh hybrid cannot serve as a functional Ig gene. Also t(11;14)(q13;q32) and t(8;14)(q24;q32) may cause disruption and nonfunctionality of the involved Ig H chain gene (3, 4). In the present study, we investigated whether translocations, deletions, or aberrant rearrangements involving the Ig H chain genes might explain defective Ig H chain production in FCC-derived lymphomas and leukemias.

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1 Abbreviations used in this paper: ALL, acute lymphocytic leukemia; FCC/CC, follicular centroblastic/centrocytic lymphomas; FCC, follicle center cell; HRP, horseradish peroxidase; NHL, non-Hodgkin's lymphoma; PCR, polymerase chain reaction; TdT, terminal transferase; TRITC, tetramethyl-rhodamin isothiocyanate.
Patients

49 patients with FCC-derived NHL collected in the Laboratory of Pathology, University of Leiden, between 1981 and 1988 were studied. Histological diagnoses were made according to the Kiel classification (5). In eight cases, multiple successive samples were available. 53 lymph node biopsies and one pleural effusion aspirate from a patient with a histologically documented history of follicular lymphoma (case 44; Table II) were studied. Blood cells of two patients with an unusual form of B cell acute lymphocytic leukemia (ALL) were analysed. In total, 19 cases of FCC-derived tumors lacked expression of Ig H chains (Table I).

Materials and Methods

Immunophenotypic Analysis

For detection of Ig expression, two methods were applied in all cases studied:

Indirect Immunoperoxidase Tests. Cryostat sections were incubated with rabbit polyclonal F(ab)2 anti-Ig L chain and IgM, IgD, IgG, and IgA H chain antisera (Dakopatts, Copenhagen, Denmark) and with a horseradish peroxidase (HRP)-conjugated swine anti-rabbit antiserum (Dakopatts) as a second layer. Staining was performed with 3-amino-9-ethylcarbazol (Aldrich Chemical Co., Beerse, Belgium). Two cases were studied with mouse mAbs raised against IgGl, -2, -3, -4, and IgAl and -2 subclasses (Central Laboratory of Blood Transfusion, CLB, Amsterdam; Dr. J. Haaymans, TNO, Rijswijk, The Netherlands) in an indirect immunoperoxidase test. The second step consisted of a rabbit anti-mouse HRP-conjugated antiserum (Dakopatts).

Direct Two-color Immunofluorescence Tests. Cryostat sections were incubated with burro, goat, or rabbit polyclonal FITC-conjugated anti-Ig antibodies against \( \kappa, \) IgM, IgD, IgG, and IgA, in combination with a tetramethyl-rhodamin isothiocyanate (TRITC)-conjugated antiserum against \( \lambda \) L chains. A separate section was incubated with an FITC-conjugated anti-\( \lambda \) antiserum. Antisera were obtained from Kallestad Laboratories, Inc. (Austin, TX), Nordic (Tilburg, The Netherlands), and Dakopatts.

For detection of B cell- and T cell-associated antigens in frozen tissue sections, a two- or three-step indirect immunoperoxidase assay was used, the latter consisting of subsequent incubation with HRP-conjugated rabbit anti-mouse and HRP-conjugated swine anti-rabbit antisera (Dakopatts). The following primary antibodies were used: CD2 (CLB-T11; CLB), CD3 (Leu-4), CD4 (Leu-3), CD5 (Leu-1), CD8 (Leu-2) (all obtained from Becton Dickinson & Co., Sunnyvale, CA), CD10 (J5), CD19 (B4), CD20 (B1) (all obtained from Coulter Electronics, Inc., Hialeah, FL), CD22 (Leu-14), and anti-HLA-Dr (Becton Dickinson & Co.). The lymphoid origin of all diffuse large cell lymphomas tested was confirmed by the staining for CD45 (Dako-LC; Dakopatts).

Both cases of ALL were immunophenotyped in suspension and on cytocentrifuge preparations (6). Primary antibodies used were: goat or burro FITC and TRITC-conjugated anti-Ig antisera (Kallestad, Nordic), for detection of cell surface Ig and cytoplasmic \( \kappa \) chains, rabbit anti-terminal transferase (TdT) (Bethesda Research Laboratories, Gaithersburg, MD), CD10 (BA3) (Hybritech, Luik, Belgium), CD19 (B4), and CD20 (B1), CD24 (BA1) (Hybritech), CD2 (Leu-5) (Becton Dickinson & Co.), and CD7 (WT1) (Sanbio, Uden, The Netherlands).

Immunogenotypic Analysis

High molecular weight DNA was isolated according to standard methods. The DNAs were digested to completion with various restriction enzymes, size fractionated in 0.7% agarose gels, and transferred to Genescreen plus® or Bioline filters. Blots were hybridized with random primer \( ^{32}P \)-labeled probes, washed in 0.3 \( \times \) SSC/0.1% SDS at 65°C, and autoradiographed at -70°C. The filters were reused up to five times with different probes after removal of the previous signal by "stripping" in 0.1 \( \times \) SSC/0.1% SDS at 100°C (controlled by overnight exposure).

The H chain rearrangements were detected with a 2.4-kb Sau IIIa Ig JH probe in Eco RI-, Hind III-, and Bam HI-digested DNA in all cases (7). In case the number of rearranged H chain alleles could not definitively be determined, additional hybridizations were done with Pst I-, Bgl II-, Sac I-, and Xba I-digested tumor DNA. The same filters were
hybridized with a 2.8-kb Eco RI–Hind III fragment of the bcl-2 gene containing the major breakpoint area and a 3' flanking 1.5-kb Hind III–Eco RI fragment to study the translocation t(14;18)(q32;q21). Rearrangements of Ig genes were studied with a 2.5-kb Eco RI ck probe in Bam HI- and Hind III-digested DNA and λ gene rearrangements with a 3.5-kb Eco RI–Hind III ck-2 probe, using Eco RI-digested DNA. The c-myc gene was studied with a 1.4-kb Eco RI–Cla I fragment containing the third exon of c-myc.

Cytogenetic Analysis

Cytogenetic analyses were performed on three peripheral blood samples, three lymph node biopsies, and one pleural fluid aspirate. The cells were cultured for 3, 5, and 7 d in the presence of PHA (Wellcome, Dartford, UK), Escherichia coli 055:B5 LPS B (Difco Laboratories, Inc., Detroit, MI) or phorbol 12-myristate 13-acetate (Sigma Chemical Co., St. Louis, MO).

Polymerase Chain Reaction (PCR)

DNA sequences of the translocation junction of t(14;18) were amplified, using a modified method as described by Lee et al. (8). Two synthetic oligonucleotides were synthesized as primer for PCR. One primer was complementary to the plus strand of the major breakpoint region of the bcl-2 gene (5'-TTTGAC CTT TAG AGA GTT GC-3'), the other complementary to the minus strand of the homologous region 3' of each JH gene (5'-ACCTGA GGA GAC GGT GAC C-3'). Samples (0.5–1 μg) of tumor DNA were denatured for 5 min at 100°C in a buffer containing 30 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl2, 0.2 mg/ml acetylated BSA, 200 μM deoxynucleotide triphosphate (each of four), and 1 μM of each primer. 1 U of heat-stable Taq-DNA-polymerase was added (Perkin-Elmer Corp., Norwalk, CT) (9). 32 cycles of denaturing (1 min; 100°C) and reannealing/extension (six cycles for 2 min, six cycles for 3 min, six cycles for 3.5 min, six cycles for 4 min, eight cycles for 5 min; 65°C) were performed, using an automated robot system (ROB 3; P & P Elektronic, Köln, FRG). Filters were made by spotting 1 μl of 100 μl of the samples on Genescreen Plus filters and by Southern blotting after size separating of 5 μl of 100 μl of the samples on 1.7% agarose gels. The filters were hybridized to JH and bcl-2 probes as described above.

Results

Immunophenotypic Analysis. 16 cases of FCC-derived tumors lacked IgH expression. 12 of these also lacked L chain expression. All lymphomas showed reactivity with at least two of the B cell–specific reagents, CD19, CD20, or CD22, but no reactivity with two panels of T cell–specific or –associated antibodies (CD2 and CD3 or CD4, CD5, and CD8). One patient presented with a composite B cell lymphoma of Ig- follicular lymphoma and Ig- lymphoblastic lymphoma. Both components were studied separately after FACS and have been described elsewhere (10) (enumerated as cases 6 and 17; Table I). Two cases of Ig- B-ALL were also studied. Case 18 presented with a relapse ALL-L3 according to the French-American-British (FAB) leukemia classification. Although the tumor cells were positive for B cell markers and CD10, they did not express Ig or TdT. Patient 19 had an Ig−, TdT− ALL (FAB-L2) with expression of B cell markers and CD10.

30 Ig H chain–positive FCC-derived NHL were studied as controls (Table II). The B cell origin of the tumor cells was demonstrated by the monotypic Ig expression in combination with reactivity with at least one of the B cell–specific reagents.

DNA and Karyotype Analysis. All cases studied showed rearrangement of one or both Ig H chain alleles (Tables I and II). The Ig L chain genes were also rearranged in 40 cases analyzed. Since in t(14;18) the bcl-2 gene on the long arm of chromosome 18 adjoining the JH genes, this translocation could be determined by detection of rearranged fragments of similar size by both the JH and the bcl-2 probes on basis of
TABLE I

Immunogenotype and translocations in IgH-
FCC-derived Lymphomas and B-ALL

| Diagnosis | Cases | H chain | L chain | BCL-2 | Other |
|-----------|-------|---------|---------|-------|-------|
| FCC/CB    | 1     | 2R      | 1R      | G     | -     | 14q'  |
| 2         | 1R    | 2R      | 1R      | +     |       | R c-myc|
| 3a/b      | 2R    | 1R      | 1R      | +     |       | c-myc |
| 4         | 1R    | 1R      | G       | +     |       |       |
| 5         | 1R    | 1R      | G       | +     |       |       |
| 6*        | 2R    | 1R      | 1R      | +     |       |       |
| 7a/b      | 2R    | 1R      | G       | +     |       |       |
| 8         | 1R    | 1R      | 1R      |       |       |       |
| DCB       | 9     | 2R      | G       | 1R    | +     |       |
| 10        | 1R    | 2R      | G       | +     |       |       |
| 11        | 2R    | 2R      | G       |       |       |       |
| 12        | 1R    | G       | 1R      |       |       |       |
| 13        | 2R    | 1R      | G       |       |       | R c-myc|
| DCB/CC    | 14    | 2R      | 1R      | G     |       |       |
| 15        | 2R    | 1R      | G       |       |       |       |
| 16        | 1R    | 1R      | ND      | +     | t(14;18) |
| LBL       | 17*   | 2R      | 1R      | 2R    | +     | t(8;14); R c-myc |
| 18        | 2R    | ND      | ND      | +     | t(8;14); R c-myc |
| 19        | 1R    | ND      | ND      | +     | -14 |  |

a/b, samples of two successive biopsies were available; R, rearrangement; -, negative; +, positive.
* Tumors 6 and 17 were found in the same patient. Case 6 represents a follicular lymphoma, in which a lymphoblastic leukemia developed (case 17).
1 Karyotype analysis: 47Y, -X, 1q', 1q', 1p', 3p', 6q', 7q', +12q', 13q', 14q', 15q', +16, -20, 22q'.
5 Karyotype analysis: 46XX, +1, -6, 14q'.
5 Karyotype analysis: 46X, -Y, -9, del(12)(p12), -13, -14, t(14;18), + markers.

Comigration. In 14 of 19 (74%) follicular centroblastic/centrocytic lymphomas (FCC/CC), four of seven (57%) diffuse CB/CC, 11 of 20 (55%) diffuse CB, and in all three lymphoblastic lymphoma/leukemia, t(14;18) was found (Tables I and II; Fig. 1 A–C). The majority of the breakpoints in the bcl-2 gene are clustered within the major breakpoint region, which is located in a 4.3 Hind III fragment (11). Involvement of this region was demonstrated by Southern blotting in 18 of 32 cases with bcl-2 rearrangement (56%) and confirmed by amplification of the breakpoint region with the PCR in 16 cases (Fig. 2). The other bcl-2 rearrangements occurred outside the major breakpoint region. No significant differences in incidence of translocations and location of the breakpoints were found between the IgH+ and IgH- follicular lymphomas. In contrast, diffuse IgH- lymphomas had more translocations than the diffuse IgH+ lymphomas (6 of 8 and 9 of 19, respectively). The numbers in each group are too small, however, to attribute significance to this finding at present (χ² test, 0.05 < p < 0.1).

In 8 of 19 Ig- cases, only one Ig H chain allele was found to be rearranged on hybridization with JH after digestion with at least three restriction enzymes. In seven of these eight cases this allele was shown to be involved in t(14;18) by comigration
Two different translocations t(14;18) were present. In the remaining 11 Ig" cases, both H chain alleles were rearranged. In 8 of these 11 cases, a t(14;18) was present involving one of the alleles. In four of these eight cases, additional rearrangement of c-myc was found on the DNA level. Cytogenetical analysis of two cases showed both the t(8;14), in which c-myc is involved, and t(14;18) together in single cells, indicating that both Ig H chain alleles were not functional.

Using the presence of restriction sites for the enzymes Bst E2, Pvu II, Rsa I, and Eco RV between the JH genes in the germline situation, restriction maps of the rearranged IgH alleles could be constructed, thus indicating which of the JH genes was involved in the rearrangement. The restriction map of case 7 strongly suggests rearrangement to the pseudo-JH3 gene (Fig. 3).
FIGURE 1. Southern blot analysis of Ig H chain and bcl-2 gene organization. DNA from Ig- lymphomas (A), an Ig- lymphoblastic leukemia (B), and Ig- lymphomas (C) was digested with the restriction enzymes indicated. Each filter was hybridized with the JH probes (lanes a) and the bcl-2 probes (lanes b). Case 18 was also hybridized with a c-myc probe (lanes c). Lane m shows the corresponding size marker. Sizes are given in kb pairs. Arrowheads indicate the germline configuration of the genes, as are also present in the contaminating normal cells in each biopsy sample. Dashes indicate the rearranged fragments. Comigrating fragments are indicated by double dashes between the lanes and are seen in all samples except case 29. In case 18, the c-myc gene is rearranged by t(8;14). No comigration of c-myc and JH is seen. The translocation has most probably taken place in an Ig switch region as in sporadic (non-African) Burkitt's lymphoma (20).
As controls, 30 IgH+ FCC-derived NHL were examined (Table II). Two rearranged H chain alleles were found in 19 cases, of which 14 had t(14;18) on one IgH allele and functional rearrangement of the other allele. One rearranged H chain allele without rearrangement of bcl-2 was demonstrated in eight cases. In two additional cases (29 and 49), however, a single rearranged H chain allele was found in

Several larger fragments are present in cases 7 and 6 (a). These are caused by the presence of JH genes 3' of the bcl-2/JH join. Since each of these genes contains the sequence that is recognized by the primer for the JH genes, these segments are also amplified. Digestion of the amplified DNA with BstE2, which cuts 5' of each JH gene (except JH2), digests away these "tails" of the bcl-2/JH junction, resulting in the abolishment of extra fragments on hybridization with the bcl-2 probe (6). Sizes are given in bp.
FIGURE 3. Restriction map of case 7. T(14;18) has taken place on JH4 (allele A). The other Ig allele (B) is nonfunctionally rearranged on the pseudo-JH3 gene.

FIGURE 4. Analysis of a biclonal lymphoma (case 30). For Southern blot analysis, filters were hybridized with the JH probe (lanes a) and the bcl-2 probe (lane b). Arrowheads indicate the germline configuration of the genes; dashes indicate the rearranged bands. Double dashes between the lanes indicate comigrating bands, of which two are found. In amplification with PCR: upon hybridization with the bcl-2-probe, two fragments of 230 and 200 bp are seen (arrow) (lane c).
combination with rearrangement of bcl-2 in the major breakpoint region. In case 29, this finding was confirmed with eight endonucleases. In both cases, no comigration of bcl-2 and JH was present, however, and the translocation breakpoint could not be amplified with PCR (Fig. 1C), indicating either a variant translocation breakpoint on chromosome 14 or association of bcl-2 with an unrelated chromosomal region. In one case (43) rearrangement of c-myc was found. No association with JH was present.

Case 30 is of special interest. Immunophenotypical analysis with Ig subclass-specific antibodies showed uniform expression of IgG1 and λ and patchy positivity for IgA2. Hybridization with JH using four restriction enzymes indicated four rearranged bands. Also, two rearranged κ L chain alleles and three rearranged λ L chain alleles were found. These findings suggest a biclonal origin of this tumor. Two different rearrangements of bcl-2 were found and comigrated each with a different JH fragment. Moreover, by amplifying the bcl-2/JH breakpoint with PCR, the two different breakpoints could be demonstrated (Fig. 4).

Discussion

Although FCC-derived lymphomas correspond to mature stages in the physiological B cell development and should thus express Ig on their cell membranes, a considerable percentage lack Ig expression (1, 2). Why these tumor cells do not produce Ig is not clear. Apart from deficiencies at the RNA or protein level or in regulatory events, the unavailability of a functional Ig H chain gene may be a cause for the lack of Ig production in these tumors.

We have studied causes of defective Ig H chain synthesis on the gene level in 17 IgH- FCC-derived lymphomas and two cases of ALL. The latter were strongly suggested to be FCC related by the exceptional presence of t(14;18) and their unusual phenotype. In seven cases, a t(14;18) that disturbs the Ig H chain gene was present while the other Ig allele was either deleted or in germline. The absence of a functional IgH allele therefore indeed excludes Ig H chain production in these cases. In four other cases the t(14;18) was accompanied by rearrangement of the c-myc gene on chromosome 8. Karyotype analysis in two of four cases identified an underlying t(8;14). Involvement of both Ig H chain genes in these translocations identifies this as the cause of lacking Ig production. In the two other cases a similar mechanism may be involved, although cytogenetic proof of t(8;14) could not be given, since only frozen material was available. Analysis of the separated components of the composite lymphoma (samples 6 and 17) revealed that consecutive oncogene activation and clonal evolution led to the development of the lymphoblastic lymphoma out of a precursor cell of the follicular tumor constituent (9). The lymphoblastic component had both translocations, while the follicular component, which was also Ig-, only had the t(14;18). Since only one Ig H chain allele was involved in t(14;18), another mechanism must account for defective IgH production in this tumor constituent.

Translocation and activation of c-myc have been associated with leukemic progression in FCC-derived lymphomas (12). Two cases with t(8;14) and t(14;18) indeed presented as lymphoblastic leukemia. Two cases with combined c-myc and bcl-2 rearrangements and one with c-myc rearrangement only, however, were nonleukemic diffuse centroblastic lymphomas. These different clinical and morphological forms indicate that translocation and activation of c-myc may be involved in progression of follicular lymphoma, though not necessarily to leukemic forms.
In 8 of 19 Ig⁻ cases, at least one rearranged IgH allele was present and shown not to be involved in a translocation (14;18) to the major and minor breakpoint regions in the 3' region of the bcl-2 gene. We did not study the 5' region of bcl-2, where very few breakpoints are reported (13). In a recent study, antibodies were raised against the bcl-2 protein products. Expression of bcl-2 could be shown in almost all follicular lymphomas studied, including 4 of 26 lymphomas in which no bcl-2 DNA rearrangement could be demonstrated (14). The authors suggest that these tumors had a t(14;18) with breakpoints on chromosome 18 outside the regions detected by their probes. In view of these results, therefore, we cannot exclude that yet more IgH⁻ tumors had t(14;18) as the cause of defective Ig synthesis.

As discussed above, several other mechanisms directly related to the recombinational process may create nonfunctional IgH genes. The involvement of the pseudo-JH3 gene in case 7 may be an example of inactivation of one IgH allele by t(14;18) and the other by nonfunctional rearrangement leading to defective Ig production. Indications for defects in transcription were given by Siminovitch et al. (15), who described several diffuse B cell lymphoma cell lines without Ig H chain expression, in which one or both H chain alleles were rearranged. Truncated Ig H and L chain mRNAs were seen on Northern blots in one case, suggesting transcriptional defects (15). The contribution of other possible mechanisms in defective Ig production may be revealed by cloning and sequencing of IgH alleles in Ig⁻ tumors and needs further research.

In 28 of 30 IgH⁺ lymphomas, at least one rearranged IgH allele was present and not involved in a translocation, accounting for Ig production. Although cases 29 and 49 showed a concomitant bcl-2 rearrangement with a breakpoint in the major breakpoint region, in none of the cases was comigration of JH and bcl-2 seen. PCR confirmed that JH and bcl-2 were not associated. This suggests a variant translocation of bcl-2, leaving the Ig H chain gene intact and available for Ig synthesis. Possible mechanisms for this are translocations to a VH region upstream from the functionally rearranged VDJ segment, or a bcl-2 translocation to another gene, which has a similar influence on bcl-2 expression during B cell maturation. This finding agrees with the findings of Doucette and Chaganti (16), who described translocations of bcl-2 to VH and switch regions. In case 43 with μ chain production, similarly, rearrangement of c-myc was accompanied by only one H chain rearrangement. In this case, a variant translocation of the c-myc gene to chromosome 2 or 22, as can be found in Burkitt's lymphoma, may be present (17).

We have not studied the mechanisms involved in the defective Ig L chain expression of the analyzed tumors. Only 4 of 22 Ig H chain-negative tumors had L chain expression. It is very unlikely that similar translocations and deletions are involved in both the L chain and the H chain genes in single tumors. More likely, regulatory events may play a role (18, 19).

In conclusion, various molecular defects on the gene level may account for defects in the Ig H chain synthesis in FCC-derived lymphomas. Our data show that among these defects t(14;18), translocation, which is essentially related to this type of lymphoma, plays a major role.

**Summary**

Although follicle center cell (FCC) lymphomas represent mature B cells, a considerable percentage do not have detectable Ig production. We have used Southern
blotting and the polymerase chain reaction (PCR) to study the involvement of translocations t(14;18) and t(8;14) in causing defective Ig production in 16 Ig⁺ FCC-derived lymphomas and three Ig⁻ B cell acute lymphoblastic leukemias. In 6 of 19 cases, a t(14;18) was present with the other allele either deleted or in germline. In two cases a t(14;18) and a t(8;14) affected both Ig alleles, as confirmed by karyotyping. In two other cases, rearrangement of both bcl-2 on chromosome 18 and c-myc on chromosome 8 were found as well. Although cytogenetic proof was not available, the latter was probably involved in t(8;14). Restriction map analysis of one more case showed rearrangement on the pseudo-JH3 gene on one allele and t(14;18) on the other. Thus, in 11 of 19 cases, defective Ig H chain production could be explained by the inactivation of both Ig H chain genes due to translocation of one allele, in combination with deletions or defective rearrangements of the other allele. In contrast, in 28 of 30 Ig⁺ lymphomas, one functional Ig H chain allele was found, either in, or not in, combination with t(14;18). In two cases a single rearranged Ig H chain allele was found in combination with rearrangement of bcl-2. No comigration of the single Ig rearrangement with bcl-2, however, was found both by Southern blotting and PCR, suggesting a variant bcl-2 translocation, which leaves the Ig H chain allele functionally intact.

We thank Drs. Y. Tsujimoto and S. J. Korsmeyer for making available the 18q21/bcl-2 probes to us and Dr. Ph. Leder for the Ig probes. We gratefully acknowledge Drs. J. J. M. van Dongen, A. M. Hagemeijer-Hausman, and M. E. F. Prins, Department of Cell Biology and Genetics and Department of Clinical Pathology, Erasmus University, Rotterdam, The Netherlands, for supplying material of cases 1, 16, and 45 and for cytogenetical analysis of these cases; Mr. E. J. Dreef for immunophenotypical analysis; and Mr. R. M. L. Heruer and Mr. K. G. van der Ham for excellent photographic assistance.

Received for publication 11 October 1988.

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