Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 2916–2920, May 1987
Immunology

High-frequency expression of a conserved κ light-chain variable-region gene in chronic lymphocytic leukemia

(crossreactive idiotype/monoclonal antibody)

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Communicated by Ernest Beutler, December 29, 1986 (received for review November 25, 1986)

ABSTRACT **Malignant B lymphocytes from several patients with chronic lymphocytic leukemia (CLL) were examined for reactivity with murine monoclonal antibody 17.109. This antibody, prepared against the rheumatoid factor (RF) paraprotein Sie, recognizes a crossreactive idiotype on 48% of human IgM RF paraproteins, but does not react with IgM paraproteins without RF activity or substantially with normal pooled immunoglobulin. The 17.109-reactive idiotype is a marker for a κ III variable-region gene, designated V_κRF, that is conserved in outbred human populations. In a limited study of 31 CLL patients, the leukemic cells from 5 of 20 patients with κ light chain-expressing CLL were recognized by the 17.109 monoclonal antibody. Despite having malignant cells specifically reactive with this antibody, patients with 17.109-positive CLL did not have elevated serum levels of circulating antibody bearing 17.109-reactive determinants. Total RNAs isolated from the CLL B lymphocytes, or from hybridomas produced by fusing the CLL cells with the WI-L2-729-HF_2 cell line, were fractionated electrophoretically and examined by blot hybridization. Under stringent hybridization conditions capable of discerning a single base-pair mismatch, RNA from the 17.109-idiotype-positive CLL cells hybridized to synthetic oligonucleotide probes corresponding to framework and complementarity-determining regions in the V_κRF gene. The high frequency of the 17.109-associated idiotype and the V_κRF gene in CLL suggests that the disease may arise from B lymphocytes that express a restricted set of inherited immunoglobulin variable-region genes with little or no somatic mutation.**

Many mechanisms work to enhance the diversity of the antibody repertoire. The site of antigen binding, the antibody variable region, is formed by two polypeptide chains, each encoded by discontinuous genetic elements on different chromosomes (1–3), that rearrange during B-cell ontogeny (4, 5). Subsequent mutation of the expressed variable-region genes (*V* genes) generates antibodies with primary structures differing markedly from those possible through simple rearrangement of the genetic elements present in the germ-line DNA (6–12). Combined, these processes of somatic rearrangement and mutation generate an antibody repertoire of enormous diversity.

Testament to the success of these mechanisms is the strikingly small number of inherited human antibody *V* genes. For example, the human genome may contain only 25–50 κ light-chain *V* genes (13, 14), which have been divided into four variable-region subgroups (15, 16).

Analysis of antibody-producing B-cell tumors inept in the mechanism(s) of somatic mutation may allow identification of idiotypic determinants encoded by *V* genes prior to somatic mutation. Waldenstrom macroglobulinemia may represent such a B-cell tumor. In 1973, Kunkel *et al.* (17) demonstrated that IgM rheumatoid factor (RF) cryoglobulins from patients with this disorder often share crossreactive idiotypes (CRIs) (17). Using carefully absorbed rabbit antisera, they identified two mutually exclusive CRIs on RFs from unrelated individuals. The major Wa idiotypic group comprises nearly 65% of monoclonal RF proteins. Subsequent sequence analyses revealed that the Wa-CRI-positive macroglobulins have very homologous light chains that belong to the κ IIIb variable-region sub-subgroup (18–21).

To analyze further the RF-associated idiotypic antigens, a monoclonal antibody was prepared against the IgM RF cryoglobulin Sie (22). Reactive with 48% of human IgM-RF paraproteins but not with IgM paraproteins without RF activity or substantially with normal pooled immunoglobulin, this monoclonal antibody, 17.109, recognizes a CRI that is expressed by isolated κ light chains (23). Studies with antisera raised to synthetic peptides, corresponding to discrete epitopes within the κ variable region of 17.109-reactive proteins, showed that the crossreactive κ chains share identical second and third complementary-determining regions (CDRs) (24). The recent isolation, from human placental DNA, of a κ III *V* gene (designated V_κRF) that encodes an amino acid sequence identical to the variable-region sequence of four 17.109-reactive paraproteins from unrelated individuals strongly suggests that the CRI is a marker for a *V* gene that apparently is conserved in the outbred human population (25).

Similar to B cells of Waldenstrom macroglobulinemia, the malignant B lymphocytes from patients with chronic lymphocytic leukemia (CLL) may express a restricted set of immunoglobulin *V* genes. In 1972, Preud'homme and Seligmann (26) observed that CLL B cells have a high incidence of RF-like activity in their surface immunoglobulin (sIg). CLL B cells have IgM and sometimes IgD on the cell surface, but generally not other immunoglobulin isotypes (27). Furthermore, CLL cells also bear receptors for mouse erythrocytes and express the pan-T-cell surface antigen CD5 (28–30). B cells with this surface phenotype are enriched in precursors for IgM RF (30).

To test the hypothesis that CLL B cells may express CRIs that are the product of a limited and conserved κ *V*-gene repertoire, we examined CLL B lymphocytes from unrelated individuals for sIg reactive with monoclonal antibody 17.109. In an initial survey, 5 out of 20 κ-positive CLL cell populations reacted with the 17.109 anti-idiotype. That the reactive CLLs express similar, if not identical, κ light chains is supported by blot hybridization analyses of RNA from

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Abbreviations: CDR, complementarity-determining region; CLL, chronic lymphocytic leukemia; CRI, crossreactive idiotype; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; PE, R-phycoerythrin; RF, rheumatoid factor; sIg, surface immunoglobulin; *V* gene, variable-region gene.

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17.109-positive cells, using a panel of specific oligonucleotide probes.

MATERIALS AND METHODS

Antibodies. Hybridomas producing monoclonal antibodies specific for the constant region of human IgM (31) were acquired from the American Type Culture Collection. Hybridoma 17.109 (22) and hybridomas producing monoclonal antibodies to human IgG or to κ light chain or λ light chain were developed in our laboratory (unpublished results). Hybridomas were injected into pristane-primed BALB/c mice, and the resulting ascites were purified by ammonium sulfate precipitation, QAE ion-exchange chromatography, and, finally, by high-performance liquid size chromatography. Purified antibodies were conjugated either to fluorescein isothiocyanate (FITC; Calbiochem–Behring) or to R-phycoerythrin (PE; Cyanotech, Woodinville, VA). Coupling to PE was achieved by introducing an average of three sulfhydryl groups onto the purified antibody, using *N*-succinimidyl *S*-acetylthioacetate with subsequent deacetylation in hydroxylamine (32). After introduction of maleimide groups onto PE with *m*-maleimidobenzoyl *N*-hydroxysuccinimide ester (Calbiochem) (33), the modified antibody and PE were equilibrated in 0.01 M phosphate buffer (pH 6.4), mixed in equal molar ratios, and allowed to react at room temperature for 30 min under argon atmosphere. Unreacted sites were blocked with *N*-ethylmaleimide and then reduced in 2-mercaptoethanol. After dialysis against phosphate-buffered saline, pH 7.2 (PBS), the PE-antibody conjugates were size-separated by HPLC. PE-antibody conjugates were tested in ELISA and retained >95% specific binding activity. Fluorochrome-conjugated and biotinylated monoclonal antibodies to CD5 (Leu-1) or to CD3 (Leu-4), and PE-avidin, used to detect biotinylated antibodies, were purchased from Becton Dickinson.

Fluorescence-Activated Cell Sorter (FACS) Analyses. Two-color immunofluorescence studies were performed on a FACS 440 equipped with logarithmic amplifiers, and cell sorting was performed on a FACStar (Becton Dickinson). Data analyses on the FACS 440 utilized a Digital Equipment (Maynard, MA) VAX 750 mainframe computer and the "Electric Desk" program developed by W. Moore of Stanford University.

RNA Blot Hybridization. Total RNA was extracted from CLL cells or CLL–WI-L2-729-HF_2 hybridomas by use of guanidinium isothiocyanate as described (34). For blot analyses, 20 μg of total RNA was loaded per lane of a 1.8% agarose gel containing 2.2 M formaldehyde, 40 mM morpholinopropanesulfonic acid (pH 7.0), 10 mM sodium acetate, and 1 mM EDTA (35). Gels were run for 480 volt·hours. RNA was transferred onto Hybond-N membranes (Amersham/Searle) and then crosslinked with UV light. Filters were prehybridized for a minimum of 4 hr at 49°C in 6× SSPE (20× SSPE is 3.6 M NaCl/200 mM sodium phosphate, pH 7.4/20 mM EDTA, pH 7.4) containing 0.1% $NaDodSO_4$, 5× Denhardt's solution (1× is 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin) and 0.2 mg of yeast RNA per ml. Subsequently, each labeled oligonucleotide probe was added and allowed to hybridize for at least 24 hr at 49°C, except oligonucleotide D (Fig. 3), which was hybridized at 25°C. Hybridized filters were washed extensively at room temperature in 0.1% $NaDodSO_4$/6× SSPE and then for 1 min at the stringent wash temperature determined for each oligonucleotide (Table 2) (36). Washed filters were exposed to x-ray film (X-Omat; Kodak) for 1–5 days at −70°C with intensifying screens.

Oligonucleotides. Synthetic oligonucleotides were synthesized on a Pharmacia Gene Assembler using β-cyanoethylphosphoramidite chemistry. Oligonucleotides were purified and 5′-end-labeled, using [γ-^{32}P]ATP and polynucleotide kinase as described (36).

ELISA. Enzyme-linked immunosorbent assays (ELISA) for IgM and 17.109 idiotype were performed on polystyrene microtiter plates as described (37).

Cells. Blood was collected into heparinized syringes from normal volunteers and from patients satisfying diagnostic criteria for B-cell CLL (27). Mononuclear cells were purified on Ficoll-Hypaque. Cells not analyzed the same day were frozen in fetal bovine serum (FBS) containing 10% dimethyl sulfoxide for storage in liquid nitrogen. Prior to immunofluorescence analyses, washed cells were cultured at 37°C for 4–6 hr in RPMI-1640 medium supplemented with 7% FBS. Cell viability typically was >95% prior to FACS analysis.

The production of hybridomas formed by fusing CLL cells with the human B-lymphoblastoid cell line WI-L2-729-HF_2 has been reported (38). Hybridomas were cultured in RPMI-1640 with L-glutamine and 10% FBS at 37°C in 7% CO_2.

RESULTS

FACS Analyses of CLL. We examined the surface-antigen phenotype of peripheral blood lymphocytes from 31 patients with B-cell CLL (Table 1). Consistent with this primary diagnosis, 76–99% of peripheral lymphocytes from all but one of the patients expressed CD5 in addition to sIg. Twenty patients (65%) had cells expressing κ light chains. Of these 20 patients, 5 had malignant B cells that reacted with the 17.109

Table 1. Surface-antigen phenotype and ELISA data of plasma samples from CLL patients

Patient	sIg phenotype*	sIg$^+$ for 17.109†	% CD3$^+$ PBLs	Plasma IgM, μg/ml	
				Total	17.109†
HAH	IgM(κ)	+	<1	680	5
HUR	IgM(κ)	−	6	2100	4.2
WAN	IgM(κ)	−	4	570	1.6
EHM	IgM(κ)	−	<1	230	<0.8
BRO	IgM(κ)	+	<1	310	5.1
BAL	IgM(κ)	−	<1	40	<1
DER	IgG(κ)	−	4	1200	4.7
LES	IgM(κ)	−	24	500	4.2
LEE	IgM(κ)	−	5	1600	<1
HIC	IgM(κ)	+	<1	400	<1
MAS	IgM(κ)	−	<1	430	<0.8
GRI	IgM(κ)	−	2	400	<1.0
KEM	IgM(κ)	−	5	100	<0.8
POL	IgM(κ)	−	7	30	<0.8
WAT	IgM(κ)	−	<1	620	1.1
BLA	IgM(κ)	−	3	NT	NT
SMI	IgM(κ)	+	2	NT	NT
RYA	IgM(κ)	+	3	NT	NT
FUH	IgM(κ)	−	<1	NT	NT
WET	IgM(κ)	−	<1	NT	NT
FIS	IgM(λ)	−	3	600	1.2
MAJ	IgM(λ)	−	<1	200	<1
SLU	IgM(λ)	−	14	800	1.2
NEI	IgM(λ)	−	3	210	<0.8
EDW	λ	−	<1	400	<1.0
SUT	IgM(λ)	−	<1	140	<0.8
AND	IgM(λ)	−	<1	NT	NT
ESC	IgM(λ)	−	5	NT	NT
GRO	IgM(λ)	−	NT	NT	NT
HOD	IgM(λ)	−	NT	NT	NT
WHI	IgG(λ)	−	NT	NT	NT
Normal (n = 31)				1850 ± 1150	76 ± 102

Included are data (mean ± SD) from plasma obtained from 31 age-matched control volunteers. PBLs, peripheral blood lymphocytes; NT, not tested.

*sIg phenotype of malignant B lymphocytes.

†CLL B-cell reactivity with PE-17.109.

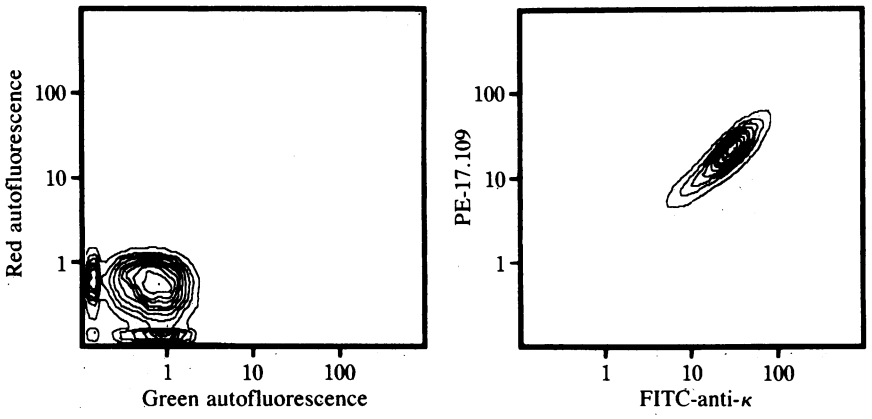

FIG. 1. Immunofluorescence detected at 575 nm (red, ordinate) and 525 nm (green, abscissa) of peripheral blood lymphocytes from patient HIC. (*Left*) Unstained cells. (*Right*) Cells stained with PE-17.109 and FITC-anti-κ light chain.

antibody (Fig. 1, Table 1). No individuals with λ light chain-expressing CLL had lymphocytes reactive with 17.109 (Table 1). The CLL cells did not react with a nonspecific IgG2b mouse monoclonal antibody that served as an isotype control for 17.109. Furthermore, <0.5% of the circulating lymphocytes from two disease-free control volunteers reacted with the 17.109 antibody (data not shown).

The dual immunofluorescence staining patterns of cells with PE-17.109 and FITC-anti-κ antibodies indicated that the 17.109 anti-idiotype reacted specifically with the κ light chains of the malignant lymphocytes. Although the staining intensity of CLL cells with PE-17.109 varied between patients, the level of staining correlated with that achieved with a non-cross-blocking monoclonal antibody against the κ light chain constant region (Fig. 1). Moreover, the diagonal fluorescence distributions seen after staining with PE.17.109 and FITC-anti-κ are consistent with both antibodies reacting with the same cell surface molecule.

ELISA of Plasma Samples from Patients with CLL. Plasma samples from several patients were examined for total IgM and 17.109-positive IgM (Table 1). The levels of circulating IgM and of 17.109-positive IgM were generally lower in the CLL patients than in normal age-matched controls. The serum concentrations of IgM reactive with the 17.109 anti-idiotype did not differ significantly between CLL patients with 17.109-positive and -negative tumors. Furthermore, mixing of CLL plasma with 17.109-reactive IgM paraprotein did not interfere with the ELISA for the CRI.

CLL Hybridomas with WI-L2-729-HF$_2$. In order to immortalize 17.109-positive cells for further study, CLL cells from two patients were fused with the human B-lymphoblastoid fusion partner WI-L2-729-HF$_2$. This cell line does not express sIg reactive with 17.109. However, hybridomas of WI-L2-729-HF$_2$ with 17.109-positive CLL cells stained significantly with the 17.109 antibody (Fig. 2 *Center*). Moreover, these hybridomas secreted IgM κ that reacted with the anti-idiotype in ELISA (38).

Analyses of RNA Using Synthetic Oligonucleotides. Short synthetic oligonucleotide probes can be used to delineate immunoglobulin κ *V* genes. The human κ *V* genes can be categorized into four major subgroups based on amino acid sequence homologies (16). Recently, these homologies have been found to extend to the nucleic acid level (39–42). Each κ variable-region subgroup has characteristic framework sequences that may serve to distinguish it from other κ variable-region subgroups. Cross-hybridization of a κ *V*-gene probe between different subgroups can be avoided by designing short synthetic nucleotides specific for those framework sequences that serve to distinguish one variable-region subgroup from another (T.J.K., unpublished observations).

Short synthetic oligonucleotides, complementary to the sense strand of V_κ III framework regions of V_κRF, were used to examine the RNA of the 17.109-positive CLL and CLL hybridomas (probes A, C, and D, Fig. 3 and Table 2). In addition, a synthetic oligonucleotide corresponding to the second CDR of V_κRF was used to analyze the electrophoretically separated RNAs (probe B, Fig. 3 and Table 2).

Hybridization with this battery of oligonucleotides revealed that the 17.109-positive CLL cells and hybridomas express highly homologous κ light-chain mRNAs. Under the hybridization conditions employed, both the V_κRF framework and hypervariable-region oligonucleotides hybridized with RNAs isolated from 17.109-positive CLL cells or hybridomas (Fig. 4, lanes 1–4). Negative controls included RNA extracted from the cell lines POL, GM607A, LES, and WI-L2-729-HF$_2$ (lanes 5–8). Patient POL has CLL with light chains that belong to the κ I variable-region subgroup, as determined by RNA blot analyses with specific oligonucleotides (T.J.K., unpublished observations). GM607A is a human B-lymphoblastoid cell line expressing a κ II variable-region subgroup (42). WI-L2-729-HF$_2$ expresses light chains

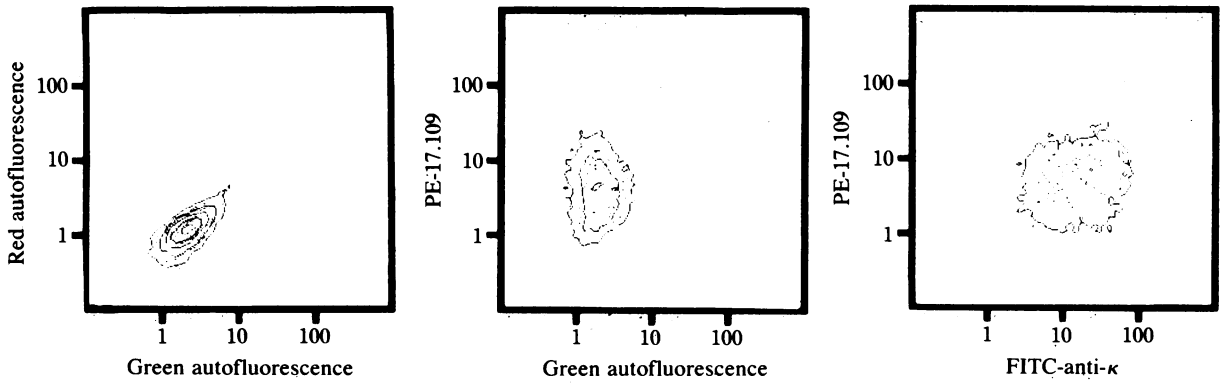

FIG. 2. Immunofluorescence of hybridoma BRO. (*Left*) Unstained cells. (*Center*) PE-17.109-stained cells. (*Right*) PE-17.109- and FITC-anti-κ-stained cells.

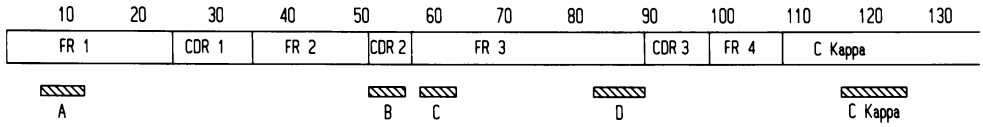

FIG. 3. Oligonucleotide probes specific for κ light-chain variable region are represented below a schematic diagram of the κ variable region with amino acid numbering according to Kabat *et al.* (16). FR1, FR2, FR3, and FR4 correspond to the first, second, third, and fourth variable-region frameworks, respectively. CDR1, CDR2, and CDR3 correspond to the first, second, and third complementarity-determining regions, respectively. C Kappa designates the κ light-chain constant region. Oligonucleotides A, B, C, D, and C Kappa (C_κ) are depicted below the positions to which they are specific.

that belong to the κ IV variable-region subgroup (T.J.K., unpublished observations). Although the κ *V* gene of LES belongs to the κ IIIa variable-region sub-subgroup, it differs from the V_κRF gene by only one nucleotide in the regions corresponding to oligonucleotides A, B, and D (Table 2) (43). Confirming that adequate κ RNA was present for hybridization, all RNA preparations hybridized with a synthetic κ constant-region oligonucleotide probe (Figs. 3 and 4).

FACS Sorting of 17.109-Positive CLL Hybridomas. Sorting experiments with CLL hybridomas further confirmed the association between positive staining with 17.109 antibody and κ light chains expressing V_κRF-associated sequences. Hybridomas generated with WI-L2-729-HF_2 have sIg derived from both the CLL cells and the WI-L2-729-HF_2 fusion partner (36). The formation of such mixed molecules at the cell surface is reflected in the heterogeneous FACS contour map of 17.109-reactive hybridomas stained with PE-17.109 and FITC-anti-κ (Fig. 2). Sorting for cells expressing sIg with κ light chains with or without reactivity to 17.109 allowed us to isolate two distinct populations. The RNA from each was analyzed using individual oligonucleotide probes. The RNA from hybridoma cells with κ light chains predominantly derived from the WI-L2-729-HF_2 fusion partner did not hybridize substantially to oligonucleotides A–D. Under the same conditions, RNA from cells sorted for 17.109 expression hybridized strongly (data not shown).

DISCUSSION

In this paper we present evidence for the high-frequency expression in CLL of an idiotypic antigen that is a marker for a conserved κ *V* gene (V_κRF) or a small gene family. The 17.109-reactive idiotype was found on 25% (5 out of 20) of the CLL clones with κ light chains on the cell surface. Hybridization studies with synthetic oligonucleotides showed that expression of the 17.109-reactive idiotype is associated with the production of κ light-chain mRNA with variable-region framework and second CDR sequences that are identical to the cloned V_κRF gene. The results suggest that the 17.109-positive CLL cells utilize the conserved V_κRF gene, or a very homologous gene, with little or no somatic mutation.

Either incompetence of the CLL B cells to mutate their expressed immunoglobulin V genes or the failure of mutated cells to expand may explain the relatively high frequency of the 17.109-reactive idiotype in this malignancy. Somatic mutation of immunoglobulin *V* genes has been estimated to occur at a frequency of 10^{-3} per base per generation during secondary immune responses (12). Such a high rate of mutation usually generates some variant clones that bind antigens more efficiently, resulting in their clonal expansion. Such a somatic mutation mechanism apparently is operative in some B-cell tumors (44, 45). The eventual result is the permutation and dilution of CRI determinants that are markers for inherited *V* genes. Consistent with this hypothesis is the low frequency of 17.109 idiotype-positive B cells in the peripheral blood of normal adults. "Immunologically incompetent" CLL B lymphocytes, on the other hand, may express *V* genes that have not diverged from the inherited repertoire. It may be anticipated that other CRIs that are markers for conserved *V* genes will be expressed in high frequency in those lymphoid tumors that are not under environmental pressure to mutate their expressed *V* genes. Similarly, anti-idiotypic antibodies that have been selected deliberately for their ability to recognize CLL cell populations from unrelated individuals (46, 47) may actually represent serologic probes for evolutionary conserved light- or heavy-chain *V* genes.

The high frequency of 17.109-positive CLL B lymphocytes also suggests that the malignant cell may express a restricted set of *V* genes. It has been estimated that humans have at least 25 functional κ *V* genes (13, 14). The utilization of one *V* gene at a frequency of 25% seems unlikely if each gene has an equal probability of being expressed. Perhaps the V_κRF gene is used repeatedly by the normal $CD5^+$ B-cell subpopulation that may be subject to malignant transformation in CLL. Alternatively, the high frequency of 17.109 among CLL populations may reflect the preferential expression of this particular κ gene(s) by B cells at an immature stage of differentiation. It is also possible that the anatomic sites where RF synthesis preferentially occurs are also regions where the CLL process is initiated. Finally, expression of sIg with RF activity may increase the probability of B-cell transformation into CLL. For example self-reactive B cells may be stimulated continually by self antigens, resulting in clonal expansion and increased susceptibility for chance transformation. On the other hand, RF may serve to focus antigen–antibody complexes containing a transforming agent onto B cells bearing sIg with RF activity. For these reasons, the eventual resolution of the mechanisms that account for the high frequency of 17.109 expression in CLL may provide important insights concerning the pathogenesis of this common adult leukemia.

The recognition that CLL cells express conserved idiotypic determinants may afford a partial answer to the problems associated with immunotherapy. The idiotypic determinants

Table 2. Oligonucleotide probes (see Fig. 3)

Oligonucleotide	Sequence (5′ → 3′)	Temp.,* °C	Position†
A	G GGT GCC TGG AGA CTG	57	6–11
B	GT GGC CCT GCT GGA TG	57	50–55
C	AA CCT GTC TGG GAT GC	54	58–63
D	ACA GTA ATA CAC TGC AAA AT	37	82–88
C_κ	CTC ATC AGA TGG CGG GAA GAT GAA	54	116–124

Sequences are interrupted as per codon.
*Final washing temperature used.
†Amino acids encoded by the sequence to which the oligonucleotide is specific, using the position numbers according to Kabat *et al.* (16).

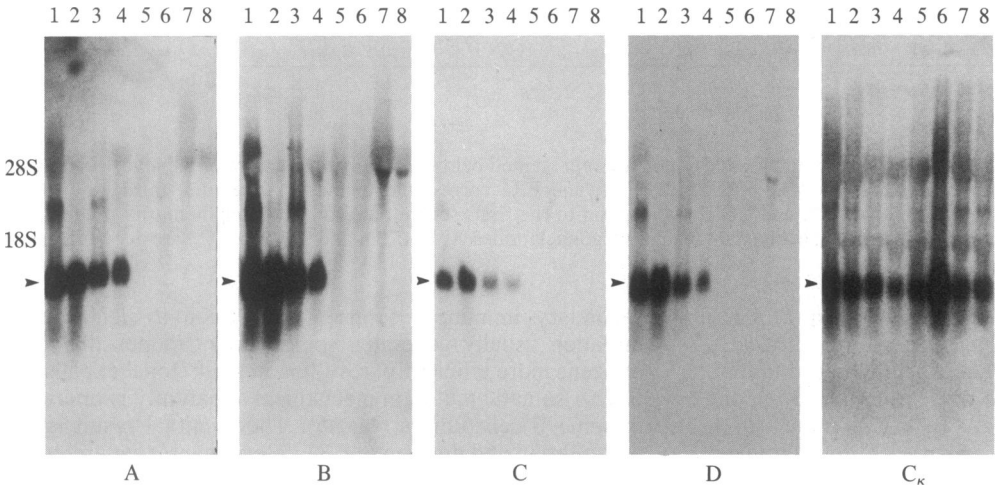

FIG. 4. Blot-hybridization analyses of total RNA isolated from CLL cells HIC (lane 1) and HAH (lane 2); hybridomas SMI (lane 3), BRO (lane 4), and POL (lane 5); cell line GM607A (lane 6); CLL cells LES (lane 7); or hybridoma fusion partner WI-L2-729-HF_2 (lane 8). Below each autoradiograph is the designation of the oligonucleotide used to probe the electrophoretically separated RNA. Arrowhead depicts position of the antibody κ light-chain mRNA. Positions of 28S and 18S rRNA markers are indicated.

on malignant B lymphocytes represent potential tumor-specific targets for active or passive immunization. However, the necessity to generate idiotypic-specific monoclonal antibodies against each patient's malignant clone makes this form of therapy extremely costly and time-consuming. Clinical studies comparing the role of immunoglobulin isotype, mode of monoclonal antibody delivery, utility of antibody-toxin conjugates, and the relative advantages of single vs. multiple monoclonal antibody therapy have been confounded by the need to tailor a unique reagent for each patient studied. The recognition of conserved CRIs expressed by malignant B lymphocytes may permit the production of batteries of monoclonal antibody reagents, and perhaps the eventual development of synthetic idiotype vaccines, suitable for the immunotherapy of a large number of CLL patients.

We appreciate the cooperation of other physicians in the Hematology/Oncology Division of the Scripps Clinic Medical Group, in particular Drs. William Miller and Lawrence Piro, in providing clinical samples from their patients with CLL. We thank Joseph Weber for his superb technical assistance. T.J.K. is a scholar of the Leukemia Society of America and recipient of an Investigator Award from the Arthritis Foundation. S.F. is the recipient of a Research Career Development Award (AG00279) from the National Institute of Aging. This work was supported in part by Grants AR38475, AR25443, AR07144, AG04100, and RR00833 from the National Institutes of Health. This is publication no. 4644BCR from the Research Institute of Scripps Clinic.

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