Two Acquired Immunodeficiency Syndrome-Associated Burkitt’s Lymphomas Produce Specific Anti-i IgM Cold Agglutinins Using Somatically Mutated V\_H4-21 Segments

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We analyzed the reactivity and the structure of the V\_H and V\_L segments of two IgM monoclonal antibodies (MoAbs) produced by spontaneously in vitro outgrowing cell lines, HBL-2 and HBL-3, established from two acquired immunodeficiency syndrome (AIDS) patients with Epstein-Barr virus (EBV)-negative Burkitt’s lymphoma (BL). These B-cell clones were representative of the respective neoplastic parental clones, as determined by immunophenotypic and molecular genetic analysis. The IgM MoAbs were highly specific for the i determinant on red blood cells (cold agglutinins), but bound none of the other eight self and nine foreign antigens (Ags) tested, including those most commonly recognized by natural antibodies or autoantibodies. Structural analysis showed that the IgM MoAb V\_Hi sequences were 93.5% and 84.2% identical with that of the germline V\_H4-21 gene, which encodes the vast majority of cold agglutinins that are specific for the i/l carbohydrate Ag and are produced under chronic lymphoproliferative conditions. The HBL-2 MoAb V\_H4-21 gene segment was juxtaposed with 20P3 and J\_6 genes and paired with a Va1 segment, the sequence of which was 95.5% identical to that of the germline Humvl17 by natural antibodies or autoantibodies. Structural analysis showed that the IgM MoAb V\_Hi segment sequences were 93.5% and 84.2% identical with that of the germline V\_H4-21 gene, which encodes the vast majority of cold agglutinins that are specific for the i/l carbohydrate Ag and are produced under chronic lymphoproliferative conditions. The HBL-2 MoAb V\_H4-21 gene segment was juxtaposed with 20P3 and J\_6 genes and paired with a Va1 segment, the sequence of which was 95.5% identical to that of the germline Humvl17.

AUTOIMMUNE PHENOMENA can occur in association with several human B-cell disorders, such as cold agglutinin disease,1-3 lymphoma,4,5 and the B-cell expansion and hypergammaglobulinemia occurring in human immunodeficiency virus (HIV)-infected patients.6,7,8 Although the precise role of different self antigens (Ags) in the B-cell clonal selection associated with the above pathologic conditions remains to be defined, circumstantial evidence for a role of self Ags in clonal expansion and selection in autoimmune humans and mice has been provided.8-10.16 The crucial role of Ags in inducing clonal expansion and selection in the normal B-cell repertoire is well documented.11-21 Recently, it has been suggested that Ag stimulation also plays a role in the B-cell expansion and selection preceding and/or associated with development of lymphomas of various histologic types.5,6,22-24

The assessment of a potential role for Ags in the clonal B-cell expansion and selection associated with lymphoma or leukemia entails, first of all, the definition of the specificity and of V\_H and V\_L segment structure of the tumor-derived antibody. The analysis of antibody specificity and V\_H and V\_L segment structure depends on the availability of a homogeneous in vitro growing and Ig-producing tumor cell population representative of the in vivo neoplastic clone. This is a critical requirement in view of the findings of oligoclonal or polyclonal, not necessarily neoplastic, B-cell populations accompanying the predominant neoplastic clone, as found particularly in biopptic specimens of Burkitt’s lymphoma (BL) emerging in patients with acquired immunodeficiency syndrome (AIDS).23-26 Possibly due to the improved treatment and longer survival rate, these patients display a 60-fold increased incidence, relative to that expected for the general population, of lymphomas, mainly of the Burkitt’s type.27

We analyzed the Ag reactivity and the structure of the V\_H and V\_L segments of the IgM monoclonal antibodies (MoAbs) produced by two spontaneously in vitro outgrowing cell lines established from 2 AIDS patients with Epstein-Barr virus (EBV)-negative BL.28 The absolute identity between the in vitro growing monoclonal cell lines and their respective in vivo neoplastic clones was established by immunophenotypic and molecular genetic analysis. The IgM MoAbs from both cell lines strongly bind to the i Ag on red blood cells (RBCs; cold agglutinins), but to none of the other self and foreign Ags tested. The structural correlate for such Ag-binding specificity was provided by segments encoded by V\_H4-21 and Va1 genes in somatically mutated configuration.
Thus, a process of selection by the self may have played a role in the B-cell clonal expansion preceding and/or associated with the development of BL in these AIDS patients.

MATERIALS AND METHODS

Generation and characterization of the monoclonal AIDS BL cell lines. The HBL-2 and HBL-3 MoAb-producing cell lines were established using B lymphocytes spontaneously outgrowing from two tumors histologically and immunophenotypically classified as small noncleaved cell lymphoma (SNCL). Both SNCL arose in patients with AIDS. Immunophenotypic analysis was performed by fluorescence flow cytometry of isolated cells using a FACScan (Becton Dickinson Corp, Mountain View, CA) and a panel of labeled murine MoAbs, including those to CD3, CD4, CD5, CD19, HLA-DR, CD10, CD21, and CD52. The clonality of the cell lines and their absolute relatedness to the tumors were identified by Ig gene rearrangement analysis using a JH probe on HindIII, EcoRI, and BamHI DNA digests. The c-myc translocations were detected by cytogenetic analysis. The status of the c-myc locus was analyzed by hybridization of EcoRI- and HindIII-digested DNA to the human c-myc probe MC413RC, representative of the third exon of the c-myc gene. The presence of the EBV genome was investigated using a probe specific for the EBV genomic termini (5.2 kb BamHI-EcoRI fragment isolated from the fused BamHI terminal fragment N1-het). The presence of HIV sequences was investigated using the X7N2 probe on EcoRI and HindIII-digested DNA. The c-rnyc translocations were detected by cytogenetic analysis using a JH probe on HindIII, and Sac I DNA digests, and that of HTLV-I sequences was investigated using the X7N2 probe on EcoRI and HindIII-digested DNA to the human c-rnyc probe (5' CCGAA'ITCATGGAGGGGGAA-(AG)TTC(AG)TC (residues 84 to 105) of the portion of the leader sequence of VHIII family, [5' GGGAAATCTAGGACTGGACCTCGAGGAGG(A)GC(T)CT - TCT(GTGC 3')] of HTLV-I). The IgM MoAb specific for the B cell line-derived MoAb. The IgM MoAbs produced by the HBL-2 and HBL-3 cell lines were analyzed for their binding to polyclonal human IgGFc fragment (Organon Teknika-Cappel, Malvern, PA); calf thymus DNA (Sigma Chemical Co, St Louis, MO); insulin (Sigma); human recombinant tumor necrosis factor-α (TNF-α), TNF-β, and interleukin-1β (IL-1β; BASF Biotech Corp, Cambridge, MA); human thyroglobulin; HIV-1 and cytomegalovirus (CMV) and parvovirus B19 recombinant glycoproteins; lipopolysaccharide (LPS) and P-galactosidase from Escherichia coli (Sigma); phosphorylcholine chloride (Sigma); Pneumococcus polysaccharides, including types 1, 3, and 4; and tetanus toxoid (Massachusetts Public Health). The clonality of the cell lines and their absolute relatedness to the tumors were determined by DNA sequence identity searches were performed using the GenBank database and the FASTA method.

Analysis of the AIDS BL cell line-derived MoAb. The IgM MoAbs produced by the HBL-2 and HBL-3 cell lines were analyzed for their binding to polyclonal human IgGFc fragment (Organon Teknika-Cappel, Malvern, PA); calf thymus DNA (Sigma Chemical Co, St Louis, MO); insulin (Sigma); human recombinant tumor necrosis factor-α (TNF-α), TNF-β, and interleukin-1β (IL-1β; BASF Biotech Corp, Cambridge, MA); human thyroglobulin; HIV-1 and cytomegalovirus (CMV) and parvovirus B19 recombinant glycoproteins; lipopolysaccharide (LPS) and P-galactosidase from Escherichia coli (Sigma); phosphorylcholine chloride (Sigma); Pneumococcus polysaccharides, including types 1, 3, and 4; and tetanus toxoid (Massachusetts Public Health). The clonality of the cell lines and their absolute relatedness to the tumors were determined by DNA sequence identity searches were performed using the GenBank database and the FASTA method.

Analysis of the putative germline IgV{\beta} segment that gave rise to the expressed HBL-3 V{\beta} gene. Genomic DNA was extracted from the monoclonal HBL-3 B cells and autologous fibroblasts obtained from the same biopsy sample used for the generation of the tumoral HBL-3 cell line. B-cell or fibroblast genomic DNA (100 ng) was supplemented with the appropriate sense and antisense oligonucleotide primers (10 pmol each). PCR amplification was performed in a 50 μL reaction volume using Taq polymerase under denaturing, annealing, and extending conditions of 94°C (1 minute), 52°C (1 minute), and 72°C (2 minutes), respectively. PCR products were sized and isolated on low melting agarose gel and ligated into pCR 1000 plasmid vector (Invitrogen Corp). The ligation mixture was used to transform INV-AE competent cells according to the manufacturer's protocol. Recombinant clones were selected according to the length of the insert and sequenced by the dideoxy chain termination method using the Taq Track Sequencing Kit (Promega Corp, Madison, WI). Each V{\beta} or V{\alpha} sequence was derived from the analysis of at least three independent clones. Differences in nucleotide sequences among different recombinant clones were observed in few cases (<0.001/base) and such variants were excluded from the sequence analysis. DNA sequences were analyzed using the software package of the Genetics Computer Group of the University of Wisconsin, version 7.1, and a Model 6000-416 VAX computer (Digital Equipment Corp, Marlboro, MA). DNA sequence identity searches were performed using the GenBank database and the FASTA method.

Analysis of the putative germline IgV{\beta} segment that gave rise to the expressed HBL-3 V{\beta} gene. Genomic DNA was extracted from the monoclonal HBL-3 B cells and autologous fibroblasts obtained from the same biopsy sample used for the generation of the tumoral HBL-3 cell line. B-cell or fibroblast genomic DNA (100 ng) was supplemented with the appropriate sense and antisense oligonucleotide primers (10 pmol each). PCR amplification was performed in a 50 μL reaction volume using Taq polymerase under denaturing, annealing, and extending conditions of 94°C (1 minute), 52°C (1 minute), and 72°C (1 minute), respectively, for 30 cycles. The oligonucleotides used were as follows: (1) the sense V{\delta}4-21 FRI primer, encompassing a portion (residues 10 to 27) of the FR1 sequence of germline V{\delta}4-21 gene and differing in two nucleotides from the corresponding sequence of the expressed HBL-3 V{\beta} gene (5' CTA-CAGCAGTTGGGGGCGCA 3'); (2) the sense HBL-3 leader primer, encompassing a portion of the leader region of the HBL-3 V{\beta} gene and differing in one nucleotide (C instead of G at position -31) from the corresponding area of the germline V{\beta}8 gene, the member of the V{\beta}1V gene family displaying the highest degree of identity with the V{\beta}4-21 gene; and (3) the antisense V{\delta}4-21 FR3 primer, consisting of the reverse complement 5' GTGCGGCGGGGCGCCA 3' of a R3 sequence (residues 250 to 257) shared by the expressed HBL-3 V{\beta} gene and the germline V{\delta}4-21 gene. Part of the amplified DNA was fractionated through a 1.5% agarose gel, transferred to a nylon membrane (Hybond; Amersham Life Sciences, Arlington Heights, IL) and hybridized at 48°C with the HBL-3 complementarity-determining region 1 (CDR1) oligonucleotide probe labeled with [γ-32P]ATP (DuPont NEN Research Products, Boston, MA) by T4 polynucleotide kinase. The HBL-3 CDR1 oligonucleotide encompassed a FR1-CDR1 sequence 5' GTTTCATGATTTACTCTTGAGCA 3' (residues 84 to 105) of the expressed HBL-3 V{\beta} gene, differing in seven bases from that of the
corresponding area of the germine V_{\gamma4-21} gene. After hybridization, the membrane was washed twice with 2\times SSC/0.1% sodium dodecyl sulfate (SDS) at room temperature for 30 minutes and twice with 1\times SSC/0.1% SDS at 54°C for 30 minutes. Autoradiography was performed using Kodak XAR-5 film (Eastman Kodak Co, Rochester, NY). Other amplified DNA was inserted into the pCR II plasmid vector (Invitrogen Corp) for cloning and sequencing.

RESULTS

Characterization of the IgM MoAb-producing HBL-2 and HBL-3 cell lines. The features of the HBL-2 and HBL-3 cell lines established from 2 AIDS patients with SNCCL and their identity with the respective primary tumoral tissues have been reported and are summarized in Table 1. Both tumor samples and respective cell lines expressed surface Ig \( \mu \) and \( \lambda \) chains and the B-cell-restricted marker CD19. This was consistent with the B-cell origin of the cell lines and with the histologic diagnosis of SNCCL. In addition, the surface expression of CD10 but not CD21 was consistent with the cell line phenotype of sporadic BL. The monoclonality of the AIDS BL cell lines and their absolute relatedness to the respective tumors was formally established by the analysis of the Ig H chain gene rearrangements and that of the c-myc oncogene translocations. The Southern blotting analyses of EcoRI- and HindIII-digested DNA were consistent with two distinct patterns of c-myc activation. In the HBL-2 cells, the breakpoint was located 5' (3 to 5 kb) to the c-myc first exon; in the HBL-3 cells, the breakpoint was within a ~4-kb region 3' of the c-myc exon 3. Both HBL-2 and HBL-3 cells were negative for EBV, HIV, and HTLV-I sequences and so were their respective original tumor cells.

The Ag-binding activity of the IgM MoAbs produced by the HBL-2 and HBL-3 cell lines. The HBL-2 and HBL-3 IgM MoAbs specifically bound to the i determinant on human erythrocytes, as shown by their strong agglutination of cord (smallest agglutinating doses: 4 and 0.6 ng/10^7 RBCs, respectively), but not adult (no agglutination by HBL-2 MoAb; HBL-3 MoAb smallest agglutinating dose, 38.8 ng/10^7 RBCs) papain-treated human RBCs. The specificity of the HBL-2 and HBL-3 IgM MoAbs was further strengthened by the MoAbs’ failure to bind to any of the eight self and nine foreign Ags tested, including IgG Fc fragment, human thyroglobulin, ssDNA, phosphorylcholine, insulin, human recombinant TNF-\( \alpha \), human recombinant TNF-\( \beta \), human recombinant IL-1\( \beta \), \( \beta \)-galactosidase and LPS from E. coli, tetanus toxoid, HIV-1, recombinant glycoproteins of CMV and Parvovirus B19, and Pneumococcus polysaccharides (Table 1). Binding to each of these Ags by HBL-2 and HBL-3 MoAbs yielded an absorbance of less than 0.05 at 492 nm; negative and positive controls were always less than 0.05 and more than 1.00, respectively. The HBL-3 but not the HBL-2 MoAb expressed the cross-reacting idiotype defined by the anti-idiotypic 9G4 MoAb.

The HBL-2 and HBL-3 MoAb \( V_H \) segments. Figure 1 depicts the nucleotide (A) and deduced amino acid (B) sequences of the HBL-2 and HBL-3 IgM-MoAb \( V_H \) genes and that of the closest reported germine \( V_H \) gene. The differences between sequences are summarized in Table 1. The HBL-2 and HBL-3 MoAb \( V_H \) gene sequences were 93.5% and 84.2% identical, respectively, to that of \( V_H4-21 \) gene, a member of the \( V_HIV \) gene family. Accurate inspection of the \( V_H4-21 \)-related sequences available in the GenBank showed that the HBL-2 \( V_H \) gene sequence displayed an identical nucleotide difference at position 82, A instead of T, with that of the expressed fetal 20P3 gene. The HBL-3 \( V_H \) gene displayed a stretch of similarity to that of the closest germline D4246 and JH genes are depicted in Fig 1. Twelve of the 19 nucleotide differences displayed by the HBL-2 \( V_H \) gene sequence resulted in putative amino acid replacements, yielding replacement to silent (R:S) mutation ratios of 5.0 in the CDR and 1.1 in the framework regions (FR). Nineteen of the 46 nucleotide differences displayed by the HBL-3 \( V_H \) sequence resulted in putative amino acid replacements, yielding R:S mutation ratios of 2.2 in the CDR and 0.3 in the FR.

The HBL-2 and HBL-3 MoAb \( D \) and \( J_H \) genes. The nucleotide and deduced amino acid sequences of the HBL-2 and HBL-3 IgM MoAb \( D \) and \( J_H \) genes, and those of their closest germine \( D_45-46 \) and \( J_H \) genes are depicted in Fig 1. The HBL-2 MoAb \( D \) gene sequence displayed some identity with that of the expressed fetal 20P3 \( D \) gene; the HBL-3 MoAb \( D \) gene displayed a stretch of similarity to that of the

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**Table 1. Features of the AIDS BLs**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Source</th>
<th>Histology</th>
<th>EBV DNA</th>
<th>HIV DNA</th>
<th>HTLV-I DNA</th>
<th>c-myc</th>
<th>H</th>
<th>L</th>
<th>Ag Specificity</th>
<th>( V_H ) Gene</th>
<th>Nucleotide (amino acid) identity* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBL-2</td>
<td>Pleural fluid</td>
<td>SNCC</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>t(8;14)</td>
<td>( \mu )</td>
<td>( \lambda )</td>
<td>i</td>
<td>( V_H4-21 )</td>
<td>93.5 (88.8)</td>
</tr>
<tr>
<td>HBL-3</td>
<td>Liver mass</td>
<td>SNCC</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>t(8;22)</td>
<td>( \mu )</td>
<td>( \lambda )</td>
<td>i</td>
<td>( V_H4-21 )</td>
<td>84.2 (60.4)</td>
</tr>
</tbody>
</table>

* The complete sequences of the genomic germline \( V_H \) genes have been reported as compared in the Results.

† The sequence of the genomic germline VH4-21 gene has been reported by Sanz et al.

‡ Expected numbers of R mutations calculated as reported in the Results.
reverse complement of the germ line DXP1 gene, suggesting a possible inverted D gene joining origin (Fig 1C). Both expressed D genes were flanked by untemplated nucleotide additions. The entire length of the D segment ranged from 13 nucleotides in HBL-2 to 36 nucleotides in HBL-3. The HBL-2 and HBL-3 MoAs used truncated and mutated forms of Jh6 and Jh5 genes, respectively (Fig 1C). The deduced amino acid sequences of the D-Jh genes are depicted in Fig 1D, as segregated in CDR3 and FR4 stretches, according to Kabat et al. The CDR3 sequences were highly divergent in length and composition. The HBL-2 and HBL-3 FR4 sequences were invariable in length and displayed two and one amino acid replacements, respectively.

The HBL-2 and HBL-3 MoAb VH and Jh genes. Figure 2 depicts the nucleotide (A) and deduced amino acid (B) sequences of the HBL-2 and HBL-3 MoAb VH genes and those of the closest reported germ line VH genes. The differences between sequences are summarized in Table 1. HBL-2 and HBL-3 MoAs used two members of the VH1 subgroup, the Humigl117 and Humigl111 genes, respectively. When compared with the germ line gene, the HBL-2 VH1 gene sequence displayed nine and four nucleotide differences in the CDRs and FRs, respectively, yielding four and two amino acid replacements, and R:S mutation ratios of 1.2 and 1.0, respectively. When compared with the germ line gene, the HBL-3 VH1 gene sequence displayed 39 nucleotide differences. These were scattered throughout the CDRs and FRs, yielding a total of 19 amino acid replacements and R:S mutation ratios of 1.1 and 0.8, respectively. Figure 2 depicts the nucleotide (C) and deduced amino acid (D) sequences of the MoAb Jh segments and their respective germ line Jh templates. The HBL-2 MoAb used a Jh2/Jh3 segment in germ line configuration; the HBL-3 MoAb used a Jh1 segment with five nucleotide mutations resulting in three amino acid replacements.

Somatic mutations in the HBL-3 MoAb VH segment. Because of conservation of the VH4-21 gene in humans, the high number of nucleotide differences displayed by the HBL-3 VH gene sequence when compared with that of the VH4-21 germ line gene, and the detection of mutations in the HBL-3 MoAb Jh1 and Jh2 segments, we hypothesized that the HBL-3 MoAb VH segment consisted of a somatically mutated form of the VH4-21 gene. PCR amplifications were performed using ad hoc designed oligonucleotide primers and genomic DNA from the HBL-3 cell line or autologous fibroblasts. The sense VH4-21 FR1 primer, encompassing an FR1 sequence (residues 10 to 27) shared by the VH4-21 segment and the expressed HBL-3 VH gene, was used in conjunction with the antisense VH4-21 FR3 primer, encompassing an FR3 sequence (residues 250 to 269) shared by the germ line VH4-21 and the expressed HBL-3 genes. The two combined primers amplified DNA from both fibroblasts and HBL-3 cells. The molecular size of the amplified product (~260 bp) was consistent with that of the sequence spanning residues 10 to 269 of the VH4-21 gene sequence (Fig 3A, lanes 1 and 2). The same antisense VH4-21 FR3 primer was also used to amplify fibroblast DNA, in conjunction with the sense VH3-3 leader primer, encompassing a stretch of the leader sequence of HBL-3 VH gene (residues -49 to -25) and differing in only one nucleotide from that of the corresponding area of the V58 gene, the VH4 family member displaying the highest degree of identity with the VH4-21 gene. The molecular size of the amplified product (~400 bp) was consistent with that of the sequence spanning residues -49 to 269 of the HBL-3 VH gene, including the untranslated intervening intron (Fig 3A, lane 3).

The three DNA amplification products were analyzed for their ability to hybridize with the [γ-32P]-labeled HBL-3 CDR1 oligonucleotide. This encompassed a stretch of the HBL-3 VH gene FR1-CDR1 sequence that displayed seven putative mutations when compared with the corresponding germ line VH4-21 gene sequence. The [γ-32P]-labeled HBL-3 CDR1 oligonucleotide strongly hybridized with the ~260 bp DNA amplified from the HBL-3 cell line (Fig 3B, lane 1), but not with the ~260 or ~400 bp DNA amplified from the autologous fibroblasts (Fig 3B, lanes 2 and 3, respectively). To identify the autologous germ line VH gene that putatively gave rise to the expressed HBL-3 VH gene, the product amplified from fibroblast DNA using the sense VH4-21 FR1 and the antisense VH4-21 FR3 primers was cloned...
and sequenced. The sequences of the eight independent clones were all identical to each other and to that of the V<sub>H</sub>4-21 germline gene throughout the overlapping area (residues 28 through 249) (Fig 1A and B; HBL-3 FR1/FR3 sequence). These experiments proved that the expressed HBL-3 V<sub>H</sub> segment was somatically point-mutated, and suggested that V<sub>H</sub>-21 is the germline gene that gave rise to it.

**Fig 1.** Top clusters: nucleotide (A) and deduced amino acid (B) sequences of the V<sub>H</sub>4-21 genes used by the HBL-2 and HBL-3 IgM MoAbs. The top sequence is given for comparison and represents the published germline VH<sub>4</sub>-21 (see Materials and Methods for details). Bottom clusters: nucleotide (C) and deduced amino acid (D) sequences of the D<sub>1</sub> and J<sub>4</sub> genes used by the HBL-2 and HBL-3 IgM MoAbs. Germline D<sub>1</sub> genes are given for comparison. Dashes indicate identity.
that expected by chance only, it is likely that pressure to maintain the germline-encoded protein structure was exerted. Conversely, if a DNA segment displays a number of R mutations higher than that expected by chance only, it is likely that pressure to maintain the germline-encoded protein structure was exerted. Therefore, if a DNA segment displays a number of R mutations higher than that expected only by chance, it is likely that pressure to select R mutations was exerted.

Fig 2. Top clusters: nucleotide (A) and deduced amino acid (B) sequences of the VA genes used by the HBL-2 and HBL-3 IgM MoAbs. In each cluster, the top sequence is given for comparison and represents the published germline VA sequence displaying the highest degree of identity with the expressed VA genes. Dashes indicate identity. Solid lines on the top of each cluster depict CDRs. The Humlv117 and Humlv1L1 genes belong to the VA1 subgroup. Bottom clusters: nucleotide (C) and deduced amino acid (D) sequences of the JA genes used by the HBL-2 and HBL-3 MoAbs. Dashes indicate identity. The present sequences are available from EMBL/GenBank/DDBJ under accession numbers L29113 and L29114.
to the CDRs \( (q = 0.23 \times 0.75) \) or FRs \( (q = 0.77 \times 0.75) \), and \( k \) is the number of observed R mutations in the CDRs or FRs.\(^{55}\) The likelihood that the excess R mutations arose by chance in the \( \mathrm{V}_{\text{H}} \) segment CDR were \( P = .11 \) in HBL-2 MoAb and \( P = .06 \) in HBL-3 MoAb. The probability that the scarcity of R mutations in the \( \mathrm{V}_{\text{H}} \) segment FR resulted from chance were \( P = .03 \) in HBL-2 MoAb and \( P = .00000002 \) in HBL-3 MoAb. In their original report on the application of the binomial distribution model to the analysis of the R point-mutations in Ag-selected IgV segment CDRs, Shlomchik et al\(^{52}\) suggested that the observed number of FR R mutations should be doubled to account for the fact that some of these mutational events will never be observed because they are deleterious to the Ig structure. Although this correction was inferred from some experimental observations, it is approximate and may not be applicable as such to all Ig \( \mathrm{V}_{\text{H}} \) genes. When the analysis of the \( \mathrm{V}_{\text{H}} \) segment somatic mutation pattern was performed with the adjustment of doubling the number of observed FR R mutations, the probabilities that excess R mutations arose by chance in the \( \mathrm{V}_{\text{H}} \) segment CDR were \( P = .49 \) and \( P = .35 \) in HBL-2 MoAb and HBL-3 MoAb, respectively.

**DISCUSSION**

In the present studies, we established from biopitic specimens of 2 AIDS patients with BL two MoAb-producing cell lines representative of the respective tumors, and analyzed the Ag-binding activity and the V segment structure of these MoAbs. We found that both IgM MoAbs were cold agglutinins highly specific for the i blood group determinant, and both MoAbs bore Ag-combining sites consisting of point-mutated \( \mathrm{V}_{\text{H}4-21} \) segments in conjunction with \( \mathrm{V}_{\text{AL}} \) segments.

The exquisite specificity of the HBL-2 and HBL-3 IgM MoAb cold agglutinins for the i repetitive N-acetyllactosamine units was strengthened by the MoAb failure to bind any of the other eight self Ags and nine foreign Ags tested. The putative use of the \( \mathrm{V}_{\text{H}4-21} \) gene segment by the HBL-2 and HBL-3 MoAbs is consistent with the use of the same segment by the majority of the reported cold agglutinins from patients with idiopathic cold agglutinin disease, FL, and Waldenström’s macroglobulinemia.\(^{1,2,5,5,6}\) A primary role of the \( \mathrm{V}_{\text{H}4-21} \) segment in the binding to the i Ag is further supported by the divergence in composition and length of the H chain CDR3 sequences as well as by the heterogeneity of the \( \mathrm{V}_{\text{H}} \) segments of the present two IgM and the 10 reported \( \mathrm{V}_{\text{H}4-21} \) cold agglutinins, which use a \( \mathrm{V}_{\text{I1}} \) segment three times, a \( \mathrm{V}_{\text{II}} \) segment once, a \( \mathrm{V}_{\text{IIIa}} \) segment once, a \( \mathrm{V}_{\text{IIIb}} \) four times, and a \( \mathrm{V}_{\text{AL}} \) segment once.\(^{1,5,4,5,6}\) Thus, the \( \mathrm{V}_{\text{H}4-21} \) gene restriction in the cold agglutinin system may result from a selection process based on an inherent affinity of this \( \mathrm{V}_{\text{H}} \) gene product for the i/i carbohydrate structure.\(^{1,2,5,4,5,6}\) Nevertheless, the \( \mathrm{V}_{\text{H}4-21} \) segment is not an absolute requirement for i/i-dependent RBC agglutination, because cold agglutinins using \( \mathrm{V}_{\text{H}} \) segments of the \( \mathrm{V}_{\text{HIII}} \) family have been reported.\(^{57}\) The restricted usage of the \( \mathrm{V}_{\text{H}4-21} \) by anti-i/i cold agglutinins is intriguing and may be related to the overrepresentation of the \( \mathrm{V}_{\text{H}4-21} \)–expressing clones in the normal B-cell repertoire, as determined by the \( \mathrm{V}_{\text{H}4-21} \)–related 9G4 idiotype studies in the circulating blood of adults, as well as cord blood and fetal tissues.\(^{55}\) In this regard, it is not known whether these circulating \( \mathrm{V}_{\text{H}4-21} \)–expressing B cells also have anti-i/i specificity. If this were the case, one could speculate that the abundant representation of \( \mathrm{V}_{\text{H}4-21} \)–expressing B cells in the periphery results from positive selection by i/i Ag, which are present not only on RBCs but...
also on lymphocytes. Expression of the i Ag on human erythrocytes is developmentally regulated. It is maximal in fetal and neonatal life and decreases in adult life, in which the expression of the I Ag prevails.

An Ag-driven process of clonal selection may play a role in the emergence and/or expansion of certain neoplastic B lymphocytes. Consistent with their putative germinal center origin, FL B cells, which represent the neoplastic equivalents of the elements recruited in a secondary Ag-specific response, display somatic mutations that resemble in nature and distribution those characteristic of an affinity maturation process. A recent thorough documentation of the somatic mutation and clonal evolution of an FL expressing a V\(_{\text{H}}\)4-21 gene, the antigenic specificity of which had not been determined, showed at least three amino acid mutations, the anti-i/I “characteristic” Gly to Asn mutation at position 31, a Val to Ile mutation at position 71, and a Ser to Thr mutation at position 83, which are shared by the HBL-2 and HBL-3 MoAb V\(_{\text{H}}\)4-21 segments. A role for clonal selection by self Ag during the evolution of anti-“Pr\(_2\)”-specific B-cell lymphoma has been documented in detail. The Pr\(_2\) Ag is a sialoglycoprotein and provides, along with the multiple N-acetyllactosamine i/I Ag, the target for autoimmune phenomena that occur in association with several human clonal B-cell disorders.

The sequences of the DNA amplified from the HBL-3 MoAb-producing cell line and autologous fibroblast genomic DNA, using the HBL-3 leader, V\(_{\text{H}}\)4-21 FR1, and V\(_{\text{H}}\)4-21 FR3 primers, as well as the differential hybridization of the HBL-3 CDR1 oligonucleotide (encompassing an FR1-CDR1 sequence of the HBL-3 MoAb VH segment) with the above amplification products, formally proved the mutated status of the HBL-3 VH segment, and suggested that V\(_{\text{H}}\)4-21 was the germline gene that gave rise to it. The somatically mutated status of the HBL-3 and, possibly, HBL-2 MoAbs was further strengthened by the high degree of conservation of the V\(_{\text{H}}\)4-21 gene sequence in humans, and the extension of the point-mutations to the, in general, highly conserved, J\(_{\text{H}}\) and/or J\(_{\text{A}}\) segments. An Ag-selection of the point-mutations in the HBL-2 and HBL-3 MoAb VH segments was suggested by the differential R:S mutation ratios in the CDR and FR (HBL-2 MoAb, 5.0 and 1.1, respectively; HBL-3 MoAb, 2.2 and 0.3, respectively) and the accumulation in the CDR of the HBL-2 and/or HBL-3 VH segments of amino acid replacements that are shared by other anti-i/I cold agglutinins and might have increased the affinity of the V\(_{\text{H}}\)4-21 segment for the i Ag, including the Gly to Asp mutation at position 31, which is shared by the FS-1, FS-2, FS-4, and KA V\(_{\text{H}}\)4-21 cold agglutinins.

The Ser to Thr mutation at position 35, which is shared by FS-1, FS-4, and FS-6 cold agglutinins, and the His to Tyr at position 53, which is shared by the FS-3 cold agglutinin. However, a positive clonal selection of R mutations in the HBL-2 and HBL-3 MoAb VH segment CDRs was not further substantiated by the statistical analysis according to the binominal distribution model with the correction for FR R mutations, as proposed by Shlomchik et al.

This finding may be consistent with a putatively inherent anti-i/I activity of the unmutated V\(_{\text{H}}\)4-21 gene product, and, perhaps, a clonal selection against R mutations in the HBL-2 and HBL-3 MoAb VH segments CDRs, similar to that shown for other B-cell tumor anti-RBC autoantibodies. The substitution of the Val with an Ile in the HBL-3 MoAb VH segment FR1 Ala-Val-Tyr (residues 23 to 25) triplet, which provides the structural correlates for the anti-idiotypic 9G4 antibody binding, as recently shown by Potter et al., is possibly responsible for the lack of 9G4 reactivity of the HBL-2 MoAb.

In the present AIDS-associated BLs, it is unclear whether the initiation of the anti-i/I Ag autoantibody response constituted a crucial event in the neoplastic transformation. The putative anti-self Ag clonal expansion and selection may have preceded the genetic accident, ie, c-myc proto-oncogene chromosomal translocation. Alternatively, in these BLs, the specific B-cell expansion and selection may have followed the chromosomal translocation, resembling the series of events that have been paradigmatically illustrated in relationship to bcl-2 proto-oncogene chromosomal t(14;18) translocation by Zelenetz et al. in a FL for which, however, a specific Ag could not be identified. Knowledge of the sequential order of activating, proliferating, and transforming events, including c-myc translocation and activation, Ag-dependent B-cell amplification, somatic hypermutation, and clonal selection, is crucial for a better understanding of the molecular pathogenesis of AIDS BL and, possibly, other BLs. These issues could be best addressed by the use of a tumor-specific Ig H chain CDR3 sequence oligonucleotide to identify tumor-related Ig V\(_{\text{H}}\)-D-J\(_{\text{H}}\) sequences in nonmalignant B-cell progenitors.

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Two acquired immunodeficiency syndrome-associated Burkitt's lymphomas produce specific anti-i IgM cold agglutinins using somatically mutated VH4-21 segments

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