Variable Region Gene Analysis of Pathologic Human Autoantibodies to the Related i and I Red Blood Cell Antigens


To investigate the molecular basis of the autoimmune response to the related i and I carbohydrate antigens, we studied cold agglutinins (CA) from B-cell clones and from the peripheral circulation of patients with lymphoproliferative syndromes. Sequence analyses of expressed variable region genes indicate that both anti-i and anti-I specificities from B-cell clones from two patients are encoded by the V_4,21 or a very closely related V_4, heavy chain gene, whereas the expressed light chain genes differed. The anti-i-secreting B-cells express unmutated germline-encoded V_4,21 and V_I gene sequences. The V_r region gene encoding anti-i has the closest homology (97%) to the V_4,21 germline gene and differs at the protein level by only three amino acids. In contrast, while the V_r region gene encoding anti-I is most homologous (98%) to the V,III, kvi328 germline gene, there are seven amino acid differences due to nonrandom replacement mutations, which suggests a role for antigen-mediated selection in the anti-i response of this individual. These studies were extended by a structural survey of 20 additional serum CA using antipeptide antibodies specific for determinants in V_r and V_v regions. All anti-i and anti-I CA were shown to express V_4, heavy chains, and 14 of 17 CA expressed a previously described V_4, second hypervariable region determinant, termed VH4-HV2a. We also found that 13 of 14 anti-i CA used V,III light chains, while the anti-I CA used light chains from at least three V_r families. Taken together, the data show that anti-i and anti-I CA probably both derive from the V_4,21 gene (or a closely related gene). Furthermore, the restricted V_r and different V_v gene use in anti-i and anti-I CA may reflect the close structural relationship of the i and I antigens.

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THE i AND I red blood cell (RBC) antigens are oligosaccharide determinants composed of repeating N-acetyl lactosamine units attached to either protein or ceramide.1 Previous studies have shown that umbilical cord RBCs contain mostly i and small amounts of I determinants. During the first 18 months after birth, however, the proportion of i antigen determinants continues to decrease, coinciding with an increase in I antigen expression. This transition is due presumably to a branching enzyme that attaches sugars by glycosidic linkage to galactose residues of the unbranched structure (i antigen) to form the complex branched structure (I antigen).1

The corresponding cold reactive anti-i and anti-I antibodies are also known as cold agglutinins (CA).2 They comprise a heterogeneous group of binding specificities that recognize different determinants of the oligosaccharide chain.3 Serologically, the anti-i and anti-I specificities are broadly defined by their differential reactivities to cord (predominantly i-expressing) versus adult (predominantly I-expressing) RBCs. In normal adult sera, the specificity of CA is almost always anti-i, while the rare autoanti-i specificity is usually associated with certain disease states (B-cell neoplasia, Epstein-Barr virus [EBV] infection).

The pathologic counterparts of the anti-i autoantibodies are generally derived from clonal B-cell expansions that may progress to a frank lymphoma. Limited NH2-terminal amino acid sequence analyses of the anti-i response in particular have shown a restricted V_rIII use.4 Heavy chain-associated cross-reactive idiotypes (CRI) have been described,56 but the structural basis of these markers has not been examined. However, by use of primary sequence-dependent antibodies to variable (V) region determinants we have recently shown that anti-i CA derive from the V_r family, and that most are also recognized by an antibody directed against a specific V_r second complementarity determining region (CDR) sequence.8 Because the i and I antigens are structurally related, we hypothesized that the corresponding anti-i and anti-I autoantibodies might be encoded by the same or similar Ig V region genes.

To analyze the structural basis of anti-i and anti-I binding, we first studied two patients, one with anti-i and the other with anti-i-induced hemolysis. Both individuals have idiopathic CA disease, ie, chronic relapsing autoimmune hemolysis caused by a monoclonal anti-erythrocyte autoantibody but without evidence of an underlying B-cell neoplasm. In one individual, a chromosomally aberrant clone could be identified in peripheral blood (PB), which is a feature typical of CA disease.60 We generated lymphoblastoid B-cell lines from both patients and compared the clones and their supernatants with PB mononuclear cells (PBMC) and serum from the corresponding patient. The serologic, DNA hybridization, and karyotype analyses established that the secreted CA are closely related to the pathogenic serum autoantibodies. The nucleic acid sequences were then determined to examine the diversity of the expressed V region genes encoding these antibody specificities and to seek evidence for antigen-mediated
clonal selection. We next extended our studies to a panel of 20 additional serum anti-i and anti-I CA to further assess heavy (H) and light (L) chain usage in CA disease.

MATERIALS AND METHODS

Patient Selection

Subjects were selected based on referral to a central blood bank/immunoserology laboratory. Binding specificity was performed by standard clinical laboratory techniques to detect high titer IgM cold agglutinins to the i and l related series of determinants. Subjects were omitted if subsequent evaluation indicated the presence of a postinfectious syndrome, eg, acute infectious mononucleosis or recent Mycoplasma pneumoniae infection. Thereby, patients were identified with monoclonal and high-titer CA associated with chronic CA syndrome, and secondary to B-cell lymphoma, well differentiated lymphoma, and Waldenstrom’s macroglobulinemia. Samples were designated either anti-i- or anti-I-binding specificities based on the predominance of cold reactive agglutination with O+ umbilical cord cells or adult O+ RBCs, respectively. Test erythrocytes were also treated with ficin to show that binding was not decreased by protease treatment.

Establishment of EBV-Transformed Clones

EBV transformation of PB lymphocytes (PBL) was accomplished as previously published.

Briefly, 10^7 monoclonal cells were suspended in 4 mL RPMI 1640 medium, supplemented with 15% fetal bovine serum (FBS) and gentamycin (10 μg/mL). The EBV-containing supernatant of the B95-8 Marmoset cell line was freeze-thawed and filtered. One milliliter of this supernatant was added to the lymphocyte suspension. The lymphocytes were then cultured for 24 hours at 37°C in 5% CO2 in 95% humidified air. The transfected cells were washed in phosphate-buffered saline (PBS), pH 7.4, and subcloned by limiting dilution in 96-well plates. Wells were screened for CA activity as previously published. Cell lines that were considered clonal based on statistical analyses were also karyotyped and the secreted CA purified from supernatant for further studies (see below).

Purification of Cold Agglutinating Antibodies

CA were purified from serum by repetitive cold-warm-cold incubation with packed RBCs followed by heat elution at 37°C. CA with anti-i fine-binding specificity were isolated from serum with adult cells, and CA with anti-I activity were purified on umbilical cord blood cells. In certain cases, large-scale purifications were performed by removal of IgG with a protein G-sepharose 4B column before affinity purification of CA on a desialated-human IgG-sepharose 4B column, as described.

Purified IgM proteins were assayed for CA activity, and all CA were without rheumatoid factor (RF) or antinuclear binding activity. IgM CA from culture supernatant and from the heat eluate were purified by passage over anti-human IgM (μ-chain-specific)-cyagen bromide (CNBr)-activated Sepharose columns (Cooper Biomedical, Malvern, PA; Pharmacia, Piscataway, NJ). The bound proteins were eluted with 0.2 mol/L glycine, pH 2.5, rapidly neutralized, and then dialyzed against PBS. The isotype of eluted antibody was then evaluated by the Ouchterlony method (Cooper Biomedical) and by agarose electrophoresis followed by immunofixation.

Monoclonal IgM Proteins

The LES, monoclonal IgM-RF, was isolated from the peripheral circulation of a patient with a B-cell chronic lymphocytic leukemia that is surface phenotype negative for CD5. The V_H gene encoding this autoantibody has been isolated from a genomic library of circulating cells, and the sequence has been reported. The L16 lymphoblastoid cell line produces a polyreactive autoantibody that includes anti-DNA activity and was the kind gift of Dr T. Logtenberg (Utrecht, The Netherlands). This IgM is directly encoded by the germline V_H6 gene. The C6B2 hybridoma produces an IgM antibody with anti-DNA, rheumatoid factor, and antibacterial polysaccharide activity and was donated by Dr S. Hoch (Boston, MA). The Ab26 cell line produces an IgM polyreactive antibody and was the gift of Dr J.D. Capra (Dallas, TX). Deduced V_H sequences are listed in Table 1. In addition, monoclonal RF and other purified monoclonal Ig from patients with lymphoproliferative syndromes have been previously described.

Cytofluorimetry and Karyotype Analysis

Cytofluorimetry was performed using a FACSCAN analyzer (Becton Dickinson, Mountain View, CA) equipped with a 488 nm argon laser, as previously described.

In the karyotype studies, 20 to 40 metaphases were counted, and at least three analyses were performed on each specimen. Additional karyotypic analyses were performed to characterize chromosomally abnormal EBV-transformed clones.

Southern Blot Analysis

Genomic DNA (5 to 10 μg) was extracted from PBL and the EBV-transformed B-cell clones digested with EcoRI, HindIII, and BamHI restriction enzymes.

The digested DNA was then separated electrophoretically on 0.5% agarose gels, blotted onto nylon membranes, and hybridized to various DNA probes. The probes were cloned genomic DNA fragments for the human H chain joining region (J_H), as well as a probe specific for terminal repeat fragments of linear EBV DNA. The probes were labeled with 32P by random priming using T, DNA polymerase (Stratagene, San Diego, CA). Hybridization was performed in 7% sodium dodecyl sulfate (SDS)/0.5 mol/L NaHPO4, pH 7.2, at 42°C; membranes were washed with decreasing salt concentrations with a final wash in 0.1% SDS/0.1X SSC at 65°C.

Sequencing and cDNA Cloning of V Region Genes

The experimental approach for the cloning and sequencing of the expressed Ig V genes has previously been described.

Briefly, first-strand cDNA synthesis from 1 μg total cellular RNA was performed with reverse transcriptase (Life Sciences, St Petersburg, FL) and an oligonucleotide primer specific for the μ chain constant region (Cμ): 5’-CGA GGG GGA AAA AAA GGG TGT GGC C-3’. For the cell line 15B from CAP, we used a 21-βp oligonucleotide primer corresponding to the Ig diversity-joining region (D-J) junction of clone 15A (patient CAP) (see Fig 2).

Second-strand synthesis was performed by polymerase chain reaction (PCR) amplification with AmpliTaq recombinant stable Tag polymerase (Cetus Perkins-Elmer, Norwalk, CT), the same Cμ or D-J primer, and a 5’ primer complementary to the conserved portion of published leader sequences of the V_H gene family: 5’-ATG AAA CAC CTG TGG TTC TT-3’. The same approach was used to isolate the V_J gene sequences. The primers corresponded to κ chain constant region and leader sequences of the V_J and V_J3 families (indicated in Figs 3 and 4). For the isolation of the V_L germline sequences (V_J and kv328) from granulocyte DNA, we constructed oligonucleotide primer corresponding to primers encoding V leader and CDR3 regions that were shared among the expressed V_L genes and the published relevant (closest homology) germline gene sequences (indicated in Figs 3 and 4). Thirty cycles were performed in a programmable thermocycler (Coy Laboratory Products, Inc, Ann Arbor, MI), with soak temperatures of 94°C, 55°C, and 72°C for 1
minute each followed by 9 minutes at 72°C. Blunt-ended double-stranded amplified DNA was then ligated with T, ligase (International Biotechnologies, Inc, New Haven, CT) into the phosphatased Sma I site of pBS M13 (Stratagene) and transfected by electroporation (Biorad Laboratories, Rockville Center, NY) into the XL-1 blue strain of Escherichia coli. Clones were screened by blue/white selection and restriction analysis of plasmid DNA. Clones containing the expected 450-bp size insert were sequenced by dideoxy nucleotide termination method with Sequazyme (United States Biochemical Corp, Cleveland, OH) using both “+” and “reverse” universal primers. Human mu and kappa oligonucleotide primers were synthesized by an oligonucleotide synthesizer (Applied Biosystems, Inc, Foster City, CA) using phosphotriester chemistry.

Generation of Antipeptide Antibodies

Immunodominant portions of the first framework region (FR1) of Ig H and L chains were used for the creation of antipeptide antisera that are specific for the V each gene family, as previously described. Antisera were also generated to portions of the second CDR of all reported germline V, genes. Certain sequences of these synthetic peptides are compiled in Table 1. Antisera were also used that identify sequences from the first domains of H and L chain constant regions. Peptides were conjugated by their carboxy-terminal cysteine to the carrier, keyhole limpet hemocyanin, before immunization of rabbits, as described.

Immunoblot Reactivity of Antipeptide Antibodies

To show the binding specificity of the peptide-induced reagents, Western immunoblot analyses were performed with 4 μg/lane of Ig proteins, as previously described. Immoblot reactive of the antipeptide antibodies directed at Ig constant regions, V, CDR, and V, family-specific reagents has been previously reported. Certain sequences of these synthetic peptides are compiled in Table 1. These were first preadsorbed with a related peptide at 0.1 μg/mL before immunoblot analyses. Primary sequence defined specificity was documented if anti-Ig reactivity of an antipeptide serum was completely abolished after preincubation with immunizing peptide at 0.01 μg/mL.

Monoclonal Anti-Idiotype Reactivity

The IgG2a rat antibody, 9G4, was produced by a rat-mouse heterohybridoma after immunization of a Lou rat with the IgM-λ, SA. Binding activity of the 9G4 antibody with paraproteins and inhibition of red blood cold agglutination has been described, but the structural basis of the expression of the 9G4 idiotype has not been investigated. For assays of binding activity of the monoclonal anti-idiotype, polystyrene microtiter plates were precoated over-night at 4°C with affinity-purified polyclonal goat antihuman IgM (Boehringer Mannheim, Indianapolis, IN) at 10 μg/mL in phosphate-buffered saline (PBS), pH 7.0. For the study of the RID CA, the wells were precoated with either antihuman κ or λ (Boehringer Mannheim). Plates were quenched for 1 hour at room temperature (RT) with 1% bovine serum albumin (BSA)-PBS, then serial dilutions of purified IgM proteins in BSA-PBS were added, beginning at 2 μg/mL. After incubation at RT for 4 hours, plates were washed with 0.05% Tween 20 PBS, then wells were incubated with an anti-idiotype antibody for 4 hours at RT. After washing, plates were incubated with either alkaline phosphatase (AP)-labeled affinity-purified F(ab')2 goat antinegat IgG (Calbiochem, La Jolla, CA) or AP-labeled affinity-purified F(ab')2 goat antirat IgG. Replete wells without anti-idiotype or isotype control were incubated with AP-labeled affinity-purified goat antihuman IgM.
Plates were subsequently developed with 50 mmol/L sodium carbonate, pH 9.8, containing p-nitrophenyl phosphate and 1 mmol/L magnesium chloride. The IgM-λ, SA, and the IgM-κ, VOG, were used as positive controls for the 9G4 assays.

Statistical Analyses

Statistical detection of positive selection in the CDR was performed using a previously reported binomial probability model. Binding reactivity of the monoclonal anti-idiotype was designated positive if the value for an Ig protein was greater than 4 SD above the mean A405 of negative proteins. Statistical significance was determined by the two-tailed Fisher exact test.

RESULTS

Establishment of CA-Secreting B-Cell Clones

PBL exposed to EBV were seeded at 1 to 3 × 10⁶ cells/well of 96-well microtiter plates. After 5 weeks of tissue culture, the supernatants were assayed for CA activity. CA activity was detected in only 2 of 418 wells from different plates, suggesting that these wells likely contained the derivatives of a single CA-secretating, transformed B cell. Subcloning at less than 10 cells/well was not feasible for the two cell lines derived from patient CAP (15A and 15B). Limiting dilution of cell line 15A at 10 cells/well yielded 10 of 235 wells with growth; 6 of 10 also tested positive for CA activity. This distribution suggested that these sublines were clonal.

For patient VOG, approximately 64% of wells plated showed colony growth, of which 56% had CA containing supernatant. Two clones were established by repeated limiting dilution at 0.1 cell/well; these two clones are referred to as 20A and 20C. An additional cell line was clonal at 10 cells/well and is referred to as 20B.

The three cell lines derived from patient VOG (20A, 20B, and 20C) are all stable Ig-producing clones (5 μg/mL). To prove that these clones represent independent transformations of different B cells, we performed DNA hybridization studies with a probe specific for the terminal repeat segments of EBV. When this probe is hybridized to the EcoRI-BamHI-digested DNA from independent EBV-transformed B-cell clones, restriction fragments of different size are generated, because the infecting EBV virus fuses to form a plasmid whose size is determined uniquely at the time of the fusion process. The three B-cell sublines from the patient VOG were found to be independent clones by this method (data not shown).

Relationship of EBV-Transformed B-Cell Clones to Patient Material

Serology of CA. The binding specificity of the RBC autoantibodies both from serum and clonal supernatants was determined by their differential reactivity against adult and cord RBCs. The CA derived from serum and clonal supernatant of patient CAP reacted more strongly with cord and I-negative RBCs and was therefore classified as anti-i; in contrast, the CA from serum and clonal supernatant of patient VOG preferentially reacted with I-positive RBCs and was classified as anti-I. For example, the CAP CA isolated from serum (heat eluate)/clonal supernatant reacted with adult (I) versus cord (i) erythrocytes at titers of 1:32/1:64 versus 1:64/1:512, respectively. In contrast, the VOG CA isolated from serum (heat eluate)/clonal supernatant reacted with adult (I) versus cord (i) erythrocytes at titers of 1:1024/1:128 versus 1:128/1:4, respectively. For patient VOG, the IgM-k CA was easily detectable as a homogeneous band by agarose gel electrophoresis followed by immunofixation, but for the patient CAP, demonstration of a homogeneous IgM-k band required the isolation of the CA from serum (not shown). Furthermore, only in patient VOG was a B-cell clone readily detected in PBL by karyotype and Southern blot analysis (see Fig 1). These findings indicate that both patients had evidence of monoclonal CA, although in patient CAP, the size of the circulating B-cell clone (CA-secreting) was too small for detection by chromosome or DNA hybridization analysis.

Cytofluorimetry and chromosome analysis. For the clone isolated from patient CAP, the surface phenotypes as determined by cytofluorimetry were μ, δ, κ, HLA-DR(+), CD22(+), CD5(−), whereas the three clones from patient VOG were all μ, κ, HLA-DR(+), CD22(+), CD5(−). In addition, homogeneous staining of cell populations (clone 15A and 20A) was found with the 9G4 rat monoclonal anti-idiotype (see below). Neither patient had a monotypic B-cell population in PB discernible by cytofluorimetry. Karyotype analysis of PBL and EBV-transformed clones from patient CAP showed only karyotypically normal cells. In contrast, both the PBL and the B-cell clones from patient VOG showed the same aberrant karyotype 48,XX,+3,+12 (data not shown).

DNA hybridization analyses. Southern blot analyses were performed on DNA from patients' PBL as well as the established B-cell clones. In patient CAP (Fig 1A), DNA hybridization with the Jκ probe failed to identify a clonal B-cell population in PBL and showed a germline band only. For 15A, the EcoRI digest shows one rearranged band (9.4 kb), whereas the HindIII digest shows two H chain gene rearrangements (9.6 and 3.0 kb); the latter finding suggests that the restriction fragments in the EcoRI digest are of similar size. The same restriction fragments were also present in DNA from cell line 15B. Additional Ig restriction fragments, however, were seen for 15B, indicating that this cell line was not homogeneous (not shown). In patient VOG, identical Ig gene rearrangements were observed for all clones (20A, 20B, and 20C) as well as for PBL (which also showed the germline band). The hybridization experiments for patient VOG are illustrated in Fig 1B and C using the Jκ probe, in which the clones have rearranged both H chain alleles. In the HindIII digest, the Jκ hybridizing bands are 9.6 and 4.7 kb (Fig 1A); in the EcoRI digest the restriction fragments are 28 kb and 9.4 kb.

Molecular Analyses of Expressed V Region Genes Encoding Anti-i and Anti-I

Nucleotide sequencing was performed to determine a genetic basis for the related anti-i and anti-I specificities. To control for sequencing and cloning artifacts, at least two DNA clones were isolated and sequenced in both direc-
Fig 1. DNA hybridization studies of patient material and B-cell clones. Southern blot analysis of PBL (15PB and 20PB) and B-cell clones (15A, 20A, and 20B, and 20C) isolated from patient CAP (A) and patient VOG (B and C). DNA was also isolated from PBL of a healthy individual (normal) to determine the size of the germline band. In panel B there is an additional cross-hybridizing band of 3.6 kb that is commonly seen with this probe in HindIII digests of genomic DNA. The molecular size standards and the positions of the restriction fragments are shown beside the illustrations. G, germline bands; arrows, rearranged bands.

The anti-i- and anti-I-specific B-cell clones express similar \( V_{\mu} \) genes. For clones 15A, 20A, 20B, and 20C, we used the \( V_{\mu} \) leader-C\( \mu \) primer pair for reverse transcription and PCR amplification. No amplification was observed with primers corresponding to the other \( V_{\mu} \) family leader sequences, thus supporting the idea that these are clonal cell lines. Because 15B was not a true clone but did contain a B-cell population related to 15A (see DNA hybridization studies above), we sequenced the 15A clone first and synthesized a 21-mer corresponding to the D-J region of 15A. A \( V_{\mu} \) clone was then isolated from 15B DNA by direct PCR using the \( V_{\mu} \) leader-DJ primer pair. As can be seen in Fig 2, the \( V_{\mu} \)-D-J sequences of these two clones (15A and 15B) from patient CAP are identical to each other and the \( V_{\mu} \) is also identical to the recently reported human \( V_{\mu} \)4.21 germline gene. The \( V_{\mu} \) sequences of clones 20A, 20B, and 20C are identical (Fig 2). The perfect sequence homology among the three clones, particularly in the highly variable CDR3 region, along with the identical Ig gene rearrangements and same abnormal karyotype, all confirm the clonal relatedness of these lines. When compared with known \( V_{\mu} \)4 germline sequences these rearranged \( V_{\mu} \) sequences are most homologous (97%) to the published \( V_{\mu} \)4.21 germline gene (Fig 2A and C). Although the assignment of a precursor germline gene cannot be made with certainty, we propose that the \( V_{\mu} \)4.21 gene is the likely precursor germline sequence of the expressed \( V_{\mu} \) genes based on the following arguments. The complexity of the \( V_{\mu} \)4 gene family has recently been studied by others; it is one of the smaller human \( V_{\mu} \) families and has been reported to display little polymorphism. The DNA hybridization studies with a \( J_{\mu} \) probe, shown in Fig 1 for EcoRI and HindIII digests, show that clones 15A, 20A, 20B, and 20C share a rearranged band of the same size using three different restriction enzymes (BamHI not shown). Because all of these clones also use the same \( J_{\mu} \)4, the findings strongly suggest that these clones have rear-
ranged and express the same V_{g.4.21} or a closely related gene.

Although the V_{g} genes encoding anti-i and anti-I are highly homologous, there is no similarity in the CDR3 regions (Fig 2B). A comparison of each of the D genes with published germline D gene families did not identify a homologous D segment, probably because of the large number of somatic changes occurring in the generation of D segments.\(^4\)\(^7\) The J_{D} regions were quite similar to each other and to the J_{D}4 germline sequence. The J_{D} region of patient CAP had one silent difference from the germline and was thus identical in amino acid composition to the germline J_{D}4 sequence. The J_{D} region of patient VOG, however, did show two differences resulting in amino acid replacements, compared with the published J_{D}4 germline sequence.\(^3\)

**Isolation of the V_{g} germline gene encoding the 15A anti-i.**

While the expressed V_{g} gene of the anti-i secreting cell line 15A is 92% homologous to a previously reported V_{I} germline gene, a much better match (greater than 98% homology) is found with a rearranged V_{I} gene expressed by the Walker cell line derived from a Burkitt's lymphoma.\(^4\)\(^5\) This finding suggests that the expressed V_{I} genes of the 15A cell line and the Walker cell line may originate from a different, closely related V_{I} germline gene. To test this hypothesis, we sought to isolate the relevant precursor V_{I} germline gene from patient CAP using PCR and cloning. The amplification primers were designed to limit the number of amplified V_{I} genes to those closely related to the expressed V_{I} gene of cell line 15A. The leader (sense) primer sequence was homologous to several previously published V_{I} genes including the Walker cell line. The antisense primer corresponded to the distal CDR3 region sequence (Fig 3). A more distal (3') primer that would be fairly specific for this new V_{I} gene could not be readily

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**Fig 2.** Expressed V_{R} sequences encoding anti-i and anti-I. (A) Nucleotide sequences of the expressed V_{R} region genes from anti-i (15A and 15B) and anti-I (20A, 20B, and 20C) EBV clones; (B) the D and J_{D} region gene sequences. To isolate the V_{R} region gene of 15B, a 20-bp oligonucleotide sequence (indicated with an asterisk) corresponding to the D-J junction of clone 15A (5'-CCA GTA GTC AAA GTA GTA AGC-3') was used to prime cDNA and in subsequent PCR amplification. Amino acid translation is given above nucleotide sequence and numbered according to Sanz et al.\(^4\) CDRs (CDR1, CDR2, and CDR3) and D and J_{D}4 gene segments are indicated. Homology of the nucleotide with the germline sequence on the top line is shown as a dash. Differences (in clones 20A, 20B, and 20C) from the published V_{g.4.21} germline sequence are indicated in both nucleotide and amino acid sequences. (C) The translated amino acid sequences of the published V_{g.4.21} germline and expressed V_{g} genes of the anti-i- and anti-i-specific B-cell clones.
### Fig 3. Sequence analyses of V,1 encoding anti-i.

Nucleotide sequence analysis of the expressed V,1 region gene from the 15A anti-i clone is shown on the top line. The V,1 germline precursor sequences isolated from patient CAP granulocyte DNA are indicated as 15G-2, -3, -4, -5, -7, -8, -9, -18, and -27. Isolate 15G5 is a pseudogene; X denotes nucleotide deletion. Sequences LSGV1 and 20GV1 represent relevant V,1 germline sequences isolated from two unrelated individuals using the same primers. Homology of the nucleotide with the germline sequence on the top line is shown as a dash. (*) The oligonucleotide sequences used as flanking (5' and 3') primers for amplification of the V,1 germline gene from patients' granulocyte DNA. The 5' primer corresponds to the V,1 leader sequence [5'-ATG GAC ATG AG(GA)G TCC 3']. The 3' primer corresponds to the V,1-CDR3 region of 15A: 5'-TGT GGG GTA CTG TAA C-3'.

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### Table 1

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of three separate nucleotide deletions causing a frame shift. Of the remainder, 5 of 10 are identical to the rearranged VJ of clone 15A; the other five sequences differ from each other and the rearranged Ve gene by a single base difference. The isolates from two unrelated individuals showed similar results: one isolate (20GVJ) is also identical to the rearranged VJ of 15A, while the other (LSGVJ) differs only by one nucleotide. Thus, of the 12 functional germline genes isolated from granulocyte DNA, six are identical and the other six differ only by one (although not the same) base pair. These single base-pair changes are likely to be the result of one or a combination of the following possibilities: (1) *Taq* infidelity; (2) allele-specific differences; or (3) multiple separate but greater than 99% homologous VJ genes. Based on the low sequence variation observed, theoretically, all five base-pair differences can be ascribed to *Taq* infidelity.

Our method involved repeated PCR amplification of genomic granulocyte DNA, cloning of the PCR product, and sequencing multiple independent isolates. In addition, the same procedures were performed on granulocyte DNA from two unrelated individuals to gather support for the existence of a yet-unreported new VJ germline sequence. Subsequent to the experiments described here, this VJ sequence has been isolated from a chronic lymphocytic leukemia (CLL) patient in our laboratory as well as from another CLL patient studied in the laboratory of Dr T. Kipps (La Jolla, CA; personal communication). The observed homology between the genes isolated from granulocyte DNA and the expressed VJ genes from different individuals, as well as the repeated occurrence of this sequence in the sample of clones analyzed, strongly suggests that the VJ expressed by clone 15A is identical to a new VJ germline gene, which we designate 15AVJ.

**Isolation of the VJ germline gene encoding the 20A anti-I.** The VJ chains of clones 20A, 20B, and 20C are identical to each other, and share most homology (96%) to a previously published VJIII (kv328) germline gene (Fig 4).46

In patient VOG we sought to isolate the relevant germline VJIII sequence to determine the degree of somatic diversity. Genomic granulocyte DNA from patient VOG was amplified, cloned, and sequenced on three independent occasions to confirm the existence of a yet-unreported new VJ germline sequence. Subsequent to the experiments described here, this VJ sequence has been isolated from a chronic lymphocytic leukemia (CLL) patient in our laboratory as well as from another CLL patient studied in the laboratory of Dr T. Kipps (La Jolla, CA; personal communication). The observed homology between the genes isolated from granulocyte DNA and the expressed VJ genes from different individuals, as well as the repeated occurrence of this sequence in the sample of clones analyzed, strongly suggests that the VJ expressed by clone 15A is identical to a new VJ germline gene, which we designate 15AVJ.

**Immunoblot analysis of VJ structural diversity.** To determine the structural diversity of the L chains of the circulating CA, analyses were performed with primary sequence-dependent antibodies. The reactivities of these reagents with replicate blots of the separated L chains of polyclonal IgM, the anti-I CA ODO, and seven anti-i CA are shown in Fig 5. One of the anti-i CA (RID) is shown to have both k (top panel) and \( \lambda \) chains (bottom panel), but both types of L chains were associated with similar \( \lambda \) chains (see below). The ODO sample is characteristic of anti-I CA because it has only VJIII-derived L chains. Similarly, four anti-i CA (GRO, PRO, TM, and FA) also have only VJIII-derived L chains. In contrast, the anti-i CA, LG, has \( \lambda \)-derived L chains. By this method we also confirmed, equivalent to their respective cell lines, that the affinity-purified serum CA from CAP expressed only VJ I L chains, and VOG expressed only VJIII L chains (not shown). The lack of reactivity of the L chains of PER* and RID with the \( \lambda \) family-specific reagents may have been due to the presence of variant VJ-FR1 region sequences of the known \( \lambda \) families not identified by the antipeptide antibodies, which has previously been observed in two VJIII L chains.19 Control studies suggested that it was unlikely that nonreactivity was due to inadequate amounts of the L chains to detect subsets. In summary, these studies show that 13 of 14 anti-I CA use \( V_{JIII} \)-derived L chains, while only four of eight anti-i CA use \( V_{JIII} \)-derived L chains. The L chains of
Fig 4. Sequence analyses of Vlll encoding anti-I. Nucleotide sequences of Vlll region gene from the anti-I-secreting clones are indicated as 2OA, 2OB, and 2OC; the precursor germline sequences isolated from granulocyte DNA are indicated as MG.1 through 9. Amino acid translation shares homology with the CDR3 region of the 20A light chain as well as to the CDR3-intron region of the published kv328 germline sequence.
the anti-i CA derive from at least three V gene families. There is a significant difference in the usage of non-V,III L chains in anti-I and anti-i CA ($P = .039$), and the data are compiled in Table 2.

The immunoblot finding of both $\kappa$ and $\lambda$ L chains in the RID CA specimen was unexpected, because only a monoclonal IgM-$\lambda$ was detected by immunoelectrophoresis (not shown). Therefore, enzyme-linked immunosorbent assay (ELISA) studies were performed with wells precoated with either antihuman $\kappa$ or antihuman $\lambda$ antibodies, which showed that the RID CA contains both $\kappa$ and $\lambda$ Ig that are associated with H chains with the 9G4 idiotyp (see below). This finding suggests that the RID CA may be biclonal, similar to a previously reported case of a biclonal anti-I CA.49

**Immunoblot analysis of $V_h$ structural diversity.** $V_h$ diversity was analyzed with antipeptide antibodies to FR1 determinants that are specific for the six known $V_h$ fami-
Table 2. Structural and Idiotypic Diversity

<table>
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<tr>
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<th>V6,4-HV2a</th>
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<td></td>
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<td>V,4</td>
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<tr>
<td>2. BAT</td>
<td>-</td>
<td>V,III</td>
<td>V,4</td>
<td>-</td>
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<tr>
<td>3. BON</td>
<td>-</td>
<td>V,III</td>
<td>V,4</td>
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<tr>
<td>4. HIG</td>
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<td>V,4</td>
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| Anti-i CA |         |         |     |           |           |
| 1. CAP | + | V,II | V,4 | + | + | + |
| 2. FA | - | V,III | V,4 | + | - | + |
| 3. GRO | - | V,III | V,4 | + | + | + |
| 4. LG | - | V,II | V,4 | + | + | + |
| 5. PER* | - | V,II | V,4 | - | + | - |
| 6. PRO | - | V,III | V,4 | + | + | + |
| 7. RID | - | V,II | V,4 | + | + | + |
| 8. TM | - | V,III | V,4 | + | + | + |

| Non-CA |         |         |     |           |           |
| 1. LES | + | V,III | V,4 | + | - | + |
| 2. SA | - | V,II | V,4 | - | - | + |
| 3. GRE | - | V,III | V,4 | - | - | + |

V, and V, family was determined by immunoblot analyses with antipeptide antibodies. The presence of the V6,4-FR1a and V6,4-HV2a sequences was determined by immunoblot analyses after preincubation of antipeptide antibodies with synthetic peptides with related sequences to improve fine binding specificity. Certain V, sequences have been determined, and below "Sequence" are designated (+). V, amino acid sequences were available for the anti-i CA, VOG(20A) and KAU, and the anti-i CA, CAP(15A) and the RF, LES (compiled in Table 1).

*Indicates the presence of two different patients with the same initials PER.

†These κ light chains were unreactive with the V, family-specific reagents (see text).

All of the eight anti-i CA expressed H chains that were identified only by the V6,4 family-specific antibody (examples in Fig 6); all (14 of 14) anti-i CA were identified by the V6,4 family-specific reagent, as previously reported.

As shown in Fig 6, these proteins were further characterized with reagents specific for variations in V6,4 framework sequences. After preincubation of the anti-VH4-FR1 antiserum with the VH4-FR1a peptide, reactivity with the four CA is abolished. In contrast, after preincubation of the anti-VH4-FR1a antiserum with the VH4-FR1 peptide, reactivity with the four CA is abolished. In contrast, after preincubation of the anti-VH4-FR1 peptide, the VH6,4-FR1 sequence in the H chain of C6B2 is identified, but not the H chain, which varies by two residues in the amino terminal aspect of this sequence. In this panel the anti-i CA, CAP and GRO, and the anti-i, ODO and HIG, are also identified (peptide sequences listed in Table 1).
VARIABLE REGION GENES OF ANTI-III AUTOANTIBODIES

anti-VH4-FR1a reagent (compiled in Table 2). These data suggest that almost all of the CA share a variant Vn4 first framework sequence, VH4-FR1a.

The H chains of the anti-i CA were also studied with a reagent that is specific for a Vn4-CDR2 sequence, VH4-HV2a, which is present in a subset of germline Vn4 genes reported thus far.24 We have previously shown that after preincubation of the anti-VH4-HV2a antibody with a related peptide that varied by only a single amino acid, on Vn regions that express the exact cognate sequence were identified. At the bottom of Fig 6, as predicted from the Vn4 sequence after preincubation, the anti-VH4-HV2a reagent identified the Vn4 of known sequence, C6B2 and CAP, but the LES RF was not identified. Of the eight anti-i CA, seven were identified by the anti-VH4-HV2a reagent. In related studies we also showed that the CA from serum and clonal supernatant from the patients VOG and CAP both expressed the same V gene families and unique VH4-HV2a determinant.

Among known Vn genes, only the Vn4.21 gene expresses both the VH4-FR1a and VH4-HV2a sequences (Table 1). Collectively, the data suggest that, while anti-i CA and anti-I CA have differences in Vn expression, they both have a comparable restriction to a subset of Vn4 H chains based on reactivity with the V region-specific reagents (compiled in Table 2).

Structural correlation with the 9G4 idiotype. We next assessed the expression of the H chain-associated 9G4 idiotype on the CA. The 9G4 anti-idiotype was expressed by nine of nine anti-I CA and seven of eight anti-i CA. To determine whether the 9G4 idiotype was only associated with CA, we studied a previously described panel of non-CA monoclonal IgM with 34 RF that include 10 Vn4 RF,25 but only the Vn4 IgM-RF of known sequence, LES, was recognized by the 9G4 antibody. Similarly, of 34 other monoclonal Ig that included eight other Vn4 IgM of unknown binding reactivity, only one additional IgM, GRE, was found to also express the 9G4 idiotope. The 9G4 antibody did not recognize any of the 18 Vn1, one Vn2, 29 Vn3, two Vn5, or two Vn6-derived proteins. In summary, 16 of 17 Vn4 CA, 1 of 10 Vn4-RF, and one of eight Vn4 IgM of unknown binding activity express the 9G4 idiotope. As compiled in Table 2, the expression of the 9G4 idiotope was independent of L chain usage but restricted to a subset of Vn4 H chains. The association of the 9G4 CRI with CA is highly significant, even in comparison with VH4-derived RF autoantibodies (P = 0.0007) and to Vn4-derived IgM of unknown binding activity (P = 0.0004). In addition, these studies indicate that the 9G4 antibody identifies a set of H chains that are nonoverlapping with the Vn4 H chains that express the RF-associated idiotype, LC1.26

The structural diversity of V regions of known sequence was also studied for 9G4 idiotope expression. In this examination, the 9G4 idiotype-reactive IgM include the LES RF and two CA (VOG and CAP), and they are compared with three related H chains that do not express the 9G4 idiotope (C6B2, AB26, and L16). While nearly identical in the portion encoded by the V gene segment, these VH regions are diverse in the CDR3 and FR4 that are the result of VDJ segment joints. By comparison with the known germline VH4 gene segment sequences, the CA and RF autoantibodies that express the 9G4 idiotope have the least sequence variation from the deduced sequence of the germline Vn4.21 gene.25 The recurrent identification of both the Vn4-FR1a and Vn4-HV2a sequences is also consistent with the impression that most, if not all, monoclonal anti-I and anti-i CA derive from the Vn4.21 gene (or closely related gene) (Tables 1 and 2). By comparison, of the six Vn sequences, expression of the 9G4 idiotope was associated with certain conserved residues in the FR1 region adjacent to the first CDR region, and with a few residues at the amino terminus of the second CDR. Binding of the 9G4 antibody was not inhibited with available synthetic peptides (data not shown). However, these initial data are inadequate for specifically identifying the exact idiotypic contact residues.

DISCUSSION

To understand the genetic basis of the related anti-i and anti-I specificities, we established B-cell lines from two individuals and characterized the pathologic antibodies from an additional 20 patients with anti-i and anti-I autoantibody-induced hemolysis. We subsequently determined the relationship of the pathologic serum autoantibodies to those derived from the EBV-transformed clones. In the patient VOG, the close relationship was readily shown by Southern blot and chromosome analyses. In patient CAP, however, the autoantibody-secreting clone was not detectable by these methods. In this case, we would argue that the anti-i-secreting cell lines are, in fact, representative of the pathologic serum autoantibodies based on the isolation of two of two cell lines that shared anti-i specificity and the Vn4-HV2a primary-sequence-dependent marker. These findings are also representative of our panel of circulating CA, as eight of nine anti-I CA and seven of eight anti-i CA also shared this marker.

Sequence analyses of the V region genes expressed by the CA-producing cell lines showed that both anti-i and anti-I antibodies were encoded by the same Vn4.21 gene. In contrast, the anti-i antibody used VJ I and the anti-I antibody uses VJIII. Taken together, the structural characterization of the 14 autoanti-I and eight autoanti-i CA suggests that the anti-i and anti-I autoimmune responses are highly restricted in H chain expression and may derive from a single Vn4 gene. We have also shown that the 9G4 monoclonal anti-idiotype probably identifies CA due to the expression of these Vn4 H chains.

The expressed V region genes were also examined for the number and pattern of somatic mutations to evaluate the role of antigen-mediated selection. We determined that both the Vn and Vl genes encoding the anti-i antibody are identical to germline sequences, whereas numerous base-pair differences were noted in the anti-I response. Compared with its most likely germline precursor, Vn4.21, the Vn gene encoding anti-I has only three amino acid differences (Fig 2C). Two of these amino acid substitutions are located in FR regions, and one is in a CDR. In contrast, the
V<sub>i</sub> sequence encoding anti-I has a relatively high number of amino acid substitutions (relative to the total number of base-pair changes) when compared with the likely precursor germline sequence (kv328) isolated from autologous DNA; these amino acid differences are due to a nonrandom distribution of replacement mutations in the CDRs. We applied a binomial probability model to the pattern of mutations in this V<sub>i</sub> gene as a test for positive selection. The expected fraction of replacement mutations in CDR for this V<sub>i</sub> sequence is 0.20, calculated by multiplying the relative size of the CDR (0.26) by the fraction of all mutations in CDR that could result in amino acid replacements (0.77). Using the binomial probability model, we conservatively calculated that the distribution of five replacement mutations in CDR of a (corrected) total of 11 mutations had a low probability of occurring at random (P = .05). However, if the two nucleotide substitutions in codon 51 occurred asynchronously, they would have generated two independent replacement mutations. In the latter instance, the probability of six replacement mutations occurring at random of a (corrected) total of 12 mutations would be even lower (P = .02). Thus, the statistical model provides evidence that positive selection led to the accumulation of amino acid substitutions in the light chain of this anti-I antibody. Such positive selection of replacement mutations in CDR has been observed in murine immune responses and presumably reflects selection for enhanced antigen binding.

In summary, we have found a nonrandom distribution of replacement mutations in the V<sub>i</sub> encoding the anti-I antibody, which supports a role for antigen-mediated clonal selection. However, the selective pressure may have favored conservation of the H chain germline sequence while favoring mutation of the light chain amino acid sequence. In this regard, the recently reported amino terminal protein sequence analysis of an anti-I CA KAU is in agreement with the conserved V<sub>i</sub>4-V<sub>i</sub>111 sequence. This CA also uses the V<sub>i</sub>4.21 gene, differing by one amino acid only from the translated V<sub>i</sub>4.21 sequence; the light chain is most likely derived from the kv325, V<sub>i</sub>111 gene differing by four amino acid changes, of which one is located in FR and three are in CDR.

Based on the fact that the anti-i and anti-I responses use the same or closely related V<sub>i</sub> but different V<sub>i</sub> genes, we propose a model for the relative contributions of these H and L chains to antigen binding. The V<sub>i</sub> sequence would be required for a global interaction with the i/I carbohydrate antigen complex. In contrast, the V<sub>i</sub> sequence would confer fine specificity to distinct but related determinants of this complex antigen. The D region genes, which differ between the anti-i and anti-I, may also play a role in determining specificity. Interestingly, in the immune response to the influenza virus hemagglutinin, also a complex antigen, an analogous model of differential contributions of H and L chains has been postulated based on V region gene sequence analysis.

The current studies have assessed the genetic basis of pathogenic autoantibodies to the i and I antigens using both molecular and serologic methods. The findings suggest a restricted use of the V<sub>i</sub>4.21 or a closely related gene, which implies a nonstochastic biologic selection. This observation could have occurred due to preferential rearrangement of the V<sub>i</sub>4.21 gene, but this gene does not appear to be overrepresented in cell lines described to date. The lack of somatic mutations in the V region gene sequences encoding the anti-i (CAP), as opposed to the anti-I cell lines (VOG), is intriguing and may reflect differential regulation of the immune responses to these related autoantigens. We hypothesize that the high expression of i antigen on fetal RBCs may mediate tolerance to i, either by clonal anergy or deletion of B cells with anti-i specificity. The expression of I antigen occurs much later in development so immunologic tolerance for I may therefore differ from that for i. This model would explain the low levels of anti-I autoantibodies present in the umbilical cord and adult blood of healthy individuals, and would also explain the absence of point mutations in the anti-i CA from CAP, as opposed to the somatic mutation pattern in the VOG cell lines consistent with antigenic selection. Further studies are needed to substantiate the proposed model of H and L chain contributions to antigen binding. We also plan to explore the structural and regulatory differences that might exist between physiologic and pathogenic autoimmune responses with anti-i and anti-I specificity.

NOTE ADDED IN PROOF

Two additional nucleotide sequences of cold agglutinins have recently been reported. The expressed V<sub>i</sub> genes are similar to the V<sub>i</sub>4.21 gene and both express the 9G4 idiootype. (Pascual V, et al: J Immunol 146:4385, 1991.)

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LE Silberstein, LC Jefferies, J Goldman, D Friedman, JS Moore, PC Nowell, D Roelcke, W Pruzanski, J Roudier and GJ Silverman