

## Naturally occurring anti-i/I cold agglutinins may be encoded by different VH3 genes as well as the VH4.21 gene segment.

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### Research Article

In the current study, we wished to determine if the V regions encoding the naturally occurring anti-i/I Cold Agglutinins (anti-i/I CA) differ from pathogenic anti-i/I CA that are exclusively encoded by the VH4.21 gene. After EBV transformation of B lymphocytes, we generated one anti-I secreting clone from each of two individuals; clone 4G (individual CM, PBL) and clone Sp1 (individual SC, spleen). Clone 4G expresses a VH3 gene sequence that is 92% homologous to the germline gene WHG26. Clone Sp1 also expresses a VH3 gene that is 98% homologous to the fetally rearranged M85/20P1 gene. Another clone, Sp2 (anti-i specificity), from individual SC is 98% homologous to the germline gene VH4.21. For correlation, we studied anti-i/I CA fractions purified from 15 normal sera and found no or relatively small amounts of 9G4 (VH4.21 related idotype) reactive IgM. Five cold agglutinin fractions contained large amounts of VH3-encoded IgM (compared to pooled normal IgM) by virtue of their binding to modified protein Staph A (SPA), and absorption of three CA fractions with modified SPA specifically removed anti-i/I binding specificity entirely. Collectively, the data indicate that naturally occurring anti-i/I CA may be encoded to a large extent by non-VH4.21-related genes, and that the VH4.21 gene is not uniquely required for anti-i/I specificity.

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# Naturally Occurring Anti-i/I Cold Agglutinins May be Encoded by Different V<sub>H</sub>3 Genes as Well as the V<sub>H</sub>4.21 Gene Segment

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## Abstract

In the current study, we wished to determine if the V regions encoding the naturally occurring anti-i/I Cold Agglutinins (anti-i/I CA) differ from pathogenic anti-i/I CA that are exclusively encoded by the V<sub>H</sub>4.21 gene. After EBV transformation of B lymphocytes, we generated one anti-I secreting clone from each of two individuals; clone 4G (individual CM, PBL) and clone Sp1 (individual SC, spleen). Clone 4G expresses a V<sub>H</sub>3 gene sequence that is 92% homologous to the germline gene WHG26. Clone Sp1 also expresses a V<sub>H</sub>3 gene that is 98% homologous to the fetally rearranged M85/20P1 gene. Another clone, Sp2 (anti-i specificity), from individual SC is 98% homologous to the germline gene V<sub>H</sub>4.21. For correlation, we studied anti-i/I CA fractions purified from 15 normal sera and found no or relatively small amounts of 9G4 (V<sub>H</sub>4.21 related idotype) reactive IgM. Five cold agglutinin fractions contained large amounts of V<sub>H</sub>3-encoded IgM (compared to pooled normal IgM) by virtue of their binding to modified protein Staph A (SPA), and absorption of three CA fractions with modified SPA specifically removed anti-i/I binding specificity entirely. Collectively, the data indicate that naturally occurring anti-i/I CA may be encoded to a large extent by non-V<sub>H</sub>4.21-related genes, and that the V<sub>H</sub>4.21 gene is not uniquely required for anti-i/I specificity. (*J. Clin. Invest.* 1993, 92:2821–2833.) Key words: immunoglobulin • autoantibody • red blood cell • cold agglutinin • autoimmunity

## Introduction

Autoantibodies with various specificities are frequently detectable in the sera of healthy individuals and are referred to as benign, natural, or naturally occurring. However, curiously, antibodies of the same specificity may also be associated with autoimmune disease (1, 2). For example, virtually all sera from healthy individuals contain low concentrations of naturally occurring anti-i/I IgM anti-red blood cell (RBC) antibodies or cold agglutinins (CA)<sup>1</sup> that cause no apparent immune

hemolysis. The pathogenic counterparts of these autoantibodies may cause severe immune hemolytic anemia and are generally derived from monoclonal B cell expansions that may progress to frank lymphoma (3). The anti-i and anti-I specificities of CA are broadly defined by their differential reactivities to cord (predominantly i-expressing) vs adult (predominantly I-expressing) RBCs. Normal adult sera contain a heterogeneous population of naturally occurring CA that almost always have anti-I specificity, although rarely small amounts of anti-i CA may also be detected. In contrast, pathogenic CA (e.g., derived from B cell lymphoma) may have either anti-I or anti-i specificity (4).

In the current study, we have begun to investigate the relationship between naturally occurring, nonpathogenic anti-i/I CA and pathogenic anti-i/I CA derived from clonal B cell expansions. In recent years, several laboratories have demonstrated a highly restricted V<sub>H</sub>4 gene use among pathogenic CA from different individuals. The heavy chain variable gene V<sub>H</sub>4.21 (or a closely related gene) has been found in all pathogenic monoclonal CA studied to date, suggesting that this gene may play an important role in defining the i/I specificity (5–9). Our study addressed the question whether the same restricted pattern of V gene usage by pathogenic CA might be found in naturally occurring CA. The simplest hypothesis, that the V<sub>H</sub>4.21 gene product is the principle determinant of anti-i/I CA activity, would predict that this same gene would encode naturally occurring CA with similar specificity. Alternatively, naturally occurring anti-i/I CA could be encoded by V<sub>H</sub> genes other than V<sub>H</sub>4.21. A schematic diagram illustrating these possibilities is shown in Fig. 1.

To evaluate the genetic diversity of naturally occurring CA, we have established EBV-transformed B cell clones secreting antibodies with anti-i/I binding specificity from two individuals without evidence of immune hemolysis. V<sub>H</sub> and V<sub>L</sub> region nucleotide sequence analyses of these B cell clones indicate that V<sub>H</sub> genes other than V<sub>H</sub>4.21 may encode anti-i/I specificity. These results were subsequently correlated with additional serological analyses of anti-i/I enriched CA fractions from normal sera to further understand the relationship between natural and pathogenic, monoclonal anti-i/I CA.

## Methods

*Generation of monoclonal anti-i/I secreting EBV-transformed B cell lines.* PBL were isolated by Ficoll-Hypaque gradient centrifugation from a healthy nonanemic individual (CM) without symptoms of cold agglutinin disease. Serum from this individual agglutinated untreated homologous red blood cells at 4°C up to a titer of 1:16. PBL were enriched 10% before EBV transformation by panning with murine monoclonal anti-idiotypic antibodies previously described (10). Four previous attempts to establish B cell clones secreting natural CA from normal individuals had been unsuccessful by EBV transformation methodology without panning. The anti-idiotypic antibodies used in the panning step are light chain restricted and bind both benign and

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1. Abbreviations used in this paper: CA, cold agglutinins; HA, hemagglutination; RBC, red blood cells; SPA, Staphylococcal protein A.

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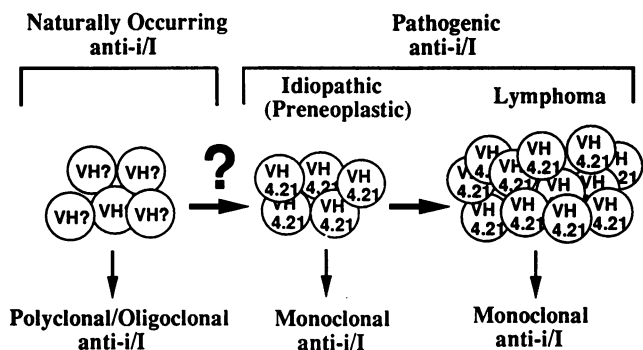


Figure 1. The relationship between naturally occurring and pathogenic anti-i/I CA. The origin of pathogenic (e.g., monoclonal) CA derived from clonal B cell expansions has been previously investigated (6, 12, 18, 25).

pathologic CA. By Western blot analysis, the anti-idiotypic antibodies bind isolated kappa light chains, and, therefore, did not bias the selection of B lymphocytes expressing selected  $V_H$  genes in these experiments. Polystyrene petri dishes were coated with a mixture of antibodies (2.0  $\mu\text{g/ml}$  IV.8, 1.0  $\mu\text{g/ml}$  IV.6, 1  $\mu\text{g/ml}$  IV.2, and 1.0  $\mu\text{g/ml}$  IV.9) suspended in PBS (0.01 M Na PO<sub>4</sub> and 0.15 M NaCl, pH 7.4) at 22°C for 1 h. Unbound antibody was removed by gentle washing four times using PBS with 1% FBS. Lymphocytes were washed twice in PBS, resuspended in 10 ml 5% FBS in PBS ( $10^5$ – $10^6$  cells/ml), and incubated in the petri dish for 1 h at 4°C. Unbound cells were removed by gentle washing four times with 1% FBS/PBS. Adherent cells were removed with flushing and scraping using 30 ml 1% FBS/PBS, pelleted, and resuspended in 5 ml ex vivo cloning media (Whittaker Bioproducts, Walkersville, MD), 1% glutamine, 10% Pen Strep, 1  $\mu\text{g/ml}$  Mito serum extender, 1  $\mu\text{g/ml}$  PHA, and 10% FBS).

PBL from donor CM and splenic lymphocytes from a second non-anemic individual (SC) were similarly enriched by panning with the rat monoclonal anti-idiotypic antibody 9G4 (11). This reagent recognizes a conformation-dependent epitope that is highly associated with  $V_H4.21$  expressing autoantibodies to the i and I antigens.

EBV transformation of PBL and splenic lymphocytes was accomplished as previously published (12). After 3–4 wk, supernatant from wells with confluent growth was added to 2–4% suspension of ficin treated adult RBC. Hemagglutination was assessed after a 30 min incubation at 4°C. Wells positive for hemagglutination were subcloned by limiting dilution (13). After subcloning, supernatants from monoclonal cell lines grown in RPMI 1640 medium were retested for hemagglutination with both ficin-treated and untreated adult (I) and cord (i) group O RBC using previously established methods (14). Serial dilutions of supernatant or CA purified from supernatant in PBS, pH 7.4, were performed to compare relative degrees of agglutination with adult and cord RBC.

**Isolation of anti-i/I CA from normal sera.** Serum was obtained from healthy individuals whose serum agglutinated untreated adult (I) RBC at 4°C at a dilution of  $\geq 1:8$ . Cold reactive autoantibodies were isolated by absorption at 4°C to allogeneic RBCs, followed by heat elution. This procedure was performed by a cold/warm/cold incubation method (15) that limits the amount of nonspecific protein in the final eluate. Split serum samples were absorbed with ficin-treated group O adult (I) and cord (i) RBCs to obtain enriched anti-I and anti-i fractions, respectively. The CA fractions are termed "enriched" because they have been isolated from sera using RBCs that are enriched for either the I (adult RBC) or i (cord RBC) antigen. Anti-i vs anti-I relative binding specificities of purified fractions were confirmed by standard methods using both ficin-treated and untreated adult (I) and cord (i) cells (14).

**Monoclonal antibodies.** The IgG2a rat antibody 9G4 recognizes a heavy chain associated idiotype highly associated with cold agglutinins

encoded by  $V_H4.21$  or a closely related  $V_H4$  heavy chain genes (6, 8, 11). Cold agglutinins VOG (IgM,  $\kappa$ ) and CAP (IgM,  $\kappa$ ) have been previously described (6). Antibody VOG with anti-I specificity, is encoded by a  $V_H4$  gene with 97% homology to the published  $V_H4.21$  germline gene (16), and expresses the 9G4 idiotype. Antibody CAP with anti-i specificity is encoded by a  $V_H4$  gene identical to the published  $V_H4.21$  germline sequence and expresses the 9G4 idiotype.

**ELISA.** Except where indicated, the following general procedures and buffers were used: Microtiter plates for ELISA (Dynatech Laboratory, Chantilly, VA) were coated (150  $\mu\text{l/well}$ ) with antibodies suspended in PBS, pH 7.4, and incubated overnight at 4°C. All subsequent incubations were for 1.5 h at 37°C. All washing steps used three washes with PBS containing 0.1% Tween 20. To decrease nonspecific binding, wells were blocked with blocking/diluent buffer (0.5% nonfat dry milk and 0.01% Thimerosol in PBS, pH 7.4), 300  $\mu\text{l/well}$ . Antibodies were diluted in blocking/diluent buffer (150  $\mu\text{l/well}$ ). All samples were tested in duplicate wells, and alkaline phosphatase-conjugated antibodies were used at 1:1,000 dilution. Alkaline phosphatase activity was measured with 5 mg *p*-nitrophenylphosphate (Sigma Immunochemicals, St. Louis, MO) per 40 ml of developing buffer (1 M Tris HCl, 0.5 mM ZnCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 0.02% NaN<sub>3</sub>, pH 8.2), 150  $\mu\text{l/well}$ , at 22°C for 30 min. Activity was quenched with 3 M NaOH, 50  $\mu\text{l}$  per well and optical density readings were measured at 405 nM (MR 700 ELISA Reader; Dynatech Laboratories).

To detect IgM in cell culture supernatants, microtiter plates were coated with rabbit anti-human IgM (Jackson Immunoresearch, West Grove, PA) blocked and washed. After incubation with supernatants or standard IgM, wells were incubated with mouse anti-human IgM (Zymed Laboratories, Inc., South San Francisco, CA), 1:1,000 dilution, and bound human IgM was detected using alkaline phosphatase conjugated goat anti-mouse IgG and IgM (Jackson Immunoresearch).

To detect light chain isotype of cell culture supernatants or purified CA, microtiter plates were coated with rabbit anti-human IgM, blocked, and washed. Duplicate wells were incubated with cell culture supernatants, or IgM kappa and IgM lambda (Tago, Burlington, CA) controls. After washing, paired wells were incubated with either alkaline phosphatase-conjugated rabbit anti-human kappa or anti-human lambda (Sigma Immunochemicals) to detect bound human immunoglobulin.

Cell culture supernatants and purified CA were tested for binding by anti-idiotypic antibody 9G4 as follows: Microtiter plates were coated with 2  $\mu\text{g/ml}$  rabbit anti-human IgM, blocked, washed and incubated with cell culture supernatant. After incubation and washing, rat monoclonal antibody 9G4 (1.25  $\mu\text{g/ml}$ ) was added. Either VOG or CAP  $V_H4.21$  proteins (6) were used as a positive control. Alkaline phosphatase-conjugated goat anti-mouse IgG and IgM (H and L) was used to detect bound rat immunoglobulin. To quantitate the relative amounts of 9G4-positive IgM in purified CA, a standard curve was established correlating the IgM concentration of a known  $V_H4.21$  expressing monoclonal IgM CA with 9G4 reactivity. Subsequently, serial dilutions of CA fractions (known IgM concentration) were tested for binding by 9G4, and the amount of 9G4 reactive IgM in CA fractions was extrapolated from the standard curve.

To detect  $V_H3$  family-encoded IgM, plates were coated with rabbit anti-human IgM (2  $\mu\text{g/ml}$ ), subsequently blocked and coated in triplicate wells with serial dilutions of purified CA (known IgM concentration), with equivalent concentrations of pooled polyclonal IgM (Calbiochem Corp., San Diego, CA) and with serial dilutions of a positive control  $V_H3$  IgM. After a 4-h incubation and washing, modified (Staphylococcal protein A (SPA) modified by iodination of tyrosines, biotin) (5.0  $\mu\text{g/ml}$ ) was added for a 2-h incubation at 22°C. Modified SPA has been shown to bind 90% of  $V_H3$ -encoded IgM via Fab-binding sites that are functionally and structurally distinct from the Fc $\gamma$ -binding sites of SPA (17). Bound IgM was detected with alkaline phosphatase-labeled streptavidin. Modified SPA reactivity of CA fractions was compared to that of pooled normal IgM (both tested at 0.25  $\mu\text{g/ml}$ ) to assess for enrichment of  $V_H3$ -encoded IgM; we did not attempt to iso-

late the total IgM fraction of each individual from whom the naturally occurring CA fractions were purified.

**Absorption of CA fractions with modified SPA.** Absorption of CA fractions with modified SPA was used to determine if depletion of V<sub>H</sub>3 IgM in CA fractions would diminish or abolish anti-I binding specificity. To this end, wells were coated overnight (4°C) with modified SPA (5 µg/ml) and blocked with 1% BSA. Each anti-i/I CA fraction (200 µl aliquot) was then added sequentially to a series of six wells with a 30-min 22°C incubation in each well for maximal absorption of V<sub>H</sub>3 IgM from the CA fraction. After removal from the last well, the CA fraction was then tested for agglutination of either untreated or ficin-treated adult RBCs (I) and compared to agglutination of an unabsorbed CA aliquot. CA fractions were also sequentially incubated with wells not coated with SPA but blocked with 1% BSA to evaluate for nonspecific binding of CA to microtiter plates. Goat anti-human IgM alkaline phosphatase was used to detect binding of V<sub>H</sub>3 IgM to plates. V<sub>H</sub>4.21 CA CAP and a V<sub>H</sub>3-encoded monoclonal IgM without RBC specificity were used as controls.

**Hemagglutination inhibition.** Serial dilutions of anti-I (VOG) and anti-i (CAP) (6) in PBS were tested against ficin treated RBCs (adult I or cord i, respectively) to establish a range of CA concentration yielding 4+ hemagglutination (HA). Hemagglutination inhibition curves were established using 50-µl aliquots of each CA (1.25 and 5.0 µg/ml) incubated with an equal volume of anti-idiotypic antibody, 9G4 (serial twofold dilutions, starting from 10.0 µg/ml). After a 30-min incubation at 37°C, ficin-treated RBCs were added and HA was assessed after a 30-min incubation at 4°C. Purified CA (0.2–6.0 µg/ml) were evaluated using high concentrations of 9G4 (> 5.0 µg/liter). HA was also

Table I. Nucleotide Sequence of Primers Used in First-strand cDNA and PCR Reactions

Application	Sequence (5'-3')
<b>cDNA synthesis</b>	
C <sub>μ</sub>	CGAGGGGGAAAAGGGTTGGGGC
C <sub>κ</sub>	AGATGGCGGGAAGATGAAGAC
HJL*	ACCTAGGACGGTCAGCTTGTT
<b>Amplification</b>	
V <sub>H</sub> 1 <sup>LEADER</sup>	ATGGACTGGACCTGGAGGGTC
V <sub>H</sub> 2 <sup>LEADER</sup>	ATGGACATACTTTGTTCAC
V <sub>H</sub> 3 <sup>LEADER</sup>	ATGGAGTTTGGGCTGAGCTGG
V <sub>H</sub> 4 <sup>LEADER</sup>	ATGAAACACCTGTGGTCTT
V <sub>H</sub> 5 <sup>LEADER</sup>	ATGGGGTCAACCGCCATCCT
V <sub>H</sub> 6 <sup>LEADER</sup>	ATGTCTGTCTCCTTCCTCAT
V <sub>κ</sub> 1 <sup>LEADER</sup>	ATGGACATGAG(GA)GTCC
V <sub>κ</sub> II <sup>LEADER</sup>	ATGAGGTCCTGCTCAGCTCCTG
V <sub>κ</sub> III <sup>LEADER 1</sup>	ATGAAACCCAGCTCAGCTTCTC
V <sub>κ</sub> III <sup>LEADER 2</sup>	CCCAGCTCAGCTTCTC
V <sub>κ</sub> IV <sup>LEADER</sup>	ATGGTGTGTCAGACCCAGGTCTTC
HLFW1*	CAGTCTGTGCTGACTCAG
4G DJ*	GGCCCCAGTAATCCCTGGGTA
4G V <sub>H</sub> CDR2	GGGTATGGTAGTAGCCACTGTC
V <sub>H</sub> 26 <sup>CDR2</sup>	GTATGTGCTACCACCACTACCACT
4G V <sub>L</sub> CDR1	CAAGTAGGTGCCAACACT
V <sub>g</sub> CDR1	TAAGTAGCTGCTAACACT
V <sub>κ</sub> IIIa <sup>CDR3/INTRON</sup>	TGTGGGAGGCCAGTTGCT
V <sub>H</sub> 3 <sup>INTRON</sup>	CTGGGCGACAATGACT

Sequences of oligonucleotide primers used for first strand cDNA synthesis and PCR amplification of V genes. \* HJL, human λ joining region; HLFW1, human λ framework 1; DJ, the junctional sequence generated by the heavy chain diversity and joining region genes.

Table II. Red Blood Cell Binding, Specificity of Serum Antibodies, and Clonal Supernatants

	Adult I ficin RBCs	Adult I untreated RBCs	Cord i ficin RBCs	Cord i untreated RBCs	Specificity
CM serum	1:128	1:16	1:64	1:4	I
4G SN	1:16	1:8	Neg	Neg	I
SC serum	1:8	1:4	1:8	1:2	I
Sp1 SN	1:8	1:4	1:2	1:2	I
Sp2 SN	1:8	1:2	1:32	1:8	i

Donor CM serum, donor SC serum and supernatants from EBV transformed B cell clones were tested for hemagglutination of RBCs. Samples were serially diluted in PBS and incubated with a 2–4% suspension of RBCs at 4°C for 30 min. Samples were designated as having either anti-i or anti-I binding specificities based on the predominance of cold reactive agglutination with umbilical cord O RBCs or adult O RBCs, respectively. Test RBCs were also treated with ficin to show that binding was not decreased by protease treatment. The dilution indicated is the highest dilution at which hemagglutination was observed. CM serum, SC serum, and 4G supernatant (4.88 µg/ml IgM) were tested “neat”; i.e., not concentrated or diluted. For Sp1 and Sp2, the clone supernatants were concentrated to 15.6 and 12.3 µg/ml IgM, respectively, for agglutination studies. Media controls (similarly concentrated) and PBS did not agglutinate RBCs.

compared to a dilutional control (antibody incubated with 50 µl PBS in place of the anti-idiotypic antibody). A decrease in the score of hemagglutination in the presence of inhibitor is expressed as percent inhibition of hemagglutination.

**Sequencing and cDNA cloning of V region genes.** The experimental approach for the cloning and sequencing of the expressed IgV genes has been previously described (18). Total RNA was isolated from EBV-transformed cell lines using thiocyanate-phenol chloroform extraction techniques. First-strand cDNA synthesis from 1 µg total cellular RNA was performed with reverse transcriptase (Life Sciences, Inc., St. Petersburg, FL) and an oligonucleotide primer specific for the μ chain constant region (C<sub>μ</sub>) (Table I). The cDNA was amplified by PCR with Amplitaq recombinant stable Taq polymerase (Perkin-Elmer Cetus Corp., Norwalk, CT), the same C<sub>μ</sub> primer and 5' primers complementary to the conserved portion of published leader sequences for each V<sub>H</sub> gene family (1–6) (Table I). The same approach was used to isolate the V<sub>L</sub> region gene sequences. The primers corresponding to κ constant region (C<sub>κ</sub>), the joining region of the λ light chain (J<sub>λ</sub>), the leader sequences of each of the V<sub>κ</sub> families (1–4) and the framework 1 region of V<sub>λ</sub> genes (consensus primer HLFW1 λ) are listed in Table I. For each clone, amplification occurred with only one of the 5' primers; e.g., corresponding to leader or framework 1 sequences. PCR amplifications were carried out using a programmable thermocycler (Coy Laboratory Products, Inc., Grasslake, MI). 30 cycles were performed with soak temperatures of 94°C, 55°C, and 72°C for 1 min each followed by 9 min at 72°C. The amplified products were blunt ended using Klenow fragment DNA polymerase I (Sigma Immunochemicals), gel purified (2% agarose), electroeluted, and phosphorylated with T<sub>4</sub> polynucleotide kinase (BRL Life Technologies, Gaithersburg, MD). Amplified DNA was then ligated with T4 ligase (International Biotechnologies, Inc., New Haven, CT) into the phosphatased SmaI site of pBS M 13<sup>-</sup> (Stratagene Inc., La Jolla, CA) and transfected by electroporation (Bio Rad Laboratories, Rockville Center, NY) into XL-1 blue strain of *Escherichia coli*. Clones were screened by blue/white selection and restriction analysis of plasmid DNA. Clones containing the insert of expected size (400–600 bp) were sequenced by dideoxynucleotide termination with Sequenase (United States Biochemical Corp., Cleveland, OH)

**A**

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<Leader----->
ATG GAG TTG GGG CTG TGC TGG GTT TTC CTT GTT GCT ATT TTA GAA G  <Intron----->
---t---a---ag---t---a---
*** *** *** *** *** ***
----->
WHG26  atgagtgagagaaacagtgatgtgtggcagttctgaccttgggtctctctttgttgcag  Leader----->
4G      GT GTC CAG TGT          GAG GTG CAG CTG GTG GAG TCT GGG
----->
WHG26  GGA GGC TTG GTA CAG CCT GGG GGG TCC CTG AGA CTC TCC TGT GCA GCC TCT GGA TTC ACC TTC AGT ACC TAT AGC ATG AAC TGG GTC CGC
4G      ---gat---
----->
WHG26  CAG GCT CCA GGG AAG GGG CTG GAG TGG GTT TCA TAC ATT AGT AGT AGT AGT ATA TAC TAC GCA GAC TCT GTG AAG GGC CGA TTC
4G      ---c---gac---gtg gc---c---cc---
----->
WHG26  ACC ATC TCC AGA GAC AAT GCC AAG AAC TCA CTG TAT CTG CAA ATG AAC AGC CTG AGA GCC GAG GAC ACG GCT GTG TAT TAC TGT GCG AGA
4G      ---t---c---g---a---
----->
4G      <JH4 Region----->
<CDR3-----> TGG GGC CAG GGG ACC CTG ATC ATC GTC TCC TCA <CH Region----->
GAT CAA CGG GGC CTA CCC AGG GAT TAC ---a---g---c--- GGG AGT GCA TCC GCC CCA ACC CTT TT
*** *** *** *** ***

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**B**

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Leader----->
M85    ATG GAG TTT GGG CTG AGC TGG AIT TTC CTT GCT ATT TTA AAA GGT GTC CAG TGT GAG GTG CAG CTG GTG GAG TCT GGG GGA GGC
SP1    ---
*** *** *** *** *** ***
----->
M85    TTG GTA AAG CCT GGG GGG TCC CTT AGA CTC TCC TGT GCA GCC TCT GGT TTC ACT TTC AGT AAC GCC TGG ATG AAC TGG GTC CGC CAG GCT
SP1    ---
----->
M85    CCA GGG AAG GGG CTG GAG TGG GTC GGC CGT ATT AAA AGC AAA ACT GAT GGT GGG ACA ACA GAC TAC GCT GCA CCC GTG AAA GGC AGA TTC
SP1    ---t---t---t---
----->
M85    ACC ATC TCA AGA GAT TCA AAA AAC AAG AAG AAG AAG AAG AAG AAG AAG AAG AAG AAG AAG AAG AAG AAG AAG AAG AAG AAG AAG AAG AAG
SP1    ---t---t---t---
----->
DXP1/JH4  GTATTACGATATTTTGACTGGTTATTATAC  <J4 Region----->
SP1      CTCGCAG-----T-GCCGAGGACTAC  TGGGCCAGGGAAACCCCTGGTCACCGTC

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Figure 2. Legend on facing page.

using both “-40” and “reverse” universal primers (19). Oligonucleotide primers were synthesized by an oligonucleotide synthesizer (Applied Biosystems, Inc., Foster City, CA) using phosphotriester chemistry. For each clone, we sequenced three to four bacterial isolates in both directions. The obtained sequences were identical and thus confirmed homogeneity of the cell populations.

## Results

### *Establishment of CA-secreting B cell clones*

PBL from normal donor CM were exposed to EBV and seeded at  $1 \times 10^3$  cells/well of 96-well microtiter plates (Corning). After 4 wk of culture, supernatants were assayed for CA activity (20). CA activity was detected in only 3 of 60 wells with growth (identified on different plates), designated 4G, 8B, and 3C. These wells were subcloned by limiting dilution (13). CA-secreting cell lines that were considered clonal based on statistical analysis were subsequently expanded. These three clones (4G, 8B, and 3C) were derived from PBL selected with the  $V_{\lambda}$ III-specific anti-idiotype. In contrast, two trials of PBL (donor CM) selection using the anti-idiotypic antibody 9G4 did not yield any CA-secreting cell lines.

EBV-transformed splenic lymphocytes from donor SC were exposed to EBV and seeded at 100, 10, and 1 cell/well. Two wells were considered to contain clonal cell lines based on statistical analysis and were designated Sp1 and Sp2. These cell lines were subcloned again at 1 cell/well, tested for CA activity, and expanded.

Southern blot analyses were performed on DNA from established clones that confirmed clonality of the cell lines (data not shown). Furthermore, clones 4G, 3C, and 8B from donor CM showed the same  $J_H$  restriction fragment pattern using three different restriction enzymes. Subsequent sequence analyses indicated that these clones were in fact identical. Consequently, we present data from only one of the clones (4G).

### *Serology of cold agglutinins secreted by B cell clones*

The binding specificity of the RBC autoantibodies was determined by their differential reactivity against adult and cord test group O RBCs (Table II) (4, 21). The CA present in the serum and in clonal supernatant of normal donor CM reacted more strongly with adult (I) RBCs than umbilical cord (i) RBCs and was therefore classified as anti-I. Similarly, CA present in the serum of donor SC and in supernatant of splenic clone Sp1 preferentially reacted with adult (I) RBCs and was also classified as anti-I. In contrast, splenic clone Sp2 reacted relatively stronger with cord (i) RBCs and was, therefore, classified as anti-i.

### *The $V_H$ genes of anti-I specific B cell clones of donor CM and SC belong to the $V_H3$ family*

The  $V_H$  sequences of clones 4G (3C and 8B) are most homologous (92%) to the published germline gene WHG26 (22) (Fig. 2A). However, based on the level of sequence complementarity,

the  $V_H$  gene WHG26 cannot reliably be ascribed as the germline precursor.

The  $V_H$  sequence of clone Sp1 was most homologous (98%) to the fetally rearranged M85/20P1  $V_H3$  gene (23) (Fig. 2B).

### *The $V_L$ genes of the anti-I specific clones of donor CM and SC are $V_{\lambda}$ I and $V_{\lambda}$ III genes, respectively*

The  $V_L$  genes from clones 4G (3C and 8B) were shown to be most closely related (96%) to the  $V_{\lambda}$ IIIa germline gene Vg (24) (Fig. 3A). Other antibody specificities encoded by rearranged genes most closely related to the Vg germline gene include a pathologic CA with anti-Pr<sub>2</sub> specificity (25) and rheumatoid factor protein (26).

The  $V_L$  gene expressed by clone Sp1 was only 94% homologous to the  $V_{\lambda}$ I germline gene L11 (27) (Fig. 3B) and its germline precursor, therefore, remains speculative, since  $V_{\lambda}$ I represents a complex  $V_L$  gene family.

### *The anti-i specific B cell clone of donor SC expresses $V_{H\lambda}$ 4.21 and $V_{\lambda}$ II genes*

The expressed  $V_H$  gene of clone Sp2 showed closest homology (98%) with the germline gene  $V_{H\lambda}$ 4.21 (8), while the expressed  $V_L$  gene was 98% homologous to a rearranged  $V_{\lambda}$ II gene ( $V_{\lambda}$ II family) (28) (Fig. 4).

### *Evidence that the expressed $V_H$ and $V_L$ genes encoding anti-I of Clone 4G are somatically diversified*

The expressed  $V_H$  and  $V_L$  genes appeared somatically mutated when compared to known germline gene sequences. To investigate whether these genes were in fact mutated rather than derived from yet unreported germline genes, we used a PCR-based approach previously described by van Es et al. (29). Specific oligonucleotide primer pairs were made corresponding to putative mutated regions of the CDRs and to the potential germline precursor sequences (Table I).

In addition, we isolated the putative germline precursor  $V_L$  gene Vg, from autologous granulocyte DNA of individual CM. A similar attempt to isolate the precursor  $V_{H3}$  gene was not performed because of the complexity of the  $V_{H3}$  family, which contains many homologous gene segments.

*$V_H$  gene analysis.* In this experiment, autologous granulocyte DNA could be amplified with primers corresponding to germline sequences but not with primers corresponding to expressed CDR2 sequences. Amplification of CM granulocyte DNA was achieved using primer pairs  $V_{H3}^{\text{LEADER}}-V_{H3}^{\text{INTRON}}$  and  $V_{H3}^{\text{LEADER}}-V_{H26}^{\text{CDR2}}$ , but not using primer pairs,  $V_{H3}^{\text{LEADER}}-4GV_{H3}^{\text{CDR2}}$  (Fig. 5A). Based on sequence homology to published  $V_{H3}$  germline genes, the  $V_{H3}^{\text{LEADER}}$  primer may be expected to amplify all  $V_{H3}$  genes. In contrast, the  $V_{H3}^{\text{INTRON}}$  primer was designed to amplify only  $V_{H26}$ -like genes. These amplifications were included as positive controls and to show the presence of commonly identified  $V_{H3}$  gene segments. The lack of amplification using  $4GV_{H3}^{\text{CDR2}}$  suggests that the expressed  $V_H$  gene of clone 4G represents a mutated rearranged  $V_{H3}$  gene. Additional analysis of the expressed  $V_{H3}$

*Figure 2.* Expressed  $V_H$  sequences encoding anti-I. (A) Sequence analysis of expressed  $V_{H3}$  genes encoding anti-I of clone 4G. The relevant reference nucleotide sequences of germline gene WHG26 (22), and  $J_{H4}$  (33) are shown on the top lines. Homology of 4G nucleotides is shown as a dash. \*Oligonucleotide sequences used as flanking primers ( $V_{H3}^{\text{LEADER}}$  and  $C_{\mu}$ ) for amplification of the  $V_H$  gene of 4G. (B) Sequence analysis of expressed  $V_{H3}$  encoding anti-I of clone Sp1. The nucleotide sequences of the relevant reference sequences, fetally rearranged M85/20P1 (23),  $D_{\lambda\text{PI}}$  (30), and  $J_{H4}$  (33) are shown on the top line. Homology of Sp1 nucleotides with reference sequences is shown as a dash. \*The oligonucleotide sequence used as a flanking primer (5'  $V_{H3}$  family specific) for amplification of  $V_H$  of clone Sp1.

**A**

```

Leader----->
Vg  ATG GAA GCC CCA GCT CAG CTT CTC CTC CTG CTA CTC TGG CTC CCA GAT ACC ACC GGA GAA ATT GTG TTG ACA CAG TCT CCA GCC ACC CTG TCT
4Gk  --- A--- --C --- *** *** *** *** ***
*** *** *** *** *** *** *** ***
Vg  TTG TCT CCA GGG GAA AGA GCC ACC CTC TCC TGC AGG GCC AGT CAG AGT GTT AGC AGC TAC TTA GCC TGG TAC CAA CAG AAA CCT GGC CAG GCT CCC
4Gk  --- --- --- A- G- --- G- -C- --- -g --- -g --- -g --- -g ---
<CDR1----->
Vg  AGG CTC CTC ATC TAT GAT GCA TCC AAC AGG GCC ACT GGC ATC CCA GCC AGG TTC AGT GGC AGT GGG TCT GGG ACA GAC TTC ACT CTC ACC ATC AGC
4Gk  --- -G --- -C ---
<CDR2----->
Vg  AGC CTA GAG CCT GAA GAT TTT GCA GTT TAT TAC TGT CAG CAG CGT AGC AAC TGG CCT CTC ACT TTC GGC GGA GGG ACC AAG GTG GAG ATC AAA
4Gk  --- --- --- -G --- -g --- -g --- -g --- -a ---
<CDR3-----> <JK4 Region----->

```

**B**

```

Leader----->
L11  ATG AGG GTC CCC GCT CAG CTC CTG GGG CTC CTG CTG CTC TGG CTC CCA G gtaaggatggagaacactggcagtttactcagcccagggtgctcagcacagcc
SP1  --- c- -t ---
*** *** *** *** *** *
----- Leader----->
L11  tggctattcagggaattcttactactacatgattgattggtggaccatttgtttgtttccaatcca GT GCC AGA TGT GCC ATC CAG ATG ACC CAG TCT CCA TCC TCC
SP1  --- -a --- -a ---
<CDR1----->
L11  CTG TCT GCA TCT GTA GGA GAC AGA GTC ACC ATC ACT TGC CGG GCA AGT CAG GGC ATT AGA AAT GAT TTA GGC TGG TAT CAG CAG AAA CCA GGG AAA
SP1  --- --- ---
<CDR2----->
L11  GCC CCT AAG CTC CTG ATC TAT GCT GCA TCC AGT TTA CAA AGT GGG GTC CCA TCA AGG TTC AGC GGC AGT GGA TCT GGC ACA GAT TTC ACT CTC ACC
SP1  --- -g --- -g --- g-g --- -g --- -a ---
<CDR3-----> <JK1 Region----->
L11  ATC AGC AGC CTG CAG CCT GAA GAT TTT GCA ACT TAT TAC TGT CTA CAA CAG TAT GAT AAT CTC CCT AGT TTC GGC CAA GGG ACC AAG GTG GAA ATG
SP1  --- --- --- -g --- -t --- ag- t-c -cg tgg ---

```

Figure 3. Expressed  $V_L$  sequences encoding anti-I. (A) Sequence analysis of expressed  $V_{HIII}$  genes encoding anti-I of clone 4G. The relevant reference nucleotide sequences of germline gene  $V_g$  (24) and  $J_4$  are shown on the top lines. Homology of 4G nucleotides is shown as a dash. Nucleotide differences from the germline sequence are indicated in capital letters to denote replacement mutations, and in lower case letters to denote silent mutations. \*The oligonucleotide sequence used as a flanking primer ( $5' V_{HIII}^{LEADER}$ ) for amplification of  $V_L$  of clone 4G. (B) Sequence analysis of expressed  $V_L$  encoding anti-I of clone Sp1. The nucleotide sequence of germline gene L11 (27), the reference sequence with closest homology to  $V_L$  of Sp1 is shown on the top lines. Homology of Sp1 nucleotides with L11 is shown as a dash. \*The oligonucleotide sequence used as a flanking primer ( $5' V_L^{LEADER}$ ) for amplification of  $V_L$  of clone Sp1.

**A**

VH4.21  
SP2

```

Leader-----
ATG AAA CAC CTG TGG TTC TTC CTC CTC CTG GTG GCA GCT CCC
*** *** *** *** *** ***

```

VH4.21  
SP2

```

          Intron
          9tgagtgctcaggaatcgggatatgaagatatgagatgctgctctgtctctgttcacag GG GTC CTG TCC CAG GTG CAG CTA CAG
          ----->
          <CDR1----->
CAG TGG GGC GCA GGA CTG TTG AAG CCT TCG GAG ACC CTG TCC CTC ACC TGC GCT GTC TAT GGT GGG TCC TTC AGT GGT TAC TAC TGG ACC TGG
----->

```

VH4.21  
SP2

```

          <CDR2----->
ATC CGC CAG CCC CCA GGG AAG GGG CTG GAG TGG ATT CAT AGT GGA AGC ACC AAC TAC AAC CCG TCC CTC AAG AGT CGA GTC
-----g-----g

```

VH4.21  
SP2

```

          <CDR3----->
ACC ATA TCA GTA GAC ACG TCC AAG AAC CAG TTC TCC CTG AAGCTG AGC TCT GTG ACC GCC GCG GAC ACG GCT GTG TAT TAC TGT GCG AGA
-----t-----g-----

```

DXP4/JH6  
SP2

```

          <CDR3----->
          <JH6 Region----->
          GTATTACGATTTTGGAGTGCTATTATACC TGGGCCAAGGACCACCGTACCCTCTCA <-----CJH----->
          GCGGGG-G-C--A-----ATCT--CGTACTACGGTC-----GGAGTGATCCGCCCCCAACCCCTTTT
          *****

```

**B**

VL2.1  
SP2

```

CAG TCT GCC CTG ACT CAG CCT GCC TCC GTG TCT GGG TCT CCT
---tg-----
*** *** *** *** ***

```

VL2.1  
SP2

```

          <CDR1----->
GGA CAG TCG ATC ACC ATC TCC TGC ACT GGA ACC AGC AGT GAT GTT GGG ACT TAT AAC TAT GTC TCC TGG TAC CAA CAG CAC CCA GGC AAA GCC
-----t-----g-----

```

VL2.1  
SP2

```

          <CDR2----->
CCC AAA CTC ATG ATT TAT GAG GGC AGT AAG CGG CCC TCA GGG GTT TCT AAT CGC TTC TCT GGC TCC AAG TCT GGC AAC ACG GCC TCC CTG ACA
-----t-----g-----

```

VL2.1  
SP2

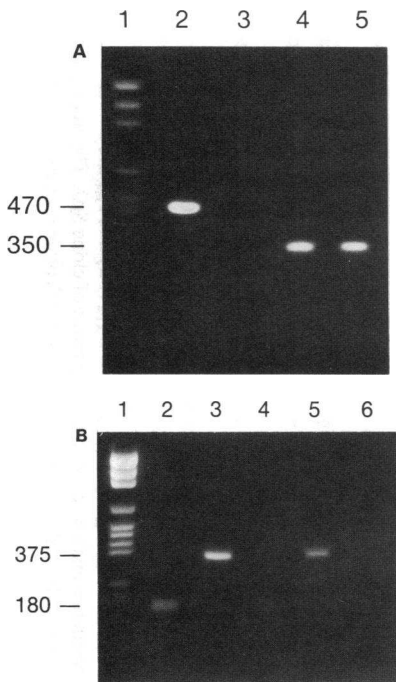
```

          <CDR3----->
          <J lambda Region----->
          ATC TCT GGG CTC CAG GCT GAG GAC GAG GAT TAT TAC TGC TGC TCA TAT GCA GGT AGT AGC ACT TTA
          -----a-----g-----g-----
          GTATTCCGGGAGGACCAGGCTGACCCTT
          *****

```

Figure 4. Expressed  $V_H$  and  $V_L$  encoding anti-i. (A) Sequence analysis of  $V_H$  encoding anti-i of clone Sp2. The nucleotide sequences of germline gene  $V_H$  4.21 (6), the reference sequence with closest homology to  $V_H$  of Sp2 is shown on the top line. Homology of Sp2 nucleotides with  $V_H$ 4.21 is indicated as a dash. Relevant D and  $J_H$  region sequences DXP4 (32) and JH6 are similarly indicated. \*The oligonucleotide sequences used as flanking primers ( $V_H$ 4LEADER and C $\mu$ ) for amplification of  $V_H$  of Sp2. (B) Sequence analysis of  $V_L$  encoding anti-i of clone Sp2. The nucleotide sequence of  $V_{\lambda}$ 2.1 (28) the closest related  $V_{\lambda}$  family gene, is shown on the top line. Homology of Sp2 nucleotides with  $V_{\lambda}$ 2.1 is indicated by dashes. \*The oligonucleotide sequences used as flanking primers (HLFW1 and HJL) for amplification of the  $V_L$  of Sp2.





**Figure 5.** (A) Evaluation of CM granulocyte DNA for the presence of target sequence  $V_H^{CRD2}$  of clone 4G. Lane 1, pGEM standards; lanes 2–4 represent CM granulocyte DNA amplified with the following primer pairs: lane 2,  $V_{H3}^{LEADER}-V_{H3}^{INTRON}$  (corresponds to 17 nucleotides located between the heptamer and nonamer sequences, 3' of the  $V_H26$  gene segment); lane 3,  $V_{H3}^{LEADER}-4G V_H^{CRD2}$ ; lane 4,  $V_{H3}^{LEADER}-V_{H26}^{CRD2}$ . Lane 5 represents 4G plasmid DNA amplified with primer pair  $V_{H3}^{LEADER}-4G V_H^{CRD2}$ . Bars indicate 470- and 350-bp

size bands. (B) Evaluation of CM granulocyte DNA for presence of target sequence  $V_L$  CDR1 of clone 4G. The 5' leader primer,  $V_{\kappa III}^{LEADER 2}$  (shorter than  $V_{\kappa III}^{LEADER 1}$ ) was constructed to approximate the length of the 3' primer  $4G V_L^{CDR1}$  to optimize amplification conditions. Lane 1, pGEM Standards; lane 2, 4G plasmid DNA amplified with primer pair  $V_{\kappa III}^{LEADER 2}-4G V_L^{CDR1}$ ; lane 3, Vg cloned from CM amplified with  $V_{\kappa III}^{LEADER 2}-Vg^{CDR1}$ ; lane 4, CM granulocyte DNA amplified with primers  $V_{\kappa III}^{LEADER 2}-4G V_L^{CDR1}$ ; lane 5, CM granulocyte DNA amplified with  $V_{\kappa III}^{LEADER 2}-Vg^{CDR1}$ ; lane 6, CM granulocyte DNA amplified with  $V_{H3}^{LEADER}-4G DJ$ . Bars indicate 375- and 180-bp size bands.

gene sequence is not possible because a putative germline precursor was not determined; the latter experiment was performed in the  $V_L$  gene analysis (see below).

**$V_L$  gene analysis.** First, oligonucleotide primers  $V_{\kappa III}^{LEADER}$  and  $V_{\kappa IIIa}^{CDR3/INTRON}$  were used to isolate and sequence germline  $V_{\kappa III}$  genes from autologous CM granulocyte DNA. Of four colonies sequenced, two were identical to germline gene Vg while two were more closely related to a highly homologous  $V_{\kappa III}$  germline gene Vh (24) (Fig. 6), thus confirming the presence of Vg in the germline repertoire of CM. Second, oligonucleotide primers were made corresponding to the putative mutated regions of CDR1 and to the potential germline precursor Vg. As expected, primer pair  $V_{\kappa III}^{LEADER}-Vg^{CDR1}$  amplified granulocyte DNA, while the primer pair  $V_{\kappa III}^{LEADER}-4G V_L^{CDR1}$  did not (Fig. 5 B). Taken together, these data suggest that the expressed  $V_L$  gene of clone 4G is a somatically mutated Vg gene.

We applied a binomial probability model to the pattern of mutations in this  $V_L$  gene as a test for positive selection (30). The expected fraction of replacement mutations in CDR from this  $V_L$  sequence is 0.22, calculated by multiplying the relative size of the CDR (0.28) by the fraction of all mutations in CDR that could result in amino acid replacements (0.79). Using the binomial probability model, we calculated that the distribution of six replacement mutations in CDR of a (corrected) total of

13 mutations had a low probability of occurring at random ( $P = 0.046$ ). Positive selection of replacement mutations in CDR has been observed in murine immune responses and presumably reflects selection for enhanced antigen binding (31). 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. It is tempting to speculate that the observed somatic mutations are the result of selection by the I antigen or another cross-reactive antigen.

#### Diversity region sequence analysis

The D region segments of the three clones (4G, Sp1, and Sp2) with specificities for the related i and I antigens are heterogeneous. Comparison of nucleotide sequences to published D region germline genes shows homology of the Sp1 D segment (11 nucleotides) with  $D_{XP1}$  (32) and homology of the Sp2 D segment (17 nucleotides) with  $D_{XP4}$  (33) (Figs. 2 and 4). The D region sequence of clone 4G could not be reliably aligned with any of the published D gene segments. The D segments from the natural anti-i/I secreting clones 4G, Sp1, and Sp2 were compared to published pathogenic anti-i/I RBC autoantibodies (6–8). However, a common D region sequence, possibly contributing to the similar serological specificities of these autoantibodies, was not apparent.

#### Evaluation of clonal supernatants for expression of 9G4 idiotype

Each of the clonal supernatants was evaluated for expression of the 9G4 idiotype (Table III). As expected, only one of these clones, Sp2, which expresses the  $V_H4.21$  gene, was bound by the monoclonal anti-idiotypic antibody 9G4 (OD > 1.0). These findings indicate that Sp2 expresses the 9G4 idiotype, which has previously been highly associated with pathologic anti-I/i autoantibodies of (pre)neoplastic B cell origin (6, 8).

#### Evaluation of naturally occurring CA purified from serum for expression of 9G4 idiotype

Purified CA, isolated from the serum of 15 individuals without immune hemolysis (including CM and SC), were evaluated by ELISA for expression of the 9G4 idiotype. For each individual, both anti-i- and anti-I-enriched fractions were evaluated. The quantity of 9G4-reactive IgM content of CA fractions was assessed by establishing a standard curve correlating the IgM concentration of a known  $V_H4.21$ -expressing monoclonal IgM CA with 9G4 reactivity. We found that of 30 fractions tested, 7 had no 9G4 reactivity, while the remainder had relatively little 9G4 reactivity (range = 1–30%). These results are summarized in Fig. 7. These data suggest that the majority of naturally occurring circulating CA are encoded by non- $V_H4.21$  immunoglobulins.

#### 9G4 fails to inhibit hemagglutination by naturally occurring anti-i/I CA purified from serum

The 9G4 antibody is known to efficiently inhibit hemagglutination by pathogenic, monoclonal anti-i/I CA encoded by the  $V_H4.21$  gene. Consequently, hemagglutination inhibition curves were first established for  $V_H4.21$ -encoded proteins VOG and CAP (6) using anti-idiotypic antibody 9G4 as the inhibitor (Fig. 8). As little as 0.15  $\mu\text{g}/\text{liter}$  and 0.62  $\mu\text{g}/\text{ml}$  of 9G4 completely inhibited hemagglutination by 1.25  $\mu\text{g}/\text{ml}$  of VOG and CAP, respectively. Complete inhibition of 5  $\mu\text{g}/\text{ml}$  of CAP was

```

Vg      Leader-----
C1&C4  Met Glu Ala Pro Ala Gln Leu Leu Phe Leu Leu Leu Trp Leu Pro A
C2&C3  ATG GAA GCC CCA GCT CAG CTT CTC TTC CTC CTG CTA CTC TGG CTC CCA G
      *** *** *** *** *** *** *** *** ***
      --- a ---
      --- a ---
Vg      <Intron-----
C1&C4  gtgaggggaacatgaggtggtttgacattagtgaaactctgcccacctgctcagcaagaataataaattcaaatatcaacaatttggctctactc
C2&C3  -----t-----
      -----t-----c-----a-g-----
Vg      Leader----->
Germ 4g (C1-C4)  sp Thr Thr Gly Glu Ile Val Leu Thr Gln Ser Pro Ala Thr
C2&C3  aaggacagttggttgattgattacatgagtgagtcattctgtttattttccaatttcag AT ACC ACC GGA GAA ATT GTG TTG ACA CAG TCT CCA GCC ACC
      -----g-----t-----
Vg      Leu Ser Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Val Ser Tyr Leu Ala Trp Tyr Gln Gln Lys
Germ 4g (C1&C4)  CTG TCT TTG TCT CCA GGG GAA AGA GCC ACC CTC TCC TGC AGG GCC AGT CAG AGT GTT AGC AGC TAC TTA GCC TGG TAC CAA CAG AAA
(C2&C3)  -----g-----
Vg      Pro Gly Gln Ala Pro Arg Leu Leu Ile Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala Arg Phe Ser Gly Ser Gly Ser Gly
Germ 4g (C1&C4)  CCT GGC CAG GCT CCC AGG CTC ATC TAT GAT GCA TCC AAC AGG GCC ACT GGC ATC CCA GCC AGG TTC AGT GCC AGT GGC TCT GGG
C2&C3  -----g-----
Vg      Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Ser Asn Trp Pro Pro Thr
Germ 4g (C1-C4)  ACA GAC TTC ACT CTC ACC ATC AGC AGC CTA GAG CCT GAA GAT TTT GCA GTT TAT TAC TGT CAG CAG CGT AGC AAC TGG CCT CCC ACA
C2&C3  -----g-----
      <CDR1----->
      <CDR2----->
      <CDR 3-----> Intron-
      *** *** *** *** *** *** *** *** ***

```

Figure 6. Sequence analysis of V<sub>H</sub>III germline precursor sequences isolated from donor CM. Nucleotide sequences of four different colony isolates from CM granulocyte DNA are indicated as C1-C4. Comparison to nucleotide sequence of gene Vg (24), the closest related germline gene is indicated by dashes. \*The oligonucleotide sequences used as flanking primers (5' V<sub>H</sub>III<sup>LEADER1</sup> and 3' V<sub>H</sub>III<sup>CDR3/INTRON</sup>) for amplification of the V<sub>H</sub>III sequences.

Table III. Ig V Region Use and Idiotype of Autoantibody-secreting Clones

Clone	Ig variable region gene		Idiotype 9G4
	V <sub>L</sub>	V <sub>H</sub>	
4G	V <sub>L</sub> III	V <sub>H</sub> 3	Neg
Sp1	V <sub>L</sub> I	V <sub>H</sub> 3	Neg
Sp2	V <sub>L</sub> λ	V <sub>H</sub> 4	Pos

V<sub>H</sub> and V<sub>L</sub> was determined by nucleotide sequence analysis for clones 4G, Sp1, and Sp2. Expression of idiotype 9G4 was determined by ELISA. Sp2 was bound by 9G4 (OD > 1.0). None of the other clonal supernatants were bound by 9G4 (OD < 0.02), but were bound by murine anti-IgM as a control to indicate adequate concentrations of clonal IgM present. (OD > 1.0) Cold agglutinin VOG (a previously described V<sub>H</sub>4.21 anti-I) (6) was used as a positive control for 9G4 reactivity (OD > 1.0). Background values are <0.02.

achieved using 2.5 μg/ml of 9G4. Naturally occurring CA were subsequently tested at concentrations of total IgM ranging from 0.11 to 6.4 μg/ml. For 14 of 14 CA evaluated, hemagglutination was not inhibited, even using high concentrations of 9G4 (5.0–15.0 μg/ml) (Fig. 8). This failure to inhibit hemagglutination using anti-idiotypic antibody 9G4 supports the finding that only a small fraction of circulating naturally occurring CA expresses the 9G4 idiotype.

#### Evaluation of naturally occurring CA for V<sub>H</sub>3-encoded IgM content

A serologic marker that would recognize all V<sub>H</sub>3 gene products is to our knowledge, currently not available. Nevertheless, we have used modified SPA, which binds to ~90% of V<sub>H</sub>3-encoded IgM through Fab-binding sites (17), to evaluate the presence of V<sub>H</sub>3 IgM in the CA purified from normal serum and also to show that the absorption with modified SPA results in

the abrogation of hemagglutination activity of the CA fractions.

Five CA fractions contained quantities of V<sub>H</sub>3 encoded IgM (OD range = 0.41–0.49) similar to the V<sub>H</sub>3 content of pooled normal human IgM (OD = 0.45) as defined by binding to modified SPA. In two of these CA fractions (including anti-I from donor CM), the modified SPA binding exceeded that of pooled IgM. The remaining CA fractions (19/24) contained modified SPA reactivity that was less than that of pooled normal IgM (OD < 0.20). The varying amount of modified SPA reactivity suggests that the IgM antibodies in these CA fractions are encoded by (a) V<sub>H</sub> genes other than V<sub>H</sub>3 (or V<sub>H</sub>4.21); (b) certain germline V<sub>H</sub>3 genes that are not bound by modified SPA, or alternatively (c) V<sub>H</sub>3 genes that have lost modified SPA reactivity caused by somatic mutations (17). A positive control antibody (representing 100% V<sub>H</sub>3-encoded IgM, at 0.25 μg/ml) gave an average OD value of 0.55. These experiments suggested that some purified CA fractions contained very high amounts of V<sub>H</sub>3-encoded IgM, which was unlikely caused by nonspecific Ig entrapment during the RBC absorption and elution procedure.

Next, we used modified SPA bound to microtiter plates to specifically absorb V<sub>H</sub>3 IgM from CA fractions and subsequently test the absorbed anti-I CA fractions for CA activity. After six sequential absorptions with SPA, three separate anti-I CA fractions (individuals CM, MS, and MG) failed to agglutinate adult (I) RBCs, whereas the unabsorbed fractions titrated to 1:16, 1:16, and 1:32, respectively. This finding suggests that the anti-I binding specificity virtually completely results from V<sub>H</sub>3 IgM in these CA fractions. As expected, SPA-bound IgM (absorbed from the CA fractions) was detected in each of six wells on the microtiter plate (Table IV). There was no absorption of IgM from the naturally occurring CA onto wells without SPA coat. There was also no absorption of a V<sub>H</sub>4.21-encoded IgM CA CAP onto wells with or without SPA coat. Together, these studies suggest that naturally occurring CA may be encoded by V<sub>H</sub>3 genes.

### Relative 9G4 reactivity of a-i versus a-I CA fractions in normal serum

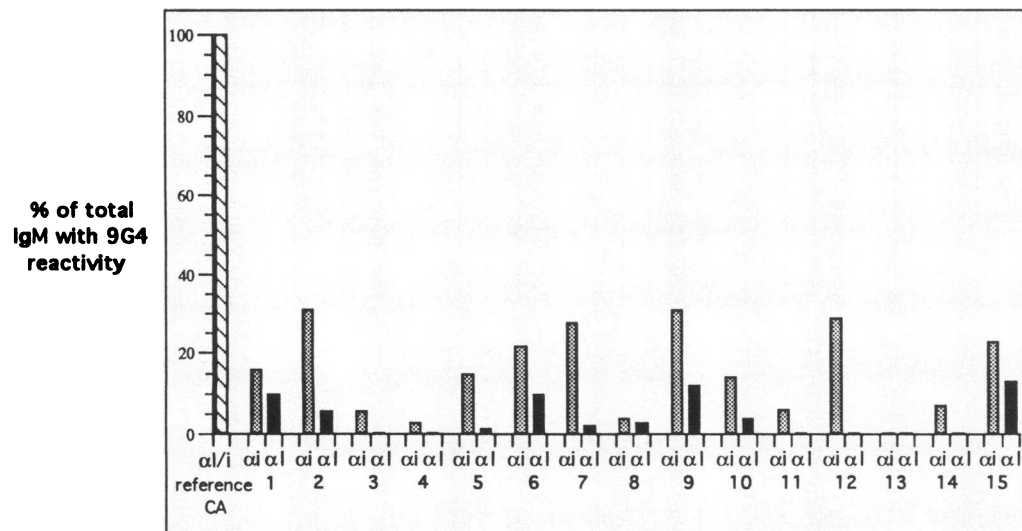


Figure 7. Relative 9G4 reactivity in enriched anti-i vs anti-I CA fractions purified from normal serum of 15 healthy individuals. The percentage 9G4 reactivity was calculated by dividing 9G4-reactive IgM content by total IgM content. The 9G4-reactive IgM content ranged from 1 to 30% of total IgM. For all individuals studied, anti-i fraction (grey bars) contained greater 9G4 content than the corresponding anti-I fraction (black bars). Individual 14 represents CM, the source of clone 4G (V<sub>H</sub>3-encoded anti-I CA) and individual 15 represents SC, the source of clone Sp1 (V<sub>H</sub>3-encoded anti-I CA) and clone Sp2 (V<sub>H</sub>4.21-encoded anti-I CA).

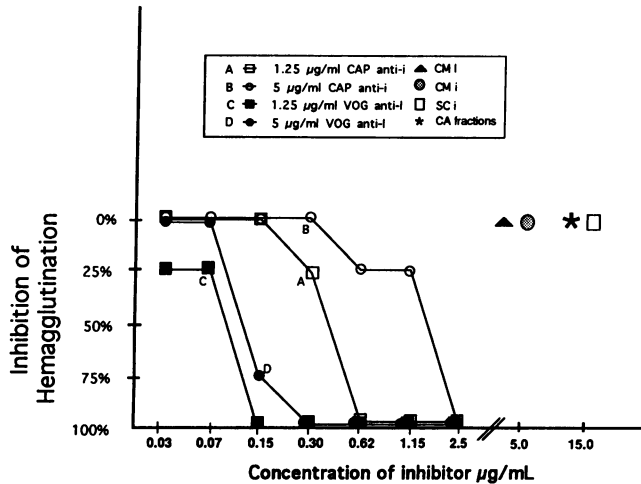


Figure 8. Hemagglutination inhibition studies. Serial dilutions of purified VOG and CAP (monoclonal  $V_{H4.21}$ -encoded anti-I and anti-i CA, respectively) were first tested to establish concentrations yielding 4+ hemagglutination. Aliquots (50  $\mu$ l) of these antibodies (both at 1.25 and 5.0  $\mu$ g/ml) were incubated with serial twofold dilutions of anti-idiotypic antibody 9G4 (beginning at 10  $\mu$ g/ml). After a 30-min incubation at 37°C, ficin treated RBCs were added and hemagglutination assessed after a 30-min incubation at 4°C. The 9G4 antibody (> 5.0  $\mu$ g/ml), was also incubated with purified naturally occurring CA (0.20–0.60  $\mu$ g/ml) for inhibition of hemagglutination. CA were incubated with adult (I) or cord (i) RBCs depending on the predetermined relative specificity. There was no inhibition of HA for any naturally occurring CA tested. CM I and CM i represent anti-I- and anti-i-enriched CA fractions, respectively purified from serum of individual CM. SC i represents anti-i-enriched CA fraction purified from serum of individual SC. \*Represents 14 anti-i- and anti-I-enriched CA fractions from seven additional healthy individuals.

## Discussion

The Ig V region gene use of pathogenic, monoclonal CA has been extensively studied by serology, protein chemistry, and recently by nucleotide sequencing, whereas the  $V_H$  and  $V_L$  region structure of naturally occurring CA has not been previously determined. Because these proteins are present in sera in much smaller quantities than the monoclonal CA, they have not been amenable to purification and subsequent analyses as performed on monoclonal CA. Thus, there is little or no information reported regarding their structure or origin. While the naturally occurring CA are probably polyclonal in origin, based on analogy with other naturally occurring autoantibodies, such as anti-DNA antibodies, the polyclonality of these CA has not been demonstrated adequately. Furthermore, because the V gene usage of naturally occurring CA is not known, the B cell origin of naturally occurring vs pathogenic CA has remained speculative.

In the current study, naturally occurring anti-I autoantibodies from two individuals were found to use  $V_{H3}$  family genes, the largest of the  $V_H$  families (34). This finding indicates that naturally occurring CA may derive from B cells that are distinct from the  $V_{H4.21}$  expressing cells, which can give rise to B cell neoplasms. The initial isolation of  $V_{H3}$  encoded anti-I autoantibodies from donor CM using  $\kappa$  chain-restricted anti-idiotypes was somewhat surprising in view of the published restricted use of  $V_{H4.21}$  or a closely related  $V_{H4}$  gene by pathogenic anti-i/I RBC autoantibodies (5–9). Therefore, we

subsequently used the 9G4 anti-idiotypic antibody (associated with the  $V_{H4.21}$  gene) to attempt to isolate 9G4-positive autoreactive B cell clones. Although no CA-secreting clones were obtained from PBL of donor CM, we did isolate from splenic lymphocytes of donor SC one clone Sp1 (anti-I), which, curiously, is encoded by a  $V_{H3}$  family gene and another clone Sp2 (anti-i), which indeed is encoded by the  $V_{H4.21}$  gene. The isolation of a clone expressing  $V_{H3}$  using the 9G4 reagent likely occurred because the panning procedure for B cell enrichment does not exclusively select idiotype-positive B cells, and because subsequent screening of the wells containing EBV transformed splenic B cells was based on CA activity and not on the expression of 9G4.

Interestingly, one of the  $V_{H3}$  genes encoding anti-I specificity is 98% homologous to the 20p1 gene (23). This gene belongs to a set of  $V_H$  genes associated with fetal ontogeny (e.g.,  $V_{H26}$  related), which are considered to be preferentially expressed during fetal development and which, in germline configuration, are associated with several natural autoantibodies (35). In certain instances, antibodies encoded by these fetally derived  $V_H$  genes with few amino acid substitutions, may crossreact with both self antigens (e.g., anti-DNA) and with bacterial polysaccharides (36). By analogy, it is possible that the few somatic changes observed in the  $V_{H3}$  gene expressed by clone Sp1, may play a role in conferring the anti-I specificity. However, the comparison of  $V_H$  region genes that encode both natural and pathogenic anti-i and anti-I CA has not revealed a shared protein sequence that would potentially contribute to anti-i/I antigen binding.

Table IV. Absorption of  $V_{H3}$  Ig from CA Fractions Using Modified SPA

CA	Hemagglutination titer of adult (I) RBCs		
	Before adsorption with SPA	After adsorption with SPA	IgM bound to SPA
CM anti-I purified from serum	1:16	0	OD 0.25
MS anti-I purified from serum	1:16	0	OD 0.17
MG anti-I purified from serum	1:32	0	OD 0.38
Anti-I from VOG ( $V_{H4.21}$ ) cell line	1:32	1:32	OD 0.0

CA fractions were sequentially incubated in a series of wells precoated with modified SPA and blocked with 1% BSA. Adsorbed CA fractions were then tested for agglutination of untreated adult (I) RBCs and compared to titration of the unadsorbed CA fraction from the same individual. Goat anti-human IgM alkaline phosphatase was added to wells after removal of the CA fractions to confirm binding of IgM to modified SPA as shown by OD values. There was no absorption of CA onto wells without modified SPA coat. There was no absorption of  $V_{H4.21}$  CA CAP onto wells with or without modified SPA coat. The signal for a positive control monoclonal  $V_{H3}$  IgM (1  $\mu$ g/ml) OD = 0.50.

The great majority of naturally occurring CA have the anti-I specificity, and when the anti-i specificity is present, it is usually found in smaller amounts (titers) relative to anti-I. Therefore, we anticipated that clones screened for CA activity would most likely have anti-I specificity, as did clone Sp1. The isolation of clone Sp2, with anti-i specificity, from the same donor SC raised the question of the relative frequency of these two specificities. Since small numbers of independent clones were isolated and since enrichment by panning was used, it is difficult to estimate the relative frequencies of anti-I vs anti-i expressing B cells, or of  $V_H4.21$ -encoded vs non- $V_H4.21$ -encoded natural occurring anti-i/I CA.

It is possible, however, to assess circulating naturally occurring CA for the relative expression of  $V_H4.21$ - vs non- $V_H4.21$ -encoded IgM, as defined by 9G4 reactivity. We found that of 30 fractions tested, 7 had no 9G4 reactive IgM, while the remainder had relatively little 9G4 reactive IgM (Fig. 7). For donor CM, from whom clone 4G (B cell clone, secreting  $V_H3$  encoded anti-I CA) was isolated, circulating anti-i and anti-I fractions contained only 4% and 0% 9G4 reactive IgM, respectively. In addition, the anti-i/I CA fractions of donor CM, as well as the remainder of anti-i/I fractions, were not inhibited by the 9G4 antibody (Fig. 8).  $V_H3$  IgM was detected in several CA fractions and was found to be enriched in donor CM anti-I fraction compared to pooled IgM. Moreover, absorption of CA fractions with modified SPA to remove  $V_H3$ -encoded Ig resulted in complete abrogation of anti-I binding specificity for each of three CA fractions evaluated (Table IV). Together, the data suggest that to a large extent, naturally occurring anti-i/I CA are encoded by non- $V_H4.21$  genes.

In summary, the current work represents an extension of our previously established model involving pathogenic CA derived from clonal B cell expansions (6, 12, 18, 25) to permit a comparison with nonpathogenic anti-i/I CA. The observed differences in V region gene use by naturally occurring vs pathogenic CA would suggest that natural/nonpathogenic anti-i/I autoantibodies may not necessarily represent the precursors of their monoclonal counterpart, and that perhaps the mechanisms leading to the development of the two different anti-i/I autoantibody populations may also differ. For example, the presence of the I antigen on *L. monocytogenes* raises the possibility that naturally occurring anti-I CA may arise from microbial stimulation, similar to naturally occurring anti-A, B, and H isoagglutinins, while pathogenic, monoclonal anti-i/I CA may primarily arise by alternative mechanisms (37, 38).

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