Molecular Characterization of a Cross-Reactive Idiotope on Human Immunoglobulins Utilizing the V_H4–21 Gene Segment

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Summary

The anti-idiotypic (anti-Id) antibody (Ab) 9G4 binds a cross-reactive idiotope (CRI) present in a select group of human autoantibodies. This Id has been localized to the portion of immunoglobulin (Ig) heavy (H) chains encoded by the V_H4–21 gene segment, a member of the human V_H4 family. This gene segment is utilized by essentially all cold agglutinin (CA) Abs with I/i specificity isolated from patients with CA disease stemming from chronic lymphoproliferative disorders. In this study, mutational analysis of a CA has been used to determine the structural basis for 9G4 binding to Abs utilizing the V_H4–21 gene segment. Recombinant CA H chain mutants were produced and their 9G4 reactivity determined. Mutants were generated by exchanging V_H4–21 sequences in the FR1, CDR1, and CDR2 with corresponding sequences from a closely related gene segment V71–2, a V_H4 family member that is associated neither with Abs having CA activity nor with Abs that react with 9G4. The results indicate that the motif AVY at amino acid positions 23–25 in FR1 defines the 9G4 idiotope. Reaction of these recombinant Abs with a polyclonal rabbit anti-CA antiserum absorbed to render it specific for a CA CRI also maps predominantly to FR1. These findings indicate that the solvent-exposed FR1 plays an important role in eliciting an immune response to Igs.

The phenomenon of cross-reactive idiotypes (CRI)[1] was first described in 1968 by Williams et al. (1) who demonstrated that many human cold agglutinins (CA) reacted with absorbed polyclonal antisera raised in rabbits against three purified human CA. The antisera did not react with other macroglobulins lacking the ability to agglutinate RBC at low temperature. This result suggested the presence of V region antigenic structures shared by CA and was the first demonstration of shared Id among nonidentical Abs derived from unrelated individuals (2).

The rat mAb 9G4 has a reactivity profile that corresponds to the absorbed polyclonal sera mentioned above. In particular, essentially all CA from patients with cold agglutinin disease (CAD) having I/i specificity show reactivity with 9G4 (3). More recently it has been shown that CA that are specific for the I/i antigens, and produced under chronic lymphoproliferative conditions, almost uniformly utilize the V_H4–21 gene segment (4–8). The V_H4–21 gene segment is also utilized by some Abs having reactivity against structurally distinct blood group antigens (9), as well as Abs with anti-DNA (10) and RF (5, 11) activities. With the exception of FOG 1, an Ab with anti-RBC D antigen specificity, all of the V_H4–21–derived Abs tested were 9G4 reactive, whereas Ig using gene segments from the other V_H families did not react with 9G4 (4, 5, 12, 13). 9G4, therefore, specifically reacts with Abs that are V_H4–21 gene segment encoded, a group of Ig which encompasses a variety of antigenic specificities. Hence, the 9G4 Id, like the CRI which reacts with the absorbed polyclonal anti-CA antisera, is a marker for a select group of Abs.

CRI were serologically defined as determinants associated with the V region of Ab molecules having the same or similar antigen-binding specificity and/or were expressed from related Ig genes (1, 2). They are determinants that can be formed from structures restricted either to the H or the L chain or

[1] *Abbreviations used in this paper:* CA, cold agglutinin; CAD, cold agglutinin disease; CRI, cross-reactive idiotope; SOE, splicing by overlap extension.

require both chains (14). Analysis of L chain, D_H and J_H segment usage indicates that the 9G4 reactive idiotope is restricted to the H chain alone. The L chains associated with CA predominantly use the VκIII family, especially anti-I CA, however, κ chains from the VκI and VκII families (4, 5, 8, 15) and λ chains have also been described, especially in association with anti-i CA (1, 5, 8, 16–18). Abs against various blood group antigens that react with 9G4, are also associated with both κ and λ chains (12). Sequence analysis of a series of eleven CA H and L chains indicates that there is no homology among these Abs in the H chain CDR3 and, whereas there is a preponderant usage of J_H4, other J_H segments are also used (4, 5, 8). Abs of both the IgM and IgG isotypes are also 9G4 reactive indicating that the C region is not a factor in forming the determinant (19). Thus, since there was nothing obviously similar in the D or J_H segments, the C_H region nor in the L chains, it was concluded that the V_H4–21 gene segment is responsible for the 9G4 CRI.

The structural basis of the CRI in V_H4–21 recognized by 9G4 has not been determined. In the present study, mutational analysis and expression of recombinant Abs has allowed us to identify and locate the amino acids involved in the idiotope recognized by 9G4. We chose to express and mutate the CA FS-7, which utilizes the V_H4–21 gene segment in germline configuration, as the focus of this study. A series of site-directed mutations indicates that the 9G4 reactive CRI is localized to amino acids 23–25 in FR1. In addition, a polyclonal rabbit anti-CA antiserum, absorbed to render it specific for a CA CRI, recognized a determinant on the recombinant Abs which also maps to FR1.

Materials and Methods

Viruses and Cells. *Autographa californica* nuclear polyhedrosis virus (AcMNPV) and recombinant viral stocks were produced and assayed in the *Spodoptera frugiperda* cell line Sf9. Sf9 cells were grown in TNM-FH medium supplemented with 10% FCS plus penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (0.25 μg/ml). Recombinant Abs were produced in Sf9 cells adapted to growth in serum-free medium (Sf-900; GIBCO BRL, Gaithersburg, MD). All procedures were essentially as previously described (20–22).

PCR Conditions. Oligonucleotides were designed for PCR-SOEing (splicing by overlap extension; 23) such that the overlap region between the primers and the portions of the primers that pair with the template had a melting temperature of 60–64°C. All of the complementary bases were added to one of the two internal primers. The external primers used for all IgM Abs were 5′ GGAATTCGCCATGGAACACCT 3′ at the 5′ end and 5′ TGCATGCATTCTAGAGCCTGTGGGCAG 3′ at the 3′ end. The total reaction volume was 100 μl, which was overlaid with 100 μl of mineral oil. Taq polymerase was used at 1 U per reaction. The PCR conditions involved two cycles of 1 min at 94°C, 2 min at 52°C, and 3 min at 72°C followed by 23 cycles of 1 min at 94°C, 2 min at 59°C, and 3 min at 72°C ending with 10 min at 72°C.

Gene Construction. Ab genes for both H and L chains of Ab FS-7 were cloned from FS-7, an immortalized human B cell line from a patient with CAD (8). Total RNA was isolated and used as template for cDNA synthesis. V regions were amplified by PCR and cloned into the Bluescript vector (Stratagene, La Jolla, CA) using standard procedures (8). As the $V_κ$ sequence was cloned minus its signal sequence, a κ signal sequence was attached and the $C_κ$ region was spliced to this product. The V_H region had been cloned with its signal sequence. The $C_μ$ region was then fused to the V_H region. The source of the κ signal and $C_κ$ region sequences were from a RF κ clone (11). The $C_μ$ region was amplified from a clone of the antithyroglobulin H chain isolated from Ab25 (24). The CDR1-FR3 V71-2 sequence was PCR amplified from clone V_H4-21 (24a) and spliced to the FR1 sequence of V71-2 and CDR3-Cμ sequence in two separate SOEing reactions to generate the entire V71-2 sequence. Primer sequences for the leader-FR1 were 5′ GGAATTCGCCATGGAACACCT 3′ and 5′ GTAGTAACTACCACTGCTGACGGAGCCACCAGAGACCGT 3′; for the V71-2 CDR1-FR3 they were 5′ GTCAGCAGTGGTAGTTACTAC 3′ and 5′ CAGCCACTGCTCTCTGCCTCTCGCACAGTAATACACAGC 3′ and for the CDR3-Cμ they were 5′ GGCAGAGAGCAGTGGCTG 3′ and 5′ TGCATGCATTCTAGAGCCTGTGGGCAG 3′. All PCR amplified fragments were run on a 1% agarose gel to separate the PCR fragment from the oligonucleotide primers before SOEing. The DNA fragments were excised from gels as agarose plugs and melted at 75°C for 2 min. 5-μl aliquots of both DNA bands were combined in a SOEing reaction in the presence of the external oligomers.

Oligonucleotide-directed Mutagenesis. All mutations were carried out by PCR-SOEing using mutagenic oligonucleotides (25). Templates included double-stranded DNA isolated from mini-plasmid preparations and single-stranded DNA. The oligomer used to generate the N-FW1 mutant was: 5′ AAGGCTTCACCAGTCCTGGGCCCGACTCCTGCAGCTG 3′; for C-FW1/CDR1 it was 5′ GTAGTAACTACCACTGCTGACGGAGCCACCAGAGACAGTGCAGGTGAG 5′; for CDR2 it was 5′ TGTAGTTGGTGCTCCCACTGTAATAGATATACCCAATCCACTC 3′; for FR1 (71-2) it was 5′ GCTCCAGTAGTAACCACTGAAGGAGCCACCAGAGACAGTGCAGGT 3′; for C-FW1 it was 5′ GCTCCAGTAGTAACCACTGAAGGAGCCACCAGGAGCCACCAGAGACAGTGCA 3′; for CDR1 it was 5′ GTAGTAACTACCACTGCTGACGGACCCACCATAGACAGC 3′; and for 25Y→25F it was 5′ GTAACCACTGAAGGACCCACCAAAGACAG 3′. The underlined bases indicate mutated sites.

Transfer Vector. The transfer vector for all constructs was pH360NX. It is essentially as described (26) except for the addition of an XbaI site. An NcoI site was created at the translation initiation site for polyhedrin synthesis and an XbaI site was generated to provide for directional cloning of foreign genes. Human Ig genes were amplified by PCR using oligomers containing NcoI and XbaI restriction enzyme sites. After PCR-SOEing, the genes were phenol/chloroform extracted, ethanol precipitated, digested with NcoI and XbaI, and purified by electrophoresis through 0.8% low melting temperature agarose gels. The DNA bands were excised as gel plugs, and ≤20 μl of melted plug were added to ligation reactions containing the NocI-XbaI–digested and –dephosphorylated transfer vector. Ligation products were transformed into *Escherichia coli* BSJ72 (TG1, $λ^r$, Sn^r; a gift from Dr. Steve Henikoff, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) and restriction enzyme digestion of mini-plasmid preparations was used to identify the clones containing the gene of interest. Single-stranded DNA was produced using the f1 origin of replication present in the vector and cloned genes were sequenced by the dideoxy chain termination method (27).

Transfection and Isolation of Recombinant Viruses. Transfer vectors ligated to Ig genes were isolated from *E. coli* BSJ72 as mini preparations. This DNA was used to transfect Sf9 cells employing linearized wild-type viral DNA and cationic liposomes (Invitrogen,

San Diego, CA). Viral supernatants were harvested at 2 d after transfection and screened by plaque assay for occlusion-minus viruses. Well isolated plaques were picked and dispersed into 1 ml of TNM-FH medium. An aliquot of the plaque (200 μl) was used to infect 2×10^5 Sf9 cells. At 3–4 d after infection, the cells were pelleted, lysed in sample buffer containing β-ME and run on a 10% SDS-polyacrylamide gel. Western blot analysis was performed using PVDF membrane (Immunobilon-P; Millipore Corp., Bedford, MA). Both ^{125}I-labeled goat anti-human IgM and IgG Abs (ICN Flow, Costa Mesa, CA) and Chemiluminescence ECL Western blotting protocol (Amersham Corp., Arlington Heights, IL) using horseradish peroxidase-conjugated anti-human IgM or IgG (EY Laboratories Inc., San Mateo, CA) were used to detect the recombinant protein products. Viruses were purified at least twice by plaque assay.

Recombinant Ab Production. Recombinant Abs were synthesized in Sf9 cells grown in serum-free medium by coinfecting cells with equal amounts of H and L chain producing viruses at an MOI of 5 for each virus. 3 d after infection, the cell supernatants were concentrated using Centriprep 100 (YM 100 membrane) concentrators or stirred ultrafiltration cells using the YM 100 membrane (Amicon, Beverly, MA). Aliquots of the concentrates were analyzed by SDS-PAGE under nonreducing and reducing conditions and Western blotting was used to detect either assembled Abs or separated H and L chains, respectively.

Isolation of Polyclonal CA from Patient Sera. Joh (IgMκ) and Sch (IgMλ) CAs were isolated by incubating patient sera with human cord blood stroma at 4°C overnight, washing the reacted cells with cold PBS and eluting the Ig from the stroma with PBS at 37°C. The eluates were then tested for CA activity by serologic titration. These CA are two of the original CA purified and described by Williams et al. (1).

Preparation of Absorbed Polyclonal Rabbit Antisera. Purified Joh and Sch CA eluates from cord blood stroma were mixed with an equal volume of CFA and injected subcutaneously into New Zealand white rabbits once a week for 1 mo. The rabbits were bled and the antisera absorbed using columns of immobilized pooled normal human IgM (Sigma Immunochemicals, St. Louis, MO) bound to Sepharose 4B (Pharmacia, Piscataway, NJ) and subsequently absorbed with three different human monoclonal IgM Waldenström's proteins linked to Sepharose 4B, which did not exhibit CA activity. The antisera were tested by ELISA for reactions with their homologous CA and other CA eluates. The anti-Joh antiserum reacted positively with the Joh eluate but did not react with the Sch CA. Conversely, the anti-Sch antiserum reacted with the Sch eluate, but did not react with the Joh CA. The absorbed antisera, which reacted with their homologous eluates, did not react with normal IgM nor with isolated IgM paraproteins by ELISA.

ELISA for 9G4 Reactivities and Testing Absorbed Anti-CA CRI Polyclonal Antisera. The isolation of 9G4 was previously described (3). The mAb SA (IgMλ) encoded by the V_H4 family member V79 was used as immunogen. Reactivity of recombinant Abs with mAb 9G4 was measured by ELISA. A sandwich assay was performed in which 96-well flat-bottomed plates were coated with 5 μg/ml 9G4 in 0.1 M carbonate buffer, pH 9.2. The plates were incubated overnight at 4°C then blocked with PBS containing 0.05% Tween 20 and 0.25% BSA for 30 min at room temperature. The plates were washed three times with water and a twofold dilution series of test Abs was added for 2 h at 37°C. After washing three times with water, bound Ab was detected using alkaline-phosphatase–conjugated goat anti-human IgM Ab (μ chain specific; Sigma Immunochemicals). This Ab was incubated for 2 h at 37°C. Unbound Ab was removed by washing three times and *p*-nitrophenyl phosphate substrate tablets were dissolved in 0.1 M diethanolamine buffer, pH 9.8, and added to the wells. The absorbance was measured at 405 nm in an ELISA plate reader (Titertek Multiskan Plus; ICN Flow). A capture assay was also used in which wells were coated with 5 μg/ml anti-rat IgG Abs, followed by 9G4 and the sample Ab which was detected using alkaline-phosphatase–conjugated goat anti-human IgM. The capture assay gave stronger reactions than the sandwich assay within a 30-min reaction time. Sandwich assays, essentially as described above, were used to test absorbed rabbit antisera with both homologous and heterologous CA.

Inhibition ELISA with Rabbit Absorbed Anti-CA CRI Polyclonal Antisera. The anti-Id reaction employed for the inhibition experiments was absorbed anti-Joh or anti-Sch reacting with another cross-reactive CA Koe, but not the individually specific Joh or Sch antigen originally employed to produce the antiserum. The Koe Ab was used in order to avoid background reactions which occur when the inducing CA is used.

The recombinant Abs were diluted to the same protein concentration and an aliquot was mixed with a 1:500 dilution of absorbed anti-CA CRI antisera and incubated for 1 h at room temperature with shaking. The mixture was added to the wells coated with Koe at 5 μg/ml and inhibition was detected by comparing the absorbance with that of the control lacking recombinant Ab.

Results

The 9G4 Idiotope Maps to the V_H Gene Segment. The structural basis for 9G4 binding to Abs containing the V_H4–21 gene segment was analyzed by expressing the CA FS-7H (IgMκ), FS-7 H chain mutants and a number of H and L chains in the baculovirus system. The influence of the L chain on 9G4 reactivity of the FS-7 H chain was analyzed by producing several combinatorial recombinant Abs in which the FS-7 Ab L chain was replaced by different L chains of both the κ and λ isotypes. This was accomplished by coinfecting monolayers of Sf9 cells with the same FS-7 H chain virus and different L chain viruses. As shown in Table 1, all of the Abs containing the FS-7 H chain, irrespective of its paired L chain, were reactive with 9G4. The Ab composed of the κ chain from the mouse anti-arsonate Ab 91A3 (28) combined with the FS-7 H chain also reacted with 9G4. The Ab containing the FS-7 κ chain paired with the Ab25 (antithyroglobulin) μ chain, which uses a V_H3 gene segment (24), did not bind 9G4. Exchange of the V_H3 gene segment utilizing RF-TS2 (IgMκ; 29) H chain, for the FS-7 H chain, converts a nonreactive Ab to a reactive one. The FS-7 H chain alone did not bind 9G4. This may indicate that there is some involvement of the L chain in stabilizing the conformation of the epitope in the H chain. The λ isotype containing Igs, anti-hepatitis B virus surface antigen antibody L chain αHBVλ OST 577 (30) paired with FS-7μ, FS-3λ (8) paired with FS-7μ and FS-3λ paired with FS-6μ (8), all bound 9G4 comparably with the κ-containing antibodies. This further substantiates the conclusion that the idiotope to which 9G4 binds is localized solely to the H chain.

Abs containing the H chain of CA FS-6 were also reactive with 9G4. The FS-6 antibody utilizes the V_H4–21 gene segment and has I antigen specificity (8). In FS-6, the V_H4–21 segment contains four amino acid mutations with respect to the germline sequence; one in each of FR1, CDR1, CDR2,

Table 1. *Reactivity of CA FS-7 and Combinatorial Abs with 9G4*

L chain	V_L family	H chain	V_H family	9G4 reactivity
FS-7κ	κ1	FS-7μ	4	+ + +
RF-TS2κ	κ3a	FS-7μ	4	+ + +
RF-TMC1κ	κ3	FS-7μ	4	+ + +
37-2κ	κ3b	FS-7μ	4	+ + +
91A3κ	κ10	FS-7μ	4	+ + +
HBVλ OST 577	λ5	FS-7μ	4	+ + +
FS-3λ	λ1	FS-7μ	4	+ + +
FS-3λ	λ1	FS-6μ	4	+ + +
FS-7κ	κ1	FS-6μ	4	+ + +
FS-7κ	κ1	FR1(4-21)	4	+ + +
FS-3λ	λ1	FR1(4-21)	4	+ + +
RF-TS2κ	κ3a	FR1(4-21)	4	+ + +
FS-7κ	κ1	V71-2	4	–
FS-3λ	λ1	V71-2	4	–
FS-7κ	κ1	Ab25μ	3	–
37-2κ	κ3b	Ab25μ	3	–
RF-TS2κ	κ3a	RF-TS2μ	3	–
HBVλ OST 577	λ5	HBVγ OST 577	3	–
–	–	FS-7μ	4	–

Combinatorial Abs were produced by coinfecting SF9 cells with the H and L chain producing indicated viruses. Purified Abs were tested for reactivity with 9G4 by ELISA. RF-TS2κ is a RF L chain (11), 37-2κ is an antiacetylcholine receptor L chain (54), HBVλ OST 577 (30) is an antihepatitis B virus surface antigen Ab, and 91A3 is a mouse anti-arsonate Ab L chain (28). Ab25μ is an anti-thyroglobulin Ab H chain (24), and FR1 (4-21) is the parental V71-2 recombinant antibody with the FR1 sequence replaced by the FR1 sequence of V_H4-21. (+ + +) OD_{405} 0.8–1.0; (–) OD_{405} <0.2.

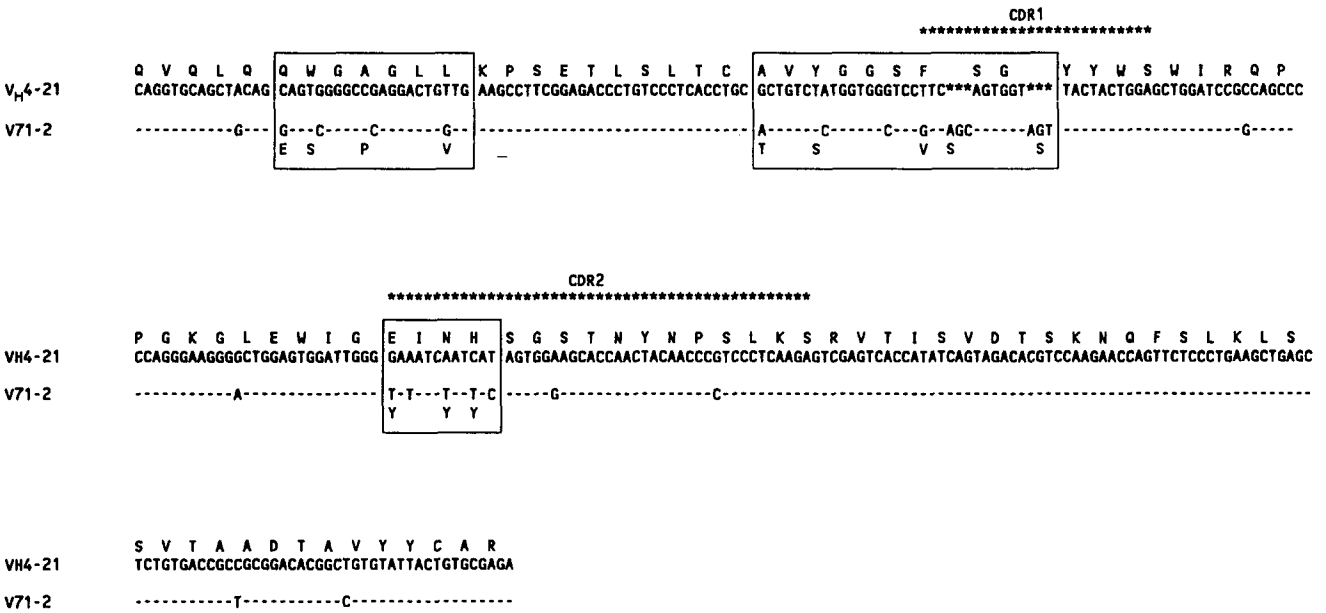

Figure 1. Comparison of the amino acid sequences of V_H4–21 and V71–2 gene segments. (*Dashes*) Identical amino acids. (*Stars* within box) Correspond to extra bp in the V71-2 sequence. Boxed areas enclose regions of sequence differences between the two gene segments and indicate the V_H4–21 amino acids that were exchanged for V71–2 amino acids.

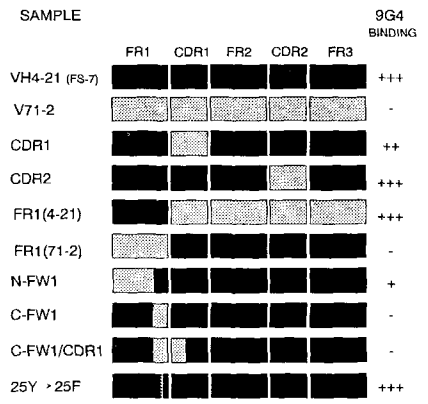

Figure 2. Generation of FS-7 mutants by interchange with V71–2 sequences. (*Solid black areas*) V_H4–21 sequences; (*hatched areas*) V71–2 sequences. The V_H4–21 amino acids were exchanged for those of V71–2 and vice-versa by PCR-SOEing mutagenesis. All H chains were paired with the FS-7κ chain. (*25Y→25F*) Exchange of tyrosine for phenylalanine at position 25. (+ + +) OD_{405} ¼1.2–1.6; (+ +) OD_{405} 0.8–1.2; (+) OD_{405} 0.4–0.8; (−) OD_{405} <0.4.

and FR3 (8). None of these mutations affects the binding of 9G4, which is in agreement with previous results (8). Since there is no sequence homology between the CDR3s of FS-7, which has 14 amino acids compared with 13 amino acids in FS-6, it is concluded that this portion of the Ab molecule is not responsible for the CRI.

The μ chains FS-7, FS-6, RF-TS2, and Ab25 have the same C region sequences, all being PCR amplified from the Ab25 clone, indicating that the idiotope is not influenced by the C region. These data definitively localize the 9G4 idiotope to the V_H4–21 gene segment.

The 9G4 Idiotope Maps to FR1. The approach used to further delineate the amino acids comprising the 9G4 idiotope involved exchanging V_H4–21 sequences with those from the V71–2 gene segment. V71–2 is a member of the V_H4 family which is not associated with Abs having CA activity nor with Abs having 9G4 reactivity (4). Comparison of the V_H4–21 and V71–2 amino acid sequences revealed three areas of sequence distinction as shown in Fig. 1. The first area involves the NH_2-terminal portion of FR1 which contains four amino acid differences. The second area encompasses the COOH-terminal portion of FR1 and part of the flanking CDR1. This involves three amino acid differences along with a V71–2 CDR1 which contains two more amino acids than the V_H4–21 CDR1. The third area involves the CDR2 where the sequences differ by three amino acids. Mutant Abs were constructed and named N-FW1, C-FW1/CDR1, and CDR2 reflecting the three areas differing between the two V_H gene segments. As shown in Fig. 2, both N-FW1 and CDR2 reacted positively with 9G4 in the ELISA, although the N-FW1 reaction was weak. The C-FW1/CDR1 mutant was nonreactive. These results indicate that the 9G4 CRI is predominantly localized to the C-FR1/CDR1 area.

The 9G4 Idiotope Maps to FR1 Amino Acid Position 23–25. As shown in Fig. 2, the ELISA results indicated that both V_H4-21 mutants which either had the entire FR1 exchanged for the V71–2 sequence (mutant FR1 [71-2]) or only

Table 2. *Inhibition of Anti-Joh Polyclonal Rabbit Antiserum Reacting with Koe CRI⁺ CA by Recombinant Abs*

Ab			
L chain	μH chain	JOH anti-Id reactivity	SCH anti-Id reactivity
FS-7κ	FS-7	+ + +	−
FS-3λ	FS-7	+ + +	−
FS-7κ	CDR1	+ + +	−
FS-7κ	CDR2	+ +	−
FS-7κ	FR1 (4-21)	+ + +	ND
FS-3λ	FR1 (4-21)	+ + +	ND
RF-TS2κ	FR1 (4-21)	−	ND
FS-7κ	FR1 (71-2)	−	−
FS-7κ	71-2	−	ND
FS-3λ	71-2	−	ND
FS-7κ	C-FW1	+ +	−
FS-7κ	C-FW1/CDR1	−	−
Koe CA positive control		+ + +	−
Wild-type virus		−	−

Recombinant Abs were adjusted to equal protein concentrations and an aliquot mixed with a 1:500 dilution of absorbed anti-Joh or anti-Sch antisera and incubated at room temperature with shaking for 1 h. The mixture was added to wells coated with 5 μg/ml CA Koe and the absorbance measured relative to the anti-Joh/Koe interaction to determine inhibition.
(+ + +) Inhibition at >0.2 μg/ml inhibitor; (+ +) inhibition at 0.8–0.2 μg/ml inhibitor; (−) no inhibition at 3.5 μg/ml inhibitor. ND, not done.

the portion of FR1 adjacent to the CDR1 exchanged for the V71–2 sequence (mutant C-FW1), were not reactive with 9G4. The nonreactive parental V71-2 antibody was converted to 9G4 reactivity when the FR1 of V71-2 was replaced with the V_H4-21 FR1 sequence in mutant FR1(4-21). The mutant CDR1, which was composed of a V_H4–21 sequence but exchanged in the CDR1 for a V71–2 CDR1 sequence, was 9G4 reactive. CDR1, therefore, is not involved in forming the 9G4 reactive idiotope. This result also indicates that the length of the CDR1, which is longer in V71–2 by two amino acids, is not a factor in 9G4 binding. This pattern of reactivity identifies the idiotypic site as comprising amino acids 23–25 of FR1 having the amino acid sequence AVY.

An inspection of published amino acid sequences indicates that the hydroxyl containing amino acids serine and tyrosine are usually found at position 25 in FR1 in both 9G4 reactive and nonreactive Ig (5–11, 13, 24, 30–32). The influence of a hydrophobic amino acid lacking a hydroxyl group at position 25 was investigated by replacing the tyrosine residue in FS-7 with phenylalanine. The Ab labeled 25Y→25F was produced and tested for 9G4 reactivity (Fig. 2). The Ab was 9G4 reactive indicating that the absence of a hydroxyl group and the change from a polar to a nonpolar residue in this position did not significantly effect the structure of the CRI.

The Reactivity of Absorbed Anti-CA CRI Polyclonal Rabbit Antiserum Maps to FR1. The recombinant FS-7 CA and mutants were incubated with absorbed anti-CA CRI polyclonal rabbit anti-Joh and anti-Sch antisera in an inhibition ELISA. As shown in Table 2, the parental 71-2 Abs, mutants FR1(71-2), C-FW1/CDR1 and FR1(4-21) paired with RF-TS2κ did not inhibit the anti-Joh/Koe reaction. The parental FS-7, CDR1 and FR1(4-21) paired with either FS-7κ or FS-3λ were the best inhibitors, with inhibition detected at 0.2–0.4 μg/ml of Ab. The remaining mutants CDR2 and C-FW1 demonstrated intermediate activity. This pattern of inhibition using the anti-Joh antiserum, with the exception of the C-FW1/CDR1 and RF-TS2 κ/FR1 (4-21) results, indicates that this polyclonal antiidiotypic antiserum is also detecting a determinant in FR1, predominantly at the NH_2-terminal end. The anti-Joh antiserum also was inhibited by the combinatorial Ab FS-3λ/FS-7μ. No inhibition was detected using the anti-Sch antiserum against any of the recombinant Abs employed in these studies. Reactivity assays of the Joh and Sch eluates with 9G4 indicate that these CA are both 9G4 reactive. It is most likely, therefore, that both of these Abs are derived from the V_H4–21 gene segment.

Discussion

In this study we investigated the primary structure and location of the 9G4 cross-reactive Id on the V_H4–21 gene segment using site-directed mutagenesis. Amino acids from the V71–2 gene segment (Id negative), which differ between the V_H4–21 and V71–2 gene segments, were substituted for the V_H4–21 sequence of FS-7, a CA which expresses a germline copy of the V_H4–21 gene segment (8). Our results clearly indicate that the FR1 region of CA Abs utilizing the V_H4–21 gene segment is responsible for binding to the anti-Id Ab 9G4, and more specifically, the amino acids AVY in FR1 have been found to be the reactive site. Replacement of the FR1 sequence of V71-2 with the V_H4-21 FR1 sequence in mutant FR1(4-21) converted the Id negative V71-2 antibody to Id positive. In mutant C-FW1/CDR1, the substitution of V_H4–21 amino acids AVY for V71–2 amino acids TVS at position 23–25, renders the Ab nonreactive with 9G4. When the V_H4–21 sequence AVY is added back to the FR1 (mutant CDR1), the reactivity with 9G4 is restored.

A comparison of published FR1 sequences from Abs of known sequence, which are either reactive or nonreactive with 9G4, is shown in Table 3. All Igs reactive with 9G4 share the AVY motif in FR1, however, when the alanine at position 23 is replaced by threonine as in Ab26, C6B2, PAG1 and 2A4 or lysine as in RF-BOR, RF-TS1, RF-TS3, 9G4 reactivity is abolished. The valine at position 24 is also important since its replacement by alanine in a number of Abs renders them nonreactive with 9G4. In addition to the sequences shown in Table 3, 14 IgM clones from B cell tumors, 10 IgG clones from normals and 21 IgM clones from patients with infectious mononucleosis were all 9G4 reactive, and all 45 clones utilized the V_H4–21 gene segment having the FR1 AVY motif (33).

This molecular localization of this idiotope also explains the negative 9G4 response of FOG 1, an anti-RBC D antigen Ab which uses the V_H4–21 gene segment (12). FR1 of FOG 1 differs from the germline sequence at position 25 and contains a histidine, a basic amino acid that renders the Ab 9G4 negative. Since many of the CA use the V_H4–21 gene segment mutated from the germline sequence (8), it is possible that a spontaneous mutation would occasionally occur in the FR1 segment recognized by 9G4. A mutation in this area does not appear to be a frequent event as a number of CA have been sequenced and other mutations have not been detected at this position (Table 3). Some mutations, however, are tolerated as the mutant 25Y→25F, in which the tyrosine at position 25 was replaced by phenylalanine, was still reactive with 9G4. Therefore, there is no absolute requirement for a hydroxyl containing amino acid at position 25 of FR1. The presence of a basic amino acid such as histidine at position 25, however, appears to be sufficient to abrogate 9G4 binding. Our conclusion that the 9G4 Id maps to FR1 is in agreement with the prediction of Silberstein et al. (5) that the 9G4 idiotope could be associated with conserved residues in the FR1 portion adjacent to the first CDR. The lack of reactivity of the H chain alone suggests that the determinant requires a specific conformation which is perhaps stabilized by the L chain.

The CA CRI was the first demonstration of shared Id among Abs from unrelated individuals (1). Since that time, other CRI have been described. Kunkel et al. (34) demonstrated that RF from unrelated individuals shared CRI. Two of these CRI have been localized to the CDR2 and CDR3 of the κ chain (35). The anti-DNA Ab-associated Id 8.12 is present in λ chains in the vicinity of the CDR1 (36). The CDR2 and/or CDR3 provide the Id determinations in Abs to *p*-azophenylarsonate (37), phosphorylcholine (38), α(1–3) α(1–6) dextrans (39, 40), and β(1–6) galactan (41). Different

Table 3. *FR1 Sequences of Abs with Known 9G4 Binding Activity*

Clone	FR1 sequences			V_H family	GL donor	Specificity	Id 9G4	Reference
FS-7	QVQLQQWGAGLLKPSETLSLTC	AVY	GGS	4	4–21	CA(I)	+	8
FS-1	----------	---	---	4	4–21	CA(I)	+	8
FS-2	--H----T----------	---	---	4	4–21	CA(I)	+	8
FS-3	----------	---	---	4	4–21	CA(i)	+	8
FS-4	----------P--	---	---	4	4–21	CA(I)	+	8
FS-5	----------	---	---	4	4–21	CA(i)	+	8
FS-6	----H------------	---	---	4	4–21	CA(I)	+	8
FS-8	----------	---	---	4	4–21	ND	+	8
Ab17	..........V--------A-	---	---	4	4–21	PR	+	24
CAP(15A)	----------	---	---	4	4–21	CA(i)	+	5
VOG(20A)	----------N-	---	---	4	4–21	CA(I)	+	5
LES	--H----------N-	---	---	4	4–21	RF	+	5
PR-TS2	----------	---	---	4	4–21	PR	+	11
KAU	----------	---	---	4	4–21	CA(I)	+	7
A483	----------	---	---	4	4–21	–	+	13
N6	----------	---	---	4	4–21	–	+	13
N26	----------	---	---	4	4–21	–	+	13
N115	----------	---	---	4	4–21	–	+	13
N127	----------	---	---	4	4–21	–	+	13
N184	----------	---	---	4	4–21	–	+	13
N187	----------	---	---	4	4–21	–	+	13
O15	----------	---	---	4	4–21	–	+	13
O26	----------	---	---	4	4–21	–	+	13
P5	----------	---	---	4	4–21	–	+	13
HY18	----------	---	---	4	4–21	CA(i)	+	6
FOM 1	----------	---	---	4	4–21	D	+	9
FOM A	----------	---	---	4	4–21	D	+	9
MAD 2	-----ES----------	---	---	4	4–21	D	+	9
T14	----------	---	---	4	4–21	DNA	+	10
FOG 1	--H-----T----------	--H	---	4	4–21	D	–*	9
Ab26	-----ES-P--V---Q------	T-S	---	4	71–4	PR	–	24
C6B2	-----ES-P--V---Q------	T-S	---	4	71–4	PR	–	5
PAG 1	-----ES-P--V-------V--	T-S	---	4	71–4	D	–	9
FOG B	-LR--ES-P--V----------	S-S	---	4	2–1	D	–	9
HAM B	-----XS-G-VVQ-GRS-R-S-	-AS	-FT	3	DP50	D	–	9
FOG 3	.-----S-G-VVQ-GRS-R-S-	-AS	-FT	3	DP50	D	–	9
BOR	E---V-S--EVK--GSSVKVS-	KAS	-DT	1	–	RF	–	31
RF-TS1	L---V-S--EVK--GSSVKVS-	KAS	-DT	1	HON-1	RF	–	11
RF-TS2	----VES-G-VVQ-GRS-R-S-	-AS	-FT	3	19.III	RF	–	11
RF-TS3	----V-S-SELK--GASVKVS-	KAS	-YT	1	4.16	RF	–	11
RF-SJ2	----V-S-G-VVQ-GRS-R-S-	-AS	-FT	3	56P1	RF	–	11
RF-AN	E----ES-G-LVQ-GRS-R-S-	VTS	-FT	3	26	RF	–	11
L16	------S-P--V---Q------	-IS	-D-	6	.	PR	–	5
2A4	-----ES-P--V---Q------	T-S	---	4	71–2	DNA	–	32
HBVOST577	----VES-G-VVQ-GRS-R-S-	-AS	-FT	3	1.9III	HBVsAg	–	30
Ab25	E---LES-G--VQ-GGS-R---	-AS	-FT	3	J00236	TG	–	24

CA(I), cold agglutinin anti-I; CA(i), cold agglutinin anti-i; PR, polyreactive; D, red blood cell D antigen; HBVsAg, hepatitis B virus surface antigen; TG, thyroglobulin.

* The only V_H4–21–derived Ig that is 9G4 negative.

CD4-specific mAbs were found by site-directed mutagenesis to have Ids located in the CDR2 and CDR3 (42), however, loss of the Id determinants did not significantly affect antigen binding. The Id in anti-α(1–6) dextran is associated with the V_H CDR2 (43), and again, loss of the Id did not affect antigen binding.

The above examples indicate that the majority of identified CRI are located in the CDRs and hence, have the potential of being closely related to the antigen-combining site. Not all anti-Id determinants on the V region of an Ab, however, are formed by the CDRs as there are a few examples where specific Ids map to framework regions. Anti-DNA CRI (thought to be the 31 Id) were directed against framework determinants (44) and a polyclonal rabbit antiserum recognized a number of mouse mAbs whose determinants were localized to FR1 and FR3 residues (45). The role of FR sequences in determining Ab specificity by influencing the three-dimensional conformation of the H chain is still poorly understood. FR1 is a solvent-exposed segment and is structurally separated from the antigen binding site (46). Residues in the FR near the CDR boundaries, however, often directly contribute to the correct folding of the Ab binding region (47–49). The location of the 9G4 reactive determinant in FR1 implies that this marker is not associated with the antigen-combining site and hence, is consistent with the findings that this site is detected on Abs with unrelated binding specificities.

The I/i carbohydrate antigens on the surface of RBCs are the most frequent specificities of CA. These antigens are composed of repetitive *N*-acetyllactosamine units which are linear in the i structure and branched in the I structure by virtue of a Gal-GlcNAc attached in β1→6 glycosidic linkage to galactose residues (50). A study by Feizi et al. (51), using 11 monoclonal anti-I Abs, determined that there are three types of blood group I specificities suggesting the presence of three major structural domains within the I molecule. None of the 11 mAbs, however, recognized the same antigenic determinant on the I structure, indicating that these Abs possess a wider range of fine specificities. This finding is consistent with the sequence data which demonstrated that Abs with CA activity, and having highly homologous V_H regions, all had different CDR3 sequences. This suggests that CA are interacting with I/i antigens through the conventional Ab combining site formed by the association of the H and L chains. Because of the structural unrelatedness of the autoantigens recognized by V_H4–21–derived Abs, it is possible, therefore, that the fine specificity of V_H4–21–derived Abs is determined by the CDR3, with involvement of the L chain, whereas the gross I/i specificity is regulated by the V_H4–21 region, as also suggested by Silberstein et al. (5).

The structural relationship between the site recognized by 9G4 and the antigen binding site is still unclear. It was demonstrated that after interaction between CA and 9G4, CA are unable to agglutinate RBCs (12, 52). This has been interpreted to mean that the I/i binding site and the 9G4 binding site are in close proximity. Alternate explanations could be that the structure of a more distally located antigen-combining site is altered after 9G4 binding, which results in the impairment of these Abs to agglutinate RBCs or that steric hindrance after 9G4 binding prevents agglutination. The localization of the 9G4 determinant to the FR1 indicates that one of the latter hypotheses is likely correct. I/i binding analysis using our recombinant FS-7 mutants, which no longer contain the 9G4 idiotope will provide additional information regarding the relationship between the 9G4 idiotope and I/i binding regions.

The absorbed polyclonal rabbit anti-Joh antiserum also detected a determinant located in FR1. The Joh CA is an IgMκ Ab with anti-I activity (53), whereas the Sch CA is IgMλ with both anti-I and anti-i activity (1, 17). Although both of these Abs were found to react positively with 9G4, they only reacted with their homologous absorbed rabbit anti-Id antiserum. This implies that the antisera are detecting determinants provided by both the H and L chains and possibly explains the lack of reactivity of mutant FR1 (4-21) paired with RF-TS2κ. It is unclear why the C-FW1/CDR1 mutant did not inhibit the anti-Joh/Koe reaction, whereas the C-FW1 and CDR1 mutants did. This may be a reflection of the composition of the polyclonal antiserum which has the ability to recognize multiple epitopes combined with a potential alteration in the three-dimensional structure of the Ab which resulted from simultaneously mutating both the FR1 and CDR1 areas.

Since both the 9G4 determinant and a CRI recognized by specifically absorbed antiserum are localized to FR1, these data underscore the influence this solvent-exposed region of the Ab plays with respect to the antigenicity of Igs. The maintenance of the AVY sequence in Abs derived from the V_H4–21 gene segment suggests that it has an important role in determining the biological activity of these Abs. The 9G4 Id is a marker for a germline gene segment that is found in 10% of all B cells in the normal human population (52). As a number of 9G4 reactive Abs are autoreactive, mechanisms that control the overproduction of these Abs must be operative under normal conditions. Some of these mechanisms may involve an idiotypic network based on determinants located in FR1 to control the quantity of Ig produced. Breakdown of the regulated synthesis of these Ig appears to lead to the overproduction of Abs with autoreactivity. Analysis of the 9G4 idiotope extends out understanding of the physical properties of a very interesting group of Abs and a genetic component involved in autoreactivity. Further expression studies are required to establish the relative positions of the 9G4 Id and the antigen-combining site. This information will potentially explain the restricted use of the V_H4–21 gene segment in CAs with anti-I/i specificity, the role of the H chain CDR3, and the contribution of L chain in determining antigen specificity.

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