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Evidence for Involvement of a Hydrophobic Patch in Framework Region 1 of Human V4-34-Encoded Igs in Recognition of the Red Blood Cell I Antigen

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The monoclonal IgM cold agglutinins that bind to the I/i carbohydrate Ags on the surface of RBCs all have Ig H chains encoded by the V4-34 gene segment. This mandatory use indicates that distinctive amino acid sequences may be involved in recognition. Critical amino acids exist in framework region 1 (FR1) of V4-34-encoded Ig, and these generate a specific Id determinant which apparently lies close to the I/i binding site. However, I binding by Id-expressing Ig can be modulated by sequences in complementarity-determining region (CDR)3. Examination of the crystal structure of an anti-I cold agglutinin has revealed a hydrophobic patch in FR1 involving residue W7 on β-strand A and the AVY motif (residues 23–25) on β-strand B. In this study we used mutagenesis to show that each of the strand components of the hydrophobic patch is required for binding the I carbohydrate Ag. In addition, the crystal structure reveals that amino acids in the carboxyl-terminal region of CDR3 form a surface region adjacent to the hydrophobic patch. We propose that the I carbohydrate Ag interacts simultaneously with the entire hydrophobic patch in FR1 and with the outside surface of CDR3. This interaction could leave most of the conventional binding site available for binding other Ags.

Cold agglutinins (CA) with specificity for the carbohydrate I/i Ag on the RBC surface are highly unusual in being encoded by a single Vg gene segment, V4-34. Although V4-34 is mandatory, a variety of different D and JH gene segments, as well as L chains from different families and isotypes, are used (1–5). The I/i Ag is composed of repetitive N-acetylgalactosamine units (Galβ1→4GlcNAc) which are linear in the I structure and branched in I (6). The strong association of a single Vg gene segment with specificity for the I/i Ag indicated that the particular structure formed by the V4-34-encoded portion of the Ab is required for I/i binding. It also suggested that the structural interaction may be of an unconventional nature involving framework regions (FR) and therefore independent of the conventional Ag binding site (7, 8).

Staphylococcal protein A (SpA) binding to V13-encoded Ig was shown by mutagenesis to interact with residues in FR1, FR3, and the adjacent residues of complementarity-determining region (CDR)3 (9). SpA has been described as the prototypical B cell superantigen that binds most members of the V3 family without a L chain preference or influence of CDR3 (10). The full extent of this interaction has recently been elucidated in a crystal structure of a Fab/SpA complex (11), which rationalizes the VHI family binding specificity of SpA and shows some similarity to the binding mode of a T cell superantigen to the Vβ FR of the TCR (12).

Therefore, B cell superantigen may be defined in structural terms as molecules that bind to a subset of VHI family-encoded Ig through interactions outside of the conventional Ag-binding site.

It is usage of this single V4-34 gene segment that probably accounts for the cross-reactive Id detected in early studies (13). The Id was identified more clearly when the rat 9G4 mAb was found to react with Igs encoded only by the V4-34 gene segment, regardless of D, JH, or L chain sequence and isotype (14, 15). The Id is not detected on isolated H chains but is present when the V4-34 H chain is paired with any L chain (14, 16). Mutational analysis revealed that the 9G4 Id is located in FR1 of V4-34-encoded Ig (16). The Id is discontinuous, involving W at residue 7 on β-strand A (17) and AVY at residues 23–25 on β-strand B (16). There was an early suspicion that FR1 might also be involved in recognition of the I Ag, because the mAb inhibited RBC agglutination (14, 18). This was confirmed when replacement of FR1 of a CA with the FR1 sequences from six VH families resulted in loss of Id specificity (19). All of the recombinant Abs with FR1 replacements had the natural CA CDR3 sequence. It was evident from these data that CA CDR3 in the absence of FR1 was not sufficient to mediate I binding. However, a modulatory role of CDR3 in Ag recognition had been indicated by the observations that Id-positive V4-34-encoded mAbs with specificity for DNA were unable to bind the I Ag (20, 21). This binding pattern appeared to be associated with basic amino acids in CDR3 that were apparently non-permissive for I binding. The role of CDR3 in I binding was also tested by mutational analysis (19). Converting the six CDR3 carboxyl-terminal amino acids of CA PS-6 to alamines abolished I binding, while replacement of the amino-terminal seven amino acids by alamines only moderately reduced binding (19). These results implicated CDR3 in I recognition.

The crystal structure of the Fab from the human I binding CA KAU has been determined (22). The KAU H chain is encoded by the V4-34-(D unidentified)-JH4 gene segments, while the L chain is A11 (k3055, V3-11b)-JL4 encoded (3). There is only one amino acid difference at position 21 in CDR1 (Gly (G) to Asp (D)) in KAU compared with the germline V4-34 sequence. The reported

*Laboratory, Southampton University Hospitals Trust, Tremona Road, Southampton, United Kingdom; and Randall Center, King’s College, London, UK.

†Molecular Immunology Group, Tenovus Laboratory, Southampton University Hospitals Trust, Southampton, United Kingdom; and Randall Center, King’s College, London, UK.

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2 Address correspondence and reprint requests to Dr. Kathleen N. Potter, Tenovus Laboratory, Southampton University Hospitals Trust, Tremona Road, Southampton SO16 6YD, U.K. E-mail address: kp1@soton.ac.uk.

3 Abbreviations used in this paper: CA, cold agglutinin; FR, framework region; SpA, staphylococcal protein A; CDR, complementarity-determining region.

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analysis of the crystal structure indicated that the KAU combining site has an extended cavity and a neighboring pocket. Residues from the hypervariable loops CDR\(_2\), CDR\(_1\), and CDR\(_3\) form the cavity, while the pocket is defined by residues from CDR\(_4\), FR\(_2\), and CDR\(_3\). The authors concluded that the nature of the site was consistent with polysaccharide binding and proposed that the I Ag is binding via the conventional combining site. Because residues in FR1 are clearly outside this conventional combining site, they explained the previous reports of inhibition of I/I binding by mAb 9G4 in terms of a conformational change or steric blocking of the conventional site by the anti-Id. However, such an explanation cannot account for the inhibition of I binding by mutagenesis of individual residues in FR1 (Ref. 19 and this paper).

To resolve this paradox, we extend the previous mutational analysis and find that both the W at position 7 and the AVY motif at positions 23–25 are simultaneously required for I binding. On the basis of the crystal structure we find that these amino acids form an extensive hydrophobic patch in FR1. We are now able to correlate the crystallographic data with the molecular data in terms of the relative locations of FR1 and CDR\(_3\) required for I binding, and we propose a superantigenic mode of recognition that involves an unconventional Ag binding site modulated by the structure of the CDR\(_3\).

**Materials and Methods**

**Viruses and cells**

Recombinant viral stocks of *Autographa californica* nuclear polyhedrosis virus were produced and assayed in the QIAprep Miniprep kit (Qiagen) and sequenced by the Sanger dideoxy method. E. coli transformed into the parental template. A total of 1 l of the digested DNA was l. The PCR-amplified DNA was isolated using the Miniprep DNA was used to transfect Sf9 cells using linearized BacPAK6 viral DNA and Bacfectin (Clontech Laboratories, Basingstoke, U.K.) as per the manufacturer’s instructions. Viral supernatants were harvested at 3 days posttransfection.

**Recombinant Ab production**

Recombinant Abs were synthesized in BTI-TN-5B1-4 (High Five) cells grown in EX-CELL 420 serum-free medium by coinfecting cells with equal amounts of H and L chain-producing viruses at a multiplicity of infection of 5 for each virus. Three days postinfection, the supernatants were centrifuged at 1600 rpm for 5 min to remove cellular material. Tris-HCl (2 M; pH 8.8) was added to neutralize the supernatants to pH 7. The resulting precipitate was removed by centrifugation at 4000 \( \times g \) for 10 min. One tablet of complete protease inhibitor mixture (Roche, Lewes, U.K.) was added per 20 ml of supernatant. The clarified supernatants were concentrated between 10- and 80-fold using Vivaspasiln concentrators (5000 MW; Sartorius, Epsom, U.K.). It has previously been determined that recombinant IgM Abs are predominantly produced as four-chain monomers and not as pentamers or hexamers in insect cells, even in the presence of J chain (19).

**Quantitative ELISA**

All recombinant Ig samples were first quantitated by capture ELISA using known amounts of IgM as a standard. Relative concentrations were calculated from the absorbances and dilutions of the samples. Microwell plate wells (Maxisorb; Fisher, Loughborough, U.K.) were first coated with goat anti-human IgM (Sigma, Poole, U.K.) as capture Ab and goat anti-human \( \kappa \)-chain Ab (Sigma) as detecting Ab.

**ELISA for 9G4 reactivity**

The reactivity of the recombinant Abs with mAb 9G4 was measured by capture ELISA as previously described (19). Reactivities of recombinant Abs were measured using equivalent amounts of Abs.

**I binding ELISA**

Recombinant IgM monomers only weakly agglutinate RBCs in a hemagglutination assay. As an alternative to hemagglutination, we used an I-acti-

tive extract from RBCs to determine the interaction between I Ag and Ab by ELISA. The I Ag was isolated by Dr. D. Roelcke (University of Hei-
delberg, Heidelberg, Germany), as previously described (19). This phenol-
saline extract was shown to correlate with I and not i activity.

**Results**

**Generation of recombinant Abs by site-directed mutagenesis**

The germline amino acid sequence encoded by the V\(_{4}\)-34 gene segment compared with that of representative members of the V\(_{4}\) family is shown in Fig. 1. It can be seen that the amino acid differences between V4-34-encoded VH and other members of the V\(_{4}\) family are primarily located in FR1, CDR1, and CDR2. Of the nine amino acid differences in FR1, five are common to all of the non-V4-34-encoded sequences which encode Iggs that neither express the 9G4 Id nor are involved in I/I binding (14–16). Based on the fact that FR1 appears to be critical for I binding (19), two mutagenic primers were used during PCR amplification, derived from FS-6 (Fig. 2). FS-6 is an IgM/\( \kappa \)CA with predominantly I specificity, based on a strong ability to agglutinate adult erythrocytes and weak agglutination of cord erythrocytes (2, 24, 25), and is unre-
active with ssDNA, dsDNA, lipid A, and IgG (21). The V4-34 gene segment is 96% homologous to germline (2), and the net charge of the CDR\(_3\) is \(-2\) (21). FS-6 had previously demonstrated strong reactivity in ELISA using the I extract (19). One mutant had W7 converted to S on \( \beta \)-strand A, while the other had the AVY(23-25) motif on \( \beta \)-strand B converted to TVS (Fig. 2).

By mutating these residues to amino acids found in the other V\(_{4}\) family sequences, correct folding of the VH domains was ensured.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Primer</th>
<th>Sequence (5→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>W7</td>
<td>1</td>
<td>CAGTTGCAGGTCATACATCAGTCGCGCCAGGACTG</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>CAGTCTCGCGCCGGCACTGATGTCAGGCACCTG</td>
</tr>
<tr>
<td>TVS</td>
<td>1</td>
<td>GTCCCTACCTGCGCTTGCTGTTGCGTCTTCAGG</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>CTGAAGGACCCACAGAGACAGTUCAGGTCAGGAC</td>
</tr>
</tbody>
</table>
The recombinant Abs were isolated from tissue culture supernatants and quantitated by capturing with anti-μ chain Abs and detecting with anti-κ chain Abs. This approach ensured that only H:L chain pairs were being measured.

Effect of FR1 mutagenesis on expression of the Id and binding to the I Ag

Reactivity of the recombinant Abs with the anti-Id rat mAb 9G4 was measured by capture ELISA using rabbit anti-human IgM Ab to detect bound Abs. Mutation of either site in FR1 severely disrupted the structure of the Id (Fig. 2A), as demonstrated by the significant reduction in 9G4 reactivity of both the W7S and AVY(23–25) constructs compared with FS-6. Some residual Id expression was detected in the AVY(23–25) FR1 construct compared with the W7S construct. Hybrid Ig FR1(V4-39), in which the FR1 sequence of V4-39 replaced the V4-34-encoded FR1 in the FS-6 CA, had previously been constructed (19) and was used as a negative control.

Ability of mutants W7S and AVY(23–25)FR1 to bind the I Ag

I binding was detected by ELISA using wells coated with I Ag. The reactivities of the recombinant Abs were conducted in the cold and detected using peroxidase-conjugated goat anti-human IgM Ab. Fig. 2B shows that when either the W7 was mutated to S or the AVY(23–25) motif was replaced with TVS, I Ag binding by both mutants was severely reduced compared with the parental FS-6 recombinant Ab. The reduced reactivities of the single mutants were comparable with the hybrid Ig FR1(V4-39), in which both the W and AVY motif have been mutated. These data indicate that both portions of the FR1 Id on β-strands A and B are critical for I Ag binding.

Structural modeling of FR1 and its proximity to CDR\_H\_3

An examination of FR1 in the crystal structure of the anti-I CA KAU by Cauerhoff et al. (22) reveal that the W at position 7 and the AVY motif at positions 23–25 lie close together. Furthermore, the side chains of W7 and the Y25 both lie totally buried in the hydrophobic core of the V\_H domain and do not contribute to the patch. Further graphic representations of the Ab/idiotope can be viewed in color at http://www.gene-memes.com/VH4-3profile.html.

The crystal structure of the KAU Ab was further examined to determine whether there was a structural explanation for the concept of “permissive” and “nonpermissive” CDR\_H\_3 sequences for I binding by V4-34-encoded Ig. Previous data indicated that the six carboxyl-terminal amino acids of FS-6 CDR\_H\_3 were involved in I binding (19). Cauerhoff et al. (22) report that the conventional Ag binding site of KAU consists of an extended cavity with a neighboring pocket, lined by residues from various CDRs, including CDR\_H\_3. However, the crystal structure also reveals that several of the side chains of CDR\_H\_3 residues point away from the conventional Ag binding site, and particularly those at the carboxyl-terminal end of CDR\_H\_3 form a surface region adjacent to the hydrophobic patch described above. This can be seen in Fig. 3A. Fig. 3B shows an orthogonal view, looking onto the conventional combining site, with all CDRs indicated. Thus, an Ag binding to residues of FR1 could certainly contact the outward-pointing residues of CDR\_H\_3. Some CDR\_H\_3 sequences could therefore permit, or even enhance, binding in this mode, while others could have side chains, especially in the carboxyl-terminal region, that would prevent interaction with the adjacent FR1 region.

Discussion

The data from previously published mutagenesis studies clearly showed that both FR1 and CDR\_H\_3 of V4-34-encoded Ig are essential for I binding (19). However, from the crystal structure of the V4-34-encoded anti-I KAU, Cauerhoff et al. (22) suggested that the I/Id Ag binds to the conventional combining site. They proposed that the small pocket formed by CDR\_H\_1, FR\_2, and CDR\_3 explains the V4-34 restriction among CAs. However, their analysis of the KAU crystal structure fails to take into account the role of FR1 in I binding. Our new data allow a resolution of this controversy and provide an interpretation consistent with both molecular and crystallographic findings.

Mutational data from this current study indicate that while the amino acid at W7 is mutated to S or the AVY(23–25) motif to TVS, I Ag binding is severely reduced. It is extremely unlikely that mutation of these particular exposed residues will have any long-range structural effects, such as in the CDRs. Although it is now well documented that mutations of FR residues immediately adjacent to the CDRs can modulate Ag binding (26–28), there are no examples of FR residues further away having any such effect. The
effects of these adjacent FR residues are readily understood in terms of local conformational effects upon residues in contact with Ag and, furthermore, their effects upon Ag binding affinity are relatively small (26). In contrast, residues W7, A23, and Y25 make no such contacts with other residues as they are exposed on the surface, and yet they have a considerable effect upon Ag affinity.

Further evidence for the fact that mutation of these FR residues is unlikely to affect CDR conformation comes from the observation that the conformational changes observed in Fab crystal structures upon conventional Ag binding do not extend beyond the CDRs themselves (although the relative alignment of V₅ and V₄ can be affected) (29, 30). Similarly, the crystal structures of superantigens bound to their targets have shown that they do not induce major conformational changes. Binding of SpA to V₄-34-encoded Fab did not alter the conformation of the combining site (11), and complex formation between Staphylococcus aureus enterotoxin B and HLA-DR1 did not induce significant conformational changes in either itself or DR1 (31). In addition, no major conformational changes were observed in either the TCR β-chain or enterotoxin C3 upon complex formation (12). These data indicate that there are no long-distance structural effects either by Ag binding through the conventional combining site or by Ag binding unconventionally outside of the combining site. Thus, we conclude that the mutations of W7, and A23 with Y25, are directly responsible for the reduced affinity for Ag, and we infer from this that both FR1 β-strands A and B are involved in this interaction.

Cauerhff et al. (22) argue that residues from the V₄-34-encoded hypervariable loops CDR H1 and CDR H2 that form the wall of the pocket are conserved in anti-I/i CAs. They report that the KAU CDR H1 loop (residues 26–32 of the V H region) folds with the group 1 canonical structure. The residues at key sites for the H1 canonical loop structure 1 are 24, 26, 27, 29, 34, and 94 (32). However, residues 24, 26, 27, 34, and 94 are present in all members of the V₄ family, as shown in Fig. 1, and are not specific to V₄-34-encoded Ig. Clothia et al. (32) indicate that the amino acid at position 29 can be F, I, or V in canonical structure 1. Although F is present only in V₄-34-encoded Ig, canonical structure 1 is maintained and is present in V₄-34-encoded Ig with both anti-I and anti-DNA specificities (21). Therefore, it does not appear that any of the residues at key sites for the H1 loop structure can be critical for I binding.

In addition, V₄-34 and V₄-i family-encoded Ig have different amino acids at positions 52 and 53 in the CDR H2 loop (residues 52–56). The V₄-34 gene segment encodes NH at these positions, while they are either YY or YH in the rest of the V₄-i family (Fig. 1). However, when the CDR H2 of a V₄-61-encoded Ig replaced the V₄-34 CDR H2 sequence (Fig. 1), the NH was replaced by YY and there was no effect on I binding (19). This indicates that the amino acids NH at positions 52 and 53 are not important for I binding. The CDR H2 exchange also removed the potential N-linked glycosylation site at NS2 (NHS), which is unique among the V₄-i family...
The nature of this hydrophobic patch, with aromatic ring structures and/or charge characteristics of the surface-exposed CDRH3 residues adjacent to FR1 are able to influence the ability of the I Ag to associate simultaneously with CDRH3 and FR1.

The I/i Ags are relatively simple structures. Both are composed of N-acetyllactosamine, which is linear in the i structure and branched in the I structure. Hence, the I Ag has determinants in common with the i Ag (6). Most CA have some level of reactivity toward both I/i Ags and are assigned an I or i specificity based on the stronger reactivity (25). It is impossible to completely rule out that both the conventional site involving the pocket and the hydrophobic patch modulated by CDRH3 might both be used in binding the branched I Ag. However, this seems unlikely in view of the sequence restriction with I binding. Using Kabat’s functional classification of anti-carbohydrate binding sites, the pocket site is thought to interact most strongly with the terminal nonreducing ends of sugar chains and could potentially bind the nonbranched portion of the I Ag. However, the antigenic determinants recognized by anti-I and anti-i Abs are thought to be larger than trisaccharides (37).

It has been observed that V4-34-encoded anti-DNA Igs do not have anti-RBC reactivity (20, 21). This means that a V4-34-encoded Ig expressing the 9G4 Id and with an intact hydrophobic patch is not necessarily a CA. Anti-DNA Igs characteristically display positively charged residues in CDRH3, and it may be that these residues required for binding DNA inhibit binding to I/i by virtue of their proximity to the hydrophobic patch. While mAb 9G4 inhibits I/i binding, it does not inhibit ssDNA binding to V4-34-encoded Ig (21), further suggesting that DNA binding activity occurs in the conventional binding site. This concept is supported by the fact that D5Trp, a W7S mutant with a structurally disrupted hydrophobic patch, retains specificity for ssDNA (17).

The interaction of an autoantigen such as I/i with the CDRs, and in particular CDRH3, outside of the conventional binding site has a precedent in the crystal structure of the complex between rheumatoid factor RF-AN and IgG-Fc (38). This structure revealed a mode of binding in which Ag recognition was achieved through CDR residues that lay along one edge of, and pointed away from,
the conventional binding site. Despite this unconventional site, CDRβ3 residues dominated the interaction, as in virtually all Ab-Ag interactions. However, as a result of this binding to the side of the Vβ Vδ domain pair, almost all of the conventional binding site remained free for the potential recognition of a different, as-yet-undefined Ag.

Thus, the pocket and cavity described in the three-dimensional structure of KAU may not be intended for the I Ag. The ultimate proof for these interactions will be in the crystal structures of V4-34-encoded Abs combined with their Ags.

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References