Active Site Structure and Antigen Binding Properties of Idiotypically Cross-reactive Anti-fluorescein Monoclonal Antibodies*

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This report includes complete V\(_\alpha\) and V\(_\lambda\) nucleotide and deduced amino acid sequences of idiotypically cross-reactive monoclonal anti-fluorescein antibodies that differed \(>10^5\)-fold in affinity. High affinity monoclonal antibody 4-4-20 and intermediate affinity antibodies 10-25, 5-14, 9-40, 12-40, and 3-24 utilized \(\geq 90%\) homologous V\(_\alpha\)IIC germ-line genes. Extensive D segment length and sequence variability were observed; however, compensatory germ-line J\(_\alpha_4\) (4-4-20 and 3-24) or J\(_\lambda_3\) (10-25, 5-14, 9-40, and 12-40) sequence lengths resulted in H chain CDR3 + FR4 to be a constant 18 amino acids. In addition, each antibody and low affinity 3-13 rearranged \(\geq 98%\) homologous V\(_\lambda_{11}\) genes to J\(_1\), except for 10-25 (J\(_5\)) and 3-13 (J\(_4\)). Resolved crystal structure of complexed fluorescein and 4-4-20 Fab fragments revealed residues His\(_{274}\), Tyr\(_{132}\), Ser\(_{181}\), Trp\(_{306}\), and Trp\(_{433}\) acted as hapten contact residues. Antibodies 5-14, 9-40, 12-40, and 3-24 primary structures possessed identical contact residues as 4-4-20 except for the substitution of His\(_{274}\) for Arg\(_{134}\). Thus, Arg\(_{134}\) was implicated in the increased affinity of monoclonal antibody 4-4-20. Finally, it was difficult to correlate extensive H chain CDR3 residue heterogeneity directly with fluorescein binding and idiotype.

Genetic elements and mechanisms involved in antibody diversity have been described (1, 2), but contributions of each to expressed immunoglobulin protein antigen-binding site structure (idiotype) and function (idiotype complementarity) are ill-defined. Investigations into select idiotypically restricted immune responses have attempted to define structural determinants involved in dominant idiotype expression (3–5). Because relatively limited information exists, it remains unclear whether similar rules pertain to antibody active site structure and function during idiotypically unrestricted immune responses. Therefore, by defining parameters required for antigen specificity and idiotype expression, unrestricted responses provide potential for a more comprehensive understanding of antibody active sites.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank\(^\text{TM}/\)EMBL Data Bank with accession number(s) J05237 and J05238.

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1 The abbreviations used are: Fl, fluorescein; mAb, monoclonal antibody; H, immunoglobulin heavy chain; L\(_\lambda\), immunoglobulin light chain; V\(_\alpha\), V\(_\lambda\), J, D, variable, constant, joining, and diversity segments, respectively; CDR, complementarity-determining region; FR, framework region.

Haptenic fluorescein (Fl\(^+\)) is dianionic at neutral to basic pH and approximates active site filling (6). When conjugated to carrier proteins, Fl elicits an unrestricted antibody response in BALB/cVb inbred mice as evidenced by lack of dominant idiotypes (7–9). In addition, since anti-fluorescein responses undergo extensive time-dependent affinity maturation (10), monoclonal antibodies ranging in affinity from \(10^6\) to \(10^8\) M\(^{-1}\) have been obtained (11). In an attempt to correlate active site structure with function, a panel of idiotypically cross-reactive monoclonal anti-fluorescein antibodies was generated and characterized. Previous reports described ligand binding patterns, idiotypic properties (8, 12–14), and preliminary primary structures (15) of high affinity prototype 4-4-20, low affinity prototype 3-13, intermediate affinity prototype 9-40 and 9-40 family members: 3-24, 5-14, 9-40, 10-25, and 12-40. mAbs 9-40 and Ig members of the 9-40 family were determined to be idiotypically related to 4-4-20 and 3-13; however, 4-4-20 and 3-13 were idiotypically distinct.

To begin to understand ligand binding and idiotypic relationships within members of the idiotype families, heavy and light chain variable region primary structures were deduced from cDNA generation, cloning, and sequencing. Except for mAbs 5-27 (V\(_\alpha_{III}\)) and 3-13 (V\(_\lambda_{IIB}\)), all antibodies rearranged highly homologous (>90%) V\(_\alpha_{III\lambda\lambda}\) subgroup genes, exhibited no D segment preference and utilized J\(_\alpha_3\) or J\(_\lambda_4\). Similarly, all antibodies rearranged >96% homologous V\(_\lambda_{I}\) II subgroup genes (except 5-27, V\(_\lambda\)) primarily to J\(_1\); however, J\(_5\) (10-25) and J\(_4\) (3-13) were also utilized. Results are discussed in terms of antigen binding and specificity in regard to the known three-dimensional structure of liganded prototype mAb 4-4-20.

MATERIALS AND METHODS

Hybridoma Cell Lines—Monoclonal anti-fluorescein secreting hybridoma cell lines were generated by polyethylene glycol fusion of hyperimmune BALB/cVb splenocytes to nonsecreting Sp2/O-Ag14 myeloma cells (16). Culture supernatants from wells or flasks with sufficient cell growth were screened for anti-fluorescein activity using the cellulose chromatographic (19) purification of polyadenylated mRNA as outlined (20). First strand variable heavy and variable light chain
**Fig. 1.** Nucleotide (top lines) and deduced amino acid (bottom lines) sequence comparison of V<sub>H</sub> cDNA generated from eight anti-fluorescein monoclonal antibodies. Dashes (-) denote identity to mAb 4-.
Heavy chain CDR3 and FR4, positions 95 through 113 and encoded by D and J names and exhibited extensive heterogeneity (Fig. 1). Diversity segments varied as follows: 4-4-20, 3 nucleotides of unknown origin; 10-25, 15 nucleotides of unknown origin; 5-14, 14 nucleotides probably contributed by Dsp25 (28, 29); 5-27, 24 nucleotides in part from Dbl162 (28, 29) with N region (30); 9-40, 15 nucleotides potentially from Dsp25 (28, 29) plus N region (30); 12-40, 15 nucleotides comprised of Dnu142 (28, 29) plus N region (30); 3-24, 11 nucleotides of undetermined origin; and 3-13 (26), 9 nucleotides derived from Dsp25 plus Dfl162-D-D joining (26, 29, 28). Further classification revealed mAbs 4-4-20, 3-24, and 13 utilized J3 (31) while mAbs 10-25, 5-14, 9-40, 3-40, and 3-12 utilized J2 (31). Single base pair substitutions in 4-4-20, 10-25, and 24-27 germ-line Ju segments yielded GlyH318, HisH31, and AlaH317, respectively. Although D segments varied in length, corresponding J3 segment length variations resulted in CDR3 plus FR4 to be a constant 18 amino residues for 4- 4-20, 10-25, 5-14, 9-40, and 24. Heavy chain variable region primary structure lengths for these antibodies, therefore, were 114 amino acid residues while 8-27 and 13-18 were 120 and 123 residues long, respectively.

Light chain variable region nucleotide and deduced amino acid sequences are shown in Fig. 2. mAbs 4-4-20, 10-25, 5-14, 9-40, 12-40, 3-24, and 13-23 V regions, were encoded by a single to a few highly homologous (29%) Vn (24) or V (23) subgroup genes (23). Light chain leader sequence primary structures, positions -19 through -1, were identical; however, at the nucleotide level a single silent substitution at 4-4-20 LeuH13 (T for C) was observed. Between residues 1 and 95, 5-14, 9-40, 12-40, and 3-13 primary structures were identical, while high affinity antibody 4-4-20 differed from these at residues ArgH21 and ValH24 instead of histidine and leucine, respectively (Fig. 2). In addition, V, differences from 5-14, 9-40, 12-40, and 3-13 occurred at 2 residues (IleH27 and PheH33) for 10-25 and only one residue (PheH33) for 3-24. At the nucleotide level, silent base substitutions were observed for 4-4-20 ValH20 (C for T) and 12-40 GlyH27 (A for G). Between positions 96 and 107, 10-25 (LeuH31, AlaH32, and LeuH106) and 3-13 (PheH26 and SerH29) primary structures differed from the remaining antibodies (Fig. 2). This resulted from 10-25 and 13-13 V, rearrangements to J5, J4, and J4 (34), respectively. The remaining antibodies rearranged their V regions to J1 (34).

Comparisons of mAbs 5-14, 9-40, and 3-13 with known V, genes revealed identity with myeloma protein TEP105 (35). Since TEPC105 was reported to be germ-line encoded (36), 5-14, 9-40, and 3-13 rearranged the same germ-line V, gene and 4-4-20, 10-25, 12-40, and 3-24 either rearranged similar V, gene(s) or the same V, gene and somatic mutations occurred following rearrangement. Further analysis revealed, surprisingly, that primary structures of mAbs 5-14, 9-40, 12-40, and 3-13 were 96% homologous to anti-blood group B substance monoclonal antibody B003 = 46/27 (25). Differences were histidine to glutamine at position L34 in CDR1, lysine to threonine at position L50 in CDR2, valine to no residue at position L94, and proline to arginine at position L95 in CDR3, respectively. The remaining antibodies (4-4-20, 10-25, and 3-24) V, regions were 94% homologous to B003 = 46/27. It remained unclear whether B003 = 46/27 and 5-14 or 9-40, which exhibited the greatest V, and V, length.
FIG. 2. Comparison of eight anti-fluorescein mAbs V, nucleotide (top lines) and deduced amino acid (bottom lines) sequences. mAbs 4-4-20 (15) and 9-40 (38) sequences were previously described. See legend to Fig. 1 for symbol designations.
identity, shared antigen cross-reactivity or similar active site quaternary structure.

Finally, mAb 5-27 rearranged a V, V (24) or V, 10 (33) subgroup gene to J,1 (34) and shared 55% primary structure identity with the other antibodies (Fig. 2). In addition, 5-27 lacked a five-amino acid insert between residues L27 and L28 that were present in 4-4-20, 10-25, 5-14, 9-40, 12-40, 3-24, and 3-13. Comparisons to known V, primary structures showed 5-27 V, to differ from anti-p-azophenylarsonate monoclonal antibody 10K26-12A1 (37) at 4 residues (ValL21 to IleL21, LysL33 to ArgL34, TrpL36 to ArgL36, and AsnL370 to LysL370, respectively) and anti-blood group substance A monoclonal antibody AC-1001 (25) at 4 residues (ValL21 to IleL21, TyrL32 to HisL34, LysL33 to ArgL33, and AsnL370 to LysL370, respectively).

**DISCUSSION**

Concurrent 4-4-20 VH and V, primary structure determination, liganded Fab fragment crystallization (39) and x-ray diffraction data resolution to 2.7 Å (40) have elucidated 4-4-20 active site amino acid residues involved in fluorescein binding. The liganded 4-4-20 active site with proper fluorescein orientation is diagrammatically represented in Fig. 3 based on x-ray diffraction studies of the complex (40). Six amino acid residues (HisL27d, TyrL32, ArgL34, SerL31, TrpL36, and TrpL33) participated in direct interactions with fluorescein. A schematic cross-sectional view of the 4-4-20 active site (Fig. 3A) details: 1) an ion pair between ArgL34 and 6’-carbon xanthenone ring enolic group; 2) proximity of HisL27d and SerL31 to 3’-carbon and 6’-carbon enolic groups, respectively; and 3) H chain residues TyrL32, TyrL33, and TyrL34 that comprised an aromatic shell for the pocket-shaped site. Prominent aromatic interactions involved in 4-4-20 binding fluorescent are depicted in Fig. 3B. Tryptophan residues were predicted to be important in fluorescein binding (41-43) and both TrpL36 and TrpL33 directly contacted the xanthene moiety, while TrpL36 also contacted the benzoate ring. In addition, TyrL34 participated in both a stacking interaction with the xanthene ring structure and a hydrogen bond with the benzoate ring carboxyl group. Finally, 4-4-20 residues HisL27d, HisL34, and ArgL36 bordered the active site mouth surrounding the 3’-carbon enolic oxygen. Although the imidazole group of HisL27d was within hydrogen bonding distance, contributions of the remaining interactions to fluorescein binding remained unclear since all potential bonds were significantly spaced. Direct contributions to 4-4-20 antigen specificity, therefore, resided primarily with V, residues (6 of 6). This differed from anti-phosphocholine myeloma protein McPC605 (4) and anti-hen egg lysozyme antibody D1.3 (44) where the majority of antigen contact residues were V, encoded. In fact, 4 of 17 D1.3 antigen contact residues were contributed by H CDRI.

mAbs 4-4-20, 10-25, 5-14, 9-40, 12-40, and 3-24 expressed similar active site structures as determined serologically (8, 12-14) which resulted from similar V, and V, gene usage (Figs. 1 and 2). Because of this, correlation between active site structure, observed fluorescein binding, and idiotypic variations was delineated in part. Primary structure inspection revealed extensive H chain CDRI heterogeneity. mAbs 5-14, 9-40, 12-40, and 3-24, however, possessed identical fluorescein contact residues as 4-4-20 except HisL34 for ArgL34. Since 4-4-20 H chain CDRI residues contributed only indirectly to antigen binding (Fig. 3A), many amino acid permutations could be allowed in this region and still maintain active site integrity. Nevertheless, these variations probably contributed to subtle affinity (±20-fold) and Qmax differences among mAbs 5-14, 9-40, 12-40, and 3-24 (12, 14). Comparison of these antibodies to 4-4-20 indicated, therefore, that increased 4-4-20 affinity (Kd = 1.7 × 10-6 M-1) and Qmax value (90.0%) resulted from the HisL34 to ArgL34 substitution (Fig. 2). Physically, a net negative charge of enolic oxygen neutralized the delocalized net positive charge of ArgL34 and stabilized the antibody-antigen complex. However, mAbs 5-14, 9-40, 12-40, and 3-24 possessed HisL34 (predominantly neutral at pH >6.5), lacked enolic moiety neutralization and, hence, exhibited lower fluorescein affinity. Active site spatial constraints were in part alleviated by mAb 4-4-20 ArgL34 which conformed to occupy similar volume as a histidine residue. From these data, antibody active site structure and specificity were individually determined by VH and V, residues, respectively. Several primarily restricted antibody responses against aromatic antigens utilized V,1-like genes; however, each expressed different VH genes and exhibited various specificities (45-50). This can be explained by VH conformational constraints on V, that resulted in different active site structure and specificity (V, antigen contact residues). For example, anti-single-stranded DNA autoantibody 04-01 (51) and 4-4-20 rearranged different VH genes (66% identity) but similar V, genes (95% identity), yet they had large groove and pocket-shaped active sites, respectively. Likewise, although mAbs 3-13 and 4-4-20 utilized similar V,1 genes (Fig. 2), V, residue (Fig. 1) contributions resulted in different active site structures and, hence, fluorescein binding mechanisms. This was evidenced by lack of idiotypic relatedness (14) and a 2.0 × 10-7-fold affinity difference (7, 13).

Although the structural correlates and number of idiotopes expressed by an antibody molecule remain unclear, investigations into several restricted antibody responses have determined idiotopes to be dynamic and complex structures. An-
tibody responses to phosphocholine (52, 53), B1355S dextran (64, 65) and β-(t-6)-d-galactan (56) localized cross-reactive idiotype expression to consensus H CDR3 sequences. For idiotypically cross-reactive anti-fluorescein mAbs, similar V region usage and mAb 4-4-20 H CDR3 active site structural contribution and location (40 and Fig. 3A) predicted potential similar conclusions. Beside invariant Tyr-containing all mAbs (except 12-40) expressed at least one additional H CDR3 Tyr residue and implicated tyrosine residues important in anti-idiotypic recognition. Previous idiototype-anti-idiotypic inhibition studies (12, 14) revealed: 1) mAbs 10-25, 5-14, and 9-40 (>94%), 5-27 (90%), 12-40 (61%), 3-24 (45%), and 3-13 (<2%) inhibition of mAb 4-4-20 idiotype-anti-idiotypic binding; 2) mAbs 4-4-20 (92%), 10-25, 5-14, 12-40, and 3-24 (>90%), 3-13 (12%), and 5-27 (5%) inhibition of mAb 9-40 idiotype-anti-idiotypic binding; and 3) mAbs 10-25, 5-14, 5-27, 9-40, 12-40, and 3-24 (20–40%), and 4-4-20 (<2%) inhibition of 3-13 idiotype-anti-idiotypic binding. H CDR3 lacked a consensus sequence, and tyrosine residues alone were not required for xenogeneic polyclonal anti-idiotypic reagent recognition. Therefore, extensive H CDR3 primary structure variations influenced, but did not abrogate, idiotype expression and mAbs 4-4-20, 3-13, 9-40, and 9-40 family probably expressed complex active site structural, rather than sequential epitopes (idiotypes). For example, 4-4-20 and 5-27 were idiotypically cross-reactive, yet their Vh and Vk primary structures were only 61 and 54% identical, respectively. mAb 5-27, however, possessed five H CDR3 Tyr residues. Interestingly, 5-27 was recently shown to be a Type II (mixed) cryoglobulin (57).

This was the second anti-fluorescein mAb that cryoprecipitates, however, potential mechanisms or similarities to mAb 18-2-3 (57) remain unknown.

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