STRUCTURE OF IDIOTYPES ASSOCIATED WITH ANTIPHENYLARSONONATE ANTIBODIES EXPRESSING AN INTRAstrain CROSSREACTIVE IDIOTYPE

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There is ample evidence that most idiotypic determinants (idiotopes) are associated with complementarity-determining regions (CDRs) of Ig molecules. For example, some, although not all, interactions between idiotopes and anti-idiopes are blocked by the ligand of the antibody expressing the idiotope (1, 2). Idiotopes whose interactions are not blocked by ligand are sometimes referred to as framework idiotopes. However, because the interaction with ligand may not involve all of the CDR residues of the antibody, even an idiotope that is not blocked by ligand may include amino acid residues in CDRs. The high degree of individual specificity of many anti-Id antibodies argues for an essential contribution of CDRs (3–6).

There is little information on the dimensions of idiotopes. Chemical modification studies and correlations with amino acid sequence variations in families of antibodies of a given specificity have implicated particular amino acid side chains, usually in CDRs, as being part of idiotopes (7). In general, such studies demonstrate that a particular modification or amino acid substitution results in loss of reactivity with anti-idiotype. Such loss could be induced, even if the modification or substitution involved only a small part of an idiotope, such as a single amino acid. The structural change could result in a significant decrease in the attractive forces between idiotope and anti-idiotope or in steric interference. Such a modification might also induce structural alterations at a different site; i.e., the side chain involved might not be part of the idiotope affected. Despite these limitations, useful information concerning the localization of idiotopes has been obtained by careful studies of this type. In the anti-p-azobenzene arsonate (anti-Ar) system an extensive study to map idiotopes was carried out by Jeske et al. (8). Their work implicated specific residues in hypervariable regions in reactivity with each of several anti-idiotopic mAbs.

A possibility suggested by Alzari et al. (9) is that the area of contact of idiotope and anti-idiotope may in general be comparable with that observed in the interaction between an epitope of a protein antigen with a specific mAb. In two instances in which interactions with protein antigens have been analyzed by X-ray crystallog-

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† Abbreviations used in this paper: Ar, azobenzene arsonate; CDR, complementarity-determining region.
raphy (10, 11), the area of contact has ranged between 700 and 800 Å²; a similar area of contact was estimated for a third antiprotein antibody (12). These dimensions correspond roughly to 25-30% of the entire surface area of an antibody comprising the six CDRs. It was found in each study that amino acid side chains present in most or all of the CDRs make contact with the epitope of the protein antigen and that few contacts are made with framework residues.

The idiotype investigated in the present report, designated CRIₐ, is associated with a large proportion of anti-Ar antibodies induced in strain A mice by immunization with the Ar conjugate of keyhole limpet hemocyanin (13, 14). The results are consistent with the view that some idiotypes of crossreactive idiotype A (CRIₐ) occupy a large proportion of the surface of an anti-Ar antibody that comprises the CDRs. Our analysis was facilitated by the recent determination of the three-dimensional structure of an A/J anti-Ar mAb, R19.9, whose Vᵣ segment and L chain are very similar to those of CRIₐ⁺ anti-Ar antibodies (15).

Materials and Methods

mAbs. A summary of properties of mAbs used is given in Table I. The CRIₐ⁺ mAbs 6-16 and CB9 show no differences in their partial Vᵣ region nucleotide sequences from the unmutated VH36-65 germline sequence (21, 22); VH36-65 and its somatic mutants are believed to encode the Vᵣ region of all CRIₐ⁺ antibodies (22). R16.7, which is strongly CRIₐ⁺, exhibits three amino acid differences in its Vᵣ segment (positions 1-98), as compared with the sequence encoded by VH36-65 (Table I). R19.9 has a Vᵣ segment and L chain sequence similar to that encoded by VH36-65. It is however, CRIₐ⁻, probably because of the large size of its D region (11 amino acids as compared with eight in canonical CRIₐ⁺ sequences). CRIₐ⁻ mAbs have Vᵣ segments corresponding to that associated with CRIₐ; However, CRI_D differs markedly from CRIₐ in its D region (Table I) and L chain; the latter corresponds to the L chain of another idiotypic family, CRI_C (20).

Table I

| mAb⁺* | Idiotype | Vᵣ amino acid sequence | Positions of substitutions | Length of D segment | Reference |
|-------|----------|------------------------|---------------------------|---------------------|----------|
| 6-16 (IgGlₓ) | CRIₐ | 56-98 | None | 8 | 8 |
| CB9 (IgGlₓ) | CRIₐ | 50-98 | None | 8 | ** |
| R16.7 (IgGlₓ) | CRIₐ | 1-98 | 51,56,85 | 8 | 16 |
| 93G7 (IgGlₓ) | CRIₐ | 1-98 | 58,59 | 8 | 17 |
| SE20.2 (IgEe) | CRIₐ | 1-98 | 58,59 | 8 | 18 |
| R19.9 (IgG2bx) | - | 16-98 | 34,55,58,59,74 | 11 | 15 |
| A11 (IgG2bx) | CRI_D | 27-98 | None | 1 | 19 |
| SM1.5 (IgMₓ) | CRI_D | 40-98 | 63 | 1 | 19 |
| DE2 (IgMₓ) | CRI_C | 27-98 | None | 1 | 19 |
| S27 (IgGlₓ) | CRI_C | 1-98 | Not applicable | 1 | 20 |
| AD8 (Rat IgG2ax) | (anti-CRIₐ Vᵣ) | | | | |

* All mAbs are of A/J origin except AD8 (rat mAb). Amino acid sequences were deduced from mRNA sequencing.

1 Positions of amino acid substitutions as compared with the sequence encoded by the VH36-65 germline gene (21,22); the Vᵣ regions of both CRIₐ and CRI_D are encoded by this gene (19).

† Number of amino acid residues.

‡ Positions 56-98 of Vᵣ have been determined for this sample. Some of the sequences have a small number of ambiguities (maximum of five).

§ E. M. Rosen and A. Nisonoff, unpublished results.

** P. F. Robbins and A. Nisonoff, unpublished results.
A cell line producing mAb AD8 was the generous gift of Dr. Peter Hornbeck (23). AD8 reacts strongly with anti-A antibodies of A/J mice and of allotype-congenic C.AL-20 mice, but not with anti-A antibodies of several unrelated strains tested, including BALB/c and C57BL/6. AD8 reacts to a significant degree with normal A/J IgG (23) and with H chains of CRI\textsubscript{A}' antibodies on a Western blot in the absence of L chains (24). AD8 also reacts strongly with members of the CRIp family, which share V\textsubscript{s} with CRI\textsubscript{A} but differ greatly in their D and V\textsubscript{L} regions (19). Thus, AD8 appears to be V\textsubscript{s}-specific.

Separation and Recombination of H and L Chains. Nonspecific IgG or an IgG mAb was reduced with 0.01 M dithiothreitol (Calbiochem-Behring Corp., La Jolla, CA) for 1 h at room temperature in Tris buffer, pH 8.0 (25). Thiol groups were then blocked with 0.06 M iodoacetamide (Sigma Chemical Co., St. Louis, MO), recrystallized from hot water. After dialysis against neutral Tris buffer, guanidine hydrochloride was added to the reduced and alkylated protein to a concentration of 7 M, pH 7.4 (26). The mixture, containing 10–20 mg of protein, was immediately passed over a 150-cm\textsuperscript{3} column of Sephacryl S-200 equilibrated with 5 M guanidine hydrochloride, pH 6.5, to separate H and L chains. The use of Sephacryl rather than Sephadex substantially reduces the time required for gel filtration (to 3–4 h). In each case two separate peaks were obtained. Recombinants were prepared immediately after gel filtration, using an approximately equimolar ratio of H and L chains. The mixture was then dialyzed extensively against borate-saline buffer, pH 8. In several instances the product was concentrated, passed over a column of Sephacryl S-300 in neutral buffer, and the protein in the major peak collected. In each case >80% of the protein was present in a single peak, which corresponded in its elution volume to that of intact IgG. When autologous chains derived from a given CRI\textsubscript{A}' antibody were recombined, the idiotypic activity recovered was, in each case, close to that of the intact molecule (see Results).

Assay for CRI\textsubscript{A} Activity. This assay was carried out as previously described (19), using 10 ng of [\textsuperscript{125}I]-labeled R16.7 as the ligand and sufficient rabbit anti-CRI\textsubscript{A} to bind ~60% of the labeled ligand. To provide carrier protein, the mixture also contained 5 \mu l of rabbit anti-OVA antiserum and 7 \mu l of normal AKR ascitic fluid. Complexes were precipitated with an excess of goat anti-rabbit IgG that had been adsorbed with mouse Ig-Sepharose. The idiotypic in unlabeled test samples was quantified by determining the amount required to cause 50% inhibition of binding of the labeled R16.7. Variations of this assay were carried out with other \textsuperscript{125}I-labeled ligands (mAb CB9, A11, or H\textsubscript{CB9L1}), and with a different anti-Id preparation (rabbit anti-6-16). The subscript N refers to nonspecific IgG.

Direct binding assays were carried out in a similar manner using varying amounts of anti-Id and a constant weight of labeled ligand; complexes were precipitated as above. In some instances direct binding assays were used to ascertain the total idiotype-binding capacity of an anti-Id preparation for a given ligand. Such assays were run with a constant level of anti-Id and increasing amounts of \textsuperscript{125}I-labeled ligand, and the plateau level of ligand binding was determined. When mAb AD8 (rat IgG2ax) was used as anti-Id reagent, immune complexes were precipitated with mouse antirat IgG. The mice used to prepare the antirat IgG were (BALB/c \times C57BL/6)\textsuperscript{F1}. A/J mice were not used because they express the AD8 idiotope in their normal Ig (23).

Rabbit Anti-Id Antibodies. Anti-Id was prepared against affinity-purified A/J anti-A antibodies by two inoculations of 500 \mu g in CFA followed by monthly inoculations of 500 \mu g in IFA. Rabbits were bled repeatedly starting 1 wk after the third inoculation. Pooled antiserum was adsorbed exhaustively with normal A/J Ig conjugated to Sepharose 4B. Rabbit anti-Id against the anti-A mAb 6-16 (CRI\textsubscript{A}*) was prepared similarly; however, the antiserum was adsorbed with normal AKR Ig-Sepharose and S27-Sepharose. The AKR strain differs in H chain allotype from A/J and also appears to be deficient in the L chain required for CRI\textsubscript{A} expression (27). mAb S27 is CRI\textsubscript{c}'; its V\textsubscript{s} and V\textsubscript{L} regions therefore differ markedly from those of CRI\textsubscript{A}\textsuperscript{*} antibodies (20). The purpose of using these adsorbents for anti-6-16 was to leave unadsorbed any anti-Id antibodies specific for the V\textsubscript{s} or V\textsubscript{L} regions.

Results

Idiotypic Activity of Hybrid Molecules Containing H\textsubscript{CRIA} and L Chains from Different Sources. Table II presents data on inhibitory capacities of intact molecules, or mole-
TABLE II

Inhibitory Capacities of Intact and Recombined Molecules in the Assay for CRIA

| Inhibitor        | Amount required for 50% inhibition | Inhibitor       | Amount required for 50% inhibition |
|------------------|------------------------------------|-----------------|-----------------------------------|
| A/J IgG          | >3,000 (6)*                         | HCB9LN          | 250                               |
| R16.7 (CRIA)     | 13                                 | HCB9-LB9        | 17                                |
| 6-16 (CRIA)      | 12                                 | HCB9-L42.9      | 19                                |
| CB9 (CRIA)       | 14                                 | HCB9-LA11       | 570                               |
| R19.9            | >3,000 (11)                         | HCB9-LS27       | 620                               |
| A11 (CRI\(_D\)) | >3,000 (17)                         | HA11-LN         | >3,000 (23)                       |
| S27 (CRI\(_C\)) | >3,000 (8)                          | HA11-LCB9       | 1,250                             |
| HNL\(_N\)\(^2\) | >3,000 (6)                          | HA11-L19.9      | >3,000 (23)                       |
| HNL-CB9          | 2,050                              | HS27-LN         | >3,000 (4)                        |
| HNL19.9          | >3,000 (9)                          | HS27-LCB9       | 1,850                             |
| HNL\(_A11\)      | >3,000 (7)                          | H19.9LN         | >3,000 (2)                        |
| HNL\(_S27\)      | >3,000 (3)                          | H19.9-LCB9      | 630                               |
| HNL19.9\(_L19.9\) | >3,000 (8)                         | H19.9-L19.9     | >3,000 (2)                        |

Assays used rabbit anti-CRIA antibodies with 10ng of unlabeled R16.7 (CRIA\(^*\)) as ligand.
* Numbers in parentheses indicate percent inhibition by the weight of inhibitor specified.
\(^2\) Recombined molecules. The subscript \(N\) refers to nonspecific A/J IgG. Other subscripts refer to the mAb that provided the H or L chain.

molecules containing recombined H and L chains, in the assay for CRIA using labeled R16.7 as ligand. 12-14 ng of unlabeled CRIA\(^*\) mAb (R16.7, 6-16, or CB9) caused 50% inhibition in the assay. For the autologous CRIA\(^*\) recombinant molecule, HCB9-LCB9, 17 ng was required; i.e., its idiotypic activity was nearly as great as that of intact CB9 (14 ng required for 50% inhibition). For hybrid molecules containing H chains from a CRIA\(^*\) mAb (CB9) and nonspecific L chains (LN), 250 ng was required for 50% inhibition. This could be accounted for by a low affinity of HCB9LN or by ~7% contamination (17/250) of HCB9 by LCB9. The low inhibitory capacities of HCB9-LA11 or HCB9-LS27 suggest that the actual contamination was 3% or less (mAb A11 and S27 are CRI\(_D\) and CRI\(_C\), respectively). L chains from R19.9 were about as effective as L chains from CB9 in restoring idiotypic activity to HCB9. This is consistent with previous data showing that R19.9, which is CRIA\(^-\), expresses L chains that are idiotypically very similar to L chains of CRIA (28), and are nearly identical in sequence to that encoded by the \(V_{\kappa10}\) germ line gene, which encodes the L chains of CRIA\(^*\) molecules (15, 29).

The high recovery of activity in the homologous recombinant, HCB9-LCB9, indicates that the method used for separating and recombining H and L chains did not disrupt idiotypic determinants. We observed a similarly high retention of idiotypic activity when H and L chains of the CRI\(_D\)\(^*\) mAb A11 or of the CRI\(_C\)\(^*\) mAb S27 were separated and recombined (data not shown).

Absence of Anti-idiotypic Antibodies with Significant Binding Affinity for CRIA-derived L Chains. The inhibition data in Table II demonstrate that molecules containing CRIA-derived L chains but H chains from other sources are very weak inhibitors in the assay for CRIA; these molecules are HNL-CB9, HA11-LCB9, and HS27-LCB9. In
addition, intact R19.9, which has CRIA-related L chains (see above), is an extremely poor inhibitor; 3,000 ng caused 11% inhibition. That the absence of activity in L chains is not due to denaturation during the recombination experiments is supported by the poor inhibitory activity of intact R19.9, and by the almost complete recovery of idioype activity in HCB9L<sub>CB9</sub>.

The requirement for appropriate L chains to provide good inhibition in the assay for CRIA is in agreement with previous studies (e.g., reference 5). This does not, however, exclude the possibility that some anti-Id antibodies react well with L chains alone. If, for example, 10% of the anti-Id population were specific for L chains alone, a maximum of 10% inhibition in the CRIA assay could be achieved by molecules containing appropriate L chains but inappropriate H chains.

To explore the possibility of direct binding of L chains, we measured the binding of <sup>125</sup>I-labeled molecules containing L<sub>CRIA</sub> by a large excess of anti-Id. The data are presented in Figs. 1 and 2. It is evident that there was virtually no detectable binding of labeled H<sub>N-L<sub>CB9</sub></sub>, even by a large excess of rabbit anti-Id antibodies directed either against affinity-purified A/J anti-Ar serum antibodies (anti-CRIA) or against the strongly CRIA<sup>+</sup> mAb 6-16. In addition, the binding capacity of each anti-Id reagent for R19.9, which has L chains characteristic of CRIA, was extremely low. The ratios of amounts of antiserum required for 50% binding of labeled R19.9 or CB9 (CRIA<sup>+</sup>) were 70:1 and 210:1, respectively, for anti-CRIA and anti-6-16(Id). These
ratios are minimal as an indication of lack of specificity for L chains alone because the V_{H} region of R19.9 is structurally related to that of CRIA^{+} antibodies (15); i.e., some of the slight binding of R19.9 may be attributable to V_{H}. It is evident that a negligible proportion of anti-Id antibodies has appreciable affinity for L chains. Nevertheless, as indicated by the above data on inhibition by recombined molecules (e.g., H_{CB9L} vs. H_{CB9L-CB9}), L chains contribute greatly to the formation of most idiotypic determinants.

Anti-Id Antibodies with Specificity for H Chains. For the reasons indicated in discussing L chains, it is difficult, from the data on inhibition, to assess the proportion of anti-Id antibodies having specificity for H chains; i.e., that react effectively with H chains in the absence of appropriate L chains. As suggested above, the interpretation of data on inhibition would be complicated by any contamination of H_{CRIA} with L_{CRIA}; such contamination would result in strong inhibition when large amounts were tested. The problem of, say, 10\% contamination is much less serious when direct binding of anti-Id to a labeled H_{CRIA}-containing preparation is measured; a maximum of 10\% could be bound. We therefore carried out direct binding measurements on labeled HL recombinant molecules.

The relevant data are shown in Figs. 1 and 2, which present results obtained with anti-CRIA, and anti-6-16(Id), respectively. Each curve was obtained by using an $^{125}$I-labeled ligand and increasing amounts of anti-Id. It is evident, first, that high percentages of recombinants containing H_{CB9} and inappropriate L chains (H_{CB9L} or H_{CB9L-S27}) were bound when sufficiently high concentrations of either anti-Id antibody were used. These high percentages cannot be explained by a small degree of contamination of H_{CB9} with residual L_{CB9}. An approximation of the proportion of anti-H_{CB9} in the anti-Id antibodies can be made by comparing the amounts of anti-Id required to bind 50\% of the maximal levels of H_{CB9L} or H_{CB9L-S27} with the amounts required to bind 50\% of the maximal level of the CRIA^{+} mAb, CB9. For anti-CRIA (Fig. 1) the ratios are 2.7:1 and 4.1:1 for H_{CB9L} and H_{CB9L-S27}, respectively. For anti-6-16(Id) the corresponding ratios are 5.7:1 and 6.7:1. These ratios will of course be affected by the affinities of the interactions. However, the actual proportion of anti-Id reactive with H chain alone probably equals or exceeds that indicated by the direct binding data, because one would not expect to elicit many antibodies with higher affinity for H chain than for the intact molecule by immunizing with the intact molecule. It is evident then that, in contrast to L chains, a significant fraction (~15-37\%) of anti-Id antibodies are reactive with H chains. Nevertheless, these data on direct binding, as well as the inhibition data in Table II, concur in indicating that the majority of anti-Id antibodies interact most effectively with molecules containing both chains.

Evidence for the Presence of Anti-Id Antibody whose Interaction with H Chains Is not Enhanced by L Chains. Despite the effective interaction of anti-Id with H chains (recombined with inappropriate L chains), the possibility remains that even those antibody molecules that react with H chains react better with the intact molecule. Experiments to address this question were carried out as follows. We labeled H_{CB9L} with $^{125}$I and added sufficient anti-Id (anti-CRIA or anti-6-16) to bind 50-60\% of the labeled ligand. Complexes were precipitated with a second antibody (see Materials and Methods). Various unlabeled molecules were tested as inhibitors of the primary interaction. The data in Fig. 3, A and B indicate that, with either anti-Id, H_{CB9L}
or H\textsubscript{CB9}L\textsubscript{S27} were about as effective as inhibitors as the intact CRI\textsubscript{A} mAbs, CB9 or 6-16. This indicates that most anti-Id molecules that do bind H\textsubscript{CB9}L\textsubscript{N} do so with an affinity comparable with that with which they interact with the intact idiotype molecule. If the anti-Id molecules involved in this interaction had relatively low affinity for H\textsubscript{CB9}L\textsubscript{N} or H\textsubscript{CB9}L\textsubscript{S27}, the unlabeled hybrid molecules would have been poor inhibitors as compared with CB9 or 6-16. Since 50–60\% of the labeled ligand was bound in the absence of inhibitor, a few-percent contamination of H\textsubscript{CB9} by L\textsubscript{CB9} could not have influenced the results significantly.

**Saturation Binding Experiments to Determine Reactivity of Anti-Id with H Chain.** The experiments of Fig. 4 represent an alternative approach to the percentage of anti-CRI\textsubscript{A} antibodies that have specificity for the H chain alone. Those direct binding
experiments utilized a constant amount of anti-6-16(Id) and increasing amounts of 125I-labeled idiotype. The data in Fig. 4 indicate that the binding capacity of anti-6-16(Id) for HCB9LN is 32 or 35% as great as its binding capacity for intact 6-16 or CB9. The corresponding percentages are 19 and 21% for HCB9Ls27. 6-16 and CB9 are CRI_{A,*}; S27 is CRI_{C,*} and has L chains unrelated to those of CRI_{A,*} antibodies. Thus, in good agreement with the data obtained with a constant amount of ligand, ~20-35% of the anti-Id antibodies are reactive with H_{CRI_{A}} combined with "inappropriate" L chains.

*Anti-Id Antibodies Specific for the V_{H} Segment.* The experiments described above indicate the presence in anti-CRI_{A} or anti-6-16(Id) of antibodies reactive with H_{CRI_{A}}; the latter of course includes V_{H}, D, and J_{H} segments. We tried to ascertain whether the V_{H} segment alone interacts effectively with some of the anti-Id molecules prepared against intact CRI_{A,*} antibody. Because it is difficult to isolate V_{H} segments, we used an indirect approach that takes advantage of the CRI_{D} class of A/J anti-Ar antibodies. Anti-Ar mAbs expressing CRI_{D} contain a germline-encoded V_{H} segment apparently identical in amino acid sequence to that of CRI_{A}, but with a much shorter D region, comprising one amino acid residue as compared with eight residues in CRI_{A} (19). The V_{L} segment in CRI_{D} appears identical to that of another idiotypic family of anti-Ar antibodies (CRI_{C}) and differs markedly from the V_{L} segments of CRI_{A} (19, 20). Our approach, then, was to ascertain the extent of reactivity of anti-CRI_{A} with CRI_{D,*} mAbs on the assumption that such crossreactivity would be directed largely to the V_{H} segment.

The validity of this approach depends on the idiotypic similarity of the V_{H} segment when present in a CRI_{A,*} or CRI_{A,*} molecule. Despite identity of amino acid sequences, it is of course possible that the nonidentity of surrounding regions, including D and V_{L}, could alter the interaction of V_{H} with anti-Id reagents. Data supporting the idiotypic similarity will be described later.

*Direct Binding of A11 by Anti-CRI_{A} or Anti-6-16(Id).* Figs. 1 and 2 show that the CRI_{D,*} mAb A11 is bound by anti-CRI_{A} or by anti-6-16(Id) but that the interaction is much weaker than that for intact CRI_{A,*} mAb. For example, the amount of anti-6-16(Id) required to bind 50% of labeled mAb A11 was ~25 times as great as that required for mAb CB9. The interaction with A11 is also weaker than that with hybrid molecules containing CRI_{A}-derived H chains and inappropriate L chains (H_{CBI,LN} or H_{CBI,Ls27}). Small amounts of anti-Id bind about two to three times more H_{CBI,Ls27} or H_{CBI,LN} than A11. When antibodies with specificity for H_{CBI,LN} were examined (by using labeled H_{CBI,LN} as ligand), a similarly weak direct interaction with A11 was observed (Fig. 3, A and B).

That A11 interacts with a smaller proportion of anti-6-16(Id) than CRI_{A,*} mAbs or H_{CRI_{A}}-containing hybrids is also shown by the saturation binding experiments of Fig. 4. The maximum degree of binding of A11 was 15-16% of the values for 6-16 or CB9 and lower than that of either H_{CRI_{A}}-containing hybrid molecule.

*Idiotype Similarity of V_{H} Present in CRI_{A} and CRI_{D}.* As already mentioned, the anti-Id mAb AD8 reacts with V_{H}DJ_{H}, and the reaction is independent of the L chain. The available data do not bear on the contribution of the D segment to reactivity with AD8. The following experiments support the reactivity of AD8 with V_{H} alone and suggest that this interaction is not influenced by the D region. They also indicate that CRI_{A,*} and CRI_{D,*} mAbs interact equally well with AD8.
Fig. 5 shows results of direct binding experiments using various labeled ligands and increasing amounts of AD8. The binding curves for CRI\textsubscript{A} mAbs 6-16 and CB9 are very similar. As indicated in Table I, the V\textsubscript{H} segments of 6-16 and CB9 are identical to the germline VH36-65 sequence in positions sequenced so far. Of particular interest is the observation that A11, SM1.5, and DE2 (all CRID\textsuperscript{*}) react as well or better with AD8 than 6-16 and CB9 (both CRI\textsubscript{A}), despite the large difference in length of their D regions; in addition, AD8 was prepared against CRI\textsubscript{A} antibodies. The fact that CRID\textsuperscript{*} mAbs bind as well as CRI\textsubscript{A} mAbs to AD8 indicates that the V\textsubscript{H} segment is responsible for the binding and that any contributions of the D region and L chain are negligible. Thus, CRI\textsubscript{A} and CRID\textsuperscript{*} mAbs express the AD8-reactive idiotope to a very similar degree.

The binding of AD8 to DE2 and SM1.5 (both CRID\textsuperscript{*}) appears to be somewhat stronger than that to the CRID\textsuperscript{*} mAb, A11 (as well as to the CRI\textsubscript{A} mAbs 6-16 or CB9). This may reflect the fact that DE2 and SM1.5 are IgM whereas A11 is IgG2b. The polymeric structure of IgM may favor bivalent attachment of AD8 with a consequent increase in avidity.

The recombinant molecule, HCB9L\textsubscript{27}, interacted strongly with AD8, providing further evidence for the predominant role of the H chain in the interaction. mAbs SE20.2 and R16.7, both of which are strongly CRI\textsubscript{A}, were bound by AD8, but relatively high concentrations of AD8 were required. The lower affinity may be correlated with amino acid substitutions in V\textsubscript{H} (see Discussion).

Specificity of AD8 as Assessed by Inhibition Measurements. Fig. 6A shows data on inhibition of binding of the \textsuperscript{125}I-labeled CRI\textsubscript{A} mAb, CB9, to AD8. Unlabeled CRI\textsubscript{A} mAbs CB9 and 6-16, the hybrid molecule HCB9L\textsubscript{27}, and A11 (CRID\textsuperscript{*}) yielded very similar inhibition curves. These data support the direct binding measurements in indicating that the specificity of AD8 is directed to V\textsubscript{H} and not to the D region or L chain, and that the V\textsubscript{H} segments in CRI\textsubscript{A} and CRID react to a similar degree with AD8.

Although mAbs SE20.2, 93G7, and R16.7 (all CRI\textsubscript{A}) are inhibitors of the interactions, the amounts required are much greater than those of 6-16 and CB9. This is in agreement with the direct measurements of binding to AD8 made with SE20.2 and R16.7 and may be attributable to somatic mutations in CDR2 (see Discussion).

The inhibition data in Fig. 6B were obtained with labeled mAb A11 (CRID\textsuperscript{*})
as ligand in place of labeled CB9 (CRI\(_A^\ast\)). The very close similarity of the results in Fig. 6, A and B are again consistent with the view that AD8 recognizes only V\(_n\), since A11 and CB9 are similar only within this segment.

**Anti-CRI\(_A\) Contains Antibodies that React Equally Well with CRI\(_A\) and CRI\(_D\).** The data in Figs. 1, 2, and 4 indicate that only a small proportion of rabbit anti-6-16(Id) reacts with A11 (CRI\(_D\)), despite the shared V\(_n\) segment. Inhibition data in Fig. 7 were obtained by using rabbit anti-6-16(Id) with \(^{125}\)I-labeled A11 as ligand. The data show that A11 (CRI\(_D\)), CB9 (CRI\(_A\)), and 6-16 (CRI\(_A\)) reacted equally well with that small subpopulation of anti-6-16(Id) that binds A11.

To summarize, results obtained with the CRI\(_D^\ast\) m\(\text{Ab}\) A11 indicate that only a small proportion of anti-CRI\(_A\) (\(\sim 15\%\); Fig. 4) reacts with V\(_n\) in the absence of the four other CDRs associated with CRI\(_A\). However, the V\(_n\) of regions of CRI\(_A^\ast\) and
GRID mAbs interact virtually identically with mAb AD8 and with that small proportion of anti-CRIA antibodies that recognize $V_{\mu}$ alone.

Discussion

The data reported here are consistent with the view (9) that some individual idiotopes may comprise a large segment of that surface area of an antibody molecule that comprises the six CDRs. X-ray crystallographic studies have shown that the total surface of this region generally comprises $\sim 2,000 \, \text{Å}^2$ and that the interaction with a single epitope of lysozyme or neuraminidase may involve direct contacts over a substantial part of the surface ($600-700 \, \text{Å}^2$) and involve all or nearly all of the CDRs of the antibody. Our results indicate that most idiotopes include amino acid residues derived from a minimum of two CDRs.

Role of L Chains. The data indicate that anti-Id antibodies prepared against a CRI* + antibody contain a very small proportion of molecules with specificity for L chains alone. This was shown by the failure of anti-Id to bind hybrid molecules containing LCRI and HCRI + from other sources (Figs. 1 and 2). In addition, such hybrid molecules were extremely poor inhibitors of the binding of CRI* + antibodies to anti-CRI (Table II). Also, mAb R19.9, whose L chains effectively reconstitute CRI when combined with HCRI, was an extremely poor inhibitor of the binding interactions of anti-CRI. The poor inhibition by the hybrid molecules containing appropriate L chains is consistent with results obtained in other idiotypic systems (5, 30-32). The direct binding measurements appear more relevant to this question because hybrid molecules would be weak inhibitors even if, say, 10–20% of the anti-Id were directed exclusively to the L chain. The maximum degree of inhibition would be 10–20% under such circumstances; nevertheless, polyclonal anti-Id specific for L chain alone could still bind a very large proportion of labeled hybrid molecules when sufficient anti-Id was tested in a direct binding assay. However, no such binding was detected.

Despite the apparent absence of anti-Id activity directed to L chains alone, the L chains play an important role in the formation of a large proportion of the idiotypic determinants. This is shown by the much greater inhibitory capacity in idiotypic assays of recombined molecules HCB9-LCB9, as compared with HCB9-LN, HCB9-LS27, or HCB9-Lsl1. The observations on direct binding and inhibition can be reconciled if one considers the relationship of the free energy of binding to the equilibrium constant for the association with anti-Id. If the L chain contributed, say, 4 kcal/mol to the energy of interaction, this would correspond to a contribution of $\sim 10^3 \, \text{M}^{-1}$ to the association constant ($K_a$) value; this would account for the very poor competition by H chains in the absence of the appropriate L chain. In addition, the $K_s$ of anti-Id for the L chain would be $\sim 10^3 \, \text{M}^{-1}$, a value too low to permit effective direct binding by concentrations of antibody available for testing.

The observation that most anti-Id antibodies require both the H and L chain for effective interaction demonstrates that at least two CDR regions, and possibly more, are required for effective binding.

Interaction of Anti-Id with H Chains. The data in Figs. 1, 2, and 4 show that some of the anti-Id antibodies are reactive with H chains in the presence of inappropriate L chains (e.g., HCB9-LN). From the saturation binding curves (Fig. 4), using increasing amounts of labeled ligand and a constant level of anti-Id, we found that
~20–35% of the anti-Id is reactive with such molecules. These percentages cannot be accounted for on the basis of contamination of H\textsubscript{CRI\textalpha} with L\textsubscript{CRI\textalpha}. The very low inhibitory capacities of H\textsubscript{CB9L\textalpha1} and H\textsubscript{CB9L\textalpha2} in the assay for CRI\textalpha (Table II) indicated that the degree of contamination of H\textsubscript{CRI\textalpha} with L\textsubscript{CRI\textalpha} was 3% or less.

The data on direct binding, which show that ~70–80% of the anti-Id requires both H\textsubscript{CRI\textalpha} and L\textsubscript{CRI\textalpha} for effective interaction, are consistent with inhibition data in indicating that most anti-Id antibodies interact with CDRs from V\textsubscript{L} and V\textsubscript{H}.

Proportion of Anti-Id Reactive with the V\textsubscript{H} Segment. The results indicate that only a small proportion of anti-CRI\textalpha reacts with the V\textsubscript{H} segment (residues 1–98) in the absence of the appropriate D and V\textsubscript{L} segments. Direct binding experiments (Figs. 1 and 2) show that a given amount of anti-Id binds ~10% as much mAb A11 as intact mAb CB9 (CRI\textalpha). A11 is a member of the CRI\textalpha\textdagger family, which shares the V\textsubscript{H} segment but not the D or V\textsubscript{L} segments with CRI\textalpha (19). Data indicating that the V\textsubscript{H} segments of CRI\textalpha and CRI\textalpha\textdagger are idiotypically very similar are discussed below. The binding of A11 to anti-CRI\textalpha was also considerably weaker than the binding of the H chain (H\textsubscript{CB9L\textalpha N} or H\textsubscript{CB9L\textalpha 27}; Fig. 1). The relatively poor interaction of V\textsubscript{H}, as compared with the entire H chain, was also demonstrated by the inhibition data in Fig. 3, A and B; mAb A11 was a poor inhibitor of the binding of labeled H\textsubscript{CB9L\textalpha N} to anti-CRI\textalpha (mAb CB9 is strongly CRI\textalpha\textdagger). These results indicate that the most effective binding to the H chain requires the D region in addition to the V\textsubscript{H} segment; the D region comprises most if not all of the solvent-exposed part of CDR3. The weak but significant interaction of the V\textsubscript{H} segment with anti-CRI\textalpha is further evidence for a requirement for multiple CDRs for effective interaction with anti-CRI\textalpha.

The fact that mAb AD8 appears to recognize the V\textsubscript{H} segment alone (as shown by its reactivity with H chains on Western blots [24] and its reactivity with CRI\textalpha\textdagger mAb) is not necessarily in conflict with the possibility that there is a large area of contact between Id and anti-Id. AD8 may interact with regions of the molecule outside CDRs in addition to one or two CDRs in the V\textsubscript{H} segment. This would not conflict with our observation that CRI\textalpha and CRI\textalpha\textdagger mAbs react almost identically with AD8, since the two families share V\textsubscript{H} framework regions as well as CDRs. In addition, it is not ruled out that AD8 reacts with some framework residues of the L chain that might be common to CRI\textalpha and CRI\textalpha\textdagger.

The data indicate that nearly all anti-CRI\textalpha antibodies react with portions of both V\textsubscript{H} and V\textsubscript{L} or with at least two CDR regions of the H chain. The areas of interaction thus defined are minimal; antibodies reactive with H and L could interact with several CDRs, and antibodies reactive with H chains might interact with all three CDRs of the V\textsubscript{H} region. In addition, none of our data exclude additional interactions with non-CDR (framework) regions of idiotype-bearing molecules. As long as the hypervariable regions play a significant role in the interaction, additional interactions with framework regions would not necessarily compromise the idiotypic specificity of an anti-antibody. Our results are consistent with the possibility that the interaction of an idiotope with anti-idiotope may take place over an area of contact comparable with that observed for those epitopes on protein antigens that have been described by X-ray crystallography (9–12). This question is being approached by X-ray crystallographic analysis of Id-anti-Id complexes.

Evidence for Retention of Structure of CDR Loops in Different Environments. It is haz-
arduous to generalize concerning constancy of the three-dimensional structure associated with a given CDR sequence in different environments. This question is of considerable interest, however, to those who try to predict the three-dimensional structure of the combining site of an antibody from amino acid sequence data (e.g., 33–39). The ability to make such predictions accurately could greatly increase our understanding of the relationship of antibody structure to specificity, because of the availability of many sequences and few crystallographic models.

Of interest in this regard is our observation that mAb AD8, which interacts with the V\textsubscript{H} segment, appears to react almost identically with unmutated CRI\textsubscript{A}\textsuperscript{*} and CRI\textsubscript{D}\textsuperscript{*} mAb, despite major differences in the D region (eight vs. one amino acid residue) and L chain (19). This observation suggests that the three-dimensional structure of CDR2 and/or CDR1 in V\textsubscript{H} is not markedly perturbed by changes in the other four CDRs of the molecule. This conclusion is in agreement with the fact that V\textsubscript{H} CDR1 and CDR2 do not make close contacts with CDRs from V\textsubscript{L} and make few contacts with V\textsubscript{H} CDR3 (15).

Another example of constancy of Id expression in a variety of environments are idiotypes present on subgroup of V\textsubscript{L}\textsubscript{21} L chains, which can be identified, when present in a variety of Ig molecules, by an anti-Id antiserum (38).

Relative Roles of H and L Chains in Forming Idiotopes. As already discussed, we found that a fraction of anti-CRI\textsubscript{A} antibodies can bind H chains of the idotype in the absence of appropriate L chains, but were unable to demonstrate direct binding to L chains in the absence of CRI\textsubscript{A}-derived H chains. A possible explanation comes from the X-ray crystallographic study of the CRI\textsubscript{A}-related mAb R19.9. An analysis of the crystallographic model shows that the CDRs of the H chain offer a larger area of contact than L chains in and around the central region of the CDR cluster. In addition, somatic recombination mechanisms produce larger sequence variations in H than in L chains, particularly in the D segment and its insertion points. A high degree of variability would be expected to correlate with increased expression of idiotypic determinants.

Residues in CDRs Exposed to Anti-Id. Examination of the three-dimensional structure of R19.9 (15) indicates that the following residues in CDRs are exposed and capable of interacting with an anti-Id antibody: V\textsubscript{H} CDR1, 31 and 32; CDR2, 55–62; CDR3, 100–107; V\textsubscript{L} CDR1, 25–32; CDR2, 50–56; CDR3, 91–95. It is noteworthy that each of the CRI\textsubscript{A}\textsuperscript{*} mAbs we have studied that react poorly with AD8 (which recognizes V\textsubscript{H}), and for which sequences are available, have one or more substitutions from the germline-encoded mAb 36–65 in the V\textsubscript{H} 55–62 region (Table I). These mAbs include R16.7, 93G7, and SE20.2, none of which has substitutions in CDR1. R19.9 (CRI\textsubscript{A} related) has three substitutions in CDR2 and one in CDR1 and does not react with AD8. Thus, loss of reactivity with AD8 correlates with substitutions in solvent-exposed areas. The data suggest strongly that AD8 interacts directly with CDR2. The unavailability of mAbs with substitutions in CDR1 but not in CDR2 prevents conclusions concerning direct contact of AD8 with CDR1.

Additional Idiotypically Relevant Features of the Three-Dimensional Structure of the CDRs of R19.9. As indicated, R19.9 has a V\textsubscript{H} region and L chain related to those of CRI\textsubscript{A} but its D region is three residues longer (11 amino acids vs. 8). Some structural features of R19.9 CDRs, derived from X-ray analysis, are the following (15). The only CDR of the L chain that interacts with an H chain CDR is CDR3, which
contacts Vₜ, CDR3. A contact is defined here by a distance of 4 Å or less. Gln89 of Vₛ, CDR3 makes contacts with Vₜ, Tyr109. Contacts between the CDR3s are important for the assembly and interaction of Vₜ and Vₜ, regions (39, 40), and one might predict that this region would often be part of an idiotope. Vₜ, CDR2 is exposed the least of all CDRs at the combining site region and might be expected to contribute little to the idiotypic structure. The combining site, a solvent-accessible area of R19.9, is similar to that of other antibodies that have been studied and can be estimated to be ≥2,000 Å². This large area offers many possibilities for Id-anti-Id interactions, which by analogy with the determined area of antigen-antibody contacts might involve an area of ~700–800 Å². All of the serological data on CRIₐ⁺ presented here are consistent with this possibility.

Summary

We have explored the structural basis of idiotopes associated with the major idiotype (CRIₐ) of A/J anti-α-azobenzenearsonate antibodies, with emphasis on the regions of contact with anti-idiotypic antibody. The analysis was facilitated by a recent description of the three-dimensional structure of the Fab portion of a CRIₐ-related antibody molecule. Direct binding measurements failed to reveal idiotopes associated exclusively with the L chain. However, the L chain participated in the formation of ~80% of the idiotopes recognized by polyclonal anti-Id. This indicates that multiple complementarity-determining regions (CDRs) participate in the formation of idiotopes. The affinity of anti-Id for CDRs on L chains must be appreciable but insufficient to permit direct binding (i.e., less than ~10⁴ M⁻¹).

Approximately 20–35% of polyclonal anti-Id reacted with high affinity with H chains recombined with non-CRIₐ-related L chains. This interaction was found to involve the D region as well as one or both CDRs in the Vₜ segment, again indicating the contribution of multiple CDRs. It is suggested that a typical idiotope may be similar in size to that of protein epitopes whose three-dimensional structures are known; such epitopes comprise a substantial fraction of the surface area occupied by the CDRs of an antibody.

The expression of an idiotope recognized by the mAb AD8, which interacts with the Vₜ segment, was found to be unaffected by major changes in the neighboring D and VL regions. This observation is relevant to efforts to predict three-dimensional structure from the amino acid sequence of CRIₐ⁺ molecules.

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Note Added in Proof: A recently completed study indicates that an anti-Id mAb and its ligand, an mAb to hen egg lysozyme, make contact over a large proportion of the area of the CDRs of the anti-Id antibody (Bentley, G. A., G. Boulot, R. J. Poljak, and M. M. Riottot, manuscript in preparation).

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