STRUCTURE OF IDIOTOPES ASSOCIATED WITH ANTIPHENYLARSONATE 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)[1] of Ig molecules. For example, some, although not all, interactions between idiotopes and anti-idiotopes 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*-azobenzenearsonate (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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[1] *Abbreviations used in this paper:* Ar, azobenzenearsonate; CDR, complementarity-determining region.

raphy (10, 11), the area of contact has ranged between 700 and 800 $Å^2$; 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 CRI_A, 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 idiotopes of crossreactive idiotype A (CRI_A) 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 V_H segment and L chain are very similar to those of CRI_A^+ anti-Ar antibodies (15).

Materials and Methods

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

Properties of mAbs Used

mAb*	Idiotype	V_H amino acid sequence	Positions of substitutions‡	Length of D segment§	Reference
6-16 (IgG1κ)	CRI_A	56-98‖	None	8	¶
CB9 (IgG1κ)	CRI_A	50-98	None	8	**
R16.7 (IgG1κ)	CRI_A	1-98	51,56,85	8	16
93G7 (IgG1κ)	CRI_A	1-98	58,59	8	17
SE20.2 (IgEκ)	CRI_A	1-98	58,59	8	18
R19.9 (IgG2bκ)	-	16-98	34,55,58,59,74	11	15
A11 (IgG2bκ)	CRI_D	27-98	None	1	19
SM1.5 (IgMκ)	CRI_D	40-98	63	1	19
DE2 (IgMκ)	CRI_D	27-98	None	1	19
S27 (IgG1κ)	CRI_C	1-98	Not applicable	1	20
AD8 (Rat IgG2aκ)	(anti-CRI_A V_H)				

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

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

§ Number of amino acid residues.

‖ Positions 56–98 of V_H 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-Ar antibodies of A/J mice and of allotype-congenic C.AL-20 mice, but not with anti-Ar 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_A^+ antibodies on a Western blot in the absence of L chains (24). AD8 also reacts strongly with members of the CRI_D family, which share V_H with CRI_A but differ greatly in their D and V_L regions (19). Thus, AD8 appears to be V_H-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^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_A^+ antibody were recombined, the idiotypic activity recovered was, in each case, close to that of the intact molecule (see Results).

Assay for CRI_A Activity. This assay was carried out as previously described (19), using 10 ng of ^{125}I-labeled R16.7 as the ligand and sufficient rabbit anti-CRI_A to bind ~60% of the labeled ligand. To provide carrier protein, the mixture also contained 3 μl of rabbit anti-OVA antiserum and 7 μl 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 idiotype 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 ^{125}I-labeled ligands (mAb CB9, A11, or $H_{CB9}L_N$), 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 ^{125}I-labeled ligand, and the plateau level of ligand binding was determined. When mAb AD8 (rat IgG2aκ) 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 × C57BL/6)F_1. 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-Ar antibodies by two inoculations of 500 μg in CFA followed by monthly inoculations of 500 μg 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-Ar mAb 6-16 (CRI_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_A expression (27). mAb S27 is CRI_C^+; its V_H and V_L regions therefore differ markedly from those of CRI_A^+ antibodies (20). The purpose of using these adsorbents for anti-6-16 was to leave unadsorbed any anti-Id antibodies specific for the V_H or V_L regions.

Results

Idiotypic Activity of Hybrid Molecules Containing H_{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 CRI_A

Inhibitor	Amount required for 50% inhibition	Inhibitor	Amount required for 50% inhibition
	ng		*ng*
A/J IgG	>3,000 (6)*	$H_{CB9}L_N$	250
R16.7 (CRI_A)	13	$H_{CB9}L_{CB9}$	17
6-16 (CRI_A)	12	$H_{CB9}L_{19.9}$	19
CB9 (CRI_A)	14	$H_{CB9}L_{A11}$	570
R19.9	>3,000 (11)	$H_{CB9}L_{S27}$	620
A11 (CRI_D)	>3,000 (17)		
S27 (CRI_C)	>3,000 (8)	$H_{A11}L_N$	>3,000 (23)
		$H_{A11}L_{CB9}$	1,250
H_NL_N‡	>3,000 (6)	$H_{A11}L_{19.9}$	>3,000 (23)
H_NL_{CB9}	2,050	$H_{S27}L_N$	>3,000 (4)
$H_NL_{19.9}$	>3,000 (9)	$H_{S27}L_{CB9}$	1,850
H_NL_{A11}	>3,000 (7)	$H_{19.9}L_N$	>3,000 (2)
H_NL_{S27}	>3,000 (3)	$H_{19.9}L_{CB9}$	630
		$H_{19.9}L_{19.9}$	>3,000 (8)

Assays used rabbit anti-CRI_A antibodies with 10ng ^{125}I-labeled R16.7 (CRI_A^+) as ligand.
* Numbers in parentheses indicate percent inhibition by the weight of inhibitor specified.
‡ Recombined molecules. The subscript N refers to nonspecific A/J IgG. Other subscripts refer to the mAb that provided the H or L chain.

cules containing recombined H and L chains, in the assay for CRI_A using labeled R16.7 as ligand. 12–14 ng of unlabeled CRI_A^+ mAb (R16.7, 6-16, or CB9) caused 50% inhibition in the assay. For the autologous CRI_A^+ recombinant molecule, $H_{CB9}L_{CB9}$, 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 CRI_A^+ mAb (CB9) and nonspecific L chains (L_N), 250 ng was required for 50% inhibition. This could be accounted for by a low affinity of $H_{CB9}L_N$ or by ~7% contamination (17/250) of H_{CB9} by L_{CB9}. The low inhibitory capacities of $H_{CB9}L_{A11}$ or $H_{CB9}L_{S27}$ 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 H_{CB9}. This is consistent with previous data showing that R19.9, which is CRI_A^-, expresses L chains that are idiotypically very similar to L chains of CRI_A (28), and are nearly identical in sequence to that encoded by the $V_{\kappa 10}$ germ line gene, which encodes the L chains of CRI_A^+ molecules (15, 29).

The high recovery of activity in the homologous recombinant, $H_{CB9}L_{CB9}$, 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 CRI_A-derived L Chains. The inhibition data in Table II demonstrate that molecules containing CRI_A-derived L chains but H chains from other sources are very weak inhibitors in the assay for CRI_A; these molecules are H_NL_{CB9}, $H_{A11}L_{CB9}$, and $H_{S27}L_{CB9}$. In

addition, intact R19.9, which has CRI_A-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 idiotype activity in $H_{CB9}L_{CB9}$.

The requirement for appropriate L chains to provide good inhibition in the assay for CRI_A 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 CRI_A 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 ^{125}I-labeled molecules containing L_{CRIA} 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_NL_{CB9}, even by a large excess of rabbit anti-Id antibodies directed either against affinity-purified A/J anti-Ar serum antibodies (anti-CRI_A) or against the strongly CRI_A^+ mAb 6-16. In addition, the binding capacity of each anti-Id reagent for R19.9, which has L chains characteristic of CRI_A, was extremely low. The ratios of amounts of antiserum required for 50% binding of labeled R19.9 or CB9 (CRI_A^+) were 70:1 and 210:1, respectively, for anti-CRI_A and anti-6-16(Id). These

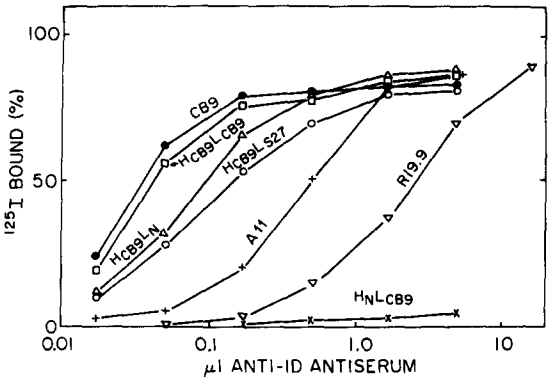

FIGURE 1. Measurements of direct binding of a series of ^{125}I-labeled ligands. The anti-Id (anti-CRI_A) was prepared in a rabbit against affinity-purified A/J anti-Ar antibodies. Complexes were precipitated with goat anti-rabbit Fc (of IgG). In all binding assays 10 ng of labeled ligand was used (except Fig. 4, in which increasing amounts of ligand were used).

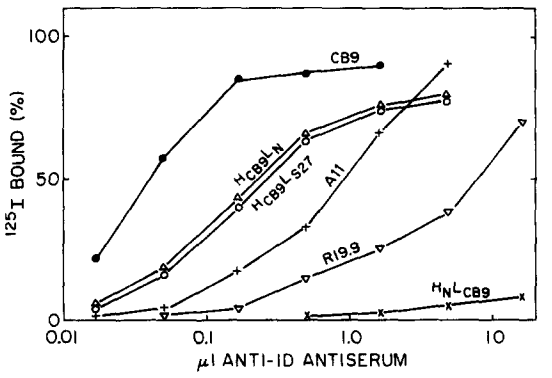

FIGURE 2. Same as Fig. 1, except that the rabbit anti-Id was prepared against mAb 6-16.

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 CRI_A^+ 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., $H_{CB9}L_N$ vs. $H_{CB9}L_{CB9}$), 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-CRI_A, 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 ($H_{CB9}L_N$ or $H_{CB9}L_{S27}$) 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 $H_{CB9}L_N$ or $H_{CB9}L_{S27}$ with the amounts required to bind 50% of the maximal level of the CRI_A^+ mAb, CB9. For anti-CRI_A (Fig. 1) the ratios are 2.7:1 and 4.1:1 for $H_{CB9}L_N$ and $H_{CB9}L_{S27}$, 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 $H_{CB9}L_N$ with ^{125}I and added sufficient anti-Id (anti-CRI_A 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, $H_{CB9}L_N$

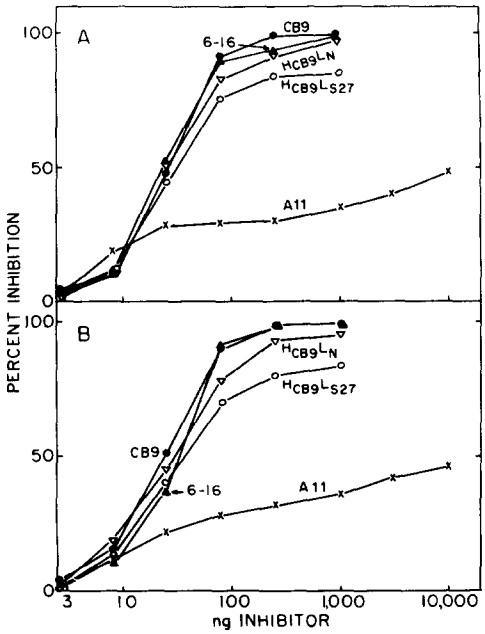

FIGURE 3. Inhibition assays; unlabeled inhibitors are specified on the graphs. (*A*) Experiments used rabbit anti-CRI_A with ^{125}I-labeled $H_{CB9}L_N$ as ligand. (*B*) Rabbit anti-Id(6-16) was used with ^{125}I-labeled $H_{CB9}L_N$ as ligand. The amount of anti-Id used in *A* and *B* was sufficient to bind 50–60% of the labeled ligand.

or $H_{CB9}L_{S27}$ were about as effective as inhibitors as the intact CRI_A^+ mAbs, CB9 or 6-16. This indicates that most anti-Id molecules that do bind $H_{CB9}L_N$ 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 $H_{CB9}L_N$ or $H_{CB9}L_{S27}$, 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_{CB9} by L_{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_A antibodies that have specificity for the H chain alone. Those direct binding

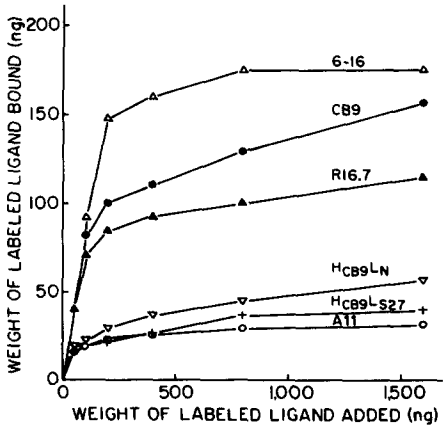

FIGURE 4. Saturation binding assays using increasing amounts of the six different ^{125}I-labeled ligands specified on the graph and 0.6 μl of adsorbed anti-6-16(Id). Immune complexes were precipitated as in Fig. 1.

experiments utilized a constant amount of anti-6-16(Id) and increasing amounts of ^{125}I-labeled idiotype. The data in Fig. 4 indicate that the binding capacity of anti-6-16(Id) for $H_{CB9}L_N$ is 32 or 35% as great as its binding capacity for intact 6-16 or CB9. The corresponding percentages are 19 and 21% for $H_{CB9}L_{S27}$. 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_{CRIA} 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_{CRIA}; 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_D^+ 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 ($H_{CB9}L_N$ or $H_{CB9}L_{S27}$). Small amounts of anti-Id bind about two to three times more $H_{CB9}L_{S27}$ or $H_{CB9}L_N$ than A11. When antibodies with specificity for $H_{CB9}L_N$ were examined (by using labeled $H_{CB9}L_N$ 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_{CRIA}-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_{CRIA}-containing hybrid molecule.

Idiotypic Similarity of V_H Present in CRI_A and CRI_D. As already mentioned, the anti-Id mAb AD8 reacts with V_HDJ_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_A^+ mAbs 6-16 and CB9 are very similar. As indicated in Table I, the V_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 CRI_D^+) react as well or better with AD8 than 6-16 and CB9 (both CRI_A^+), despite the large difference in length of their D regions; in addition, AD8 was prepared against CRI_A^+ antibodies. The fact that CRI_D^+ mAbs bind as well as CRI_A^+ mAbs to AD8 indicates that the V_H segment is responsible for the binding and that any contributions of the D region and L chain are negligible. Thus, CRI_A^+ and CRI_D^+ mAbs express the AD8-reactive idiotope to a very similar degree.

The binding of AD8 to DE2 and SM1.5 (both CRI_D) appears to be somewhat stronger than that to the CRI_D^+ mAb, A11 (as well as to the CRI_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, $H_{CB9}L_{S27}$, 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_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_H (see Discussion).

Specificity of AD8 as Assessed by Inhibition Measurements. Fig. 6 *A* shows data on inhibition of binding of the ^{125}I-labeled CRI_A^+ mAb, CB9, to AD8. Unlabeled CRI_A^+ mAbs CB9 and 6-16, the hybrid molecule $H_{CB9}L_{S27}$, and A11 (CRI_D) yielded very similar inhibition curves. These data support the direct binding measurements in indicating that the specificity of AD8 is directed to V_H and not to the D region or L chain, and that the V_H segments in CRI_A and CRI_D react to a similar degree with AD8.

Although mAbs SE20.2, 93G7, and R16.7 (all CRI_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. 6 *B* were obtained with labeled mAb A11 (CRI_D^+)

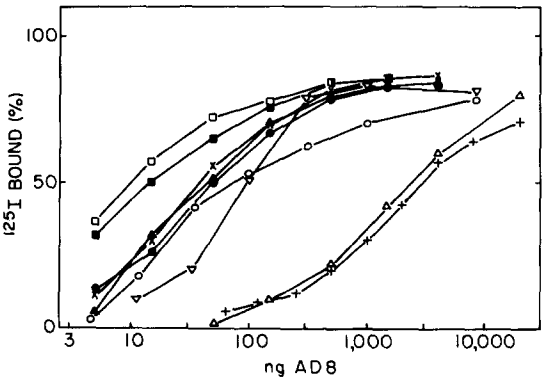

FIGURE 5. Direct binding assays; same as Fig. 1, except that mAb AD8 (rat anti-Id) was used and complexes were precipitated with mouse antirat Ig. ^{125}I-labeled ligands: (□) DE2; (■) SM1.5; (×) A11; (▲) 6-16; (●) CB9; (○) $H_{CB9}L_{S27}$; (▽): $H_{CB9}L_{CB9}$; (△) SE20.2; (+) R16.7. mAb R19.9 was completely nonreactive.

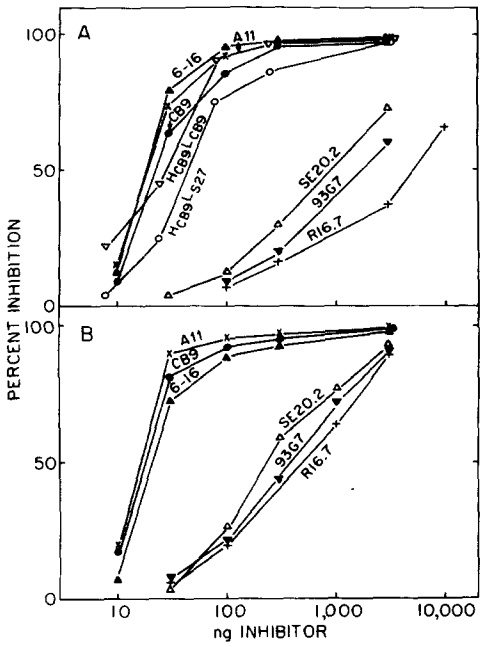

FIGURE 6. Inhibition assays; unlabeled inhibitors are specified on the graphs. The amount of anti-Id (mAb AD8) used was sufficient to bind ~50% of the labeled ligand. (*A*) The ligand is ^{125}I-labeled mAb CB9 (CRI_A^+); (*B*) the ligand is ^{125}I-labeled mAb A11 (CRI_D^+).

as ligand in place of labeled CB9 (CRI_A^+). The very close similarity of the results in Fig. 6, *A* and *B* are again consistent with the view that AD8 recognizes only V_H, 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_H 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^+ mAb A11 indicate that only a small proportion of anti-CRI_A (~15%; Fig. 4) reacts with V_H in the absence of the four other CDRs associated with CRI_A. However, the V_H of regions of CRI_A^+ and

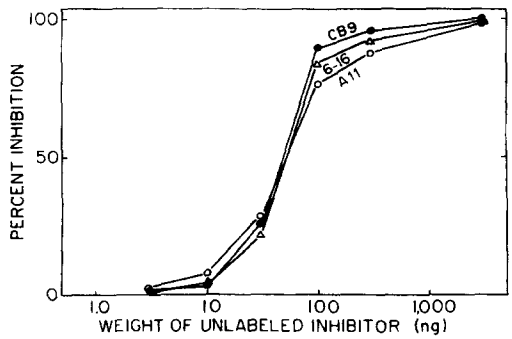

FIGURE 7. Inhibition assays; unlabeled inhibitors are specified on the graph. The anti-Id used was anti-6-16(Id), affinity purified on A11-Sepharose. The ligand is ^{125}I-labeled mAb A11 (CRI_D^+). In the absence of inhibitor, 64% of the labeled ligand was bound.

CRI_D^+ mAbs interact virtually identically with mAb AD8 and with that small proportion of anti-CRI_A antibodies that recognize V_H 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 ~2,000 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 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_A^+ 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 L_{CRIA} and H chains from other sources (Figs. 1 and 2). In addition, such hybrid molecules were extremely poor inhibitors of the binding of CRI_A^+ antibodies to anti-CRI_A (Table II). Also, mAb R19.9, whose L chains effectively reconstitute CRI_A when combined with H_{CRIA}, was an extremely poor inhibitor of the binding interactions of anti-CRI_A. 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 idiotype assays of recombined molecules $H_{CB9}L_{CB9}$, as compared with $H_{CB9}L_N$, $H_{CB9}L_{S27}$, or $H_{CB9}L_{A11}$. 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 ~10^3 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_a of anti-Id for the L chain would be ~10^3 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., $H_{CB9}L_N$). 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_{CRIA} with L_{CRIA}. The very low inhibitory capacities of $H_{CB9}L_{A11}$ and $H_{CB9}L_{S27}$ in the assay for CRI_A (Table II) indicated that the degree of contamination of H_{CRIA} with L_{CRIA} was 3% or less.

The data on direct binding, which show that ~70–80% of the anti-Id requires both H_{CRIA} and L_{CRIA} for effective interaction, are consistent with inhibition data in indicating that most anti-Id antibodies interact with CDRs from V_L and V_H.

Proportion of Anti-Id Reactive with the V_H Segment. The results indicate that only a small proportion of anti-CRI_A reacts with the V_H segment (residues 1–98) in the absence of the appropriate D and V_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_A). A11 is a member of the CRI_D family, which shares the V_H segment but not the D or V_L segments with CRI_A (19). Data indicating that the V_H segments of CRI_A and CRI_D are idiotypically very similar are discussed below. The binding of A11 to anti-CRI_A was also considerably weaker than the binding of the H chain ($H_{CB9}L_N$ or $H_{CB9}L_{S27}$; Fig. 1). The relatively poor interaction of V_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 $H_{CB9}L_N$ to anti-CRI_A (mAb CB9 is strongly CRI_A^+). These results indicate that the most effective binding to the H chain requires the D region in addition to the V_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_H segment with anti-CRI_A is further evidence for a requirement for multiple CDRs for effective interaction with anti-CRI_A.

The fact that mAb AD8 appears to recognize the V_H segment alone (as shown by its reactivity with H chains on Western blots [24] and its reactivity with CRI_D^+ 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_H segment. This would not conflict with our observation that CRI_A and CRI_D mAbs react almost identically with AD8, since the two families share V_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_A and CRI_D.

The data indicate that nearly all anti-CRI_A antibodies react with portions of both V_H and V_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_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-

ardous 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_H segment, appears to react almost identically with unmutated CRI_A^+ and CRI_D^+ 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_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_H CDR1 and CDR2 do not make close contacts with CDRs from V_L and make few contacts with V_H CDR3 (15).

Another example of constancy of Id expression in a variety of environments are idiotypes present on subgroups of $V_\kappa 21$ 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_A antibodies can bind H chains of the idiotype in the absence of appropriate L chains, but were unable to demonstrate direct binding to L chains in the absence of CRI_A-derived H chains. A possible explanation comes from the X-ray crystallographic study of the CRI_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_H CDR1, 31 and 32; CDR2, 55–62; CDR3, 100–107; V_L CDR1, 25–32; CDR2, 50–56; CDR3, 91–95. It is noteworthy that each of the CRI_A^+ mAbs we have studied that react poorly with AD8 (which recognizes V_H), and for which sequences are available, have one or more substitutions from the germline-encoded mAb 36–65 in the V_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_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_H region and L chain related to those of CRI_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 V_H CDR3. A contact is defined here by a distance of 4 Å or less. Gln89 of V_LCDR3 makes contacts with V_H Tyr109. Contacts between the CDR3s are important for the assembly and interaction of V_H and V_L regions (39, 40), and one might predict that this region would often be part of an idiotope. V_L 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 Å^2. 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 Å^2. All of the serological data on CRI_A^+ presented here are consistent with this possibility.

Summary

We have explored the structural basis of idiotopes associated with the major idiotype (CRI_A) of A/J anti-*p*-azobenzenearsonate antibodies, with emphasis on the regions of contact with anti-idiotypic antibody. The analysis was facilitated by a recent description of the three-demensional structure of the Fab portion of a CRI_A-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 $\sim 10^4$ M^{-1}).

Approximately 20–35% of polyclonal anti-Id reacted with high affinity with H chains recombined with non-CRI_A-related L chains. This interaction was found to involve the D region as well as one or both CDRs in the V_H 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 V_H segment, was found to be unaffected by major changes in the neighboring D and V_L regions. This observation is relevant to efforts to predict three-dimensional structure from the amino acid sequence of CRI_A^+ 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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