PRESENCE OF HIGHLY CONSERVED IDIOTYPIC DETERMINANTS IN A FAMILY OF ANTIBODIES THAT CONSTITUTE AN INTRASTRAIN CROSS-REACTIVE IDIOTYPE*

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Serological and primary structural analyses of murine monoclonal antibodies have shown that several idiotypes (Id) that are highly cross-reactive within an inbred strain comprise a family of closely related, but nonidentical, antibody molecules (1–11). In the case of A/J anti-*p*-azophenylarsonate (Ar)[1] antibodies, serological evidence for idiotypic heterogeneity of cross-reactive idiotype $(CRI)^+$ hybridoma products (HP) includes the wide quantitative variation in their inhibitory capacity in the standard radioimmune assay for Id. The amounts of such HP required to displace 50% of labeled specifically purified A/J anti-Ar from its rabbit anti-Id antibodies varied between 9 and 3,200 ng (6, 7). In addition, anti-Id antisera prepared against individual CRI^+ HP revealed the presence of private idiotypic determinants that were not found in other CRI^+ HP; some of these determinants were, however, present in A/J anti-Ar immune serum (5–7).

To date, structural correlates of the Ar Id have not been fully established. However, it has been shown that the third complementarity-determining region and J segments of both the heavy and light chains of the CRI^+ HP 93G7 are very similar, although not identical to the corresponding sequences in pooled CRI^+ A/J serum antibodies (L. J. McCumber, P. Estess, M. Siegelman, and J. D. Capra. Manuscript in preparation.).

We present data here that indicate that most CRI^+ HP possess at least one idiotypic determinant that is highly conserved. This was demonstrated by using anti-Id against one HP, with a second HP as the labeled ligand. Such a procedure, originally used in studies of human cold agglutinins and antiglobulins (12, 13), is designed to minimize the role of private idiotypic determinants (12–14); i.e., it is anticipated that such a system will primarily measure shared idiotypic determinants. With two such heterol-

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[1] *Abbreviations used in this paper:* ABA-HPP, *m*-azobenzene-*p'*-arsonate-3(*p*-hydroxyphenyl)-propionic acid; anti-Id(D), rabbit anti-idiotypic antibodies directed against A/J serum anti-Ar antibodies; anti-Id(93G7), rabbit anti-idiotypic antibodies directed against hybridoma product 93G7; anti-Id(R16.7), rabbit anti-idiotypic antibodies directed against hybridoma product R16.7; Ar, *p*-azophenylarsonate; BSA, bovine serum albumin; CRI, cross-reactive idiotypes; HP, hybridoma product(s); KLH, keyhole limpet hemocyanin.

ogous systems, it was found that the majority of CRI^+ HP are quantitatively equivalent to one another with respect to their inhibitory capacities, indicating they share one or more closely related public idiotypic determinant(s). Also, the binding of anti-Id mediated by such public determinants was almost completely inhibitable by hapten, indicating that these determinants are associated with the hapten-binding site.

Materials and Methods

Preparation of Hybridomas That Secreted Anti-Ar Antibodies. Hybridomas that secreted monoclonal anti-Ar antibodies were generated as previously described (4–6). In brief, A/J mice were immunized intraperitoneally with 500 μg of Keyhole limpet hemocyanin (KLH)-Ar emulsified in complete Freund's adjuvant. The animals were challenged intravenously 8 wk later with 100 μg of KLH-Ar in saline, and spleens were removed 4 d afterward for fusion. 1×10^8 splenic leukocytes and 1×10^7 nonsecreting Sp2/0-Ag14 tumor cells (15) were fused with 33% polyethylene glycol (PEG-1000; J. T. Baker Chemical Co., Phillipsburg, N. J.). Hybrid cells were selected in medium that contained hypoxanthine, thymidine, and aminopterin, as described by Littlefield (16). Culture supernates were tested for anti-Ar activity on bovine serum albumin (BSA)-Ar-coated polyvinyl microtiter plates by the method of Klinman et al. (17). The bound antibodies were quantitated by measuring the uptake of ^{125}I-labeled specifically purified rabbit anti-mouse Fab. Selected cultures that secreted anti-Ar antibodies were cloned either in soft agarose or by limiting dilution. Approximately 25% of anti-Ar HP were CRI^+. An HP was defined as CRI^+ if it caused at least 50% displacement of the ligand (labeled serum anti-Ar antibody) in the conventional assay for CRI (18). 17 HP, of which 14 were CRI^+, were used in this study. 13 of the hybrid lines arose from a single mouse (6), and the other 4 originated from two other mice (4, 5). Each hybrid was derived from a separate culture well and therefore represents the product of an independent fusion event.

Isolation of Monoclonal Anti-Ar Antibodies. Ascites fluids that contained large amounts of hybridoma antibody were generated by injecting $1–5 \times 10^6$ hybrid cells into the peritoneal cavity of Pristane (Aldrich Chemical Co., Inc., Milwaukee, Wis.) -primed (BALB/c × A/J)F_1 mice (19). Anti-Ar antibodies were specifically purified by affinity chromatography (20) on a column of bovine gamma globulin-Ar coupled to Sepharose 4B. Bound antibodies were eluted from the column with 0.5 M sodium Ar, pH 8.0. Each HP was purified on a separate column and dialyzed for 1 wk against multiple changes of buffer to remove hapten. Each protein was homogeneous by the criteria of disc electrophoresis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions. Four HP were tested and found to be homogeneous by the criterion of amino-terminal amino acid sequence analysis (5, 6).

Anti-Id Antisera. Anti-Id antisera were prepared in rabbits by injections of specifically purified A/J anti-Ar antibodies or purified HP (6, 18). The antisera were adsorbed by successive passage over two columns of Sepharose 4B to which a crude globulin fraction of normal A/J serum had been conjugated by using cyanogen bromide (21). The anti-Id antisera against HP were additionally adsorbed with the BALB/c myeloma protein MOPC-21 ($IgG_1\kappa$) coupled to Sepharose 4B. All precipitating activity against normal mouse globulin was lost after passage over the first column. Rabbit anti-Id antibodies against the myeloma protein TEPC 15, which binds phosphorylcholine, were the gift of Dr. Alan R. Brown, Rosensteil Research Center, Brandeis University, Waltham, Mass.

Assays for Id. Serological assays for idiotypic determinants expressed on various anti-Ar antibodies were performed as previously described (6, 18). HP and affinity-purified serum anti-Ar antibodies were radiolabeled with carrier-free Na[^{125}I] (Amersham Corp., Arlington Heights, Ill.) by the chloramine T method (22). Binding assays used 10 ng of ^{125}I-labeled ligand (affinity-purified HP or A/J serum anti-Ar antibody) and slightly less than an equivalent amount of anti-Id antiserum. Each assay was carried out in the presence of 20–25 μl of normal mouse serum (that contained ~80–100 μg of mouse immunoglobulin). Rabbit anti-ovalbumin was also added to each mixture to keep the amount of rabbit immunoglobulin constant. Id-anti-Id complexes were precipitated by the addition of a slight excess of goat anti-rabbit Fc that had been adsorbed with mouse immunoglobulin. The mixtures were incubated for 30 min at 37°C

and overnight at 4°C. Precipitates and supernates were separated and counted in a three-channel Packard gamma scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). [^{22}Na]Cl was included in each reaction mixture to permit quantitation of the amount of supernate that remained in the pellet; this eliminated the necessity for washing precipitates. The percentage of ^{125}I-labeled ligand in each precipitate was calculated from the ^{125}I and ^{22}Na counts in both precipitate and supernate. The presence of the Id in various unlabeled HP or in serum anti-Ar was quantitated through the capacity of the Id to inhibit the binding of the labeled ligand by the anti-Id antibodies. Varying amounts of the test samples were incubated with anti-Id for 30 min at 37°C before the addition of the labeled ligand. Id-binding assays for protein TEPC 15 were carried out as described above. In addition the reaction mixture also contained 100 μg of the myeloma protein MOPC 460 (IgA, κ).

Haptens. Phosphorylcholine chloride was obtained from Sigma Chemical Co., St. Louis, Mo. *p*-Aminobenzenearsonic acid (Eastman Kodak Co., Rochester, N. Y.) was recrystallized twice from hot water. *m*-Azobenzene-*p'*-arsonate-3-(*p*-hydroxyphenyl)-propionic acid (ABA-HPP) was synthesized by the reaction of the diazonium salt of *p*-aminobenzenearsonic acid with 3-(*p*-hydroxyphenyl)-propionic acid according to the method of Tabachnick and Sobotka (23). The resulting orange compound was further purified by chromatography on Sephadex G-15 in 0.05 M $(NH_4)_2CO_3$. Its purity was assessed by thin-layer chromatography on silica gel plates.

Results

The purpose of these experiments was to serologically define the public idiotypic determinants responsible for the reactivity shared among many A/J anti-Ar HP and A/J serum anti-Ar antibodies. Previous experiments have demonstrated that CRI$^+$ HP that differ with respect to primary structure and that possess private idiotypic specificities are able to inhibit the reaction between rabbit anti-Id and serum anti-Ar antibodies (4–7). This indicates the existence of at least one public or cross-reacting idiotypic determinant. However, the amounts of various HP required to cause 50% inhibition in the assay vary markedly (5, 6).

To study the shared idiotypic determinants, an inhibition assay system was designed that, although allowing the measurement of public determinants, minimized the role of the private determinants (12–14). This was accomplished by using anti-Id antibodies directed to one HP with a different CRI$^+$-labeled HP as ligand. The two systems investigated were: (*a*) rabbit anti-idiotypic antibodies directed against HP R16.7 [anti-Id(R16.7)] reacting with ^{125}I-labeled HP R10.8, and (*b*) rabbit anti-idiotypic antibodies directed against HP 93G7 [anti-Id(93G7)]reacting with labeled HP 121D7. The direct binding curves for these two interactions are shown in Fig. 1. Also presented are the autologous binding curves: anti-Id(R16.7) reacting with labeled HP R16.7 and anti-Id(93G7) reacting with labeled HP 93G7. It is evident that the amounts of anti-Id required to bind the heterologous HP were somewhat greater than the amounts required in the autologous interactions. However, the ratios are only of the order of 2 or 3 to 1. The differences in amounts required are not unexpected and can be accounted for by the presence of antibodies to private determinants that are operative in the autologous, but not in the heterologous, interactions. Any remaining difference must be quite small, suggesting that binding affinities for the public determinants of the autologous and heterologous proteins are very similar.

Inhibition of the Interaction of Anti-Id(R16.7) with ^{125}I-labeled HP R10.8. The data in Table I show the inhibitory capacities of a number of unlabeled anti-Ar HP in this system. Included in the table are 14 HP that are CRI$^+$ and 3 that are CRI$^-$. Also presented are the results obtained with purified unlabeled A/J anti-Ar antibodies as

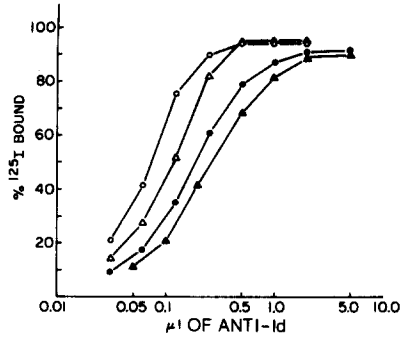

FIG. 1. Binding of 10 ng of ^{125}I-labeled specifically purified HP to autologous and heterologous rabbit anti-Id antisera. Id-anti-Id complexes were precipitated with excess goat anti-rabbit Fc. ○, HP R16.7 and anti-Id(R16.7); ●, HP R10.8 and anti-Id(R16.7); △, HP 93G7 and anti-Id(93G7); ▲, HP 121D7 and anti-Id(93G7).

TABLE I

*Inhibition by Unlabeled HP of Binding of Anti-Id Antibodies to Heterologous Labeled HP**

Unlabeled inhibitor	Anti-Id against: ^{125}I-labeled ligand:	Nanograms required for 50% inhibition R16.7 R10.8	93G7 121D7	Anti-Ar‡ Anti-Ar
Serum anti-Ar		15 (100)§	11 (100)	11 (97)
R16.7 (G1)		12 (100)	8 (97)	9 (94)
93G7 (G1)		7 (100)	9 (99)	12 (90)
R20.4 (G2b)		10 (92)	8 (92)	14 (86)
R26.5 (G3)		12 (88)	10 (99)	17 (85)
R13.4 (G3)		13 (100)	14 (97)	21 (87)
R10.8 (G2a)		8 (100)	7 (90)	180 (60)
R23.2 (G2b)		5 (100)	7 (91)	200 (66)
R9.3 (G2b)		9 (100)	10 (90)	300 (65)
121D7 (G1)		17 (88)	16 (100)	300 (71)
R17.5 (G2b)		7 (100)	7 (91)	460 (63)
R24.6 (G2a)		6 (100)	7 (93)	1,800 (52)
123E6 (G1)		>2,000 (35)	>2,000 (19)	1,900 (51)
124E1 (G1)		1,900 (51)	>2,000 (44)	2,900 (47)
R22.4 (G2a)		6 (100)	7 (92)	3,200 (49)
R18.11‖ (G3)		>2,000 (0)	>2,000 (12)	>2,000 (20)
R19.9‖ (G2b)		>2,000 (4)	>2,000 (10)	>2,000 (15)
R21.10‖ (G1)		>2,000 (0)	>2,000 (7)	>2,000 (6)

* Each test utilized 10 ng of ^{125}I-labeled ligand and slightly less than an equivalent amount of rabbit anti-Id. Immune complexes were precipitated by goat anti-rabbit Fc.

‡ Data in the last column are from reference 6.

§ The percent inhibition by 2,000 ng is in parentheses.

‖ Anti-Ar HP lacking the major CRI.

inhibitor. The data are expressed as nanograms of protein required to cause 50% inhibition in the radioimmune assay. Also included, in parentheses, are the values for percentage inhibition obtained with a large excess (2,000 ng) of inhibitor.

It is evident that 12 of the 14 CRI$^+$ HP were very similar to one another and to serum anti-Ar antibodies in their quantitative inhibitory capacities in this system. For these 12 CRI$^+$ HP, the amounts required for 50% inhibition vary between 5 and 17

ng. As shown in the last column of Table I, these HP varied widely in their inhibitory capacities in the conventional idiotypic system (rabbit anti-idiotypic antibodies directed against A/J serum anti-Ar antibodies [anti-ID(D)]reacting with labeled serum antibodies). The amounts required for 50% inhibition in the latter assay varied from 9 to 3,200 ng.

2 of the 14 CRI^+ HP, 123E6 and 124E1, were weak inhibitors of the binding of HP R10.8 by anti-Id(R16.7). These two HP are also very poor inhibitors in the conventional assay (Table I, last column).

Inhibition of the Interaction of Anti-Id(93G7) with ^{125}I-labeled HP 121D7. The results obtained in this system (Table I) are very similar to those already discussed. Again 12 of the 14 HP, with the same two exceptions, were very similar in their inhibitory capacities in terms of nanograms of protein required for 50% inhibition and the degree of inhibition by 2,000 ng of protein. Serum anti-Ar was similar in its inhibitory capacity to the 12 strongly inhibitory HP.

The results obtained in both systems when very large amounts (2,000 ng) of unlabeled HP were tested as inhibitors are also informative. In both systems the same 12 CRI^+ HP, as well as serum anti-Ar, caused almost complete displacement (88–100%) of the labeled ligand. Again these results stand in contrast to the data obtained in the anti-Id(D) vs. anti-Ar system (Table I, last column) in which most of the CRI^+ HP were incapable of causing complete inhibition when 2,000 ng were tested.

Effects of Haptens of Id-anti-Id Interactions. The data in Table II show the effects of haptens on several Id-binding reactions. The haptens tested comprised two *para* derivatives of benzenearsonate (ABA-HPP and *p*-aminobenzenearsonic acid) and, as a control, phosphorylcholine. Each hapten was also tested for its inhibitory effect on the binding of myeloma protein TEPC 15, which combines specifically with phosphorylcholine, to its rabbit anti-idiotypic antibodies.

The data of principal interest are those involving the heterologous binding reactions: anti-Id(R16.7) reacting with HP R10.8 and anti-Id(93G7) interacting with HP 121D7. These cross-interactions would be expected to principally involve public idiotypic determinants. In the two systems the compound ABA-HPP caused 84 and 88% inhibition, respectively, at a final concentration of 10 mM, indicating that a very

TABLE II

Inhibition by Haptens of Binding of Anti-Id Antibodies to Autologous and Heterologous HP

Anti-Id against	^{125}I-labeled ligand	Percent inhibition of binding; hapten						
		ABA-HPP			*p*-Aminobenzenearsonic acid			Phosphorylcholine
		1*	5	10	5	10	20	20
R16.7	R10.8	69	80	84	39	49	58	0
R16.7	R16.7	77	87	91	39	46	54	0
93G7	121D7	82	87	88	43	50	56	0
93G7	93G7	30	37	43	15	19	22	0
Anti-Ar‡	Anti-Ar	66	79	83	27	32	41	0
TEPC 15	TEPC 15			0			0	55

* Final hapten concentrations in the reaction mixtures (mM).

‡ Specifically purified serum antibodies.

large fraction of the anti-Id antibodies recognize determinants at or very near the binding sites of the ligands. The smaller hapten, *p*-aminobenzenearsonic acid was inhibitory in both systems but the degree of inhibition at a concentration of 20 mM was less (58 and 56%, respectively). Phosphorylcholine was noninhibitory in both systems, although, as expected (24), it caused significant inhibition of the binding of the myeloma protein TEPC 15 to the autologous anti-Id.

We also investigated the effects of haptens on the autologous systems (HP R16.7, HP 93G7, and serum anti-Ar reacting with their respective autologous anti-Id antibodies). The degree of inhibition by ABA-HPP in the R16.7 and serum anti-Ar systems was very high (91 and 83%), but it was considerably lower in the case of anti-Id(93G7) reacting with HP 93G7 (43% at 10 mM). In each system the single-ring compound, *p*-aminobenzenearsonic acid, inhibited significantly, but not as well as the two-ring compound, ABA-HPP. Again, phosphorycholine was noninhibitory.

As an additional control, the two phenylarsonate derivates were also tested for their inhibitory effects in the anti-Id against TEPC 15-TEPC 15 ligand system (Table II). In each case, no inhibition was observed.

Discussion

Our results bear on the serological relationships among a group of anti-Ar HP that express the major CRI but also show individual variation. The criterion for possession of the major idiotypic determinant(s) is the capacity to cause 50% displacement of the labeled ligand (purified A/J serum anti-Ar) in the conventional radioimmune assay for Id. This degree of inhibition has not been seen with antibodies other than anti-Ar and cannot be achieved with the anti-Ar antibodies of many other strains (25) or with anti-Ar antibodies from A/J mice that have been immunologically suppressed by inoculation of anti-Id before immunization (26). The individual structural variation of HP has been established by amino acid sequence analysis and by the presence of private idiotypic determinants (4–9) (L. J. McCumber, P. Estess, M. Siegelman, and J. D. Capra. Manuscript in preparation.). Such determinants are revealed when anti-Id antibodies are prepared against an individual HP. Many CRI^+ HP are relatively poor inhibitors of the binding of an anti-Id antibody to its autologous, CRI^+ HP. Some, but not all, private determinants are found in appreciable concentration in A/J anti-Ar sera.

These experiments were designed to permit analysis of the shared or public determinant(s). Anti-Id antibodies against one HP were allowed to react with another, ^{125}I-labeled CRI^+ HP. This procedure should minimize the role of private idiotypic determinants. Such a procedure was originally used in human Id systems by Williams et al. (12) and Kunkel et al. (13) who first described the phenomenon of cross-idiotypic specificity. Two cross-idiotypic systems were investigated: anti-Id(R16.7) reacting with labeled HP R10.8 and anti-Id(93G7) reacting with labeled HP 121D7. A panel of 14 unlabeled CRI^+ HP were tested as inhibitors of binding of the labeled ligand in each system, and 3 CRI^- HP's were used as controls. The results obtained in the two systems were virtually identical. 12 of the 14 HP were very strong inhibitors, and the amounts of HP required for 50% inhibition varied over a narrow range. When 2,000 ng were tested as inhibitor, each of these 12 HP caused 88–100% inhibition. The results were in marked contrast with the wide variation in inhibitory capacity seen in the conventional system, in which anti-Id(D) reacts with ^{125}I-labeled serum anti-Ar.

The results indicate that at least one cross-reactive idiotypic determinant is highly conserved and, by serological criteria, is virtually identical in 12 of the 14 CRI^+ HP studied. The large variation in inhibitory capacities in the conventional assay must be attributed to microheterogeneity with respect to shared idiotypic determinants other than the conserved determinant identified by the cross-idiotypic assays. The HP may differ with respect to the number of such determinants they possess or with respect to their binding affinities for the anti-Id antibody.

2 of the 14 CRI^+ HP were exceptional in that they were relatively poor inhibitors in the two systems designed to test for public determinants. These two proteins, 123E6 and 124E1, were also very poor inhibitors in the conventional [D-(anti-Ar antibody possessing CRI of A/J strain mice) anti-Id(D)] assay. However, proteins R24.6 and R22.4, which were also very weak inhibitors in the conventional assay (Table I, column 4), were very strong inhibitors in the assays for cross-idiotypy (Table I, columns 2 and 3). It should be noted that proteins 123E6 and 124E1 can be bound almost completely by anti-Id(D), as shown by the inhibition of their binding to BSA-Ar-coated plates by anti-Id. The fact that they are weak inhibitors may be a result of either a deficiency in the number of public idiotypic determinants or of a structural alteration of the public determinants such that they interact with the anti-idiotypic antibodies with lower affinity.

Thus, the observation that proteins 123E6 and 124E1 are poor inhibitors in the assay for cross-idiotypy suggests that public determinants can also exhibit microheterogeneity. However, the conservation of the determinants in 12 of the HP indicates that this variation is of secondary importance as compared to the variations reflected by the presence of the private idiotypic determinants.

The effects of haptens on the two cross-Id systems studied indicate that the public determinants are localized in the region of the hapten-binding site. In both systems, the hapten ABA-HPP caused at least 84% inhibition of binding. *p*-Aminobenzenearsonic acid was somewhat less effective, causing 56 and 58% inhibition in the two systems at a concentration of 20 mM. The difference in effectiveness of the two haptens may be a result of a difference in affinities or size (27). If affinity is a significant factor, it is possible that ABA-HPP would cause 100% inhibition if a sufficiently high concentration were used; i.e., that the public determinant(s) are entirely associated with the region of the combining site.

The above results on hapten inhibition contrast with the data obtained in the autologous system: HP 93G7 reacting with anti-Id(93G7). In this system, only 43% inhibition by ABA-HPP was achieved. This suggests that the anti-Id antibodies prepared against HP 93G7 recognize determinants outside the combining site as well as site-related determinants. Anti-Id(R16.7), in contrast, interacts almost entirely with site-related determinants on the autologous protein, HP R16.7. The interaction of anti-Id(D) with serum anti-Ar antibodies was inhibited to the extent of 83%, indicating that nearly all of the anti-Id recognized determinants near the hapten-binding site.

Elucidation of the genetic control of this family of cross-reactive Id should be of great interest. Serological, as well as amino acid sequence, analyses suggests that the family may be very heterogeneous. On the other hand, our data would lead to the prediction that there is a substantial portion of the amino acid sequence, at least in hypervariable regions, that is conserved. How heterogeneity is generated on a similar background, either through evolution of germ line genes or somatic mutation, may be

revealed by further amino acid sequence analyses and by studies at the level of DNA.

Summary

It has been shown that A/J anti-*p*-azophenylarsonate antibodies that share a major cross-reactive idiotype (CRI) comprise a family of closely related, but nonidentical, molecules. Our results demonstrate that 12 of 14 monoclonal hybridoma products that express the CRI have in common at least one highly conserved idiotypic determinant. It is proposed that this reflects conservation of a portion of the amino acid sequence, presumably in hypervariable regions. That the conserved determinant(s) are located in the region of the hapten-binding site is indicated by the ability of haptens to inhibit idiotype-anti-idiotype interactions involving the conserved, or public determinants.

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