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Immunology

Idiotypic analysis of monoclonal antibodies to poly(Glu^{60}Ala^{30}Tyr^{10})

(hybridoma antibody/idiotype/antibody diversity)

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ABSTRACT **Fifteen hybridoma anti-poly(Glu^{60}Ala^{30}Tyr^{10}) (anti-GAT) antibodies were analyzed for the presence of a common set of idiotypic specificities associated with murine anti-GAT antibodies, termed CGAT idiotype, which are present on the anti-GAT antibodies of all mouse strains. Thirteen of these monoclonal anti-GAT antibodies expressed a major fraction of CGAT idiotypic specificities. However, the remaining fraction of CGAT idiotypic specificities were not detected in individual or pooled hybridoma anti-GAT antibodies. Anti-idiotypic antisera made against each of the 15 hybridoma anti-GAT antibodies preferentially bound homologous ligand and showed minimal binding activity to specifically purified serum anti-GAT antibodies. Furthermore, the diversity of the hybridoma anti-GAT antibodies was demonstrated by the presence of individual idiotypic specificities on each of the hybridoma anti-GAT antibodies. However, relatedness among some of the hybridoma antibodies was also apparent since idiotypic analysis revealed that some hybridoma anti-GAT antibodies shared cross-reactive idiotypic specificities not associated with CGAT idiotype. The genetic mechanisms which could account for the generation of such antibody diversity are discussed.**

The antibody responses of inbred strains of mice to the synthetic copolymer of L amino acids, poly(Glu^{60}Ala^{30}Tyr^{10}), abbreviated GAT, are characterized by the presence of a common set of idiotypic specificities, termed CGAT idiotype (1). The CGAT idiotype was defined by the interaction between a guinea pig anti-idiotypic antiserum made against specifically purified, pooled D1.LP (*H-2^b, Ig-1^c*) anti-GAT antibodies and ^{125}I-labeled, specifically purified anti-GAT antibodies from a single D1.LP mouse (1, 2). The expression of CGAT idiotype in inbred strains of mice is independent of the *H-2*, *Ig-1*, and V_λ genetic polymorphisms (1). The CGAT idiotype has been identified in all individuals of 34 different inbred strains of mice and in 9 of 13 inbred strains of rats tested (refs. 1–4; unpublished results). Furthermore, poly(Glu^{50}Tyr^{50}) (GT)-related antigenic moieties are the functional determinants that are responsible for induction of antibodies with CGAT idiotype as evidenced by the ability of various GT-containing copolymers (e.g., [T,G]-A- -L) to generate CGAT idiotype in the corresponding responder mice (3, 4). In contrast, anti-poly(Glu^{60}Ala^{40}) (anti-GA) antibodies do not express CGAT idiotype (2).

To determine the heterogeneity of B cell clones able to produce anti-GAT antibodies with CGAT idiotypic specificities, we studied the idiotypic properties of a series of monoclonal anti-GAT antibodies specifically purified from hybridoma cell lines. These hybridoma anti-GAT antibodies were previously characterized in terms of their purity and antibody specificity (5). Idiotypic analyses of hybridoma antibodies have demonstrated the diversity of the antibody responses in other idiotypic systems (6, 7). This approach has certain advantages: (*i*) The monoclonal nature of hybridoma anti-GAT antibodies permits analysis of the clonal heterogeneity of the *in vivo* produced anti-GAT antibodies, and (*ii*) the availability of monoclonal anti-GAT antibodies enables us to prepare anti-idiotypic antisera against the hybridoma antibodies; these reagents can be used to further probe idiotypic determinants of anti-GAT antibodies. The present investigation describes the detailed analysis of the idiotypic properties associated with hybridoma anti-GAT antibodies and provides additional information with respect to the idiotypic nature of the immune response to GAT.

MATERIALS AND METHODS

Experimental Animals. Mice, 2- to 8-months old, were obtained either from the Jackson Laboratory, or from breeding colonies at Harvard Medical School (Boston, MA). New Zealand White rabbits and guinea pigs were purchased from the Animal Research Center, Harvard Medical School.

Polymers and Antigens. The linear synthetic copolymers of L amino acids—GAT (lot 6, average M_r 32,500 and lot 7, average M_r 90,800); poly(Glu^{60}Ala^{40}) (GA) (lot 1, average M_r 360,000); and poly(Glu^{50}Tyr^{50}) (GT) (lot 9, average M_r 133,000)—were purchased from Miles. The immunization and production of antisera against these antigens were carried out as described (1, 2).

Cell Fusion. The production of hybridoma cell lines secreting monoclonal anti-GAT antibodies has been described (5). The IgM and IgG hybridoma cell lines were derived from the fusion of spleen cells from individual DBA/2 and (C57BL/6 × DBA/2)F$_1$ mice with the P3-X63-Ag8 and NS-1 myeloma cell lines, respectively.

Radioiodination. Proteins were radiolabeled with carrier-free Na^{125}I (New England Nuclear) by using the chloramine-T method (8).

Purification of Anti-GA and Anti-GAT Antibodies. Anti-GAT antibodies from an individual D1.LP (no. 6.1) mouse, pooled D1.LP ascites, and pooled DBA/2 ascites were specifically purified by using a GAT-aminohexyl-Sepharose 4B column as described (1). Anti-GA antibodies from individual A/J ascitic fluid were purified from a GA-aminohexyl-Sepharose 4B column. Purification of hybridoma anti-GAT antibodies has been described (5). The purity of these products was demonstrated by analytical techniques including sucrose gradient or polyacrylamide isoelectric-focusing.

Preparations of Anti-Idiotypic Antisera. The production of guinea pig anti-idiotypic antiserum to pooled, specifically purified D1.LP anti-GAT antibodies has been described (2). To prepare anti-idiotypic antisera specific to individual hybridoma anti-GAT antibodies, guinea pigs were given three injections of 200 μg of protein solution in complete Freund's

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Abbreviations: GAT, poly(Glu^{60}Ala^{30}Tyr^{10}); CGAT, common idiotype associated with murine anti-GAT antibodies; GA, poly(Glu^{60}Ala^{40}); GT, poly(Glu^{50}Tyr^{50}); IdI, individual antigenic determinants; IdX, cross-specific determinants; IRI, idiotypic relatedness index; L, light; H, heavy.

adjuvant at 10-day intervals. Before use these antisera were absorbed with TEPC 183 (μ, κ) myeloma protein, normal (C57BL/6 × DBA/2)F_1 gamma globulins, and MOPC 21 (γ_1, κ) ascitic fluid.

Idiotype Binding Assays. Idiotype binding assays and the inhibition of idiotype binding were carried out as described (2), except that each assay mixture contained 500 μg of (C57BL/6 × DBA/2)F_1 gamma globulin, 20 μg of TEPC 183 (2 mg/ml), 20 μl of MOPC 21 ascitic fluid, 7–10 ng of ^{125}I-labeled ligand, and 3 μl of normal guinea pig serum containing various quantities of anti-idiotypic antiserum.

Determination of the Idiotype-Relatedness Index (IRI). The relative degree of idiotypic relatedness among individual hybridoma anti-GAT antibodies was determined according to the following formula:

$$\text{IRI} = \frac{\text{ng of homologous idiotype needed for 30\% inhibition of idiotype binding} \times 10^3}{\text{ng of inhibitor needed for 30\% inhibition of idiotype binding}}.$$

An IRI of 1000 represents complete idiotypic identity between the test sample and the homologous idiotype. Conversely, small IRI value indicates a lesser degree of crossreactivity between the test sample and the homologous idiotype.

RESULTS

Direct Binding of Hybridoma Anti-GAT Antibodies. The guinea pig anti-idiotypic antiserum, made against pooled, specifically purified D1.LP anti-GAT antibodies, was shown to bind ^{125}I-labeled anti-GAT antibodies obtained from an individual D1.LP mouse. This specific idiotypic interaction defines CGAT idiotypic specificities that are present in the anti-GAT antibodies of all mouse strains (1). Table 1 lists our laboratory designation of each hybridoma anti-GAT antibody, and a letter code is assigned to each hybridoma for convenience. In addition, the immunoglobulin class of each hybridoma anti-GAT antibody is indicated. To determine the percentage of native immunoglobulin in each hybridoma ligand, rabbit anti-MOPC 104E Ig (μ, λ) and rabbit anti-mouse Ig were used as positive controls. The hybridoma anti-GAT antibodies were then assayed for binding to the guinea pig anti-idiotypic antiserum. The results indicated that all but one hybridoma anti-GAT antibody (hybridoma I) was bound by the heterologous guinea pig anti-idiotypic antiserum. The binding ranged from 69 to 95%. Approximately the same fraction of these labeled hybridoma anti-GAT antibodies was bound by control anti-Ig antisera. Thus, after correction from the percentage of immunoglobulin in each ligand, over 90% of the ^{125}I-labeled hybridoma anti-GAT antibodies could be specifically bound by the anti-idiotypic antiserum. The idiotypic specificity of the system was demonstrated by the finding that there was no significant binding of anti-GAT antibodies from hybridoma I with the guinea pig anti-idiotypic antiserum, although nearly 90% of hybridoma I product could be bound by rabbit anti-mouse Ig antiserum. In addition, the anti-idiotypic antiserum failed to bind either ^{125}I-labeled TEPC-183 myeloma protein or (C57BL/6 × DBA/2) F_1 IgG under identical conditions.

Table 1. Binding of hybridoma anti-GAT antibodies to heterologous anti-iodiotypic antiserum and anti-Ig antisera

Hybridoma anti-GAT antibody			% ligand bound	
Code	Laboratory designation	Ig class	Anti-idiotypic antiserum*	Anti-Ig antisera†
A	F9-102.2	μ, κ	78 (1)	80 (2)
B	F9-238.9	μ, κ	69 (36‡)	77 (6)
C	F9-157.12	μ, κ	87 (16)	91 (2)
D	F9-195.6	μ, κ	91 (5)	91 (2)
E	F9-38.2	μ, κ	92 (67‡)	93 (7)
F	F9-231.3	μ, κ	89 (6)	89 (−2)
G	F9-32.2	μ, κ	88 (1)	91 (−3)
H	F9-94.6	μ, κ	93 (11)	94 (23)
I	F17-148.3	γ_1, κ	17 (6)	86 (1)
J	F17-142.2	γ_1, κ	89 (3)	89 (9)
K	F17-167.1	γ_1, κ	78 (13)	78 (4)
L	F17-174.3	γ_1, κ	95 (−4)	97 (−1)
M	F17-5.19	γ_1, κ	93 (−2)	93 (−1)
N	F17-59.2	γ_1, κ	93 (0)	94 (−2)
O	F17-97.1	γ_3, κ	88 (7)	92 (24)
	IgG§	—	5 (5)	65(8)
	TEPC183	μ, κ	4 (4)	89 (18)

* Heterologous guinea pig anti-idiotypic antiserum (3 μl) was used for idiotype binding experiments. Control experimental results with 3 μl of normal guinea pig serum are shown in parentheses.

† Rabbit anti-mouse Ig antisera (3 μl) was used for Ig binding experiments. The immune complexes were precipitated with excess goat anti-rabbit Ig antiserum. Control experimental results with 3 μl of normal rabbit serum are shown in parentheses.

‡ This nonspecific binding was observed in three separate trials.

§ (C57BL/6 × DBA/2)F_1 IgG purified from a DEAE-cellulose column.

Identification of CGAT Idiotypic Specificities on Hybridoma Anti-GAT Antibodies. Because the guinea pig anti-idiotypic antiserum was made against a pool of specifically purified D1.LP anti-GAT antibodies, it is possible that this antiserum contained multiple anti-idiotypic antibodies specific to idiotypic determinants associated with different anti-GAT antibody molecules. Therefore, direct binding by this anti-idiotypic antiserum of ^{125}I-labeled hybridoma anti-GAT antibody is not sufficient evidence for the identification of CGAT idiotypic specificities. Furthermore, direct binding experiments do not reveal the extent of the shared idiotypic specificities—i.e., whether some or all of the CGAT idiotypic specificities are present in the hybridoma anti-GAT antibodies. To resolve these points, we used varying quantities of unlabeled hybridoma anti-GAT antibodies to inhibit the binding of ^{125}I-labeled CGAT (^{125}I-CGAT) idiotype (anti-GAT antibodies from an individual D1.LP mouse) to the guinea pig anti-idiotypic antiserum. The results are shown in Fig. 1. Of the 15

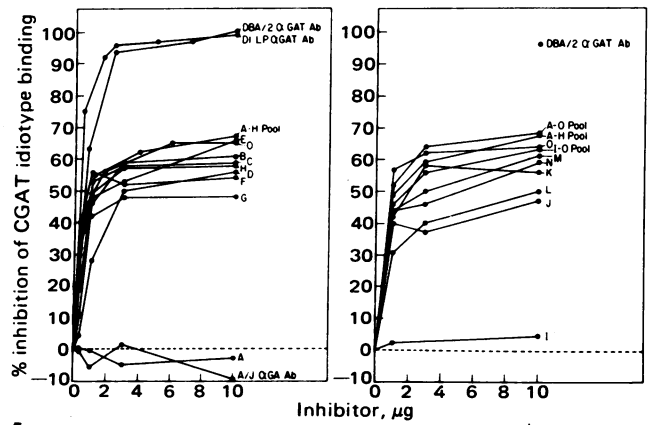

FIG. 1. Inhibition of ^{125}I-CGAT idiotype binding by hybridoma anti-GAT antibodies (Ab). (*Left*) Inhibition with 0.3–10 μg of eight IgM (A–H) and one IgG_3 (O) hybridoma anti-GAT antibodies, a pool of hybridoma (A–H) antibodies, DBA/2 and D1.LP anti-GAT antibodies (positive control), and A/J anti-GA antibodies (negative control). (*Right*) Inhibition with 1.0–10 μg of seven IgG hybridoma anti-GAT antibodies (I–O), pooled IgM (A–H), pooled IgG (I–O), as well as pooled IgM and IgG (A–O) hybridoma anti-GAT antibodies.

hybridoma anti-GAT antibodies, 13 possessed some but not all of the CGAT idiotypic specificities. This was demonstrated by the inability of each of these hybridoma anti-GAT antibodies to inhibit completely the ^{125}I-CGAT idiotype binding. A significant degree of inhibition could be obtained with 1 μg of individual hybridoma anti-GAT antibodies. However, with increasing quantities of inhibitor up to 10 μg, the degree of inhibition reached a plateau and the maximal values ranged from 45% to 70%. In contrast, nearly complete inhibition could be easily obtained with 2 μg of either D1.LP or DBA/2 anti-GAT antibodies. The specificity of the assay was demonstrated by the failure of A/J anti-GA antibodies and of hybridoma products A and I to cause detectable levels of inhibition. These results have been independently confirmed by using a rabbit anti-idiotypic antiserum against pooled D1.LP anti-GAT antibodies; all hybridoma anti-GAT antibodies except A and I yielded a maximum of 45–70% inhibition of ^{125}I-CGAT idiotype binding (data not shown).

Pools containing equal amounts of each of the hybridoma anti-GAT antibodies of the IgM and IgG class or both of the IgM and IgG classes were tested for their ability to inhibit ^{125}I-CGAT idiotype binding (Fig. 1). In all cases, the maximal degree of inhibition obtained was about 70%. This result suggests that (*i*) the CGAT specificities accounting for the 30% of the idiotypic binding not inhibited by using individual hybridoma products are not distributed among these hybridoma anti-GAT antibodies, (*ii*) CGAT idiotype contains a minimum of two distinct sets of idiotypic specificities, and (*iii*) the CGAT idiotypic specificities of each hybridoma anti-GAT antibody are similar to one another.

However, small variations in the extent of shared CGAT idiotypic specificities are suggested by the different levels of maximal inhibition of CGAT idiotype binding with each hybridoma anti-GAT antibody (Fig. 1). These conclusions are supported by the observations that the specific idiotypic binding of ^{125}I-labeled hybridoma anti-GAT antibodies from hybrids E and O with the guinea pig anti-idiotypic antiserum was completely inhibited by unlabeled homologous hybridoma anti-GAT antibody, whereas heterologous CGAT bearing hybridoma anti-GAT antibodies caused 70–100% inhibition of idiotype binding (data not shown). This implies that not all of the hybridomas share identical CGAT idiotypic specificities. This approach to analyze the idiotypic variation among individual hybridoma antibodies confirms the earlier results of Imanishi-Kari *et al.* (6).

Identification of GA-1 Idiotype. A paradoxical finding was noted in comparing the results of the direct binding and the inhibition of ^{125}I-CGAT idiotype binding assays. The guinea pig anti-idiotypic antiserum bound the anti-GAT antibody from hybridoma product A, but this monoclonal antibody did not express idiotypic specificities as determined by inhibition of CGAT idiotypic binding (Table 1 and Fig. 1). The binding of ^{125}I-labeled A to guinea pig anti-CGAT idiotypic antiserum defines a new idiotype, termed GA-1 idiotype. This idiotype is not inhibitable by the other 14 hybridoma anti-GAT antibodies except unlabeled A itself (data not shown). These results indicate that the anti-idiotypic antiserum of the guinea pig immunized with pooled D1.LP anti-GAT antibodies actually contains at least three populations of anti-idiotypic antibodies. One population of anti-idiotypic antibodies binds the major CGAT idiotypic specificities found on hybridomas other than A or I, the second recognizes CGAT determinants absent from all tested hybridoma products, and the third binds antibody from hybridoma A (unpublished results). Additional experiments (unpublished) suggest that GA-1 idiotype represents a common idiotype induced by GA antigenic moieties of GAT molecules.

Anti-Idiotypic Antisera Specific to Hybridoma Anti-GAT Antibodies. To determine the diversity of the hybridoma anti-GAT antibodies and to analyze the spectrum of idiotypes on these hybridoma anti-GAT antibodies, we prepared guinea pig anti-idiotypic antisera specific to each of the 15 hybridoma anti-GAT antibodies. After appropriate absorbtion, each anti-idiotypic antiserum strongly bound the homologous ^{125}I-labeled hybridoma anti-GAT antibodies and showed minimal binding of ^{125}I-CGAT idiotype. Moreover, these anti-idiotypic antisera did not bind ^{125}I-labeled (C57BL/6 × DBA/2) F_1 IgG or purified TEPC 183 (μ, κ) myeloma protein. As shown in Table 2, a major fraction (69–97%) of the homologous ^{125}I-labeled hybridoma anti-GAT antibodies could be bound by as little as 0.03 μl of the corresponding anti-idiotypic antiserum. In contrast, a 100-fold excess (3 μl) of these anti-idiotypic antisera did not bind more than 29% of the ^{125}I-CGAT idiotype. This is true in all cases studied. Furthermore, in another attempt to induce anti-idiotypic antiserum specific for CGAT determinants, we immunized a guinea pig with 600 μg of a pool of 13 CGAT-positive hybridoma anti-GAT antibodies. Again, the specific anti-idiotypic antiserum bound the pooled homologous ligand, but exhibited little or no binding activity to ^{125}I-CGAT idiotypic antibodies. These results indicated that CGAT idiotypic determinants on hybridoma anti-GAT antibodies are not sufficiently immunogenic to elicit significant levels of the corresponding anti-idiotypic antibodies. Instead, determinants other than CGAT idiotypic specificities must act in an immunodominant fashion, giving an anti-idiotypic antibody response directed mainly toward these non-CGAT idiotypic determinants. Moreover, these results further indicate that these major immunodominant idiotypic specificities on hybridoma anti-GAT antibodies are not frequently represented in the ^{125}I-CGAT idiotypic ligand.

Inhibition of Idiotype Binding by GAT and Related Polymers. Table 3 demonstrates the ability of GAT, GT, and GA to block the homologous hybridoma idiotype–anti-idiotypic interactions. Nine of the 14 idiotypic systems could be blocked

Table 2. Binding specificity of anti-idiotypic antisera against hybridoma anti-GAT antibodies

Anti-idiotype prepared against	Ligand bound by anti-idiotypic antiserum, %*					
	CGAT, μl		Autologous, μl		Control, μl	
	3	0.03	3	0.03	3	0.03
D1.LP anti-GAT	64	23	64	23	5	4
A	8	5	82	79	10	4
B	26	7	69	69	9	5
C	29	8	88	89	8	4
D	15	5	91	89	8	4
E	26	6	75	75	8	5
F	26	6	90	89	8	2
G	26	4	91	88	14	4
H	24	6	93	93	7	3
I	7	4	87	60	5	5
J	7	4	89	85	6	3
K	24	6	81	82	6	4
L	12	5	97	86	4	4
M	24	4	93	90	4	5
N	28	6	94	93	6	3
O†	ND‡	ND	87	87	ND	ND

* Data represent uncorrected values without subtracting background binding of normal guinea pig serum, which are given in Table 1. The background binding of CGAT ligand was 5%.

† Anti-idiotypic antiserum against hybridoma product O was further absorbed with FLOPC 21 (γ_3, κ) ascitic fluid.

‡ ND indicates not determined.

Table 3. Idiotypic interrelationships among hybridoma anti-GAT antibodies

Inhibitors	Homologous idiotype–anti-idiotypic interaction														
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
Polymers*															
GAT	2	10	−1	2	82	65	39	0	85	74	84	70	86	54	ND†
GT	14	16	22	2	90	41	28	20	10	84	88	55	79	81	ND
GA	2	6	−2	3	8	3	4	2	81	−1	4	14	3	2	ND
Hybridoma antibodies‡															
A	1000	<3	<3	<5	<0.1	<1	<3	<3	<0.1	<0.1	<3	<0.1	<3	<0.6	<5
B	<3	1000	<3	<5	1000	<1	<3	<3	<0.1	<0.1	<3	<0.1	<3	<0.6	<5
C	<3	<3	1000	<5	<0.1	<1	<3	<3	<0.1	<0.1	<3	<0.1	<3	<0.6	<5
D	<3	<3	<3	1000	3	<1	<3	<3	<0.1	<0.1	<3	<0.1	<3	<0.6	<5
E	<3	1000	<3	<5	1000	<1	16	<3	<0.1	<0.1	<3	<0.1	<3	<0.6	<5
F	<3	<3	3	<5	<0.1	1000	<3	<3	<0.1	<0.1	<3	<0.1	<3	<0.6	<5
G	<3	50	14	<5	45	<1	1000	<3	<0.1	<0.1	<3	<0.1	<3	<0.6	<5
H	<3	<3	<3	<5	<0.1	<1	<3	1000	<0.1	<0.1	<3	<0.1	<3	<0.6	<5
I	<3	<3	<3	<5	<0.1	<1	<3	<3	1000	<0.1	<3	<0.1	<3	1.2	<5
J	<3	<3	<3	<5	<0.1	<1	<3	<3	<0.1	1000	<3	<0.1	<3	<0.6	<5
K	<3	<3	<3	<5	<0.1	<1	<3	<3	<0.1	<0.1	1000	<0.1	<3	<0.6	<5
L	<3	<3	<3	<5	<0.1	<1	<3	<3	<0.1	<0.1	<3	1000	<3	<0.6	50
M	<3	<3	<3	<5	<0.1	<1	<3	<3	<0.1	<0.1	<3	<0.1	1000	<0.6	<5
N	<3	<3	4.5	<5	<0.1	<1	<3	<3	0.5	<0.1	<3	<0.1	<3	1000	<5
O	<3	<3	<3	<5	<0.1	<1	<3	<3	<0.1	<0.1	<3	<0.1	<3	<0.6	1000

* Polymers at 50 μg were used. Results are expressed as % inhibition of idiotype binding.
† ND indicates not determined.
‡ Results are expressed as IRI.

by appropriate polymers. The sensitivity of idiotype–anti-idiotypic interactions to GAT-related polymers correlates with the fine specificity patterns of the hybridoma anti-GAT antibodies (5). Thus, the idiotypic binding of hybridoma anti-GAT antibodies having preferential binding activity to GT are inhibited by GT but not GA. Conversely, GA but not GT is a potent inhibitor of the idiotypic binding of hybridoma anti-GAT antibody I, which has binding to GA and little or no detectable binding activity to GT (7).

Idiotypic Interrelationship among Hybridoma Anti-GAT Antibodies. Inhibition of idiotypic binding was carried out in crisscross fashion to determine the idiotypic "relatedness" or "individuality" of hybridoma anti-GAT antibodies (Table 3). The results are expressed as idiotypic relatedness index, IRI. The specificity of the assay system was demonstrated by the finding that the homologous unlabeled hybridoma anti-GAT antibodies were always potent inhibitors of idiotype binding, and, generally, a 300- to 10,000-fold excess of heterologous hybridoma anti-GAT antibodies did not cause significant inhibition. In agreement with the direct binding data using ^{125}I-CGAT idiotype as ligand (Table 2), each hybridoma idiotypic system primarily detected the specificities associated with the hybridoma anti-GAT antibodies but not CGAT idiotype. This conclusion is based on the observations that individual DBA/2 anti-GAT antisera and a pool of DBA/2 anti-GAT antisera, which were powerful inhibitors of CGAT idiotype binding, were either extremely weak or noninhibitory for the idiotype binding of hybridoma anti-GAT antibodies (data not shown) and that many of the CGAT-positive hybridoma anti-GAT antibodies failed to cross-inhibit the idiotype binding of other CGAT-positive hybridoma anti-GAT antibodies (Table 3). Eight out of 15 anti-idiotypic antisera (anti-A, -D, -F, -H, -J, -K, -L, -M) recognized the idiotypic determinants uniquely associated with homologous hybridoma anti-GAT antibodies, as indicated by the lack of detectable inhibition by any other hybridoma anti-GAT antibodies. Table 3 also demonstrated that each of the 15 hybridoma anti-GAT antibodies expressed "individual" idiotypic specificities. This is best illustrated by comparing the IRI values of the hybridoma anti-GAT antibodies. Complete idiotypic identity was not observed between any two hybridoma anti-GAT antibodies. Thus, it is concluded that virtually every hybridoma cell line was derived from a unique B cell clone. These idiotypes clearly resemble those "individual" idiotypes described by Kunkel (9), individual antigenic determinants ("IdI") described by Lieberman *et al.* (10), and "private" idiotypes described by Ju *et al.* (11). However, it should be noted that most of these hybridoma anti-GAT antibodies also shared CGAT idiotypic specificities (Fig. 1). Thus, a hybridoma anti-GAT antibody can possess a set of common idiotypic specificities and a set of "individual" idiotypic specificities.

Additional shared idiotypic specificities other than CGAT idiotype were detected among individual anti-GAT antibodies. This type of idiotypic crossreaction appears to be equivalent to cross-specific determinants ["IdX" as defined by Lieberman *et al.* (10)]. Seven anti-idiotypic antisera recognized homologous idiotypic determinants, some of which are present in other hybridoma anti-GAT antibodies. In general, the heterologous IRI values were low, which indicated weak and nonidentical idiotypic crossreaction. In one case (hybridomas B and E), strong idiotypic crossreactions were observed. This suggests that hybridoma cell lines B and E may be derived from closely related B cell clones. This conclusion is strengthened by the finding that the anti-idiotypic reagents made against hybridomas B and E detect distinct idiotypic determinants, one associated with the antibody combining site and the other a presumed framework idiotypic determinant (Table 3).

DISCUSSION

This paper describes the idiotypic properties of eight IgM and seven IgG hybridoma anti-GAT antibodies. Thirteen of the 15 hybridoma anti-GAT antibodies expressed CGAT idiotypic specificities. This agrees well with our previous findings which showed that the majority of the *in vivo* induced IgM and IgG anti-GAT antibodies bear CGAT idiotypic specificities (3). However, in contrast to the anti-GAT antisera which express all CGAT idiotypic specificities, each of the hybridoma anti-GAT antibodies expressed only a fraction of the CGAT idiotypic

specificities. Similar disparities between the idiotypic specificities present in antiserum vs. those on hybridoma antibodies have been reported by Imanishi-Kari *et al.* (6). The consistency of our finding that approximately the same fraction of CGAT idiotypic determinants was present on 13 different anti-GAT hybridoma antibodies emphasizes the validity of these results. Furthermore, these findings were also verified by using a rabbit anti-CGAT idiotypic antiserum, which gave identical results.

The most likely explanation for this observation is that CGAT idiotype actually consists of at least two sets of idiotypic specificities: one set, defined with hybridoma anti-GAT antibodies used in the present study, constitutes the major fraction of CGAT idiotypic determinants as evidenced by 45–70% inhibition of CGAT idiotype binding with most of the hybridoma anti-GAT antibodies. The second set of CGAT idiotypic specificities was not identified in the present study but is present in immune anti-GAT antibodies. Two possibilities might account for the inability to detect the minor fraction of CGAT idiotypic specificities in the hybridoma anti-GAT antibodies. It is possible that the minor set of CGAT idiotypic determinants may be heterogeneous, and idiotypic specificities of each of the monoclonal anti-GAT antibodies would be expected to contribute a small fraction to the second set of CGAT idiotypic specificities. Consequently, neither pooled nor individual hybridoma anti-GAT antibodies may completely inhibit CGAT idiotype binding. An alternative explanation is that the second set of idiotypic specificities represents the product of a single clone with low frequency and hence was not selected by the cell fusion process.

Because most of these hybridoma anti-GAT antibodies were shown to contain predominantly one species of L chain (5), presumably not the MOPC 21 L chain, it is unlikely that the majority of the observed individual idiotypic specificities associated with hybridoma anti-GAT antibodies are generated by the interaction between MOPC 21 light (L) chains and anti-GAT heavy (H) chains. Furthermore, the possibility that unique posttranslational modification of C regions, such as carbohydrate moieties, generated the individual determinants on the hybridomas was excluded, because most of these anti-idiotypic antisera detect determinants associated with the antibody combining sites. Collectively, this and other observations presented in this investigation strongly suggest that the idiotypic specificities of hybridoma anti-GAT antibodies reflect the idiotypic specificities of the corresponding B cell clones, rather than artifacts of the cell fusion process.

We have previously shown that antisera with GT but not GA binding specificity contained CGAT idiotypic specificities (1). The correlation between the fine specificity patterns and the expression of CGAT idiotypic specificities was also demonstrated in all 15 hybridoma anti-GAT antibodies. Thirteen CGAT-positive hybridoma anti-GAT antibodies exhibited preferential binding activity to GT polymer and the two CGAT-negative hybridoma anti-GAT antibodies selectively bound GA polymer (5). The complete concordance of CGAT idiotypic determinants and GT binding specificity among the 15 hybridoma anti-GAT antibodies tested suggests that CGAT idiotypic determinants are associated with the GT combining sites.

The result obtained with the crisscross inhibition of idiotype binding among the hybridoma anti-GAT antibodies provides additional information. The hierarchy of idiotype relatedness among hybridoma anti-GAT antibodies can be classified into four levels: (*i*) CGAT idiotypic specificities, (*ii*) strongly crossreactive idiotypes such as hybridoma products B and E, (*iii*) weakly crossreactive idiotypes, and (*iv*) individual idiotypes. The finding that each of the 15 hybridoma anti-GAT antibodies possesses a distinct group of idiotypic specificities strongly suggests that the anti-GAT antibody response is extremely heterogeneous and implies that different H or L chains or both are utilized to generate anti-GAT antibodies. In spite of such heterogeneity, the majority of these hybridoma anti-GAT antibodies shared a major fraction of CGAT idiotypic specificities and other "IdX" idiotypic specificities. To account for such complex idiotypic relationships, we propose that (*i*) the major CGAT idiotypic specificities are encoded by a structural germ line *V* gene (V_H, V_L, or both) and that (*ii*) hybridoma anti-GAT antibodies were derived from genes that had undergone a series of either random or programmed somatic mutational processes that generated new idiotypic determinants while retaining the ability to bind GT determinants on the GAT molecule and express CGAT idiotypic specificities. In support of this hypothesis was the observation that, in addition to CGAT idiotypic specificities, the hybridoma anti-GAT antibodies shared additional idiotypic specificities with one another and exhibited individual idiotypic specificities as well. This genetic process of somatic mutation gives rise to many different B cell clones, each producing anti-GAT antibodies related by their idiotypic specificity. Analysis of anti-(4-hydroxy-3-nitrophenyl) acetyl and anti-*p*-azophenylarsonate hybridoma antibodies shows similar idiotypic families (6, 7). Individual idiotypic specificities in the hybridomas may reflect variation contributed by the J segment, as suggested by Clevinger *et al.* (12). In this context, DNA sequence determinations of the genes encoding the immunoglobulin *V* genes of these hybridoma cell lines will be interesting and may provide additional information with regard to the mechanisms of generating antibody diversity.

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