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Immunology

Fine specificity of antibodies to poly(Glu^{60}Ala^{30}Tyr^{10}) produced by hybrid cell lines

(cell hybridization/monoclonal antibodies/idiotype)

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ABSTRACT **The polyethylene glycol-mediated cell fusion technique has been used to analyze the diversity of the antibody response to the terpolymer poly(Glu^{60}Ala^{30}Tyr^{10}) (GAT). Nine stable clones (all producing IgM κ anti-GAT antibodies) were isolated from a fusion between P3-X63-Ag8 myeloma cells and spleen cells from a DBA/2 mouse sensitized to GAT 5 days earlier. Seven other clones (producing IgG κ anti-GAT antibodies) were derived from another fusion between NS1 myeloma cells and spleen cells of (C57BL/6 × DBA/2)F$_1$ hybrid mice hyperimmunized with GAT. These 16 anti-GAT antibodies were grouped according to their pattern of reactivity with GAT and the two related polymers of poly(Glu^{60}Ala^{40}) (GA) and poly(Glu^{50}Tyr^{50}) (GT). Two monoclonal anti-GAT antibodies (IgM F9-102.2 and IgG F17-148.3) demonstrated crossreactivity with GA but failed to crossreact with GT determinants. In contrast, the remaining 14 hybridoma antibodies demonstrated preferential reactivity with GAT but also exhibited crossreactive binding to GT and in some cases GA. There was a correlation between the fine specificity pattern and the presence of a common anti-GAT idiotype on these antibodies. Thus, the hybridoma anti-GAT antibodies which reacted with GT shared crossreactive idiotypic determinants (CGAT) present in mouse anti-GAT immune sera. In contrast, the monoclonal F9-102.2 and F17-148.3 antibodies that failed to bind to GT lacked the major CGAT idiotypic determinants.**

Synthetic polypeptide polymers have been used successfully to study the regulation of B and T cell immunity. For instance, the *H-2* linked *Ir* gene-controlled immune responses to the terpolymer poly(Glu^{60}Ala^{30}Tyr^{10}) (GAT) have been extensively analyzed at the T and B cell levels. At the T cell level, GAT is immunogenic *in vivo* and *in vitro* in responder mice, whereas it must be coupled to an immunogenic carrier to elicit an antibody response in the nonresponder mice bearing the *H-2^{p,q,s}* haplotypes. After immunization with GAT, nonresponder mice develop suppressor T cells, which produce I region-coded GAT-specific soluble suppressor factor(s) (GAT-T$_s$F) (1, 2). The anti-GAT response has also been investigated at the B cell level by idiotypic analysis. A guinea pig anti-idiotypic antiserum, raised against purified D1.LP anti-GAT antibodies, recognizes idiotypic specificities present on the majority of anti-GAT antibodies from 34 different responder or nonresponder mouse strains, and also from some strains of inbred rats (3, 4). This crossreactive idiotype (CGAT) is associated with antibodies having preferential binding for the copolymer poly(Glu^{50}Tyr^{50}) (GT). Conversely, antibodies against the copolymer of poly(Glu^{60}Ala^{40}) (GA) bind GAT but lack CGAT idiotypic determinants (5, 6). Recent evidence from this laboratory demonstrated shared specificities between D1.LP CGAT idiotype present on anti-GAT antibodies and SJL or DBA/1 nonresponder GAT-T$_s$F (7). These data support the concept that for GAT a conserved set of variable gene regions are expressed on Ig receptors of B cells as well as on the suppressor T cell mediator molecules. To analyze the diversity and structure of the variable (V) regions of these anti-GAT antibodies and be able to compare these V regions with the antigen binding site of GAT-T$_s$F at a later stage, we have used the polyethylene glycol-induced hybridization technique (8) to obtain cell lines secreting GAT-specific antibodies. This report describes the production of hybridoma anti-GAT cell lines and characterizes the diversity of the anti-GAT response by fine specificity analysis of the hybridoma anti-GAT antibodies with various related polymers. In addition, this report correlates the expression of CGAT idiotypic determinants with the fine antigenic specificity of these hybridoma antibodies.

MATERIALS AND METHODS

Mice. Eight- to ten-week-old male DBA/2 (*H-2^d*), (BALB/c × DBA/2)F$_1$, (CD2F$_1$, *H-2$^{d/d}$*), and (C57BL × DBA/2)F$_1$ (B6D2F$_1$, *H-2$^{b/d}$*) mice were purchased from the Jackson Laboratory and maintained in our animal facilities on standard laboratory chow and chlorinated water ad lib.

Antigens and Immunizations. The random polymers poly(Glu^{60}Ala^{30}Tyr^{10}), lot 7, average M_r 90,800; poly(Glu^{50}Tyr^{50}), lot 9, average M_r 133,000; poly(Glu^{60}Ala^{40}), lot 1, average M_r 360,000; and polyGlu, lot 135, average M_r 18,000, were purchased from Miles. Mice were immunized intraperitoneally with 100 μg of GAT in 0.2 ml of saline containing 5% aluminum–magnesium hydroxides gel (Maalox) (W. H. Rorer, Ft. Washington, PA) and 2 × 10^9 heat-killed *Bordetella pertussis* bacteria (Michigan Department of Public Health, Lansing, MI) as adjuvants (Maalox–pertussis).

Mouse B Cell–Myeloma Hybridizations. The BALB/c hypoxanthine phosphoribosyltransferase-negative P3-X63-Ag8 myeloma cell line (which secretes the γ$_1$, κ MOPC 21 Ig) and its nonsecreting variant NS.1 (9) (which were kindly provided by Inga Melchers, Stanford University, CA) have been used for the hybridizations described in this paper. These lines were maintained *in vitro* in Dulbecco's modified Eagle's medium containing 4.5 g of glucose per liter without sodium pyruvate (GIBCO) and supplemented with 10% heat-inactivated fetal calf serum (Reheis Chemical Company, Phoenix, AZ, lot 61306) and penicillin/streptomycin. Fusion 9 was carried out between spleen cells obtained from an individual DBA/2 mouse, primed 5 days earlier with 100 μg of GAT (in Maalox–pertussis), and X63 myeloma cells. For fusion 17, NS1 myeloma cells were

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Abbreviations: GA, poly(Glu^{60}Ala^{40}); GAT, poly(Glu^{60}Ala^{30}Tyr^{10}); GT, poly(Glu^{50}Tyr^{50}); CGAT, crossreactive idiotype associated with anti-GAT antibodies; GAT-T$_s$F, GAT-T suppressor factor; HGPRT, hypoxanthine guanine phosphoribosyl transferase; Ir, immune response; PFC, plaque-forming cell; SRBC, sheep erythrocytes; SPRIA, solid phase radioimmunoassay.

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hybridized with spleen cells prepared 3 days after challenging (100 μg of GAT in Maalox–pertussis) B6D2F$_1$ mice primed with GAT 14 weeks earlier in the same manner. In each experiment 10^8 spleen cells were hybridized with 10^7 myeloma cells by using polyethylene glycol (PEG 1540, Baker, average M_r 1300–1600) (10), according to the technique described by Galfré *et al.* (11) with the following modifications: after fusion the cells were suspended in medium supplemented with 20% fetal calf serum and were seeded in 298 wells of microtiter plates (Linbro, no. 76.003,05) together with 3×10^4 untreated spleen cells per well as feeder cells. After 24 hr, the culture supernatants were gradually replaced by selective medium containing hypoxanthine, aminopterin, and thymidine (HAT) (12). Between 12 and 20 days after fusion vigorous growth was taken as a successful hybridization.

Screening for Anti-GAT Antibodies Produced by Hybridomas. Several techniques have been used to detect the anti-GAT antibodies produced by hybridomas.

(*i*) *Direct or rabbit anti-mouse IgG facilitated hemagglutination of GAT-coupled sheep erythrocytes (SRBC) by hybridoma culture supernatants.* This test was performed in V bottom plates (Linbro, no. 76.332.05), using 50 μl of 0.5% SRBC that were coupled to GAT by the chromium technique (13) and were suspended in phosphate-buffered saline containing 1% bovine serum albumin and 25 μl of 1:2 dilutions of test samples. For facilitation, 25 μl of a 1:100 dilution of hyperimmune rabbit anti-mouse IgG was added to each well.

(*ii*) *Plaque-forming cell (PFC) activity of hybridoma cells.* This was detected by a slide modification of the Jerne hemolytic plaque assay as described (13). Depending on the Ig class of anti-GAT antibodies produced by hybridoma cells, either direct IgM plaques [detected on GAT–SRBC coupled with poly(L-lysine) (14)], rabbit anti-μ (MOPC 104E, μλ$_1$) facilitated IgM plaques, or rabbit anti-IgG facilitated IgG plaques (both detected on GAT–SRBC coupled by the chromium chloride method) were assayed.

(*iii*) *Solid phase radioimmunoassay (SPRIA).* SPRIA was performed according to the technique of Klinman *et al.* (15). Briefly, polyvinylchloride microtiter plates (Cooke Laboratory Products Division, Dynatech Laboratories, Inc., Alexandria, VA) were coated with a 1 mg/ml solution of the appropriate antigens [GAT, GA, GT, and poly(Glu)] in phosphate-buffered saline, extensively washed with 2% fetal calf serum in phosphate-buffered saline, and incubated for 2 hr with 25 μl of hybridoma culture supernatant. After repeated washings the bound antibodies were quantified by using a ^{125}I-labeled rabbit anti-mouse Fab or anti μ, γ$_1$, γ$_{2a}$, γ$_{2b}$, κ, λ$_1$ (gifts of J. Weinberger and M. Dietz, Department of Pathology, Harvard Medical School and N. Klinman, Scripps Clinic, La Jolla, CA).

Cloning of Anti-GAT Producing Hybrids. Cells from microwells with positive tests upon screening were cultured in larger volumes and aliquots were frozen in medium containing 10% fetal calf serum and 10% dimethyl sulfoxide (Sigma). Cloning in soft agar was performed according to the technique of Coffino and Scharff (16) without the fibroblast layer. Cloning efficiency showed great variability (ranging from 0.1 to 10%) from one mass hybridoma culture to another. After 10–20 days, the isolated clones were transferred from soft agar to liquid growth medium.

***In Vivo* Passages.** CD2F$_1$ mice were injected intraperitoneally with 0.5 ml of Pristane (2,6,10,14-tetramethylpentadecane) (Aldrich). Five to thirty days later groups of mice were inoculated with $1–5 \times 10^6$ hybridoma cells derived from fusion 9. Within 20 days ascites usually developed in most of the mice.

Purification of Hybridoma Antibodies. Monoclonal anti-GAT antibodies present in hybridoma culture supernatants (10–30 μg/ml) or in ascitic fluid (up to 10 mg/ml) were precipitated with cold ammonium sulfate (pH 7.0) at a final concentration of 50% saturation, dialyzed overnight against phosphate-buffered saline, and then purified by affinity chromatography using GT- or GAT-immunoadsorbents (17). The bound antibodies were eluted with 0.1 M glycine·HCl, pH 2.5, and immediately neutralized with 1 M Tris·HCl, pH 8.0. GT- and GAT-Sepharose eluates were further purified by chromatography on a Bio-Gel A5M (Bio-Rad) column (140 × 4 cm) equilibrated with 0.15 M NaCl/0.01 M Tris/2 mM NaN_3 at pH 8.0. The IgG$_3$ κ antibodies present in the culture supernatants of clone F17-97.1 were absorbed on protein A-Sepharose (Pharmacia) (18) in the cold at pH 8.0 and eluted with 0.1 M glycine·HCl, pH 2.7.

Determination of the Presence of CGAT Idiotype on Hybridoma Anti-GAT Antibodies. The identification of CGAT idiotypic specificities on purified hybridoma anti-GAT antibodies was carried out by testing the ability of various amounts of these monoclonal antibodies to inhibit the binding of guinea pig anti-idiotypic antiserum to purified ^{125}I-labeled D1.LP anti-GAT antibodies as described (3).

RESULTS

Derivation of Hybrid Cell Lines Producing Anti-GAT Antibodies. The monoclonal anti-GAT antibodies analyzed in this study have been obtained from hybridomas derived from two hybridization experiments. One fusion (no. 9) was carried out between P3-X63-Ag8 myeloma cells and recently sensitized DBA/2 mouse spleen cells. Vigorous growth was observed in 268 of 278 wells after 3 weeks of culture. The direct hemagglutination of GAT–SRBC (which detects less than 0.1 μg of anti-GAT antibody per ml) was used as the screening assay for detecting clones producing anti-GAT antibodies. Supernatants from 82 of the 268 wells showing hybrid growth gave a positive hemagglutination with a titer ≥1:16. Upon subsequent passages, many of these hybridomas lost their anti-GAT producing activity or stopped growing. Nevertheless, 32 bulk hybrid lines were still positive after 5 weeks and 9 stable clones have been derived from separate fusion events by soft agar cloning. The characteristics of the second fusion (no. 17) between GAT hyperimmune B6D2F$_1$ spleen cells and NS1 myeloma cells were similar: hybridoma cells grew in 276 of 288 wells, and anti-GAT antibodies were detected in 94 wells by either facilitated hemagglutination of GAT–SRBC or SPRIA using a radiolabeled rabbit anti-mouse Fab ligand. Seven distinct clones were derived from this fusion. The 16 cloned cell lines obtained from these two experiments have been stable *in vitro, in vivo,* or both for more than 6 months.

Ig Classes of Hybridoma Anti-GAT Antibodies. The heavy and light chain classes of these antibodies have been determined by either (*i*) SPRIA using GAT-coated plates and ^{125}I-labeled class-specific anti-mouse Ig antisera ligands or (*ii*) the Ouchterlony immunodiffusion technique using class-specific antisera (Bionetics, Kensington, MD or gifts of R. Asofsky, National Institutes of Health, Bethesda, MD). It was apparent that, in the case of fusion 9, most of the positive hybrids detected during the initial screening and all the stable cloned lines secreted anti-GAT antibodies of the IgM κ class. In contrast, 6 of the 7 stable cloned lines derived from fusion 17 secreted anti-GAT antibodies of the γ$_1$ κ class. Clone F17-97.1 was an exception in that it produced IgG$_3$ κ anti-GAT antibodies.

Purification of Hybridoma Anti-GAT Antibodies. Monoclonal anti-GAT antibodies were purified either on GT-Sepharose (Fusion 9: lines 238.9, 32.2, 195.6, 38.2, 231.3, 157.12, 94.6,

150.3, all IgM) or on GAT-Sepharose (Fusion 9: line 102.2, IgM; and Fusion 17: lines 5.19, 59.2, 142.2, 170.1, 148.3, 174.3, 167.1, all IgG) immunoadsorbents. The IgG_3 κ antibodies produced by clone F17-97.1 were purified on protein A-Sepharose. Almost all the IgM antibodies eluted from GAT- and GT-Sepharose were further purified on a Bio-Gel A5M column. Fig. 1 shows the results of sodium dodecyl sulfate gel electrophoretic analysis of 10 specifically purified IgM and IgG hybridoma anti-GAT antibodies run under reducing conditions on a 3–13% gradient gel (19). A single heavy chain (in the μ region) was found in all the IgM anti-GAT analyzed, in spite of the use of the secreting myeloma X63 for fusion 9. Only one γ heavy chain also characterized the IgG anti-GAT antibodies because the nonsecreting myeloma NS1 was used for fusion 17. However, in several of the IgM samples two bands could be discerned in the 23,000 M_r region, probably reflecting the presence of two light chains in a given anti-GAT antibody, as reported (20). Isoelectric focusing and precipitability after ^{125}I-labeling by rabbit anti-Ig or by guinea pig anti-CGAT idiotype further confirmed the purity and the monoclonal origin of these hybridoma antibodies (unpublished results).

Fine Specificity of Anti-GAT Hybridoma Antibodies. Hybridoma anti-GAT antibodies, because of their monoclonal origin, can be used to estimate the diversity of the anti-GAT antibody responses at the combining site level. First, the fine specificity of these antibodies with respect to the related polymers GA, GT, and GAT has been investigated by using three different techniques:

(*i*) *Hemagglutination of GAT, GA, or GT coupled SRBC.* Table 1, which presents the results obtained with the IgM anti-GAT antibodies derived from fusion 9, shows that 8 of 9 of these antibodies agglutinated GAT- or GT-SRBC with comparable titers. The F9-102.2 antibodies were an exception, because they did not agglutinate GT-SRBC but did react with GA-SRBC.

(*ii*) *PFC activity of hybridoma cells.* Fig. 2 shows that, when various amounts (2500 to 93 ng per slide) of soluble antigens were used to inhibit plaque formation by GAT-specific hybridoma cells, two different patterns of inhibition could be discerned: (*i*) clone F9-157.12 facilitated IgM plaques and clone

Table 1. Hemagglutination titers of IgM anti-GAT hybridoma antibodies on GAT, GT, and GA-coupled SRBC

IgM anti-GAT antibody*	Hemagglutination titer†		
	GAT-SRBC‡	GT-SRBC§	GA-SRBC‡
F9-102.2	6	0	5
F9-238.9	7	6	0
F9-157.12	10	10	0
F9-195.6	10	10	0
F9- 38.2	8	7	0
F9-231.3	11	11	0
F9- 32.2	11	8	0
F9-150.3	12	11	0
F9- 94.6	11	9	0

* Affinity purified hybridoma IgM anti-GAT was adjusted to a protein concentration of 60 μg/ml in phosphate-buffered saline.
† $\log_2$ of hemagglutination titer.
‡ GAT and GA were coupled to SRBC as described (13).
§ GT-SRBC were prepared by using the coupling reagent dinitrodifluorobenzene (21).

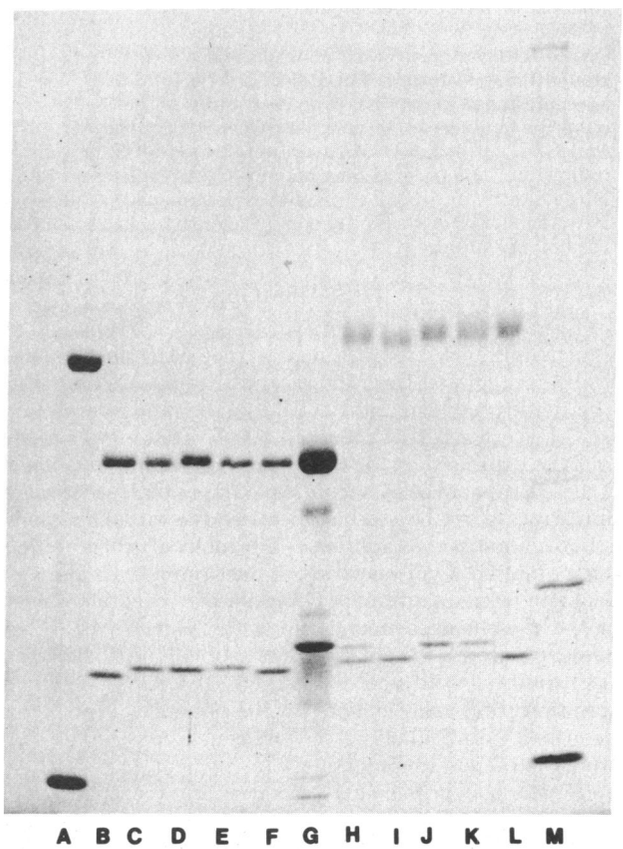

FIG. 1. Sodium dodecyl sulfate gel electrophoretic analysis of specifically purified hybridoma anti-GAT antibodies. A 3–13% gradient gel was run under reducing conditions at 240 V (constant) for 7 hr. Proteins were stained with Coomassie blue. Channels: A, markers (bovine serum albumin, cytochrome c); B–F, IgG anti-GAT F17-174.3, F17-170.1, F17-148.3, F17-59.2, F17-167.1; G, markers; H–L, IgM anti-GAT F9-195.6, F9-231.3, F9-238.9, F9-38.2, F9-94.6; M, markers.

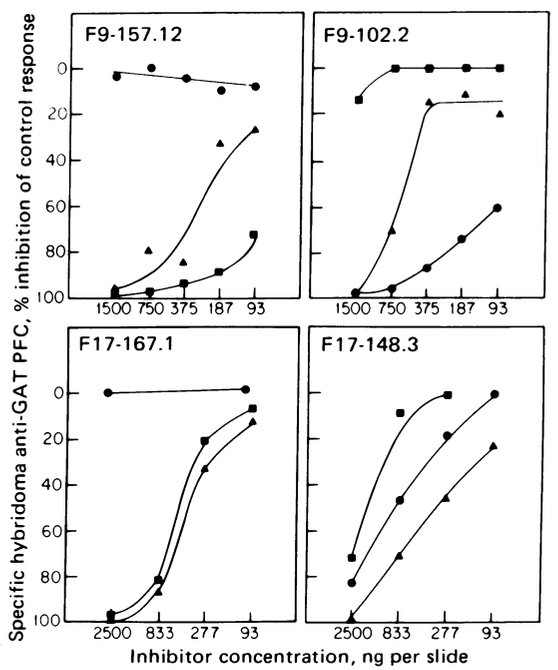

FIG. 2. Specificity of hybridoma anti-GAT PFC. Rabbit anti-μ facilitated IgM (clone F9-157.12 and F9-102.2) and rabbit anti-IgG facilitated IgG (clone F17-167.1 and F17-148.3) anti-GAT PFC were detected by using a slide modification of the Jerne hemolytic plaque assay (13) with GAT-SRBC as indicator cells. Various amounts (from 2.5 μg to 93 ng) of GAT (▲), GT (■), and GA (●) polymers were tested for inhibition of these hybridoma anti-GAT PFC.

F17-167.1 indirect IgG plaques were strongly inhibited by GT and GAT but unaffected by GA; (*ii*) conversely, GA and GAT inhibited more efficiently than GT clone F9-102.2 IgM plaques and clone F17-148.3 IgG plaques.

(*iii*) *SPRIA.* Various dilutions of affinity purified monoclonal anti-GAT antibodies were tested for their ability to bind to GAT, GT, or GA coated plates. Fig. 3 represents the specific binding to these antigens of four hybridoma anti-GAT antibodies (IgM F9-231.2 and F9-102.2; IgG F17-59.2 and F17-148.3). It is apparent that all these antibodies bind to GAT. However, they differ with regard to their GA and GT cross-reactivity. Thus, IgM F9-102.2 and IgG F17-148.3, which react with GAT and with GA, failed to show detectable levels of binding to GT. Another pattern of fine specificity is illustrated by IgM F9-231.3 and IgG F17-59.2 antibodies, which demonstrated preferential reactivity with the homologous GAT polymer and also considerable cross-reactivity to GT and GA, as is the case for the majority of the remaining clones studied. However, none of these clones exhibited crossreactivity with poly(Glu) (data not shown). Table 2 presents the SPRIA fine specificity data in detail as the amount of purified anti-GAT antibody giving an end point binding of 1000 cpm on GAT, GA, or GT coated plates. Table 2 also summarizes preliminary results that indicate that the expression of CGAT idiotypic determinants might be correlated with the fine antigenic specificity pattern of these hybridoma anti-GAT antibodies. One microgram of specifically purified hybridoma anti-GAT antibodies that exhibit crossreactivity with GT polymer was able to inhibit (≥40%) the binding of ^{125}I-labeled D1.LP anti-GAT antibodies to the guinea pig anti-CGAT antiserum. In contrast, the two GT nonreactive antibodies F9-102.2 and F17-148.3 did not exert any significant level of inhibition of idiotype–anti-idiotype binding (≤10%), indicating that they lack major CGAT idiotypic determinant(s).

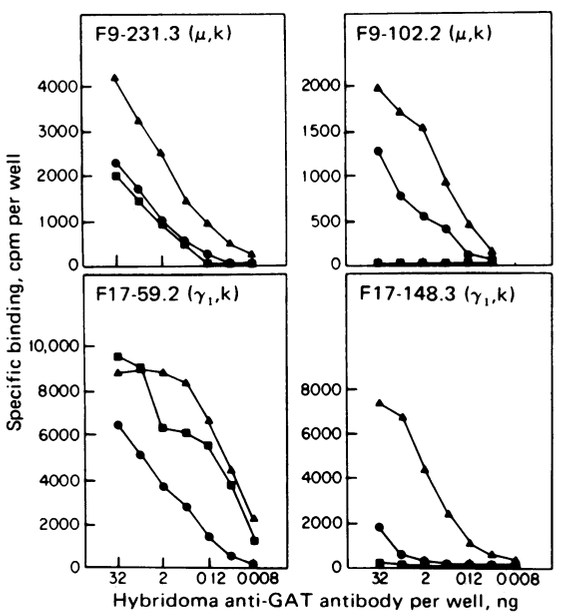

FIG. 3. Fine specificity of purified anti-GAT hybridoma antibodies. The binding of various amounts (32 ng to 8 pg per well) of affinity purified IgM (F9-231.3 and F9-102.2) and IgG_1 (F17-59.2 and F17-148.3) to GAT (▲), GT (■), GA (●) coated plates was determined by SPIRA using ^{125}I-labeled rabbit anti-mouse Fab as ligand. The nonspecific binding of these antibodies to the antigen-coated plates has been estimated by using an irrelevant purified hybridoma antibody (see legend to Table 2).

Table 2. Fine specificity of hybridoma anti-GAT antibodies and its relationship to the expression of CGAT idiotypic determinants

Hybridoma* anti-GAT antibody	$Ig^{\dagger}$ (κ) class	End point binding, ng‡ GAT	GT	GA	Presence of CGAT determinants§
F9-102.2	IgM	0.5	<8300	16	−
F9-238.9		0.5	0.02	2	+
F9-157.12		0.01	6.5	1	+
F9-195.6		0.25	16	2.5	+
F9- 38.2		0.5	1.5	0.25	+
F9-231.3		0.12	2	2	+
F9- 32.2		0.03	8	32	+
F9-150.3		0.12	8	8	+
F9- 94.6		0.25	8	1	+
F17-148.3	IgG_1	0.12	130	16	−
F17-142.2		0.01	0.02	0.5	+
F17-167.1		0.03	0.03	0.12	+
F17-174.3		0.12	0.01	4	+
F17- 5.19		0.008	0.008	4	+
F17- 59.2		<0.008	<0.008	0.04	+
F17- 97.1	IgG_3	0.5	0.5	120	+

* Specifically purified hybridoma anti-GAT antibodies.

† See *Materials and Methods.*

‡ Various amounts (from 8.3 μg to 8 pg per well) of purified hybridoma anti-GAT antibodies were tested for their ability to bind to GAT, GT, or GA coated plates. The bound antibodies were quantified by using ^{125}I-labeled rabbit anti-mouse Fab. The binding of identical amounts of purified hybridoma IgG anti-$Ig5^a$ antibodies (clone 11-6.3, derived in the Department of Genetics, Stanford University, CA) to these antigen-coated plates has been considered as background and was subtracted from each experimental value. The data are expressed as the amount of purified anti-GAT antibodies giving a specific binding of 1000 cpm per well.

§ Anti-GAT antibodies (1 or 10 μg) were tested for their ability to inhibit the binding of guinea pig anti-CGAT antiserum to ^{125}I-labeled D1.LP anti-GAT antibodies as described (3). −, Less than 10% of inhibition of idiotype binding; +, more than 40% of inhibition of idiotype binding.

DISCUSSION

GAT represents a model to study the T or B cell functions that regulate immune responses in an *Ir* gene controlled system. It has been suggested from the recent demonstration of shared specificities between anti-GAT antibodies and nonresponder GAT-T_sF extracts that a limited set of variable region genes could be expressed on the GAT specific receptor(s) molecules of these lymphoid cells. According to this hypothesis, structural similarities might be expected between the variable regions of anti-GAT antibodies and GAT-T_sF molecules. To approach these questions GAT-specific cell lines provide a unique way to obtain large quantities of GAT-specific receptors. The aim of the present experiments, using the polyethylene glycol-induced cell fusion technique, was to obtain monoclonal anti-GAT antibodies in order to characterize their fine antigenic specificity and analyze them for the presence of the recently described CGAT idiotype. Several conclusions can be drawn from the present investigation:

(*i*) After both primary and secondary immunizations to GAT, DBA/2 and $B6D2F_1$ responder mice, respectively, generate a large number of activated B cells, which readily fuse with P3-X63-Ag8 or NS1 myeloma cells. Whether these cells represent the actual precursors of the anti-GAT plasma cells remains to be determined. Recent evidence by Köhler and Shulman (20) and Reth *et al.* (22) support such a view.

(*ii*) The hybridization technique represents an indirect approach for estimating the diversity of the antibody response. There is a striking homogeneity in the Ig class distribution of

the hybridoma anti-GAT antibodies in each of the 2 hybridization experiments reported here. Thus, the 9 clones isolated from fusion 9 between recently sensitized DBA/2 mouse and P3-X63-Ag8 myeloma cells all secrete IgM κ anti-GAT antibodies. Earlier data from this laboratory have shown a failure to detect primary direct IgM anti-GAT PFC responses (13, 23) using chromium chloride-coupled GAT-SRBC as indicator cells. Recently, however, conditions have been described which allow the detection of such primary IgM anti-GAT responses (14). The fact that these hybridomas can generate either direct [using poly(L-lysine) to couple GAT to SRBC] or rabbit anti-μ facilitated (on GAT-SRBC coupled with chromium chloride) IgM anti-GAT PFC confirm these findings. Although the precise nature of the parental B cells of these hybrids is not known (immature, membrane IgM bearing B cell precursors or differentiated IgM secreting B or plasma cells), these data suggest that μ, κ antibodies represent a large fraction of the DBA/2 anti-GAT B cell response 5 days after immunization. In contrast, all the stable clones that were isolated from fusion 17 between hyperimmunized B6D2F$_1$ spleen cells and NS1 myeloma cells secrete IgG κ anti-GAT antibodies. Thus, although both fusions were not carried out in identical strains, it is possible to conclude that, as for many other antigens, a μ to γ shift occurs in the course of anti-GAT B cell responses. Most (7 of 8) of the hybridoma IgG anti-GAT antibodies belong to the γ_1 subclass. This stands in accord with earlier observations indicating that the majority of anti-GAT IgG PFC secreted γ_1, κ antibodies (13), and with the restricted heterogeneity of anti-GAT antibodies present in the immune sera of several strains.

(*iii*) The fine specificity analysis of the hybridoma anti-GAT antibodies to the closely related polymers GAT, GT, GA, and poly(Glu) was determined by three techniques and can be summarized as follows: (*a*) Two anti-GAT antibodies (IgM F9-102.2 and IgG F17-148.3) were crossreactive with the GA but not with the GT polymer, (*b*) the remaining monoclonal antibodies (14 of 16) exhibited preferential binding to the homologous GAT polymer and had distinct relative affinities for GA and GT, and (*c*) none of these anti-GAT antibodies showed detectable levels of binding to poly(Glu). Whether these fine specificity data actually reflect the specificity of the combining site of the IgM antibodies produced by the parental B or plasma cells remains to be determined. Thus, although structural data ruled out any 19S IgM carrying both μ and MOPC 21 γ_1 heavy chains, it is clear that the two parental light chains could contribute to a heterogeneity of the combining sites of the F9 IgM antibodies. Nevertheless, these data indicate that similar patterns of fine specificity characterize primary and secondary anti-GAT Ig responses.

(*iv*) Finally, preliminary evidence indicates that most of these anti-GAT antibodies inhibit the CGAT idiotype–antiidiotype binding. It appears that both DBA/2 IgM and B6D2F$_1$ IgG anti-GAT antibody responses are markedly restricted in terms of CGAT expression. These results agreed with the demonstration of CGAT idiotypic specificities on the majority of the anti-GAT antibodies (3) and with the Ig class distribution analysis of this idiotype (5), which demonstrated its presence on antibodies with γ_1, γ_{2a}, γ_{2b}, and μ C_H regions. These results indicate now that CGAT determinants can also be associated with IgG$_3$ antibodies (F17-97.1 clone).

In this study a correlation exists between anti-GT binding and the presence of CGAT idiotype in 14 monoclonal anti-GAT antibodies. Conversely, IgM F9-102.2 and IgG F17-148.3, which do not react with GT, do not express major determinants of this idiotype. These results stand in accord with the determinant specificity analysis of the CGAT idiotype (5). It would be of interest to determine whether each CGAT$^+$ monoclonal anti-GAT antibody expresses all the CGAT specificities present on mouse anti-GAT antibodies, and whether the idiotypic specificities expressed in each hybridoma antibody are identical. Preliminary evidence from the idiotypic analysis of individual CGAT$^+$ hybridoma anti-GAT antibodies indicate that these antibodies possess distinct idiotypic specificities. Similarly, evidence from an analysis of the diversity of the primary and secondary anti-4-hydroxy-3-nitrophenyl acetyl B cell responses in C57BL/6 mice (22, 24) suggests that, although most of the clone products are closely related and belong to the same idiotypic family, they are nevertheless distinct by one or several idiotypic specificities. Finally, even with the limitation of a light chain heterogeneity, hybridoma anti-GAT antibody should allow a precise dissection of the specificities recognized by the anti-CGAT antiserum.

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