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Immunology

Structural bases for public idiotypic specificities of monoclonal antibodies directed against poly(Glu^{60}Ala^{30}Tyr^{10}) and poly(Glu^{60}Ala^{40}) random copolymers

(amino acid sequence/hybridomas/Ig diversity)

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ABSTRACT **NH_2-terminal amino acid sequences of heavy and light chains of seven poly(Glu^{60}Ala^{30}Tyr^{10}) (GAT) specific hybridoma products derived from DBA/2 and (DBA/2 × BALB/c)F_1 hybrid mice and those of BALB/B polyclonal antibodies have been determined over the first 40 residues. Comparison of these sequences with those of nine other GAT or poly(Glu^{60}Ala^{40}) (GA) specific hybridoma products previously reported allowed the following conclusions. (*i*) Sequences of hybridoma H and L chains are present in the pool of polyclonal antibodies. (*ii*) The public CGAT (or pGAT) idiotypic specificities are strictly confined to antibodies exhibiting limited heterogeneity with regard to both the variable heavy (V_H) and the variable κ ($V_κ$) sequences that may be accounted for by one and two germ-line genes, respectively. (*iii*) The public idiotypic specificities GA-1, expressed by some anti-GAT and most anti-GA antibodies, make use of the same (or similar) V_H germ-line genes as the CGAT or pGAT antibodies but possess a distinctive $V_κ$ sequence. (*iv*) Antibodies expressing neither of the alternative public specificities mentioned above appear to be more heterogeneous and express V_H and $V_κ$ sequences that were found to differ from the basic structures defining the CGAT (pGAT) or GA-1 correlates. It is concluded that CGAT (or pGAT) and GA-1 public idiotypic specificities are germ-line markers of both V_H and $V_κ$ regions, an observation in agreement with previously reported serological data.**

In various inbred strains of mice, the random terpolymer (Glu^{60}-Ala^{30}Tyr^{10})$_n$ (GAT) elicits specific antibodies, most of which express crossreactive, or public, idiotypic specificities. These were initially defined by using heterologous anti-idiotypic antisera prepared against polyclonal anti-GAT antibodies from D1.LP (1, 2) or BALB/c (3, 4) mice. The guinea pig antiserum against D1.LP anti-GAT antibodies identified idiotypic markers that were called CGAT (1, 2), whereas idiotypic specificities of BALB/c anti-GAT antibodies detected by a rabbit antiserum were termed pGAT (3, 4). For simplification, it may be considered that both reagents identify similar or identical public idiotypes, hereafter referred to as pGAT or CGAT idiotypes.

The CGAT idiotype was induced after immunization with various glutamic acid- and tyrosine-containing polymers (5) but was absent in anti-GA antisera (6). The presence of CGAT or pGAT specificities in all inbred strains of mice tested (1, 2, 5, 7) as well as the biochemical analysis by isoelectric focusing or two-dimensional gel electrophoresis of a collection of IgG1 BALB/c anti-GAT antibodies (8) suggested the conservation of a common set of anti-GAT-associated V_H and $V_κ$ germ-line genes. Analysis of monoclonal antibodies made it possible to define the CGAT family in more precise terms (9, 10) and to describe, within CGAT$^-$ anti-GAT antibodies, a family of idiotypic determinants also expressed in several anti-GA antibodies and for that reason termed GA-1 idiotype (11). In addition, broader public specificities, expressed only by a limited number of mouse strains, were also described (7, 12). Finally, a number of individual or private specificities were identified on discrete monoclonal antibodies (10, 13).

NH_2-Terminal sequence determination performed on various DBA/2 (14) and BALB/c (15) anti-GAT and anti-GA monoclonal antibody heavy chains provided evidence for a strong conservation of the V_H structures expressing either the CGAT or the GA-1 specificities. On the other hand, expression of the pGAT specificities have been shown to require both the H and the L chains of the corresponding idiotype (16, 17). Because of the apparent conservation of the CGAT/GA-1 system, we have undertaken the H and L sequence analysis of various anti-GAT and anti-GA monoclonal antibodies derived from BALB/c, DBA/2, and (BALB/c × DBA/2)F_1 hybrid mice. The analyzed antibodies were selected so that several samples expressing the following phenotypes would be represented: C-GAT$^+$ (or pGAT$^+$), GA-1$^-$; CGAT$^-$, GA-1$^+$; and CGAT$^-$, GA-1$^-$.

Nineteen NH_2-terminal sequences (8 V_H and 11 $V_κ$) are reported in the present communication. Added to the previously reported data, we have now at hand 16 V_H–V_L pairs representative of the above serological categories which can be compared in their NH_2-terminal section, covering, on the average, residues 1–43. They allow definition of the structural requirements of the major idiotypic specificities. In addition, NH_2-terminal sequences of polyclonal anti-GAT H and L chains are also presented and are in complete agreement with data derived from monoclonal antibodies.

MATERIALS AND METHODS

Derivation of Hybridomas and Purification of GAT-Specific Monoclonal Antibodies. Monoclonal antibodies were obtained from eight distinct series of hybridoma lines, each derived from a different fusion. Experimental conditions have been reported in detail for the F9-anti-GAT (9), F27-anti-GAT (11), and the G5, G6, G7, and G8-anti-GAT (13) series.

The hybridoma lines of the H series were established by fu-

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Abbreviations: GAT, random terpolymer (Glu^{60}Ala^{30}Tyr^{10})$_n$; GA, random copolymer (Glu^{60}Ala^{40})$_n$; CGAT (or pGAT) and GA-1, crossreactive public idiotypic specificities.

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sion of the nonsecreting myeloma X63Ag8.653 (18) with spleen cells from mice primed intraperitoneally with 100 μg of GAT (average M_r, 80,000; Vega–Fox, Tucson, AZ) in 5% Maalox (aluminum and magnesium hydroxide; Rorer, Paris) and 25% heat-killed *Bordetella pertussis* vaccine (Michigan Department of Public Health, Lansing, MI). Mice were immunized either once (100 μg of GAT in Maalox/vaccine on day 5, for fusion H56, DBA/2) or twice [100 μg of GAT in Maalox/vaccine on day 18 followed by an intravenous booster of 50 μg of GAT in saline on day 3, for fusion H51 with (BALB/c × DBA/2)F_1 hybrid mice]. Cloned hybridomas were grown in ascites in pristane-primed (BALB/c × DBA/2)F_1 hybrid mice. Monoclonal antibodies were specifically purified from ascitic fluids on a GAT-aminohexyl-Sepharose column as described (19). Major CGAT and GA-1 idiotypic specificities were determined as reported (9, 11) and are given in Table 1 for series H51 and H56. The main characteristics of all monoclonal antibodies used in this study are summarized in Table 2.

Sequence Determination Procedures. Sequence determination was performed on fully reduced H and L chains that had been separated and checked for purity as described (14). NH_2-Terminal sequence analyses were carried out in a Beckman 890 C automatic sequencer using Beckman program no. 122974 operated with 0.33 M Quadrol in the presence of Polybrene (20). PITC amino acids were converted into their phenylthiohydantoin derivatives in 30% (vol/vol) aqueous trifluoroacetic acid made 0.1% in dithiothreitol. Phenylthiohydantoin derivatives were identified by HPLC on a C_{18} μBondapak column with a Waters apparatus equipped with an automatic injector (WISP; Waters, Paris, France). In addition, an aliquot of each phenylthiohydantoin derivative was hydrolyzed with hydriodic acid (21) and the corresponding amino acid was identified on a 121 M Beckman amino acid analyzer.

Table 2. Characteristics of hybridoma proteins

Protein	Inbred strain	Immunizing antigen	Isotype	Public idiotypic specificities: CGAT or pGAT	Public idiotypic specificities: GA-1*
G5-Bb 2.2	BALB/c	GAT	γ1κ	+	ND
G7-Ab 2.9	BALB/c	GAT	γ1κ	+	ND
G8-Ad 3.8	BALB/c	GAT	γ1κ	+	ND
G8-Ca 1.7	BALB/c	GAT	γ1κ	+	ND
G6-Bd 2.6	BALB/c	GAT	γ1κ	±	ND
H51-5.2	BALB/c × DBA/2	GAT	γ1κ	+	−
H51-129.2	BALB/c × DBA/2	GAT	γ1κ	+	ND
H51-85.2	BALB/c × DBA/2	GAT	μκ	−	−
H51-81.5	BALB/c × DBA/2	GAT	μκ	−	−
H51-31.6	BALB/c × DBA/2	GAT	γ1κ	−	−
H51-54.33	BALB/c × DBA/2	GAT	γ1κ	−	−
H56-406.48	DBA/2	GAT	μκ	+	−
F9-102.2	DBA/2	GAT	μκ	−	+
F27-105.12	DBA/2	GA	γ1κ	−	+
F27-243.4	DBA/2	GA	γ1κ	−	+
F27-127.12	DBA/2	GA	γ1κ	−	−

* ND, not determined.

RESULTS AND DISCUSSION

Structural Correlates to the Public CGAT (or pGAT) Idiotypic Specificities. NH_2-Terminal sequences of the H and the L chains of $CGAT^+$ monoclonal anti-GAT antibodies prepared from DBA/2 (H56.406.48), or (DBA/2 × BALB/c)F_1 hybrid mice (H51.5.2 and H51.129.2) have been determined and compared with sequences of antibodies with similar specificities derived from BALB/c mice and expressing the pGAT idiotype (Fig. 1). The three V_H sequences of the H series were remarkably homologous to the BALB/c derived G5 Bb 2.2 prototype sequence. This sequence was identical in all monoclonal antibodies of the G series, obtained from four separate fusions. According to the proposals of Potter (22), all sequences pertain to the same subgroup, which appears to be close to the V_H II subgroup (23). It should be stressed that the anti-GAT, $CGAT^+$ antibody H chains contain Asn-Ile-Lys at positions 28–30 which seems to be unique so far.

Monoclonal antibodies do not necessarily reflect representative examples of serum antibodies. For example, H chains from monoclonal anti-arsonate antibodies expressing the CRI idiotype lacked amino acid residues that were found at homologous positions in polyclonal antibodies (24, 25). In the case of the anti-GAT antibodies, the major sequence determined in the monoclonal H chains was indeed expressed in the polyclonal antiserum (Fig. 1). The proportion of this sequence in the polyclonal unblocked anti-GAT H chains was estimated to be on the order of 66%, as calculated from the height of peaks of phenylthiohydantoin derivatives identified by HPLC. The remain-

Table 1. Characteristics of H51 and H56 monoclonal anti-GAT antibodies

Hybridoma product*	Ig isotype	Expression of CGAT or GA-1 idiotypic specificities: Direct idiotype binding,† % ligand bound, 4 μl	Direct idiotype binding,† % ligand bound, 0.4 μl	Competitive inhibition of idiotype binding‡: CGAT, Amount, μg	CGAT, % inhibition	GA-1, Amount, μg	GA-1, % inhibition	Phenotype: CGAT	Phenotype: GA-1
H51.129.2	γ1κ	**99**	**61**	35	**45**	35	0	+	−
H51.85.2	μκ	**91**	**29**	7	0	5.4	0	−	−
H51.31.6	γ1κ	19	0	20	0	31	8	−	−
H51.32.39	γ1κ	27	7	15	0	15	0	−	−
H51.81.5	μκ	0	7	3	0	42	0	−	−
H51.143.2	γ1κ	29	8	30	0	40	0	−	−
H51.54.33	γ1κ	16	4	15	9	20	2	−	−
H51.5.2	γ1κ	**93**	**16**	95	**43**	140	0	+	−
H56.406.48	μκ	**97**	**97**	20	**55**	27	6	+	−

* All fusions with immune spleen cells from (BALB/c × DBA/2)F_1 except H56.406.48 was fusion with DBA/2.
† Idiotype binding of 10 μg of ^{125}I-labeled ligand by 4 or 0.4 μl of guinea pig anti-idiotypic antiserum to D1.LP anti-GAT antibodies. Binding >30% (4 μl of antiserum) and >10% (0.4 μl of antiserum) was considered as significant (**boldface** values).
‡ % inhibition of idiotype binding was tested as described with an amount of guinea pig anti-idiotypic serum giving 20–30% idiotype binding and 20 μg of ^{125}I-labeled D1.LP anti-GAT antibodies or F9.102.2 HP as reference ligands for CGAT and GA-1, respectively.

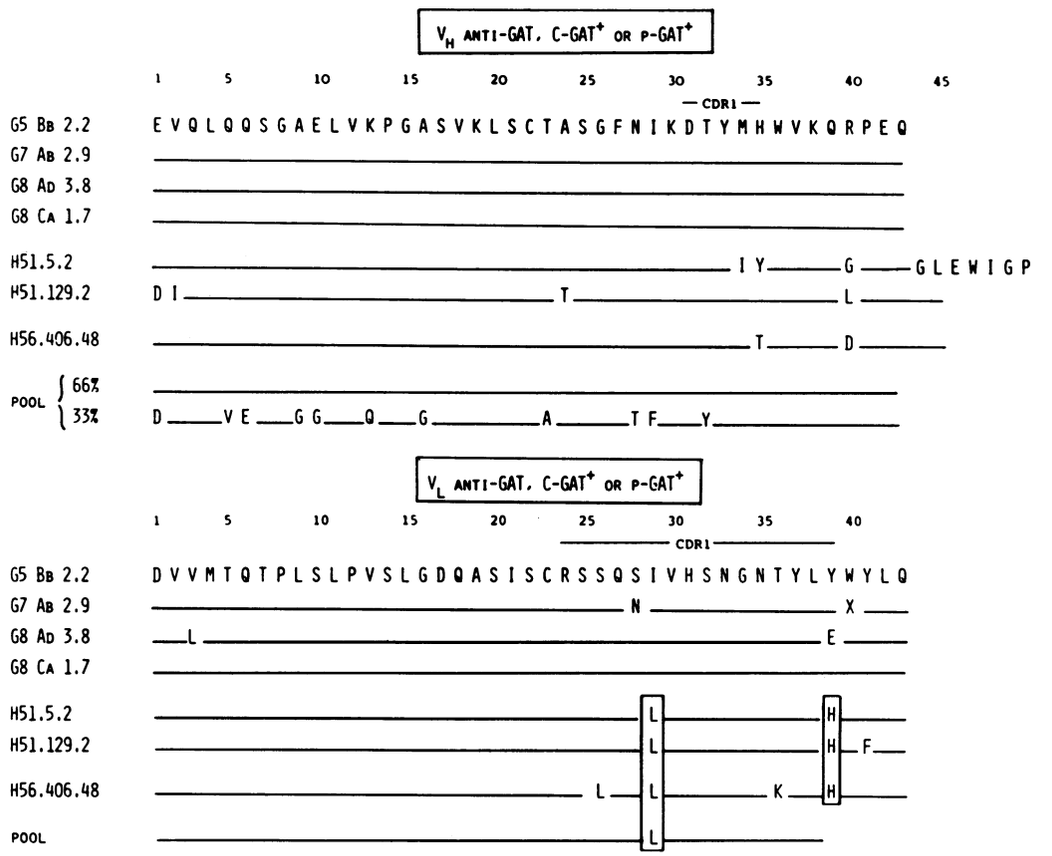

FIG. 1. NH_2-Terminal amino acid sequences of H and L chains of monoclonal antibodies bearing CGAT and pGAT idiotype.

ing 33% clearly diverged from the previous sequence and seems rather close to the V_H III subgroup (23) which is found in CGAT$^-$ anti-GAT antibodies.

We reported previously (15) that anti-GAT, pGAT$^+$ hybridoma product also has highly conserved L chain sequences, although the overall repertoire of mouse V_κ chains is large. Sequences reported in the present paper for the L chains of the H series confirm this conservation. It should be observed, however, that all sequences have a leucyl residue at position 29 and a histidyl at position 39, which were not found in the BALB/c sequences. This would lead to the suggestion that at least two germ-line genes might be coding for these two sets of discrete structures. Alternatively, it is possible that these genes are allelic; however, the sequence containing leucine-29 was also identified in polyclonal BALB/B anti-GAT light chains. This sequence was clearly identical to that of the H51 series. It was also found identical to the V_κ I-A sequence identified and highly conserved in several BALB/c myeloma protein L chains (26). Interestingly, homologous L chains isolated from NZB myeloma proteins contained a tyrosyl residue at position 39, instead of histidine, as found in three anti-GAT light chains of the G series.

Structural Correlates of Anti-GAT or Anti-GA Antibodies That Expressed the Public GA-1 Idiotype Specificities. A small proportion of anti-GAT antibodies were found to express a distinct set of idiotypic specificities, called GA-1. The GA-1 idiotypes were associated with the majority of anti-GA antibodies (11). As reported (14), GA-1$^+$, DBA/2 monoclonal anti-GA antibodies (F27 series) had a V_H NH_2-terminal sequence that was close to the V_H anti-GAT, CGAT$^+$ basic sequence but also to the V_H of F9.102.2, a GCAT$^-$, GA-1$^+$ anti-GAT hybridoma product.

Sequences of the corresponding L chains are reported in this paper (Fig. 2). DBA/2 sequences of the F27 and F9 series appear to be homologous but clearly differ from those of the V_κ 1-A subgroup. This observation suggests that, if CGAT$^+$ (or pGAT$^+$) and GA-1$^+$ idiotypes use the same basic V_H repertoire, the expression of one or the other specificity will depend ultimately on the nature of the V_κ chain. This interpretation completely agrees with previous reports (16, 17) which presented evidence that both the V_H and the V_κ chains were required in order for the pGAT (or CGAT) specificities to be expressed. The same requirement may therefore apply to the expression of the GA-1 specificities.

V_H and V_κ Repertoires Expressed in CGAT$^-$, GA-1$^-$ anti-GAT or Anti-GA Antibodies Are Encoded by Other Germ-Line Genes and Present a Much Higher Level of Diversity. Fig. 3 gives sequences of V_H and V_κ regions derived from anti-GAT or anti-GA antibodies that expressed neither of the major public specificities or gave only a weak inhibition in the pGAT radioimmunoassay (G6, see ref. 15). No two heavy chains were identical, and they all differed from the basic sequences associated with the expression of the CGAT or GA-1 specificities. It should be noted the V_H and V_κ sequences of the H51.85.2 HP that could be bound with multispecific guinea pig anti-idiotypic antibodies did not exhibit GA-1 idiotype. This hybridoma product was only partially homologous to the GA-1$^+$ sequences. It therefore seems highly unlikely that it might be derived from the same germ-line gene but, on the basis of additional data (not shown), it represents a member of another common GA-related idiotype. The G6 and F27.127.12 heavy chains were close to the V_H I subgroup (23) which contains the dinitrophenyl-binding proteins M 460 (27) and M 315 (28). Most κ chains are also different. However, the V_κ sequence of the G6 anti-GAT antibody is homologous to the C-GAT$^+$ V_κ chains and therefore may account for the partial inhibition in the pGAT

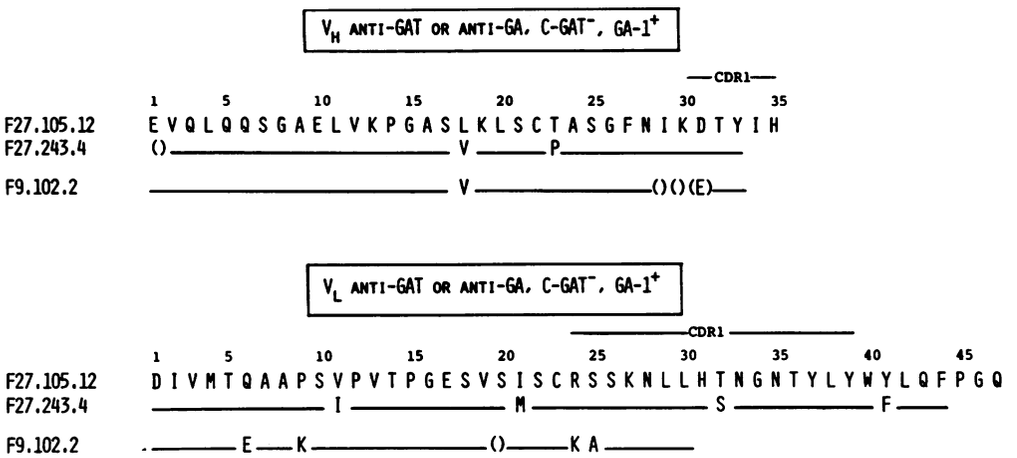

FIG. 2. NH_2-Terminal amino acid sequences of H and L chains of monoclonal antibodies bearing GA-1 idiotype.

radioimmunoassay because all CGAT⁻, GA-1⁻ monoclonal antibodies resemble each other more than they resemble G6.

It may be observed that in this CGAT⁻, GA-1⁻ group the extent of diversity is much broader than in any of the previous groups. Such a situation, reminiscent of other systems such as the arsonate (29–31), might simply witness the usual degeneracy of recognition in any antibody response.

Estimation of the Minimum Number of Germ-Line Genes That Are Involved in the Expression of the Public CGAT and GA-1 Specificities Expressed by Anti-GAT or Anti-GA Antibodies. Structural data fully support previous serological analyses that were reported for the GAT/GA system and are in agreement with the proposals that public idiotypes may represent germ-line markers (32). In essence, anti-GAT and anti-GA antibodies may be classified into three groups: (*i*) anti-GAT antibodies expressing the pGAT or CGAT idiotypes, (*ii*) anti-GAT or anti-GA antibodies expressing the GA-1 idiotype, and (*iii*) anti-GAT and anti-GA antibodies that express neither of these specificities. Correlation of structures with these three serological categories has been substantiated in this study (Fig. 4).

The first two groups use the same V_H repertoire which is related to the V_H II subgroup and which clearly defines at least one germ-line gene. The presence of the unique sequence Asn-Ile-Lys at positions 28–30 seems to be a good marker for this gene. Although it is still extremely difficult, if not impossible, as yet to extrapolate from amino acid sequence to the three-dimensional structure that, in most cases, conditions the expression of an antigenic determinant, it is not impossible that this tripeptide might represent one of the structures involved in the expression of one of the public idiotypes defined by the anti-pGAT antisera because it is close to the antibody-combining site and because the idiotype–anti-idiotype binding was inhibited by a M_r 3,000 fragment cleaved from the antigen GAT (16). However, we cannot exclude participation of other regions (D or J) of the V_H chain which are important for idiotype expression (33).

We propose to call this germ-line gene the "V_H-CGAT" gene. The existence of several repeats at positions 29 and 39 of the

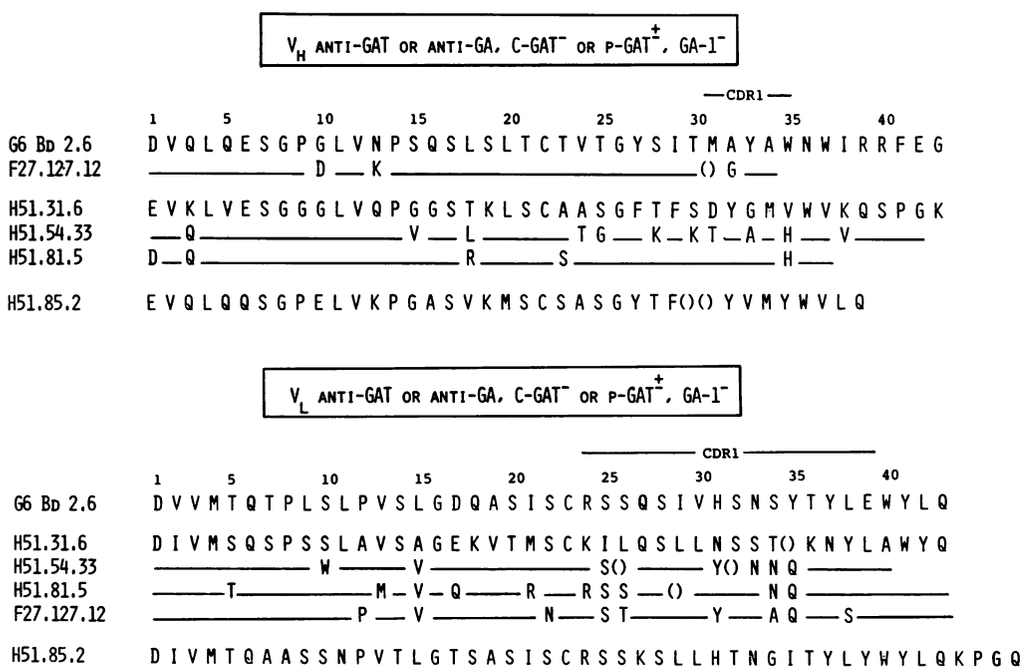

FIG. 3. NH_2-Terminal amino acid sequences of H and L chains of anti-GAT or anti-GA monoclonal antibodies which bear neither CGAT nor GA-1 idiotype. [Another sequence determination run allowed us to correct the previous reported sequence of V_κ G6 Bd 2.6 (15) and to reach position 43.]

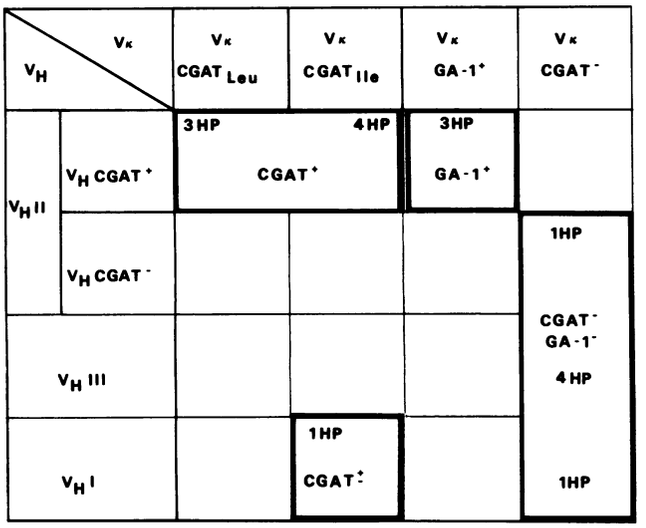

FIG. 4. Germ-line genes expressed in the GAT and GA systems.

light chains involved in the expression of the pGAT/CGAT specificities imposes a minimum of two V_κ germ-line genes, which we propose to refer to as V_κ CGAT-Leu and V_κ CGAT-Ile. Interaction of the polypeptide products of either of the two V_κ genes with a H chain encoded by the V_H-CGAT gene will result in the synthesis of an anti-GAT, $CGAT^+$ (or $pGAT^+$) antibody (Fig. 4).

The anti-GAT or anti-GA antibodies that express the GA-1 idiotype may also use the same V_H-CGAT gene or a related member of the same gene family. Although not as restricted as the anti-GAT, $CGAT^+$ antibodies, the antibodies of the F series, expressing the GA-1 idiotype, use V_κ sequences that may be derived from a single gene. Because no two identical V_κ repeats have been identified so far, this may be only taken as a provisional and minimal estimate of the number of germ-line genes.

Finally, diversity is maximal in the last group of anti-GAT and anti-GA antibodies expressing neither of the public idiotypic specificities. The minimum number of V_H germ-line genes for this group rank among the three major subgroups generally defined.

The probable very low number of germ-line genes involved in the expression of anti-GAT or anti-GA antibodies which bear one of the major public idiotypic specificities definitely ranks these markers as identifying germ-line-encoded structures.

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