MOLECULAR DISSECTION OF THE MURINE ANTIBODY RESPONSE TO STREPTOCOCCAL GROUP A CARBOHYDRATE

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Immunoglobulin genes are composed of several genetic elements (1, 2). In the mouse, a lymphocyte-specific rearrangement event juxtaposes one of a large number of V_H gene segments (3) with one of 10–20 D gene segments (4) and one of four J_H gene segments to encode the variable region of Ig heavy (H) genes. Similarly, one of a large number of V_κ gene segments (5) is combined with one of four functional J_κ gene segments to encode the variable region of κ light chains. Heavy and light chain gene products associate to form antibody molecules.

The variable regions of antibody molecules contain two functionally important structures, antigen binding sites and idiotypic determinants. Although antibody populations selected for individual specificities are structurally heterogeneous, it is not yet clear how much of V, D, and/or J gene segment diversity may be associated with antibodies of a single antigen-binding specificity or bearing a single idiotypic determinant.

Antibodies to streptococcal group A carbohydrate (GAC)[1] provide a particularly interesting model to study the relationship between gene segment diversity and variable region structures. Although essentially all anti-GAC antibodies are specific for the hapten sugar, *N*-acetyl glucosamine (6), these antibodies are strikingly more diverse than antibodies to other polysaccharide antigens, such as *C*-polysaccharide (7, 8) and $\alpha(1 \rightarrow 3)$ dextran (9). Furthermore, anti-GAC antibodies express a multiplicity of idiotypes, allowing them to be divided into four groups: (*a*) IdX^+, $IdI\text{-}1^+$, $Id5^-$; (*b*) IdX^+, $IdI\text{-}1^-$, $Id5^-$; (*c*) IdX^-, $IdI\text{-}1^-$, $Id5^+$; (*d*) IdX^-, $IdI\text{-}1^-$, $Id5^-$. The IdX, IdI-1, and Id5 determinants are all expressed by free κ chains (10, 11, and our unpublished data). In contrast to IdX and IdI-1, which are expressed in an all-or-none fashion by anti-GAC mAb, other idiotypic determinants (e.g., IdI-3a) are expressed to a varying degree by

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[1] *Abbreviation used in this paper:* GAC, streptococcal group A carbohydrate.

most anti-GAC mAb (12). Unlike IdX and IdI-1, IdI-3a is intimately associated with the antigen-binding site, in that binding of anti-IdI-3a to anti-GAC mAb is fully inhibitable by *N*-acetyl glucosamine (10).

In this paper we show that at least four V_κ, one V_λ, two V_H, three J_κ, one J_λ, and three J_H gene segments encode anti-GAC antibodies. Each pattern of IdX, IdI-1, and Id5 public idiotypes identifies antibodies using a single V_κ gene segment and does not correlate with expression of particular V_H gene segments. Thus, in the murine antibody response to GAC, a multiplicity of V and J gene segments is compatible with anti-GAC binding structures and with the IdI-3a idiotope, but a single V_κ gene segment appears to encode each set of public idiotypic determinants.

Materials and Methods

Anti-GAC and idiotype-specific hybridomas were produced as described (12, 13). Isotype and GAC binding were determined by radioimmunoassay as described elsewhere (13).

Genomic DNA was isolated from liver or hybridoma cells, by standard techniques (14). 10 μg of liver DNA or 20 μg of hybridoma DNA was digested for 1.5–3 h with 2–3 U of restriction endonuclease per microgram of DNA, followed by a second digestion with an equal amount of enzyme (New England Biolabs, Beverly, MA; Bethesda Research Laboratories, Gaithersburg, MD; Boehringer Mannheim, Indianapolis, IN; International Biotechnologies, New Haven, CT; or Pharmacia Fine Chemicals, Piscataway, NJ). Digested DNA was subjected to Southern analysis using the procedure described by Maniatis et al. (14).

The 5′V_κ-25-39 probe is a 536 bp Eco RI–Nsi I fragment or a 420 bp Dra I–Nsi I fragment, both consisting of leader and noncoding sequence from the HGAC 39 rearranged κ gene (15 and Fig. 1).[2] The J_κ5 probe is a Hae III–Sau 3AI fragment (positions 1,995–2,328; 16 and Fig. 1). The J_H4 probe is a Hind III–Eco RI fragment, or subfragments, Hind III–Xba I or Xba I–Eco RI (positions 727–2,316, 727–1,639, and 1,639–2,316, respectively; 17 and Fig. 1). J_H4 and J_κ5 probes were subcloned from J_H and J_κ BALB/c DNA in pBR322; the 5′V_H-9 probe, which is identical to the 5′ HGAC 9 probe (18 and Fig. 1) was excised from M13mp8. Probes were labeled by nick translation (14) or by random hexamer primer extension (19).

After hybridization, nitrocellulose filters were washed three times in 0.4 × SSC (14) at 68°C and exposed to Kodak XAR-5 film with or without intensifying screens. Nitrocellulose filters were reused after washing at room temperature, twice in 0.25 M NaOH for 15 min, once in 0.1 M Tris-HCl (pH 7.5) for 10 min, and once in 0.01 M Tris-HCl for 10 min. Filters were then soaked in 5× SSC, prehybridized, and hybridized. In all cases, radioactive probe from the previous hybridization was completely removed by this washing protocol.

Results

At Least Four V_κ and One V_λ Gene Segments Encode Anti-GAC Antibodies. Preliminary experiments suggested that the GAC-specific hybridomas HGAC 39 and HGAC 47 rearranged distinct V_κ gene segments. The rearranged V_κ gene segments from these two cell lines and their germline counterparts were cloned and sequenced (15), confirming that HGAC 39 and HGAC 47 rearranged distinct, albeit related, V_κ gene segments. These V_κ gene segments are closely related to the V_κ24B gene segment of Joho et al. (20) and to the V_κ25 proteins

[2] Lutz, C. T., and J. M. Davie. Genetics and primary structure of V_κ gene segments encoding antibody to streptococcal group A carbohydrate.

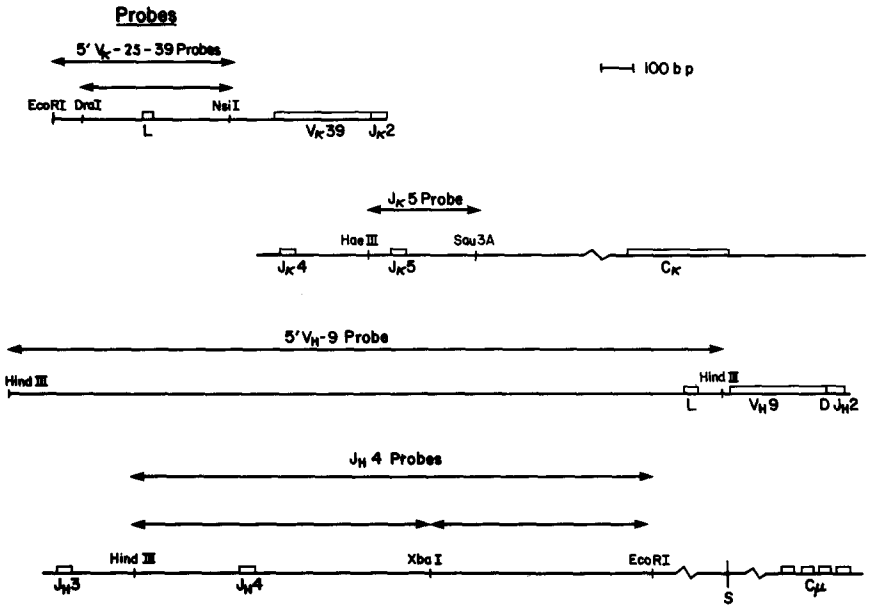

FIGURE 1. Hybridization probes used in this study. The 5′V_κ-25-39 probes are from the HGAC 39 rearranged V_κ gene segment and the 5′V_H-9 probe is from the rearranged HGAC 9 V_H gene segment; both probes are of A/J origin. The J_κ5 and J_H4 probes are from germline BALB/c DNA.

reported by Herbst et al. (20a). Because all these sequences belong to the V_κ25 group of Potter et al. (20b and L. A. D'Hoostelaere, personal communication), we name them V_κ-25-39 and V_κ-25-47. We examined other anti-GAC hybridoma cell lines using a J_κ5 probe, which hybridizes to the germline J_κ region and to all rearranged V_κ-J_κ gene segments. These results suggested that HGAC 8, 39, 47, and 91 have each rearranged distinct V_κ gene segments. For example, Bgl II digestion produced three J_κ5-hybridizing bands for each hybridoma: two are shared by the SP2/0 fusion partner and one is a rearranged band specific to each hybridoma (Fig. 2). HGAC 8, 39, 47, 91, showed specific rearranged bands of 14, 5.0, 2.9, and 3.9 kb, respectively (Fig. 2, Table I). Each of the four hybridomas differed from the others following at least two restriction endonuclease digestions (Table I), consistent with use of four distinct V_κ gene segments. Furthermore, the 5′V_κ-25-39 probe hybridized to the rearranged band in both HGAC 39 and HGAC 47 (which are ~92% homologous in this probe region[2]), but not to a rearranged band in HGAC 8 or HGAC 91 (Fig. 2). In addition, some anti-GAC mAb contain λ light chains (13). Thus, at least five distinct light chain V gene segments appear to encode anti-GAC antibodies.

We analyzed V_κ gene segments used by 25 additional anti-GAC hybridomas. In this analysis we have adopted the following assumptions: (*a*) When a hybridoma displayed two non-SP2/0 rearranged bands, the rearranged band similar to those of other anti-GAC hybridomas was expressed and the other dissimilar band was nonproductively rearranged. V and J gene segments often rearrange nonproductively in B cells (21, 22). (*b*) When rearranged bands from two

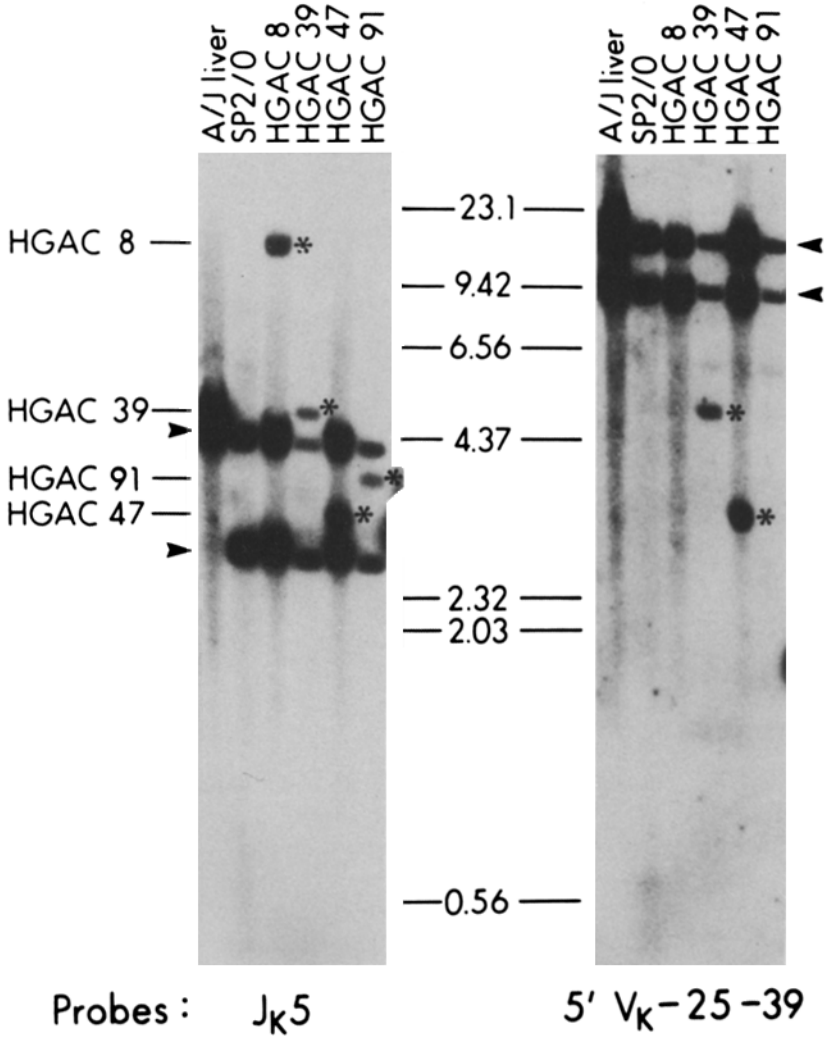

FIGURE 2. Four distinct V_κ gene segments are used by anti-GAC hybridomas. Shown are Southern blots with Bgl II. Arrowheads denote the location of germline or SP2/0 hybridizing bands. The position of each specifically rearranged band is indicated. Size markers are ^{32}P-labeled Hind III fragments of λ phage DNA. *Left*: Filter hybridized with the $J_\kappa5$ probe. *Right*: The same filter was stripped free of probe and hybridized with the 5'V_κ-25-39 probe (Eco RI–Nsi I).

hybridomas differed by a small consistent amount with several different restriction endonucleases, the same V gene segment was paired with different J gene segments. Use of different J gene segments by the same V can result in discrete size shifts of up to 1.3 kb. (*c*) When rearranged bands from two hybridomas migrate similarly with all restriction endonucleases except one, the change is due to somatic mutation. Somatic mutation occurs frequently in the vicinity of rearranged V gene segments (23).

The results of the Southern analysis of V_κ gene segments permitted the

TABLE I

Southern Blot Analysis of Specific J_κ5-hybridizing Bands

Hybridomas (by V_κ group)	Relative mobility of bands after digestion of DNA with:				
	Bgl II	Hind III	Hind III and Eco RI	Sst I	Xba I
			kb		
V_κ-25-39*					
HGAC 39	5.0	3.5	3.4	2.6	6.4
HGAC 34	5.0	3.5	3.4	2.6	ND
HGAC 54	5.0/1.3	3.5/~17	3.4/8.0	2.6/2.3	6.4/9.5
HGAC 58	5.0	3.5	3.4	2.6	6.4
HGAC 89	4.4/5.0	3.0/3.5	2.6/3.2	2.0/1.1	5.6/4.9/3.9
V_κ-25-47*					
HGAC 47	2.9	~11	2.9	2.1	0.6
HGAC 44	2.9	~11	2.9	2.1	ND
HGAC 50	2.9	~11	2.9	2.1	ND
HGAC 92	4.0	~11	4.0	3.2	1.6
HGAC 103	4.0	~11	4.0	3.2	ND
HGAC 125	3.6	~11	3.6	2.7	1.3
HGAC 128	3.6	~11	3.6	2.7	1.3
HGAC 129	ND	~11	3.6	2.7	ND
IdXP-1	3.9/1.5	~11/7.4	3.9/7.9	3.1/3.8	ND
IdXP-2	3.5	~11	3.6	2.8	ND
V_κ-24A-8					
HGAC 8	~14	~11	8.2	1.7	—
HGAC 9	~14	~11	8.2	1.7	ND
HGAC 11	~14/2.9	~11/5.8	8.2	1.7/2.6	ND
HGAC 35	~14/2.2	~11/3.9	8.2/3.5	1.7/3.3	ND
HGAC 53	~14/3.2	~11/3.0	8.2	1.7/3.0	ND
HGAC 57	~17/3.1	~11/5.8	8.2	1.7/2.7	ND
HGAC 62	~14	~11	8.9	2.4	3.9
HGAC 85	~14	~11	8.2	1.7	ND
HGAC 86	~14	~11	8.2	1.7	ND
HGAC 96	~14/~11	~11	8.2/2.5	1.7/3.2	ND
HGAC 99	~14/3.3	~11/5.0	8.2/4.8	1.7/6.9	3.2
Id5P-8	~14	~11	9.2	2.7	1.25
Id5P-11	~14	~11	8.2	1.7	—
Id5P-13	~14	~11	9.2	2.7	1.25
Id5P-24	~14/2.9	~11/3.5	9.2/3.6	2.7/4.2	1.25/2.9
Id5P-40	~14/1.6	~11	9.2	2.7/6.2	1.25/1.5
V_κ-2-91					
HGAC 91	3.9	5.9	3.9	7.7	8.1
HGAC 93	3.9	5.9	3.9/2.0	7.7	8.1
HGAC 98	3.9	5.9	3.9	7.7	ND
HGAC 101	3.9	5.9	3.9	7.7	ND
HGAC 102	3.9	5.9	3.9	7.7	ND
Unrelated					
IdI-3aP-1.1	1.5	3.5	3.2	3.2	ND

When more than one J_κ5-hybridizing band appears in addition to germline- and SP2/0-derived bands, both are listed. When one of these bands migrates similarly to bands in other hybridomas, it is listed first. Low relative mobilities can be estimated only approximately, denoted by tildes.

* Denotes a rearranged band that hybridized to the 5′V_κ-25-39 probe for each member of the group; in all cases it matched the specific J_κ5-hybridizing band from that hybridoma.

clustering of the anti-GAC panel into four groups (Table I). One group of five hybridomas showed rearranged J_κ5-hybridizing bands similar to HGAC 39 that also hybridized to the 5′V_κ-25-39 probe, consistent with use of V_κ-25-39. A second group of eight hybridomas gave hybridization patterns similar to that of HGAC 47 with both the J_κ5 and the 5′V_κ-25-39 probes and likely use V_κ-25-47. 11 hybridomas displayed rearranged J_κ5-hybridizing bands similar to HGAC 8, which failed to hybridize to the 5′V_κ-25-39 probe. Because one member of this group, HGAC 9, shows NH_3-terminal amino acid homology to the deduced amino acid sequence of V_κ24A (18, 20, and data not shown), we label the gene segment rearranged by this group of hybridomas V_κ-24A-8. These sequences are also homologous to the 7S34.1 protein designated V_κ27 by Chang et al. (23a). Five hybridomas displayed rearranged J_κ5-hybridizing bands, which comigrated with the specific band of HGAC 91 and which did not hybridize to the 5′V_κ-25-39 probe, consistent with the use of the same V_κ gene segment. Because these sequences are homologous to the V_κ2 group (20b and N. J. Phillips, personal communication), we name them V_κ-2-91.

The simplest interpretation of the data presented thus far is that four V_κ gene segments encode anti-GAC antibodies in these 29 hybridomas. Other interpretations of the data would increase the number of V_κ gene segments that are used in the anti-GAC response. In addition, small differences in size of the rearranged bands within the groups suggest that at least three J_κ segments are used by these antibodies.

At Least Two V_H Gene Segments Encode Anti-GAC Antibodies. Perlmutter et al. (18) cloned and sequenced the rearranged V_H gene segment from HGAC 9 and demonstrated that at least two V_H gene segments were used by nine anti-GAC hybridomas. To extend this observation we examined 28 anti-GAC hybridomas with J_H4 and 5′V_H-9 probes. The J_H4 probe detects the germline J_H region and all productively rearranged J_H regions. The anti-GAC hybridomas fell into two groups, the HGAC 9 and HGAC 39 groups (Fig. 3 and Table II). Eight hybridomas displayed hybridization patterns like HGAC 9, and likely have rearranged V_H-9. Five additional hybridomas displayed hybridization patterns similar to HGAC 9 after all digestions except Eco RI and Eco RI plus Bgl I. These reflect alleleic differences surrounding the V_H-9 locus, because each subgroup is derived from strains that differ at the Igh-1 locus (24). Therefore 13 hybridomas appear to have rearranged V_H-9. Because HGAC 9 has rearranged V_H-9 to J_H2, we can deduce from the M_r of rearranged bands that other hybridomas have paired V_H-9 with J_H1, J_H2, J_H3, and J_H4 (Table II).

15 anti-GAC hybridomas had J_H4 and 5′ V_H-9 hybridization patterns similar to HGAC 39, but different from HGAC 9 (Fig. 3 and Table II); these hybridomas thus appear to have rearranged the same V_H, which we label V_H-39. Small, consistent differences in the size of restriction endonuclease fragments suggest that this group of hybridomas has paired V_H-39 with three different J_H gene segments.

V_κ Gene Segment Correlates with κ Chain–associated Public Idiotype. Id5, IdX, and Idl-1 public idiotypic determinants are expressed by free κ chains (10, 11, and data not shown). Table III shows that all IdX^+, $Idl\text{-}1^+$, anti-GAC mAb are encoded by V_κ-25-39; all IdX^+, $Idl\text{-}1^-$, anti-GAC mAb are encoded by V_κ-25-47;

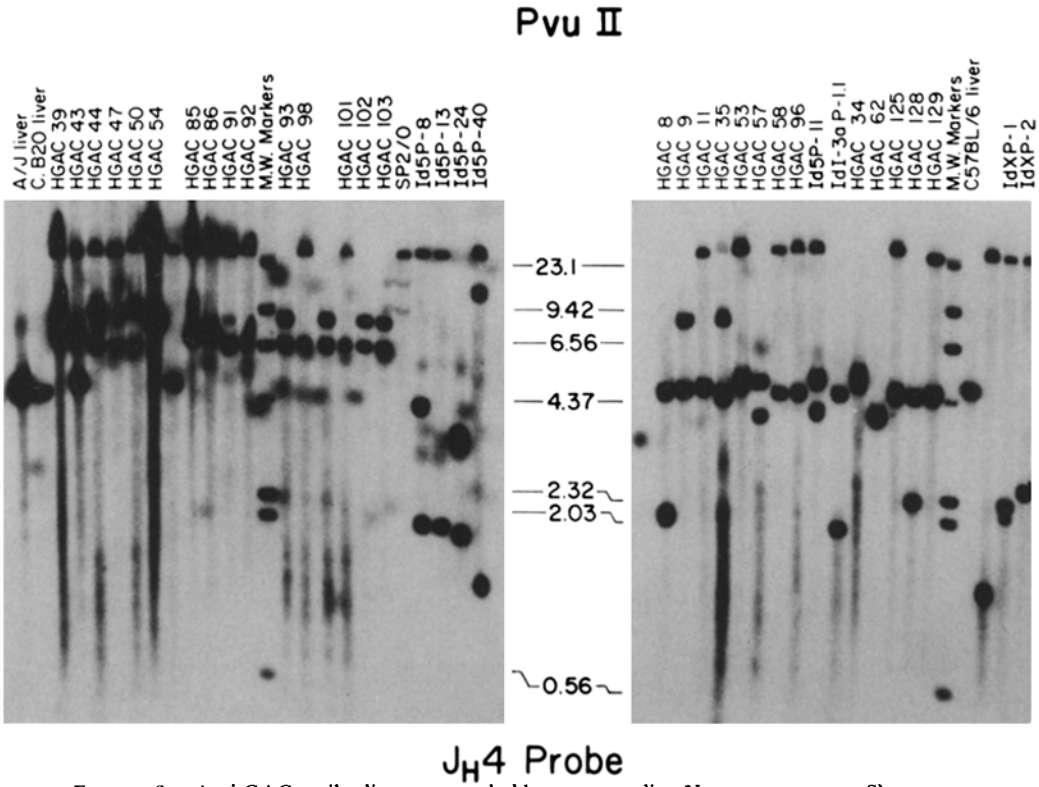

FIGURE 3. Anti-GAC antibodies are encoded by two germline V_H gene segments. Shown are Southern blots using Pvu II, with the J_H4 (Hind III–Xba I) probe. Arrowheads denote the location of germline or SP2/0 hybridizing bands. Brackets indicate the location of rearranged V_H-39 and V_H-9 gene segments. Molecular mass standard is ^{32}P-labeled Hind III fragments of λ DNA. HGAC 8, 9, 34, 35, 53, 57, 58, 62, 96, 125, 128, and 129 have specific bands that migrate similarly to the germline J_H band (4.9 kb). However, because all of these hybridomas have lost their A/J, C57BL/6, or C.B20 germline J_H regions, as seen on all other digests, the 4.9 kb J_H4-hybridizing bands seen here cannot be ascribed to germline J_H DNA. Unmarked lanes represent irrelevant hybridoma DNA.

and all $Id5^+$ anti-GAC mAb are encoded by V_κ-24A-8. The perfect correlation between idiotypic pattern and V_κ gene segment is broken only by HGAC 85, which has rearranged V_κ-24A-8, but which secretes an $Id5^-$ anti-GAC antibody. We suspect that somatic mutation destroyed the Id5 idiotypic determinant in HGAC 85; single amino acid substitutions can destroy idiotypes without changing hapten-binding affinity (25). The IdX^-, $IdI\text{-}1^-$, $Id5^-$ idiotype is associated with secretion of a λ light chain (HGAC 43), and with the V_κ-2-91 gene segment. Finally, there is no correlation of any of these determinants with V_H gene segment. For example, the V_H-39 gene segment encodes anti-GAC antibody with each of the four idiotypic patterns.

The correlation between idiotype and V_κ gene segments extends to immunoglobulins that do not bind GAC. We injected A/J mice with pokeweed mitogen, fused their spleen cells with SP2/0, and screened the resulting hybridomas for idiotype-positive Ig. Two independent hybridomas, IdXP-1 and IdXP-2, secrete IdX^+, $IdI\text{-}1^-$, $Id5^-$ products that do not bind GAC; IdXP-1 secretes a complete

TABLE II

Southern Blot Analysis of Specific J_H4-hybridizing Bands

Hybridomas (by V_H group)	Relative mobility of bands after digestion of DNA with:					
	Pvu II	Sst I	Xba I	Eco RI	Eco RI Bgl I	Dra I
			kb			
V_H-39						
HGAC 39	7.2	5.1	2.9	7.1	7.1	2.2
HGAC 43	7.2	5.1	2.9	7.1	7.1	2.2
HGAC 44	6.6/9.2	4.5	2.3/6.1	6.6/4.0	6.6/4.0	1.5/1.7
HGAC 47	6.6	4.5	2.3	6.6	6.6	1.5
HGAC 50	6.6/9.6	4.5/3.5	2.3/6.2	6.6/7.0	6.6	1.8/2.4
HGAC 54	7.6	5.4	3.4	7.5	7.5	2.7
HGAC 85	7.2	5.1	2.9	7.1	7.1	2.2
HGAC 86	7.2	5.1	2.9	7.1	7.1	1.8
HGAC 91	6.6	4.5	2.3	6.6	6.6	1.8
HGAC 92	6.6	4.5	2.3	6.6	6.6	1.5
HGAC 93	6.6/8.5	4.5/3.2	2.3/3.6	6.6/4.7	6.6/4.7	1.5/1.7
HGAC 98	6.6	4.5	2.3	6.6	6.6	1.5
HGAC 101	6.6	4.5	2.3	6.6	6.6	1.5
HGAC 102	6.6/8.4	4.5/3.2	2.3/3.6	6.6/4.7	6.6/4.7	1.5/1.7
HGAC 103	6.6/8.4	4.5/3.2	2.3/3.6	6.6/4.7	6.6/4.7	1.5/1.7
V_H-9*						
HGAC 8	5.2/2.1	3.8/6.1	4.0/2.7	5.6/3.9	5.6/3.9	1.7/3.3
HGAC 9	5.2/8.7	3.8/4.4	4.0/6.0	5.6/3.6	5.6/3.6	1.7/1.8
HGAC 11	5.2	3.8	4.0	5.6	5.6	1.7
HGAC 35	4.8/8.8	3.4/4.4	3.7/1.6	5.2/4.4	5.2/4.4	1.3/1.5
HGAC 53	5.6	4.3	4.3	5.9	5.9	2.0
HGAC 57	5.2/4.2	6.1/2.4	4.0/4.5	5.6/4.5	5.6/1.8	1.8/1.9
HGAC 58	4.8	3.4	3.7	5.2	5.2	1.3
HGAC 96	4.8	3.4	3.7	5.2	5.2	1.3
HGAC 34‡	5.2/5.5	3.8	4.0/5.6	8.6/5.3	8.6/2.8	2.0/3.0
HGAC 62‡	4.2	2.8/4.7	3.2/2.6	8.0/5.7	8.0/2.6	0.8/1.7
HGAC 125‡	4.6	3.4	3.8	8.3	8.3	1.3
HGAC 128‡	4.6/2.3	3.4/3.6	3.8/6.0	8.3/4.1	8.3/4.1	1.3/3.2
HGAC 129‡	4.6/~25	3.4/5.0	3.8/6.0	8.3/~16	8.3/5.3	1.3/~11
Id5P-11	4.3	3.0	3.3	4.8	4.8	0.8
IdI-3aP-1.1	4.8/1.8	3.4/3.0	3.7/5.1	5.2/5.8	5.2/5.3	1.3
Unrelated						
IdXP-1	2.2/2.0	9.5/2.6	4.2/2.2	4.8/3.3	4.8/3.3	1.5
IdXP-2	2.4	4.1	6.0	5.4	5.4	2.1
Id5P-8	1.8/4.4	9.0/8.7	1.8/4.4	5.5/5.7	5.5/5.7	1.2/2.6
Id5P-13	1.8	9.0	1.8	5.5	5.5	1.2
Id5P-24	3.6/1.7	5.9/4.1	~22/1.9	3.1/2.4	3.1/2.4	4.2/3.5
Id5P-40	~14/1.2	3.9/3.4	4.5/2.5	9.2/5.5	9.2/5.5	4.0/1.6

When more than one J_H4-hybridizing band appeared in addition to germline- and SP2/0-derived bands, both are listed as in Table I. Tildes denote approximate (low) mobilities.

* Denotes a rearranged band that hybridized to the 5′V_H-9 probe for each member of the V_H-9 group; in all cases it matched the specific J_H4-hybridizing band from that hybridoma.

‡ Denotes C.B20 or C57BL/6 origin; all other hybridomas were derived from A/J.

TABLE III

Relation of Idiotypic Determinants to V_κ and V_H Gene Segments

Hybridoma group	IdI-3a idiotope expression*	V_κ	V_H
HGAC 39 (IdX^+, $IdI\text{-}1^+$)‡			
HGAC 39	+++	V_κ-25-39	39
HGAC 34	−	25-39	9
HGAC 54	++	25-39	39
HGAC 58	+++	25-39	9
HGAC 89	+++	25-39	ND
HGAC 47 (IdX^+, $IdI\text{-}1^-$)§			
HGAC 47	−	V_κ-25-47	39
HGAC 44	++	25-47	39
HGAC 50	−	25-47	39
HGAC 92	−	25-47	39
HGAC 103	−	25-47	39
HGAC 125	++	25-47	9
HGAC 128	−	25-47	9
HGAC 129	+++	25-47	9
IdXP-1	−	25-47	Unrelated
IdXP-2	−	25-47	Unrelated
HGAC 8 ($Id5^+$)‖			
HGAC 8	+/−	V_κ-24A-8	9
HGAC 9	+	24A-8	9
HGAC 11	++	24A-8	9
HGAC 35	++	24A-8	9
HGAC 53	++	24A-8	9
HGAC 57	−	24A-8	9
HGAC 62	+++	24A-8	9
HGAC 86	ND	24A-8	39
HGAC 96	+/−	24A-8	9
HGAC 99	−	24A-8	ND
Id5P-8	ND	24A-8	Unrelated
Id5P-11	−	24A-8	9
Id5P-13	−	24A-8	Unrelated
Id5P-24	−	24A-8	Unrelated
Id5P-40	−	24A-8	Unrelated
Others			
HGAC 43	++	None-λ	39
HGAC 85	−	V_κ-24A-8	39
HGAC 91	−	V_κ-2-91	39
HGAC 93	++	2-91	39
HGAC 98	++	2-91	39
HGAC 101	+++	2-91	39
HGAC 102	++	2-91	39
IdI-3aP-1.1	++	Unrelated	9

All HGAC mAb bind GAC antigen; none of Id mAb bind GAC except Id5P-11.

* The level of expression of the IdI-3a idiotope, as measured in competition radioimmunoassay with HGAC 54 antibody as the labeled proband (12).

‡ From four independent fusion experiments.

§ From five independent fusion experiments.

‖ From nine independent fusion experiments.

Ig molecule, whereas IdXP-2 secretes κ light chain in the absence of detectable Ig heavy chain (data not shown). IdXP-1 and IdXP-2 had $J_\kappa 5$ and $5'V_\kappa$-25-39 hybridization patterns similar to HGAC 47, consistent with rearrangement of V_κ-25-47 to $J_\kappa 1$ and $J_\kappa 2$, respectively (Table I). The $5'V_H$-9- and J_H-4-hybridizing bands of IdXP-1 and IdXP-2 did not resemble one another or the V_H-39 or V_H-9 gene segments (Table II). This observation further strengthens the association of the IdX^+, $IdI\text{-}1^-$ idiotype with the V_κ-25-47 gene segment, but not with any V_H gene segment.

We also isolated several hybridomas that secrete $Id5^+$ Ig. These hybridomas secrete IgM, IgG2a, IgG2b, and IgG3, in contrast to members of our anti-GAC panel, which only secrete IgM or IgG3. We tested five hybridomas derived from three separate animals that showed distinct Ig IEF patterns (data not shown); three secrete IgG2a (Id5P-8, -13, -24) and two secrete IgG3 (Id5P-11 and -40). Table I shows that rearranged, $J_\kappa 5$-hybridizing bands of all five resembled V_κ-24A-8. Only one hybridoma, Id5P-11, secretes a GAC-binding antibody; it displayed $5'V_H$-9- and $J_H 4$-hybridizing bands similar to HGAC 9, consistent with V_H-9 rearranged to $J_H 4$. The other four $Id5^+$ hybridomas do not bind GAC and their hybridization patterns did not resemble the V_H-39 or V_H-9 rearranged gene segments (Table II). This observation strengthens the correlation between the Id5 idiotype and the V_κ-24A-8 gene segment.

The IdI-3a Idiotope Is Associated with Several V Gene Segments. The IdI-3a idiotope is expressed to a varying degree by most anti-GAC mAb (12) and is intimately associated with the GAC binding site (10). Not surprisingly, we found no correlation between IdI-3a and either V_H or V_κ (Table III). IdI-3a was associated with all possible V gene segments, including V_λ. HGAC 128 and 129 rearranged the same V_H, J_H, V_κ, and J_κ gene segments, yet expressed totally different levels of IdI-3a (Table III). Further, IdI-3aP-1.1, an $IdI\text{-}3a^+$, non-GAC-binding hybridoma, had a light chain hybridization pattern unlike any of the anti-GAC hybridomas (Table I). In contrast, IdI-3aP-1.1 had a V_H-9- and $J_H 4$-hybridizing pattern similar to HGAC 9 (Table III). Thus, expression of IdI-3a is compatible with extensive diversity of V and J gene segments.

Discussion

Multiple V_H and V_L Gene Segments Encode Murine Anti-GAC Antibodies. A hallmark of anti-GAC antibodies is extensive structural diversity. Comparison of the IEF profiles of 17 $IgG_3\kappa$ mAb (11 of which are included in the present study) showed no duplicate spectrotypes (13). Indeed, based on IEF patterns, Briles and Carroll (26) estimated that the A/J mouse strain can produce ~200 distinct clonotypes in response to GAC. Our results provide at least a partial explanation for this extensive diversity. 30 GAC-specific hybridoma cell lines rearranged four V_κ, one V_λ, two V_H, three J_κ, one J_λ, and four J_H gene segments. This is a minimal estimate because highly homologous gene segments may share the same restriction endonuclease patterns (27). Also, we have interpreted isolated differences with single restriction endonucleases as being due to somatic mutation, and assumed that the rearranged band that was shared by several other hybridomas was always the expressed gene segment. Interestingly, there appears to be no restriction in the pairing of V_H and V_L gene segments (Fig. 4 and Table

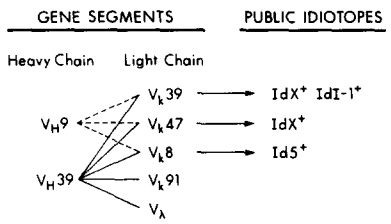

FIGURE 4. The pairing of V_H and V_L gene segments observed in anti-GAC hybridomas and their relationship to IdX, IdI-1, and Id5 public idiotypic determinants.

III). Thus, all five V_L gene segments are paired with V_H-39, and three of the five are paired with V_H-9. Assuming that combinatorial joining of GAC-specific antibody V and J gene segments occurs randomly, and that combinatorial association of GAC-specific heavy and light chains is similarly promiscuous, at least 104 distinct germline antibody species contribute to the murine GAC-specific repertoire. Thus, much of the molecular heterogeneity in this antibody response as defined by Briles and Carroll can be explained without recourse to somatic hypermutation or D gene segments.

Although the V region gene segments of anti-GAC antibodies are distinct, some are clearly related. Rearranged V_H-39 (HGAC 39) and V_H-9 (HGAC 9) gene segments share 57 of 59 NH_3-terminal amino acids (18). The germline V_κ-25-39 and V_κ-25-47 nucleotide sequences are 95% homologous.[2] The NH_3-terminal 74 amino acids of V_κ-24A-8 rearranged by HGAC 9 are 86% homologous to the V_κ-25-39 of HGAC 39 (18). In contrast, V_κ-2-91 rearranged by HGAC 91 and HGAC 101 is only 75% homologous to V_κ-25-39 in nucleotide sequence encoding mature protein (N. J. Phillips, personal communication).[2] Moreover, both germline V_λ gene segments are <50% homologous to V_κ-25-39 (data not shown). Thus, the two V_H gene segments are very homologous, and three out of five of the V_L gene segments are homologous. Antibodies to other naturally occurring carbohydrate haptens such as *C* polysaccharide (28), $\alpha(1 \rightarrow 3)$ dextran (29), and $\beta(1 \rightarrow 6)$ galactan (30–32) are encoded by few, relatively homologous V gene segments. In contrast, antibodies to synthetic haptens such as 2-phenyl-5-oxazolone (33–35) and azophenylarsonate (36–38) are encoded by a relatively large number of nonhomologous V gene segments. The dichotomy between naturally occurring and synthetic haptens is consistent with the hypothesis that bacterial pathogens provide selective pressure to maintain germline V gene segments that encode antibody specific for naturally occurring haptens (28, 39). The availability of a germline-encoded close fit may allow one or a few germline V gene segments to dominate the immune response to naturally occurring haptens. An alternative hypothesis is that there are a limited number of antibody sequences that can effectively compete with water to bind hydrophilic, naturally occurring carbohydrate haptens, whereas a large number of antibody sequences may effectively bind hydrophobic synthetic haptens.

Molecular Basis of Idiotypy in Anti-GAC Antibodies. It has been proposed that public idiotypic determinants may be directly encoded by germline elements (8, 40). Our data on IdX, IdI-1, and Id5, which are expressed by free κ chains, are clearly consistent with this hypothesis. V_κ-25-39 encodes IdX^+, $IdI\text{-}1^+$ Ig, V_κ-25-

47 encodes IdX$^+$, IdI-1$^-$ Ig, and V_κ-24A-8 encodes Id5$^+$ Ig, apparently without the requirement for particular J_κ, V_H, or J_H gene segments (Tables I–III and Fig. 4), or for somatic mutation. The IdX and IdI-1 idiotypic determinants are encoded by rearranged V_κ gene segments identical in sequence to the germline V_κ-25-47 and V_κ-25-39 (N. J. Phillips, unpublished data).[2]

In contrast to IdX and IdI-1, the IdI-3a idiotope is intimately associated with the *N*-acetyl glucosamine–binding site (10). In addition, anti-GAC mAb express IdX or IdI-1 in an all-or-nothing fashion but express IdI-3a to varying degrees (12). As might be predicted, IdI-3a expression did not correlate with any V or J gene segment (Table III). However, because all IdI-3a$^+$ Igs are encoded by the closely related V_H-39 and V_H-9 gene segments, IdI-3a expression may require these gene segments with additional contributions provided by D regions, junctional sequences, or somatic hypermutation. More non-GAC-binding, IdI-3a$^+$ hybridomas will have to be analyzed to answer this question.

Selective Expression of Individual GAC-specific Antibody Clones. Finally, we note that diversity of GAC-specific antibody gene segments makes the phenomenon of clonal dominance more complex (41). Although the murine anti-GAC repertoire includes hundreds of chemically distinct species with >100 potentially accountable by germline V and J gene segments, individual mice typically express one, or at most a few antibody species distinguishable by IEF analysis. With a germline-encoded repertoire of perhaps 10^8 distinct antibodies, any individual clone is likely represented only once in the preimmune murine B cell population. GAC-specific antibodies, by virtue of their germline diversity, may well be represented in 100 or more preimmune B cells. Perhaps somatic hypermutation is responsible for the dominance of unique B cell clones that produce antibodies with high affinity for GAC. However, the continued maintenance of clonal dominance in animals exposed to GAC may require more than simple competition for antigen and might additionally reflect biases in rearrangement frequencies (42) or regulatory effects of an underlying immune network (43). The availability of V_H and V_L probes for anti-GAC antibodies will permit the analysis of the physiology of clonal dominance in molecular detail.

Summary

Monoclonal antibodies (mAb) to streptococcal group A carbohydrate (GAC) are encoded by a minimum of two V_H, four J_H, four V_κ, three J_κ, one V_λ, and one J_λ gene segments. The IdX, IdI-1, and Id5 idiotypic determinants are expressed by anti-GAC mAb and are found on free κ chains. Each pattern of these determinants is encoded by a distinct V_κ gene segment, apparently without the requirement for a particular J_κ, V_H, or J_H gene segment, or somatic mutation. In contrast, the binding site–associated idiotypic determinant IdI-3a does not correlate with any single V or J gene segment.

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References

1. Tonegawa, S. 1983. Somatic generation of antibody diversity. *Nature (Lond.).* 302:575.
2. Honjo, T. 1983. Immunoglobulin genes. *Ann. Rev. Immunol.* 1:499.
3. Brodeur, P. H., and R. Riblet. 1984. The immunoglobulin heavy chain variable region (Igh-V) locus in the mouse. I. One-hundred Igh-V genes comprise seven families of homologous genes. *Eur. J. Immunol.* 14:922.
4. Kurosawa, Y., and S. Tonegawa. 1982. Organization, structure, and assembly of immunoglobulin heavy chain diversity DNA segments. *J. Exp. Med.* 155:201.
5. Cory, S., B. M. Tyler, and J. M. Adams. 1981. Sets of immunoglobulin V_κ genes homologous to ten cloned V_κ sequences: Implications for the number of germline V_κ genes. *J. Mol. Appl. Genet.* 1:103.
6. Coligan, G. E., W. C. Schnute, and T. J. Kindt. 1975. Immunochemical and chemical studies on streptococcal group-specific carbohydrates. *J. Immunol.* 114:1654.
7. Claflin, J. L. 1976. Uniformity in the clonal repertoire for the immune response to phosphorylcholine in mice. *Eur. J. Immunol.* 6:669.
8. Claflin, J. L., S. Hudak, and A. Maddalena. 1981. Anti-phosphocholine hybridoma antibodies. I. Direct evidence for three distinct families of antibodies in the murine response. *J. Exp. Med.* 153:352.
9. Hansburg, D., D. E. Briles, and J. M. Davie. 1976. Analysis of the diversity of murine antibodies to dextran B1355. I. Generation of a large, pauci-clonal response by a bacterial pathogen. *J. Immunol.* 117:569.
10. Greenspan, N. S., and J. M. Davie. 1985. Serologic and topographic characterization of idiotopes on murine monoclonal anti-streptococcal group A carbohydrate antibodies. *J. Immunol.* 134:1065.
11. Fulton, R. J., and J. M. Davie. 1984. Influence of the immunoglobulin heavy chain locus on expression of the VK_1^{GAC} light chain. *J. Immunol.* 133:465.
12. Greenspan, N. S., and J. M. Davie. 1985. Analysis of idiotope variability as a function of distance from the binding site for anti-streptococcal group A carbohydrate antibodies. *J. Immunol.* 135:1914.
13. Nahm, M., B. L. Clevinger, and J. M. Davie. 1982. Monoclonal antibodies to streptococcal group A carbohydrate. I. A dominant idiotypic determinant is located on V_κ. *J. Immunol.* 129:1513.
14. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
15. Deleted in proof.
16. Max, E. E., J. V. Maizel, Jr., and P. Leder. 1981. The nucleotide sequence of a 5.5 kilobase DNA segment containing the mouse κ immunoglobulin J and C genes. *J. Biol. Chem.* 256:5116.
17. Gough, N. M., and O. Bernard. 1981. Sequences of the joining region genes for immunoglobulin heavy chains and their role in generation of antibody diversity. *Proc. Natl. Acad. Sci. USA.* 78:509.
18. Perlmutter, R. H., J. L. Klotz, M. W. Bond, M. Nahm, J. M. Davie, and L. Hood. 1984. Multiple V_H gene segments encode murine antistreptococcal antibodies. *J. Exp. Med.* 159:179.
19. Feinberg, A. P., and B. Vogelstein. 1983. Addendum: A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:266.
20. Joho, R., H. Gershenfeld, and I. L. Weissman. 1984. Evolution of a multigene family of V_κ germline genes. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:185.

20a. Herbst, H., J. Y. Chang, R. Aebersold, and D. G. Braun. 1982. Murine $V_\kappa 25$ isotype

sequence: Monoclonal antibody 2S1.3 specific for the group A streptococcal polysaccharide. *Hoppe-Seyler's Z. Physiol. Chem.* 363:1069.

20b. Potter, M., J. B. Newell, S. Rudikoff, and E. Haber. 1982. Classification of mouse V_κ groups based on the partial amino acid sequence to the first invariant tryptophan: Impact of 14 new sequences from IgG myeloma proteins. *Mol. Immunol.* 19:1619.

21. Tsukamoto, A., I. L. Weissman, and S. V. Hunt. 1984. Allelic exclusion in rat kappa immunoglobulin chains: extent of J_κ rearrangement in normal B lymphocytes. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:975.

22. Alt, F. W., G. D. Yancopoulos, T. K. Blackwell, C. Woods, E. Thomas, M. Boss, R. Coffman, N. Rosenberg, S. Tonegawa, and D. Baltimore. 1984. Ordered rearrangement of immunoglobulin heavy chain variable region segments. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:1209.

23. Gearhart, P. J., and D. F. Bagenhagen. 1983. Clusters of point mutations are found exclusively around rearranged antibody variable genes. *Proc. Natl. Acad. Sci. USA.* 80:3439.

23a. Chang, J.-Y., H. Herbst, R. Aebersold, and D. G. Braun. 1983. A new isotype sequence (V_κ27) of the variable region of κ-light chains from a mouse hybridoma-derived anti-(streptococcal group A polysaccharide) antibody containing an additional cysteine residue. *Biochem. J.* 211:173.

24. Parsons, M., L. A. Herzenberg, A. M. Stall, and L. A. Herzenberg. 1986. Mouse immunoglobulin allotypes. *In* Handbook of Experimental Immunology. Fourth Edition. D. M. Weir, editor. Blackwell Scientific Publications, Oxford, United Kingdom. 97.1–97.17.

25. Radbruch, A., S. Zaiss, C. Kappen, M. Brüggemann, K. Beyreuther, and K. Rajewsky. 1985. Drastic change in idiotypic but not antigen-binding specificity of an antibody by a single amino-acid substitution. *Nature (Lond.).* 315:506.

26. Briles, D. E., and R. J. Carroll. 1981. A simple method for estimating the probable numbers of different antibodies by examining the repeat frequencies of sequences or isoelectric focusing patterns. *Mol. Immunol.* 18:29.

27. Seikevitz, M., S. Y. Huang, and M. L. Gefter. 1983. The genetic basis of antibody production: a single heavy chain variable region gene encodes all molecules bearing the dominant anti-arsonate idiotype in the strain A mouse. *Eur. J. Immunol.* 13:123.

28. Perlmutter, R. M., S. T. Crews, R. Douglas, G. Sorensen, N. Johnson, N. Nivera, P. J. Gearhart, and L. Hood. 1984. The generation of diversity in phosphorylcholine-binding antibodies. *Adv. Immunol.* 35:1.

29. Clevinger, B., J. Shilling, L. Hood, and J. M. Davie. 1980. Structural correlates of cross-reactive and individual idiotypic determinants on murine antibodies to α-(1 $\rightarrow$ 3) dextran. *J. Exp. Med.* 151:1059.

30. Hartman, A., and S. Rudikoff. 1984. V_H genes encoding the immune response to β-(1,6)-galactan: somatic mutation in IgM molecules. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:3023.

31. Pawlita, M., M. Potter, and S. Rudikoff. 1982. κ-chain restriction in anti-galactan antibodies. *J. Immunol.* 129:615.

32. Rudikoff, S., M. Pawlita, J. Pumphrey, E. Mushinski, and M. Potter. 1983. Galactan-binding antibodies. Diversity and structure of idiotypes. *J. Exp. Med.* 158:1385.

33. Kaartinen, M., G. M. Griffiths, A. F. Markham, and C. Milstein. 1983. mRNA sequences define an unusually restricted IgG response to 2-phenyloxazolone and its early diversification. *Nature (Lond.).* 304:320.

34. Griffiths, G. M., C. Berek, M. Kaartinen, and C. Milstein. 1984. Somatic mutation and the maturation of immune response to 2-phenyloxazolone. *Nature (Lond.).* 312:271.

35. Berek, C., G. M. Griffiths, and C. Milstein. 1985. Molecular events during maturation of the immune response to oxazolone. *Nature (Lond.).* 316:412.
36. Margolies, M. M., A. Marshak-Rothstein, and M. L. Gefter. 1981. Structural diversity among anti-*p*-azophenylarsonate monoclonal antibodies from A/J mice: Comparison of Id^- and Id^+ sequences. *Mol. Immunol.* 18:1065.
37. Milner, E. C. B., and J. D. Capra. 1982. V_H families in the antibody response to *p*-azophenylarsonate: Correlation between serology and amino acid sequence. *J. Immunol.* 129:193.
38. Slaughter, C. A., D. J. Jeske, W. A. Kuziel, E. C. B. Milner, and J. D. Capra. 1984. Use of the J_H4 joining segment gene by an anti-arsonate antibody that bears the major A-strain cross-reactive idiotype but displays diminished antigen binding. *J. Immunol.* 132:3164.
39. Perlmutter, R. M., B. Berson, J. A. Griffin, and L. Hood. 1985. Diversity in the germline antibody repertoire. Molecular evolution of the T15 V_H gene family. *J. Exp. Med.* 162:1998.
40. Eichmann, K., and T. J. Kindt. 1971. The inheritance of individual antigenic specificities of rabbit antibodies to streptococcal carbohydrates. *J. Exp. Med.* 134:532.
41. Briles, D. E., and J. M. Davie. 1980. Clonal nature of the immune response. II. The effect of immunization on clonal commitment. *J. Exp. Med.* 152:151.
42. Yancopoulos, G. D., S. V. Desiderio, M. Paskind, J. F. Kearney, D. Baltimore, and F. W. Alt. 1984. Preferential utilization of the most J_H-proximal V_H gene segments in pre-B-cell lines. *Nature (Lond.).* 311:727.
43. Rajewsky, K., and T. Takemori. 1982. Genetics, expression, and function of idiotypes. *Ann. Rev. Immunol.* 1:569.