

Analysis of Mutations in Immunoglobulin Heavy Chain Variable Region Genes of Microdissected Marginal Zone (MGZ) B Cells Suggests that the MGZ of Human Spleen Is a Reservoir of Memory B Cells

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Summary

The splenic marginal zone (MGZ), which surrounds the mantle zone (MTZ) in human splenic white pulp, contains a phenotypically and morphologically distinct population of B cells. The origin of MGZ B cells is uncertain. Whereas some experiments in rodents have suggested that they are a distinct cell lineage responsible for the immune response to T-independent type 2 antigens, others have suggested that they are memory B cells derived from a germinal center (GC) response. The progeny of a GC reaction is expected to have rearranged immunoglobulin (Ig) genes that are mutated. The distribution of mutations would be expected to reflect the selection of Ig by its affinity for antigen. We have analyzed rearranged Ig heavy chain variable region (V_H) 6 and V_H 4.21 genes in MGZ and MTZ B cells microdissected from frozen sections of human spleen to determine whether these genes have the properties of an affinity-selected memory B cell population. MTZ B cells contained germline Ig V_H genes, confirming previous reports and providing an internal control for mutational analysis. MGZ B cells contained Ig V_H genes that were mutated, showing that these cells had been subjected to a mutational mechanism characteristically active in the GC. The rearranged V_H 6 genes showed patterns of mutation indicative of an antigen selection process, whereas the distribution of mutations in V_H 4.21 genes was not characteristic of gene selection by conventional T-dependent antigen. Our studies provide the first evidence that the human splenic MGZ is a reservoir of memory B cells.

The marginal zone (MGZ)¹ of the spleen is a microanatomically defined lymphoid compartment at the border of the white and red pulp. The major cellular components of the MGZ are B cells and macrophages (1–3). In humans and rodents, MGZ B cells are characteristically IgM⁺, IgD⁻, and they strongly express CD21 (1, 4–6). They are not in cell cycle and do not express antigens associated with cellular activation (7, 8).

Two different functions have been ascribed to MGZ B cells in rodents: the antibody response to T-independent type 2 (TI-2) antigens and B cell memory. It has been well documented that an intact splenic MGZ is required for an efficient response to TI-2 antigen (9–11). A number of experiments have suggested that the B cells in the MGZ (12) and the B cells responding to TI-2 antigens (13) differ developmentally from other B cell populations, and they have prompted the suggestion that MGZ B cells are of a distinct

lineage. However, in vitro comparison of the ability of murine MGZ B cells and follicle center B cells to respond to T-dependent (TD), TI-1, and TI-2 antigens have failed to show any major differences, suggesting that responsiveness to TI-2 antigens is due to factor(s) in the microenvironment of the MGZ (14). The role of MGZ macrophages in the response of MGZ B cells to TI-2 antigens has not yet been resolved. Although MGZ macrophages sequester TI-2 antigens (3), the possibility that this function is one of antigen elimination only has not been excluded (15, 16).

Evidence that memory B cells reside in the splenic MGZ is derived from experiments in rats using immunocytochemistry to follow the migration of hapten-specific B cells. These cells localize in the MGZ after primary antigen challenge and selectively migrate out of the MGZ in response to the appropriate secondary challenge (7).

Less is known of the properties of MGZ B cells in humans, although there is circumstantial evidence linking human MGZ B cells with the response to TI-2 antigens. Splenectomized patients have an impaired antibody response to TI-2 antigens (17). Also, the response to TI-2 antigens in newborns and

¹ Abbreviations used in this paper: Fw, framework region; GC, germinal center; MGZ, marginal zone; MTZ, mantle zone; R, replacement; s, silent; TD, T dependent; TI-2, T independent type 2; V_H , heavy chain variable region.

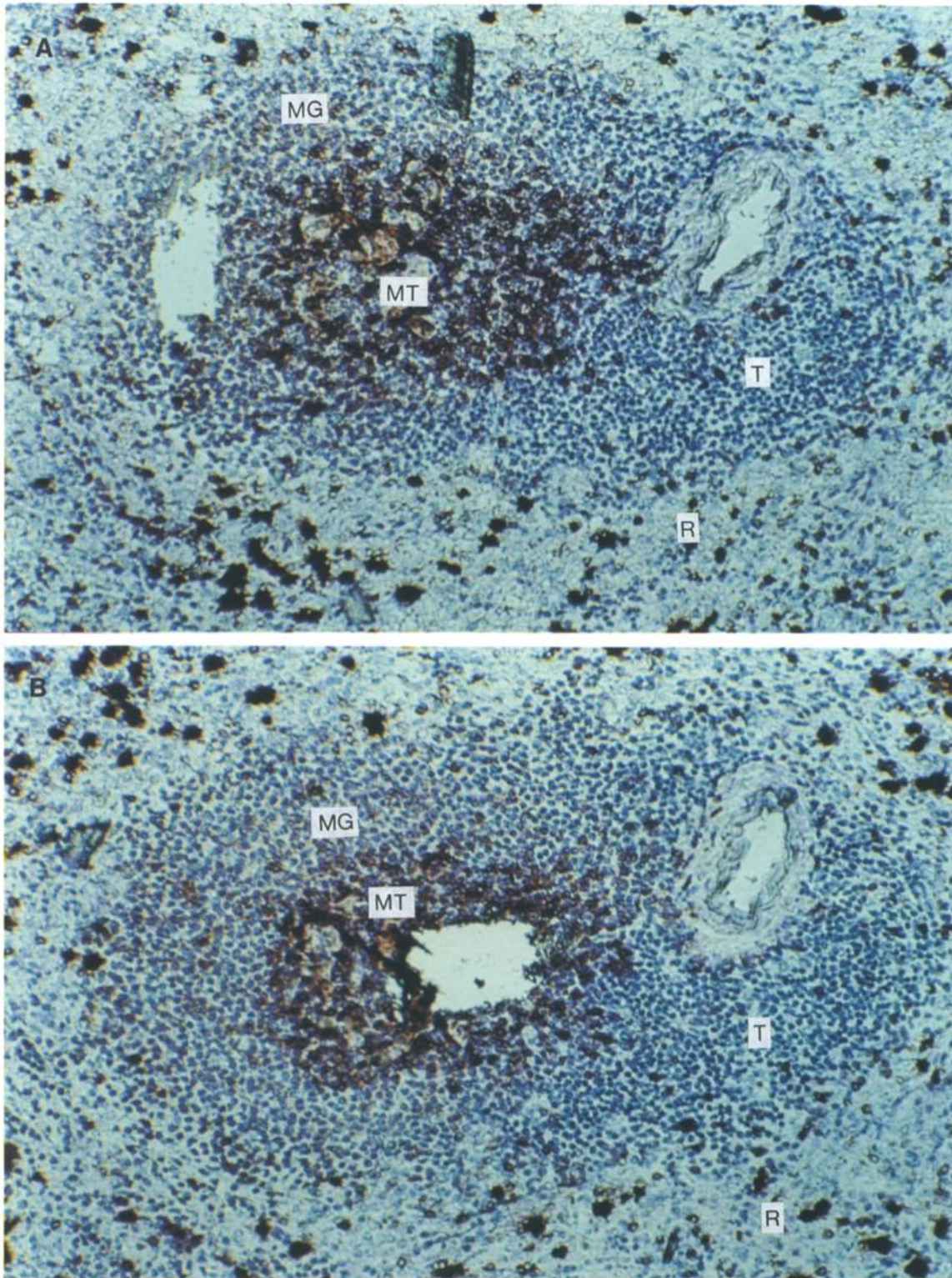


Figure 1. Serial sections of spleen immunohistochemically stained to identify IgD⁺ MTZ B cells (*brown*). Microdissected areas of (A) MGZ, and (B) MTZ cells are illustrated. The location of MGZ (MG), MTZ (MT), T cell area (T), and red pulp (R) are indicated. Original magnification: 50.

infants up to the age of two is limited; this corresponds with the age at which a fully developed MGZ is seen (18). Other than the supporting observation that MGZ B cells accumulate over the first years of life (18), there is no published evi-

dence that the human MGZ is a reservoir of memory B cells.

Memory B cells are the progeny of a germinal center (GC) response to TD antigens. In the GC, Ig genes are somatically mutated, and the B cells expressing the mutated Ig genes

are subsequently selected for high affinity binding to antigen (19). Hence, memory cells are expected to have mutated Ig heavy chain variable region (V_H) genes reflecting this process (19–21). In this study, we have sequenced rearranged Ig genes from microdissected MGZ and mantle zone (MTZ) B cells. We studied V_H 4.21 and V_H 6 because these are highly conserved genes (22), enabling comparison with germline sequence without the problem of discriminating between mutation and polymorphic variation. We have observed that MGZ but not MTZ B cells have mutated Ig genes. Human MGZ B cells have therefore been through a GC response, and the majority are likely to be memory B cells.

Materials and Methods

Normal Human Splenic Tissue. Normal splenic tissue from a 36-yr-old male (patient A) and from a 41-yr-old female (patient B) was removed during pancreatectomy. The tissue was snap frozen in liquid nitrogen soon after surgery.

Immunohistochemistry and Microdissection. 7- μ m, serial, frozen sections were prepared and kept at -20°C for use within 24 h. Sections were stained as previously described (23) using mAbs L26 (CD20), DRC1 (follicular dendritic cells), IgD, UCHT1 (CD3), and 9G4 (V_H 4.21⁺ cells). All antibodies, with the exception of 9G4, were obtained from Dako (High Wycombe, Bucks, UK). 9G4 was kindly provided by Dr. F. K. Stevenson (Tenovus Laboratory, Southampton, UK).

After staining, the sections were left unmounted at room temperature overnight to air dry. Stained serial sections were examined to determine the distribution of MGZ and MTZ B cells. Both are CD20⁺, but only MTZ B cells are IgD⁺. All areas of white pulp studied contained GC, but these were not always cut through in the sections used for microdissection. Approximately 20–40 cells from either the MGZ or MTZ were then dissected from IgD-stained sections (Fig. 1). Microdissection was performed under 50% ethanol using glass capillary tubing with a Leitz micromanipulator (Leica UK Ltd., Milton Keynes, Bucks, UK). The samples were collected in 0.6-ml Eppendorf tubes and allowed to dry at room temperature overnight.

Preparation of Samples for PCR. DNA was released from microdissected samples by treating with 10 μ l of a solution containing proteinase K at 200 μ g/ml in 1 \times Taq polymerase buffer (Promega, Southampton, UK) at 37°C for 48 h. The proteinase K was inactivated at 95°C for 10 min and 5 μ l of the resulting solution was used for PCR.

PCR. All reactions were performed using a PCR machine (OmniGene; Hybaid, Teddington, UK). To amplify Ig V_H -D_H-J_H regions for cloning and sequencing, nested PCR reactions were performed, amplifying \sim 350 bp of rearranged H chain genes, from framework one (Fw1) to J_H. In the first reaction, leader region 5' primers for a V_H 6 or a V_H 4, family-specific, consensus sequence were used, together with a 3' J_H region primer, J_HA, 5'-ACC-TGAGGAGACGGTGACCAGGGT-3' (25). "Hot start" PCR amplification was carried out as previously described (23), using 5 μ l of DNA sample. Optimal concentrations of MgCl₂ used were 3.5 mM for V_H 6 and 1.35 μ M for V_H family 4. Denaturation, annealing, and extension phases were carried out at 94°C for 40 s, 65°C for 45 s, and 72°C for 2 min, respectively. 30 cycles of PCR were performed, followed by a final elongation step of 10 min at 72°C .

The nested reaction was performed with a Fw1 primer specific for either the V_H 6 gene (5'-CCTGTGCCATCTCCGGGGACATG-3') or the V_H 4.21 gene (5'-AGCTACAGCAGTGGG-

GCG-3'), together with a J_H region 3' primer, situated upstream of the first J_H region primer (J_HB, 5'-GTGACCAGGGTACCT-TGGCCCCAG-3') (26). Of the first round PCR products, 2 μ l was amplified as above, using a concentration of 1.5 mM MgCl₂ and with an annealing temperature of 55°C . The success and specificity of the reaction were checked by running 15 μ l of the final PCR products on a 3.5% agarose gel (Nusieve; Flowgen Instruments Ltd., Sittingbourne, UK) and visualizing using ethidium bromide.

Cloning and Sequencing. PCR products were end filled with Klenow (Promega), purified, and cloned into the vector pCR-Script SK(+) using Wizard Preps (Promega) and the pCR-Script cloning method (Stratagene Ltd., Cambridge, UK) as previously described (23). Before preparation for sequencing, plasmids were checked for correct insert size by PCR. A small sample of each clone was boiled in 10 μ l water, and 1 μ l of this was amplified using T3 and T7 primers under standard conditions using 30 cycles of PCR with an annealing temperature of 45°C . Of the products, 10 μ l was checked on agarose gels, and 5- μ l aliquots of the remainder were used for sequencing.

Clones were sequenced in both directions using a sequencing kit (Sequenase; Amersham International, Little Chalfont, UK). PCR products were prepared for sequencing by sequential treatment with exonuclease 1 and shrimp alkaline phosphatase, as recommended in the Sequenase protocol.

Analysis of sequences was performed using GCG software (Genetics Computer Group, Madison, WI). PCR error was calculated by sequencing a number of different clones of the same gene (as determined by comparison of CDR3 regions). The PCR error rate for these methods was found to be $<0.2\%$. Where possible, mutations in each gene were checked by sequencing another clone of the same gene. Calculations of whether mutations were random or selected were performed according to the following formula: Expected number of replacement (R) (or silent [s]) mutations in a particular region = total number of mutations \times proportion of total sequence in the region \times the expected proportion of R (or s) for that region. The values for the expected proportion of R or s mutations in CDR regions were taken to be 0.805 and 0.195, respectively, while those expected for the Fw regions were taken to be 0.739 and 0.261, respectively (27).

Results and Discussion

Microdissection and PCR of Tissue. Successful PCR with both V_H 4.21 and V_H 6 primer sets yielded clear amplification products of \sim 320 bp with no contaminating product, allowing cloning without prior purification. Of the 21 different microdissected samples used for V_H 4.21 amplification, 19 were successfully amplified. However, only 15 successful amplifications of V_H 6 genes were obtained from 44 different microdissected samples. This success rate of only 38% is a likely consequence of the infrequent V_H 6 gene usage in the adult B cell population (28). Agarose electrophoresis of PCR products yielded multiple bands of the V_H 4.21 gene but usually only a single band of the V_H 6 gene, again reflecting the relative frequency of gene usage. As a consequence, multiple copies of V_H 6 genes from the same microdissection were cloned, allowing PCR error to be eliminated from the sequence analysis. However, the V_H 4.21 clones from single microdissections were variable, and multiple clone analysis of a single gene was not always possible.

MGZ Ig V_H 6 Genes Are Mutated. A total of 31 V_H 6

	22	29	31	33	34	35	37	42	46	54	59	60	61	62	64	65	68	69	70	72	73	74	Source	Frame	No. of Clones	
VH6	C	V	S	S	A	A	N	S	G	Y	W	Y	N	D	A	V	K	S	R	T	I	N				
	TGT	GTC	AGC	AGT	GCT	GCT	AAC	TCC	GGC	TAC	TGG	TAT	AAT	GAT	GCA	GTA	AAA	AGT	CGA	ACC	ATC	AAC				
MG6-1	---	---	-A-	-A-	AT-	---	---	---	---	---	---	---	---	---	---	---	---	G--	---	---	---	---	MGZ B	IN	2	
MG6-2	---	---	---	GT-	---	---	---	---	---	---	---	---	---	---	---	C--	-G-	---	---	-c	---	---	---	MGZ B	IN	5
MG6-3	-T-	---	---	---	---	---	---	---	---	---	---	C--	-G-	T--	---	-Cg	-G-	G--	---	---	---	---	---	MGZ A	IN	3
MG6-4	---	---	---	---	-T-	CG-	-t	---	---	---	---	---	---	---	---	-g	-t	G--	---	---	-t	G--	-t	MGZ A	IN	2
MG6-5	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-G-	G--	G--	MGZ A	OUT/S	2
MG6-6	---	-g	---	---	---	---	---	C--	-C-	-TA	--A	-c	---	CT-	---	---	---	-G-	---	-T-	-G-	---	G--	MGZ B	OUT/S	3
MG6-7	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	MGZ B	OUT/S	-
MT6-1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	MTZ B	IN	-
MT6-2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	MTZ B	IN	3
MT6-3	---	---	---	---	---	---	---	---	-t	---	---	---	---	---	---	---	---	---	---	---	---	---	---	MTZ A	IN	2
MT6-4	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	MTZ A	OUT	2
MT6-5	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	MTZ A	S	2
MT6-6	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-g	---	---	---	---	---	MTZ B	OUT/S	2
MT6-7	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	MTZ B	OUT	-

	75	78	81	82	85	86	87	90	92	96	97	101	Source	Frame	No. of Clones
VH6	P	S	Q	F	Q	L	N	T	E	V	Y	R			
	CCA	TCC	CAG	TTC	CAG	CTG	AAC	ACT	GAG	GTG	TAT	AGA			
MG6-1	---	---	---	---	---	t	---	---	---	---	---	---	MGZ B	IN	2
MG6-2	---	---	---	---	a	t	-G-	---	---	---	---	---	MGZ B	IN	5
MG6-3	---	---	G--	C--	---	C	---	---	---	---	---	G--	MGZ A	IN	3
MG6-4	-c	---	---	---	---	---	---	---	---	-C	---	---	MGZ A	IN	2
MG6-5	G--	-t	---	---	---	---	---	---	---	C-a	---	---	MGZ A	OUT/S	2
MG6-6	---	---	---	---	---	---	-G-	---	---	---	C--	---	MGZ B	OUT/S	3
MG6-7	---	---	---	---	---	---	---	---	---	---	---	---	MGZ B	OUT/S	-
MT6-1	---	---	---	---	---	---	---	---	---	---	---	---	MTZ B	IN	-
MT6-2	---	---	---	---	---	---	---	---	---	---	---	---	MTZ B	IN	3
MT6-3	---	---	---	---	---	---	---	---	---	---	---	---	MTZ A	IN	2
MT6-4	---	---	---	---	---	---	---	---	---	---	---	---	MTZ A	OUT	2
MT6-5	---	---	---	---	---	---	---	---	---	---	---	---	MTZ A	S	2
MT6-6	---	---	---	---	---	---	---	---	---	---	---	---	MTZ B	OUT/S	2
MT6-7	---	---	---	---	---	---	---	---	---	---	---	---	MTZ B	OUT	-

Figure 2. Comparison of Ig V_H 6 genes from splenic MGZ and MTZ B cells. The germline V_H 6 sequence, numbering of codons, and position of CDRs (*bold type*) are as published by Tomlinson et al. (24). Codons unmutated from germline are not shown. Identity with the germline sequence is represented by dashes, replacement mutations are represented by uppercase letters, and silent mutations are represented by lowercase letters. The source of the B cells from which the Ig genes were amplified is shown, with A and B indicating two different spleens. In-frame V_H 6 genes, which are likely to be from the expressed allele, are marked in the Frame column as IN; others are marked OUT and/or S (terminated). Where more than one copy of each gene was sequenced, the number of multiple clones is shown. These sequence data are available from EMBL under accession numbers X84172-X84178 and X84186-X84192.

clones were sequenced, representing seven different rearranged Ig V_H genes from each of the MGZ and MTZ (Fig. 2). Multiple copies of single rearranged genes were identified by comparison of their CDR3 region sequences (sequence data not shown). Of these rearranged genes, 50% are probably from unexpressed alleles, as their V_H regions are either not in the same reading frame as the J_H regions, or they contain a termination codon in CDR3 (Fig. 2). These are included in the results, as the somatic mutation process, if occurring, affects both alleles (29). The in-frame rearrangements, with no termination codons in CDR3, are probably expressed alleles (30).

Most MTZ Ig V_H sequences (MT6-1 to MT6-7) are unmutated, although two of these clones, MT6-3 and MT6-6, do have a single silent mutation each (Fig. 2). These are not likely to be an artifact of the PCR process, as both these mutations have been identified in two separate clones. This result confirms that the V_H 6 genes from the individuals studied do not vary from published germline sequences. The germline configuration of Ig V_H 6 genes in MTZ B cells is consistent with other studies showing that MTZ B cells are virgin B cells (29). Of the seven MGZ gene rearrangements sequenced, one (MG6-7) did not show any mutations. Multiple clone analysis was not possible for this gene; hence, it

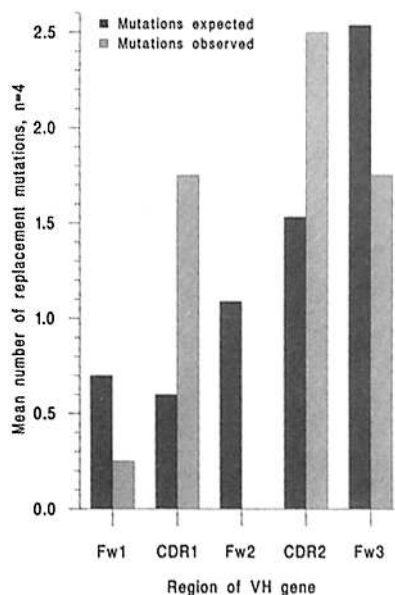


Figure 3. Comparison of the mean number of observed replacement mutations for in-frame MGZ Ig V_H 6 gene regions with the mean number expected for equivalent randomly mutated sequences.

	11	16	17	18	19	20	21	22	23	26	27	29	30	31	32	34	35	40	41	43	46	47	Source	Frame	No. of Clones
VH4.21	L	E	T	L	S	L	T	C	A	G	G	F	S	G	Y	W	S	P	P	K	E	W			
MG4-1	CTG	GAG	ACC	CTG	TCC	CTC	ACC	TGC	GCT	GGT	GGG	TTC	AGT	GGT	TAC	TGG	AGC	CCC	CCA	AAG	GAG	TGG	MGZ A	IN	-
MG4-2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	MGZ A	IN	-
MG4-3	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	MGZ A	IN	-
MG4-4	-A-	---	---	---	---	---	---	---	---	---	---	---	---	AC-	---	---	---	T-	---	---	---	---	MGZ B	IN	-
MG4-5	---	---	---	---	---	---	---	-G-	---	---	---	---	-G	---	-T-	---	---	T-	---	C-	---	---	MGZ B	IN	2
MG4-6	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	MGZ B	IN	-
MG4-7	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	MGZ B	-	-
MG4-8	---	---	---	---	---	---	---	---	---	-A-	---	-A-	---	-A-	---	---	---	---	---	---	---	---	MGZ B	OUT	2
MG4-9	A--	---	---	---	---	---	---	-A-	-A-	-A-	---	-A-	---	-A-	---	---	-C-	---	---	-c	---	---	MGZ B	OUT	2
MT4-1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	MTZ B	IN	-
MT4-2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	MTZ B	IN	2
MT4-3	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	MTZ A	-	-
MT4-4	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	MTZ A	-	-
MT4-5	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	MTZ A	-	-
MT4-6	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	A--	---	---	---	---	---	---	MTZ B	OUT/S	-
MT4-7	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	MTZ B	OUT	-

	48	49	50	51	52	53	58	60	64	68	69	71	72	75	76	78	81	82	83	92	93	94	Source	Frame	No. of Clones
VH4.21	I	G	E	I	N	H	N	N	K	T	I	V	D	K	N	F	K	L	S	V	Y	Y			
MG4-1	ATT	GGG	GAA	ATC	AAT	CAT	AAC	AAC	AAG	ACC	ATA	GTA	GAC	AAG	AAC	TTC	AAG	CTG	AGC	GTG	TAT	TAC	MGZ A	IN	-
MG4-2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	MGZ A	IN	-
MG4-3	---	---	---	---	---	---	---	---	-T-	---	---	C-	---	-C	---	---	---	---	---	---	---	---	MGZ A	IN	-
MG4-4	---	---	---	---	---	---	---	---	-G-	---	---	C-	---	---	---	---	---	G-	-T-	---	-t	---	MGZ B	IN	-
MG4-5	---	---	-C	---	---	-G-	---	---	-G-	---	---	C-	---	---	---	---	---	G-	-T-	---	-t	---	MGZ B	IN	2
MG4-6	---	---	---	---	---	-c	---	---	---	---	---	---	---	---	---	-C-	---	---	---	---	---	---	MGZ B	IN	-
MG4-7	---	---	---	---	---	---	---	---	-t	---	---	---	---	---	---	---	---	---	---	---	---	---	MGZ B	-	-
MG4-8	---	---	-g	---	---	---	---	-C-	---	---	---	---	---	---	---	---	---	---	---	C-	---	---	MGZ B	OUT	2
MG4-9	---	---	---	---	-T-	---	---	C-	---	---	---	T-	-G	---	G-t	-C-	-C	---	-C-	-G-	-G-	C-	MGZ B	OUT	2
MT4-1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	MTZ B	IN	-
MT4-2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	MTZ B	IN	2
MT4-3	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	MTZ A	-	-
MT4-4	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	MTZ A	-	-
MT4-5	---	---	---	---	---	---	---	---	T-	---	---	---	---	---	---	---	---	---	---	---	---	---	MTZ A	-	-
MT4-6	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	MTZ B	OUT/S	-
MT4-7	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	MTZ B	OUT	-

Figure 4. Comparison of Ig V_H 4.21 genes from splenic MGZ and MTZ B cells. The germline V_H 4.21 sequence, numbering of codons, and position of CDRs (*bold type*) are as published by Tomlinson et al. (24). Codons unmutated from germline are not shown. Identity with the germline sequence is represented by dashes, replacement mutations by uppercase letters, and silent mutations by lowercase letters. Clone MG4-9 has large deletions, which are left blank. The source of the B cells from which the Ig genes were amplified is shown, with A and B indicating two different spleens. In-frame V_H 4.21 genes that are likely to be from the expressed allele are marked in the Frame column as IN; others are marked OUT and/or S (terminated). Those not marked could not be designated IN or OUT because of poor quality of the CDR3 sequence. Where more than one copy of each gene was sequenced, the number of multiple clones is shown. These sequence data are available from EMBL under accession numbers X84163-X84171 and X84179-X84185.

is not clear whether this is significant. The remaining six gene rearrangements had heavily mutated V_H 6 genes, and all mutations were confirmed by multiple clone analysis (Fig. 2). This is the first evidence that human MGZ B cells have been subjected to somatic mutation.

V_H 6 Ig Genes from MGZ Show Characteristics of Antigen Selection. Out-of-frame Ig gene rearrangements, or those with termination codons in CDR3, have not been used when considering whether the distribution of mutations in Ig V_H 6 genes resembles that expected from Ig genes in affinity-matured B cells. Analysis of the distribution of mutations in the remaining sequences (MG6-1 to MG6-4) shows that a higher number of replacement mutations is observed in the CDRs and a lower number of replacement mutations is observed in the Fw regions than would be expected if the genes had been randomly mutated with no selective pressure (Figs. 2 and 3). Therefore, it can be concluded that Ig V_H 6 genes

from MGZ B cells have patterns of mutation indicating selection by antigen in the GC. In light of these findings, it is likely that the MGZ B cells containing these Ig genes are memory B cells.

MGZ Ig V_H 4.21 Genes Are Mutated. A total of 20 V_H 4.21 clones were sequenced, representing nine different rearranged genes from the MGZ and seven from the MTZ. No V58 sequences, which could also be amplified with the Fw1 primer used, were observed. Of the 20 V_H 4.21 sequences, 50% were clearly in frame, with no termination codons in CDR3. These are therefore considered to be the expressed allele. Of the seven MTZ sequences, five were identical to the published germline V_H 4.21 sequence (Fig. 4). Again, this agrees with previously published findings that B cells from the MTZ are virgin B cells. The number of repeat sequences from different clones was limited; therefore, the possibility of PCR error as a cause of the single replacement

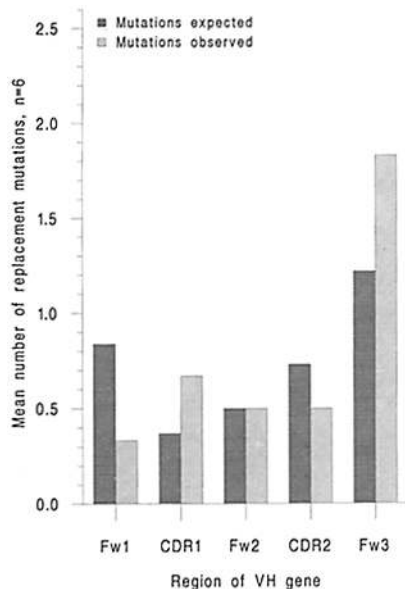


Figure 5. Comparison of the mean number of observed replacement mutations for in-frame MGZ Ig V_H 4.21 gene regions with the mean number expected for equivalent randomly mutated sequences.

mutations seen in clones MT4-5 and MT4-7 cannot be discounted. All nine of the MGZ genes were mutated, and seven had two or more mutations (Fig. 4).

Clones MG4-4 and MG4-5 have a different sequence from Fw1 to CDR2 but have the same CDR3 and Fw3 regions (Fig. 4). It is possible that these sequences are clonally related. However, if the shared mutation in Fw2 codon 40 is considered to be coincidental, then the apparent relatedness of these clones would be more likely to be due to a PCR hybrid artifact.

Ig V_H 4.21 Genes from MGZ Do Not Show Characteristic Evidence of Antigen Selection. The distribution of replacement mutations was analyzed in the clones likely to be expressed alleles, MG4-1 to MG4-6. In contrast to the V_H 6 genes, the frequency of replacement mutations observed in Fw2 and Fw3 of V_H 4.21 was the same as or higher than would be expected, and the frequency of replacement mutations in CDR2 was lower than would be expected, if the acquisition of the mutations were random and the population were under no selective pressure (Fig. 5). These regions, therefore, do not have the distribution of mutations characteristically acquired during affinity maturation. However, CDR1 does have more replacement mutations than would be expected and Fw1 has less mutations than would be expected if the process were random, under no selective pressure. The presence of mutated V_H 4.21 sequences in the MGZ B cells suggests that they have been subjected to a mutational mechanism characteristically active in the GC. How-

ever, in contrast with the rearranged V_H 6 genes from MGZ B cells, there is little evidence of the conventional TD antigen selection of V_H 4.21 genes with variable CDRs and intact Fw regions that would normally be expected in the GC.

It is unlikely that cells using Ig V_H 4.21 genes would undergo mutation in the GC reaction and differentiate into memory B cells without having been through a selection process. If antigen has been involved in the selection of these V_H 4.21-containing cells, it is not likely to be in the conventional manner. It has been suggested that Ig V_H 4.21 may bind a putative B cell superantigen (Ig-sAg) (31). Cold agglutinin antibodies with I/i specificity all use V_H 4.21 but have widely varied D_H, J_H, and L chain genes (32, 33). In conventional Ag-antibody interactions, it is generally agreed that all these factors combine to produce specificity (30). However, a known Ig-sAg, *Staphylococcus aureus* protein A, has been shown to bind to V_H 3 family genes in Fw region (34) rather than the CDRs. If B cells using V_H 4.21 bind Ig-sAg, then they would not be expected to acquire the same patterns of mutation in CDRs and Fw regions of the Ig V_H genes as observed in the conventional GC response to TD Ag. Interestingly, the Fw1 region of V_H 4.21 was found to be conserved in this study.

The presence of mutated V_H genes in the MGZ B cell population, which is mainly IgM⁺, does not concur with the general idea that memory cells are isotype switched. B cells expressing IgA and IgG are present in the MGZ, but only as relatively minor components (1, 35). Mutated IgV_H and IgV_L sequences from IgM⁺ cells have been observed by others (36-39) and also are considered to be associated with an IgM⁺, IgD⁻ B cell subset (39). Evidence to support the idea that somatic mutation and isotype switching are separate events in the GC, with some mutation of V genes preceding isotype switching, has been derived from a study of clonally related genes that use different constant regions (including C_μ) and that show progressive somatic mutation (40). This, together with data presented here, would imply that the MGZ harbors mainly early memory B cells. However, it has also been suggested that IgM⁺ memory cells may be responsive to a particular group of antigens or that they could represent a distinct pathway of memory B cell development (38, 39). These ideas may be relevant, considering the distinctive functional properties ascribed to the MGZ in earlier studies.

Conclusions. MGZ B cells have mutated Ig V_H genes, whereas MTZ B cells do not. This is the first evidence that human MGZ B cells are memory B cells. The V_H 6 Ig genes studied showed the characteristic pattern of mutation expected from an antigen-selected product of a GC reaction. V_H 4.21 genes, however, did not exhibit a conventional pattern of mutation.

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