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Sequence analysis of rearranged IgV_H genes from microdissected human Peyer's patch marginal zone B cells

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SUMMARY

The Peyer's patches of the terminal ileum are a source of IgA plasma cells in the intestinal lamina propria of experimental animals. They are also thought to harbour IgA memory cells. However, the microanatomical location of Peyer's patch memory cells, and whether they are also present in man is not known. Human Peyer's patches have a pronounced marginal zone (MGZ) of sIgD-negative B cells. In this study we analysed the sequence of polymerase chain reaction-amplified, rearranged IgV_H genes from microdissected MGZ B cells, to determine whether this is a site of B-cell memory in Peyer's patches. We observed that the majority of Peyer's patch MGZ B cells contain heavily mutated IgV_H genes and are therefore clearly memory B cells. Sequences of rearranged mutated genes in the MGZ have a pattern of replacement and silent mutations expected of selected products of the affinity maturation process. Related clones, with identical CDR3 but different patterns of mutation, are seen. This suggests that either these memory cells are formed as the germinal centre selection process proceeds, or a memory cell has re-entered the germinal centre for further rounds of mutation. Interestingly, in one patient, the MGZ in the Peyer's patches also contains a proportion of B cells with unmutated IgV_H 4.21 genes.

INTRODUCTION

Marginal zone (MGZ) B cells are sIgD-negative/low B cells which surround the mantle zone of sIgD-positive B cells in normal human spleen and Peyer's patches.[1,2] They are also present as a relatively minor component of the B-cell population in tonsil.[3] The splenic MGZ B-cell population in rats is thought to include both mature virgin B cells and postfollicular memory cells.[4,5] It is thought that responses to T-independent type 2 (TI-2) antigens are localized in this population.[6] There is evidence that a similar localization of responsiveness to TI-2 antigens is also present in man, since splenectomy significantly impairs the ability of an individual to respond to TI-2 antigens.[7] We have recently presented evidence that memory B cells are present in the human splenic MGZ, since B cells microdissected from the MGZ have the mutated immunoglobulin V_H genes expected of B cells which have been through the process of affinity maturation which characteristically occurs in the germinal centre.[8]

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Abbreviations: CDR, complementarity-determining region; D_H, heavy-chain diversity region; dNTP, deoxynucleotide triphosphate; Fw, framework region; J_H, heavy-chain joining region; MGZ, marginal zone; R, replacement mutations; s, silent mutations; TI-2, T-independent type-2; V_H, heavy-chain variable region.

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The Peyer's patches of the terminal ileum are the source of a substantial proportion of IgA plasma cells in the lamina propria.[9,10] In comparison to splenic MGZ B cells, relatively little is known of the MGZ B cells in Peyer's patches. This is due not only to the difficulty in obtaining normal human Peyer's patches for experimental work, but also to the absence of a defined MGZ in Peyer's patches of rodents, where IgD-positive cells account for the majority of B cells surrounding the germinal centre.[11] Memory B cells expressing surface IgA (sIgA) have been observed in the Peyer's patches of rodents[12,13] and a secondary IgA response can be observed after Peyer's patch immunization.[14] However, the precise microanatomical location of memory B cells, and whether they are present in human Peyer's patches, is not known.

In this study we have microdissected B cells from immunohistochemically stained sections of human Peyer's patches in order to determine whether the MGZ of the Peyer's patch, like the MGZ of the spleen, is a site of memory B cells. The V_H4.21 gene and V_H genes from families 5 and 6 were studied because they are relatively non-polymorphic[15] and therefore interpretation of any mutations would be uncomplicated by polymorphic variants. Also, the V_H4.21 gene is known to be quite widely expressed[16] and is likely to be present in the small samples obtained by microdissection. Since microscopic samples were used, we studied rearranged genomic genes and not RNA. This precluded the use of constant region primers in order to determine the isotype of expressed genes. Since the isotype of cell populations is of interest, in the context of the degree of mutation of their immunoglobulin V_H genes, where

possible we have analysed the surface immunoglobulin expressed in the MGZ of the Peyer's patches in the patients studied, using immunohistochemistry.

MATERIALS AND METHODS

Normal human tissue

Terminal ileum, which was both macroscopically and histologically normal, was obtained from three patients (A, B and C). Patient A, a 56-year-old male, had undergone resection of a segment of terminal ileum for adhesions. Patients B and C, males aged 66 and 64 respectively, had undergone right hemicolectomy for carcinoma of the colon. Immediately after surgery, specimens of terminal ileum were snap frozen in liquid nitrogen and stored at −70° until required.

Immunohistochemistry

Serial, 7 μm, frozen sections were prepared and kept at −20° for use within 24 hr. Frozen sections were stained using indirect immunoperoxidase as previously described using monoclonal antibodies (mAb); CD20 (L26), IgD, IgM, IgA, IgG and CD3 (UCHT1). All primary and secondary antibodies were obtained from Dako Ltd (High Wycombe, Bucks, UK). Sections to be used for analysis of surface immunoglobulin expression were mounted. Those stained to show sIgD expression, to be used for microdissection, were left unmounted.

Quantitative analysis of surface immunoglobulin expression

The MGZ was identified by locating a zone of CD20-positive cells which was predominantly sIgD-negative, and which surrounded a mantle zone of CD20-positive, sIgD-positive cells in serial sections. The isotype of surface immunoglobulin used by MGZ B cells in blocks to be used for microdissection was quantified in cases A and B by counting the number of peroxidase stained cells in a sample of at least 200 MGZ cells. The number of cells which could be confidently counted varied between slides. It was not possible to quantify isotypes for case C due to shortage of adequately stained sections.

Microdissection and preparation of samples for polymerase chain reaction (PCR)

Serial sections were examined to determine the distribution of CD20-positive, sIgD-negative MGZ B cells. Approximately 10–30 cells from the MGZ were then dissected from IgD stained sections, taking care to avoid sIgD-positive cells (Fig. 1). Microdissection and preparation of DNA for PCR was performed as previously described.[8]

PCR

To amplify Ig V_H–D_H–J_H regions for cloning and sequencing, a nested PCR reaction was performed, amplifying approximately 350 base-pairs (bp) of rearranged heavy-chain gene, from framework one (Fw1) to J_H. In the first reaction, consensus-sequence, leader region 5′ primers for the V_H 4, 5 and 6 families were used,[17] together with a 3′ J_H region primer, J_HA, 5′-ACCTGAGGAGACGGTGACCAGGGT-3′. The nested reaction was performed either with a Fw1 primer specific for the V_H 4.21 gene (5′-AGCTACAGCAGTGGGG-CG-3′), or with family-specific consensus Fw1 primers for families 5 and 6,[18] together with a J_H region 3′ primer, situated upstream of primer J_HA (J_HB, 5′-GTGACCAGGGTACCTT-GGCCCCAG-3′). PCR reaction conditions were as previously described.[19]

Cloning and sequencing

Second-round PCR products were end-filled with Klenow, purified and cloned into the vector pCR-Script SK(+) using Wizard™ Preps (Promega, Southampton, Hants, UK) and the pCR-Script™ cloning method (Stratagene Ltd, Cambridge, UK) as previously described.[19] A small sample of each clone was boiled in 10 μl water and 1 μl of this was used for amplification with T3 and T7 primers under standard conditions. Of the products, 10 μl were checked for size and specificity on agarose gels and 5-μl aliquots of the remainder were prepared for sequencing by sequential treatment with exonuclease 1 and shrimp alkaline phosphatase, as recommended in the Sequenase™ protocol. Clones were sequenced in both directions using the Sequenase™-sequencing kit (Amersham Intl Plc, Bucks, UK). Sequences were compared against the GenBank database using the 'blast' algorithm.[20] The PCR error rate for these methods was previously found to be less than 0·2%, as calculated by sequencing multiple identical clones.[19] Where possible, mutations in each cloned immunoglobulin gene were checked by sequencing another clone of the same immunoglobulin gene. Calculations of whether mutations were random or selected were performed according to the formula: expected number of R (replacement) [or s (silent)] mutations in a particular region = total number of mutations × proportion of total sequence in the region × 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.[21]

RESULTS

Immunohistochemical analysis of immunoglobulin isotype expression in Peyer's patch MGZ used for microdissection

The percentage of cells expressing CD20, sIgD, sIgM, sIgA and sIgG in the MGZ of Peyer's patches from patients A and B which were used for microdissection is shown in Table 1. Due to the small number of available sections, patient C was not included in this analysis. The MGZ is (by definition) predominantly CD20-positive, sIgD-negative, though occasional sIgD-positive cells are seen (Fig. 1). Approximately

Table 1. Percentages of MGZ B cells expressing different immunoglobulin isotypes in the two patients studied. The numbers in parentheses indicate the total number of cells counted

Antibody	Patient A	Patient B
L26	94% (300)	95% (300)
IgD	7% (300)	6% (400)
IgM	20% (900)	22% (200)
IgA	23% (200)	7% (700)
IgG	27% (300)	ND

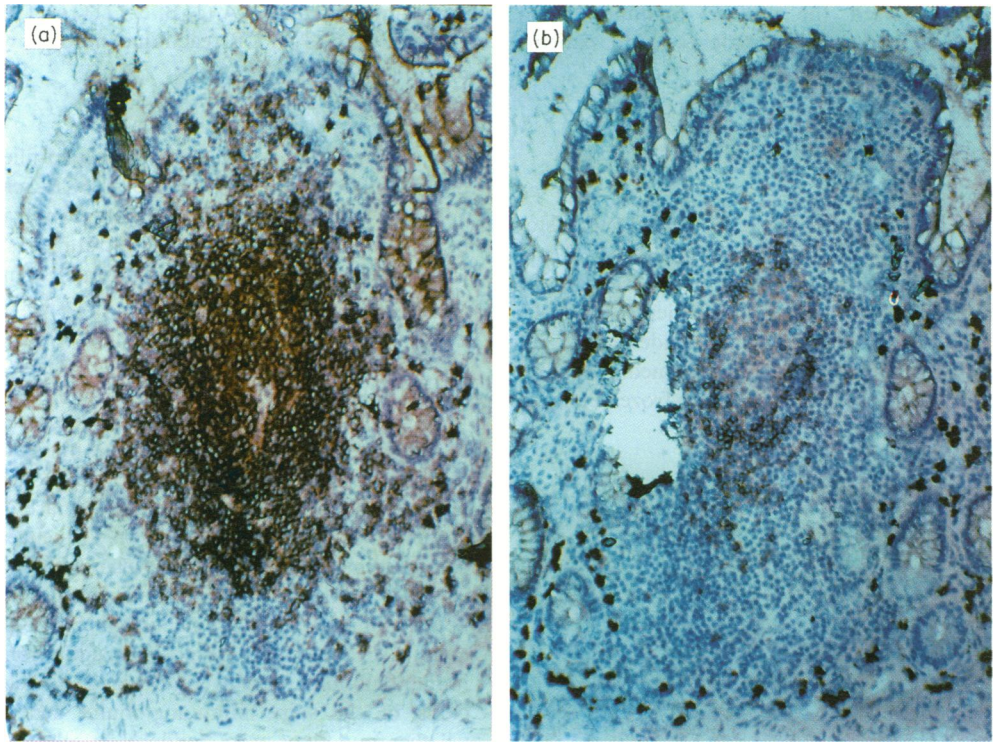

Figure 1. Serial sections of Peyer's patch from the terminal ileum of patient A, immunohistochemically stained (brown) to identify CD20-positive (a) and sIgD-positive (b) cells. The microdissected area of CD20-positive, sIgD-negative cells is shown (b); original magnification ×37·5.

20% of the MGZ B-cell population was sIgM-positive in both patients, which was consistent with the percentage observed by staining a wider panel of specimens in paraffin sections to detect sIgM-positive cells (data not shown). In patient A, similar numbers of sIgA-positive and sIgG-positive cells were also detected. In patient B, the percentage of sIgA-positive cells was distinctly lower. sIgG expression was not quantifiable in this patient due to excessive background staining.

Microdissection of tissue and PCR

Areas of CD20-positive sIgD-negative cells were microdissected from IgD-stained Peyer's patches avoiding any sIgD-positive cells (Fig. 1). PCR amplification of the DNA extracted from these samples resulted in single or a small number of discrete bands, with no other contamination, which could be cloned and sequenced without prior purification (data not shown).

Most MGZ IgV_H genes of Peyer's patch are mutated

A summary of all the different immunoglobulin genes identified is shown in Table 2. Different immunoglobulin genes are defined by their unique sequences. Multiple copies of immunoglobulin genes were identified by comparison of their CDR3 regions (CDR3 sequences not shown). Clonal heterogeneity, where immunoglobulin genes with the same CDR3 regions (that is, the same V_H–D_H–J_H rearrangement), but which have mutational differences which were considered to be too large to be attributable to PCR error, was considered to be indicative of *in vivo* B-cell clonal expansion.

A total of 37 sequences, with 20 different CDR3 regions, representing 20 different immunoglobulin gene rearrangements, from 13 different microdissected areas, was obtained (Table 2 and Fig. 2). Clonal heterogeneity, where immunoglobulin gene sequences have identical CDR3 with different somatic mutations, was seen in two of these rearrangements, shown as PM1 a-b and PM2 a-c in Fig. 2. This brings the total number of different immunoglobulin genes to 23, 18 of which were obtained by amplification with the V_H4.21 primers. Of these, PM15 was found to have more homology with V_H58, this is a gene closely related to V_H4.21 which can also be PCR amplified with the primer used. Due to the relative scarcity of V_H5 and V_H6 genes in the normal population, only four V_H5 family immunoglobulin genes and one V_H6 immunoglobulin gene were isolated, in addition to the 17 V_H 4.21 genes and 1 V_H58 gene (Fig. 2, Table 2).

Of the immunoglobulin genes sequenced, eight are probably the expressed allele, as the V_H region is in the same reading frame as the J_H region and there are no stop codons. Of the other immunoglobulin genes, five were clearly out of frame, while two contained stop codons and could not therefore be expressed. We were unable to determine whether immunoglobulin genes PM13, 14, 17, 18 or 19 were likely to represent used alleles due to poor quality or truncation of CDR3 sequence.

Eighteen of the 23 immunoglobulin genes were mutated in comparison with the appropriate germline immunoglobulin V_H sequences. The immunoglobulin genes isolated from patient B

Table 2. Summary of immunoglobulin gene sequences isolated from Peyer's patch MGZ B cells. The name of the immunoglobulin gene is in column 1. Where two immunoglobulin genes share the same name, but have a different alphabetical suffix, they are the same V_H–D_H–J_H gene rearrangement, but with different mutations. The closest matched germline immunoglobulin V_H gene for each immunoglobulin gene is shown and the patient source is indicated as A, B, or C. If more than one plasmid clone with the same sequence was identified, this is indicated in the fourth column. Also indicated is the number of mutations different from the germline immunoglobulin V_H gene that each immunoglobulin gene has, and whether the V_H and J_H of each immunoglobulin gene are in reading frame

Immunoglobulin gene	Germline immunoglobulin V_H gene	Source	Clones	Mutations	In frame?
PM1a	V_H4.21	A	1	18	Yes
PM1b	V_H4.21	A	1	20	Yes
PM2a	V_H4.21	A	1	16	Yes
PM2b	V_H4.21	A	1	16	Yes
PM2c	V_H4.21	A	2	14	Yes
PM3	V_H4.21	B	2	7	Yes
PM4	V_H4.21	B	1	7	Yes
PM5	V_H4.21	C	3	19	Yes
PM6	V_H4.21	A	1	0	Yes
PM7	V_H4.21	A	2	0	Yes
PM8	V_H4.21	A	1	35	No
PM9	V_H4.21	A	1	18	No
PM10	V_H4.21	B	4	7	No
PM11	V_H4.21	B	3	3	No
PM12	V_H4.21	A	2	0	No
PM13	V_H4.21	A	1	0	No
PM14	V_H4.21	A	2	0	No
PM15	V_H58	A	1	33	Yes
PM16	V_H251	C	3	27	No
PM17	V_H251	C	1	17	No
PM18	V_H251	C	1	14	No
PM19	V_H32	C	1	17	No
PM20	V_H6	A	1	26	No

had less mutations in the V_H region when compared with germline immunoglobulin V_H sequences than the immunoglobulin genes isolated from patients A and C (Table 2). The mean number of mutations per mutated immunoglobulin V_H gene from patients A and C was similar (21·7 in A, 18·8 in C) compared with six for patient B. However, in patient A, five of the V_H4.21 immunoglobulin V_H genes were unmutated when compared with the germline V_H4.21 sequence.

The distribution of mutations, along the 10 V_H 4.21 immunoglobulin genes which were likely to be used, was analysed by comparing the mean number of replacement mutations in each Fw and CDR region with the mean number of replacement mutations which would be expected if the mutational process were random with no selective pressure (Fig. 3). Conservation of Fw1 and Fw2, and concentration of replacement mutations in CDR1 and CDR2, was observed. The distribution of silent mutations throughout the immunoglobulin V_H gene was random.

DISCUSSION

Most of the immunoglobulin V_H gene sequences from microdissected Peyer's patch MGZ B cells were mutated in all patients studied. This suggests that, as in the spleen, memory B cells which had been subjected to the hypermutation process characteristic of the germinal centre response constitute the majority of B cells in the Peyer's patch MGZ. However, some unmutated V_H4.21 sequences were also detected in the population sampled by microdissection. Although some of the unmutated sequences included alleles which were not used by the B cells, the absence of mutations remains significant since the mutational mechanism acts on both alleles of the immunoglobulin V_H genes as B cells undergo a germinal centre response.[23]

Of the mutated immunoglobulin V_H genes sequenced, the V_H4.21 genes PM1 a-b, which share the same CDR3, and PM2a-c, which share the same CDR3, show mutational differences which are too large to be attributable to PCR error, and so are thought to be evidence of *in vivo* B-cell clonal expansion. Evidence of related B-cell clones has previously been shown in the germinal centre of human tonsil,[23] and has provided evidence that the germinal centre reaction is a progressive process of mutation and selection. The presence of related B-cell clones in the MGZ would indicate that the passage of memory cells into the MGZ pool occurs at different points along the hypermutation and selection process. This finding may also be evidence that memory cells re-enter the germinal centre for further rounds of mutation, as has previously been suggested.[24]

In this study, patients A and C have a similar number of mutations per immunoglobulin gene. It is interesting to note that the immunoglobulin V_H genes obtained from patient B,

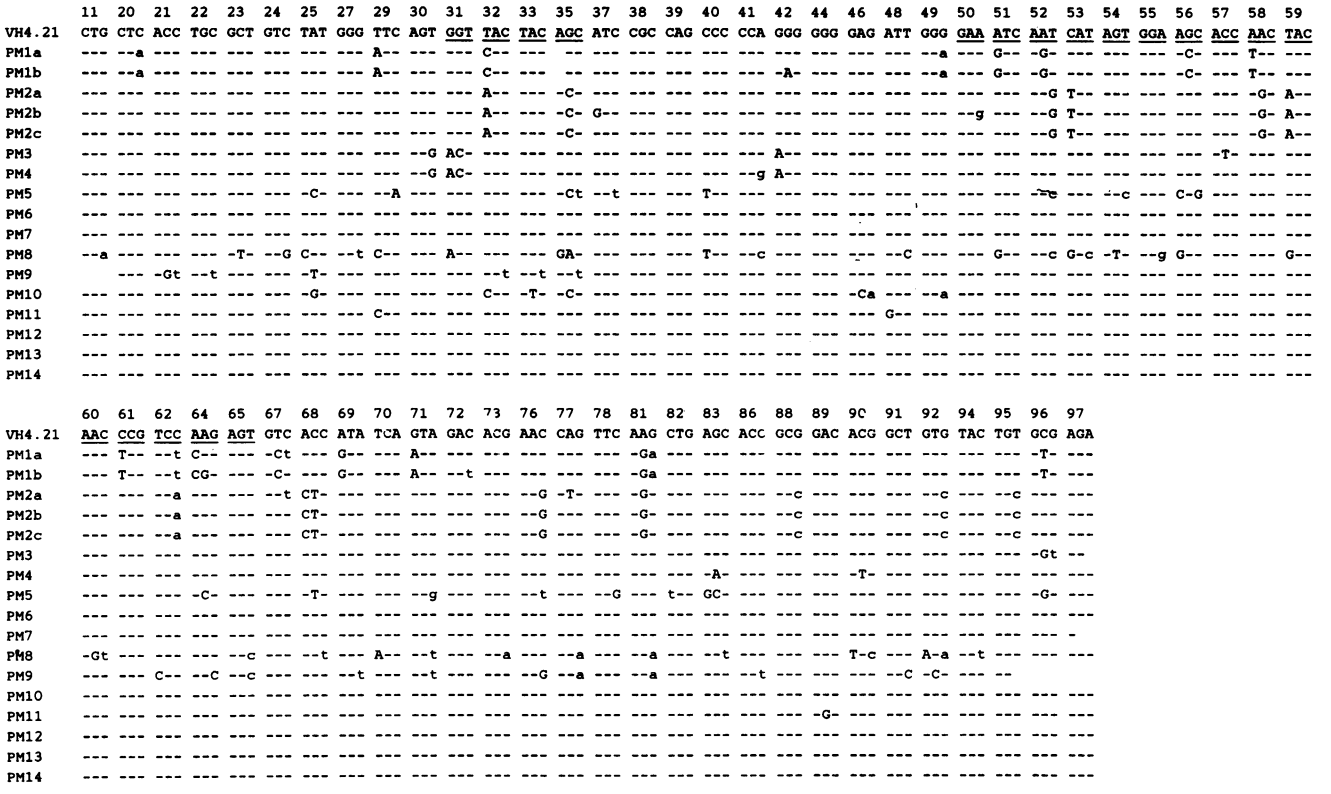

Figure 2. Immunoglobulin V_H4.21 genes from Peyer's patch MGZ B cells. The germline sequence, numbering of codons, and position of CDR (underlined) are as published by Tomlinson *et al.*[22] Codons unmutated from germline are not shown. Identity with the germline sequence is represented by dashes, replacement mutations by uppercase letters and silent mutations by lower case letters.

where a lower number of sIgA expressing cells are seen in the MGZ when compared to patient A, are mutated to a lesser extent. This may be due either to a difference in the state, or history, of activation of lymphoid tissue at the time of sampling, or to individual variation. Overall, the degree of mutation seen in mutated immunoglobulin V_H genes of the Peyer's patch MGZ is greater than that previously reported for immunoglobulin V_H genes in the MGZ of the spleen.[8] This may be due to individual variation, but could be due to differences in the anatomical site of the follicle, since the gut is continually exposed to antigen. The immunohistochemical evidence presented here has shown that, in contrast to the mainly IgM-positive cells of splenic MGZ, a substantial proportion of Peyer's patch MGZ B cells are isotype switched. Cells which are isotype switched are further along the maturation pathway and might be expected to show more evidence of affinity maturation than sIgM-positive cells.[25]

When considering whether the immunoglobulin V_H genes have been mutated under selective pressure in the germinal centre, only those V_H4.21 immunoglobulin genes that are known to be in frame (PM1 to PM7) have been included in the analysis. There is conservation of Fw1 and Fw2, with a concentration of replacement mutations in CDR1 and CDR2 (Fig. 3). This biased distribution of mutations is suggestive of selection. The mutations in Fw3, however, are consistent with accumulation by a random process. These findings are in contrast to previous findings from the MGZ B cells of the spleen, where mutated V_H4.21 immunoglobulin genes did not show any evidence of antigen selection at all.[8] This difference may be explained by variations in the use of the V_H4.21 gene. This gene has been implicated as a target for super-antigen binding, where the affinity for a particular antigen is not dependent on the CDR.[26] A V_H4.21 immunoglobulin gene which binds superantigen would not show the pattern of mutations expected from a normal antigen-selected gene. However, a V_H4.21 immunoglobulin gene which is responding to conventional antigen would be expected to show a distribution of mutations suggestive of selection, such as that observed here.

Cells which are sIgD-negative with germline immunoglobulin V_H sequences have been previously reported in human tonsil and peripheral blood,[27,28] but not in as great a proportion of the total population as that observed in the Peyer's patch MGZ in this study. The presence of unmutated rearranged genes would normally indicate that the cells sampled were virgin B cells which had not been through the antigen selection and memory cell differentiation processes in a germinal centre reaction. The finding of IgD-negative B cells with germline immunoglobulin V_H genes in the MGZ of the Peyer's patch is consistent with the hypothesis that the MGZ B-cell population includes mature sIgD-negative virgin B cells, as has been shown in rats.[4]

CONCLUSIONS

The majority of the Peyer's patch MGZ B cells contain mutated immunoglobulin V_H genes and are therefore considered to be

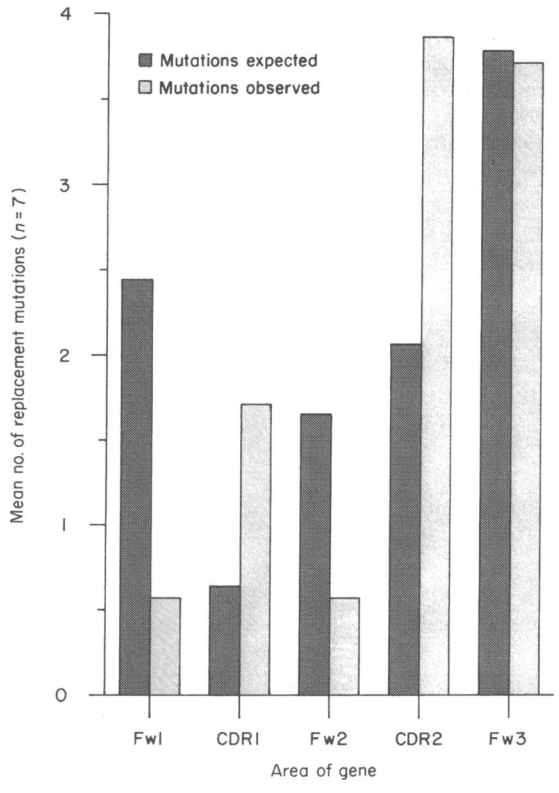

Figure 3. Comparison of the mean number of observed replacement mutations for in-frame immunoglobulin V_H4.21 gene regions from B cells of the Peyer's patch MGZ with the mean number expected for equivalent randomly mutated sequences.[21]

memory cells. These findings agree with those previously reported for the MGZ of the spleen, although the extent of mutation differs. However, unlike the MGZ of the spleen, the B-cell population in Peyer's patch MGZ contains a proportion of sIgD-negative cells which have unmutated immunoglobulin V_H4.21 genes. The presence of related B-cell clones, whose immunoglobulin V_H genes have the pattern of replacement and silent mutations expected of an affinity matured immunoglobulin gene, is evidence of continual memory formation during the germinal centre process and/or re-entry of memory B cells into the germinal centre.

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