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Structural Analysis of the V_H-D-J_H Segments of Human Polyreactive IgG mAb:

Evidence for Somatic Selection[1]

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Abstract

Polyreactive (natural) antibodies are primarily IgM and account for a major proportion of circulating Ig in humans. They use various V gene segments, in general, in germ line (unmutated) configuration. To analyze the V_H regions of polyreactive antibodies, with particular attention at their somatically mutated status, we generated five IgG (three IgG1 and two IgG3) mAb (using B cells from a healthy subject, a patient with insulin-dependent diabetes mellitus and a patient with SLE), which bound with various efficiencies a number of different self and foreign Ag. Gene cloning experiments showed that the V_H region sequences were unique to each IgG mAb. The H chain complementary determining region (CDR3) of two IgG (mAb10 and mAb426.4.2F20) displayed an identical stretch of five amino acids (RFLEW), but the other three IgG mAb CDR3 were divergent in both length and composition. The V_H gene sequences of two IgG, mAb426.4.2F20 and mAb410.7.F91, were 99% identical to those of the germ line V_H4.11 and V_H4.21 genes, respectively. Those of the remaining three IgG mAb displayed a number of differences (93.6 to 95.9% identity) when compared with the germ line V_H4.18, V_H4.11, and hv1263 gene sequences. These and the V_H4.21 gene have been found to encode polyreactive IgM and IgA and, in mutated configuration, monoreactive high affinity autoantibodies and antibodies induced by foreign Ag. When compared with the respective framework region, the CDR of three IgG mAb V_H segment sequences displayed a significantly higher: 1) frequency of total nucleotide differences (6.1×10^{-2} vs 4.5×10^{-2} difference/base); 2) frequency of putative nucleotide changes yielding amino acid replacements (5.6×10^{-2} vs 1.4×10^{-2} replacement change/base); and 3) ratio of overall putative replacement to silent (R:S) mutations (11.0 vs 0.4). Thus, the distribution and nature of the nucleotide differences were consistent with a process of somatic mutation and Ag-dependent clonal selection. This was formally proved in IgG mAb426.12.3F1.4 and IgG mAb10 by differentially targeted polymerase chain reaction amplification and cloning and sequencing of the germ line genes that gave rise to the expressed V_H segments, using DNA from polymorphonuclear cells of the same subjects whose B cells were used for the generation of these IgG mAb. Somatic mutations might have been responsible for bringing about polyreactivity in

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originally monoreactive antibodies or, more likely, they accumulated in originally polyreactive antibodies, which after undergoing a process of Ag selection, retained polyreactivity and may have or may have not acquired a higher affinity for the selecting Ag.

Sera of healthy humans and animals contain antibodies that react with a variety of Ag present on pathogenic microorganisms, including bacteria and viruses and with self-Ag. Because their emergence is independent of known or intentional immunization, these antibodies have been termed “natural antibodies or auto-antibodies” (1–6). Most natural mAb generated form humans and mice are polyreactive, i.e., they bind multiple Ag, dissimilar in nature, such as polysaccharides, nucleic acids, haptens, and proteins, including structural cellular and tissue components and soluble hormones (1–6). A single polyreactive mAb displays different affinities for different Ag (7–10). These are in general low, although in some polyreactive mAb, affinities of the same order of magnitude as those of specific antibodies induced by foreign Ag or those of autoantibodies found in patients with autoimmune diseases have been measured (7, 9–11).

Despite their mostly low intrinsic affinity, polyreactive antibodies display in general a high avidity for Ag due to the multivalency of their predominant Ig class, IgM (12). Because of their broad range of reactivity and high avidity, polyreactive antibodies may play a major role in primary line of defense against invading bacteria or viruses before the specific immune response is generated and in the clearance of debris, such deriving from dead cells, or, possibly some toxic substances. Analysis of the primary structure of the V regions of polyreactive primarily IgM natural antibodies has shown that these are in germ line configuration (13–17). This has led to the hypothesis that natural polyreactive antibodies do not accumulate somatic point mutations. As a consequence, their effectiveness in binding Ag would dramatically decrease, due to a decrease in overall avidity after Ig class switch and substitution of the μ, with a γH chain (18).

In these studies, we generated five polyreactive human IgG mAb with distinctive Ag binding activities. The IgG mAb V_H region genes were similar to those used by polyreactive IgM and/or specific high affinity antibodies or autoantibodies. When compared with the sequences of the closest germ line genes, those of the V_H genes of three IgG mAb displayed a number of differences, the distribution and nature of which suggested an Ag-dependent selection process. In two IgG mAb, such differences were formally proved to represent somatic mutations. Thus, these findings are consistent with the hypothesis that natural polyreactive IgM antibody-producing cells may class switch to IgG while undergoing an Ag-directed selection process, which is underpinned by somatic hypermutation.

Materials and Methods

Generation of the polyreactive IgG mAb-producing cell lines

PBMC obtained from a healthy subject, a patient with IDDM[5], and a patient with SLE were depleted of T lymphocytes, infected with EBV, and then distributed in microcultures in presence of irradiated feeders (19, 20). After 3 wk, the microculture fluids were tested for IgG to human insulin or ssDNA or insulin, ssDNA, and tetanus toxoid (7–11). IgG mAb-producing cell lines were established from EBV-transformed lymphoblasts by sequential subculturing after fusion with the F3B6 human-mouse hybrid cells (19, 20). Concentrated mAb were prepared from culture fluids (7–11). Their Ag binding activity and K_d different Ag were measured as reported (8–11).

Cloning and sequencing of the expressed Ig V_H genes

mRNA was extracted from mAb-producing cells and first strand cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (21). The degenerate sense HA1, HA3a, HA4a oligonucleotide primers, and the antisense Cγ primer were synthesized and used to amplify the V_H gene cDNA. The sequence of HA1 [5′ ATGGACTGGACCTGGAGG(AG)TC(CT)TCT(GT)C 3′] was highly similar to a portion of the leader sequences of the members of V_HI gene family; the sequence of HA3a [5′ ATGGAG(CT)TTGGGCTGA(CG)TTT(CT)T 3′] was highly similar to a portion of the leader sequences of members of the V_HIII gene family; the sequence of HA4a [5′ ATGAA(AG)CA(TC)CTGTGGTTCTT(CT)(AC)T(CT)CT(CG)C 3′] was highly similar to a portion of the leader sequences of the V_HIV family genes. The sequence of the antisense (HI-1) Cγ primer [5′ TAGTCCTTGACCAGGCAGCC 3′] was the reverse complement of a 5′-portion of the IgG constant region gene conserved in all four human IgG subclasses. PCR was performed in a 50 μl of volume using the primers described above and Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) under the condition previously described (21). PCR-amplified DNA fragments were separated on 2% low melting agarose gel by electrophoresis. Each amplified DNA fragment was excised, purified, and then cloned into pCR1000 plasmid vector (Invitrogen, La Jolla, CA) (21). Dideoxy sequencing was conducted using double-stranded plasmid DNA prepared from the selected bacterial clones (21). Each V_H gene sequence was derived from the analysis of at least four independent clones. Nucleotide differences in sequences among different recombinant clones were observed in few cases (<0.001 difference/base). Such variants were excluded from analysis. Sequences were analyzed using the software package of the Genetics Computer Group of the University of Wisconsin, Release 6, and a Model 6000–410 VAX computer (Digital Equipment Corp., Marlboro, MA). Identity searches of the expressed V_H genes were performed using the FASTA program with the GenBank database.

[5]Abbreviations used in this paper: IDDM, insulin-dependent diabetes mellitus; CDR, complementary determining region; FR, framework region; K_d, dissociation constant; PMN, polymorphonuclear cells; PCR, polymerase chain reaction; R, replacement (mutation); RF, rheumatoid factor; S, silent (mutation).

PCR amplification of Ig V_H genes from B cell hybridoma and autologous PMN DNA

To determine whether the nucleotide differences displayed by the mAb426.12.3F1.4V_H gene sequence compared with that of the closest reported germ line V_H gene represented somatic point mutations, we performed PCR amplification of V_H gene segments from genomic DNA of the mAb426. 12.3F1.4–producing cells and autologous PMN using combinations of the ad hoc designed 58–5, 58–3, and 12.3–3 oligonucleotide primers. The sequence of the sense 58–5 [5′ GCACTGTCTCTGGTGGCTCC 3′] primer encompassed a FR1 stretch (residues 65 to 84) shared by the mAb426.12.3F1.4 and the germ line V_H4.11 genes (22). The antisense 58–3 [5′ GGCCGTGTCCGCAGCGGTCA 3′] primer was the reverse complement of a FR3 sequence (residues 253 to 273) of the germ line V_H4.11 gene and differed in two bases from the expressed mAb426.12.3F1.4 V_H gene. The antisense 12.3–3 [5′ TCGCGGACGTGTCCACTGACA 3′] oligonucleotide primer was the reverse complement of a part of the FR3 sequence of the expressed mAb426.12.3F1.4 V_H gene (residues 206 to 226). This sequence differed from the corresponding sequence of the V_H 4.11 gene in four bases. Genomic DNA (100 ng) was subjected to PCR (50 μl volume) with 100 ng of the 58–5 oligonucleotide and 100 ng of either the 12.3–3 or the 58–3 oligonucleotides. Each cycle consisted of denaturing, annealing, and extension steps of 1,2, and 2 min, respectively. Denaturing and extension temperatures were 94 and 72° C, respectively. Annealing temperatures were 61 and 57°C for the 12.3–3 and 58–3 primers, respectively. After 30 cycles, the products amplified using DNA extracted from autologous PMN or from mAb426.12.3F1.4–producing cells were fractionated on 1.7% agarose gel containing 1 μg/ml of ethidium bromide. DNA was transferred to a filter membrane and hybridized with the ^{32}P-labeled oligonucleotide 12.3–3 at 61°C (21). Filter was washed twice with 2 × SSC/0.5% SDS at room temperature for 10 min and twice with 1 × SSC/0.5 × SDS at 61°C for 20 min before exposure to Kodak XAR film (Eastman Kodak, Rochester, NY). The PCR product amplified from autologous PMN DNA using the 58–5 and 58–3 primer pair and that amplified from the mAb426.12.3F1.4–producing cell DNA using the 58–5 and 12.3–3 primer pair were also fractionated on 2% low melting agarose gel. The DNA fragments were isolated, cloned, and sequenced.

To determine whether the nucleotide differences displayed by the mAb10 V_H gene sequence compared with that of the closest reported germ line V_H gene represented somatic point mutations, we performed PCR amplification of V_H gene segments from genomic DNA of the mAb10–producing B cell hybridoma and autologous PMN using combinations of the ad hoc designed V_H4.18/V_{2-1}FR1-CDR1 and mAb10V_HCDR1 oligonucleotide primers, and the HBL-3 FR3 primer. The sequence of the sense V_H4. 18/V_{2-1} FR1-CDR1 [5′ GCTCCATCAGCAGTAGTAGT 3′] primer encompassed a FR1-CDR1 stretch (residues 80 to 99) of the germ line V_H4.18 gene (22), which differed by four nucleotides from that of the corresponding area of the mAb10 V_H gene. The sequence of the sense mAb10V_HCDR1 oligonucleotide primer [5′ GCTCCATCAGTACCAGTACT 3′] encompassed a FR1-CDR1 stretch (residues 80 to 99) of the mAb10 V_H gene, which was different by four nucleotides from that of the corresponding area (same as that of V_H4.18/V_{2-1} FR1-CDR1) of the germ line V_H4.18 gene (Fig. 2A). The antisense HBL-3 oligonucleotide belonged to our collection of primers and was the reverse complement [5′ TCGCGGACGTGTCCACTGACA 3′] of a FR3 sequence (residues 256 to 275) identical in the germ line V_H4.18 gene and the mAb10

V_H gene except for a G instead of an A at position 270 (Fig. 2A). Genomic DNA (100 ng) was subjected to PCR (50 μl vol) with 100 ng of the HBL-3 FR3 and 100 ng of either the $V_H4.18/V_{2-1}$ FR1-CDR1 or the mAb10V_H CDR1 oligonucleotide primer. Each cycle consisted of denaturing, annealing, and extension steps of 1, 2, and 2 min, respectively. Denaturing and extension temperatures were 94° and 72°C, respectively. Annealing temperatures were 60°C for both the $V_H4.18/V_{2-1}$ FR1-CDR1 and the mAb10V_H CDR1 oligonucleotide primers. After 30 cycles, the products amplified using DNA extracted from autologous PMN or from mAb10–producing B cell hybridoma were fractionated on 1.7% agarose gel containing 1 μg/ml of ethidium bromide or on 2% low melting agarose gel for cloning and sequencing.

Results

Establishment of the IgG mAb-producing cell lines and mAb Ag binding features

EBV-transformed B cells from a healthy subject and two autoimmune patients (one with IDDM and one with SLE) were selected for production of IgG to human recombinant insulin (mAb10) (23) or ssDNA (mAb410.7.F91 and mAb410.30F305) or ssDNA, insulin, and tetanus toxoid (mAb426.4.2F20 and mAb426.12.3F1.4) and used to generate mAb-producing cell lines. All IgG mAb were poly-reactive. Their IgG subclasses, L chain types, and K_d values for insulin, ssDNA, and tetanus toxoid are listed in Table I. Their dose-saturable bindings to a panel of self and foreign Ag are depicted in Figure 1.

Sequences of the IgG mAb V_H segments

The nucleotide and deduced amino acid sequences of the five IgG mAb V_H genes are depicted in Figure 2A and B, respectively. Their comparisons with the sequences of the closest germ line V_H genes are summarized in Table I. The mAb10 V_H gene sequence displayed the highest degree of identity with that of the germ line $V_H4.18$ gene (22). Nucleotide differences were five in the CDR and 14 in the FR, yielding four and six amino acid differences, respectively. The mAb426.4.2F20 and mAb426.12.3F1.4 V_H gene sequences displayed the highest degree of identity with that of the germ line $V_H4.11$ gene (22). The mAb426.4.2F20 V_H gene contained three nucleotide differences, two in the CDR2 and 1 in the FR1, resulting in one and one amino acid differences, respectively. The mAb426.12.3F1.4 V_H gene displayed 12 nucleotide differences, of which three were in the CDR and nine in the FR. All three differences in the CDR resulted in amino acid differences, whereas only three of the nine in the FR did. The mAb410.7.F91 V_H gene sequence differed from that of the germ line $V_H4.21$ gene in three nucleotides. These yielded a single amino acid difference. The mAb410.30F305 V_H gene sequence displayed the highest similarity to that of the germ line (V_HI) hv1263 gene (24). The four differences in the CDR and the eight in the FR resulted in three and two amino acid differences, respectively.

Thus, although the mAb426.4.2F20 and mAb410.7.F91 V_H gene sequences were virtually identical with those of the respective germ line genes, the V_H genes of the IgG mAb10, mAb426.12.3F1.4, and mAb410.30F305 displayed a high number of differences when compared with the respective germ line gene sequences. Table II shows the total number of

putative changes and R:S mutation ratios in the V_H genes of the five polyreactive IgG mAb, in those of the highly substituted three IgG, mAb10, mAb426.12.3F1.4, and mAb410.30F305, and in those of the virtually unmutated two IgG, mAb426.4.2F20 and mAb410.7.F91. In the three highly substituted IgG V_H segments, the overall putative nucleotide changes were 12 in the CDR and 31 in the FR, yielding a frequency of 6.1×10^{-2} and 4.5×10^{-2} nucleotide change/base, respectively. These putative changes entailed a significantly higher number of replacement mutations in the CDR than the FR, 5.6×10^{-2} vs 1.4×10^{-2} R mutation/base, respectively ($p < 0.01$, χ^2 test), and a significantly higher overall R:S mutation ratio (11.0 in CDR vs 0.4 in FR) ($p < 0.01$, χ^2 test).

D and J_H gene sequences and CDR3 configuration

The nucleotide and deduced amino acid sequences of the D and J_H segments of the polyreactive IgG mAb are depicted in Figure 3A and B, respectively. The mAb410.7.F91 D segment contained a stretch of nucleotides highly similar to most of the DXP′1 gene sequence (25); mAb10 and mAb426.4.2F20 D segments contained nucleotide sequence stretches identical to portions of the germ line DXP′4 gene (25); mAb426.12.3F1.4 and mAb410.30F305 D segments contained short nucleotide sequences identical with areas of the DK4 gene (25). The numbers of putative N segment additions at the 5′- and 3′-flanks of the D genes were highly variable. The overall lengths of the D segments ranged from 17 to 58 nucleotides. The nucleotide sequence of mAb426.12.3F1.4 J_H segment was identical, except for one base to that of J_H3 (26); those of the mAb10, mAb410.7.F91, and mAb410.30F305 J_H segments consisted of truncated forms of the putatively predominant haplotype of the J_H4 germ line gene, J_H4b (27), with two replacement mutations in the mAb10 J_H sequence. The mAb426.4.2F20 J_H segment consisted of a truncated form of the putatively major haplotype of the germ line J_H6 gene (26), J_H6c (27).

The deduced amino acid sequences of the expressed D-J_H segments were segregated into CDR3 and FR4 according to Kabat et al. (28) and are depicted in Figure 3C. The IgG mAb CDR3 were divergent in length (8 to 24 amino acids; mean value, 16.4 ± 6.5 SD) and structure, with the exception of those of the two IgG mAb using the DXP′4 gene. In these IgG (mAb10 and mAb426.4.2F20), the DXP′4 gene was used in the same reading frame, yielding an identical H chain CDR3 stretch of five amino acids (RFLEW). The FR4 structure of the five polyreactive IgG mAb was conserved.

Somatic point mutations in the polyreactive IgG mAb426.12.3F1.4 V_H segment

The polyreactive mAb426.12.3F1.4 V_H gene sequence displayed 12 differences when compared with that of the germ line $V_H4.11$ gene. To assess whether these nucleotide differences resulted from somatic mutation or were due to the use of a germ line V_H gene related to but not identical with $V_H4.11$, we performed the following experiments. We synthesized the sense 58-5 oligonucleotide primer encompassing a FR1 sequence shared by the $V_H4.11$ and mAb426.12.3F1.4 V_H genes (Fig. 2A). We also synthesized the antisense 12.3-3 primer, the sequence of which was identical with the reverse complement of a 5prime;-portion of the FR3 region of mAb 426.12-3F1.4 V_H sequence, but differed in four nucleotides from the reverse complement of the corresponding $V_H4.11$ sequence. We chose this FR3 area because it was the only one throughout the mAb426.12.3F1.4 V_H gene that

contained at least four nucleotide differences when compared with the V_H4.11 gene sequence. The theoretically preferable V_H CDR2 area contained only two nucleotide differences (Fig. 2A). Somatic mutations have been found to commonly occur in the FR3 5′-area concomitant with mutations in the CDR2, as suggested by the analysis of mAb generated through the response against various Ag in mice (29–34) and humans (21, 35–38). In addition, a relative lack of conservation in this FR3 sequence among V_HIV gene family members has been shown (22, 39–41), suggesting that the use of the 12.3-3 primer would have preferentially targeted those V_HIV genes different in this area from V_H4.11, thus maximizing the discrimination power of our analysis. The PCR involving the 58-5 and 12.3-3 primers resulted in amplification of DNA from the mAb426.12.3F1.4–producing B cell hybridoma DNA, but not from autologous PMN DNA. The molecular size of the amplified product (~160 bp) was consistent with that of the sequence spanning residues 65 to 226 (161 bp) of the expressed V_H gene (Fig. 4A). The sequence of this DNA fragment was identical with the corresponding sequencing of the mAb426.12.3F1.4 V_H gene (Fig. 2A, 58-5/12.3-3 sequence). This suggested that the expressed V_H gene was somatically mutated. This was confirmed by the results of the subsequent experiments. We synthesized the antisense 58-3 oligonucleotide primer, the sequence of which was identical with the reverse complement of a V_H4.11 FR3 sequence and differed in two bases from the reverse complement of the corresponding mAb426.12.3F1.4 V_H gene sequence. PCR involving the 58-5 and 58-3 primer pair yielded products of the expected and identical size (~200 bp) when applied to the amplification of not only B cell hybridoma, but also autologous PMN DNA (Fig. 4B). This demonstrated that the failure to amplify any DNA from autologous PMN DNA using the 12.3-3 primer was not due to flaws inherent to the DNA preparation and provided the material necessary for differential hybridization with the 12.3-3 oligonucleotide as a probe. The 12.3-3 nucleotide hybridized with DNA amplified from B cell hybridoma but not with that amplified from PMN (Fig. 4C). The DNA fragment PCR amplified from the PMN DNA was used to identify, by cloning and sequencing, the putative germ line gene that gave rise to the expressed mAb426.12.3F1.4 V_H gene. Five independent clones were analyzed and found to contain an identical V_H gene sequence. This (G411) was identical with the sequence of the V_H4.11 gene throughout the overlapping area (169 bp). These experiments proved that the expressed mAb 426.12.3F1.4 V_H gene was somatically mutated and strongly suggested that V_H4.11 was the germ line gene that gave rise to it.

Somatic point mutations in the polyreactive IgG mAb10 V_H segment

The polyreactive mAb10 V_H gene sequence displayed 19 differences when compared with that of the germ line V_H4.18 gene. To assess whether these nucleotide differences resulted from somatic mutation or were due to the use of a germ line V_H gene related to but not identical with V_H4.18, we performed the following experiments. We synthesized the sense V_H4.18/$V_{2\text{-}1}$ FR1-CDR1 oligonucleotide primer encompassing a FR1-CDR1 sequence of the germ line V_H4.18 gene, which differed by four residues from that of the corresponding area of the mAb10 V_H gene (Fig. 2A). We used this primer in conjunction with the antisense HBL-3 FR3 primer, the sequence of which was the reverse complement of a FR3 sequence shared by the germ line V_H4.18 gene and the mAb10 V_H gene, except for a G instead of an A at position 270. The PCR involving this pair of primers yielded a DNA amplification product when using DNA from autologous PMN but not when using DNA from the

mAb10–producing B cell hybridoma. The molecular size of the amplified DNA (~ 195 bp) was consistent with that of the sequence spanning residues 80 to 275 of the V_H4.18 gene (Fig. 5A). In addition, the sequence (from five independent clones) of the amplified DNA fragment was identical with that of residues 80 through 275 of the germ line V_H4.18 gene, except for the G of the HBL-3 primer (Fig. 2A, G418 sequence). The results of the above experiments were consistent with the hypothesis that the mAb10 V_H segment was somatically mutated. To demonstrate that the failure to amplify DNA from the mAb10-producing B cell hybridoma DNA when using the V_H4.18/V2-1 FR1 primer was not due to flaws inherent to the genomic DNA preparation, and to provide a "positive" finding in favor of mutations in the mAb10 V_H segment, we synthesized the sense mAb10V_HCDR1 oligonucleotide primer encompassing a FR1-CDR1 sequence of the mAb10 V_H segment that differed from that of the V_H4.18/$V_{2\text{-}1}$ FR1-CDR1 oligonucleotide by four bases (Fig. 2A). The PCR involving the mAb10V_HCDR1 primer in conjunction with the antisense HBL-3 FR3 primer yielded an amplification DNA product using DNA from the mAb10-producing B cell hybridoma but not DNA from autologous PMN. The molecular size of the amplified fragment (~195 bp) was consistent with that of the sequence spanning residues 80 to 275 of the mAb10 V_H gene (Fig. 5B). In addition, the sequence (from five independent clones) of the amplified fragment was identical with that of residues 80 through 275 of the mAb10 V_H gene, except for the G of the HBL-3 primer (Fig. 2A, mAb10V_HCDR1/FR3 sequence). These experiments showed that the expressed mAb10 V_H gene was somatically mutated and strongly suggested that V_H4.18 was the germ line gene that gave rise to it. The somatically mutated status of the mAb10 V_H segment was further emphasized by the two mutations, a T replacing an I and a T replacing a S at the seventh and fourth but last residues, respectively, in the mAb10 J_H4b FR4 (Fig. 3).

Discussion

Polyreactive natural antibodies are primarily IgM and are thought to rely on the use of V regions in germ line configuration for the recognition of multiple Ag. To test the hypothesis that polyreactivity may be also a function of somatically mutated antibodies, we generated five human polyreactive IgG mAb and sequenced the genes encoding their V_H segments. The choice of the IgG class was dictated by the hypothesis that if polyreactive somatically mutated antibodies existed, these would be more numerous among IgG than IgM. Thus, selection for IgG would increase the probability of generating polyreactive mAb with mutated V regions. The results of our experiments showed that: 1) the V_H genes used by the five polyreactive IgG mAb were identical with or closely related to those used by natural polyreactive IgM antibodies, as well as high affinity specific autoantibodies and antibodies induced by foreign Ag; 2) the Ag binding features and the V_H-D-J_H sequences were distinctive in each polyreactive IgG mAb; and 3) the V_H segments of three IgG mAb contained somatic mutations, which displayed a distribution and a nature similar to those found in Ig V regions that underwent a process of Ag-dependent selection.

Findings in mice and humans have suggested that the Ag binding properties of individual polyreactive IgM mAb are distinctive (4–12, 14). Like polyreactive IgM, the present polyreactive IgG mAb display discrete Ag binding activities for different self and foreign Ag. The functional "uniqueness" of each polyreactive IgG probably reflects the structural

heterogeneity of the Ag binding sites of these mAb. Previous observations have shown that while polyreactive, mainly IgM, human antibodies can use various arrays of different V_H and V_L segments, as well as different D, J_H, or J_L segments (5, 13, 15–17, 21, 37, 38, 42–44), they may display some preference for the use of the V_HIV family genes (15, 17, 44a). Accordingly, four of the five polyreactive IgG mAb used three different V_HIV genes, the sequences of which are similar to those of the germ line V_H4. 11, V_H4.18, and V_H4.21 genes. These V_H genes, however, along with the hv1263 (V_HI) gene used by the fifth polyreactive IgG mAb are used also by monoreactive high affinity mAb to encode reactivities for different self and foreign Ag (5, 17, 21, 37, 38, 43, 44). For example, germ line and variously mutated forms of the V_H4.21 gene are used by various polyreactive and monoreactive antibodies and autoantibodies (44); variously mutated forms of the V_H4.18 gene are used by the monoreactive RF IgM mAb 61 (17) and the H3 IgG1 mAb, which neutralizes both HSV-1 and HSV-2 (45). Variously mutated forms of the hv1263 gene are used by monoreactive high affinity IgG RF mAb (46), the IgG1 mAb 57 (21), which neutralizes rabies virus in vivo and in vitro (9), and by the high affinity IgG1 mAb 49 to insulin generated from an IDDM patient (23) (H. Ikematsu et al., manuscript in preparation). Thus, the pool of V_H genes used by the present polyreactive IgG autoantibodies overlaps, at least partially, with that of V_H genes recruited in high affinity immune responses to foreign Ag in healthy subjects or to self Ag in autoimmune patients (4, 5, 43).

Some unmutated V_H segments are inherently capable of binding certain foreign Ag, as exemplified by the frequent selection of the V_HOx1 segment in the BALB/c mouse response to 2-phenyl-5-oxazolone (29, 47) and that of the S107 V_H segment in the response of the same mouse strain to phosphorylcholine (32) or self Ag, as exemplified by the V_H11 segment use of the autoantibodies to DNA in BALB/c mice (48) and the 3H9 V_H segment use of DNA-selected autoantibodies in SLE-prone MRL/*lpr* mice (49). The somatically generated H chain CDR3, however, appears to be the major contributor to the overall function of the Ag binding site, at least in the case of relatively large proteinic Ag (50, 51). The H chain CDR3 structures of two IgG, mAb10 and mAb426.4.2F20, which were generated from different subjects, shared an identical five amino acid stretch, RFLEW, but those of the other three IgG mAb were highly divergent in composition and length and did not allow for the identification of any obvious common motif. Thus, if consistent with what has been suggested by the in vitro expression of a human IgM RF H and L chain genes (52) and by a census of 84 natural polyreactive and Ag-induced monoreactive murine mAb (53), the H chain CDR3 provides the correlate for polyreactivity in our five different IgG mAb, it does so by virtue, in most cases, of discrete primary structures. A thorough evaluation, however, of the contribution of the different H chain regions to the overall structure and activity of a polyreactive IgG Ag binding site must take into account also the configuration of the V_L segment.

This study is one of the first evidences that the V_H regions of polyreactive antibodies can be somatically mutated. The relatively high degree of allelic polymorphism of members of certain human V_H multigene families and the presence of different but highly homologous members in a single haplotype can make it difficult to assess the somatically mutated status of expressed V_H genes in the outbred human population by mere inspection of the already

reported closest germ line genes. Some individual Ig V_H genes, however, including V_H4.18 and V_H4.21 (V_HIV family) and hv1263 (V_HI family) are highly conserved among humans (22, 24, 40, 43, 54). The data obtained from differential PCR amplification, Southern hybridization, and genomic DNA sequencing confirmed that the mAb426.12.3F1.4 and mAb10 V_H segments contained more than eight and seven somatic point mutations, respectively. The patient B (Table I) source of the germ line G411 gene also provided the B cells used for the generation of the IgG mAb426.4.2F20, which probably used the same V_H4.11 or V_H4.11-like gene used by the mAb426.12.3F1.4. Thus, at least two of the three nucleotide differences (from the germ line G411 gene) found in the polyreactive IgG mAb426.4.2F20 V_H gene also represented somatic point mutations (Fig. 2A and B). Finally, the somatically mutated status of the IgG mAb410.30F305 V_H segment was suggested not only by the high degree of conservation of the hv1263 gene (24, 43), but also by the fact that none of the six amino acid differences displayed by the mAb410. 30F305 V_H gene deduced amino acid sequence (compared with that of the germ line hv1263 gene) are shared by the 51P1 sequence, possibly the closest gene to hv1263 and by the fact that only one (residue 33) of these six amino acid differences is shared by any of the reported expressed hv1263 segment sequences, including that of the mAb57 (21).

Thus, the present experiments suggested that although some polyreactive IgG antibodies can be virtually unmutated, possibly reflecting the germ line configuration of the IgM-producing clones from which they arose, other polyreactive IgG antibodies bear the imprints characteristic of a selective antigenic pressure. They, however, could provide little clue to the natural history of the polyreactive somatically mutated IgG-producing cell clones. High R:S mutation ratios in the Ig V segment CDR and low R:S ratios in the FR are features characteristic of the clonotypes that have been recruited and positively selected during the maturation of an antibody response to a foreign Ag or to a self Ag in the course of a chronic autoimmune disease (37, 38, 46, 47, 55–60). At different stages of such antibody and autoantibody responses, similar features, however, are also displayed by relatively large numbers of clones with a reduced affinity for the selecting Ag (47, 61, 62). These clones are possibly negatively selected (by Ag), do not contribute to affinity maturation, and are part of the tremendous (clonotypic) “wastage” accompanying any Ag-directed affinity maturation process (47, 61, 62). Other clones with reduced affinity may emerge as a result of a positive selection by stimuli, other than those provided by the Ag driving the overall response, such as an anti-idiotypic interaction. Thus, the predominant distribution of the mutations in the three polyreactive IgG mAb410.30F305, mAb10, and mAb426.12.3F1.4 V_H segment CDR and high R:S ratio (11.0 in CDR vs 0.8 in FR) suggest that these antibodies underwent a process of Ag-directed selection. They, however, do not allow for any inferences as to whether the clones producing these IgG participated in vivo to an affinity maturation to any of the Ag tested or to any other undetermined Ag. In this respect, the relatively high affinity for ssDNA displayed by the three “highly substituted” IgG mAb possibly reflects the strong ssDNA binding activity in general displayed by natural IgM, IgA, and IgG polyreactive autoantibodies (5–9, 63), including the remaining two “minimally” substituted IgG mAb (mAb426.4.2F20 and mAb410.7.F91), rather than suggesting that DNA was the in vivo selecting Ag.

Somatic mutations could accumulate in the V segments of an originally polyreactive antibody without significantly affecting its ability to bind multiple Ag. An alternative but not mutually exclusive possibility, is that somatic mutations accumulated in a inherently monoreactive “specific” antibody and conferred it the ability to bind multiple Ag. Although we cannot formally rule out this possibility, we believe the first to be far more likely. To the best of our knowledge, accumulation of somatic mutations has never been reported to result in acquisition of polyreactivity by antibodies undergoing an Ag-directed selection in the course of various murine responses to foreign or self Ag (47, 55–60). Rather, polyreactivity has been shown to be a function, in general, of unmutated IgM V_H segments. It has been speculated that application of antigenic pressure to polyreactive natural antibodies in unmutated configuration can result in the accumulation of somatic point mutations, somatic selection, loss of polyreactivity, and, possibly, class switch (52). In humans, a strong evidence for somatic hypermutation of clonally related V_H-D-J_H sequences expressed with IgM, IgG, and IgA in the spleen has been recently provided (64). The present findings are consistent with the hypothesis that application of antigenic pressure to germ line natural IgM autoantibodies can lead to the accumulation of somatic point mutations and class switch to IgG without significantly affecting the antibody ability to bind multiple Ag. A similar mechanism has been suggested to underlie the generation of some IgG clones emerging, after the second injection with Ag, during the affinity maturation of the response to *p*-azophenyl arsonate (Ars) from (originally germ line) Id^{CR+} (also called CRI-A) polyreactive natural IgM autoantibody-producing cells, as shown by Naparstek et al. (65; see clones hV_H65-219 and 31–62 in group III of Table I). It also has been called into question to account for the polyreactivity of the human IgM and IgG mAb specific for blood group Ag, emerging in vivo after alloimmunization (66).

These and our present findings would be consistent with our preliminary data suggesting that in a polyreactive antibody different, although partially overlapping, V segment structures can mediate the binding to different Ag (Ichiyoshi et al., manuscript in preparation).

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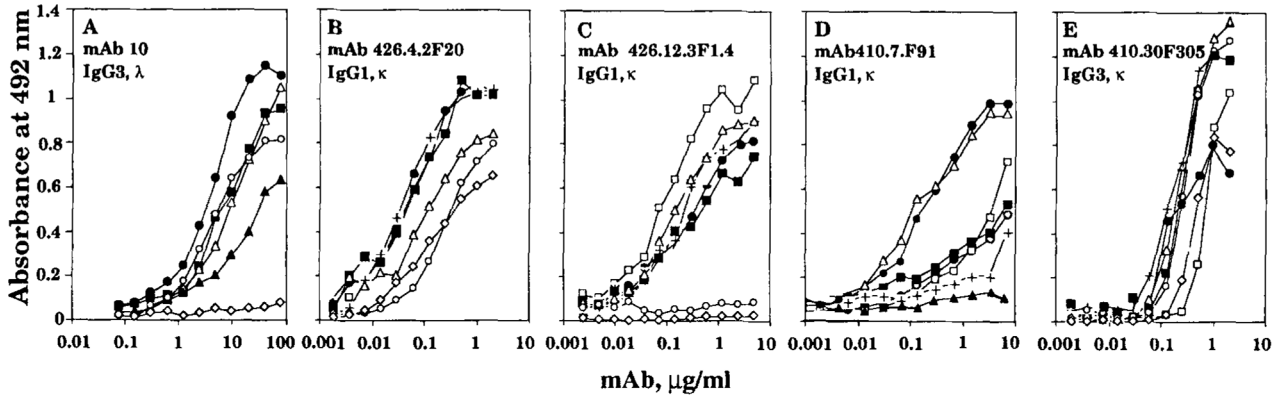

FIGURE 1.

Dose-saturable binding of the polyreactive IgG mAb. The Ag binding activity of each mAb is expressed as optical absorbance at 492 nm. The following Ag were used: human recombinant insulin (●), tetanus toxoid (■), ssDNA (△), phosphorylcholine (+), human recombinant TNF-*α* (○), thyroglobulin (▲), and BSA (◇).

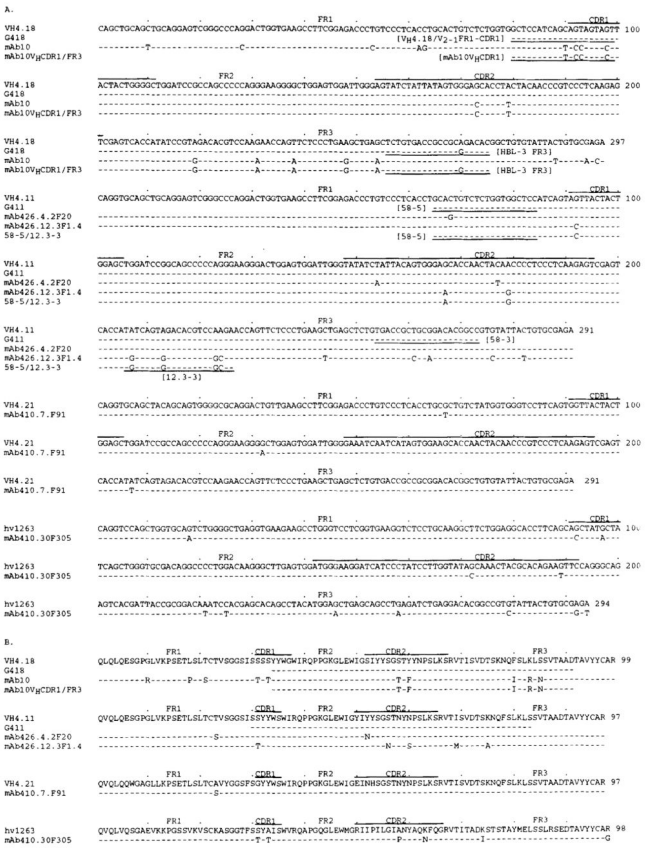

FIGURE 2.

Nucleotide (*A*) and deduced amino acid (*B*) sequences of the V_H genes used by the polyreactive IgG mAb. In each cluster, the top sequence is given for comparison and represents the closest published germ line V_H gene. Dashes indicate identities. Solid lines above each cluster depict CDR. G418 and G411 are the germ line sequences derived from healthy subject A and IDDM patient B, respectively (see text). The sequences or complementary sequences of the primers used for PCR amplification of genomic DNA are underlined. The present sequences are available from the EMBL/GenBank under accession numbers L23515, L23516, L23517, L23518, and L23519.

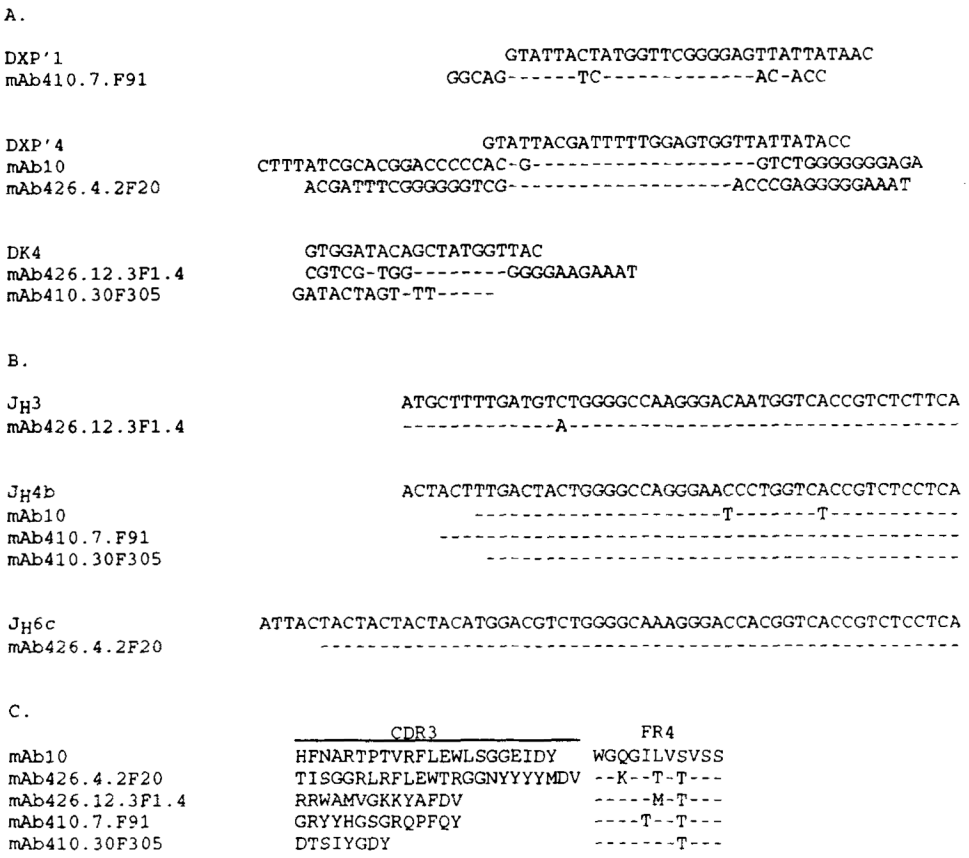

FIGURE 3.

Nucleotide (*A* and *B*) and deduced amino acid (*C*) sequences of the D and J_H segments of the polyreactive IgG mAb. The top sequence in each cluster is given for comparison. Dashes indicate identities. Solid lines above the deduced amino acid sequences depicts the CDR3. The present sequences are available from the EMBL/GenBank under accession numbers L23515, L23516, L23517, L23518, and L23519.

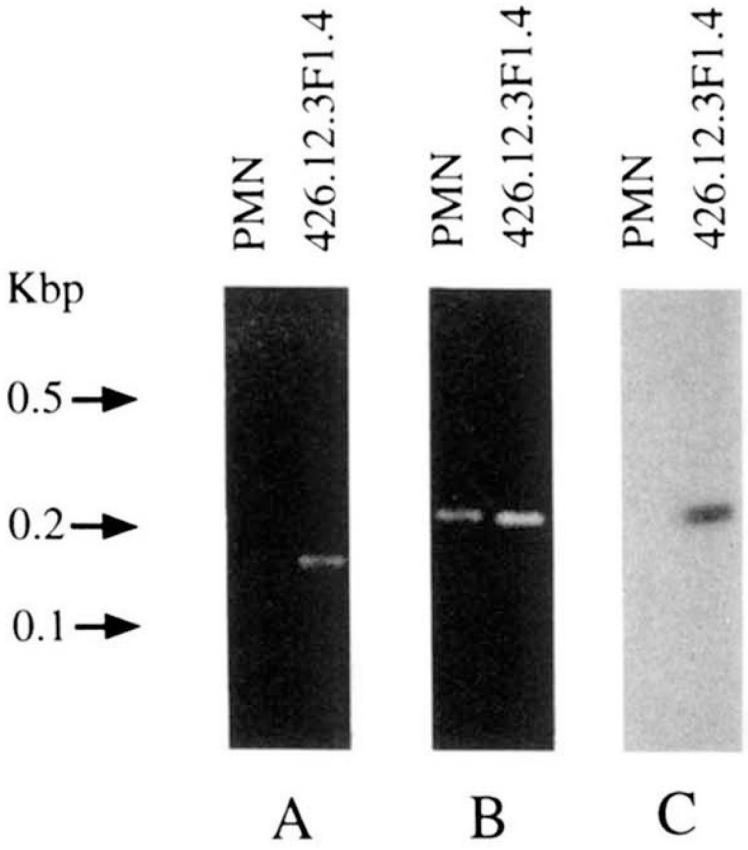

FIGURE 4.

PCR and Southern blot analysis of somatic mutations in the mAb 426.12.3F1.4 V_H gene. *A*, ethidium bromide staining of DNA PCR amplified using the 58-5 and 12.3-3 primers and genomic DNA extracted from autologous PMN (left lane) or the mAb426.12.3F1.4–producing B cell hybridoma (right lane). *B*, ethidium bromide staining of the DNA PCR amplified using the 58-5 and 58-3 primers and genomic DNA extracted from autologous PMN (left lane) or the mAb426.12.3F1.4–producing B cell hybridoma (right lane). *C*, Southern blot hybridization of the PCR-amplified products shown in *B* with the ^{32}P-labeled 12.3-3 oligonucleotide probe.

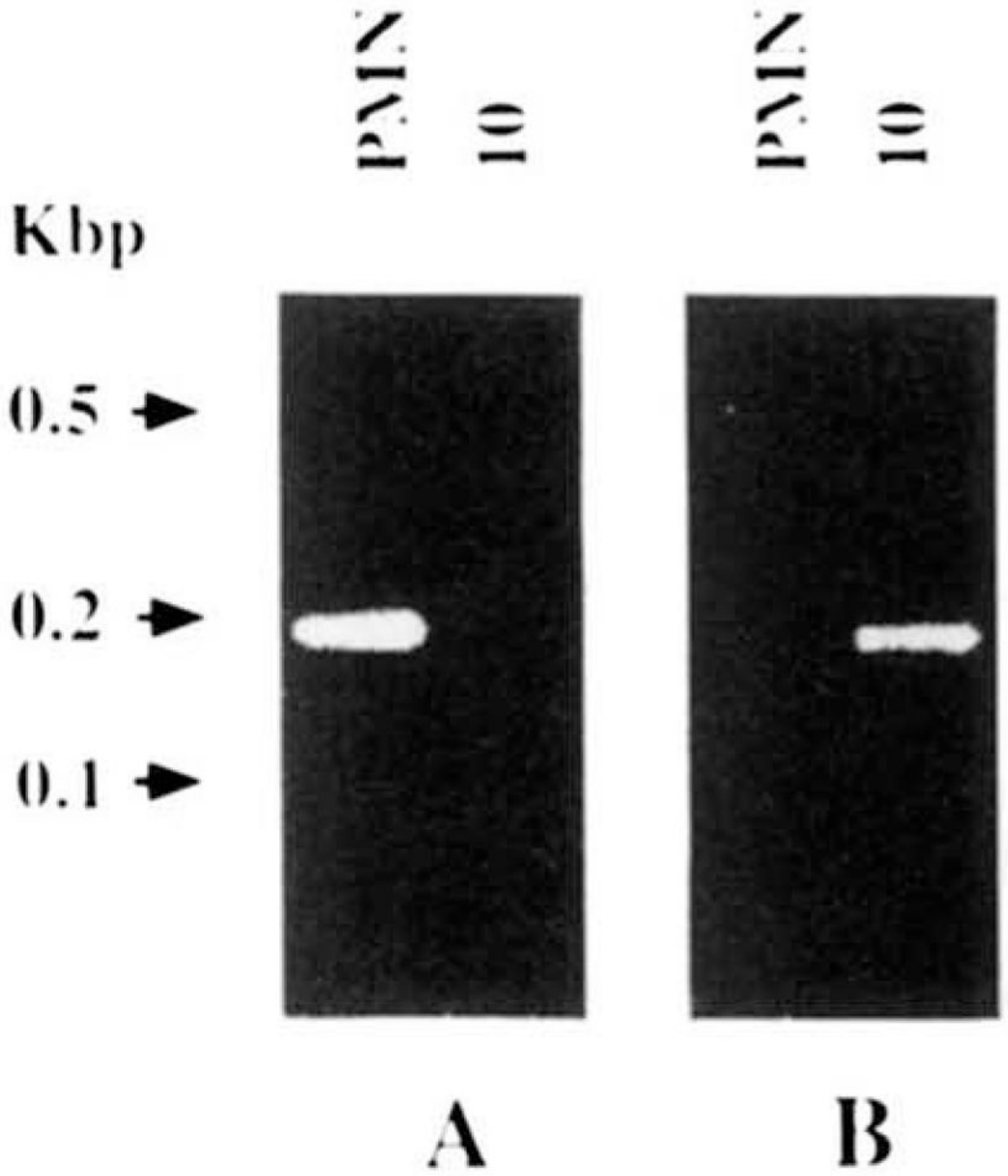

FIGURE 5.

PCR analyses of somatic mutations in the mAb10 V_H gene. *A*, ethidium bromide staining of DNA PCR amplified using the $V_H4.18/V_{2-1}$ FR1-CDR1 and HBL-3 FR3 primers and genomic DNA extracted from autologous PMN (left lane) or the mAb10-producing B cell hybridoma (right lane). *B*, ethidium bromide staining of the DNA PCR amplified using the mAb10V_H CDR1 and HBL-3 primers and genomic DNA extracted from autologous PMN (left lane) or the mAb10-producing B cell hybridoma (right lane).

Table I

Ag binding features and V_H, D, and J_H genes of human polyreactive IgG mAb

Clone	Source	Chains	K_d (M)			V_H Segment							D Gene[b]	J_H Gene[c]
						Closest Gene		%	Nucleotide Differences		R:S Mutation Ratio			
		HL	Insulin	ssDNA	Tetanus Toxoid	Family	Member[a]	Identity	CDR	FR	CDR	FR		
mAb10	Healthy subject A	γ3, λ	1.0×10^{-5}	2.0×10^{-7}	1.0×10^{-5}	V_HIV	V_H4.18	93.6	5	14	4:1	6:8	DKP4	J_H4
mAb426.4.2F20	IDDM patient B	γ1, κ	2.0×10^{-5}	6.8×10^{-7}	3.9×10^{-7}	V_HIV	V_H4.11	99.0	2	1	1:1	1:0	DKP4	J_H6
mAb426.12.3F1.4	IDDM patient B	γ1, κ	4.0×10^{-5}	1.7×10^{-8}	1.0×10^{-6}	V_HIV	V_H4.11	95.9	3	9	3:0	2:7	DK4	J_H3
mAb410.7.F91	SLE patient C	γ1, κ	1.9×10^{-6}	1.0×10^{-8}	1.0×10^{-6}	V_HIV	V_H4.21	99.0	0	3	0:0	1:2	DXP′1	J_H4
mAb410.30F305	SLE patient C	γ3, κ	2.5×10^{-4}	8.3×10^{-8}	1.7×10^{-6}	V_HI	hv1263	95.9	4	8	4:0	2:6	DK4	J_H4

[a] The sequences of the germ line V_H4.18, V_H4.11, and V_H4.21 genes have been reported by Sanz et al. (22); the sequence of the hv1263 gene has been reported by Chen et al. (24).

[b] D genes have been reported by Ichihara et al. (25).

[c] J_H genes have been reported by Ravetch et al. (26).

Table II

Mutations in human polyreactive IgG mAb V_H segments

	CDR	FR
5 (all) IgG mAb		
Total (R and S) mutations[a]	14	35
Total mutations (mutation/base)	4.3×10^{-2}	3.0×10^{-2}
R mutations (mutation/base)	3.7×10^{-2}	1.0×10^{-2}
R:S mutation ratio	6.0	0.5
3 IgG mAb (mAb10.mAb426.12.3F1.4, and mAb410.30F305)		
Total (R and S) mutations[a]	12	31
Total mutations (mutation/base)	6.1×10^{-2}	4.5×10^{-2}
R mutations (mutation/base)	5.6×10^{-2}	1.4×10^{-2}
R:S mutation ratio	11.0	0.4
2 IgG mAb (mAb426.4.2F20 and mAb410.7.F91)		
Total (R and S) mutations[a]	2	4
Total mutations (mutation/base)	1.6×10^{-2}	0.7×10^{-2}
R mutations (mutation/base)	0.8×10^{-2}	0.4×10^{-2}
R:S mutation ratio	1.0	1.0

[a] In mAb10, mAb426.12.3F1.4, and mAb426.4.2F20 V_H segments mutations were formally verified.