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Autoreactivity in Human IgG+ Memory B Cells

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SUMMARY

More than half of the nascent B cells in humans initially express autoreactive antibodies. However, most of these autoantibodies are removed from the repertoire at two checkpoints before maturation into naive B cells. A third checkpoint excludes remaining autoantibodies from the antigen-experienced IgM+ memory B cell pool. Nevertheless, low-affinity self-reactive antibodies are frequently found in the serum of normal humans. To determine the source of these antibodies, we cloned and expressed antibodies from circulating human IgG+ memory B cells. Surprisingly, we found that self-reactive antibodies including anti-nuclear antibodies were frequently expressed by IgG+ memory B cells in healthy donors. Most of these antibodies were created de novo by somatic hypermutation during the transition between mature naive and IgG+ memory B cells. We conclude that deregulation of self-reactive IgG+ memory B cells may be associated with autoimmunity.

INTRODUCTION

Random immunoglobulin (Ig) gene recombination frequently produces self-reactive antibodies: as many as ~75% of newly generated B cells in the bone marrow show reactivity to human epithelial larynx carcinoma HEp-2 cells in clinical anti-nuclear antibody (ANA) ELISA or immunofluorescence (Wardemann et al., 2003). A substantial fraction of these autoantibodies are polyreactive and many recognize nuclear self-antigens. However, polyreactive antibodies and true ANAs are tightly regulated by central and peripheral self-tolerance mechanisms. Thus, few self-reactive naive B cells persist, and these express polyreactive antibodies or HEp-2 reactive antibodies with only low amounts of reactivity for cytoplasmic antigens but not nuclear antigens (Wardemann et al., 2003).

Mature naive B cells responding to antigen differentiate into antibody-secreting plasma cells and memory B cells (McHeyzer-Williams and McHeyzer-Williams, 2005; Meffre et al., 2000; Radbruch et al., 2006; Rajewsky, 1996; Shapiro-Shelef and Calame, 2005). In humans, two types of memory B cells have been described: IgM+ memory B cells and class-switched memory B cells (Agematsu et al., 1997; Klein et al., 1998; Tangye et al., 1998). Little is known about the origin of IgM+ memory B cells, but by analogy to the mouse, it has been proposed that these cells are products of T cell-independent immune responses (Weller et al., 2001, 2004). We have shown that the transition from naive B cells into circulating IgM+ memory B cells is accompanied by efficient counterselection against self-reactive naive B cells before the onset of somatic hypermutation and that the few self-reactive IgM+ memory B cells present in the circulation of healthy humans gain self-reactivity as a result of somatic hypermutation (Tsuiji et al., 2006).

In contrast, development of most class-switched memory B cells depends on the germinal center reaction and T cell help. Ig class switching is accompanied by extensive Ig gene somatic hypermutation that could change antibody affinity or create self-reactive antibodies (Ray et al., 1996; Shlomchik et al., 1990; van Es et al., 1991).

To examine the establishment of self-tolerance in class-switched IgG+ memory B cells, we cloned, expressed, and measured the reactivity of 141 antibodies from human IgG+ memory B cells isolated from peripheral blood of three healthy donors. Surprisingly, we found that self-reactive antibodies including true ANAs and polyreactive antibodies were substantially enriched in circulating IgG+ memory B cells relative to naive B cells and IgM+ memory B cells.

Our finding that a large number of circulating IgG+ memory B cells normally express autoantibodies suggests that abnormalities in the regulation or activation of peripheral self-reactive IgG+ memory B cells may contribute to the development of autoimmunity in susceptible individuals.

RESULTS

Features of IgG+ Memory B Cell Antibodies

To characterize the antibody gene repertoire of IgG+ memory B cells, we isolated single IgG+CD27+CD19+CD10− peripheral blood cells from three healthy donors and cloned their IgH and IgL chains (Figure 1; see Figure S1 and Tables

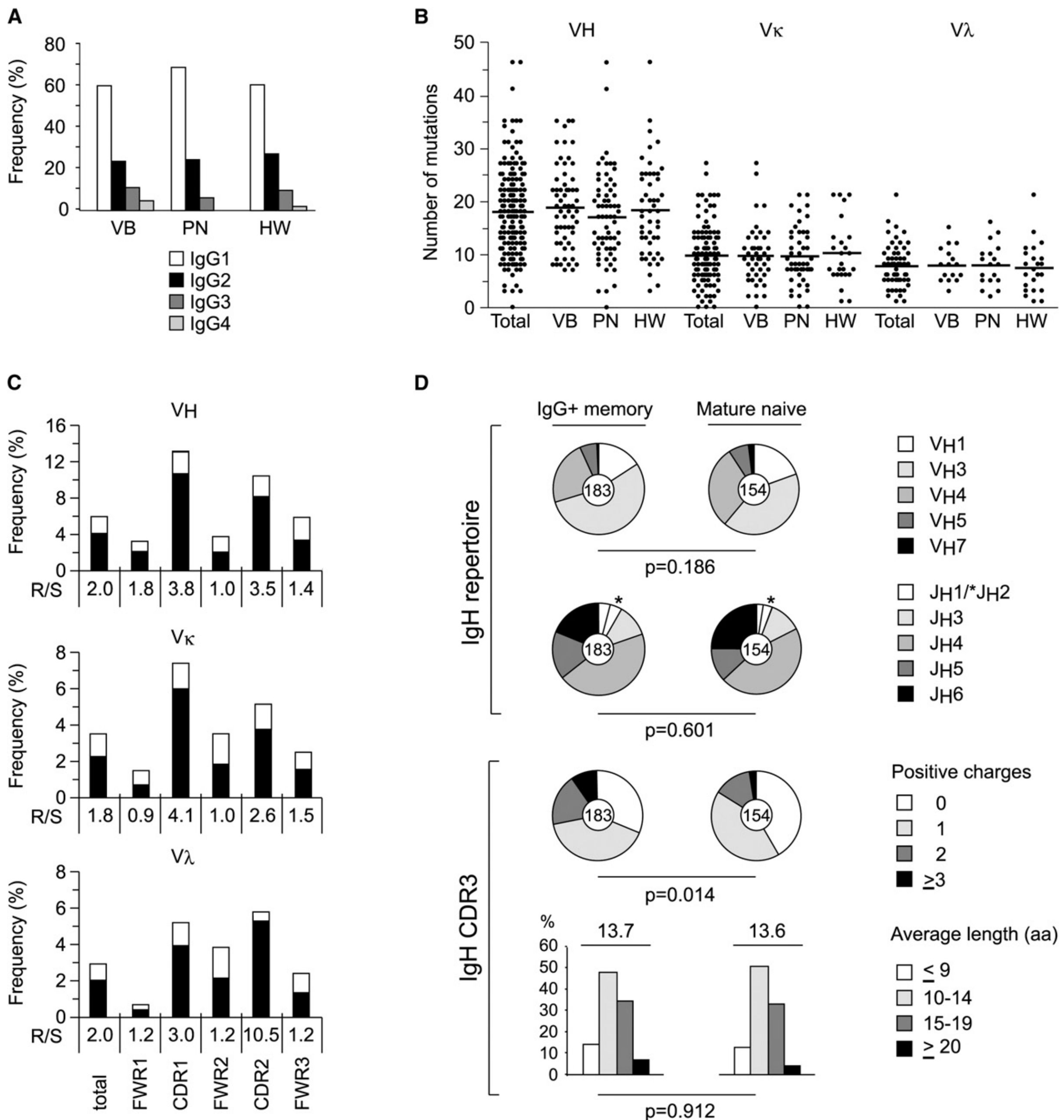

Figure 1. IgH and IgL Chain Gene Features from IgG Memory B Cell Antibodies

(A) IgG subclass distribution in single IgG⁺CD27⁺ memory B cells from three healthy donors.

(B) The number of mutations in VH, Vκ, and Vλ genes in antibodies from IgG⁺ memory B cells.

(C) The frequency of mutations in VH, Vκ, and Vλ genes in antibodies from IgG⁺ memory B cells calculated from the number of replacement (R; black bar) and silent (S; white bar) nucleotide exchanges per base pair in FWRs and CDRs. The R/S ratio for each region is indicated.

(D) IgH V and J gene repertoire and IgH CDR3 positive charges and length in amino acids (aa) of antibodies from IgG⁺ memory B cells compared to mature naive B cells. Pie charts depict VH and JH family usage and the proportion of IgH CDR3s with 0, 1, 2, or ≥3 positive charges. p values for VH and JH usage and IgH CDR3-positive charges were calculated by 2 × 5 Fisher exact test. Bar graphs show frequencies of IgH CDR3s with 9 aa (white bars), 10–14 aa (light gray bars), 15–19 aa (dark gray bars), and ≥20 aa (black bars). p values for IgH CDR3 aa length were calculated by two-tailed nonparametric t test. The absolute number of sequences analyzed is indicated in the center of each pie chart. Values for mature naive B cells in this and other figures were published previously and are shown here for comparison (Tsuiji et al., 2006; Wardemann et al., 2003).

S1–S3 in the Supplemental Data available online; Agematsu et al., 1997; Klein et al., 1998; Plebani et al., 1989; Tangye et al., 1998). B cells from all three donors showed an Ig subclass distribution reflecting that of human serum IgG antibodies with IgG1>IgG2>IgG3>IgG4 (Figure 1A; on average 63.4% IgG1, 25.5% IgG2, 8.9% IgG3, and 2.2% IgG4) and

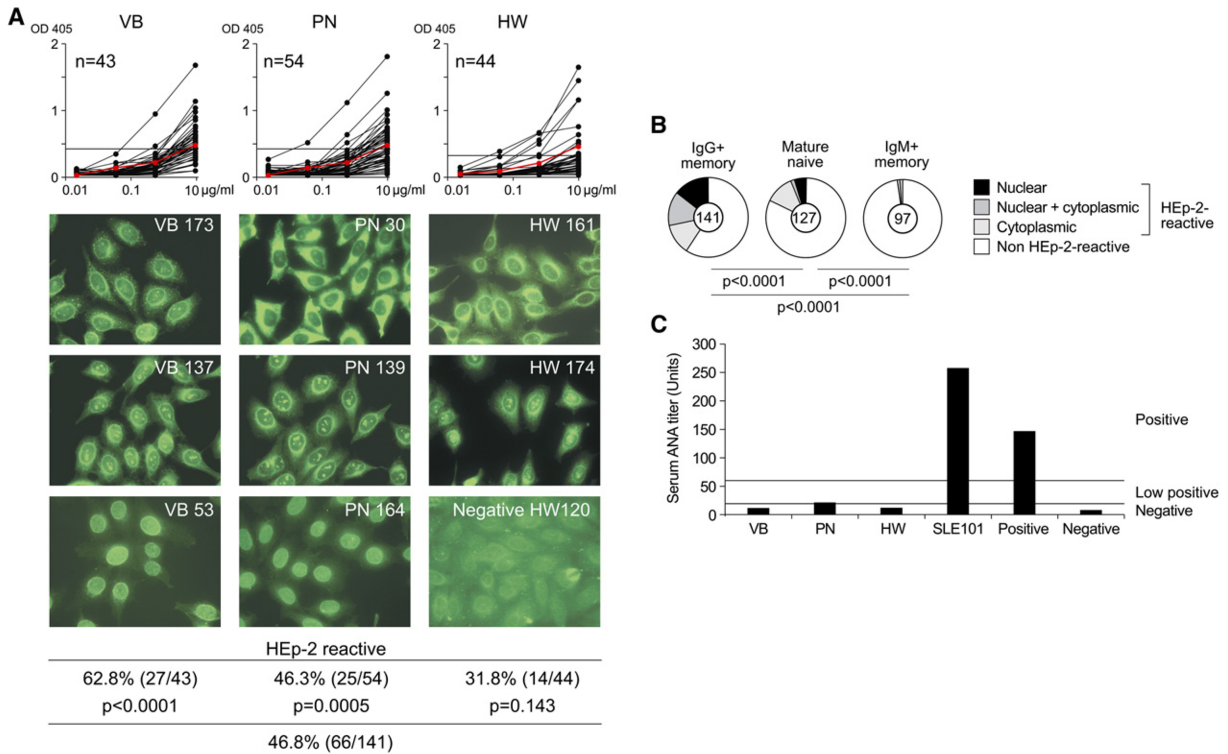

Figure 2. Self-Reactive Antibodies Are Enriched in the IgG+ Memory B Cell Pool

(A) IgG+ memory B cell antibodies from healthy donors were tested for self-reactivity by HEp-2 cell lysate ELISA and IFA. Horizontal line shows cut-off OD_{405} for positive reactivity determined by comparison to low positive control serum (red line). Typical HEp-2 cell IFA staining patterns of antibodies cloned from IgG+ memory B cells are shown. Data shown are representative for at least two independent experiments. p values were calculated by 2 × 2 Fisher exact test.

(B) Pie charts summarizing the frequency of HEp-2 self-reactive IgG+ memory B cell antibodies with nuclear (black), nuclear plus cytoplasmic (dark gray), and cytoplasmic (light gray) IFA staining patterns, and the frequency of nonreactive antibodies (white) in comparison to mature naive and IgM+ memory B cell clones (Tsuiji et al., 2006; Wardemann et al., 2003). The number of tested antibodies is indicated in each pie chart center. p values are in comparison to mature naive B cells and IgM+ memory B cells (Tsuiji et al., 2006; Wardemann et al., 2003) and were calculated by 2 × 5 Fisher exact test.

(C) Serum IgG ANA amounts of three healthy donors and one patient with systemic lupus erythematosus (SLE101; Yurasov et al., 2005) were determined by HEp-2-ANA-ELISA. The manufacturer's instructions were followed to calculate relative units based on internal positive and negative control sera. Horizontal lines show positive, low positive, or negative cut-off titres as indicated.

were somatically mutated with an average 18.0 ± 8.1, 9.7 ± 5.4, and 7.7 ± 4.0 mutations for IgH, Igκ, and Igλ V genes, respectively (Figures 1B and 1C). There were no major differences in IgV gene-mutation frequencies among IgG isotypes (data not shown), and as expected, replacement mutations were more frequent than silent mutations in heavy and light chain V gene complementary-determining regions (CDRs) compared to framework regions (FWRs), suggesting antigen-mediated selection (Figure 1C). Overall, IgH, Igκ, and Igλ light chain gene usage was similar between IgG memory and IgM mature naive B cell antibodies, but antibodies with positively charged IgH CDR3 regions, a feature associated with self-reactivity, were enriched in IgG+ memory B cells (Figure 1D; p = 0.014; Jang et al., 1998; Radic et al., 1993).

IgG+ Memory B Cells Frequently Express Self-Reactive Antibodies

Human epithelial cell line, HEp-2 enzyme-linked immunosorbent assay (ELISA), and indirect immunoflourescence assay (IFA) are used as diagnostic tests to detect self-reactive antibodies in the serum of patients with autoimmune diseases (Egner, 2000). We used both assays to determine the frequency of self-reactive IgG memory B cell antibodies and to define their subcellular staining patterns (Figure 2 and Tables S1–S3). Nearly half of the 141 IgG memory B cell antibodies from three healthy donors showed low amounts of HEp-2 reactivity (Figure 2A; 46.8%). Although there was individual variation (Figure 2A; 62.8% for VB, 46.3% for PN, and 31.8% for HW), the frequency of HEp-2 self-reactive antibodies was higher in IgG+ memory B cells in all donors compared to antibodies from mature naive B cells and IgM+ memory B cells (Figure 2B; Tsuiji et al., 2006; Wardemann et al., 2003). Some mature naive B cells do express HEp-2-reactive antibodies, but these are rarely true ANAs and usually recognize cytoplasmic antigens (Wardemann et al., 2003). In contrast, a major fraction of the self-reactive antibodies produced by IgG+ memory B cells from all three donors showed true ANA staining patterns by IFA including

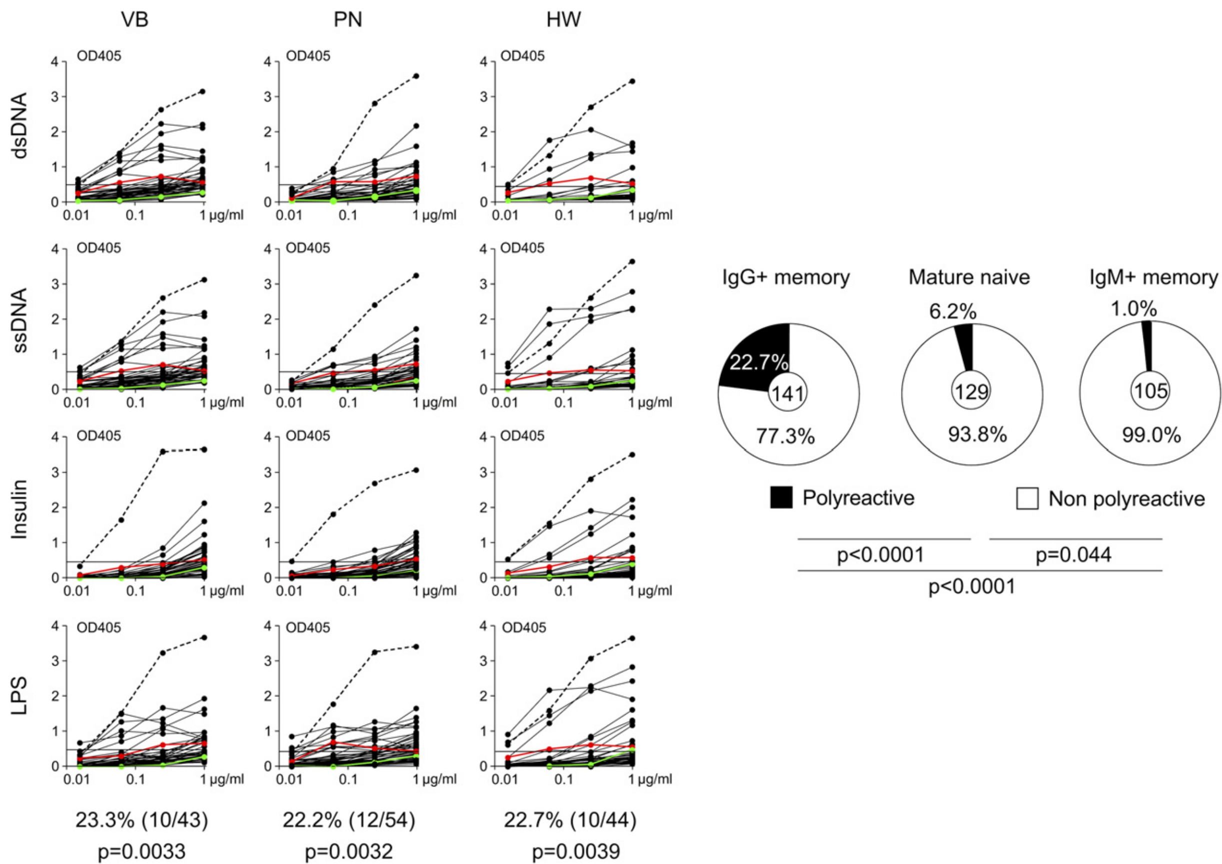

Figure 3. Polyreactive Antibodies Contribute to the IgG+ Memory B Cell Compartment

IgG+ memory B cell antibodies from healthy donors were tested for polyreactivity by ELISA with dsDNA, ssDNA, LPS, and insulin. Dotted lines represent the high-positive control antibody ED38 (Meffre et al., 2004). Horizontal lines show cut-off OD_{405} for positive reactivity as determined by comparison to the negative control antibody mGO53 (green line) and low-positive control eiJB40 (red line; Wardemann et al., 2003). Data shown are representative for at least three independent experiments. Pie charts show the frequency of polyreactive clones from IgG+ memory B cells from all three donors compared to mature naive B cell and IgM+ memory B cell antibodies (Tsuiji et al., 2006; Wardemann et al., 2003). The number of tested antibodies is indicated in the center. p values are in comparison to mature naive B cells and IgM+ memory B cells (Tsuiji et al., 2006; Wardemann et al., 2003) and were calculated by 2 × 2 Fisher exact test.

nuclear and nucleolar patterns typically associated with autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis, or scleroderma (Figure 2). It is important to note that all three donors studied showed normal serum ANA IgG titres compared to patients with SLE (Figure 2C). We conclude that in healthy humans, self-reactive antibodies including ANAs are more abundant in the IgG+ memory B cells than in mature naive B cells and IgM+ memory B cells.

Polyreactive Antibodies Expressed by IgG+ Memory B Cells

To screen for polyreactive antibodies, we tested the 141 IgG memory B cell antibodies for reactivity against a panel of defined antigens including double-stranded and single-stranded DNA (dsDNA and ssDNA), insulin, and lipopolysaccharide (LPS; Figure 3). We found that on average, 22.7% of the antibodies were polyreactive (Figure 3; 23.3% for VB, 22.2% for PN, and 22.7% for HW), and these antibodies recognized several strains of bacteria and bacterial antigens (Figure S2 and Tables S1–S3). In addition, a substantial fraction of IgG memory B cell antibodies reacted with several strains of bacteria but not with self-antigens (20.9% for VB, 20.4% for PN, and 15.9% for HW; Figure S2 and Tables S1–S3), and 1/141 antibodies showed high specificity with *Staphylococcus aureus* but not with any of the other antigens tested (HW 224; Figure S2 and Table S3). We conclude that the frequency of polyreactive antibodies is significantly higher in the IgG+ memory B cell compartment than in mature naive B cells (22.7% versus 6.2% in mature naive; $p < 0.0001$; Figure 3; Wardemann et al., 2003) or IgM+ memory B cells (22.7% versus 1.0% in IgM+ memory; $p < 0.0001$; Figure 3; Tsuiji et al., 2006), with no substantial differences between isotype subclasses (Tables S1–S3 and data not shown).

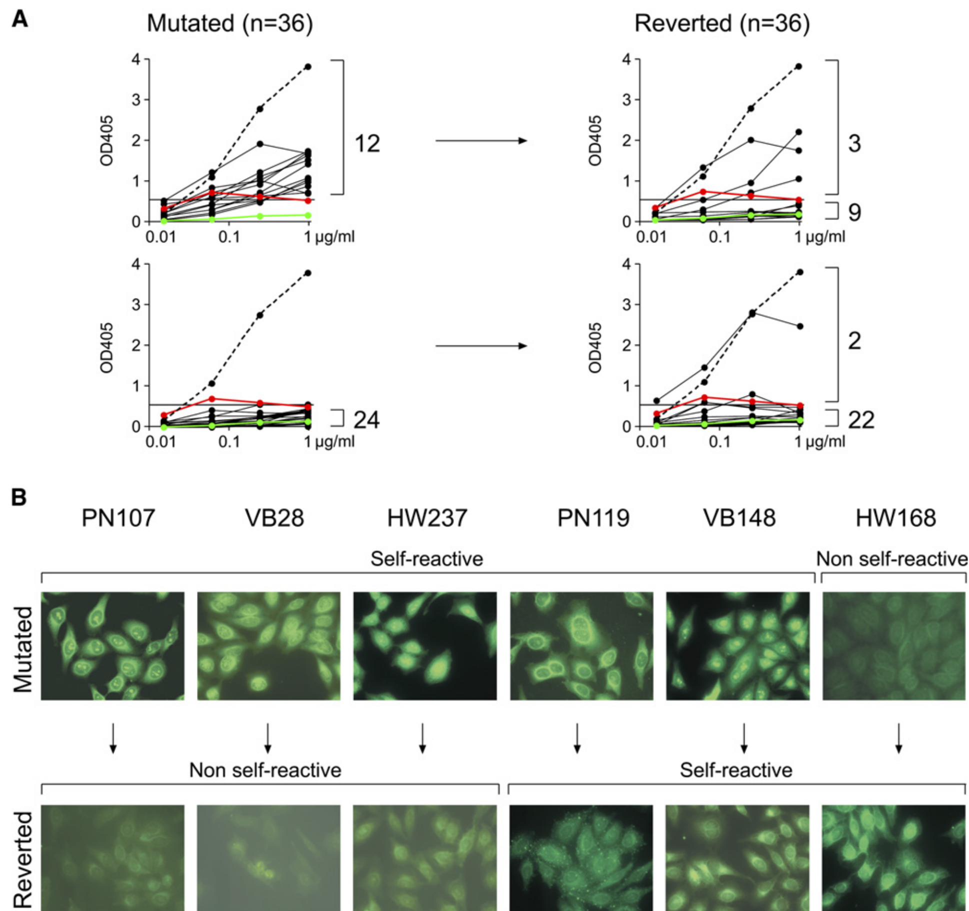

Figure 4. Somatic Hypermutation Contributes to Self-Reactivity in IgG Memory B Cell Antibodies

(A) IgH and IgL chains from IgG+ memory B cell antibodies were reverted into their germline counterparts by PCR. Recombinant polyreactive (top left) and nonpolyreactive (bottom left) IgG+ memory B cell antibodies and their germline counterparts (right) were tested for polyreactivity by ELISA with dsDNA, ssDNA, insulin, and LPS. Representative graphs with dsDNA as antigen are shown. Dotted lines represent the high-positive control antibody ED38 (Meffre et al., 2004). Horizontal lines show cut-off OD_{405} for positive reactivity as determined by comparison to the previously published control antibodies mGO53 (negative control: green line; Wardemann et al., 2003) and eiJB40 (low-positive control: red line; Wardemann et al., 2003). Data shown are representative for ELISA reactivity measured with all four antigens. Reactivity with each antigen was tested in at least three independent experiments.

(B) Typical HEp-2 cell IFA staining patterns of mutated IgG+ memory B cell antibodies (top) and their germline counterparts (bottom). Data shown are representative of two independent experiments.

Somatic Hypermutation Creates Polyreactivity and Self-Reactivity

The increase in self-reactivity during the transition between mature naive and IgG+ memory B cells might be due to a selective advantage for pre-existing self-reactive cells, or selection for cells with self-reactive antibodies produced by somatic hypermutation. To determine the origin of the self-reactive antibodies, we reverted the somatic mutations of 36 randomly chosen self- and polyreactive and nonreactive IgG memory B cell antibodies to their unmutated germline forms by PCR (Table S4; Herve et al., 2005; Tsuiji et al., 2006) and tested the recombinant antibodies for polyreactivity with dsDNA, ssDNA, insulin, and LPS (Figure 4 and Table S4 and data not shown). Out of these 36 antibodies, 12 were initially polyreactive (Figure 4A, top left). Of these, 3 (25%) still exhibited polyreactivity in the corresponding germline form, while the other 9 (75%) were completely negative (Figure 4A, top right). Of the remaining 24 antibodies that were not polyreactive in their mutated form (Figure 4A, bottom left), the vast majority (91.6%; 22/24) were also not polyreactive in the absence of mutations (Figure 4A, bottom right). We found only two antibodies out of the initial 36 that showed polyreactivity in the germline but not in the mutated form (Figure 4A, bottom right). Similar results were obtained when HEp-2 cell reactivity was analyzed by IFA and ELISA (Figures 4B; Figure S3 and Table S4 and data not shown). We conclude that most self-reactive and polyreactive IgG antibodies originate from precursors that acquired reactivity by somatic hypermutation.

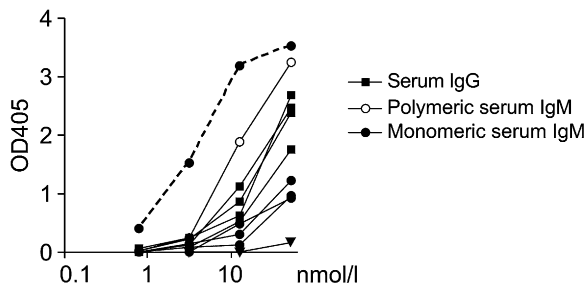

Figure 5. Monomeric IgM from Human Serum Is Less Self-Reactive than Serum IgG

Polymeric IgM from pooled human plasma of healthy donors (open circles) and from serum of two healthy donors (PN, HW) was reduced under mild conditions to its monomeric IgM subunits (filled circles). Serum IgG antibodies from pooled human plasma of healthy donors and from three healthy donors (PN, HW, VB) were purified with Protein-G beads (squares). Equal molar quantities of the antibodies were tested by ELISA for reactivity against dsDNA, but data shown are also representative for ELISA reactivity with ssDNA, insulin, and LPS. Reactivity ELISAs with individual antigens were repeated at least three times. Control antibodies were the highly polyreactive ED38 (dotted line; Meffre et al., 2004) and negative mGO53 (triangles; Wardemann et al., 2003).

Serum IgM versus IgG

Most polyreactivity in human serum has been attributed to IgM and not IgG (Coutinho et al., 1995; Guilbert et al., 1982; Seigneurin et al., 1988). However, secreted IgM is a pentamer, which has greater avidity than monomeric antibodies such as IgG. To determine the role of avidity in polyreactivity, we reduced and purified monomeric human IgM from serum of pooled donors and from two of our individual donors (Figure 5; Figure S4). When tested at equal molar ratios in polyreactivity ELISAs with dsDNA, ssDNA, insulin, and LPS, monomeric IgMs were less reactive than purified serum IgG antibodies (Figure 5 and data not shown). In contrast, the pentameric IgM antibodies were more reactive than corresponding IgGs (Figure 5 and data not shown). Thus, the increased avidity of multimeric IgM is essential for their higher polyreactivity. We conclude that in humans, monomeric IgMs such as those found in the B cell antigen receptor expressed on naive and IgM+ memory B cells are less polyreactive than the corresponding IgGs found on IgG+ memory B cells.

DISCUSSION

Isolated VH genes cloned from unseparated peripheral human B cells show autoreactivity (Lecerf et al., 1998) when expressed in bacteria. However, the reactivity of the intact antibodies from which the VH genes were cloned could not be determined because they were expressed in the absence of light chains that play a very important role in determining autoantibody reactivity (Wardemann et al., 2004). Furthermore, the representation of autoreactive B cells could not be assesed by such approaches because the amount of IgG mRNA produced varies with the stage of B cell differentiation and because cloning from pools of cells would lead to overrepresentation by cells producing higher amounts of IgG mRNA.

To examine the development of B cell tolerance in humans, we cloned antibodies from developing, naive and memory B cells and tested them for reactivity against a panel of defined antigens and HEp-2 cells (Tsuiji et al., 2006; Wardemann et al., 2003). We found that cells expressing self-reactive antibodies are efficiently excluded from the naive and circulating IgM+ memory B cell pool (Tsuiji et al., 2006; Wardemann et al., 2003). Surprisingly, development of IgG memory involved selective enrichment of B cells that produce antibodies that react with self-antigens. Many of these self-reactive antibodies were authentic ANAs as determined by IFA on HEp-2 cells.

As many as one out of three healthy humans shows ANA reactivity at a screening dilution of 1/40 in clinical ELISA tests with HEp-2 cells (Egner, 2000). However, the cellular origin of these ANAs has never been determined. Although memory B cells do not produce high amounts of secreted antibodies, they develop into plasma cells that do (Bernasconi et al., 2002; Radbruch et al., 2006). Our experiments suggest that the ultimate source of the high amounts of self-reactive serum antibodies found in clinical ELISA assays is the large number of memory B cells that produce such antibodies.

What is the origin of the ANA expressing IgG+ memory B cells in normal humans? Only a few IgG memory B cell antibodies were polyreactive in their germline form, reflecting the paucity of such cells in the naive B cell compartment (Wardemann et al., 2003). In addition, there were no substantial differences in Ig gene usage between mature naive B cells and IgG+ memory B cells including VH4-34, which is thought to have intrinsic autoreactivity (Cappione et al., 2005). Thus, there appears to be no initial selection for or against low autoreactivity in B cells entering the germinal center (GC). Instead, most of the selection occurs after somatic hypermutation either in the GC or after B cells emerge from the GC and enter the long-lived IgG memory compartment. Irrespective of the compartment in which the cells are selected or the mechanism, it is likely that self-reactivity in the IgG+ memory B cell compartment represents a by-product of affinity maturation for binding to unknown foreign antigens (Casson and Manser, 1995; Diamond and Scharff, 1984; Liu et al., 1989; Pulendran et al., 1995, Han et al., 1995; Shokat and Goodnow, 1995; Wellmann et al., 2005). Consistent with this idea, polyreactive IgGs are highly mutated with replacement/substitution ratios indicating antigen-mediated selection (Berek et al., 1991; Weiss and Rajewsky, 1990). In addition, there are well-known examples of highly specific human antibodies that are also polyreactive, e.g., broadly neutralizing HIV antibodies (Haynes et al., 2005; Muster et al., 1993; Zwick et al., 2001).

In mice, both low- and high-affinity B cells are initially recruited to GCs, but competition ensures that only high-affinity clones become memory cells (Paus et al., 2006; Shih et al., 2002). Our experiments suggest that there is also no initial selection against polyreactivity in seeding GCs. Thus, patients with SLE and rheumatoid arthritis

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that show abnormally high numbers of mature naive B cells producing self-reactive and polyreactive antibodies may also have larger numbers of such cells in GCs, but whether this will impact on the nature of the memory repertoire in these patients remains to be determined (Samuels et al., 2005; Yurasov et al., 2005, 2006).

Finding large numbers of polyreactive B cells in the IgG memory compartment was surprising because most polyreactivity in sera has been attributed to IgM (Coutinho et al., 1995; Guilbert et al., 1982; Seigneurin et al., 1988). However, any direct comparison of monomeric IgG and pentameric IgM would naturally favor IgM because of the greater avidity of the latter. Comparison of monomeric serum IgM to IgG shows that it is the IgG fraction that contains higher polyreactivity.

In mice, natural IgM antibodies with low self-reactivity and polyreactivity are produced by B1 and marginal zone B cells and they are protective early in immune responses to a variety of pathogens (Baumgarth et al., 2000; Haas et al., 2005; Martin et al., 2001). The human equivalents of the mouse B1 cells have not been defined, and human marginal zone B cells differ from mouse marginal zone B cells in that they show signs of antigen-mediated selection, display a memory B cell phenotype, and circulate in the bloodstream (Weller et al., 2001, 2004). Furthermore, the relative roles of IgM and IgG antibodies in early protection against infection have not been determined in mouse or human. However, a role for IgG in early protection in humans is suggested by increased susceptibility to bacterial infection in patients unable to produce IgG as a result of B cell-intrinsic hyper-IgM syndrome, its reversal by passive transfer of pooled IgG, and by our finding that IgGs are frequently reactive against a number of different species of bacteria or bacterial antigens (Alachkar et al., 2006; Quartier et al., 2004). In addition to their role in immune protection, natural IgM antibodies have also been implicated in prevention of autoimmunity by binding and clearing of apoptotic cells (Kim et al., 2002). A similar role for IgG in preventing autoimmunity is suggested by the finding that patients with hyper-IgM type 2 syndrome are also highly susceptible to autoimmunity (Quartier et al., 2004). Despite the lower overall avidity of IgGs, these molecules may be important in early protection against infection and in prevention of autoimmunity because they can engage both the complement system and Fc receptors (Carroll, 2004; Ravetch and Clynes, 1998). This feature enables a series of important protective effector functions not available to IgMs, which do not bind to conventional Fc receptors.

In summary, our data demonstrate that a large number of circulating IgG+ memory B cells normally produce low-affinity nonpathogenic autoantibodies. How many of these cells eventually develop into plasma cells and contribute to steady-state antibody titers remains to be determined. Under normal conditions, autoreactive IgG+ memory B cells may be anergic. Alternatively, an additional and yet-to-be-defined checkpoint may control the differentiation of self-reactive IgG+ memory B cells into antibody-secreting plasma cells (Han et al., 1995; Shokat and Goodnow, 1995; Pulendran et al., 1995; Klinman, 1996). Nevertheless, abnormalities in checkpoint regulation or activation of peripheral self-reactive IgG+ memory B cells may contribute to the development of autoimmunity in susceptible individuals (Bernasconi et al., 2002; Kaneko et al., 2006; Mackay et al., 2006; Radbruch et al., 2006).

EXPERIMENTAL PROCEDURES

Single B Cell Sorting

All samples were obtained after signed informed consent in accordance with IRB-reviewed protocols at the Rockefeller University. Control data from mature naive B cells were previously published and are shown for comparison. Single CD19+CD10−CD27+IgG+ B cells were isolated as described (Wardemann et al., 2003) after staining with anti-CD19-APC, anti-CD10-PE, anti-CD27-FITC, anti-IgG-Biotin (BD Biosciences Pharmigen), and Streptavidin-PECy7 (Caltag Laboratories).

PCR Amplification and Expression Vector Cloning

Single-cell cDNA synthesis was performed as described with Superscript III at 50°C (Wardemann et al., 2003). Igγ, Igk, and Igλ chain genes were amplified in two rounds of nested PCR (T.T. and H.W., unpublished data; Tsuiji et al., 2006; Wardemann et al., 2003; Yurasov et al., 2005, 2006). All PCR products were sequenced and analyzed for Ig gene usage and CDR3 analysis, number of V gene mutations (IgBlast; Tables S1–S3), and IgG isotype subclass (http://imgt.cines.fr). Second PCR products for Igλ genes contained restriction sites allowing direct cloning into expression vectors. For Igγ and Igk genes, restriction sites for expression vector cloning were introduced after sequencing by gene-specific primers and first PCR products as template. All IgH and IgL chain genes were sequenced after cloning to confirm entity with the original PCR products.

Antibody Production, ELISA, and Indirect Immunofluorescence Assay

Antibodies were expressed and tested for polyreactivity with ds/ssDNA, insulin, and LPS and for self-reactivity with HEp-2 cells by ELISA and IFA as described (Wardemann et al., 2003). ELISA against bacteria was described previously (Tsuiji et al., 2006). Threshold values for reactivity are indicated in the graphs and were set in all assays with our previously published control antibodies mGO53 (negative), eiJB40 (low positive), and ED38 (high positive) for polyreactivity ELISAs and additional positive and negative control sera for HEp-2 reactivity (Meffre et al., 2004; Wardemann et al., 2003). Data shown are representative for at least two independent experiments.

Reversion of Hypermutated Sequences to Germline

Antibodies for reversion experiments were chosen randomly and are listed in Table S4. Mutated IgH and IgL chain genes were reverted to their germline sequence by two separate PCR reactions for the V gene and the (D)J gene followed by a third overlap PCR to fuse the two PCR products as described (Herve et al., 2005; Tsuiji et al., 2006). All reverted IgH and IgL chain PCR products were sequenced before and after cloning into the corresponding expression vectors to confirm lack of mutations. Recombinant mutated and unmutated antibodies were expressed and tested for polyreactivity and self-reactivity in comparison as described above.

Statistics

p values for Ig gene repertoire analyses, analysis of positive charges in IgH CDR3, and antibody reactivity were calculated by 2×2 or 2×5 Fisher Exact test or chi-square test. p values for IgH CDR3 length were calculated by Student's t test.

Serum IgM and IgG Purification and Reduction of IgM

IgM was purified from serum of three healthy donors as described (Cambier and Butler, 1974). In brief, 20 ml of clarified whole serum

were dialyzed against 0.01 M potassium phosphate buffer (pH 5.4) for 24 hr at 4°C. Precipitates were dissolved in 4 ml 0.01 M acetate buffer (pH 5.4) containing 0.15 M NaCl. 0.1 M $ZnSO_4$ was added drop-wise to a final concentration of 10 mM. The preparations were stirred at 25°C for 2 hr prior to centrifugation at 3000 rpm for 15 min. Supernatants were subjected to a Superdex200 column (GE Healthcare) equilibrated with 0.1 M Tris-HCl buffer (pH 8.6) containing 0.1 M NaCl, and eluted fractions were collected. IgG was purified from human serum of the same three donors with Protein G beads (GE Healthcare) according to the manufacturer's instructions.

To obtain IgM subunits, IgM was reduced in 0.5 M Tris buffer (pH 8.6) containing 10 mM cysteine for 2–4 hr at room temperature. The optimal incubation conditions were determined empirically for each IgM preparation. The reaction was stopped with 10% molar excess of iodoacteamide and applied to a Superdex200 column (GE Healthcare) equilibrated with sodium borate buffer (pH 8.6). Size and purity of the preparations was monitored by nonreducing SDS-PAGE (see Figure S5). Monomeric IgM concentration was determined by serial dilution on SDS-PAGE with total human IgG (Sigma) standards. Equal molar amounts of IgM (180 kDa) and IgG (156 kDa) were used in indirect ELISAs with dsDNA, ssDNA, insulin, and LPS as antigens as described above (Wardemann et al., 2003). Reactive antibodies were detected with anti-human IgM plus IgG HRP antibody (Jackson ImmunoResearch) and confirmed in independent experiments with either anti-human kappa-HRP or anti-human lambda-HRP (Biosource).

Supplemental Data

Supplemental Data include four figures and four tables and can be found with this article online at http://www.immunity.com/cgi/content/full/26/2/205/DC1/.

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