Anti-hinge antibodies (AHAs) are an autoantibody subclass that, following proteolytic cleavage, recognize cryptic epitopes exposed in the hinge regions of immunoglobulins (Igs) and do not bind to the intact Ig counterpart. AHAs have been postulated to exacerbate chronic inflammatory disorders such as inflammatory bowel disease and rheumatoid arthritis. On the other hand, AHAs may protect against invasive microbial pathogens and cancer. However, despite more than 50 years of study, the origin and specific B cell compartments that express AHAs remain elusive. Recent research on serum AHAs suggests that they arise during an active immune response, in contrast to previous proposals that they derive from the preexisting immune repertoire in the absence of antigenic stimuli. We report here the isolation and characterization of AHAs from memory B cells, although anti-hinge-reactive B cells were also detected in the naive B cell compartment. IgG AHAs cloned from a single human donor exhibited restricted specificity for protease-cleaved F(ab')_2 fragments and did not bind the intact IgG counterpart. The cloned IgG-specific AHA-variable regions were mutated from germ line-derived sequences and displayed a high sequence variability, confirming that these AHAs underwent class-switch recombination and somatic hypermutation. Consistent with previous studies of serum AHAs, several of these clones recognized a linear, peptide-like epitope, but one clone was unique in recognizing a conformational epitope. All cloned AHAs could restore immune effector functions to proteolytically generated F(ab')_2 fragments. Our results confirm that a diverse set of epitope-specific AHAs can be isolated from a single human donor.

The development of B cells occurs in a stepwise process whereby the early stages are defined by a series of gene segment rearrangements leading to the ordered and sequential assembly of the B cell antigen receptor (BCR) (1). B cells can also secrete the BCR as soluble antibodies. The generation of the BCR heavy chain (HC) is regulated by a process known as V(D)J recombination. In this process, the variable (V), diversity (D), and joining (J) segments recombine to form unique HC variable regions. The generation of the light chain (LC) is regulated in a similar manner by VJ recombination. Antigen-inexperienced, naive B cells express HC and LC V-regions that are non-mutated from the original germ line-derived sequence and are typically characterized as being IgD^{high}, IgM^{low}, and CD27^{neg}. During the process of an immune response, B cells that encounter antigen can undergo a process known as class-switch recombination (CSR) and develop into a number of later stage B cells such as plasmablasts, plasma cells, and memory B cells (2). IgG memory B cells are characterized by the cell-surface expression of IgG and CD27. As such, peripheral human B cells are often defined as antigen-inexperienced, naive (IgD^{pos}, CD27^{neg}), unswitched memory (IgD^{pos}, CD27^{pos}), and switched memory (IgD^{neg}, CD27^{pos}) (3). During B cell development, the majority of self-reactive B cells are eliminated prior to maturation into antigen-inexperienced, naive B cells (4). However, a breakdown in tolerance can lead to autoreactive B cells that secrete antibodies that recognize self-antigens (i.e. autoantibodies).

There are several types of autoantibodies that engage immunoglobulins (Igs) (5). One of the best characterized types binds to the Fc portion of IgGs and is termed rheumatoid factor. Anti-IgG autoantibodies can also bind to the variable region, which are known as anti-idiotype autoantibodies. An entire class of autoantibodies recognizes post-translationally modified proteins. These are known as anti-modified protein antibodies (AMPAs), including anti-citrullinated antibodies and anti-carbamylated protein (6). Among the AMPAs, there is another type of autoantibody that binds to cryptic epitopes exposed after proteolytic cleavage in the hinge regions of Igs, known as anti-hinge antibodies (AHA) (7). This type of autoantibody was first characterized in the 1960s as the serum-binding fraction that specifically recognized F(ab')_2 fragments generated with pepsin and were termed “pepsin agglutinators” (8). The majority of studies characterizing anti-hinge antibodies were performed using sera derived from human patients (8–14). A recurrent finding regarding the specificity of AHAs was that the

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This article contains Fig. S1.

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3 The abbreviations used are: BCR, B cell antigen receptor; AHA, anti-hinge antibody; CDC, CDC-dependent cytolysis; HC, heavy chain; LC, light chain; ADCC, antibody-dependent cell-mediated cytolysis; PBMC, peripheral blood mononuclear cell; SEC-MALS, size-exclusion chromatography–multiance laser light scattering; RA, rheumatoid arthritis; AMPA, anti-modified protein antibody; SPR, surface plasmon resonance; CSR, class-switch recombination; CFM, continuous flow microspotter; PE, phycoerythrin; SPRi, SPR imager; PAMP, pathogen-associated molecular pattern; 7-AAD, 7-aminoactinomycin D.
Molecular characterization of anti-hinge antibodies

Results

Anti-hinge–reactive B cells were detected in naive and memory B cell compartments by flow cytometry using antigen tetramers

Given the often low frequency of antigen-specific B cells in circulating blood, distinguishing these rare events from assay noise is often required. Antigen tetramers have been used to increase the avidity of BCR labeling and enable reliable detection of specific B cells with very low frequencies (26). We generated antigen tetramers by incubation of PE-tagged streptavidin with biotinylated MMP3-cleaved human IgG1 F(ab’)_2 at a molar ratio of 1:4 and in 10 steps to avoid excess of free streptavidin–PE. These tetramers were then used along with a panel of mAbs for the identification of hinge-specific B cells. To eliminate non-specific binding, we enriched B cells from PBMCs to get rid of as many irrelevant cells as possible and included 7-AAD, a viability dye, to eliminate non-specific binding that often occurs with non-viable cells. Cells were detected with CD19+, CD27+, IgG-, and MMP3-generated F(ab’)_2 (which will be termed F(ab’)_2 MMP3) antigen streptavidin–PE tetramer (Fig. 1, a–e). A mixture of streptavidin–PE with a non-biotinylated F(ab’)_2 MMP3 fragment at a molar ratio of 1:4 was used as an isotype control (Fig. 1f). Anti-hinge–reactive B cells were detected in antigen-inexperienced, naive B cells (IgD_pos CD27_neg), as well as class-switched (IgD_neg CD27_pos) and unswitched (IgD_neg CD27_pos) memory B cells in 10 healthy donors (Table 1). The frequency of anti-hinge–reactive B cells in naive B cells, switched memory B cells, and unswitched memory B cells ranged from 0.06 to 0.15, 0.05 to 0.15, and 0.1 to 0.4%, respectively. The average frequency of anti-hinge–reactive B cells in naive B cells, unswitched memory B cells, and switched memory B cells were 0.11, 0.23, and 0.09%, respectively (Table 1). The results demonstrated that B cells at numerous developmental stages expressed BCRs capable of binding to F(ab’)_2 MMP3 fragments under avidity-binding conditions.

Single memory B cell sorting using MMP3-cleaved F(ab’)_2 fragment tetramer of a human IgG1

We next sought to isolate and clone out AHAs from memory B cells for further characterization. The sorting strategy of hinge-specific single memory B cells is shown in Fig. 2, a–i. One × 10^7-enriched human B cells were stained with streptavidin–PE conjugated to biotinylated F(ab’)_2 MMP3 tetramer or non-biotinylated negative control followed by detecting with CD19, CD27, IgD, and IgG to specifically select class-switched memory B cells (CD19_pos CD27_pos IgD_pos IgG_pos). F(ab’)_2 MMP3-positive memory B cells represented 0.11% of class-switched memory B cells, whereas no B cells were detected with the negative control. Anti-hinge positive B cells were sorted into single wells, and the IgG heavy and light chains were cloned out from single cells as described under “Materials and methods.”

ELISA-based AHA specificity

Cloned IgGs were next screened for specificity to F(ab’)_2 fragments generated with either MMP-3 or MMP-7 or with peptide analogues of the lower hinge region spanning amino

C terminus was critical for binding (15, 16). Numerous in vitro studies have demonstrated that AHAs that interact with cell-bound F(ab’)_2 fragments can provide a surrogate Fc region and recruit immune effector functions such as complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC), and antibody-dependent cellular phagocytosis (9, 15, 17, 18). Furthermore, several in vivo studies have demonstrated that anti-hinge antibodies that engage proteolyzed IgGs can facilitate platelet clearance in rats and dogs (15), xenograft tumor suppression in mice (18), and reduction in Staphylococcus aureus colony-forming units in rabbits (19). The origin of AHAs has proven enigmatic, and the early descriptions suggested that AHAs are a part of the “natural” immune repertoire with germ line encoded V-region sequences (20). More recent studies have demonstrated that AHAs are composed of multiple isotypes and IgG subclasses (9) and that AHAs recognize subclass- and protease-restricted neoepitopes (10). These studies suggested that as opposed to being a part of the natural immune repertoire, AHAs developed as a part of an immune response to inflammatory or infectious conditions.

There is increased interest in the characterization of AHAs with regard to the development of antibody-based therapeutics because the presence of AHAs has confounded several preclinical and clinical therapeutic programs. For instance, a preclinical cynomolgus study using a pepsin-generated F(ab’)_2 fragment against GPIIbIIIa (αIIbβ₃) intended to block platelet activation resulted in severe thrombocytopenia in 5 of 18 monkeys due to AHAs (21). More recently, pre-existing autoantibodies recognizing the C terminus of an anti-TNFR1 domain keys due to AHAs (21). More recently, pre-existing autoantibodies recognizing the C terminus of an anti-TNFR1 domain resulted in cytokine secretion by B cells at various stages of memory B cell compartments by flow cytometry using antigen tetramers. The frequency of anti-hinge–reactive B cells in naive B cells, switched memory B cells, and unswitched memory B cells ranged from 0.06 to 0.15, 0.05 to 0.15, and 0.1 to 0.4%, respectively. The average frequency of anti-hinge–reactive B cells in naive B cells, unswitched memory B cells, and switched memory B cells were 0.11, 0.23, and 0.09%, respectively (Table 1). The results demonstrated that B cells at numerous developmental stages expressed BCRs capable of binding to F(ab’)_2 MMP3 fragments under avidity-binding conditions.

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For the peptides, all cysteines (i.e., Cys-220, Cys-226, and Cys-229) were changed to serines to avoid inter- and intra-disulfide bonds. ELISA analysis on F(ab')2 fragments demonstrated that one clone (1-A6) showed robust binding to F(ab')2 fragments whereas five clones demonstrated binding to F(ab')2 fragments (1-A3, 1-A5, 1-A6, and 1-A7) (data not shown), and 1-A8 (Fig. 3, a and b). Clone 1-A7 was very poorly expressed (low microgram quantities from a 1.0-liter expression run), so it was not carried forward for characterization beyond the initial screen and sequence analysis.

Fig. 3c depicts the ELISA-binding results against peptide analogues of the lower hinge region. Surprisingly, clone 1-A6 did not show detectable binding to the MMP3 cleavage-site peptide, whereas clones 1-A3, 1-A5, and 1-A8 demonstrated robust binding to the MMP7 cleavage-site peptide analogue in agreement with the ELISA result shown in Fig. 3b. Clone 1-B3 did not show detectable binding to either F(ab')2 fragments or peptides and was included in further analyses as a non-binding control mAb. These results implicated that clone 1-A6 perhaps recognized a conformational epitope and that clones 1-A3, 1-A5, and 1-A8 recognized a linear epitope as demonstrated by their ability to bind to both a F(ab')2 fragment as well as a peptide analogue of MMP7-cleaved hinge.

Table 1

| Donor | % CD27− | % CD27+ | % CD27+ |
|-------|---------|---------|---------|
|       | CD27− IgD+ | CD27+ IgD+ | CD27+ IgD− |
| 1     | 0.099    | 0.132   | 0.079   |
| 2     | 0.138    | 0.370   | 0.113   |
| 3     | 0.150    | 0.192   | 0.107   |
| 4     | 0.094    | 0.291   | 0.109   |
| 5     | 0.143    | 0.210   | 0.150   |
| 6     | 0.147    | 0.294   | 0.078   |
| 7     | 0.059    | 0.198   | 0.018   |
| 8     | 0.105    | 0.120   | 0.047   |
| 9     | 0.057    | 0.108   | 0.092   |
| 10    | 0.071    | 0.362   | 0.092   |
| Mean ± S.D. | 0.106 ± 0.036 | 0.228 ± 0.097 | 0.088 ± 0.037 |

Acids Ala-231 to Gly-237 (Eu numbering (25)). For the peptides, all cysteines (i.e. Cys-220, Cys-226, and Cys-229) were changed to serines to avoid inter- and intra-disulfide bonds. ELISA analysis on F(ab')2 fragments demonstrated that one clone (1-A6) showed robust binding to F(ab')2 MMP3 whereas five clones demonstrated binding to F(ab')2 MMP7 (1-A3, 1-A5, 1-A6, and 1-A7) (data not shown), and 1-A8 (Fig. 3, a and b). Clone 1-A7 was very poorly expressed (low microgram quantities from a 1.0-liter expression run), so it was not carried forward for characterization beyond the initial screen and sequence analysis.

**Figure 1. Detection of anti-hinge-reactive B cells in the antigen-inexperienced, naive; unswitched memory; and switched memory B cell compartments.** a–d, enriched B cells from a single human peripheral blood donor were gated on IgD−, CD27− (antigen-inexperienced, naive); IgD−, CD27+ (unswitched memory); and IgD+, CD27+ (switched memory B cell compartments). e, anti-hinge-reactive B cells from all three compartments were detected with PE-labeled streptavidin-F(ab')2 MMP3 tetramers. f, isotype control for AHA detection. Data are representative of a single donor where 10 total donors were assessed (Table 1).
**Molecular characterization of anti-hinge antibodies**

![Image](image_url)

**Figure 2. Gating strategy for sorting anti-hinge–reactive single B cells from the switched memory B cell compartment.** To sort anti-hinge–reactive B cells, enriched B cells were initially gated by forward and side scatter (a); cells were further gated for singlets (b and c); dead cells were excluded (d); and CD19<sup>+</sup> B cells (e) were further gated to select for the IgD<sup>−</sup> and CD27<sup>+</sup> switched memory B cell compartments (f). From this gating strategy, IgG<sup>+</sup> (g), anti-hinge-reactive B cells (h) were single-cell sorted in the wells of a 96-well plate for cloning of antibody HC and LC i, isotype control for AHA detection.

**Figure 3. ELISA detection of AHAs binding to F(ab′)<sub>2</sub> fragments or peptide analogues of the human IgG1 hinge region lower hinge region.** F(ab′)<sub>2</sub><sub>MMP3</sub> fragments (A) or F(ab′)<sub>2</sub><sub>MMP7</sub> fragments (B) were coated on a 96-well plate, and AHAs were serially diluted. AHAs were detected with an HRP-conjugated anti-Fc reagent. Data are representative of three independent experiments. C, hinge analogue peptides were biotinylated 14-mers where the free C termini is indicated on the x axis. SCR represents a scrambled peptide. DS933 serum represents ELISA reactivity of the serum from the donor from which the AHAs were derived. Determinations were performed in triplicate wells with error bars representing S.D. Data are representative of three independent experiments.
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Figure 4. ELISA detection of AHAs binding to F(ab’)2 fragments and Fab’ fragments to assess linear versus conformational epitope-binding dependence. F(ab’)2 MMP3 fragments (A) or Fab’ MMP3 fragments (B) were coated on a 96-well plate, and AHAs were serially diluted. AHAs were detected with an HRP-conjugated anti-Fc reagent. F(ab’)2 MMP7 fragments (C) or Fab’ MMP7 fragments (D) were coated on a 96-well plate, and AHAs were serially diluted. AHAs were detected with an HRP-conjugated anti-Fc reagent. Data are representative of two independent experiments.

contained AHAs reactive to the -PEL peptide and that any serum AHAs specific for the linear -PAP peptide were below the level of detection.

To further explore the specificity of the AHAs for either a linear or conformational epitope by ELISA, we reduced the F(ab’)2 fragments to generate Fab’ fragments. Whereas the F(ab’)2 fragments could present both a linear and conformational epitope, the Fab’ would more likely present only a linear epitope similar to the peptide analogues of the lower hinge. As shown in Fig. 4, a and b, clone 1-A6 displayed a greater than 100-fold reduction in binding to the Fab’MMP3 fragment relative to the F(ab’)2 MMP3 fragment. In contrast, clones 1-A3, 1-A5, and 1-A8 displayed a less than 3-fold reduction in binding to the Fab’MMP7 fragment relative to the F(ab’)2 MMP7 fragment (Fig. 4, c and d). Similar to Fig. 3b, clone 1-A6 bound to the Fab’MMP7 fragment but showed nearly undetectable binding to the Fab’MMP3 fragment. These results further support that clone 1-A6 recognizes a conformational epitope present in a F(ab’)2 fragment that is not fully preserved in either the peptide hinge analogues or a single HC-containing Fab’ fragment.

Sequence analysis of AHA

The CDR-H3 and CDR-L3 sequences of AHA clones 1-A3, 1-A5, 1-A6, 1-A7, 1-A8, and 1-B3 are shown in Tables 2 and 3. The clones 1-A3, 1-A5, and 1-A7 reactive to the -PAPEL peptide were all derived from VH1–18 and had similar CDR-H3 sequences. Clones A6 and A8 were derived from VH3-48 and VH3-73, respectively. Notably, none of the AHAs had full germ line sequences and had percent homologies ranging from 83.7% (clones 1-A5, 1-A6, and 1-A7) to 94.0% (clone 1-A8) for their heavy chains. These results demonstrated that the these human AHAs were not part of the natural antibody repertoire, and the mutated CDR3 sequences coupled with CSR indicated that these antibodies were generated as a part of an active immune response.

Monovalent affinity of full-length AHA

Specificity of AHAs against F(ab’)2 epitopes was evaluated in vitro by Biacore surface plasmon resonance (SPR). Affinity was assessed using an IgG capture format, which enables detection of monovalent binding of full-length antibody to target antigen. The binding of the AHAs to intact IgG could not be tested by this method because the AHAs were captured with an anti-human Fc reagent that would also bind to intact IgG, so only binding to F(ab’)2 fragments was assessed. Sensorgrams representing the interaction of each AHA with epitopes generated by digestion with MMP3, MMP7, GluV8, or IdeS are shown in Fig. 5. Each of the four AHAs displayed binding to epitopes terminating in either -PAP or -PAPEL with K_D values in the nanomolar range. No measurable interaction was observed between any of the AHAs against epitopes created by specific cleavage of F(ab’)2 with either GluV8 or IdeS, consistent with the hinge peptide analogue-binding data (Fig. 3c). Although all four AHAs bound to either of the two epitopes -PAP or -PAPEL, each antibody differed in monovalent affinity (~2–17-fold) for each cleaved terminus, revealing further sub-specificity of the individual AHAs. Antibody 1-A6 showed single digit nanomolar affinity to the -PAP epitope yielded by MMP3 cleavage and at least 10-fold lower affinity for the -PAPEL epitope yielded by MMP7 cleavage. The other AHAs, 1-A3, 1-A5, and 1-A8 showed higher affinity for the -PAPEL epitope. Based on these data, there are clear differences in specificity among these AHAs.
This clone demonstrated high sequence identity to clone 1-A3. Clones 1-A3, 1-A6, and 1-A8 and omitted clone 1-A5 because AHAs to restore CDC and ADCC. For these assays, we used the cleavage site, we next wanted to assess the ability of the engagement by both Fab arms. We initially set up this assay using Biacore SPR; however, amine-based capture of the F(ab')2 fragments, and these human AHAs showed differential binding to different F(ab')2 fragments depending on the cleavage site, we next wanted to assess the ability of the AHAs to restore CDC and ADCC. For these assays, we used clones 1-A3, 1-A6, and 1-A8 and omitted clone 1-A5 because this clone demonstrated high sequence identity to clone 1-A3. CDC assays were performed using the target cell line WIL2-S, AHAs, and anti-CD20 antibodies. Fig. 7A demonstrated that intact anti-CD20 could elicit CDC, but the AHAs alone or F(ab')2 fragments generated with MMP3, MMP7, GluV8, and IdeS were not capable of driving CDC. Clones 1-A3 and 1-A8 were capable of restoring CDC to F(ab')2, fragments but not to any of the other F(ab')2 fragments (Fig. 7, B and D). Clone 1-A6 was only capable of restoring CDC to F(ab')2,MMP3 (Fig. 7C). These results demonstrated that these AHAs were capable of restoring CDC, but only to F(ab')2 fragments for which they showed the highest binding affinity.

ADCC assays were performed using PBMCs as effector cells, WIL2-S target cells, AHAs, and anti-CD20 antibodies. Similar to the CDC assay, Fig. 7E demonstrated that intact anti-CD20 could elicit ADCC, but the AHAs alone or F(ab')2 fragments generated with MMP3, MMP7, GluV8, and IdeS were not capable of ADCC. Clones 1-A3 and 1-A8 demonstrated the most potent ADCC against F(ab')2,MMP7, but a lower level of ADCC was detected against F(ab')2,MMP3 (Fig. 7, F and H). Clone 1-A6 had the most potent ADCC against F(ab')2,MMP3 but also detectable activity against the F(ab')2 fragment generated with MMP7 and GluV8 (Fig. 7G). These results demonstrated that AHAs could restore ADCC to F(ab')2 fragments generated with different proteases.

Immune complex characterization of AHA by SEC-MALS and electron microscopy

Although many previous studies have characterized AHA binding using ELISA, SPR, and cell-based binding formats, no studies have characterized the stoichiometry and architecture of AHA binding to F(ab')2 fragments to date. We next sought to investigate AHA binding stoichiometry by single-particle negative stain electron microscopy (EM) and SEC-MALS analysis. For these studies, we used the highest affinity clone 1-A6 and MMP3-generated F(ab')2 fragments. Reference free 2D classification showed that F(ab')2,MMP3 presents a significant flexibility of the hinge region, because the two Fab arms could spatially arrange in different V-shaped formations, including a complete 180° angle (Fig. 8A). The full-length AHA clone 1-A6 was visualized in the typical Y-shape with the characteristic open conformation of the CH2 domains within the Fc region (Fig. 8B) (27). Images of the F(ab')2,MMP3–1-A6 complex obtained incubating equimolar amounts of F(ab')2,MMP3 and 1-A6 indicated that a single AHA could engage up to 2 F(ab')2,MMP3 fragments with either a 1:1 or 1:2 stoichiometry (Fig. 8C). These results were corroborated by SEC-MALS analysis (Fig. S1).
Discussion

Although it has been reported for over 50 years that proteases can specifically cleave IgGs in the hinge region, and that there are pre-existing antibodies against the cryptic epitopes generated by IgG proteolysis, there is still continued interest in AHAs as many detailed studies recently assessed AHAs in different disease contexts. For chronic inflammatory autoimmune disorders such as RA, it was shown that there is an increased incidence of AHAs in RA patients compared with healthy controls (12). These authors later demonstrated that AHAs could facilitate complement activation to cleaved IgG4s, a subclass usually devoid of complement activity, and postulated that AHAs could exacerbate tissue damage in inflamed joints (13). Another study assessed the presence of both cleaved IgGs and AHAs in inflammatory bowel patients being treated with the anti-TNF mAb, infliximab (28). The authors showed that infliximab-treated patients with higher incidences of both cleaved IgGs and AHAs had poorer clinical outcomes compared with patients with lower levels. Together, these studies indicated that AHAs could be pathologic in cases of chronic inflammatory autoimmune disorders. In contrast to autoimmune disorders, it has been suggested that AHAs could restore effector function to IgGs cleaved by invasive microorganisms and cancers (7). Indeed, vaccination of rabbits with a peptide with a C terminus corresponding to the epitope exposed after *S. aureus* GluV8 proteolysis resulted in reduced *S. aureus* colony-forming units in infected rabbits compared with control immunized animals (19). A breast cancer xenograft study showed that a rabbit AHA could suppress tumor growth when targeting a proteolytically-cleaved anti-HER2 antibody (18). However, despite the many studies on AHAs, there has yet to be a detailed characterization of the B cells reactive to cleaved IgGs as well as a detailed molecular characterization of AHAs produced by these B cells.

This study, for the first time, definitively demonstrates that AHAs can be isolated from switched memory B cells and that these human AHAs have V-region sequences consistent with being generated in an active immune response. Anti-hinge-reactive B cells were also found in the antigen-inexperienced, naive B cell compartment (Fig. 1 and Table 1), suggesting that these cells had not been eliminated through B cell tolerance mechanisms. Therefore, although the previous suggestions that AHAs were composed of germ line V-regions were not accurate, it is now certain that subsets of anti-hinge-expressing B cells are found within the switched memory B cell compartment and contain mutated CDR-L3s and CDR-H3s (Tables 2 and 3).

Previous analyses with polyclonal serum and a rabbit monoclonal AHA showed that the AHAs bound to a linear epitope that could be mimicked by peptide analogues of the hinge region (9, 15). Indeed, the only crystal structure of an AHA, a rabbit mAb specific for the IdeS cleavage site, demonstrated that a peptide analogue of an IdeS-cleaved hinge inserted into complementarity-determining regions of the mAb (16). In this
study, clones 1-A3, 1-A5, and 1-A8 were capable of binding both F(ab')2 MMP-7 -PAP and a peptide analogue of MMP7-cleaved hinge (epitope -PAPEL), indicative of linear epitope recognition. In contrast, clone 1-A6 was only capable of binding to F(ab')2 MMP-3 and F(ab')2 MMP-7 by ELISA but did not demonstrate detectable binding to peptide analogues of either the MMP3- or MMP7-cleaved hinge. It could be argued that the lack of binding to the -PAP peptide was due to the C229S mutation that is just 4 amino acids from the C terminus. However, this is likely not the case with the -PAPEL peptide, where C229S is 6 amino acids from the C terminus, as clones 1-A3, 1-A5, and 1-A8 were all capable of binding to this peptide, whereas clone 1-A6 did not. These data suggest that clone 1-A6 uniquely engages a conformational epitope rather than a linear epitope. This is consistent with a previous report where a human phage-derived AHA recognized a conformational hinge epitope (29).

The ability to bind to both -PAP and -PAPEL may be due, in part, to the presence of a proline 3 amino acids from the C terminus in both epitopes. Our initial intention was to clone out AHAs reactive to the -PAP epitope using the F(ab')2 MMP-7 tetramer. However, it was surprising that the majority of the AHAs were reactive to the -PEL epitope. As described above, the proline at position P3 is conserved in both epitopes. Considering that there was serum reactivity to the -PEL peptide, whereas the -PAP reactivity was at baseline (Fig. 3c), we instead isolated a subset of MMP7-specific antibodies that were cross-reactive to the MMP3 epitope under avidity-based binding conditions. Therefore, we believe that had we sorted for the F(ab')2 MMP-7 tetramer, we would have generated a larger pool of antibodies. A hallmark of AHAs is their ability to engage cell-surface-bound cleaved IgGs and provide a surrogate Fc region to restore effector function. The hybridoma-derived rabbit AHA was highly specific for the IdeS cleavage site and did not restore ADCC or CDC to F(ab')2 GluV8 or F(ab')2 MMP-7 fragments. The AHAs in this report were all capable of restoring both CDC and ADCC, but with different degrees of specificity. CDC was

|  | F(ab')2 MMP-3 -PAP | F(ab')2 MMP-7 -PAPEL | F(ab')2 IdeS -PAPELLG | intact IgG1 |
|---|---|---|---|---|
| 1-A3 | 179.7 +/- 18.9 | n.m. | n.m. | n.m. |
| 1-A5 | 219.7 +/- 8.0 | n.m. | n.m. | n.m. |
| 1-A6 | 0.007 +/- 0.002 | 23.2 +/- 1.9 | 0.042 +/- 0.005 | n.m. |
| 1-A8 | 189.2 +/- 12.5 | n.m. | 0.18 +/- 0.011 | n.m. |
| 1-B3 | n.m. | n.m. | n.m. | n.m. |

**Figure 6. Comparison of SPRi sensorgrams for the interaction between AHAs and immobilized protease-cleaved F(ab')2 fragments.** Sensorgrams represent real-time binding interactions as a change in refractive index plotted as response units against time in seconds. Avidity-based binding was achieved using a F(ab')2 fragments capture format. A concentration series of each AHAs from 33 to 900 nM was flowed as mobile analyte, and binding was analyzed using a Langmuir 1:1 model. Affinity values are reported as nanomolar dissociation constants (Kd) ± S.D. n = 3. n.m. refers to no measured specific binding.
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Figure 7. Cell-based CDC and ADCC assays of AHAs on anti-CD20 F(ab’)2 fragments generated with the proteases MMP3, GluV8, MMP7, and IdeS. CDC assays were performed with WIL-2 cells as described under the "Materials and methods." A, CDC with intact anti-CD20, AHAs 1-A3, 1-A6, and 1-A8 as well as anti-CD20 F(ab’)2 MMP3, F(ab’)2 GluV8, F(ab’)2 MMP7 and F(ab’)2 IdeS. B-D, CDC with 1-A3 (B), 1-A6 (C), and 1-A8 (D) against anti-CD20 F(ab’)2 MMP3, F(ab’)2 GluV8, F(ab’)2 MMP7, and F(ab’)2 IdeS. CDC assays were performed in triplicate wells, and error bars represent S.D. Data are representative of three independent experiments. ADCC assays were performed with WIL-2 cells and PBMC effector cells as described under "Materials and methods." E, ADCC with intact anti-CD20, AHAs 1-A3, 1-A6, and 1-A8 as well as anti-CD20 F(ab’)2 MMP3, F(ab’)2 GluV8, F(ab’)2 MMP7, and F(ab’)2 IdeS. ADCC assays were performed in triplicate wells, and error bars represent S.D. Data are representative of three independent experiments.

Only restored against the single F(ab’)2 fragment for which the AHA had the highest affinity. In contrast, the AHAs were able to restore ADCC to multiple F(ab’)2 fragments. This observation was consistent with a previous report where monoclonal antibodies with affinity-purified against one F(ab’)2 fragment could restore function to a F(ab’)2 fragment generated with different proteases (9). Therefore, monoclonal human antibodies cannot only bind to multiple F(ab’)2 fragments, single AHAs can restore ADCC function to multiple F(ab’)2 fragments.

Although AHA detection is often elevated in patients with chronic inflammatory autoimmune disorders, the presence of AHAs can readily be detected in normal human serum. The observation of CSR and mutated CDR-L3s and CDR-H3s in the present AHAs suggests these cells received T cell help during affinity maturation. Because BCRs reactive to F(ab’)2 MMP3 fragments were detected in the antigen-inexperienced, naive peripheral B cell compartment, this further suggests that cleaved hinge-reactive BCRs are not removed by either central or peripheral B cell tolerance mechanisms. This may be due to the fact that normal B cell development checkpoints in the bone marrow or periphery are likely devoid of high levels of IgG-cleaving proteases; thus cleaved IgGs would not be present and recognized as self-antigen in the absence of co-stimulation and subsequent removal from the B cell repertoire. However, naive B cells that encounter cleaved IgGs in autoimmune inflammatory environments or pathogen inflammatory environments likely encounter additional secondary stimuli such as the presence of damage-associated molecular patterns and pathogen-associated molecular patterns (PAMPs), respectively. We now
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Antibodies and proteases

The anti-CD20 mAb, rituximab, and anti-gD human IgG1 monoclonal antibodies were generated by Genentech. Proteases MMP3 and MMP7 were purchased from Enzo Life Sciences; *S. aureus* V8 glutamyl endopeptidase (GluV8) was obtained from Thermo Fisher Scientific; IdeS protease was produced by Genentech.

Antigen tetramer preparation

Anti-gD human IgG1 was digested with MMP3 at molar ratio 10:1 for 96 h at 37 °C in TBS buffer (50 mM Tris, 150 mM NaCl, pH 7.6) plus 5 mM CaCl2. The reaction was stopped by adjustment to 10 mM EDTA. MMP3-cleaved anti-gD F(ab′)2 fragment was purified using MAbSelect Sure (GE Healthcare) and labeled with biotin at 1:1 molar ratio by EZ-Link NHS-PEG4-biotinylation kit (Thermo Fisher Scientific). Biotinylated MMP3-cleaved F(ab′)2 anti-gD was further incubated with premium-grade phycoerythrin-labeled streptavidin (Thermo Fisher Scientific) at 4:1 molar ratio on ice with 10-step labeling. In each step, 10% of the phycoerythrin-labeled streptavidin was incubated with biotinylated F(ab′)2 fragment for 10 min on ice before adding another 10% of phycoerythrin-labeled streptavidin to maximize tetramer formation.

Generation of F(ab′)2 fragments

Rituximab was digested with either IdeS or GluV8 at pH 7.4 in phosphate-buffered saline (PBS). Digestion of rituximab by MMP7 was done under the same condition as MMP3 cleavage. IgG cleavage was assessed by electrophoresis in SDS denaturing solution without reduction of disulfide bonds. The reactions were stopped by adjustment to 1 mM iodoacetamide for IdeS or solution without reduction of disulfide bonds. The reactions showed the previously published cleavage sites for MMP3 (between Pro-232 and Glu-233) (30, 31), GluV8 (between Glu-233 and Leu-234) (31), MMP7 (between Leu-234 and Leu-235) (30, 31), and IdeS (between Gly-236 and Gly-237) (32) (data not shown).

Generation of Fab′ fragments

To reduce a portion of the F(ab′)2MMP3 and F(ab′)2MMP7 fragments, ~300 μl of 0.5 mg/ml F(ab′)2 fragment was incu-
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Figure 9. Schematic of proposed mechanism for how anti-hinge–specific B cells receive T cell help. A, anti-hinge–specific B cell binds to a cleaved IgG that is opsonized on a pathogen. PAMPs from the pathogen provide co-stimulation to the B cell. The B cell then internalizes and processes and presents antigen, including the pathogen antigen even though the BCR bound to cleaved IgG. B, B cell presentation of pathogen antigen on MHCII elicits cognate T cell help and further co-stimulation (e.g. through CD40–CD40L interactions) resulting in CSR and affinity maturation of the anti-hinge specific B cell. C, legend for the symbols depicted in A and B.

bated with dithiothreitol at a final concentration of 5 mM. Samples were incubated overnight at 37 °C after which the free cysteines were blocked by addition 1 mM iodoacetamide. Each sample was buffer-exchanged with PBS using Eppendorf spin columns (GE Healthcare) to remove residual iodoacetamide. Reduction of the disulfide bonds was assessed using an Agilent Bioanalyzer. Additional confirmation of the generation of the Fab’/H11032 fragment reduction was performed by size-exclusion chromatography analysis.

Phenotype of anti-hinge–reactive B cells by flow cytometry

Human whole blood was obtained from 10 healthy volunteers (Genentech Inc.). Human B cells were enriched from PBMCs by depletion of non-B cells using magnetic cell sorting with human B cell isolation kit (Miltenyi Biotec). B cells were first incubated with 5 μg/ml streptavidin-PE conjugated or unconjugated biotinylated F(ab’)2 anti-gD MMP3 tetramer for 30 min on ice, washed off unbound antigen, and then followed by CD19-allophycocyanin/Cy7 and CD27-brilliant violet 510 (Biolegend), IgD-allophyocyanin, IgG-R-phycocerythrin/Cy7, CD16, CD32 (clone FLI8.26), and CD64 (BD Biosciences). Dead cells were excluded by 7-AAD staining (BD Biosciences).

Single-cell sorting

PBMCs were isolated from a Leuko Pak (AllCells) using Ficoll-Paque gradient fractionation (GE Healthcare). PBMCs were then frozen in 90% fetal bovine serum (FBS) supplemented with 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen until thawed for use. B cells were enriched from thawed PBMCs and stained as indicated under “Phenotype of anti-hinge–reactive B cells by flow cytometry.” Cells were sorted with a fluorescence-activated cell sorter Aria II (BD Biosciences) into a cooled 96-well PCR plate prefilled with RNA lysis buffer.

Cloning of anti-hinge autoantibodies from single B cells

The cloning of human immunoglobulin heavy and light chain variable segments from single human B cells was described elsewhere in detail (33). A brief description follows. Single B cells were sorted into 14 μl of reverse transcriptase (RT) lysis buffer and stored frozen at −100 °C. To generate first strand cDNA synthesis, each well was adjusted to contain a final concentration of 0.24 M hexamers and anchored dT primer mixture (Random Primer Mix S1330S, New England Biolabs, Ipswich, MA) and 7.7 units/μl of Superscript III (Thermo Fisher Scientific, Waltham, MA). The plates were incubated for three continuous 30-min intervals at 45, 50, and 55 °C. Each well was diluted with 7 μl of water prior to dispensing 1.4 μl of the RT reaction mix for the first PCR using a previously described touchdown cycling PCR protocol. A second PCR with nested primers that were designed to enable Gibson cloning into expression vectors was performed as described. Second PCR products were treated with exonuclease I and shrimp alkaline phosphatase (New England Biolabs) for 30 min at 37 °C followed by a 15-min inactivation step at 70 °C. Three μl of the treated second PCR products were ligated with pre-digested immunoglobulin heavy (IgG1) and light (κ) chain mammalian expression vectors following a modified Gibson cloning proce-
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Apparent affinity of anti-hinge autoantibodies measured by SPRi and CFM method

Using CFM technology from Wasatch Microfluidics, anti-gD whole antibody and F(ab′)2 fragments digested with IdeS, MMP3, MMP7, and GluV8 were printed onto a sensor chip (SPR Sensor prism CMD 200m, Xantec Bioanalytics) at 10 μg/ml in 10 mM sodium acetate buffer at pH 4.5 via amine coupling (amine coupling kit, GE Healthcare). Subsequently, the chip surface was quenched with ethanolamine and rinsed with HBS/TE buffer (100 mM HEPES, pH 7.2, 0.15 M NaCl, 0.05% Tween 20, 3 mM EDTA) and MilliQ water. The kinetics of anti-hinge antibody binding to immobilized F(ab′)2 fragments was carried out using IBIS MX96 SPRi. Anti-hinge antibodies 1-A3, 1-A5, 1-A8, and 1-B3 were captured and recorded at four different concentrations (900, 300, 100, and 33 nM) with an association time of 3 min and a dissociation time of 20 min at a flow rate of 8 μl/s, whereas 1-A6 antibody was captured at eight different concentrations starting from 300 nM and serially diluted by 1:3. To remove the bound antibodies and to prepare the sensor chip for the next analytical cycle, the chip surface was regenerated by the injection of 10 mM glycine, pH 2.0, for 30 s. The avidity constant was calculated using a 1:1 Langmuir binding model with software supplied by the manufacturer to calculate the kinetics and binding constants.

Peptides

The 14-mer peptides of human IgG1 hinge sequence were synthesized in Genentech and Eli Lilly Biopharmaceutical in the format of acetyl-Lys (biotin)–(hinge-14-mer peptide)–α-acid as described previously (9).

ELISA

ELISAs for anti-hinge autoantibodies binding to proteolytic fragments of mAbs were adapted from the method previously described (9). Briefly, proteolytic cleaved anti-gD F(ab′)2 fragments were coated on 96-well plates at 10 μg/ml in PBS for 1 h at 37 °C. Plates were then washed and blocked with 3% bovine serum albumin (BSA) in PBS. Detection of bound human antibodies was by horseradish peroxidase (HRP)-conjugated anti-human IgG specifically for Fc domain (Life Technologies, Inc.). The plates were developed using TMB One component HRP microwell substrate and stop solution (SurModics).

ELISAs for detection of anti-hinge autoantibodies binding to peptide analogues of the human IgG1 hinge were performed as follows. ELISA plates were coated with NeutrAvidin biotin-binding protein (Thermo Fisher Scientific) at 10 μg/ml at 4 °C overnight. Peptides were incubated at 10 μg/ml for 1 h. The blocking, washing, and detection steps were the same as the ELISAs of F(ab′)2 fragment binding described above.

Immune complex formation of F(ab′)2 fragments and anti-hinge autoantibodies

Immune complexes were formed by combining MMP3-cleaved F(ab′)2 fragment and anti-hinge autoantibodies at a molar ratio 1:1 at 1 mg/ml in PBS and incubated on ice for 1 h. Sample contents were analyzed by size-exclusion chromatography on an HPLC system connected to a multilabel light scatter instrument (Waters XBridge BEH SEC 200Å column, Wyatt Dawn Heleos II). The instruments were run at room temperature with PBS as the running buffer. Molar mass and peak integration were determined from the multilabel light scatter instrument using supplied software (Wyatt ASTRA 6).

Negative stain electron microscopy (EM)

Approximately 100 ng of freshly prepared F(ab′)2 fragment was flowed over immobilized IgG (30 μl/min for 2 min) and allowed to dissociate for 10 min. Between each injection, the capture surface was regenerated with a 60-s injection of 10 mM glycine, pH 2.0. A 3-fold concentration series of each F(ab′)2 fragment ranging from 2 to 500 nM was used to analyze binding to AHA antibodies. All sensorgrams produced for each IgG interaction with F(ab′)2 fragments were analyzed using a 1:1 Langmuir binding model with software supplied by the manufacturer to calculate the kinetics and binding constants.

Monovalent affinity measurement by SPR

Binding kinetics and affinity for anti-hinge antibodies 1-A3, 1-A5, 1-A6, and 1-A8 were evaluated by surface plasmon resonance on a BIAcore T200 instrument (GE Healthcare). The assay format involved antibody capture on a Series S CM5 chip. Briefly, amine coupling was used to create a human IgG capture surface (anti-human Fc mAb) following instructions provided with the GE Healthcare human IgG capture kit. Each antibody was captured in parallel (n = 3) on flow cells 2–4 leaving flow cell 1 as a subtractive reference. Capture levels of IgG were targeted between 50 and 100 resonance units, after which individual F(ab′)2 fragments were flowed over immobilized IgG (30 μl/min for 2 min) and allowed to dissociate for 10 min. Between each injection, the capture surface was regenerated with a 60-s injection of 10 mM glycine, pH 2.0. A 3-fold concentration series of each F(ab′)2 fragment ranging from 2 to 500 nM was used to analyze binding to AHA antibodies. All sensorgrams produced for each IgG interaction with F(ab′)2 fragments were analyzed using a 1:1 Langmuir binding model with software supplied by the manufacturer to calculate the kinetics and binding constants.
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bated for 30 s on a freshly glow discharged 400-mesh grid covered with a thin layer of continuous carbon (Electron Microscopy Sciences). After incubation, the samples were stained with 2% (w/v) uranyl acetate solution (SPI Supplies) and blotted dry. Samples were then imaged on a TEM JOEL 1400 equipped with a US1000 CCD camera and operated at 80 kV. Images were acquired at a magnification of 30,000 using a defocus of approximately negative 1 μm. A total of 1743, 6258, and 3454 particles were collected, respectively, from the F(ab')MMP3, 1-A6, and F(ab')MMP7=1-A6 samples, using the e2boxer.py software included into EMAN2 distribution (34) using a box size of 128 or 152 pixels. Particles were subjected to reference free 2D classification using the software suite Relion (35).

CDC assay

CDC assay was adapted from the method previously described (15). Briefly, WIL2-S cells were plated in 96-well U-bottom plates at 8 × 10^4 cells/well in 50 μl of RPMI 1640 supplemented medium with 10% heat-inactivated FBS (Life Technologies, Inc.). Cells were incubated at room temperature with 50 μl of increasing concentrations of rituximab or rituximab F(ab')2 fragments generated with IdeS, MMP3, MMP7, or GluV8 cleavage in the presence or absence of a fixed concentration of 5 μg/ml anti-hinge autoantibody. After 1 h, 50 μl of a 20% rabbit complement (AbD Serotec) solution was added, mixed by pipetting up and down, and then incubated at 37 °C for 60 min. Plates were centrifuged at 200 × g for 5 min, and 50 μl of supernatant was mixed with 50 μl of lactate dehydrogenase detection kit (Roche Applied Science) for 15 min at room temperature in the dark. Plates were read on a SpectraMax (Molecular Device) at 490 and 650 nm. Percent lysis was calculated as (sample release − WIL2-S in complement solution alone)/(maximal release from Triton X-100 lysis − WIL2-S in complement solution alone) × 100%.

ADCC assay

ADCC assay was performed with modification from a previously described method (15). Briefly, 0.5 × 10^6 human PBMCs with FcyRIIIA-158V/F polymorphisms were used as effector cells, and 1 × 10^4 BATDA-labeled (PerkinElmer Life Sciences) WIL2-S cells were used as target cells in a 50:1 ratio. Cells were incubated with increasing concentrations of Rituximab or F(ab')2 fragments of Rituximab generated with IdeS, MMP3, MMP7, GluV8, or IdeS cleavage in the presence or absence of a fixed concentration of 5 μg/ml anti-hinge antibody. Human PBMCs, labeled WIL2-S cells, and the indicated antibody concentrations were combined in 200 μl total in RPMI + 10% heat-inactivated FBS in U-shaped 96-well plates, centrifuged for 2 min at 200 × g, and incubated at 37 °C. After 2 h, plates were centrifuged at 400 × g for 5 min, and 20 μl of supernatant was mixed with 200 μl of DELPHIA Europium-based reagent (PerkinElmer Life Sciences). Relative fluorescence units were measured using EnVision Multimode Plate Reader (PerkinElmer Life Sciences). Percent lysis was calculated as (sample release − spontaneous release)/(maximal release from Triton X-100 lysis − spontaneous release) × 100%.

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