Human Anti-IgG1 Hinge Autoantibodies Reconstitute the Effector Functions of Proteolytically Inactivated IgGs

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Human Anti-IgG1 Hinge Autoantibodies Reconstitute the Effector Functions of Proteolytically Inactivated IgGs

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A number of proteases of potential importance to human physiology possess the ability to selectively degrade and inactivate Igs. Proteolytic cleavage within and near the hinge domain of human IgG1 yielded products including Fab and F(ab')2, possessing full Ag binding capability but absent several functions needed for immune destruction of cellular pathogens. In parallel experiments, we showed that the same proteolytically generated Fabs and F(ab')2 become self-Ags that were widely recognized by autoantibodies in the human population. Binding analyses using various Fab and F(ab')2, as well as single-chain peptide analogues, indicated that the autoantibodies targeted the newly exposed sequences where proteases cleave the hinge. The point of cleavage may be less of a determinant for autoantibody binding than the exposure of an otherwise cryptic stretch of hinge sequence. It was noted that the autoantibodies possessed an unusually high proportion of the IgG3 isotype in contrast to Abs induced against foreign immunogens in the same human subjects. In light of the recognized potency of IgG3 effector mechanisms, we adopted a functional approach to determine whether human anti-hinge (HAH) autoantibodies could reconstitute the (missing) Fc region effector functions to Fab and F(ab')2. Indeed, in vitro cellular assays, purified HAH autoantibodies restored effector functions to F(ab')2 in both Ab-dependent cellular cytotoxicity and complement-dependent cytotoxicity assays. The results indicate that HAH autoantibodies selectively bind to proteolytically cleaved IgGs and can thereby provide a surrogate Fc domain to reconstitute cell lytic functions. The Journal of Immunology, 2008, 181:3183–3192.

The Fab and Fc regions of Igs are attributed with resistance to proteolysis under most physiological conditions. However, our group and others have shown that several proteases secreted or expressed in environments where Igs are present can cleave Igs in the hinge region, resulting in a number of cleavage products, including Fab or F(ab')2 (1–3). These fragments retain the ability to bind to their respective epitopes but lose Fc-mediated effector functions that are dependent on Fc receptor interactions with Fc receptors, complement, etc. Pathogenic cell-associated proteolytic cleavage of Igs has thereby been proposed as a potential mechanism for evasion of host immune surveillance (2, 4, 5).

Proteases have been associated with tumor invasion, inflammation, and metastasis (6, 7), as well as with bacterial infections. The matrix metalloproteinases (MMPs)3 matrilysin (MMP-7) and stromelysin-1 (MMP-3) were reported to cleave all subclasses of human IgG in a step-wise process (4). Certain bacterial proteases have been shown to possess strict selectivity for mammalian IgGs (8–10). The IgG-degrading enzyme of Streptococcus pyogenes (IdeS) specifically cleaves human IgG1 between glycine 236 and glycine 237 (EU numbering) (11) in the lower hinge (1, 9, 12). IdeS-mediated cleavage of Igs bound to the surface of S. pyogenes inhibited killing of the bacteria by phagocytosis (1, 2). Numerous mucosal pathogens such as Streptococcus sanguis, Haemophilus influenzae, and Neisseria meningitides express proteases that cleave IgA1 in the hinge region, blocking its effector functions. Microbial proteases that cleave IgM have also been identified (13, 14). Collectively, these investigations suggested that extracellular proteolysis of host Igs might represent a pathogenic tactic for avoidance of host humoral immune functions. Our group recently showed that a broad group of human proteases including MMP-3, cathepsin G, plasmin, and human neutrophil elastase (HNE), as well as GluV8 from Staphylococcus aureus, and IdeS all catalyzed specific in vitro cleavages of the human IgG hinge (1).

Proteases present at sites of inflammation, infection, and tumor environments (15, 16) that are capable of cleaving Igs may expose epitopes in the hinge region that would be hidden in intact Igs. Such cryptic epitopes could allow for activation of anti-hinge autoreactive B cells. Autoreactive B cells are thought to be eliminated from the repertoire by three processes, namely, anergy, receptor editing, or deletion (17–19). Yet, healthy individuals have circulating B cells with specificity for autoantigens (20). Autoantibodies exist that recognize different domains of Igs, such as the Fc domain-binding rheumatoid factor (22). There is also evidence for autoantibodies that recognize proteolytically cleaved Igs in the form of Fab and F(ab')2 (23–25). However, most earlier anti-fragment autoantibody studies used Fab and F(ab')2 generated with papain and pepsin (26, 27),
proteases of questionable relevance for most human tissue and disease. Many of these studies did not indicate whether the anti-Fab and anti-F(ab')2 autoantibodies were specific for variable, hinge, or Fab constant regions. In one study, it was suggested that these autoantibodies function to negatively regulate Ag-engaged B cells by binding to FcγRIIB and inducing B cell apoptosis (28).

The presence of autoantibodies to proteolytically cleaved IgG1 was also documented in human clinical trials or preclinical testing using therapeutic monoclonal Fab or F(ab')2, respectively (23, 29, 30). In five human subjects treated with a human/murine chimeric Fab (papain-generated), serum anti-human hinge reactivity was observed to increase soon after administration. The timing of the increased immune reactivity was suggestive of a memory recall response (23), since immune responses to the fully murine version increased immune reactivity was suggestive of a memory recall obtained as previously described (1). Proteolytic digestions of IgGs by providing a surrogate Fc domain. We document the existence of anti-hinge autoantibodies in healthy human donors that are specific for the hinge region of IgG1. To study the effect of these autoantibodies in vitro, we affinity purified HAH autoantibodies from pooled human IgG preparations (IVlg). HAH autoantibodies were tested in both Ab-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) assays and were shown to restore both functions to cell-bound F(ab')2 at Ab concentrations that correspond to in vivo levels.

Materials and Methods
Proteases and IgG digestions
Proteolytic enzymes used to generate Fab and F(ab')2 that lack Fc-mediated effector function, we hypothesize that human anti-hinge (HAH) autoantibodies counteract proteolytic cleavage of IgGs by providing a surrogate Fc domain. We document the existence of anti-hinge autoantibodies in healthy human donors that are specific for the hinge region of IgG1. To study the effect of these autoantibodies in vitro, we affinity purified HAH autoantibodies from pooled human IgG preparations (IVlg). HAH autoantibodies were tested in both Ab-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) assays and were shown to restore both functions to cell-bound F(ab')2 at Ab concentrations that correspond to in vivo levels.

Pepsines and IgG digestions
Proteolytic enzymes used to generate Fab and F(ab')2 of mAbs were produced or obtained as previously described (1). Proteolytic digestions of mAbs were performed at physiological pH and ionic strength as previously described (1). IgG cleavage was assessed by Agilent Biosizing microcapillary electrophoresis in SDS denaturing solution without reduction of disulfide bonds (31). Purification of mAb fragments used protein A adsorption steps and yielded Fab and F(ab')2 without detectable levels of Fc-containing materials. Designations of individual purified fragments include a subclass to denote the protease responsible for the cleavage (e.g., F(ab')2,smmp-3).

Abs and protein reagents
mAbs included fully human, recombinant humanized Abs or human/murine chimeric Abs possessing human constant domains and hinge regions. The mAb1 (anti-viral Ag) is a human IgG1. The mAb2 (anti-human cytokine) and mAb3 (anti-human CD4/CD61) are human/murine chimeric IgG1s possessing human constant regions and hinge domains. The mAb4 (anti-human CD142) is a CDR-grafted humanized IgG1. The mAb5 (anti-human CD20), a chimeric IgG1, is a product of Genentech. The mAbs contained k L chains. The above mAbs were used as substrates for proteolytic digestion to Fab and F(ab')2. IgG concentrated from large pools of human plasma (IVlg) was a product of Octapharma AB.

EBV Ag was obtained from BioPacific. Staphylokinase was from Affinity BioReagents. Tetanus toxoid was a product of List Biological Laboratories. Streptokinase was obtained from Sigma-Aldrich.

ELISA
ELISAs for human Ig binding to proteolytic fragments of mAbs were adapted from the format previously described (1). In brief, mAbs, Fab, and F(ab')2 were coated on 96-well plates at 10 μg/ml. Single donor serum and pooled healthy donor serum were incubated on Ag-coated wells beginning at 1/50 dilutions. Detection of bound human Abs was by isotype-specific, HRP-conjugated secondary Abs; anti-IgG1 mAb (Zymed; 1/3200), anti-IgG2 mAb (Zymed; 1/1000), anti-IgG3 mAb (Zymed; 1/3000), anti-IgG4 mAb (Sanquin; 1/100,000), anti-IgM mAb (Zymed; 1/1200), anti-IgA mAb (Nordic Immunol.; 1/600), goat anti-IgA (Zymed; 1/1000), and anti-IgD mAb (Nordic Immunol.; 1/24,000). Isotype standards of IgG1, IgG2, IgG3, IgG4, IgM, IgE, and IgD were from myeloma serum and were obtained from Contrex Research & Technology. Human serum-derived IgA was obtained from Jackson Immunoresearch Laboratories. Isotype standards were directly coated in microtiter wells at 0.1 μg/ml to serve both as specificity controls and as reactivity standards against which to normalize human serum IgG binding. The plates were developed using SIGMAFAST OPD (Sigma-Aldrich) and stopped by acidification with HCl.

ELISAs for detection of IgG3 binding to peptide analogues of the human IgG1 hinge were performed as follows. Microtiter plate wells were precoated with 10 μg/ml streptavidin (Pierce) overnight at 4°C. With the exception of the omission of casein from the blocking buffer, the washing and detection steps were otherwise as previously described for the fragment ELISA (1). Peptides were added to wells at 1 μg/ml in a volume of 50 μl. Pooled human serum was added to microtiter wells at a dilution of 1/50 in PBS (pH 7.4) containing 3% BSA. Experiments were conducted at 2.5 g/ml. Detection of bound Abs used an anti-IgG3-specific secondary as described above.

Pepitides
The 14-mer hinge peptides were synthesized on a Rains SMPS-110 using standard Fmoc protocols for HBTU activation. Acetyl-Lys(Fmoc)-OH (Bachem) was coupled to the N-terminal residue and, after Fmoc deprotection, biotinylated with N-(Biotinloyloxy) succinimide (Bachem). Fmoc-Glu(biotinyl-PEG3)-OH (a spacer of three ethylenoxy units (PEG3) inserted between the biotin and the ϖ-carboxy group of glutamic acid; Novabiochem) was coupled to Rink resin. Peptides were deprotected and cleaved from resin by acidolysis using trifluoroacetic acid cleavage mixture then purified by preparative reverse-phase HPLC as previously described (32). Two sets of peptides were prepared: Acetyl-Lys(biotin)-(hinge-14-mer peptide)-α-α-amide (hinge-14-mer peptide)-Glu(biotinyl-PEG3)-amide. Analytical reverse-phase HPLC and capillary electrophoresis showed a high degree (>97%) of peptide homogeneity (data not shown). Surface-enhanced laser desorption-ionization and matrix-assisted laser desorption-ionization mass spectrometry gave the expected molecular masses.

Human materials
Human donor blood cells for use in ADCC assays were collected from employee volunteers at Centocor with all necessary permissions and approvals. The protocol and informed consent form, as well as any relevant study-related documentation, were approved by a third party Institutional Review Board. Healthy human serum samples were commercially obtained from Bioreclamation and used as individual test samples or as pools.

Isolation of HAH autoantibody
Autoantibodies specific for the hinge region of IgG were affinity purified from IVlg (Octapharma AB). In brief, mAb3 was digested with either 0.02% I2E(s) (Genovis) or GluV8, and the resultant F(ab')2 of mAb3 were purified from the Fc fragment over Mabsellect Protein A (GE Healthcare). A total of 200 mg of each mAb3 F(ab')2 was coupled to NHS-activated Sepharose 4 Fast Flow (GE Healthcare) and 5g of IVlg was applied to the Sepharose-coupled mAb3 F(ab')2. Bound Abs were eluted with 0.1 M glycine (pH 2.5), and the pH was neutralized with 2 M Tris (pH 7.0) (1/10 volume). Cross-reactive anti-whole IgG Abs were removed by further chromatography on 40 mg of intact mAb3 coupled to NHS-activated Sepharose 4 Fast Flow. The unbound flow through on the IgG column was termed the HAH autoantibody.

ADCC assay
ADCC assays were performed as previously described (33). In brief, PBMCs were purified from human blood and used as effector cells for ADCC assays. MDA-MB-231 human breast carcinoma cells (ATCC) were used as target cells with a ratio of 1 target cell to 50 effector cells. Target
cells were pre-labeled with BAPTA (PerkinElmer) for 20 min at 37°C, washed twice, and resuspended in DMEM, 5% heat-inactivated FBS, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, penicillin (500 U/ml), streptomycin (500 U/ml), and 2 mM L-glutamine (all from Invitrogen). Target (1 × 10^6 cells) and effector cells (0.5 × 10^6 cells) were combined, and 100 µl of cells were added to the wells of 96-well U-bottom plates. An additional 100 µl was added with or without Abs. All samples were performed in triplicate. The plates were centrifuged at 200 g for 3 min, incubated at 37°C for 2 h, and then centrifuged again at 200 g for 3 min. A total of 20 µl of supernatant was removed per well, and cell lysis was measured by the addition of 200 µl of the DELPHA Europium-based reagent (PerkinElmer). Fluorescence was measured using an Envision 2101 Multilabel Reader (PerkinElmer). Data were normalized to maximal cytotoxicity with 0.67% Triton X-100 (Sigma-Aldrich) and minimal control containing only cells and complement alone. Data were fit to a sigmoidal dose-response model using GraphPad Prism v5. EC50 values were calculated from the 4-parameter regression analyses.

CDC assay

WIL2-S cells (ATCC) were used as target cells for CDC assays. A total of 50 µl of cells were added to the wells of 96-well plates for a final concentration of 8 × 10^3 cells per well in RPMI, 5% heat-inactivated FBS, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, penicillin (500 U/ml), streptomycin (500 U/ml), and 2 mM L-glutamine. An additional 50 µl was added to the wells with or without Abs and the plates were incubated at room temperature for 2 h. A total of 50 µl of 10% rabbit complement (Invitrogen) was added to the wells, and the plates were incubated for 20 min at 37°C. All samples were performed in triplicate. The plates were centrifuged at 200 g for 3 min, 50 µl of supernatant was removed to separate plates, and CDC was measured with LDH cytotoxicity detection kit (Roche). Absorbance was measured using a Spectra max Plus 384 (PerkinElmer). Data were normalized to maximal cytotoxicity with Triton X-100 (Sigma-Aldrich) and minimal control containing only cells and complement alone. Data were fit to a sigmoidal dose-response model using GraphPad Prism v5. EC50 values were calculated from the 4-parameter regression analyses.

Statistics

Statistics for Fig. 1A were determined as follows. Eight isotypes were measured in four donors and compared with controls on 6 days. On each day, three replicates from each donor were tested. Repeated measures ANOVA was conducted on data expressed as percent of the isotype control. Three repeated effects were included in the model: isotype, day, and replicate within day. Pairwise comparisons between the isotypes were evaluated. Adjustments for multiple comparisons were made using the Tukey-Kramer method. Adjusted p values of 0.05 or less were considered significant.

Results

Human serum autoantibodies bind to the hinge region of proteolytically cleaved IgG1

The major goal of this study was to establish functional properties for the HAH autoantibodies that recognize proteolytically exposed epitopes in IgG. We generated a panel of purified Fab and F(ab’)2 from mAbs as Ags for autoantibody binding. The mAbs included a human IgG1, as well as humanized and human/murine chimeric Abs – each possessing the same human IgG1 hinge and constant region amino acid sequences. A F(ab’)2 was generated from human IgG1κ mAb1, using IdeS as previously described (1). Fig. 1 depicts the ELISA binding reactivity in diluted serum from a small test group (n = 4) of individual healthy donors to this mAb1 F(ab’)2. Fig. 1A shows that reactivity was found within the IgG1, IgG2, IgG3, IgM, and IgA isotypes, whereas no anti-fragment reactivity was detected for IgG4, IgE, or IgD. IgA and IgM anti-fragment autoantibodies have been reported previously (34). The remaining studies focused on IgG isotypes since pooled human IgG (e.g., IVIg) was used extensively for characterization and as a source of autoantibodies.

Fig. 1B depicts the average binding of IgG isotypes to mAb1 F(ab’)2 from pooled serum from 20 healthy donors, as well as from IVIg (IgG purified from a plasma pool of >3500 donors). The binding results for these two pools of increased size confirmed that among the IgG isotypes, reactivity was detectable with IgG1, IgG2, and IgG3, but not IgG4. The serum pool and IVIg contained detectable IgG1 and IgG2 reactivity, but IgG3 was the major component among autoantibody reactivity to this human F(ab’)2.

Table 1.

Comparisons of human serum Ab isotypes binding in ELISA to IgG fragments and exogenous Agsa

<table>
<thead>
<tr>
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<th>Anti-IgG1</th>
<th>Anti-IgG2</th>
<th>Anti-IgG3</th>
<th>Anti-IgG4</th>
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</thead>
<tbody>
<tr>
<td>mAb2 F(ab’)2 MMP-3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>mAb2 F(ab’)2 MMP-12</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>mAb2 F(ab’)2 Glu. Endopep.</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>mAb2 F(ab’)2 Pepsin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>mAb2 F(ab’)2 IdeS</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Staphlokinase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Streptokinase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EBV Capsid Ag</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Tetanus Toxoid</td>
<td>+</td>
<td>+</td>
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a Pooled serum from 20 healthy donors was tested at serial 2-fold dilutions starting at 1/50. The IgG fragments, staphlokinase and EBV were coated at 10 µg/ml; streptokinase and tetanus toxoid were coated at 4 µg/ml.

The reactivity was assigned based on the highest dilution at which the serum pool resulted in 10% of the OD of the directly coated isotype standard and replicate within day. From standard wells containing directly coated isotype standards and replicate within day. Pairwise comparisons between the isotypes were evaluated. Adjustments for multiple comparisons were made using the Tukey-Kramer method. Adjusted p values of 0.05 or less were considered significant.
The isotype profile of HAH autoantibodies was compared with human immune responses elicited to three commonly encountered exogenous immunogens; EBV capsid Ag, streptokinase, and staphylokinase, as well as tetanus toxoid. The isotype distribution toward these Ags was investigated in pooled serum from 20 healthy donors using a serial dilution-based criterion to compare reactivity. ELISA results in Table I point to IgG1 and IgG2 as the primary isotypes directed to exogenous Ags, as well as IgG4 in the case of tetanus toxoid. IgG3 was the weakest among the isotypes detected against these “non-self” Ags. In contrast, autoantibody reactivity to various F(ab′)2s of mAb2, generated by cleavage with five different proteases, MMP-3, MMP-12, GluV8, pepsin, and IdeS, indicated a bias toward the IgG3 isotype. The remaining experiments focused exclusively on IgG3; a departure from earlier studies that did not report a specific isotype (35, 36) or that concentrated on other isotypes such as serum IgA (34).

To further test whether IgG3 HAH autoantibodies were selective for the cleaved IgG hinge rather than to other elements of Ab structure, we used a group of different mAb Fab and F(ab′)2 fragments as Ags, as well as their intact IgG1 parents. The fragments were produced by proteases catalyzing cleavage at the peptide bonds indicated in Fig 2C. The purity and molecular size of representative proteolytically generated mAb fragments for ELISA were confirmed by bio-sizing as shown in Fig 2D. Levels of IgG3 anti-fragment reactivity above background were evident in individual healthy donor sera (n = 20) toward many of the human IgG1 fragments in the ELISA (Fig 2A). Autoantibody recognition was evident against each of the three Fabs of mAb4 generated by enzymatic cleavage in the upper hinge – by papain, HNE, and human plasmin. The highest incidence of IgG3 reactivity in the upper hinge was against the HNE-generated fragment.

IgG3 HAH autoantibody reactivities to F(ab′)2 of “human” IgG1s were consistently elevated as also illustrated in Fig. 2A. Anti-F(ab′)2 reactivity was found against each of the mAbs, mAb2, mAb3, mAb4, and mAb5, and was independent of the proteases used for digestion since MMP-3, MMP-12, GluV8, pepsin, and IdeS were each represented. The detection of IgG3 HAH autoantibody binding to intact versions of these four mAbs was at baseline for 19 of the 20 healthy donors, in distinct contrast to the fragments. No comparable binding of IgG3 HAH autoantibodies was detected to the cleaved Fc domain of IgG1 mAbs (data not shown). These findings indicated that C-terminal cleavage sites of proteolyzed IgG1 Abs were primary targets for binding by IgG3 HAH autoantibodies.

**Large pools of human plasma-derived IgGs possess anti-fragment autoantibody reactivity**

In Fig. 2B, the investigation of IgG3 binding to mAbs and to proteolytically generated fragments was extended to IVIg as the source of HAH autoantibodies; the bar heights indicated the mean reactivity in the pool of >3500 donors. The binding patterns to this panel of IgG1 Ags were consistent with the results in the individual donors (Fig. 2A). This indicated that IgG3 HAH autoantibodies spanning serine 219 to valine 240, and the positions of cleavage by individual proteases are indicated (1). **D.** Agilent biosizing analysis of representative proteolytically generated Fab and F(ab′)2. Microcapillary electrophoresis was performed under nonreducing conditions as previously described (1).
against F(ab')2, but not to intact IgG, were comparable in a much larger healthy population. The results supported that the IgG3 reactivity was specific for the cleavage-related epitopes in these mAbs and not to other nonhinge targets such as idiotypic determinants or constant region structures. Thus, proteolysis of IgG by human and bacterial extracellular proteases exposed antigenic determinants that were recognized by IgG3 HAH autoantibodies.

**The HAH autoantibody specificity to the IgG1 hinge was further defined using peptide analogues**

Because only seven positions were identified in the hinge as protease cleavage sites, we wanted to determine whether other C-terminal amino acids within the hinge could be bound by autoantibodies. In an attempt to address the positional gaps, a peptide analog approach was adopted. A series of advancing 14-mer peptide hinge analogues were synthesized that encompassed C-terminal positions from aspartic acid 221 to leucine 251 (Eu numbering) (11). Peptides were synthesized with an N-terminal Ac-lysine (N-ε-biotin) coupling to facilitate immobilization on streptavidin-coated microtiter wells and to expose the C terminus of the peptide. Each consecutive C-terminal position within the hinge and adjoining constant region was represented in this single-chain peptide series.

Fig. 3 presents the ELISA results of pooled serum from 20 healthy donors binding to the peptide series. A relatively low level of IgG3 HAH autoantibody reactivity was detected to the upper hinge region encompassing aspartic acid 221 to histidine 224; “DKTH.” There was no detectable HAH autoantibody binding to peptides with C-termini in and immediately flanking the core hinge (threonine 225 through alanine 231, “TCP-PCPA.” Substantial IgG3 anti-peptide reactivity was found for hinge analogues with C-termini at positions of the lower hinge from proline 232 to glycine 236 and the adjoining constant domain from glycine 237 to phenylalanine 241, “PELLGGPSVF.” Within this latter sequence were the C-terminal positions corresponding to protease cleavage sites in IgG1 by MMP-3 and MMP-12 (proline 232), by cathepsin G and GluV8 (glutamic acid 233), by MMP-7 and pepsin (leucine 234), and IdeS (glycine 236). Similar “maps” of anti-IgG1-hinge-peptide-analog reactivity were established for individual donor serum and IVIg (data not shown). Peptide analogues of IgG1 exhibited a peak zone of Ag reactivity centered at the hinge/CH2 junction (also the IdeS cleavage point) between glycine 236 and glycine 237. However, some of the highly targeted peptides possessed C-termini corresponding to putative F(ab')2s where no extracellular proteases are known to directly fragment IgG1. The pattern of binding of IgG3 HAH autoantibodies to hinge peptide analogues with free C-termini was not paralleled in peptides that encompassed the same range of amino acid sequence, but constructed with free N-terminal amino acids. The latter series was conjugated with biotin on the C terminus of the 14-mer peptide and was assayed in ELISA in similar fashion to the previous group. IgG3 HAH autoantibody binding was minimal or undetectable to the latter series of hinge analogues (A. Schmidt, unpublished data). Thus, the results indicated substantial IgG3 HAH autoantibody binding to the C-terminal analogues of IgG1 Fab and F(ab')2, but no comparable reactivity to the cleaved end analogues of free Fcs.

**HAH autoantibodies were isolated by affinity adsorption on proteolytically generated F(ab')2**

IVIg was used as the source for the affinity purification of HAH autoantibodies. F(ab')2 IdeS or F(ab')2 GluV8 of mAb3 were separately attached to affinity matrices, and 5 grams of human IVIg Abs were applied to the respective columns. In each case, only a small fraction of Abs was bound and then eluted under acidic conditions (0.1M glycine (pH 2.5)). The pH-neutralized eluates from the F(ab')2 columns were further absorbed on a column of intact mAb3 IgG1 to minimize the presence of Abs to nonhinge determinants. In two replicate affinity chromatographies on the F(ab')2 IdeS matrix, the protein yields of HAH autoantibody IgGs were 0.037 and 0.032% of the total IgG applied to the columns. The purified HAH autoantibody preparations were several hundred-fold enriched in IgG3 anti-hinge binding compared with the starting IVIg based on ELISA reactivity (per milligram total IgG; data not shown). This result supported the previous findings that HAH Abs are enriched in IgG3 isotype.

**HAH autoantibodies reconstituted ADCC to mAb F(ab')2**

When tested in several cell-based assay systems, unfraccionated IVIg contributed directly to cell killing independent of cell-bound F(ab')2. Thus, affinity-isolated HAH autoantibodies were tested for their ability to reconstitute Fcy-mediated immune functions to cell-bound, effector-impaired F(ab')2. The mAb4 binds to CD142 present on the plasma membrane of MDA-MB-231 cells (37). Incubation of MDA-MB-231 cells with the intact IgG of mAb4 and isolated human PBMCs resulted in mAb concentration-dependent cell killing of the target cells in an ADCC assay (Fig. 4A). Nearly full cell killing was achieved at 200 ng/ml IgG, whereas no specific cell lysis was evident with F(ab')2 of mAb4 generated with IdeS, GluV8, or MMP-3 at any concentration up to 36 μg/ml. A control human IgG (mAb1) that was not specific for either MDA-MB-231 cells, intact IgG, or any fragment of IgG did not restore ADCC either alone or in combination with any of the mAb4 F(ab')2 (Fig. 4B). However, a dose-dependent restoration of ADCC function was observed when serial dilutions of HAH autoantibodies, purified by affinity chromatography on the F(ab')2 IdeS were added to a fixed 200 ng/ml of any of the three F(ab')2s (Fig. 4C). Maximum restoration was observed at HAH autoantibody concentrations 1 μg/ml and higher, and an EC50 was calculated for each reconstituted curve. A similar concentration-dependent pattern of restored ADCC function was observed with the addition of the preparation of HAH that had been affinity purified on F(ab')2 IdeS to each of the three F(ab')2 in contact with MDA-MB-231 cells (Fig. 4D). Additionally, using the opposite titration strategy, serial dilution of the respective F(ab')2 into a fixed HAH autoantibody concentration of 5 μg/ml (the estimated physiological concentration), yielded EC50 values for F(ab')2 plus HAH autoantibodies that were similar to intact mAb4 IgG (Fig. 4, E and F). Addition of...
either HAH autoantibody preparation, in the absence of mAb4 F(ab')2 IdeS, was without effect. The results indicated that both preparations of affinity-purified HAH autoantibodies reconstituted ADCC activity to low concentrations of F(ab')2 generated with IdeS, GluV8, and MMP-3.

**HAH autoantibodies reconstitute CDC to mAb F(ab')2**

The affinity-isolated, HAH autoantibodies were tested for their abilities to restore CDC function to F(ab')2 IdeS of mAb5. The mAb5 IgG1 Ab binds to CD20 expressed on the human B cell line WIL2-S and induces cell lysis in the presence of complement. Near maximum lysis of WIL2-S cells was obtained at ~1 µg/ml mAb5 IgG, whereas none of the mAb5 F(ab')2 (generated with IdeS, GluV8, or MMP-3) demonstrated an effect at up to 27 µg/ml (Fig. 5A). The nonspecific control, mAb1, had no activity alone or in combination with any of the F(ab')2 (Fig. 5B). Addition of HAH autoantibodies isolated by affinity adsorption on mAb3 F(ab')2 IdeS or mAb3 F(ab')2 GluV8 reconstituted complement-mediated cell lysis to the three different F(ab')2 of mAb5 with one exception (Fig. 5, C and D). HAH isolated on mAb3 F(ab')2 GluV8 did not restore CDC to mAb5 F(ab')2 IdeS. Higher concentrations of HAH were required for CDC restoration compared with ADCC, and the reason for this is uncertain. When the opposite titration strategy was performed with serial dilution of the respective F(ab')2 into a fixed 5 µg/ml mAb3 alone added to mAb4 F(ab')2, generated with IdeS (Δ), GluV8 (●), or MMP-3 (○). E, ADCC activity using HAH GluV8 alone (●) or a constant concentration of 5 µg/ml HAH ideS alone added to mAb4 F(ab')2, generated with IdeS (Δ), GluV8 (●), or MMP-3 (○). F, ADCC activity using HAH GluV8 alone (●) or a constant concentration of 5 µg/ml HAH ideS alone added to mAb4 F(ab')2, generated with IdeS (Δ), GluV8 (●), or MMP-3 (○). Bar heights correspond to the mean of triplicate wells ± SD. The results are a representative of three independent experiments.

**FIGURE 4.** Cell based ADCC assays of HAH affinity purified on either F(ab')2 IdeS (designated as HAH ideS) or on F(ab')2 GluV8 (designated as HAH GluV8). All assays were performed with human PBMCs from the blood of healthy donors and with the cell line MDA-MB-231 at a ratio of 50 PBMCs to 1 target cell. A, ADCC activity against MDA-MB-231 cells using intact mAb4 (●) and F(ab')2 of mAb4 (anti-CD142) generated with IdeS (Δ), GluV8 (●), or MMP-3 (○). B, ADCC activity using an intact IgG (mAb1) nonspecific for MDA-MB-231 cells (●) or intact IgG of mAb1 added to a constant concentration of 0.2 µg/ml mAb4 F(ab')2 generated with IdeS (Δ), GluV8 (●), or MMP-3 (○). C, ADCC activity using intact HAH ideS alone (●) or HAH ideS added to a constant concentration of 0.2 µg/ml mAb4 F(ab')2 generated with IdeS (Δ), GluV8 (●), or MMP-3 (○). D, ADCC activity using HAH GluV8 alone (●) or HAH GluV8 added to a constant concentration of 0.2 µg/ml mAb4 F(ab')2 generated with IdeS (Δ), GluV8 (●), or MMP-3 (○).
Discussion

We recently investigated a number of human and bacterial proteases that catalyze specific cleavages of human IgGs (1). These physiologically relevant proteases targeted both the upper and lower IgG1 hinge to generate fragments including Fab and F(ab’)_2 and the separated Fc domain. Moreover, the in vitro fragmentation patterns in purified systems were analogous to ex vivo patterns of endogenous IgG breakdown in synovial fluid samples from human subjects with rheumatoid arthritis. Related observations also led to speculations that widespread, targeted fragmentation of host Igs in pathologic, protease-rich environments could cause localized immune dysfunction (2, 4, 5). In the current study, we investigated a potential mechanism by which the host might combat proteolytic activity using HAHIdeS alone (EC50 = 2.5 µg/ml) or HAHGluV8 added to a constant concentration of 1 µg/ml mAb5 F(ab’)_2 generated with IdeS (EC50 = 1.5 µg/ml) or MMP-3 (EC50 = 4.8 µg/ml) (23, 24). A functional role for mAb5 F(ab’)_2 (designated as HAHideS) or on F(ab’)_2 GluV8 (designated as HAHGluV8). All assays were performed with the WIL2-S cell line as described in Materials and Methods. A, CDC activity against WIL2-S cells using intact mAb5 (■) and F(ab’)_2 of mAb5 (anti-CD20) generated with IdeS (△), GluV8 (◇), or MMP-3 (○). B, CDC activity using an intact IgG (mAb1) nonspecific for WIL2-S cells (□) or intact IgG of mAb1 added to a constant concentration of 1 µg/ml mAb5 F(ab’)_2 generated with IdeS (△), GluV8 (◇), or MMP-3 (○). C, CDC activity using HAHideS alone (■) or HAHGluV8 added to a constant concentration of 1 µg/ml mAb5 F(ab’)_2 generated with IdeS (△), GluV8 (◇), or MMP-3 (○). D, CDC activity using HAHGluV8 alone (■) or HAHGluV8 added to a constant concentration of 1 µg/ml mAb5 F(ab’)_2 generated with IdeS (△), GluV8 (◇), or MMP-3 (○). E, ADCC activity using HAHideS alone (■) or a constant concentration of 5 µg/ml HAHideS alone added to mAb5 F(ab’)_2 generated with IdeS (△), GluV8 (◇), or MMP-3 (○). F, ADCC activity using HAHGluV8 alone (■) or a constant concentration of 5 µg/ml HAHGluV8 added to mAb5 F(ab’)_2 generated with IdeS (△), GluV8 (◇), or MMP-3 (○). Bar heights correspond to the mean of triplicate wells ± SD. The results are a representative of three independent experiments.

We focused on autoantibody binding to mAb IgG1 fragments with a view toward characterizing functional consequences of autoantibody/fragment interactions. Specifically, the studies concentrated on autoantibody association with Fab and F(ab’)_2 since these fragments retain their abilities to bind to the specific Ags on cell surfaces. By the use of human and “humanized” mAb substrates, as well as peptide analogues, the present approach departed from the majority of earlier investigations that focused on polyclonal, serum-derived human IgGs as substrates for papain (vegetable-derived) or pepsin (from the digestive tract). Since the latter proteases are not normally present in human vasculature or tissue,
they may be less relevant to pathways leading to immune compromise in pathological (proteolytic) environments. Additionally, the use of mAbs facilitated the identification of enzymatic cleavage points (I) and minimized potential autoantibody interactions with diverse idiotypic determinants in polyclonal preparations.

Fab of IgG were generated by different proteases at three positions in the upper hinge (I). When Fabs of the same parent IgG1 were derived with plasmin, HNE, and papain (C-termini at lysine 222, threonine 223, and histidine 224, respectively) and tested as Ags, autoantibody binding from healthy donor serum was most pronounced toward the fragment produced by HNE. HNE is a potent, serine protease that is released from neutrophil granules, is present in extracellular inflammatory environments (16), and possesses broad substrate reactivity including generation of Fab from IgG1 (Fig. 2D) and F(ab′)2, from IgG2 and IgG4 (41). A relatively diminished level of autoantibody binding was seen toward papain-generated Fab even though the two fragments differed in length by only a single amino acid (depicted in Fig. 2C). The heightened autoantibody reactivity to mAb3 FabHNE compared with Mab3 Fabpapain implies that HAH autoantibodies may preferentially recognize Ags generated by physiologically relevant proteases. Nevertheless, the reactivity to mAb3 Fabpapain was higher than the reactivity to intact mAb3 IgG1 (Fig. 2A).

One human/murine chimeric Fab has been studied with regard to human immune responses following therapeutic administration (23). In subjects treated with a papain-generated Fab, five individuals were observed to develop early increased anti-hinge autoantibodies titers. The boost in autoantibody levels was evident at the earliest determination for three subjects (2 wk) and one subject (10 days). The early onset of specific anti-hinge autoantibodies were temporally different from anti-murine variable region responses to the same Ab or to the fully murine Fab version of the Ab that occurred at 4–6 wk (14 of 15 subjects). This difference in timing suggested a memory recall response to the hinge epitope in contrast to the likely primary response to the murine variable region components. Normal (baseline) anti-hinge autoantibody levels to papain-generated Fab appear to be among the lowest for any IgG fragment (Fig. 2), and few autoimmune consequences have been associated with initial treatments with mAb3 Fab against the αIIbβ3 receptor on platelets (42, 43). A relatively low level of HAH autoantibody binding to papain-generated Fab compared with pepsin-generated F(ab′)2 has previously been observed (26). Clinical investigations with other therapeutic Fabs possessing different C-termini with higher reactivity for human autoantibodies (e.g., the HNE cleavage site) have not been reported.

Without question, the most antigenic sites were those exposed in or adjacent to the lower hinge by proteases that generated F(ab′)2s. It also became apparent that IgG3 was highly represented among the human anti-F(ab′)2 autoantibodies, similar to reports of IgG3 anti-Fab autoantibodies in two diseases (27, 40). It is not clear what factors might bias anti-hinge autoantibodies toward IgG3. The persistence and high incidence of IgG3 anti-hinge autoantibodies is indicative of a class-switching event and suggests that autoreactive B cells were selected for by exposure to cleaved hinge self Ags. Although IgG3s are known to possess potent ADCC- and CDC-mediated effector functions (44–47), it is uncertain whether this is related to a selection of IgG3 for the targeting of these self-Ags (in contrast to the use of other IgG isotypes vs foreign challenges; Table I). This prompted an investigation of IgG3-enriched HAH autoantibody binding to F(ab′)2s on cells for reconstitution of the absent effector functions of the fragments (see further below).

The binding of HAH autoantibodies to a series of peptide analogues of the IgG1 hinge provided a profile of reactivity to all potential-terminal cleavage positions on Fabs and F(ab′)2s (whether or not these are known to be directly generated by proteases). Peptides terminating in the upper hinge, analogues of Fabs, were bound to a lower extent than might have been suggested by the actual Fab with the same termini. The reasons are uncertain but could include a different conformational presentation of the epitope in Fab related to orientation, flexibility, or the association of the L chain. The most antigenic peptides possessed C-termini in the lower hinge “PELLGG” sequence within which several proteases have been identified to cleave IgG1. Also within this sequence resides the “LL” motif that is central to the recognition of IgG1 by FcyRs on immune effector cells (48–51) and “LLG” found important for complement (52). This extended lower hinge sequence is highly conserved among several human IgG isotypes as well as IgGs of mice and many other mammals (53). Pepsin-generated F(ab′)2s also terminate within the antigenic zone identified above at leucine 237, and one preclinical study involving administration of such a F(ab′)2 was informative regarding anti-hinge autoantibodies. When a humanized anti-platelet αIIbβ3 monoclonal F(ab′)2 was injected into 18 nonhuman primates (30), an acute clearance of circulating platelets was noted in 5 of the treated animals. The platelet clearance was in many cases profound (>75% from baseline) and rapid (within 24 h). The authors showed that the monkeys exhibiting platelet clearance each possessed pre-existing anti-hinge autoantibodies to the humanized F(ab′)2s, and that the autoantibodies were similar in specificity to HAH autoantibodies. No long-term follow-up of the animals was reported to allow an assessment of potential recall responses to this Ag, and isotype characterizations of the autoantibodies were not presented. Nevertheless, in addition to raising issues regarding future development of F(ab′)2 for human therapy, this primate study provided insight into the potential action of anti-F(ab′)2 autoantibodies in association with a cell-bound F(ab′)2.

It proved important to separate HAH autoantibodies from the large excess of nonspecific Abs in IVlg since the latter were found to contain various anti-cell-specific Abs that interfered with our cell-based functional assays. To this end, purifications of HAH autoantibodies from IVlg were separately performed by affinity adsorption on two strongly antigenic fragments: immobilized FabH3 F(ab′)2 IdeS or on mAb3 F(ab′)2 GluVen. The procedures typically yielded HAH-autoantibody preparations containing less than 0.04% of the total starting IgG, with several hundred-fold increased binding to F(ab′)2 (compared with starting IVlg), and with an isotype profile enriched in IgG3. Binding to purified F(ab′)2 and to hinge peptide analogues confirmed that the reactivity of these preparations displayed some overlapping reactivity to fragments generated with different physiologically relevant proteases. The exact point of cleavage may be less important for autoantibody binding than the exposure of an otherwise cryptic stretch of hinge sequence.

Although, our main emphasis was on HAH autoantibodies against Fab and F(ab′)2, that retain reactivity for target cells, we did attempt to find related IgG3 autoantibody binding to purified Fab domains from proteolytically digested IgG1s. However, minimal binding to purified, protease-generated Fcs was observed, and this was confirmed by near background IgG3 binding against single-peptide analogues of the hinge containing exposed N-termini. The preferential autoantibody binding of IgG3 to peptides oriented with free C-termini differed from a previous and detailed epitope analysis (54). That work showed that two recombinant, human, single-chain FvS with anti-hinge specificity were bound to multiple synthetic double-chain peptides (and variants) containing the core C-P-P-C hinge sequence with a free N terminus corresponding to threonine 223 in the upper IgG1 hinge (54, 55). Nevertheless,
when tested on actual fragments of IgG, those same two scFvs exhibited primarily anti-F(ab')₂ reactivity with little or no anti-Fc binding (in accord with our own findings with serum IgG3) (55). The authors concluded through additional peptide binding approaches that human serum IgG and IgA anti-hinge autoantibodies most likely bound to conformational epitopes in the hinge (26, 34). Despite the diversity of strategies and previous findings on the specificity of anti-hinge autoantibodies, we chose to focus on autoantibodies that directly bind to the exposed hinge in Fab and F(ab')₂ and their peptide analogues, because these fragments retain cellular Ag-binding reactivity and could thereby persist in local environments. Functional HAH autoantibody activities to F(ab')₂ were testable in in vitro cellular assays, whereas it was difficult to envision a similar functional test for anti-Fc autoantibodies.

As the final component of the present investigation, the affinity-purified and enriched HAH autoantibody preparations were shown to restore in vitro ADCC and CDC lysis to cell-bound F(ab')₂ in concentration-dependent manners. The reconstitution of in vitro effector functions occurred with HAH autoantibodies in the ~1–10 μg/ml range when titrated against a fixed 200 ng/ml F(ab')₂. When HAH autoantibody was present at a fixed 5 μg/ml and F(ab')₂ was varied, partial restoration was seen at as low as 1 and 10 ng/ml of the fragment in ADCC and CDC, respectively. These concentrations are compatible with the estimates of anti-hinge autoantibodies of ~5 μg/ml (~0.04% of total IgG in circulation) as calculated from the recoveries on F(ab')₂ affinity columns. The two preparations of HAH autoantibodies, separately purified from IVIg on mAb3 F(ab')₂ GluV8 or on mAb3 F(ab')₂ IdeS- differed slightly in their ability to reconstitute ADCC and CDC to cell-bound F(ab')₂ that had been generated with MMP-3, GluV8, or IdeS. Those findings are consistent with multiple Ab populations with differential specificities and/or affinities to fragments of different lengths, possibilities that could be expected for Ab preparations from large pools of serum donors. Further, the restoration of effector functions by HAH autoantibodies was repeatedly demonstrated in vitro using F(ab')₂ generated by several enzymes from mAbs that targeted cells expressing different Ags, including CD142 and CD20 (Figs. 4 and 5), noncleavable surface-bound TNF (56) (data not shown) and Epacam (57) (data not shown). The present reconstitution experiments in ADCC and CDC were performed in purified systems. This was prompted, in part, by the inherent cell killing activity of unfraccionated IVIg, presumably due to specific Abs against human tumor lines within pooled human Ig. A recent study indicated that high concentrations of serum IgG inhibited ADCC mediated by mAbs in certain in vitro systems (58). These observations suggest the need for further studies regarding the influence of excess IgG on autoantibody functions.

The present results may have relevance to in vivo conditions. Namely, the HAH autoantibodies could be envisioned to bind to proteolytically derived fragments of endogenous IgGs that target pathogenic cells and thereby restore Fc effector functions. Additionally, a recent study hypothesized that anti-hinge natural Abs amplify complement activity to Ag-bound F(ab')₂ by enabling C3b complex binding to the conformationally stabilized hinge of the fragment (59). Although that pathway may be less applicable to ADCC, it serves to further highlight the potential complexity and contribution of anti-hinge Abs to humoral autoimmune.

The presently proposed model gains appeal if IgG-degrading proteases are secreted from and act locally around the targeted cell (bacterial, tumor, etc.). Specific proteolysis of the IgG1 hinge does not usually impact the Ag binding function of the resulting Fab and F(ab')₂. Thus, if specific proteolysis occurred to a cell-targeting Ab in vivo, the cell-bound fragment might well remain bound to its antigenic site to shield the cell from subsequent targeting Abs.

Anti-hinge autoantibodies could provide an additional layer of humoral immune protection to combat lost effector functions when endogenous Abs encounter localized, extracellular protease activity.

In summary, human circulation contains autoimmune reactivity directed to cryptic epitopes in the IgG1 hinge exposed by proteolytic enzymes; particularly by enzymes that are relevant to pathological conditions. The serum anti-hinge autoantibodies, in large part of IgG3 isotype, were shown to bind to cell-bound F(ab')₂ and to restore complement and ADCC cell killing functions in vitro to otherwise inactive F(ab')₂. We propose that anti-hinge autoantibodies could represent an immune countermeasure against the proteolytic inactivation of host IgGs.

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Disclosures

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associated with depletion of the natural generic anti-idiotypic (anti-Fab\(^\beta\)) system.


