Cleavage of IgGs by proteases associated with invasive diseases
An evasion tactic against host immunity?

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Introduction

Antibodies are integral components of the host immune response. The structure of the IgG isotype contains two antigen-binding Fab arms that are joined to a single Fc domain by the hinge region (Fig. 1). This unique structure allows antibodies to recognize antigen by the variable regions on the Fab arms and elicit immune effector functions through Fc domain interactions with Fcγ receptor-bearing immune cells.1 Fc receptor-engaged cells can then eliminate pathogenic micro-organisms or invasive cancer cells via antibody-dependent cellular cytotoxicity (ADCC) or antibody-dependent cellular phagocytosis (ADCP). Antibodies can also destroy pathogens or cancerous cells by complement-dependent cytotoxicity (CDC) whereby antibodies bound to the cell-surface initiate deposition and activation of early complement components leading to the formation of a membrane attack complex and subsequent lysis of the target cell.2 The advent of monoclonal antibody (mAb) therapeutics has provided a means to exploit these dynamic properties of antibodies by defining a target on cancerous cells, e.g., CD20 (rituximab), HER2 (trastuzumab) or EGFR (cetuximab), which can then in some cases recruit immune effector cells or complement to eliminate the targeted cell. Indeed, several clinical studies involving mAb cancer therapeutics have shown that patients who have higher affinity Fc receptor polymorphisms (H131 on FcγRIIa and V158 on FcγRIIIa) have a longer progression-free survival than patients bearing the lower affinity Fc receptor polymorphisms (R131 on FcγRIIa and F158 on FcγRIIIa) of those receptors.5,6 These findings indicate that, in vivo, at least one mechanism of action for anti-cancer therapeutic mAbs is recruitment and activation of innate immune effector cells or complement.

A key component for either endogenous antibodies or therapeutic mAbs to link antigen to immune effector cells is structural integrity of the hinge region. Although antibodies are generally regarded as highly resistant to proteolytic-mediated breakdown, our group and several others have shown that antibodies are in fact susceptible to proteolytic breakdown by multiple physiologically-relevant bacterial or mammalian proteases.6-8 Of particular interest is that proteolytic sensitivity has been mapped primarily to short stretches of amino acids within either the upper, or perhaps more importantly, within the lower hinge/CH2 region, a highly conserved stretch of amino acids critical for binding to the Fc family of receptors.9-21 Several of the proteases that are capable of cleaving IgGs within the lower hinge/CH2 are also associated with either pathogenic bacteria, e.g., glutamyl endopeptidase V8 (GluV8) of Staphylococcus aureus or immunoglobulin-degrading...
enzyme of Streptococcus pyogenes (IdeS), or invasive cancers (several matrix metalloproteinases). An unexpected finding was that even a single proteolytic cleavage of the lower hinge/CH2 could to render the cleaved antibody incapable of engaging FcγRs. These results suggest that proteolytic processing of the antibody hinge region could uncouple the ability of antibodies to link cell-surface antigen to immune effector cells.

In this review, we discuss several aspects connected with the proteolytic cleavage of the hinge region of both recombinant monoclonal and endogenous antibodies. We highlight the identification of proteases associated with pathogenic microorganisms or invasive cancers that are capable of cleaving antibodies. We also discuss the fact that several groups have identified a class of autoantibodies that bind specifically to proteolytic cleavage sites in the hinge, an observation consistent with generation and immune recognition of IgG cleavage epitopes in vivo. The idea that antibody cleavage may function as an immune evasion mechanism by rendering an antibody incapable of linking antigen to Fc receptors on immune effector cells is also considered. Finally, we evaluate the implications that antibody hinge cleavage has on mAb therapeutics for which a functional Fc domain is considered integral for efficacy.

**Proteolytic Cleavage of IgG in the Hinge Region**

The susceptibility of IgGs to proteolytic enzymes was exploited over 50 years ago for the generation of discrete fragments that greatly enabled the elucidation of immunoglobulin structure. Porter used papain to digest rabbit gamma globulin in the upper hinge to separate the two antigen-binding Fab fragments from the crystallizable Fc. Nisonoff et al. employed pepsin to cleave rabbit IgG in the lower hinge/CH2 region to generate the F(ab′)2 fragment. Those same highly potent enzymes found repeated applications for the production of specific fragments from polyclonal IgGs of various animal species and for the digestion of mAbs, e.g., mouse, rat, human. The utility of papain and pepsin derived from their selectivity for cleaving in the upper and lower hinge domains of IgGs, respectively. However, neither protease normally occurs, or is active within, human circulation or tissue. Accordingly, we and others have investigated a variety of human and bacterial extracellular proteases that in theory could encounter IgGs in pathological settings. Emphasis was placed on the human IgG1 isotype as the substrate since it (1) represents the predominant human IgG isotype, (2) possesses substantial effector functions, and (3) represents the major fraction of mAbs in therapeutic use. The IgG2 isotype has been reported to be more resistant to proteolysis by pepsin, although a systematic survey of physiologically-relevant proteases has not been published.

It should also be noted that the IgG hinge exhibits instabilities that are not related to proteolysis but may reflect a biophysical lability of this structural domain. Those include spontaneous peptide bond hydrolysis, attack by oxygen radicals and metal-ion catalyzed cleavages of the human IgG1 hinge under various in vitro storage conditions. These phenomena carry obvious importance for the development of therapeutic formulations of mAbs. This review is limited to enzymatic proteolytic pathways and does not extend to non-proteolytic mechanisms of IgG breakdown.

Although earlier crystallographic studies had difficulty in visualizing the lower hinge region due to flexibility, it has been possible to gain information on hinge conformation from the solved crystal structures of intact IgGs. All of the Fcγ family of receptors can interact with human IgG1, and these interactions perhaps function to further stabilize the lower hinge region allowing an assessment of amino acid contact points between FcRs and the Fc. Multiple points of interaction within the CH2 domain and FcγRs have been documented, but there is also a critical stretch in the lower hinge/CH2 region required for FcγR-binding ranging from E233-L234-L235-G236-G237-P238, (EU numbering). Although contact between each of these amino acids and FcRs have not been visualized directly by crystallography, mutational studies implicate a requirement for each member of this sequence. Sequences in the lower hinge/CH2 interface (in particular L234 and L235) and amino acids further down the CH2 region have also been implicated in C1q binding. Figure 1 presents a diagram of a representative IgG1 mAb and indicates the central position of the hinge between the Fab and Fc domains. The specific points where proteases cleave...
are depicted in the figure and several of these cleavage points fall within the critical stretch of E233-P238 (highlighted in red). It is not surprising that among the IgG isotypes that participate in Fcγ receptor-mediated effector pathways, there exists a strong sequence homology of the lower hinge of different IgG isotypes and among diverse animal species.

Until recently, few proteolytic enzymes were identified that exhibited the rapid fragmentation of IgGs catalyzed by papain or pepsin. This almost certainly contributed to the perception that IgGs are resistant to attack under physiological conditions. Nevertheless, a number of individual reports of IgG cleavage by alternative proteases accumulated over time. Of particular interest were those proteases known to be secreted or expressed by alternative proteases accumulated over time. Of particular interest were those proteases known to be secreted or expressed by alternative proteases accumulated over time. Of particular interest were those proteases known to be secreted or expressed by alternative proteases accumulated over time. Of particular interest were those proteases known to be secreted or expressed by alternative proteases accumulated over time.

| Protease | Source | Human IgG1 peptide bond specificity | Protease description |
|----------|--------|-------------------------------------|----------------------|
| Gelatinase A (MMP-2) | Human | Unknown but results in a single-cleaved IgG intermediate approximately 138 kDa | Matrix metalloproteinase, clan MA(M), family M10A |
| Stromelysin 1 (MMP-3) | Human | P232-E233 | Matrix metalloproteinase, clan MA(M), family M10A |
| Matrix (MMP-7) | Human | L234-L235 | Matrix metalloproteinase, clan MA(M), family M10A |
| Gelatinase B (MMP-9) | Human | Unknown but results in a single-cleaved IgG intermediate approximately 138 kDa | Matrix metalloproteinase, clan MA(M), family M10A |
| Macrophage metalloelastase (MMP-12) | Human | Unknown but results in a single-cleaved IgG intermediate approximately 138 kDa | Matrix metalloproteinase, clan MA(M), family M10A |
| Collagenase-3 (MMP-13) | Human | Unknown but results in a single-cleaved IgG intermediate approximately 138 kDa | Matrix metalloproteinase, clan MA(M), family M10A |
| Cathepsin G | Human | E233-L234 | Serine endopeptidase, clan PA(S), family S1A |
| Pseudolysin | Pseudomonas aeruginosa | Unknown | Matrix metalloproteinase, clan MA(E), family M4 |
| Mirabiliysin | Proteus mirabilis | Unknown | Matrix metalloproteinase, clan MA(M), family M10B |
| Glutamyl endopeptidase I (GluV8) | Staphylococcus aureus | E233-L234 | Serine endopeptidase, clan PA(S), family S1B |
| Immunoglobulin-degrading enzyme of Streptococcus (IdeS) | Streptococcus pyogenes | G236-G237 | Cysteine endopeptidase, family C |
| Streptopain (SpeB) | Streptococcus pyogenes | Cleavage occurs in the upper hinge | Cysteine endopeptidase, clan CA, family C10 |
| Trepolisin | Treponema denticola | Unknown | Serine endopeptidase, clan SB, family S8A |

Table 1. Mammalian and bacterial enzymes capable of cleaving human IgG1
the mixture on protein A/G matrices served to remove unbound trypsin or iodoacetamide for cysteine proteases, adsorption of which was often employed due to the rapid action of that protease. Otherwise, under these catalytic conditions, the time spans for conversion of IgG to the final $\text{F(ab')}_2$ product were often measured in hours for this group of proteases, compared to the faster rates commonly observed for papain and pepsin. The comparatively slower rates of cleavage of the lower hinge consistently revealed a step-wise sequence in which the respective heavy chains were clipped in separate and distinct events. In the case of several proteases, e.g., IdeS, MMP-3, the cleavage of the first chain was often significantly faster than the cleavage of the second. The mechanistic or conformational explanations for the disparate rates of cleavage of the same peptide sequence must await additional structural and kinetic analysis; however, it would seem likely that a loosening of structure or increased inter-domain flexibility would accompany the single cleavage and influence the proteolytic susceptibility of the second heavy chain. Despite the dangers in extrapolating from in vitro, solution-phase kinetics to physiological settings, the results suggested that the single-cleaved intermediate might be the favored IgG derivative to accumulate in protease-rich, pathological sites. As will be discussed, proteolysis of the one heavy chain in the lower hinge turned out to be unexpectedly disabling of the ADCC and CDC functions of the entire IgG. In contrast to the FcR binding regions mentioned above, residues responsible for binding to the neonatal Fc receptor (FcRn), which is involved in extending IgG half-life, are located between the CH2 and CH3 regions of the Fc. We had found that a single cleavage in the lower hinge/CH2 region did not affect FcRn binding or in vivo mAb half-life compared to intact IgG.

Cleavage of one IgG1 heavy chain lower hinge did not induce a detectable change in molecular mass under native or non-denaturing conditions. Size exclusion chromatographic methods yielded indistinguishable elution behaviors for intact and the single-cleaved derivative. The single-cleaved IgG derivatives of many mAbs were nevertheless amenable to purification from proteolytic digest that had progressed to full depletion of IgG, yet retaining substantial fractions of the single-cleaved derivative. After terminating proteolysis, e.g., with EDTA for metalloproteases or iodoacetamide for cysteine proteases, adsorption of the mixture on protein A/G matrices served to remove unbound $\text{F(ab')}_2$, and the bound single-cleaved IgG and Fc components were eluted and subsequently separated by size exclusion methods. The similarity of the biophysical characteristics of intact and single-cleaved IgGs supported the conclusion that the associated heavy chain Fc domains do not readily dissociate due to the strong non-covalent interactions in the CH3 domains.

Those observations led to predictions that the detection of single cleaved IgGs in vivo would be challenging since the majority of IgG detection systems, e.g., immuno-histochemistry and flow cytometry, commonly employ immune-detection of the Fc domain. This difficulty prompted us to generate specific antibody reagents for cleaved IgG detection. We reported a method to generate polyclonal rabbit anti-hinge antibodies using peptide analogs of the cleaved IgG1 hinge. Additionally, studies have shown that these antibodies only recognize cleaved IgGs, and not intact IgGs, both by western blot analysis and flow cytometry.

It was recognized that $\text{F(ab')}_2$ derivatives usually lose little, if any, antigen binding capability compared to the parent IgGs. As would be predicted, the single-cleaved IgG derivative of several mAbs also showed no diminution of antigen binding. In addition, proteolysis of IgG was shown to occur on cell surfaces to which a mAb was directed, and it was noted that a single-cleaved intermediate accumulated preferentially and was not displaced from the cell surface. Indeed, only limited data exist for the presence of IgG breakdown products in biological samples and those were primarily limited to severely inflammatory settings and focused on Fab and $\text{F(ab')}_2$ products. It can only be speculated whether localized secretion of IgG-degrading proteases represents a mechanism to quell the inherent inflammatory potential of antibodies. We and others have had our attention drawn to the group of proteases that are expressed by invasive and pathological cells, e.g., tumor cells and bacteria, although enzymes from non-pathological sources, e.g., neutrophil elastase and other human extracellular proteases, might also contribute to IgG breakdown. Additional investigations would be needed to further define the settings for normal, healthy protease-mediated degradation of IgGs. However, the presence of anti-hinge autoantibodies in normal healthy individuals is suggestive that such breakdown may be a normal occurrence.

**Autoantibodies Directed Against Cleaved IgG1 Hinge**

Since it has been demonstrated that IgGs can serve as substrates for multiple mammalian and bacterial proteases in vitro, the question arose as to whether or not IgGs are cleaved in vivo. Proteolysis of IgGs in vivo could potentially reveal cryptic epitopes within the cleaved IgG that would be hidden within the intact IgG. Exposure of these cryptic epitopes could result in activation of anti-hinge specific B cells, leading to the production of autoantibodies capable of binding to cleaved IgGs in individuals where IgG proteolysis had occurred. Autoreactive B cells are typically thought to be eliminated by three mechanisms—deletion, receptor editing or anergy, however, autoreactive B cells and the autoantibodies that they produce are often readily observed in otherwise healthy individuals. The three regions on Iggs to which autoantibodies could bind are the Fab regions, the Fc region, or the hinge region. The two best characterized anti-immunoglobulin autoantibody types are rheumatoid factor, which binds to the intact Fc domain, and anti-idiotypic autoantibodies, which bind to the variable regions. Over the years however, several groups have also identified autoantibodies directed...
against the hinge region of cleaved IgGs. These anti-hinge autoantibodies primarily bind to the exposed C-terminal residues of both Fab and F(ab’)_2 fragments.

The second therapeutic mAb approved for use in humans, abciximab, directed against GPIIb/IIIa expressed on platelets, is a proteolytically-generated Fab fragment. The parent antibody of abciximab is cleaved in the upper hinge region between amino acids H224 and T225 by the protease papain. Abciximab is a chimeric monoclonal Fab fragment comprising murine heavy and light chain variable regions, while the constant regions of the heavy and light chain are human. An early concern about administering the chimeric Fab fragment to human subjects was the induction of human anti-chimeric antibodies (HACA) against the foreign sequences present in the murine variable regions. Although some post-treatment antibody responses were detected against the murine variable regions, the most prevalent immune reactivity was that present prior to dosing and directed against an otherwise cryptic epitope exposed in the human C-terminal cleaved residue within the upper hinge region. This finding was unexpected given the fact that amino acid residues in the upper hinge region of IgG1 are present in all endogenous IgG1s. Further analysis revealed that the latter type of autoantibodies bind to the exposed C-terminus of the Fab and not to the intact IgG counterpart. Of particular interest was the timing of the anti-hinge response in one early clinical trial with patients receiving abciximab. The serum titer of anti-hinge autoantibodies in five of the human subjects increased over pre-administration titers shortly after treatment with abciximab. Immune responses to the murine variable regions in other subjects were not detected until 4–6 weeks after administration, much slower than the anti-hinge autoantibody responses. These results suggested that the rapid increase in titer to the exposed C-terminal hinge residue in abciximab was perhaps an anamnestic response, in contrast to the more likely de novo immune response to the murine variable regions. Although papain is a non-mammalian protease, and it would seem unlikely that individuals would be exposed to papain in vivo, there may exist functional equivalents in human tissues. In addition, several mammalian proteases, such as plasmin and human neutrophil elastase, cleave IgGs in the upper hinge region at amino acid residues adjacent to the papain cleavage site. It seems likely that in some cases where human subjects received abciximab, the patients exhibited anti-upper hinge autoantibodies that were capable of cross-reacting with the papain cleavage site, which led to a memory recall response that increased the subject anti-hinge autoantibody titers. Therefore, those subjects that had pre-existing anti-hinge autoantibodies were probably exposed to cleaved IgGs generated by endogenous proteases that had generated a neoepitope and elicited an immune response to the cleaved hinge.

A preclinical study in cynomolgus monkeys demonstrated the presence of anti-hinge autoantibodies directed to the cleaved lower hinge in animals treated with a humanized anti-GPIIa/IIIb monoclonal F(ab’)_2 fragment generated with pepsin. The presence of autoantibodies was encountered in this study because treatment of monkeys with the F(ab’)_2 fragment resulted in acute platelet clearance in several animals. The authors indicated that the IgG isotype autoantibodies were not anti-idiotypic antibodies, but instead appeared to be directed against the C-terminal end of F(ab’)_2, exposed by pepsin cleavage. The authors did not identify F(ab’)_2 fragments present in the monkeys, but concluded that at some point prior to the study, the monkeys had been exposed to such endogenously-generated fragments leading to an immune response, supporting the hypothesis that antibodies undergo cleavage within the lower hinge in vivo.

These human clinical trial analyses and monkey preclinical studies identified the presence of autoantibodies that were capable of cross-reacting with cryptic epitopes exposed by the proteases papain and papain (the papain cleavage site between L234 and L235 is the same cleavage site as human MMP-7). We recently conducted an in vitro study to characterize human anti-hinge autoantibodies capable of recognizing IgGs cleaved with proteases associated with highly inflammatory sites such as bacterial infections and invasive cancers. The study indicated the presence of autoantibodies that bound to human IgG1 Fab fragments generated with plasmin (human) and human neutrophil elastase, as well as human IgG1 F(ab’)_2 fragments generated with the proteases MMP-3 (human), MMP-12 (human), GluV8 (S. aureus), and IdeS (S. pyogenes). Importantly, there was minimal to no detection of autoantibodies to the intact IgG1 parent antibodies of the Fab and F(ab’)_2 fragments, indicating that sequences within the hinge region are only detectable by autoantibodies when exposed by proteolytic cleavage. The specific residues within the upper and lower hinge region where autoantibodies bound were further defined by using peptide analogues of the IgG1 hinge. Low reactivity was detected to peptides with C-terminal amino acid residues corresponding to the upper hinge, while no reactivity was detected to peptides truncated within the core hinge sequence, T225 through A231 (TCP CCPA). The highest reactivity was detected against peptides terminated at positions within the lower hinge/CH2 region encompassing P232 through F241 (PELLGGPSVF), containing the same stretch of amino acids that were previously described as critical for FcγR binding to IgGs. Therefore, this study confirmed the presence of anti-hinge autoantibodies that were selective for C-terminal positions as generated with physiologically relevant proteases.

Numerous additional studies over the years pointed to autoantibodies that bind to the C-terminal end of F(ab’)_2 fragments. Studies have correlated the titer of anti-hinge autoantibodies with pathological conditions such as cold agglutination, HIV, rheumatoid arthritis, and systemic lupus erythematosus. Others have suggested that natural anti-hinge autoantibodies bound to antigen-engaged F(ab’)_2 fragments serve to augment complement activity. One group speculated that anti-hinge autoantibodies can serve in an immunoregulatory role by inducing B cell apoptosis in antigen-engaged B cells by binding to the inhibitory receptor FcγRIIB. Our own work suggested that anti-hinge autoantibodies can provide a surrogate Fc domain to F(ab’)_2 fragments generated with physiologically relevant proteases and restore ADCC and CDC effector functions in vitro. Although the biology of anti-hinge autoantibodies has not fully been defined in vivo, their widespread presence supports the hypothesis that antibodies can be cleaved...
by physiologically relevant proteases in vivo in either the upper hinge or lower hinge/CH2 regions.

**IgG Cleavage as a Potential Immune Evasion Mechanism**

Both invasive microorganisms and cancerous cells employ multiple mechanisms to evade host immune responses. Most of the proteases discussed here are potentially present or expressed in the extracellular tissue milieu and around growing tumors or certain bacterial infections. Because of the loss-of-function associated with the displacement of the Fc region from the antigen binding Fab arms, several groups have suggested that IgG proteolysis could function as immune evasion mechanism.\(^{41,45,73,74}\)

However, a strikingly similar defense strategy was described more than 35 years ago for certain bacteria that inhabit the oral cavity and gastrointestinal tract.\(^{75}\) IgG does not normally cross from circulation into the GI tract and would not be expected to exert immunoregulatory functions there. However, the secretary IgA isotype readily enters that space and has been attributed with anti-infective functions against mucosal pathogens. Thus, when it was observed that a number of these pathogens expressed IgA-degrading proteases (without similar actions on IgG), it was postulated to be a potential defense strategy against host immune surveillance in the GI tract.\(^{41}\) The preferred sites of cleavage of secretary IgAs by various microbial proteases have been localized specifically to the hinge region.\(^{76}\) These investigators generated a number of positional variants and deletion mutants to define certain alterations that could confer protease resistance.\(^{77,78}\) The effects of such changes on IgA function with regard to pathogen removal were not specifically discussed. A related strategy to confer protease resistance to the IgG hinge would likely be hampered by overlap between the protease susceptibility sites and the highly conserved hinge sequences involved in Fcγ receptors and complement recognition.

Mechanistically, it was speculated that IgA proteases could facilitate the establishment of a localized zone of immunodeficiency around the microbe,\(^{42}\) and that Fab cleavage products, by retention of anti-microbial antigen binding capacity, could obstruct the access of intact IgAs to the bacterial surface.\(^{79}\) Proteolysis of IgA by microbes that colonize the mucosal compartment may be analogous to the proposed loss of IgG effector functions mediated by IgG-degrading proteases in tissues.

The potential implications for the effects of proteolysis of IgG in vivo on local immune surveillance were heightened when we noticed that the Fc-mediated effector functions of cell targeting mAbs were almost completely abolished following single cleavage of one lower hinge chain.\(^{3}\) MMP-3 and IdeS were employed most often for this demonstration, but other enzymes with selectivity for the lower hinge induced a similar loss of Fc function. The experimental system was replicated numerous times with different mAbs including those directed against a cellular cytokine, a canine platelet integrin receptor, tissue factor expressing human tumor cells, EpCAM on tumor cells, T cell markers on both human and murine cells, and epidermal growth factor bearing cells. In addition to the loss of ADCC and CDC activities in vitro in the presence of immune effector cells, single-cleaved mAbs vs. a canine platelet surface integrin receptor and CD4 on murine T cells also proved unable to mediate cell clearance in vivo.\(^{3}\) Thus, the limited cleavage of the single lower hinge domain, without any detachment of the Fc, rendered mAbs incapable of directing immune effector cell function, e.g., natural killer cells expressing FcγRIIIa, monocytes, neutrophils, macrophages. A parallel loss of complement-mediated cell killing accompanied the single proteolytic event was demonstrated with several mAbs.\(^{3}\) The underlying structural cause(s) for the loss of IgG Fc-mediated function were not completely clear, but seemed related to chain scission at positions disabling for FcγR binding or the induction of conformational changes to prevent effective recognition of the IgG by FcγR-bearing effector cells.

Several published reports document microbial proteolytic defenses against host immunity, in particular with S. pyogenes and S. aureus. S. pyogenes is highly pathogenic (it is the organism associated with necrotizing fasciitis) and expresses a particularly potent extracellular IgG-degrading proteases.\(^{8,74}\) The IdeS protease exhibits a high degree of specificity for cleavage in the lower hinge of IgG and is rapid in its action. Its characteristics against human IgGs suggested that it as a likely and important virulence factor promoting S. pyogenes pathogenicity. The Bjork group showed that IdeS-expressing S. pyogenes supernatants were capable of cleaving IgGs, and that bacteria opsonized with IdeS-cleaved human IgGs were resistant to phagocytosis by macrophages.\(^{75}\) In addition, other organisms that infect human tissue, such as S. aureus, also express proteases that induce the single cleavage of human IgGs in the lower hinge.\(^{7}\) Together, these examples support the conclusion that microbial proteolytic inactivation of host IgG, in particular, the limited cleavage described here that supports the conclusion that microbial proteolytic inactivation of host IgG, in particular, the limited cleavage described here that (1) difficult to detect; (2) disabling with regard to IgG effector function; and (3) blocks further IgG binding to the cellular target, could represent a subtle and unappreciated means by which invasive bacteria gain substantial advantage against host immune response.

We also speculate that this pathway of proteolytic defense against host immunity may have been mirrored by certain cancers. Many studies over the years have addressed the interplay between tumors and the immune system. Schreiber and colleagues have written a series of reviews detailing immune/tumor interactions and have named this interplay “cancer immuno-editing.” They describe three main phases, elimination, equilibrium and escape, that occur throughout tumor development.\(^{80}\) Early immune events mediate tumor elimination, the equilibrium stage occurs when tumor destruction is incomplete, and escape occurs when the tumor can evade or co-opt the immune system. Proteases have been associated with many aspects of tumor immunity, where in some cases proteolytic events contribute to tumor suppression, but they have also been documented to contribute to tumor invasiveness, as well as tumor immune evasion.\(^{81}\) Several studies have recently characterized how the metalloproteinasises ADAM10 and ADAM17 can enhance immune evasion of tumor cells.\(^{82,83}\) Genotypic or cellular stress on tumor cells induces ligands of the NKG2D (natural killer group 2, member D) receptors that can activate NK cells and function as co-stimulators to T cells. The
activated immune cells will then eliminate tumor cells bearing NKG2D ligands. ADAM10 and ADAM17 can shed the membrane NKG2D ligand called MICA from the surface of tumor cells, which results in NKG2D receptor internalization and degradation, ultimately allowing the tumor cells to evade the cellular immune response. Although these studies did not specifically address the ability of these proteases to cleave IgGs bound to tumor cells, they do point to the ability of metalloproteinases to actively cleave substrates on the surface of cancerous cells within the tumor microenvironment.

Since our group and others have documented that several proteases associated with cancer, in particular the matrix metalloproteinases, have the ability to cleave IgGs,\textsuperscript{6-7,9,49} we speculate that antibody cleavage could be another method of tumor immune evasion. In order for IgG proteolysis to function as an immune evasion mechanism, anti-tumor IgGs must be capable of binding surface antigens present on the tumor cells. Several studies have documented specific anti-tumor IgGs directed against autologous host tumor cells,\textsuperscript{84,85} yet the role of endogenous tumor reactive antibodies and their subsequent activation of innate immune cells by Fc/FcR interactions remains a question.\textsuperscript{80} However, the observation that genetic polymorphisms in FcγRIIa and FcγRIIIa correlate with the clinical outcome of patients with B-cell lymphoma (rituximab),\textsuperscript{6,86} colorectal cancer (cetuximab)\textsuperscript{3,87} and breast cancer (trastuzumab)\textsuperscript{5} implicate a role for antibody Fc/FcγR interactions in cancer immune surveillance, at least with anti-cancer mAb therapeutics. A more recent study indicated that anti-cancer mAb therapies can function to augment endogenous antibody responses to tumor surface antigens, in particular the antigen HER2.\textsuperscript{84} Our in vitro studies have shown that the predominant form of cleaved IgG most likely present on the surface of the tumor cell would be the single-cleaved intermediate.\textsuperscript{7} Due to the difficulty of identifying the single-cleaved intermediate under native conditions, the presence of the single heavy chain cleavage would most likely go undetected; however, we have identified the presence of single-cleaved IgGs in breast cancer tumor extracts.\textsuperscript{9} We note that, to date, no published reports exist that document in vivo cleavage of therapeutic mAbs in patients. A series of studies have shown that tumor-bearing individuals had blocking antibodies that correlated with tumor immune evasion.\textsuperscript{89-91} It is interesting to speculate that these blocking antibodies might in part be cleaved IgGs that functioned to mask tumor-associated antigens and were unable to elicit Fc-mediated immune responses. Clearly, additional studies will need to be undertaken to address the potential of IgG cleavage as an immune evasion mechanism.

**Potential Implication of Hinge Cleavage for Antibody Therapeutics**

We have focused on endogenous IgGs and aspects of host immunity for which susceptibility to proteolysis might disable immune functions in pathological settings; however, within this paradigm, there is an obvious connection to numerous mAb therapies, particularly those directed against cell surface targets on invasive tumors and bacterial infections. It was recently estimated that as many as 25 mAbs have been approved worldwide and 26 mAbs are in late-stage clinical trials, 13 of which are for treatment of cancer.\textsuperscript{92} These agents have shown varying degrees of clinical success and it has been pointed out that further explorations into improving clinical outcomes are warranted.\textsuperscript{93}

As discussed above, tumor cells and bacteria can generate proteolytic extracellular environments and express enzymes that cleave IgGs. A number of tumor- and bacterial-expressed proteases disable IgGs by the subtle single-cleavage route that we have described. So far, we can only conjecture that mAb therapies may be less effective than expected within the hostile proteolytic environments into which they are directed. The proteolytic pathways of IgG inactivation that we have observed are subtle, difficult-to-detect and potentially block subsequent immune surveillance. To be sure, there are caveats that apply to the early stage research findings and implications that we have suggested.

**Conclusions**

Accumulating in vitro evidence implicates human IgG1 as a substrate for physiologically-relevant proteases associated with pathological microorganisms and invasive cancers. Additionally, the widespread presence of autoantibodies that recognize cleavage sites with C-terminal exposed residues in the hinge region suggests that such immune epitopes in cleaved IgG1 are routinely generated in vivo. In vitro and in vivo studies have shown that the cleavage of IgG1, even a single proteolytic cleavage in one lower hinge heavy chain, can render IgG1 inert in terms of Fc-mediated effector functions associated with target cell elimination. Cumulatively, these observations suggest that proteolytic cleavage within the hinge region of IgG1 can help influence the immune evasion capacity of virulent microorganisms and invasive cancers and tip the balance from immune containment to pathology.

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References

1. Nimmerjan F, Raven J, Fc receptors. Gamma receptors as regulators of immune responses. Nat Rev Immunol 2002; 2:34–47.

2. Carter P. Improving the efficacy of antibody-based cancer therapies. Nat Rev Cancer 2001; 1:118-29.

3. Bibeau F, Lopez-Crapez E, Di Fiore F, Thezenas S, Ychou M, Blanchard F, et al. Impact of FcgammaRIIa-FcgammaRIIIa polymorphisms and KRAS mutations on the clinical outcome of patients with metastatic colorectal cancer treated with cetuximab plus irinotecan. J Clin Oncol 2009; 27:1122-9.

4. Cartron G, Dacheux L, Salles G, Solal-Celigny P, Bardos P, Colombat P, et al. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcgammaRIIIa gene. Blood 2002; 99:754-8.

5. Musolino A, Naldi N, Bortesi B, Pezzuolo D, Capelli M, Missale G, et al. Immunoglobulin G Fragments C receptor polymorphisms and clinical efficacy of trastuzumab-based therapy in patients with HER-2 neu-positive metastatic breast cancer. J Clin Oncol 2006; 24:1789-96.

6. Gearing AJH, Torpe J, Miller K, Mangan M, Varley PG, Dudgeon T, et al. Selective cleavage of human IgG by matrix metalloproteinases, matrixin and stromelysin. Immunol Lett 2002; 81:41-8.

7. Ryan MH, Petrone D, Nemeth JF, Barthan E, Bjork J, Jordan RE. Proteolysis of purified IgGs by human and bacterial enzymes in vitro and the detection of specific proteolytic fragments of endogenous IgG in rheumatoid synovial fluid. Mol Immunol 2008; 45:1837-46.

8. Vincents B, von Pawel-Rammingen U, Björck L, Jordan RE. Proteolysis of purified IgGs by human and bacterial enzymes in vitro and the detection of specific proteolytic fragments of endogenous IgG in rheumatoid synovial fluid. Mol Immunol 2008; 45:1837-46.

9. Brezski RJ, Vafa O, Petrone D, Tam SH, Powers G, et al. Antibody therapeutics: isotype and glyco modification of specific proteolytic fragments of endogenous IgG in rheumatoid synovial fluid. Mol Immunol 2008; 45:1837-46.

10. Gearing AJ, Torpe J, Miller K, Mangan M, Varley PG, Dudgeon T, et al. Selective cleavage of human IgG by matrix metalloproteinases, matrixin and stromelysin. Immunol Lett 2002; 81:41-8.

11. Ryan MH, Petrone D, Nemeth JF, Barthan E, Bjork J, Jordan RE. Proteolysis of purified IgGs by human and bacterial enzymes in vitro and the detection of specific proteolytic fragments of endogenous IgG in rheumatoid synovial fluid. Mol Immunol 2008; 45:1837-46.

12. Vincents B, von Pawel-Rammingen U, Björck L, Jordan RE. Proteolysis of purified IgGs by human and bacterial enzymes in vitro and the detection of specific proteolytic fragments of endogenous IgG in rheumatoid synovial fluid. Mol Immunol 2008; 45:1837-46.

13. Bibeau F, Lopez-Crapez E, Di Fiore F, Thezenas S, Ychou M, Blanchard F, et al. Impact of FcgammaRIIa-FcgammaRIIIa polymorphisms and KRAS mutations on the clinical outcome of patients with metastatic colorectal cancer treated with cetuximab plus irinotecan. J Clin Oncol 2009; 27:1122-9.

14. Cartron G, Dacheux L, Salles G, Solal-Celigny P, Bardos P, Colombat P, et al. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcgammaRIIIa gene. Blood 2002; 99:754-8.

15. Musolino A, Naldi N, Bortesi B, Pezzuolo D, Capelli M, Missale G, et al. Immunoglobulin G Fragments C receptor polymorphisms and clinical efficacy of trastuzumab-based therapy in patients with HER-2 neu-positive metastatic breast cancer. J Clin Oncol 2006; 24:1789-96.

16. Gearing AJH, Torpe J, Miller K, Mangan M, Varley PG, Dudgeon T, et al. Selective cleavage of human IgG by matrix metalloproteinases, matrixin and stromelysin. Immunol Lett 2002; 81:41-8.

17. Ryan MH, Petrone D, Nemeth JF, Barthan E, Bjork J, Jordan RE. Proteolysis of purified IgGs by human and bacterial enzymes in vitro and the detection of specific proteolytic fragments of endogenous IgG in rheumatoid synovial fluid. Mol Immunol 2008; 45:1837-46.

18. Bibeau F, Lopez-Crapez E, Di Fiore F, Thezenas S, Ychou M, Blanchard F, et al. Impact of FcgammaRIIa-FcgammaRIIIa polymorphisms and KRAS mutations on the clinical outcome of patients with metastatic colorectal cancer treated with cetuximab plus irinotecan. J Clin Oncol 2009; 27:1122-9.

19. Cartron G, Dacheux L, Salles G, Solal-Celigny P, Bardos P, Colombat P, et al. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcgammaRIIIa gene. Blood 2002; 99:754-8.
61. Norvell A, Mandik L, Monroe JG. Engagement of the antigen-receptor on immature murine B lymphocytes results in death by apoptosis. J Immunol 1995; 154:4404-13.

62. Radic MZ, Erikson J, Liwin S, Weigert M. B lymphocytes may escape tolerance by revising their antigen receptors. J Exp Med 1993; 177:1165-75.

63. Tiller T, Tsuji M, Yurasov S, Velinzon K, Nussenzenwg MC, Wardemann H. Autoreactivity in human IgG memory B cells. Immunity 2007; 26:205-13.

64. Wardemann H, Yurasov S, Schueller A, Young JW, Meffre E, Nussenzenwg MC. Predominant autoantibody production by early human B cell precursors. Science 2003; 301:1374-7.

65. Knight DM, Wagner C, Jordan R, McAleer MF, Wardemann H, Yurasov S, Schaefer A, Young JW, Tiller T, Tsuiji M, Yurasov S, Velinzon K, Nussenzweig MR. Natural generic anti-idiotype (anti-F(ab')2) system. J Exp Med 1993; 177:1165-73.

66. Terness P, Navolan D, Dufier C, Welschof M, Opelz G. Immunossuppressive anti-immunoglobulin autoantibodies: specificity, gene structure and function in health and disease. Cell Mol Biol 2002; 48:271-8.

67. Yano S, Kaku S, Suzuki K, Terazaki C, Sakayori T, Kawakami T, et al. Natural antibodies against the immunoglobulin F(ab')2 fragment cause the elimination of antigens recognized by the F(ab')2, from the circulation. Eur J Immunol 1995; 25:3128-33.

68. Perselijn J, Stevens RH. Anti-Fab antibodies in humans. Predominance of minor immunoglobulin G subclasses in rheumatoid arthritis. J Clin Invest 1985; 76:723-30.

69. Susal C, Oberg HH, Daniel V, Dorr C, Terness P, Huth-Kuhne A, et al. Isotypes and IgG subclasses of anti-Fab antibodies in human immunodeficiency virus-infected hemophilia patients. Vox Sang 1994; 66:37-43.

70. Terness P, Kirschfink M, Navolan D, Dufier C, Kohl I, Opelz G, et al. Striking inverse correlation between IgG and anti-F(ab')2, and autoantibody production in patients with cold agglutination. Blood 1995; 85:548-51.

71. Williams RC Jr, Malone CC, Huffman GR, Silvestris F, Croker BP, Ayoub EM, Maisengill S. Active systemic lupus erythematosus is associated with depletion of the natural generic anti-idiotpe (anti-F(ab')2) system. J Rheumatol 1995; 22:1075-85.

72. Fumia S, Goede JS, Fischler M, Lugninhahl A, Frick S, Fodor P, et al. Human F(ab')2-containing immune complexes together with anti-hinge natural antibodies stimulate complement amplification in vitro and in vivo. Mol Immunol 2008; 45:2951-61.

73. Collin M, Olofsson A. Effect of Spelld and Endosomal Stresses on rearrangements of human immunoglobulins. Infection Immun 2001; 69:7187-9.

74. von Pawel-Rammingen U, Johansson BP, Bjorkl B, Ide, A novel streptococcal cytoxin protease with unique specificity for immunoglobulin G. EMBO J 2002; 21:1607-15.

75. Mehta SK, Plau AG, Calvacano NJ, Tomasi TB Jr. Human immunoglobulin A: Production of an Fc fragment by an enteric microbial proteolytic enzyme. J Immunol 1973; 111:1274-6.

76. Senior BW, Woolf JM. Effect of mutations in the human immunoglobulin A1 (IgA1) hinge on its susceptibility to cleavage by diverse bacterial IgA1 proteases. Infect Immun 2005; 73:1515-22.

77. Senior BW, Woolf JM. The influences of hinge length and composition on the susceptibility of human IgA to cleavage by diverse bacterial IgA1 proteases. J Immunol 2005; 174:7792-9.

78. Senior BW, Woolf JM. Sites in the CH3 domain of human IgA1 that influence sensitivity to bacterial IgA1 proteases. J Immunol 2006; 177:3913-9.

79. Hajishengallis G, Nikolova E, Russell MW. Inhibition of the antigen-receptor on immature murine B lymphocytes results in death by apoptosis. Eur J Immunol 1995; 25:3128-33.

80. Caroll TE, Takahashi T, Resnick LA, Oettgen HF, Old LJ. Cell surface antigens of human malignant melanoma: mixed hemadsorption assays for humoral immunity to cultured autologous melanoma cells. Proc Natl Acad Sci USA 1976; 73:3278-82.

81. Sahin U, Tureci O, Schmitt H, Kochlowski B, Johannes T, Schmitz R, et al. Human neoplasms elicit multiple specific immune responses in the autologus host. Proc Natl Acad Sci USA 1995; 92:11810-3.

82. West JK, Levy R. Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. J Clin Oncol 2003; 21:3940-7.

83. Zhang W, Gordon M, Schultheis AM, Yang DY, Nagashima F, Azuma M, et al. FCGR2A and FCGR3A polymorphisms associated with clinical outcome of epidermal growth factor receptor expressing metastatic colorectal cancer patients treated with single-agent cetuximab. J Clin Oncol 2007; 25:3712-8.

84. Taylor C, Hershman D, Shah N, Suciu-Foca N, Petrylak DP, Taub R, et al. Augmented HER-2-specific immunity during treatment with trastuzumab and chemotherapy. Clin Cancer Res 2007; 13:5133-43.

85. Sjo gren HO, Hellstrom I, Bansal SC, Hellstrom KE. Suggestive evidence that the “blocking antibodies” of tumor-bearing individuals may be antigen—antibody complexes. Proc Natl Acad Sci USA 1973; 68:1572-5.

86. Tamerius J, Hellstrom I, Hellstrom KE. Evidence that blocking factors in the sera of multiparous mice are associated with immunoglobulins. Int J Cancer 1975; 16:456-64.

87. Tamerius J, Nepom J, Hellstrom I, Hellstrom KE. Tumor-associated blocking factors: isolation from sera of tumor-bearing mice. J Immunol 1976; 116:274-30.

88. Reichert JM, Antibodies to watch in 2010. Mabs 2010; 2:84-100.

89. Strobl WR. Optimization of Fc-mediated effector functions of monoclonal antibodies. Curr Opin Biotechnol 2009; 20:685-91.