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EVIDENCE THAT THE HINGE REGION PLAYS A ROLE IN MAINTAINING SERUM LEVELS OF THE MURINE IgG1 MOLECULE

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Abstract—The site of the murine IgG1 molecule that regulates catabolism has recently been shown to encompass amino acids that are located at the CH2–CH3 domain interface. The CH2 and CH3 domains are connected to each other by a relatively flexible "mini-hinge" region, and flexibility in this region could clearly affect the orientation of the domains with respect to each other. The internal movement of the CH2 domain depends on the absence/presence of the hinge disulphide. The increased mobility of the CH2 domain relative to the CH3 domain in a hingeless IgG or Fc fragment may result in a conformational change at the CH2–CH3 domain interface and alter the accessibility of the residues that are involved in catabolism control. To investigate this possibility, four Fc fragments which differ in the presence/absence of hinge disulphides and hinge sequences have been analysed in both *in vivo* pharmacokinetic studies and *in vitro* by limited proteolysis with pepsin. The data show that the presence of hinge disulphide(s) in the Fc fragment results in a longer intravascular half life but a higher susceptibility to pepsin attack. This, taken together with the knowledge that pepsin cleaves close to the CH2–CH3 domain interface, suggests that the longer half life of disulphide linked Fc fragments relative to unlinked fragments may be due to conformational differences in this region of the IgG molecule, and these conformational changes may affect the accessibility of the catabolic site for binding to putative protective Fc receptors.

Key words: IgG catabolism, recombinant Fc fragment, hinge region, limited proteolysis, conformational change.

INTRODUCTION

The early work of Spiegelberg and Weigle demonstrated that the Fc region of an IgG molecule is important for its serum persistence (Spiegelberg and Weigle, 1965). More recently, site-directed mutagenesis of a recombinant Fc-hinge fragment has been used to demonstrate that the site of the murine IgG1 molecule controlling catabolism encompasses amino acids located at the CH2–CH3 domain interface (Kim *et al.*, 1994*a*,*b*). This site overlaps with binding sites for Staphylococcal protein A (SpA; Deisenhofer, 1981; Kato *et al.*, 1993), the neonatal intestinal transfer receptor (FcRn; Raghavan *et al.*, 1994; Kim *et al.*, 1994*c*) and rheumatoid factors (Stone *et al.*, 1989; Bonagura *et al.*, 1992). The CH2–CH3 domain interface consists of two hydrophobic patches with a solvent accessible surface area of 778 A^2 (Deisenhofer, 1981; Burton, 1985; Padlan, 1990). The domains are linked to each other by a "mini-hinge" region that is relatively flexible and susceptible to enzymatic attack by several proteases (Turner and Bennich, 1968; Connell and Porter, 1971; Stewart *et al.*, 1973). Therefore, in addition to the flexibility of the hinge region which may be involved in regulating effector functions (Oi *et al.*, 1984; Dangl *et al.*, 1989), the Fc region has a second region of flexibility at the CH2–CH3 domain interface. Consistent with this, selective reduction of hinge disulphides has been observed to affect the spatial relationship and mobility of the CH2 domain relative to the CH3 domain (Romans *et al.*, 1977; Chan and Cathou, 1977; Seegan *et al.*, 1979). This increased mobility of the CH2 domain could conceivably affect the accessibility of the CH2–CH3 domain interface, which in turn might affect the rate of catabolism of the Fc fragment (or IgG molecule).

Our observation that a recombinant Fc-hinge fragment has a longer half life than an Fc fragment without hinge (produced by papain digestion of a murine IgG1 antibody) prompted us to further investigate the role of the hinge region in the control of IgG catabolism. In this report, the pharmacokinetics of four Fc fragments which differ in either hinge sequence or the absence/presence of disulphide bridge(s) in the hinge region have been analysed. The enzyme pepsin, which is known to cleave at the CH2–CH3 domain interface (Turner and Bennich, 1968; Bennich *et al.*, 1974) has also been used as a probe to determine the relative accessibility of the susceptible residues in this region of each of the Fc fragments. The data indicate that the IgG hinge plays an indirect role in

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Abbreviations: IgG, immunoglobulin G; SpA, Staphylococcal protein A; TCA, trichloroacetic acid.

the control of catabolism by modulating the conformation of the CH2–CH3 domain interface. This observation has implications in the design of experiments for the engineering of therapeutic proteins with the aim of increasing their *in vivo* half lives.

MATERIALS AND METHODS

Bacterial strains and hybridomas

E. coli BMH71-18 (Rüther *et al.*, 1981) was used as expression host. The murine RFB4 antibody (Campana *et al.*, 1985) was used as a source of glycosylated IgG1.

Construction of plasmids for the expression of Fc fragments

A plasmid for the expression of the Fc-hinge fragment derived from the murine IgG1 molecule has been described previously (Kim *et al.*, 1994*a*). To construct plasmids for the expression of the Fc without hinge (designated Fc) and Fc with synthetic hinge (Fc-cys), a similar procedure to that used for the Fc-hinge fragment was used (Kim *et al.*, 1994*a*). The 5′ oligonucleotides used in the PCR differed from those used for the construction of the Fc-hinge encoding fragment and were as follows: Fc 5′ primer: 5′ ATC **ACC ATG** GGC *CGA AGT ATC ATC TGT CTT CAT C* 3′ (*Nco*I site indicated in bold and codons of 5′ end of CH2 domain shown in italics); Fc-cys 5′ primer: 5′ ATC **ACC ATG** GCC *GGC TGC GGA GGT GCA TCA* GAA GTA TCA TCT GTC TTC ATC 3′ (*Nco*I site indicated in bold and codons of synthetic hinge shown in italics).

The 3′ oligonucleotide has been described previously (CH3BstFor; Kim *et al.*, 1994*a*). The synthetic hinge in Fc-cys has the amino acid sequence gly-cys-gly-gly-ala-ser. All plasmid constructions were sequenced using the dideoxynucleotide method (Sanger *et al.*, 1977) and Sequenase (USB Biochemicals, Cleveland, OH) prior to expression analysis. Recombinant proteins were purified as described previously (Kim *et al.*, 1994*a*) using Ni^{2+}-NTA-agarose followed by SpA-Sepharose. Bound Fc-hinge, Fc or Fc-cys fragments were eluted from the SpA-Sepharose using 0.1 M glycine pH 3.0 and 0.145 M NaCl. Eluates were immediately neutralized by addition of 1/10 volume of 1 M Tris–HCl pH 8.0 and dialysed into phosphate buffered saline.

Preparation of a glycosylated Fc fragment by papain digestion

RFB4 antibody was digested with papain at pH 7 in the presence of 10 mM cysteine and 5 mM Na_2EDTA at 37°C for 5 hr prior to separation using DEAE Sepharose (Pharmacia, Uppsala, Sweden) followed by SpA-Sepharose (Goding, 1983).

Radiolabelling of the Fc fragments

Proteins were radiolabelled with $Na^{125}I$ (Amersham, Arlington Heights, IL) using the Iodo-Gen reagent (Fraker and Speck, 1978) as described previously (Kim *et al.*, 1994*a*). Free iodine was removed by gel filtration using Sephadex G-25M and the final volume of each protein adjusted to 0.5 ml. Radiolabelled proteins (10^6–10^7 cpm/μg) contained less than 5% free iodine.

Pharmacokinetic studies

Pharmacokinetic analyses were carried out using previously described methods (Kim *et al.*, 1994*a*). Data analysis was carried out using a non-compartmental model (Perrier and Mayerson, 1982) using the PKCAL program (Schumaker, 1986). The half lives were calculated using 0–24 hr (α-phase) and 24–96 hr (β-phase) intervals of time following injection. For the clearance curves, average values of percentage of radioactivity remaining for all mice within each group are shown.

High performance liquid chromatography (HPLC)

The molecular weights of the radiolabelled Fc (with and without hinge) fragments (10^6 cpm/μg) were determined using HPLC and a SEC-250 (Bio-Rad, Melville, NY) column in 0.1 M sodium phosphate buffer pH 6.8. The radiolabelled fragments were added to a mixture of protein standards containing bovine serum albumin (68 kDa), ovalbumin (46 kDa), trypsin inhibitor (20 kDa) and cytochrome c (14 kDa) and the molecular weight of the radiolabelled Fc fragment determined from its retention time relative to the standards.

Limited proteolysis of Fc fragments

Radiolabelled proteins (10^4 cpm/μg) were incubated at a concentration of 1 mg/ml in 0.1 M sodium acetate buffer (pH 3.9) with 5 μg/ml pepsin (Sigma, St Louis, MO) at 37°C. Aliquots of the digestions were withdrawn at 10, 20, 40, 80 and 160 min after addition of pepsin. Immediately after removal of the aliquot, the pH was adjusted to pH 8 by addition of Tris–HCl buffer (pH 8) to a final concentration of 0.2 M and frozen at −20°C prior to further analysis. The digestions were analysed using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), and to analyse the release of fragments that were non-precipitable by trichloroacetic acid (TCA) the following procedure was used: 10 μl of the digest was added to 250 μl BSA (1 mg/ml in phosphate buffered saline, pH 7.4) and TCA added to a final concentration of 10% v/v. Following incubation for 10 min on ice, the precipitate was separated from the soluble material by centrifugation and the radioactivity present in soluble and precipitated fractions determined by gamma counting. Kinetics of digestions were expressed as the percentage of radioactivity in the soluble fraction as a function of time. The percentage of radioactivity released for each time point (Fig. 3) is the average of three experiments.

SDS–PAGE

Fc fragments were analysed using 15% gels run under reducing (5% 2-mercaptoethanol) and non-reducing conditions. Gels were stained with Coomassie brilliant blue (R250, Sigma). To determine the percentage of undigested Fc at different time points, gels were

Table 1. Fc derivatives used in this study

Fc fragment	Sequence of hinge region [a]	Molecular mass (kDa) Phosphate buffer (HPLC)	SDS–PAGE	SDS–PAGE + 2-mercaptoethanol
Recombinant Fc with hinge (Fc-hinge)	VRPDCGCKPCI	55	50–55	30
Recombinant Fc without hinge (Fc)	No hinge	55	30	30
Recombinant Fc with synthetic hinge (Fc-cys)	GCGGAS	55	50–55	30
Fc obtained by papain digestion (Fc-pap)	No hinge	55	30	30

[a] In one letter amino acid code.

autoradiographed and scanned using a laser densitometer (Ultroscan XL, Pharmacia).

RESULTS

Radiolabelled proteins (Fc-hinge, Fc, Fc-cys and Fc-pap; see Table 1) were analysed using SEC-250 columns in the presence of size standards (Materials and Methods). All Fc fragments had molecular weights of about 55 kDa as previously reported for the recombinant Fc-hinge fragment (Kim *et al.*, 1994*a*). Consistent with the Fc-hinge and Fc-cys fragments being disulphide linked homodimers, these two proteins migrated as 50–55 kDa proteins when analysed using SDS–PAGE under non-reducing conditions, and as 30 kDa proteins when reduced (Fig. 1). In contrast, Fc and Fc-pap migrated as 30 kDa proteins under both reducing and non-reducing conditions (Fig. 1), indicating that the dimers observed using SEC-250 columns are non-covalently linked. The glycosylated Fc produced by papain digestion (Fc-pap) of the antibody RFB4 does not have an interheavy chain disulphide bridge, as the papain has cleaved the heavy chain at a position C-terminal to the hinge. For reasons that are not clear, attempts to produce an Fc-pap fragment with the hinge region failed. The data concerning the presence/absence of the hinge region and the molecular size of the four fragments used in this study are summarized in Table 1.

Pharmacokinetics of the Fc fragments

The clearance curves of the Fc fragments (Fig. 2) are biphasic with short α phases (representing equilibration between the intra- and extravascular space) and longer β phases (representing elimination of the equilibrated protein from the intravascular space). For comparative purposes, the clearance curve of the glycosylated murine IgG1 molecule (RFB4; Campana *et al.*, 1985) is also shown. From these data it is clear that the Fc-hinge and Fc-cys have similar β phase half lives to both each other and murine IgG1, whereas the half lives of Fc-pap and Fc are significantly shorter (Table 2). The values for mean residence time are consistent with the calculated values for the β phase half lives. Thus, the fragments can be placed into two groups: one containing Fc fragments that are covalently linked as a homodimer by at least one intermolecular disulphide bridge, and a second group containing non-covalently linked homodimers. The Fc fragments in the second group have a shorter β phase half life than those in the first. This suggests that the conformation of the CH2–CH3 domain interface which encompasses the "catabolic site" (Kim *et al.*, 1994*a*) is dependent on the presence of a disulphide bridge in the hinge region.

Proteolytic digestion

To assess possible differences in conformation of the Fc fragments in the two groups, the rates of digestion by pepsin has been assessed, as susceptibility to cleavage has been proposed to reflect an "open" conformation that is accessible to solvent and protease attack (Tao and Morrison, 1989). Furthermore, the Glu333–Lys334

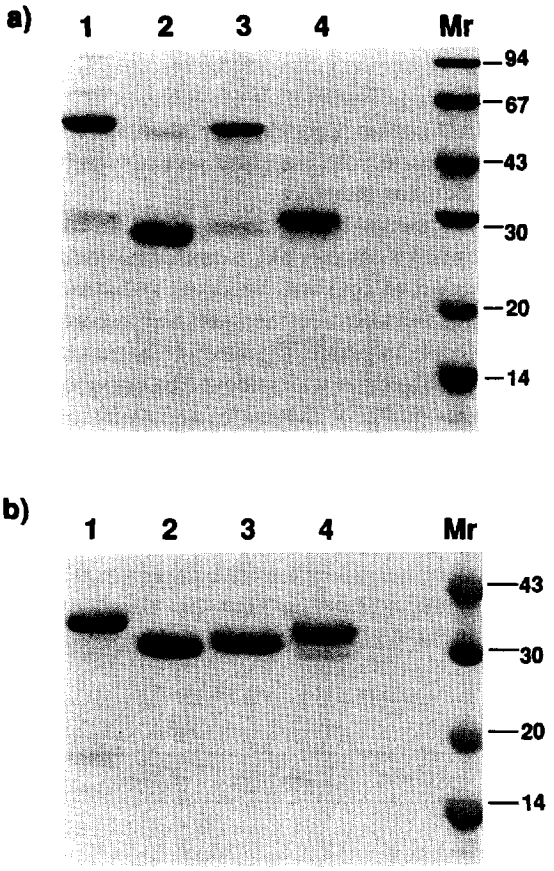


Fig. 1. 15% SDS–PAGE analysis of Fc fragments. (a) Lane 1, Fc-hinge; lane 2, Fc; lane 3, Fc-cys and lane 4, Fc-pap, run under non-reducing conditions. (b) Lanes 1–5, same as for (a) but run under reducing conditions. For both (a) and (b), M_r = molecular weight standards with sizes in kDa on the right margin.

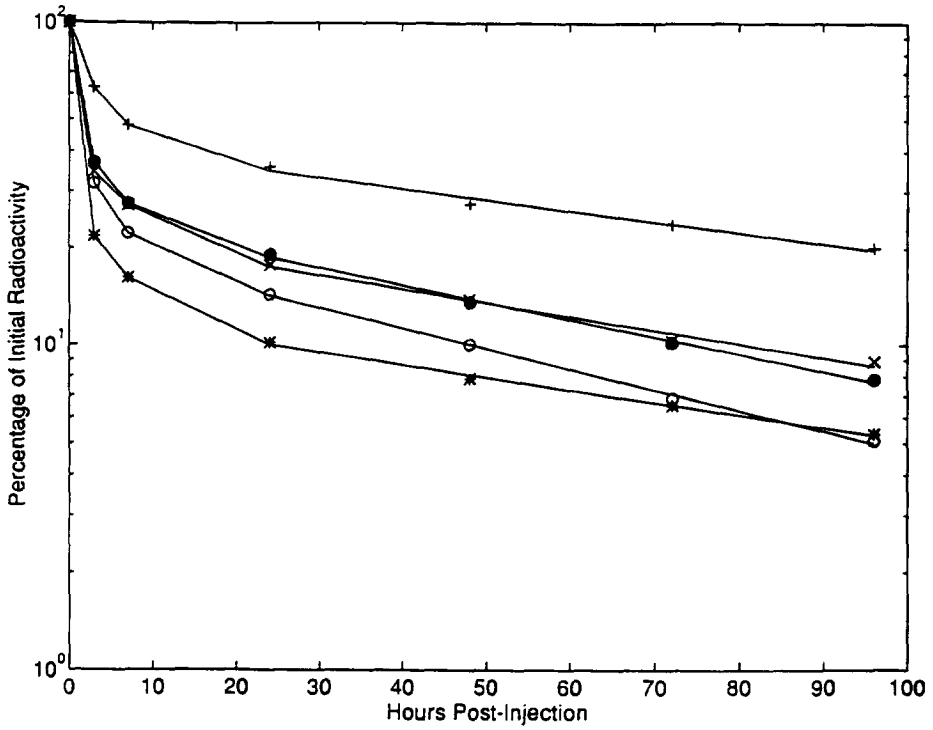


Fig. 2. Clearance curves of the Fc fragments. +; murine IgG1; *, Fc-hinge; ○, Fc; ×, Fc-cys; ●, Fc-pap.

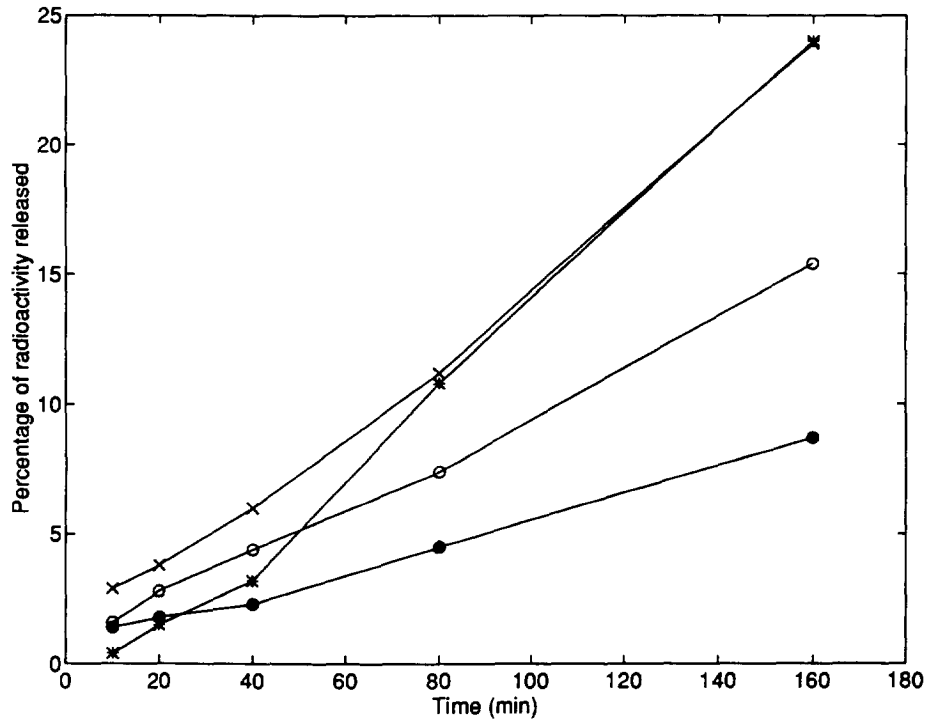
peptide bond near the CH2–CH3 domain interface (the "mini-hinge" region) is known to be susceptible to pepsin attack (Turner and Bennich, 1968; Bennich *et al.*, 1974), indicating that pepsin is an appropriate reagent to probe possible changes in conformation in the vicinity of the CH2–CH3 domain interface.

The Fc fragments were subjected to limited pepsin digestion at pH 3.9. At this pH, a conformational change in the Fc region of IgG was reported (Abaturov *et al.*, 1969), and this modification increases the susceptibility of the IgG molecule to papain digestion (Ghetie and Buzila, 1972). Limited digestion was achieved by using low pepsin concentrations (0.5% of substrate) and short incubation times. The kinetics of digestion of each fragment was determined by measuring the amount of released material that was non-precipitable by TCA as a function of time (Fig. 3a). From these data, it is clear that the Fc-hinge and Fc-cys fragments are more sensitive to pepsin attack than Fc and Fc-pap, indicating that by constraining the N-termini of the CH2 domains by one or more -S—S- bridges, the Fc fragment is more susceptible to pepsin digestion. The sequence of the hinge itself plays no role in this enhanced susceptibility since a synthetic hinge (sequence: gly-cys-gly-gly-ala-ser) can be used to replace the "natural" IgG1 hinge to produce an Fc with indistinguishable properties. The decrease in the percentage of non-digested Fc fragment as a function of time follows the same pattern as the release of TCA non-precipitable material (Fig. 3b). This again indicates that Fc-hinge and Fc-cys are more susceptible than Fc-pap and Fc to digestion. There is a good correlation (coefficient = 0.98) between β phase half life and rate of pepsin proteolysis and this is represented graphically in Fig. 4. Susceptibility to pepsin proteolysis for each fragment was evaluated by calculating the slope of the curves of release of TCA non-precipitable material (radiolabelled) against time (Fig. 3a). Digestion products were also analysed by SDS–PAGE using reducing conditions (Fig. 5). The use of non-reducing conditions would result in a different fragmentation pattern for the

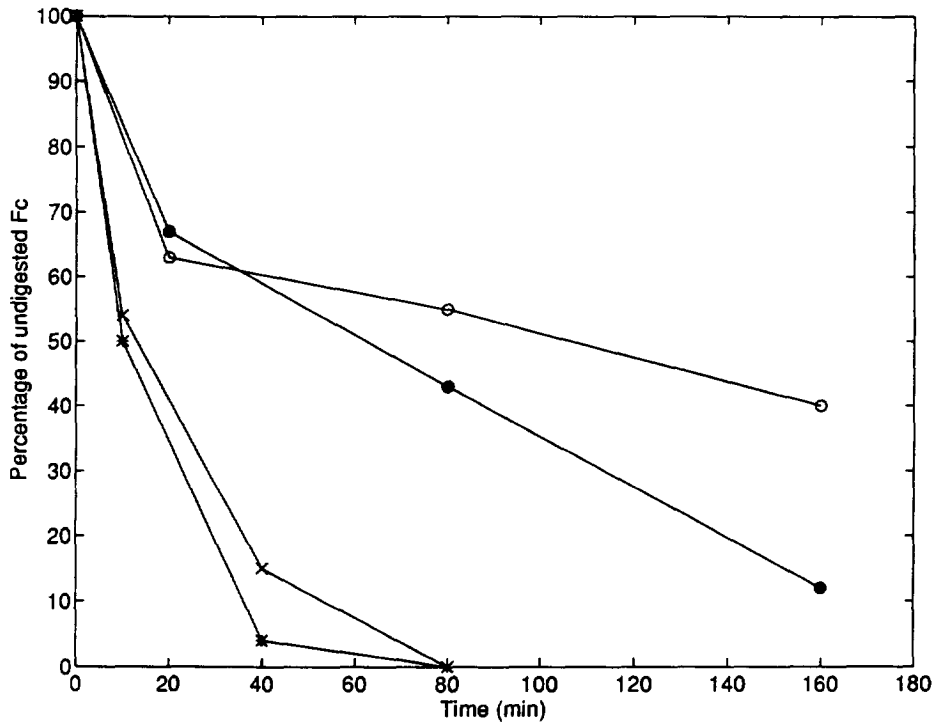
Table 2. Pharmacokinetics of the Fc derviatives

Fragment	Number of animals	α phase $t_{1/2}$ (hours)	β phase $t_{1/2}$ (hours)	MRT [a]
IgG1	3	20.1 ± 0.4	85.3 ± 4.3	117.1 ± 6
Fc-hinge	12	9.8 ± 0.8	79.8 ± 9.5	99.9 ± 12.7
Fc	12	11.0 ± 11.1	48.7 ± 7.8	61.3 ± 9.3
Fc-cys	8	12.6 ± 0.5	72.0 ± 7.1	94.3 ± 9.9
Fc-pap	8	13.1 ± 0.9	56.1 ± 5.8	73.2 ± 8.4

[a] MRT, mean residence time. Statistical significance (student *t*-test) of β phase $t_{1/2}$s. Significant differences (i.e. $p < 0.01$): IgG1 versus Fc or Fc-pap; Fc-hinge versus Fc or Fc-pap: Fc-cys versus Fc or Fc-pap. Insignificant differences (i.e. $p > 0.01$): Fc-hinge versus IgG1 or Fc-cys; Fc-pap versus Fc.



(a)



(b)

Fig. 3. Analysis of the kinetics of pepsin digestion of the Fc fragments. (a) Release of TCA non-precipitable material as a function of time. (b) Decrease of non-digested Fc fragment as a function of time. For both (a) and (b), *. Fc-hinge; ○, Fc; ×, Fc-cys; ●, Fc-pap.

disulphide linked dimers compared with the non-covalently linked dimers. The Fc-derived fragments that are released by pepsin digestion have a molecular weight ranging from 14–20 kDa, and presumably represent mixtures of CH2 and CH3 domains at different stages of proteolytic fragmentation.

DISCUSSION

Previous work has identified amino acid residues located at the CH2–CH3 domain interface as being involved in IgG catabolism control (Kim *et al.*, 1994*a*,*b*). High resolution structural studies of a human Fc (Deisenhofer, 1981) suggest that the extended switch

peptide linking the CH2 and CH3 domains may allow the CH2 and CH3 domains to change orientation with respect to each other. In support of this, reduction of the hinge region of complete IgG antibodies has been shown to result in increased mobility of the CH2 domain at the CH2–CH3 switch region (Romans *et al.*, 1977; Seegan *et al.*, 1979; Klein *et al.*, 1981; Nezlin, 1990). Clearly, mobility in this region could result in a change in the accessibility of the residues that regulate catabolism and our current data support this.

Our findings related to the increased clearance rates (β phase half life approximately 70% of that of Fc-hinge fragments) of Fc fragments not covalently linked by hinge region disulphides suggest that CH2–CH3 switch region flexibility results in a decreased functional activity of the catabolic site residues and a more compact conformation of the Fc fragments. The flexibility could affect the conformation of the residues at the CH2–CH3 domain interface, resulting in decreased binding to putative Fc receptors (Brambell *et al.*, 1964) that protect IgGs against degradation by a mechanism that is as yet unclear. Consistent with this concept is the observation that reduced and alkylated IgG is also defective in intestinal transcytosis (Matre, 1977; Van der Meulen *et al.*, 1980; McNabb *et al.*, 1976; Burton, 1985), together with our more recent observations that the catabolic site of murine IgG1 is closely related to the site that interacts with FcRn (Kim *et al.*, 1994*c*).

Limited proteolysis has been used previously by other groups to probe changes in conformation (Neurath, 1980; Lo Bello *et al.*, 1993; Jamison *et al.*, 1994; Wyss *et al.*, 1993). Thus, to probe possible changes in the conformation of Fc induced by the presence or absence of interchain disulphide bridge(s), these Fc fragments were analysed by limited proteolysis. The Fc fragments analysed fall into two groups: the first contains a recombinant Fc-hinge fragment and Fc-cys fragment with a genetically engineered hinge region. This latter fragment was designed to eliminate the possibility of any effects of IgG1 hinge residues *per se*, rather than intermolecular disulphide bridges, on the pharmacokinetics and pepsin sensitivity. The second group comprises two hingeless Fc fragments of faster *in vivo* clearance rates than those in the first group. Pepsin was chosen for these studies because at low pH it is known to attack a site in the neighbourhood of the CH2–CH3 domain interface, splitting the Glu333–Lys334 peptide bond (Bennich *et al.*, 1974; Turner and Bennich, 1968). Limited digestion also yielded a clear fragmentation pattern that facilitated comparison of the kinetics of digestion for each Fc fragment. Pepsin is known to attack other regions of the Fc fragment (in an intact IgG molecule), and these include the C-terminus of the hinge region and many sites in the CH2 domain (Turner and Bennich, 1968; Bennich *et al.*, 1974). These multiple sites of attack result in the complex nature of proteolytic products seen (Fig. 5).

The data show that increased plasma clearance correlates with decreased susceptibility to pepsin digestion. Thus, in contrast to other proteins (Brinkmann *et al.*, 1992), higher resistance to (pepsin) proteolysis is not correlated with increased serum persistence for Fc fragments. This is reminiscent of our earlier data showing that a mutant Fc-hinge fragment with a greatly reduced serum half life relative to the wild type Fc-hinge is not more susceptible to proteolysis (Kim *et al.*, 1994*a*). Taken together, the results suggest that the hinge disulphide

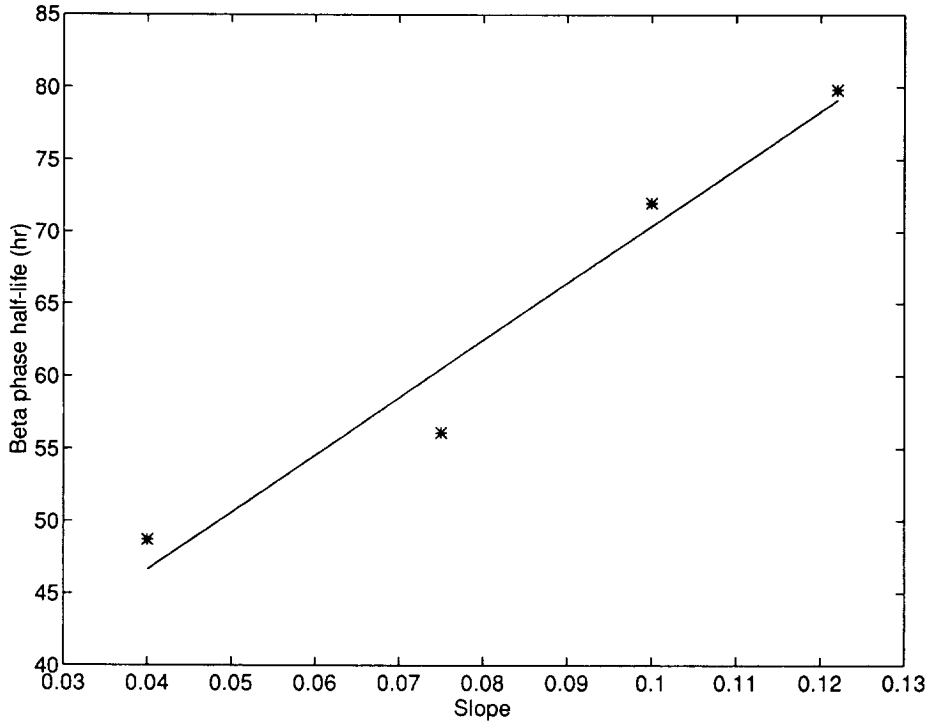


Fig. 4. Correlation between A phase half life and rate of proteolysis of different Fc derivatives. Slope = initial slopes of curves shown in Fig. 3.

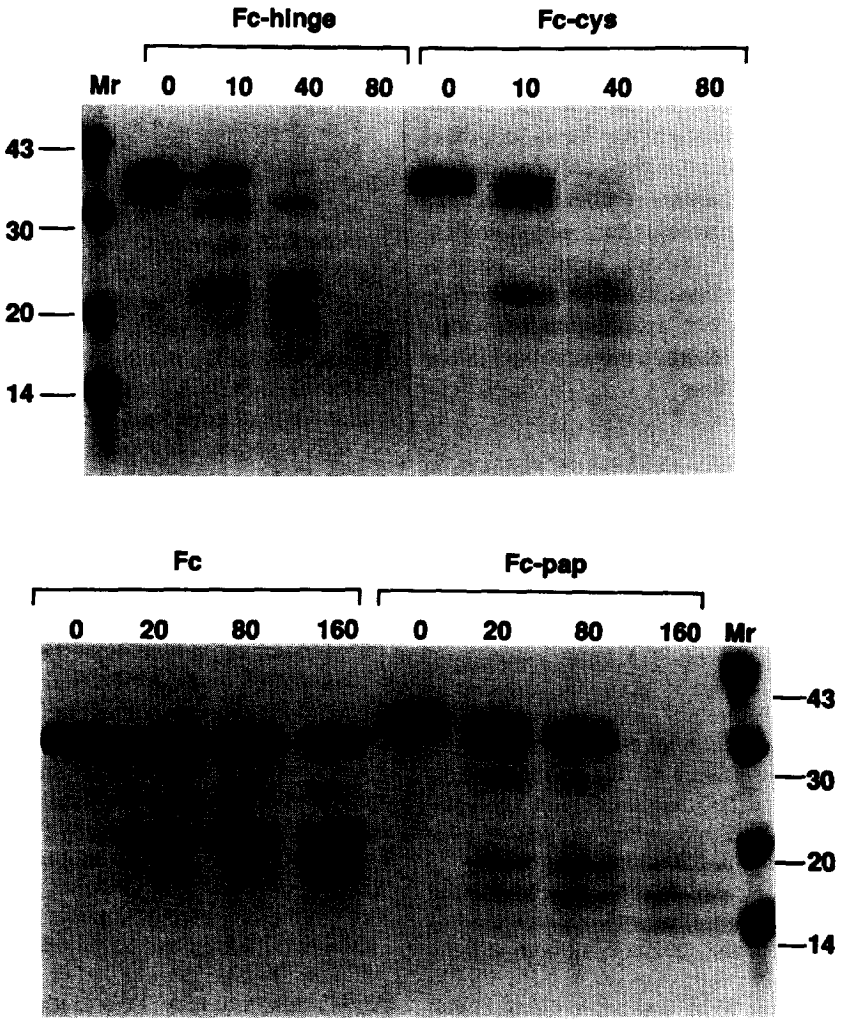


Fig. 5. SDS–PAGE analysis of pepsin digestion of the Fc fragments under reducing conditions.

plays a role in constraining the movement of the CH2 domain relative to the CH3 domain, and removal of the covalent tether results in an increased mobility of the CH2 domains. This in turn changes the conformation at the CH2–CH3 domain interface which is manifested as an increase in clearance rate (due to alteration of the catabolic site) and decreased susceptibility to pepsin digestion.

Unexpectedly, all four fragments can be purified using SpA-Sepharose, indicating that SpA can still bind despite an alteration in the conformation of the CH2–CH3 domain interface. However, in this study, the relative affinities of the Fc fragments for SpA-Sepharose have not been analysed, and it is therefore conceivable that the affinity of the Fc fragments without -S—S- bridges are decreased but not to the extent that SpA binding is ablated.

The similar digestion profiles of Fc and Fc-pap indicate that the presence of carbohydrate does not affect the susceptibilty to pepsin proteolysis of these fragments. This observation is in contrast to the findings of Tao and Morrison (1989) who observed that aglycosylated human IgG1 and IgG3 were more susceptible to proteolysis than the glycosylated counterparts. These apparent discrepancies may be due to the difference in the molecular species used (Fc versus complete IgGs), differences in the conditions used for proteolysis and/or isotypic/species differences.

In summary, our data demonstrate that alteration of the conformation of the CH2–CH3 domain interface by ablation of the hinge -S—S- bridge(s) affects the β phase half life of a murine Fc fragment. The requirement for an intact hinge disulphide in serum persistence of the Fc fragments, together with our earlier data (Kim *et al.*, 1994*a*,*b*) indicates that for the genetic manipulation of therapeutic proteins to increase their *in vivo* half life, the IgG hinge region should be included with both the CH2 and CH3 domains. The role of the hinge disulphide bridges in maintaining serum IgG levels seems to be of minor physiological significance *in vivo*, since intravascular IgG molecules are in an oxidising environment and this preserves the disulphide bonds. However, even in the presence of the hinge region in an IgG molecule, exposure to low pH can also induce conformational changes in the CH2–CH3 domain interface (Abaturov *et al.*, 1969) and increase susceptibility to proteolysis (Connell and Porter,

1971; Stewart *et al.*, 1973; Ghetie and Buzila, 1972). This suggests that even with intact hinge disulphide bridges, the Fc region has a degree of rotational freedom that allows such changes to occur in the "mini-hinge" region (Timofeev *et al.*, 1978). Although we have identified non-physiological factors that alter the conformation of the CH2–CH3 domain interface and as a result, affect the serum persistence of Fc fragments, more physiological conditions may (reversibly) induce similar conformational changes. Our observations may therefore suggest future experiments that are directed towards understanding the molecular interactions involved in the *in vivo* control of IgG catabolism.

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