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Catabolism of the Murine IgG1 Molecule: Evidence that Both CH2-CH3 Domain Interfaces are Required for Persistence of IgG1 in the Circulation of Mice

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Site-directed mutagenesis of a recombinant Fc-hinge fragment has previously been used to identify a region of the murine IgG1 molecule that controls catabolism, and this site encompasses amino acid residues at the interface of the CH2 and CH3 domains. In the current study the nature of this 'catabolic site' has been further analysed using recombinant techniques. Fc-hinge, CH2-hinge, CH2 and CH3 fragments have been expressed in *Escherichia coli*, purified and analysed in pharmacokinetic studies in mice. The CH2-hinge has been analysed as both a monomer and dimer, and the dimer has a longer β phase half-life (61.6 h) than the monomer (29.1 h). This suggests that two catabolic sites per Fc fragment are required for serum persistence. The need for two functional sites per molecule has been confirmed by the analysis of a hybrid Fc-hinge fragment comprising a heterodimer of one Fc-hinge with the wild type (WT) IgG1 sequence and a mutant Fc-hinge with a defective catabolic site (mutated at His310, Gln311, His433 and Asn434). This hybrid is cleared with a β phase half-life of 37.9 h and this is significantly shorter than that of the WT Fc-hinge fragment (82.9 h). In contrast to the CH2-hinge dimer, the CH3 domain is cleared rapidly (β phase half-life of 21.3 h) indicating that the region of this domain (His433 and Asn434) previously identified as being involved in the control of catabolism is not sufficient in the absence of the CH2 domain for the serum persistence of an IgG fragment. The data extend our earlier observations concerning a region of the murine IgG1 molecule that is involved in the control of catabolism and have implications for the design of engineered antibodies for therapy.

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INTRODUCTION

Despite the extensive use of antibodies in diagnosis and therapy [1], relatively little is known about the molecular mechanisms by which gammaglobulins (IgGs) persist in the serum [reviewed in 2]. To get a better understanding of IgG catabolism, we previously demonstrated that a site of the murine IgG1 molecule which regulates catabolism ('catabolic site') is located at the interface of the CH2 and CH3 domains and overlaps with the protein A (SpA) binding site [3]. Using site-directed mutagenesis, amino-acid residues that are conserved in both human and murine IgGs [4], and are located in both the CH2 (Ile253, His310, Gln311) and CH3 domains (His433, Asn434), were found to be involved in controlling the pharmacokinetics of a recombinant Fc-hinge fragment in mice. The finding that a catabolic site of murine IgG1 encompasses residues in both CH2 and CH3 domains is in contrast with the widely accepted concept that the CH2 domain is solely responsible for the control of IgG catabolism [5]. This concept was based on earlier work, primarily by Dorrington and coworkers [6, 7], in which proteolysis was used to generate CH2 and CH3 domain fragments, and the CH2 domain had a half-life similar to that of an Fc fragment, whereas the CH3 domain fragment was cleared rapidly. However, more recent data [8] have suggested that CH3 sequences might be involved and this is consistent with our observations [3].

Recombinant DNA technology allows the generation of proteins of a predetermined size, and in this study the genes encoding the CH2-hinge fragment, CH2 domain or CH3 domain have been tailored for expression as secreted pro-

teins from recombinant *E. coli* cells. The CH2-hinge fragment is expressed predominantly as a dimer in which the two domains are held together by a disulphide bond. In contrast, the CH2 domain is expressed as a monomer and the CH3 domain as a dimer held together by non-covalent forces [9]. The CH2, with and without hinge region, and CH3 fragments have been expressed, characterized and used in pharmacokinetic studies. The production of a CH2-hinge dimer and CH2 domain monomer has allowed the analysis of whether the number of catabolic sites per protein molecule, i.e. one or two, affects the catabolic rate. In addition, the data concerning the number of catabolic sites per molecule have been extended by analysing a hybrid Fc-hinge heterodimer that contains a wild type Fc-hinge fragment associated with a mutant Fc-hinge which has a defective catabolic site (the HQ-310/HN-433 mutant described previously [3]). The results of this study expand the existing knowledge of the factors that are involved in controlling the serum elimination rates of IgGs, and could be of value in designing therapeutic antibodies/proteins with predetermined pharmacokinetics.

MATERIALS AND METHODS

Bacterial strains and hybridomas. For the expression of recombinant proteins, *E. coli* BMH 71-18 [10] was used as host. The genes encoding the Fc-hinge, CH2-hinge, CH2 and CH3 fragments were isolated from the 9E10 hybridoma [11]. Mouse IgG1 monoclonal antibody specific for the human CD22 marker (RFB4) was prepared by Abbott Biotechnology (Needham Heights, MA, USA). This antibody does not cross-react with murine tissue or peripheral blood cells [12].

Plasmid constructions. Plasmid constructions were undertaken in order to the express of immunoglobulin constant region domains with His_6 peptide tags. Total RNA was extracted from 1×10^7 9E10 [11] hybridoma cells using previously described methodology [13]. cDNA synthesis was primed using oligonucleotides CH3forBst or CH2forBst (see below) for the isolation of either the CH3 domain gene/Fc-hinge fragment genes or the CH2-hinge/CH2 domain genes respectively. The genes were amplified from the cDNA using the polymerase chain reaction (PCR, [14]) and the following primers: (a) CH3 domain, CH3bakNco = 5'ATCACCATGGCC-*GGCAGACCGAAGGCTCCACAG3'* and CH3forBst = 5'TACA-GGTGACC*TTACCAGGAGAGTGGGAGAGGCT3'*; (b) CH2-hinge, HingebakNco = 5'ATCACCATGGCC*GTGCCCAGGGAT-TGTGGTTG3'* and CH2forBst = 5'ATCAGGTGACC*TTGGTTT-TGGAGATGGTTTT3'*; (c) CH2 domain, CH2bakNco = 5'ATCA-CCATGGCC*GAAGTATCATCTGTCTTCATC3'* and CH2forBst; (d) Fc-hinge fragment, HingebakNco and CH3forBst. A typical PCR comprised: 3 units Promega *Taq* polymerase, 25 pmol each primer, 0.2 mM dNTPS, 1 × Promega PCR buffer, 10 µl cDNA synthesis reaction in a final volume of 100 µl. Cycling conditions were: 94°C (0.5 min), 55°C (0.5 min), 72°C (1 min) for 30 cycles using a Techne temperature cycling block. Italicized sequences in the oligonucleotide sequences indicate the regions of the oligonucleotides that anneal to murine IgG1 constant region genes [15]. The oligonucleotides each encode either an *Nco*I or *Bst*EII restriction site, indicated by the underlined sequences, which allows restriction enzyme digestion of the PCR products followed by gel purification and ligation as *Nco*I–*Bst*EII fragments into VαpelBHis [16]. The ligated DNA was then transformed into *E. coli* BMH 71-18 [10]. The sequences of the inserts of all plasmid constructions were analysed using the dideoxynucleotide method [17] and either Sequenase (USB Biochemicals, Cleveland, OH, USA) for single stranded DNA templates or Femtomole kits (Promega, Madison, WI, USA) for double-stranded DNA templates.

A plasmid construction undertaken to express the D1.3 Fv fragment with a His_6 peptide tag. The plasmid pSW1-VHD1.3-VKD1.3-TAG1 [18] was restricted with *Bst*EII and the following oligonucleotide duplex ligated into gel purified (Bio 101, La Jolla, CA, USA) linearized plasmid: 5'GTCACCGTCTCCTCACAT-CACCATCACCATCACTAATAA3' and 3'GCAGAGGAG-TGTAGTGGTAGTGGTAGTGATTATTCAGTG5' (coding strand indicated by underlined sequence). This results in the tagging of the VHD1.3 domain with a carboxy terminal His_6 peptide, which allows purification of the expressed and secreted D1.3 Fv using Ni^{2+}-NTA-agarose (Qiagen) and the methodology described by Ward [16].

PCR mutagenesis and the oligonucleotide primer 5'ATCA-CCCGGGGTTGCGTCTGAGCTGT*CGCC*ACCTC3' in combination with HingebakNco was used to convert His285 to Ala285 (EU numbering, [19]) in the Fc-hinge fragment. Mutated bases are indicated in the sequence by italic. The WT Fc-hinge fragment gene was used as a template and the PCR product digested with *Nco*I and *Sma*I (underlined in primer) and used to replace the corresponding fragment in the WT gene. The mutant derivative (H-285) was sequenced using the dideoxynucleotide method [17] and Sequenase (USB Biochemicals) prior to protein expression.

Expression and purification of recombinant proteins. E. coli BMH 71-18 transformants harbouring the plasmids shown in Fig. 1 were grown up and induced for expression as described previously [16]. The recombinant proteins were isolated from the periplasm by osmotic

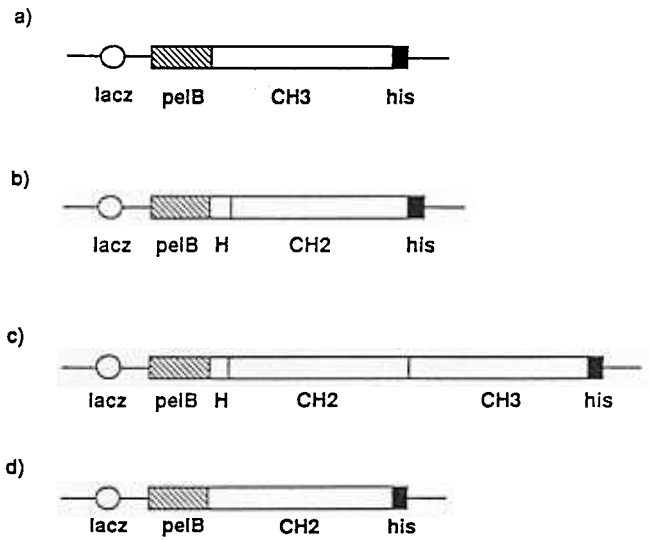

Fig. 1. Schematic representation of the plasmids used for the expression and secretion of immunoglobulin constant region fragments in *E. coli*. (a) CH3 domain; (b) CH2-hinge fragment; (c) Fc-hinge fragment; (d) CH2 domain. The lacz promoter is represented by open circles, the pelB leader by hatched boxes, the immunoglobulin domains [hinge region (H) and CH2, CH3 domains] by open boxes and the His_6 peptide tag (his) by black boxes.

shock followed by affinity purification using Ni^{2+}-NTA-agarose [16, 20]. The anti-lysozyme D1.3 Fv fragment containing the VHD1.3 domain tagged with a His_6 peptide was purified in the same way.

Preparation of CH2 dimer. CH2-hinge fragments that were isolated from recombinant *E. coli* cultures using Ni^{2+}-NTA-agarose comprised two species, -S-S- linked dimer (25 kDa) and monomer (12.5 kDa). Dimer was separated from monomer by gel filtration on Sephadex G-75 (Pharmacia-LKB Biotech, Piscataway, NJ, USA) equilibrated with 50 mm phosphate buffer plus 3 mm Na_2EDTA (PBE) pH 7.5.

Preparation of CH2-hinge monomer by reduction and alkylation of CH2-hinge dimer. 0.5 ml CH2-hinge dimer (2 mg) in PBE was incubated with dithiothreitol (DTT) at a final concentration of 2 mm for 1 h at room temperature in the dark. The reduced CH2-hinge was then passed through a Sephadex G-25 column equilibrated with PBE, and concentrated to approximately 0.5 ml prior to treatment with iodoacetamide at a final concentration of 10 mm at room temperature for 2 h. The reduced and alkylated CH2-hinge was passed through a Sephadex G-25 column and concentrated to approximately 1 mg/ml prior to use.

Polyacrylamide gel electrophoresis (PAGE). The expressed and purified proteins were analysed using SDS-PAGE gels [21] run under reducing and non-reducing conditions. β-mercaptoethanol (5% v/v) was used as reductant in reducing gels. Gels were stained with Coomassie brilliant blue R-250 (Sigma, St Louis, MO, USA).

Immunoblotting. Electrophoresed proteins were transferred from PAGE gels onto nitrocellulose membranes as described previously [22] and c-myc tagged proteins detected using 9E10 antibody [11] that had been biotinylated using an Amersham biotinylation kit. Bound 9E10 was detected using streptavidin-horse radish peroxidase (HRP) conjugate (ICN Immunochemicals, Irvine, CA, USA) and HRP detected as described previously [18]. For the detection of the murine Fc-hinge fragment, anti-mouse Fc-HRP conjugate (ICN Immunochemicals) was used.

Construction of a plasmid for the expression of a hybrid Fc-hinge fragment. An *Nco*I–*Bst*EII fragment encoding the mutant HQ-310/HN-433 Fc-hinge was ligated into *Nco*I-*Bst*EII restricted VαpelB tag1 [23]. The insert of the resulting plasmid was ligated as an *Eco*RI fragment into pBGS19 ([24], and kanamycin resistant) and the orientation checked using the PCR [14]. A plasmid with the correct orientation with respect to the lacz promoter was isolated and used to transform competent *E. coli* cells in combination with a plasmid conferring ampicillin resistance and designed to express the WT Fc-hinge fragment [3]. Doubly transformed clones were selected for both ampicillin (100 μg/ml) and kanamycin (40 μg/ml) resistance.

Isolation of hybrid Fc-hinge fragment. Kanamycin/ampicillin resistant clones harbouring plasmids encoding the WT Fc-hinge fragment and HQ-310/HN-433 mutant were grown up and expression induced as described previously [16]. The osmotic shock fractions were passed through Ni^{2+}-NTA-agarose columns and bound protein eluted and dialysed into phosphate buffered saline (PBS). This resulted in purification of WT Fc-hinge homodimers (tagged with His_6 peptide) and WT/mutant Fc-hinge heterodimers. The mutant Fc-hinge homodimers did not bind to the column as they are tagged with c-myc peptides (data not shown). The protein mixture was then passed through a column comprising the 9E10 antibody covalently linked to protein A-Sepharose (Pharmacia-LKB Biotech, Piscataway, NJ, USA) by dimethyl pimelimidate [25]. To ensure that the column had no free SpA that would bind to WT Fc-hinge homodimers (without c-myc tags), the column was analysed for binding to radiolabelled IgG1 and no binding was detected. Following passage of the WT Fc-hinge homodimers and WT/mutant Fc-hinge heterodimers through the column and washing with 10 column volumes of PBS, heterodimers were eluted with 50 mm diethylamine. Eluates were immediately neutralized by addition of 0.1 × fraction volume of 1 M Tris-HCl pH 7.4.

Radiolabelling of proteins. The Iodo-Gen procedure [26] was used to radiolabel proteins with ^{125}-INa (Amersham, Arlington Heights, IL, USA) for use in pharmacokinetic studies. The free iodine was removed by two successive gel filtrations/centrifugations on Sepharose G-25 using a tuberculin syringe column. The final volume of radioactive protein was adjusted to 0.5 ml with phosphate buffered saline containing 1 mg/ml bovine serum albumin. The specific activities of the radiolabelled proteins were approximately 10^7 cpm/μg with less than 5% free iodine. The radioactive proteins were stored at 4°C for less than one week prior to injecting into mice for clearance studies.

Pharmacokinetic studies. Mice (either BALB/c, BAB14 or CB20) of 20–25 gm weight were used and were given 0.01% NaI in drinking water one day prior to injection and throughout the entire period of monitoring the clearance of the radiolabelled proteins. Radiolabelled proteins were injected intravenously through the tail vein in a volume not larger than 150 μl with a radioactive load of 2–100 × 10^6 cpm. The mice were bled with heparinized 44.7 μl capillary pipettes from the retro-orbital sinus 3 min following injection and at the time points afterwards as indicated in Fig. 2. The radioactivity of 44.7 μl whole blood or 25 μl plasma was determined using a gamma counter at the end of each experiment (96 h) when all the counts were measured at the same time. All radioactive counts that were used to calculate half-lives were more than 20 times above background (30 cpm). As it was not possible to inject precise volumes of radiolabelled protein into each animal, the results are expressed relative to the radioactivity in a given volume of blood at the 3 min time point (this amount was taken as the total injected dose). For the calculation of the α and β phases, non-compartmental modelling was applied [27] using a program provided by Dr K. Vyas, Merck, Sharp and Dohme, West Point, Pennsylvania, USA [28]. Some of the results obtained for the α and β phases using non-compartmental pharmacokinetics were verified using a two-compartment Siphar/Base program. The pharmacokinetic characteristics for the majority of recombinant proteins or the complete IgG1 molecule were analysed in two to four independent experiments in which each preparation was injected into 3 to 4 animals (for each protein analysed, the number of mice are shown in Table 1). For analysis of each of the D1.3 Fv fragment and CH2 domain, only one experiment with four mice was carried out. Data from one representative experiment is shown in Fig. 2.

Precipitation with TCA. 100 μl of plasma was harvested from mice 24 h following injection with radioactive IgG fragments, and was diluted with 100 μl of phosphate buffered saline and 200 μl 20% TCA added. Following 30 min incubation at room temperature, the mixture was centrifuged and the radioactivity in an aliquot of the supernatant measured. To calculate the percentage of non-precipitable radioactivity, the total radioactivity in the supernatant was divided by the total radioactivity of the initial sample. All TCA precipitations were carried out using plasma samples from two mice for each protein.

Analyses of plasma samples using HPLC. The molecular size of the radioactive proteins as they persisted in the serum of mice was determined by carrying out HPLC on SEC-250 columns of plasma samples. The plasma samples were harvested 24 h post-injection and the method described previously [3] was used.

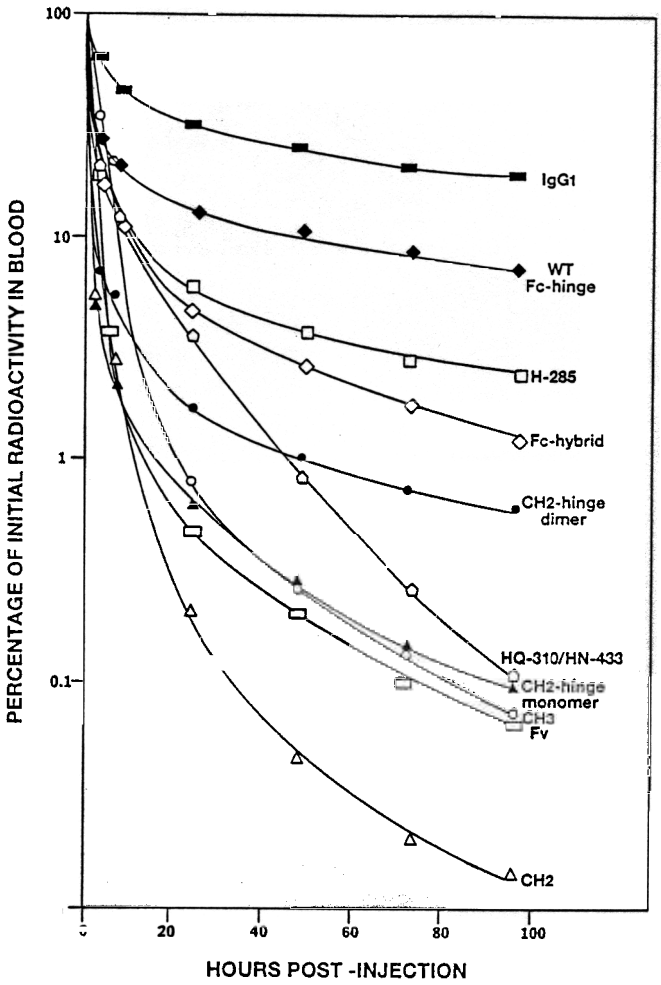

Fig. 2. Clearance curves of the IgG1 molecule and IgG1 derived fragments.

RESULTS

The PCR was used to isolate and tailor the genes encoding fragments derived from the murine IgG1 molecule 9E10 [11] for ligation into the expression plasmids (Fig. 1). These antibody fragments were expressed and secreted from recombinant *E. coli* cells, and the carboxy-terminal His_6 peptide tags allowed purification using Ni^{2+}-NTA-agarose [20]. The recombinant fragments were purified in yields of 2, 1–1.5, 2 and 0.5 milligrams per litre of culture for the CH3 domain, CH2-hinge fragment, CH2 fragment and Fc-hinge fragment respectively.

The CH2-hinge fragment is expressed and purified as a mixture of monomer and -S-S- linked dimer (Fig. 3). Structural analyses of immunoglobulins indicate that the CH2 domains in the Fc region of an antibody molecule form few interdomain interactions [9, 29, 30], and the relative weakness of these interactions (compared with those between the CH3 domains, for example) probably results in a low proportion of expressed dimer. In the native immunoglobulin molecule, carbohydrate residues are interposed between the two CH2 domains [9, 29, 30]. Analyses using both reducing and non-reducing PAGE indicate that the recombinant CH2-hinge dimer is covalently linked by -S-S- bridges (Fig. 3), and the -S-S- bridges are presumably formed between cysteine residues located in the hinge region. This dimer was separated from the monomer as described using a G-75 column (Fig. 4) prior to use in pharmacokinetic studies. In contrast to the CH2-hinge fragment, the CH2 domain is expressed as a monomer that has no free -SH groups as would be expected for a protein that has only intramolecular -S-S- bridges and no hinge region. The CH3 domain is expressed and purified as a homodimeric protein (Fig. 3 and data not shown). The CH3 dimer is non-covalently linked as the CH3 protein has a mobility that corresponds to a monomer when analysed using reducing and non-reducing PAGE (Fig. 3). The CH3

Table 1. Half-lives of recombinant Fc-hinge fragments and Fc derived fragments in mice

Derivative	Number of mice	Half-life (h) α-phase	Half-life (h) β-phase
IgG1	7	19.2 ± 1.2	89.2 ± 10.6
CH2-hinge dimer	10	6.3 ± 1.2	61.6 ± 10.7
CH2-hinge monomer (reduced & alkylated)	7	4.3 ± 0.1	29.1 ± 1.2
CH2	4	2.9 ± 0.1	25.5 ± 2.3
CH3	9	3.4 ± 0.1	21.3 ± 2.3
Fv	4	3.4	24.1 ± 3.5
WT Fc-hinge	9	10.5 ± 0.8*	82.9 + 10.0*
HQ-310/HN-433 mutant Fc-hinge	7	5.8 ± 0.2*	15.6 ± 0.8*
Fc-hybrid	16	7.3 ± 1.2	37.9 ± 7.1
H-285 mutant Fc-hinge	8	8.7 ± 1.1	76.0 ± 14·6

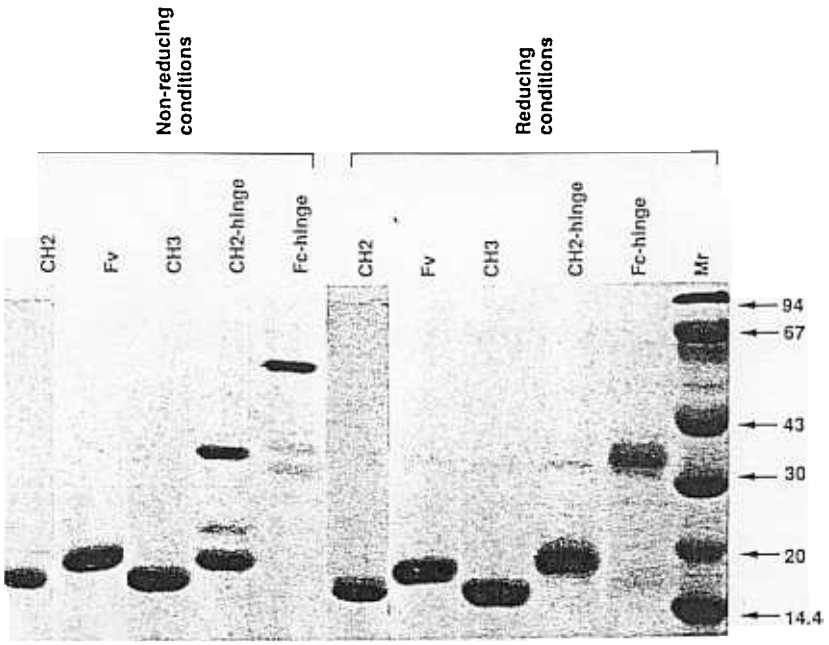

Fig. 3. 15% SDS polyacrylamide gel analyses of purified recombinant proteins using non-reducing and reducing conditions (as indicated). Fv = D1.3 Fv fragment and Mr = molecular weight standards (sizes shown on the right margin in kDa).

domains are closely apposed in the immunoglobulin structure and form many interdomain interactions [9, 29, 30], and these interactions would be expected to drive and stabilize the dimerisation of the CH3 domains. The Fc-hinge fragment is purified as an -S-S- linked dimer as described previously [3]. The purified D1.3 Fv fragment has also been analysed by PAGE (Fig. 3) using both reducing and non-reducing conditions. For the D1.3 Fv, the heavy and light chain variable domains co-migrate under the gel conditions used and are therefore visualized as a single band.

To analyse the pharmacokinetic behaviour of the immunoglobulin fragments *in vivo*, the recombinant proteins were radiolabelled with ^{125}I and the levels of serum radiolabel monitored as a function of time (Fig. 2). The clearance rates were compared with those of intact murine IgG1 (expressed and purified from the hybridoma RFB4) and the bacterially expressed D1.3 Fv fragment [18] derived from the murine D1.3 antibody [31]. The clearance curves are shown in Fig. 2 and are biphasic for all the proteins that have been analysed. The α phase represents, in part at least, equilibration between the vascular and extravascular space and the β phase represents the elimination of equilibrated protein from the intravascular space, that is, the biological half-life. For all fragments the serum levels were analysed for at least 96 h following injection. For the more rapidly cleared fragments, this was made possible by radiolabelling the proteins to high specific activity. To ensure that the proteins were not extensively proteolysed after entering the circulation, TCA precipitations were performed on plasma harvested 24 h after injection of Fc-hinge, CH2-hinge dimers, CH2, CH3 and D1.3 Fv fragments. This showed a moderate amount of nonprecipitable radioactivity for all of the fragments (8.9% for CH2-hinge dimers, 11.9% for CH2, 13.7% for CH3, 17.7% for D1.3 Fv and 3.3% for Fc-hinge) and this is probably due to de-iodination of the proteins. The α and β phase half-lives of the IgG1 molecule and recombinant fragments are shown in Table 1. For the smaller CH3, CH2 with or without hinge and Fv fragments the α phase half-lives are short. These short α phase half-lives can be explained, at least in part, by the lower molecular mass of these fragments that results in a shorter equilibration time between the intra- and extravascular space. In contrast, the β phases are much longer and therefore accurate values of the biological half-lives are readily obtained from the clearance curves using computerized methods. The half-lives of the β phases of the IgG1 molecule and Fc-hinge fragment are similar. The β phase half-life of the CH2-hinge dimer is less than that of the Fc-hinge fragment, whereas the β phase half-life of the CH3 domain is shorter than that of the CH2-hinge dimer, and similar to that of the D1.3 Fv fragment (Fig. 2 and Table 1). Furthermore, the monomeric CH2 domain has a β phase half-life of 25.5 h, in contrast to that of 61.6 h for the CH2-hinge dimer. The CH2 domain lacks the hinge region and therefore to exclude the possibility that the presence of the hinge might account for the longer half life the CH2-hinge dimer was converted into a monomer by reduction followed by alkylation and the protein analysed using a G-75 column (Fig. 4) prior to pharmacokinetic analyses. The β phase half-life of 29.1 h for the CH2-hinge monomer is not significantly different from that of the CH2 domain (no hinge), and this indicates that amino acids in the hinge region are not involved in the control of catabolism of the murine IgG1 molecule.

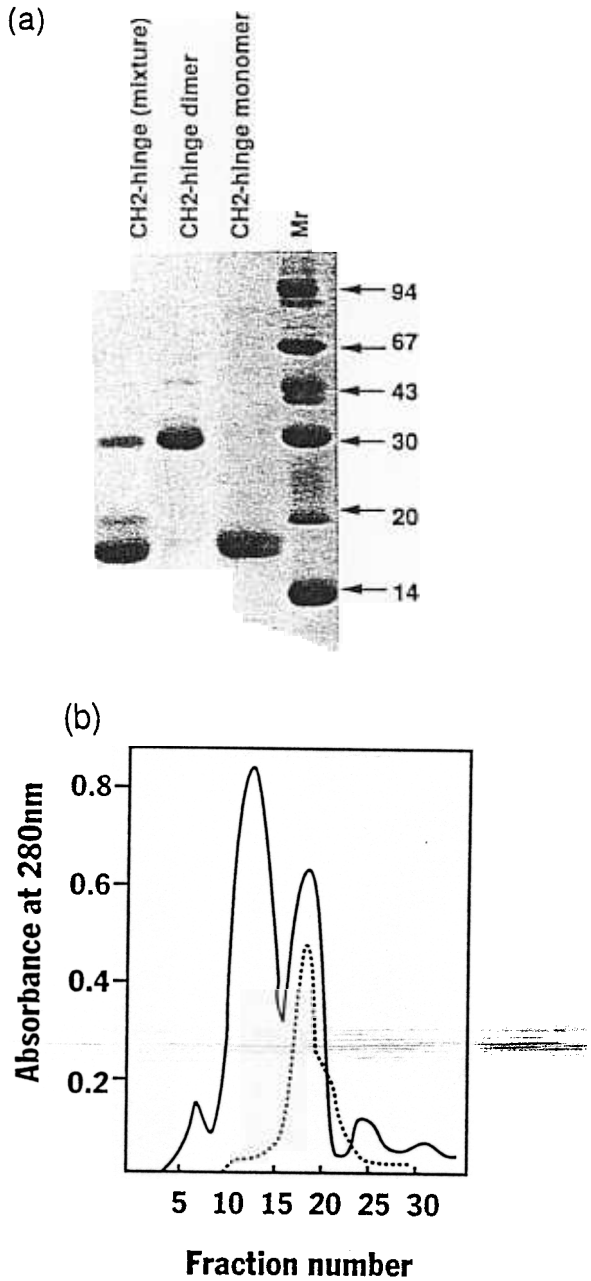

Fig. 4(a). 15% SDS polyacrylamide gel analysis of the purification of the CH2-hinge dimer and monomer. Molecular weight standards (Mr) are shown on the right margin in kDa.
(b) separation of CH2-hinge dimer from CH2-hinge monomer (solid line) and analysis of CH2-hinge dimer following reduction and alkylation (dotted line) using Sephadex G-75 columns.

HPLC analyses of serum samples of mice injected with the CH2-hinge fragments (monomer or dimer) indicated that the proteins persisted in the serum with molecular weights of 18 kDa and 30 kDa for the monomer and dimer respectively (Fig. 5). HPLC analyses of the purified CH2-hinge dimer and CH2-hinge monomer prior to injection into mice indicate similar molecular weights (data not shown), and these are higher than predicted from the corresponding amino acid

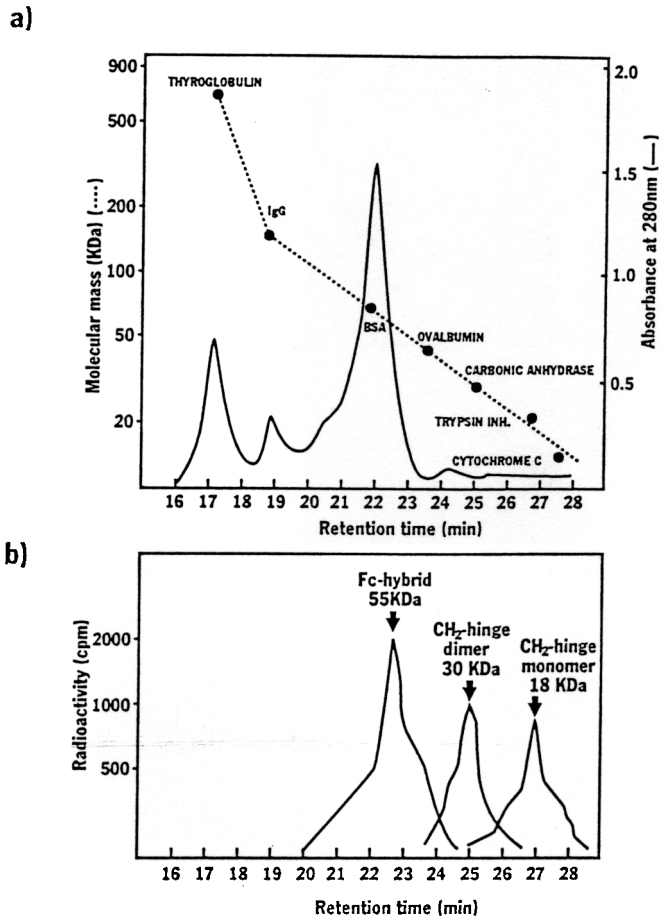

Fig. 5. HPLC analyses on an SEC-250 column of plasma samples from mice at 24 h post-injection of radiolabelled Fc-hybrid, CH2-hinge dimer and CH2-hinge monomer (made by reduction and alkylation). (a) chromatographic profile of mouse plasma and calibration curve for molecular weight determination.
(b) distribution of radioactivity in plasma samples of mice injected with radiolabelled fragments.

sequences [15]. The anomalous migration is presumably owing to the effects of molecular shape.

The data obtained for the pharmacokinetics of the CH2-hinge monomers and dimers prompted us to analyse whether one or two functional catabolic sites per Fc-hinge dimer are necessary for serum persistence *in vivo*. In contrast to the CH2-hinge fragments, the stable dimerisation of Fc-hinge fragments mediated by CH3 domain interactions clearly ruled out the possibility that Fc-hinge monomer could be prepared for pharmacokinetic analyses. Therefore, to address this question a hybrid Fc-hinge heterodimer was made, and this comprised a heterodimer of the WT Fc-hinge and HQ-310/HN-433 mutant Fc-hinge. The comparison of dimeric Fc-hinge fragments that differ only in the number of functional catabolic sites eliminates any possible effects that size differences might have on the pharmacokinetics. For the isolation of the hybrid, the WT and mutant Fc-hinge fragments were co-expressed in *E. coli*. The WT and mutant were

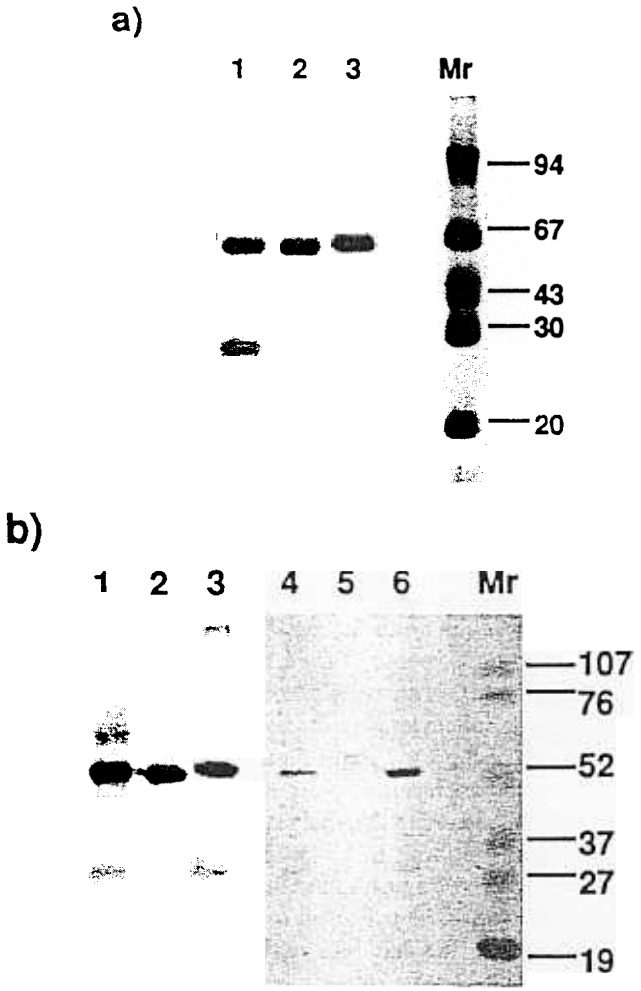

Fig. 6. Analysis of the purification of the Fc-hybrid protein. (a) 10% SDS polyacrylamide gel using non-reducing conditions of lane 1, eluate from Ni^{2+}-NTA-agarose gel; lane 2, flow-through from 9E10-Sepharose; lane 3, eluate from 9E10-Sepharose; Mr = molecular weight standards, with molecular weights shown in kDa on the right margin. (b) Lanes 1–3, immunoblot of duplicate gel as in Fig. 6(a), using anti-mouse-HRP for detection; lanes 4–6, immunoblot of duplicate gel as in Fig. 6(a), using biotinylated 9E10 followed by streptavidin-HRP for detection; Mr, prestained molecular weight standards with molecular weights shown in kDa on the right margin.

tagged with two different carboxy-terminal peptides, namely His_6 and c-myc respectively. This allowed purification of the hybrid by using two sequential columns (Ni^{2+}-NTA-agarose followed by 9E10-Sepharose). The first column results in the isolation of WT Fc-hinge homodimers (tagged with His_6 peptides) and WT-mutant heterodimers, but removes mutant Fc-hinge homodimers (tagged with c-myc peptides) as c-myc tagged proteins do not bind to Ni^{2+}-NTA-agarose (data not shown). The WT homodimer/hybrid mixture was depleted of WT Fc-hinge homodimers by passage through 9E10-Sepharose. Immunoblotting analyses of the purification steps used to isolate the hybrid protein, using both anti-mouse HRP and biotinylated 9E10 (Fig. 6) are consistent with the hybrid comprising a heterodimeric mixture of WT and mutant Fc-hinge fragments. This hybrid has been designated Fc-hybrid.

The Fc-hybrid was radiolabelled and injected into mice and the pharmacokinetics analysed (Fig. 2 and Table 1). The data show that both the α and β phase half-lives are significantly shorter than those of the WT Fc-hinge fragment but longer than that of the HQ-310/HN-433 mutant. In addition, the hybrid persists in the serum as a 55 kDa protein that is not non-covalently associated with other serum proteins (Fig. 5). As a further control in these experiments, a mutation was made in the Fc-hinge fragment that converted a conserved histidine at position 285 (EU numbering, [19]) to alanine, and this mutant (H-285) was radiolabelled and analysed pharmacokinetically (Fig. 2 and Table 1). The β phase half-life is statistically indistinguishable from that of the WT Fc-hinge. This histidine is located in a loop on the external surface of the CH2 domain [9] and is distal to the CH2-CH3 domain interface. The analysis of this mutant demonstrates that mutation of a histidine residue in the Fc-hinge that is distal to the CH2-CH3 domain interface does not result in a decrease in β phase half-life, and indicates that mutation of surface exposed histidines *per se* is not the cause of the more rapid clearance of the HQ-310/HN-433 mutant.

DISCUSSION

This study extends our earlier analysis [3] of a region of the murine IgG1 molecule that is involved in controlling catabolism. In this earlier study, specific mutations of conserved, surface exposed residues at the CH2-CH3 domain interface were found to affect the catabolic rate of recombinant Fc-hinge fragments. Circular dichroism data indicated that the mutations did not result in misfolding of the protein [3]. Findings from the current study indicate that for serum persistence of the murine IgG1 molecule, two catabolic sites per Fc are necessary. Data derived from two experiments support this. The first involves a comparison of the pharmacokinetics of a dimeric CH2-hinge fragment and monomeric CH2 and CH2-hinge fragments. Both monomers (with and without the hinge) have significantly more rapid catabolic rates than the dimer. However, it could be argued that the size differences between the monomer and dimer are responsible for the observed effects. Therefore, to clearly demonstrate that two sites per Fc-hinge are necessary for serum persistence, pharmacokinetic analysis of an Fc-hinge heterodimer (hybrid) was carried out. This hybrid comprised one WT Fc-hinge chain and one mutant Fc-hinge chain. The mutant (HQ-310/HN-433) has been described previously and as a homodimer has a β phase half-life that is similar to that of an Fv (Table 1). The Fc-hybrid has been made by co-expression of WT Fc-hinge and mutant Fc-hinge fragments in *E. coli* followed by purification using two affinity columns sequentially. The Fc-hybrid has a β phase half-life of 37.9 h, and this is significantly less that that of 82.9 h

for the WT Fc-hinge homodimer but longer than that of the HQ-310/HN-433 mutant. This indicates that two functional catabolic sites located at the CH2-CH3 domain interfaces of the dimeric Fc are necessary for an Fc/IgG molecule to be maintained in the circulation with pharmacokinetics that are similar to those of a WT Fc-hinge homodimer. However, the longer β phase half-life of the hybrid relative to that of the HQ-310/HN-433 mutant suggests that the hybrid retains some of the pharmacokinetic characteristics of the WT homodimer. The possibility that two catabolic sites per Fc are needed for the control of catabolism of IgGs was suggested by others who showed that SpA-B fragment complexed with Fc in a stoichiometry of one SpA-B fragment molecule per Fc were cleared rapidly from the circulation [32], but at this time no catabolic site had been localized to specific amino acid residues.

The mechanism of catabolism of IgG is currently unclear, and the data presented herein and in an earlier report [3] support the hypothesis of Brambell and colleagues [33]. This hypothesis postulates the presence of cellular receptors that bind to and protect the IgG molecules from degradation [33]. These receptors are saturable, and any unbound IgG is destined for catabolism. To date, the nature of the receptors, the cell types in which they are expressed and their subcellular location are uncharacterized. Our findings indicate the necessity for two functional catabolic sites per Fc for serum persistence, and this suggests that the increase in avidity of Fc fragments brought about by the presence of two sites per molecule results in improved binding to 'protective' receptors. Thus, within the framework of the Brambell hypothesis, the hybrid molecule competes relatively poorly with the endogeneous IgGs for binding and is degraded rapidly. However, the longer β phase half-life of the hybrid relative to that of the HQ-310/HN-433 mutant [3] suggests that the hybrid does retain some binding affinity for the protective receptors. The testing of this possibility awaits the isolation and characterization of the putative receptors using *in vitro* binding studies.

Interestingly, it has recently been suggested that dimeric IgA stimulates transcytosis by cross-linking of the poly-Ig receptor [34, 35], and by analogy, cross-linking of protective receptors by bivalent IgGs/Fcs may be involved in the control of IgG catabolism. Alternatively, the difference in half-lives for the WT Fc-hinge and Fc-hybrid may solely be a reflection of the different avidity of binding to protective receptors. If the latter is the case, this is in contrast to the findings of a study in which the human FcγRI receptor has been shown to bind to a murine IgG1-IgG2a hybrid, demonstrating that the interaction between receptor and ligand requires only one of the IgG2a heavy chains [36].

In earlier experiments concerning the catabolic rates of Fc-hinge fragments and mutant derivatives, mutations in the CH2 domain were found to have a greater effect on the pharmacokinetics than those in the CH3 domain [3]. Consistent with these data, the CH2-hinge dimer has a β phase half-life (61.6 h) that is significantly less than that of the Fc-hinge fragment but greater than that of an Fv or CH3 fragment, and this suggests that sequences additional to those in the CH2 domain are necessary to constitute this catabolic control site of the IgG1 molecule. The more rapid clearance of the CH2-hinge dimer relative to the Fc-hinge fragment is also consistent with the findings of a study in which the half-lives of murine IgG2a/2b hybrids were analysed [8], leading to the conclusion that regions within both the CH2 and CH3 domains are involved in catabolic control. Further evidence supporting the involvement of CH3 domain residues in catabolism control is derived from earlier experiments of Arend and Webster [37] in which proteolytic removal of a carboxyterminal peptide from the CH3 domain resulted in the rapid elimination of the truncated Fc fragment. However in our study, the isolated CH3 domain has a half-life that is similar to that of an Fv, suggesting that the CH3 domain residues that are involved in catabolism control are alone not sufficient to confer serum persistence. Alternatively, the correct folding of the region of the CH3 domain that is involved in catabolism control may be dependent on the presence of the CH2 domain.

In summary, for serum persistence the requirement for two functional catabolic sites per murine IgG1 molecule has been demonstrated using CH2-hinge dimer/monomer and an Fc-hybrid. This finding has important implications for the design of experiments to modify the pharmacokinetics of therapeutic proteins by tagging with IgG domains. Tagging of a protein with an Fc-derived fragment containing only one functional catabolic site would be predicted to be ineffective in significantly extending the half-life, and this may explain the rather short half-life of a recently described CD4-CH2-CH3-Pseudomonas exotoxin chimera [38]. The similarities between the catabolism of immunoglobulins in man and mouse [39] indicate that the data from this study can be extended to the persistence of antibodies in the circulation of man, and this may be of value in the design and engineering of IgGs for use in therapy.

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REFERENCES

1 Waldmann TA. Monoclonal antibodies in diagnosis and therapy. Science 1991;252:1657–62.
2 Zuckier LS, Rodriguez LD, Scharff MD Immunologic and pharmacologic concepts of monoclonal antibodies. Semin Nucl Med 1989;29:166–86.
3 Kim J-K, Tsen M-F, Ghetie V, Ward ES. Identifying amino acid residues that influence plasma clearance of murine IgG1 fragments by site-directed mutagenesis. Eur J Immunol 1994;24:542–8.
4 Kabat EA, Wu TT, Perry HM, Gottesman KS, Foeller C.

Sequences of proteins of Immunological Interest. Bethesda, MD: U.S. Department of Health and Human Services, 1991, 661–96.
5 Burton DR. Immunoglobulin G: Functional sites. Molec Immunol 1985;22:161–206.
6 Ellerson JR, Yasmeen D, Painter RH, Dorrington KJ. Structure and function of immunoglobulin domains. III. Isolation and characterization of a fragment corresponding to the Cγ2 homology region of human immunoglobulin G1. J Immunol 1976;116:510–7.
7 Yasmeen D, Ellerson JR, Dorrington KJ, Painter RH. Structure and function of immunoglobulin domains. IV. The distribution of some effector functions among the Cγ2 and Cγ3 homology regions of human immunoglobulin G[1]. J Immunol 1976;116: 518–26.
8 Pollock RR, French DL, Metlay JP, Birshtein BK, Scharff MD. Intravascular metabolism of normal and mutant mouse immunoglobulin molecules. Eur J Immunol 1990;20:2021–7.
9 Deisenhofer J, Colman PM, Epp O, Huber R. Crystallographic studies of a human Fc fragment. II. A complete model based on a Fourier map at 3.5 A resolution. Hoppe-Seyler's Z Physiol Chem 1976;357:1421–34.
10 Rüther U, Koenen M, Otto K, Müller-Hill B. pUR322, a vector for cloning and rapid chemical sequencing of DNA. Nucl Acids Res 1981;9:4087–98.
11 Evan GI, Lewis GK, Ramsay G, Bishop JM. Isolation of monoclonal antibodies specific for human c-myc oncogene product. Mol Cell Biol 1985;5:3610–6.
12 Campana D, Janossy G, Bofill M *et al.* Human B cell development I. Phenotypic differences of B lymphocytes in the bone marrow and peripheral lymphoid tissue. J Immunol 1985;134: 1524–9.
13 Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory, 1989.
14 Saiki RK, Gelfand DH, Stoffel S *et al.* Primer-directed amplification of DNA with a thermostable DNA polymerase. Science 1988;239:487–91.
15 Honjo T, Obata M, Yamawaki-Kataoka Y *et al.* Cloning and complete nucleotide sequence of mouse immunoglobulin γ1 chain gene. Cell 1979;18:559–68.
16 Ward ES. Secretion of T cell receptor fragments from recombinant *Escherichia coli* cells. J Mol Biol 1992;224:885–90.
17 Sanger F, Nicklen S, Coulsen AR. DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 1977;74: 5463–7.
18 Ward ES, Güssow DH, Griffiths AD, Jones PT, Winter G. Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli.* Nature 1989; 341:544–6.
19 Edelman GM, Cunningham BA, Gall WE, Gottlieb PD, Rutishauser U, Waxdal MJ. The covalent structure of an entire γG immunoglobulin molecule. Proc Natl Acad Sci USA 1969;63: 78–85.
20 Hochuli E, Bannwarth W, Döbeli H, Gentz R, Stüber D. Genetic approach to facilitate purification of recombinant proteins with a novel metal chelate adsorbent. Bio/technol 1988;6:1321–5.
21 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227: 680–5.
22 Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979;76:4350–4.
23 Ward ES. Expression and secretion of T-cell receptor Vα and Vβ domains using *Escherichia coli* as a host. Scand J Immunol 1991;34:215–20.
24 Spratt BG, Hedge PJ, te Heesen S, Edelman A, Broome-Smith JK. Kanamycin-resistant vectors that are analogues of plasmids pUC8, pUC9, pEMBL8 and pEMBL9. Gene 1986;41:337–42.
25 Harlow E, Lane D. Antibodies: A Laboratory Manual, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory, 1988.
26 Fraker PJ, Speck JC. Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycouril. Biophys Res Comm 1978;80:849–57.
27 Perrier D, Mayerson M. Noncompartmental determination of the steady state volume of distribution for any mode of administration. J Pharm Sci 1982;71:372–3.
28 Schumaker RC. PKCALC: a basic interactive complex program for statistical and pharmacokinetic analysis of data. Drug Metab Rev 1986;17:331–48.
29 Silverton EW, Navia MA, Davies DR. Three-dimensional structure of an intact human immunoglobulin. Proc Natl Acad Sci USA 1977;74:5140–4.
30 Deisenhofer J. Crystallographic refinement and atomic models of human Fc fragment and its complex from fragment B of protein A from *Staphylococcus aureus* at 2.9 and 2.8 A resolution. Biochemistry 1981;20:2361–70.
31 Amit AG, Mariuzza RA, Phillips SEV, Poljak RJ. Three-dimensional structure of an antigen-antibody complex at 2.8 A resolution. Science 1986;233:747–53.
32 Dobre M-A, Dima S, Moraru I. Fate of protein A released in circulation after extracorporeal perfusion of plasma over immobilized protein A. Rev Roum Biochim 1985;22:3–10.
33 Brambell FWR, Hemmings WA, Morris IG. A theoretical model of γ-globulin catabolism. Nature 1964;203:1352–5.
34 Kaetzel CS, Robinson JK, Chintalacharuvu KR, Vaerman J-P, Lamm ME. The polymeric immunoglobulin receptor (secretory component) mediates transport of immune complexes across epithelial cells: a local defense function for IgA. Proc Natl Acad Sci USA 1991;88:8796–800.
35 Song W, Bomsel M, Casanova J, Vaerman J-P, Mostov K. Stimulation of transcytosis of the polymeric immunoglobulin receptor by dimeric IgA. Proc Natl Acad Sci USA 1994;91:163–6.
36 Koolwijk P, Spierenburg GT, Frasa H, Boot JHA, van de Winkel JGJ, Bast BJEG. Interaction between hybrid mouse monoclonal antibodies and the human high affinity IgG FcR, huFcγRI, on U937. Involvement of only one of the mIgG heavy chains in receptor binding. J Immunol 1989;143:1656–62.
37 Arend WP, Webster DE. Catabolism and biologic properties of two species of rat IgG2a Fc fragments. J Immunol 1977;118:395–400.
38 Batra JK, Kasturi S, Gallo MG *et al.* Insertion of constant region domains of human IgG1 into CD4-PE40 increases its plasma half-life. Mol Immunol 1993;30:379–86.
39 Waldmann TA, Strober W. Metabolism of immunoglobulins. Prog Allergy 1969;13:1–110.

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