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Chimeric Human-Mouse IgG Antibodies with Shuffled Constant Region Exons Demonstrate that Multiple Domains Contribute to *in Vivo* Half-Life

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ABSTRACT

Structural features that determine the differing rates of immunoglobulin catabolism are of great relevence to the engineering of immunologically active reagents. Sequences in the C_H2 and C_H3 region of IgG have been shown to regulate the rate of clearance through their interaction with FcRn. In an attempt to probe additional structural features that regulate antibody half-life, we have investigated two families of chimeric antibodies, composed of identical murine heavy and light antidansyl variable regions joined to human κ light-chains and wild-type or shuffled human IgG heavy-chain constant regions. These antibodies were iodinated, and their clearance was studied in severe combined immunodeficient mice hosts by whole-body radioactivity measurements. Clearances of the wild-type and recombinant antibodies were biphasic. In a panel of immunoglobulins derived from IgG_2 and IgG_3, as successive domains were varied from γ_2 to γ_3, β-phase half-life gradually decreased from 337.0 h to 70.6 h. Statistical analysis suggested that the composition of each of the three domains affected half-life, and no single region of the molecule by itself determined the rate of clearance. In the second panel of immunoglobulins derived from IgG_1 and IgG_4, the construct with the amino terminus portion of the molecule derived from IgG_4, joined within the C_H2 domain to the COOH terminus portion of IgG_1, had a half-life paradoxically greater than either IgG_1 or IgG_4 ($P < 0.012$). All four IgG_1/IgG_4 constructs demonstrated presence of the concentration catabolism phenomenon, which is a unique hallmark of immunoglobulin catabolism. The contribution of all three constant region domains to immunoglobulin half-life may be due to distant conformational effects in addition to direct binding to protective receptors, and emphasizes the importance of distant sequences on the rate of immunoglobulin catabolism. Interesting possibilities regarding mechanisms controlling immunoglobulin metabolism are raised by the hybrid γ_4/γ_1 molecule with a half-life greater than either parental immunoglobulin. Understanding the relationships between the structure of these molecules and their clearance rate will further our ability to produce immunoglobulins with improved pharmacokinetic properties.

INTRODUCTION

Over the last 2 decades, the concept of immunologically-targeted diagnostic and therapeutic agents has advanced from a laboratory curiosity to human clinical trials and ultimately to U. S. F. D. A. approval, in selected instances (1). Four radiolabeled murine monoclonal antibodies are presently approved for human diagnostic use, and a number of other immunological agents are in the developmental process for diagnostic and therapeutic applications (1, 2). A limitation of many of these reagents is that they contain at least portions of murine immunoglobulins, which can lead to the production of human antimouse antibodies, affecting subsequent biodistribution (3) and potentially interfering with antigen binding. For this reason, recombinant DNA techniques have been applied to redesign these foreign antibody molecules, making them more human-like, by "chimerization" (4) or "humanization" (3, 5, 6). Antibody constant-region structure also determines the rate of intravascular clearance, which is a crucial variable in pharmacokinetics of antibody delivery. Modification of half-life by antibody engineering has not been as greatly explored, in part because the molecular structures that control immunoglobulin homeostasis have only recently begun to be elucidated in detail (7–9).

From an experimental point of view, a study of the regulation of metabolism of human antibodies would be most naturally examined in man; however, relevant studies cannot be easily performed in human subjects due to ethical and safety concerns, and animal investigation must instead represent the initial mode of study. We have previously reported on a model system (10) for studying the half-life of iodinated chimeric antibodies in SCID mice (11) that lack a "mouse antihuman antibody" response. Four chimeric proteins were studied, composed of identical murine antidansyl variable regions and human κ light-chains and IgG heavy-chains. Whole-body half-lives for IgG_{1-4} were determined to be 170.0 ± 15.5, 312.3 ± 30.8, 59.8 ± 4.18, and 71.0 ± 3.34 h, respectively, and the concentration-catabolism phenomenon, a hallmark of normal IgG catabolism (12, 13), was preserved. In this model system, the terminal or β phase of whole-body half-life was statistically indistinguishable from that of intravascular half-life (10). In an attempt to probe the structural correlates of antibody catabolism, we now extend our observations to include chimeric antibodies with successively shuffled or otherwise rearranged human constant regions, which have been useful tools in elucidating the structural basis of other immunoglobulin properties (14, 15).

MATERIALS AND METHODS

Immunoglobulins. Two panels of immunoglobulins were studied, γ_2/γ_3 constructs derived from portions of the IgG_2 and IgG_3 molecule and γ_1/γ_4 constructs derived from IgG_1 and IgG_4 (Table 1). These recombinant immunoglobulins are composed of identical murine heavy and light antidansyl variable regions joined to human κ light-chains and wild-type or recombinant human IgG heavy-chain constant regions. Construction and expression of the immunoglobulins has been described previously (15). In the γ_2/γ_3 constructs, the heavy-chain constant region domains are positioned appropriately within the immunoglobulin molecule, but derive variably from either γ_2 or γ_3. In this panel, the hinge region always originates from the same isotype as the C_H1 domain, resulting in eight possible permutations of the heavy-chain constant region domains, of which six were available for analysis in the present study. The second panel of recombinant γ_1/γ_4 constructs is composed of shuffled IgG_1 and IgG_4 constant-region domains, with DNA rearrangement performed in the mid-C_H2 domain sequences, resulting in a total of four permutations (Table 1).

Proteins were gently iodinated with ^{125}I by the Iodogen (Pierce Chemical Co., Rockford, IL) method (16) to a specific activity of approximately 1–2 μCi/μg as described previously (10). Free iodine was separated from the iodinated proteins by passage over a size exclusion column (Sephadex G-25; Pharmacia Fine Chemicals, Piscataway, NJ).

Animals. Adult SCID[2] mice were used to study half-life, according to our previously described model (10). Thyroid uptake of radioiodine was blocked

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[2] The abbreviation used is SCID, severe combined immunodeficient.

Table 1 *Composition and half-life of wild-type and recombinant immunoglobulins*

β-phase whole-body half-life (mean ± S.D.) for two families of iodinated recombinant human immunoglobulin constructs, injected intravenously into SCID mice.

	Composition					
Family	C_H1	Hinge	C_H2	C_H3	Name	Half-life (h)
γ_2/γ_3	2	2	2	2	IgG_2	337.0 ± 13.7
	2	2	3	2		292.6 ± 21.9
	2	2	2	3		195.5 ± 6.2
	3	3	2	3		128.1 ± 3.2
	3	3	3	2		102.6 ± 16.9
	3	3	3	3	IgG_3	70.6 ± 2.9
γ_1/γ_4	1	1	1	1	IgG_1	199.0 ± 9.8
	1	1	1/4	4		172.1 ± 4.3
	4	4	4/1	1		281.5 ± 8.2
	4	4	4	4	IgG_4	77.3 ± 10.1

by the addition of three drops of Strong Iodine (Lugol's) Solution U. S. P. (Regional Service Center, Inc., Woburn, MA) to the drinking water for 1 week before and during the duration of the study. Prior use of this protocol has effectively blocked thyroid uptake in clearance and imaging studies. Each of the six immunoglobulins of the γ_2/γ_3 panel were studied in three mice, whereas the four γ_1/γ_4 constructs were studied in five mice.

Whole-Body Half-Life Determination. The basic method of administration and measurement has been detailed previously (10). Each mouse received injections in the tail vein of approximately 0.1 μCi of activity in a volume of 0.1 ml of PBS. Whole-body retention of the radiolabeled antibodies was followed using one of two configurations of whole-body counters, in a variation of our previously described method (10). For the γ_2/γ_3 panel, counts were obtained using an early-vintage commercial whole-body animal counter (ARMAC Scintillation Detector; Packard Instruments, Downers Grove, IL), whereas for the γ_1/γ_4 constructs, a custom-designed well-type NaI scintillation detector (Wm. B. Johnson and Associates, Fairea, WV) was interfaced to a multichannel analyzer (Packard Series 35; Packard Instruments). Whole-body measurements were taken immediately after injection of immunoglobulin, and at frequent intervals thereafter over a span of 2 weeks. At the conclusion of half-life measurements in the γ_1/γ_4 constructs, three of five mice in each of the four groups received i.p. injections of 50 mg of reconstituted human γ-globulin (Cohn Fraction II and III; Sigma Chemical Co.). An additional whole body measurement was performed 2 days thereafter, to evaluate for a change in the rate of catabolism.

Whole body measurements were corrected for background radioactivity and isotopic decay, and the retained activity was described as a percentage of that present immediately after injection. Terminal (β-phase) half-life of whole body disappearance of the labeled antibody was calculated by nonlinear regression of the data points from 2 days onward, using a commercially available pharmacokinetic software package (PCNONLIN; SCI Software, Inc., Lexington, KY), as described (10).

Tests of Statistical Significance. Because of the small sample size and variable SD, nonparametric tests were initially used. The overall effect of antibody composition on half-life was evaluated by the Kruskal-Wallis test, and pair-wise comparisons were performed using the Wilcoxon Rank-Sum test. For the panel of γ_1/γ_4 constructs, six multiple comparisons were made among the four groups, and significance was determined by comparison with the adjusted type-1 error probability. For the 15 pairwise comparisons of the

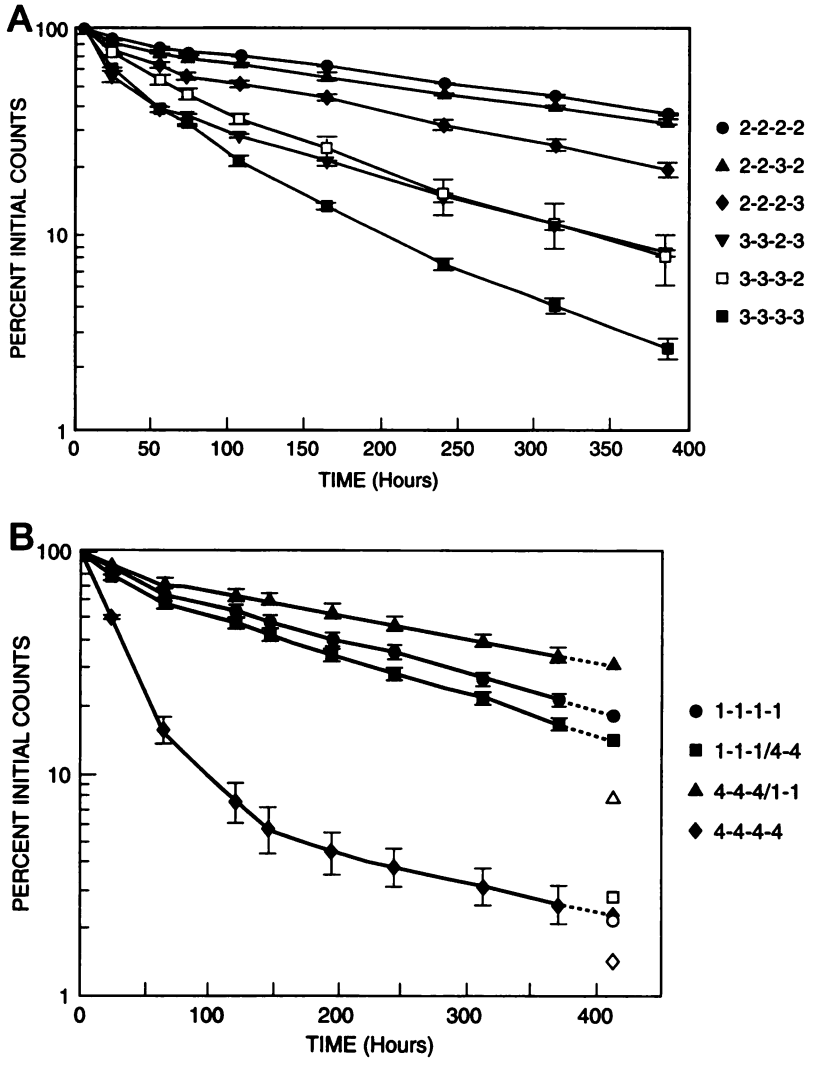

Fig. 1. Semilog plot of retained whole-body radioactivity of the recombinant antibodies expressed as the percentage of initial counts. The error bars signify one SD. *A*, panel of six γ_2/γ_3 antibodies. Each point represents the mean of three mice. *B*, panel of four γ_1/γ_4 antibodies. Each point represents the mean of five mice, with the exception of the final points which represent the mean of three animals treated with 50 mg of human γ-globulin IP (△, □, ○, ◇) or the mean of the two untreated animals (▲, ●, ■). In all four groups, the whole-body clearance of radiolabeled antibody was accelerated in the animals administered human γ-globulin, consistent with the normally described concentration-catabolism phenomenon.

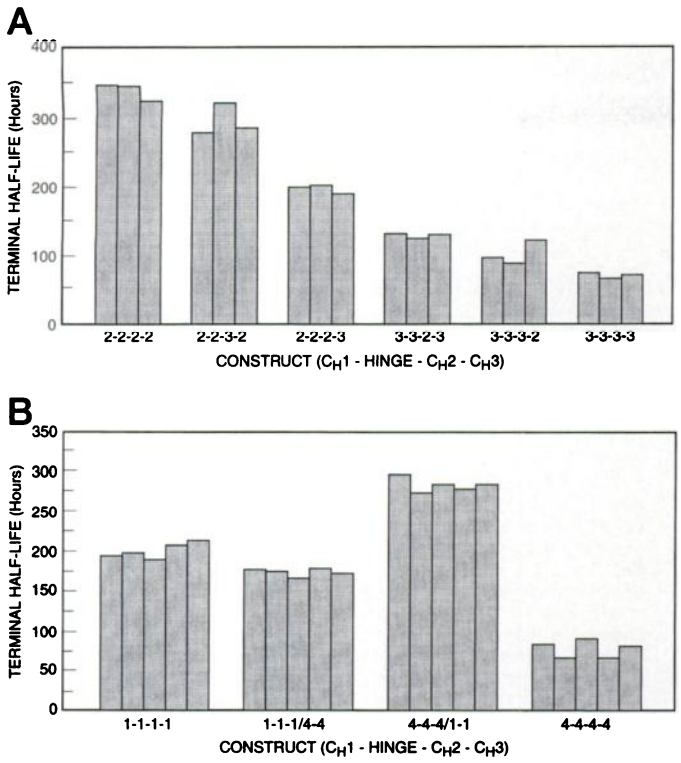

Fig. 2. Terminal (β) phase half-life of whole-body clearance of the iodinated antibody. *A*, half-life of the γ_2/γ_3 antibodies with the three animals in each group (*bar*). The half-life gradually decreases as the composition progressed from IgG_2 to IgG_3. *B*, half-life of the γ_1/γ_4 panel with each of the five animals/group (*bar*). Of note, the terminal half-life of the 4–4-4/1–1 recombinant molecule exceeds that of both wild-type parental immunoglobulins.

six γ_2/γ_3 constructs, the small sample size precluded attaining clear-cut significance using the highly conservative nonparametric techniques, and analysis of boderline significance was further supplemented by performing a one-way ANOVA with 15 pairwise comparisons.

RESULTS

Clearances of the wild-type and recombinant antibodies was biphasic, with a more rapid initial clearance followed by a slower subsequent rate, conforming to a model of first-order kinetics (Fig. 1, *A* and *B*). Individual β-phase half-lives are displayed in Fig. 2, *A* and *B*, and grouped statistical parameters are listed in Table 1. In the γ_2/γ_3 panel, as successive domains were varied from γ_2 to γ_3, half-life gradually decreased from 337.0 h to 70.6 h. The overall comparison among the six groups was significant with a P of 0.0054. All of the 15 pairwise comparisons were identically significant with borderline Ps of 0.0495, as compared with the adjusted P of 0.05/15. Although half-life values of animals within different groups did not overlap each other and were, therefore, maximally significant, the borderline significance was attributed to the small group size ($n = 3$) in this nonparametric test. A less conservative parametric analysis using ANOVA suggested that significant results would be obtained in larger cohorts for each of the pairwise comparisons ($P < 0.003$), with the exception of 3–3-3–2 *versus* either 3–3-2–3 or 3–3-3–3.

In the panel of γ_1/γ_4 constructs, the antibody with γ_4 amino terminus spliced to γ_1 COOH terminus within the C_H2 domain (4–4-4/1–1) had a half-life of 281.5 h, greater than either parental immunoglobulin (half-lives of 199.0 and 77.3 h for IgG_1 and IgG_4, respectively), whereas the antibody with γ_1 amino terminus spliced to γ_4 COOH terminus (1–1-1/4–4) had an intermediate half-life of 172.1 h. The overall group comparison was significant ($P = 0.0005$), as were all six pairwise comparisons relative to the adjusted type-1 error P of 0.0083 (0.05/6; $P = 0.0122$). Finally, all four $IgG1/IgG_4$ constructs demonstrated presence of the concentration catabolism phenomenon, evidenced by an accelerated rate of clearance after γ-globulin administration at 15 days (Fig. 1*B*).

DISCUSSION

Intravascular and whole-body clearance of administered antibodies are key variables in describing pharmacokinetic behavior. Knowledge of structural features that control half-life is invaluable in designing immunological molecules for diagnostic or therapeutic human use. Despite this importance, mechanisms and structures that regulate immunoglobulin half-life have only recently begun to be elucidated (7, 8, 17–19).

An early model of IgG regulation, proposed by Brambell *et al.* (20), attempts to address the unusual concentration-catabolism phenomenon unique to IgG catabolism, whereby increasing IgG levels result in an accelated rate of catabolism. Brambell (21) hypothesized a specific, saturable receptor for immunoglobulin, which conferred protection from degradation. The identity of such a receptor has recently been shown by several groups (17–19) to be the MHC class I-related receptor, FcRn, which binds sequences in the C_H2 and C_H3 region of the IgG molecule implicated in regulation of murine immunoglobulin metabolism (7, 8).

An animal model of antibody clearance is an important first step to probing the catabolic behavior of human antibodies. Nonetheless, one must interpret the results in this heterologous system with some degree of caution because it is possible that human immunoglobulins may not interact appropriately with the putative mouse receptors. For example, in man, the IgG subclasses have half-lives of approximately 25 days, with the exception of IgG_3, which has a half-life of 7 days (12). In the SCID mouse, both IgG_3 and IgG_4 are noted to have shorter half-lives than IgG_1 and IgG_2 (10), suggesting that the regulation of the intravascular metabolism of the human isotypes is, at best, only partially reflected in the mouse model. Nonetheless, preservation of the concentration-catabolism phenomenon, a unique feature of IgG metabolism (12, 13), does speak for some fidelity to normal catabolic pathways.

As a general rule, previous work defining the structural correlates of antibody functional behavior have associated various attributes with specific loci on the molecule. For example, complement binding occurs on an exposed face of the C_H2 domain (15), whereas hinge-proximal sequences in C_H2 have been associated with binding to the human FcγRI receptor (22). In contrast, the present investigation suggests that all three regions contribute incrementally to the overall rate of catabolism. The C_H2-C_H3 domain interface, which interacts with the putative "Brambell" receptor mentioned above (17–19), has been demonstrated to be of importance in regulating metabolism of IgG_1 in mice (7, 8). Our findings do not contradict this conclusion, but instead emphasize that multiple regions are likely involved in the regulation of immunoglobulin half-life, either exerting their effects by conformational changes on the C_H2 and C_H3 regions, altered susceptibility to proteolysis, or possibly by additional receptor interactions that have yet to be elucidated. A previous study of intrinsically labeled mutant murine immunoglobulin with hybrid heavy chains (23) has shown that the determinant of subclass-specific catabolic rate in mice resides in the COOH-terminal end of the C_H2 and in the C_H3 domain, paralleling the conclusions of Kim *et al.* (7, 8). A further conclusion of that study, consistent with the present observation, is that deletion of any of the constant domains of IgG_{2b} results in the shortening of intravascular half-life either directly or by inducing conformational changes elsewhere in the molecule (23).

A provocative finding with the γ_1/γ_4 panel of antibodies is the prolonged half-life of the γ_4/γ_1 recombinant molecule, consistently observed with different preparations of antibodies and differing sources of SCID mice (data not shown). Because binding by the putative "Brambell" receptor, FcRn, is influenced by sequences in the C_H2 region (7, 8), it may not be surprising that an intradomain mutation in this region would have a profound effect on antibody clearance. It, however, remains unexplained exactly how these changes lead to prolongation of half-life. Possibly the 4-4-4/1-1 antibody has a higher affinity for the protective receptor than either IgG_1 or IgG_4. To our knowledge, binding constants for the interaction of human constant regions with murine FcRn have not been determined. Alternatively, the increased half-life could originate from a change in the number of normal regulatory regions on the immunoglobulin molecule (a "dosage" effect), or may instead reflect a novel mechanism, possibly through conformational changes or additional interactions with a specific receptor.

The presently described whole-body half-lives of the wild-type immunoglobulins are similar to the immunoglobulin whole-body half-life values described for SCID mice in our initial study, ranging between 8% and 18% higher than initially described, and in the same relative rank order (10). This minor difference is within the range of intragroup variation typically noted on measurements performed on different occasions, possibly related to variation in animal size, metabolic rate, and other environmental factors. Our measurements may also be compared with those of other investigators who have measured the clearance of specific immunoglobulins in SCID mice, by differing methods. Bazin *et al.* (24), using immunological means, found that the serum half-life of an IgG_3 antihuman Rh(D) antibody in SCID mice was 6.3 ± 0.5 days. Hassan *et al.* (25), assaying by sandwich ELISA, demonstrated that human donor and mouse-human chimeric IgG_3 had serum half-lives of approximately 7 days in SCID mice. Although these values are longer than those noted in our present and previous studies (10), variation in apparent immunoglobulin half-life based on the method of assay is a well known phenomenon (12, 13) and is a possible cause of the difference in results. In contrast to the various techniques of assaying serum by immunological means, we have used whole-body measurements of iodinated proteins, because it avoids perturbation of the intravascular pool by periodic bleeding, which can artifactually affect the apparent clearance rate. In concordance with our findings, Bazin *et al.* (24) and Hassan *et al.* (25) demonstrated that injection of human serum resulted in an accelerated clearance of IgG_3 (concentration-catabolism phenomenon).

In summary, we have shown that all three of the constant region domains have an incremental effect on the half-life of IgG_2 and IgG_3. This effect may, in fact, be mediated by interaction of a single region of the immunoglobulin molecule with a specific receptor, as suggested by Kim *et al.* (7, 8), with conformational effects on the receptor binding site mediated by the other regions of the immunoglobulin molecule. The hybrid γ_4/γ_1 molecule, with a half-life greater than either parental immunoglobulin, raises interesting possibilities regarding mechanisms controlling immunoglobulin metabolism. Additional studies of selected immunoglobulins bearing single amino acid changes will define the relationship between the affinity for FcRn and the observed serum half-life and may suggest additional elements that regulate *in vivo* half-life. Understanding the mechanisms and pathways of antibody clearance will result in the ability to produce immunoglobulins with improved pharmacokinetic properties.

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