Human Myeloma IgG Half-Molecules

CATABOLISM AND BIOLOGICAL PROPERTIES

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ABSTRACT A human IgG1 myeloma protein that has a deletion in the third constant domain of the heavy chain (Cγ3) and forms two-chain half-molecules was studied for its in vivo turnover and its ability to fix C1q and hemolytic complement, to bind to human lymphocytes, neutrophils, and monocytes, and to induce a passive cutaneous reaction in guinea pigs. In both man and monkeys, the half-molecule was rapidly catabolized and in part excreted into the urine. The half-life in man was 4.3 days and the fractional turnover 165% per day; 7.6% of the intravascular pool was excreted into the urine per day. Although the 7S four-chain myeloma protein could not be obtained in a pure form, the elimination from the serum of a partially purified preparation suggested that it was also rapidly catabolized. The unaggregated half-molecule neither formed complexes with C1q, bound to human lymphocytes, neutrophils, and monocytes, nor elicited a reverse passive cutaneous reaction in guinea pigs. In contrast, the aggregated half-molecule fixed hemolytic complement and bound to the human white cells similarly to an intact IgG1 myeloma protein. In order to explain the biological activities of this half-molecule, it is postulated that IgG1 may have several (at least two) submolecular sites for a given biological activity that are localized on both the Cγ2 and Cγ3 domains. Proteins having both sites would be capable of binding to C1q and Fc cell receptors in unaggregated form whereas proteins having a site on only one domain, such as the half-molecule, must be aggregated in order to obtain this binding.

INTRODUCTION

Immunoglobulin half-molecules consisting of one heavy and one light chain are rare. IgG half-molecules were first detected in the serum of colostrum-deprived piglets (1, 2), and later IgA half-molecules were found in the urine of mice bearing certain mineral oil-induced IgA plasmacytomas (3). Hobbs and Jacobs reported the first patient with a plasmacytoma who produced half-molecules (4), and since then four more such patients have been detected (5-7). The half-molecules production does not appear to be associated with a distinct clinical syndrome since three patients had extramedullary plasmacytomas (5), one plasma cell leukemia (6), and one classical multiple myeloma (M. Seligmann, personal communication). Nevertheless, all these patients had in common that they excreted large amounts of half-molecules into their urine. Detailed structural analysis of the paraprotein of our patient (8) revealed that it lacked the noncovalent interactions characteristic for the Fc portion of the γ-chain and that it had a deletion in the third constant domain (Cγ3)[1] of the heavy chain. The size of the deletion was 5,000-8,000 daltons. It probably involved the intrachain disulfide loop of the Cγ3 domain but not the carboxyterminal structure of the gamma chain. The half-molecule expressed the genetic marker Gm(f) which is localized on the Cγ1 domain but lacked the corresponding marker Gm(non-a) as well as another marker on the Cγ3 domain. The "hinge" peptide containing the inter-heavy-heavy chain disulfide bonds appeared intact and its amino acid composition was identical to the corresponding peptide of IgG1 proteins. This explained why the patient formed also a small amount of four-chain 7S type myeloma protein. In order to investigate the biological properties of this unusual myeloma protein, we measured its rates of elimination from the circulation and excretion into the urine, its capacity to fix complement, and its ability to bind to the Fc receptors of human white cells and guinea pig mast cells.

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[1] *Abbreviations used in this paper:* Cγ2, Cγ3, the second and third constant domains, respectively, of the heavy chain; RPCA, reverse passive cutaneous anaphylaxis; TCA, trichloroacetic acid.

METHODS

Isolation of proteins. The myeloma protein of patient K. N. was isolated from his plasma and urine by a combination of DEAE-cellulose chromatography and Sephadex G-200 gel filtration (8). The half-molecule was obtained from either serum or urine in a relatively pure form whereas the 7S IgG serum fraction contained only about 35% myeloma protein, the remaining portion being normal IgG (8). IgG1 (Ma) and IgG2 (Do) myeloma proteins and normal human IgG were isolated by DEAE-cellulose chromatography using a 0.01 M phosphate buffer, pH 8.0. An IgM macroglobulin (Vi) was isolated by a combination of euglobulin precipitation, Pevikon block electrophoresis, and Sephadex G-200 gel filtration. The Fab fragment was isolated from either normal IgG or the half-molecule after digestion with papain for 18 h (8).

Aggregation of proteins. Three methods were used to aggregate the proteins for the different test methods. For complment fixation, the proteins were aggregated either by addition of 200 μg/5 mg of bis-diazotized benzidine (9) or by heating at 63°C for 5–30 min until the solution became slightly turbid. For the experiments designed to measure the binding to different cell types, the proteins were aggregated with a $F(ab')_2$ preparation of a rabbit anti-human Fab fragment antiserum (10). The $F(ab')_2$ anti-Fab antiserum precipitated immunoglobulins of all classes and light chain types when tested by double gel diffusion analysis.

Radioiodination. All proteins were radioiodinated with either ^{125}I or ^{131}I by using modifications of the chloramine T procedure. For turnover studies, protein in 5-mg aliquots was dissolved in 1 ml 0.05 M phosphate buffer, pH 7.0, and was labeled with 10 μg of chloramine T/mg by incubating for 5 min (11). The uptake of radioactivity varied between 35 and 60% and the specific radioactivity was about 0.1 μCi/μg. 95–97% of the half-molecule and 98–99% of the 7S IgG preparations were precipitable as protein bound radioactivity at a concentration of 10% trichloroacetic acid (TCA). For determination of the protein's ability to bind to cells, they were labeled with ^{125}I by a chloramine T procedure that yielded high specific activity of 20–40 μCi/μg (12). The radioactivity of the ^{131}I- and ^{125}I-labeled proteins as well as mixtures of the two isotopes were determined in a dual channel scintillation counter (Baird Atomic, Inc., Cambridge, Mass.).

Turnover studies. The protocol that has previously been described was used for turnover studies (13, 14). One study of the half-molecule isolated from the serum of patient K. N. was performed by injecting it into the author and other studies were done in monkeys of the macaca family (*M. mulatta, M. nemestrina,* and *M. speciosa*). No difference was observed in the different species. All labeled proteins were injected within 2 days after labeling. To remove any aggregates and to render the monkeys unresponsive to human IgG (14), the proteins were centrifuged at 40,000 rpm for 60 min immediately before injection. In man, 10 min after injection the first blood sample was drawn from the vein of the opposite arm used for injection and in monkeys after 5 min from the opposite leg (vena saphena). The plasmas of these first blood samples were used as the value for the 100% plasma concentration on day 0.

Urine was collected into plastic containers over 24-h periods, an aliquot cleared by centrifugation, and the total and protein bound radioactivity determined. 0.1 ml of normal serum was added per milliliter of urine as protein carrier in order to determine the protein bound radioactivity precipitable by 10% TCA.

The plasma half-lives and the percent intra- and extravascular distribution was calculated from the semilogarithmic plot of the plasma elimination curve. The total body half-life in man was calculated from the slope of the semilogarithmic plot of the radioactivity retained in the body (total injected radioactivity minus accumulated radioactivity excreted into the urine). The whole body elimination could not be determined in this manner in monkeys because complete daily urine collections could not be made. The fractional turnover rate was calculated from the radioactivity excreted into the urine per day expressed as percent of the average intravascular pool in the same 24-h period (15).

In order to determine the molecular size of the protein precipitable with TCA in the urine, the urine of a monkey injected with ^{125}I-labeled half-molecule was collected during the first 6 h after injection. 2 ml monkey serum from the same animal was added as protein carrier; the mixture was then dialyzed overnight against 0.001 M phosphate buffer, pH 8, containing toluene as a preservative and lyophilized. The lyophilized material was dissolved in saline and applied to a Sephadex G-200 column.

Complement fixation. The ability of the proteins to bind the complement component C1q was determined by analytical ultracentrifugation.[2] The immunoglobulin preparation was first cleared of aggregates by centrifugation at 100,000 *g* for 60 min in a swinging bucket rotor of a Beckman model L preparative ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) and then mixed with purified C1q kindly provided by Dr. H. J. Müller-Eberhard. The final protein concentrations were 3 mg myeloma protein preparation and 1 mg C1q. The mixture was then centrifuged at 52,000 rpm in a Beckman model E analytical ultracentrifuge equipped with schlieren optics. The sedimentation rates were calculated by standard procedures, corrected for temperature and solvent but not to zero protein concentration. It had previously been shown that IgG1, IgG2, IgG3, but not IgG4 myeloma proteins form complexes with C1q sedimenting at a rate of about 13S as compared to 7S and 11S for IgG and C1q, respectively. All the C1q participates in the complex formation whereas the excess IgG sediments behind the 13S complex as a 7S protein peak.

The fixation of CH_{50} units was performed according to a standard method (16). The aggregated proteins were diluted before testing to 100 μg protein per test with complement buffer, and the same quantity of unaggregated protein served as control.

Binding to white blood cells. The binding of the radiolabeled unaggregated and aggregated myeloma protein preparations and control IgG1, IgG2, and IgM proteins to isolated lymphocytes, neutrophils, and monocytes was determined as previously described (10). 40 ng of labeled protein was added to 5×10^6 cells in a volume of 0.1 ml and incubated for 30 min at 4°C before washing. For aggregation, 1 μg of the rabbit $F(ab')_2$ anti-Fab antiserum was used.

Reverse passive cutaneous anaphylaxis (RPCA). The ability of the myeloma proteins to bind to guinea pig skin and induce RPCA reaction was studied as described by Terry (17). Hartley guinea pigs were injected at several sites with 0.1 ml of a saline solution containing 0.1, 1, and 10 μg of protein. The animals were injected 3 h later with 0.5 ml of a goat anti-human IgG antiserum and 0.5 ml of a 1% Evans blue solution. The skin sites were examined

[2] Schumaker, V., H. L. Spiegelberg, and H. J. Müller-Eberhard. To be published.

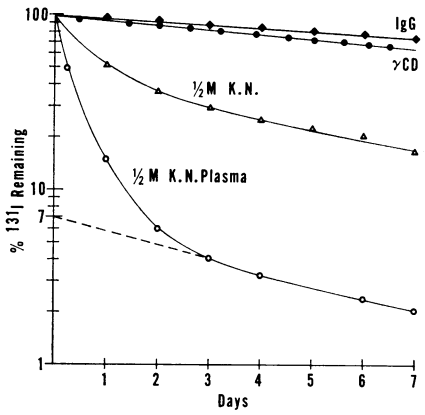

FIGURE 1 Whole body (△——△) and plasma (○——○) elimination curve of ^{131}I-labeled half-molecule K. N. in man. The whole body elimination curves of normal IgG and a gamma chain disease protein (γCD) determined by the same method (14) are shown for comparison.

after 30 min when the animals were sacrificed, skinned, and the blueing reaction measured inside the skin.

RESULTS

Turnover studies. The whole body and plasma elimination curves in man of the ^{131}I-labeled half-molecule isolated from the serum of patient K. N. are shown in Fig. 1. The turnover calculations are presented in Table I together with values obtained for normal IgG and gamma chain disease proteins in a previous study performed under the same conditions (14). The half-molecule was rapidly eliminated from the circulation and catabolized, the whole body half-life being 4.3 days and the plasma half-life 3.8 days. About 7% of the injected radioactivity remained in the intravascular compartment. An average of 165% of the intravascular pool was catabolized per day or 6.7% per h. Although the fractional turnover varied slightly from day to day (148–182%), it was no greater during the first 3 days after injection (155, 182, and 161%) than later. The semilogarithmic plot of the whole body elimination was, however, not linear and indicated that the half-molecule was more rapidly eliminated during the first few days after injection. About 10% of the radioactivity excreted daily into the urine was precipitable with TCA, which amounted to an average of 7.6% of the intravascular pool per day.

Additional turnover studies in monkeys showed almost identical plasma elimination curves to those seen in the human study (Fig. 2). In different monkeys, the half-lives of the injected half-molecule varied between 3 and 4 days, 6–7% remained in the intravascular compartment and between 9 and 15% of the radioactivity excreted into the urine was precipitable with TCA. The 7S IgG fraction of the patient K. N. was also injected paired-labeled with normal IgG, although it contained only 35% of 7S four-chain myeloma protein (8). A portion of this preparation was more rapidly eliminated from the circulation (Fig. 2) and catabolized as indicated by higher excretion of radioactivity over that of the normal IgG. 3–5 days after injection this difference became insignificant and the plasma half-life of the 7S IgG myeloma protein fraction was only slightly shorter than that of normal IgG. No significant amounts of radioactivity excreted into the urine of either preparation were precipitable with TCA during the entire period studied (0.1–0.3%). After equilibration 28–30% of the 7S IgG myeloma protein fraction and 42–49% of the normal IgG remained in the intravascular compartment.

In order to determine if the half-molecule or the 7S IgG myeloma protein fraction were fragmented during the labeling procedure, they were labeled with ^{125}I, mixed with ^{131}I-labeled normal IgG, and applied to a Sephadex G-200 column. All protein eluted as single peaks, the half-molecule at the position of 5S proteins which is characteristic of the 4.3S half-molecule, and the

TABLE I

Catabolism in Man of IgG Half-Molecule K. N. as Compared to Three Gamma Chain Disease Proteins (γCD) and Normal IgG

Protein	Whole body $t_{\frac{1}{2}}$	Percent intravascular	Percent of plasma pool	
			Catabolized	Excreted
	days		*per day*	
½M K. N.	4.3	6.7	165	7.6
γCD*	25.8	25.5	8.9	0.8
IgG*	30.6	41.3	5.2	<0.1

* Average of four volunteers (14).

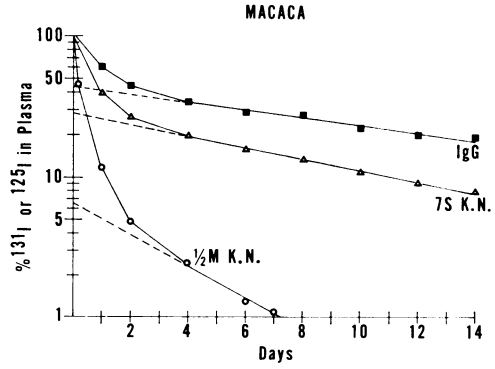

FIGURE 2 Representative plasma elimination curves of normal IgG, the 7S IgG myeloma protein fraction (7S K. N.), and the myeloma half-molecule (½M K. N.) from monkeys. Two proteins were usually injected paired-labeled with ^{131}I and ^{125}I.

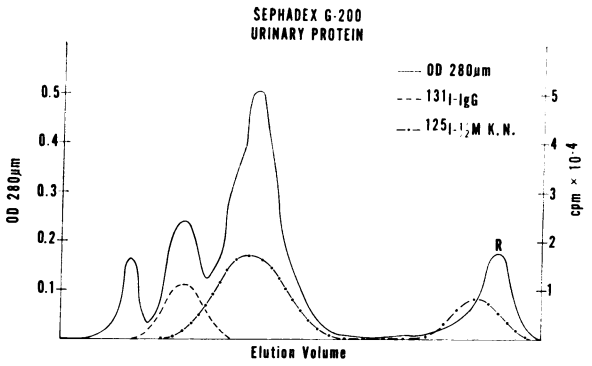

FIGURE 3 Elution profile of mixed normal monkey serum and dialyzed and lyophilized urinary protein dissolved in saline of a macaca monkey injected with ^{125}I-labeled half-molecule. The elution position of salts from this column is marked by "R." A small amount of ^{131}I-labeled normal human IgG was added to determine where IgG eluted. The urine was collected during the first day after injection of the ^{125}I-labeled half-molecule K. N.

7S IgG myeloma protein fractioned with the normal IgG, suggesting no significant change in molecular weights of the labeled proteins. A similar experiment was performed in order to determine if the protein-bound radioactivity in the urine of monkeys represented intact half-molecules or fragments thereof. As can be seen in Fig. 3, the protein-bound radioactivity of the urine eluted as a single peak at the position of 5S proteins, such as albumin, which is characteristic for the half-molecule. A second peak of ^{125}I eluted just before the reagent peak and this radioactivity was not TCA precipitable. It most likely represented either free ^{125}I or ^{125}I bound to small peptides or tyrosine or both that were not removed by dialysis during the overnight period.

The predominant equilibration of the half-molecule into the extravascular space could have been the result of an autoantibody-like activity of the Fab fragment to a tissue antigen. In order to test this hypothesis, the elimination of the Fab fragment of the half-molecule was compared with that of normal Fab fragments. However, the two preparations, after being paired-labeled and injected into monkeys, were eliminated and excreted into the urine in an almost identical manner. The plasma elimination curves did not differ significantly during the first 3 days after injection from those of the half-molecule but afterwards were more rapid with half-lives of 1.8–2.2 days. The portion that remained in the intravascular compartment was 12–13% as determined by extrapolation of the plasma elimination curve. 10–13% of the radioactivity excreted in the urine was TCA precipitable.

Complement fixation. The ability of the half-molecule and the 7S IgG myeloma protein fraction to form complexes with C1q is shown in Fig. 4. The half-molecule failed to form a significant amount of complexes. The sedimentation rate of the faster moving protein peak in the mixture was 11.1S, not significantly higher than 10.8S for the C1q control preparation. In contrast, the 7S IgG myeloma protein fraction formed complexes; all of the C1q sedimented as a protein peak of a 13.2S rate, characteristic for complexes formed between C1q and normal IgG or IgG1, 2, and 3 myeloma proteins. As described before (8) the 7S IgG myeloma protein preparation contained 65% normal IgG; therefore, we could not conclude that the 7S myeloma protein-bound C1q.

In order to determine if the aggregated half-molecule preparation could fix hemolytic complement, 100-μg aliquots of either bis-diazotized benzidine or heat-aggregated half-molecule as well as IgG1 and IgG2 myeloma proteins were tested for quantitative complement fixation. Different preparations of aggregates of the half-molecule fixed 66–78%, the IgG2 myeloma protein fixed 49–68%, and the IgG1 protein 95–100%. None of the unaggregated proteins fixed significant amounts of hemolytic complement, indicating that they were not anticomplementary.

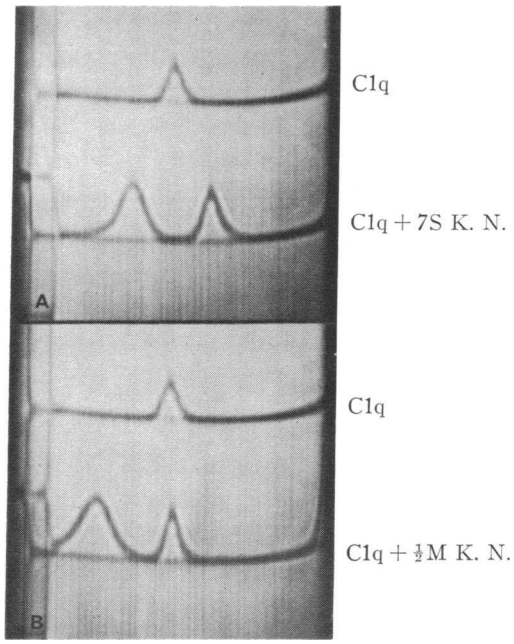

FIGURE 4 Ultracentrifugal analysis of the complement component C1q and mixtures of C1q and the 7S IgG myeloma protein fraction K. N. (A) and the half-molecule K. N. (B). The uncorrected sedimentation rates were 10.8S for the isolated C1q preparation, 13.2S for the complex, and 6.7S for the 7S K. N. preparation (A), 11.1S for the C1q peak, and 4.3S for the half-molecule peak in the mixture of these proteins (B). The picture was taken 48 min after reaching two-thirds of maximum centrifugation speed (52,600 rpm).

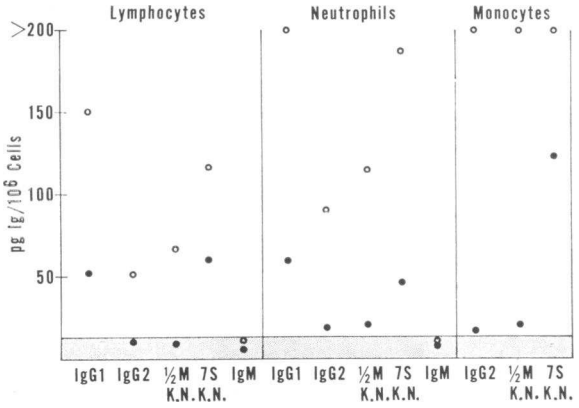

FIGURE 5 Binding of ^{125}I-labeled myeloma proteins of IgG1 and IgG2 subclasses, the half-molecule K. N., the 7S IgG serum fraction K. N., and a IgM macroglobulin in unaggregated form (●) and after aggregation (○) with a rabbit F(ab')$_2$ anti-human Fab antiserum. The shaded area indicates binding which was considered insignificant because it was also obtained with other proteins such as bovine serum albumin (14).

Binding to lymphocytes, neutrophils, and monocytes. The binding of unaggregated and aggregated half-molecules, 7S IgG myeloma protein fraction, IgG1 and IgG2 myeloma protein, and an IgM macroglobulin are shown in Fig. 5. In the unaggregated form, only insignificant amounts of the half-molecule bound to these white cells, the quantity being similar to those of the IgG2 myeloma protein and the IgM macroglobulin (10). The 7S IgG myeloma protein preparation bound to a similar extent as the IgG1 myeloma protein. However, as mentioned above it contained 65% normal IgG, and therefore we could not evaluate its performance alone. After aggregation, significant quantities of the half-molecule bound to all three cell types, the absolute amounts being similar to those of the IgG2 myeloma protein which also bound significantly only in aggregated form. In contrast, binding of the IgM macroglobulin, which served as a negative control, did not increase significantly after aggregation.

RPCA in guinea pigs. In order to determine if the half-molecule could bind to guinea pig mast cells and elicit a RPCA reaction, the half-molecule and the IgG1 myeloma protein were injected into guinea pigs 0.1–10 μg per skin site. The half-molecule did not elicit an RPCA reaction in any of the six animals tested. In contrast, the IgG1 myeloma protein usually produced a slight reaction after 0.1 μg was injected per site, a reaction of 8-35 mm in diameter at 1 μg, and after 10 μg a large and diffuse blueing reaction measuring over 40 mm in diameter.

DISCUSSION

These experiments demonstrate that an IgG1 myeloma protein that has a deletion in the Cγ3 domain and forms half-molecules differs greatly from intact IgG1 myeloma proteins in its secondary biological functions (18). The half-molecule was very rapidly catabolized and in part excreted into the urine. The unaggregated half-molecule failed to form complexes with the complement component C1q and to bind to Fc receptors of human lymphocytes, neutrophils, and monocytes or to guinea pig mast cells. In contrast, the aggregated half-molecule fixed hemolytic complement and bound to lymphocytes, neutrophils, and monocytes, indicating that it did not lack all the structures on the Fc fragment responsible for these biological activities. The covalent four-chain 7S myeloma protein could not be obtained in pure form because the patient's serum contained only a small quantity of such molecules. However, a significant portion of the 7S myeloma protein preparation was rapidly catabolized, suggesting that the 7S myeloma protein also lacked the structures responsible for a slow turnover. Whether an essential structure was missing or the deletion caused a change in the overall configuration resulting in lack of expression of the active site are presently unknown. The ability of the aggregated proteins to fix complement and bind to cells suggests that the sites responsible for secondary biological functions are not totally absent but that the affinity of the remaining site in the unaggregated protein is too low to bind effectively. It appears from these studies that only a relatively intact Fc fragment can mediate the secondary biological functions normally.

The short survival and excretion into the urine of the half-molecule were markedly different from those of Fc fragments and gamma chain disease proteins (14, 19) but were similar to those of the half-molecule's Fab fragment and of Bence Jones proteins (20). One explanation for the fast turnover and excretion of the half-molecule could have been fragmentation and alteration occurring during the labeling procedure. However, no evidence for fragmentation or aggregation of the labeled myeloma protein was obtained. Furthermore, Fc fragments and gamma chain disease proteins labeled under the same conditions showed long plasma half-lives and a low rate of excretion into the urine (14). Moreover, the patient's clinical manifestations also indicated that the half-molecule turns over rapidly. The serum of the patient showed only a modest 1.5 g/100 ml myeloma spike despite general myeloma involvement, and he excreted 300 mg/100 ml of half-molecules into the urine in the absence of overt renal failure (6). It appears most likely, therefore, that the half-molecule as well as 7S myeloma protein had rapid turnovers because they did

not express sites on their Fc fragments that are necessary for the characteristic slow catabolism of IgG.

The biphasic whole body elimination curve is not fully understood. Although it could be the result of alteration of a large portion of the labeled protein, it could also be the result of the rapid turnover of the half-molecule. Many assumptions that are generally made in turnover studies are not true for short-lived proteins (15, 20), and biphasic whole body elimination curves have also been seen with Bence Jones proteins (20). One condition for accurately studying the kinetics of elimination is the rapid exchange between intra- and extravascular compartments. The fact that this condition was probably not fulfilled in this study could explain the biphasic curve and notable extravascular distribution of the half-molecule. Since only protein of the intravascular compartment (or in rapid exchange with it) is catabolized (15), many more half-molecules were catabolized during the first few days after intravenous injection. Later, a large portion moved to the extravascular compartment and therefore was not catabolized, resulting in a slower overall whole body elimination curve. Evidence for this explanation comes from the relatively constant daily fractional turnover rate which measures only the turnover of the intravascular pool.

The half-molecule with its deletion in the $C\gamma3$ domain is a unique protein in which to study the localizations of active sites within the Fc fragment. Recently, a number of investigators have studied Fc subfragments having either only the $C\gamma2$ domain (21, 22) or only the $C\gamma3$ domain (23) for their biological activities. Controversial findings have, however, been reported from different laboratories. Interaction with complement components has been localized to the $C\gamma2$ domain by some investigators (22, 24, 25) whereas others recovered activity in peptides of both the $C\gamma2$ and $C\gamma3$ domain (26). Similarly, the sites reacting with the Fc cell receptors have been reported to be in the $C\gamma2$ domain (27) and in the $C\gamma3$ domain (25, 28, 29). The activities of the half-molecule may help to resolve this controversy. If it is assumed, as shown in the scheme of Fig. 6, that a given function has several sites on both domains and that at least two sites must be in close proximity in order to express activity, the behavior of the Fc subfragments as well as the half-molecule could be explained. In this hypothesis IgG1 and IgG3 would have sites on both domains and could therefore react with C1q and cells in unaggregated form. In contrast, the half-molecule would have sites only on the $C\gamma2$ domain and could therefore react only when aggregated. IgG2 and IgG4 proteins would have also sites on only one domain since they behave similarly to the half-molecule with respect to cell binding (10). They might have additional sites of a lower affinity on the other domain, and those could be responsible for the subtle quantitative differences in cell binding between IgG2 and IgG4 proteins (10) and the differences between IgG1, IgG3, and IgG2 proteins in complement fixation (18, 30) and the observation that IgG4 Fc fragments but not intact IgG4 can fix C1 (31). Since the different domains have probably evolved by gene duplication as shown by the structural homology to one another (32), it seems not unlikely that both may have sites capable of reacting with complement components or Fc cell receptors. To test this hypothesis conclusively the presence or absence of activity in the domains of the four IgG subclasses should be be demonstrated with either Fc subfragments or myeloma proteins having a deletion in another area of the Fc fragment.

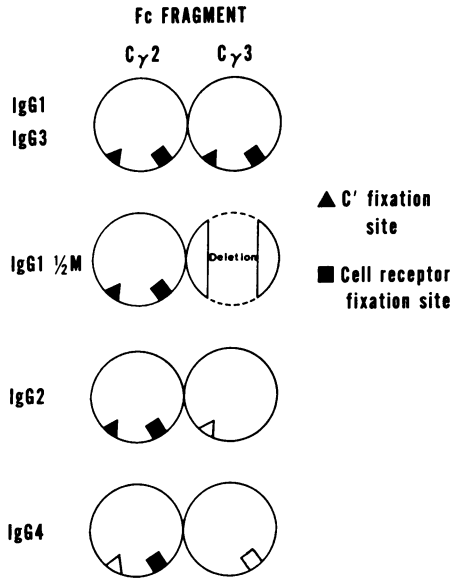

FIGURE 6 Scheme of hypothetical localization of sites responsible for complement fixation (▲) and cell binding (■). IgG1 and IgG3 would have sites on both domains but IgG2 and IgG4 on only one domain (arbitrarily assigned to the $C\gamma2$ domain). Only proteins having sites on both domains can bind to C1q and to cells whereas those with sites on only one domain, including the IgG1 half-molecule, bind only in aggregated form. IgG2 and IgG4 may have additional sites having a lower affinity to complement (△) or cells (□), and these may be responsible for the more subtle differences between the subclasses' abilities for complement and cell fixation (10, 30, 31).

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