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Immunology

Expression of biological effector functions by immunoglobulin G molecules lacking the hinge region

(complement/Fc receptors/protein A/conformation/segmental flexibility)

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ABSTRACT **Several biological effector functions mediated by sites on the Fc region of human IgG1 have been studied in two variant IgG1κ monoclonal proteins (Dob and Lec) which contain deletions corresponding to the entire hinge region of the heavy chains. Neither Dob nor Lec protein in aggregated form was able to activate the classical complement pathway, and this was shown to be due to an inability to bind the first component of complement (C1). By rosette inhibition assays, Dob and Lec proteins were shown to have no measurable affinity for Fc receptors on human B cells or neutrophils. Dob and Lec proteins had a much reduced affinity for Fc receptors on the murine macrophage-like cell line $P388D_1$ when compared to normal human IgG1. Furthermore, the hinge-deleted proteins were able to compete with murine IgG2b for $P388D_1$ receptors but not with murine IgG2a. In contrast, the binding of Dob and Lec proteins to protein A from *Staphylococcus aureus* was entirely normal. The functional consequences of the hinge deletion were parallel to those seen when normal IgG1 was reduced and alkylated. It was concluded that the functional impotency of Dob and Lec proteins was related to the close association between the Fab and Fc regions in these molecules and the limited degree of segmental flexibility permitted in the absence of the hinge region. The data also suggest a major role for the Cγ2 domain (C is the constant region) in mediating effector functions in normal IgG1.**

The hinge region of IgG, composed of a stretch of ≈15 amino acid residues joining the Cγ1 and Cγ2 homology regions (C is the constant region) in each of the paired heavy (H) chains and encoded by a separate exon (1), plays a number of interrelated structural roles in the molecule. The interchain disulfide bonds that link the H chains are located in this region, and these bonds serve to maintain the quaternary relationship between the paired Cγ2 domains that do not show significant noncovalent association (2). Segmental motion within the IgG molecule, which permits the Fab regions to assume a variety of orientations in space relative to the Fc region, is mediated by the hinge (3). Segmental flexibility, which allows variation in the distance between the antibody-combining sites located at the distal ends of the Fab regions, serves the important function of allowing bivalent attachment of antibodies to antigenic determinants even if the latter form a fixed array (for example, on a cell surface) (4). Finally, the hinge region serves as a "spacer," thus minimizing the population of conformational states in which close association between the Fab and Fc regions is achieved. In fact, as we shall see, the hinge region and its constituent disulfide bonds maintain a delicate balance between the degree of segmental flexibility required for optimal expression of antigen binding and unrestricted flexibility, which would compromise the expression of biological effector functions.

Although it is established that the sites mediating effector functions are within the Fc region (for a review, see ref. 5), it has become apparent that the expression of these functions can be modulated by one-dimensionally distant regions of IgG. Reduction of IgG under conditions in which only interchain disulfides are cleaved results in partial or complete abrogation of its ability to mediate effector functions (5). When Fc fragments are reduced, loss of activity is much less dramatic, and some functions [e.g., the binding of the first component of complement (C1) (6)] are insensitive to reduction of Fc but are completely lost when IgG is reduced. To explain the effects of reduction on C1 binding, Isenman *et al.* (6) proposed that cleavage of the disulfide bonds in the hinge region permitted greater segmental flexibility within IgG, leading to steric blockade of the C1-binding site on each Cγ2 region by the adjacent Fab. Another possible model to account for the effects of reduction, particularly if the activity of Fc fragments is affected, suggests that cleavage of the hinge-region disulfides results in a change in the quaternary relationship between the Cγ2 domains which, in turn, could affect Cγ2–Cγ3 association (5).

In order to explore the role of the hinge region further, we have studied some biological properties of variant human IgG1 monoclonal proteins in which the hinge region is deleted. Three such proteins have been described: IgG1κ Dob (7), IgG1κ Lec (8), and IgG1λ McG (9). The structural defect in these molecules is identical and involves a deletion of residues 216–230 of the normal γ1 sequence, which corresponds to the entire hinge region. The interpretation of the functional data has been greatly facilitated by the availability of a molecular model for the Dob protein based on low-resolution x-ray crystallographic data obtained with this protein in conjunction with high-resolution data available for several IgG fragments (ref. 10; see *Discussion*).

MATERIALS AND METHODS

Proteins and Fragments. Monoclonal IgG1 proteins and IgG1 Lec protein were isolated from myeloma sera by $(NH_4)_2SO_4$ precipitation followed by DEAE-cellulose chromatography. IgG1 Dob protein, a cryoglobulin, was purified by successive cryoprecipitations. Murine IgG2a and IgG2b were obtained as described (11). Published methods were used to obtain $F(ab')_2$ and Fab fragments (2), which were further purified by passage over a protein A-Sepharose CL-4B column (Pharmacia, Dorval, Quebec). The purity of proteins and fragments was assessed by immunoelectrophoresis and double diffusion in agar with

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Abbreviations: C region, constant region; L, light chain; H, heavy chain; C_L, constant region of the light chain; C1, first component of complement; EA, erythrocyte–antibody.

monospecific and polyvalent antisera and by NaDodSO$_4$/polyacrylamide gel electrophoresis. Protein concentrations were determined spectrophotometrically with the following extinction coefficients $A^{1\%}_{280}$): IgG1, IgG1 Lec, F(ab')$_2$, and Fab, 14.0; and IgG1 Dob, 16.0 (7). IgG1 was reduced with 10 mM dithioerythritol (pH 8.6) and alkylated with 24 mM iodoacetamide or with iodo[^{14}C]acetamide (Amersham; 57 Ci/mol; 1 Ci = 3.7 × 10^{10} becquerels). Radioiodination was performed by the iodine monochloride method (12). Prior to use in biological assays, all proteins were freed of aggregates either by gel filtration on Sephacryl S-300 in 0.15 M NaCl/40 mM phosphate, pH 7.2 (or Veronal-buffered saline) or by centrifugation at 100,000 × *g* for 1 hr in an airfuge (Beckman).

Complement Assays. C1 binding by monomeric proteins was determined by the method of Augener *et al.* (13) as modified by Isenman *et al.* (6). Complement fixation by IgG1 Br, Dob, Lec, and Fab was measured by a modification (14) of the microcomplement fixation assay of Wasserman and Levine (15). Proteins were adsorbed onto polystyrene latex particles under conditions that gave half-monolayer coating (16). The extent of coating with the three IgG1 preparations was comparable, as judged by agglutination titers obtained with washed particles and an antiserum to IgG. No anticomplementary activity was seen with either uncoated latex particles or monomeric proteins.

Cell Preparations. Human B cells were isolated from heparinized peripheral blood of three patients with chronic lymphocytic leukemia by Ficoll–Hypaque density centrifugation (ρ = 1.078 g/ml). The extent of contamination by T cells and monocytes was less than 2%, as assessed by rosetting with sheep erythrocytes and by nonspecific esterase staining, respectively. Neutrophils were prepared from the blood of healthy donors by the method of Boyum (17) and were 98% pure. The mouse macrophage-like cell line P388D$_1$ was grown in spinner culture as described (11). All cell suspensions were adjusted to 2 × 10^6 cells per ml in RPMI 1640 medium or minimal essential medium containing 10% (vol/vol) fetal calf serum; viability was consistently >95% as judged by trypan-blue exclusion.

Assays for Binding to Fc Receptors. The cytophilic activity of normal and hinge-deleted IgG1 towards human cells was assessed by inhibition of erythrocyte–antibody (EA)-rosette formation between these cells and human D-positive erythrocytes sensitized with anti-Rh$_0$(D) immunoglobulin (Connaught Laboratories, Toronto, Canada). Briefly, 0.1 ml of the appropriate cell suspension was incubated with 0.1 ml of increasing molar concentrations of the various ligands for 45 min at room temperature; 50 μl of EA (2 × 10^8 cells per ml) was added, and the suspension was centrifuged at 70 × *g* for 3 min and allowed to stand at 4°C for 1 hr. The cells were gently resuspended, and any cell surrounded by four or more erythrocytes was scored as a rosette. Each set of ligand concentrations was examined in duplicate, and each experiment was repeated at least twice. The inhibition of the specific binding of radiolabeled human and murine immunoglobulins to P388D$_1$ cells was determined as described (11).

Binding to Immobilized Protein A. Approximately 1.0 mg each of normal IgG1, IgG1 Dob, or IgG1 Lec was applied separately to a 2-ml column of *Staphylococcus aureus* protein A-Sepharose CL-4B in sodium acetate buffer (pH 5.0). Bound protein was eluted at constant ionic strength (μ = 0.15) with a gradient between 5.0 and 3.5. Binding assays were also performed using a mixture of 1 mg of unlabeled protein and trace amounts of the same protein mildly reduced and alkylated with iodo-[^{14}C]acetamide.

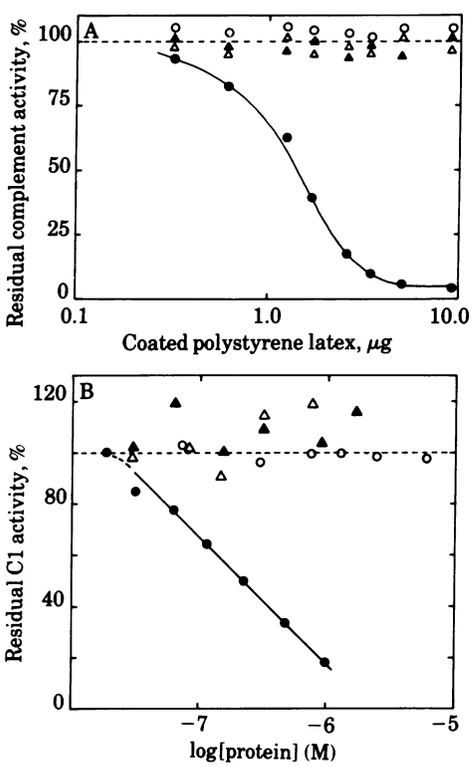

FIG. 1. (*A*) Fixation of complement by a normal human IgG1 monoclonal protein (Br) coated onto polystyrene latex beads to form a half monolayer (●). In contrast, beads coated under identical conditions with either IgG1 Dob (▲) or Lec (△) protein failed to fix complement over the same concentration range. Fab fragment from IgG1 Br was used as a negative control (○). (*B*) In the C1-binding assay of Augener *et al.* (13), IgG1 Br showed a dose-dependent binding (●) whereas Dob (▲) and Lec (△) did not at comparable concentrations. Again, Fab fragment served as a negative control (○).

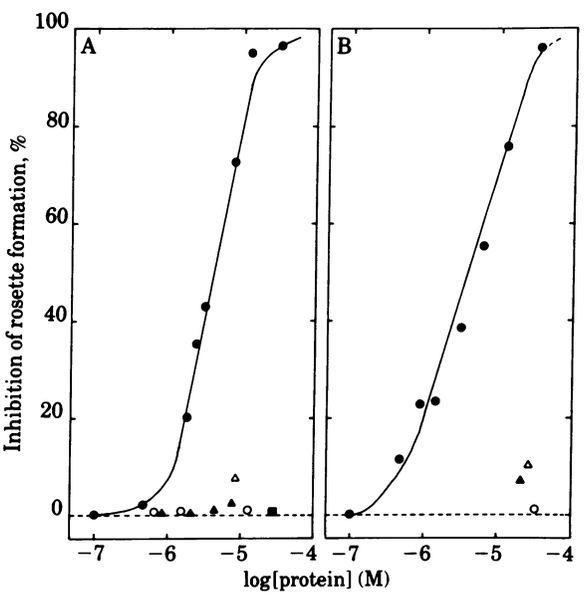

FIG. 2 (*A*) EA-rosette formation between human B cells (chronic lymphocytic leukemic cells carrying monoclonal surface IgMλ) and anti-Rh$_0$(D)-coated human Rh$^+$ erythrocytes was inhibited by IgG1 Br (●) in a dose-dependent fashion. Dob (▲) and Lec (△) failed to show significant inhibition, as did reduced and alkylated IgG1 Br (○). F(ab')$_2$ fragment of IgG1 Br was used as a negative control (■). (*B*) EA-rosette formation by human neutrophils was inhibited by IgG1 Br (●) but not significantly by high concentrations of Dob (▲), Lec (△), or reduced and alkylated IgG1 Br (○).

RESULTS

C1 Binding and Complement Activation. Polystyrene latex beads coated with a half-monolayer of a normal protein, IgG1 Br, fixed complement in a dose-dependent fashion, whereas beads coated under identical conditions with either Dob or Lec protein did not show any complement consumption (Fig. 1*A*). The Augener assay, which measures the interaction of immunoglobulins with macromolecular C1, was used to determine if the failure of Dob and Lec to activate complement was due to their inability to bind C1. Increasing concentrations of IgG1 Br bound progressively more C1 (Fig. 1*B*). In contrast, the hinge-deleted proteins showed no ability to bind C1 over the same concentration range and behaved like the Fab fragment from IgG1 Br used as a negative control.

Interaction with Fc Receptors. EA-rosette inhibition assays were performed to determine whether the hinge-deleted proteins were able to interact with Fc receptors on human B cells and neutrophils (Fig. 2). Monomeric IgG1 Br was able to inhibit rosette formation by B cells over the approximate concentration range 5×10^{-6}–5×10^{-4} M, with 50% inhibition occurring near 5×10^{-5} M (Fig. 2*A*). Mild reduction and alkylation of IgG1 Br completely abolished the inhibitory activity. No significant inhibition was observed with either Dob or Lec over a similar range of concentrations. Entirely comparable results were obtained with neutrophils (Fig. 2*B*); the inhibition by IgG1 Br was lost upon mild reduction, and the hinge-deleted proteins showed little or no inhibition even at high concentrations.

The murine macrophage-like cell line $P388D_1$ possesses Fc receptors for murine IgG2a and IgG2b, and human IgG1 binds to both classes of receptor (18). As shown in Fig. 3*A*, 50 μg of unlabeled IgG1 Br completely inhibited the specific binding of ^{125}I-labeled IgG1 to $P388D_1$ cells, but a comparable amount of mildly reduced and alkylated IgG1 Br failed to show any significant inhibition. Both Dob and Lec were able to inhibit ^{125}I-labeled IgG1 Br binding in a dose-dependent fashion, but only to about one-half the level achieved with normal IgG1. This phenomenon was explored further by determining the extent to which the hinge-deleted proteins could inhibit the binding of radiolabeled murine IgG2b (Fig. 3*B*) or IgG2a (Fig. 3*C*) to $P388D_1$ cells. Whereas 50 μg of IgG2b completely inhibited the binding of its radiolabeled counterpart, only 50–60% inhibition of ^{125}I-labeled IgG2b binding was achieved with 100 μg of either Dob or Lec. For IgG2a, however, neither Dob nor Lec was able to inhibit the binding of ^{125}I-labeled IgG2a significantly, even at concentrations as high as 200 μg.

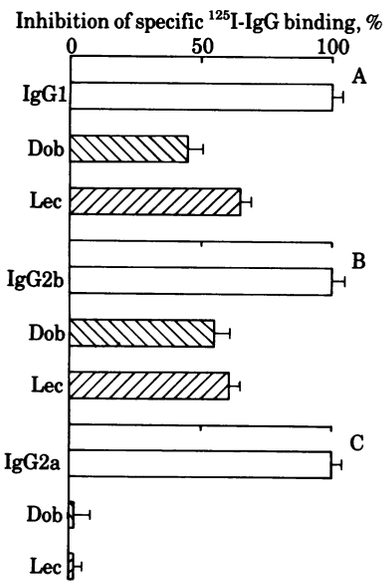

FIG. 3. (*A*) Specific binding of ^{125}I-labeled human IgG1 Br (1 μg/well) to $P388D_1$ cells was completely inhibited by 50 μg of IgG1 Br per well but only partially inhibited by 200 μg of either Dob or Lec per well. (*B*) Binding of ^{125}I-labeled murine IgG2b (1 μg/well) was completely inhibited by 50 μg of IgG2b per well and partially inhibited by 200 μg of either Dob or Lec per well. (*C*) In contrast, 200 μg of Dob or Lec per well did not inhibit the specific binding of ^{125}I-labeled murine IgG2a (1 μg/well), whereas complete inhibition was achieved with 50 μg of IgG2a per well. Each histogram represents the mean ± SEM of at least three separate assays.

Interaction with Protein A. Fig. 4 shows the results of a series of experiments designed to determine the pH dependence of the interaction between protein A-Sepharose and IgG1. There was no measurable difference between the pH values at which IgG1 Br and either of the hinge-deleted proteins were eluted from the protein A-Sepharose; maximal elution occurred at pH 4.1. Although mild reduction and alkylation did not affect the elution characteristics of IgG1 Br (Fig. 4), reduced and alkylated Dob and Lec did elute reproducibly at a slightly higher pH (maximum at pH 4.45) (data not shown).

DISCUSSION

The data presented clearly show that several biological activities mediated by sites on the Fc region of IgG1 are either not expressed or severely compromised in the hinge-deleted IgG1 variants. There is a marked parallelism between the functional consequences of not having a hinge region in Dob and Lec proteins and the absence of hinge-region disulfides in reduced IgG1. The notable exception to the above was observed with protein A binding, which was insensitive to both the absence of the hinge region and reduction. The extent to which these observations can be interpreted is due to the availability of a three-dimensional structure for Dob (10) and precise information on the nature of the structural defect in both Dob and Lec (7, 8).

X-ray diffraction data for Dob, collected to ≈6-Å resolution, have suggested that the overall molecular envelope was approximately T-shaped (19). Higher resolution data could not be obtained because of the susceptibility of the crystals to radiation damage. In order to obtain a more detailed picture of the molecule, Silverton *et al.* (10) took the αC coordinates of human Fc fragment (20) and murine McPC603 Fab fragment (21) and rotated and translated them into the Dob electron-density map until an optimal fit was obtained. A space-filling model of the resultant structure is shown in Fig. 5. Two IgG molecules with

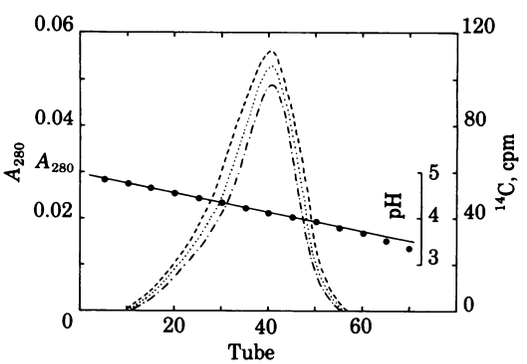

FIG. 4. Approximately 1.0 mg of IgG1 Br (·····) and Dob (-----) was bound separately to a 2-ml column of protein A-Sepharose CL-4B in sodium acetate buffer (pH 5.0). Elution was achieved with a pH gradient between 5.0 and 3.5 at constant ionic strength (μ = 0.15). IgG1 Br reduced and alkylated with iodo[^{14}C]acetamide eluted over the same pH range (·····).

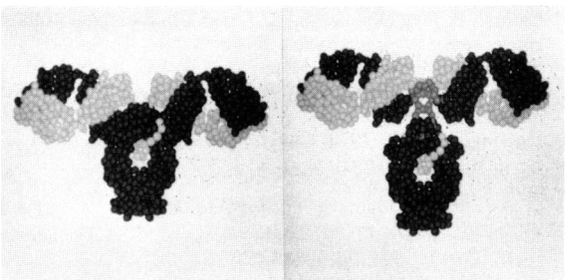

FIG. 5. (*Left*) Space-filling model of IgG1 Dob. (*Right*) Hypothetical model of IgG1 Dob in which a γ1 hinge region has been introduced. An Evans and Sutherland Picture System II was used to construct the hinge, with programs written by R. J. Feldmann. The hinge is anchored at the Fab end by the disulfide bond between the γ and light (L) chains and, at the Fc end, by proline 238, whose coordinates come from the Fc fragment structure (20).

normal hinge regions have been studied by x-ray diffraction [IgG1 Kol (22) and IgG2 Zie (23)]; in both cases, sufficient electron density to account for the Fc region could not be identified. This led to the suggestion that the Fc regions occupied more than one position in the crystal lattice, causing disorder. Because we are particularly interested in the spatial relationships between Fab and Fc in normal and hinge-deleted IgG1, Fig. 5 also shows a model of Dob in which a hypothetical normal hinge region has been introduced.

In the context of the present study, there are two aspects of the Dob structure that are particularly relevant. The first is the marked similarity between the conformations of the Fc region of Dob and the isolated Fc fragment. Although minor differences might escape detection, there is no evidence that the failure of Dob to express several biological functions is due to major conformational changes in either Cγ2 or Cγ3 or to alterations in the quaternary relationships between these domains. In addition, sequence analysis of both Dob and Lec points to a deletion of residues 216–230 as the only structural defect, with the Fab and Fc regions having an otherwise normal structure. The second aspect concerns the close approach of portions of Fc and parts of the Fab regions. This is especially so between the constant region of the light chain (C_L) and Cγ2 (2 contact distances less than 6.5 Å and 43 less than 10 Å) and between Cγ1 and the carbohydrate prosthetic group linked to Cγ2 (8 contacts less than 6.5 Å and 24 less than 10 Å). Some less extensive contacts are also made between Cγ1 and Cγ2. These interactions almost certainly account for the fact that diffraction from Fc was obtained for Dob inasmuch as the opportunity for segmental motion is limited. It seems logical to attribute the functional impotency of Dob and Lec to the unique relationship between Fab and Fc in these molecules.¶

The site of protein A binding to Fc is known precisely from the x-ray crystallographic studies of Deisenhofer *et al.* (24); it is located at the junction between Cγ2 and Cγ3. The protein-A binding studies presented here clearly show that the Cγ2-Cγ3 junction is not significantly affected by the atypical Fab–Fc interactions that exist in Dob and, presumably, in Lec. Furthermore, we can infer that those biological activities that are absent or severely compromised in Dob and Lec and that cannot be attributed to sites uniquely located on either the Cγ2 or the Cγ3 domain [i.e., binding to Fc receptors on neutrophils (25), placental syncitiotrophoblasts (26),‖ and $P388D_1$ macrophages (18)] also do not involve a site at the Cγ2-Cγ3 junction.

The sites on Fc that bind the globular "head" regions of C1q (27) are located on the Cγ2 domains (2) and probably involve residues near the proximal ends of these domains (28). The failure of Dob and Lec to bind C1 and their consequent inability to activate the complement system** may reasonably be accounted for on the basis of the unusually close approach of the C_L and Cγ2 domains. Although the interaction is weak, C_L covers a portion of the outer surface of Cγ2, forming a crevasse approximately 10 Å wide between the domains (Fig. 5). The globular heads of C1q have dimensions of 50 × 70 Å (29), which would effectively limit their access to a binding site in or near the crevasse. Steric blockade has also been proposed by Isenman *et al.* (6) to account for the loss of C1 binding in reduced IgG1 and for the failure of intact human IgG4 to interact with C1 even though binding sites are demonstrable on $Fc\gamma_4$ fragments.

Although in the crystal of Dob the crevasse referred to above is too narrow to permit the intrusion of another globular protein, model-building studies indicated that some segmental flexibility was possible in "free solution." Nevertheless, the hinge deletion, together with the L-L disulfide bond, will hold the Fab nearer to Fc than in a normal IgG1. The construction of a hypothetical hinge model for Dob (Fig. 5) led to the realization that another factor (namely, the rigidity of the hinge) might account for some of the properties of a normal IgG1. The hinge contains the sequence Cys-Pro-Pro-Cys-Pro, which imposes a powerful constraint on its structure. The two cysteines must form two disulfide bridges with the corresponding cysteines of the other H chain and, as a result, must occupy approximately equivalent positions along the axis of the chains. These requirements are satisfied by a *trans* polyproline helix having a 3-fold screw axis for each chain. The covalent association of the rigid helical structures from each chain should result in a stiff "spacer" segment that would act to separate the Fab and Fc regions. The length of this spacer would be expected to be at least 4 times the helical repeat distance of 3.1 Å, or 12.4 Å. In comparing the Dob structure with and without a hinge (Fig. 5), one finds a substantial increase in the width of the crevasse, from ≈10 Å to 20 Å. This would presumably allow access of effector molecules to Cγ2. In solution, the spacer would also make it more difficult for large areas of Fab to interfere with access to Fc.

The rigid hinge segment provides a natural explanation for the effects of reduction and alkylation. Breaking the inter-H-chain disulfides will remove the 3-fold constraint, resulting in the collapse of the rigid structure of the individual strands and freeing the Fab fragments to interact more with Fc. The increased separation of combining sites upon reduction could be as much as 50 Å simply as a result of stretching the polypeptide chains of the hinge in opposite directions. This could account for the electron microscopic observations of Seegan *et al.* (30) and the behavior of reduced incomplete antibodies described by Romans *et al.* (31).

Steric effects probably also account for the failure of Dob and Lec to interact with Fc receptors in B cells and neutrophils as measured by EA-rosette inhibition. The precise location of the

¶ This relationship probably also accounts for the failure of both Dob and Lec to express the expected allotypic markers associated with Cγ1 and Cκ (7, 8). On the basis of the residue present at position 214 in Cγ1, Dob should be G1m 17 (residue 214 is lysine) and Lec should be G1m 3 (residue 214 is arginine), but they type as G1m −17 and −3, respectively. Similarly, the Km markers on Cκ, which are sensitive to γ-L interaction, are not expressed by Dob and Lec. Dob should be Km 3 and Lec should be Km 1, 2.

‖ In ref. 26, it was shown that IgG1 Dob bound to the syncitiotrophoblast Fc receptor with a much lower affinity than its normal counterparts.

** An early study on IgG1 McG (29) showed that this protein also did not fix complement.

binding sites for these receptors on Fc is not known, although for neutrophils neither Cγ2 nor Cγ3 fragments could inhibit rosette formation (25). A major involvement of Cγ2 seems to be suggested by the present data (i.e., the Cγ2-Cγ3 junction is unaffected by the hinge deletion), but we cannot distinguish between a Cγ2 site stabilized by Cγ3 or a site formed jointly by the paired Cγ2 domains.

The results obtained with the $P388D_1$ macrophages are particularly interesting because they suggest that the murine IgG2a and IgG2b receptors on these cells recognize topographically distinct regions on their respective ligands. There is evidence that $P388D_1$ possesses two classes of Fc receptor, each showing a preference for one of the IgG2 subclasses, although IgG2a will bind to IgG2b receptors and vice versa (11). Human IgG1 binds to both receptors, but Dob and Lec were only able to compete with IgG2b. Diamond *et al.* (32) suggested that IgG2b binds to mouse macrophage via a site on the Cγ2 domain based on studies with a molecule having a γ2b-γ2a hybrid heavy chain secreted by a variant of the MPC11 myeloma cell line. Our data suggest that both subclasses bind to $P388D_1$ via separate sites on Cγ2, with the IgG2a site being located nearer the Fab–Fc interface. The inability of either Cγ2 or Cγ3 fragment from human IgG1 to inhibit IgG binding further suggests that quaternary factors are also important (18).

The studies described in this paper highlight the crucial role played by the hinge region and its constituent disulfides in allowing IgG to perform its biological effector functions. An essential feature of this role is to limit segmental flexibility; yet this flexibility seems to be important for optimal antigen binding. Thus, the hinge serves to balance the antigen-dependent and antigen-independent flexibility requirements of the molecule. The polymorphism exhibited by the hinge (e.g., in the human IgG subclasses) suggests an evolutionary pressure to maintain linkage between specific hinge and C-region exons, contributing to the differential expression of effector functions. The biological significance of this asymmetric distribution of functions among closely related molecules, however, remains unclear.

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