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Comparison of Intact Antibody Structures and the Implications for Effector Function

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I. Introduction

Immune systems of vertebrates rely on antibody molecules, structurally dynamic glycoproteins, for coupling foreign body recognition with elimination processes. An intact immunoglobulin G (IgG) molecule is composed of four polypeptide chains, two identical light (L) chains and two identical heavy (H) chains, with variable (V) and constant (C) regions, which form two Fab segments and one Fc segment for a total of 12 domains. The domains associate in pairs (V_L:V_H, C_L:C_{H^1}, C_{H^2}:C_{H^2}, and C_{H^3}:C_{H^3}), producing six globular units within the three segments. Hypervariable loops (L1, L2, L3, H1, H2, and H3) make up the antigen-binding site on the variable domain pair (V_L:V_H) of each Fab, whereas effector sites are located primarily on the Fc (for further review, see Davies and Chacko, 1993).

Inherent flexibility of the immunoglobulin yields a spectrum of molecular conformations, making crystallization problematic. Consequently, progress in intact IgG structural elucidation has been painfully slow, spanning the past 30 years. Eight intact IgG crystals have so far been reported: Dob (Terry *et al.*, 1968), Mcg (Edmundson *et al.*, 1970), Kol (Palm and Colman, 1974); Zie (Ely *et al.*, 1978), Mab231 (Larson *et al.*, 1991; Harris *et al.*, 1995), Mab4B7 (Stura *et al.*, 1994), Mab24-404.1, and Mab61.1.3 (Harris *et al.*, 1995). Five of these yielded structures, or partial structures; Zie, Mab4B7, and Mab24-404.1 are the exceptions. Other classes of intact immunoglobulin, i.e., IgA, IgD, IgE, and IgM, have not been crystallized, only IgG.

As opposed to entire Ig, fragments of antibodies, such as Fabs, Fc, and Fvs, are more readily crystallized, and from their analyses the β-barrel topologies of individual antibody domains have been defined at high resolution. Fragment studies have, in addition, provided insight into mechanisms of antigen binding by the complementarity-determining regions (CDRs), and of some effector protein interactions with the Fc (for reviews, see Colman, 1988; Sheriff, 1993; Wilson and Stanfield, 1994; Braden and Poljak, 1995; Edmundson *et al.*, 1995; Davies and Cohen, 1996; Padlan, 1996). Structural information on the intact molecule is currently confined to Dob, Mcg, Kol, Mab231, and Mab61.1.3. In every case, the IgG was visualized in its free state, not associated with either antigen or effector.

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Comparison of the five intact antibodies whose structures are known delineates to some extent the dynamic range of IgG, and it serves to clarify some features of antigen clearance through effector function processes. This is of relevance in deducing mechanisms by which binding of antigen-bearing targets is allied with the activities of effectors such as C1q and Fcγ receptors. Our intent here is to summarize the structures of the intact antibodies available, both visually and geometrically (Table I), and to describe the dispositions of biologically relevant sites on the molecules.

II. Hinge-Deleted Dob and Mcg

Dob, Mcg, and Kol are human myeloma proteins obtained from patients. Both Dob and Mcg are of the IgG_1 subclass but contain "genetic" hinge deletions (Table II). A mere seven amino acids of the C_{H^2} gene product (structural lower hinge residues in normal antibodies) serve as a hinge bypass peptide. Thus these IgGs, without upper and core hinge regions, have no inter-heavy-chain disulfide bonds. Light chains instead are linked by disulfide bridges between their terminal cysteines. This further restricts the molecule's flexibility, particularly that of the Fabs. The deleted hinge also causes the Fc to be drawn tightly against the Fab segments. As a consequence, Dob and Mcg are conformationally restricted and their structures exhibit compact, twofold symmetrical T shapes (Figs. 1 and 2, see color plate) (Silverton *et al.*, 1977; Guddat *et al.*, 1993).

Although the natural antigen is unknown for both Dob and Mcg, the latter is known to bind a variety of synthetic peptides on solid supports (Edmundson *et al.*, 1995), thus normal antigen-binding properties are not believed altered. Effector functions are, however, obstructed (see below). While Dob and Mcg are unable to perform in the same capacity as antibodies possessing hinges, it is still an open question as to whether they play some meaningful role in the immune system. About 1% of a normal human IgG pool consists of hinge-deleted antibodies. These could represent genetic recombination errors (Steiner and Lopes, 1979), but this is still very speculative.

In Fig. 2, a model of Mcg, based on a 3.2 Å resolution crystal structure determination (Guddat *et al.*, 1993), is presented. The two halves of the molecule are related by an exact (crystallographic) twofold axis. Lack of hinge polypeptides, and the presence of an L–L disulfide bond joining light chain termini, not only impose a compact T shape on the molecule, but they also result in noncanonical domain pairing for V_L:V_H, C_L:C_{H^1}, C_{H^2}:C_{H^2}, and C_{H^3}:C_{H^3}. Consequently, Fabs, as well as the Fc of Mcg, do not have the characteristic quaternary structures that are found for other antibody fragments, or for Fab and Fc segments within intact antibodies

containing normal hinges. Furthermore, the Fabs are inclined slightly toward the Fc. Thus the angle between Fabs is obtuse at 185°, and the hinge angle between each Fab and the Fc is acute at 87.5°.

Intact, hinge-deleted Dob is a lower resolution structure for which only alpha carbon coordinates are available. It was originally modeled at 6 Å by fitting Fab and Fc segments, determined from fragment crystals by X-ray diffraction, into the electron density for the Dob crystals (Silverton *et al.*, 1977). Like Mcg, this model has a crystallographic dyad relating halves, an angle between Fabs of 185°, and hinge angles of 87.5° (Fig. 1). Domains were later fitted to 4 Å resolution X-ray data (Sarma and Laudin, 1982) but, as yet, coordinates from that investigation are not available.

III. Partial Structure of Kol

Similar to Dob and Mcg, the immunoglobulin Kol is a human IgG_1, but possesses normal hinge polypeptides (Table II). Crystals of intact Kol yielded an electron density map that revealed only approximately two-thirds of the molecule (Marquart *et al.*, 1980). The Fc segment was crystallographically disordered. The two Fab segments were defined, however, in the Fourier maps, including also the upper and core hinge regions. Statistical disorder appeared with the lower hinge and propagated to the Fc, which, probably as a rigid body, occupied at least several orientations in the crystal lattice. This suggested that, for antibodies with normal hinges, the disposition of the Fc is, in general, not fixed with regard to the Fab segments, but is free to assume a variety of positions, perhaps even an unlimited number.

Though the structure of intact antibody Zie has not been determined, evidence indicates that the Fc in its crystal is also mobile (Ely *et al.*, 1978), as for Kol. Zie, of human subclass IgG_2, was crystallized in its intact form, but $F(ab')_2$ fragments made from Zie were crystallized in addition. Crystals of the two were essentially isomorphous, with mean differences in corresponding intensities nearly within experimental error. This implied that the Fc did not contribute significantly to the diffraction pattern of the intact IgG. The observation that both Kol and Zie had multiple conformations of Fc in their crystals proved a discouraging reminder of how the flexibility of immunoglobulins could hinder crystallographic analyses.

For Kol, as for Dob and Mcg, an exact, crystallographic twofold axis related the Fab segments (Fig. 3, see color plate). Although the Fc portion of the molecule was not visible, the angle between long axes of the Fabs, defined as the line connecting the centers of masses of V_L:V_H and C_L:C_{H^1}, was 132°, lending a Y shape to the molecule (Fig. 3A). Figure 3B shows that the Fab long axes do not intersect but are offset by 11 Å. The corresponding translation offset of Fab axes is 9 Å for Mcg, 10 Å for

TABLE I
COMPARISON OF INTACT ANTIBODY STRUCTURES

Antibody Characteristic	Dob	Mcg	Kol	Mab231	Mab61.1.3
PDB code	Not deposited[a]	1MCO	2IG2	1IGT	1IGY
Source	Human myeloma	Human myeloma	Human myeloma	Mouse hybridoma	Mouse hybridoma
Isotype	$IgG_{1\kappa}$	$IgG_{1\lambda}$	$IgG_{1\lambda}$	$IgG_{2a\kappa}$	$IgG_{1\kappa}$
Antigen	Unknown	Unknown	Unknown	Canine lymphoma	Phenobarbital
Number of residues in hinge[b]	7	7	22	23	17
Number of inter-heavy-chain disulfides in core hinge	0	0	2	3	3
Activates complement	No	No	Unknown	Yes	No
Special solubility characteristic	Cryoglobulin	Euglobulin	Cryoglobulin	Cryoglobulin-like	None
Crystal conditions	0.1 *M* Tris, pH 8, 4°C	Deionized water, 20°C	1.5 *M* AS, pH 5	4% PEG 3350, pH 8, 18°C	12% PEG 3350, pH 5, 23°C
Resolution of structure	4.0 Å	3.2 Å	3.0 Å	2.8 Å	3.2 Å
Space group	Monoclinic, *C*2	Orthorhombic, $C222_1$	Trigonal, $P3_221$	Triclinic, *P*1	Monoclinic, $P2_1$
Asymmetric unit	½ Ab	½ Ab	½ Ab	1 Ab	1 Ab
Overall symmetry	Exact dyad	Exact dyad	Exact dyad	None	None
Angle between Fabs	185°	185°	132°	172°	115°
Translation offset between Fab long axes	0 Å	9 Å	11 Å	10 Å	6 Å
Fab elbow angles	147°	118°	174°	159° and 143°	155°
Hinge angles	87.5°	87.5°	n/a[c]	66° and 113°	78° and 123°
Angle between Fab dyad and Fc dyad	0°	0°	n/a	128°	107°
Translation offset of Fab dyad and Fc dyad	0 Å	0 Å	n/a	26 Å	0 Å
Translation between Fabs along Fab dyad	0 Å	0 Å	0 Å	0.9 Å	9 Å
Distance between H3s	156 Å	120 Å	142 Å	151 Å	118 Å
Distance from C_H's to H3 of Fab1 and Fab2	99 Å	95 Å	n/a	131 Å and 119 Å	108 Å and 126 Å
Overall conformation of structure	Symmetric T	Symmetric T	Symmetric Y	Asymmetric, distorted T	Asymmetric, distorted Y

[a] Dob alpha carbon coordinates, at 6 Å resolution, were kindly provided by David R. Davies for this analysis.
[b] For Dob and Mcg these residues are considered a hinge "bypass;" see Table II also.
[c] n/a, not applicable.

TABLE II
HINGE SEQUENCES FOR INTACT IgG STRUCTURES[a]

Antibody	Upper	Core	Lower
Dob	----------	-----	APELLGG
Mcg	----------	-----	APELLGG
Kol	EPKSCDKTHT	CPPCP	APELLGG
Mab231	EPRGPTIKP	CPPCKCP	APNLLGG
Mab61.1.3	VPRDCG	CKPCICT	VPEV
	←——Genetic hinge——→		
	←————Structural hinge————→		

[a] Heavy chain sequences are compared from amino acid 226 to 250 [in Kabat numbering (Kabat *et al.*, 1991)] corresponding to the hinge polypeptides. All Cys in the hinge core form inter-heavy-chain disulfide bonds. For Kol and Mab61.1.3, the Cys of the upper hinge supplies the heavy chain's contribution to the heavy–light chain disulfide bridge. For Mab231, Cys-128 of the C_H^1 domain engages in a disulfide with the light chain terminus Cys-214. The structural lower hinge residues are genetically part of the C_H^2 gene product.

Mab231, and 6 Å for Mab61.1.3 (Table I). The Dob structure, at 6 Å resolution, was modeled with no offset. This, however, preceded the higher resolution structures when an offset was unanticipated.

In Kol, the normal human IgG_1 hinge is such that the terminal cysteine of each light chain bonds with a cysteine in the upper hinge of the molecule. Two inter-H-chain disulfides form the hinge core, producing a polyproline helix through the assembly (Fig. 3A). The packing of the Kol molecule in the unit cell utilized hypervariable loops (CDRs) interacting with the upper and core hinge regions of crystallographically related molecules, thereby stabilizing those sections of the hinge. Thus packing contacts were likely responsible for the clarity with which those portions of the hinge polypeptides appeared in electron density maps.

IV. Mab231

Obtaining a well-ordered crystal is to arrange conformationally identical molecules in a periodic, three-dimensional array. Even with a conformationally variable molecule, however, this can be achieved if the crystal selects only a single conformer for incorporation. In such cases, X-ray crystallography may yield a single "still image" of this select conformation if all segments are immobilized in the crystal through favorable packing interactions. Mab231 and Mab61.1.3 (murine hybridoma Mabs) have normal hinge polypeptides representative of their respective subclasses, IgG_{2a} and IgG_1. In both cases the three-dimensional structures of the entire molecules were deduced by X-ray crystallography (Harris *et al.*, 1992, 1997, 1998). They exhibit strikingly different conformations, both adopted as a consequence of specific, stabilizing crystal lattice interactions. These X-ray crystallographic images provide but two states, among a likely multitude, that are possible for IgG molecules with normal hinge polypeptides.

Mab231, specific for canine lymphoma cells, exhibits a distorted T shape (Fig. 4, see color plate). The angle between Fab long axes is 172°, and the Fc is obliquely disposed with hinge angles of 66° and 113°, while making an angle of 128° with respect to the plane of the Fabs. The structure contains no global symmetry operators as were found for Dob, Mcg, and the ordered portion of Kol. Though the structure appears overall asymmetric, local pseudotwofold axes (dyads) do pertain within or between segments of the IgG. One dyad relates only constant domain pairs of the Fabs. C_L:C_H^1 of Fab1 is related to C_L:C_H^1 of Fab2 by a rotation of 179.4°. A second, independent, pseudodyad relates heavy chains composing the Fc.

Fab elements have been shown, by earlier crystallographic studies of Ig fragments, to be flexible at their switch peptides, which connect variable and constant domains. The Fab "elbow" is defined as the angle between the pseudotwofold axes relating V_L to V_H, and C_L to C_H^1. Elbow angles have been observed from 127–227° (for a review, see Wilson and Stanfield, 1994). In Mab231, one Fab assumes an elbow angle of 159° while that of the second is less obtuse at 143°. As a consequence of the 16° difference in elbow angles, V_L:V_H pairs are not related by the local dyad, which relates Fabs by their C_L:C_H^1 pairs. For convenience, we will refer to the local dyads within Mab231 as the "Fab dyad" and the "Fc dyad," though both are only approximate. As noted above, the angle between the Fab dyad and the Fc dyad is 128°. Moreover, the dyads do not intersect one another, but are offset by 26 Å (Fig. 4). This translation further emphasizes the independent nature of the Fc with respect to the Fabs.

The canine lymphoma antibody packed in the crystallographic unit cell such that hypervariable loops of each Fab contacted Fc segments of lattice-related molecules, specifically at the switch peptide junctions between C_H^2 and C_H^3 domains. Contacts between CDRs and the Fcs of adjacent molecules stabilized both Fabs as well as the Fc segment. This was not the case for crystals of antibody Kol, and is probably why its Fc segment was not visible in electron density maps. The Mab231 hinge polypeptides, on the other hand, did not benefit from packing contacts, and although their paths were ultimately determined from difference Fourier maps, they were seen to exhibit high thermal factors indicative of some statistical disorder (Harris *et al.*, 1997).

V. Mab61.1.3

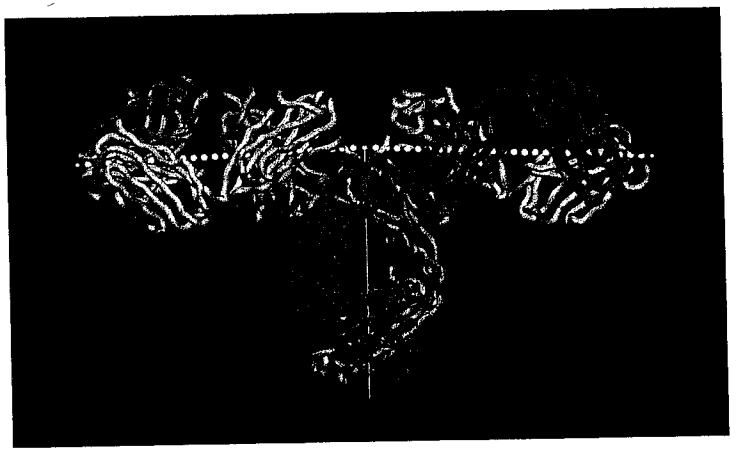
A model of the intact monoclonal antibody against phenobarbital, Mab61.1.3, is shown in Fig. 5 (see color plate) (Harris *et al.*, 1998). Like the IgG_{2a} for canine lymphoma, this IgG_1 conformation exhibits no overall symmetry, though it too possesses local dyads relating segments. Hinge angles, elbow angles, the angle between Fabs, and the angle between dyads are different, thus providing another "still image" of an IgG. This antibody displays a distorted Y shape with an angle between Fab long axes of 115°. The Fc is oriented at 107° with respect to the plane of the Fabs.

Heavy chains of the Fc segment are approximately related by a local twofold axis, similar to Mab231 and, indeed, to all Fc fragment structures. As for other structures of Fc segments, the twofold axis tends to degenerate with respect to the C_{H^2} domains, and for Mab61.1.3 the rotation relating C_{H^2} domains is about 175°. IgG_1 Fab segments both have elbow angles of 155° and they are related by a rotation of 179.7°. There is, however, a relative translation of the Fabs along the rotation axis of 9 Å (Fig. 5A), thus it is more properly described as a screw axis. For Dob, Mcg, and Kol, exact crystallographic symmetry precludes any possibility for such a translation between Fabs. For Mab231, which crystallized in a triclinic unit cell lacking any symmetry operators, the translation is 0.9 Å, which is but marginally significant. The structure of Mab61.1.3 thus identifies yet another degree of freedom available to the IgG. Again, for convenience, we may refer to the axis relating Fab segments as the "Fab dyad," but with the provision of a 9 Å translational component.

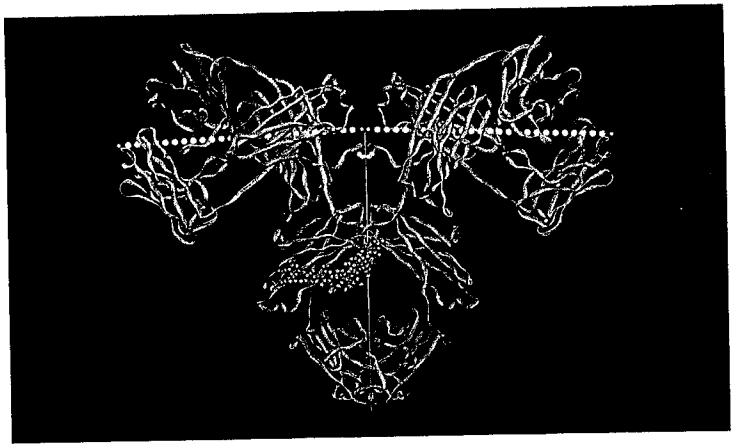
In contrast to Mab231, the IgG_1 Fab dyad exactly intersects the dyad relating Fc heavy chains, that is, there is no translational offset. The 107° angle between the Fab and the Fc dyads and the differing hinge angles of 78° and 123° establish the independence of segments within the molecule. The angle between Fab segments of 115° is the sharpest angle observed for intact antibody structures and, indeed, may represent the limit for this subclass. Any angle much more acute would result in unacceptable steric interactions between murine IgG_1 Fabs.

The distribution of molecules in crystals of Mab61.1.3 was such that the V_H domain of every Fab was in contact with the V_H domain of a nearby IgG. These interactions involved, specifically, the CDR regions. Intermolecular β ribbons were formed as a result of hydrogen bonding between the strand following hypervariable loop H2 (residues 55–59)[1] and the corresponding strand of the V_H of an adjacent antibody in the unit cell. While Fabs engaged in V_H lattice contacts, their V_L domains contacted the Fc of another antibody. These specific interactions stabilized the three

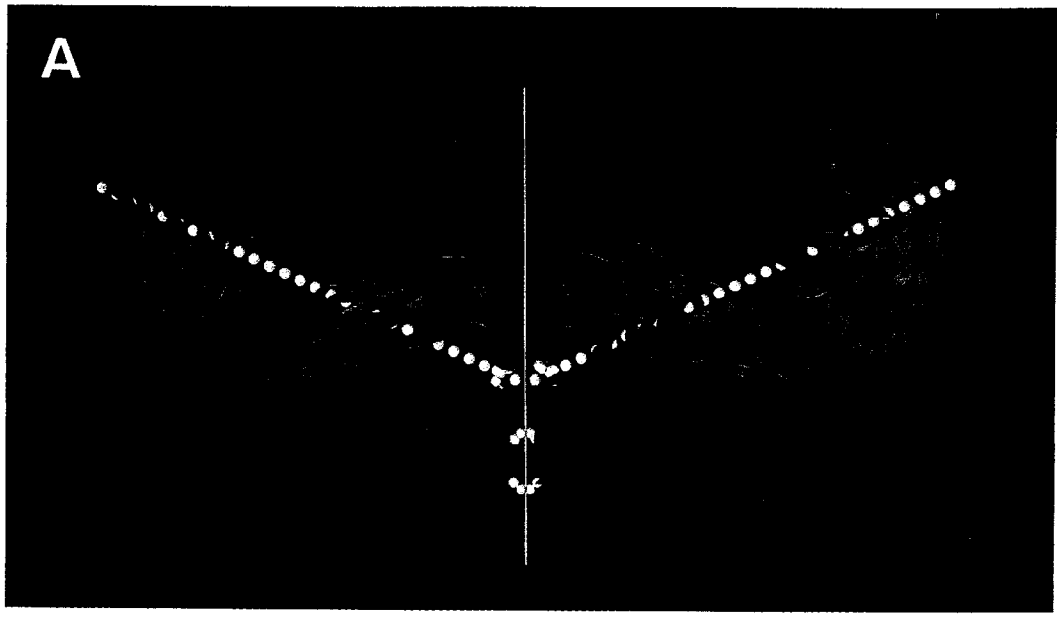
[1] The residue numbering convention used herein is that of Kabat *et al.* (1991).

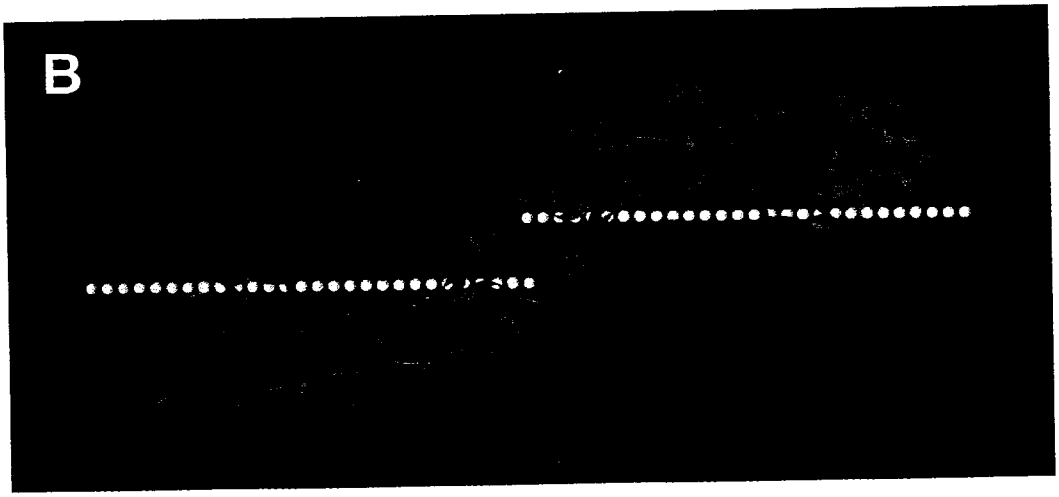


CH. 5, FIG. 1. The 6 Å resolution crystal structure of the hinge-deleted immunoglobulin Dob. Light chains are in gold, and heavy chains are two shades of orange. The crystallographic twofold axis is shown by a solid white line. The long axes of the two Fabs are indicated by white spheres. As seen here, the angle between Fab long axes is 185°. The angles between each Fab long axis and the Fc dyad, termed hinge angles, are 87.5°. The molecule exhibits a compact, symmetrical T shape.

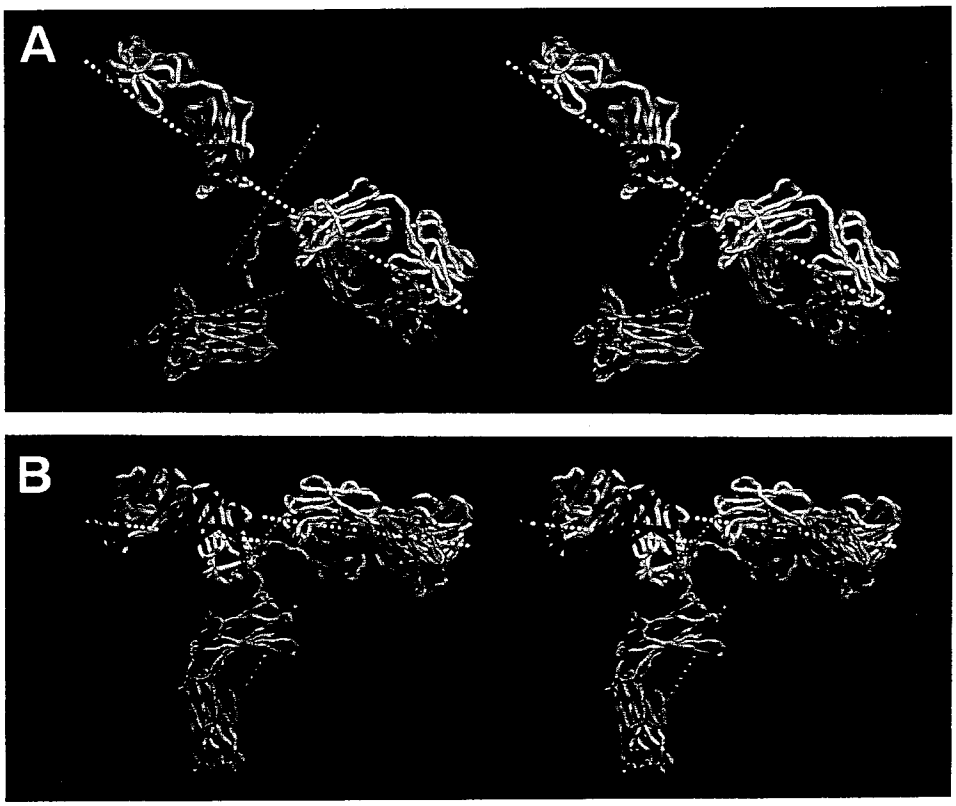


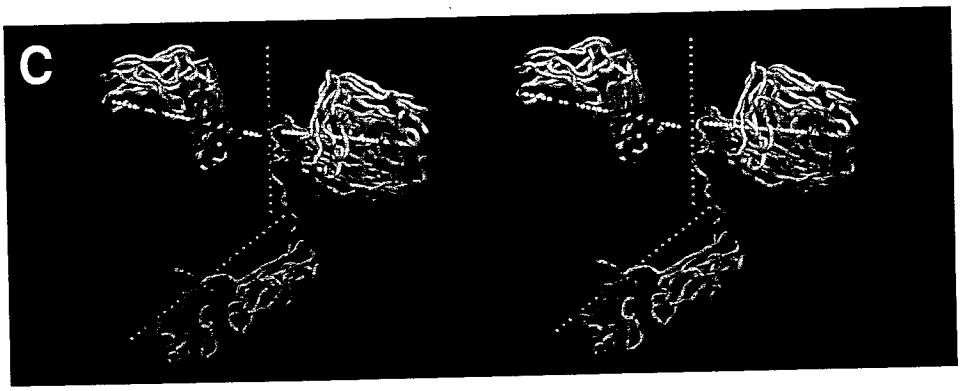
CH. 5, FIG. 2. Molecular structure of the hinge-deleted antibody Mcg at 3.2 Å resolution. Light chains are beige, and heavy chains are colored purple and blue. The two oligosaccharide chains are indicated in red and orange. An interchain disulfide bond, which connects light chain termini, is shown in yellow. No inter-heavy-chain disulfides appear in genetic hinge-deleted antibodies. The crystallographic dyad that relates halves of the IgG is displayed as a lime green line. Like Dob, Mcg assumes a compact, symmetrical T shape.



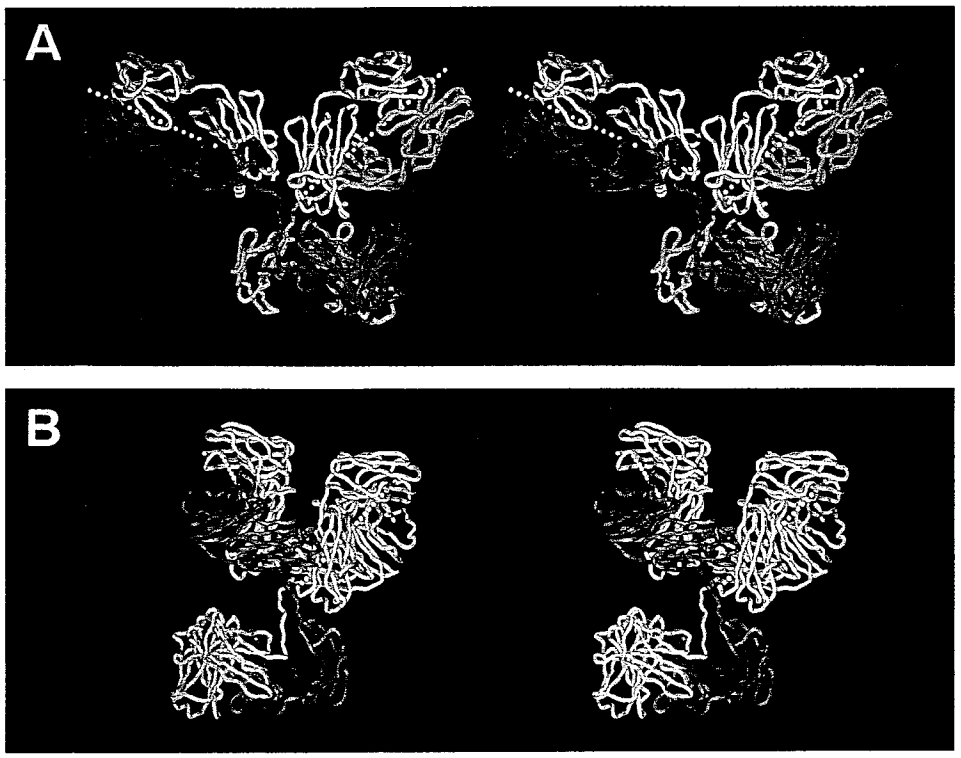


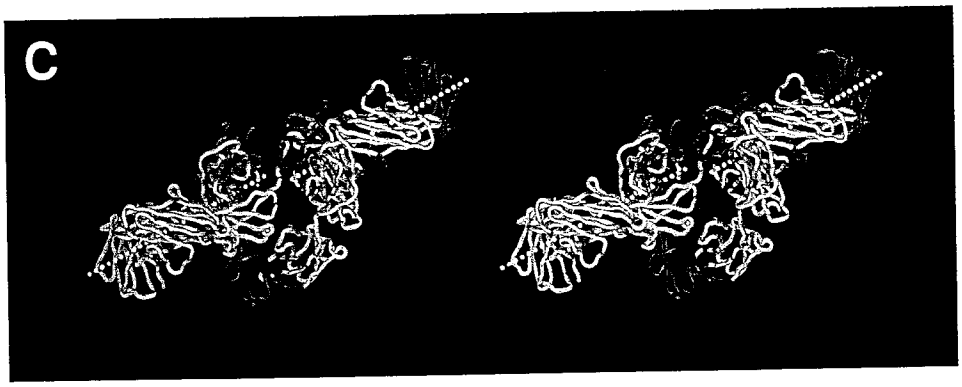
CH. 5, FIG. 3. Partial structure of the intact immunoglobulin Kol based on 3 Å resolution x-ray diffraction data. Fab segments and the upper and core hinge regions were visible in electron density maps, whereas the lower hinge region and Fc were not. Light chains are pink, and heavy chains of the two Fabs are red. (A) The two light–heavy chain disulfide bonds of the upper hinge, and the two inter-heavy-chain disulfide bonds of the core hinge, are highlighted in yellow. The crystallographic dyad is indicated by a pale pink line. The angle between Fab long axes is 132°, yielding a symmetric Y shape. (B) It is evident that Fab long axes do not intersect but are offset by 11 Å.



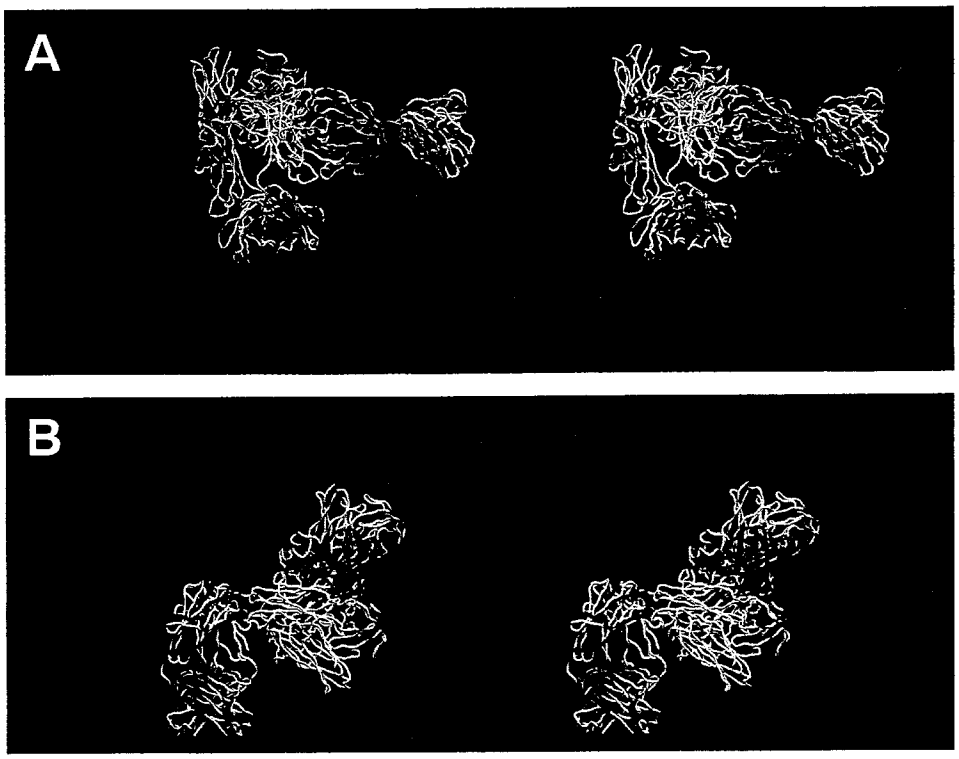


CH. 5, FIG. 4. The 2.8 Å resolution structure of Mab231, an IgG_{2a} against canine lymphoma cells. Light chains are lime green; heavy chains are orange and red. Fab long axes are white, and the local Fab dyad and Fc dyad are both in blue. (A) The molecule is viewed perpendicular to the pseudodyad relating C_L:C_H1 domain pairs of the Fab segments. The Fab long axes are nearly colinear, with an angle between them of 172°. Fab long axes do not intersect, but as seen in B are offset by 10 Å. (B) The IgG_{2a} is viewed approximately perpendicular to the pseudodyad relating heavy chains of the Fc. In both A and B it is apparent that the Fab and Fc dyads do not intersect but are offset by a translation of 26 Å. In B, hinge angles are clearly observed: the angle between one Fab long axis and the Fc dyad is 66°, and the hinge angle between the other Fab and the Fc is 113°. (C) The angle between the Fab and Fc dyads, 128°, is most evident. The Fc segment assumed an orientation 128° with respect to the plane of the Fabs. The conformation of Mab231 is best described as a distorted T shape.

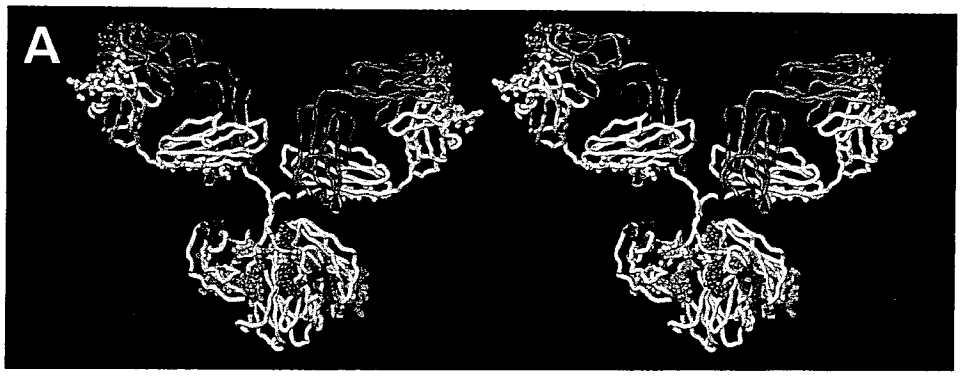


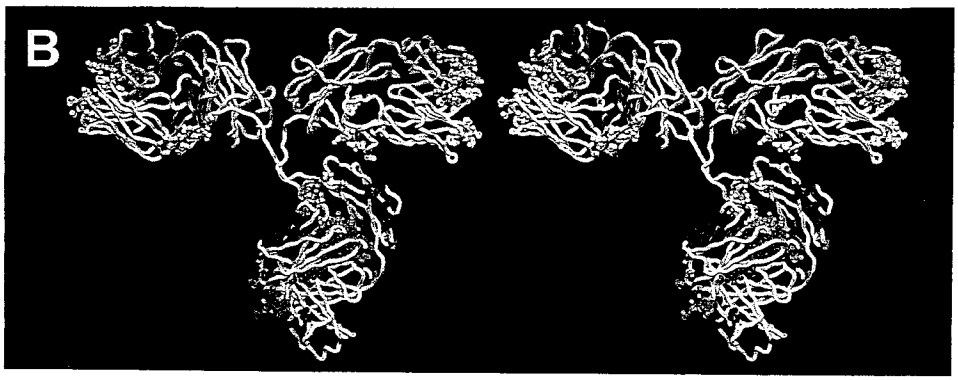


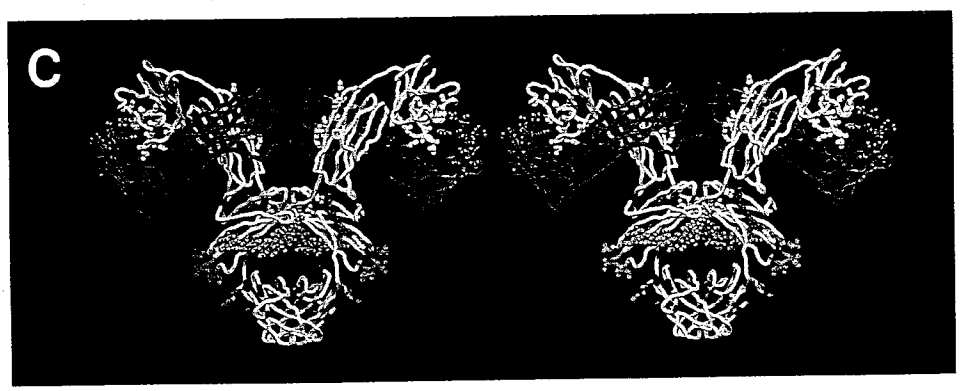
CH. 5, FIG. 5. Crystallographic structure of Mab61.1.3, an IgG_1 specific for phenobarbital, based on 3.2 Å resolution x-ray diffraction data. Light chains are yellow, and heavy chains are shown in cyan and blue. Fab long axes are white, and the local Fab dyad and Fc dyad are in red. (A and B) The angle between Fabs is seen as being a rather closed 115°, with the Fc segment oriented roughly perpendicular (107°) to the plane of the Fabs. The two local pseudodyads for this Mab exactly intersect, in contrast to Mab231, which exhibits a 26 Å offset. The angle between dyads is 107° for Mab61.1.3, in contrast to Mab231, where it is 128°. In A, a relative translation of 9 Å between Fabs along their dyad can be seen, and in C, there is a translation offset of 6 Å for their long axes. (C) The molecule is viewed along the pseudodyad relating Fab segments, which best illustrates the differing hinge angles. The hinge angle between one Fab long axis and the Fc dyad is 78°, whereas that between the second Fab and the Fc is 123°. The conformation of Mab61.1.3 is best described as a distorted Y shape.



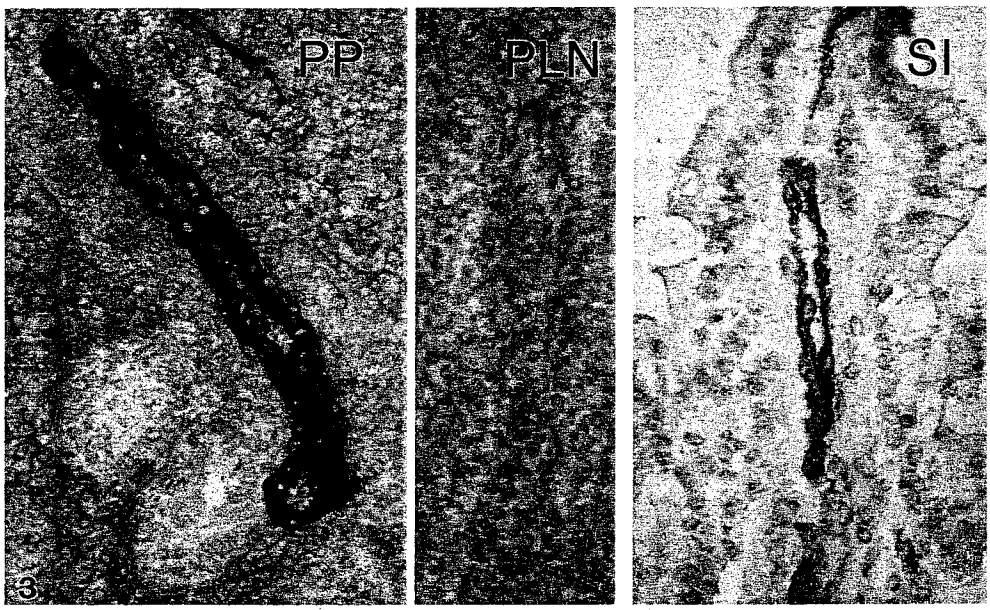
CH. 5, FIG. 6. Comparison of conformations for the two intact monoclonal antibodies, Mab231 and Mab61.1.3. Intact Mab231 is displayed in red and Mab61.1.3 in yellow. (A) The C_L:C_H1 domains of one Fab of Mab61.1.3 are superimposed (as a unit) onto the corresponding domains of Fab2 from Mab231. Variable domains do not superimpose but are shifted by 12°, as a consequence of differing Fab elbow angles (Table I). (B and C) The C_H3:C_H3 domain pairs of the two Fc segments are superimposed. The conformations of the two antibodies are quite unique.



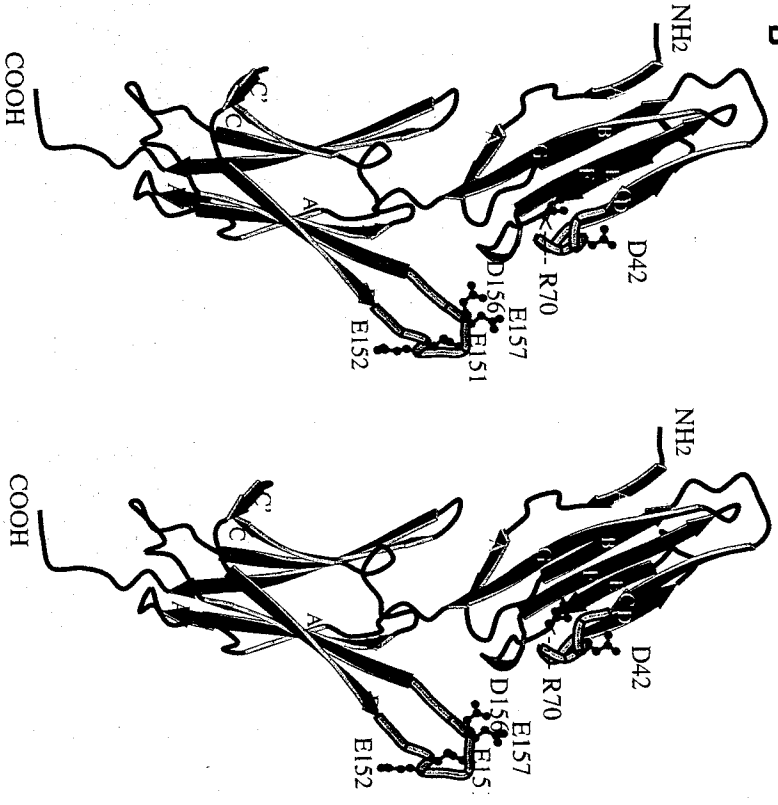




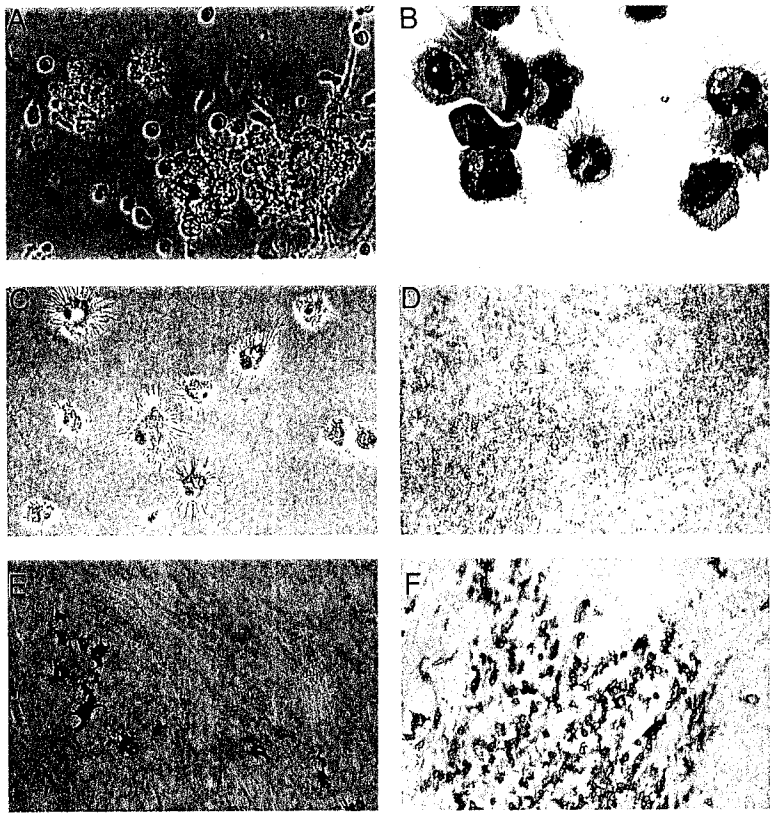
CH. 5, FIG. 7. Mapping of the distribution of IgG residues involved in key antigen and effector molecule interactions. Structures of (A) Mab61.1.3, (B) Mab231, and (C) Mcg. In all cases heavy chains are white, while light chains are blue, lime green, and purple, respectively. Antigen binding sites are indicated by ball and stick at the extremes of the Fab arms. The four lower hinge residues (cyan) immediately preceding Pro-251 are involved in Fcγ receptor binding. Lower hinge amino acids have also been implicated in Clq binding specificity. Core Clq binding residues (orange), carried by all IgG isotypes, are Glu-337, Lys-339, and Lys-341 (or Arg-341 in murine IgG_1). A hinge-proximal loop containing Pro-350 (magenta) is suggested to participate in both Clq and Fcγ receptor interaction. At the C_H2-C_H3 junction, bacterial proteins A and G have common contact points shown in green; residues specific for protein A are in red and for protein G, dark purple. Protein G has a second interaction site on C_H1 domain (yellow). Rheumatoid factor and rat neonatal Fc receptor binding sites overlap the area where proteins A and G contact C_H2-C_H3 (green). Oligosaccharides (gray) are generally required for proper effector functioning but have not, as yet, been implicated in forming binding sites. (A) For Mab61.1.3, it should be noted that although lower hinge residues are colored cyan, human FcγR1 does not bind mouse IgG_1 because these residues lack the critical Leu-Leu-Gly-Gly-Pro-251 binding sequence present in mouse IgG_{2a} and human IgG_1. (C) For comparison purposes, effector sites are mapped on Mcg, but note that hinge-deleted Mcg does not have normal effector function capabilities like molecules with hinges.



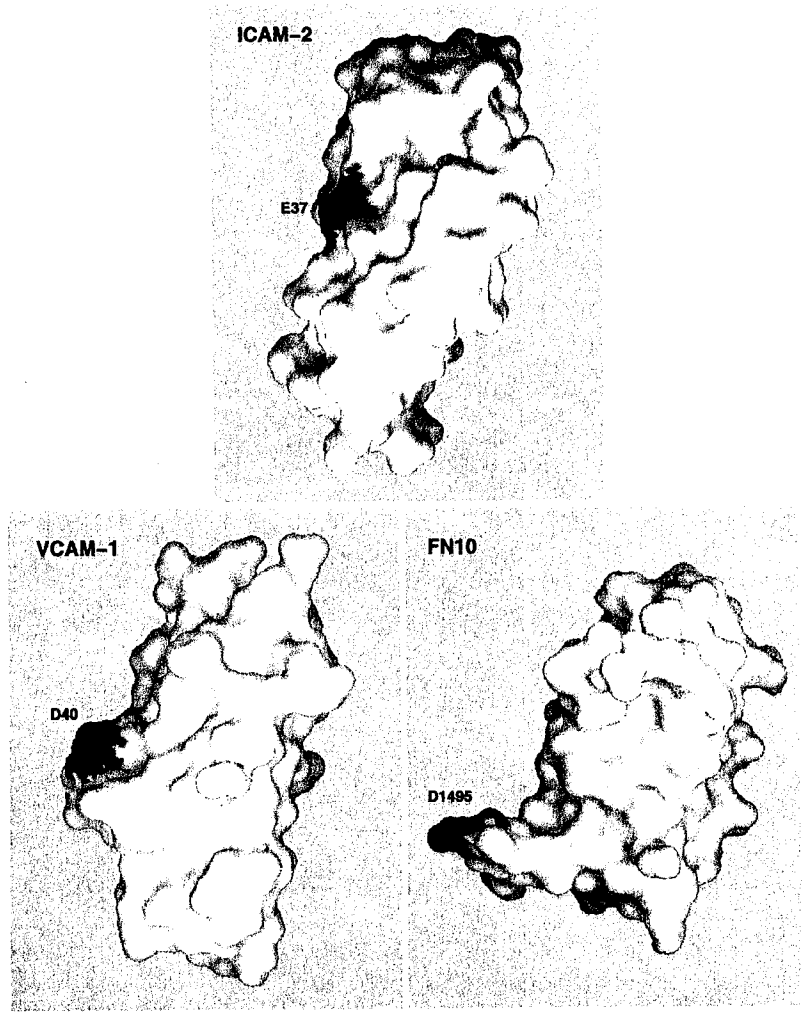
CH. 6, FIG. 3. Selective expression of the mucosal vascular addressin, MAdCAM-1, on postcapillary venules involved in lymphocyte homing to gastrointestinal sites. Immunohistologic staining reveals MAdCAM-1 on high endothelial venules in Peyer's patches (PP) and on postcapillary venules in the small intestinal lamina propria (SI). In contrast, high endothelial venules in axillary lymph nodes (PLNs) are negative.



CH. 6, FIG. 5B. Stereo view of the crystal structure of an N-terminal two-domain fragment of human MAdCAM-1 (68). The two most significant loops involved in $\alpha 4\beta 7$ binding, the CLDTS-containing CD loop on domain 1 and the negatively charged DE β ribbon, or "antenna," on domain 2, are highlighted in yellow (with purple stripes). The key D42 side-chain in domain 1 is shown as a stick-and-ball model with red oxygens; R70, important for maintenance of the structure of the CD and EF loops (the EF loop, with a short piece of helix in D1, is in blue), is shown with its oxygens in blue. The most critical residues in the domain 2 DE antenna, D156, and E151, E152, and E157, are also highlighted.



CH. 7, FIG. 2. Morphology of dendritic cells. (A) Cluster of DCs obtained by culturing blood $CD34^+$ hematopoietic progenitors with GM-CSF and TNF (×40). (B) Giemsa staining of a cytocentrifuged DC cluster (×100). (C) During culture of $CD34^+$ hematopoietic progenitors, a fraction of DCs is strongly adherent to the vessel surface (Giemsa staining, ×40). (D) Interdigitating DCs within the T-cell-rich areas of tonsils (blue, CD3 staining of T cells; red, CD83 staining of DCs); a germinal center is identified on the upper right corner. (E) CD1a DC_{imm} (red) infiltrate breast carcinoma tissue. (F) CD83 DC_{mat} (red) surround breast carcinoma together with $CD4^+$ T cells (blue); the purple color of the DCs indicates that they also express CD4.



CH. 8, FIG. 2. Molecular surface representations of integrin-binding domains for ICAM-2, VCAM-1, and FN10. The critical acidic residues involved in integrin binding are shaded red. Note that the binding regions in VCAM-1 and FN10 protrude from the protein surface; in contrast, the corresponding binding region of ICAM-2 is on a flat plane. [Reprinted by permission from *Nature* (Casasnovas *et al.*, 1997), copyright 1997, Macmillan Magazines Ltd.]

segments of the IgG. Though the hinge polypeptides were free of any intermolecular packing contacts, stabilization of the Fabs and Fc significantly restricted the conformation of the hinge. The 9 Å translation between Fabs stretched the hinge through its upper section to the first inter-heavy-chain disulfide. The core hinge was further stabilized by making an "intramolecular" contact with one glycosylated C_{H^2} loop and its attendant oligosaccharide. Because of this immobilization due to tension, electron density for the murine IgG_1 hinge was considerably more interpretable at an early stage of X-ray analysis than was the hinge region for the IgG_{2a}.

In Fig. 6A (see color plate), a C_L:C_{H^1} domain pair of the IgG_1 antiphenobarbital antibody (Mab61.1.3) is superimposed on Fab2 C_L:C_{H^1} of the IgG_{2a} canine lymphoma antibody (Mab231). A 12° shift in variable domains resulting from the different elbow angles, 155° for Mab61.1.3 and 143° for Fab2 of Mab231, is evident. Fab1 of Mab231, on the other hand, has an elbow angle of 159°, which is closer to those of Mab61.1.3 (Table I). In Fig. 6A–C, it can be seen that the two IgG conformations are substantially different, yet both exhibit two pronounced local dyads. Because the dispositions of segments are unique, those of corresponding dyads are as well.

These Mab structures provide images of two entire IgG molecules possessing normal hinge polypeptides (Figs. 4–6). The angle between the long axes of the Mab61.1.3 Fabs is 115°, producing a Y appearance, whereas that between Mab231 Fabs is 172°, yielding a T shape. In both cases, the Fc is obliquely oriented with respect to the Fabs and thus "distorts" the Y and T. In solution, antibodies transform within a spectrum of conformations. Taking the conformations of these two intact antibodies as limits, a range of antigen combining-site distances of about 120 to 150 Å would be expected (Table I). Fab segments can, however, adjust to accommodate many diverse antigen arrangements, for example, those presented on the surfaces of lymphoma cells. As evidenced by the phenobarbital antibody, the Fabs can also make translational adjustments to optimize antigen targeting. For Mab61.1.3 this Fab translation was only 9 Å, but murine IgG_1 has a relatively short upper hinge. Antibodies with longer upper hinge polypeptides could likely undertake greater translational adjustments in collaboration with rotational and hingelike movements.

VI. Biological Implications and Effector Functions

Antigen clearance mechanisms are either cellular, through binding of Fc to Fcγ receptors FcRI, FcRII, and FcRIII on leukocytes, or humoral, dependent on activation of the complement cascade. Fc effector interactions which lead to elimination occur only after the antibody has established contact with antigen. For the case of complement, antibody Fc–Fc aggrega-

tion must also occur, following antigen contact, in order to activate C1 and subsequently the cascade. Details as to how effector functions are mediated are, in general, lacking. How antigen binding, given the flexible nature of the antibody molecule, triggers effector responses at the Fc is a particular mystery. Some possibilities have been reviewed by Metzger, who discusses (1) an allosteric model, (2) a distortive model, and (3) a purely associative or aggregation model (Metzger, 1978). Burton, on the other hand, describes (1) a disordered adapter model and (2) an ordered adapter model, both of which are distinct from constructs implying allosteric conformational changes upon IgG complexation with antigen (Burton, 1990a; Burton and Woof, 1992). Recombinations of different elements of these proposals are possible, whereas specific features of the models are difficult to assess by physical methods.

The flexible nature of the antibody allows for the Fc to move out of the plane of the Fabs, while adjusting in a variety of ways to both target and effector. The intact monoclonal antibody structures, Mab231 and Mab61.1.3, discussed above, exemplify the possibilities (Figs. 4–6). Both exhibit dislocated Fcs, yielding arbitrary conformations that could permit Fc–Fc association. This would fulfill the minimum binding requirement for two of the six heads of C1q needed for complement activation, and conformations that allow clear access to Fcγ receptor sites in the lower hinge regions. Although these few structures do not provide definitive answers to the salient questions regarding effector function, information does emerge by mapping, as in Fig. 7 (see color plate), the distribution of key residues involved in antigen and effector interactions. Our discussion of IgG effector functions will be primarily restricted to human IgG_1, murine IgG_1, murine IgG_{2a}, and human hinge-deleted IgG_1, which are the isotypes of the structures reviewed here (Table I).

The Fcγ receptor family has been characterized for human and mouse immunoglobulins and consists of FcγRI, FcγRII, and FcγRIII in each species. The critical IgG recognition sequence for the Fcγ receptor has been identified as lower hinge residues 247–251, Leu-Leu-Gly-Gly-Pro, of the C_{H^2} gene product (EU 234–238). Particular weight has been assigned to the two leucines. This sequence has been implicated as a binding site, or overlapping binding site, for all three human Fcγ receptors, and for mouse FcγRII as well (Duncan *et al.*, 1988; Lund *et al.*, 1991, 1992; Sarmay *et al.*, 1992; Morgan *et al.*, 1995). Human FcγRI binds with high affinity to both monomeric human IgG_1 and murine IgG_{2a} but fails to bind to murine IgG_1 (Duncan *et al.*, 1988). Sequence 247–251 is identical for human IgG_1 and murine IgG_{2a} (Table II), and the isotypes are virtually indistinguishable in terms of their affinities for human FcγRI. Murine IgG_1, having a lower hinge that is three residues shorter, does not contain the binding sequence (Table II). Variation in lower hinge sequences for the IgG subclasses may dictate specificity of antibody interaction for mouse and human Fcγ receptors (Lund *et al.*, 1992).

Dob and Mcg contain the crucial Leu-Leu-Gly-Gly-Pro lower hinge sequence, because they have only genetic hinge deletions (Table II). FcγRI has some binding affinity for Mcg [J. M. Woof and D. R. Burton, as quoted in Burton (1990b)], but not for intact Dob (Klein *et al.*, 1981). An engineered chimeric IgG_3 having a genetic hinge deletion, but a disulfide bond inserted at the N terminus of the lower hinge, showed high activity for FcγRI-mediated phagocytosis. Hinge-deleted chimeric IgG_3 without an H–H disulfide bridge yielded low to no activity (Brekke *et al.*, 1993, 1996; Michaelsen *et al.*, 1994). Thus the key role of the "genetic" hinge (upper and core structural hinge), required for high-efficiency FcγR signaling, appears to be maintenance of at least one inter-H-chain disulfide bond. This joins heavy chains just prior to the lower hinge residues. The genetic hinge acting as a long spacer, or endowing Fab–Fab flexibility, seems unnecessary for FcγR interaction (Michaelsen *et al.*, 1994).

Optimal association with human FcγRI suggests that lower hinge residues act in combination with a hinge-proximal C_{H^2} loop, sequence 344–350 (EU numbering 325–331), containing a particularly significant Pro-350 (Canfield and Morrison, 1991). The loop likely forms part of the binding platform for FcγRI. In the canine lymphoma antibody structure, the lower hinge polypeptide of one heavy chain does in fact make contact with C_{H^2} loop residues 344–350 on that same heavy chain. This confirms that lower hinge residues can be in intimate contact with the hinge-proximal loop containing Pro-350 (EU Pro-331) (see Fig. 7B). In Mcg, accessibility of Leu-Leu permits receptor binding (see above), but Pro-350 was noted to be sterically blocked by the bypass peptides (Edmundson *et al.*, 1995).

A locus essential for C1q binding has been identified as three specific polar residues of the C_{H^2} domain (Duncan and Winter, 1988). All IgG isotypes contain this "core" C1q site (Fig. 7). In most IgGs, including human IgG_1 and mouse IgG_{2a}, the residues are Glu-337, Lys-339, and Lys-341. For mouse IgG_1 residue 341 is an arginine. Because antibody subclasses show differing abilities to activate complement, specificity must reside elsewhere on the IgG. It has been assigned to the C_{H^2} gene product, implying residues in the structural lower hinge or C_{H^2} domain (Clackson and Winter, 1989; Tao *et al.*, 1991, 1993; Greenwood *et al.*, 1993; Brekke *et al.*, 1994; Morgan *et al.*, 1995). Lower hinge residues and the hinge-proximal C_{H^2} loop that were found to be important for FcγR binding have additionally been implicated in C1q specificity (Morgan *et al.*, 1995; Tao *et al.*, 1993; Brekke *et al.*, 1994). Again, within this C_{H^2} loop, Pro-350 appears to be of particular importance. Oligosaccharides have also been

shown essential for activation (Nose and Wigzell, 1983), as are paired C_{H^3} domains (Utsumi *et al.*, 1985).

Human IgG_1 and mouse IgG_{2a} are strong activators of complement, whereas mouse IgG_1 is poor, and genetic hinge-deleted human Dob and Mcg fail altogether. Fluorescent depolarization studies of normal IgG subclasses, and the examples of Dob and Mcg, encouraged the idea that hinge flexibility was correlated with an ability to activate complement (Oi *et al.*, 1984; Dangl *et al.*, 1988). A genetic hinge-deleted, chimeric IgG_3, with Ala-Ala-Cys-Ala introduced between Ala-244 and Pro-245 of the lower hinge region, was shown, however, to have an even greater complement-mediated lysis (CML) activity than wild-type IgG_3. The corresponding hinge-deleted IgG_3 without an inserted disulfide bond had essentially no CML activity (Brekke *et al.*, 1993, 1996; Michaelsen *et al.*, 1994). The genetic hinge (upper and core hinge) functioning as a flexible spacer was, therefore, shown unnecessary for complement activation. The only role of the "genetic" hinge for complement activation appears to be maintenance of an inter-H-chain disulfide bond(s) linking heavy chains at the N terminus of the lower hinge. This result corroborates a longtime observation that reduction of normal IgG, under conditions where only interchain disulfides are cleaved, causes loss of Fc-mediated effector functions (Dorrington, 1978).

Because Mcg and Dob lack an inter-heavy-chain disulfide, hinge bypass segments (lower hinges) are not drawn together seven residues N terminal to the structural C_{H^2} domain. These residues, instead, take a path that could obstruct access to residues involved in the binding of C1q, and/or their path may be such that C_{H^2} domains do not maintain correct orientations for activation. Although the core C1q-binding motif of Mcg is exposed (Fig. 7), the hinge-proximal loop containing residues 344–350 is sterically obstructed by the bypass residues (Edmundson *et al.*, 1995). The fact that Mcg does not have a canonical C_{H^2}:C_{H^2} relationship lends some credence to the proposal that C_{H^2} domains may not be oriented in an appropriate manner for C1q interaction.

Removal or degradation of the immunoglobulin oligosaccharides has also been shown to alter significantly not only complement interactions, but most effector functions in general (Nose and Wigzell, 1983). IgGs lacking carbohydrates lose ability to activate complement, to bind Fc receptors, and to induce antibody-dependent cellular cytotoxicity (ADCC). Galactose deficiencies, in particular, have been associated with rheumatoid arthritis and other chronic diseases (Parekh *et al.*, 1985; Tsuchiya *et al.*, 1989; Furukawa and Kobata, 1991). Even though sugar residues have not been directly implicated in effector binding sites, there is abundant evidence that they play an essential role in the function of Fc. We now know from the structure of Mab61.1.3 that core and lower hinge polypeptides

can contact the oligosaccharide, as well as the C_{H^2} polypeptide loop that N links the carbohydrate chain (Harris *et al.*, 1998). This contact, although likely transient in solution, could be important for effector function (see Fig. 7A).

The interfaces between C_{H^2} and C_{H^3} domains within the Fc are additional regions of physiological interest, because several biological macromolecules are known to recognize and bind to this area, or to include it in overlapping sites. Biochemical studies have indicated the interface to be important in regulating both transcytosis and catabolism (Ghetie and Ward, 1995). Crystallographic studies have identified the C_{H^2}–C_{H^3} junction as the site of interaction for rheumatoid factor (Corper *et al.*, 1997), rat neonatal Fc receptor (Burmeister *et al.*, 1994; Vaughn *et al.*, 1997), and *Staphylococcus aureus* proteins A (Deisenhofer, 1981) and G (Sauer-Eriksson *et al.*, 1995). Ig-binding proteins A and G are considered Fc receptors; however, both proteins utilize secondary binding sites on the Fab segments as well (Derrick and Wigley, 1992). These molecules, A and G, are found on the surfaces of bacteria and are relevant to bacterial virulence (Kastern *et al.*, 1990; Raeder and Boyle, 1993). Protein A has been shown to bind hinge-deleted Dob (Klein *et al.*, 1981) and Mcg, even though the Fc interaction site on Mcg is enlarged due to its noncanonical C_{H^2}–C_{H^3} interface (Edmundson *et al.*, 1995).

In all crystallographic structures of Fc fragments, or Fc segments within intact antibodies, the first loop of the C_{H^2} domain (residues 264–267) remains stationary with respect to the interface with C_{H^3}, while the remainder of the C_{H^2} domain may show appreciable movement. This loop appears to serve as a "pivot" allowing the C_{H^2} domain to undergo rigid body motion with respect to the C_{H^2}–C_{H^3} interface (Harris *et al.*, 1997). While the interface is maintained, the C_{H^2}–C_{H^3} pivot could modulate effector functions by altering the spatial relationship of binding sites.

Although segmental flexibility and spacer properties of the genetic hinge (upper and core hinge) were shown of little importance in complement activation and FcγR signaling, these features do affect antigen accessibility. The lengths of the upper hinge polypeptides dictate Fab–Fab flexibility and thus give differing "reach" capabilities, rotational and translational, during antigen complexation. IgGs are able to interact with epitopes of varying dispositions as a consequence of genetic hinge diversity. This diversity may be most important when the IgG is membrane bound on B cells (Brekke *et al.*, 1995). In this case its reach would be almost entirely dependent on the genetic hinge sequence.

VII. Concluding Remarks

The intersegmental flexibility illustrated by the intact antibody structures is well established, and was previously documented using a variety of

lower resolution methods, such as electron microscopy and fluorescence depolarization spectroscopy (Wrigley *et al.*, 1983; Roux, 1984; Wade *et al.*, 1989; Oi *et al.*, 1984). These high-resolution crystallographic images do, however, allow a more careful analysis and exact definition of degrees of freedom (Table I and Figs. 1–6). Mutational studies pertinent to hinge sequence and effector interactions, taken in the structural context of the intact antibody models, suggest that some ideas regarding antibody flexibility should possibly be reevaluated.

The genetic hinge, composed of the upper and core structural hinge residues (Table II), is primarily responsible for providing Fab–Fab movements for antigen binding. It does not appear to play a major role in effector processes, except to provide at least one inter-heavy-chain disulfide bond, thereby tethering the chains, and thus the segments. Variability in genetic hinge sequences provides various reaching capabilities for the IgG subclasses, and this, perhaps, is its primary function. The flexible-spacer role of the genetic hinge is apparently unnecessary for effector processes, including complement activation, Fcγ receptor signaling, and antibody-dependent cellular cytotoxicity (see review, Brekke *et al.*, 1995, 1996).

Polypeptides C terminal to the genetic hinge are structurally regarded as the lower hinge (Table II), but they are encoded by the C_H2 exon. These residues alone provide the flexibility necessary for effector functions. C1q and FcγR recognition sites on IgG are accessible even when the upper and core hinge regions are deleted, as long as a disulfide link is inserted, and properly formed, at the N terminus of the lower hinge. Residues essential for C1q and FcγR specificity and activation are included within the C_H2 gene product, and are composed of the lower hinge and C_H2 domain (Fig. 7). The interface between C_H2–C_H3 domains in the Fc is an additional locus for interaction with a variety of biological molecules, some of which are involved in effector mechanisms (Fig. 7). A critical C_H2 loop at the interface serves as a structural "pivot" for rigid body motions of C_H2 domain(s) with respect to the C_H3:C_H3 pair.

One somewhat unexpected result to emerge from the crystallographic studies discussed above, consistent with fragment studies as well, is the independence of the two C_H2s. These are the only domains in the antibody not paired through noncovalent interactions; all other domain pairings exhibit extensive contacts. The C_H2s are glycosylated in a manner that sequesters the carbohydrates, unlike most glycoproteins. They are clearly the most mobile of the domains, but, at the same time, they carry a variety of effector sites. In some cases, C_H2 domains seem to function in concert with lower hinge residues, and in others cases in collaboration with C_H3s. Disulfide bridging of heavy chains in the hinge core does impose

limits on C_H2 movements that are, perhaps, significant for proper orientation of C_H2 domains during effector activation.

The intact antibodies with hinge polypeptides, Kol, Mab231, and Mab61.1.3, taken together, provide a dynamic range of motion for immunoglobulin G during antigen recognition and subsequent effector functions. Although the crystal structures represent "snapshots" or "still images" of IgG, in solution the antibodies can be considered as transforming between these representative conformations, as well as a myriad of others. Genetic hinge-deleted antibodies such as Dob and Mcg are clearly the most rigid IgG molecules, but these also, in solution, would be expected to have some flexibility, albeit severely reduced.

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Lymphocyte Trafficking and Regional Immunity

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I. Introduction

Lymphocytes are migratory cells, trafficking from their sites of origin in the bone marrow and thymus, and homing to and recirculating through specialized lymphoid and extralymphoid tissues in the periphery (*1–14*). Like all leukocytes, lymphocytes develop with characteristic trafficking properties. Lymphocytic components of the innate immune system, such as natural killer cells, as well as subsets of $\gamma\delta$ T cells and specialized lymphoid dendritic populations, appear to be preprogrammed with particular tissue or inflammation tropisms during their development in primary lymphoid organs. For lymphocytes of the adaptive immune system, comprising the bulk of $\alpha\beta$ receptor-expressing T cells and of B cells in the adult animal, initial homing properties—a pronounced tropism for secondary lymphoid organs, including lymph nodes (LNs), Peyer's patches (PPs), and spleen—also seem to be determined developmentally, prior to or in association with emigration from the thymus or bone marrow (see, for example, Ref. *15*). However, whereas most leukocytes pass through the blood only once in their migration to terminal sites of participation in inflammatory responses, these T and B cells recirculate continously from blood to lymph. Moreover, antigen-dependent stimulation appears to reprogram the trafficking properties of naive B and T cells in the periphery, inducing or selecting for memory and effector cells that not only home much more effectively than their naive precursors to extralymphoid sites of tissue inflammation, but that can also display striking selectivity for trafficking to the gastrointestinal tract, skin, or other specific tissues or organs (*1, 4–10, 12–14, 16–18*) (reviewed in Refs. *19–21*). For example, B and T immunoblasts and T memory cells induced in gastrointestinal lymphoid tissues traffic preferentially into the intestinal wall and/or gut-associated lymphoid organs (PPs, appendix, and mesenteric LNs; see below). Conversely, immunocytes generated in response to cutaneous immunization home better to inflamed skin (see Fig. 1).

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