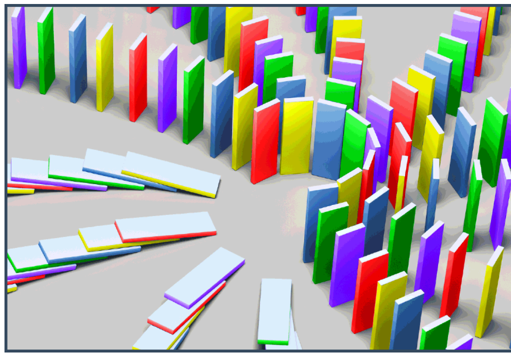
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Human IgG2 Can Form Covalent Dimers[1]

Esther M. Yoo, Letitia A. Wims, Lisa A. Chan, and Sherie L. Morrison[2]

Unlike IgA and IgM, IgG has not yet been shown to form covalent polymers. However in the presence of specific Ag, murine IgG3 has been shown to polymerize through noncovalent interactions. In contrast to the noncovalent oligomers found with murine IgG3, we have detected covalent dimers in three different recombinant human IgG2 Abs produced in myeloma cells. Both IgG2,κ and IgG2,λ can form dimers. In addition, analysis of pooled human γ globulin and several normal sera revealed the presence of IgG2 dimers. The IgG2 dimers are in contrast to the noncovalent IgG dimers found in pooled sera of multiple donors resulting from idiotype/anti-idiotype (Id/anti-Id) interactions. Cyanogen bromide cleavage analysis suggests that one or more Cys residues in the γ2 hinge are involved in dimer assembly. The potential role of IgG2 dimers in immunity against carbohydrate Ags is discussed. *The Journal of Immunology,* **2003, 170: 3134–3138.**

The prototypic IgG molecule is composed of two H and two L chains. The IgG monomers are held together by inter-H chain disulfide bond(s) in the hinge regions and by disulfide bonds between the H and L chains. Human IgG4 differs from the prototypic IgG in that HL half-molecules are observed which interact noncovalently to form H_2L_2 molecules (1–5). In IgG4, the inter-H chain disulfide bonds in the hinge are labile and Cys^{226} and Cys^{229} (EU numbering) can form either inter- or intra-H chain disulfide bonds (6, 7). The equilibrium between inter- and intra-H chain bonds explains the presence of HL half-molecules and the functional monovalency of IgG4 (8). IgG has not yet been reported to form covalently linked polymers as is seen with IgA and IgM. However, murine IgG3 (mIgG3)[3] has been shown to self-associate through noncovalent interactions (9–13). One consequence of the self-association of mIgG3 is enhanced Ag binding (12). This enhancement of binding was concentration dependent and isotype specific. In addition, self-association required intact Fcγ3 regions, indicating that the noncovalent interaction was occurring in the Fc portion of IgG3 (13).

IgG dimers have been detected in pooled plasma from multiple donors but not in plasma from single donors. The majority of these dimers are thought to be idiotype (Id)/anti-Id complexes that associate noncovalently through the F(ab) regions (14, 15). Although all four human IgG isotypes have been shown to dimerize in pooled plasma, IgG3 is over-represented and IgG2 is under-represented in these Id/anti-Id interactions (14). In contrast to the dimers found in pooled plasma, we report that human IgG2 can form covalent dimers. Several different recombinant mAbs with the human γ2 constant region were found to be secreted as monomers and dimers and included both IgG2,κ and IgG2,λ. In addition, dimers of IgG2 were detected in pooled human γ globulin (Miles IgG) as well as in serum from individuals, indicating that IgG2 polymers are formed in vivo. Analysis of cyanogen bromide (CNBr)-cleaved polypeptide fragments of IgG2 suggested that one or more Cys residues in the hinge participate in the formation of IgG2 dimers.

Materials and Methods

Production and analysis of recombinant Abs and human sera

Chimeric IgG2,κ anti-*Streptococcus mutans* and IgG2,λ anti-dansyl (DNS) were produced in NS0/1. Chimeric IgG2 κ anti-DNS was produced in P3 × 63Ag8.6533. Abs were purified from culture supernatant by DNS-Sepharose affinity chromatography (16) or using protein A (17) as described previously. Protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL). Recombinant Abs and human sera were treated with 0.125 mM iodoacetamide (Sigma-Aldrich, St. Louis, MO) on ice or at 37°C for 1 h to block free thiols. Loading buffer (25 mM Tris, pH 6.7, 0.2% SDS, 10% glycerol, 8 μg/100 ml bromphenol blue) was added and the samples were boiled for 3 min before analysis by SDS-PAGE on 4% Tris-glycine or 5% sodium phosphate-buffered (PO_4) gels. To examine H and L chains separately, the purified proteins were reduced by treatment with 0.15 M 2-ME at 37°C for 30 min and analyzed on 12.5% Tris-glycine gels. IgG2 was detected by staining with Coomassie blue or by Western blotting (see below).

Western blotting to detect human IgG

Proteins were transferred to Immobilon-P polyvinylidene fluoride membrane (Millipore, Bedford, MA) according to the semidry method of Towbin et al. (18). Nonspecific sites were blocked by incubating the membrane for 1 h at room temperature in PBS containing 0.2% (v/v) Tween 20 and 5% (w/v) nonfat dried milk. Mouse mAb HP6070 specific for human γ1, HP6002 specific for human γ2, HP6050 specific for human γ3, or HP6035 specific for human γ4 (kindly provided by Dr. R. G. Hamilton (Johns Hopkins University, Baltimore, MD)) was used as a primary reagent for 1 h at room temperature or overnight at 4°C. After washing, the blot was probed with peroxidase-conjugated sheep anti-mouse IgG (Sigma-Aldrich) for 1 h at room temperature or overnight at 4°C. The peroxidase-conjugated Ab was detected using the Supersignal Substrate system (Pierce) as recommended by the manufacturer.

Fast protein liquid chromatography (FPLC) analysis for IgG2 polymers

One milligram of Miles IgG was separated by gel filtration on a 30 × 1.5 cm Superose 6 column (Pharmacia, Piscataway, NJ) in PBS plus 0.02% NaN_3 and eluted in 600-μl fractions. The fractions containing dimers were then analyzed by nonreducing SDS-PAGE on 4% Tris-glycine gels and Western blotting was performed as described above.

Department of Microbiology, Immunology, and Molecular Genetics and Molecular Biology Institute, University of California, Los Angeles, CA 90095

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[2] Address correspondence and reprint requests to Dr. Sherie L. Morrison, Department of Microbiology, Immunology, and Molecular Genetics and Molecular Biology Institute, University of California, 405 Hilgard Avenue, Los Angeles, CA 90095. E-mail address: sheriem@microbio.ucla.edu

[3] Abbreviations used in this paper: mIgG3, murine IgG3; CNBr, cyanogen bromide; DNS, dansyl; FPLC, fast protein liquid chromatography; Id, idiotype.

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Analysis of CNBr-cleaved IgG2

A total of 30–75 μg of recombinant IgG2 was dried and 80 μl of 88% formic acid (Sigma-Aldrich), 5 μl of 5 M CNBr in acetonitrile (Sigma-Aldrich), and 15 μl of dH_2O were added to the sample and incubated at room temperature overnight in the dark. dH_2O (1.2 ml) was then added, and the samples were dried under vacuum. After the addition of loading buffer, the samples were boiled for 3 min and analyzed by SDS-PAGE on 8% Tris-glycine gels under nonreducing conditions. For analysis in the second dimension, the gel lane of interest was excised and reduced with 5% (w/v) DTT (Sigma-Aldrich) in dH_2O for 30 min at 37°C. The gel lane was placed flush against the top of a 14% Tris-glycine gel with the more rapidly migrating peptide fragments at the right and separated by size on SDS-PAGE. Peptide fragments were visualized using silver staining (Bio-Rad, Hercules, CA) as recommended by the manufacturer. To stain for polypeptides containing carbohydrates, CNBr-cleaved IgG2 was separated by SDS-PAGE under nonreducing conditions on 8 and 14% Tris-glycine gels. The gels were stained using the Pro-Q Fuchsia Glycoprotein Gel Stain kit (Molecular Probes, Eugene, OR) as recommended by the manufacturer. CandyCane glycoprotein m.w. standards (Molecular Probes) containing glycosylated and nonglycosylated proteins were used as positive and negative controls for the stain.

Results

Recombinant chimeric IgG2 is secreted as covalent dimers

Chimeric Abs containing a murine variable region and human γ2 constant region were expressed in myeloma cell lines NS0/1 or P3 × 63Ag8.653. An anti-*Streptococcus mutans* IgG2,κ was found to be secreted as a monomer species of ~150 kDa as well as a higher m.w. species with a m.w. of that expected for an IgG dimer when subjected to SDS-PAGE (Fig. 1*A*). To verify that the higher m.w. band was dimers of IgG2 rather than a contaminant or a different protein associated with IgG2, the protein sample was reduced and run on SDS-PAGE. Staining with Coomassie blue revealed only H and L chains, confirming that the higher m.w. band was indeed IgG2 dimer (data not shown). Two other recombinant IgG2 proteins were tested by Western blotting using the HP6002 anti-human γ2-specific Ab (Fig. 1*B*) for their ability to form dimers. Dimers and monomers were observed for an anti-DNS IgG2,κ as well as for an anti-DNS IgG2,λ. The specificity of the detecting Ab was confirmed since recombinant IgG1, IgG3, and IgG4 with the same specificity for DNS failed to be recognized by the HP6002 Ab (data not shown). In addition, analysis of the anti-DNS IgG2,κ and IgG2,λ by gel filtration chromatography revealed two peaks, corresponding to dimer and monomer (data not shown). These data suggest that dimer formation occurs through the γ2 H chain since Abs with different variable regions and different isotypes of L chain were found to dimerize.

IgG2 dimers are present in normal human serum

To test for the presence of IgG2 dimers in human serum, Miles IgG was analyzed by gel filtration chromatography. A polymer peak was detected by FPLC (Fig. 2*A*). The polymer peak could represent both covalent dimers as well as noncovalent Id/anti-Id complexes since Miles IgG contains immunoglobulins from numerous donors. To determine whether covalent IgG2 polymers are present, the dimer fractions were analyzed by SDS-PAGE and Western blotting. A prominent band corresponding to IgG2 dimers is seen in the Miles polymer fractions whereas only a very faint band corresponding to dimer is seen in unfractionated Miles IgG (Fig. 2*B*).

We also tested unpooled serum from individuals for the presence of IgG2 polymers by Western blotting. In all individuals tested, IgG2 dimers were detected (Fig. 3*A*). In some samples, multiple bands corresponding to the dimer were observed. These bands may be due to the differences in mobility of IgG2,κ and IgG2,λ or variability in glycosylation. A band migrating at the position expected for H_3L_3 was also frequently observed. Three representative serum samples as well as recombinant IgG2,κ were treated with iodoacetamide as described in the *Materials and Methods* to block any free thiol groups and prevent disulfide bond formation during sample preparation. As shown in Fig. 3*B*, dimers were still present in sera treated with iodoacetamide, indicating that dimer assembly does not occur during sample preparation.

To verify that the bands detected by Western blotting were indeed IgG2, we performed a "competitive" Western blot (Fig. 3*C*). Either 5 or 10 μg of soluble recombinant IgG2,κ protein was

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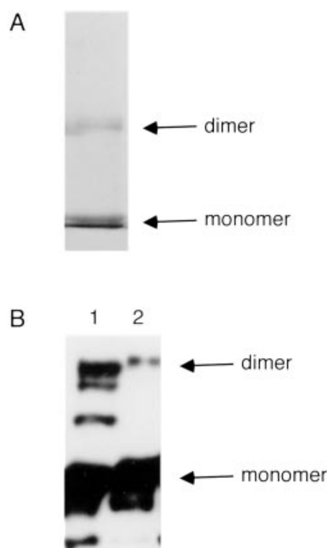

FIGURE 1. Analysis of recombinant IgG2 proteins. Three different recombinant IgG2 proteins were analyzed for the presence of dimers by nonreducing SDS-PAGE on 4% Tris-glycine gels. *A,* Anti-*S. mutans* IgG2,κ. *B, Lane 1,* anti-DNS IgG2,κ; *lane 2*, anti-DNS IgG2,λ. The gel in *A* was stained with Coomassie blue while *B* was visualized by Western blotting using a mouse anti-human γ2 chain-specific Ab followed by a peroxidase-conjugated sheep anti-mouse IgG.

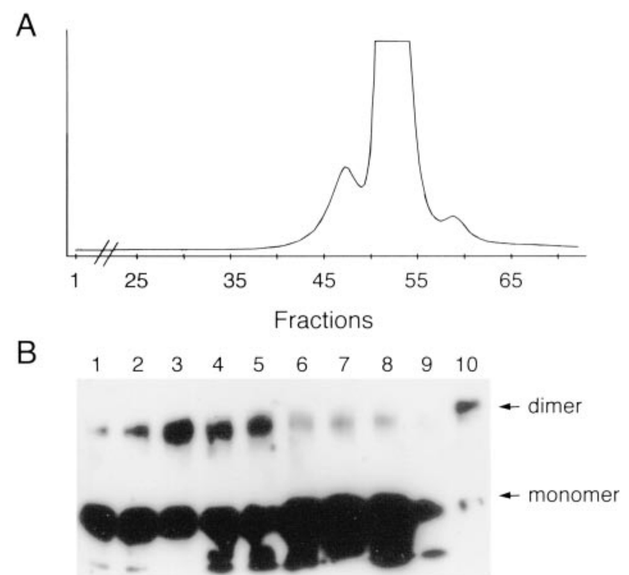

FIGURE 2. Analysis of Miles IgG. *A,* IgG dimers were detected by size exclusion FPLC. The fractions numbers are indicated on the *x*-axis. *B,* Western blotting was performed on the FPLC fractions containing dimers. *Lanes 1–8* show the FPLC dimers fractions 41–48, respectively; *lane 9* shows unfractionated Miles IgG, and *lane 10* shows recombinant IgG2,κ.

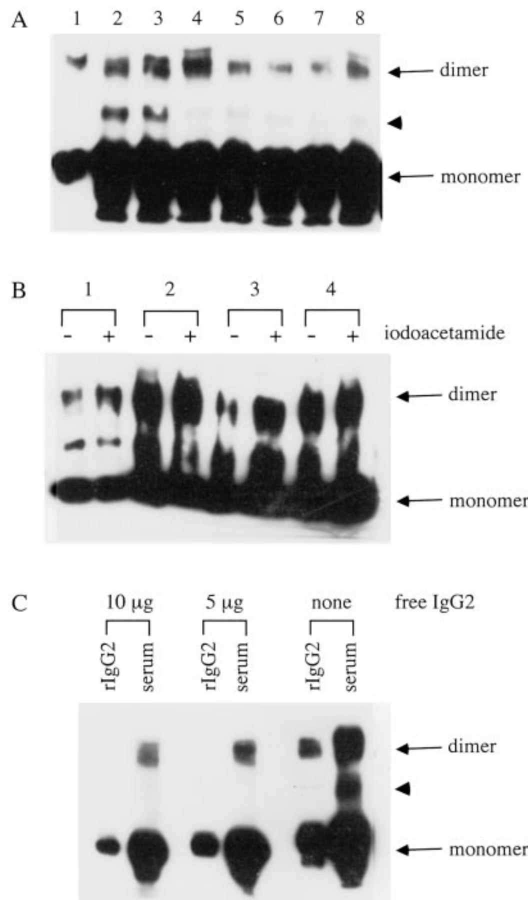

FIGURE 3. Analysis of normal human serum for IgG2 polymers. Western blotting was performed on sera from different individuals. The blots were probed using a mouse anti-human γ2 chain-specific Ab followed by a peroxidase-conjugated sheep anti-mouse IgG. *A, Lane 1*, rIgG2,κ; *lanes 2–8*, sera from seven human individuals. *B, Lane 1*, rIgG2,κ; *lanes 2–4*, sera from three individuals (same as those in *A lanes 4, 3*, and *8*, respectively) were untreated (−) or treated (+) with iodoacetamide to block any free thiol groups. *C,* Competitive Western blot done in the presence of 10 μg, 5 μg or no soluble IgG2. rIgG2 is recombinant anti-DNS IgG,κ. The arrow head represents a protein species of the size expected for H_3L_3. *B*, The serum shown in *lane 4* was used for the competition studies.

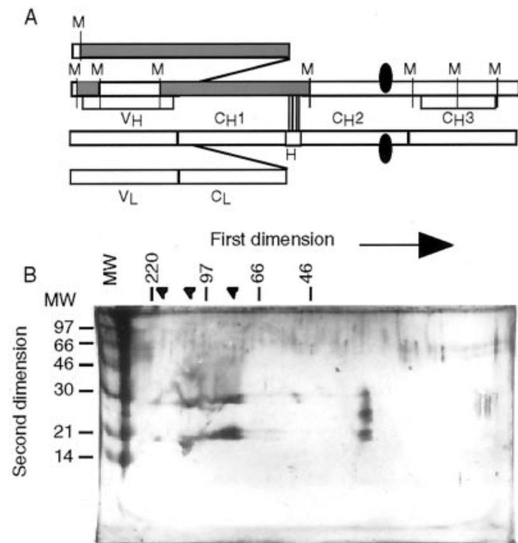

FIGURE 4. Analysis of CNBr-cleaved IgG2. A. A schematic representation of CNBr-cleaved anti-DNS IgG2,λ. The positions of methionine residues are noted by an M. The polypeptide fragments that are covalently associated through disulfide bonds after cleavage with CNBr are noted by thin lines. There are four Cys residues in the hinge (H) and all are shown by a line as making inter-H chain bonds, although that does not appear to be the case. The fragments that are covalently associated into the larger m.w. complexes after CNBr cleavage are shown as filled. The C_H2 associated carbohydrate is indicated by a dark oval. *B,* Analysis of IgG2 fragments after cleavage with CNBr by SDS-PAGE. CNBr-cleaved IgG2 was fractionated by SDS-PAGE, the gel was cut, and the lane was excised and reduced in DTT. The reduced polypeptide fragments were then separated by size in the second dimension. The 23-kDa fragment represents the L chain and the 18-kDa fragment represents the C_H1/hinge. The three major bands corresponding to fragments of 170, 130, and 85 kDa in the first dimension are noted by the arrow heads.

added to the blots containing recombinant protein or serum along with the anti-human γ2 HP6002. The free recombinant IgG2 should compete with the IgG2 on the membrane for the HP6002 Ab. In the absence of free IgG2, both the recombinant IgG2 and human serum sample revealed strong polymer signals. In contrast, the signals for both monomeric and polymeric recombinant and serum IgG2 were significantly decreased in the presence of soluble IgG2.

The Cys residue(s) in the hinge is (are) involved in IgG2 dimer formation

To ascertain which Cys residue(s) is (are) involved in the formation of dimers, recombinant IgG2,λ was cleaved using CNBr and analyzed. The cleaved protein was subjected to nonreducing SDS-PAGE in the first dimension and reducing SDS-PAGE in the second dimension. A schematic representation of the cleavage products are shown in Fig. 4*A*. The hatch boxes in Fig. 4*A* represents the majority of the L chain and parts of the V_H, all of C_H1 and the hinge of the H chain; these fragments are covalently linked through disulfide bonds. For simplicity, they will be called the L chain and C_H1/hinge, respectively. When the IgG2 monomer is cleaved by CNBr, a fragment of 85 kDa with (L chain-C_H1/hinge)$_2$ held together by inter-H chain and inter-H and L chain disulfide bonds is expected. In addition to this band, two other major bands of ~130 and ~170 kDa were also present when IgG2 was analyzed in the first dimension under nonreducing SDS-PAGE (Fig. 4*B*). Following reduction, all three bands resolved into fragments of 23 and 18 kDa corresponding to the sizes predicted for the L chain and C_H1/hinge, respectively. Therefore, we believe that the 170 kDa band represents the cleavage product of IgG2 dimers consisting of (L chain-C_H1/hinge)$_4$ and that the 130 kDa band represents (L chain-C_H1/hinge)$_3$. It is possible that the latter band represents an intermediate between IgG2 monomers and dimers (see *Discussion*).

In addition, the fragments were analyzed by Pro-Q Fuchsia staining, which stains glycoproteins but does not stain nonglycosylated proteins. The Ab contains the single conserved glycosylation site at position 297 within C_H2. While the fragment migrating at 18 kDa in the first dimension consisting of the IgG2 Fc containing the *N*-linked carbohydrate was stained, the 170-, 130-, and 85-kDa bands were not stained by Pro-Q Fuchsia, again indicating that these bands were not part of the Fc portion of IgG2 (data not shown). Therefore, our data indicate that one or more Cys residues in the L chain or C_H1/hinge is involved in the formation of IgG2 dimers.

Discussion

Our analysis of recombinant IgGs, Miles IgG, as well as serum from single human donors clearly indicates that human IgG2 can

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Table I. *Sequence comparison of human IgG hinge regions*

Isotype	Sequence of Hinge Region[a]
Human γ1	EPKSCDKTHT**CPPC**P
Human γ2	ERKC**CVECPPC**P
Human γ3 (exon 1)	ELKTPLGDTTHT**CPRC**P
Human γ3 (exons 2, 3, 4)	EPKSCDTPPP**CPRC**P
Human γ4	ESKYGPP**CPSC**P

[a] CXXC motif is in bold. The sequence is that of the genetic hinge.

be secreted as dimers. The dimers were observed under denaturing conditions in SDS, indicating that the polymers are covalently associated. This is in contrast to mIgG3 (12, 13) and IgG dimers found in pooled human plasma, which associate noncovalently. Interestingly, it has been reported that covalent polymers, albeit at low levels, are also present in pooled plasma (14). Since these covalent dimers were not analyzed further, it is difficult to speculate if some of them are indeed IgG2 dimers.

Unlike the human γ constant exons, which share >90% homology, the human IgG hinge sequences are only ~50–70% homologous. This degree of homology is less than that found in the flanking intronic sequences (19–21). A sequence comparison of the human IgG hinges is shown in Table I. The hinge of human IgG3 is unusual in that it is encoded on four separate exons (20) although hinges containing only two and three exons have been reported (22, 23). Unlike the other γ hinge regions, the γ2 hinge contains four Cys residues. These four Cys residues were assumed to be involved in forming inter-H chain disulfide bonds (24, 25) although no definitive data have been presented demonstrating that this is indeed the case. In fact, human IgG2 has been reported to contain free SH-group(s) although the position of the free Cys has not been elucidated (26). Therefore, it is possible that not all four Cys form inter-H chain disulfide bonds and/or that some of the inter-H chain disulfide bonds are unstable, as is the case for human IgG4 (6, 7).

Our experiments using CNBr cleavage and analysis of recombinant IgG2 on two-dimensional gels revealed that it is the L chain-C_H1/hinge fragment that is involved in the formation of dimers. Although the L chain and C_H1 do contain Cys residues, all of them are involved in the formation of intrachain or inter-H and L chain disulfide bonds and none are free. Therefore, we believe that it is one or more of the Cys residues in the hinge region that is involved in forming disulfide bonds between two IgG2 molecules. However, because of their close spacing, we have not been able to determine which of the Cys residue(s) is (are) responsible for dimerization. All four human γ isotypes contain a Cys-X-X-Cys motif in the hinge, in which X is a non-Cys residue (Table I). Interestingly, IgG2 contains two Cys-X-X-Cys motifs in tandem.

In addition to (L chain-C_H1/hinge)$_2$ and (L chain-C_H1.hinge)$_4$ CNBr-cleaved fragments characteristic of the monomer and dimer, respectively, two-dimensional gel analysis also revealed the presence of (L chain-C_H1/hinge)$_3$ fragments. These fragments may be derived from an intermediate in a pathway in which monomeric IgG2 (H_2L_2)→(H_3L_3)→dimeric IgG2 (H_4L_4). Alternatively, they may be derived from species present in a pathway in which monomeric IgG2 (H_2L_2)→dimeric IgG2 (H_4L_4)→(H_3L_3). Indeed, species migrating at the position of H_3L_3 are observed in some samples (Figs. 1*B* and 3). Both of these cases are possible if the inter-H chain disulfide bond is labile, as in the case of human IgG4.

IgG2 is the major IgG isotype elicited against carbohydrate Ags in humans (27, 28) and IgG2 deficiency is frequently associated with increased susceptibility to infections by encapsulated bacterial pathogens (29–32). The Ab isotype restriction against polysaccharide Ags has also been observed in other mammalian species. In mice IgG3 is the predominant IgG subclass elicited by bacterial polysaccharide Ags (33). Mice lacking IgG3 are more susceptible to fatal sepsis following infection with *Streptococcus pneumoniae* although a protective IgG1 response can be induced by a *S. penumoniae* glycoconjugate. In rats IgG2c (34), which has a high degree of sequence identity to mIgG3 (35) and is antigenically similar (36), is the predominant isotype. The equine anti-pneumococcal Ab response has been reported to be restricted to IgG1 (37). Although the anti-carbohydrate Ab response tends to be restricted to a particular IgG subclass that usually does not contribute significantly to the Ab response against protein Ags, other isotypes such as IgA and IgM have also been observed to be specific for polysaccharide Ags (38–41).

Anti-carbohydrate Abs are usually of low intrinsic affinity (10^3–10^6 M^{-1}) compared with Abs specific for protein Ags (10^5–10^{10} M^{-1}). The ability of human IgG2 to dimerize and hence increase its avidity may represent an adaptation that permits more effective binding to the surfaces of bacteria and may help to more effectively trigger effector functions such as complement activation and Fc receptor binding. Such a mechanism has been suggested for mIgG3, which forms noncovalent polymers in the presence of Ag (12, 13) and has been shown to be more effective at binding to a multivalent polysaccharide Ag and triggering complement activation and opsonization than a V region identical IgG1 (42). Anti-carbohydrate IgGs in rats (43) and horses (37) have also been reported to have the propensity to form noncovalent aggregates; however, the nature of the IgG self-association in rats and horses has not been elucidated.

Several lines of evidence also suggest that the anti-carbohydrate response is evolutionarily conserved. In mice, the Cγ3 gene is thought to be the least polymorphic Ig H chain gene, and it is the only H chain C region locus for which no allotypes have been described in inbred mice (44, 45). In humans, IgG2 is thought to have arisen earlier in evolution than the other IgG isotypes. Nearly all primate immunoglobulins including those of some prosimians share common antigenic determinants with human IgG2. In contrast, human IgG1 shares determinants only with immunoglobulins of the great apes (46). In addition, there are fewer allotypes of γ2 and γ4 (47) than γ1 and γ3 (48) in humans. These data suggest that the isotypes involved in the anti-carbohydrate response contain unique features not present on other IgGs such as the ability to polymerize that are advantageous in the generation of effective immune responses against low affinity carbohydrate Ags.

In summary, we have now demonstrated that human IgG2 can form covalent polymers. Our studies suggest that Cys residues located in the hinge region are used for polymer formation. The availability of additional covalently associated variable regions may make it a more effective isotype, especially against Ags that elicit only low affinity Abs.

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