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STUDIES OF AGLYCOXYLATED CHIMERIC MOUSE-HUMAN IgG

Role of Carbohydrate in the Structure and Effector Functions Mediated by the Human IgG Constant Region

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Chimeric mouse-human IgG was used to study the structural and functional roles of the carbohydrate present in the C\textsubscript{2} domain of human IgG molecules. To remove this N-linked carbohydrate, Asn-297, the oligosaccharide attachment residue, was changed to either Gln (a conservative replacement) or His for IgG1 or Lys for IgG3 (nonconservative replacements) by site-directed mutagenesis. Carbohydrate-deficient antibodies are properly assembled and secreted and bind Ag and protein A. However, aglycosylated IgG are more sensitive to most proteases than their corresponding wild-type IgG, indicating some conformational changes have occurred. Aglycosylated IgG do not bind to the human Fc\textgamma RI and do not activate C; depending on the isotype, C1q binding ability is either completely lost (IgG1) or dramatically decreased (IgG3). The serum half-life in mice of aglycosylated IgG1-Gln remains the same as wild-type IgG1, 6.5 ± 0.5 days, whereas aglycosylated IgG3-Gln has a shorter half-life, 3.5 ± 0.2 days, compared to that of wild-type IgG3, 5.1 ± 0.4 days. These results indicate the carbohydrate interposed between C\textsubscript{2} domain of human IgG is necessary to maintain the appropriate structure for the maintenance of many of the effector functions dependent on the C\textsubscript{2} domain.

Ab\textsuperscript{3} of the IgG isotype are the most abundant of the serum Ab. IgG Ab are glycoproteins composed of two identical H chains and two identical L chains. The L chain folds into two functional domains [\textit{V\textsubscript{L}} and C\textsubscript{\textit{L}}], the H chain into four [\textit{V\textsubscript{H}}, C\textsubscript{\textit{H}}1, C\textsubscript{\textit{H}}2, C\textsubscript{\textit{H}}3]; carbohydrate is located in the C\textsubscript{\textit{H}}2 domain. This C\textsubscript{\textit{H}}2 carbohydrate is found at the same position in various species (1), suggesting it plays an important role in maintaining the structure and/or functions of IgG (2). There are strong trans interactions between the protein domains with \textit{V\textsubscript{H}} interacting with \textit{V\textsubscript{L}}, C\textsubscript{\textit{H}}1 with C\textsubscript{\textit{L}} and the two C\textsubscript{\textit{H}}3 domains interacting with each other. However, no interaction is seen between the two C\textsubscript{\textit{H}}2 domains; instead the carbohydrate residues are interposed between them (3, 4). The N-terminal region of an molecule is responsible for Ag recognition, whereas most of the effector functions are performed by the C-terminal region.

In previous studies, endo-/exoglycosidases (5–7) or Tm vectors and site-directed mutagenesis. The mutated H chain gene with its specific L chain gene was used to generate transfectoma cell lines producing aglycosylated Ab in large quantities. The structural and functional contributions of the carbohydrate components were then tested by comparing the wild-type and mutant human IgG. Our results are consistent with previous studies of C activation and FcR binding using mouse proteins (8, 9). We show that aglycosylated human IgG1 and IgG3 are completely deficient in their ability to activate C and to bind human Fc\textgamma RI; with aglycosylated IgG1 the ability to bind C1q is completely lost, however with aglycosylated IgG3 there is significant residual C1q binding. In addition, we show that depending on the IgG subclass the serum half-life of chimeric human IgG in mouse may or may not be affected by carbohydrate depletion. We find evidence for the widely held view that the presence of carbohydrate serves to protect Ig against proteolysis; however we find that the altered susceptibility to proteolysis depends on the protease, and for some there is no increase in susceptibility.

MATERIALS AND METHODS

Vectors and site-directed mutagenesis. The Salt-BamHI fragment containing the constant region gene of human IgG1 (3.3 kb) or IgG3 (3.7 kb) was cloned into M13mp19. Site-directed mutagenesis of this fragment was performed using the two-primer method (11) and the mutation confirmed by sequence analysis (12). The mutated Salt-BamHI fragment was then cloned into heavy chain expression vectors, pSV2AH-gpt with the expressed \textit{V\textsubscript{H}} gene from mouse anti-DNS hybridoma (13).

Transfectoma production. P3X63 Ag8.653, an Ig nonproducing mouse myeloma cell line, was transfected simultaneously with H and L chain genes by protoplast fusion as previously described (14).
Transfectants were selected with G418 (GIBCO, Grand Island, NY) at 1.0 mg/ml and surviving clones screened for Ab production by ELISA using DNS/BSA-coated plates. The amount of bound chimeric Ab was determined utilizing alkaline phosphatase-conjugated polyclonal goat Ab (Sigma Chemical Co., St. Louis, MO) against human IgG constant regions. Clones producing large quantities of anti-DNS Abs were expanded and maintained in Iscove’s modified Dulbecco’s medium (IMDM) containing 5% calf serum.

**Quantitation and characterization of chimeric Ab.** To determine Ab production, 1 x 10^6 cells were grown for 24 h and the Ab concentration in the culture supernatant measured by ELISA. To characterize the assembly, secretion and m.w. of Ig, cells were labeled with [35S]-Met and chased with or without Tm (4 mg/ml, Boehringer Mannheim, Indianapolis, IN). Ab molecules in the supernatants were immunoprecipitated with polyclonal rabbit Ab against human Fe and Staphylococcus aureus protein A (IgG sorb, The Enzyme Center, Malden, MA) and analyzed by sodium SDS/PAGE.

For Ab purity determination, 2 x 10^6 transfectoma cells were grown to saturation in IMDM and treated with affinity column.

**Protease digestion.** To test susceptibility to proteases, 5 µg of IgG were incubated with 0.05 µg of the protease (papain, pepstatin, chymotrypsin, or pronase) in the appropriate buffer as described [16] at 37°C in a volume of 20 µl. Papain digestion was at pH 8.0, chymotrypsin at pH 7.7, pronase and trypsin at pH 7.6 and pepstatin at pH 4.5. At the indicated time 4 µl of 5x loading buffer (125 mM Tris-Cl, pH 6.7, 10% SDS, 50% glycerol) was added to the mixture and heating in a boiling water bath for 2 min. Digestion products were separated by SDS/PAGE and stained with Coomassie blue. The gel was scanned by a densitometer (Ultra Scan XL, LKB, Bromma, Sweden) and the percentage of intact IgG remaining calculated.

**Half-life in mouse serum.** Radiolabeled chimeric Ab were incubated with groups of 5-10 mice in the presence of 35S-Met and purified using affinity chromatography. Four to five female BALB/c mice were injected i.v. with labeled Ab (4-5 x 10^6 cpm). Blood was sampled for 14 days post-injection with blood capillaries kept at 1°C. The radioactivity of intact IgG remaining in each sample was calculated as: (counts from each sample/counts from the samples bled at 3 min after injection) x 100. The percentage of radioactivity remaining was plotted against bleeding time. The half-life of each mouse was calculated from the slope of the line fitted to the data points from 48 h on.

**Clq binding and complement consumption.** Clq binding was determined using a solid phase assay in which increasing amounts of Ab were incubated with fixed amounts of Ag. Ag complexes coated onto microtiter plates. Microtiter wells were coated of 125I-labeled human Clq were added to fixed amounts of Ag/Ab complexes. The percentage of C consumption was calculated as: (cpm of Ag + Al + C/cpm of Ab + C) x 100.

**FcR binding.** An enzymatic assay has been devised to quantify the binding affinities of chimeric Ab to FcRII present on human monocytes and macrophages [15]. Human monocyte-like cells were prepared from U937 cell line by treatment with various concentrations (twofold serial dilution starting from 50 µg/ml). After 2 h incubation, β-galactosidase conjugated-DNS was added and incubated for another 2 h. The cells were then washed by centrifugation through a sucrose pad and β-galactosidase bound to IgG quantitated using the substrate, o-nitrophenyl galactosidase. Scatchard analysis was employed to determine the association constant of IgG for the receptor and the number of receptors per cell.

**RESULTS**

Chimeric mouse-human Ab in which the variable region from the mouse hybridoma 27-44 with specificity for the hapten DNS chloride was joined to human IgG1 or IgG3 constant regions were used in this study. In these chimeric Ab there is a site for carbohydrate addition at Asn 297 in the C2 domain. Site-directed mutagenesis was used to remove the carbohydrate attachment signal by replacing Asn 297 with either Gln or His (IgG1) or Lys (IgG3).

The mutated and wild-type H chain genes were cloned into the pSV2-gpt expression vector and transfected with their specific chimeric L chain gene into the Ig nonproducing myeloma cell line, P3X63 Ag8.653, by protoplast fusion. The resulting transfectoma cell lines secreting 10 to 40 µg of Ab/10^6 cells/24 h. No difference was seen in the production level of mutant and wild-type IgG.

To confirm that the C2 glycosylation site was absent from mutant IgG, transfected cell lines were biosynthetically labeled with [35S]-Met in the presence or absence of Tm, an inhibitor of N-linked carbohydrate addition. Culture supernatants were immunoprecipitated using anti-IgG specific Ab and analyzed on SDS/PAGE. All IgG, both mutant and wild type, were secreted as H2L2 tetramers; comparison of the mutant H chains with their corresponding wild types showed them to have a smaller apparent m.w. This difference in apparent m.w. disappeared when transfectoma cells were grown in medium containing Tm [data not shown] and the m.w. of Tm-treated wild-type Ab were the same as that of the aglycosylated mutants. These results therefore demonstrated that there was no Fab-associated carbohydrate. This is consistent with the known amino acid sequence of the variable regions (V. Tl. Ol, unpublished results) and C1r,1 and C, which contain no carbohydrate additional signals (Asn-X-Thr/Ser).

Ab were purified from culture supernatants using a DNS isomer-coupled affinity column as described [15]. Properly assembled H2L2 tetramers were present in more than 95% purity as was shown by SDS/PAGE analysis and no aggregated IgG was found in the preparation as shown by gel filtration on FPLC. These purified Abs were used in the following assays.

**Aglycosylated IgG shows enhanced sensitivity to a number of proteases.** Susceptibility to cleavage by protease is thought to reflect an open conformation in the protein molecule leading to increased solvent accessibility. To determine if removal of the carbohydrate resulted in an altered susceptibility to proteolytic attack, aglycosylated and wild-type IgGs were incubated with papain, pepstatin, trypsin, or pronase and digestion products were separated on SDS/PAGE (Fig. 1). Representative time points chosen to illustrate the sensitivity and digestion patterns with the different enzymes are shown in Table I and Fig. 1. The results obtained varied both with the protease and with the isotype of the Ig.

Aglycosylated human IgG1 were, surprisingly, found to be slightly more resistant to papain cleavage than wild type (Table 1, Fig. 1A, lane 3 and 4). In this gel system
Fab migrates with an apparent \( M \), of about 50 kDa as does aglycosylated Fc from IgG1. Aglycosylated Fc from IgG3 migrates with an apparent \( M \), of about 70 kDa, the glycosylated Fc of IgG1 and IgG3 are approximately 60 and 80 kDa, respectively. The Fc of IgG3 is larger than that of IgG1 because of the presence of the extended hinge region. When other enzymes were used, aglycosylated IgG1 was found to be more sensitive to cleavage but to varying degrees depending on the enzyme. When pepsin, which cleaves in Fc to generate (Fab'), was used aglycosylated IgG1 was much more sensitive (Table I, Fig. 1A, lanes 13 and 14). We see two sites of cleavage by pepsin (Fig. 1C, lanes 13 and 14). Chymotrypsin, which generates cleavage products similar to pepsin (Fig. 1A, lanes 7 to 10), is also much more effective in cleaving aglycosylated IgG1 (Table 1). Pronase also cleaves IgG1 after the hinge to generate (Fab'), (Fig. 1A, lanes 5 and 6) but the resulting Fc fragment is stable. Aglycosylated IgG1 is only slightly more sensitive to pronase cleavage. Aglycosylated IgG1 is also slightly more sensitive to trypsin (Fig. 1A, lanes 11 and 12) but from the data we cannot determine the cleavage site.

In general IgG3 is more sensitive to proteolytic attack than IgG1 (Table I). When papain is used a novel cleavage site after the hinge region is observed in aglycosylated molecules (Fig. 1B and D, lane 4); cleavage then takes place both before and after the hinge. Pepsin has a single, identical cleavage site in both wild-type and aglycosylated IgG3 (Fig. 1B and D, lanes 13 and 14). Chymotrypsin appears to cleave the aglycosylated protein at two sites (Fig. 1B, lanes 8 and 10), the cleavage after the hinge is predominant early with a second cleavage taking place before the hinge at a later time to generate Fab: in the wild-type protein the cleavage after the hinge takes place very slowly so the primary products are Fab and Fc (Fig. 1B, lanes 7 and 9). Pronase also cleaves IgG3 both before and after the hinge (Fig. 1B and D, lanes 5 and 6) whereas trypsin is seen to have multiple cleavage sites within the extended hinge of IgG3 (Fig. 1B and D, lanes 11 and 12), the number and size of the fragments sug-

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### TABLE I

<table>
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<tr>
<th>Protease</th>
<th>Time (h)</th>
<th>IgG1 (% intact)</th>
<th>Time (h)</th>
<th>IgG3 (% intact)</th>
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<tr>
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<td>15.2</td>
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*aProtease digestion products fractioned by SDS/PAGE were scanned by a densitometer and the intact IgG remaining was calculated. Each value represents the mean from triplicate experiments.*
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gests discrete cleavage sites between the hinge exons.

Aglycosylated IgG is severely impaired in its effector functions. To determine if aglycosylated human IgG shows any change in its ability to activate C, fixed amounts of Ab, and guinea pig C were incubated with increasing amounts of Ag (DNS/BSA). Both the conservative and nonconservative aglycosylated mutants of IgG1 and IgG3 completely lost the ability to activate C (Fig. 2A).

Clq binding is the first step in the activation of the classical C cascade (18). To determine whether the ability to bind Clq was lost in aglycosylated human IgG, increasing amounts of human 125I-Clq were added to a saturating amount of Ag/Ab complex. The bound Clq was then determined and plotted against input Clq. Both IgG1 carbohydrate mutants completely lost their ability to bind Clq (Fig. 2B). The IgG3 mutants, although decreased in their ability to bind Clq, still exhibit significant Clq binding at high concentrations of input Clq.

To determine the binding affinities of the chimeric Abs for the FcR present on the human monocyte-like cell line U937, cells were incubated for 2 h with monomeric chimeric IgG at various concentrations (twofold serial dilutions starting at 50 µg/ml). β-Galactosidase conjugated with DNS was then reacted with the FcR-bound IgG; unbound β-galactosidase was removed by centrifugation the cells through a sucrose pad and the FcR-bound IgG quantitated by determining the amount of enzyme attached. Scatchard analysis was used to calculate the binding constant of the IgG for the FcR. Using this assay, it has been shown that chimeric IgG1 and IgG3 bind to U937 cells with $K_a = 1 \times 10^9 M^{-1}$ (15), the same as reported for the corresponding human IgG subclasses (19). Both carbohydrate mutants of IgG1 and IgG3 did not show any detectable binding to U937 cells. The sensitivity of the assay is $K_a = 1 \times 10^6 M^{-1}$, which means aglycosylated human IgG has a more than 1000-fold decrease in binding affinity for U937 cells.

Half-life of chimeric IgG in mouse serum. To determine the half-life of wild-type and aglycosylated IgG-Gln in mouse serum, 35S-Met labeled IgG was injected i.v. into BALB/c mice and the radioactivity remaining in the serum at the indicated times measured. The average decay curve for four to five mice was determined (Fig. 3). In the first 48 h there was a rapid loss of Ab from the circulation caused at least in part by equilibrium between the blood and the extravascular spaces. After 48 h there was a slow clearance of Ab from the blood and this was used to calculate the half-life of Ab. In the case of IgG1, no difference has been found in the half-life of wild-type and aglycosylated Ab, both are in the range of 6.5 ± 0.5 days. However, aglycosylated IgG3 has a shorter half-life of 3.5 ± 0.2 days than does wild-type IgG3 which has a half-life of 5.1 ± 0.4 days. Thus, the effect of absence of carbohydrate on serum half-life depends on the isotype of the Ab and for some human isotypes there is no detectable change; these Ab do not contain carbohydrate in their Fab.

DISCUSSION

Chimeric Ab with a mouse V region and human C region are indistinguishable from normal human Ab in their ability to perform effector functions (20) and are therefore appropriate for the study of the biologic importance of carbohydrate in human IgG Ab.

To produce large quantities of proteins completely free of carbohydrate, we elected to make aglycosylated IgGs by site-directed mutagenesis. In this study the oligosac-
The IgG molecule consists of compact protein domains with the hinge region serving as a spacer separating the CH1 domains from CH2 domains. Extensive trans interactions between Ig domains stabilize the molecule. However, crystallographic studies have shown that the two CH2 domains do not interact directly but instead are pushed apart by the two N-linked branched carbohydrate chains [3, 4]. An interesting question is what will be the structure of CH2 in the absence of carbohydrate. In particular will the two domains collapse together? If the structure of CH2 changes what will be the impact on the rest of the molecule? The paucity of cts interactions between domains makes it reasonable to expect that the structure of other domains, especially CH1, on the other side of the hinge, would not be strongly altered by the depletion of carbohydrate in CH2. One approach to investigating the altered structure of CH2 is to determine its sensitivity to various proteases. Solvent accessible regions are presumed to be the principle target of attack by proteases and altered accessibility would correlate with changes in conformation. If the structure preceding the hinge remains unchanged, sensitivity to enzymes that cleave in this region should also remain unchanged. Indeed we do find a more profound increase in sensitivity of aglycosylated IgG to enzymes that cleave after the hinge than those cleaving within or before the hinge. Differences are seen between enzymes cleaving in the same region; presumably they cleave at different sites whose conformation is variously affected by the absence of carbohydrates.

Papain cleaves IgG1 before the hinge and the aglycosylated molecule is slightly more resistant to cleavage. Aglycosylated IgG3 is more sensitive to papain cleavage as the consequence of a novel cleavage site after the hinge. Trypsin cleaves IgG3 within the hinge and there is not much alteration in sensitivity in the aglycosylated protein, consistent with the concept that the change in CH2 is not transmitted through the hinge. The aglycosylated IgGs show a profound increase in sensitivity to cleavage by pepsin and chymotrypsin, both of which cleave in CH2. Presumably in the native molecules the carbohydrate either shields the protein chain from cleavage or in its absence there is a change in conformation making the IgG more easily cleaved. However, surprisingly, pronase that also cleaves IgG1 in CH2 shows only a slight increase in cleavage efficiency in the absence of carbohydrates; probably pronase cleaves in a slightly different region that is either not shielded by carbohydrate or whose conformation is not altered in the absence of carbohydrate. IgG3 is generally more sensitive to proteolytic attack than IgG1 most likely as a consequence of its long extended hinge region.

Altered ability to carry out effector functions would also be indicative of changes in structure. The ability to activate C and to bind to FcγRII, both of which are associated with CH2 [23], are profoundly affected by the absence of carbohydrate.

Previous studies showed carbohydrate-depleted mouse monoclonal IgG had a decreased ability to activate C, but that at high concentrations these Ab did activate C1 (9) and mediate C-mediated cell lysis (8). In contrast, our results show that aglycosylated IgG1 and IgG3 completely lose the ability to activate C. The differences between the two studies can most probably be explained by contamination by intact IgG in the initial studies and point out the value of being able to produce Ab completely free of carbohydrate; alternatively we cannot exclude the possibility of differences between Ab from different species or that Asn-297 itself is involved in C activation and that its substitution further decreases the ability of aglycosylated IgG to activate C.

Glu-318, Lys-320, and Lys-322 located on a β-strand of CH2 domain have been shown to contribute to the recognition site for C1q binding (24), the first step in C activation. Even at high concentrations, C1q does not bind to either IgG1 carbohydrate deficient mutant, explaining the inability to activate the C cascade. However, at high concentrations of input C1q, the IgG3 mutants, especially IgG3-Gln, bind C1q about half as well as wild-type IgG1. IgG1 can efficiently activate C, but the IgG3-Gln mutant does not show any ability to activate C. This lack of correlation between C1q binding and C activation has been observed [23] and probably reflects the fact that C1q binding is not the sole requirement for C activation. A certain configuration for C1q or additional structures on the Ag/Ab complex may be necessary.

Binding of IgG to a variety of cell types is important for...
the clearance of foreign Ag by phagocytosis and Ab-dependent cellular cytotoxicity. The aglycosylated IgG1 and IgG3 proteins did not show any detectable binding to FcγRI indicating a greater than 1000-fold decrease in the binding affinity, greater than that reported previously (9). It has been demonstrated recently that Leu-235 is the major determinant in the binding to the high affinity FcγRI (25). Leu-235 is located in a region whose structure our protease experiments suggest is affected by carbohydrate depletion.

Mouse-human chimeric IgG1 has been produced in yeast cells (26); this Ab molecule exhibits Ab-dependent cellular cytotoxicity but does not activate C. It has been shown that the core structure, Man₃GlcNAc₂, of N-linked carbohydrate is the same in the glycoproteins of yeast and mammalian cells (27). However, the outer chain structures are quite different; IgG made from animal cells has three terminal carbohydrate residues. N-acetylgalactosamine, galactose, and sialic acid, in the outer chain, whereas up to 30 mannose residues are present in the outer chain of yeast secreted N-linked glycoproteins. These results suggest: 1) carbohydrate plays a different role in maintaining the structures required for C1q and Fc receptor binding; 2) the core structure of C₃,2 carbohydrate is necessary and sufficient to maintain Fc-binding structures and that variation in the composition and length of the terminal carbohydrate residues does not interfere with FcR binding; 3) the elongated outer-chain mannose residues may destroy or cover the structures necessary for C1q binding and thus inhibit C activation.

The protein A binding site is located between the C₃,2 and C₅,3 domains (4) and is present in both wild-type and aglycosylated IgG1 (data not shown). Thus the interdomain conformation between C₃,2 and C₅,3 is largely if not completely intact in the absence of carbohydrate.

IgG has a long serum half-life compared to other glycoproteins. The structure responsible for retaining IgG in the circulation was assigned to the Fc region (28), and has been suggested to be the C₅,2 domain (29). Presence of terminal sialic acid on the oligosaccharide chains protects many serum glycoproteins from clearance by the asialoglycoprotein receptor on hepatocytes that recognizes terminal galactose (30). However, the role of galactose residues in the catabolism rate of IgG is somewhat controversial (28, 29, 31). In our studies both wild-type IgG1 and IgG1-Gln have the same half-life, 6.5 ± 0.5 days, which is in the same range as the reported half-life of monoclonal mouse IgG1, IgG2a, and IgG3 (34). If IgG were indeed removed from the circulation via the asialoglycoprotein receptor, aglycosylated IgG would be expected to have a longer half-life than its wild-type parent. These results suggest therefore that the asialoglycoprotein receptor does not play a role in clearing human IgG1 in mice; the increased sensitivity of the aglycosylated IgG1 to proteases also does not result in a shorter serum half-life. The IgG3-Gln mutant has a shorter half-life of about 3.5 ± 0.2 days compared to wild-type chimeric IgG3 with a half-life of 5.1 ± 0.4 days, the same as mouse IgG2b (32). Taken together these results suggest that carbohydrate does not play a major role in IgG catabolism, but that structures whose conformation is influenced by carbohydrate or sensitivity to proteolytic attack may play some role in IgG3. It is not clear why IgG1 and IgG3 mutants behave differently. It may be that aglycosylated IgG3 has a greater conformational change than aglycosylated IgG1. It may also be that different mechanisms are involved in the clearance of different isotypes.

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