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Effect of C2-Associated Carbohydrate Structure on Ig Effector Function: Studies with Chimeric Mouse-Human IgG1 Antibodies in Glycosylation Mutants of Chinese Hamster Ovary Cells

Ann Wright and Sherie L. Morrison

The complex biantennary oligosaccharide at Asn297 of IgG is essential for some effector functions. To investigate the effect of carbohydrate structure on Ab function, we have now expressed mouse-human chimeric IgG1 Abs in Chinese hamster ovary (CHO) cells with defined defects in carbohydrate biosynthesis. We had previously shown that IgG1 Abs produced in the cell line Lec 1, which attaches a high-mannose intermediate carbohydrate, were severely deficient in complement activation, showed a slightly reduced affinity for FcyRI, and had a reduced in vivo half-life. We have extended these studies by producing the same dansyl-specific IgG1 in cell lines deficient in attachment of sialic acid (Lec 2) and galactose (Lec 8). IgG1-Lec 1, IgG1-Lec 2, and IgG1-Lec 8 all showed varying reactivity with a mAb specific for an epitope in the amino terminal region of C\(_{\text{H}2}\), suggesting that the conformations of these proteins were altered by the different carbohydrate structures. Functionally, IgG1-Lec 2 and IgG1-Lec 8 were comparable to wild type with respect to in vivo half-life, affinity for FcyRI, and capacity for complement-mediated hemolysis. While IgG1-Lec 2 was essentially identical to wild type in its capacity to interact with individual components of the classical complement activation pathway, IgG1-Lec 8 demonstrated equivalent maximal binding at lower concentrations and was preferentially bound by mannose-binding protein. Although IgG1-Lec 1 was deficient in activation of the classical pathway, it had a superior capacity to activate the alternative pathway. These studies demonstrate that Abs bearing C1\(_{\text{H}2}\)-linked carbohydrate of differing structures have different functional properties. The Journal of Immunology, 1998, 160: 3393–3402.

The presence of carbohydrate in the C1\(_{\text{H}2}\) domain of IgG has been shown to be critical for Fc-mediated functions such as complement activation and the engagement, through Fc receptor binding, of phagocytic cells (1–3). The carbohydrate itself, which is attached at Asn297 and which is seques-
tered between the heavy chains, has a complex biantennary struc-
ture composed of a core saccharide structure (Fig. 1) consisting of
two \(\alpha\)-mannosyl residues attached to a \(\beta\)-mannosyl-di-N-acet-
ylchitobiose unit (4). The outer arms arise from the terminal pro-
cessing of the oligosaccharide in the Golgi; although the overall
structure of the carbohydrate is conserved, considerable heteroge-
neity is seen in the identity of the terminal sugar residues. Analysis
of carbohydrates isolated from normal human serum IgG has
yielded up to 30 different structures. Somewhat fewer structures
have been enumerated in other mammals (5), but the biantennary
structure is conserved.

The absence of C1\(_{\text{H}2}\)-associated carbohydrate is thought to cause
conformational changes in the C1\(_{\text{H}2}\) and hinge regions that are un-
favorable to the interaction with effector molecules and thus result
in loss of function. A growing body of evidence suggests that
certain alterations in carbohydrate structure also can affect Ab
function. In diseases such as rheumatoid arthritis, a higher than
normal incidence of agalactosyl structures (which seems to be spe-
cific for IgG Fc-associated carbohydrate) has been documented (6,
7). It has been proposed that this structure is more mobile than the
structure normally seen in this region and thus may induce changes
in the quaternary structure of the glycoprotein, contribute to the
immunogenicity of the Ab, or may itself contribute to aberrant Ab
function (8, 9). In the disease state, however, this structure is only
one of numerous glycoforms observed.

Recently, systematic efforts have been undertaken to produce
and characterize proteins with defined alterations in carbohydrate
structure. Because it is becoming increasingly commonplace to
express recombinant proteins in heterologous host cells, it is fun-
damentally important whether changes in carbohydrate structure
due to species-specific glycosylation or cell growth conditions af-
fect function. Several approaches have been attempted to alter the
glycosylation state of IgG Abs: inhibition of glycosylation by cul-
turing cells in the presence of the drug tunicamycin (2, 10, 11);
treatment of glycoproteins with specific glycosidases that remove
the entire oligosaccharide or specific residues (12, 13); or site-
directed mutagenesis to remove either the carbohydrate addition
site (3) or residues within the C1\(_{\text{H}2}\) region that contact the core
oligosaccharide residues (14). These studies have confirmed that
the presence of carbohydrate is essential to Ab function. However,
site-directed mutagenesis alters the sequence of the protein, while
glycosidase treatment is rarely completely efficient and the reac-
tion conditions may adversely affect the protein: these are variables
that are difficult to evaluate and may obscure interpretation of
results.

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The contribution of carbohydrate structure to Ab function has been investigated in this laboratory using Abs produced in glyco-
sylation mutants of Chinese hamster ovary (CHO) cells. Com-
pared with glycosidase digestion, this approach is advantageous in that: 1) homogeneous carbohydrate structures (at least in terms of which residues they lack) are produced without the potential com-
plication of damage to the protein backbone through enzyme treat-
ment; and 2) carbohydrate structures can be attached that are not easily produced by glycosidase treatment. In previous work, we
expressed mouse-human chimeric IgG1 Abs in Lec 1 cells, which
are deficient in N-acetylglucosaminyltransferase I and thus attach
a truncated, Man, GlcNAc, structure not normally seen on IgG
(15). The resulting glycoprotein was severely deficient in comple-
ment activation and C1q binding and showed reduced affinity for
FcγRI receptors. Moreover, the in vivo half-life of these Abs was
greatly reduced compared with Abs produced in mouse myeloma
cells or in wild-type CHO cells.

We now extend these studies to characterize IgG1 Abs of the
same chimeric structure produced in the CHO glycosylation mu-
tants Lec 2 and Lec 8, each of which is deficient in transporting
one of the nucleotide sugars to the Golgi (16). Lec 2 is defective
in the transport of CMP-sialic acid, while Lec 8 lacks transport of
UDP-galactose, leading to the synthesis of complex carbohydrate
structures lacking sialic acid and galactose, respectively. Since
both cell lines attach complex but truncated carbohydrates, the
proteins they produce can be evaluated for the contribution of spe-
cific sugar residues to glycoprotein function.

Materials and Methods

Cell lines

The CHO cell lines Pro-5, Lec 1, Lec 2, and Lec 8, originally derived by
Dr. Pamela Stanley (16), were obtained from the American Type Culture
Collection (Rockville, MD). The cells were maintained at 37°C under 5%
CO2, in Iscove’s modified Dulbecco’s medium (IMDM; Irvine Scientific,
Irvine, CA) supplemented with 5% FCS (HyClone Laboratories, Logan,
UT). The cells were maintained in monolayer culture in tissue culture-
treated petri dishes (Falcon Labware, Lincoln Park, NJ) and harvested by
treatment with trypsin (Life Technologies, Grand Island, NY). The human
monocyte-like cell line U937 was maintained in IMDM supplemented with
5% iron-supplemented bovine calf serum (HyClone).

Transfection of CHO cells

The CHO cells were transfected with expression vectors encoding Ig genes
under the control of the SV40 promoter and enhancer as previously de-
scribed (15). The chimeric lgs contained human γ1 and κ constant regions,
while the murine variable regions conferred specificity for dansyl.

Large-scale cell culture and protein purification

Transformedos were cultured in roller bottles (Fisher Scientific, Pittsburgh,
PA) in IMDM supplemented with 2% α-calf serum (HyClone). Proteins
were purified by affinity chromatography as previously described (15). Pro-
tein concentration was determined either with the bicinchoninic acid (BCA)
assay (Pierce Chemical Co., Rockford, IL) or by visualizing on a
SDS-polyacrylamide gel in comparison with a standard of known
concentration.

Fc receptor binding assay

The capacity of Abs to interact with high affinity Fc receptors on mono-
nuclear cells was measured in a competitive inhibition assay as previously
described (15), using the monocyte-like cell line U937 cultured for 2 days
in IMDM supplemented with 5% FCS and 100 U/ml IFN (the gift of
Jorge Gavilondo-Cowley, Centro de Ingenieria Genetica y Biotecnologica,
La Habana, Cuba) to increase Fc receptor expression. The affinity constants

3 Abbreviations used in this paper: CHO, Chinese hamster ovary cells; DNS-BSA, dansyl coupled to BSA; BCA, bicinchoninic acid; HBS, HEPES-buffered saline; MAC, membrane attack complex; IMDM, Iscove’s modified Dulbecco’s medium; MBP, mannose-binding protein; ADCC, Ab-dependent cell-mediated cytotoxicity.
kind gift of Dr. R. A. B. Ezekowitz (19), was diluted 1:1500. After over-
night incubation and washing with PBS, horseradish peroxidase substrate
(0.4 mg/ml o-phenylenediamine, 3 mM H2O2 in 50 mM citrate-phosphate
buffer, pH 5; reagents obtained from Sigma Chemical Co., St. Louis, MO)
was added to the plates incubated with the MFB-specific Ab and the plates
were read at 490 nm. All other Abs were obtained from Quidel (San Diego,
CA). Mouse anti-human C1q (diluted 1:10,000); goat anti-human C4 (diluted
1:5000); goat anti-human C3 (1:5,000); mouse anti-human factor Bb (1:
500); and mouse anti-human sc5b-9 (1:10,000). In these assays, the plates
were incubated with alkaline phosphatase-conjugated swine anti-goat
(Boehringer Mannheim, Indianapolis, IN) or rabbit anti-mouse IgG
(Zymed, South San Francisco, CA) as appropriate. After incubation for one
and a half hours, the plates were washed and developed with p-nitrophenyl phos-
phate (Sigma Chemical Co.), and absorbance was measured at 410 nm. All
measurements were taken on a Dynatech Model 700 plate reader.

Analysis of conformations of the CHO-produced Abs by anti-
human IgG1 mAb

Ag-coated plates were prepared as described above. Culture supernatants
of CHO-produced Abs were diluted so that the starting concentrations
were equivalent, and serial twofold dilutions were added (100 µl/well) to each
of two plates and incubated overnight at 4°C. For comparison, the super-
natants from the murine cell line producing the same chimeric dansyl-
specific IgG1, as well as the cell line producing the aglycosylated IgG1 Ab
IgG1-Gln297 (3), was included on each plate. After washing with PBS, one plate
was then incubated with alkaline-phosphatase-conjugated anti-human kappa
(Sigma Chemical Co.; diluted 1:50,000 in PBS + 1% BSA) for 1 h at
37°C and then developed with p-nitrophenylphosphate in the usual way. The
other plate was incubated (this and all subsequent reactions 100
µl/well) with the human yl-specific Ab, HP6070 (20) (the kind gift of Dr.
Robert G. Hamilton, Johns Hopkins University School of Medicine, Bal-
timore, MD; diluted 1:1,000 in PBS + 1% BSA) for 2 h with shaking at
room temperature and then washed with PBS. The samples were then in-
cubated with a 1:100 dilution (in PBS + 0.5% Tween-20) of biotinylated
goat anti-mouse Ab (Kierkegaard and Perry Laboratories, Gaithers-
burg, MD) for 2 h at room temperature. After washing, alkaline phosphatase-
conjugated streptavidin was added for 1 h, and binding was detected with
p-nitrophenylphosphate.

Lectin binding

Con A preferentially binds to terminal α-D-mannosyl and α-D-glucosyl
residues, but in earlier studies we had found that it bound to carbohydrate
expressed in the Ab variable region regardless of the oligosaccharide struc-
ture (21). We employed two different assay conditions to ascertain whether
the C3g2-associated carbohydrate was accessible to lectin. A solid-phase
assay was conducted to measure the recognition by Con A of Abs bound
downs, coated plates. Using the same dilution scheme that was used for the
isotype-binding study, supernatants (the four CHO-produced Abs as
prepared in this laboratory by Letitia Wims) followed by precipitation
with identical Ag specificity that differ from each other only in the
structure of the C3g2-associated carbohydrate.

Fc receptor binding

To determine binding to FcγRI, radiolabeled Abs mixed with in-
creasing amounts of homologous unlabeled competitor were incub-
ated with U937 cells that had been treated with IFN-γ to increase the
expression of Fc receptors, and the bound radioactivity was
determined. Scatchard analysis of the data yielded the Kd values
listed in Table I each of which represents the mean of four experi-
ments. The Fc receptor-binding behaviors of IgG1-Pro-5, IgG1-
Lec 2, and IgG1-Lec 8 are very similar and are in agreement with
the previously published Kd for the same Ab produced in myeloma
cells (22). The Kd for Lec 1 had previously been shown to be
reduced by approximately four- to sixfold, and that observation is
confirmed in the present study. Therefore, although the lack of
sialic acid and galactose from the carbohydrate does not affect
FcγRI binding, the aberrant carbohydrate produced by Lec 1 does
result in impaired FcγRI binding.

In vivo half-life

BALB/c mice were injected i.p. with radiolabeled Abs, whose
clearance was monitored by whole body counting. The kinetics of
elimination of IgG1-Lec2 and IgG1-Lec 8 were very similar to those of IgG1-Pro-5 (Fig. 2): ~40 to 50% of the Ab was rapidly
cleared in the α-phase, with the remainder eliminated with a
β-phase t1/2 of ~6 days. In contrast, 80% of IgG1-Lec 1 was cleared
in the α-phase, with the remainder showing an in vivo t1/2 of
2.8 days. Previous studies had shown that clearance of the man-
nosylated IgG1-Lec 1 could be blocked temporally by the coin-
jection of mann (15). The results indicate that whereas the Lec
1-associated carbohydrate clearly affects the in vivo persistence of
that Ab, the clearance kinetics of the other Abs are comparable.

Complement-mediated cytolysis

Abs were incubated with Ag-coated target cells and serum to measure
their ability to activate the complement cascade and lyse target
cells. The lytic profile and maximal lytic capacity of IgG1-
Pro-5, IgG1-Lec 2, and IgG1-Lec 8 Abs were comparable in the

<table>
<thead>
<tr>
<th>Ab</th>
<th>Kd (M⁻¹)</th>
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<tbody>
<tr>
<td>IgG1-Pro-5</td>
<td>1.19 × 10⁻⁹ ± 0.85</td>
</tr>
<tr>
<td>IgG1-Lec2</td>
<td>1.82 × 10⁻⁹ ± 0.158</td>
</tr>
<tr>
<td>IgG1-Lec8</td>
<td>0.78 × 10⁻⁹ ± 0.024</td>
</tr>
<tr>
<td>IgG1-Lec1</td>
<td>8.31 × 10⁻⁹ ± 0.33</td>
</tr>
</tbody>
</table>

* Radiolabeled IgG1 Abs were incubated in the presence of increasing amounts of cold competitor with U937 cells that had been stimulated with IFN-γ as described. The affinity constants, determined by Scatchard analysis (17), are the average of four experiments.
presence of either human or guinea pig serum. The results shown in Figure 3, obtained with human serum, are the mean of three experiments. As demonstrated previously, the Lec 1-produced Ab does not promote target cell lysis.

Detection of binding of specific complement components by ELISA

A series of ELISAs was performed in which Abs were immobilized on plates through binding to Ag such that the Fc regions were “free” to interact with subsequently added reagents. This approach would more closely mirror actual immune complex formation than would directly binding Abs to plastic. To insure that equal amounts of Ab were compared, they were quantitated both by BCA and by densitometry of Coomassie blue-stained SDS gels, which yielded comparable results (data not shown). All data shown are representative experiments that were repeated at least three times, and in all of them, the background values were subtracted (serum alone).

Classical pathway activation. Assays for the detection of classical pathway activation were performed under conditions of low serum concentration (0.125%) and in the presence of Ca$^{2+}$ and Mg$^{2+}$. Upon formation of Ag-Ab complexes, the initial step in classical complement activation is the binding of the C1 subunit C1q to the Ab Fc regions. In subsequent steps, C4 is cleaved by a proteolytic enzyme generated by C1 activation. The deposition of C4b, a product of this cleavage event, can be detected by a C4-specific Ab. These reactions contribute to the binding and activation of C3, which results in the release of various anaphylactic and chemotactic peptides, and ultimately, the formation of the membrane attack complex (MAC; complement proteins C5b-9), which causes target cell lysis. We investigated the capacities of these Abs to trigger these binding events throughout the classical activation pathway.

At maximal Ab concentrations, C1q binding was manifested at approximately equal levels by the Pro-5-, Lec 2-, and Lec 8-produced Abs; however, as Ab concentration decreased below 2 μg/ml, the IgG1-Lec 8 consistently bound C1q better than IgG1-Pro-5 and IgG1-Lec 2, which had comparable binding behavior. The Lec 1-produced Ab bound C1q at reduced but detectable levels only at the highest concentrations tested, as previously observed (Fig. 4A). A similar pattern was observed for C4b deposition (Fig. 4B), although for C4 the Lec 1-produced Ab appeared to bind comparably at the highest concentration tested. In contrast, IgG1-Lec 1 failed to bind C3 (4C) and showed greatly reduced binding of sc5b-9 (MAC, Fig. 4D; note that for C3 binding and MAC formation, a lower maximum Ab concentration of 2.5 μg/ml was used). IgG4 failed to bind any of these components, consistent with its inability to activate complement by the classical pathway.

In the presence of EDTA, which chelates divalent cations, C3 binding was completely abolished for all Abs, confirming that the observed binding is dependent on complement activation (4E).

Alternative pathway activation. To observe the activation of complement via the alternative pathway, it is necessary to chelate the calcium ions required for the classical pathway. Furthermore, the assays were conducted in the presence of 5%, rather than 0.125%, serum. Alternative pathway activation was analyzed by detecting the binding of C3, the precursor protein of the alternative pathway, factor Bb, a component of the C3 and C5 convertases, and sc5b-9, the MAC. Under these conditions, all of the Abs tested, including IgG4, showed detectable binding of C3. IgG1-Lec 1, which is generally inactive in the classical pathway, showed substantially more binding under these conditions than any others. IgG1-Pro-5, IgG1-Lec 8, and IgG4 reached equivalent maximal C3 binding, although at different concentrations (Fig. 5A). At the highest Ab concentration tested, IgG1-Lec 2 bound somewhat better than the others but less well than IgG1-Lec 1. A similar hierarchy for maximal binding of sc5b-9 was observed (Fig. 5B). IgG1-Lec 1 showed the most binding at all concentrations tested, while IgG1-Lec 2 showed intermediate binding at the highest concentration tested. IgG1-Pro-5 and IgG1-Lec 8 again showed equivalent maximal binding, with IgG1-Lec 8 reaching maximal binding at a lower concentration than did IgG1-Pro-5. While it is completely inactive in the classical pathway, IgG4 showed detectable binding but at a lower maximal level than the other Abs.

Factor Bb binding was tested with an Ab specific for a unique epitope generated when factor B is cleaved (Quidel) under the same conditions as the other components and was tested additionally in the presence of NiCl$_2$. Nickel is reported to stabilize the formation of the C3b, Bb C3 convertase and thus enhance its detection (23). In addition, the maximal Ab concentration was increased to 10 μg/ml. Whether factor Bb binding was measured in the presence of nickel (Fig. 5C) or magnesium (Fig. 5D), binding was observed, and at maximal binding, the degree of substrate development was approximately the same. However, the binding behavior of the Abs varied under the different conditions. In the
presence of Mg$^{2+}$, IgG-1 Lec 1 and IgG1-Lec 8 showed equivalent maximal binding, while that of IgG1-Lec 2 was slightly increased. IgG1-Pro-5 and IgG4 showed reduced binding. At a lower Ab concentration, IgG1-Lec 8 bound better than the other Abs. In the presence of Ni$^{2+}$, however, the binding of IgG1-Lec 1 was suppressed to the level of IgG4; IgG1-Pro-5 and IgG1-Lec 2 bound comparably; and IgG1-Lec 8 showed a significantly increased binding compared with the others.

Binding of MBP

It has been proposed that agalactosyl IgG Abs have the ability to activate complement through binding MBP (24). It has been suggested that the agalactosyl carbohydrate is more mobile than the more fully glycosylated structure and that the terminal N-acetylglucosamine residues might be accessible to MBP. The relative capacity of the different Abs to bind MBP was measured (Fig. 6, A–C) using a series of serum concentrations ranging from 0.5 to 5%. In 5% serum, the Pro-5-, Lec 2-, and Lec 8-produced Abs showed comparable concentration-dependent binding (Fig. 6A). However, in 1% serum (Fig. 6B) and 0.5% serum (Fig. 6C), IgG1-Lec 8 consistently bound MBP better than the other Abs. At the highest concentration tested, IgG1-Lec 2, IgG1-Lec 8, IgG1-Lec 1, and the aglycosylated Ab IgG1-Gln297 showed equivalent binding; however, at subsequent dilutions IgG1-Lec 2 and IgG1-Gln297 showed decreased binding compared with IgG1-Lec 1 and IgG1-Lec 8, which showed comparable binding. These results suggest that recognition by this mAb is affected by the altered carbohydrate structures attached by the different cell lines. Testing the same dilutions in parallel with an anti-human kappa Ab confirmed that equivalent amounts of Ab were bound to the plate (Fig. 7B).

Lectin binding

We had previously proposed that the rapid in vivo clearance of IgG1-Lec 1 is mediated by interaction of mannose-specific receptors with the exposed Fc-associated carbohydrate (26). To determine whether the carbohydrate of IgG1-Lec 1 or any other glycosylation variant is in fact exposed and accessible to lectin, Abs bound to Ag-coated plates were incubated with the lectin Con A, which was chosen because it can interact with a variety of oligosaccharide structures. Binding of biotinylated Con A was detected using alkaline phosphatase-conjugated streptavidin. The Con A
bound most strongly to IgG1-Lec 1, which expresses sugars bearing terminal mannose residues. The lectin bound IgG1-Lec 2 at an intermediate level and showed weaker but significant interaction with IgG1-Pro-5 and IgG1-Lec 8 (Fig. 8). No reactivity with the aglycosylated Ab IgG1-Gln297 was observed. This result indicates that in these Abs the Fc-associated carbohydrate is accessible to Con A.

We also investigated the binding of Con A to biosynthetically labeled Abs in solution. Supernatants were incubated with Con A-Sepharose in excess, and Ab was recovered by immunoprecipitation from the unbound or bound fraction. These are depicted in Figure 9 as pairs of unbound fractions (odd numbers) and fractions eluted from Con A (even numbers). Approximately 60% of IgG4 is retained by Con A through the high-mannose carbohydrate that is accessible on the surface of the VH CDR2 domain (lanes 1 and 2; note the secreted HL molecules characteristic of the IgG4 isotype). In contrast, the aglycosylated IgG1-Gln297 shows essentially no binding to Con A (lanes 3 and 4); the small percentage of binding likely reflects residual trapping of the Ab by the resin. Under these conditions, the CHO-produced Abs bind in varying degrees to Con A. Approximately 60% of IgG1-Pro-5 (lanes 5 and 6) and IgG1-Lec 2 (lanes 7 and 8) is eluted from Con A. One-half of IgG1-Lec 8 (lanes 9 and 10) is retained, while approximately one-third of IgG1-Lec 1 (lanes 11 and 12) is retained by Con A. Although the Cg2-associated carbohydrates of these Abs remain accessible to lectin in solution, a different hierarchy of binding is observed than when Abs are bound to Ag-coated plates.

Discussion

Numerous studies have shown that the C1q2-associated oligosaccharide plays an essential role in IgG1-mediated Ab effector activity (1–3, 12–15). However, few studies have addressed the issue of how differences in the structure of this carbohydrate impact Ab function. We have approached this problem by expressing Abs of identical amino acid sequence in CHO cells with defined defects in their glycosylation machinery. Thus, it is possible to evaluate the contribution of individual sugar residues to carbohydrate-associated Ab activity without exposing the glycoprotein to glycosidase treatment.

In earlier studies, we had shown that Abs produced in the CHO glycosylation mutant Lec 1 were correctly assembled and secreted and retained Ag specificity. The CH2-associated carbohydrate was Endoglycosidase H-sensitive, consistent with the failure of this cell line to process the Man9-GlcNAc2 intermediate because of a defect in N-acetylgalcosaminyltransferase 1. We have extended these studies by producing the identical IgG1 dansyl-specific Abs lacking sialic acid (IgG1-Lec 2) and galactose (IgG1-Lec 8). In contrast to those produced by IgG1-Lec 1, the glycoforms present on IgG1-Lec 2 and IgG1-Lec 8 are normally seen on serum Abs.
Indeed, IgG1 Abs lacking galactose are proposed to manifest aberrant activity that might contribute to inflammatory disorders (7–9). All Abs have been evaluated in comparison with the identical Ab produced in myeloma cells (15).

The Fc-associated carbohydrates of IgG Abs are normally buried the CH2 domains of the two heavy chains, and in previous studies we have found that the Fc-associated carbohydrate is not accessible to Con A using myeloma-produced Abs (27). In contrast, the oligosaccharides attached by the various CHO cells do appear to be at least partially exposed under similar conditions. Although a hierarchy of Con A binding was observed with Abs bearing carbohydrates of differing structures, it is not clear at this point whether this is due to differences in access to the carbohydrate or in the affinity of the carbohydrate for Con A. In solution, the more truncated structures attached by IgG1-Lec 8 and IgG1-Lec 1 may be less exposed. Under these conditions, the identity of the exposed residues and whether they are intermediate or terminal could not be determined. Nevertheless, it can be concluded that the altered or truncated carbohydrate attached by these cell lines can be accessible to lectin and may contribute directly to the conformational changes detected in the epitope recognized by the isotype-specific Ab and to the demonstrated differences in the capacities of the Abs to interact with effector proteins.

Carbohydrate is thought to play a role in vivo glycoprotein targeting and clearance with exposed sugar residues such as galactose- or mannose-targeting glycoproteins to specific receptors on hepatocytes (28) or macrophages (29), respectively. Indeed, we have shown that the rapid clearance of IgG1-Lec 1 is temporarily inhibitable by the coinjection of yeast mannan (15). In contrast to IgG1-Lec 1, the in vivo half-lives of IgG1-Lec 2 and IgG1-Lec 8 are virtually identical to each other and to that of the Ab produced in Pro-5. That these more fully glycosylated Abs manifest identical in vivo clearance suggests that the differences in oligosaccharide profiles do not confer any differences in the kinetics of clearance among these Abs. Perhaps those Abs with exposed carbohydrate residues are removed in the α-phase of clearance. The region of the Fc that controls Ab catabolism has been localized to the CH2-CH3 domain interface (28), a region that is not considered sensitive to variations in glycosylation at CH2 (2). Indeed, the kinetics of clearance of aglycosylated and normally glycosylated mouse-human chimeric IgG1 Abs are similar (29).

The capacity of IgG to interact with effector systems such as complement and Fc receptor-bearing phagocytes is encoded within the CH2 domain of the constant region. The binding site on IgG1 for the high affinity receptor FcγRI has been localized to amino acid residues in the lower hinge region and the hinge-proximal bend between two β-strands within CH2 (22, 30); the presence of carbohydrate is essential for this interaction (3). While the attachment of a high-mannose carbohydrate had a significant but minor impact on FcR binding to IgG1-Lec 1, the sialic acid- and galactose-deficient Abs were essentially identical to the wild type with respect to FcγRI binding. There is disagreement as to the contribution of galactose to FcγRI binding. Our findings are in general agreement with those of Boyd et al. (13), who degalactosylated the CHO-derived IgG1 Campath-1H and found its capacity to mediate Ab-dependent cell-mediated cytotoxicity (ADCC) was unaffected. In contrast, Tsuchiya et al. found that radiolabeled human IgG showed reduced binding to FcγRI when treated with β-galactosidase (12). In this case, the human IgG was not fractionated into subclasses, and it is possible that the reduction in binding was contributed by one of the other subclasses present. Kumpel et al. found that human IgG3 anti-D Abs mediated ADCC of RBC more efficiently when they were fully galactosylated (i.e., contained galactose on each arm of the biantennary carbohydrate (31)). However, no difference in FcγR-mediated superoxide release by U937 cells was observed between agalactosylated IgG3 and Ab that was enzymatically degalactosylated (32). Taken together, these results suggest that the effect of galactose on FcγR-mediated activity is subtle, dependent on Ab isotype, and varies depending on the function tested.

To evaluate the Fc-associated binding of complement components, we took advantage of the fact that these Abs are specific for a well-defined hapten, which, when coupled to BSA, can readily be adsorbed to plastic. In contrast to such methods as coating plates with Abs or Ig fragments, this more closely approximates the
Ab-Ag binding event that initiates the classical complement pathway. Purified Ig preparations were quantified by BCA and visualization by SDS-PAGE to insure that the Ab concentrations compared were identical.

While the Lec 1-produced Ab was unable to perform complement-mediated hemolysis, the Lec 2- and Lec 8-produced Abs were as effective as wild type. The reduced but significant binding of C1q and C4b by IgG1-Lec 1 appears to be insufficient to allow for the subsequent events in the complement cascade. Consistent with the results seen in the direct lysis assay, maximal C1q binding of IgG1-Pro-5, IgG1-Lec 2, and IgG1-Lec 8 is comparable, but IgG1-Lec 8 shows increased affinity for C1q at lower Ab concentrations. This hierarchy of binding is also seen at the sequential binding of C4b; it is even more apparent at the binding of C3b and the formation of SC5b-9, the terminal attack complex. Therefore, in these studies IgG1 lacking galactose is the most effective in activating the complement cascade.

Other studies of the interaction of degalactosylated Abs with complement have obtained variable results. Tsuchiya et al. reported that human IgG that was degalactosylated with streptococcal β-galactosidase showed reduced binding to C1q bound to ELISA plates, compared with untreated Ab (12). More recently, Boyd et al. observed that a tumor-specific Ab subjected to the same treatment was reduced in its ability to carry out complement-mediated lysis by tumor cells (13). Both treatments required long term incubations at acidic pH. In agreement with our studies, when Lund et al. (14) used site-directed mutagenesis of residues in CH2 that contact carbohydrate to alter the degree of oligosaccharide processing, they found that the least galactosylated Abs were the most effective in complement activation. Taken together, these reports indicate that the absence of galactose does affect IgG effector function but that different outcomes are seen depending on whether the Ab is degalactosylated enzymatically or whether the glycoprotein is initially formed without galactose.

A recent study suggested that the surface accessibility of the N-linked oligosaccharide might inhibit C1q binding (33) and that removal of the oligosaccharide by PNGase F hydrolysis resulted in increased C1q, C4b, and C3b deposition. Our results indicate that the sugars attached to IgG1-Lec 1 and IgG1-Lec 8 are both accessible, but while the interaction of IgG1-Lec 1 with complement proteins is impaired, that of IgG1-Lec 8 is enhanced. Thus, the accessibility of the sugar per se appears to be insufficient to explain its contribution to the properties of the Ab glycoprotein; this issue clearly warrants further investigation.
Activation of the alternative activation pathway occurs independently of C1 activation. Indeed, the IgG1-Lec 1 Ab, which is deficient in C1 activation, manifested superior binding of C3 and sc5b-9 formation under conditions that inhibit the classical and promote the alternative pathway. Binding of factor Bb, a component of both the active C3 and C5 convertases of the alternative pathway, is a distinctive indicator of the activation of the alternative pathway. However, the component decays rapidly, and its binding is difficult to measure. It was reported that substitution of Ni²⁺ for Mg²⁺ in the formation of the convertase stabilizes the structure and facilitates detection (23). We tested for deposition of factor Bb in the presence of either cation. Interestingly, factor Bb shows little binding to IgG1-Lec 1 in the presence of Ni²⁺, while it binds the other CHO-produced Abs. In the presence of Mg²⁺, IgG1-Lec 1 is bound as well as IgG1-Lec 2 although less well than IgG1-Lec 8. The results obtained with the other complement proteins, suggesting that use of this cation to detect factor Bb binding may be misleading. However, even in the presence of Mg²⁺, factor Bb binding does not correlate with the formation of the sc5b-9 complex. Apparently, the interaction of factor Bb with IgG1-Lec1, while too unstable to measure, is sufficient to allow for the efficient formation of the sc5b-9 complex.

It has recently been proposed that degalactosylated Abs may contribute to inflammation through binding of MBP (24). MBP contains carbohydrate recognition domains that recognize terminal fucose, mannose, glucose, and N-acetylglucosamine, but not galactose. MBP bears structural similarities to C1q and, through binding to Ab Fc regions, might initiate complement activation. Degalactosylated IgG and Fc fragments incubated with MBP show enhanced deposition of C4b compared with untreated Abs (24). By incubating Ag-immobilized Abs with various dilutions of serum, we found that the hierarchy of MBP binding to Ab is strikingly similar to that of C1q. Indeed, IgG1-Lec 8 bound MBP more efficiently than the other Abs. However, IgG1-Pro-5 and IgG1-Lec 2 bound nearly as well as IgG1-Lec 8 at the highest serum concentration tested. Because we tested the binding of MBP present in serum rather than in isolation, we cannot rule out the possibility that other serum factors may contribute to the binding of MBP to Ab Fc regions; nevertheless, these findings suggest that MBP binds preferentially, but not uniquely, to agalactosyl Abs. Although the terminal mannose residues on IgG1-Lec 1 were accessible to Con A, MBP bound this Ab poorly, perhaps due to steric inhibition of the interaction.

These studies were motivated, in part, by the suggestion that aberrantly glycosylated Abs might contribute to inflammatory conditions, and our findings tend to support that possibility. While IgG1-Pro-5 and IgG1-Lec 2 appear to function "normally," IgG1-Lec 1 and IgG1-Lec 8 acquired novel effector activity. IgG1-Lec 1 appears to acquire the capacity to activate complement via the alternative pathway, which, when activated inappropriately, might cause inflammation. The potential in vivo consequence of this might, however, be offset by its rapid clearance from the circulation. The preferential enhanced binding of MBP and C1q to IgG1-Lec 8 might contribute to an enhanced capacity for complement activation. It might very well be the case that the agalactosyl IgG1 contributes to tissue damage through complement activation.

In summary, these studies highlight the importance, as well as the complexity, of understanding how Fc-associated carbohydrate structure affects the biologic activity of IgG; even subtle changes can alter the interaction of Abs with effector molecules such as complement proteins. With the advent of the commercial production of recombinant proteins, it has been shown that the degree of carbohydrate processing in glycoproteins can be affected not only by species-specific differences in the glycosylation apparatus of the host cell (34–37) but also by culture conditions (31, 38, 39); thus, the potential effects of carbohydrate structure on the function of the recombinant protein must be understood. It is possible to study the functional consequence of any carbohydrate structure of interest by producing the protein in the appropriate glycosylation mutant cell line (41). With the knowledge acquired from the systematic analysis of such glycosylation variants, it should be possible not only to produce IgGs with enhanced or novel properties but also to understand how certain alterations in glycosylation might contribute to disease.

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References


