In vivo trafficking and catabolism of IgG1 antibodies with Fc associated carbohydrates of differing structure

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We have now produced mouse–human chimeric IgG1 in wild-type Chinese hamster ovary (CHO) cell lines Pro-5 as well as in the glycosylation mutants Lec 2, Lec 8, and Lec 1. Analysis of the attached carbohydrates shows those present on IgG1-Lec 1 were mannose terminated. Carbohydrate present on IgG1-Lec 8 was uniformly biantennary terminating in N-acetylglucosamine. The glycosylation profiles of IgG1-Lec 2 and IgG1-Pro-5 were heterogeneous. Only IgG1-Pro-5 was sialylated with sialic acid present on only a small percentage of the carbohydrate structures. When the in vivo fate of antibodies labeled with 125I-lactotyramine was determined, it was found that the majority of all of the antibodies, irrespective of the structure of their attached carbohydrate, is catabolized in the skin and muscle. However, the attached carbohydrate structure does influence the amount that is catabolized in the liver and the liver serves as a major site for the catabolism of proteins bearing carbohydrate with the Lec2 (with terminal galactose) or Lec1(with terminal mannose) structure.

Key words: Fc carbohydrate/IgG catabolism

Introduction

A fundamental understanding of the sites and mechanisms of degradation of plasma proteins is critical if we are to both understand the mechanisms of maintaining the steady-state levels of these proteins and to produce proteins with optimal pharmacokinetic properties. Identification of the site of catabolism of the protein and the features of the protein that might target it to that site and control its rate of degradation will provide useful information for the design of therapeutic proteins.

Antibodies are glycoproteins that have been extensively studied both because of their ability to specifically recognize foreign antigens and because of their diverse functional properties. Antibodies of different isotypes have different properties including different rates of in vivo clearance. Among the isotypes, IgG is relatively long-lived in vivo with a serum half-life in humans exceeding 21 days reported for some subclasses (Roitt, 1994). IgG is also distinguished by the fact that its rate of clearance is dependent on its serum concentration with increasing concentration associated with more rapid clearance (Brambell et al., 1964).

The amino acid sequence of an antibody plays a role in determining its serum clearance. Different subclasses of IgG clear at different rates. In addition amino acid changes within the Fc region of the IgG can have profound effects on the rate of serum clearance (Kim et al., 1994, 1999; Medesan et al., 1997; Zuckier et al., 1998; Hornick et al., 2000). In addition to amino acid sequence, the structure of the attached carbohydrate is thought to play a role in protein targeting and clearance (Mattes, 1987; Varki, 1993; Dwek, 1995). We have shown that IgG1 antibodies whose Fc-associated carbohydrate has terminal mannose residues exhibit more rapid clearance than more fully glycosylated IgG1 antibodies (Wright and Morrison, 1994). The present study is designed to further investigate the influence of the structure of the Fc-associated carbohydrate on the sites of clearance and organ targeting of IgG1.

The kinetics of protein clearance is traditionally measured by radiolabeling proteins with isotopes such as 125I and observing the rate of clearance of radioactivity either from the body of the experimental animal or from individual organs. However, when the protein is degraded the free label is rapidly lost from the tissue making it difficult or impossible to identify the site of catabolism of the protein.

To address these issues alternative labeling procedures using glycoconjugates have been developed. Glycoconjugates that are relatively large, hydrophilic, and resistant to lysosomal degradation remain for extended periods at the site of release from the protein and can serve as biologically inert tracers to identify in vivo sites of protein uptake and catabolism (Thorpe et al., 1993; Thorpe and Baynes, 1994). Termed residualizing labels (R-labels), compounds such as dilactitol tyramine (DLT) have been developed which can be coupled to candidate proteins through mild reductive amination procedures that minimize damage to the protein and effects on the biological activity of the protein. In addition the residualizing labels can themselves be easily radiiodinated.

Several approaches have been used to alter the glycosylation state of IgG antibodies. These include inhibition of glycosylation by culturing cells in the presence of the drug tunicamycin an inhibitor of N-linked glycosylation (Leatherbarrow et al., 1985; Walker et al., 1989), treatment of glycoproteins with specific glycosidases that remove the entire oligosaccharide or specific residues (Tsuchiya et al., 1989; Boyd et al., 1995) or site-directed mutagenesis to either remove the carbohydrate...
addition site (Tao and Morrison, 1989) or residues within the Cg2 region that contact the core oligosaccharide residues (Lund, et al., 1996). However, a persisting concern with many of these approaches is that the treatment itself alters the conformation of the protein and hence influences its catabolism.

Production of immunoglobulin in Chinese hamster ovary (CHO) cells with defined glycosyltransferase mutations provides an alternative approach to studying the contribution of carbohydrate structure to antibody function. Compared to glycosidase digestion, this approach is advantageous in that: (1) homogeneous carbohydrate structures (at least in terms of what residues they lack) are produced without the potential complication of damage to the protein backbone through enzyme treatment; and (2) carbohydrate structures can be attached that are not easily produced by glycosidase treatment.

We have now expressed mouse–human chimeric IgG1 antibodies in wild-type Pro-5 CHO cells as well as in the glycosylation mutants Lec 1, Lec 2, and Lec 8. Lec 1 cells are deficient in N-acetylglucosaminyltransferase I and are expected to attach a truncated, Man,GlcnAc2 structure not normally seen on IgG. Lec 2 cells are defective in the transport of CMP-sialic acid and should synthesize a complex carbohydrate structure lacking sialic acid. Lec 8 cells fail to transport UDP-galactose, and should attach a complex carbohydrate structure lacking galactose to IgG1 (Stanley, 1984, 1987a,b). We have determined the structure of the carbohydrate attached to the purified antibodies used in this study to verify that mutant CHO cells do indeed attach carbohydrates of expected structure. We have now labeled these well-characterized proteins with a residualizing label and compared their organ targeting and clearance pathways. All proteins are catabolized throughout the body with the skin the major site of catabolism. In addition, the liver is a significant site of catabolism of IgG1 bearing the Lec 2 carbohydrate with terminal galactose and the Lec 1 carbohydrate with terminal mannose.

Results

Analysis of the oligosaccharide fractions by amide column HPLC

When the 2AB-labeled oligosaccharide fractions obtained from each IgG sample were subjected to anion-exchange column chromatography, all IgGs except for IgG1-Pro-5 gave only a neutral fraction. Although acidic components of IgG1-Pro-5 occupied 8% of total oligosaccharides and were converted to neutral oligosaccharides by sialidase digestion, further structural study could not be performed due to the limited amounts of sample available. Neutral oligosaccharide mixtures from each IgG clone were analyzed by amide column HPLC. IgG1-Pro-5 and IgG1-Lec 2 samples contained three major components eluting in peaks a, b, and c in Figure 1A and Figure 1B, respectively. Only peak d, which showed the same elution position with peak a in Figure 1A and 1B, was observed in IgG1-Lec 8 sample (Figures 1C). On the other hand, IgG1-Lec 1 showed five peaks (peaks e–i, in Figure 1D) with different elution positions than peaks a, b, c, or d. In order to more precisely clarify the difference, further structural analysis was performed. It should be noted that each peak in Figure 1D showed approximately one glucose unit difference. Because the results obtained from both IgG1-Pro-5 and IgG1-Lec 2 samples were essentially the same, only the data for IgG1-Pro-5 shown in Figure 1A will be described here.

Structural analysis of the oligosaccharides by sequential exoglycosidase digestion

Upon incubation with diplococcal β-galactosidase, peak c in Figure 1A was converted to a peak eluting at the same position as peak a, with the release of two galactose residues as shown in Figure 2A. On the other hand, peak b released one galactose residue and moved to the elution position of peak a (Figure 2B). Peak a in Figure 1A, which is totally resistant to
from the column earlier than the GlcNAc\(\beta\)\(\alpha\)β, 1978), it was concluded that all the Gal et al. GlcNAc cleave only the GlcNAc\(\beta\) substrate specificity of diplococcal \(\beta\)-galactosidase, which cleaves the Galβ1→4GlcNAc linkage but not the Galβ1→3GlcNAc or the Galβ1→6GlcNAc linkage (Paulson et al., 1978), it was concluded that all the Galβ1→GlcNAc groups in the oligosaccharides in the peaks c and b should occur exclusively as the Galβ1→4GlcNAc group.

Digestion of the components in Figure 2A and in Figure 2B with diplococcal \(\beta\)-N-acetylhexosaminidase, which can cleave only the GlcNAc\(\beta\)→2Man linkage but not the GlcNAc\(\beta\)→4Man or the GlcNAc\(\beta\)→6Man linkage (Yamasita et al., 1981), released two N-acetylglucosamine residues and the new product eluted at the same position as authentic Man\(\alpha\)1→6(Man\(\alpha\)1→3)Man\(\beta\)1→4GlcNAc\(\beta\)1→4(Fuc\(\alpha\)1→6)GlcNAc-2AB (Figure 2C). From these results, it was concluded that the oligosaccharides of IgG1-Pro-5 IgG in Figure 1A are biantennary complex-type sugar chains containing ≤Galβ1→4GlcNAc\(\beta\)→2 as outer chains. Based on the enzymatic digestion described above it was concluded that the Galβ1→4GlcNAc\(\beta\)→2 group of oligosaccharides located in area b represent two different isoforms whose distribution could be assigned by LA-RCA HPLC, because the Galβ1→4GlcNAc\(\beta\)→2Man\(\alpha\)1→6(GlcNAc\(\beta\)→2Man\(\alpha\)1→3)Man\(\beta\)1→4GlcNAc\(\beta\)1→4(Fuc\(\alpha\)1→6)GlcNAc isomer was eluted from the column earlier than the GlcNAc\(\beta\)→2Man\(\alpha\)1→6(Galβ1→4GlcNAc\(\beta\)→2Man\(\alpha\)1→3)Man\(\beta\)1→4GlcNAc\(\beta\)1→4(Fuc\(\alpha\)1→6)GlcNAc isomer (Harada et al., 1987). The percent molar ratio of both isomers was calculated from the corresponding peak (data not shown). The percentage of oligosaccharides with zero, one, and two galactose residues on IgG1-Pro-5 and IgG1-Lec 8 was calculated from the peak areas, a, b, and c, respectively, and the results are summarized in Table I.

On the other hand, peak d in Figure 1C is totally resistant to \(\beta\)-galactosidase treatment. When it was digested with diplococcal \(\beta\)-N-acetylhexosaminidase, it released two N-acetylglucosamine residues and the new product eluted at the same position as authentic Man\(\alpha\)1→6(Man\(\alpha\)1→3)Man\(\beta\)1→4GlcNAc\(\beta\)1→4(Fuc\(\alpha\)1→6)GlcNAc-2AB (Figure 2C). From these results, it was concluded that the oligosaccharides of IgG1-Lec 8 in Figure 1C are biantennary complex-type sugar chains containing only GlcNAc\(\beta\)→2 group as outer chains.

When oligosaccharides of IgG1-Lec 1 (peaks f–i in Figure 1D) were digested with A. saitoi α-mannosidase, which can cleave only the Man\(\alpha\)1→2Man linkage (Yamasita et al., 1980), peaks f–i were all converted to the same position as component e, which showed the same mobility as an authentic Man\(\alpha\)1→6(Man\(\alpha\)1→3)Man\(\alpha\)1→6(Man\(\alpha\)1→3)Man\(\beta\)1→4GlcNAc\(\beta\)1→4GlcNAc-2AB with the release of one, two, three, and four Man\(\alpha\)1→2 residues (Figure 2D). The results indicated that oligosaccharides of IgG1-Lec 1 are a series of high mannose type containing one, two, three, and four Man\(\alpha\)1→2 residues linked to Man\(\alpha\)1→6(Man\(\alpha\)1→3)Man\(\beta\)1→4GlcNAc\(\beta\)1→4GlcNAc-2AB (component e) instead of complex type sugar chains as found in other clones.

### Table I. Structure of the carbohydrate present on IgG1 produced in different cell lines

<table>
<thead>
<tr>
<th>Structure</th>
<th>Pro5</th>
<th>Lec2</th>
<th>Lec8</th>
<th>Lec1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galβ1→4GlcNAcβ1→2Manα1</td>
<td>16.8%</td>
<td>24.1%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Galβ1→4GlcNAcβ1→2Manα1</td>
<td>40.5%</td>
<td>34.7%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Galβ1→4Fucα1→6GlcNAcβ1→4GlcNAcβ1→6Manα1→2Manα1</td>
<td>8.2%</td>
<td>11.3%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Galβ1→4Fucα1→6GlcNAcβ1→4GlcNAcβ1→6Manα1→2Manα1</td>
<td>34.5%</td>
<td>29.9%</td>
<td>100%</td>
<td>0%</td>
</tr>
</tbody>
</table>

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On the basis of these results, the structures of oligosaccharides on IgG1 produced by the wild type Pro-5 CHO cells and the glycosylation mutants Lec 2, Lec 8, and Lec 1 were elucidated and are summarized in Table I.

In vivo clearance of IgG1 with different carbohydrate structures

Mice injected intravenously with protein iodinated through the attached dilactotyramine moiety were sacrificed 3 or 6 days following injection, their organs were harvested, and the associated radioactivity was determined. The results are shown in Figure 3A and are expressed as the total radioactivity present in a particular organ compared to the total radioactivity present at the time of sacrifice. Values represent the average of two mice. For all of the proteins, there is significant residual radioactivity in the blood, skin, and muscle. For IgG1-Lec 2 and IgG1-Lec 1 there is also significant quantities of radioactivity present in the liver at days 3 and 6.

Radioactivity associated with intact protein is TCA precipitable whereas radioactivity associated with DLT that has been removed from catabolized proteins is TCA soluble. To further investigate the sites of catabolism, the organs were processed as described above and the TCA precipitable (Figure 3B) and TCA soluble (Figure 3C) radioactivity determined. Several things are noteworthy. Virtually all radioactivity present in the blood is associated with intact protein indicating that the blood represents a recirculating pool, not a site of catabolism. In contrast, skin and muscle contain significant quantities of radioactivity associated with both intact and catabolized proteins. Therefore, for both of these organs intact proteins

**Fig. 2.** Exoglycosidase digestion products of oligosaccharides. Solid triangles are the same as those in Figure 1. White triangles indicate the elution positions of authentic oligosaccharides: I, Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc2AB; II, Manα1→6(Manα1→3)Man−α1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc2AB. (A) Peak c in Figure 1A incubated with diplococcal β-galactosidase; (B) peak b in Figure 1A incubated with the same enzyme; (C) components in (A) and (B); peak a in Figure 1A, and peak d in Figure 1C incubated with diplococcal β-N-acetylhexosaminidase; (D) components e–i in Figure 1D incubated with A. saitoi α-mannosidase.

**Fig. 3.** Percentage of the counts remaining in different organs. Animals were injected as described with proteins labeled with 125I-dilactotyramine, and then sacrificed 3 or 6 days after injection. Organs were harvested and the amount of radioactivity present determined and expressed as a percentage of the total radioactivity present in the mouse at the time of sacrifice (A). Organs were processed and the amount of TCA precipitable radioactivity (B) or TCA soluble radioactivity (C) determined; data are expressed as percentage of the total radioactivity present in the mouse at the time of sacrifice and represent the average of two mice. Little radioactivity was present in the heart, lungs, and stomach, and those organs are not shown.
accumulate but only a portion of the accumulated proteins is catabolized. It remains to be determined if the intact proteins are returned to the blood for recirculation or if they remain permanently associated with the skin and muscle. The majority of the counts associated with the liver are TCA soluble, suggesting that proteins targeted to the liver are rapidly catabolized. Although less radioactivity is present, a similar pattern is seen with the spleen with the majority of the associated radioactivity TCA soluble again suggesting that antibodies targeted to that organ are rapidly catabolized. The majority of the radioactivity present in both the intestine and kidney is also TCA soluble and may represent radioactivity that is in the process of being eliminated. The values for the heart, lungs, and stomach suggest that these organs are not a major site of catabolism.

This treatment of the data indicates that the majority of all of the antibodies irrespective of the structure of their attached carbohydrate is catabolized in the skin and muscle. However, the attached carbohydrate structure does influence the amount that is catabolized in the liver, and the liver serves as a major site for the catabolism of proteins bearing carbohydrate with the Lec 2 (with terminal galactose) or Lec 1 (with terminal mannose) structure.

We also investigated the distribution of the radioactivity in the body on a weight basis (Figure 4). When the weight of the organs is considered, the radioactivity is seen to be broadly distributed but with reduced accumulation on a weight basis in the stomach, intestine and muscle. Comparison of TCA precipitable (Figure 4B) and TCA soluble (Figure 4C) radioactivity suggests that catabolism occurs in all organs although some of the TCA soluble counts present in the blood, stomach intestine, and kidney may represent catabolic products that are in the process of being excreted.

**Discussion**

The contribution of the oligosaccharide moieties to the structure, function and stability of glycoproteins has been studied extensively in many systems. For example, the C57–produced recombinant carboxydy is essential for some IgG1– and IgG3–mediated effector activities (Tao and Morrison, 1989; Umana et al., 1999). The carbohydrate associated with these antibodies is a complex biantennary structure with considerable heterogeneity in the terminal residues seen in serum IgG. Nevertheless it has been suggested that certain glycoforms are correlated with disease (Parekh et al., 1985; Rademacher et al., 1988). It is becoming increasingly common to express recombinant proteins in heterologous host cells which may attach carbohydrate structures that are subtly but distinctively different from those produced by the native cell (Lifely et al., 1995). For several reasons, therefore, it is important to understand whether specific changes in carbohydrate structure due to aberrant glycosylation, species-specific glycosylation, or cell growth conditions affect the functional properties of glycoproteins.

CHO cells are frequently used for the production of recombinant human glycoproteins. They do not attach the immunogenic α(1→3) galactose residue and the oligosaccharide structures obtained from CHO-produced proteins such as erythropoietin and tissue plasminogen activator closely correspond to those derived from the native protein (Smith et al., 1990; Goochee et al., 1991). The derivation of several CHO cell lines with well-characterized glycosylation mutants has yielded a useful system in which to produce and evaluate glycoproteins with defined carbohydrate constituents.

These studies have shown that the CHO expression system with its Lec mutants is suitable for producing IgGs with altered carbohydrate structure. IgG1-Lec 1 (high-mannose intermediate) has an oligomannose structure as expected. Antibodies produced in Lec 8 (agalactosyl) have the most homogeneous carbohydrate structure suggesting that Lec 8 would be an appropriate expression system for production of proteins when uniform carbohydrate structure is desired. Although multiple different glycoforms are present on IgG1-Pro-5 and IgG1-Lec 2, the latter two antibodies showed far less heterogeneous glycosylation than the ~30 glycoforms reported for human serum immunoglobulin. Pro-5 was the only cell line capable of producing sialylated IgG. However, only 8% of the carbohydrate structure present on IgG produced by Pro-5 had terminal sialic acid, similar to the extent of sialylation seen on human IgG (Rademacher et al., 1985). A higher percentage of IgG1-Lec 2 than of IgG1-Pro-5 bears carbohydrate with two terminal galactose residues while Pro-5–produced IgG1 has more structures with two terminal GlcNAc residues than Lec 2–produced IgG1. With the exception of the Lec 1–produced
antibodies, all carbohydrate structures present on the antibodies are seen on normal human immunoglobulins.

The rate at which a therapeutic agent is catabolized plays an important role in determining its efficacy. Although these studies have shown that the glycoform present on the Fc of the antibody molecule can influence its biolocalization the major sites of protein localization for all of the IgGs, 3 and 6 days after injection are the blood, liver, skin, and muscle. The blood represents a recirculating pool of intact protein. The catabolism of IgG occurs throughout the body. When the total amount of catabolism is considered, the skin and muscle catabolize the most, irregardless of the structure of the attached carbohydrate. These results are largely in agreement with those of Henderson et al. (1982), who found that rat IgG is catabolized at several sites in the body.

The liver also serves as a major catabolic site, however the structure of the attached carbohydrate plays a significant role in determining the amount of IgG that is catabolized there. Within the liver several receptors are candidates for IgG clearance. One, the asialoglycoprotein receptor, binds terminal galactose residues of desialylated glycoprotein and mediates endocytosis and eventual degradation of these ligands. Seventy percent of the carbohydrate structures present on IgG1-Lec 2 contain terminal galactose residues, allowing it to be recognized and degraded through this receptor pathway. It is noteworthy that IgG1-Pro-5–produced proteins exhibit decreased liver targeting; only 65% of the glycoforms have galactose residues with 8% having sialic acid. More IgG1-Pro-5 protein is seen in the liver on day 6 than on day 3; possibly removal of sialic acid occurs in the circulation. Another candidate receptor is the mannose binding receptor expressed on Kupffer and sinusoidal endothelial cells (Lennartz et al., 1987; Stahl, 1992; Takahashi et al., 1998). It would appear likely that IgG1-Lec 1 proteins are recognized by this receptor. Indeed, earlier studies (Wright and Morrison, 1994) have shown that administration of yeast mannan delays the clearance of IgG1-Lec 1. The mannose receptor could also contribute to the clearance of IgG1-Lec 8 since a recent study has demonstrated the binding and uptake of agalactosyl IgG through the mannose receptor (Dong et al., 1999). Virtually all radioactivity present in the liver at both day 3 and day 6 is TCA soluble.

Recently the neonatal Fc receptor (FcRn) responsible for transport of immunoglobulin across the neonatal rodent intestine has been proposed to play a role in the control of the half-life of IgG. FcRn resembles a Class I MHC molecule in structure. In β2-microglobulin (β2-m) deficient mice which fail to express FcRn, the half-life of IgG is much shorter (Ghetie et al., 1996; Israel et al., 1996). The expression of FcRn is widespread and includes the capillary endothelium (Story et al., 1994; Blumberg et al., 1995; Israel et al., 1996, 1997; Leach et al., 1996). IgGs lacking carbohydrate continue to be recognized by FcRn (Hobbs et al., 1992). Thus, it may be expected that alterations in carbohydrate structure would not affect recognition by FcRn.

FcRn shows a pH-dependent binding exhibiting high affinity for IgG at pH 6 and low affinity at neutral pH (Raghavan et al., 1995). The concept has evolved that FcRn binds IgG that has been taken up in the fluid phase in the acidified endosome and diverts it from trafficking to the lysosome (Junnghans and Anderson, 1996). This model postulates a saturable receptor in the salvage pathway consistent with the observation that the half-life of IgG decreases with increasing serum concentration. In this model the cells involved in salvaging IgGs are also responsible for IgG breakdown. Our data support the presence of a recycling receptor since significant quantities of both TCA precipitable and TCA soluble radioactivity are present at sites likely to express this receptor.

When the amount of residual counts per gram is considered several organs would appear to play an active role in catabolism on a per weight basis. Especially prominent is the spleen. Within the spleen there would be macrophages bearing the mannose receptor, and as discussed previously these may play a role (Gross et al., 1988; Smedsrod et al., 1990). Accordingly, of the antibodies described in this study, IgG1-Lec 1 was targeted to and catabolized most efficiently in the liver and spleen. In addition, the spleen would have cells bearing Fc receptors. However, it is not clear that Fc receptors play an active role in determining catabolic rate since IgGs lacking carbohydrate, and therefore not recognized by Fc receptors, clear at rates similar to what is observed with IgGs that are recognized by Fc receptors (Tao and Morrison, 1989). For the heart and lungs, it may be the vascular endothelium that is participating in catabolism. For the kidney, the radioactivity may represent degraded protein in the process of being eliminated.

These studies have confirmed that the Lec mutants of CHO cells attach glycoforms of the expected structures and provide a convenient expression system for the production of glycoproteins with defined glycoforms attached. We have found that antibodies are catabolized at many sites throughout the body. Although the amount catabolized at each site is influenced by the structure of the attached carbohydrate, the general pattern of catabolism remains similar for all of the glycoforms.

Materials and methods

Proteins
Dansyl-specific mouse-human chimeric IgG1 antibodies were produced in the CHO cell lines Pro-5, Lec 2, Lec 8, and Lec 1 as described previously (Wright and Morrison, 1998). The CHO cell lines Lec 1, Lec 2, and Lec 8, were originally derived by Dr. Pamela Stanley (Stanley, 1984). All CHO lines were acquired from the American Type Culture Collection. Antibodies were purified from culture supernatants by affinity chromatography as described previously.

Materials
Diplococcal β-galactosidase and β-N-acetylhexosaminidase were purchased from Boehringer Mannheim (Mannheim, Germany). α-Mannosidase specific for ManT1→2 linkage was purified from Aspergillus nuioti as described previously (Yamashita et al., 1980).

 Liberation of the N-glycans of IgG as oligosaccharides
Each IgG was subjected to 9 h hydrazinolysis as described previously (Takasaki et al., 1982). After N-acetylation, oligo-
saccharide fraction was labeled with 2-aminobenzamide (2-AB) using the 2-AB labeling kit (Oxford GlycoSystems, Abingdon, UK) to obtain 2AB-labeled oligosaccharides.

Analytical methods

Anion-exchange column chromatography and paper chromatography were performed as described previously (Chiba et al., 1997). Amide column HPLC was carried on GlycoSep N (Oxford GlycoSystems), and the acetonitrile/250 mM ammonium acetate (pH 4.0) ratio was changed linearly from 80:20 to 47:53 (v/v) over 132 min after injection at a flow rate of 1.0 ml/min at 30°C. Neutral oligosaccharides were subjected to HPLC using an LA-40 A. communis agglutinin 120 column (Seikagaku Co., Tokyo). After elution with 16 ml of 10 mM phosphate buffer, pH 7.4, 140 mM NaCl (PBS), the bound oligosaccharides were eluted with a gradient of PBS containing 0–2 mM lactose, at a flow rate of 0.8 ml/min at 30°C.

Glycosidase digestion

Oligosaccharides were incubated with one of the following mixtures at 37°C for 18 h: diplococcal β-galactosidase (10 mU) in 50 µl of 0.3 M citrate–phosphate buffer (pH 6.0); diplococcal β-N-acetylgalactosaminidase (8 mU) in 50 µl of 0.3 M citrate–phosphate buffer (pH 6.0); A. saitoi α-galactosidase (0.15 µg) in 30 µl of 0.1 M acetate buffer (pH 5.0). One drop of toluene was added to all reaction mixtures to inhibit bacterial growth during incubation. Digestion was terminated by heating the reaction mixture in a boiling water bath for 3 min and product was analyzed by amide column HPLC.

Oligosaccharides

GlcNAcβ1→2Manβ1→6(GlcNAcβ1→2Manβ1→3)Manβ1
→4GlcNAcβ1→4(Fucβ1→6)GlcNAc-2AB and
Manβ1→6(Manβ1→3)Manβ1→6(Manβ1→3)Manβ1→4
GlcNAcβ1→4GlcNAc-2AB were prepared from human myeloma IgG (Endo et al., 1989) and bovine pancreatic ribonuclease B (Liang et al., 1980), respectively, by hydrazinolysis followed by labeling with 2AB as described above. Manβ1→6(Manβ1→3)Manβ1→4(GlcNAcβ1→4
(Fucβ1→6)GlcNAc-2AB was obtained from GlcNAcβ1→2Manβ1→6(GlcNAcβ1→2Manβ1→3)Manβ1→4(GlcNAcβ1→4(Fucβ1→6)GlcNAc-2AB by diplococcal β-N-acetylgalactosaminidase digestion.

Synthesis of the residualizing label dilactitol tyramine (DLT)

Synthesis of the glycoconjugate dilactitol tyramine (DLT) was performed according to Strobel et al. (1985). Briefly, a 5-ml reaction mixture containing 130 mM tyramine, 1.3 M lactose, and 520 mM sodium cyanoborohydride (NaBH₄CN; molar ratios of the reactants 1:10:4) was combined in 0.2 M potassium borate buffer, pH 9, and incubated overnight at 65°C. The glycoconjugate was diluted and then purified from the reactants by cation-exchange chromatography on Dowex 50-X2 (Bio-Rad). Fractions were eluted with 0.05 M ammonium acetate followed by 1 M ammonium acetate, pH 7. The amount of protein in the fractions was determined using A₂₈₀ absorbance; the presence of carbohydrate was determined using the anthrone reaction. The molar ratio of sugar to tyramine was determined for each fraction, and those exhibiting a 2:1 ratio were pooled and concentrated by lyophilization. The DLT was dissolved in distilled water and the concentration estimated by absorbance at 280 nm, using an extinction coefficient of 1360 M⁻¹ cm⁻¹ as described previously (Strobel et al., 1985). The concentration of DLT was then adjusted to a concentration of 100 mM and stored at −20°C.

Radioiodination and coupling of DLT to the antibodies

The proteins were labeled with Na¹²⁵I (Amersham) using the Iodobead method (Pierce). Radiolabeled DLT (1 μM) was incubated at 37°C with 4 U galactose oxidase (Sigma), which converts DLT to the aldehyde form. Purified immunoglobulin (~20 mg, resuspended in PBS buffer pH 7.4) was added to the aldehyde as well as 40 mM NaBH₄CN and incubated for up to 2 h at 37°C. The iodinated antibody was then separated from the free conjugate by chromatography on Sephadex G-50 equilibrated with PBS 7.4 and 1% BSA (bovine serum albumin, Sigma). The final radiolabeled protein was ~95% precipitable in 10% TCA.

Animals

Female BALB/c mice (~3 months old, Taconic Farms, Germantown, NY) were fed water treated with 0.1 mg/ml potassium iodide for at least 1 week before proteins were injected. Groups of four mice were injected intravenously (tail vein) with equal amounts of iodinated antibody. To determine whole body radioactivity each mouse was placed, at regular intervals, in a NaI crystal with a model JS-5A scaler/ratemeter attached (Ronceverte, WV). Measurements were begun immediately after injection. After 3 days, half the mice from each group were sacrificed. The remaining mice were sacrificed 6 days post-injection.

Mice were weighed and counted immediately before sacrifice using ether anesthesia. The abdominal area was shaved so that skin samples could be removed. The animals were exsanguinated and the blood allowed to clot. Organs were rapidly dissected out, rinsed with ice-cold PBS, and placed in labeled, preweighed Eppendorf tubes in a dry ice-ethanol bath. Tissues were kept cold to minimize protein degradation. Radioactivity remaining in each organ was measured and analysis of precipitable protein was performed within 24 h.

Analysis of organ distribution of proteins

Processing of tissues was performed as described previously (Thorpe and Baynes, 1994). Tissues were homogenized and aliquots were incubated with an equal volume of 40% TCA. To estimate the percentage of the degraded protein in the sample radioactivity was measured in both the supernatant and pellet. Radioactivity in the supernatant was calculated as the percentage of the total radioactivity of both the supernatant and pellet.

For skin and muscle, the radioactivity in the recovered aliquots was measured. Total radioactivity was calculated by multiplying the radioactivity of the aliquots per gram tissue by total tissue weight, estimated at 18% or 45% of the total body weight, respectively (Moldoveanu et al., 1988). TCA precipitability of the protein was calculated as described above.

In all cases the calculated specific radioactivity associated with the organ or tissue was corrected by subtraction of the blood-borne radioactivity. Based on published records of blood volumes of organs in mice, expressed as ml/kg of body weight (Altman and Dittmer, 1971), these values were adjusted.
for the weights of the experimental animals and accordingly subtracted from the total radioactivity recovered from each organ.

**Interpretation of results**

An estimate of the overall distribution of the distribution of the protein in the body was calculated as a percentage of the remaining for each organ. The contribution of each organ or tissue to degradation (catabolism) of the protein was calculated by multiplying the percentage of the remaining dose recovered in each tissue by the acid-soluble fraction of radioactivity. This was expressed as the % dose catabolized/tissue. To estimate the relative catabolic efficiency of each tissue, each of these values was divided by the weight of the appropriate tissue.

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**Abbreviations**

2AB, 2-aminobenzamide; HPLC, high-performance liquid chromatography; CHO, Chinese hamster ovary; RCA 120, *Ricinus communis* agglutinin 120; DLT, dilactitol tyramine; FcRn, neonatal Fc receptor.

**References**


