Anti-galactose-α-1,3-galactose IgE from allergic patients does not bind α-galactosylated glycans on intact therapeutic antibody Fc domains

To the Editor:

Manufacture of monoclonal antibodies (mAb) and related biologics is performed in eukaryotic cell lines to allow proper glycosylation, which is often critical for function. The first mAbs were produced in the mouse-derived cell lines SP2/0 and NS0, which have the genetic makeup to produce proteins containing nonhuman glycan structures, such as galactose-α-1,3-galactose (α-Gal) and *N*-glycolylneuramic acid[1], that can adversely affect safety and half-life. The best example is Erbitux (cetuximab), produced in SP2/0 cells, clinical use of which has been associated with α-Gal–specific, IgE-mediated anaphylactic responses[2]. In addition, patients with IgE antibodies to α-Gal may experience allergic reactions to red meat[3,4]. The quantity of α-Gal required to induce hypersensitivity reactions is not known; however, it is clear that binding of IgE antibodies against α-Gal is an important trigger in this pathology[2].

Another host cell frequently used for mAb manufacture is the Chinese hamster ovary (CHO) cell line. For years it was thought that CHO cells lack the machinery to synthesize α-Gal and therefore represent a more suitable production system for protein therapeutics, although the ability of CHO cells to produce the α-Gal epitope in recombinant proteins was previously reported[5]. Recently, Bosques *et al.*[6] have identified a functional CHO ortholog of *N*-acetyllactosaminide-3-α-galactosyltransferase-1, showing that the therapeutic product abatacept (Orencia, CTLA4-IgG fusion protein), produced in CHO, contains α-Gal residues. Knowing this, they argue that proteins manufactured in CHO cells need to be monitored and controlled for α-Gal levels.

To date, therapeutic mAbs manufactured in various production cell lines have been used to treat numerous patients without direct evidence for the α-Gal IgE-mediated hypersensitivity reactions observed with cetuximab; however, cetuximab is not the only therapeutic mAb for which IgE-mediated hypersensitivity-like reactions have been observed[7]. For cetuximab, the problem appears to relate to its α-Gal–containing glycosylation in the Fab domain. Therapeutic mAbs usually lack such Fab glycosylation and carry glycans only in their Fc domain[8]. There are several possible explanations for the absence of IgE-mediated hypersensitivity reactions associated with therapeutic mAbs produced in murine cell lines other than cetuximab. First, these mAbs may contain fewer α-Gal glycans; or second, glycans in the Fc domain may not be sufficiently accessible for IgE binding; or third, glycans present in the Fab domain may differ in α-Gal glycan composition compared with glycans in the Fc domain. Finally, an IgE-mediated reaction might be attenuated by competition with natural α-Gal IgG or IgM antibodies.

Here we studied whether IgE antibodies against α-Gal can interact with α-Gal moieties on six representative therapeutic mAbs produced in the three different mammalian production systems: SP2/0 (cetuximab; and Remicade, infliximab), NS0 (Simulect, basiliximab; and Synagis, palivizumab) and CHO (Vectibix, panitumumab; and zalutumumab) (**Supplementary Table 1**). First, we quantified the amount of α-Gal present on these mAbs. Antibody glycans were enzymatically released using *N*-glycanase F and characterized using high-performance anion exchange chromatography with pulsed amperometric detection (HPEAC-PAD) analysis, as described in **Supplementary Methods**. Under native conditions, *N*-glycanase F only releases glycans from the Fc domain (asparagine 297) and therefore the enzyme treatment was also performed in the presence of hexadecyltrimethylammonium bromide (CTAB) detergent to release glycans at other potential sites[8]. Sodium dodecyl sulfate PAGE (SDS-PAGE) analyses of *N*-glycanase F–treated mAbs demonstrated a clear shift in molecular weight of the heavy chain of ~2 kDa, indicating the release of the glycans. Only for cetuximab an additional weight shift was observed for the heavy chain when enzyme treatment was performed in the presence of CTAB, supporting previous evidence that cetuximab harbors additional glycans outside the Fc domain. For all other mAbs no additional glycans were detected when glycans were released in the presence of CTAB (**Supplementary Fig. 1**).

As expected, α-Gal–containing glycans were identified in SP2/0- and NS0-derived mAbs (**Table 1 and Supplementary Fig. 2**). The glycan species identified for cetuximab were identical to mass spectrometry results previously reported[1]. For CHO-derived mAbs, the level of α-Gal–containing glycans was below the limit of quantification. To translate our data to the findings of Bosques *et al.*[6], abatacept was included, and here we did detect α-Gal moieties; however, we were not able to quantify all the α-Gal glycan species due to their complex character not typically observed on therapeutic mAbs. No α-Gal was observed in basiliximab. Alpha-galactose residues are enzymatically linked by α(1,3)-galactosyltransferase to a β(1,4)-galactose-acceptor substrate. We observed that basiliximab contains low levels of β(1,4)- galactose substrate, which explains the lack of α-Gal moieties in basiliximab's glycan profile.

To identify differences between Fab and Fc glycans in more detail, isolated cetuximab Fab, $F(ab')_2$ and Fc fragments were analyzed. The analyses confirmed our own and previous findings of Qian *et al.*[1] that most of the α-Gal in cetuximab is located in the Fab domain (Fab 990 nmol α-Gal/μmol IgG versus Fc 140 nmol α-Gal/μmol IgG) (**Table 1** and **Supplementary Figs. 1,3**). The Fab glycans accounted for 24.4% of bi α-galactosylated, biantennary complex–type glycan structures, whereas glycans in the Fc predominantly contained mono-α-galactosylated glycans (mono-α-galactosylated glycans: cetuximab, 6.5%; infliximab, 8.7%) and almost no bi-α-galactosylated, biantennary α-galactosylated glycans (0.4% for cetuximab; 1.1% for infliximab). The extent of α-Gal content in carbohydrate chains of the complex type is an outcome of competition between the enzymes that cap the carbohydrate chain either with α-Gal or sialic acid, the activity of which may vary depending on culture conditions[9]. The ratio between bi-α-galactosylated and mono-α-galactosylated glycans observed in biologics may therefore differ.

To assess whether these therapeutic mAbs are able to bind α-Gal–specific

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IgE, two radioimmunoassays (RIAs) were set up. Serum from 20 individuals with allergy to red meat was used as a source for α-Gal IgE antibodies (**Supplementary Table 2**). In the first RIA, binding of IgE to antibody-coated sepharose beads was tested and compared with a positive control of human serum albumin (HSA)-expressed α-Gal. Only cetuximab and α-Gal-HSA demonstrated binding of IgE above background levels (**Fig. 1a**). The good correlation of IgE reactivity to cetuximab with that to α-Gal-HSA confirmed the α-Gal specificity of the IgE antibodies bound ($r^2 = 0.89$; **Supplementary Fig. 4**). In the second RIA, putative interactions of α-Gal IgE with the therapeutic mAbs were investigated in a competition format. Using pooled serum of four α-Gal IgE–positive patients, inhibition of α-Gal IgE binding to cetuximab-coated sepharose was tested by titration of the individual therapeutic mAbs. In this experimental design, only cetuximab and α-Gal-HSA demonstrated interactions with IgE, as they competed for IgE binding (**Fig. 1b**). The α-Gal specificity of these IgE antibodies was confirmed by the observation that in a similar setting an excess of cetuximab completely blocked the binding of IgE to α-Gal-HSA-coated sepharose (**Supplementary Fig. 5**). These experiments demonstrated that although infliximab, palivizumab and abatacept contain detectable levels of α-Gal, these therapeutics do not interact with α-Gal IgE. As expected, therapeutics without detectable α-Gal levels (that is, zalutumumab, panitumumab and basiliximab) do not show reactivity with α-Gal IgE.

To further dissect α-Gal IgE reactivity in terms of apparent affinity and epitope recognition, the intact mAbs of cetuximab and infliximab and their isolated Fab, $F(ab')_2$ and Fc fragments were tested in the cetuximab-sepharose inhibition-RIA (**Fig. 1c**). Cetuximab $F(ab')_2$ fragments demonstrated a similar reactivity with α-Gal IgE as intact cetuximab, indicating that most of the binding is attributed by the cetuximab Fab domain. A notable difference, however, was observed in α-Gal IgE reactivity between cetuximab $F(ab')_2$ and Fab fragments (IC_{50} of 24 μg/ml versus 140 μg/ml, respectively). This difference is likely explained by the presence of two α-Gal–containing glycan sites in the cetuximab variable heavy (V_H) domain, one in each of the Fab arms[2], which allows bivalent binding of α-Gal IgE, thereby increasing its binding avidity.

Table 1 Level of galactose-α-1,3-galactose present in mAbs from different production systems

Production system	Sample	Treatment to release glycans for analyses	Galactose-α1,3-galactose peak area (nC × min)	α-Gal content (μmol per μmol IgG)
SP2/0	Cetuximab			1.132
		N-glycanase F	4.5	
		N-glycanase F-CTAB	24.7	
	Infliximab			0.245
		N-glycanase F	5.4	
		N-glycanase F-CTAB	5.8	
NS0	Palivizumab			0.153
		N-glycanase F	4.3	
		N-glycanase F-CTAB	4.6	
	Basiliximab			<LOQ
		N-glycanase F	<LOQ	
		N-glycanase F-CTAB	<LOQ	
CHO	Zalutumumab			<LOQ
		N-glycanase F	<LOQ	
		N-glycanase F-CTAB	<LOQ	
	Panitumumab			<LOQ
		N-glycanase F	<LOQ	
		N-glycanase F-CTAB	<LOQ	
	Abatacept			0.06[b]
		N-glycanase F	–	
		N-glycanase F-CTAB	–	
SP2/0	Cetuximab $F(ab')_2$	*N*-glycanase F-CTAB[a]	24.0	0.990
	Cetuximab Fab	*N*-glycanase F-CTAB[a]	25.1	0.603
	Cetuximab Fc	*N*-glycanase F-CTAB[a]	4.5	0.140
	Infliximab Fab	*N*-glycanase F-CTAB[a]	<LOQ	<LOQ
	Infliximab Fc	*N*-glycanase F-CTAB[a]	5.7	0.217

<LOQ: below limit of quantification.

[a]Analyzed amount is proportional to amount present in intact IgG. [b]Calculated from Bosques *et al.*[6]

Interestingly, both the cetuximab and infliximab Fc domain did not significantly interact with α-Gal IgE, although they contained detectable levels of α-Gal. We considered several explanations for the apparent absence of IgE binding to Fc domain–associated α-Gal. It is possible that the amount of α-Gal in the Fc domain is insufficient for the detection of α-Gal IgE reactivity or it may be less accessible for binding. Alternatively, in contrast to the bi-α-galactosylated Fab glycans on cetuximab, the mainly mono-α-galactosylated Fc glycans might not, or might only partially, recapitulate the dominant epitope recognized by α-Gal IgE, resulting in decreased binding affinity.

To address these possibilities, we first studied whether glycans in the Fc domain were less accessible for IgE binding. We therefore developed a method to expose the glycans in the infliximab Fc domain using Proteinase K digestion, and then assessed binding in the cetuximab-sepharose inhibition-RIA (**Fig. 1d**). Remarkably, considerable binding of α-Gal IgE was observed to 'exposed' infliximab Fc glycans, albeit at relatively high concentrations. Again, no α-Gal IgE reactivity to intact infliximab was observed, suggesting an α-Gal glycan shielding effect in the Fc domain. A similar finding has previously been observed for Fc-associated mannose[10].

Even so, besides the shielding effect in intact Fc, we noted an additional difference in the binding of α-Gal IgE antibody to cetuximab and infliximab, as exemplified by an increased apparent affinity for proteinase K–digested cetuximab relative to similarly digested infliximab when plotted according to their α-Gal content (IC_{50} 1.4 μM versus 4.6 μM α-Gal, respectively; **Fig. 1d**). As mentioned, one of the most striking differences between the α-Gal glycan structures present on these mAbs is that cetuximab contains predominantly bi-α-galactosylated biantennary complex glycans mainly located in the Fab domain, a finding previously also observed by Qian *et al.*[1], whereas infliximab largely contains

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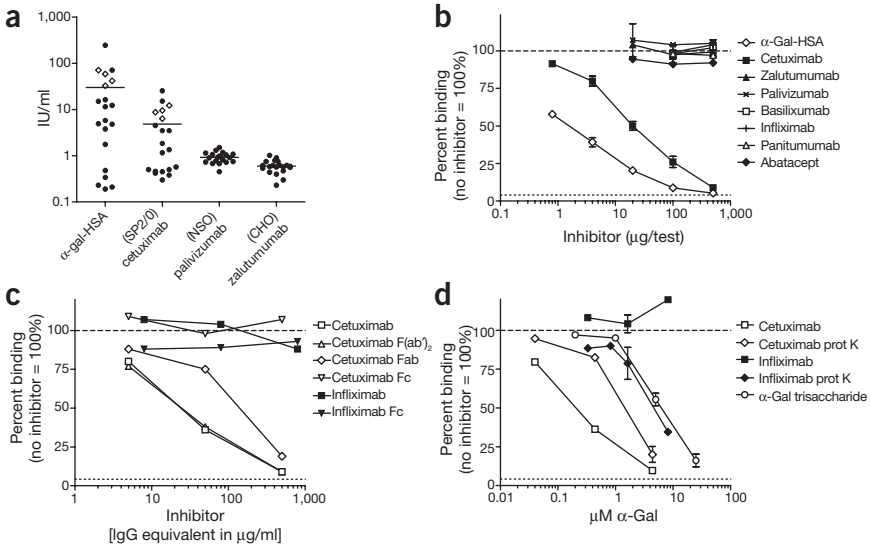


Figure 1 Binding of IgE antibodies in serum samples of 20 meat allergy patients to therapeutic mAbs in different production systems and the interaction of cetuximab, infliximab or isolated fragments thereof with α-Gal–specific IgE measured in an IgE inhibition RIA. (**a**) IgE RIA. Binding of meat allergy patient serum IgE to therapeutic mAb or α-Gal-HSA coupled to sepharose. Binding of IgE was observed to cetuximab (SP2/0) and α-Gal-HSA, but not to zalutumumab (CHO) or palivizumab (NSO). The horizontal lines indicate geometric mean values. The sera that were pooled for use in the IgE inhibition-RIA are indicated (◇). (**b**) IgE inhibition-RIA. The interference of cetuximab, zalutumumab, palivizumab, basiliximab, infliximab, panitumumab, abatacept or α-Gal-HSA with α-Gal IgE binding to cetuximab-sepharose. Pooled serum of four α-Gal IgE-positive patients was used to compete with and set as 100% binding. Only cetuximab and α-Gal-HSA competed for IgE binding to cetuximab-sepharose. No competition was observed for the other tested therapeutic mAbs. (**c**) Inhibition of α-Gal IgE binding by cetuximab, cetuximab $F(ab')_2$, cetuximab Fab, cetuximab Fc, infliximab and infliximab Fc. No competition was observed for cetuximab Fc fragments, infliximab or infliximab Fc fragments, whereas a strong reduction in binding was observed for cetuximab, cetuximab $F(ab')_2$ and cetuximab Fab. The amount of $F(ab')_2$, Fab or Fc fragments used is plotted equivalent to their proportions in intact IgG (e.g., 1 μg/ml IgG ≅ 0.67 μg/ml Fab fragments). (**d**) The interaction of infliximab (0.2 mg/ml, 1 mg/ml and 5 mg/ml), cetuximab (0.02 mg/ml, 0.1 mg/ml and 0.5 mg/ml) or proteinase K–digested fragments thereof with α-Gal IgE measured using an IgE inhibition-RIA. Micromolar (IgG-bound)-α-Gal is plotted on the *x* axis. Linear galactose-α-1,3-galactose-β-1,4-GlcNac trisaccharides were tested as a comparison to mono-α-galactosylated complex type glycans. A notable difference in IC_{50} was observed between cetuximab, proteinase K–digested cetuximab and proteinase K–digested infliximab/or α-Gal trisaccharide.

mono-α-galactosylated glycans located in the Fc domain. To specifically assess α-Gal IgE binding to mono-α-galactosylated glycans, we performed the IgE inhibition-RIA in the presence of a linear α-Gal polysaccharide (galactose-α1,3-galactose-β1,4-GlcNac). This polysaccharide is the equivalent of the three terminal monosaccharide residues present on each of the two antennas of a typical complex α-Gal glycan, and likely represents a substantial part of the epitope recognized. Notably, we demonstrated that α-Gal IgE possessed a similar affinity for this isolated trisaccharide compared with the mono-α-galactosylated glycans on proteinase K–digested infliximab (IC_{50} 6.4 μM α-Gal). This observation supports the notion that mono-α-galactosylated glycans only partially define the dominant epitope recognized by α-Gal IgE antibodies.

In conclusion, of the six intact mAbs tested, only cetuximab binds α-Gal–specific IgE antibodies as present in the serum of red-meat allergy patients; this binding is restricted to α-Gal moieties in its Fab domains. Notably, neither cetuximab nor infliximab Fc domains bound such IgE antibodies, even though they contained detectable α-Gal moieties. This absence of binding may be explained by the three following observations. First, α-Gal in the Fc domain is protected by shielding as indicated by the emergence of binding after glycan exposure by means of proteolytic degradation of the amino acid back bone. Second, we observed an increased affinity of α-Gal IgE antibodies for bi α-galactosylated biantennary complex glycans, present in the cetuximab Fab domain, compared with mono-α-galactosylated glycans detected in Fc domains. Finally, the presence of α-galactosylated glycans in both Fab arms of cetuximab appear to provide a more avid binding surface for IgE, potentially by allowing bivalent IgE binding. Taken together, our data suggest that therapeutic mAbs that are only glycosylated in their Fc domains and produced in rodent cell lines do not bind α-Gal–specific IgE antibodies. Further research is warranted to address whether this correlates to the absence of α-Gal IgE-induced anaphylactic reactions in patients.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturebiotechnology.com.

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Supplementary information for manuscript

IgE antibodies against galactose-α-1,3-galactose from allergic patients do not bind α-galactosylated glycans attached to the Fc domain of intact therapeutic mAbs

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Supplementary Tables and Figures

Supplementary Table 1

Studied antibodies and their production system and glycosylation sites.

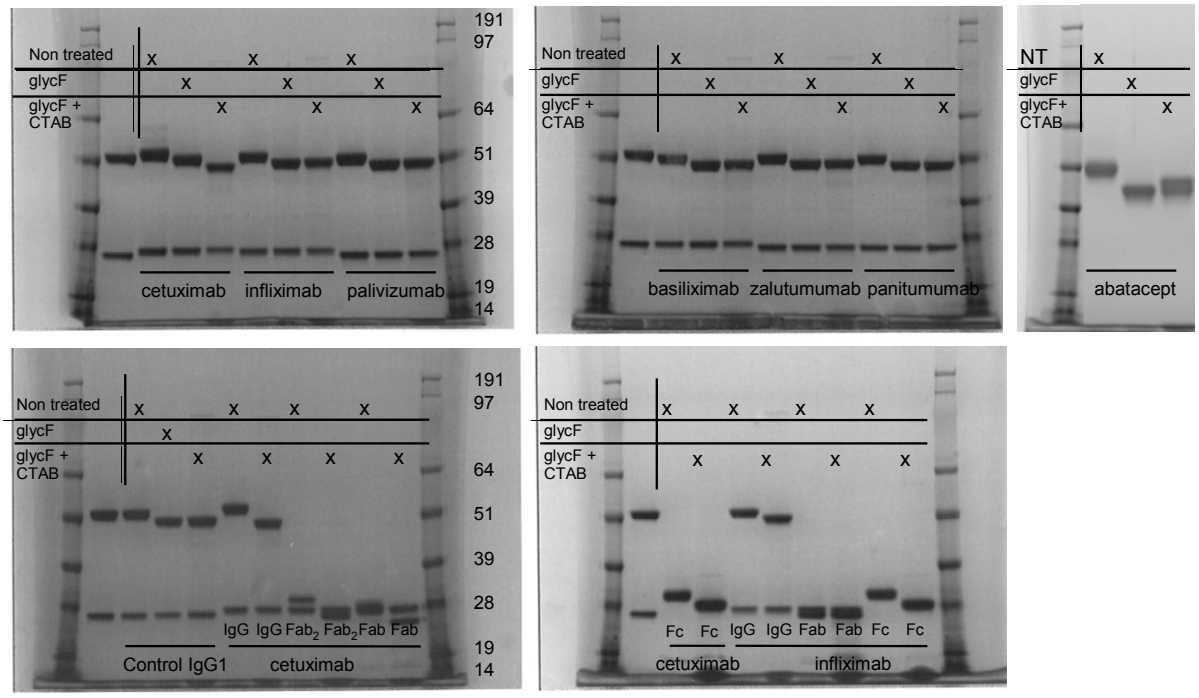
Trade name	Generic	Company	Target	Format/ Subclass	Therapeutic category	Glycosylation site	approval	Production cell line
Erbitux	cetuximab	ImClone/ Merck	EGFR	Chimeric IgG1κ	Oncological	Fc + HC Fab	US/EU	SP2/0
Remicade	infliximab	Centocor	TNF-alpha	Chimeric IgG1κ	Immunological	Fc	US/EU	
Simulect	basiliximab	Novartis	CD25	Chimeric IgG1κ	Immunological	Fc	US/EU	NS0
Synagis	palivizumab	MedImmune	Respiratory syncytial virus	Humanized IgG1κ	Anti-infective	Fc	US/EU	
Vectibix	panitumumab	Amgen	EGFR	Human IgG2	Oncological	Fc	US/EU	CHO
HuMax-EGFr	zalutumumab	Genmab	EGFR	Human IgG1κ	Oncological	Fc	Phase III	
Orencia	abatacept	Bristol-Myers Squibb	CD80/CD86	Fusion CTLA-4 human IgG1 Fc	Immunological	Fc + CTLA-4 domain	US/EU	

HC: heavy chain

Supplementary Table 2

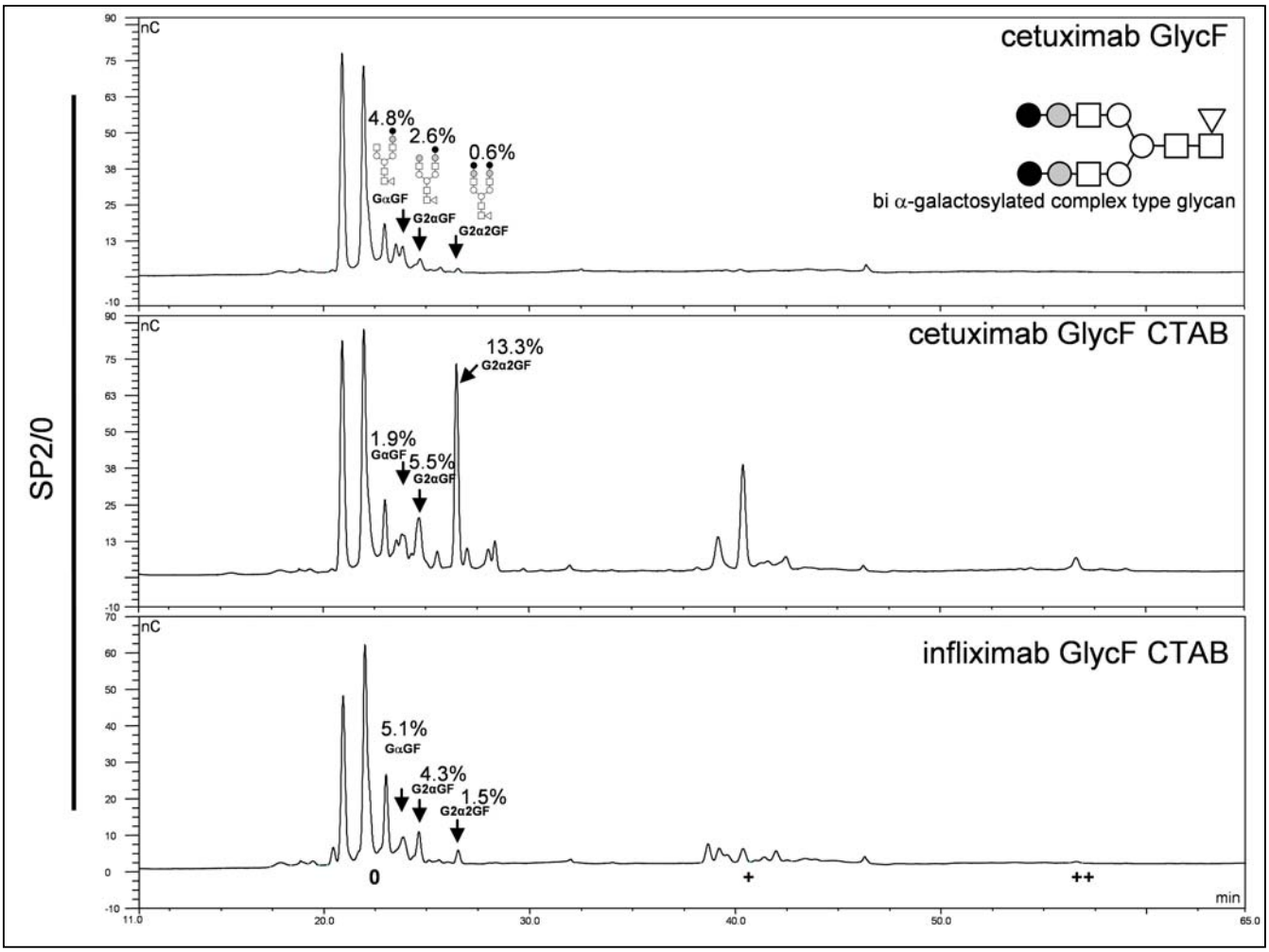
Characteristics of meat allergy patients positive for galactose-α-1,3-galactose IgE.

Variable		Case subjects N = 20
Age (yr)		
	median	50
	range	13-77
Sex		
	Male: female ratio	8:12
	Male (%)	40
Race or ethnic group (no.)		
	white	19
	black	0
	Other	1
total IgE titer (G.M.)		113 (63-202)

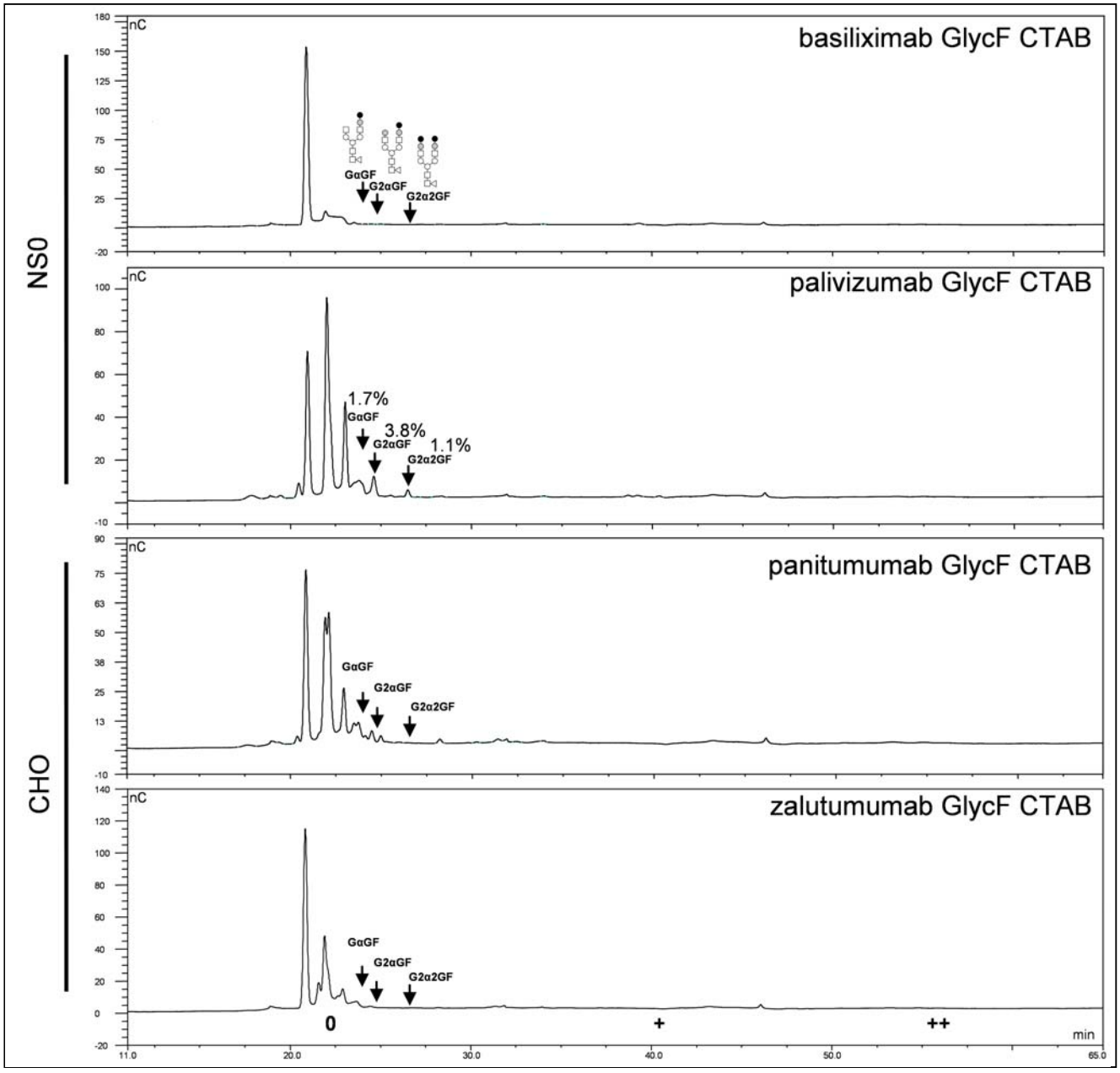


Supplementary Figure 1. Reduced SDS-PAGE analyses of samples used for glycan analyses by HPAEC-PAD. Antibodies or fragments thereof were enzymatically treated to release the glycan moieties from the amino acid backbone. Samples were treated with N glycanase F (64 hr, 37ºC) or with CTAB detergent, boiled at 95ºC and N glycanase F. Samples were loaded under reducing conditions on a 4-15% acrylamide gel. N glycanase F predominantly released glycan structures in the antibody Fc domain. N Glycanase F combined with CTAB treatment released all N-linked antibody glycans. For abatacept, which has a lower pI than the other therapeutics, CTAB treatment slightly interfered with migration into the gel presumably due to dissociation of SDS. (Bash J. et al., Biochimica et Biophysica Acta 577(1), 1979: 125-131). The masses (KDa) of the molecular weight marker are indicated in the upper and lower left panel.

Supplementary Figure 2

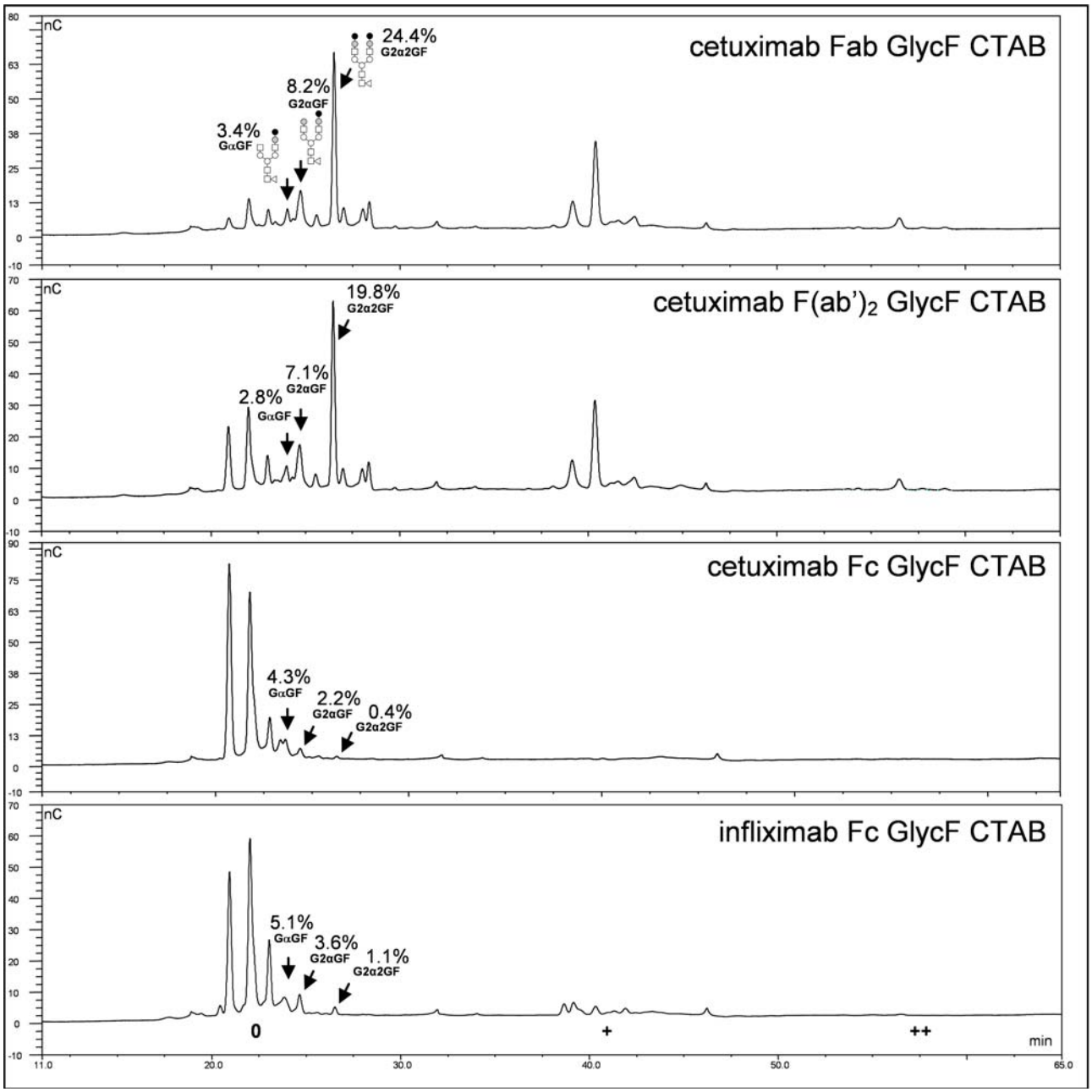


Supplementary Figure 2 continued



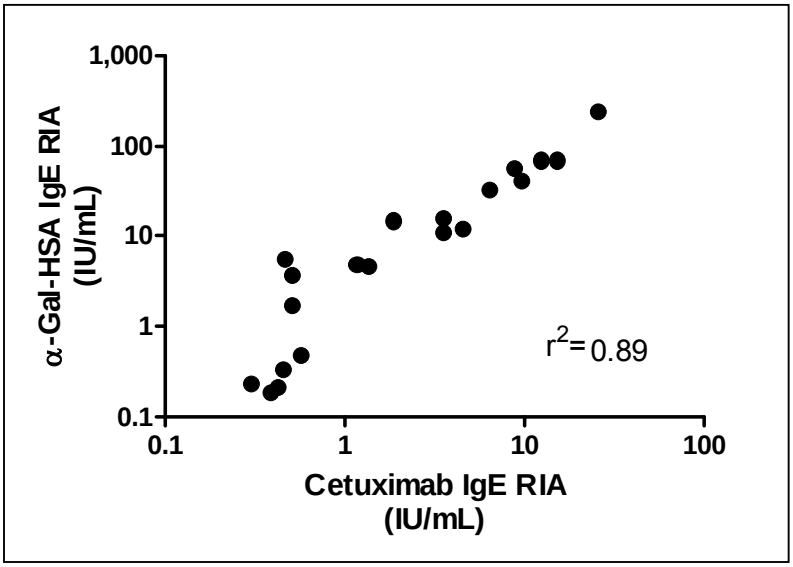
Supplementary Figure 2. Analysis of the N-linked glycosylation profile of cetuximab, infliximab, basiliximab, palivizumab, panitumumab and zalutumumab. N-linked glycans of therapeutic mAb were enzymatically released using N glycanase F or N glycanase F + CTAB treatment and analyzed by HPAEC-PAD. The retention time (min) is indicated on the abscissa, the detector response (nC) is indicated on the ordinate. Peaks eluting at (+) and (++) represent mono-sialylated and di-sialylated variants of neutral glycans eluting at (0). Explanation of symbols: squares, *N*-acetylglucosamine; open circles, mannose; gray circles, β1,4-galactose; black circle, α1,3-galactose; triangle, α1,6-fucose.

Supplementary Figure 3



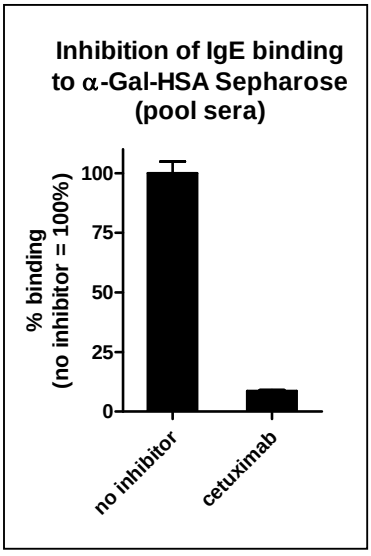
Supplementary Figure 3. Glycan profile of cetuximab Fab, F(ab')2, Fc and infliximab Fc. N-linked glycans of cetuximab Fc, Fab and F(ab')2 and infliximab Fc were enzymatically released using glycanase F + CTAB treatment and analyzed by HPAEC-PAD. The retention time (min) is indicated on the abscissa, the detector response (nC) is indicated on the ordinate. Peaks eluting at (+) and (++) represent mono-sialylated and di-sialylated variants of neutral glycans eluting at (0).

Supplementary Figure 4



Supplementary Figure 4. Correlation of IgE binding to cetuximab and α-Gal-HSA. Binding of meat allergy patient serum IgE to cetuximab plotted against IgE binding to α-Gal-HSA. There is a good correlation between IgE to cetuximab and IgE binding to α-Gal-HSA.

Supplementary Figure 5



Supplementary Figure 5. The interaction of cetuximab (2 mg/test) with α-Gal specific IgE measured using a α-Gal-HSA-sepharose RIA. Cetuximab competed for IgE binding to α-Gal-HSA-sepharose.

Supplementary information

Methods

Antibodies and reagents

The therapeutic antibodies cetuximab (ImClone/Merck), palivizumab (MedImmune), infliximab (Centocor), basiliximab (Novartis), panitumumab (Amgen) and abatacept (Bristol-Myers Squibb) were obtained commercially. Zalutumumab (Genmab) The reagents Galα1-3Galβ1-4GlcNAc-HSA (α-Gal-HSA, product nr. NGP2334) and Galα1-3Galβ1-4GlcNAc trisaccharide (product nr. GN334) were purchased from Dextra LTD, Reading, UK.

IgE RIA

Serum IgE to cetuximab was measured by a radioimmunoassay (RIA) using cetuximab coupled to Sepharose and detected by radiolabeled anti-IgE. 2 mg of cetuximab was coupled to 100 mg of CNBr-activated Sepharose (Amersham Biosciences, Uppsala, Sweden). Ten microliters of serum was incubated with 0.5 mg cetuximab-Sepharose in a total volume of 1 ml of PBS-AT (PBS, pH 7.4, supplemented with 0.3% bovine serum albumin, 0.1% Tween-20, 10 mM EDTA and 0.05% (w/v) NaN_3) and incubated overnight on a vertical rotor. After washing five times with PBS-T (PBS supplemented with 0.1% Tween-20 and 0.05% (w/v) NaN_3), ^{125}I-labeled anti-IgE was added and after overnight incubation and washing, radioactivity was measured. The results were expressed as arbitrary units as compared with known standards. IgE to palivizumab, zalutumumab, and α-Gal-HSA were measured analogously, except that α-Gal-HSA was coupled at 1 mg/100 mg Sepharose.

IgE inhibition-RIA

A serum pool (10 µl) was incubated with cetuximab-Sepharose in the presence of different inhibitors, including cetuximab and fragments thereof. Serum pool and inhibitor were mixed and incubated for 1 hour before Sepharose was added and incubated as described above in a constant total volume. Detection of IgE was carried out as described above. Binding is expressed as relative to binding with no inhibitor present. It was also tested whether cetuximab could inhibit all of the IgE binding to α-Gal-HSA. Ten µl of serum pool was incubated overnight with 0.25 mg of

α-Gal-HSA-Sepharose in the presence of cetuximab (2 mg), and IgE was detected similarly.

Digestion of infliximab and cetuximab with pepsin, papain, and proteinase K

$F(ab')_2$ fragments of cetuximab were generated by digestion of 8 mg of cetuximab with 80 µg of pepsin at pH 3.5 in 0.1 M sodium citrate buffer for 16 hours at 37 ^{o}C. After neutralization with 1 M Tris and dialysis to PBS, traces of undigested antibody were removed by protein A affinity chromatography.

Fab and Fc fragments of cetuximab and infliximab were prepared by digesting 8 mg of antibody with 80 µg of DTT-preactivated papain for 3 hours at 37 ^{o}C. Fab and Fc fragments were separated from traces of undigested material by size exclusion chromatograpy (Superdex 16/10 High-load column, GE Healthcare) and Fab was separated from Fc using a MonoQ column (10 mM Tris pH 8, linear gradient from 0 – 0.35 mM NaCl).

Proteinase K digestion of infliximab and cetuximab was carried out by overnight incubation at 50 ^{o}C (1:100 w/w), and proteinase K was inactivated by brief incubation at 80 ^{o}C.

Analysis of antibody attached glycan structures

N-linked glycans were released by two methods. 200 µg IgG, 110 µg Fab/$F(ab')_2$ fragments or 55 µg Fc fragments were used for analyses. 1) The samples were buffer exchanged to 20 mM sodium phosphate buffer, pH 7.2 (NaPi) using Amicon Ultra-0.5 Centrifugal Filter Devices (Millipore, Billerica, MA, USA) and digested with 50 mU/mL N-glycanase (Glyko, Hayward, CA, USA) for 16 hours at 37 ºC. This procedure predominantly released N-linked glycans located in the IgG Fc domain attached to Asn^{297}. 2) The samples were buffer exchanged to NaPi buffer, 10% (w/v) Hexadecyltrimethylammonium bromide (CTAB, Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and heated for 5 min at 95 ºC in a heating block. Next, the samples were cooled to 37 ºC and digested for 64 h with N-glycanase enzyme. This procedure also released N-linked glycans outside the Fc domain.

N-Linked glycosylation profiling by High-pH Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD).

Samples were centrifuged at 13,000 rpm for 10 sec before analysis by HPAEC-PAD. HPAEC-PAD analysis was performed on a 2 mm internal diameter CarboPac PA1

column (Dionex, Sunnyvale, CA) with CarboPac PA100 guard, using a linear elution gradient of 0-175 mM sodium acetate in 150 mM sodium hydroxide at a flow rate of 1 ml/min. PAD occurred with the quadropulse and peaks were integrated with Chromeleon Software (Dionex, Sunnyvale, CA). For each profile, 70 µg digest was loaded.

Characterization of the HPAEC-PAD profile was done as follows. First, MALDI-TOF was used to determine which glycans were present in the released glycan pool as described previously (Peipp M, Blood 2008 Sep 15;112(6):2390-9). Peak assignment to glycan structures was done using GlycoMod software (www.expasy.ch/tools/glycomod). Next, glycan structures were assigned to peaks in the HPAEC-PAD profile on the basis of comparison to the elution of glycans with known structure and to elution of released glycans incubated with exoglycosidases. Specifically, glycans containing α-Gal moieties were assigned on the basis of sensistivity to α(1,3)-galactosidase and α(1,4)-galactosidase or *N*-acetylhexosaminidase. The GαGF, G2αGF, and G2αG2F peaks areas obtained from the chromatograms were used to calculate the moles of terminal α-linked Gal per mole protein.

SDS-PAGE

All test items were analyzed on SDS-PAGE (4–12% Bis-Tris; Invitrogen) under nonreducing conditions at neutral pH according to the manufacturer's instructions. The gels were stained with Coomassie (Invitrogen) and digitally imaged using the GeneGenius (Synoptics).