Supplementary Information

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A Non-human Sialic Acid on Recombinant Glycosylated Biotherapeutic Products: Implications for Immunogenicity and Efficacy, and a Proposed Solution

Darius Ghaderi, Rachel E. Taylor, Vered Padler-Karavani,

Sandra Diaz, and Ajit Varki.

Supplementary Table 1. Sialic acids on Cetuximab and Panitumumab

Antibody	Total sialic acid (mol/mol antibody)	% Neu5Gc	Neu5Gc Content (mol/mol antibody).
Cetuximab	1.84	95.5	1.77
Panitumumab	0.22	<0.1	<0.1
Murine IgG	0.50	>99.9	0.50

Bound sialic acids were released by mild acid, derivatized with 1,2-diamino-4,5-methyl dioxybenzene (DMB), and analyzed by HPLC as described in Methods.

Supplementary Table 2. Neu5Gc Content of Some Biotherapeutic Agents

Therapeutic Agent	Company	Cell line*	Neu5Gc content (mol/mol)**
Alemtuzumab Campath® Mabcampath®	Genzyme Corp.	CHO	0.001
Bevacizumab Avastin®	Genentech Inc.	CHO	0.001
Cetuximab Erbitux®	Merck & Co., Inc. ImClone Systems Inc.	Sp2/0	1.77
Daclizumab Zenapax®	F. Hoffmann-La Roche Ltd.	Sp2/0	0.081
Erythropoietin Procrit®	Amgen	CHO	0.014
Panitumumab Vectibix®,	Amgen	CHO	<0.001
Rituximab Rituxan® Mabthera®	Genentech Inc	CHO	0.01
Trastuzumab Herceptin®,	F. Hoffmann-La Roche Ltd. Genentech Inc. (US)	CHO	0.005

Information regarding therapeutic agents, companies and cell lines were obtained by searching publicly accessible and/or official web sites

* CHO, Chinese Hamster Ovary cells; Sp2/0, murine myeloma cells.

**Samples were subjected to base treatment with 0.1 M NaOH (final) at 37˚C for 30 min to remove any O-acetyl esters, and the sialic acids were then released by acid hydrolysis with 2 M acetic acid (final), at 80˚C for 3 hrs. Samples were filtered, derivatized with 1,2-diamino-4,5-methyl dioxybenzene (DMB), and analyzed by HPLC. Measured values of non acid-treated controls (free sialic acids) were subtracted.

Supplementary Table 3. Overview of FDA-approved Biotherapeutic Agents, Likely Glycosylation Status, and Sources

		Source (Number of products)								
Type of Agent	**Glycosylated (%)**	***E. Coli****	**Yeast**	**CHO**	**Hybridoma /Myeloma**	**BHK**	**HEK293/ HT-1080**	**Animal Serum**	**Milk**	**Others**
Monoclonal Antibodies	92% (24/26)	2		11	10					Murine ascites
Fc-Fusion Proteins	80 % (4/5)	1		4						
Hormones	33 % (7/21)	7	3	6						Pig, Murine cell line (mouse C127)
Cytokines	26 % (5/19)	14		5						
Clotting Factors	100 % (8/8)			4		3	1			
Enzymes	81 % (13/16)	2*	1	9			1			Sheep, Bovine, Pig
Anti-Sera	100 % (4/4)							4		
Enzyme inhibitor	100 % (1/1)								1	

Information regarding common FDA-approved biotherapeutic agents and sources was obtained by searching publicly accessible web sites only, as of April 2010. The list is not meant to be comprehensive nor can its accuracy be guaranteed, and it was added at the explicit request of the reviewers and editors.

HEK293, Human Embryonic Kidney cells; HT-1080, human fibrosarcoma cells; CHO, Chinese Hamster Ovary cells; BHK, Baby Hamster Kidney cells; Myeloma/Hybridoma, murine myeloma or hybridoma cells.

***Xiaflex®** Collagenase is produced in *Clostridium histolyticum.*

Although there are no published studies of the sialic acid types of most of the products listed, it is reasonable to predict the following ascending order of *relative* Neu5Gc content, based on cell-type or source: HEK293 < CHO < BHK < Animal sources < Myeloma/Hybridoma. The *absolute* Neu5Gc content will also depend on the extent of glycosylation and sialylation of a given biotherapeutic agent. Biotherapeutics produced in *E. coli, Clostridium histolyticum* and yeast should not carry sialic acids.

Supplementary Table 4. Details of Examples of FDA-approved Biotherapeutic Agents, Cell Sources for Production and Likely Glycosylation Status of the Agents

Agent	Marketing Company	Glycoprotein (Yes/No)	Production Source*
Monoclonal Antibodies			
Actemra® Tocilizumab	Genentech Inc., Hoffmann-La Roche Ltd.	Yes	CHO cells
Avastin® Bevacizumab	Genentech Inc., Hoffmann-La Roche Ltd.	Yes	CHO cells
Campath® (US), Mabcampath® (EU) Alemtuzumab	Genzyme Corp.	Yes	CHO cells
Herceptin® Trastuzumab	F. Hoffmann-La Roche Ltd, Genentech Inc.	Yes	CHO cells
Humira® Adalimumab	Abbott Laboratories	Yes	CHO cells
Rituxan® Rituximab	Genentech Inc, Biogen Idec	Yes	CHO cells
Simponi® Golimumab	Centocor Ortho Biotech Inc., Merck & Co	Yes	CHO cells
Stelara™ Ustekinumab	Centocor Ortho Biotech Inc.	Yes	CHO cells
Vectibix® Panitumumab	Amgen	Yes	CHO cells
Xolair® Omalizumab	Genentech Inc., Novartis Pharmaceuticals Corp. Tanox Inc.	Yes	CHO cells
Zevalin® Ibritumomab tiuxetan	Biogen Idec., Bayer Schering Pharma AG	Yes	CHO cells
Cimzia® Certolizumab pegol	UCB	No (PEGylated)	*E. coli*
Lucentis® Ranibizumab)	Genentech Inc., Novartis Pharmaceuticals Corp.	No	*E. coli*
Bexxar® Tositumomab-I131	GlaxoSmithKline	Yes	Hybridoma, mammalian
Orthoclone Okt3® Muromonab-CD3	Centocor Ortho Biotech Inc.	Yes	Murine ascites, hybridoma
Soliris®	Alexion	Yes	Murine myeloma

Eculizumab	Pharmaceuticals, Inc		cell line
Ilaris® Canakinumab	Novartis Pharmaceuticals Corp.	Yes	Murine Sp2/0-Ag14 fused hybridoma cell line
Mylotarg® Gemtuzumab ozogamicin	Wyeth Pharmaceuticals	Yes	NS0 mouse myeloma cells
Arzerra® Ofatumumab	GlaxoSmithKline	Yes	NSO mouse myeloma cells
Synagis® Palivizumab	Abbott Laboratories, MedImmune Inc.	Yes	NSO mouse myeloma cells
Tysabri® Natalizumab	Élan Pharmaceuticals, Biogen Idec.	Yes	NSO mouse myeloma cells
Erbitux® Cetuximab	ImClone Systems Merck & Co., Inc., Bristol-Myers Squibb	Yes	Sp2/0 mouse myeloma cells
Remicade® Infliximab	Centocor Ortho Biotech Inc.	Yes	Sp2/0 mouse myeloma cells
Reopro® Abciximab	Centocor Ortho Biotech Inc., Eli Lilly & Co.	Yes	Sp2/0 mouse myeloma cells
Simulect® Basiliximab	Novartis Pharmaceuticals Corp.	Yes	Sp2/0 mouse myeloma cells
Zenapax® Daclizumab	F. Hoffmann-La Roche Ltd., PDL (Protein Design Labs) BioPharma	Yes	Sp2/0 mouse myeloma cells
Fc-Fusion Proteins			
Amevive® Alefacept	Astellas Pharma Inc.	Yes	CHO cells
Arcalyst® Rilonacept	Regeneron Pharmaceuticals Inc.	Yes	CHO cells
Enbrel® Etanercept	Amgen, Wyeth Pharmaceutical	Yes	CHO cells
Orencia® Abatacept	Bristol-Myers-Squibb	Yes	CHO cells
Nplate® Romiplostim	Amgen	No	*E. coli*
Hormones			
Follistim® Follitropin beta	Merck & Co	Yes	CHO cells
Gonal-F® Follitropin alfa	EMD Serono, Inc.	Yes	CHO cells
Luveris®	EMD Serono, Inc.	Yes	CHO cells

Luteinizing hormone			
OP-1 Putty Osteogenic Protein-1 (BMP-7)	Stryker Biotech	Yes	CHO cells
Ovidrel® Choriogonadotropin α	EMD Serono, Inc.	Yes	CHO cells
Thyrogen® Thyrotropin alfa	Genzyme Corp	Yes	CHO cells
Byetta®	Eli Lilly & Co., Amylin	No	*E. coli*
Genotropin® Somatropin	Pfizer, Inc.	No	*E. coli*
Humalog®	Eli Lilly & Co.	No	*E. coli*
Humanotrope® Somatropin	Eli Lilly $ Co	No	*E. coli*
Humatrope® Somatropin	Eli Lilly & Co.	No	*E. coli*
Humulin®	Eli Lilly & Co.	No	*E. coli*
Kepivance® (palifermin) keratinocyte growth factor	Biovitrum AB	No	*E. coli*
Lantus®	Sanofi Aventis Pharmaceuticals, Inc.	No	*E. coli*
Norditropin® Somatropin	Novo Nordisk Pharmaceuticals, Inc.	No	*E. coli*
Nutropin® Somatropin	Genentech Inc	No	*E. coli*
Omnitrope® Somatropin	Novartis Pharmaceuticals Corp.	No	*E. coli*
Serostim®, Saizen®, Zorbtive™ Somatropin	EMD Serono, Inc.	Yes	Murine cell line (mouse C127)
Emdogain® tooth enamel proteins	Staumann USA	Yes	Pig
Novolin® and analogs	Novo Nordisk Pharmaceuticals, Inc.	No	*Saccharomyces cerevisiae* (yeast)
Novolog®, NovoRapid®	Novo Nordisk Pharmaceuticals, Inc.	No	*Saccharomyces cerevisiae* (yeast)
Regranex® Platelet-derived growth factor (PDGF)-BB	Johnson & Johnson Co.	No	*Saccharomyces cerevisiae* (yeast)
Cytokines			
Aranesp® Darbepoetin alfa	Amgen	Yes	CHO cells
Avonex®	Biogen Idec, Inc.	Yes	CHO cells

Interferon beta-1a			
NeoRecormon® Epoetin beta	Hoffmann-La Roche Ltd.	Yes	CHO cells
Procrit® , **Epogen®** Epoetin alfa	Amgen, Centocor Ortho Biotech Inc.	Yes	CHO cells
Rebif® Interferon beta-1a	Pfizer, Inc., EMD Serono, Inc.	Yes	CHO cells
Actimmune® Interferon gamma-1b	Intermune Inc.	No	*E. coli*
Betaseron® Interferon beta-1b	Bayer HealthCare Pharmaceuticals	No	*E. coli*
Extavia® Interferon beta-1b	Novartis Pharmaceuticals Corp.	No	*E. coli*
Infergen® Interferon alfacon-1	Three Rivers Pharmaceuticals	No	*E. coli*
Intron® A Interferon alfa-2b	Merck & Co	No	*E. coli*
Kineret® (anakinra) interleukin-1 receptor antagonist (IL-1Ra)	Biovitrum AB	No	*E. coli*
Neulasta® Pegfilgrastim	Amgen	No (PEGylated)	*E. coli*
Neumega® Des-Pro Interleukin-11	Wyeth Pharmaceuticals	No	*E. coli*
Neupogen® Recombinant G-CSF	Amgen, Hoffmann-La Roche Ltd.	No	*E. coli*
Ontak® (denileukin diftitox) IL-2/diphtheria toxin fusion protein	Eisai Co., Ltd.	No	*E. coli*
Pegasys® Peginterferon alfa-2a	Hoffmann-La Roche Ltd.	No (PEGylated)	*E. coli*
Pegintron® Peginterferon alfa-2b	Merck & Co	No (PEGylated)	*E. coli*
Proleukin® Aldesleukin (IL-2)	Novartis Pharmaceuticals Corp.	No	*E. coli*
Roferon A® Interferon alfa-2a	Hoffmann-La Roche Ltd.	No	*E. coli*
Clotting Factors			
Helixate FS Coagulation factor VIII	ZLB Behring	Yes	BHK cells
Kogenate FS Coagulation factor VIII	Bayer Schering Corp.	Yes	BHK cells
NovoSeven®,	Novo Nordisk	Yes	BHK cells

Coagulation Factor VIIa			
Advate® Antihemophilic factor	Baxter International Inc.	Yes	CHO cells
BeneFIX® Coagulation Factor IX	Wyeth Pharmaceuticals	Yes	CHO cells
ReFacto® Antihemophilic Factor	Wyeth Pharmaceuticals	Yes	CHO cells
Xyntha® Coagulation factor VIII	Wyeth Pharmaceuticals	Yes	CHO cells
Xigris® Drotrecogin alfa (Activated Protein C)	Eli Lilly & Co.	Yes	HEK293
Enzyme Inhibitor			
ATryn® (Antithrombin/ATIII)	GTC Biotherapeutics	Yes	Goat milk
Enzymes			
Amphadase® Hyaluronidase (bovine)	Amphastar Pharmaceuticals	Yes	Bovine
Activase®, Cathflo Activase®, Actilyse® Alteplase	Genentech Inc, Boehringer Ingelheim Pharma KG	Yes	CHO cells
Aldurazyme® Laronidase	Genzyme Corp	Yes	CHO cells
Cerezyme® (Imiglucerase)	Genzyme Corp.	Yes	CHO cells
Fabrazyme® agalsidase-β	Genzyme Corp	Yes	CHO cells
Hylenex®, Cumulase® Hyaluronidase	MediCult A/S, MidAtlantic Diagnostics, Inc., Halozyme Baxter Healthcare	Yes	CHO cells
Myozyme® Alglucosidase alfa	Genzyme Corp	Yes	CHO cells
Naglazyme® N-acetylgalactosamine 4-sulfatase	BioMarin Pharmaceutical Inc.	Yes	CHO cells
Pulmozyme® Human DNase	Genentech Inc, Hoffmann-La Roche Ltd.	Yes	CHO cells
TNKase® Tenecteplase	Genentech Inc	Yes	CHO cells
Xiaflex®	Auxilium	No	*Clostridium*

Collagenase	Pharmaceuticals Inc.		*Histolyticum*
Fortical® Calcitonin (salmon)	Upsher Smith Laboratories	No	*E. coli*
Elaprase® Idursulfase	Shire Pharmaceuticals	Yes	human cell line (HT-1080)
Pancreaze® Pancrelipase	Ortho McNeil Janssen	Yes	Pig
Elitek® Rasburicase	Sanofi Aventis Pharmaceuticals, Inc.	No	*Saccharomyces cerevisiae* (yeast)
Vitrase® Hyaluronidase (ovine)	ISTA Pharmaceuticals	Yes	Sheep
Antisera			
Atgam® Anti-thymocyte globulin	Pfizer, Inc.	Yes	Equine Serum
Thymoglobulin® Anti-thymocyte globulin	Genzyme Corp.	Yes	Rabbit Serum
CroFab® [Crotalidae Polyvalent Immune Fab (Ovine)]	Savage Laboratories	Yes	Sheep Serum
DigiFab® Digoxin Immune Fab (Ovine)	Savage Laboratories	Yes	Sheep Serum

Details of information used to generate Supplementary Table 3, with more information regarding common FDA-approved biotherapeutic agents. Information and sources obtained by searching publicly accessible web sites only, as of April 2010. The list is not meant to be comprehensive nor can its accuracy be guaranteed, and it was added at the explicit request of the reviewers and editors.

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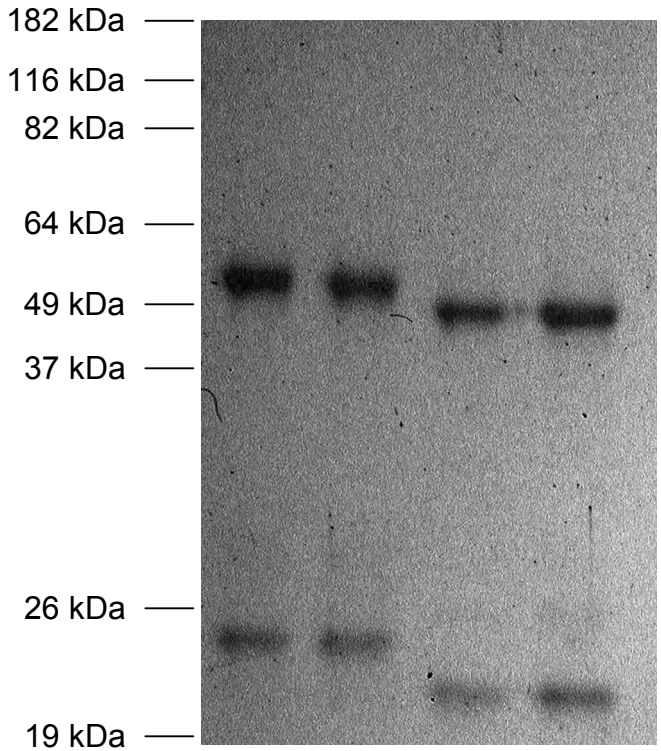
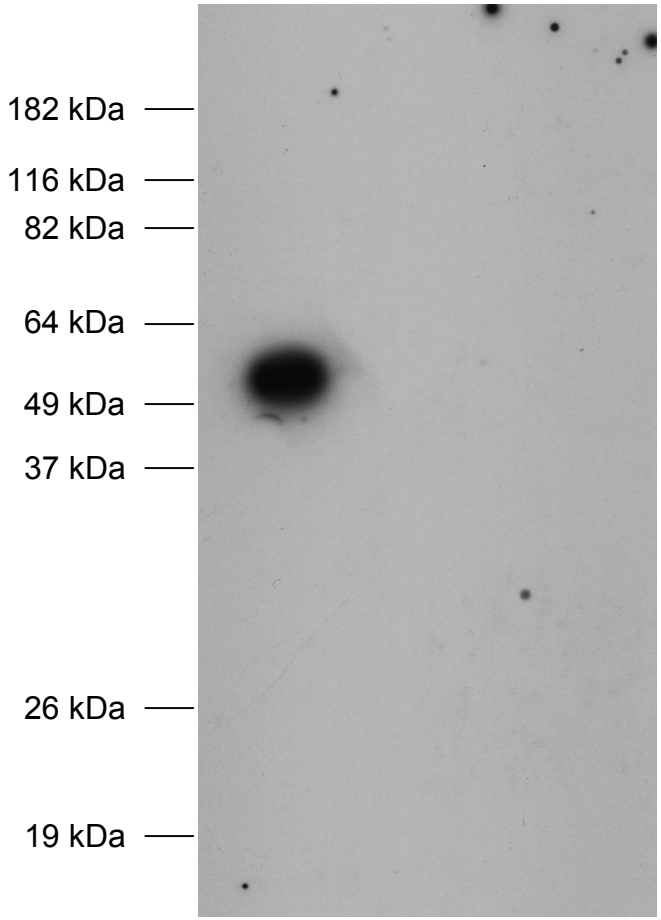
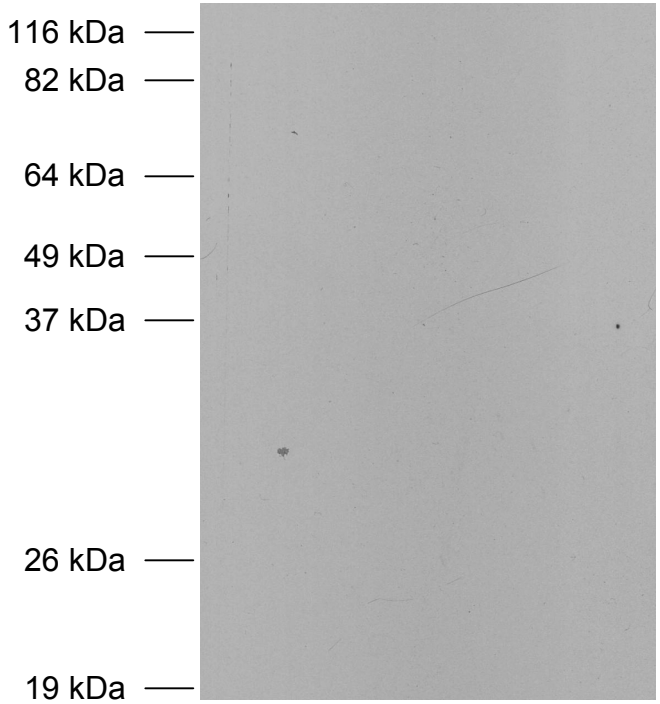
Supplementary Figure 1: SDS-PAGE and Coomassie Staining of Biotherapeutic Antibodies. 1 μg of sialidase treated or untreated Cetuximab (Cet) and Panitumumab (Pan) were separated by SDS-PAGE and Coomassie stained, see Figure 1B, upper panel. The full gel is shown here. The lower bands are light chains.



Supplementary Figure 2: Western-Blot Detection of Neu5Gc on Biotherapeutic Antibodies by Anti-Neu5Gc IgY Antibodies. 1 μg of sialidase treated or untreated Cetuximab (Cet) and Panitumumab (Pan) were separated by SDS-PAGE and blotted. Neu5Gc was detected using an affinity-purified chicken anti-Neu5Gc IgY. For more details, see Figure 1B. Middle Panel. The full gel is shown here.



Supplementary Figure 3: Western-Blot Detection of Neu5Gc on Biotherapeutic Antibodies by Anti-Neu5Gc IgY Antibodies. 1 μg of sialidase treated or untreated Cetuximab (Cet) and Panitumumab (Pan) were separated by SDS-PAGE and blotted. As a control, non-specific IgY was used. For more details, see Figure 1B, lower panel. Full gel is shown here.



Supplementary Figure 4: Western-Blot Detection of Neu5Gc on Biotherapeutic Antibodies by IgG Antibodies from Normal Human Serum. 1 μg of sialidase treated or untreated Cetuximab (Cet) and Panitumumab (Pan) were separated by SDS-PAGE and blotted. Neu5Gc was detected using biotinylated human anti-Neu5Gc IgG. For more details, see Figure 1E. Full gel is shown here.