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Analysis of lysine clipping of a humanized Lewis-Y specific IgG antibody and its relation to Fc-mediated effector function

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Abstract

During the analytical characterization of the humanized Lewis-Y specific monoclonal antibody IGN311 (IgG1/κ) used for passive anti-cancer therapy in humans, isoelectric focusing (IEF) experiments revealed that IGN311 batches produced in serum-containing and serum-free medium, respectively, displayed different banding patterns. The additional bands in the IEF pattern correlated with additional peaks observed by subsequent cation exchange (CEX)-HPLC analysis. Since the IEF pattern is one of the specification criteria in the quality control of monoclonal antibodies and a non-matching pattern may be indicative for lot-to-lot inconsistency, this phenomenon was investigated in detail. First, we investigated whether a difference in antibody glycosylation was the cause for the observed charge heterogeneity. De-*N*-glycosylation experiments demonstrated that charge heterogeneity observed in the IEF pattern is not a consequence of glycosylation. In contrast, sample treatment by carboxypeptidase B, removing the carboxy-terminal lysine residues from the two heavy chains of the antibody, resulted in reduced charge heterogeneity eliminating the two most basic bands observed in IEF. These data were supported by reversed phase HPLC–MALDI-TOF-MS analysis of enzymatically cleaved peptides of the antibody as well as by carboxy-terminal sequencing of the heavy chains. It was demonstrated that the differences in the IEF banding pattern were due to lysine clipping occurring during the production of the antibody. The antibody batch produced under serum-free conditions was less affected by lysine clipping. Both antibody variants – clipped and unclipped – elicited the same potency in a complement dependent cytotoxicity (CDC) assay demonstrating that lysine clipping of IGN311 does not impair Fc-mediated effector functions.

Keywords: IEF; mAb; Charge heterogeneity; Lewis-Y; Lysine clipping

1. Introduction

Monoclonal antibodies (mAbs) intended for therapeutic use require a comprehensive characterization of their structural integrity, purity and stability [1]. Upon mAb manufacturing, molecular alterations of the product can occur during fermentation, purification, formulation and storage [2]. Since alterations in the product most likely affect its biological profile and are therefore of regulatory concern, analytical methods have to be in place which are capable of identifying such modifications as well as assessing the impact on the biological/therapeutic Ab function(s) [3].

The IEF pattern of an Ab represents the 'fingerprint' of the charge heterogeneity within the preparation and is therefore used as a criterion for batch-to-batch comparison and as critical quality parameter in stability studies [4]. Charge microheterogeneity in immunoglobulins can result from different *N*-glycosylation (e.g. varying amounts of charged sugars like sialic acid) or from other post-translational modifications. Such modifications may comprise of amino acid modifications in the mAb binding region including deamidation and/or oxidation processes, aggregation, disulphide bond fidelity and hinge region fragmentation [5]. Additionally, amino-terminal glutamic

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acid dehydration and C-terminal lysine heterogeneity have been observed in mAb preparations [6]. All these mAb modifications are of regulatory concern because they may result in decreased antigen binding, increased immunogenicity and altered effector functions depending on the mode-of-action.

The removal of the carboxy-terminal lysine from the heavy chains is routinely observed upon the characterization of monoclonal antibodies [7,8] and is caused by intracellular enzymes [9]. From a regulatory aspect, this 'lysine clipping' is not regarded as critical under the condition that a potency assay is available that proofs the quality of the mAb [1]. The term "potency" refers to the specific ability or capacity of a product to achieve its intended biological effect [3]. Regarding effector functions mediated via the Fc-part of the IgG molecule, the complement dependent cytotoxicity (CDC) assay is an accepted method to evaluate potency of monoclonal antibodies [10].

IGN311 is a humanized mAb that is currently in clinical development intended for the treatment of Lewis-Y antigen overexpressing malignancies and has been recently tested in a clinical Phase I study in patients with various tumors of epithelial origin [11]. The anticipated mode-of-action of IGN311 is the lysis of Lewis-Y positive tumor cells via Fc-mediated mechanisms like CDC and antibody dependent cellular cytotoxicity (ADCC) [12] as well as inhibition of signal transduction due to binding to Lewis-Y glycosylated growth factor receptor [13]. For a clinical Phase I study applying a therapeutic mAb, the FDA recommends to report the range of charge heterogeneity caused by lysine clipping and for Phase II/III studies, it is recommended to monitor lysine clipping within the different batches of drug substance/drug product and to assess the impact on formulation development. Additionally, the knowledge of the degree of lysine clipping should be included into lot-to-lot comparability studies [5].

The current work aims at the elucidation of charge heterogeneity of three batches of IGN311 applying isoelectric focusing (IEF) and cation exchange (CEX)-HPLC. Furthermore, different enzymatic treatments, RP-HPLC–MALDI-TOF-MS as well as C-terminal sequencing were used to confirm the nature of potential mAb alterations. In addition, the biological activity of the different batches was compared using CDC.

2. Experimental

2.1. Samples and materials

IGN311, a fully humanized IgG1/κ mAb specific for the Lewis-Y antigen, was derived from murine ABL364 antibody (BR55-2 hybridoma) by CDR grafting [12]. IGN311 Batches I and II were produced in a hollow fibre reactor by SP2/0 cells in serum containing medium. The cell culture harvest was filtered (0.22 μm) and purified by Protein-A affinity chromatography (MabSelect, Amersham). Briefly, bound IgG was eluted with 0.15 NaCl, 20 mM Na-citrate (pH 3.6), neutralized and subsequently virus inactivation was performed for 1 h at pH 3.5. The pH was adjusted to pH 6.0 and the sample was applied to a cation (CM Sepharose, Amersham) – and anion (Q-Sepharose 6FF, Amersham) – exchange chromatography procedure. Finally, diafiltration against buffer containing 10 mM $NaPO_4$, 156 mM NaCl (pH 6.0) was performed. In contrast, IGN311 Batch III was produced by SP2/0 cells in medium without serum in a stirred tank reactor and purified by Protein-A chromatography. Briefly, the sample was diluted 4-fold with PBS_{def}, filtered (0.22 μm, Millex GV) and subsequently loaded onto a ProsepA (Amersham) affinity column. After washing the column with 10 CV of running buffer (PBS_{def}), elution was performed with buffer containing 0.1 M glycine and 0.2 M NaCl, pH 2.8. Ab containing fractions were collected and neutralized with 1 M $NaHPO_3$. Ab fractions were pooled and dialyzed (Slide-a-Lyzer, Pierce, MWCO 3500) against buffer containing 10 mM $NaPO_4$ and 156 mM NaCl, pH 6.0. Finally, the Ab solution was again filtered. The purity of all batches was determined by SEC-HPLC and concentration was determined by measuring the absorbance at 280 nm. Antibody samples were stored at 4 °C.

Buffer chemicals and MALDI-matrix compounds were purchased in the highest available quality from Fluka (Buchs, Switzerland) or Sigma–Aldrich (St. Louis, MO, USA). Water of ultra-high quality was prepared with an Elgastat UHQ apparatus (Elga LabWater, Siershahn, Germany). Ten millimolar phosphate buffer (pH 7.2) was obtained from PAA, Linz, Austria.

2.2. Cation exchange chromatography (CEX-HPLC)

For CEX-HPLC, a 1 ml Mono S column HR5/5 (Pharmacia) was used which is based on a beaded hydrophilic polystyrene/divinyl benzene resin (10 μm particle size). The charged group on the gel is $–CH_2–SO_4$ and the protein capacity of the column is 20–50 mg. IGN311 (500 μg in 10 ml) was loaded onto the column using an Aekta Explorer System (Amersham Biosciences, Uppsala, Sweden) and eluted with 10 mM sodium phosphate buffer in the presence of 10 mM NaCl applying a linear pH gradient (eluent A at pH 6.8, eluent B at pH 9.2) ranging from 0 to 100% B over 60 min with a flow rate of 1 ml/min (i.e., totally 60 column volumes). Protein elution was monitored by measuring the absorbance at 280 nm.

2.3. Isoelectric focusing

All reagents and devices used for IEF were obtained from Amersham Biosciences. The mAb solution was diluted to 1 mg/ml with 10 mM phosphate buffer, pH 7.2, and 15 μl were loaded onto an 'Ampholine PAGplate 3.5–9.5′ IEF gel. IEF-PAGE was performed at 1500 V, 50 mA and 30 W for 1.5 h using the Multiphor II flatbed electrophoresis system. After the run the gel was fixed with a solution containing 12% TCA and 3% sulpho-salicylic acid and stained with PhastGel Blue R.

2.4. De-N-glycosylation by PNGase F

Two milligrams of the various IGN311 batches were treated with PNGase F (New England Biolabs, MA, USA) in 10 mM Tris–Acetate buffer, pH 6.5, followed by 1 h incubation at 37 °C. Subsequently, samples were analyzed by IEF according to the protocol described above.

2.5. Carboxypeptidase B (CP-B) treatment

The IGN311 sample solution was diluted with 10 mM phosphate buffer, pH 7.2, to a concentration of 3 mg/ml and 35 μl of this solution was mixed with 2 μl CP-B solution (1 mg/ml, Roche, IN, USA) and incubated for 2 h at 37 °C. The CP-B treated antibody was diluted with 10 mM phosphate buffer pH 7.2 to a concentration of 1 mg/ml and analyzed by IEF.

2.6. Peptide mass analysis by RP-HPLC and MALDI-TOF-mass spectrometry

For enzymatic digestion of IGN311, 1 nmol of Ab in solution was denatured by addition of the 1.5-fold volume of a solution containing 8 M guanidinium hydrochloride/0.4 M ammonium bicarbonate, pH 8.2. Subsequently the Ab was reduced by addition of 45 mM DTT (one fourth of the volume of the Ab solution) and incubation at 55 °C for 40 min, and then alkylated by addition of 100 mM iodoacetamide (one fourth of the volume of the Ab solution) and incubation in the dark for 20 min. The solution was diluted with water of ultra-high quality (1.5-fold of volume). For the enzymatic cleavage, 2.5 μg trypsin (modified, sequencing grade, Roche Diagnostics, Basel, Switzerland) dissolved in water or 2.5 μg endoproteinase Glu-C dissolved in 100 mM ammonium bicarbonate pH 7.8, was added and digestion was carried out at 37 °C overnight. The digested samples were split into aliquots containing approximately 50 pmoles of peptides, lyophilized by vacuum-centrifugation and stored at −20 °C.

Samples containing the digested peptide mixtures were submitted either directly to MALDI-TOF-MS or were pre-separated by capillary RP-HPLC and spotted for subsequent MALDI-MS analysis. For spotting, 20 pmoles of the Ab digest were injected into the HPLC system and the eluted fractions were directly deposited every 0.5 min onto a MALDI-target pre-spotted with matrix solution (10 mg/ml 2,4,6-trihydroxyacetophenone and di-ammonium hydrogen citrate in methanol/0.1% TFA mixed 2/1). The elution gradient for the RP-HPLC was as follows: eluent A was 0.1% TFA in water, eluent B was 0.1% TFA in acetonitrile; initially 2% B for 5 min, linear gradient to 20% B in 25 min, linear gradient to 90% B in further 30 min. HPLC was carried out using an Agilent Series 1100 system (Waldbronn, Germany) equipped with a micro-cell for UV detection at 214 nm. The C18 column (inner diameter 300 μm, particle size 5 μm, pore size 300 Å) was purchased from Vydac (Hesperia, CA, USA) and operated at a flow rate of 3 μl/min achieved by a flow splitter.

Samples for MALDI-TOF-MS measurements were prepared as follows: 10 pmoles of the mAb digest were applied onto the target using the dried-droplet technique. As matrix solution 10 mg/ml 2,4,6-trihydroxyacetophenone and di-ammonium hydrogen citrate in methanol/0.1% TFA was used. Peptide mass analysis was carried out using a linear TOF instrument (“AXIMA-LNR”, Shimadzu Biotech–Kratos Analytical, Manchester, UK). Spectra were taken in the positive ion-mode and typical parameter settings were used [14]. Briefly, a pulsed nitrogen laser with wavelength of 337 nm, an accelerating voltage of 20 kV and molecular ion-optimized delayed extraction were used. Data acquisition and processing were performed by using Kratos software Version 2.3.5. Calibration was done externally using standard peptides and the over-all spectra were created by adding up 300–500 unselected spectra of single laser shots in a fully automated procedure.

2.7. Lectin affinity chromatography glycopeptide analysis by multistage MALDI-MS

Lectin affinity chromatography for pre-separation and enrichment of glycopeptides was done by means of Con-A Sepharose (Amersham Biosciences, Uppsala, Sweden) following the procedure described previously [15]. Fifty microlitres of the solution of the tryptic peptide mixture containing about 1 nmol of Ab were loaded, and after washing with background buffer the glycopeptides were eluted by 200 μl elution buffer (0.3 M α-D-methylmannopyranoside in 20 mM ammonium acetate, pH 8.5), collected in a vessel and manually spotted onto a MALDI target. MS analysis was performed by single-stage MS (using the above described procedure) and subsequently also by multistage MS using a hybrid instrument combining a quadrupole ion trap (QIT) with a reflector TOF analyzer (“AXIMA-QIT”, Shimadzu Biotech–Kratos Analytical). MALDI-MS experiments gave fragmentation spectra under low energy collision induced dissociation conditions particularly showing fragmentation of the glycan structures. Typical parameter settings were used: accelerating voltage 4030 V, ion trap continuously flooded with helium ($5–6 \times 10^{-5}$ mbar) for ion cooling combined with short argon pulses for “ultra-cooling” and enhancement of the fragmentation efficiency, respectively. MS^n spectra were obtained by adding up the signals of 4000 unselected single laser shots.

2.8. C-terminal sequencing of the mAb heavy chain

The C-terminal protein sequence of the heavy chain (hc) was determined by Eurosequence (Groningen, The Netherlands). Briefly, the Ab hc was isolated by RP-HPLC after reduction and alkylation [16] of the Ab sample. The sequence analysis was performed by C-terminal degradation with an automatic sequenator (Model 477A, Applied Biosystems). Step-wise released ATH-amino acids were identified by on-line HPLC based on their elution times.

2.9. Complement dependent cytotoxicity

Complement mediated cell lysis activity was determined using a ^{51}Cr-release assay with the Lewis-Y positive SKBR-3 breast cancer cell line as target. SKBR-3 cells were incubated for 2 h with 100 μCi of ^{51}Cr, washed three times with medium and plated at a density of 25×10^3 cells per well into 96-well microtiter plates together with serial dilution of the sample to be analyzed (72 ng–75 μg/ml) and complement-active serum from a volunteer donor. The plate was incubated for 1 h at 37 °C in a CO_2 incubator (5%). Supernatants were collected and the released ^{51}Cr was determined using a Cobra 5005 gamma counter (Packard, IL, USA). Spontaneous release and maxi-

mum release were measured after incubation of representative samples with medium alone and with detergent (SDS), respectively. Percentage of lysis obtained with Batches II and III was calculated relative to Batch I.

3. Results and discussion

3.1. CEX-HPLC and IEF analysis as indicators for charge heterogeneity

Three batches of IGN311 derived from different production processes were compared by a set of analytical techniques. Two consecutive Batches I (B1) and II (B2) produced in serum-containing medium and purified using Protein-A followed by ion-exchange chromatography were analyzed and compared to a third Batch III (B3) which was grown under serum-free conditions and purified by Protein-A only. CEX-HPLC of the three IGN311 batches revealed chromatograms with a different number of additional peaks besides the main peak. Whereas the pattern resulting from peaks eluting prior to the main peak was very similar for Batches B1 and B2 produced in serum-containing medium, B3 produced in serum-free medium showed a less pronounced pattern in this elution area (Fig. 1) which might be explained by less impurities due to the serum-free process. In this context, in another mAb preparation the deamidation of the light chain asparagine 30 residue to aspartate in one or both light chains has been demonstrated by Harris et al. [17] to be responsible for two acidic forms in B1 and B2. In addition, when compared to B1, the B2 preparation showed an additional small peak (designated K1) besides the main peak (designated K0) and B3 displayed two additional peaks: K1 (as present in B2) and a further peak designated K2. IEF gel electrophoresis (shown as insets in Fig. 1) revealed the presence of four distinct bands in B1 located within the pH range from 7.77 to 8.01. B2 showed an additional band with an approximate p*I* value of 8.1, and B3 two additional bands—one corresponding to the band (p*I* 8.1) observed in B2 and a second at a p*I* greater than 8.1. These additional bands located on the more basic side (indicative for molecules with increased positive charge) thus correlate well with the additional peaks present in the CEX chromatograms of B2 and B3. The observed elution profile was reproducible with respect to qualification criteria identified in the course of a time/operator different assay qualification.

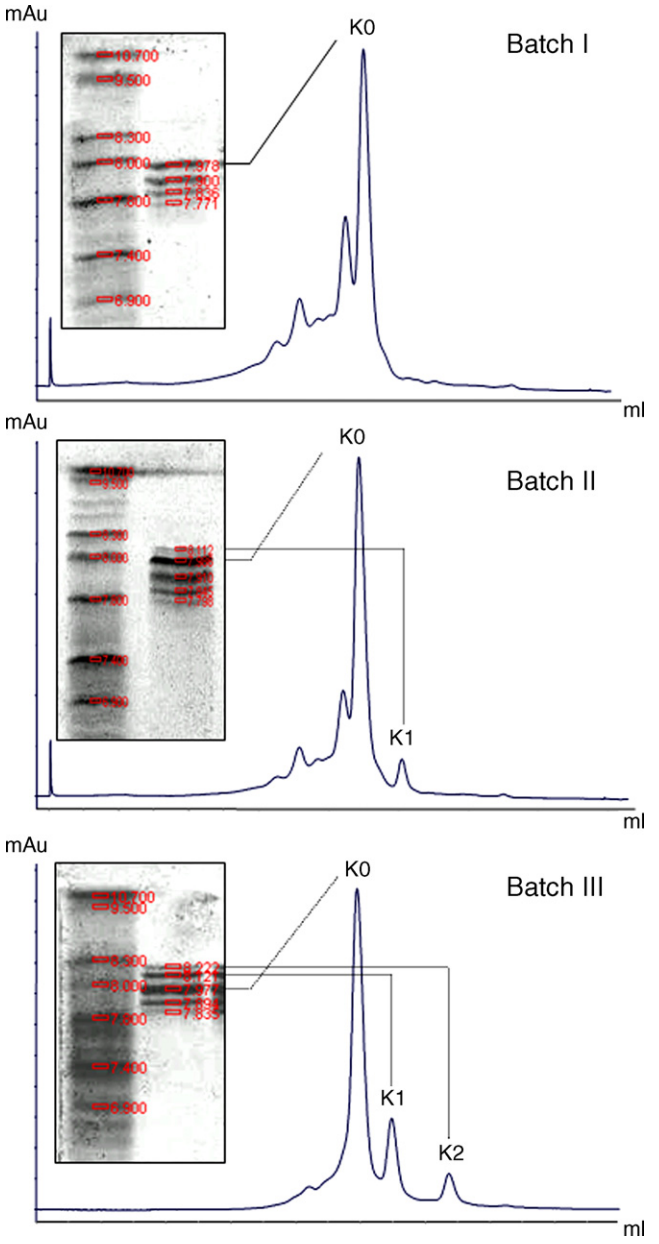

Fig. 1. CEX-HPLC chromatograms of three IGN311 batches are shown. Absorbance was measured at 280 nm. The corresponding IEF-PAGE patterns are shown as insets. Peaks at the CEX-HPLC chromatograms corresponding to antibody molecules containing no (K0), one (K1) and two (K2) carboxy-terminal lysines, respectively, and their corresponding bands in the IEF-gel are indicated.

3.2. Glycosylation is not the cause of charge heterogeneity

To investigate whether this charge heterogeneity is caused by differences in glycosylation, the three batches were treated with PGNase F. This glycosidase cleaves N-linked glycoproteins between the asparagine residues and the innermost GlcNAc of high mannose, hybrid and complex oligosaccharides [18]. The IEF pattern of the glycosylated and the de-*N*-glycosylated forms of the different batches are shown in Fig. 2. Although the pattern shifted as a whole towards a more acidic p*I* after de-*N*-glycosylation for all batches, the number and spacing of the bands did not change indicating that the charge heterogeneity observed within the different IGN311 batches is not the consequence of variation in the glycosylation between the different batches. The observed p*I* shift of all bands towards the acidic side was expected as a consequence of the conversion of Asn to Asp upon de-*N*-glycosylation leading to one free carboxylic group per glycosylation site.

The finding of this de-*N*-glycosylation experiment was supported by the analysis of the glycan structures attached to the IGN311 Ab. Trapping of the glycopeptides obtained by tryptic digestion of the mAb by Con-A affinity chromatography and subsequent analysis by single-stage MALDI-MS (Fig. 3) revealed the presence of three glycan variants attached to the peptide EEQYNSTYR, and containing four *N*-acetyl-

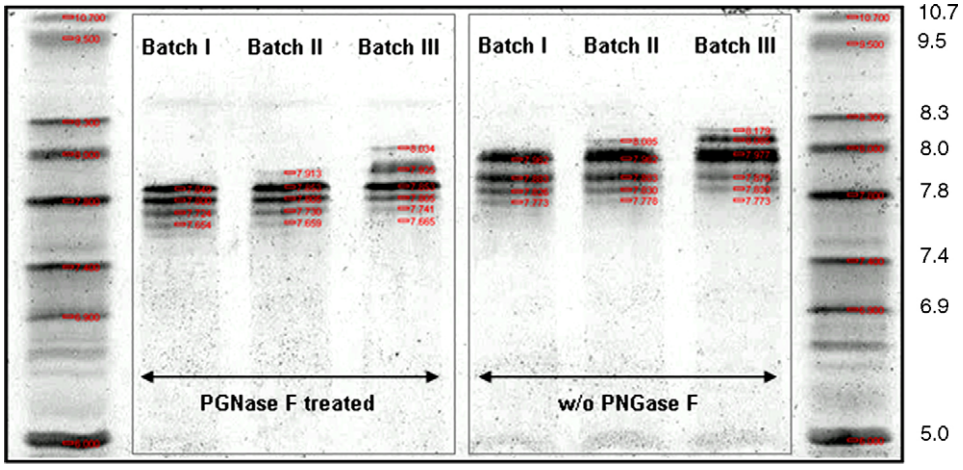

Fig. 2. IEF-PAGE pattern of IGN311 antibody samples before and after de-glycosylation by PNGase F treatment. The outer lanes show the IEF marker.

hexosamines, one deoxy-hexose and 3, 4 and 6 hexoses, respectively (M_r of the glycan moieties: 1445.6, 1607.8 and 1932.2 units, respectively). MS^2-spectra taken from the same spots by means of MALDI-Ion Trap-MS confirmed one *N*-acetyl-hexosamine that is attached to the peptide backbone and is fucosylated (data not shown). From these data the hybrid-type glycan structures depicted in Fig. 3 are deduced which indicate the absence of any charged sugar like sialic acid. This finding is similar to the situation reported for mAb 1B7-11 [14] where not the glycosylation patterns – but the modifications of the polypeptide chains – were found to generate charge heterogeneity.

3.3. Identification of C-terminal lysine clipping as cause of charge heterogeneity

Another potential source of charge heterogeneity in Ab preparations is the presence of a different number of charged amino acids. This is of particular relevance for the lysine present at the carboxyl-terminus of the two heavy chains [6] which can be clipped by carboxypeptidases during the fermentation process—a phenomenon commonly observed for therapeutic proteins [6,7]. According to the amino acid sequence of the IGN311 heavy chain, a maximum of two carboxy-terminal lysines per Ab are expected. To assess the contribution of C-terminal amino acid clipping, the three IGN311 batches were treated by carboxypeptidase B (CP-B). After enzyme treatment, the IEF pattern of B2 and B3 changed whereas the pattern of B1 remained unchanged indicating that this batch represents a completely clipped variant even without CP-B treatment (Fig. 4, Panel A). In contrast, the changes in the IEF pattern observed in Batches II and III, respectively, after CP-B treatment indicate that these batches represent variants with a C-terminal lysine on either one (B2) or both heavy chains (B3), although with relatively low abundance. The IEF experiment revealed that neither

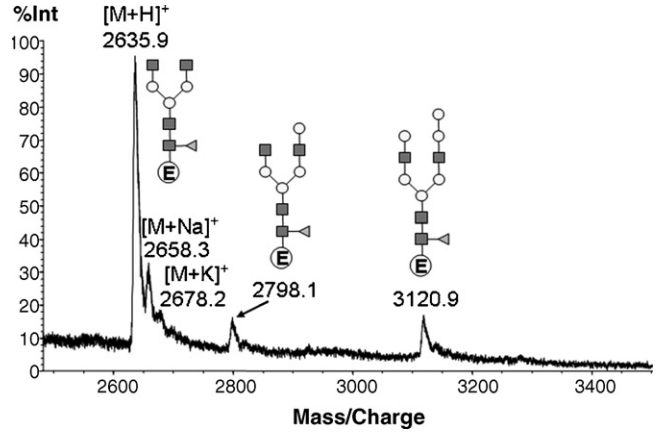

Fig. 3. MALDI-linear TOF-MS spectrum (positive ion mode) of Con-A affinity chromatography enriched tryptic glycopeptides from IGN311 showing three N-linked glycan variants attached to the peptide EEQYNSTYR. Further experimental conditions are given in Section 2. *Symbols*: Triangle, fucose; square, *N*-acetyl-glucosamine; circle, mannose.

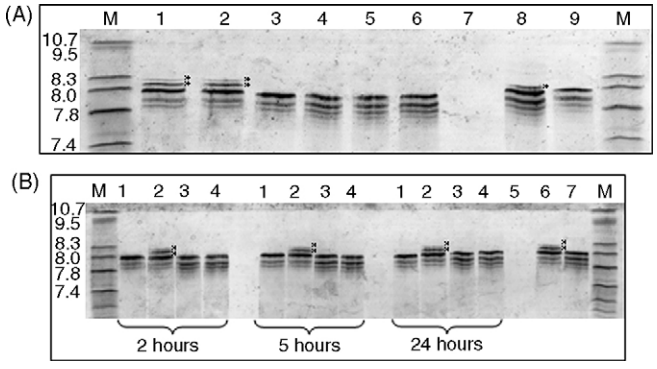

Fig. 4. IEF-PAGE pattern of IGN311 antibody samples before and after treatment with carboxypeptidase B (CP-B). Panel (A), comparison of the Batches B1 to B3. Samples were treated with CP-B for 2 h at 37 °C. *Lane description*: M, marker; lane 1, B3 untreated; lane 2, B3 incubated at 37 °C without CP-B; lane 3, B3 treated with CP-B; lane 4, B1 untreated; lane 5, B1 incubated at 37 °C for 2 h without CP-B; lane 6, B1 treated with CP-B; lane 7, CP-B only; lane 8, B2 untreated; lane 9, B2 treated with CP-B. Asterisks indicate IEF bands that correspond to an antibody molecule with either one or two lysines. Panel (B), kinetics of CP-B digest performed at 37 °C. *Lane description*: M, marker; lane 1, B3 treated; lane 2, B3 incubated at 37 °C without CP-B; lane 3, B1 treated; lane 4, B1 incubated at 37 °C without CP-B; lane 5, CP-B only; lane 6, B3 untreated and not incubated at 37 °C; lane 7, B1 untreated and not incubated at 37 °C. Asterisks (one or two, respectively) indicate the IEF bands that correspond to a mAb molecule with one or two C-terminal lysines, respectively.

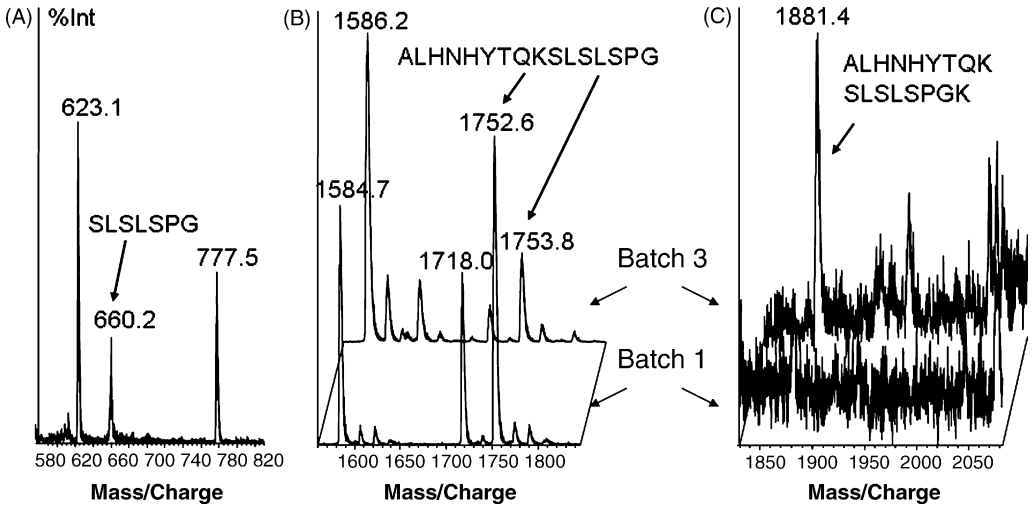

Fig. 5. Cut-outs of MALDI-linear TOF-MS spectra of IGN311 peptides obtained after enzymatic cleavage: (A) tryptic peptides from B1 without pre-separation demonstrating the presence of the lysine-clipped peptide SLSLSPG (M_r = 660.2) and the absence of the lysine-containing peptide SLSLSPGK (M_r = 788.5); (B and C) Glu-C cleaved peptides from B1 and B3, respectively, after RP-HPLC separation; the spectra from the eluate fraction collected over the time interval 43.0–43.5 min: (B) indicate the presence of the lysine-clipped peptide ALHNHYTQKSLSLSPG (M_r = 1752.6 and 1753.8, respectively) in both Batches, those collected over the time interval 41.5–42.0 min (C) document the presence of the lysine-containing peptide ALHNHYTQKSLSLSPGK in B3 (M_r = 1881.4) and its absence in B1.

the enzyme preparation without mAb (lane 7) nor mAb treatment at 37 °C without enzyme resulted in bands in the p*I* range of IGN311. Fig. 4, Panel B shows the enzyme kinetics (incubation times of 2, 5 and 24 h) of CP-B for Batches B1 and B3. Even after 24 h of incubation the IEF pattern was identical to the 2 h treatment indicating that digestion is complete after 2 h.

To confirm that the three batches differ in their carboxy-terminal lysine content, the amino acid sequence of their carboxy-terminus was determined by C-terminal sequencing (data not shown). Sequencing revealed that B1 and CP-B treated B2 and B3 contained glycine but no lysine at detectable amounts as the C-terminal amino acid, whereas in the untreated B3, the C-terminus exhibited lysine as terminal amino acid with an abundance of about 15% and glycine with an abundance of 85%. In the untreated B2 no peptides with a C-terminal lysine were identified. Although this would have been expected on the basis of the data obtained by CEX-HPLC and IEF analysis, the relative abundance of these peptides (expected with about 5%) was obviously too low for the sensitivity of the C-terminal sequencing method.

C-terminal lysine-clipping was further confirmed by mass spectrometric analysis of the C-terminal peptides of the heavy chain obtained after enzymatic cleavage of the Ab with trypsin and Endo-Glu-C, respectively, the later cleaving at the C-terminal side of glutamic acid. In the MALDI-TOF spectrum of the tryptic digest peptide mixture of B1 (Fig. 5A) a (small) peak was detected with a molecular mass of M_r = 660.2 which is the theoretical M_r value of the C-terminal peptide lacking the terminal lysine (M_r = 660.4). No signal was identified that corresponds to the C-terminal peptide containing the terminal lysine (M_r = 788.5). Because the presence of basic moieties in analytes generally leads to better ionization yields when working with MALDI-MS in the positive ion mode, ionization suppression can be excluded with high probability. It is therefore more likely that the signal was below the limit of detection due to the absence of the considered peptide or its very low abundance. The analysis was repeated with Glu-C treated peptides that have been pre-separated by capillary-HPLC prior to MALDI-TOF-MS in order to increase sensitivity. Signals for the C-terminal peptide without lysine, ALHNHYTQKSLSLSPG (theoretical M_r = 1752.9) were detected in eluate fractions of both Batches B1 and B3 (Fig. 5B). No signal corresponding to the theoretical mass (M_r = 1881.0) of the peptide including the terminal lysine was found in the eluate fractions of B1, however, within the B3 preparation such a peak was identified with low intensity (Fig. 5C). This low intensity was in accordance with our expectations because of the low abundance of about 15%.

In summary, our data indicate that IGN311 manufactured under serum-free conditions (B3) exhibited lysine clipping to a lesser extent suggesting that addition of serum to the cell culture may introduce (or induce) additional carboxypeptidase activity resulting in partial or complete lysine clipping.

3.4. Lysine clipping does not affect complement dependent cytotoxicity

Finally, we investigated whether lysine clipping affects the ability of the Ab to destroy Lewis-Y expressing tumor cells by CDC. The qualified bioassay that is used as release/stability assay measuring the CDC revealed that lysine clipping from both batches, B2 and B3, did not affect the lytic potential in the CDC assay (Fig. 6). Relative to B1, which is used as reference, B2 showed a lytic potential of 67% (unclipped) and 66% (clipped) and B3 showed a lytic potential of 90% (unclipped) and 88% (clipped). CDC specifications for IGN311 product were set to ±50% relative to an in-house "golden standard"—based on this criterion, it can be concluded that: (i) the batches tested did not significantly differ regarding their CDC activity and (ii) that all three batches range within the specifications set for IGN311.

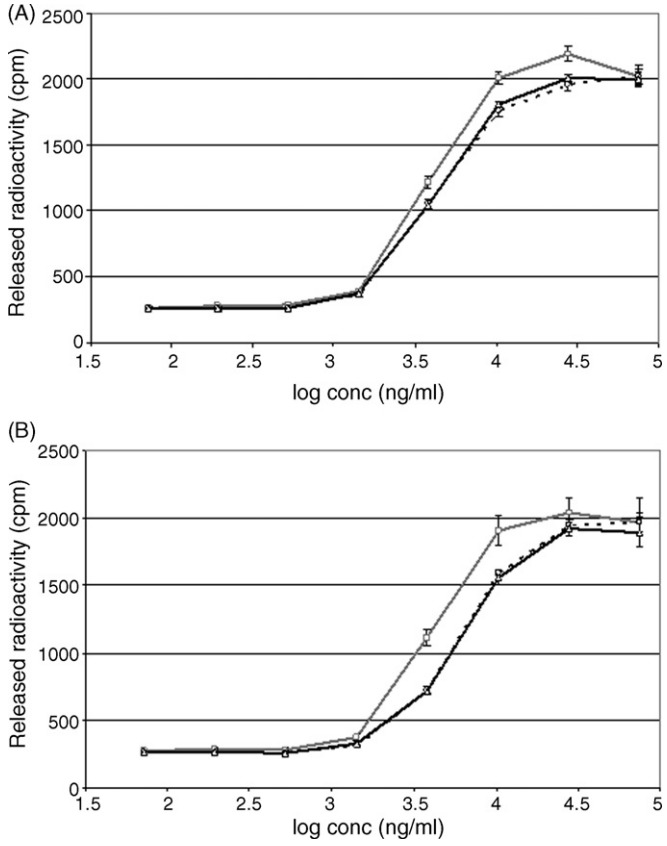

Fig. 6. CDC assay. ^{51}Cr labelled human target cells (SKBR 3) were incubated with IGN311 in the presence of human serum as complement source. Released radioactivity following cell lysis was measured as a function of the concentration of IGN311 applied for cell lysis. Samples were tested in triplicates and the mean values are shown. Batch I (fully lysine-clipped) was used as reference in both panels. Upper panel (A), comparison of Batch II to Batch I. Grey line, Batch I; black solid line, Batch II; black dotted line, Batch II CP-B treated. Lower panel (B), comparison of Batch III and Batch I. Grey line, Batch I; black solid line, Batch III; black dotted line, Batch III CP-B treated.

4. Conclusion

Three different batches of recombinant therapeutic mAb IGN311 obtained by fermentation in serum containing and serum-free media, respectively, were characterized by a variety of physico-chemical methods. IEF analysis revealed the presence of four to six protein bands differing slightly in their p*I* values. Two of these bands were identified by means of CEX-HPLC, MALDI-MS and C-terminal sequencing as Ab populations devoid of one and two lysine(s), respectively, at the C-terminal position of the two heavy chains.

In contrast to previous reports dealing with the characterization of mAbs – that focused exclusively on physico-chemical methods [7] – we here extend this test portfolio by a biological potency assay that mimics the IGN311 in vivo mode-of-action—namely the killing of tumor cells by complement dependent cytotoxicity. Furthermore, we have analyzed two consecutive batches produced in a serum-containing fermentation process to a batch produced by a serum-free fermentation process. The presented results demonstrate that the capability to mediate CDC tumor cell lysis was not significantly changed even when using batches derived from different fermentation processes resulting in different degrees of C-terminal lysine clipping. This finding is important in a regulatory perspective, suggesting that lysine clipping in the humanized monoclonal Lewis-Y specific antibody IGN311 does not influence the biological activity of this therapeutic antibody.

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