FcyRIII (CD16) and GD2 binding assay for an IgG using surface plasmon resonance (SPR; Biacore) as a surrogate for evaluation of Antibody-Dependent Cell-mediated Cytotoxicity (ADCC)

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Abstract
Two Biacore binding assays were established and validated at Rentschler to be used as a surrogate for evaluation of Antibody-Dependent Cell-mediated Cytotoxicity (ADCC). The combination of both Biacore binding assays with the cell-based ADCC assay was shown in different studies. The GD2 binding assay allows to evaluate the specific IgG binding to the antigen on the target cell (= GD2 ganglioside, which is overexpressed by target cells). To evaluate the binding of the Fc region of the IgG to FcyRIII (CD16) receptor on an effector cell, a natural killer cell (NK), the CD16 binding assay can be used. It could be shown that the GD2 Biacore- and the CD16 Biacore-method together are able to substitute an ADCC assay for characterization of the lytic potential of an IgG. In the course of validation it was shown in terms of Accuracy and Precision the two Biacore methods are superior to a cell-based ADCC assay. Therefore, the two Biacore methods are used for release and stability testing.

Introduction
ADCC Mode of Action
The mode of action of ADCC is described schematically in Figure 1, in principle consisting of two binding interactions. Antibody binding to a target molecule via the variable regions (CDRs) and the interaction with the FcyRIII/CD16a (CD16) molecule via the Fc-domain (constant region). Accordingly, IgG binds to its target antigen GD2, overexpressed on the surface of the tumor cells. After binding of FcyRIII/CD16a (CD16) expressed on Natural killer cells (NK) to the IgG Fc domain, multiple cross-linking of the target and effector cells occurs. This interaction is strongly influenced by N-linked oligosaccharides in the IgG Fc region, and triggers activation of ADCC (Figure 1). Killing of target cells is an endpoint of this pathway activation and lysis of target cells is used as readout in classical ADCC bioassays. Donor peripheral blood mononuclear cells (PBMCs) or the natural killer cell (NK) subpopulation are used as effector cells.

Biacore Binding Assays
GD2 Binding Assay
To determine the binding characteristics of the monoclonal IgG to the specific antigen (GD2) on the target cells a binding assay with Biacore C was set up. The IgG is injected over a sensor surface on which a known amount of antigen is immobilized. The injected IgG sample interacts with the immobilized GD2 (Figure 2). The detected signal (RU) is plotted against the respective concentration (+ dose-response-curve). To determine the activity relative to reference, IgG samples are diluted into the working range of the method between 2.5 and 20 μg/mL (Figure 2). The IgG/GD2 complex is removed in two regeneration steps by 20 μL 15 mM NaOH.

CD16 Binding Assay
To determine the binding characteristics of the Fc region of the IgG antibody to CD16 receptor a steady-state affinity assay with Biacore T200 was set up. The IgG is captured in a first step by Protein A (ProA) which is covalently bound on the surface of a Biacore CM5 Sensor Chip via amine coupling (Figure 3). The injected CD16 interacts with the ProA-captured IgG (Figure 3). Changes in amounts of molecules bound to the chip are detected in real time and data are presented in a sensorgram. To determine the binding affinity constant by steady-state-model (considering only the on-rate), CD16 is injected in different concentrations and the detected signals (RU) are plotted against their respective concentration (M) (Figure 3). After each measurement cycle the IgG/CD16 complex is removed from the ProA surface by 15 μL 10 mM Glycine/HCl, pH 1.7. For reference a further ProA flow cell without captured IgG is used.

Tests to show equivalence of ADCC Cell Based Assay and Combined Biacore Methods
Binding behaviour after deglycosylation
N-linked glycosylation of IgGs is essential for the mechanism of action of ADCC. Therefore 5 IgG samples showing different amounts of N-linked oligosaccharides (0, 25, 50, 75 and 100%) were generated by digestion with Peptide-N-Glycosidase F and investigated for GD2 and CD16 binding and the potency to trigger cellular cytotoxicity (ADCC). The results of CD16 Biacore assay correlate to the results obtained with the ADCC assay shown in Figure 4. GD2 binding was not affected by deglycosylation.

Stability testing under temperature stress
To use the combined Biacore methods as a comparable substitute for the cell based ADCC within GMP routine, it is essential to show that the assays are able to monitor changes within stability studies. Therefore the same IgG sample where stored at different temperatures and where subsequently analyzed by the GD2 Biacore assay and the CD16 Biacore assay. The results of this study are shown in Table 1.

Release testing and characterization
Product characterization and release testing within quality control are necessary and are critical parts within development of a biopharmaceutical manufacturing processes to show quality as well as comparability between different IgG batches. Therefore, five IgG batches were analyzed with the GD2 and CD16 Biacore assays as described above and for the potential of lysis tumor cells via ADCC. The results of this study are shown in Table 2.

Conclusion
Antibodies can be seen as "biological bridge" which connects target cells to effector cells in order to form immunological synapses which trigger lysis of the target cell. The target antigen is recognized by the variable domains of the antibody, while the CD16 receptor on effector cells interacts with antibody constant domains. This mechanism is understood as antibody mediated cellular cytotoxicity. The result of a cell based ADCC assay is a number describing the amount of lysed cells in comparison to the seeded cells. The result of such cellular biosafety is the product of both involved binding mechanisms: Binding to the target cell and to the effector cell, while the assay can not distinguish between both. Furthermore two types of cellular preparations are required, which leads to a high degree of intra- and inter-assay variability resulting in broad confidence intervals, which only hardly can detect small amounts of degradation products in an antibody sample. Therefore a GD2 and a CD16 Biacore binding assay were established at Rentschler to be used as a surrogate for evaluation of Antibody-Dependent Cell-mediated Cytotoxicity (ADCC). The combination of both binding methods provides a precise insight into both mechanisms and can substitute the cell based ADCC assay, since they comply with the following requirements:

- Distinguish between both involved binding mechanisms and elucidate which moiety of the antibody becomes degraded
- Give insight into binding behaviour of both involved binding reactions
- Reproducibly and precisely give quantified binding properties
- Detect small amounts of possibly degraded product

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