Antibody-dependent cell-mediated cytotoxicity (ADCC) is one of the immune system’s primary defence mechanisms, and therapeutic antibodies – such as Herceptin – use this mechanism to kill target cells following binding (see Figure 1). The process is triggered when the Fcγllla (CD16a) receptor, found on the surface of cells of the immune system, interacts with the bound antibody via its Fc region, forming a bridge between the target cell and the immune system cells. Following formation of this bridge, lysis of the target cells is mediated through the release of cytotoxic granules or by expression of cell death-inducing molecules.

Different immune system cells (leukocytes) have the ability to mediate ADCC as effector cells. They include natural killer (NK) cells, monocytes, macrophages, neutrophils, eosinophils and dendritic cells. Typically, most in vitro studies used to evaluate ADCC activity are conducted using peripheral blood mononuclear cells (PBMCs), in which NK cells are the principal effectors of ADCC activity.

**Method of Action**

Antibodies have many touch points with cells of the immune system and have the potential to display multiple mechanisms of action (MOA). However, understanding the contribution of different effector functions of the immune system, in particular ADCC, on overall clinical outcomes is poorly understood. All antibody therapeutics can mediate ADCC activity provided two conditions are met: the molecule can bind the Fcγllla receptor and the antibody target is cell-associated. A specific role for ADCC has been implied in the MOA of oncology products – for example, trastuzumab, rituximab and cetuximab – but its function is poorly understood in other indications, particularly that of autoimmune disease.

This issue is probably best highlighted when considering the theoretical capabilities of different anti-tumour necrosis factor (TNF)-α therapeutics and their efficacy in Crohn’s disease (2-5). ADCC has been suggested in some publications as one of the secondary mechanisms where these therapeutics can resolve the disease. The primary action of anti-TNF-α drugs is the neutralisation of the inflammatory...
mediator TNF-α, thereby reducing the body’s response to this potent cytotoxic agent. In the case of some drugs, it is believed that they can destroy the TNF-α producing cells by means of ADCC and, in this way, help to resolve the condition.

Extensive in vitro data supports the ability of infliximab to mediate ADCC activity. However, it is also acknowledged that, to date, there are no published reports describing the induction of ADCC by a TNF antagonist in patients, despite some studies detailing an association between polymorphisms in the Fcyl1a receptor and biological response to infliximab in Crohn’s disease (6-8). Thus, some have proposed the potential for ADCC as a MOA, others suggest no link (9).

Glycoengineered Antibodies

Key to the ability of an antibody’s effectiveness at inducing ADCC activity is the presence or absence of sugar (glycan) residues in the molecule; these are added as a post-translation step. The occurrence of certain glycoforms in the Fc domain of the antibody directly influences the binding kinetics to the Fcyl1a receptor. In vitro data has shown glycoforms that lack a core fucose residue at Asn297 demonstrate a significant increase in Fcyl1a receptor-binding activity, and this improved binding translates into increased ADCC activity when compared to fucosylated counterparts (10, 11). Lida et al has suggested that completely afucosylated therapeutics antibodies can evade the inhibitory effects of plasma immunoglobulin G (IgG) on Fcyll1a receptor binding on NK cells, with higher affinity to exhibit much greater ADCC in vivo in humans (12).

The therapeutic advantages associated with improvements to Fcyl1a receptor binding have resulted in the creation of a number of glycoengineered antibodies. This has been achieved either by modifying the structure of the Fc-glycan or by engineering the Fc-polypeptide backbone (13). The approach allows for the creation of new molecules with the potential to improve clinical outcomes for existing and novel antibody targets. At least 15 glycoengineered antibodies are in development, including biobetter versions of trastuzumab, cetuximab, rituximab and infliximab, with two glycoengineered products — mogamulizumab and obinutuzumab — currently licensed (14).

Performance Standards

The use of assays to measure ADCC activity is increasing, yet these tests still remain one of the most challenging bioassay formats to manage. As critical decisions on the structure and action of therapeutics are made on assay data, ADCC methodologies should be developed to an acceptable performance standard, such as ICH Q2 (R1) (15). They should include an assessment of accuracy, precision, specificity, linearity and range, and recognise that the actual scope is dependent upon the intended use. Above and beyond understanding these basic performance requirements, knowledge of the method’s sensitivity is critical.

Within the context of ADCC, sensitivity is defined as the ability of the method to detect the impact on activity of specific product physiochemical attributes. Such attributes are defined on a case-by-case basis, and are typically those that may arise due to variability in the production process, or that have a significant impact on product quality or performance. Most frequently, these are linked to the Fc-glycan composition of the antibody. There are a vast number of different design options for ADCC assays, each with various pros and cons that can directly affect the main outputs. Care is needed to define and optimise the experimental parameters and interpret the data that is generated.

Design Decisions

ADCC methodologies can be broadly divided into classical methods – in which the death of target cells is measured directly – or surrogate methods that rely upon a reporter cell line in which a signal cascade is activated following functional activation of the Fc receptor, and where the response is measured via a suitable reporter (16). Beyond this selection, there are two key decisions to be made when designing an ADCC assay: selection of the target cells and selection of the effector cells.

The most commonly adopted approach for target cell line selection is the use of a continuous cell line that endogenously over-expresses the antigen of interest. Continuous cell lines are more practical to use in assays, as this improves consistency. In addition, over-expression can enable clustering of the antibody Fc domain to improve Fcyl1a receptor binding. Primary cells may be used either in a native or activated state, but certain molecules require the creation of engineered cells to provide appropriate levels of antigen, in order for ADCC activity to be measured in vitro.

The use of continuous and engineered cell lines that over-express the required antigen are incredibly helpful tools, and can allow highly accurate determination
of the potential for an antibody to elicit ADCC. It is recognised, however, that these models do represent an artificial environment and results may not be fully translatable to an in vivo outcome; so evaluation of activity using biologically-relevant primary cells is recommended as part of the assessment.

Traditionally, ADCC assays are carried out using a great many different types of cells, both as the effector and target. As there is no universally applied standard methodology, this makes the comparison of data between methods or studies very difficult. In particular, cell preparations contain NK cells which are the principal effectors of ADCC activity, but may also include monocytes and other cells. It has been proposed that using mixed cell populations better models the in vivo conditions – compared with the use of a single cell type – and many different preparation methodologies have been utilised to obtain effector cells.

**Awareness of Sensitivity**

Varying preparations can impart differences to the degree of biological relevance and sensitivity. Commonsense would suggest that the most biologically relevant effector population is whole blood (6, 17). However, here, the relative abundance of NK cells to target cells is low, and a wide range of soluble factors and cell types would interfere with the assay’s performance, compromising sensitivity. Instead, the most commonly applied effector cell preparation is PBMCs, which contain a mixture of lymphoid and myeloid cells. Alternative approaches can also be applied to enrich the PBMC preparation with lymphoid cells alone – thus reducing the presence of myeloid cells. This preparation reduces the biological relevance of the applied methodology, but can result in increased sensitivity through the removal of cell types that either compete with NK cells for binding of the antibody Fc, or remove the target antigen from the cell membrane via monocyte shaving (18).

The most sensitive ADCC formats are achieved with an effector population consisting solely of an enriched NK faction. This limits the lack of competition from other cell types or soluble blood factors that can impede the NK-mediated activity, and assay responses are at the most optimal. Generally speaking, and irrespective of the effector cell population selected, the magnitude of ADCC activity observed in an assay is proportional to the quantity of effector cells used. Increasing the signal to noise can also yield improvements in accuracy, but this needs to be considered against a loss of sensitivity if the overall response begins to approach a saturation point in the method; high levels of target cell death do not always correlate with optimum method sensitivity.

When selecting the effector cell population, consideration should be given to the genetic polymorphisms that exist for the Fcylla receptor. Here, a point mutation at nucleotide 559 results in the substitution of valine by phenylalanine at amino acid 158, and causes proteins with different binding affinities for IgG. The V/V variant of the protein demonstrates a much higher binding affinity for IgG, than the F/F variant, and this can be clearly observed in Fcylla receptor-binding studies (19).

**Role of Polymorphisms**

General assay performance can be dependent upon polymorphisms, leading to changes in the magnitude of response influencing the method’s signal to noise, which can, in turn, affect accuracy. More importantly, differing sensitivity of the method is imparted by the polymorphisms and can lead to vastly different results being obtained from the same sample. It is crucial that the sensitivity of individual ADCC assay formats is fully understood prior to sample assessment, in order to understand the impact of design choices on the observed results.

Table 1 provides a broad overview of the impact on sensitivity that could be expected when differing effector cell preparations are utilised in the assay for antibodies with varying degrees of fucosylation.

The sensitivity imparted by the different effector cell preparations on the assay is further influenced by the Fcylla polymorphism of the donor. Figure 2 details the relative ADCC activity of a test antibody – containing elevated levels of fucosylation – to a reference antibody, both molecules having comparable antigen binding activity.
The ADCC activity of the test antibody was compared to the reference antibody using a range of effector cell preparations on at least three separate occasions, from a minimum of three different donors, spanning each of the possible FcγRIIa polymorphisms with the precision of all results within 25% coefficient of variation. An insensitive assay incapable of detecting differences in fucosylation would yield a relative ADCC activity result of approximately 100%; the lower the relative ADCC activity, the greater the sensitivity.

A general reduction in assay sensitivity is observed commensurate with the complexity of the applied effector cell preparation. Beyond this, and of particular interest, is the varying influence of the FcγRIIa polymorphisms. This suggests that within a more complex effector cell preparation, NK cells expressing the high affinity FcγRIIa receptor are better able to compete with other cell types in the preparation, than their lower affinity counterparts.

**Proving Biosimilarity**

Biosimilars have revolutionised the biotechnology industry for many reasons, not least due to the extensive requirements for analytical characterisation that are now needed to show comparability between innovator and biosimilar copies. It is generally accepted that the innovator and biosimilar will be different due to the complex nature of the production process; however, analytics seek to show that these molecules are sufficiently similar to provide the same clinical outcome when used to treat disease. The most critical evaluation is that of biological function, through assays that replicate the likely function in vivo, with ADCC and related assessments forming an integral component.

The first biosimilar monoclonal antibody to receive global approval was Remsima/Inflectra. ADCC evaluation studies carried out as part of the comparability profile of the molecule have provided insight into how regulators judge the value of these assays, and have become a benchmark for industry (6). The EMA noted that Remsima was comparable to Remicade in relation to all major physiochemical and biological activities, with the only noted issue of concern being a higher level of fucosylated molecules in the biosimilar, compared to the innovator drug. This difference resulted in a reduction in FcγRIIa binding of the biosimilar, leading to a decrease in ADCC activity in some of the assay formats tested.

Consequently, the developer has sought to understand the clinical significance of these differences in a range of adapted FcγRIIa receptor and ADCC methods to encompass a greater degree of biological relevance. Lipopolysaccharide-stimulated monocytes were used in place of an engineered cell line and, within this format, no ADCC activity was detected. This finding suggested that ADCC activity could only be observed using cell lines engineered to express membrane-bound TNF-α at a level higher than would be expected under physiological conditions. Therefore,
ADCC activity is likely to be limited in inflammatory settings in vivo. Further evaluation of whole blood as an effector cell population presented comparable responses between the two molecules, confirming the results of other ADCC methods used in the study. The question as to the relevance of ADCC to clinical efficacy for this category of antibody still remains to be resolved.

Understanding the Therapeutic

It is clear that ADCC assays are key to understanding the issues of comparability and similarity for biosimilars, but are also essential for making design decisions when developing molecules to target a specific activity. Recreating in vivo an assay which models the complex immune system interaction of ADCC is clearly difficult, and data interpretation is surrounded by pitfalls.

There are two diametrically opposed issues which impact these assessments: the desire to have as sensitive a method as possible – capable of picking up even the slightest impact on activity – and the need to have an assay which represents the complexity of the in vivo environment and is predictive of clinical efficacy. One assay methodology cannot, with the current state of technology, fulfil both these criteria; scientists should be prepared to use an orthogonal approach, building data one step at a time to understand the therapeutic and how it fits with the clinical objective.

References
4. Kaymakcalan Z et al, Comparisons of affinities, avidities, and complement activation of adalimumab, infliximab and etanercept in binding to soluble and membrane tumour necrosis factor, Clinical Immunology 131: pp308-316, 2009
5. Arora T et al, Differences in binding and effector functions between classes of TNF-antagonists, Cytokine 45: pp124-131, 2009
12. Ida I et al, Two mechanisms of the enhanced antibody-dependent cellular cytotoxicity (ADCC) efficacy of non-fucosylated therapeutic antibodies in human blood, BMC Cancer 9(58), 2009
17. Mossier K et al, Increasing the efficacy of CD20 antibody therapy through the engineering of a new type 2 anti-CD20 antibody with enhanced direct and immune effector cell-mediated B-cell cytotoxicity, Blood 115(22): pp4,393-4,402, 2010