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Antibody-Dependent Cellular Phagocytosis: The Mechanism of Action That Gets No Respect

A Discussion About Improving Bioassay Reproducibility

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Abstract

Antibody-dependent cellular phagocytosis (ADCP), which relies on macrophages to attack and devour tumor cells following antibody binding, is a potentially useful mechanism of action (MOA) for antibody drug developers and vaccine makers to consider in determining product efficacy. Unfortunately, it is often ignored in favor of more accessible MOAs driving biological function such as antibody-dependent cellular cytotoxicity (ADCC) because the assays are tedious to prepare, perform, and reproduce. This article will summarize a number of those challenges and discuss how novel approaches, such as a reporter-gene assay that replaces the macrophages with an engineered cell line, are more reliable in measuring this neglected MOA. We will also discuss why this could help developers better understand how their biosimilars compare to the originator product.

Complex Bioanalyses

Functional bioassays designed to assess an antibody's mechanism of action (MOA), which regulators require in investigational new drug (IND) filings, provide an excellent example of the challenges that researchers face in reproducing results. Antibody-dependent cellular phagocytosis (ADCP), a neglected MOA and the primary focus of this article, offers a good example of how difficult it can be to measure the biological activity of certain concentrations of proteins in primary cell cultures or isolated cell lines. The bioanalytical process is time-consuming and so complex that many laboratories simply aren't equipped to perform the functional assays. Others have tried and given up in frustration. For fulfillment of statistical criteria described in both the European Pharmacopoeia (Ph. Eur.)^[2] and US Pharmacopoeia (USP)^[3], a full dose-response curve is required and it has to be comparable to the reference dose-response curve with regard to slope and asymptotes. But due to blood donor variability, and to a lesser extent, other factors (e.g., related to assay setup and readout), it is nearly impossible to match the comparability criteria with a primary cell-based assay. Fortunately, there are alternative *in vitro* approaches, notably a novel reporter-gene bioassay that will be described later on which represents a simpler, faster, and less variable method of measuring a particular antibody MOA.

An Alphabet of Bioassays

Certainly, ADCP isn't the only option for determining MOA, and we will consider their distinct characteristics and applicability in this paper. Antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and programmed cell death (apoptosis) are three well-documented MOAs that the immune system employs to single out and kill specific pathogens and cancer or infected cells. They are the frontrunners in antibody functional analysis. ADCC, for instance, is widely thought to be one of the mechanisms behind the modest

Introduction

There is little doubt about the impact biologics have had and will continue to have on the global drug market. Over a quarter century ago, the development of game-changing monoclonal antibodies (mAbs) like trastuzumab (for metastatic breast cancer) and rituximab (for hematological cancers and autoimmune disorders) opened up new and powerful treatment regimens. Today, the rapidly expanding field of therapeutic antibodies, along with cytokines and growth factors, comprise about 30% of the global drug market. With close to 1,000 biologics and vaccines currently in development, this percentage can only climb further. MAbs lead the pack with over 370 products in various stages of development.^[1]

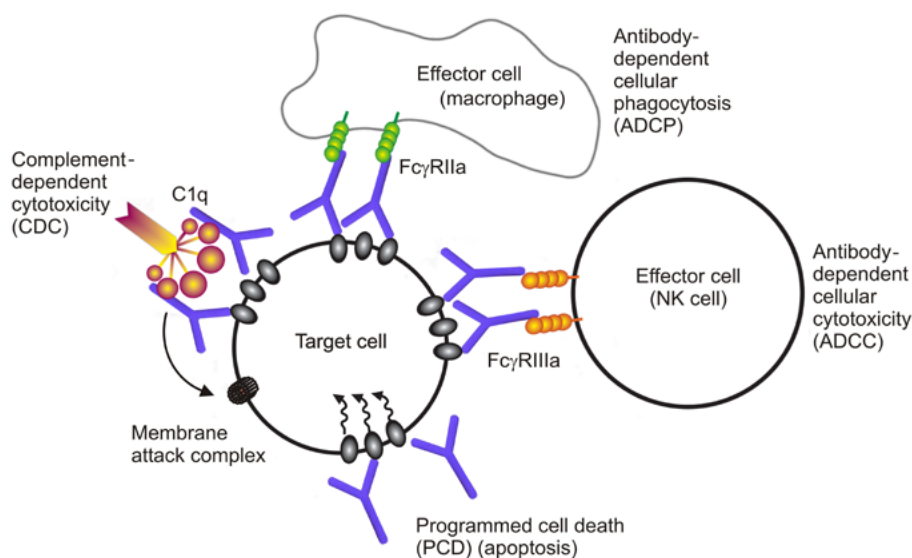


FIGURE 1. Classic functional bioassays designed to assess an antibody's mechanism of action.
(Image courtesy of Charles River Laboratories.)

33% vaccine-induced protection seen in a landmark AIDS vaccine trial known as RV144^[4], the only trial thus far to demonstrate efficacy against HIV. The effectiveness of a specific mAb that works via the classical immunological pathways to eliminate cancer cells, inflammatory disease targets, or virus-infected cells is determined by its efficacy for a certain MOA. The binding of the epitope to the antigen target, and the binding of effectors to the corresponding receptors on the constant chain of the therapeutic mAbs each impact the strength of induction of the respective MOA.

All of these MOAs function in different ways, so evaluating a particular antibody can be tricky. In ADCC, antibodies act as a bridge between an infected or cancer cell and an immune cell. The tips of the antibody (the fragment antigen-binding [Fab] region) bind to the target cell while the opposite end of the antibody binds to proteins on the surface of immune cells which can then kill the infected cell. The antibody releases preformed toxic proteins via a mechanism that secretes and then discharges the proteins to the outside of the target cell. When a protein complex known as C1q binds the antibody, CDC is induced. This binding triggers the formation of a membrane attack complex that punches holes into the surface of a target cell. In contrast, ADCP relies on macrophages to devour the target cells. Programmed cell death (apoptosis) occurs when a family of enzymes inside a target cell set off a cascade effect following the binding of the mAb to its target on the surface of the infected cell.

Different functional bioassays exist to analyze these MOAs. ADCC assays use target cells that express antigens specific to the pathogen that the antibody is targeting, effector cells such as natural killer (NK) cells, and a source of antibody such as serum, plasma, or mAbs. ADCC assays measure the death of the target cells by NK cells in the presence of antibody, usually by measuring the release

of a dye or another compound that the cells release once they die.^[5] CDC assays use flow cytometry or luminescence methods to estimate tumor damage and measure the functionality of the pathway induction by reading the mAb dose-dependent cell death caused by the formation of the membrane attack complexes.

ADCC, ADCP, CDC, and apoptosis are so-called “classical pathway” MOAs (**Figure 1**). For ADCC, CDC, and ADCP, a two-step mechanism is involved: (1) binding of the antibody specifically to the target—a suitable target cell line mimicking the patient’s tumor; and (2) binding of the effector cell to the Fc receptors. But ADCP is a far more complicated MOA function to quantify. In ADCP, receptors on the macrophages or other phagocytic cells bind to antibodies that are attached to target cells, including tumor cells. Macrophages are crucial to the efficacy of many antibodies because they perform the process of ADCP.^[6] One of those receptors, FcγRIIIa (CD32a), is thought to be the dominant player in the induction of ADCP by macrophages.

Measuring ADCP mediated through this receptor is important in determining the bioactivity of a mAb and any biosimilars that follow. It was found recently that ADCP is enhanced *in vivo* by simultaneous treatment with immunomodulatory agents, so being able to measure ADCP has suddenly become even more important.^[7]

However, measuring FcγRIIIa-mediated ADCP using primary monocytes or macrophage cells is a far more tedious process than ADCC where commercially available assays to measure cytotoxicity can be used, and blood donor cells can be prepared within a few hours. In ADCP, the macrophages must be derived from primary monocytes isolated from blood donors, and unlike NK effector cells for ADCC, need to be differentiated in culture to macrophages for nearly a week. The effector macrophages and target cells must then be made distinguishable through the use of cell surface markers and labeling, and

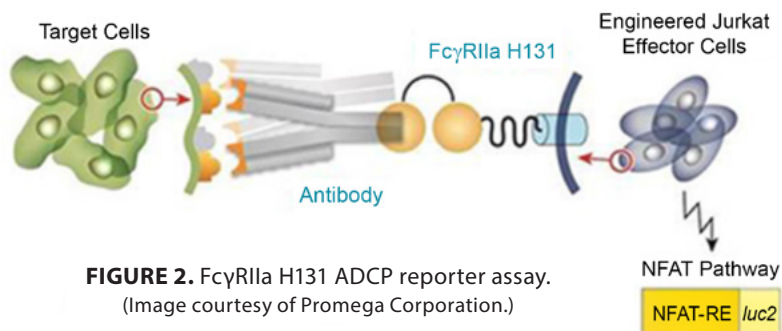


FIGURE 2. FcγRIIIa H131 ADCP reporter assay.
(Image courtesy of Promega Corporation.)

fluorescence-activated cell sorting (FACS) analyses must be performed in order to generate dose-response curves to determine ADCP activity. Many labs simply do not have the flow cytometry equipment or cell differentiation and flow cytometry expertise to perform these tests. In addition, effector cells from individual donors cannot be pooled due to major histocompatibility (MHC) restrictions, resulting in inherent greater assay variability than in ADCC assays where pooling can and is often used to minimize variability.^[8]

The reproducibility problems presented by these functional bioassays are formidable. Having a well-controlled, easy-to-use, and reproducible assay in place as early as possible is a cornerstone in preventing delays in drug development. MOA-reflecting assays are more than just a tool for lot release and stability testing. They are used for confirmation of biosimilarity between the generic and the innovator product, for accelerated stress condition testing, and as supportive data for confirmation of successful scale-up. In some cases, they are also used as a supportive method for the selection of suitable clones at the beginning of biologic drug development. Authorities require a full validation of the method at an earlier phase in drug development, which places additional pressure on developers to have an assay in place that is suitable for fulfillment of validation criteria.

A Nimble Tool: The Reporter-Gene Bioassay

With regulators demanding data on the impact of ADCP in therapeutic antibody performance, biopharmaceutical developers are on the hunt for faster and more reliable bioassays to assess this MOA. One such tool that our lab has been studying for several years is a novel reporter-gene assay. The tool was developed by Wisconsin-based [Promega](#). Charles River established the primary ADCP assay using flow cytometry readouts for confirmation and comparison purposes. Drug developers are rapidly adopting Fc effector function reporter-based assays to measure ADCC and ADCP activity during therapeutic antibody development and production because they are able to provide a consistent and reliable mechanism of action-based measure of mAb bioactivity and stability. The multiple product formats, thaw-and-use cells, and simple workflow overcome the limitations of traditional primary cell-based assays.^[9]

In the reporter-gene assay we worked with, primary macrophages were replaced with an engineered Jurkat reporter cell line stably expressing FcγRIIIa (H131) and NFAT-RE/*luc2* (**Figure 2**). As with primary macrophages, the same signaling for ADCP was activated when the FcγRIIIa was bound by antibody that was bound to a target cell, meaning that the reporter assay reflected the *in vivo* molecular pathway for Fc receptor-mediated phagocytosis via macrophages.

Methodology

Rituximab, an early mAb known to induce ADCP activity through FcγRIIIa, offered a good test case for how the reporter-gene assay could be used to measure antibody effector function. The antibody works by binding to the protein CD20 antigen on the surface of mature B cells and B cell tumors, then recruits other soldiers of the immune system to kill malignant and normal mature B cells.

In order to assess ADCP function in the reporter gene assay, target cells were first incubated with a titration of rituximab. Once the therapeutic antibody was bound to the receptor on the target cell surface, the engineered effector cells were added. ADCP pathway activation ensued and resulted in the production of luciferase through activation of the reporter gene NFAT-RE/*luc2*. Luciferase activity was measured following a 4–24 hour induction period, after addition of the luciferase assay reagent. The dose-dependent response in the microtiter plate-based assay was then used to quantify the relative biological activity of the therapeutic antibody compared to the dose-response curve of a suitable reference item. Free antibody did not induce any phagocytosis response in the patient nor in the *in vitro* assay. The engineered Jurkat effector cells made in a thaw-and-use format were convenient and helped reduce assay run-to-run variability.

The bioassay sidestepped a number of obstacles that one encounters with more classic assays. It eliminated the need to source, purify, perform multi-day cytokine differentiation of macrophage effector cells, and it avoided the variability of different primary cell donors. The thaw-and-use engineered effector cell method allowed same-day results without culturing or having to worry about cell bank concerns—and the test was simple.

The assay was found to perform well (data not shown). Validation studies conducted according to the ICH Q2(R1)

guideline for different pairs of therapeutic mAbs and target cells fulfilled all requirements.^[10] Biocomparability studies found that the ADCP reporter bioassay captured differences in bioactivity between the brand name (innovator) rituximab—a strong inducer of ADCP—and third-generation follow-on molecules of rituximab. The bioassay was also highly specific. It detected responses when antibody binding occurred and none when non-relevant isotype antibody couldn't bind to target cells lacking the required surface receptor. The bioassay was able to quantify the ADCP induction of a variety of drug and research antibodies, and it was stability-indicating in detecting loss of biological activity of rituximab in this MOA under heat-stress conditions. This ability of bioassays to detect loss of biological activity is important for therapeutic antibodies that are prepared, formulated, stored, and transported before being administered.

Specific Issues With the ADCP Assay

Our efforts to advance a primary donor cell-based ADCP assay to a level where the biostatistics could be applied to the data in accordance with EP and USP statistics chapters were unsuccessful. Because ADCP is a two-step process—the macrophages need to first recognize and bind to the therapeutic antibody bound to the target cell leading to phagocytosis—flow cytometry turned out to be more difficult than expected. Using constitutively stained target cells makes it difficult to distinguish between binding and phagocytosis because the signals in the flow cytometer remain the same for both. Moreover, the dynamic range of the assay is quite limited, even for therapeutic antibodies known to be strong inducers of ADCP. This is the reason why alternative staining approaches capable of distinguishing between binding and phagocytosis were successful in confirming phagocytosis but were not suitable for setting up a complete assay. For instance, in the staining approach we used, target cells remained colorless in a neutral environment but became red in an acidic environment (inside macrophages) once phagocytosis occurred.

Conclusion

Our attempts to optimize the reproducibility and dynamic range of the primary cell-based assay in accordance with good manufacturing practice (GMP) requirements have fallen short, confirming why descriptions of ADCP function in the scientific literature are so sparse. We conclude that primary ADCP assays might be suitable for strong inducers of this MOA for confirmation purposes on a non-GMP level, but reporter-gene assays are potentially a better alternative. They are stability-indicating bioactivity assays with good reproducibility and validatability. Therefore, reporter-gene assays are a more suitable tool during routine lot release and stability testing and for reliable biocomparability testing.

In any case, assessing ADCP function is becoming increasingly important in therapeutic antibody development as

regulators are seeking a more complete picture of the potential pathways that therapeutic antibodies use to generate a certain response, particularly for biosimilars and biobetters that are modified to optimize the efficacy of the antibody on its intended target.

Changes to the binding epitopes may have a severe impact on the MOA of a follow-on biologic as compared to the innovator. Thus, in addition to simple binding tests of the Fc receptors to confirm a certain effector function potential, it becomes more relevant whether there is a significant impact on the MOA of a follow-on biologic mediated by the particular Fc receptor. For instance, in at least one case we have looked at, the innovator therapeutic mAb was known to induce ADCC but was a weak inducer of ADCP, yet the mAb being compared to the innovator had a completely different MOA—ADCC was not relevant, but ADCP was significantly enhanced.

Therefore, it is understandable that regulators want a more complete picture of potential pathways that therapeutic antibodies are using to generate a specific response. It is particularly relevant for biosimilars to confirm similarity in all potentially relevant MOAs. With biobetters, which have been modified to optimize the efficacy of the antibody on its intended target, these improved properties must be verified. Developing advanced tools to assess these different functions will be key to the future of therapeutic antibody development.

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