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Surrogate CD16-expressing effector cell lines for determining the bioactivity of therapeutic monoclonal antibodies

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ABSTRACT

Traditional antibody dependent cellular cytotoxicity (ADCC) assays use donor derived natural killer (NK) or peripheral blood mononuclear cells, but donor genetic variability and the technically challenging nature of the assay means that alternative in vitro assay formats are required. We explored the utility of two reporter gene cell lines, the J2 and J9, as surrogate effector cells for ADCC assays. Both express the ADCC relevant Fcγ receptor CD16, crosslinking of which leads to firefly luciferase expression. For anti-CD20 rituximab and anti-HER2 trastuzumab (both IgG1 monoclonal antibodies, mAbs) a dose dependent firefly luciferase response was observed exclusively in the presence of their respective targets, representing the molecular interaction which potentiates ADCC activity. Importantly, both surrogate effector and NK cell based assays gave statistically similar values for rituximab ADCC activity. Increased engagement with target cell bound mAbs was determined to be cytotoxic for the J2 and J9 cell lines at the assay end point (at which luciferase expression is measured). However, use of the J9 cells containing the constitutively expressed *renilla* luciferase gene enabled data normalisation and corrected for fluctuations in both cell number and viability providing an advantage over currently available surrogate effector cell-lines. Abrogated ADCC activity with IgG4 mAbs, but enhanced activity with an IgG1 non-fucosylated mAb, was seen with the J9 cell line, as expected. Additionally, two rituximab products (biosimilars in development) with similar binding by flow cytometry, *N*-glycan profiles using HPLC and CD16 binding by surface plasmon resonance showed comparable ADCC activity to Mabthera. The ADCC activity of another anti-CD20 mAb, ofatumumab, reported only with primary cell based assays to date was also measured. This is the first report of a dual reporter gene based ADCC assay.

1. Introduction

The therapeutic use of monoclonal antibodies (mAbs) is now well established. Most of the ~50 approved mAb products are used in the treatment of malignant and autoimmune diseases [1]. Biosimilar mAbs, which must demonstrate high similarity to the innovator product on which they are based, and novel and more complex antibody variants, have recently also gained approval. The therapeutic value of mAbs derives from their various mechanisms of action including preventing receptor overstimulation, ligand neutralisation, direct apoptosis, complement dependent cytotoxicity (CDC) and antibody dependent cellular phagocytosis [2]. Antibody dependent cellular cytotoxicity (ADCC) is another important mechanism through which mAbs activate immune effector cells leading to accelerated apoptosis of target cells. ADCC requires crosslinking of Fc receptors present on immune effector cells, primarily the Fcγ receptor IIIA (FcγR IIIA), CD16 displayed by natural killer (NK) cells [3]. A CD16 gene dimorphism gives rise to high [Valine 158; V158] and low [Phenylalanine 158; F158] affinity receptors [4]. It has been found experimentally that Fc-FcR affinity influences the level of ADCC activity [5]. Importantly, the implication that Fc affinity may impact upon the therapeutic benefit of mAbs has been reported. Weng and Levy [6] in a large study, calculated that progression free survival two years after rituximab treatment for larger follicular lymphoma was almost three times greater for homozygous V158 patients than F158 patients.

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Currently, most therapeutic mAbs are of IgG1 subclass, although several IgG2 and IgG4 antibodies have also been developed, the latter being used when effector cell activity is not required [7]. Since the mammalian cell expression systems that are employed for production of most mAbs are sensitive to environmental factors, post-translational modifications are carefully monitored, as these can have important implications for both the efficacy and safety of the final product [8]. This extends to the composition of the N linked glycans present on a mAb which confers structural stability, but also influences ADCC activity [9]. For example, whilst deglycosylation completely inhibits ADCC, defucosylation results in increased binding affinity to CD16a which mediates greater ADCC activity [10].

Traditionally, target cell death due to ADCC activity has been studied *in vitro* using peripheral blood mononuclear cells (PBMCs), or semi-purified NK cells. Such assays can be challenging, with complications arising not least from inter-donor variability of cells [11]. Both the immune status and genetic background of a donor, including possession of the 158 V or 158F CD16a alleles, and copy number variation at the CD16 gene locus, can give rise to significant variability in ADCC activity [12]. Difficulties with ADCC assays can also result from the readouts used. Chromium 51 (^{51}Cr) release has conventionally been used as a marker for target cell death, yielding highly sensitive results with low background signal [13]. In order to eliminate concerns associated with radioactivity, other markers have been evaluated, for example, calcein-acetoxymethyl, a fluorescent dye which is well retained by pre-loaded target cells. However, in comparison with the ^{51}Cr assay, spontaneous release of the marker is still high, reaching up to 40% over the course of the assay [14]. Additionally, culture media contains compounds that can auto-fluoresce, which also contributes to non-specific noise [15]. The release of intracellular enzymes lactate dehydrogenase (LDH), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are also often used as markers of cell death. However, it must be appreciated that LDH is not released exclusively by target cells, but also by effector cells, resulting in difficulties with interpretation.

Increasingly, the limitations of using donor derived effector cells are being overcome by the use of transfected NK or T cell lines. The NK92 cell line, for example, has been transfected to enable stable expression of the high or low affinity CD16a receptor [16]. ADCC activity has also been measured as a function of expression of a luciferase reporter gene upon CD16 receptor engagement. Parekh et al. [17] described the application of an engineered CD16-expressing Jurkat T cell line, in which luciferase gene expression is controlled by the nuclear factor of activated T cells (NFAT) transcription factor. For both NK and T cells, a CD16-induced increase in intracellular calcium that results from cross linking by antibodies allows translocation of NFAT to the nucleus, where it can bind to the promoter regions of ADCC relevant genes [18]. Recently, based on their previous work with a transfected human cell line, containing an NFκB firefly luciferase reporter gene construct and *renilla* luciferase gene, Lallemand et al. [19] produced two similarly engineered Jurkat T cell lines, the J2 and J9 lines for use as effectors for ADCC function. Both cell lines express CD16 and contain the NFAT controlled luciferase reporter gene. Luciferase transcription occurs under the control of the chimeric promoter NFAT Activator Protein 1 (AP-1), the latter of which is a heterodimer of Fos and Jun proteins activated by protein kinase C. Like NFAT, AP-1 is also regulated by downstream signals [20]. In order to account for any fluctuation in cell number, the J9 cell line contains an additional *renilla* reporter gene expressed by SV40, a promoter of constitutively expressed T cell genes.

In this paper, the utility of the J2 and J9 cell lines as surrogate effector cells for measuring ADCC activity of mAbs is demonstrated. Initial optimisation and characterisation of the assay were carried out using the well-established ADCC-mediating mAb rituximab, which targets B cell lymphocyte antigen CD20 and is widely used in combination with chemotherapy for the treatment of non-Hodgkin's lymphoma, chronic lymphocytic leukaemia and also treatment of rheumatoid arthritis [21]. Trastuzumab, which targets human epidermal growth factor receptor 2 (HER2) over-expressed in ~25% of breast and some stomach cancers, and cetuximab against epidermal growth factor receptor (EGFR), efficacious in colon, head, neck and lung cancers, were then used to demonstrate ADCC efficacy with adherent cell lines [22,23]. The value of J9 cells, which constitutively express the *renilla* luciferase reporter gene, in normalising the data is examined: this is a feature which is currently not present in other available reporter gene cell lines used to determine ADCC. Having confirmed the assay's sensitivity to glycosylation-both the presence of the *N*-glycan and fucosylation- the J9-based assay was used to compare the ADCC activity of two rituximab biosimilar products in development in conjunction with other analytical techniques. Finally, the fully human anti-CD20 antibody ofatumumab was examined, revealing that the J9 ADCC assay results corroborate with a limited report of its increased ADCC activity compared to rituximab.

2. Materials and methods

2.1. Reagents

Anti-HER2 trastuzumab (Herceptin), anti-CD20 antibodies rituximab (Mabthera), (both from Roche, Basel, Switzerland) and ofatumumab (Arzerra; Novartis, Basel, Switzerland), and anti-EGFR cetuximab (Erbitux; Merck, Darmstadt, Germany) and panitumumab (Vectibix; Amgen, CA, USA) are therapeutic products. The IgG1 and IgG4 rituximab and trastuzumab isotypes, non-glycosylated and non-fucosylated rituximab were from InvivoGen (CA, USA). Other reagents used were dead cell apoptosis kit containing annexin V and propidium iodide (Fisher Scientific, Loughborough, UK), anti-Human IgG (Fc gamma-specific) PE, anti-Human CD28 APC and Brilliant Violet 421 anti-Human CD56 (NCAM) (Affymetrix UK Ltd, High Wycombe, UK); anti-human CD16 (Cambridge Bioscience, Cambridge, UK); anti-human CD19 PE (BioLegend, CA, USA). IgG from human serum, used as a standard for HPLC *N*-glycan analysis, was obtained from Sigma Aldrich (MO, USA).

2.2. Cell culture

Human B cell lines: WIL2-S (lymphoblastoid cell line), Raji (derived from a Burkitt's lymphoma patient) and Daudi (lymphoblastoid) cells were cultured in complete medium: RPMI (Roswell Park Memorial Institute) 1640 (Sigma-Aldrich) containing 10% Foetal calf serum (FCS, Labtech, South American origin), penicillin/streptomycin (1.25 mg/ml), 1% glutamine supplemented (both from Sigma Aldrich). For Daudi cells, the medium was supplemented with 1 mM sodium pyruvate (Sigma Aldrich). SKBR3 cells from ATCC (Middlesex, UK) were cultured in McCoy's 5A medium (Sigma Aldrich) and A431 cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium, Sigma Aldrich) both with 10% FCS; penicillin streptomycin (1.25 mg/ml) 1% glutamine supplemented. The transfected J2 and J9 (ENS Cachan, Villejuif, France) were cultured in RPMI complete medium with 150 μg/ml Hygromycin and 50μg/ml Zeocin, and specifically 12.5 mM Puromycin for J9 cells (all from InvivoGen). PBMC's were separated from blood (obtained from leukocyte cones (NHSBT, Colindale)) using Histopaque-10771 (Sigma Aldrich). Following negative selection of NK cells with human NK cell Isolation Kit (Miltenyi Biotec, Bergisch Galdbach, Germany), NK cells were cultured in NK cell culture media (55% DMEM; 1% sodium pyruvate (Sigma Aldrich)); 30% F-12