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Research paper

High throughput screening for antibody induced complement-dependent cytotoxicity in early antibody discovery using homogeneous macroconfocal fluorescence imaging

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ABSTRACT

Complement-dependent cytotoxicity (CDC) represents an important Fc-mediated effector function of antibodies and is a quality often sought in candidates for therapeutic antibody development in cancer. Antibodies inducing potent CDC are relatively rare as the ability to induce CDC is strongly dependent on the antigen and epitope recognized as well as antibody isotype. To allow the identification of antibodies with optimal CDC characteristics in early stages of antibody discovery, we developed a homogeneous high throughput CDC assay, compatible with 384 and 1536 well formats and which therefore allows direct functional screening of very large panels of antibodies. Results obtained with our newly developed CDC method are consistent with those obtained with conventional assays. The assay proved to be robust, reliable over a wide reading window, easy to perform with low hands-on, high throughput, cost effective and applicable to crude hybridoma samples as typically available in early hybridoma discovery. In conclusion, we developed a novel high throughput assay for the identification of therapeutic antibody lead candidates with optimal CDC characteristics from large antibody libraries.

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1. Introduction

Antibodies are taking an increasingly important place in the arsenal of therapeutic drugs and are finding their way into the clinic for treatment of many different diseases including cancer, inflammatory diseases, transplantation and infectious disease. Approximately two dozen monoclonal antibodies have been approved for human use worldwide and over 160 are in clinical trials (Parren and van de Winkel, 2008). The growing insight in the mechanisms of action of therapeutic antibodies is demanding a greater scrutiny of specific functional activities of antibodies very early in development in order to select the best possible drug candidates. This trend, combined with the desire to screen very large numbers of candidates, requires the development of novel sensitive high throughput assays suitable for assaying antibody function.

Therapeutic antibodies may engage different mechanisms of action to induce a therapeutic effect. Complement-Dependent Cytotoxicity (CDC) represents an important activity for antibodies directed against cell bound tumor targets. CDC is one of the most potent cell killing mechanism that antibodies can employ and therefore represents a highly desired activity for therapeutic antibodies with cytotoxic activity. Complement activation is initiated by C1q binding to the antibody Fc fragment. The ability of antibodies to induce complement activation is strongly dependent on the antibody isotype and epitope recognized. The human isotypes IgG1 and

Abbreviations: CDC, Complement-dependent cytotoxicity; FACS, Fluorescent Activated Cell Sorter; FMAT, fluorometric microvolume assay technology; NHS, Normal Human Serum; IgG, Immunoglobulin G; HuIgG, Human IgG; HMFI, homogeneous macroconfocal fluorescence imaging.

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IgG3 activate complement particularly well, in contrast to IgG2 and IgG4. The ability to induce CDC is also critically dependent on the epitope recognized in which density and spatial positioning of antibody epitopes and distance of the epitope from the cell membrane are known to affect CDC potency (Bindon et al., 1988; Xia et al., 1993; Teeling et al., 2006). Antibodies of the same CDC-inducing antibody isotype but directed against distinct epitopes therefore often show very large differences in their ability to induce complement activation and cell lysis. Induction of CDC by antibodies is finally modulated by target cell characteristics, such as complement regulatory molecules including CD55 and CD59 (Gorter et al., 1996, Varsano et al., 1998, Weng and Levy, 2001), and resistance molecules such as mortalin (Pilzer and Fishelson, 2005) which should be taken into account in screening design. To identify antibodies which potently induce CDC it is therefore highly desirable to screen antibodies against various cell types in early discovery. Different technologies are currently employed to determine the CDC-inducing capabilities of tumor-directed antibodies. The classical method to detect complement-mediated lysis is by assessing chromium ^{51}Cr) release (Sier et al., 2004). Other methods make use of assessing cell viability such as the MTT (Wulf et al., 2006) and Alamar blue assay (Gazzano-Santoro et al., 1997). Finally, cell killing may be quantified by assessing DNA staining of dead cells by propidium iodide using flow cytometry (Gratama et al., 1983). Although these assays have proven to be reliable in CDC detection they are unsuitable for screening of large libraries due to assay handling, serum usage and throughput limitations as well as relatively high costs.

In this paper we describe the development of a high throughput fluorescence assay in which detection of biological activity is performed by following a fluorescent signal using homogeneous macroconfocal imaging in FMAT. Our assay is useful for integration into early antibody discovery to screen for antibodies with pre-defined CDC characteristics.

2. Material and methods

2.1. Reagents

Human antibodies against human CD38 and KLH were developed at Genmab (Utrecht, The Netherlands). Chrompure Human IgG was purchased from Jackson Immuno (Newmarket, UK). TOPRO-3 Iodide was purchased from Molecular Probes/Invitrogen (Leiden, The Netherlands) and DRAQ5™ was obtained from Biostatus (Shepshed, UK). Rituximab (MabThera) was obtained from Roche (Woerden, The Netherlands). Alamar blue was purchased from Biosource (Breda, The Netherlands) and NHS was obtained from Sanquin (Amsterdam, The Netherlands). Sodium azide was obtained from Sigma-Aldrich chemicals (Zwijndrecht, The Netherlands) and BSA fraction V was obtained from Roche (Mannheim, Germany). Triton-X100 was obtained from Riedel de Haen (Seelze, Germany).

2.2. Cell lines

CHO-CD38 cells, Chinese Hamster Ovary cells expressing human CD38, were kindly provided by Prof. M. Glennie (Tenovus Research Laboratory, Southampton General Hospital, Southampton, UK). Daudi-luc cells were generated as described in Bleeker et al., 2008.The cells were cultured in RPMI 1640 (Cambrex Bioscience, Verviers, Belgium) culture medium supplemented with 10% FCS (Optimum C241, Wisent Inc., St. Bruno, QC, Canada), 2 mM L-glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin, 1 mM sodium pyruvate (all purchased from Gibco BRL, Life Technologies, Paisley, Scotland). Medium was refreshed twice a week. Before use, cells were split and seeded out at $1–1.5\times10^6$ cells/ml to ensure viability and optimal growth.

2.3. Detection of CDC using macroconfocal fluorescent imaging

For the large volume assay, CHO-CD38 or Daudi-luciferase cells were plated into 96 well black clear bottom assay plates (Greiner) at 15,000 cells per well in RPMI 1640 (Cambrex) plus 0.1% BSA (Roche, Mannheim, Germany). Subsequently, 50 µl antibody dilution in RPMI 1640 plus 0.1% BSA was added to each well, and the samples were incubated for 15 min at room temperature. This was followed by addition of 50 µl 60% Normal Human Serum in RPMI 1640 plus 0.1% BSA to each well and incubation for 45 min at 37 °C, 5% CO2 and 80% humidity. As positive and negative controls, rituximab and anti-KLH or Chromepure human IgG were used respectively in assay buffer (0% Lysis) and Triton-X-100 (100% Lysis).

For control staining, 50 µl of DRAQ5 (180 nM), or TOPRO-3 (6 nM) was added to the particular control wells, and 50 µl of TOPRO-3 plus DRAQ5 (16 nM/180 nM) mixture was added to test wells. All dye combinations were diluted in FMAT buffer (PBS, 0.1% BSA and 0.02% Sodium Azide). After a 4–10 h incubation, detection was performed using an 8200 Cellular Detection System (Applied Biosystems, Foster City CA, USA).

Miniaturized assays were performed in 384 black clear bottom assay plates (Greiner) and 1536 well polystyrene plates (Nunc, Rochester USA) using a Sciclone ALH 3000 workstation (Caliper Life Sciences, Hopkinton, MA, USA) equipped with a 384 low volume head. The low volume assays differ in volumes and not in concentrations of cells, antibodies, serum and dyes. For experiments using 384 and 1536 well plates, the volumes for each addition were respectively 15 µl and 2 µl, resulting in 4500 cells per well for 384 well plates and 600 cells per well for 1536 well plate assays. The final volumes for the assays are 60 µl for 384 well and 8 µl for 1536 well plates. In order to prevent evaporation the plates were sealed with aluminium foil using the plateloc sealer (Agilent Technologies, Velocity 11 Automation Solutions, Santa Clara, CA, USA).

2.4. CDC detection with Alamar blue

50 µl CHO-CD38 or Daudi-luciferase cells were plated into 96 assay plates (Greiner) at 25,000 cells per well (in RPMI 1640 plus 0.1% BSA). Subsequently, 50 µl antibody dilution in RPMI 1640 plus 0.1% BSA was added to each well, and the samples were incubated for 15 min at room temperature. This was followed by addition of 20 µl 100% Normal Human Serum and 25 µl of Alamar blue solution to each well, and incubation at 37 °C, 5% CO_2 and 80% humidity for 5 h. As controls we used anti-KLH or Chromepure human IgG diluted in assay buffer to determine maximal response level (no lysis).

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