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A method to quantitate the neutralizing capacity of anti-therapeutic protein antibodies in serum and their correlation to clinical impact

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ABSTRACT

A robust, quantitative method for assessing the neutralizing capacity of anti-therapeutic protein antibodies was developed and tested using 4 analytical assay platforms typically used for detection of anti-drug neutralizing antibodies. The method described here utilized titration of increasing concentrations of therapeutic protein into serum containing anti-therapeutic protein antibodies, either positive control antibodies or clinical samples. Neutralizing capacities were calculated by determining the EC_{50} from the titration curves. The neutralizing capacity of purified anti therapeutic protein antibodies was expressed in terms of "μg of drug neutralized per μg of anti-TP antibody" present. In the case of serum originating from clinical study subjects, the neutralizing capacity of the samples was expressed as "μg of drug neutralized per mL of serum". A relative shift in EC_{50} values was observed as the amount of serum or antibody was changed resulting in a proportional shift of the calculated neutralizing capacity. Application of this approach using different assay platforms was consistent providing evidence for its potential to be a useful approach to characterize, qualify and compare neutralizing positive control antibody preparations or clinical samples. Using this methodology, we were able to draw a clear correlation between the neutralizing capacity and the effect of these antibodies on a clinical pharmacodynamic (PD) marker. Determination of the neutralizing capacity of antibody positive samples from subjects in a clinical study indicated direct correlation of pharmacodynamic results with non-response, partial response and response to high, mid and low neutralizing capacities respectively.

1. Introduction

The immunogenicity of therapeutic proteins (TPs) is an important point of consideration during the development and commercialization process [1]. Immune responses to TPs that resemble an endogenously expressed protein can have serious implications for patient safety as demonstrated by the noted incidence of antibody-mediated pure red cell aplasia (PRCA) associated with erythropoiesis-stimulating agents (ESAs) [2]. In other cases, immune responses to TPs have been noted to result in reduction of efficacy and bioavailability of the therapeutic(s) in the clinical setting [3–6]. Consequently, it is a regulatory expectation to perform immunogenicity testing for TPs during early and late stage development to gain approval for marketing.

Immunogenicity testing typically employs a two-tiered testing strategy where study samples at appropriate study time points are tested for anti-drug antibodies (ADAs) first in an immunoassay that is well-suited for the detection of binding antibodies. Samples found to contain binding ADAs are subsequently tested for their ability to inhibit or neutralize the biological activity of the drug in a suitable neutralizing antibody (NAb) assay [7,8]. Depending upon the risk associated with unwanted immunogenicity of the therapeutic, further characterization of the antibodies may be done including isotyping, quantitation or titer determinations [9]. It has been recommended by some regulatory agencies that the neutralizing capacity of anti-therapeutic protein antibody responses be quantified in a meaningful manner [10].

The semi-quantitative determination of the neutralizing capacity of antibodies is conducted extensively during vaccine development [11,12] and during assessment of immune responses to cytokine therapies such as interferons [13,14]. Expression of neutralizing capacity of antibodies is often done in terms of (a) titer, expressed as the dilution of serum to the extent the neutralizing antibody can be detected, or (b) percent neutralization

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of a known concentration of drug [7,15,16]. Obtaining titer values by performing serial dilution of the sample is relatively easier of the abovementioned two approaches; however these values may be inadequate for comparison and correlation of clinical effects of NAbs within and/or across studies. The approach of evaluating percent neutralization requires use of a robust drug standard curve that can be used for drug quantitation. To calculate the percent neutralization, a known amount of the drug is spiked into serum containing varying concentrations of anti-drug antibody [17]. In this approach there is the potential of interference by serum factors that could enhance or inhibit the drug's activity resulting in confounding results. In each case the relevance of the measurement (e.g. correlation to clinical effects) and comparability of the results between platforms or drug products is challenging. Consequently, there is a need to develop an approach that provides a reliable and biologically relevant quantitative measurement of the neutralizing capacity of anti-drug antibodies that is not significantly impacted by the method or the preparation of positive control antibody used to ascertain this value.

Here, we describe a method to quantify the neutralizing capacity of purified monoclonal and polyclonal anti-therapeutic protein antibodies and antisera by expressing the amount of drug neutralized per mass concentration of antibody or unit volume of serum. We demonstrate that the method is applicable across assays that detect neutralizing antibodies to multiple therapeutic proteins as well as across assay platforms.

2. Materials and methods

Recombinant human growth factors, cytokines, biotinylated antibodies and cytokine detection ELISA kits were obtained from R&D Systems (Minneapolis, MN, USA). Murine monoclonal anti-phosphotyrosine antibody clone 4G10 was obtained from Upstate Biotechnology (Lake Placid, NY, USA). Therapeutic proteins 1, 2, 3, 4 and 5 (TP1, TP2, TP3, TP4, TP5) were obtained from Amgen Inc. (Thousand Oaks, CA, USA). Pooled normal human serum (PNHS) and serum from normal individual human subjects were obtained from Bioreclamation (Hicksville, NY, USA). MSD Blocker A solution, MSD Antibody diluent, avidin coated MSD 6000 96-well microtiter plates, ruthenium conjugated goat anti-mouse IgG, ruthenium conjugated streptavidin, MSD PR 100 96 well microtiter plates, streptavidin-coated MSD PR 100 microtiter plates and MSD 4X T-Buffer with surfactant were obtained from Mesoscale Discovery (MSD, Gaithersburg, MD, USA). Sodium orthovanadate, bovine serum albumin 35% solution, 30% H_2O_2 solution, Tween-20 and protease inhibitor cocktail were obtained from Sigma–Aldrich (St. Louis, MO, USA). Cell lysis Buffer containing 0.15 M NaCl, 0.05 M Tris–HCl (pH 7.4), 6 mM sodium deoxycholate, 0.5% NP-40 was prepared internally (Amgen Inc.).

Prior to use, sodium orthovanadate was diluted 1:10 with 1× DPBS and activated for at least 15 min by the addition of H_2O_2 to a final dilution of 0.18% H_2O_2. Protease inhibitor cocktail was added to the cell lysis buffer at a 1:200 dilution and activated orthovanadate at a final concentration of 30 μM, prior to the addition to cells. Antisera and affinity-purified rabbit polyclonal anti-therapeutic protein antibodies (pAb) were obtained from Amgen Inc. Monoclonal anti-TP1 antibodies (mAb) clone 2 and 3 were obtained in-house (Amgen Inc). Anti-TP1 monoclonal antibody clone 1 was obtained from STEMCELL Technologies Inc. (Vancouver, Canada). ^{3}H-Thymidine was obtained from Amersham Biosciences (Piscataway, NJ, USA).

Serum samples from NAb-positive subjects from a TP5 clinical study was obtained for characterization of neutralizing capacity and antibody titer. Samples were used within the guidelines of the informed consent for antibody testing for these samples.

2.1. Cell culture

Cell lines were obtained from ATCC and banked and stored in-house (Amgen Inc).

Adherent cells (COS-1 and A431) were maintained in high glucose DMEM (Gibco-BRL, Carlsbad, CA, USA) with 10% fetal bovine serum (Hyclone, South Logan, Utah, USA) and 100 U/mL penicillin, 100 μg/mL streptomycin and 0.292 mg/mL glutamine in 0.0085% saline and 0.10 mM citrate buffer (Gibco-BRL) in a 36 ± 2 °C incubator with 5 ± 1% CO_2 and 90 ± 10% relative humidity. Cells were passaged every 3 to 4 days at 1×10^5, 2×10^5 or 4×10^5 cells/mL in a 225-cm^2 flask. Cell monolayers were washed twice with Dulbecco's PBS (DPBS, without magnesium or calcium chloride, (Gibco-BRL) followed by the addition of 5–15 mL trypsin-EDTA (Gibco-BRL). Cells were incubated with trypsin-EDTA for up to 10 min until detached. Growth medium was added at twice the trypsin volume and cells were counted for an assay or seeded in a 225 cm^2 flask.

Growth factor-dependent 32D suspension cell lines were maintained in RPMI (Gibco-BRL) containing 15% heat inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.292 mg/mL glutamine in 0.0085% saline and 0.10 mM citrate buffer and 10 U/mL recombinant human growth factor (Amgen Inc.) in a 36 ± 2 °C incubator with 5 ± 1% CO_2 and 90 ± 10% humidity. Cells were passaged every 2 to 3 days at 5×10^4, 1.5×10^5 or 3×10^5 cells/mL in a 75-cm^2 flask.

2.2. ^{3}H-thymidine incorporation assay

TP1-dependent 32D suspension cells were washed 3 times to remove growth factor by pelleting the cells with centrifugation at 1000–1200 rpm (200–300 × *g*) for 3–5 min. Cells were resuspended at a concentration of 5×10^5 mL^{-1} in growth medium in a 75-cm^2 flask and incubated for 15–32 h prior to use in the assay. Affinity-purified rabbit polyclonal anti-TP1 antibodies, rabbit anti-sera or anti-TP1 monoclonal antibodies were added to PNHS or individual subject human serum at various concentrations and diluted to a final concentration of 1:20 with growth medium.

TP1 was added at varying concentrations and allowed to incubate at ambient temperature for at least 30 min prior to addition to cells plated at 20,000 cells/well in U-bottom 96 well microtiter plates (Becton Dickson, Franklin Lakes, NJ, USA). Once added, the serum mixtures and cells were incubated for 44 ± 1 h. ^{3}H-thymidine was prepared in growth medium and 2 μCi was added to all wells and allowed to incubate for 4 ± 1 h. Cells were harvested onto filter plates (Perkin Elmer, Waltham MA, USA) using a Perkin Elmer Cell Harvester. The plates were allowed to dry prior to the addition of 40 μL of Liquid Scintillation Fluid (Perkin-Elmer). The plates were sealed and the scintillation counts per minute (CPM) were measured using a TopCount Liquid Scintillation and Luminescence Plate Reader (Perkin-Elmer).

2.3. ATP activity proliferation assay

Growth factor-dependent suspension cells were prepared as described in Section 2.2. Rabbit polyclonal, affinity purified anti-TP1 antibodies, rabbit anti-sera or anti-TP1 monoclonal antibodies were added to PNHS or individual subject human serum at various concentrations and diluted to a final concentration of 1:20 with growth medium. TP1 was added at varying concentrations and allowed to incubate at ambient temperature for at least 30 min prior to addition to cells plated at 5000 cells/well in U-bottom 96 well microtiter plates (Becton Dickson). Once added, the serum mixtures and cells were incubated for 44 ± 1 h. Cell Titer-Glo reagent (Promega Corporation, Madison, WI, USA) was prepared according to manufacturer's protocol and added in 100 μL volumes to all