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Research paper

Development and validation of cell-based luciferase reporter gene assays for measuring neutralizing anti-drug antibodies against interferon beta

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ABSTRACT

Neutralizing anti-drug antibodies (NAbs) against therapeutic interferon beta (IFNβ) in people with multiple sclerosis (MS) are measured with cell-based bioassays. The aim of this study was to redevelop and validate two luciferase reporter-gene bioassays, LUC and iLite, using a cut-point approach to identify NAb positive samples. Such an approach is favored by the pharmaceutical industry and governmental regulatory agencies as it has a clear statistical basis and overcomes the limitations of the current assays based on the Kawade principle. The work was conducted following the latest assay guidelines. The assays were re-developed and validated as part of the "Anti-Biopharmaceutical Immunization: Prediction and analysis of clinical relevance to minimize the risk" (ABIRISK) consortium and involved a joint collaboration between four academic laboratories and two pharmaceutical companies. The LUC assay was validated at Innsbruck Medical University (LUC_{IMU}) and at Rigshospitalet (LUC_{RH}) Copenhagen, and the iLite assay at Karolinska Institutet, Stockholm. For both assays, the optimal serum sample concentration in relation to sensitivity and recovery was 2.5% (*v*/*v*) in assay media. A Shapiro–Wilk test indicated a normal distribution for the majority of runs, allowing a parametric approach for cut-point calculation to be used, where NAb positive samples could be identified with 95% confidence. An analysis of means and variances indicated that a floating cut-point should be used for all assays. The assays demonstrated acceptable sensitivity for being cell-based assays, with a confirmed limit of detection in neat serum of 1519 ng/mL for LUC_{IMU}, 814 ng/mL for LUC_{RH}, and 320 ng/mL for iLite. Use of the validated cut-point assay, in comparison with the previously used Kawade method, identified 14% more NAb positive samples. In conclusion, implementation of the cut-point design resulted in increased sensitivity to detect NAbs. However, the clinical significance of these low positive titers needs to be further evaluated.

1. Introduction

Long-term repetitive treatment with biopharmaceuticals can give rise to anti-drug antibodies, a proportion of which will be neutralizing (NAb) and will block the effects of the drug. For people with multiple sclerosis (MS) receiving interferon beta (IFNβ) treatment, quantification of NAbs may give an early indication of a loss of drug efficacy. If the titers are persistently high, a shift to alternative drugs is recommended to ensure continuous treatment benefit (Polman et al., 2010). However, there are variations between laboratories in the NAb assays and the validation processes used. In this study the currently used reagents and cell culture methods were harmonized between laboratories and the assays developed and validated in parallel.

All bioassays used to measure NAbs against IFNβ are based on the inhibition of cellular responses to IFNβ. However, different read-outs have been adopted over time, e.g. by measuring the cytopathic effect during viral challenge or quantification of IFNβ-receptor specific signals like

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myxovirus resistance protein A expression at the protein or mRNA level (Pungor et al., 1998; Bertolotto et al., 2007).

A more direct read-out is achieved by determining the IFN-induced gene expression in cells stably transfected with a firefly luciferase reporter-gene. Two versions of the luciferase reporter-gene bioassay are currently used for clinical routine testing of anti-IFNβ NAbs. One luciferase-based bioassay (LUC), which was described by Lam and colleagues 2008 (Lam et al., 2008), depends on the propagation of a human fibrosarcoma cell line HT1080 transfected with a luciferase reporter-gene controlled by an IFN-inducible promoter (Uze et al., 1994). The other assay is available as a commercialized kit (iLite®, Biomonitor/Euro Diagnostica) where a luciferase reporter-gene, linked to the interferon-stimulated response element from the interferon stimulated gene 15, is transfected into the promonocytic cell line U937 (clone PIL5) (Lallemand et al., 1996, 2008), which has been growth arrested with the antimitotic drug vinblastine. In the kit these cells are provided frozen and are thawed and dispensed on the day the assay is run. In both bioassays the Kawade method is used to calculate the NAb titer, defined as the reciprocal value of the serum dilution giving a ten-fold reduction in the IFNβ stimulation, and expressed in ten-fold reduction units (TRU/mL). A two-step NAb testing procedure is used in the routine clinical setting. Samples are firstly screened to identify positive samples and the positive samples are titrated and reanalyzed to obtain a titer value.

Both luciferase-based bioassays have been validated before (Lam et al., 2008; Lallemand et al., 2008) and the iLite assay is CE marked (Biomonitor). However, several new recommendations on how to harmonize and perform immunogenicity testing using bioassays have been published (Gupta et al., 2007, 2011; Shankar et al., 2008; Mire-Sluis et al., 2004) since these assays were validated. Important factors not considered in the earlier validation of these assays were the serum matrix effect and the use of a statistically based cut-point approach. Here the assays were developed to include a constant matrix concentration, revalidated according to these latest recommendations (Gupta et al., 2007, 2011; Shankar et al., 2008; Mire-Sluis et al., 2004), and a comparison was made between the different sites involved.

During validation, both the LUC assay at the Innsbruck Medical University (LUC_{IMU}) and Rigshospitalet in Copenhagen (LUC_{RH}) and the iLite assay proved to be specific and showed high sensitivity. The LUC assay was successfully revalidated at both sites whilst the iLite validation was stopped when significant cell batch to batch variations were obtained in the sensitivity and the magnitude of the response to IFNβ.

2. Materials and methods

2.1. Serum samples

Serum samples were selected based on the following criteria; healthy human donors and MS patients receiving IFNβ treatment with known NAb status as defined by routine analysis using the standard iLite (Biomonitor) assay. A human serum mixed gender pool from 50 healthy individuals, treatment naïve to IFNβ, was supplied by Sera Laboratories [SeraLabs] International Ltd., UK. The pooled serum (SeraLabs) was used for all development work, as the negative control (NC) and as an additive for the assay medium. For the assessment of minimum required dilution (MRD), assay and specificity cut points, and sensitivity (iLite) a different set of 54 human serum samples from healthy individuals was collected (Stockholm blood center, Sweden). Confirmed sensitivity assessment in the LUC assay was performed using different sets of ten serum samples from healthy individuals (Rigshospitalet, Denmark; Innsbruck blood donating service, Austria). For a comparison of the Kawade versus cut-point assays, samples from 30 MS patients on IFNβ treatment recruited from four neurological hospital centers in Sweden were used (IFNβ treatment at time of sampling was: 10% Avonex, 20% Rebif, 70% Betaferon). To determine the impact of the increased sensitivity, 49 MS patients (Rigshospitalet, Denmark) on IFNβ treatment that tested NAb negative for more than a year, were used (IFNβ treatment at time of sampling was: 76% Avonex, 22% Rebif, 2% Betaferon). Written informed consent was given by all blood donors.

2.2. Neutralizing anti-drug antibody assays and equipment

Assay development and validation was performed using modified versions of the LUC bioassay (Lam et al., 2008; Farrell et al., 2008), and the commercially available *iLite*™ anti-human IFNβ-1a bioassay (Biomonitor/Euro Diagnostica) (Lallemand et al., 2008). In both assays patients' serum, together with recombinant IFNβ, is added to the cells. The IFNβ stimulates the IFNβ receptor and induces transcription of the luciferase gene. Luciferase expression is inversely correlated with the amount of NAbs present in the serum sample. The LUC assay was basically performed as previously described (Farrell et al., 2008) and the iLite assay according to the manufacturers protocol (Biomonitor) with modifications described below. Luciferase activity was measured using DTX 880 Multimode Detector (Beckman Coulter) for LUC_{IMU}, LUMIstar optima (BMG Labtech) for LUC_{RH}, and GloMax® 96 Microplate Luminometer (Promega) for iLite. The signal magnitude was expressed in terms of relative luminescence units (RLU).

2.3. Reagents

In the LUC assay, the cell culture media consisted of DMEM + GlutaMAX (product code: 61965, GIBCO, Life Technologies) supplemented with 10% fetal bovine serum (FBS) (product code: 10500, GIBCO, Life Technologies), HAT Media Supplement Hybri-Max (product code: H0262, Sigma-Aldrich), 0.45 g $NaHCO_3$ (Sigma-Aldrich), and 1% Antibiotic–Antimycotic (product code: A5955, Sigma-Aldrich). DMEM supplemented with 1% pooled human serum (SeraLabs) (essential for the prevention of the loss of IFNβ-1a through non-specific absorption to plastic surfaces) was used as the assay media. The recombinant human IFNβ-1a (Avonex) preparation was kindly donated by Biogen Idec and had an activity of 12×10^6 international units per milliliter (IU/mL). Luciferase expression was measured by the addition of Steady-Glo® Luciferase Assay System (product code: E2520, Promega). Cells were grown in 175 cm^2 flasks (product code: 10246131, Fisher Scientific). White-walled 96-microwell plates with clear bottoms were used as the assay plates (product code: 655098, Greiner Bio-One Ltd.) and polypropylene 96-microwell NUNC plates (product code: 732–2662, VWR) as sample preparation plates.

iLite™ cells, *iLite*™ recombinant human IFNβ-1a (950 IU/mL), *iLite*™ Bright-Glo™ luciferase assay buffer and substrate, and white-walled 96-microwell culture plates were provided with the *iLite*™ IVD kit 85–88 (Biomonitor). In addition to the provided reagents, RPMI (product code: 61870010, Invitrogen), supplemented with 1% Penicillin Streptomycin (product code: 15140122, Invitrogen) and 1% pooled human serum (SeraLabs), was used as the assay diluent. Polystyrene 96-well round bottom plates were used for sample preparation (code: 3799, Corning).

A rabbit anti-human IFNβ polyclonal antibody (product code: 500-P32B, PeproTech) was used as positive control for both the LUC and iLite assays.

Immunodepletion, for the assessment of assay specificity, was performed in 1.2 μm Durapore membrane filter-plates (product code: MSBVN1210, Merck Millipore) using Protein G/A Agarose beads (product code: IP10, Calbiochem Millipore) and 96-well V-bottom collection plates (product code: MSCPNPP00, Merck Millipore).

2.4. Cell lines and cultivation

The LUC assay utilizes the human fibrosarcoma cell line HT-1080 (ATCC) clone HL116 (Uze et al., 1994) and the iLite assay uses division-arrested, frozen promonocytic U937 (clone PIL5) cells that do not require cell culturing (Lallemand et al., 2008). Both cell lines carry

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