Clinica Chimica Acta 453 (2016) 147–153

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Clinical laboratory application of a reporter-gene assay for measurement of functional activity and neutralizing antibody response to infliximab

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ARTICLE INFO

Article history:
Received 17 September 2015
Received in revised form 10 December 2015
Accepted 11 December 2015
Available online 12 December 2015

Keywords:
Infliximab
Neutralizing antibody
Reporter-gene assay
Cell-based assay
TNF-α antagonist

ABSTRACT

Background: TNF-α antagonists such as infliximab are effective for the treatment of inflammatory bowel disease and other inflammatory and autoimmune diseases. Development of an immune response and subsequent neutralizing antibodies against these protein-based drugs is a major impediment that contributes to therapeutic failure, or adverse effects such as hypersensitivity reactions. As opposed to empirical dose-escalation strategies, rational and cost-effective evaluation of clinical non-responsiveness includes measurement of serum drug levels, and detection of drug-specific antibodies. We present the validation and 2-y experience using a functional, cell-based reporter gene assay (RGA) developed for measuring the biological activity and antibody response to serum infliximab.
Methods: The RGA was used to test 4699 clinical specimens from patients suspected of therapeutic failure. In contrast to binding assays, which detect an overall antibody response, the RGA specifically detects those antibodies that have drug-neutralizing function, and thus, poses higher risk for therapeutic failure.
Results: The RGA presented here is currently the only functional clinical test available to measure serum infliximab activity and neutralizing antibodies.
Conclusions: Due to its accuracy and precision, and suitability for high-throughput testing, this robust platform can be applied to any TNF-α antagonist, providing an invaluable tool for the clinical management of patients with treatment failure.

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1. Introduction

The use of tumor necrosis factor (TNF-α) antagonists, such as infliximab, has revolutionized therapy in patients with chronic inflammatory and autoimmune diseases, such as rheumatoid arthritis, psoriasis, ulcerative colitis and Crohn's disease [1,2]. Infliximab is a chimeric monoclonal antibody against TNF-α, which is given to patients who fail conventional medical therapy, and is used for induction and maintenance of clinical remission. However, although infliximab is well tolerated for most patients, not everyone responds to therapy. About a third of patients experience primary nonresponse, which is caused by a rapid clearance of infliximab in the absence of any immune reaction to the drug [3]. Among those who show initial response to the drug, response vanishes over time in approximately half of them [4]. The most common cause of this secondary nonresponse is the production of antibodies against infliximab, which neutralize the effect of the drug [5].

The development of an immune response to a therapeutic protein is not uncommon, and while it occurs more frequently with foreign proteins, it can also occur with repeated injections of fully human proteins. In addition to decreasing efficacy, these antibodies may also adversely affect the safety of treatment and can potentially cause immune complex-mediated allergic reactions (serum sickness), or rarely, the development of autoantibodies due to cross-reactive epitopes [6]. According to a recent comprehensive review, 25.3% of all patients treated with infliximab, a chimeric molecule combined of both mouse and human sequences, developed drug-specific antibodies [7].

The clinical management of patients who become nonresponsive to infliximab often follows a sequence of empirical steps, beginning with increasing the dose of infliximab, or shortening dose intervals, to address possible low drug concentrations [8,9]. If this has no effect, it is followed by switching to another type of TNF-α antagonist, to address possible emergence of antibodies against the drug. When that fails, therapy is changed to a different biological class of drug, for example, interleukin 6 antagonists, as the treatment history suggests the involvement of non-TNF-α-mediated inflammatory pathways. In this empiric strategy, no early attempt is made to identify the mechanism for loss of response to infliximab, or to investigate if the symptoms are due to causes other than TNF-α-mediated mechanisms.

Abbreviations: HMSA, homogenous mobility shift assay; HPLC, high-performance liquid chromatography; IFN-γ, interferon γ; NFκB, nuclear factor-κB; RGA, reporter gene assay; sCD40L, soluble CD40 ligand; TNF-α, tumor necrosis factor-α.

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http://dx.doi.org/10.1016/j.cca.2015.12.015
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A rational and mechanistic alternative to this empiric strategy is to apply laboratory testing to measure infliximab and drug-specific antibody levels, in order to select the best treatment based on the most likely mechanism responsible for loss of response. Support in favor of the test-guided strategy comes from recent studies showing significantly reduced average treatment costs per patient, compared to the empirical approach, without differences on clinical efficacy [10–14].

Several methodologies are currently available for measuring serum infliximab levels and anti-infliximab antibodies in clinical laboratories. These are primarily binding assays, and include solid-phase ELISA methods [15], and a liquid phase, HPLC-based homogenous mobility shift assay (HMSA) [16]. Recently a reporter-gene assay (RGA) has become available [17], which allows for the measurement of serum infliximab activity, and detection of functional, neutralizing antibodies to infliximab by using a cell-based test.

2. Materials and methods

2.1. Materials

iLite™ TNF-α-sensitive reporter cells and TNF-α were obtained from Euro Diagnostica. *iLite*™ TNF-α-sensitive cells are division-arrested erythroleukemic K-562 cells transfected with a NFκB-regulated firefly luciferase reporter gene construct. In addition, a Renilla luciferase reporter gene is also co-transfected under the control of a constitutive promoter that allows TNF-α induced firefly luciferase to be normalized relative to Renilla luciferase expression. Infliximab was purchased from Janssen Biotech, Inc. Infliximab calibrators (5 to 40 μg/ml), infliximab diluent (8% normal human serum in RPMI 1640), and infliximab antibody diluent (40% normal human serum in RPMI), as well as test controls, were produced by the Reagent Laboratory of ARUP Laboratories. Dual-Glo® Luciferase Assay System (Promega Corp.) was used for detection.

2.2. Patient sera

Patient sera were submitted for clinical testing to ARUP Laboratories, most commonly for investigating treatment failure. For the purpose of this publication, the patient data was de-identified, and used as aggregate for our analysis, therefore informed consent was not required. ARUP has an IRB in place that allows us to use de-identified specimens from clinical testing for validation of assay methods.

2.3. RGA method

2.3.1. Infliximab

Serum samples (diluted 1:50, 1:150, 1:450 and 1:1350), infliximab calibrators and controls are pre-incubated with a fixed amount of TNF-α (16 ng/ml) for 30 min at 37°. Subsequently *iLite*™ TNF-α-sensitive cells are added and further incubated for 3 h at 37°. Following incubation, Dual-Glo® Luciferase Assay System reagents are added according to manufacturer instructions and bioluminescent signals of firefly and Renilla luciferases are recorded using the MicroBet TriLux Luminescence Counter (PerkinElmer). TNF-α-induced activation of the firefly luciferase reporter gene construct is inversely proportional to the amount of infliximab present in samples. After normalization of firefly luciferase expression relative to the Renilla luciferase level, serum infliximab concentration is calculated according to the calibration curve established with known concentrations of infliximab.

2.3.2. Neutralizing antibodies

For measurement of neutralizing infliximab antibodies, all patient samples undergo primary screening. Serum samples diluted 1:20 along with negative and positive controls are pre-incubated with fixed amount of infliximab (320 ng/ml) for 30 min at 37°, 5% CO_2. This allows infliximab antibody, when present, to bind to the added infliximab. Following incubation, TNF-α (16 ng/ml) is added for 30 min at 37° (5% CO_2). Subsequently, iLiteTM cells are added, and DualGlo® reagents are used to determine bioluminescent signals as described above. Samples showing positive anti-infliximab activity in the screen undergo confirmatory testing using serial two-fold dilutions, to determine endpoint titer. For each sample, firefly luminescence normalized by Renilla luminescence is plotted against serum dilution factors to determine antibody titer.

2.4. Test performance evaluation

Accuracy of infliximab RGA method was determined by performing the assay with normal human serum spiked with 3 different concentrations of infliximab (4.6 μg/ml, 9.3 μg/ml and 37 μg/ml). Linearity was assessed by preparing serial dilutions (1:2) of a sample containing 37 μg/ml infliximab using normal human serum. Precision was evaluated on three serum samples containing three different infliximab concentrations for three consecutive days. Limit of detection was calculated as mean plus two standard deviations of nine replicas of assay diluent. Upper limit of the reference interval for the drug test was calculated as central 95% of the infliximab concentrations of 96 sera of apparently healthy donors from ARUP clinical laboratory collection. Linearity of the neutralizing infliximab antibody test was performed by running a sample with high neutralizing antibody concentration (titer 1:5000) serially diluted 1:2 with normal human serum; precision was determined by running three samples for three consecutive days. Lower limit of the reference interval for the neutralizing anti-infliximab antibody test was confirmed by running the assay on 96 sera of apparently healthy donors from ARUP clinical laboratory collection. Effect of potential interfering substances was evaluated in three infliximab-containing samples and three neutralizing infliximab antibody-containing sera spiked with hemoglobin (1250 mg/dl), bilirubin (7.25 mg/dl) and triglycerides (2000 mg/dl). In clinical laboratory practice, patient serum samples are visually inspected before the assay and samples with visible hemolysis or excessive lipidemia are excluded.

2.5. Statistical analysis

Data were analyzed using the EP Evaluator software (Data Innovations, LLC, S. Burlington, Vermont). For method comparison Deming regression analysis was performed, which assumes that both methods are subject to measurement error.

3. Results

3.1. Principles of the RGA

The principles of the RGA for measuring infliximab activity, and neutralizing antibodies to the drug are illustrated on Fig. 1. The development and technical details of this method were described earlier [17]. Briefly, the RGA uses reporter cells that carry a TNF-α inducible, NFκB regulated firefly luciferase reporter-gene construct. When TNF-α is added to the cells, the reporter gene turns on and generates firefly luciferase expression, which is normalized to the expression of the Renilla luciferase gene carried within the same reporter cell. To measure the infliximab activity, serum mixed with a fixed concentration of TNF-α is added to the cells. If present, infliximab will block the activity of TNF-α, and the amount of infliximab present will inversely correlate to the amount of luminescence produced by the cells. The presence of biologically active infliximab in the serum can then be calculated using a calibration curve.

To measure the concentration of neutralizing antibodies to infliximab, the serum is pre-incubated first with a known concentration of infliximab, and the assay is performed as described above. If the serum does not have neutralizing antibodies, the infliximab added to the assay will block the activity of TNF-α, and it will not turn on the

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