Methods 44 (2009) 104–108

Contents lists available at ScienceDirect

Methods

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

A quantitative ELISA for bioactive anti-Nogo-A, a promising regenerative molecule for spinal cord injury repair

M. Douglas Baumann [a,b,e], James W. Austin [d], Michael G. Fehlings [d], Molly S. Shoichet [a,b,c,e,*]

[a] Department of Chemical Engineering and Applied Chemistry, University of Toronto, 200 College Street, Toronto, Ont., Canada M5S 3E5
[b] Institute of Biomaterials and Biomedical Engineering, 164 College Street, Toronto, Ont., Canada M5S 3G9
[c] Department of Chemistry, University of Toronto, 80 St. George Street, Toronto, Ont., Canada M5S 3H6
[d] Krembil Neuroscience Centre, Toronto Western Research Institute and Department of Surgery, University of Toronto, 399 Bathurst Street, Toronto, Ont., Canada M5T 2S8
[e] Terrence Donnelly Center for Cellular and Biomolecular Research, University of Toronto, 514-160 College Street, Toronto, Ont., Canada M5S 3E1

ARTICLE INFO

Article history:
Accepted 25 July 2008
Available online 12 August 2008

Keywords:
ELISA
Anti-Nogo-A
11c7
Bioactivity
Neuroregeneration

ABSTRACT

The detection and quantification of bioactive anti-Nogo-A mAbs, which is of interest for the treatment of spinal cord injury, has previously been accomplished using cellular or indirect immunoassays. In one such assay the presence of Nogo-A inhibits neurite outgrowth from the PC12 neuronal cell line: pre-treatment with anti-Nogo-A overcomes this inhibition and the concentration of anti-Nogo-A is correlated with the reduction in growth inhibition. In the current work we demonstrate the first anti-Nogo-A sandwich ELISA utilizing a Nogo-A fragment in the role of capture agent and the anti-Nogo-A mAb 11c7 as the soluble analyte. Because the Nogo-A fragment contains the amino acid sequence against which 11c7 was raised, we postulate this combination reproduces the native binding mechanism and results in the detection of bioactive anti-Nogo-A. In support of this hypothesis, we have found good agreement between the inhibitory action of the Nogo-A fragment and myelin proteins used in existing PC12 cell assays. Importantly, unlike the several days required for cellular assays the ELISA is a fast and easy to use method for the detection and quantification of bioactive 11c7 in the range of 500–6000 pg/mL.

1. Introduction

Traumatic spinal cord injury (SCI) affects 10,000 new patients annually in North America, often with devastating emotional and financial impact on the patient and family [1]. Although there is yet no cure for SCI, promising advances in tissue engineering and an enhanced understanding of the cellular mechanisms after injury have stimulated translation of basic discoveries into clinical trials [2,3].

Since the 1980s it has been known that central nervous system (CNS) neurons can extend neurites after injury when provided with a permissive environment such as that in a peripheral nerve graft [4]. The endogenous inhibition of the CNS has been recognized by the interaction of cell surface receptors on CNS neurons with myelin [5,6]. The transmembrane protein Nogo-A was identified as a primary contributor to neurite inhibition, and application of antibodies raised against this molecule resulted in significant axonal growth *in vitro* and in animal models of SCI in both rat [7] and macaque monkey [8]. These and other studies demonstrated that anti-Nogo-A partially functions by blocking the interaction of Nogo-A with the Nogo receptor (NgR) and co-receptors p75 [9,10], TROY [11,12] and LINGO-1 [13] located on CNS neurons. Delivery of anti-Nogo-A results in increased neuroregeneration and thus represents an important regenerative strategy after SCI. Significantly, at least one mAb is being evaluated clinically in Europe where it is being delivered by external pump and catheter [14]. While our long term goals include developing a localized intrathecal delivery strategy [15,16], the focus herein is on a new method related to facilitate quantification of bioactive anti-Nogo-A by ELISA.

Various anti-Nogo-A antibodies have been reported in the literature including IN-1 [17] and 11c7 [18], among others. The mAb 11c7 was raised in mice against a synthetic peptide corresponding to the rat aa (amino acids) 623–640 of Nogo-A [18], which are within the so-called central inhibitory domain [14]. The *in vitro* presence of 11c7 has been determined by Western blot [19] or an indirect immunoassay utilizing brain sections [20], but more commonly by one of a variety of cell based assays utilizing PC12 neurons or 3T3 fibroblasts [18,19,21]. While cellular assays are valued because they quantify bioactive anti-Nogo-A, they are time intensive. The development of a fast and

* Corresponding author. Address: Terrence Donnelly Center for Cellular and Biomolecular Research, University of Toronto, 514-160 College Street, Toronto, Ont., Canada M5S 3E1. Fax: +1 416 978 4317.
E-mail addresses: douglas.baumann@utoronto.ca (M.D. Baumann), jaustin@uhnres.utoronto.ca (J.W. Austin), michael.fehlings@uhn.on.ca (M.G. Fehlings), molly.shoichet@utoronto.ca (M.S. Shoichet).

1046-2023/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymeth.2008.07.007

easy to use quantitative assay for bioactive 11c7 is therefore of interest to those working in SCI research for the determination of circulating antibody concentration *in vivo* and the development of drug delivery systems *in vitro*.

Enzyme linked immunosorbent assays (ELISA) have been widely used to quantify antigen concentrations in the range of picograms per milliliter [22]. In the current application, sandwich (or capture) ELISA is of special interest because the analyte is captured from solution by specific antigen/antibody binding, not by adsorption to a substrate. This feature allows the native binding of 11c7 to Nogo-A to be reproduced.

Rat Nogo-A is 1163 aa long with two regions known to inhibit neurite outgrowth. At least one of these, corresponding to aa 544–725 [18,23] is commercially available as a recombinant rat Nogo-A/Fc chimera: this inhibitory sequence is fused to the Fc region of human IgG [24]. We have used this Nogo-A chimera as the capture agent in a sandwich ELISA because it contains the 18 aa sequence used to generate 11c7 [18]. Thus in this newly developed ELISA, Nogo-A acts as the substrate-bound antigen and 11c7 as the soluble antibody analyte.

Given that binding of anti-Nogo-A to Nogo-A ameliorates abortive sprouting and allows neurons to extend neurites over Nogo-A containing substrates [7,8], the bioactivity of 11c7 is thus defined by its ability to bind Nogo-A. Because the ELISA utilizes the central inhibitory domain of Nogo-A as the capture agent for 11c7, mimicking the *in vivo* mechanism, we propose that immunoreactive 11c7 is bioactive.

Based on this principle we developed an ELISA for the anti-Nogo-A 11c7 and demonstrated that the assay is both quantitative and a measure of bioactivity. To our knowledge, this is the first non-cellular assay to measure bioactive anti-Nogo-A 11c7: its primary benefits over earlier methods are speed, ease of use and quantitative results.

2. Description of methods

2.1. Development of the anti-Nogo-A ELISA

With reference to a publicly available ELISA development guide [22], grid experiments were used to evaluate a total of 16 analytical conditions in the following ranges: capture antibody, 0.25–4.0 μg/mL (rat Nogo-A/human IgG Fc chimera, R&D Systems, Minneapolis, USA); analyte, 375–10,000 pg/mL (mouse anti-rat Nogo-A 11c7, Novartis, Basel, CH); and detection antibody, diluted 1:500–1:4000 (donkey anti-mouse IgG coupled to horse radish peroxidase, R&D Systems). The completed sandwich ELISAs were then incubated with tetramethylbenzidine and hydrogen peroxide (R&D Systems) and quenched with 1M sulfuric acid (Sigma–Aldrich, Oakville, CA). All assays were run in 96-well, high binding, flat bottom micro titer plates (Greiner Bio-One, Monroe, USA), read at 450 nm and optically blanked at 540 nm using a VersaMax microplate reader (Molecular Devices, Sunnyvale, USA).

All buffers were made with distilled and deionized water prepared using a Millipore Milli-RO 10 Plus and Milli-Q UF Plus at 18 MΩ resistance (Millipore, Bedford, USA). Phosphate buffered saline powder was purchased from MP Biomedicals (pH 7.4, 9.55 g/L, Solon, USA); Tween 20, bovine serum albumin (BSA), and all other reagents were purchased from Sigma–Aldrich unless otherwise noted.

The grid experiments yielded multiple acceptable assay conditions (Fig. 1) and demonstrated the assay could be tuned to give larger linear ranges (shallower slope) or higher sensitivity (steeper slope). Under optimized conditions linear responses as long as 1000–6000 pg/mL or as short as 500–2000 pg/mL were obtained: the shorter range provided three fold better sensitivity. When used at dilutions of 1:1000 or lower, the detection antibody

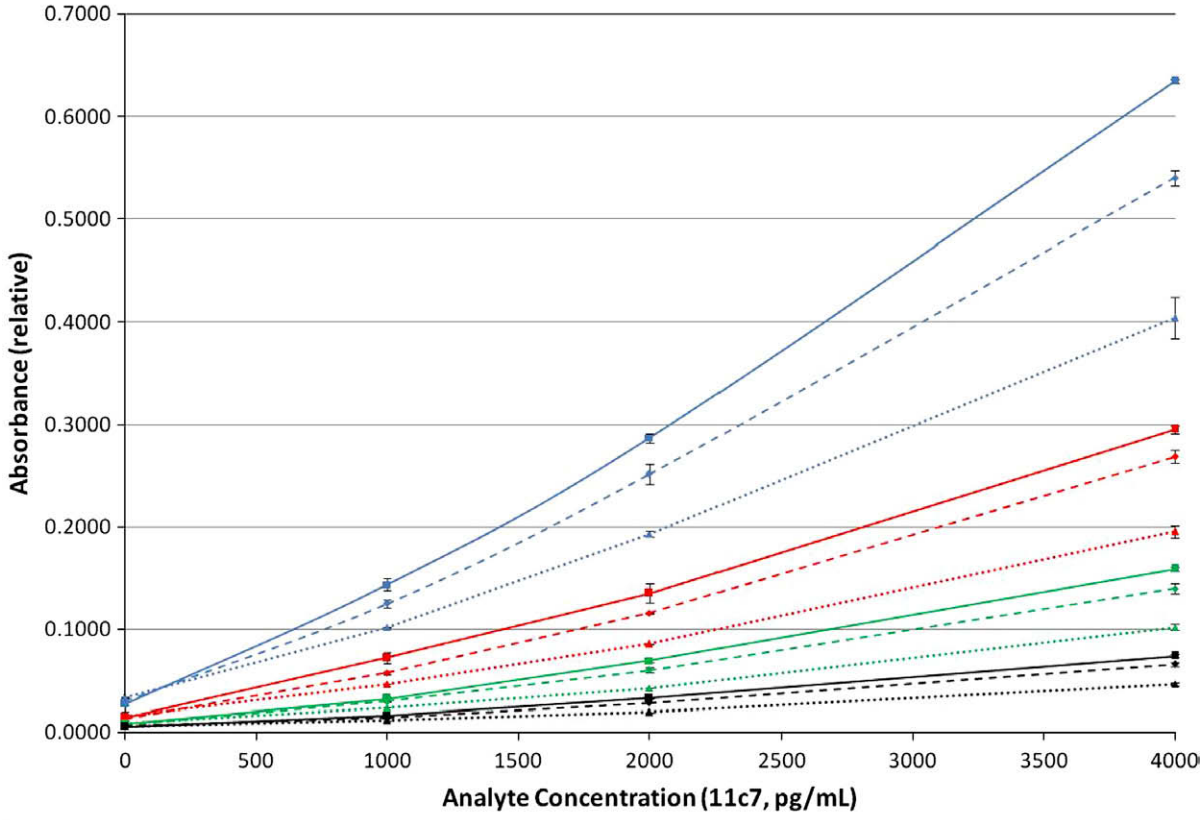

Fig. 1. Typical results from a grid experiment evaluating 12 ELISA conditions. Absorbance values have not been blanked in order to illustrate the inherent background. Color denotes detection Ab dilution: blue, 1:500; red, 1:1000; green, 1:2000; black, 1:4000. Line style denotes capture Ab concentration: solid, 1 μg/mL; dashed, 2 μg/mL; dotted, 4 μg/mL. The data are $n=2$, error bars are ±1 standard deviation. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)