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Research paper

Development of a new antibody to the human inhibin/activin βB subunit and its application to improved inhibin B ELISAs

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Abstract

Inhibin B has emerged as a clinically useful analyte for studies of reproductive function in both men and women. The antibody to the βB subunit (C5) used in current commercially available assays (DSL and OBI) was raised in this laboratory to a synthetic peptide from the βB subunit. These assays require pre-treatment of samples with hydrogen peroxide to oxidise two methionines in the epitope to the sulfoxide for full immunoreactivity. It was also claimed that this antibody cross-reacted significantly with inhibin A leading to a 0.5% cross-reaction of inhibin A in the current generation of immunoassays. Both of the above immunoassays required overnight incubation with sample. The development of improved antibodies to the βB subunit has proved difficult due to the conservation of the βB subunit between species. We describe the development of new monoclonal antibodies to the βB subunit, by immunisation of mice with recombinant *X. laevis* activin B using the RIMMS method of immunisation. The result has been the development of highly specific antibodies in a short time period. One of these antibodies 46A/F is shown to be a highly effective capture antibody in a human inhibin B ELISA, without any sample pre-treatment. The results of the validation of an improved inhibin B assay using 46A/F as the capture antibody are shown, with comparison to one of the commercially available inhibin B assays. Overall, the inhibin B assay is simplified and the performance improved by using this new antibody 46A/F. It was further shown that the cross-reaction of inhibin A in both the OBI and DSL inhibin B ELISAs is ten fold less than previously reported. This can be attributed to the poor quality of recombinant inhibin B available for use as standard in 1996. Although the present generation of inhibin B assays has proved adequate to enable the physiological function of inhibin to be determined and novel clinical applications found, the simplification of the assay made possible by the improved antibody should make possible a new generation of more rapid, sensitive, convenient and robust tools for routine use.

Keywords: Activin B; Inhibin B; ELISA; Immunoassay; Monoclonal antibodies; RIMMS

Abbreviations: DSL, Diagnostic Systems laboratories; ELISA, enzyme-linked immunosorbent assay; FCS, foetal calf serum; FSH, follicle stimulating hormone; HBR, heterophilic blocking reagent; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; NIBSC, National Institute for Biological Standards and Control; OBI, Oxford Bio-Innovation; PEG, polyethylene glycol; PMS, post-menopausal serum; PBS, phosphate buffered saline; RIMMS, repetitive immunisations multiple sites; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TMB, tetramethylbenzidine.

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1. Introduction

Inhibins are members of the transforming growth factor β (TGF-β) superfamily and are dimeric in structure (Evans and Groome, 2001; Robertson et al., 2004a,b). They regulate the reproductive system by acting on the pituitary gland and blocking the synthesis of the FSH-β subunit and therefore the secretion of FSH (Burger and Igarashi, 1988; Attardi et al., 1992; Burger et al., 1998; Knight and Glister, 2001). Inhibins are heterodimeric molecules containing an α subunit and either a βA or βB subunit, which are connected to each other by a disulfide bond. If the dimer consists of a βA subunit the molecule is called inhibin A, and if it consists of a βB subunit the molecule is called inhibin B (Miyamoto et al., 1985; Robertson et al., 1985; Mason et al., 1986). Activins contain two β subunits and can be homodimeric or heterodimeric depending on the arrangement of their subunits (Ling et al., 1986; Vale et al., 1986). Two βA subunits make activin A, two βB subunits make activin B and a βA subunit attached to a βB subunit make activin AB (Vale et al., 1986, 1988).

The measurement of inhibins in biological fluids has led to insights into its physiology, such as the pattern of inhibins in the menstrual cycle (Groome et al., 1996). Some of the many applications include; Down's syndrome screening (inhibin A), male infertility testing (inhibin B), ovarian reserve/menopause onset (inhibin B) and ovarian cancer (inhibin αC subunit and inhibin B) (Illingworth et al., 1996; Muttukrishna et al., 2000; Robertson et al., 2002; Wald et al., 2003).

Two commercial inhibin B immunoassays are available, from DSL and OBI (both Beckman Coulter companies). Both assays use the same pair of monoclonal antibodies raised to synthetic peptides by Groome and co-workers in this laboratory over 10 years ago. The capture antibody (C5), was raised to a peptide from the βB subunit of inhibin, and R1 the detection antibody was raised to a peptide from the α subunit of inhibin. Both assays require a methionine oxidation step with hydrogen peroxide to allow the C5 antibody to recognise its epitope. The current cross-reactivity of the inhibin B assays with inhibin A was reported to be approximately 0.5% (Groome et al., 1996). Adequate sensitivity of the present assays requires overnight incubation with the sample. This manuscript describes the development of antibodies to the βB subunit of inhibin/activin which are superior tools for immunoassay and immunohistochemistry. We also compared the cross-reactivity of inhibin A in the currently available OBI and DSL inhibin B ELISAs with our new assay using recombinant preparations of inhibin B from R&D systems as standard.

2. Materials and methods

2.1. Monoclonal antibodies to the βB subunit of inhibin/activin

2.1.1. Immunisation

Mice were immunised in a similar procedure to that described by Wong et al. (1993) using recombinant mature *Xenopus laevis* activin B, to produce monoclonal antibodies from lymph node fusions. The immunisation protocol also incorporated the repetitive immunisations, multiple sites (RIMMS) technique. Immunisations were given subcutaneously in proximity to draining lymph nodes (Caterson et al., 1983, Wring et al., 1999). Initial immunisations were performed using Freund's complete adjuvant, with subsequent boosts using RIBI adjuvant. *X. laevis* and human mature inhibin/activin βB subunit share 96.5% identity (Pearson et al., 1997) with only four amino acids different. The *X. laevis* activin B was expressed in *E. coli* as inclusion bodies, refolded to native, dimeric form and purified by a combination of reverse phase and ion exchange chromatographies (Department of Biochemistry, University of Cambridge).

2.1.2. Development of monoclonal antibodies

Sp2/0 myeloma cells were fused to the B-lymphocytes obtained from the lymph nodes using PEG (Harlow and Lane, 1988). Cloning and re-cloning of the initial antibody secreting cell lines were performed in ClonaCell methylcellulose (StemCell Technologies SARL, London, UK, cat: 03804) with individual colonies being picked. Antibody purification was achieved by Protein G (Millipore, Billerica, Massachusetts, US) affinity chromatography (Harlow and Lane, 1988). The ability of the antibodies to recognise the βB subunit was assessed by screening them using a solid-phase antibody capture ELISA against *X. laevis* activin B (as used for immunisation), human activins A and B and human inhibin B (R&D Systems Europe Ltd, Abingdon, Oxfordshire, UK).

2.2. Monoclonal antibody to the α subunit of inhibin

2.2.1. Fragmentation of R1

Monoclonal antibody R1 (IgG2a) raised to the alpha subunit of inhibin (Groome et al., 1990), was digested into $F(ab')_2$ fragments by using lysyl endopeptidase (Wako Chemicals GmbH, Neuss, Germany, cat: 125-02543) as described by Yamaguchi et al. (1995). Any intact R1 and Fc fragments were removed by purification with Protein A (Millipore, Billerica, Massachusetts, US), and whole R1 to $F(ab')_2$ reduction was monitored by SDS-PAGE. The $F(ab')_2$ fragments were treated with 2-mercaptoethylamine