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Molecular and Cellular Endocrinology 180 (2001) 79–86

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Molecular and Cellular Endocrinology
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Development of an inhibin α subunit ELISA with broad specificity

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Received 8 November 2000; accepted 9 January 2001

Abstract

Inhibin immunoassays with a sufficiently broad specificity to detect all α subunit-containing forms are of value in detecting and monitoring various ovarian cancers. Assays to date with this specificity are not readily amenable to wide diagnostic application. The objective of this study was to develop a sensitive two-site ELISA using α subunit-directed monoclonal antibodies (Mabs) able to detect all forms of inhibin to replace a previously described α subunit-directed immunofluorometric assay (IFMA). In this study, the major inhibin epitopes in the two polyclonal antisera used in the αC IFMA were initially identified and Mabs were raised to these regions. These Mabs in conjunction with the inhibin α subunit R1 Mab (Groome) were used to develop α subunit ELISAs with high sensitivity. Application of these assays to human serum and human follicular fluid following fractionation by an immunoaffinity/preparative PAGE/electroelution procedure which separated inhibins according to their molecular weights, indicated that the specificity of the various ELISAs differed between Mab combinations with preferences noted for either the α subunit or dimeric forms. A combination of Mabs in an ELISA was identified which provided data which matched that obtained with the αC IFMA and which may be useful as a replacement inhibin assay in clinical studies.

Keywords: Inhibin α subunit; Ovarian cancer

1. Introduction

Inhibin consists of two subunits (α and βA or βB) which are produced as dimeric precursor forms prior to processing at serine peptidase sensitive sites to form mature bioactive 30 kDa forms (Vale et al., 1990; Burger, 1992; Baird and Smith, 1993; Mason et al., 1996). The bioinactive α subunit can undergo processing to form two fragments termed Pro-αC and αN. Recent years have seen the development of specific ELISAs to measure the individual forms of inhibin (inhibin A, B, and Pro-αC) (Groome and O'Brien, 1993; Groome et al., 1994, 1996a,b). These assays supersede earlier immunoassays which were directed to the αC region of the α subunit which were able to detect all inhibin molecules including inhibin dimer and α subunit monomers (McLachlan et al., 1986; Robertson et al., 1996, 1997). While these new specific assays have clearly led to advances in understanding the physiology of inhibin particularly in relation to the importance of the dimeric biologically active forms, there is still a need for sensitive and rapid α subunit-directed assays which detect all α subunit forms.

This need was identified in the application of inhibin assays to monitor serum inhibin in various types of ovarian cancer (Lappohn et al., 1989; Healy et al., 1993). Studies undertaken by this group (Burger et al., 1996; Robertson et al., 1999a) using inhibin A, B, Pro-αC ELISAs, an inhibin (Monash) radioimmunoassay (RIA) and a newly developed two-site immunofluorometric assay (αC IFMA) showed that the α subunit-directed RIA and the αC IFMA, had the highest detection rate for ovarian cancer. Of the ovarian cancers assessed, granulosa cell tumours produced inhibin A or B and free α subunit, while mu-

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0303-7207/01/$ - see front matter
PII: S0303-7207(01)00518-4

cinous tumours preferentially produced free α subunits. The Pro-αC ELISA gave results which ranged between those obtained by the dimeric and α subunit assays. The conclusion from these studies was that α subunit-directed assays which detected all dimeric and monomeric inhibin forms were preferred to more specific assays in application to ovarian cancer.

The suitability of the α subunit-directed assays to detect all inhibin forms was supported by fractionation studies in which serum and human follicular fluid (hFF) were separated into their molecular weight forms by an immunoaffinity/preparative PAGE/electroelution procedure (Robertson et al., 1996, 1997). These data showed that the α subunit-directed assays detected the majority of known inhibin forms.

The αC IFMA detected 100% granulosa cell tumours and 90% mucinous tumours, and in combination with a common tumour marker, CA125, which has high specificity for other ovarian cancers, e.g. serous, the two assays detected 90% of all cancers (Robertson et al., 1999b). While the αC IFMA had the advantages of higher assay sensitivity and higher practicability compared to earlier assays, wider application of this assay was hampered by the limited availability and difficulties in reproducibility between ovine antiserum stocks.

The purpose of the present study was to develop a two-site immunoassay procedure using monoclonal antibodies to the αC region of the α subunit which detected all dimeric and monomeric forms of inhibin. To achieve this purpose, the major inhibin epitopes detected by the antisera used in the αC IFMA were identified. Monoclonal antibodies directed to these epitopes were produced and tested in an ELISA format and these assays were assessed for their specificity in detecting inhibin forms.

2. Identification of epitopes to the α subunit of inhibin

Two polyclonal antisera (# 41 and # 128) were used in the αC IFMA (Robertson et al., 1997, 1999b). These antisera were initially raised in two sheep (# 41 and # 128) to a fusion protein of the αC region with subsequent boosts with recombinant human 30 kDa inhibin A. To identify the inhibin epitopes, 31 overlapping biotinylated peptides of the inhibin α subunit (amino acids 233–366 designated aa 1–123 in this study, Mason et al., 1996) were synthesised (Mimotopes Pty Ltd, Clayton) each 14 amino acids (aa) long with an overlap of 2 or 4 aa. A 4 aa spacer consisting of a serine–glycine repeat with an N-terminal biotin was attached to the N terminus of each peptide.

The inhibin epitopes identified by these antisera were assessed by three methods. The first method assessed the ability of the biotinylated peptides to bind the # 128 and # 41 antisera. The peptides were initially bound to streptavidin-coated plates, the antiserum added and the bound antiserum detected by the binding of an anti-ovine IgG serum labelled with horse radish peroxidase. Four peptide regions corresponding to aa 1–30, 33–62, 63–96, 97–134 with elevated binding were identified (data not shown).

For the second method, RIA procedures using the # 41 and # 128 antisera as primary antibodies and iodinated human recombinant 30 kDa inhibin A as tracer were employed. This more specific approach assessed the ability of the peptides to compete with iodinated inhibin for the antisera. The RIA was optimised for each antiserum to give a sensitive response. As seen in Fig. 1, high affinity binding peptides (in terms of ED_{50} values) were seen in three of the four regions observed with the first method with highest affinities in regions I and IV. From these data three major epitope regions were identified termed # 5 (aa 9–22), # 20 (aa 69–82) and # 30 (aa 109–122). These peptide regions were designated according to the peptide number which gave peak responses in these assays.

To assess the relative contribution of these peptide regions in the αC IFMA, three peptides corresponding to peptides # 5, # 20 and # 30 above were synthesised with high purity and known mass (Mimotopes). These peptides were then used individually or in combination to absorb out the corresponding antibodies in # 41 and # 128 antisera prior to testing them in the αC IFMA. In a typical experiment, peptides (0.1–1 μM) were preincubated with either the # 128 antiserum (which was used as coating antibody) or biotinylated antiserum # 41 (used as label) for 1 h at room temperature and then incubated together in the presence of a fixed concentration of inhibin A (2 ng) for 1 h at room temperature. The plate was washed, Europium (Eu)-labelled streptavidin added, incubated and the bound Eu-detected in a time-resolved fluorometer. These data showed that peptides # 5 and # 20 competed with inhibin for As # 128 and peptides # 5 and # 30 for As # 41 (data not shown).

In a further set of experiments, peptides at a fixed maximal suppressing dose were incubated in the presence of increasing doses of inhibin A. As seen in Fig. 2, the three peptide regions represented by peptides # 5, # 20 and # 30 are responsible for > 95% of inhibin binding in the αC IFMA. In terms of As # 41, peptide # 5 and # 30 are the major epitopes. Preabsorption of As # 128 only with peptides # 5, # 20 and # 30 resulted in partial suppression only (55%), indicating that there is another major α subunit-directed antibody in As # 128 which has not been identified. Interestingly, the addition of peptide # 5 alone without inhibin led to significant binding in the αC IFMA (data not shown) indicating that there are at least two different epitope regions on peptide # 5 detected by the # 128 and # 41 antisera.

These results indicate that the αC IFMA detects 3–4 major epitope regions in the αC region with one/two sites present in the N terminal region of the αC region and the other two sites in separate regions of the molecule.

3. Characterisation of monoclonal antibodies raised to the human inhibin α subunit

In order to raise monoclonal antibodies (Mabs) to these epitopes, mice were immunized with the human αC fusion protein as originally used as antigen to raise antisera in sheep (Forage et al., 1987). For these studies the fusion protein was initially immunopurified using the Groome R1 α subunit directed Mab. Mabs were produced using previously described hybridoma methodology (Groome and O'Brien, 1993; Groome et al., 1994, 1996a,b). In initial experiments the fusion protein provided a large number of positive clones which were initially assessed for binding to inhibin A and α subunit. Mabs with high affinity for inhibin were then identified. The specificity of these Mabs was then assessed using overlapping peptides to the αC region similar to that used above to characterise the ovine antisera. These Mabs were localised to the three regions of the α subunit identified with the ovine antisera above corresponding to peptide # 5 (Mab PO # 6, PO # 22 aa 3–26), an amino acid region alongside and overlapping peptide # 20 (PO # 12, # 14,aa 73–96) and peptide # 30 (PO # 9, PO # 19, # 23, # 25, # 26, aa109–126). The characteristics of these Mabs are presented in Table 1. The PO # 6, # 22 Mabs detected inhibin α subunit sequences similar to that seen by the R1 Mab (aa 3–26, Groome) but were not considered further because of their poor affinity for inhibin. The specificity of the other two groups of Mabs are unique with PO # 14 and PO # 23 showing the highest affinity for inhibin. Interestingly, PO # 23 bound the same peptide region as did the rabbit # 1989 antiserum in the Monash # 1989 RIA (aa 109–126). This aa sequence differs from that previously considered to be the inhibin epitope for the Monash # 1989 RIA (aa 94–109, Lambert-Messerlian et al., 1995).

4. Assessment of Mabs directed to the α subunit of inhibin

Mabs PO # 14 and PO # 23 were tested in an ELISA system based on the procedure employed previously with inhibin A, B and Pro-αC (Groome and O'Brien, 1993; Groome et al., 1994, 1996a,b). The ELISA consisted of one antibody coated onto 96-well microtitre

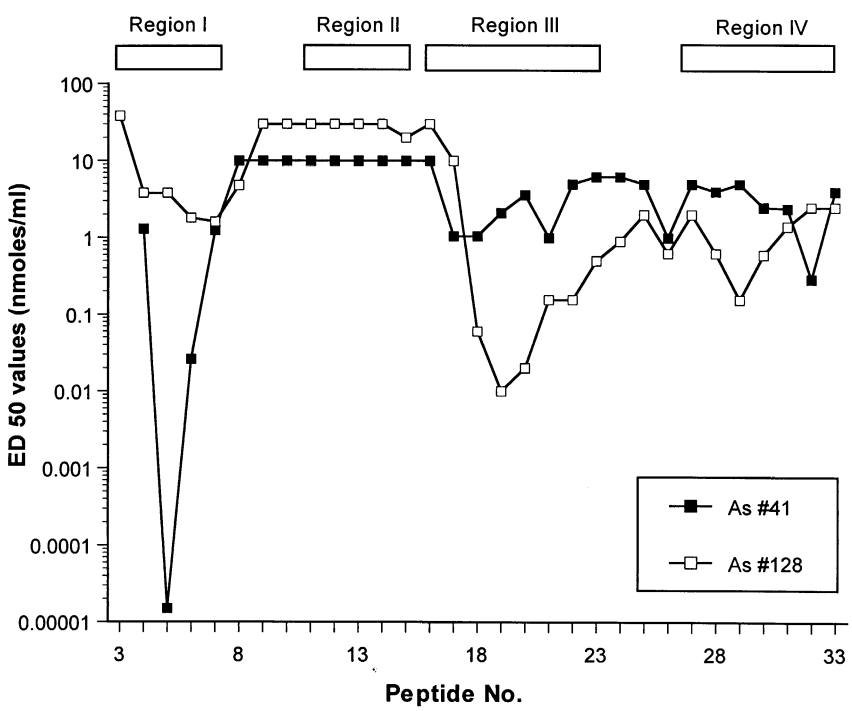

Fig. 1. ED_{50} values for α subunit peptides in the inhibin RIA with either # 41 or # 128 antiserum. Four regions (Region I aa 1–30, Region II aa 33–58, Region III aa 59–96, Region IV aa 97–134) were originally identified based on a solid phase ELISA (see text). Regions I, III and IV show evidence of competition with iodinated inhibin for the antisera.

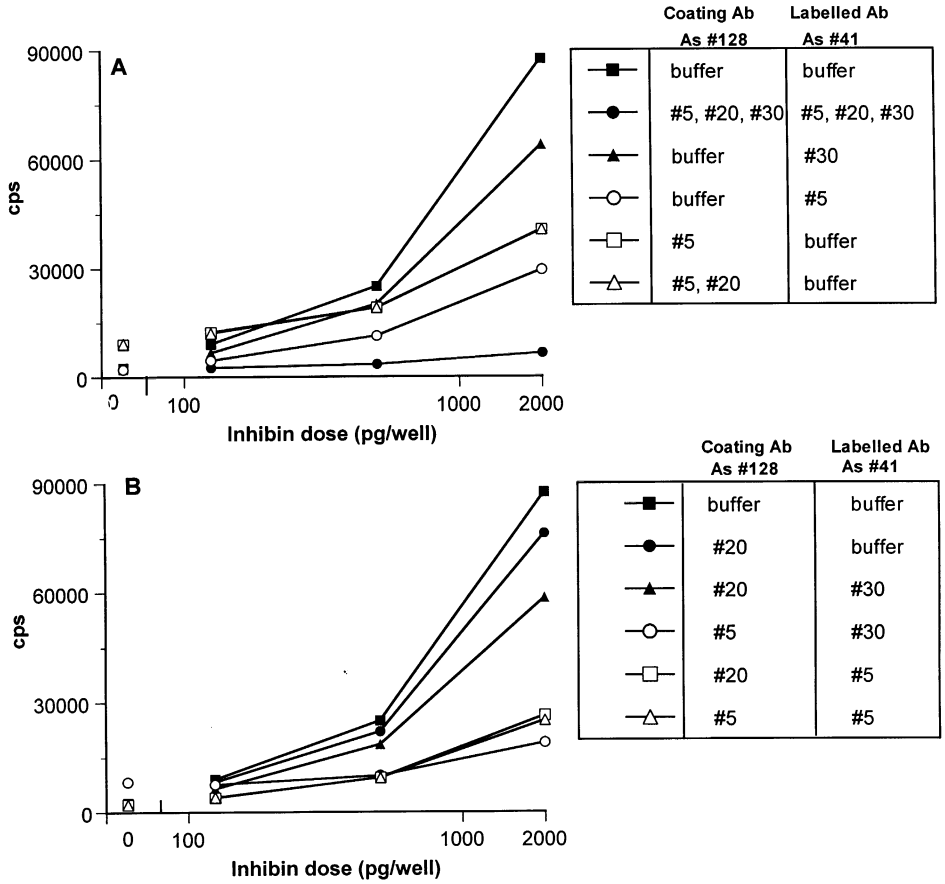

Fig. 2. Assessment of the contribution of the various antibodies in antiserum # 128 and # 41 to the αC IFMA. An inhibin A standard curve was set up in the αC IFMA. In parallel wells either the coating antiserum # 128 or labelled antibody was preincubated with peptides directed to the various inhibin α subunit epitopes in the antisera. In comparison with no added peptide (filled square) the addition of a combination of peptides # 5, # 20, # 30 (filled circle) led to total inhibition of response. Various peptide combinations led to partial responses related to the extent the various antibodies to these peptides contributed in the αC IFMA.

plates, inhibin and samples added and incubated overnight at room temperature. Alkaline phosphatase (AP) labelled second antibody was added and incubated for a further 2 h. The enzymatic response was amplified with an AMPAK kit (Dako, Carpinteria, CA). Serum samples were initially boiled in the presence of 6% SDS prior to assay as undertaken by Groome et al. (1994, 1996b) to offset any nonspecific effects reported previously with this assay design.

Two assay systems were initially devised with PO # 14 or PO # 23 as coating antibodies, AP linked R1 (Table 1) as label and recombinant human (rh) 30 kDa inhibin A (WHO 91/624) as standard. As seen in Table 2, sensitive responses to inhibin A were observed in both assays with different specificities in terms of their ability to detect rh-30 kDa inhibin B (R&D Systems) and human Pro-αC (used as standard in the Pro-αC ELISA, Oxford Bio-innovation Ltd, Upper Heyford, UK) although activin A gave a nondetectable response ($\leq 0.2\%$) (Table 2). Mab PO # 14 (coating) and PO # 23 (label) were not effective in an ELISA combination and not proceeded with further.

5. Fractionation of serum and human follicular fluid inhibin

In order to characterise further the specificity of these inhibin α ELISAs (Mab combinations 14-R1, 23-R1), human follicular fluid (hFF) and serum from women stimulated with gonadotrophins as part of an IVF program (IVF serum) were fractionated according to their molecular weight by a combined immunoaffinity, preparative PAGE and electroelution procedure. This procedure is a modification of previously described procedures (Robertson et al., 1996, 1997). In brief, sample in the presence of protease inhibitors was initially fractionated on an immunoaffinity column consisting of an immobilised Ig fraction of # 41 antiserum. The bound inhibin forms were eluted with guanidine hydrochloride which was removed using disposable C18 reverse phase columns with 100% acetonitrile as eluate. The eluate was lyophilised before fractionation on 10% Tris/Trycine/SDS–PAGE (Schagger and von Jagow, 1987). The inhibins were eluted from the polyacrylamide gel with an electroelution device (Whole Gel

Table 1
Characteristics of the Mabs raised to the αC region of the α subunit of inhibin[a]

Mab	Deduced epitope amino acid (aa) sequence	Affinity for inhibin A (ED_{25} nmol/l)
R1	aa 3–24	1.6
PO # 6, # 22	aa 3–24	*
PO # 12	aa 73–96	37
PO # 14	aa 73–96	14.8
PO # 9	aa 109–123	*
PO # 19	aa 109–123	12
PO # 23	aa 109–123	5.5
PO # 25	aa 109–123	9.4
PO # 26	aa 109–123	*
#1989 RIA	aa 109–123	0.19

[a] Amino acid (aa) 1 represents aa 233 of the α subunit precursor chain (Mason et al., 1996). The ED_{25} values were determined in an RIA format using iodinated inhibin A as tracer and inhibin A (91/624) as standard. The corresponding epitope sequence and affinity of inhibin for the rabbit antiserum (# 1989) used in the Monash RIA is included.

* Limited competition corresponding to a very low affinity for inhibin A.

Eluter, Biorad, Hercules, USA) using Tris/Hepes as elution buffers. Coloured protein markers (Sigma Aldrich, St Louis, MO) of known molecular weight were run in parallel. The eluted samples were assayed using the various immunoassays. The use of the electroelution device provided greater throughput than the earlier procedure employed (Robertson et al., 1996, 1997).

The reproducibility of this procedure was assessed by the repeat fractionation ($n = 4$) of a serum pool. The coefficient of variation of the molecular weight values calculated for the peak activities of inhibin A and Pro-αC in the eluted samples from the four runs was $< 10\%$. Recoveries of inhibin A and Pro-αC were 60% for the immunoaffinity step, 56 and 41%, respectively, for the Prep-PAGE step, 31 and 25% respectively, overall.

The patterns of α subunit immunoactivity as measured by the αC IFMA and 14-R1 and 23-R1 ELISAs for hFF (Fig. 3) and IVF serum (Fig. 4) using inhibin A (WHO 91/624) as standard showed marked differences between assays. The molecular weight profile of hFF was included as a recognised inhibin source and as reference for subsequent fractionation studies, below. α subunit profiles of IVF serum as determined by 14-R1 and Pro-αC ELISAs (i.e. Mab combinations INPRO-R1 and INPRO-14) showed a close similarity (Fig. 4) while a comparison of the α subunit profile (23-R1)

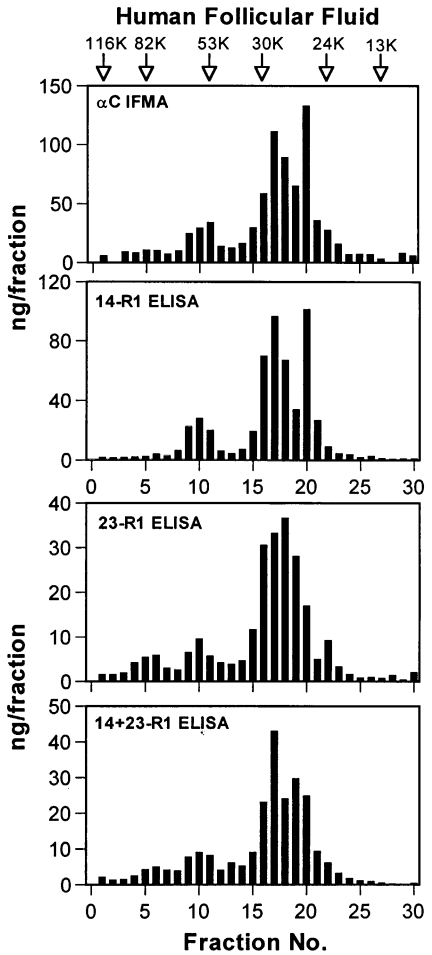

Fig. 3. Molecular weight profiles of α subunit inhibin forms in human follicular fluid as determined in various assays. Samples were fractionated through an immunoaffinity/preparative PAGE/electroelution procedure and the molecular weight patterns of inhibin using inhibin α subunit ELISAs and αC IFMA were determined.

Table 2
Validity criteria of the inhibin α ELISAs using Mab combinations 14-R1, 23-R1 and 14+23-R1[a]

		14-R1 ELISA	23-R1 ELISA	14+23-R1 ELISA
rh-inhibin A	WHO 91/624	100	100	100
rh-inhibin B	R&D systems	320	253	138
Pro-αC	ELISA standard	98.5	41.5	38.5
rh-activin A	PHIMR preparation	<0.2	<0.2	<0.2
Sensitivity and working range	Inhibin A (WHO 91/624) as standard (pg/well)	1.5–100	0.8–100	0.6–100

[a] Specificity data are presented in relation to the inhibin A standard (= 100) as means of two experiments.

with dimeric inhibin A (E4-R1) and inhibin B (C5-R1) profiles also showed similarities, particularly in relation to the presence of high molecular weight forms. A quantitative comparison of the levels of α subunit immunoactivity determined with the 14-R1 and 23-R1 ELISAs showed that the 14-R1 ELISA was detecting threefold higher the α subunit levels compared to the 23-R1 ELISA.

These data suggested that there is a difference in specificity between the two ELISAs with the 14-R1 ELISA more readily detecting the α subunit forms while the 23-R1 ELISA preferentially detected the dimeric forms. To clarify this point further, the Pro-αC and dimeric inhibin forms in hFF were initially fractionated by immunoabsorption to an immobilised column of the INPRO antiserum which is directed to the Pro region of the inhibin α subunit (Groome et al., 1996b). The absorbed Pro-containing proteins were then fractionated by the Prep-PAGE/electroelution procedure above. The nonabsorbed hFF fraction containing predominantly dimeric inhibin was recycled through the INPRO column until Pro-αC immunoactivity in the eluate was nondetectable and then fractionated through the immunoaffinity/Prep-PAGE/electroelution procedure.

As seen in Fig. 5, 14-R1 and 23-R1 ELISAs showed largely similar patterns of immunoactivity between INPRO absorbed and non absorbed samples, although quantitatively inhibin levels with the 14-R1 ELISA were 5.5× higher for the INPRO bound-inhibin α subunit profile compared to the 23-R1 ELISA and, conversely the 23-R1 ELISA levels were 2.7× higher compared to the 14-R1 ELISA for the non-absorbed sample profile. These data suggested that although the two inhibin α subunit ELISAs detected all inhibin α subunit and dimeric forms, the 14-R1 ELISA more readily detected the Pro-αC forms and 23-R1 ELISA more readily detected the dimeric forms.

Since the purpose of this study was to devise a replacement assay for the αC IFMA, these data would suggest that neither 14-R1 or 23-R1 ELISAs are in principle an appropriate alternative as they are too selective in the forms of inhibin they detect. This conclusion led to the suggestion that a combination of PO # 14 and PO # 23 as capture antibodies in combination with R1 as label may provide a more appropriate replacement assay. Application of 14 + 23-R1 ELISA to the fractionated inhibin samples presented in Figs. 3 and 4 gave results which provided a mix of the 14-R1 and 23-R1 inhibin patterns which more closely

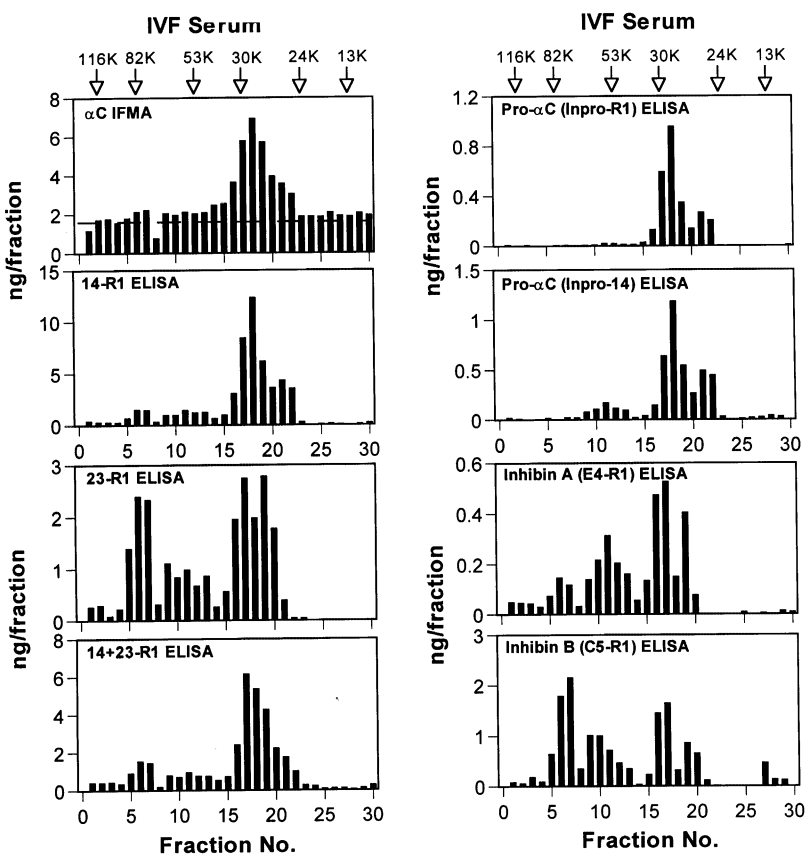

Fig. 4. Molecular weight profiles of Pro-αC and inhibin A in IVF serum as determined by various assays. Samples were fractionated through an immunoaffinity/preparative PAGE/electroelution procedure prior to assay. Horizontal dashed line in the αC IFMA profile refers to the sensitivity of the assay.

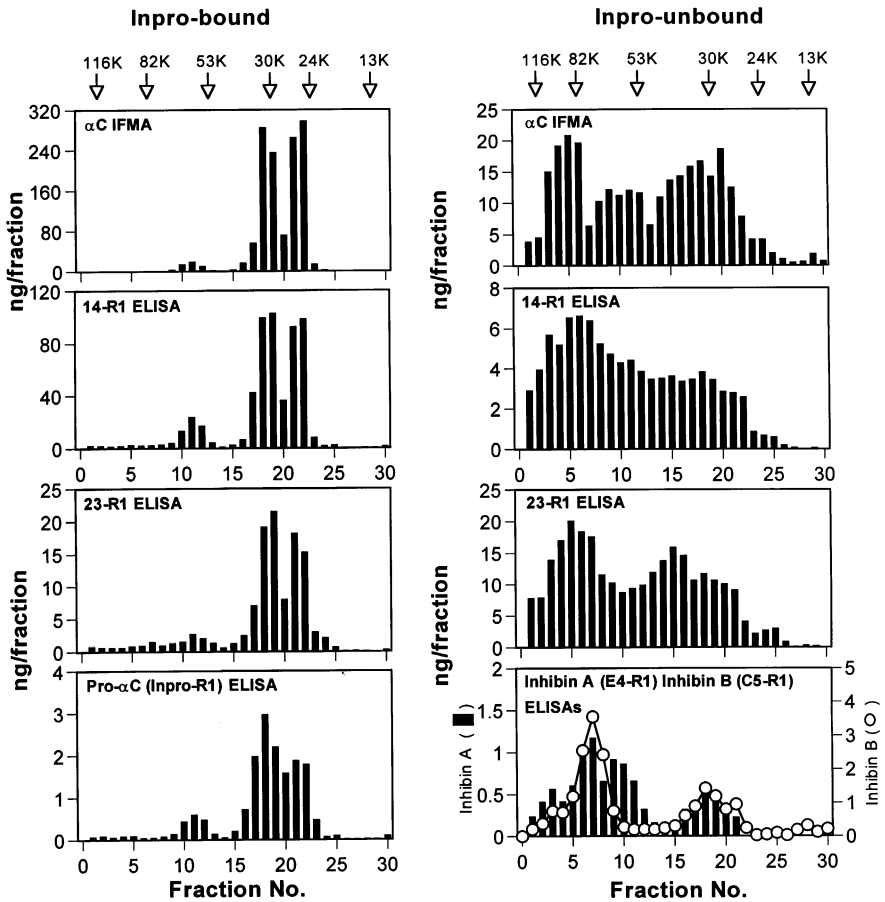

Fig. 5. Molecular weight profiles of α subunit inhibin forms and inhibin A and B in human follicular fluid (hFF) following separation into Pro-containing and non-Pro-containing inhibin forms. hFF was initially fractionated through an INPRO Mab immunoaffinity column to isolate the Pro-containing inhibin forms. Both Pro-containing forms (almost exclusively α subunit) and the remaining non-Pro-containing forms (representing dimeric inhibin A and B forms) were fractionated through a preparative PAGE/electroelution procedure and the molecular weight patterns of inhibin using various assays were determined.

resembled those obtained with the αC IFMA. It is interesting to note that the 14 + 23/R1 values were not an average of values from the other two ELISAs (Table 1). In addition, the 14 + 23-R1 ELISA was apparently more sensitive when using 30 kDa inhibin A as standard. It is thus proposed that the 14 + 23-R1 ELISA may be the assay of choice for an α subunit assay.

Studies are currently underway to assess which of these assay formats are of clinical value in monitoring ovarian cancer. Preliminary data shows that inhibin levels in postmenopausal serum are nondetectable using these assays while inhibin levels in women during the reproductive years are readily detectable. These findings support the conclusion that these assay(s) are appropriate replacements for the αC IFMA.

To what extent the quantitative differences in inhibin levels detected by the 14-R1, 23-R1 and 14 + 23-R1 ELISAs are attributed to the choice of inhibin standard is unclear. The 30 kDa inhibin standard is clearly not representative of the large molecular weight range in inhibin forms or the Pro-αC forms present in serum. Thus the high molecular weight forms may be either under- or over-estimated depending on their affinities for the Mabs in the ELISAs. A more representative standard which also contains high molecular weight inhibin forms may lead to more quantitatively similar data between assays although it is difficult to envisage how such a standard would be formulated in practice.

6. Applications of the α subunit Mabs to other assays

Since the PO # 14 and PO # 23 Mabs showed different specificities for inhibin, it was of interest to see what would be the effect of replacing the R1 antibody with either of these Mabs on the specificity of the inhibin A, B and Pro-αc ELISAs. A combination of a Mab to the βA subunit (E4 Mab) with either R1, PO # 14 or PO # 23 resulted in largely similar profiles with the E4-R1 detecting the 100 k forms a little better than the other E4 combinations (data not shown). In the case of Pro-αC, high molecular weight forms of Pro-αC were

more readily detected with the PO # 14 Mab than with R1 (Fig. 4). The PO # 14 and # 23 antibodies were not able to combine with the C5 βB subunit Mab using the inhibin B ELISA. Overall these data indicate that the PO # 14 and possibly PO # 23 Mabs are detecting inhibin species not seen previously. To what extent these observations are physiologically important has yet to be resolved.

Acknowledgements

This study was funded in part by a Program Grant (98/3212) of the National Health and Medical Research Council of Australia.

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