Biological and Immunological Characterization of Inhibin Forms in Human Plasma*

D. ROBERTSON, H. G. BURGER, J. SULLIVAN, N. CAHIR, N. GROOME,
E. PONCELET, P. FRANCHIMONT,† T. WOODRUFF, AND J. P. MATHER

*Prince Henry's Institute of Medical Research (D.R., H.G.B., J.S., N.C.), Clayton, Victoria, Australia;
School of Biological and Molecular Sciences (N.G.), Oxford Brookes University, Oxford, United
Kingdom; Department d'Endocrinologie (E.P., P.F.), Universite de Liege, Liege, Belgium; Cell Biology
(T.W., J.P.M.), Genentech Inc., South San Francisco, California*

ABSTRACT

A number of immunoassay methods have been developed recently to detect specifically the bioactive α-βA subunit inhibin dimer (inhibin A) in human plasma. However, the specificity of these assays in terms of their ability to detect the range of inhibin forms found in plasma and their relationship to bioactivity have not been investigated. Inhibin was fractionated from human follicular fluid (hFF) and serum/plasma from women stimulated with gonadotropins (IVF serum), and from postmenopausal and male plasma, using a combined immunoaffinity/preparative SDS-PAGE procedure. The molecular weight profile of inhibin was established by inhibin in vitro bioassay, three α-βA subunit specific immunoassays, and three α subunit-directed immunoassays that detect the α subunit as well as inhibin A and B forms.

In hFF inhibin forms of 33, 36, 55 and 66K were detected by in vitro bioassay and by most immunoassays except for 33 k inhibin, which was nondetectable by one α-βA ELISA. The α subunit-directed assays also detected activity in the 29–31K region, in some assays in considerably high levels. In IVF serum in vitro bioactivity and immunoactivities were detected between 27 and 100K with the α-βA assays failing to detect all bioactive forms. Alpha subunit-directed assays gave similar immunoactive profiles. Neither in vitro bioassay nor α-βA assays detected activity in post-menopausal plasma or male plasma, while α subunit-directed assays showed peaks predominantly at 36 k, although at low levels.

It is concluded that dimeric inhibin A specific assays detected bioactive inhibin forms in hFF and to a lesser extent in IVF serum. Alpha subunit-directed assays correlated poorly with in vitro bioassay in hFF because of the high α subunit levels in this sample. The higher correlation between these assays in IVF serum suggested that there was little free α subunit. The 36K form in male plasma may be free α subunit or inhibin B. (*J Clin Endocrinol Metab* **81:**669–676, 1996)

I NHIBIN is a dimer of two subunits designated α and β, of which the latter has two forms, βA and βB. The dimers are thus termed inhibin A and B (1–4). These subunits are produced as larger precursors that dimerise to form bioactive inhibin, which is then processed to produce smaller forms. Both subunits have also been found as free forms. The α-β dimers, in contrast to the free α subunit, are biologically active in suppressing FSH secretion. By contrast, β-β subunit dimers (activins) stimulate FSH secretion.

Much research has been undertaken to characterize the biological activities of 30K inhibin A (produced by recombinant methods). In addition, a number of immunoassays have been developed, including two site methods, to specifically measure the 30K inhibin A dimer in biological fluids (5–8). It has become apparent, however, that circulating inhibin probably exists as either α-βA or α-βB forms (8) as well as higher molecular weight unprocessed, partially processed, and free α subunit forms (9–11). As a consequence the specificity of these assays is unclear in regard to serum inhibin. The aim of this study was to identify the biologically active forms in human follicular fluid (hFF), serum/plasma

by in vitro bioassay using ovine pituitary cell cultures and compare their molecular weight pattern with those obtained by α-βA specific ELISAs, and α subunit-directed immunoassays that detect the free α subunit as well as inhibins A and B. This study arose out of discussions at the Ares-Serono Symposium on "Inhibin and Related Proteins" held at Siena, Italy in June, 1993 (see reference 8).

Materials and Methods

Molecular weight identification of inhibin forms

The α subunit, either in the free form or as an α-β subunit dimer, is cleaved from a ~65K precursor (consisting of three regions, pro(aa1–18)-αN(aa19–248)-αC(aa249–366), to form pro-αC and αN, or αN-αC. Similarly, the β subunit precursor is cleaved to form the pro-β and β subunits (1, 2). In addition, the αC subunit can be either mono- (α*) or diglycosylated (α**), resulting in inhibin forms differing in molecular mass by approx 3K (12). As a consequence, a range of inhibin molecular weight forms have been predicted and identified. The more generally well-known forms are 26 and ~30K pro-αC (α*, α**), 31 and 34K inhibin (α*-β, α**-β), ~45K (αN-αC), 50–60K (αN-αC/β and αN/proβ-β) and more than 90K (αN-αC/proβ-β).

Preparations

Human recombinant activin A, B, inhibin B, follistatin, and culture medium containing human recombinant inhibin A and B, activin A and B, and inhibin α subunit were obtained from Genentech (San Francisco, CA) (see ref 6 for further details). The human inhibin standard (recombinant 31–34K inhibin A, 91/624) was obtained from the NIBSC, Blanche Lane, Potters Bar, Herts UK.

Human follicular fluid (hFF)

Follicular fluid (hFF) was collected from women undergoing gonadotropin stimulation at the time of egg pick-up from Monash IVF (Clayton, Victoria, Australia). The hFF was placed on ice within minutes of collection, centrifuged (to remove blood and debris) within ~1 hr, and snap frozen in solid CO_2/ethanol. A pool (20–30 mL hFF/patient from five patients) was prepared for immunoaffinity purification with aliquots kept for assessing recoveries.

IVF serum

Sera from women undergoing gonadotropin stimulation as part of an IVF program (designated as IVF serum) were obtained from Monash IVF (Richmond, Victoria, Australia). The sera collected were remains saved following hormone measurements and were frozen within hours of collection. Serum samples from women with serum estradiol values of more than 2 nm were combined to give a serum pool (100 mL) for use in the inhibin immunopurification with aliquots kept for assessing recoveries.

Postmenopausal plasma

Blood in heparinized tubes was collected from 12 postmenopausal or ovariectomized postmenopausal women, centrifuged within 1 h of collection, and snap frozen in solid CO_2/ethanol. All plasma samples were initially shown to have nondetectible inhibin levels as determined by Monash RIA. Plasma from all subjects was pooled (94 m:l) for immunoaffinity chromatography, with aliquots kept for assessing recoveries.

Male plasma

Blood (50–80 mL/subject) was collected on ice in heparinized tubes from five normal males, centrifuged immediately at 4C, and the plasma snap frozen in solid CO_2/ethanol. Plasma from all subjects was pooled (100 mL) for immunoaffinity chromatography, with aliquots kept for assessing recoveries.

The collection of human samples was approved by the Monash Medical Centre Human Research and Ethics Committee.

Fractionation procedure

The fractionation procedure is based on a previously described method (11). Some modifications (listed in brief below) were introduced to cope with the larger volumes of sample used in this study. A caprylic acid immunoblobulin G (IgG) fraction obtained from sheep immunized against inhibin α-C subunit fusion protein (13), was coupled by N-alkyl carbamate linkage to a gel matrix (Reacti-Gel 6X, Pierce, Rockford, Illinois). hFF (100 mL) or plasma/serum pools (94–100 mL), plus an equal volume of 0.2 mol/L phosphate buffer, pH 7.0, containing protease inhibitors, 2 mmol/L p-chloromercurobenzoate, 2 mmol/L phenyl-methylsulphonyl fluoride, and 20 mmol/L ethylenediaminetetraacetic acid was added to the gel (20 mL coupled gel/100 mL hFF, 10 mL gel/100 mL serum/plasma) and incubated overnight at 4 C on a turning wheel. The supernatant was discarded and the gel washed with 20 mmol/L phosphate buffer pH 7.0. 6 m guanidine hydrochloride (GnHCl, 5 × 2 mL) was then added and eluted material collected. The GnHCl fractions were pooled, acidified with an equal volume of 0.1% trifluoroacetic acid and fractionated by reverse phase (RP) high performance liquid chromatography (HPLC) to remove the GnHCl. Inhibin-containing fractions from the RP-HPLC run were pooled, lyophilized with SDS (2% final concentration in reconstituted sample), and reconstituted in 500 μl 0.1 M TRIS/HCl pH 8.0. The samples were fractionated on 10% SDS-PAGE (20 × 20 cm gels), the gel slices (0.25 cm slices, 58–60 slices/gel) were electroeluted and the SDS removed by methanol precipitation. Each electroeluted sample was reconstituted in 5 mL sterile Dulbecco's phosphate buffer containing 0.1% bovine serum albumin before subaliquotting for the various assays. The aliquots were snap frozen in solid CO_2/ethanol. In some cases, aliquots were lyophilized for transport purposes. The recoveries of immunoactivity (Monash RIA) through the fractionation procedure were hFF, 17.9%; IVF serum, 27.1%, male plasma, 35%. In a previous study (11) it was shown that, by using this fractionation procedure, the recovery through the immunoaffinity step was more than 90%, with 50–70% recoveries at the RP-HPLC step

and ~30% recoveries at the preparative PAGE, electroelution, methanol precipitation steps. Immunological recovery for postmenopausal plasma was not determined as the immunoactivity in the starting material was below the sensitivity of the assay. The ovine *in vitro* bioassay was utilized because of its previous use in application to serum (14, 15) and its increased sensitivity (Table 1) compared to the rat *in vitro* bioassay.

hFF Prep-PAGE pools

Fractions from the hFF prep-PAGE were pooled according to the profile of *in vitro* bioactivity and immunoactivity for further analysis. Details of these pools (designated I-VII) are presented in the legend to Fig 5 (see below).

Assays

The World Health Organization human inhibin A reference preparation (91/624) was used as standard in all assays. The unitage of this preparation was expressed in terms of its nominal vial inhibin content (5 μg) rather than a specified unitage for two reasons, firstly the preparation has yet to be assigned a unitage by the WHO and secondly, a mass unitage was used in this study to enable assessments of specificity between assays.

Inhibin in vitro bioassays

An ovine inhibin *in vitro* bioassay was employed based on the effects of graded doses of inhibin on FSH release by ovine pituitary cells in culture (14). The within-assay variation, based on the average index of precision, was 0.15.

A rat inhibin *in vitro* bioassay was also used to test the various preparations for FSH suppressing and stimulating activity. The method was based on the method of Scott (*et al.* (16), except FSH release rather than FSH cell content was used as assay endpoint. The within-assay variation, based on the average index of precision, was 0.11.

This modification was introduced because the inhibin dose response range was broader using FSH release as the endpoint.

Groome α-βA subunit dimer ELISA

The inhibin α-βA subunit dimeric ELISA of Groome and O'Brien (7) was used with modifications (17). The monoclonal antibody (E4) to the inhibin βA subunit peptide (amino acids 82–114) was employed as capture antibody with Fab-alkaline phosphatase conjugate of monoclonal antibody (R1) to the α subunit peptide (amino acids 1–32) of human inhibin as label. The alkaline phosphatase activity was amplified using the AMPAK kit (Dako Corp, Carpinteria, CA). Samples were initially treated with hydrogen peroxide as described (17). Further details about the specificity and sensitivity of the assay are presented in Table 1. The assays were undertaken in Melbourne, Australia.

Medgenix α-βA subunit ELISA

This two-site ELISA (Medgenix Diagnostics, Fleurus, Belgium) consists of an affinity chromatography-purified polyclonal antibody to inhibin βA subunit (amino acids 91–103) as capture antibody and a horseradish peroxidase conjugate to a monoclonal antibody to inhibin α subunit (amino acids 1–17) as label (8). All samples were assayed, as described in the kit protocol, in the presence of inhibin-free serum provided separately by the company. Further details of the assay are presented in Table 1.

Medgenix α-α subunit ELISA

A two-site ELISA (Medgenix Diagnostics) was employed using an immunoaffinity purified polyclonal antibody raised to inhibin α subunit peptide (amino acids 15–32) as capture antibody and a horseradish peroxidase conjugate to a monoclonal antibody raised to inhibin α subunit (amino acids 1–17) as label. (8, 18). The assay procedure was followed exactly as outlined in the kit protocol. Further details of the assay are presented in Table 1. The Medgenix reagents were provided in kit form with assays undertaken in Melbourne, Australia.

Genentech inhibin A (Ck/Ck) ELISA

This two-site inhibin A ELISA uses affinity purified chicken polyclonal antibodies raised to purified human recombinant 34k inhibin A as both capture and detection (biotinylated) antibodies (6). The bound biotinylated antibody was detected by horseradish peroxidase (coupled to streptavadin). This assay detects all forms of inhibin A dimers with crossreactivity with free α subunit and inhibin B.

Genentech α-βA subunit ELISA(9A9/CK)

This format uses a mouse monoclonal antibody (9A9) raised to recombinant human activin A as capture antibody and biotinylated chicken antibody (described above) as detection antibody (6). This assay does not detect inhibin B and free α subunit. This assay was applied to hFF fractions only based on its lower sensitivity. Studies utilizing Genentech assays were undertaken at Genentech.

Monash inhibin RIA

Inhibin was measured by a heterologous RIA (15) with bovine 31K inhibin as tracer and an antiserum (#1989) raised against bovine 31K inhibin. This assay shows cross-reaction with bovine 58K inhibin, pro-αC, and pro-αN-αC inhibin subunits but not with αN subunit, activin, or follistatin (19). The immunoactivity of inhibin following reduction was less than 0.5%, indicating the RIA detected a conformational epitope. Cross-reaction studies using peptides to the αC subunit identified a carboxy terminal sequence (93–108 aa) with 0.002% cross-reaction, while other peptides showed no cross-reaction (20).The inhibin-free serum provided in the Medgenix α-βA assay was used as diluent for the inhibin standard and serum dilutions in the assay of plasma/serum.

Statistical analyses

Prep-PAGE fractions were assayed at one dose level in all immunoassays. Prep-PAGE samples assayed in the in vitro bioassay, hFF prep-PAGE pools, and samples used in the specificity studies were assessed at multiple doses and analyzed where appropriate by parallel line assay statistics.

Results

Specificity of the in vitro bioassays and immunoassays

The sensitivity and specificity of the various inhibin assays presented in Table 1 are expressed in terms of the World Health Organization inhibin A standard. All preparations in the specificity studies were bioactive as tested in the rat in vitro bioassay. In the ovine in vitro bioassay inhibin B, follistatin and activin A and B were much less active compared with inhibin A. The α-βA ELISAs detected inhibin A but did not detect activin A, B, follistatin, or inhibin B. The α subunit-directed assays, including Genentech Ck/Ck ELISA and Monash RIA, detected inhibin A, B, and corresponding culture media as well as the media from an α subunit producing cell line, but not activin A, B, or follistatin.

Fractionation of hFF and serum/plasma

To investigate the ability of these assays to detect the various inhibin forms found in hFF and serum/plasma, these samples were fractionated by preparative-PAGE to resolve the various molecular weight forms of inhibin. The patterns of in vitro and immunological activities are presented in Figs. 1–5, and the molecular weights of peak activities are presented in Table 2.

In vitro bioactivity

Following hFF fractionation, peaks of in vitro bioactivity with molecular wts of 33, 36, 55, 66K, with low levels of more than 100K (Figs. 1–4, Table 2) were detected. The in vitro bioactive profile in IVF serum showed a similar pattern to hFF, with evidence of 36K, 55K, and 66K forms; however, additional forms of ~29K, 45K, and more than 100K were noted with the apparent absence of a 33K component. No in vitro bioactivity was detected in either postmenopausal or male plasma.

α-βA immunoactivity

The Groome and Medgenix α-βA ELISAs gave similar hFF patterns of immunoactivity (correlation coefficient, r = 0.97, Table 3) comparable with the in vitro bioactivity (r = 0.86, 0.76 respectively, Table 3), detecting the 33–36 and 55–66K forms.

TABLE 1. Assessment of the specificities of the in vitro bioassays and immunoassays used in this study

	In vitro bioassay (ovine)	In vitro bioassay (rat)	Groome α–βA	Genentech (9A9/CK) α–βA	Medgenix α–βA	Genentech (CK/CK) inhibin A	Medgenix α–α	Monash RIA inhibin A
Sensitivity (pg/mL) (Inhibin A, 91/624)	50	200	4	400	80	1000	780	360
Preparation								
Inhibin A (WHO)	100%	100%	100%	100%	100%	100%	100%	100%
Inhibin B	0.9	17.2	< 0.1	< 0.1	< 0.1	82	16.7	12.3
Activin A	not active[a]	stim[b]	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Activin B	not active[a]	stim[b]	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	0.6
Follistatin	not active[a]	2.5[b]	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Culture Medium (ng/mL)								
Inhibin A	> 1170	1188	2650	800	1860	52000	41600	3600
Inhibin B	12	246	3.4	< 0.1	4.8	4800	2860	498
Activin A	stim	stim	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.5
Activin B	stim	stim	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
α subunit	0.032	4.8	0.74	< 0.1	~2.9	900	105	70

The sensitivities of the various assays in terms of inhibin A (91/624) reference preparation are also included.
[a] not active at 10 ng/mL.
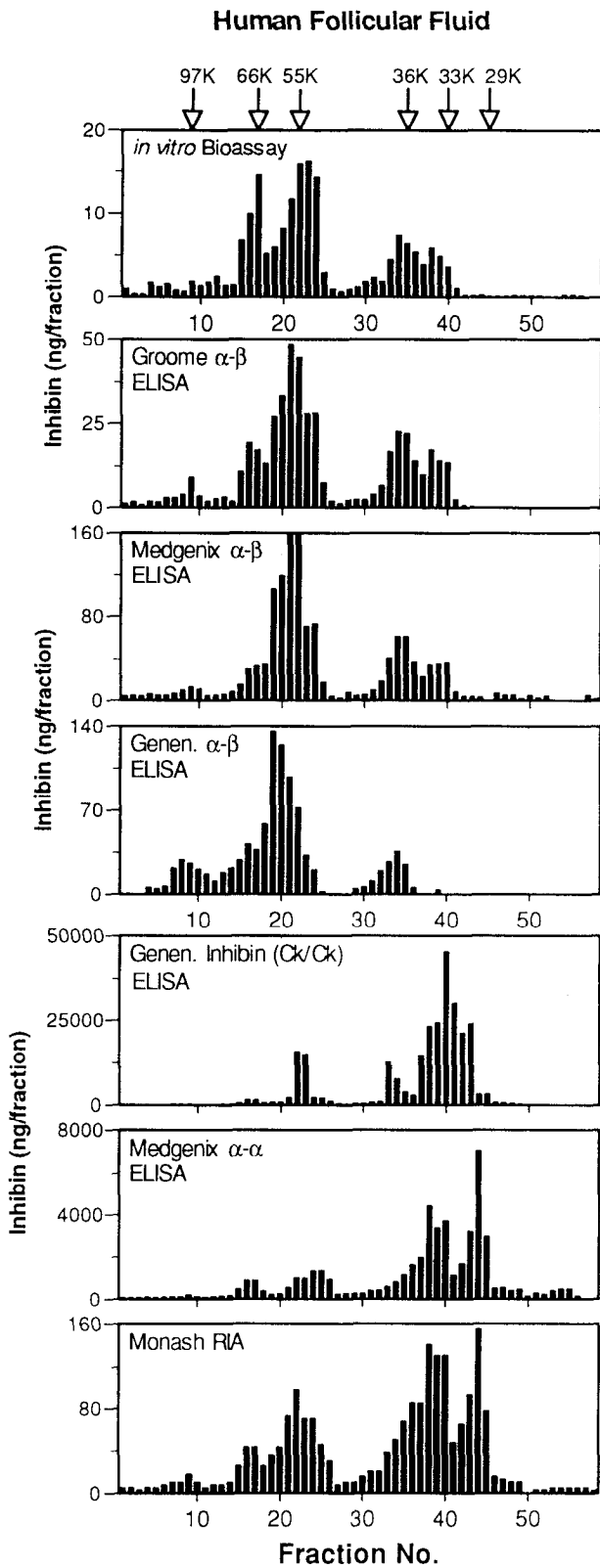[b] at 5ng/ml.

Human Follicular Fluid

FIG. 1. Molecular weight distribution of inhibin *in vitro* bioactivity and immunoactivities in human follicular fluid fractionated through a combined immunoaffinity preparative SDS-PAGE procedure. The inhibin activity was recovered from the gel by an electroelution procedure.
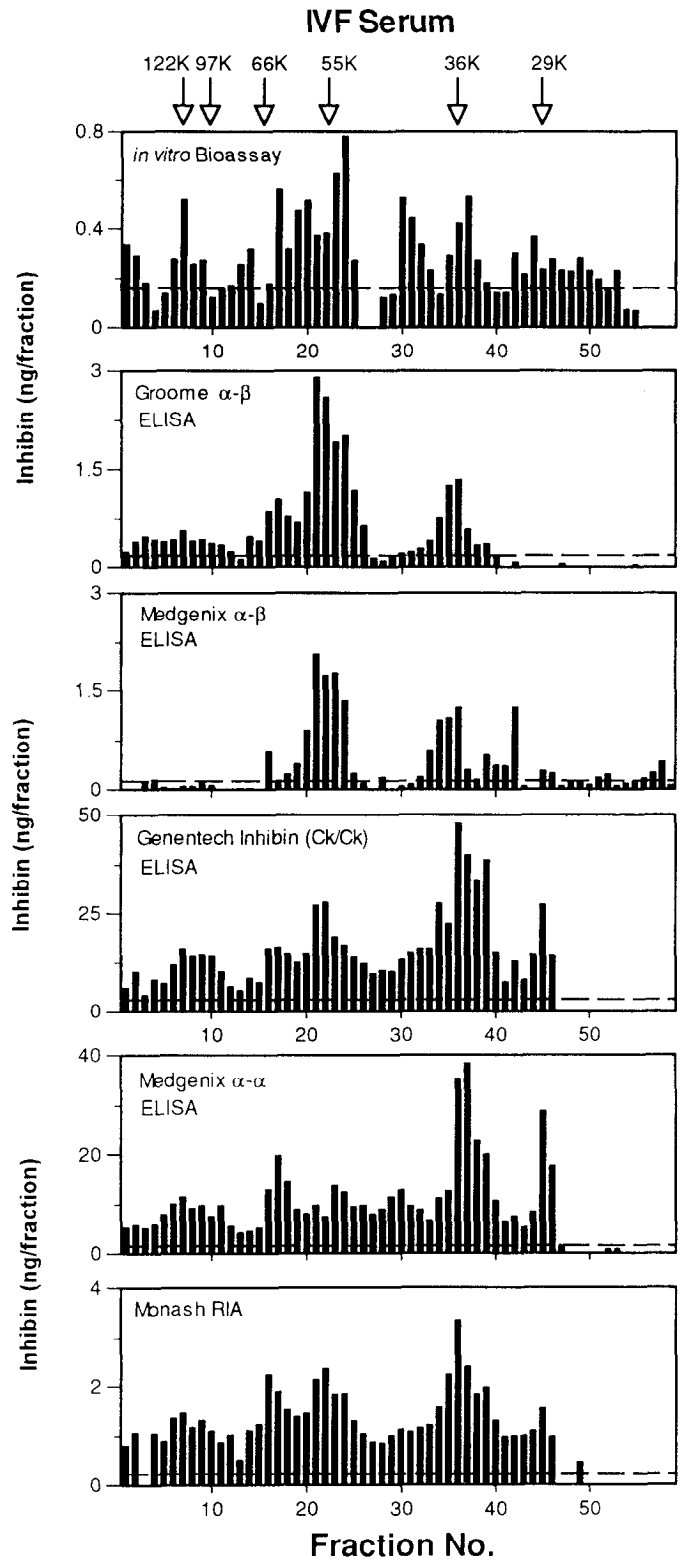
IVF Serum

FIG. 2. Molecular weight distribution of inhibin *in vitro* bioactivity and immunoactivities in serum from women stimulated with gonadotropins (IVF serum) fractionated through a combined immunoaffinity preparative SDS-PAGE procedure. The *horizontal dashed line* refers to the level of detection of the assay.
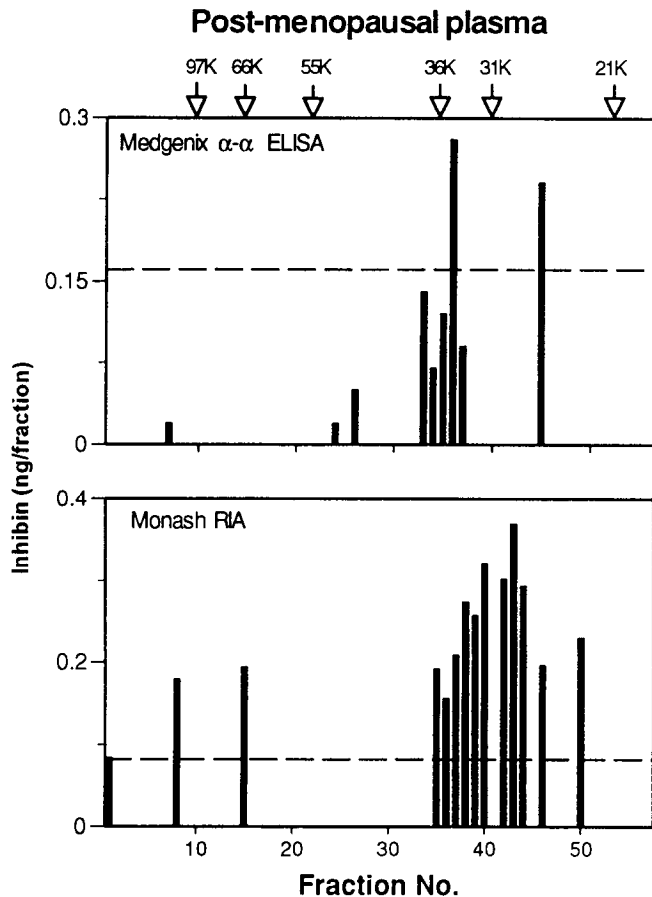
Post-menopausal plasma

FIG. 3. Molecular weight distribution of inhibin immunoactivities in serum from women after menopause fractionated through a combined immunoaffinity preparative SDS-PAGE procedure.

The Genentech α-βA ELISA differed from the other ELISAs by detecting ~100K forms but not detecting the 33K form.

The patterns of immunoactivity (Groome and Medgenix ELISAs) and *in vitro* bioactivity in fractionated IVF serum were poorly correlated (r < 0.38), although immunoactivity levels measured by the two α-βA assays were highly correlated (r = 0.83). 55K and 36K peaks were detected by both *in vitro* bioassay and ELISAs, although 43K and ~29K bioactive peaks were less readily detected by either immunoassay. Medgenix α-βA ELISA did not detect the 100K forms but detected activity in the 30K region. Neither Groome nor Medgenix α-βA ELISAs detected any activity in postmenopausal and male plasma.

α subunit-directed immunoassays

In hFF, in addition to the molecular weight forms detected by *in vitro* bioassay, immunoactivity was detected in the 29K region. The correlations between *in vitro* bioassay and immunoassays were poor (r < 0.55), while correlations between immunoassays were good (r = 0.82, 0.92, Table 3). Similar patterns of immunoactivity were detected in fractionated IVF serum with poor correlations with *in vitro* bioactivity (r < 0.27) and moderate correlations between immunoassays (r = 0.52, 0.65). Immunoactivity in the 29–36K region was observed in fractionated male and postmenopausal plasma,
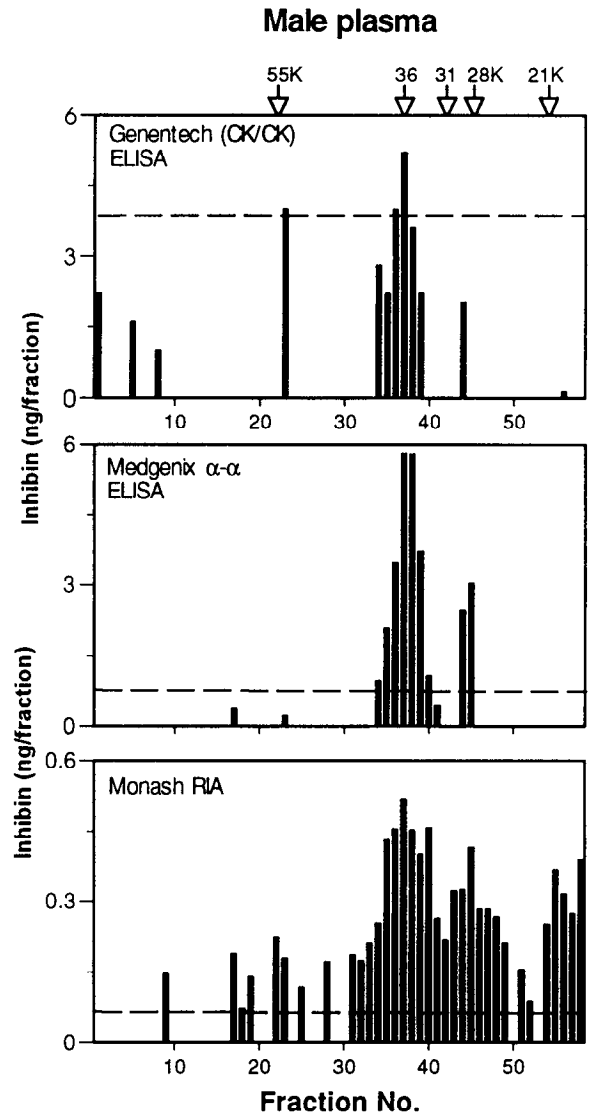
Male plasma

FIG. 4. Molecular weight distribution of inhibin immunoactivities in serum from male plasma fractionated through a combined immunoaffinity preparative SDS-PAGE procedure. The *horizontal dashed line* refers to the level of detection of the assay.

with higher levels detected by RIA particularly in the 29K region, although the levels overall were very low.

hFF inhibin pools

The Prep-PAGE hFF fractions were combined into pools (I-VII) for further assay characterization. These fractions were assayed at multiple dilutions in the respective assays, and activities were determined by parallel line assay statistics. As seen in Fig. 5, the B:I ratios for the various fractions in the molecular weight region 36K-97K were largely similar for each immunoassay, although the average B:I ratio showed major differences (100-fold) between immunoassays.

Discussion

As seen in previous studies, inhibin exists as a variety of molecular weight forms in hFF and serum (11, 21–23). In this
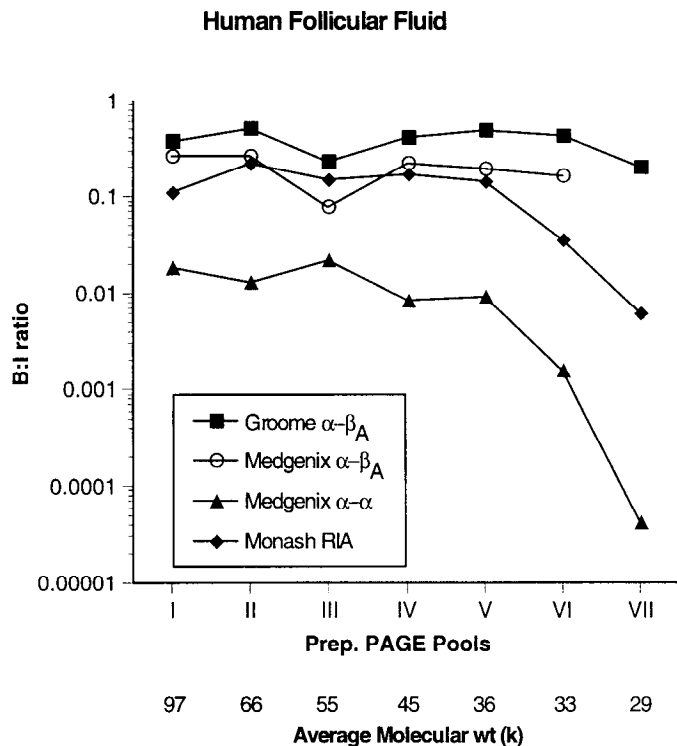
Human Follicular Fluid

FIG. 5. Ratio of *in vitro* bioactivity (B) to immunoactivity (I) for the different immunoassays for inhibin pools representing the major hFF inhibin fractions (Fig. 1). Pool I fractions 6–10 (mean mol wt 97K); II, 15–18 (66K); III, 19–22 (55K); IV, 23–26 (45K); V, 33–37 (36K); VI, 38–41 (33K); VII 42–45 (29K).

study, hFF bioactivity is found predominantly in the 30–70K region, with little evidence of higher molecular weight or unprocessed inhibin forms. In serum, the pattern appears more complex with evidence of both higher and lower molecular weight forms. The dimeric assays largely reflected inhibin bioactivity, although there were differences between ELISAs. The α subunit-directed assays emphasized to varying extents the presence of the free α subunit in the samples.

Human follicular fluid inhibin

The four major regions of *in vitro* bioactivity (33, 36, 55, and 66K) identified in hFF have been attributed previously to mono- and diglycosylated 30K inhibin A (33K and 36K inhibin in this study) as found in recombinant human inhibin A (12), αN-αC/β (55K), and either pro-αN-αC/β or αC/proβ-β (66K). These forms are expected to be both bioactive and detectable by the α-βA and α subunit-directed immunoassays. It was thus surprising that the 33K form of bioactive inhibin was not detected by the Genentech α-β ELISA, because one would not expect that the specificity of this assay would be influenced so markedly by differences of glycosylation. It was also noted that, in contrast to the other α subunit-directed assays, the Genentech Ck/Ck, which utilized the same α subunit antiserum as the Genentech α-βA ELISA, did not detect the 29K immunoactive form (presumably pro-αC) in hFF. An alternative possibility is that the 33K and 29K forms consist of NH_2-terminally clipped αC subunits [as reported for ovine inhibin (24) and human pro-αC (22)], and thus the the Genentech α-βA and Ck/Ck ELISAs would not detect these forms, because the antibody

epitope(s) in these assays would be located at the NH_2 terminal region of the αC subunit. The site of the epitope in these assays has not yet been identified, although it is known that an inhibin epitope is located in this region of the molecule. In fact, there may be a number of epitopes in this region, because the other ELISAs also utilize antisera raised to the NH_2 terminal region of the αC subunit; in fact, the Medgenix α-α ELISA utilizes two antibodies to the 1–17aa and 15–32aa region of the αC subunit. Aside from Genentech Ck/Ck assay where the epitopes are unknown, the RIA is the only assay with its epitope, which is conformationally directed, in the carboxy terminal region of the the αC subunit (20), and not in the NH_2 terminal region like the others. It is of interest in this regard that the RIA is able to readily detect molecular weight forms of less than 30K in male and postmenopausal plasma not readily detected by the other α subunit-directed assays, suggesting that even these latter assays may be unable to detect all inhibin α subunit forms. In addition, the suggestion that the 33K inhibin is a processed form of 36K inhibin rather than a monoglycosylated form is supported by the presence of the bioactive 36K but not 33K form in IVF serum in contrast with hFF, suggesting that processing of 36K inhibin may be occurring in hFF but not in serum, rather than the alternative possibility that the monoglycosylated form is found in hFF but not secreted into the circulation.

IVF serum inhibin

The pattern of inhibin bioactivity and immunoactivity in IVF serum is different from hFF in several respects. The presence of high molecular weight forms (>70K) in serum and not in hFF is attributed to hFF sample variability of unknown cause, as previous hFF pools have shown evidence of higher molecular weight forms (11). The presence of the 45K bioactive and possibly immunoactive (cf Medgenix α-α ELISA profile) peak in IVF serum is not readily found in hFF, and the presence of bioactivity (<30K) suggests alternative, yet unknown, processing. In addition, the levels of free α subunit appear to be much less compared with patterns of α subunit immunoactivity determined in hFF. These results are in agreement with previous studies comparing inhibin *in vitro* bioactivity and immunoactivity (RIA) levels in IVF serum (15), where similar values and responses to gonadotrophin treatment were observed. To differentiate between inhibin dimer and free α subunit in these chromatograms will require the development of free α subunit specific assays.

Male and postmenopausal serum inhibin

The profile of inhibin immunoactivity in male plasma (the levels were too low to be detected by bioassay) showed a preponderance of activity at 36K. In previous studies, higher proportions of higher molecular weight inhibin were detected in male plasma (11). Some possible explanations for this difference may relate to subject variability, sensitivity to proteolytic cleavage, etc; further studies are clearly needed. Is the 36K material, which is not detected by the α-βA ELISAs, inhibin B? The development of specific assays for inhibin B will resolve this question.

The levels of inhibin measured by Monash RIA across the

TABLE 2. Molecular weights of peak fractions of *in vitro* bioactivity as determined using ovine pituitary cells in culture and immunoactivity following fractionation of hFF, serum from women stimulated with gonadotropins (IVF serum), postmenopausal plasma, and male plasma presented in Figs. 1–4

Assay	Human follicular fluid						
In vitro bioassay	(100K)[a]	66K	55K		36K	33K	
α-βA ELISAs							
Groome	(100)[a]	66	55		36	33	
Medgenix		66	55		36	33	
Genentech	100		60		36		
α subunit-directed assays							
Genentech (Ck/Ck)			55		36	(33)[a]	29?
Medgenix		66	55		(36)[a]	33	29
Monash RIA	(100)[a]	66	55		36	33	29
	IVF serum						
In vitro bioassay	120	75–50		45	36		27–30
α–βA ELISAs							
Groome	>100	66	55		36		
Medgenix		(66)[a]	55		36	33	
α subunit-directed assays							
Genentech (Ck/Ck)	>100	66	55		36		29
Medgenix	>100	66	55	45	36		29
Monash RIA	>100	66	55	(45)[a]	36		29
	Postmenopausal plasma						
α subunit-directed assays							
Genentech (Ck/Ck)					36		29
Monash RIA					36		29
	Male plasma						
α subunit-directed assays							
Medgenix					36		29
Genentech (Ck/Ck)			55		36		
Monash RIA			55		36		29	20

Presumed structures: 100 and 120K = αN-αC/proβ-β; 55 and 66K = (Pro)-αN-αC/β or αC/proβ-β; 43k = ?; 33–36k = αC/β, 29K = Pro-αC; 20K = αC?

[a] () small peak or shoulder.

TABLE 3. Regression coefficients comparing inhibin values obtained between assays across human follicular fluid and IVF serum chromatograms

	Human follicular fluid		IVF serum	
In vitro bioassay (x axis) *vs.*				
Groome α-βA	y = 2.32x + 1.14	r = 0.86[a]	y = 2.12x + 0.016	r = 0.38[c]
Medgenix α-βA	y = 6.84x + 1.32	r = 0.76[a]	y = 0.067x+1.39	r = 0.03NS
Genentech α-βA	y = 3.09x + 16.3	r = 0.45[b]		
Genentech (Ck/Ck)	y = 516x + 2211	r = 0.25NS	y = 13.7x + 12.2	r = 0.19NS
Medgenix α-α	y = 60.4x + 476	r = 0.27NS	y = 16.6x + 5.92	r = 0.27NS
Monash RIA	y = 4.64x + 17	r = 0.55[b]	y = −0.79x + 2.57	r = 0.28NS
Groome α-βA (x axis) *vs.*				
Medgenix α-βA	y = 3.23x − 5.3	r = 0.97[a]	y = 0.46x +0.57	r = 0.83[a]
Genentech α-βA	y = 1.88x + 7.42	r = 0.74[a]		
Monash RIA (x axis) *vs.*				
Genentech (Ck/Ck)	y = 24.2x − 170	r = 0.92[a]	y = 16.2x −7.76	r = 0.65[c]
Medgenix α-α	y = 197x −294	r = 0.82[a]	y = 12.2x + 7.93	r = 0.52NS

Values above assay sensitivity only are included in the analysis.
NS, not significant.
[a] $P < 0.001$.
[b] $P < 0.01$.
[c] $P < 0.05$.

postmenopausal plasma chromatogram were detectable primarily because of the 5–10-fold concentration of inhibin by the fractionation procedure. In contrast, immunoactivity in the original serum was below the sensitivity of the assay. Does the ovary after menopause produce low levels of inhibin?

B:I Ratios

The B:I ratios for pools collected over the hFF chromatogram (Fig. 5) show marked differences between assays and between pools. The differences between pools within assay, particularly with the α subunit-directed assays, was attributed to the pres-

ence of bioinactive α subunits in the lower molecular weight range as discussed above. However the marked differences between assays, as clearly seen in the more than 36K molecular weight region (Pools I–V) where predominantly dimeric inhibin is found, is most likely caused by the choice of the standard employed. As an example, the large overestimation of the levels of immunoactivity in the Medgenix α-α assay in all of these pool fractions is more likely caused by a reduced sensitivity of the 30K inhibin A standard in the assay. Although the reasons are unknown, oxidation of inhibin has been shown to modify its immunoactivity (25), and it is possible, as suggested by these authors, that the standard and sample may be differentially oxidized, leading to discrepant results. What ever the reason, these findings highlight the need for appropriate standards, particularly when inhibin assays are to be used in a clinical setting.

The observation that inhibin B was relatively inactive in the ovine *in vitro* bioassay (0.9% compared with inhibin A) and to a lesser extent in the α subunit-directed assays was a surprise and raised the question that inhibin B was only partially detected in the fractionated samples by these assays. Studies specifically directed to investigate inhibin B using an appropriate *in vitro* bioassay and immunoassay are clearly an important next step.

It is recognized however, that although the antiserum used as immunoabsorbant was raised to the full length αC subunit, there are forms of inhibin present in the samples that are most likely not detected by this antiserum or by the various assay methods. For example, αN subunit fragments, which have been identified in bovine follicular fluid (19), would be expected to be found at least in follicular fluid and not be detected by the immunoabsorbant step or by any of the assay methods.

Conclusions

First, α-βA assays generally give a good indication of bioactivity in hFF but less so in serum. Their failure to detect all apparent molecular weight forms in serum appears to be related to differential processing of inhibin, and thus loss of immunoactivity, and to the possible presence of inhibin B; issues which require future attention. Second, α-α subunit directed assays show a wide range of specificities in their ability to detect inhibin subunit and dimeric forms in hFF; however, their profiles in serum suggest that the free α sub-unit may not be a major component.

References

1. **Vale WW, Hseuh A, Rivier C, Yu J.** 1990 The inhibin/activin family of hormones and growth factors. In: Sporn MA and Roberts AB, eds. Peptide growth factors and their receptors: handbook of experimental physiology. Vol 95. Berlin: Springer-Verlag; 211–248.
2. **Burger HG.** 1922 Inhibin. Reprod Med Rev. 1:1–20.
3. **Baird DT, Smith KB.** 1993 Inhibin and related peptides in the regulation of reproduction. Oxf Rev Reprod Biol. 15:191–232.
4. **Moore A, Krummen LA, Mather JP.** 1994 Inhibins, activins, their binding proteins and receptors: interactions underlying paracrine activity in the testis. Mol Cell Endocrinol. 100:81–86.
5. **Knight PG, Groome N, Beard AJ.** 1991 Development of a two site immuno-radiometric assay for dimeric inhibin using antibodies against chemically synthesised fragments of the α and βsubunit. J Endocrinol 129:R9-R12.
6. **Baly DL, Allison DE,, Krummen LA, et al.** 1993 Development of a specific and sensitive two-site enzyme-linked immunoabsorbent for measurement of inhibin-A in serum. Endocrinology. 132:2099–2108.
7. **Groome NP, O'Brien M.** 1993 Two-site immunoassays for inhbin and its subunits. Further applications of the synthetic peptide approach. J Immunol Methods. 165:167–176.
8. **Poncelet E, Franchimont P.** 1994 Two site enzymo-immunoassays of inhibin. Ares-Serono Symposia Series-Frontiers in Endocrinology. 3:45–54.
9. **Knight PG, Beard AJ, Wrathall HM, Castillo RJ.** 1989 Evidence that the bovine ovary secretes large amounts of monomeric inhibin α subunit and its isolation from bovine follicular fluid. J Mol Cell Endocrinol. 2:189–200.
10. **Schneyer AL, Sluss PM, Whitcomb RW, Martin KA, Sprengel R, Crowley WF.** 1991 Precursors of α-inhibin modulate FSH receptor binding and biological activity. Endocrinology. 129:1987–1999.
11. **Robertson DM, Sullivan J, Watson M, Cahir N.** 1995 Inhibin forms in human plasma. J Endocrinol. 144:261–269.
12. **Tierney ML, Goss NH, Tomkins SM, et al.** 1990 Physicochemical and biological characterization of recombinant human inhibin A. Endocrinology. 126:3268–3270.
13. **Forage RG, Brown RW, Ring JM, et al.** 1987 The cloning and expression of inhibin genes: α subunit use as a fecundity vaccine. In: Burger HB, de Kretser DM, Findlay JK, Igarashi M, eds. Inhibin: non-steroidal regulation of follicle stimulating hormone secretion. Serono Symposium. New York: Raven Press; 42:89–103.
14. **Tsonis CG, McNeilly AS, Baird DT.** 1986 Measurement of exogenous and endogenous inhibin in sheep serum using a new and extremely sensitive bioassay for inhibin based on inhibition of ovine pituitary FSH secretion *in vitro.* J Endocrinol. 110:341–348.
15. **Robertson DM, Tsonis CG, McLachlan RI, et al.** 1988 Comparison of inhibin immunological and *in vitro* biological activities in human serum. Journal of Clinical Endocrinology and Metabolism. 67:438–448.
16. **Scott R, Burger HG, Quigg.** 1980 A simple and rapid *in vitro* bioassay for inhibin. Endocrinology. 107:1536–1542.
17. **Groome NP, Illingworth PJ, O'Brien M, et al.** 1994 Detection of dimeric inhibin throughout the human menstrual cycle by two-site enzyme immuno-assay. Clin Endocrinol (Oxf). 40:717–723.
18. **Poncelet E, Eliard P.** Construction of a sensitive two-site immunoenzymatic assay for human inhibin in serum and other biological fluids. Abstracts of the 2nd joint ESCO-ESHRE meetng Milan, 1990, p 110–111.
19. **Robertson DM, Giacometti M, Foulds LM, et al.** 1989 Isolation of inhibin α-subunit precursor proteins from bovine follicular fluid. Endocrinology. 125:2141–2149.
20. **Lambert-Messerlian GM, Isaacson K, Crowley Jr WF, Sluss P, Schneyer AL.** 1994 Human follicular fluid contains pro- and C-terminal immunoreactive α-inhibin precursor proteins. J Clin Endocrinol Metab. 78:433–439.
21. **Robertson DM, Foulds LM, de Vos F, Leversha L, de Kretser DM.** 1990 Identification of inhibin and inhibin-related proteins in human follicular fluid. Reprod Fertil Dev 2:327–335.
22. **Hasegawa Y, Miyamoto K, Sugino H, Takio K, Inoue M, Ibuki Y.** 1994 Progress with human and rat inhibin. Ares-Serono Symposia Series- Frontiers in Endocrinology. 3:5–24.
23. **Muttukrishna S, Fowler PA, Groome NP, Mitchell GG, Robertson WR, Knight PG.** 1994 Serum concentrations of dimeric inhibin during the spontaneous human menstrual cycle and after treatment with exogenous gonadotrophin. Hum Reprod. 9:1634–1642.
24. **Bardin CW, Morris PL, Chen C-L, et al.** 1987 Testicular inhibin:structure and regulation by FSH, androgens and FGF. Serono Symposia. 42:179–190.
25. **Knight PG, Muttukrishna S.** 1994 Measurement of dimeric inhibin using a modified two-site immunoradiometric assay specific for oxidised (met-o) inhibin. J Endocrinol. 141:417–425.