Immunological characterization of human luteinizing hormone with special regard to a common genetic variant

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Abstract

Immunoassays are widely used for measuring gonadotropins, including luteinizing hormone (LH), as these are both specific and sensitive. Because LH is microheterogeneous it has been claimed that the specificity of monoclonal antibodies used in two-site immunoassays can limit their clinical utility. Furthermore, we reported earlier a common genetic variant form of LH due to amino acid alterations in the LH β gene that is poorly or not recognized by antibodies directed against epitopes present in the intact molecule. We here report the result of an LH epitope mapping using 30 different monoclonal antibodies. The antigenic area affected by the amino acid alterations is fairly large, as antibodies to the two intact domains are able to sandwich each other. Combinations of α - β or β - β

antibodies generally provide alternatives for unbiased detection of circulating LH and some have reasonably good discrimination of human chorionic gonadotropin. The β - β combinations exhibit a peculiarity in urine determinations as they detect the urinary β -core fragment. Our aims were to study the altered immunoreactivity caused by the amino acid changes and to design two-site LH assays fully capable of recognizing the biologically active LH variant. We also conclude that the variability in recognizing the universally occurring LH variant is the most important factor contributing to the widely documented LH immunoassay discrepancies.

Journal of Endocrinology (2001) **168**, 107–116

Introduction

The human (h) glycoprotein hormones luteinizing hormone (LH), chorionic gonadotropin (hCG) and follicle-stimulating hormone (FSH) are required for normal reproduction. These hormones, along with thyroid-stimulating hormone (TSH), are all α / β -heterodimers with a common α -subunit, whereas the β -subunit is unique and confers the hormone specificity. Both subunits are glycosylated at specific residues and the degree of glycosylation modulates the biological activity of the gonadotropins (Gharib *et al.* 1990). These hormones appear in blood and urine in a variety of forms ranging from heterodimeric intact molecules to small fragments (Birken *et al.* 1996).

Sensitive and specific assays of the gonadotropins are essential for assessment and treatment of fertility problems. LH and FSH are presently mostly determined by two-site immunoassays frequently using monoclonal antibodies (mab) recognizing different epitopes on the hormone molecule (Odell & Griffin 1987, Pettersson & Söderholm 1990). Variations in detectability of hormones by antibodies can depend on many factors affecting the epitope presentation

such as posttranslational modifications, internal cleavages or fragmentations, interactions with other proteins or mutations. It is also possible that mabs may recognize the same epitope but with different affinities. Epitopes are generally thought to consist of linear and/or conformational segments of the polypeptide chain. As the glycoproteins are highly microheterogeneous due to variations in the attached oligosaccharides, it is conceivable that these may influence the ligand detection of the mabs. The effect of carbohydrate heterogeneity on the immunoreactivity of antibodies against glycoprotein hormones remains highly controversial (Costagliola *et al.* 1994b, Berger *et al.* 1996). In addition it has been reported that glycosylation of the α -subunit is essential for expression of the domains involved in hTSH immunoreactivity (Papandreou *et al.* 1991).

The high specificity of mabs may occasionally limit their clinical utility. For LH this was demonstrated by Pettersson *et al.* (1991) who reported that some commercial two-site immunoassays utilizing mabs recognizing the intact LH α / β -heterodimer failed to detect a variant form of LH. This common genetic variant LH form (V-LH) was later shown to be due to two point mutations in the LH β -subunit gene which both alter the amino acid sequence,

Trp8 Arg and Ile15 Thr (Furui *et al.* 1994, Pettersson *et al.* 1994, Nilsson *et al.* 1998). Because of the amino acid change in codon 15, introducing a glycosylation consensus sequence, the V-LH is suggested to have an extra carbohydrate chain at Asn13 similar to that exhibited by hCG (Talmadge *et al.* 1984) and by recombinantly produced V-LH (Suganuma *et al.* 1996).

The LH polymorphism due to these mutations is now known to be widely distributed in different populations, varying between zero and 52% in carrier frequency (Nilsson *et al.* 1997, 1998). The half-life of V-LH in circulation is shorter than that of wild type LH (WT-LH), but it has a higher *in vitro* bioactivity (Haavisto *et al.* 1995, Suganuma *et al.* 1996). The homozygotes for V-LH in the Finnish population are apparently healthy whereas Japanese findings have been connected to infertility (Suganuma *et al.* 1995, Takahashi *et al.* 1998). Some other clinical effects have also been reported. Fourteen-year-old boys heterozygotic for V-LH have a slower progression of puberty as shown by testicular volume and height development (Raivio *et al.* 1996). V-LH also seemed to protect obese women from developing symptomatic polycystic ovarian syndrome (PCOS; Tapanainen *et al.* 1999), but this observation is not consistent in all studied populations (Rajkhowa *et al.* 1995).

The two point mutations causing the anomalous immunoreactivity obviously alter the epitope site which is recognized by intact LH specific mabs. Since mabs directed to the intact LH molecule often have low cross-reactivities with hCG, these have been the preferred mabs used in commercial immunoassays highly specific for LH. In this study, we tested 32 LH specific monoclonal antibodies in all possible two-site combinations in order to construct a relative epitope map. A major aim was to investigate which epitopes or epitope groups are affected by V-LH and to what extent. Using this information our intention was to identify two-site combinations with fully preserved recognition of V-LH combined with a low cross-reactivity with hCG. Since selection of antibody combinations may profoundly affect recognition of LH forms in urine, we also wanted to test the selected mab combinations with urine samples.

Materials and Methods

Reagents

A total of 32 monoclonal antibodies against LH was included in this study. These were all purchased in purified form from various commercial sources (see Table 1), except INN-bLH-1, which was obtained in ascites fluid and purified by protein G chromatography. Based on the specifications provided by the manufacturers, two antibody pairs, M21241 and 090-10266, and M21301 and 090-11412, respectively, were most likely identical. Since this proposition was supported by the results of this study we refer to these pairs by the first code mentioned here.

The antibodies which recognize the α -subunit are termed anti α -subunit antibodies, the corresponding β specific antibodies anti β -subunit antibodies and intact specific antibodies are referred to as intact LH specific antibodies.

All standards used, except LH β (WHO International Reference Preparation (IRP) 75/551), were commercial DELFIA standards (Wallac Oy, Turku, Finland). Concentrations of the standards used were 324 mIU/l TSH, 256 IU/l FSH, 10 000 IU/l hCG and 25 ng/ml LH β . Specific activities evaluated by commercial DELFIA immunoassay kits were: 6.4 IU/mg for TSH (2nd IRP, 80/558), 2100 IU/mg for FSH (2nd IRP, 78/549), 9000 IU/mg for hCG (1st IRP 75/537) and 7300 IU/mg for LH (1st IRP 68/40).

Samples

Serum and urine samples were obtained from apparently healthy nonpregnant volunteers. Serum samples were collected from 5 men, 18 premenopausal women at different phases of the menstrual cycle, and from 4 postmenopausal women. Urine samples were collected randomly from 32 women. The V-LH sample was obtained from a premenopausal woman previously described by us (Pettersson *et al.* 1992). The blood samples were allowed to clot and the tubes were centrifuged before the sera were separated and stored at -20°C until analyzed. Urine samples were stored at 4°C .

Coating of microtiter strip wells with monoclonal antibodies

Polystyrene microtiter strips (Eflab, Helsinki, Finland) were coated with purified immunoglobulin G (IgG) by physical adsorption as follows. To each well was added 1 μg purified IgG in 100 μl 0.1 mol/l NaH_2PO_4 and incubated at 35°C for 16–20 h. The wells were incubated, after a wash step, with 300 μl 0.1 mol/l NaH_2PO_4 containing 6% sorbitol and 0.1% bovine serum albumin for 1 h at room temperature. The blocking solution was then aspirated, the strips were covered with adhesive tape and stored in tight plastic bags with a humidifier at 4°C for not more than 6 months.

Labeling of antibodies

The detection mabs were labeled as previously described (Lövgren *et al.* 1984). In short, a 5 mg/ml solution of antibody and a 120- to 1400-fold molar excess of europium-isothiocyanatophenyl-diethylene-triamine-tetraacetic acid in 0.1 mol/l borate buffer (pH 8.6) were incubated for 16–20 h at 4°C . The labeled antibody was then purified by gel filtration on a Sepharose 6B/Sephadex G50 column (Pharmacia, Uppsala, Sweden) with 50 mmol/l Tris-HCl, pH 7.75 containing 150 mmol/l NaCl and 7.75 mmol/l NaN_3 as elution buffer. The Eu/IgG ratio of the labeled antibodies varied from 2:1 to 7:9 and the protein concentrations according to OD₂₈₀

varied between 10 and 95 µg/ml. Two tracer antibodies, 8d10 and 5301 from commercial DELFIA kits, had Eu/IgG ratios of 9.9 and 14.2 respectively.

Protocol for immunofluorimetric assays (IFMA)

The following protocol was used for assaying serum and urine samples. First, 25 µl of sample or standard solution were pipetted into each coated well followed by 100 µl DELFIA assay buffer (Wallac Oy). Urine samples were pipetted after the assay buffer since this provided better assay precision. The plates were then incubated for 90 min at room temperature with continuous shaking, and washed once. Following addition of 50 ng labeled tracer antibody in 150 µl assay buffer, the wells were incubated for 30 min as above. The strips were then washed six times and 200 µl enhancement solution were added (Wallac Oy). After shaking for 5 min and incubating for 15 min, the fluorescence was measured in an Arcus fluorimeter (Wallac Oy).

Screening of the monoclonal antibodies

All the antibodies were tested in two-site assays with a 210 IU/l LH standard and a blank by the following assay procedure. To coated, aspirated microtiter wells were pipetted 25 µl of standard or blank solution, in duplicate, followed by 100 µl assay buffer. The plates were then continuously shaken for 90 min at room temperature and washed once. After adding 50 ng labeled tracer antibody in 150 µl assay buffer to each well, the strips were incubated for 60 min and thereafter treated as described earlier.

Adsorption tests

The 30 mabs were tested for their ability to identify LHβ, the other glycoprotein hormones and V-LH by adsorption tests. The microtiter wells coated with the different mabs were first washed three times and 100 µl LHβ, TSH, FSH, hCG standard solution or V-LH serum sample were pipetted into the wells. The samples were then allowed to adsorb to the capture antibody for 3 h at room temperature with continuous shaking. After removing the samples from the wells, the concentrations of the unbound hormones were assayed with the following assays: DELFIA LHSpec for LHβ, DELFIA TSH for TSH, DELFIA FSH for FSH and DELFIA hCG for hCG (Wallac Oy). The V-LH serum sample was assayed with the reference method 5301/8d10 (Pettersson *et al.* 1992).

Affinity determinations

The affinity constants were determined for five Eu-labeled mabs as previously described (Pettersson & Söderholm 1991). The procedure was essentially as described above for the IFMAs. In the first incubation 25 µl WT- or V-LH serum sample were incubated with the capture antibody of the respective IFMA combination. After a wash step, the

LH bound to the capture antibody was titrated by increasing amounts of the labeled mab (12.5–400 ng/well). After subtracting the non-specific binding from the total binding, the data were used to calculate the affinities according to the method of Scatchard (1949).

Statistics

The results of the affinity and correlation studies were analyzed using Statview computer program (Abacus Concepts, Inc., Berkeley, CA, USA). The *P* values of the affinity analysis were calculated by a paired *t*-test, *P* values below 0.05 were considered statistically significant.

Results

Immunological characterization of LH

The antibodies capabilities to bind to two different sites on the hormone molecule simultaneously were studied using a high LH standard (210 U/l) and a blank. For construction of the epitope map, we combined 30 different monoclonal antibodies in two-site assays in all possible ways. An epitope chart based on these results is shown in Fig. 1. Mabs capable of binding to the analyte simultaneously (=different epitopes) are shown as non-overlapping ellipses. Antibodies that were unable to form a detectable sandwich signal (overlapping epitopes) are presented as partly overlapping ellipses. For many antibodies it was impossible to find any differences regarding their ability to sandwich the other antibodies. These antibodies are shown with their codes within the same ellipse. Using these 30 mabs we detected 20 different binding patterns (=ellipses in Fig. 1). These were grouped into six different epitope domains, two of which were directed against the α-subunit, two against the β-subunit, and two epitope domains, fully separated from each other, only reactive with the intact LH dimer. From this epitope map it is evident that many principally different two-site combinations can be constructed; alpha-alpha, beta-beta, intact-intact, alpha-beta, alpha-intact and beta-intact.

Cross-reactivities

The cross-reactivities of the mabs with LHβ and the other gonadotropins, hCG, FSH and TSH are presented in Table 1. These were determined after adsorbing the hormones to a capture antibody, and then measuring the levels of each gonadotropin, by a suitable assay, in the supernatants. Of the mabs which recognize epitope sites on the β-subunit, none reacted with FSH and one, 23c191, strongly cross-reacted with TSH. Five mabs, 094-10355, 5302, M94184, 5301 and 094-10711 cross-reacted with hCG. The six anti α-subunit antibodies reacted with all other glycoprotein hormones to varying degrees. The mabs which are specific for the intact LH

Figure 1 Epitope map of human luteinizing hormone. All 30 mabs were tested as both capture and detecting antibody in all possible combinations against each other in two-site assays. Six different epitope domains can be seen, two on each subunit and two on the intact molecule. Antibodies designated as being directed to the same epitope, that is, when they are written within an ellipse, will not bind to the hormone or its subunits simultaneously. Those antibodies which are directed to different epitope domains, presented in groups, were able to bind simultaneously to the LH molecule. Antibodies within an epitope domain and with overlapping epitopes, recognize the LH molecule poorly when they are used simultaneously. In other words, when antibodies from the same ellipse are used in a two-site assay no signal can be measured, whereas a good or even excellent signal level is obtained by combining non-overlapping antibodies from two different epitope domains.

heterodimer exhibited no binding of LH β , and showed no significant cross-reactivity to hCG, FSH or TSH in this test.

Figure 2 shows the percentage of LH adsorption from V-LH serum by 30 mabs. The inability of antibodies directed to the intact sites on the LH molecule to recognize V-LH is obvious. Five mabs, M21241, M97185, M97183, MCA 395 and INN-bLH-1, did not bind V-LH at all whereas other intact LH specific mabs, 543, M97181, M97182, M94183 and M94187, showed varying restricted reactivities with V-LH. Although mabs directed to the α - and β -domains as groups showed a higher reactivity with V-LH, the recognition by some mabs (e.g. M94137, 1035 and 5303) was clearly reduced.

Performance of selected antibody combinations

On the basis of the same results that were used for construction of the LH epitope map, 22 antibody combinations, preferably with subunit specific antibodies, were chosen for further studies (Table 2). A central criterion for selecting these combinations was the analytical performance, i.e. combining antibodies of good affinity against independent epitopes. Alpha-alpha combinations were excluded as they cross-reacted with all four glycoprotein hormones.

The cross-reactivities of the selected 22 mab combinations to recognize TSH, FSH, hCG and LH β , shown in Table 2, were determined with a high standard

Table 1 Description of the 30 commercial monoclonal antibodies used in this study. Information provided by the manufacturer is the affinity and the IgG class. The cross-reactivities, which were determined in this study, are given as + when over 90% of the glycoprotein in the sample was adsorbed, $\pm$ when 90–30% was adsorbed, and as – when below 30% had been adsorbed by the antibody

	Monoclonal antibody	Manufacturer	Affinity by manufacturer (mol/l)	IgG class	Cross-reactivities				
					LH	LH β	hCG	TSH	FSH
Epitope site									
Alpha 1	8d10	Wallac Oy	2×10^{11}		+	–	+	+	+
	1-04*	Scantibodies	2×10^{11}	IgG1	+	–	$\pm$	$\pm$	–
	M94137	Fitzgerald	8×10^9	IgG1	+	–	–	–	–
Alpha 2	5501	Medix Biochemica	2×10^9		+	–	$\pm$	$\pm$	–
	1035				+	–	$\pm$	$\pm$	$\pm$
	M94136	Fitzgerald	4.0×10^{10}	IgG	+	–	+	+	+
Beta 1	23c191	Wallac Oy	2.4×10^{10}	IgG1	+	+	–	+	–
	094-10355	OEM Concepts	8×10^{10}	IgG1	+	+	$\pm$	–	–
	5302	Medix Biochemica	1×10^{11}		+	+	$\pm$	–	–
	5303	Medix Biochemica	3×10^{11}		+	+	–	–	–
	5304	Medix Biochemica			+	+	–	–	–
Beta 2	M21301	Fitzgerald	3.7×10^{10}	IgG1	+	+	–	–	–
	M94182	Fitzgerald	$>2.0 \times 10^9$	IgG1	+	$\pm$	–	–	–
	M94184	Fitzgerald	$>5.0 \times 10^{10}$	IgG1	+	+	+	–	–
	M94186	Fitzgerald	$>4.0 \times 10^{10}$	IgG	+	$\pm$	–	–	–
	M97184	Fitzgerald	$>4.1 \times 10^{10}$	IgG1/IgG2a	+	$\pm$	–	–	–
	5301	Medix Biochemica	0.4×10^{10}	IgG1	+	+	+	–	–
	094-10711	OEM Concepts	2×10^{11}	IgG1	+	+	+	–	–
Intact 1	MCA 394*	Serotec		IgG2b	+	–	–	–	–
	M21241	Fitzgerald	3.2×10^{10}	IgG1	+	–	–	–	–
Intact 2	M97181	Fitzgerald	$>1 \times 10^9$	IgG1	+	–	–	–	–
	M97185	Fitzgerald	$>4.1 \times 10^9$	IgG1	+	–	–	–	–
	M97182	Fitzgerald	$>6.2 \times 10^9$	IgG1	+	–	–	–	–
	M94183	Fitzgerald	$>2.0 \times 10^9$	IgG1	+	–	–	–	–
	M94187	Fitzgerald	2×10^{10}	IgG	+	–	–	–	–
	3-03*	Scantibodies	3×10^9	IgG1	+	–	–	–	–
	M97183	Fitzgerald	$>1.0 \times 10^9$	IgG1	+	–	–	–	–
	MCA 395*	Serotec		IgG1	+	–	–	–	–
	543	Argene Biosoft	7.7×10^6		+	–	–	–	–
	INN-bLH-1*	Serotec		IgG	+	–	–	–	–

Scantibodies, Santee, CA, USA; Fitzgerald, Concord, MA, USA; Medix, Kauniainen, Finland; OEM Concepts, Toms River, NJ, USA; Serotec, Oxford, Oxon, UK; Argene Biosoft, Varilhes, France.

*Not currently available.

concentration of the hormone preparation. It is worth noting that the combinations with the intact LH specific mab, M94187, showed very low cross-reactivities with hCG. The three combinations with the anti β -subunit mab 5303, which is close to the intact 1 domain, had the lowest cross-reactivities with hCG as a group. The TSH standard contained trace amounts of LH which is obvious when the cross-reactivities for 23c191/5301 using two different preparations are compared (Pettersson & Söderholm 1990). The LH β preparation was apparently also contaminated with some intact LH, explaining the cross-reactivity with the intact/alpha combination. In this study, good combinations for detecting V-LH with low hCG cross-reactivity were 23c191/5301, 5304/5301 and 5304/8d10.

Correlations

Individual serum samples from premenopausal women at different phases of the menstrual cycle, postmenopausal

women and male samples, $n=27$, as well as 32 female urine samples were measured by the 22 mab combinations (data not shown). Of these combinations, the results from seven assays were chosen for correlation analyses, of which 5301/8d10 was used as reference assay (shown in Fig. 3). The overall correlations for the serum samples were all very good, $R>0.987$, independent of the type of anti-subunit combination. Urine and serum samples correlations were very similar when the β/α combinations were correlated with the β/α reference combination. The two β/β combinations, 23c191/5301 and M94184/23c191, detected variably, but predominantly, twofold (ratio 2.09) higher immunoreactivities in the urine samples compared with the β/α assays. For the two β/β combinations the urine sample correlations were 0.470 and 0.860, and for the other combinations they were >0.994 . The two β/β combinations correlated excellently to each other regarding the serum samples, $R=0.9997$, and moderately for the urine samples, $R=0.830$. The average measured LH

Figure 2 Adsorption of variant LH from a serum sample by 30 mAbs. Antibodies directed to the intact sites on the LH molecule did not recognize V-LH or recognized it poorly compared with subunit specific mAbs. Mabs M21241, M97185, M97183, MCA 395 and INN-bLH-1 did not bind V-LH at all whereas the other mAbs at the intact sites reacted poorly with V-LH.

concentration ratio of the two β/β combinations was 1.92 (data not shown). The regression line between them was $y_{M94184/23c191} = -2.81 + 0.61 \cdot x_{23c191/5301}$, showing a marked difference in slope.

Affinities of five mAbs to WT- and V-LH

We determined the affinity constants of five labeled mAbs (1 α , 2 β and 2 intact) using serum samples from three individuals of WT-LH and three individuals homozygous for V-LH. The average affinities $\pm$ s.d. of the five mAbs for the two groups of serum LH are presented in Table 3. No significant differences in affinity constants were seen for the labeled anti α -subunit or anti β -subunit mAbs. The affinity of the intact LH specific antibody M94187 was, however, significantly lower for V-LH compared with WT-LH (0.18 ± 0.04 E9 vs 0.6 ± 0.13 E9; $P < 0.028$). With the intact LH specific mAb M21241, the affinity constant for WT-LH was 1.88 ± 0.21 E9 but no binding was detected for the V-LH samples.

Discussion

The pronounced heterogeneity of the glycoprotein hormones, which predominantly is a result of variations in the

carbohydrate moieties, is well documented (Burgon *et al.* 1996, Phillips *et al.* 1997). Whether or not this heterogeneity is reflected in the way antibodies recognize glycoprotein hormones has been highly controversial (Costagliola *et al.* 1994b, Mitchell *et al.* 1995, Berger *et al.* 1996). The explanation for the anomalous immunoreactivity of the LH variant described by us in 1991 (Pettersson & Söderholm 1991) was, however, shown to be two amino acid alterations in the LH β gene (Furui *et al.* 1994, Pettersson *et al.* 1994, Nilsson *et al.* 1998). The main aim of this study was to investigate a large selection of commercially available anti LH mAbs for their reactivity with WT- and V-LH. This is important both for estimating the extent of the antigenically modified area in V-LH as well as for identifying antibody combinations that can provide equal recognition of these two biologically active forms (WT- and V-LH) while still minimizing the co-identification of hCG. Further, we intended to evaluate and compare the performance of the selected pairs of antibodies using serum and urine samples. Our study shows that a large area of the V-LH epitope determining region is altered, as all intact LH specific antibodies exhibited restricted reactivity for this LH form. We also identified three immunoassays fulfilling our criteria, and excellent correlations were obtained with α - β and β - β

Table 2 Cross-reactivities of gonadotropins with 22 different two-site LH assays performed in two steps. The standard concentrations of the gonadotropins used were: 324 mIU/l TSH, 256 IU/l FSH, 10 000 IU/l hCG and 25 ng/ml LH β

	Detector antibody	$\alpha/\beta/\text{I}$	Cross-reaction (w/w in %)			
			TSH	FSH	hCG	LH β
Capture antibody						
M21301	5303	β/β	1.73	0.01	0.00	61.74
M94187	M21301	I/β	1.09	0.01	0.05	7.46
	M94184	I/β	1.16	0.02	2.35	7.06
	23c191	I/β	1.22	0.00	0.06	7.85
	5302	I/β	1.22	0.01	1.09	7.53
	5303	I/β	1.21	0.00	0.01	6.79
	8d10	I/α	1.17	0.00	2.45	6.51
M94184	23c191	β/β	1.38	0.00	15.85	72.54
	5303	β/β	1.65	0.01	0.80	61.67
	8d10	β/α	1.80	0.01	45.38	7.99
23c191	5301	β/β	1.76	0.00	1.85	71.15
23c191*	5301	β/β	0.002	0.003	1.0	66
5301	23c191	β/β	1.59	0.01	14.35	65.21
	M94136	β/α	1.46	0.01	44.02	8.00
	8d10	β/α	1.37	0.00	44.17	7.95
	5501	β/α	1.64	0.00	52.47	7.25
5302	5301	β/β	1.54	0.00	25.96	63.55
	8d10	β/α	1.24	0.00	35.59	7.72
5303	M94186	β/β	1.76	0.00	0.03	71.27
	5301	β/β	1.61	0.00	0.06	64.11
	8d10	β/α	1.32	0.86	0.02	8.06
5304	5301	β/β	1.60	0.01	0.34	64.83
	8d10	β/α	1.20	0.04	0.30	7.90

*In this experiment reported earlier (Pettersson & Söderholm 1990) an ultrapure preparation of TSH and FSH was used.

combinations using serum samples. In applying these assays to urine samples, large differences were obtained between α - β and β - β combinations as well as between differently configured β - β assays.

For the immunological characterization of LH we combined 30 different monoclonal antibodies in all possible ways in order to investigate which antibodies were able to bind simultaneously to the LH molecule. Immunological mapping has been used for developing measuring techniques and has also helped to understand topographical relationships among major surface features (Alonso-Whipple *et al.* 1988). In our epitope map six different epitope domains were distinguished, two on the intact hormone molecule, two on the β -subunit and two overlapping sites on the α -subunit (Fig. 1). Earlier, Krichevsky *et al.* (1994) located seven antibody-binding regions on LH using an extensive library of mabs developed in their laboratory, and Costagliola *et al.* (1994a,b) distinguished 21 clusters of reactivity on the surface of the LH heterodimer. It should be acknowledged, however, that mab epitopes, as evident also from this study, rarely form totally discrete units or epitope domains but rather are arranged into a continuum of binding patterns.

In the present study we have shown that antibodies which recognize the two independent intact LH dimer epitope domains, as well as some α - and β -antibodies in

their close vicinity (e.g. M94137, 1035 and 5303), show variably restricted reactivity to V-LH. The restricted reactivity ranges from complete inability to recognize the variant to a decrease in the affinity constant relative to WT-LH. This indicates that a fairly large topographic area of the LH β molecule is affected by the amino acid changes in Trp8 Arg and Ile15 Thr. The altered immunoreactivity is probably due to conformational changes in the protein because both mutations render the NH₂-terminus more hydrophilic. Another possibility is that the putative additional carbohydrate residue at Asn13 could block the epitopes on the intact LH molecule.

In order to achieve a low cross-reactivity with hCG and good recognition of V-LH, several aspects have to be considered in selecting mabs for a two-site LH immunoassay. Combinations using an anti α -subunit specific antibody as capture mab are excluded, as these would be influenced by all glycoprotein hormones. In a combination with an anti β -subunit specific capture mab and an anti α -subunit specific detecting mab, the specificity of the assay relies totally on the anti β -subunit specific mab. One has to remember that some anti β -subunit specific mabs are so close to the intact LH epitope domains that they do not recognize V-LH properly. An example of this in our study is the anti β -subunit specific mab 5303, which has a very low cross-reactivity

Figure 3 Comparison of six different immunoassays utilizing subunit specific antibodies with the reference β/α method 5301/8d10. The results for the serum samples correlate well with R values over 0.92. Urine samples give higher concentrations with the β/β combinations, due to recognition of fragmented LH β molecules causing the lower correlations. The regression coefficients can be seen from the equations for each regression line. The slopes for the serum sample lines were relatively similar for all combinations and varied between 0.80 and 1.07. The higher LH values obtained with the β/β combinations for the urine samples are reflected by the decreased slopes, 0.54 and 0.73 for 23c191/5301 and M94184/23c191 respectively.

Table 3 Average affinity constants of three serum samples for five monoclonal antibodies. Capture mabs were 090-11412 in combination with 8d10, M94187 and M21241, 5301 with 23c191, and 23c191 with 5301. The value was not obtained for the intact antibody M21241 when samples from individuals homozygous for the point mutations in the LH β gene were analyzed. Generally, intact (int) antibodies reacted poorly with V-LH. The affinities are expressed as E9

	8d10 (α)	M94187 (int)	M21241 (int)	23c191 (β)	5301 (β)
WT-LH	33.8 $\pm$ 0.21	0.60 $\pm$ 0.13	1.88 $\pm$ 0.21	4.78 $\pm$ 0.12	4.11 $\pm$ 1.19
V-LH	35.1 $\pm$ 6.65	0.18 $\pm$ 0.04*		9.61 $\pm$ 0.78	3.23 $\pm$ 0.53

* $P=0.028$ compared with WT-LH samples.

with hCG but recognizes V-LH poorly. Combinations employing anti β -subunit specific mabs for capture and detection not only measure the biologically active dimer, as β/α combinations do, but also recognize free β subunits and fragmented forms to varying degrees. Unfortunately, they often have high cross-reactivities with hCG although reasonably specific mabs for LH β are available, such as 23c191, M21301 and M94186 in this study. Intact LH specific mabs should be excluded, as these do not recognize V-LH and WT-LH similarly.

Apart from having a closely similar α -subunit, the amino acid sequence homology of hCG β and LH β is as high as 80 percent (Talmadge *et al.* 1984) and thus the construction of LH assay designs not recognizing hCG has been difficult. The amino terminal amino acid differences of the LH β subunit and the lack of Asn13 carbohydrate moiety, and its association with the α -subunit evidently constitute a strong antigenic determinant that has enabled the development of LH antibodies highly discriminative with regard to hCG. This specificity advantage explains the popularity of antibodies against the intact heterodimer in numerous commercial tests (Costagliola *et al.* 1994b). Unfortunately, such assays were too specific in the sense that V-LH, due to its similarity to hCG, was equally poorly detected.

Costagliola *et al.* (1994a,b) reported a study which was designed to understand the causes of discrepancy among LH immunoassay methods. They suggested that the immunoreactivity of LH depends more on the purification than upon the origin of the preparation (pituitary or urinary). Comparison of 11 commercial mab-based kits indicated that discrepancy among LH assay kits was related to variation in kit calibration and in epitope specificity of the mabs used. However, they also reported five kits, all using intact LH specific detector antibodies, which misrecognized circulating LH in some subjects.

Regarding measurement of LH concentrations in urine, it has to be considered that urine to a large degree also contains free subunits, as well as fragmented forms of LH β (Kovalevskaya *et al.* 1995, Birken *et al.* 1996). The performance of intact LH specific mabs is similar, with dimeric LH found in both urine and serum, and they show a restricted reactivity towards V-LH. The two β - β mab combinations reported here can detect several-fold higher urine LH concentrations relative to the reference mab

combination detecting only intact LH, and with fairly poor correlations. The regression line between them showed a moderate correlation ($R=0.83$) and a substantial difference in slope (0.61). This amply illustrates the variability of different antibody combinations in recognizing the various fragments of the LH β -subunit present in urine.

From the comparison of numerous two-site combinations involving the different antigenic domains and their performance with patient specimens, we conclude that the single most important factor in explaining LH immunoassay discrepancies is the lack of ability to detect variant LH. Combinations with β - β and α - β specific mabs generally correlate excellently to each other although some anti β -subunit specific mabs in close vicinity to the intact domain show similar problems as assays involving intact LH specific mabs. This conclusion should not, however, be interpreted so as to exclude the possibility that other sources of LH heterogeneity can influence immunoassay behavior. It should also be kept in mind that the excess reagent situations usually applying to sandwich assays make possible affinity differences between isoforms less influential.

It is of interest to compare the findings of the present study with the hCG/LH receptor interaction model suggested by Cosowsky *et al.* (1995). According to this hypothesis the groove formed by the β 1-, β 3- and α 2-loops mediates the ligand binding to a segment of the receptor. The putative additional carbohydrate moiety at Asn13 may also, according to the same model, contribute to hormone efficacy by providing 'extra bulk', enhancing the interaction with the extra-cellular domains of the receptor. The large immunologically affected area in the α - β interface of V-LH, the likelihood of an additional sugar moiety, and the reported findings of potentiated receptor interaction of V-LH (Haavisto *et al.* 1995, Sukanuma *et al.* 1996) could conceivably fit the model proposed by Cosowsky *et al.*, although this remains speculative at the present stage.

Despite numerous investigations (Rajkhowa *et al.* 1995, Sukanuma *et al.* 1995, Raivio *et al.* 1996, Tapanainen *et al.* 1999), no major pathologies have been clearly associated with the presence of V-LH in hetero- or homozygous form. This is not surprising in view of the high prevalence of this polymorphism found in various populations around the world. Being fully biologically active, the variant LH

should, therefore, be determined in routine LH assays with equimolar potency as WT-LH.

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Received 21 March 2000

Revised manuscript received 10 August 2000

Accepted 6 September 2000