Measurement of serum and peritoneal fluid LH concentrations as a diagnostic tool for human endometriosis

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A rapid, sensitive enzymeimmunoassay for the measurement of LH concentrations in serum and peritoneal fluid samples of healthy women and women with endometriosis is reported. The ligand (LH) was captured by a readily available, widely used and well-characterized monoclonal antibody (mAb, 518B7) generated against the β subunit of bovine LH. This mAb, although specific for LH, shows very little species specificity and detects LH by radioimmunoassay in humans. A polyclonal antiserum raised in rabbits against hCG was conjugated to horseradish peroxidase and was used as the second antibody signal. This anti-hCG antiserum crossreacts with LH. The enzymeimmunoassay uses the standard human LH (hLH) preparations (NIADDK-hLH-I-3, AFP-827OB) and results are based on the relative concentrations of LH in serum and peritoneal fluid. Total assay time was < 3 h. The range of the standard curve was 0.002–0.500 ng LH per well and the lowest concentration of hLH that could be distinguished from zero concentration was 0.15 ± 0.02 ng ml⁻¹ serum and 0.058 ± 0.021 ng ml⁻¹ peritoneal fluid. Clinical

diagnostic parameters for the LH enzymeimmunoassay showed a sensitivity of 85.71%, specificity 92.50%, efficiency 88.54%, positive predictive value 94.11% and negative predictive value 82.22%. The study was retrospective. Serum LH concentrations of women with endometriosis were 13.67 ± 7.21 ng ml⁻¹, whereas serum LH concentrations of women in the control group were 4.52 ± 2.03 ng ml⁻¹. One-way ANOVA showed significant differences ($P < 0.001$) between women with endometriosis and control groups. Women in the control group had peritoneal fluid LH values of 5.65 ± 2.43 ng ml⁻¹, whereas peritoneal fluid LH values of 64.06 ± 16.44 ng ml⁻¹ were obtained in women with endometriosis ($P < 0.001$). A cycle-dependent pattern of serum and peritoneal fluid LH concentration was observed in women in the control group, which was not observed in the peritoneal fluid of the group with endometriosis. The application of this assay to serum or peritoneal fluid samples provides the attractive possibility that it could be included in the panel of markers used for diagnosis of endometriosis.

Introduction

Endometriosis is defined as the presence of endometrial glands and stroma in ectopic locations (Sampson, 1924). Peritoneal fluid of women undergoes alterations in volume (Drake *et al.*, 1980; Koninckx *et al.*, 1980a) and composition during the menstrual cycle (Syrop and Halme, 1987). The peritoneal fluid microenvironment is sensitive to the influences of endometriosis due in part to the presence of activated macrophages (Halme *et al.*, 1984). Specific changes include alterations in peritoneal fluid proteins (Haney *et al.*, 1981; Badawy *et al.*, 1984; Nothnick *et al.*, 1994), cytokines (Weinberg *et al.*, 1991; Khorram *et al.*, 1993; Ryan *et al.*, 1995; Punnonen *et al.*, 1996; Harada *et al.*, 1997; Hsu *et al.*, 1997), growth factors (Oosternlynck

et al., 1994; Huang *et al.*, 1996; Taskin *et al.*, 1996) and hormones (Loumaye *et al.*, 1984; Bouckaert *et al.*, 1986; Chew *et al.*, 1987; Barry-Kinsella *et al.*, 1994). Such alterations have been linked to infertility with effects on sperm function (Sueldo *et al.*, 1987; Coddington *et al.*, 1992; Aeby *et al.*, 1996), embryo toxicity (Morcos *et al.*, 1986; Taketani *et al.*, 1992) and uterine receptivity (Hahn *et al.*, 1986; Lessey *et al.*, 1994). Furthermore, such changes may be useful for the diagnosis of endometriosis in some women.

LH participates in the regulation of the menstrual cycle leading to folliculogenesis, ovulation, and eventual fertilization and embryo implantation. Measurement of serum LH concentrations in women has been an important procedure for understanding the endocrine changes around the time of implantation and for detection of the preovulatory surge (Yoshida *et al.*, 1987). During the menstrual cycle, serum LH concentrations peak at the time of

ovulation (Dufau *et al.*, 1976). The role of LH in human peritoneal fluid has not yet been elucidated.

A cycle-dependent pattern of peritoneal fluid LH concentrations has been observed during the menstrual cycle with concentrations that are greater than or equal to those of serum (Bouckaert *et al.*, 1986; Syrop and Halme, 1987), although this has not been observed in all studies (Kim-Brjörlund *et al.*, 1991). On the basis of its carbohydrate (sialic acid) content, the presence of LH in peritoneal fluid can potentially be explained by diffusion from the ovarian vasculature (Bouckaert *et al.*, 1986). Previous reports have also documented higher peritoneal fluid LH concentrations in women with endometriosis (Chew *et al.*, 1990), although no study has compared concomitant serum and peritoneal fluid LH concentrations in normal patients and those with endometriosis. Serum LH concentrations are increased in women with luteinized unruptured follicle syndrome, many of whom have endometriosis (Koninckx *et al.*, 1980b). The aims of the present study were: (i) to validate a well-established sandwich LH enzymeimmunoassay in several species (Illera *et al.*, 1995a, 1996, 1997; Munro *et al.*, 1997) for estimating the concentrations of LH in human serum and peritoneal fluid; and (ii) to establish whether the measurement of serum and peritoneal fluid LH concentrations could be a diagnostic tool for human endometriosis. The assay can be completed in < 3 h, is simple to perform and could potentially be included in the panel of markers used for detection of endometriosis.

Materials and Methods

Subjects

The study was performed retrospectively on two groups of women. The first group consisted of women (*n* = 32; aged 24–38 years) who were seen in the Division of Reproductive Endocrinology and Fertility at the University of North Carolina for investigation of infertility. Each of these women was diagnosed with endometriosis by laparoscopy, at which time peritoneal fluid, serum and endometrial biopsies were obtained. Each patient signed informed consent to participate in these studies and all samples used in this study were obtained in accordance with the Committee for the Protection of Human Subjects at the University of North Carolina. Histological dating was determined on each endometrial sample according to the dating criteria of Noyes *et al.* (1950). The stage of endometriosis was recorded according to the revised classification of The American Society for Reproductive Medicine (ASRM, 1997). The second group consisted of women with regular menstrual cycles (*n* = 20; control group) who underwent laparoscopic tubal ligation either by cautery or clips. None of the women entered in this study had received hormone therapy within the previous 30 days and none had been exposed to injectable progestins during the previous 6 months. Both groups were divided into three categories according to the stage of the menstrual cycle as

Table 1. Demographics of patients with and without endometriosis

	Control	Endometriosis*
Number of patients	<i>n</i> = 20	<i>n</i> = 32
Age	28.11 ± 4.2	31.16 ± 6.78
Stage of cycle		
Early proliferative	5 (25.00%)	9 (28.12%)
Proliferative	9 (45.00%)	9 (28.12%)
Secretory	6 (30.00%)	14 (43.75%)

*Stage of endometriosis: stage I: *n* = 5 (15.62%); stage II: *n* = 14 (43.75%); stage III: *n* = 6 (18.75%); stage IV: *n* = 7 (21.89%).

follows: early proliferative (days 1–7), proliferative (days 8–14), and secretory phase (days 15–27); this division was based on the last menstrual period and endometrial histology dating according to Noyes *et al.* (1950). More detailed information on the women studied is shown (Table 1).

Non-paired serum and peritoneal fluid samples obtained from the patients diagnosed as endometriosis positive (*n* = 56 samples) and from healthy women (*n* = 40 samples) that came to the Division of Reproductive Endocrinology and Fertility at the North Carolina University Hospital were used for determining the clinical diagnostic parameters.

Collection of blood and peritoneal fluid samples

Serum was obtained by venepuncture at the time of laparoscopy. Laparoscopy was performed for evaluation of endometriosis or at the time of tubal ligation. Peritoneal fluid samples (2–10 ml) were obtained from the cul-de-sac at the time of laparoscopy, using a metal suction cannula attached to a 10 cc syringe. Volumes of peritoneal fluid ranged from 2 to > 10 cc. The sample was not used if blood mixed with peritoneal fluid was found at the time of laparoscopy. Samples were placed on ice before being transported to the laboratory and were centrifuged immediately at 2000 *g* for 15 min at 4°C. Supernatants were divided into aliquots and stored at –80°C until assayed.

Preparation of hCG–horseradish peroxidase conjugate

Antiserum for the enzyme-labelled, second antibody signal was polyclonal anti-hCG antiserum (R76; Department of Population Health and Reproduction, University of California, Davis, CA) raised against purified hCG (CR-121; R. Canfield, Department of Medicine, Columbia University, New York, NY). The purified IgG fraction of the anti-hCG antiserum (R76) was conjugated to sodium-periodate-activated horseradish peroxidase (HRP; EC 1.11.1.7., type VI, Sigma Chemical, St Louis, MO) by a modification of the method of Wilson and Nakane (1978), as described by Munro *et al.* (1997).

The optimum concentration and dilution of this

conjugate was determined by checkerboard titration, as the coating concentration of the 518B7 had been established previously in this laboratory and by Spearow and Trout (1987). Appropriate dilution of the anti-hCG-horseradish peroxidase was chosen to achieve both sufficient colour development and low non-specific binding in the enzyme-immunoassay.

Enzymeimmunoassay

Dynatech Immulon I (Fisher Scientific, Santa Clara, CA) or Nunc Maxisorb (Krape, Madrid) 96-well microtitre plates were coated with 100 μ l (250 ng ml⁻¹ in 0.05 mol sodium bicarbonate l⁻¹, pH 9.6) of the primary capture antibody, a purified mouse monoclonal antiserum, clone 0220-518B7 (518B7) raised against the β subunit of bovine LH (J. Roser, Department of Animal Science, University of California, Davis, CA), except for the first two wells (1a and 1b) which were used as plate/assay blanks. The plates were sealed and incubated overnight at 4°C or stored at 4°C until used. Before use, non-bound antiserum was removed from the wells by washing the plates five times with wash solution (0.15 mol NaCl l⁻¹ containing 0.05% (v/v) Tween 20).

Before assay, standards (NIADDK-hLH-I-3, AFP-827OB, range 0.002–0.500 ng well⁻¹), serum (diluted between 1:10 and 1:20), peritoneal fluid (diluted 1:20) and all internal controls were diluted in MOPS assay buffer (0.04 mol 3-(N-morpholino) propanesulfonic acid l⁻¹ containing 0.015 mol NaCl l⁻¹, 0.01 mol EDTA l⁻¹, 0.05% (v/v) Tween 20, 0.005% (v/v) chlorhexidine digluconate and 0.1% (w/v) BSA, pH 7.2 (Sigma Chemical)). A 25 μ l aliquot of MOPS assay buffer was pipetted across the entire plate, followed immediately by addition of 100 μ l of appropriate standards, buffer (zero standard), diluted samples (serum or peritoneal fluid) and assay controls. Plates were sealed and incubated for 1 h, after which time non-bound LH was removed from the wells by washing the plates as described previously. The enzyme-labelled second antibody, R76, (anti-hCG:HRP, 100 μ l) was diluted to 1:1500 in MOPS assay buffer and pipetted across the entire plate. The plates were again sealed and the second antibody conjugate reaction was allowed to proceed for a further 1 h. Non-bound second antibody was removed by washing, as described, followed by addition of 100 μ l substrate (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS) (Sigma Chemical) or 100 μ l 3,3',5,5'-tetramethyl benzidine dihydrochloride (TMB) (Pierce, Rockford, IL). Conversion of the substrate by the enzyme, manifested as a colour change, was stopped after appropriate colour development (approximately 60 min for ABTS, 15–20 min for TMB) by the addition of 100 μ l stop solution (0.15 mol hydrofluoric acid l⁻¹ for ABTS; 2.0 mol sulphuric acid l⁻¹ for TMB). Absorbance was measured in an automatic plate reader (Biotek Instruments Inc., Winooski, VT) at 405 nm for ABTS, 450 nm for TMB, and data were transferred to an interfaced computer (IBM) for analysis. Calibration curves were constructed by using weighted least-squares linear

regression according to the method of Rodbard and Lewald (1970) to yield concentrations.

Comparison of other hLH assays: radioimmunoassay and hLH enzymeimmunoassay kit for determination of LH in serum and peritoneal fluid

Samples of serum and peritoneal fluid ($n = 19$) from healthy women and women with endometriosis were selected randomly from the groups of subjects included in the present study. LH was assayed both by a traditional radioimmunoassay (Sufi *et al.*, 1986) and by a commercial enzyme-immunoassay kit (Boehringer Mannheim, Barcelona). The results of these assays were compared with values determined by our enzymeimmunoassay. The purpose of these assays was to determine whether similar results could be obtained with other currently available LH assay systems.

Assay validation

The specificity of the combination of the two antibodies (518B7 and R76) in the sandwich enzymeimmunoassay was tested by calculating the percentage of crossreactivity to related protein hormones. Curves generated from assays of LH standards and serially diluted serum and peritoneal fluid samples were compared for parallelism in the enzyme-immunoassay. Human serum from women taking oral contraceptives (oral contraceptive serum) and containing a known added concentration of LH (40 ng ml⁻¹) was also diluted serially and compared with the standard dose-response curve for parallelism. Sensitivity was determined as the least amount of hormone that could be distinguished from zero concentration of standard as defined by the mass at 2 SD above the mean of the zero absorbance. The precision of the assay was assessed by determination of intra-plate (24 aliquots of the same sample or control within the same plate), intra-assay (measurement of the same sample or control on different plates (inter-plate) in the same assay), and interassay coefficients of variation. Both inter- and intra-assay coefficients of variation were calculated by the method of Rodbard (1974). Accuracy of the assay was determined by the percentage recovery of exogenous, known amounts of LH that had been added to different pools of serum known to initially contain no LH, and to pools of control serum or peritoneal fluid.

Statistical analyses and calculations

LH concentrations either in peritoneal fluid or serum of both groups of patients (women with endometriosis and healthy women) were compared by ANOVA and Student's *t* test with the Bonferroni adjustment. LH concentrations were compared with the phase of the menstrual cycle in both groups. *P* values < 0.05 were considered significant. An LH concentration either in serum or peritoneal fluid > 2 SD above the mean value for healthy women was considered abnormal.

The sensitivity, specificity, diagnostic efficiency, and predictive value of the enzymeimmunoassay for LH in either serum or peritoneal fluid were calculated according to the methods of Wulff (1981) and Smiley and Peterson (1993). The sensitivity of a test expresses the probability that a woman with endometriosis will give a positive test. Sensitivity is determined in a population of subjects known to have endometriosis. Specificity of a test is the probability that a patient will give a negative test in the absence of endometriosis. Specificity is measured in a population of subjects known to be free of endometriosis. Efficiency of a test is the percentage of correct results regardless of whether they are positive or negative. Efficiency expresses the combined positive and negative accuracy of a test. As the diagnostic probabilities are relevant to the clinic diagnostic they have also been calculated. Predictive values predict the probability of the presence (positive predictive value) or absence (negative predictive value) of endometriosis, given a positive or a negative test, and are measured in a population of subjects that contains patients with and without endometriosis. Diagnostic probabilities are dependent on the sensitivity and specificity of the assay.

Results

Characterization of antibodies

The primary capture antibody (518B7) chosen for this enzymeimmunoassay, which was generated against the β subunit of bovine LH, has been well characterized for detection of LH and chorionic gonadotrophin (CG) in the serum of diverse mammalian species (Matteri *et al.*, 1987; Illera *et al.*, 1996; Munro *et al.*, 1997). The enzymeimmunoassay crossreacted with human LH standard, which gave a dose–response curve parallel to the hCG standard dose–response curve and a minimum detection of 0.05 ng LH per well. However, the enzymeimmunoassay did not crossreact with other human protein hormones (FSH, thyroid stimulating hormone (TSH)) tested, as evidenced by a lack of colour development in the assay. As the specificity of the sandwich enzymeimmunoassay is dependent on the determinant-specific monoclonal capture antibody, in this case 518B7, this allows for the use of a second, enzyme-labelled antiserum that does not need to have a high degree of specificity. Hence, the polyclonal second antiserum, R76, was not tested for crossreactivity. However, this anti-hCG signal antiserum (R76) had a sensitivity of > 0.015 ng hCG (WHO preparation 75/537) per tube in the competitive radioimmunoassay described by Lasley *et al.* (1985).

Conjugate assessment

Recovery of enzyme activity after conjugation was 87%. A final dilution of 1:1500 of the anti-hCG–horseradish peroxidase conjugate in assay buffer was found to give both adequate colour development and low non-specific binding in the assay.

Validation of enzymeimmunoassay for LH in serum and peritoneal fluid of healthy women and women with endometriosis (sensitivity, precision, accuracy and parallelism test)

The range of the standard dose–response curve was 0.002–0.500 ng LH per well. The lowest concentrations of hLH that could be distinguished from zero concentration were 0.15 ± 0.02 ng ml⁻¹ serum (100 μ l, 1:10 dilution) and 0.058 ± 0.021 ng ml⁻¹ peritoneal fluid (100 μ l, 1:20 dilution). The precision of the enzymeimmunoassay was assessed in a 5.0 ng ml⁻¹ standard preparation of LH, as well as in selected pooled serum and peritoneal fluid samples. The intra-plate (within-plate, 24 aliquots of the same sample) coefficient of variation for a 5 ng ml⁻¹ LH standard averaged 4.3%, whereas for replicate measurements of serum samples and peritoneal fluid the coefficients of variation were 5.4 and 3.7%, respectively. Mean intra-assay coefficients of variation for pools of high, medium and low LH concentrations in serum and peritoneal fluid were 5.4, 7.1 and 9.8%, respectively, whereas the average inter-assay coefficients of variation for the same pools were 6.4, 8.8 and 12.1%, respectively.

The accuracy of the enzymeimmunoassay was determined by measuring the recovery rates of known amounts of LH (0, 1, 5, 10 and 25 ng ml⁻¹) that had been added to different pools of serum obtained from women taking oral contraceptives (oral contraceptive serum), as this pool of serum was readily available. The recovery rates (mean $\pm$ se) were 96.54 ± 1.87 for the high (10.0 and 25.0 ng ml⁻¹) and 94.58 ± 4.65 for the low (0.0–5.0 ng ml⁻¹) concentrations of hLH. The recovery rates for pooled control serum or peritoneal fluid to which 10 ng hLH ml⁻¹ had been added were 95.98 ± 3.23 ($n = 15$) and 93.77 ± 4.45 ($n = 15$), respectively.

LH standard concentrations were diluted in MOPS assay buffer and oral contraceptive serum, and each standard dose–response curve was compared in the assay to study the effects of human serum. A high control sample (40 ng LH ml⁻¹ in oral contraceptive serum) was also diluted serially in both MOPS assay buffer (decreasing volume of human serum per well) and oral contraceptive serum (constant volume of human serum per well) and these dilutions were run in parallel with the standard dose–response curves (Fig. 1). All of the dose–response curves are parallel, indicating that there is little or no effect of human serum on the LH enzymeimmunoassay. The standard curves are also parallel and identical whether LH standards are diluted in MOPS assay buffer or oral contraceptive serum.

In the same way, a single peritoneal fluid sample diluted serially in MOPS assay buffer gave a dose–response curve parallel to the LH standard dose–response curve (Fig. 2). All standards are diluted in MOPS buffer, and serum and peritoneal fluid samples are diluted at a point on the curve where there is still parallelism in the dilution curve (normally 1:10 or 1:20 for serum and 1:20 for peritoneal

Fig. 1. Serial dilutions of a known concentration (40 ng ml^{-1}) of human LH (hLH) diluted in serum from women taking contraceptives (oral contraceptive serum; ■) or in MOPS buffer (□). The displacement curves produced are parallel to those of standard LH dose-response curves in which LH standards were diluted in either oral contraceptive serum (○) or MOPS buffer (●).

fluid) to use the enzymeimmunoassay in its simplest form for the measurement of LH in human serum or peritoneal fluid.

The simple regression analysis of serum and peritoneal fluid LH concentrations measured by the three different assays yielded the following results: a high correlation was found ($r = 0.98$) when the enzymeimmunoassay was compared with the enzymeimmunoassay kit. However, correlation was low when the enzymeimmunoassay was compared with the radioimmunoassay ($r = 0.95$), although that means only a divergent result from two subjects instead of one subject.

The clinical diagnostic validation parameters gave the following results: sensitivity of LH enzymeimmunoassay was 85.71% (of 56 endometriosis samples assayed, 48 were positive and eight were negative), specificity was 92.50% (of 40 healthy samples assayed, 37 were negative and 3 were positive) and efficiency was 88.54% (of 96 samples assayed, 85 were diagnosed correctly and 11 were false negative (eight samples) or false positive (three samples)). The diagnostic accuracy measured was as follows: positive predictive value was 94.11% and negative predictive value was 82.22%.

Concentrations of LH in serum and peritoneal fluid during the menstrual cycle of healthy women and women with endometriosis

Serum LH values (mean $\pm$ SD) of healthy women were $4.52 \pm 2.03 \text{ ng ml}^{-1}$, whereas women with endometriosis had serum LH values of $13.67 \pm 7.21 \text{ ng ml}^{-1}$ ($P < 0.001$). Healthy women had peritoneal fluid LH values of $5.65 \pm 2.43 \text{ ng ml}^{-1}$, whereas peritoneal fluid LH values in women with endometriosis were significantly higher

Fig. 2. Serial dilutions of human serum (◆) and human peritoneal fluid (■) in MOPS buffer were parallel with the LH standard dose-response curve, also in MOPS buffer (□).

($64.06 \pm 16.44 \text{ ng ml}^{-1}$; $P < 0.001$). These comparisons were calculated by using absolute LH values independent of the stage of the menstrual cycle.

LH concentrations varied depending on the stage of the menstrual cycle for both serum and peritoneal fluid and for healthy women and women with endometriosis. Healthy women had the following serum and peritoneal fluid values during the different phases of the menstrual cycle assayed: early proliferative: 2.17 ± 0.22 and $2.69 \pm 0.32 \text{ ng ml}^{-1}$, respectively; proliferative: 6.46 ± 0.23 and $7.89 \pm 0.43 \text{ ng ml}^{-1}$, respectively; secretory: $3.92 \pm 0.60 \text{ ng ml}^{-1}$ and $4.78 \pm 0.34 \text{ ng ml}^{-1}$, respectively. For women with endometriosis the following serum and peritoneal fluid concentrations were measured at each stage of the cycle: early proliferative: 7.73 ± 0.71 and $50.35 \pm 4.01 \text{ ng ml}^{-1}$, respectively; proliferative: 22.66 ± 2.37 and $76.27 \pm 4.51 \text{ ng ml}^{-1}$, respectively; secretory: 11.64 ± 0.79 and $65.07 \pm 4.40 \text{ ng ml}^{-1}$, respectively. For both groups of women, serum and peritoneal fluid LH concentrations were highest in the proliferative phase (with the LH peak occurring during this phase) and lowest in the early proliferative phase. Comparisons made between serum versus serum, and peritoneal fluid versus peritoneal fluid LH concentrations at each phase of menstrual cycle indicated that there were significant differences in LH concentrations in all the cases analysed ($P < 0.05$), except for the case of peritoneal fluid versus peritoneal fluid in the proliferative and secretory phases of women with endometriosis. No significant differences were observed when comparing serum versus peritoneal fluid LH concentrations at all the stages of the menstrual cycle of healthy women, although peritoneal fluid LH concentrations were slightly higher compared with those of serum. In contrast, in women with endometriosis there were significant differences ($P < 0.001$) between serum and peritoneal fluid LH concentrations in all the cases analysed, with peritoneal fluid LH concentrations more than three times higher than those of serum.

The different phases of the menstrual cycle are depicted (Fig. 3). During each phase, comparisons between serum LH and peritoneal fluid LH concentrations of both groups were performed, and significant differences were observed in all the cases analysed ($P < 0.001$).

Serum samples showed a positive test at 8.58 ng mL^{-1} (mean + 2 SD) of LH and peritoneal fluid samples at $10.51 \text{ ng LH mL}^{-1}$ (mean + 2 SD). These results indicate that in the case of healthy women only three of 40 samples analysed overlapped with a positive test (false positives). These cases were peritoneal fluid samples of healthy women in the proliferative phase of the menstrual cycle. Only eight of 56 samples from women with endometriosis overlapped with a negative test. All these cases were diagnosed in serum samples, six of them in the early proliferative phase and two in the secretory phase of the menstrual cycle, both being results in the secretory phase near the limit of a positive test (8.16 and $8.26 \text{ ng LH mL}^{-1}$, respectively). All the analyses performed in peritoneal fluid samples of women with endometriosis tested positive.

Discussion

Minimally invasive methods are needed increasingly for the development of markers to diagnose endometriosis in women. Hormone assays have been used for the diagnosis of several pathological disorders associated with infertility, including luteal phase defect (Jones and Madrigal Castro, 1970) and polycystic ovary syndrome (Homburg, 1996). While several markers for the diagnosis of endometriosis have been described (Pittaway, 1989; Lessey *et al.*, 1994), the use of hormone assays has also been examined (Andrews, 1975; Chew *et al.*, 1990; Khorram *et al.*, 1993; Barry-Kinsella *et al.*, 1994; Colacurci *et al.*, 1996). The detection of serum or peritoneal fluid LH concentrations could provide a useful tool for examining the hormonal events associated with endometriosis in women. In the present study, women with endometriosis were observed to have increased peritoneal fluid and serum LH concentrations compared with healthy women without evidence of endometriosis.

Enzymeimmunoassays are generally less expensive, easier and safer to perform than other assay techniques (Munro and Lasley, 1988), and enzymeimmunoassay methods permit the use of antibodies with limited binding affinities and specificities. In contrast to competitive assays, reagents in enzymeimmunoassay systems can be used in excess. The present data demonstrate that the enzymeimmunoassay described in this report can be used for the measurement of LH concentrations in serum and peritoneal fluid from healthy women and women with endometriosis.

Monoclonal antibody techniques and the ability to select immunoglobulins that recognize individual epitopes have allowed specific, sensitive and precise immunoassay systems to be developed. The monoclonal antiserum, 518B7, can be produced in virtually unlimited quantity, providing a stable source of high quality antiserum.

Fig. 3. Comparison between peritoneal fluid and serum LH concentrations in healthy women and women with endometriosis at different stages of the menstrual cycle. Peritoneal fluid (■) and serum (▨) samples from healthy women. Peritoneal fluid (□) and serum (▩) samples from women with endometriosis. Values are mean $\pm$ SD. Values with different symbols (a versus b and * versus **) within the same stage of the menstrual cycle are significantly different ($P < 0.001$).

Although the enzyme-labelled signal antibody is a polyclonal antiserum that has finite availability, this is unlikely to limit the general concept presented in this study. As the final LH recognition and specificity of the assay are determined by the specific monoclonal capture antibody, 518B7, and the signal antiserum does not require a high degree of specificity, there are probably a number of signal antibodies (including mAbs) that will perform as well as, if not better, than R76. This simple practical non-competitive enzymeimmunoassay does not require plate-blocking or overnight steps and, most importantly, does not require the chemical conjugation of extremely limited supplies of purified pituitary hormone preparations.

A basic enzymeimmunoassay requires an efficient and stable enzyme labelling of the second or signal antibody, as well as the ability to avoid matrix effects. The second enzyme conjugate used in the present study has retained 100% of its original activity when stored in 60% (v/v) glycerol at both 4°C and -15°C for 24 months (Matteri *et al.*, 1987). The precision of this assay system is partially due to its ability to permit unbound materials to be separated from bound proteins by a simple but thorough washing step. This robustness in binding obviates the need for complicated separation steps required in other assay systems. The elimination of interference and the virtual absence of LH competitors are demonstrated by the recovery rates and parallelism of known amounts of LH when compared with the standard dose-response curves in both peritoneal fluid and serum. This non-isotopic enzymeimmunoassay for hLH, which can be completed in $< 3 \text{ h}$, offers a stable, rapid and improved assay system compared with previously reported methods or commercially available enzymeimmunoassay kits, and has been developed and validated with materials that are readily available to all laboratories.

This fact is of particular importance to laboratories that are unable to acquire the necessary licences or do not have the facilities to handle radioisotopes.

Our results in terms of diagnostic validation parameters demonstrated that the present enzymeimmunoassay has an increased sensitivity, specificity and efficiency comparable and even higher than other methods reported for human LH (Bouckaert *et al.*, 1986; Chew *et al.*, 1990; Kim-Brjör Lund *et al.*, 1991). The explanation of the false positives and negatives found in either serum or peritoneal fluid is related more to the variations of LH concentrations during the different phases of the menstrual cycle than to the technique used. Owing to this cycle-dependent variation, the clinician must take into account the day of the menstrual cycle on which the sample was taken in order to avoid false positive results. Such false positive results may occur if the serum or peritoneal fluid of healthy women is obtained coincident with the LH peak in the late proliferative phase. False negatives might also be determined in the serum of women with endometriosis during the early proliferative stage. The number of cases examined in the present study does not allow the diagnostic ranges for each stage of the menstrual cycle to be established but the results indicate that there are optimal times for measurement.

LH concentrations varied with the different phases of the menstrual cycle, both in serum and peritoneal fluid, and in healthy controls significant differences in LH concentrations during the menstrual cycle were observed in both peritoneal fluid and serum. LH concentrations are also higher in peritoneal fluid than in serum from the same patient, although these differences were not significant. Bouckaert *et al.* (1986) also reported higher concentrations of LH in peritoneal fluid than in serum and the differences were significant. In contrast, Kim-Brjör Lund *et al.* (1991) found higher concentrations of LH in the serum of healthy women compared with peritoneal fluid, except during the early luteal phase when concentrations were equal. These authors attributed the differences in the reported results to the methods of dating of the oestrous cycle, the accuracy of the hormone assay and to the size of the study groups, explanations that could also be applied to the present data.

In the present study, in women diagnosed with endometriosis peritoneal fluid LH concentrations were significantly higher compared with serum LH concentrations during all the stages of the menstrual cycle, and both serum and peritoneal LH concentrations were significantly higher than in healthy women. However, the cycle-dependent pattern of LH concentrations was observed in serum only. Previous reports, using radioimmunoassay, have also demonstrated increased peritoneal fluid LH concentrations in women with endometriosis (Chew *et al.*, 1990). Although this technique does not distinguish bioactive from immunoactive LH, the high concentrations observed may account for the stimulatory effect of peritoneal fluid from endometriosis patients on granulosa cell progesterone secretion described *in vitro* (Whitehead *et al.*, 1996). Furthermore, as gonadotrophin receptors have

been reported in the endometrium (Reshef *et al.*, 1990), the high LH concentrations may account for the alterations in the endometrial protein patterns noted in the peritoneal fluid of women with endometriosis (Joshi *et al.*, 1986).

Bioactive substances such as LH in peritoneal fluid, especially when present at high concentrations, could also account for the infertility associated with endometriosis. Increased LH could contribute to the ovulatory disturbances noted in endometriosis, including luteinized unruptured follicle syndrome (Dhont *et al.*, 1984; Dmowski *et al.*, 1986), luteal phase defect (Grant, 1966; Li *et al.*, 1991; Hiram and Ochiai, 1995) and anovulation (Soules *et al.*, 1976; Hahn *et al.*, 1986), as peritoneal fluid from rabbits with surgically induced endometriosis blocked implantation in otherwise normal animals. Similar data from human peritoneal fluid into mice corroborates the effects of human peritoneal fluid from endometriosis patients on implantation (Illera *et al.*, 1995b). Altered expression of endometrial integrins has been observed in women with endometriosis (Lessey *et al.*, 1994). It would be of interest to correlate such changes with concentrations of peritoneal LH in these women with apparent defects in uterine receptivity.

The enzymeimmunoassay described in the present study is likely to be useful beyond obstetrical diagnosis and biomedical research, and the application of this assay to serum and peritoneal fluid samples provides the attractive possibility that such a test could be included into the panel of markers for endometriosis.

hCG standard preparations were received from J. O'Connor (CR-127, Irving Center for Clinical Research, New York, NY) and the World Health Organization (1st International Reference Preparation, 75/537, National Institute for Biological Standards and Control, Holly Hill, Hampstead, London). Human luteinizing hormone (hLH, NIADDK-hLH-I-3 (AFP-827OB)) was obtained from the National Hormone and Pituitary Program, NIADDK, Bethesda, MD. This research was supported by NICHD/NIH through cooperative agreement U54 HD-30476 (BL) as part of the Specialized Cooperative Centers Program in Reproduction Research, the National Cooperative Program on Markers of Uterine Receptivity for Blastocyst Implantation (HD 34824; BL).

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