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Endometrial dating and determination of the window of implantation in healthy fertile women

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Objective: To reassess endometrial morphological criteria of normality identifying the best morphological and molecular "implantation window" indicators in normal women.

Design: Prospective clinical study.

Setting: Assisted reproductive unit.

Patient(s): Fourteen healthy volunteers.

Intervention(s): Blood sampling for LH, E_2, and progesterone (P4) determinations. Daily vaginal ultrasounds. Two endometrial biopsies per volunteer, 7 days apart, during luteal phase.

Main Outcome Measure(s): Endometrial dating, pinopodes formation, immunohistochemical determination of integrins ($\alpha v\beta 3$, $\alpha 4\beta 1$), leukemia inhibitory factor (LIF), interleukin-1 receptor type I (IL-1R tI), mouse ascites Golgi (MAG), the transmembrane mucin (MUC-1), and P4 receptor expression.

Result(s): In 26 of 28 biopsies observers agreed; in two biopsies there was a discrepancy (difference of 72 hours). With use of LH peak, 24 of 26 samples were in phase, and 2 were 3 days behind. Pinopodes appeared on days 20–21 and persisted through day 28 in small groups or larger areas. $\beta 3$ Integrin was highly expressed in luminal and glandular epithelium from day 22 through 28; 48 hours thereafter pinopodes appeared. $\alpha 4$ Subunit exhibited luminal epithelium reaction positivity on days 22–23 and glands on days 18–23. LIF and IL-1R tI showed weak, erratic expression. MAG antibodies showed luminal epithelium expression up to day 22 and glands up to day 25. MUC-1 showed positivity during the whole luteal phase. P4 receptors were positive through day 20 and at the end of the luteal phase.

Conclusion(s): The three most cited markers that frame the window of implantation do not correlate in our material. Pinopodes are present from day 20 on; $\beta 3$ and $\alpha 4$ integrin subunits indicate a window opening on days 22–23. (Fertil Steril® 2000;73:788–98. ©2000 by American Society for Reproductive Medicine.)

Key Words: Endometrium, window of implantation, dating, pinopodes, integrins, LIF, IL-1r-R tI, MAG, MUC-1, progesterone receptors

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The classic work of Noyes et al. (1, 2) on endometrial morphology that has served clinicians so well for 50 years is losing power in the evaluation of human endometrium in normal and abnormal circumstances (luteal phase defects). Conflicting views on the timing and interpretation of the endometrial biopsies (3, 4), recent technological developments, and a new type of information being required to fulfill actual clinical needs have made the classical endometrial evaluation by biopsy outmoded. The ability to better evaluate the menstrual cycle and to time its events with greater precision, the more recently acquired capabilities to determine endometrial morphology changes using electron microscopy, the incorporation of techniques that focus on critical molecular aspects of endometrial development, and the need to answer specific questions posed by programs of assisted reproduction for the so-called "window of implantation," seem to indicate the necessity of new and updated methods to judge the endometrium.

The need to chronologically define the critical events of the menstrual cycle in a very

precise manner has been emphasized by the results obtained by Shoupe et al. (5). These investigators used four different menstrual cycle parameters, namely, ultrasound demonstration of ovulation, LH surge, basal body temperature shift, and starting of menstrual flow after the biopsy, as reference points for endometrial dating. An agreement of ± 2 days was found in 96%, 85%, 77%, and 65% of the samples, respectively.

The question of the window of implantation has been addressed in several different ways. Martel et al. (6) and Nikas et al. (7), using scanning electron microscopy on the uterine luminal epithelium, demonstrated the presence of specialized cell surface formations called pinopodes, apparently involved in the mechanisms of transduction of the surface epithelium and in the exchange of fluids and low molecular weight proteins. Simultaneous changes occur in epithelial glycocalixes and in the surface negative charge (8–10). Development of pinopodes has been linked to the adhesion of blastocysts to the luminal epithelium (11), suggesting endometrial nidation receptivity (12). Progesterone stimulates the appearance of pinopodes, whereas estrogens result in their regression (6). Psychoyos and Nikas (13) and Lessey and Damjanovich (14) have shown stage-dependent changes in pinopode formation during normal and stimulated menstrual cycles.

During the normal menstrual cycle the greatest appearance of pinopodes occurs on days 19, 20, and 21 of a 28-day cycle (13). In stimulated cycles using clomiphene citrate (100 mg/d) for 5 consecutive days, followed by hMG on days 6, 8, and 10 of the cycle and subsequent administration of hCG (5,000 IU), the endometrium showed an advancement in the development of pinopodes compared with the spontaneous menstrual cycle. With use of this protocol, formation of pinopodes was observed on days 16, 17, and 18. (Psychoyos A. The implantation window: basic and clinical aspects. Plenary Lecture. J Assist Reprod Genet 1993;10 Suppl:9 [abstract]).

The timing of pinopode formation in stimulated cycles then appears to be advanced several days compared with what is observed during the spontaneous cycle and seems to be a morphological marker for the window of implantation; the need to use scanning electron microscopy for their identification precludes its use in daily clinical work. The issue of pinopode formation at the level of the endometrial surface epithelium and its relevance to the window of implantation were recently reviewed by Psychoyos and Nikas (13).

Besides the morphological marker mentioned above, the use of molecular techniques and immunohistochemistry has helped to identify several biomarkers in the human endometrium that seem to participate in the implantation process in a positive (facilitatory) or in a negative (inhibitory) fashion. Some of them are on the surface of the luminal epithelium, representing significant factors in the preembryo-endometrium apposition, adhesion, and attachment, and some others have been described at the level of the extracellular matrix of the endometrial stroma, representing also significant factors at the time of trophoblast invasion.

Although a fair number of biomarkers have been described in the literature, we will review only a few that seem to have clinical significance and have been investigated in this study.

The first biomarker is represented by adhesion molecules of the integrin category present during the secretory phase. According to Lessey et al. (14, 15) and Tabibzadeh et al. (16), one integrin detected by immunohistochemistry and flow cytometry in the glandular epithelium during postovulatory days 5 and 6, αv β3 vitronectin receptor expression, may indicate the opening of the window of implantation. Subunit αv is present at the epithelial level during the early and late secretory phase and at the stromal level during the whole cycle; β3 is present at the epithelium during the late secretory phase and in the stroma during the whole menstrual cycle. In the luminal epithelium, the expression of αvβ3 and of the β3 subunit starts abruptly on day 20 of the cycle (theoretical opening of the window of implantation), continues until the end of the cycle, and persists during early pregnancy. Integrin α4β1 appears in the glandular epithelium on day 14 and disappears on day 24 (closing of the window of implantation). It was not found at the level of luminal epithelium.

The second marker belongs to the cytokine family and is heavily implicated in the implantation process. It is a polypeptide growth factor known as leukemia inhibitory factor (LIF) that belongs to the family of epidermal growth factors (EGF). In mice endometrium, LIF was necessary for normal implantation because embryos from transgenic mice with no LIF expression are unable to implant but show normal development in the in vitro system (17). In the human female, LIF has been found in the endometrium at the theoretical time of implantation (18), with maximal expression between days 19 and 25 of an ideal cycle (19). It is possible that in abnormal or stimulated cycles the expression of LIF in the human may be different, as is the case with other markers, because endometrium in these circumstances is advanced (20).

Furthermore, LIF markedly decreases trophoblast production of hCG protein and expression of β-hCG messenger RNA (mRNA) and increases the expression of oncofetal fibronectin trophouteronectin (TUN) mRNA, which is responsible for adhesion to endometrial integrins (18, 19). This finding indicates that LIF may be also an important regulator of human embryonic implantation by modulating trophoblast differentiation (21). The LIF would be necessary, therefore, in the adhesion phase, through the stimulation of trophoblastic differentiation (villous to invasive syncytiotrophoblast).

The third biomarker selected was interleukin-1 receptor type I (IL-1R tI); this biomarker is expressed during the whole menstrual cycle, with maximal levels during the early and late luteal phase in the epithelial and stromal endometrial cells. The binding of IL-1 to maternal IL-1R tI is a necessary step in implantation. The abundance of this receptor through the luminal epithelium is required for adequate embryo attachment (22).

The fourth marker used is MAG (mouse ascites Golgi), a blood group A–related epitope expressed on an endometrial gland mucin (presumably MUC-1) in a menstrual cycle–dependent manner. It is normally expressed in the glandular Golgi on day 5, is secreted beginning on day 16, appears on the apical surface of the human luminal epithelium on day 17, and lasts until day 19; then it is absent. MAG may be related to the initial steps of the implantation cascade (23). The MAG abnormalities may be related to unexplained infertility.

A fifth marker is represented by a transmembrane mucin, MUC-1, described as an inhibitor of blastocyst attachment and having specific expression in the uterine epithelium of rodents, rabbits, pigs, baboons, and humans. MUC-1 expression (protein and mRNA) declines significantly during the receptive uterine state and is high during the nonreceptive phase in the mouse (24). In the human, its role is still unclear; it may inhibit the initial phases of implantation by steric hindrance, or it may promote them by carrying carbohydrate recognition structures that mediate cell-to-cell interaction. MUC-1 and protein are detected during the proliferative phase and increase after ovulation, when the core protein is found both in the cytoplasm and at the apical surface of the epithelial cells and immunoreactive mucin is present in the uterine luminal epithelium during the receptive phase.

Secretory MUC-1 increases during the receptive phase in the uterine flushes (25). During this phase, the luminal epithelium contains a mosaic of cells with patches that display not only the core protein but also highly sulfated lactosaminoglycan structures, originating a localized reduction of electronegativity that may favor the interaction of the epithelium with the attaching blastocyst.

Finally, endometrial receptors for E_2 and P4 are essential for hormonal action and for the expression of some of these markers. Both receptors show maximal expression in the glandular epithelium and stroma during late proliferative and early secretory phases. After day 19, there is an abrupt disappearance of these receptors from the glands, most likely due to the effect of P4; meanwhile, they persist in the stroma (26).

The work of endocrinologists, pathologists, basic scientists, and clinicians on all aspects of the menstrual cycle has created discrepancies in definitions and descriptions of chronological events that need to be reconciled, if we want to avoid further difficulties in communicating the generated knowledge. Furthermore, it is extremely important when referring to menstrual cycle day to state the event on which it is based.

This work was performed to fulfill several objectives: [1] to reevaluate the accuracy of proposed endometrial morphological parameters, when new methodology is used to assess the endocrine and functional events of the normal menstrual cycle; [2] to attempt to establish the best available molecular tools to determine the chronological appearance and normality of the theoretical endometrial "window of implantation" during the human normal menstrual cycle; and [3] to correlate the classic knowledge with the new findings obtained.

MATERIALS AND METHODS

Volunteers

Fourteen healthy, fertile, female volunteers between 25 and 35 years of age, with normal, regular menstrual cycles and normal pelvic examinations, having had neither endocrine treatment on the month preceding the investigation nor tubal or ovarian surgery and not using an intrauterine device, were recruited for this project. Volunteers were also screened for endocrine normality with serum determinations of FSH, LH, and E_2 on day 3 of the menstrual cycle in which the research was performed. Normal values were defined as <10 mIU/mL for FSH and LH and E_2 <50 pg/mL. All volunteers were advised to refrain from sexual intercourse or to use barrier contraception during the month of investigation. Transvaginal ultrasound was also performed on that day to ensure ovarian normality. All of them were required to keep a basal temperature chart (BTC) as a general orientation tool to the menstrual cycle.

The research project was submitted to and approved by the Scientific, Ethics, and Institutional Review Committees of Centro de Estudios en Ginecologia y Reproduccion, and the volunteers signed appropriate approved consent forms.

Menstrual Cycle Monitoring

After the baseline endocrine investigation on day 3 of the menstrual cycle was obtained, volunteers were requested to start a BTC and to return on the periovulatory period for further studies. For the purpose of this study and to reconcile endocrine and clinical data, we call the day of LH peak day 14 or day 0. That day will correspond to the day after administration of hCG (day of maximum level of hCG after an afternoon or evening injection) in the IVF programs and to day 14 (day 15 being the first day of progesterone [P4] administration by convention) in the oocyte donation programs for anovulatory or suppressed patients with artificially created endometrial cycles.

Monitoring of the periovulatory period started on day -3 (day 11) with transvaginal ultrasound (US) for follicle size and endometrial characteristics using transvaginal examination performed with a 7.5-mHz transducer and an Aloka SSD-680 unit (Aloka Co. Ltd., Tokyo, Japan); LH levels were determined twice daily until the peak was detected.

Peripheral blood was obtained during the luteal phase every other day for serum progesterone (P4) and E_2 determinations, starting on day $+1$ and ending with the menstrual period. Only results of day 21 (peak function of the corpus luteum) are included in this article.

Endometrial Biopsies

Two endometrial biopsy samples (1 from the anterior and 1 from the posterior aspect of the uterine fundus) were

obtained without anesthesia, from each volunteer, with a Pipelle de Cornier (Prodimed, Neuilly-en Thelle, France). The biopsies were performed 7 days apart, starting on day +1 and ending on day +14 of the luteal phase. The biopsies performed for each volunteer were scheduled to cover the 14 days of the luteal phase and to ensure that two biopsies were performed for each day for two different volunteers. Tissue samples were immediately divided in three portions: one was placed in buffered formalin fixative for light microscopy and for MAG, MUC-1, and P4 receptors (PrR) studies by immunohistochemistry; one was fixed in glutaraldehyde for scanning electron microscopy investigation; and one was immediately immersed in isopentane and placed rapidly in liquid nitrogen for integrin and LIF immunohistochemistry determinations.

Laboratory Procedures

Hormone Determinations

Serum FSH and LH levels were determined with use of chemiluminescence (ACCESS Immunoassay System, Sanofi Diagnostics Pasteur, Redmond, WA), and E_2 levels were determined with the Coat-a-count IRMA kit (Diagnostic Products Corporation; Los Angeles, CA), a solid phase immunoradiometric assay. Interassay coefficients of variation are 3.1%, 3.6%, and 5.9%, respectively. Intra-assay coefficient of variation depends on circulating hormone levels (low, medium, or high) and are 3.5%, 3.1%, and 4.3% for FSH; 3.8%, 3.6%, and 5.4% for LH, respectively; and 4.9% for E_2.

Light Microscopy

Tissue was fixed in 4% neutral formaldehyde solution, embedded in paraffin, and stained with hematoxylin-eosin and Periodic acid–Schiff-hematoxylin. The material was prepared to be used for immunohistochemistry. Observation was done "blindly" for clinical and laboratory data, and endometrial dating was performed with use of Noyes et al. criteria (1, 2) as more fully detailed in the report by Hendrickson and Kempson (27), by two or three professionals trained in this type of diagnosis. Only portions of each biopsy from "functionalis" layer were used for dating. The most advanced area was used to assign the final date. When glands dating did not match stromal dating, two separate dates were given together with the percent of the glands showing dyssynchrony. More than 50% dyssynchrony was considered clinically significant (27).

Scanning Electron Microscopy

Tissue was fixed for 2–4 hours by immersion in phosphate buffered (0.1 M, pH 7.4) 3% glutaraldehyde, postfixed for 2 hours in 1.3% osmium tetroxide, and dehydrated in a graded series of acetone (30%–50%–70%–100%). The samples were dried in a Balzers CDP 030 critical point drying apparatus (Balzers Union Ltd., Balzers, Liechtenstein) with CO_2 as transition fluid, coated with gold-palladium in a Balzers Union SCD 040, and observed in a Philips 515 scanning electron microscope (Philips, Netherland BV, Eindhoven, the Netherlands).

Immunohistochemistry

A series of purified mouse monoclonal antibodies were used for immunohistochemical procedures. These reacted with the following human antigens: [1] CD61 (reacts with integrin β3); it behaves as a vitronectin receptor and is also expressed on platelets and endothelial cells (Pharmingen, San Diego, CA); [2] CD49d (reacts with the integrin α4 chain, which is mainly expressed as a heterodimer $\alpha 4\beta 1$) (Pharmingen); [3] a polyclonal rabbit antibody to mouse/human leukemia inhibitory factor (LIF; Pharmingen); [4] IL-1 receptor (reacts with type 1 IL-1 receptor) (Biogenesis, Pollen, United Kingdom).

The endometrial biopsy specimen was snap frozen in isopentane and liquid nitrogen. Air-dried cryostat sections (4- to 6-μm thick) were fixed in cold acetone. An immunoperoxidase technique using the avidin-biotin complex (Universal ABC kit; Vector Laboratories, Burlingame, CA) was applied.

Briefly, sections were washed in phosphate buffered saline (PBS), incubated with 0.3% H_2O_2 in methanol to eliminate endogenous peroxidase, washed in PBS, and incubated with the blocking normal horse serum. After a 40-minute incubation with the antibodies selected, sections were successively incubated with the biotinylated antimouse/rabbit serum and ABC complex. Development of the reaction in 3-3′ diaminobenzidine-H_2O_2 resulted in a brown reaction product. Primary antibodies were used at 1/100 (CD61 and

FIGURE 1

Surface of an endometrial biopsy specimen during the luteal phase (day 25). Cells with microvilli (MV) cover most of the surface. Ciliated cells (C) are interspaced between them. Pinopodes (P) are seen among the other cell types. Notice that some pinopodes show a few remaining microvilli in their surface (*). Original magnification, ×8,800.

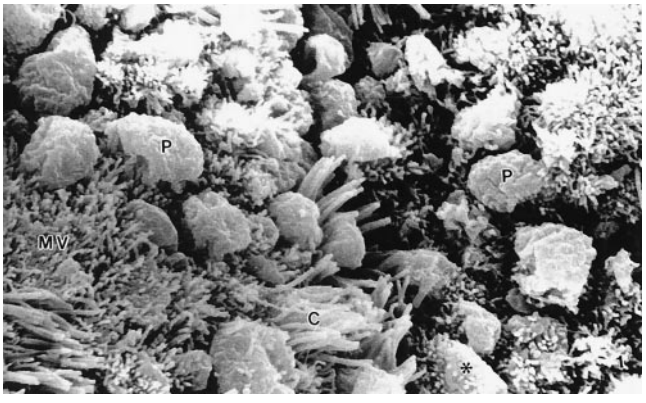

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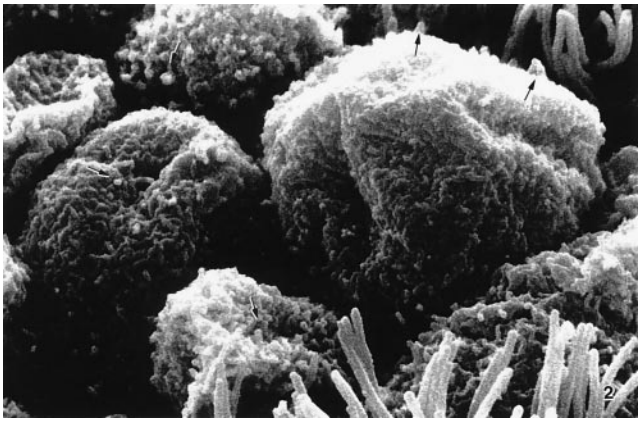
FIGURE 2

Pinopodes at higher magnification. In different areas the remains of preexisting microvilli can be observed (→). Original magnification, ×20,000.



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CD49d) and 1/50 dilution (LIF, IL-1r). Sections were counterstained with hematoxylin.

The procedure was performed at 4°C except for room temperature incubation with sera. Negative controls were obtained by incubating sections with PBS or with normal rabbit or mouse IgG (1/50 and 1/100, respectively) as primary antibodies. Staining intensity was graded as: negative (−), weak (±), moderate (+), or strong (++) and separately recorded for endometrial luminal and glandular epithelium, stromal cells, and vascular endothelium.

Mouse ascites antibodies were used at 1:1,000 dilution as described previously (23). Anti-A, B, and O (H) monoclonal antibodies from Dako Corp. (Carpinteria, CA) were used; the former at a dilution of 1:1,000 and the latter two at 1:250. Biotinylated anti-mouse alpha, gamma, and mu specific secondary antibodies from Vector Laboratories were used at a final concentration of 2.25 μg/mL, as instructed by the manufacturer. Antiepithelial membrane antigen antibody from Dako was used at a dilution of 1:300. Antiprogesterone receptor antibody was a gift from Abbott Laboratories (Abbott Diagnostic Division, South Pasadena, CA) at a dilution of 1:10,000.

Five-micrometer sections from paraffin-embedded tissue were placed on glass slides previously coated with a film of 1% poly-d-lysine, 30–70,000 molecular weight (Sigma Laboratories, St. Louis, MO), dried for 30 minutes at temperatures ≤60°C, and stored at room temperature until used. Immunoperoxidase staining was performed with the avidin-biotin detection method with kits from Vector with diaminobenzidine (Sigma) as the chromagen. Slides were counterstained with hematoxylin. All immunohistochemical studies included known positive control slides.

RESULTS

Results for 14 volunteers were analyzed. The mean (±SD) age of the group was 30.86 ± 3.16 years.

Basal endocrine investigation on day 3 of the menstrual cycle showed a mean (±SD) FSH level of 6.98 ± 2.35 mIU/mL; LH was 4.46 ± 1.63 mIU/mL; FSH-LH ratio was 1.74 ± 0.85; and E_2 level was 27.77 ± 10.13 pg/mL. One patient's basal FSH level was excluded from computations because of an elevated value (19.25 mIU/mL), but her age (26 years old) and all remaining parameters of cycle monitoring were normal; therefore, she was not excluded from the

FIGURE 3

Graphic representation of the characteristics and expression of pinopodes during the luteal phase (the two half panels in each day represent the two biopsies from two different volunteers).

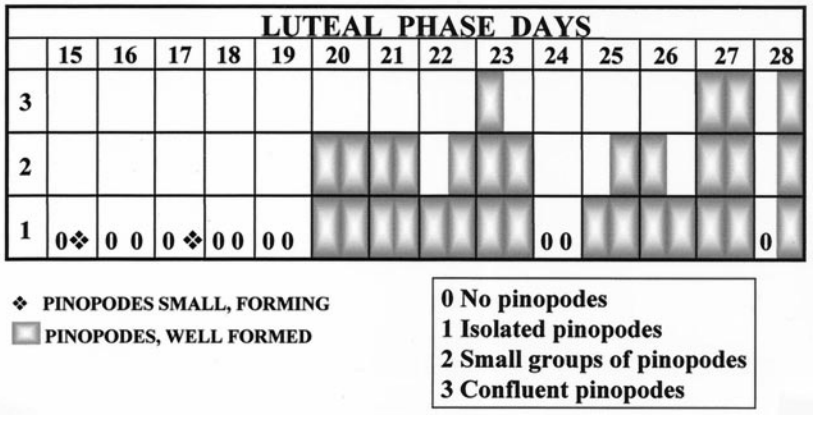

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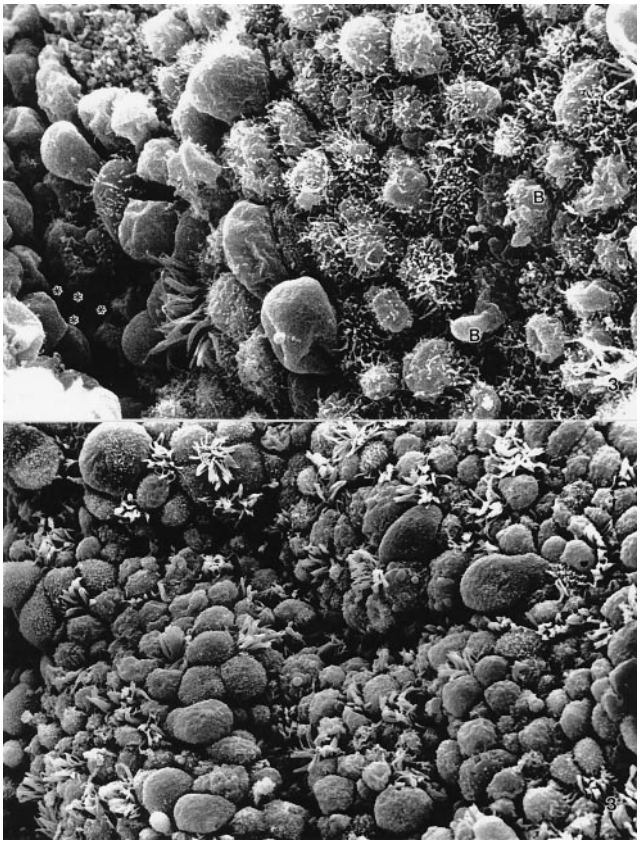
FIGURE 4

Top: A cluster of pinopodes surrounds the opening of an endometrial gland (*). There are numerous cells with microvilli, some of them with budding structures (B) probably indicating the initial stages of pinopode formation (luteal phase, day 20, original magnification, ×5,000). *Bottom:* The surface of the endometrium is extensively covered by pinopodes. Cells with microvilli are very rare (same patient, luteal phase, day 27, original magnification, ×2,500).



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series. A similar problem arose with another volunteer (32 years old) with slightly elevated E_2 levels (72 pg/mL) and all remaining monitoring parameters within normal limits. All calculations were made including and excluding these two patients, and the results showed no differences.

LH peak level on normalized day 14 was 45.83 ± 12.31 mIU/mL, significantly higher than the peak level on day 13 (25.55 ± 14.07 mIU/mL) ($P=.001$).

The mean (±SD) duration of follicular phase measured from the first day of the cycle to the day of LH peak was 12.93 ± 1.33 days. The luteal phase lasted exactly the same: 12.93 ± 1.69 days. The total cycle length was 25.85 ± 2.11 days, further reinforcing the normalcy of the menstrual cycle chronology. Six volunteers had luteal phases of <26 days: two had 25-day cycles, two had 24-day cycles, and two had 23- and 22-day cycles, respectively. The mean (±SD) maximum follicular size diameter measured by transvaginal ultrasound on day 14 was 21.21 ± 2.58 mm. The mean (±SD) endometrial thickness on that day was 9.86 ± 1.46 mm. All endometria presented a trilaminar pattern.

Endocrine evaluation of the luteal phase on calculated day 21 showed a mean E_2 level of 162.86 ± 41.57 pg/mL and a mean (±SD) P4 level of 15.18 ± 4.69 ng/mL, both within normal limits according to the published literature.

Light Microscopy

Of the 28 biopsy specimens (14 volunteers) used in this project, 26 showed agreement in pathological diagnosis in terms of day of the cycle (at least 2 of the 3 reviewers agreeing within 48 hours) and 2 showed a discrepancy (72 hours' difference). One of the pathologists reported eight cases of stromal-glandular discrepancy, but only two reached clinical significance (>50% of the glands involved). These two patients were included because of our previous experience in an oocyte donation program, in which artificially prepared endometria almost invariably showed this type of picture with no reflection in implantation and/or pregnancy results (Acosta AA, unpublished observations).

In all the cases the more advanced dating characteristics were used after discussion.

Using the day of LH peak to date the luteal phase, of the 26 biopsies without classic pathological dating discrepancy, 24 showed ≤48 hours' difference, and in two the dating showed an endometrium 3 days behind. When the luteal phase was dated by the 1st day of the subsequent menstrual period, 22 biopsies were in phase, 3 were 3 days behind, and 1 was 4 days behind.

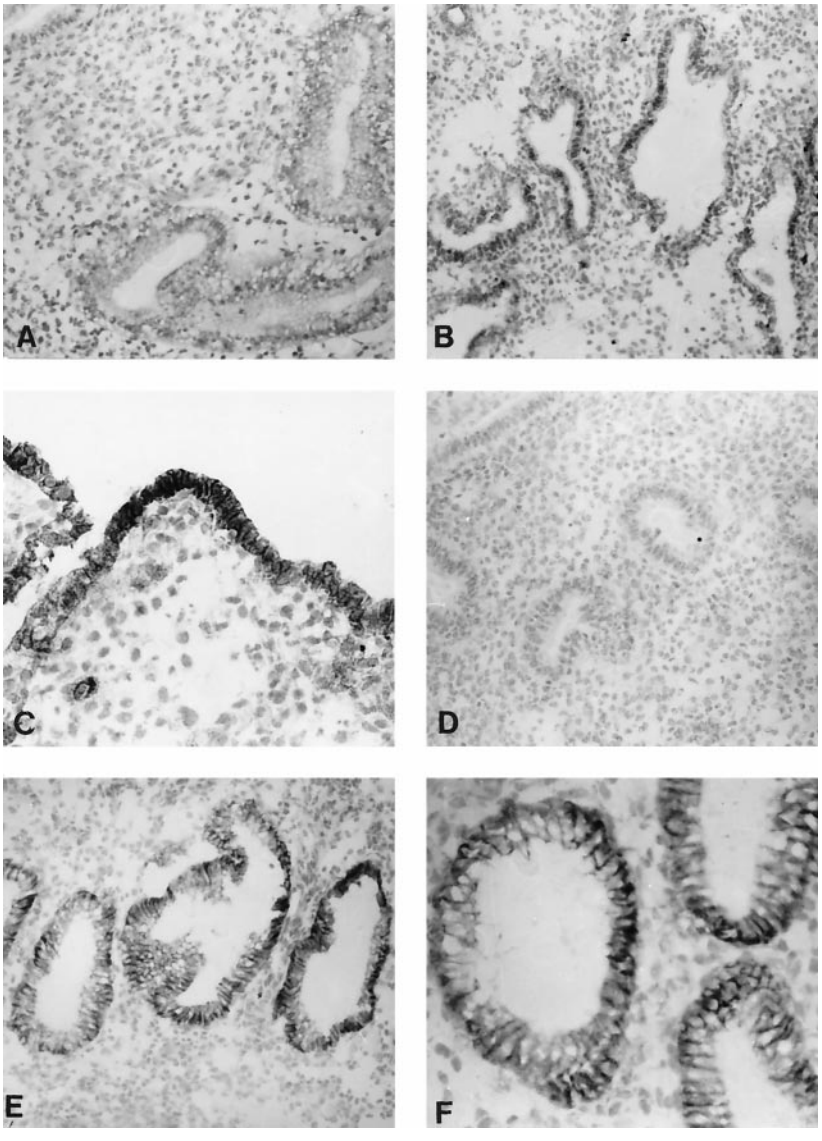
Scanning Electron Microscopy

Secretory endometrium has two different types of cells: ciliated, which appear isolated, and show characteristic bundles of cilia, and cells with microvilli that are predominant and cover most of the endometrial surface. These cells usually have a dome-like appearance and fine microvilli covering most of luminal aspect (Fig. 1). Pinopodes appear during the luteal phase and are spheroidal protrusions without microvilli (Fig. 2); they seem to disappear as pinopodes develop and enlarge.

During the initial part of the luteal phase (days 15–19), they are very small, or completely absent (Fig. 3). Well-formed pinopodes start being seen on days 20 and 21, when they are isolated and frequently localized around the opening of endometrial glands (Fig. 4, top); between days 22 through 26 they form small groups or larger areas of confluence. During the last part of the cycle (days 27–28), large confluent areas can still be observed (Fig. 3 and 4, bottom). Full coverage of the endometrial surface by pinopode formation was not visualized at any time. In some patients well-formed

FIGURE 5

Immunohistochemistry of β3 (**A–C**) and α4 (**D–F**) integrin subunits in cryostat sections of endometrial biopsy specimens at different days of the luteal phase. No expression of β3 integrin subunit was detected at day 20 (A), whereas a strong reaction was observed in the glandular (B) and luminal epithelium (C) at day 24. No expression of α4 integrin subunit was detected in the glandular and luminal epithelium at day 15 (D), whereas a strong reaction was observed in the glandular epithelium at day 22 (E) and at day 23 (F). Original magnification, $\times$150 (A, B, D, and E); $\times$300 (C and F).



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pinopodes could be seen in the two endometrial biopsy specimens spanning a 7-day period (Fig. 4).

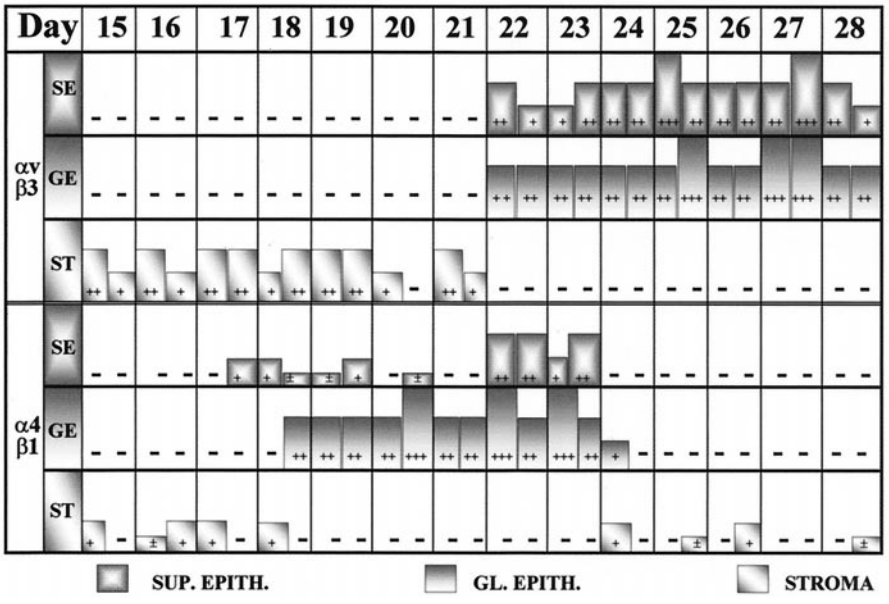
Immunohistochemistry

The expression pattern of $\alpha v\beta 3$ (Fig. 5, A–C) and $\alpha 4\beta 1$ (Fig. 5, D–F) integrins and its chronology and that of LIF and IL-1R tI in human endometrial biopsies is illustrated in Figures 6 and 7. Two samples of each day of the menstrual cycle (from day 15 to day 28) were processed. In general, similar results were observed in the two samples of each day of the cycle. Positive immunoreactive cells were easily identified by the presence of a cytoplasmic brown reaction product, contrasting with the nuclear hematoxylin stain. Lack of staining was observed in control samples.

As seen in Figure 6, epithelial cells displayed a clear cut different expression of the two integrin subunits (β3 and α4) in human endometrium at different days of the menstrual cycle. β3 Integrin subunit was highly expressed in the luminal and

FIGURE 6

Graphic representation of integrins expression ($\alpha v\beta 3$ and $\alpha 4\beta 1$) in superficial and glandular epithelium and in the stroma during the luteal phase. "Sup. epith." = superficial epithelium; "gl. epith." = glandular epithelium.



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glandular epithelium from day 22 up to day 28. A variation in the intensity of the reaction between moderate and strong was detected. No expression of integrins in the epithelium was seen before day 22. From day 15 to day 21 a faint positive reaction was present in the stroma including lymphoid cells. Endothelial cells exhibited a positive reaction throughout the cycle.

Glandular epithelium highly expressed $\alpha 4$ integrin subunit from day 18 up to day 23. Luminal epithelium exhibited a strong positive reaction on days 22 and 23 only. However, a fainter and discontinued expression was also present on day 17 through 19. Lack of $\alpha 4$ reactivity was observed after day 24 with the exception of weak staining in endothelial cells of small vessels and some cells of the stroma.

Changes in LIF and IL-1R tI expression pattern did not correlate with changes in the menstrual cycle stages (Fig. 7). Weak and discontinuous immunoreactivity was observed in LIF expression in the luminal epithelium during all days of the menstrual cycle studied (days 15–28). In contrast, the glandular epithelium was weekly positive only from days 18–27 of the cycle.

IL-1R tI was weakly expressed in stromal cells throughout the cycle and in a more discontinuous and faint way in the cells of the luminal epithelium.

For MAG determinations (Fig. 8), only six patients were blood type A, despite efforts made to recruit all type A volunteers. Only blood type A or AB erythrocytes are able to absorb the anti-MAG antibodies; therefore, we can compute results only in those biopsies. These volunteers had biopsies performed on days 15 (2 biopsies), 16 (2 biopsies), 18, 20, 22 (2 biopsies), 23 (2 biopsies), 25, and 27. All biopsy specimens were positive on the surface epithelium up to day 22; at the level of the glandular epithelium all were positive up to day 20; on days 22 and 23 one of the two biopsies performed was positive, and the single specimen on day 25 was also positive (Fig. 8). In previous work it was found that only staining in the glands seems to be clinically relevant.

MUC-1 showed positivity during all of the luteal phase, and therefore no cyclic variation was identified (Fig. 8). The one volunteer who was negative reflects a technical problem, and the determination could not be repeated because of lack of residual material.

Progesterone receptors were positive from days 15 through 20, except on day 19; a return to positivity was seen sporadically in the last part of the luteal phase (Fig. 8). Surface and glandular epithelia show a clear follicular pattern, and they have been taken into consideration.

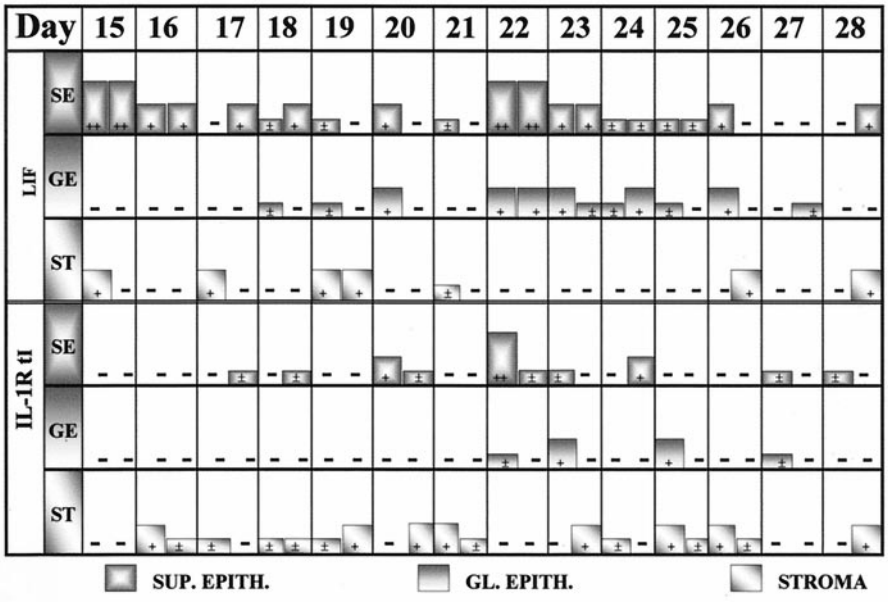
DISCUSSION

The idea of a window of implantation around day 20 is certainly not new; in 1945, Hertig (28) stated that implantation of the blastocyst occurs on the 20th day of the cycle, at the earliest.

The concept of markers or biomarkers for the window of

FIGURE 7

Graphic representation of leukemia inhibitory (LIF) and IL-1R tI expression during the luteal phase. "Sup. epith." = superficial epithelium; "gl. epith." = glandular epithelium.



Acosta. Window of implantation. Fertil Steril 2000.

implantation is a difficult one to define. Because the initial phases of actual human implantation (apposition, adhesion, attachment and penetration through surface epithelium and basement membrane) have not been visualized, the real chronology of this period of endometrial receptivity is theoretical and based mainly on indirect evidence (timing of human embryo development "in vivo" and "in vitro," timing of embryo hatching, data on migration of human embryos through the female reproductive tract, presence of late stages of preimplantation embryos in the endometrial cavity, period of early and late successful transfers in assisted reproduction, and first detection of implantation by peripheral blood determinations of sensitive indicators).

It is possible also that the time required by the embryo to reach that stage could be different under in vitro than under in vivo conditions and that the presence of the embryo in the uterine cavity could induce special local characteristics. Such a physiological mechanism would make it practically impossible to determine the window of implantation when the embryo is not present.

Based on these highly speculative elements, it is doubtful that the markers present at the theoretical time of the window of implantation are the real indicators of receptivity in the human.

Furthermore, a review of the past experience seems to indicate that some of the biomarkers identified not only define the window of implantation but also play a physiological role at apposition and attachment; therefore, they should be present at the level of the surface epithelium (functional markers). A second category seems to play no role at the early stages of implantation but is present during the assumed period of receptivity (pure markers) or has a function at more advanced stages of nidation (late functional markers).

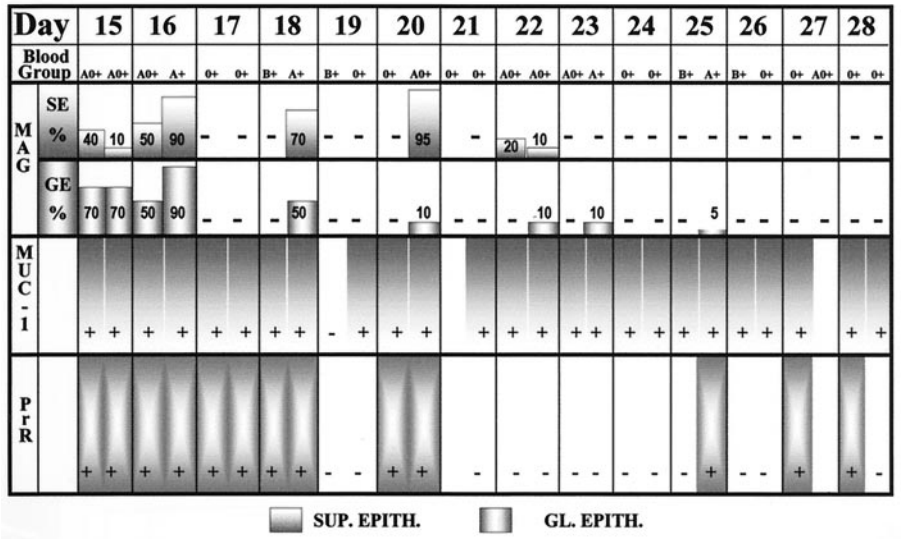
In this article we have tried to eliminate some of the confounding variables of past research by carefully selecting volunteers with normal menstrual cycles (according to present standards) and by accurately dating the ovulation time and the luteal phase. All parameters of menstrual cycle quality have been normal.

The Noyes criteria gives at least a 48-hour variability in dating the luteal phase and do not seem to be accurate enough to relate the different events of the window of implantation to them.

The formation and significance of pinopodes has been extensively studied, mostly by a single group. They indicate, as summarized by Nikas and Psychoyos (29), that the pinopodes have a limited life span that does not exceed 48 hours and that fully developed pinopodes may appear for a single day (one biopsy sample) within days 19–21, counting, as we did, from the day of LH peak (day 14). Our results are somewhat different. Well-formed pinopodes appear in small groups on day 20 and persist for the rest of the luteal phase,

FIGURE 8

Graphic representation of MAG, MUC-1, and PrR expression in superficial and glandular epithelium during the luteal phase (note the blood groups of the patients for MAG determinations, in the top panel row). "Sup. epith." = superficial epithelium; "gl. epith." = glandular epithelium.



Acosta. Window of implantation. Fertil Steril 2000.

sometimes becoming more confluent but at no time covering the entire endometrial surface. In several patients, pinopodes were observed in both biopsy specimens, taken 7 days apart, indicating that their temporal duration may be much longer than 48 hours. These observations may indicate that if the window of implantation is a short period, pinopodes may signal the opening but not the total duration.

With regard to expression of integrins, the extensive work of Lessey indicated the importance of the coexpression of two of them, $\alpha v\beta 3$ and $\alpha 4\beta 1$, in framing the endometrial window of implantation. Lessey's work (30) indicates the presence of $\beta 3$ on the surface epithelium coincident with the opening of the window (cycle days 19–20 postovulation); this corresponds to days 20–21 of our study. In the legend of Figure 3 of that work, the author states: "Note the appearance of surface integrins by day 22 or receptive endometrium" without stating whether it refers to the LH surge or ovulation day.

Our findings indicate the appearance of this integrin also on day 22 in both the luminal and glandular cells. With regard to $\alpha 4$, Lessey states (15, 31) that it appears in the glandular epithelium on day 14 and disappears on days 24–25 (days 15 and 25–26, respectively, of our study); it has not been visualized on the surface epithelium. In our investigation it appears on day 18–19 and disappears on day 23–24, 2 days earlier than in Lessey's report. Contrasting with their results, a strong expression was detected in our study, also on days 22 and 23 on the surface epithelium. Taking all our results together, the window of implantation using these markers seems to be restricted to days 22 and 23.

With regard to LIF expression, Senturk and Arici (32), in a review that includes their own results, report maximal expression between days 19 and 25 (by menstrual history and endometrial dating) in the luminal and glandular epithelium. In our experience, LIF was present in both luminal and glandular epithelium without a definite luteal phase pattern.

The interleukin system at the human endometrium-embryonic level has been extensively studied by Simon et al. (33). IL-1R tI is expressed at the mRNA level in the human endometrial epithelium throughout the menstrual cycle and at increased levels during the luteal phase; its precise role is still undefined. Its neutralization by the antagonist (IL-1ra) prevents implantation in mice. Our findings indicate weak expression at the surface epithelium and stromal level throughout the cycle and irregular presence in very few days of the late luteal phase on the glandular cells.

According to Kliman et al. (23), MAG appears on the surface epithelium on day 17 in the form of scattered patches of apical secretion and lasts until day 19, judged by endometrial dating. In our material of blood group A+ volunteers, MAG is present in all of them until day 22 with different degrees of expression.

MUC-1 is positive all through the luteal phase and does not show any identifiable variation. This is in accord with

previous results indicating that the only variation along the cycle is the intensity and the subcellular localization.

The maximal concentration of P receptor (PgR) occurs in the middle to late proliferative phase in the glandular epithelium, and the concentration decreases during the luteal phase (34). In our material, PgR disappears by day 20, and there is an irregular reappearance in some of the samples after day 25 but at the level of the stroma. The evaluation of the expression of PgR in our work does not distinguish between the A and B isoforms that seem to activate genes differentially (35).

Even if the indicators presently identified to mark the window of implantation are reliable, several questions remain to be answered before the clinician and the patient can benefit from these findings. How is the clinician supposed to obtain and use this information in the menstrual cycle of interest? Are we dealing with a chronologically variable event within each patient (intercycle variability), between different patients (interpatient variability), or under special pathologic or clinical conditions (ovulation induction or stimulation) as has been implicated in the literature? Is the length or duration of the window of implantation modifiable by therapeutic means? If so, can this be used to improve endometrial conditions for natural implantation or for transfer under ART conditions? Can the window of implantation be investigated during the cycle of treatment? How can this information be used to improve the results of ART?

In conclusion, the three markers that have been identified in this paper as being present in the luteal phase, which is compatible with a theoretical window of implantation, do not have synchronous expression: pinopodes indicate an opening of the window on day 20, β3 and α4 integrins seem to indicate the existence of a window on days 22–23 of the normalized menstrual cycle. Thus, all previous questions remain open.

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