PHYSIOLOGY

Evaluation of Endometrial Inflammation by Quantification of Macrophages, T Lymphocytes, and Interleukin-1 and -6 in Human Endometrium

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Purpose: Our purpose was to determine the impact of endometrial inflammation on fertility.

Methods: The study population included 49 women: 5 women bearing an intrauterine device, 33 sterile patients, and 11 fertile women. Endometrial biopsies were performed between day 17 and day 21 of the cycle. Immunostaining was performed with an ABC kit. Monoclonal antibodies were CD68 for macrophages, CD3 for T lymphocytes, and B-E8 for interleukin (IL)-6; IL-1 α and IL-1 β were stained with polyclonal antibodies. A double immunofluorescence staining was performed to identify endometrial cells. Macrophage and lymphocyte rates were defined as the percentage of stained cells by field. Double staining was compared among macrophages, T lymphocytes, and anti-IL antibodies in each group.

Results: The macrophage rate was high for women bearing an intrauterine device, intermediate for sterile patients, and low for fertile women ($P = 0.03$). There was a lack of double staining between activated T lymphocytes and anti-IL-6 antibody in tubal sterility and endometriosis. Endometrial

macrophages were stained with anti-IL antibodies for all groups.

Conclusions: Endometrial macrophages seem to be an interesting parameter for evaluating endometrial inflammation. In tubal infertility and endometriosis, the lack of expression of IL-6 in T lymphocytes could participate in impaired embryo implantation.

KEY WORDS: endometrium; fertility; interleukins; macrophages; T lymphocytes.

INTRODUCTION

Among infertility diagnoses, implantation failure is infrequent, probably because of the lack of understanding, and 15.3% of sterilities are unexplained (1). Thus, it is probable that among patients with unexplained infertility, a number have implantation failure (2,3).

Endometrial inflammation is one of the known mechanisms of intrauterine device (IUD) contraceptive activity (4) and probably of endometriosis (5,6). In in vitro fertilization (IVF), patients with tubal sterility implant less than patients with unexplained or male sterility (7–9). Chronic tubal inflammation can possibly diffuse to the endometrium and then inhibit implantation (10,11).

The morphological analysis of endometrium during the implantation period is not specific enough to appreciate the implantatory quality of human endometrium. Macrophages and T lymphocytes are the main cells of the immune system in the endometrium (12). These cells produce interleukin IL-1 α and IL-1 β and IL-6 (12). These cytokines are known to be involved

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in the growth and differentiation of many tissues and take part in the inflammatory response (13).

We hypothesize that chronic or subacute endometrial inflammation could explain a part of female infertility. Our aim was to detect possible inflammation in infertile women by counting inflammatory cells (endometrial macrophages and T lymphocytes) and their production of cytokines (IL-1 and -6).

MATERIALS AND METHODS

Patient Population

Forty-nine women with normal menstrual cycles were included. Three groups of patients were selected. The first group included women bearing an IUD ($n = 5$). Fertile women comprised the second group (two spontaneous and ongoing pregnancies, no contraception, and no pelvic inflammation story) ($n = 11$), and the third group included sterile women (history of involuntary infertility of ≥ 2 years without male factor) ($n = 33$). Sterile patients were subdivided in to three groups: unexplained ($n = 18$), tubal ($n = 9$), and sterility with endometriosis ($n = 6$). The infertility was defined as unexplained if the following criteria were fulfilled: anatomical normal uterus and patent tubes demonstrated by hysterosalpingogram and laparoscopy with chromotubation, a history of regular cycles, and evidence of ovulation as judged by serum progesterone concentration. Tubal infertility was defined by x-rays and laparoscopic criteria as extensive inflammatory disease in the proximal part of the tube with diverticules extending to more than 2 cm of the isthmus (salpingitis isthmica nodosa), bad prognosis hydrosalpinges (disturbed mucosal folds, irregular walls), or bifocal tubal obstruction (proximal and distal). Sterility with endometriosis was defined by the presence of endometriosis lesions on the peritoneal tissue and/or ovaries (revised American Fertility Society score ≥ 3) without tubal pathology.

Women with general acute or chronic inflammatory disease, those using oral contraceptives and/or anti-inflammatory drugs, and those with inadequate luteal-phase progesterone levels (< 2.5 ng/ml) were excluded from the study.

Informed consent was obtained from each woman.

Sample Collection

Endometrial samples were obtained by a pipelle endometrial suction curette (14). All biopsies were

performed on days 17–21 of the menstrual cycle. Two biopsies were performed for each patient. One was immediately snap-frozen by immersion in liquid nitrogen and stored in OCT compound embedding medium (Tissue-Tek, Miles Inc., Elkhart, IN) at -80°C until use. The other one was examined histologically to establish the menstrual cycle phase according to the criteria of Noyes *et al.* (15). Each woman had a blood sample to determine the plasma concentration of progesterone.

Based on progesterone level and histological information, samples were assigned to luteal phase. Any biopsy which did not correspond to a luteal phase was excluded.

Immunohistochemical Labeling of ILs (IL-1 α , IL-1 β , and IL-6)

Seven-micrometer thick frozen sections were prepared on a cryocut (2800 Frigocut; Reichert Jung). Serial sections were collected onto gelatin-coated glass slides, immediately fixed in 0.9% buffered formalin for 10 min, and then washed in 10 mM phosphate-buffered saline (PBS), pH 7.4.

All further processing was performed with the same technique for each primary antibody on serial section. To inhibit endogenous tissue peroxidase, slides were incubated with a solution of methanol (150 ml) and hydrogen peroxide (2 ml; 30%) for 30 min and then washed with PBS for 10 min.

After 20 min of treatment with serum-blocking solution (horse normal serum at 1.5%), serial sections were incubated with either mouse monoclonal antibody against IL-6 (BE-8; CTS, Besançon, France) or rabbit polyclonal antibodies against IL-1 α or IL-1 β (Genzyme Laboratories, Cambridge, MA) used as primary antibodies at a concentration of 4, 5, and 5 $\mu\text{g/ml}$, respectively, for 30 min.

Then primary antibody–antigen complexes were visualized using a commercial avidin–peroxidase–biotin system (kit ABC Vectastain, Vector Laboratories Inc., Burlingame, MA). Serial sections were successively incubated with biotinylated second antibodies (anti-mouse or anti-rabbit at 0.5%) for 30 min, avidin–peroxidase conjugate for 30 min, and the chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical Co., St. Louis, MO) for 7 min.

Sections were counterstained with Mayer's hematoxylin for 3 min, progressively deshydrated with alcohols, and then mounted with mounting medium and covered with a glass coverslip. The entire procedure was performed at room temperature.

Controls

Negative controls were generated by substituting the primary antibodies with normal mouse immunoglobulin of the same isotype, IgG1 (MOPC 21; Organon Teknika Inc., West Chester, PA), or rabbit normal serum (Sigma Chemical Co.) at the equivalent protein concentration.

Positive control cells used in this study were cells from bronchioloalveolar lavage of smoking patients.

Immunostaining of Endometrial Macrophages and T Lymphocytes

Endometrial macrophages were labeled with a monoclonal antibody against a specific epitope of human macrophage (anti-CD68; Dakopotts, Glostrup, Denmark) at a concentration of 1 µg/ml.

Endometrial activated T lymphocytes were labeled with a specific monoclonal antibody (anti-CD₃ [fluorescein Isothiocyanate] monoclonal antibody; Sigma Chemical Co.) according to an immunofluorescence direct procedure.

Fixed sections were transferred in NH₄Cl, 50 mM, for 10 min to inhibit formalin binding. All procedures were performed at room temperature. Rabbit serum was used as blocking solution at a 1:40 dilution for 20 min and slides were then incubated with anti-CD₃ FITC monoclonal antibody (1:5 dilution) for 1 hr. After 1 hr of washing in PBS, sections were mounted with polyvinyl alcohol and then covered with a glass coverslip.

Characterization of Cells Containing IL by Double Immunofluorescence Staining

To characterize cells containing IL-1α, IL-1β, and IL-6, we used a double immunofluorescence method using green fluorescence to recognize the type of cell and the red fluorescence for ILs (respectively isothiocyanate fluorescein [ITCF] and isothiocyanate rhodamine [ITCR]; Genzyme).

Briefly fixed, sections were first transferred in NH₄Cl, 50 mM, for 10 min. All procedures were performed at room temperature in two steps. First, goat serum-blocking solution (1:40 dilution), primary antibodies (anti-ILs at equivalent concentrations), and fluorescent second ITCR antibody (goat anti-mouse, 1:32 dilution, or goat anti-rabbit, 1:16 dilution) were applied for, respectively, 20 min, 1 hr, and 1 hr.

In the second step, endometrial sections were incubated for 20 min with rabbit serum-blocking solution

(1:40 dilution) and either 1 hr with anti-CD 68 monoclonal antibody (antimacrophages) and fluorescent second ITCF antibody (rabbit anti-mouse, 1:60 dilution) or 1 hr with anti-CD 3 direct ITCF (1:5 dilution) to label T lymphocytes. Sections were then washed for 1 hr in PBS and mounted with polyvinyl alcohol before being covered with a glass coverslip.

Using two wavelengths of excitation (546 and 485 nm), the immunofluorescence double staining revealed, on the same slide, the cells in red and the ILs in green.

Assessment of Staining

Assessment of immunohistochemical staining was performed with a light microscope at a magnification of 1200 (objective, ×40). Slides were scored blindly by two people. Ten nonoverlapping fields of view were examined per biopsy in a systematic random sampling pattern. The intensity of staining of the endometrial components was evaluated by a quantitative scoring system. Results are expressed as the percentage of stroma cells stained per field.

Assessment of immunofluorescence staining was performed using two wavelengths (546 and 485 nm).

Statistics

The data were analyzed by analysis of variance and the Kruskal-Wallis test. We used a BMDP logical, 1993 version (Statistical Software Inc, Los Angeles, CA). Significance was defined as *P* less than 0.05.

RESULTS

Forty-nine samples of the 57 included in this study were analyzed. Eight were excluded: five in the sterile group because of a low plasma level of progesterone and one in each group because histologic study was not feasible. Age and plasma level of progesterone were similar in the three groups (Table I).

Tissue Localization of the Labeling

The immunohistochemical staining of endometrial macrophages and ILs is cytoplasmic, brown, and finely granular. All the stained cells are in the endometrial stroma and there is no staining in the epithelial cells of glands. Negative controls are exempt of staining.

Table I. Comparison of Age and Progesterone Level in Patients with Sterility vs. Fertile Women vs. Women Bearing an IUD

	Mean $\pm$ SD			
	IUD	Sterility	Fertility	
Number of patients	5	33	11	
Age (yr)	35.2 $\pm$ 5.3	31.2 $\pm$ 4.7	32.2 $\pm$ 4.7	NS ^a
Progesterone (ng/ml)	12.7 $\pm$ 7.8	15 $\pm$ 7.2	13.5 $\pm$ 10.7	NS

^a Not significant.

Modification of the Labeling According to Groups

The percentage of endometrial macrophages per field is statistically different ($P = 0.03$, overall comparison between groups) between women bearing an IUD (0.08 ± 0.04), sterile patients (0.05 ± 0.03), and fertile women (0.04 ± 0.02) (Fig. 1). The number of macrophages is high in women with an IUD, low in fertile women, and intermediate in patients with sterility. The percentage of endometrial macrophages is higher in the group of tubal infertile patients compared with the other groups (unexplained or sterility with endometriosis), but this difference is not significant.

The immunostaining of activated T lymphocytes has shown a statistically significant variation among the three groups ($P = 0.0025$). The percentage of endometrial activated T lymphocytes is high in women with an IUD (0.07 ± 0.03), low in sterile patients (0.04 ± 0.02), and intermediate in fertile women (0.05 ± 0.02) (Fig. 2).

There is no significant variation in the IL rates among the three groups of women. Rates of IL-6 and IL-1 α are low in women with an IUD and high in fertile women; these cytokine rates are intermediate in sterile patients.

Double Labeling According to Group

Double staining of activated T lymphocytes and ILs has shown a lack of IL-6 staining in T lymphocytes in patients with tubal sterility or endometriosis (Fig. 3). In the other cases, there is always double staining between activated T lymphocytes and IL-6 ($P < 0.01$).

Activated T lymphocytes were staining with IL-1 α but without significant variation among the groups of women.

The percentage of T lymphocytes stained with the anti-IL-1 β antibody is higher in patients with tubal sterility than in the other groups.

Fig. 1. Number of macrophages expressed as the percentage (%) of stromal cells per field immunostained using an anti-CD68 monoclonal antibody as described under Materials and Methods. Each point represents an endometrial sample obtained in the luteal phase of women bearing an IUD, sterile patients, and fertile women. The transversal line represents the median, and the p is the value of the significance of the difference between the three groups using the Kruskal-Wallis test.

Fig. 4. Number of macrophages having IL-6 in each endometrium of the groups studied: number of macrophages having IL-6 expressed as the percentage (%) of stromal cells per field immunostained using both an anti-CD68 monoclonal antibody and an anti-IL-6 monoclonal antibody as described under Materials and Methods. Each point represents an endometrial sample obtained in the luteal phase of women bearing an IUD (I), patients with unexplained sterility (U.S), patients with tubal sterility (T.S), sterile patients with endometriosis (E.S), and fertile women (F). The transversal line represents the median, and p is the value of the significance of the difference between the five groups using the Kruskal–Wallis test.

For IL-1 β , there is no significant difference, but in fertile women, the percentage of double staining is higher than in the other group.

DISCUSSION

Macrophage and activated T lymphocyte rates are significantly higher in women bearing an IUD than in sterile patients or fertile women during the implantation period of the menstrual cycle. The increase in these inflammatory cells has been shown in the uteri of mice bearing an IUD (16). Endometrium with IUD could be a pattern to quantify the inflammation and the implantation failure related. However, we cannot give a threshold of significance to define sterility or fertility in women.

The variation of endometrial T lymphocytes in normal fertile women has already been reported (17). However, concerning these variations, interpretation can be difficult because of the large number of T cells described in the literature. King *et al.* demonstrated that the number of large granular lymphocytes increased after ovulation (18). In our study, the presence of an IUD in the uterine cavity seems to stimulate and increase the number of T lymphocytes CD3+. There is a difference between fertile women and sterile women, with a low count for the sterile ones. We can

hypothesize that the lack of T lymphocytes is one of the mediators of infertility. However, we cannot make conclusions about the mechanism of this establishment.

Cytokines secreted by macrophages or T lymphocytes could take part in endometrial inflammation to prevent embryo implantation. Nevertheless, the mechanism of these endometrial cells activities is unknown.

There is no difference among the three groups of patients for the quantification of ILS. The quantitative study of these cytokines seems not to be useful in evaluating endometrial inflammation during the implantation period, as there are no significant differences between the group with unexplained sterility and the group of fertile women. Evaluation is difficult because the cytokine expression is under the regulatory influence of systemic steroid signals (19). Moreover, cytokine staining at this time of the menstrual cycle is weak (20). The distribution of cytokine staining is variable during the menstrual cycle (12).

In our study, we found cells stained by anti-IL antibodies only in the stromal part of human endometrium during the implantation period. The role and regulation of these cytokines are unknown in human endometrium during this period. These cytokines interact and inhibit or enhance much of production, like prostaglandins (21).

The immunofluorescence double staining study showed a lack of staining of IL-6 on activated T lymphocytes in patients with tubal sterility or endometriosis. Therefore, this lack of staining could be related to an excess of IL-6 consumption in this type of pathology or a lack of stimulation of T lymphocytes (22). IL-6 is perhaps only the image of a pathology and not the mediator of this pathology. The exact function of these activated T lymphocytes and others, and their potential role for cytokine production are not known (23).

We hypothesize that this finding in patients with tubal sterility or in the group with endometriosis could be related to either a pathology of the IL-6 receptor or trouble with stimulation of T lymphocytes (24). At this point in the research, we cannot draw a definitive conclusion, and further studies will be necessary to explain this phenomenon.

Quantification of endometrial macrophages seems to be an interesting parameter for evaluating endometrial inflammation during the implantation period. In tubal infertility and endometriosis, the lack of expression of IL-6 in T lymphocytes could participate in impaired embryo implantation. Mechanisms of these interactions are still unclear.

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REFERENCES

- De Mouzon J, Lancaster P: World collaborative report 1993 of the International Working Group for Registers on Assisted Reproduction. 15th World Congress on Fertility and Sterility, Montpellier, France, Sept 17–22, 1995
- Klentzeris LD, Bulmer JN, Trejdosiewicz LK, Morrison L, Cooke ID: Beta-1 integrin cell adhesion molecules in the endometrium of fertile and infertile women. *Hum Reprod* 1993;8:1223–1230
- Klentzeris LD, Bulmer JN, Warren MA, Morrison L, Li TC, Cooke ID: Lymphoid tissue in the endometrium of women with unexplained infertility: Morphometric and immunohistochemical aspects. *Hum Reprod* 1994;9:646–652
- Ezra Y, Birkenfeld A, Levij IS: Endometrial reaction to intra-uterine device in pregnancy. *Gynecol Obstet Invest* 1989;28:5–7
- Halme J, Becker S, Haskill S: Altered maturation and function of peritoneal macrophages: Possible role in pathogenesis of endometriosis. *Am J Obstet Gynecol* 1987;156:783–789
- Fakin H, Bagett B, Holtz G, Tsang K, Lee JC, Williamson HO: Interleukin-1: A possible role in the infertility associated with endometriosis. *Am J Obstet Gynecol* 1987;47:213–217
- Sims JA, Jones D, Butler L, Muasher SJ: Effect of hydrosalpinx on outcome in in-vitro fertilization. *American Fertility Society*, 1993, p S95 (abstr P-032)
- Kassabji M, Sims JA, Butler L, Muasher SJ: Reduced pregnancy outcome in patients with unilateral or bilateral hydrosalpinx after in-vitro fertilization. *Eur J Obstet Gynecol* 1994;56:129–132
- Dechaud H, Arnal F, Humeau C, Bonifacj C, Hedon B: Embryo implantation rate changes with the etiology of infertility. Abstract P327, 15th World Congress on Fertility and Sterility, September 17–22, 1995, Montpellier, France. *Contracept Fertil Sex* 1995;9 (Suppl 23):S144
- Mansour RT, Aboulghar MA, Sevour GI, Riad R: Fluid accumulation of the uterine cavity before embryo transfer: A possible hindrance for implantation. *J In Vitro Fert Embryo Transfer* 1991;8:157–159
- Ben Rafael Z, Orvieto R: Cytokines—Involvement in reproduction. *Fertil Steril* 1992;58:1093–1099
- Tabibzadeh S: Human endometrium: An site of cytokine production and action. *Endocr Rev* 1991;12:272–290
- Benedetti F, Massa M, Robbioni P, Ravelli A, Burgio GR, Martini A: Correlation of serum interleukin-6 levels with joint involvement and thrombocytosis in systemic juvenile rheumatoid arthritis. *Arth Rheum* 1991;34:1158–1163
- Hill GA, Herbert CM, Parker RA, Wentz AC: Comparison of late luteal phase endometrial biopsies using a Novak curette or pipelle endometrial suction curette. *Obstet Gynecol* 1989;73:443–445
- Noyes RW, Hertig A, Rock J: Dating the endometrial biopsy. *Fertil Steril* 1950;1:3–25
- Takeuchi K, Mori A, Yamamoto S, Sonoda T, Nagata Y: Effect of the secretions from the IUD-bearing uterus on peri-implantation mouse embryos. *Contraception* 1990;41:655–662
- Klentzeris LD, Bulmer JN, Warren MA, Morrison L, Li TC, Cooke ID: Endometrial lymphoid tissue in the timed endometrial biopsy: morphometric and immunohistochemical aspects. *Am J Obstet Gynecol* 1992;167:667–674
- King A, Wellings V, Gardner L, Loke YW: Immunocytochemical characterization of the unusual large granular lymphocytes in human endometrium throughout the menstrual cycle. *Hum Immunol* 1989;24:195–205
- Tabibzadeh S, Sun XZ: Cytokines expression in human endometrium throughout the menstrual cycle. *Hum Reprod* 1992;7:1214–1221
- Kauma S, Dennis M, Strom S, Eierman D, Turner BS: Interleukin-1 β , human leukocyte antigen HLA-DR alpha and transforming growth factor- β expression in endometrium, placenta and placenta membranes. *Am J Obstet Gynecol* 1990;163:1430–1437
- Tabibzadeh S, Kaffka KL, Satyaswaroop PG, Kilian PL: IL-1 regulation of human endometrial function: presence of IL-1 receptor correlates with IL-1 stimulated PGE₂ production. *J Endocrinol Metab* 1990;70:1000–1006
- Wong GG, Clarck SC: Multiple actions of interleukin 6 within a cytokine network. *Immunol Today* 1988;9:137–139
- King A, Loke YW: Uterine large granular lymphocytes: A possible role in embryonic implantation? *Am J Obstet Gynecol* 1990;162:308–310
- Kishimoto T, Akira S, Taga T: Interleukin-6 and its receptor: A paradigm for cytokines. *Science* 1992;258:593–597