Effect of menstruation and intrapelvic injection of endometrium on inflammatory parameters of peritoneal fluid in the baboon (*Papio anubis* and *Papio cynocephalus*)

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OBJECTIVE: Our purpose was to test the hypothesis that menstruation and intrapelvic injection of endometrium for the induction of endometriosis affect inflammatory parameters in peritoneal fluid from baboons.

STUDY DESIGN: In the first part of this study, 107 laparoscopies were performed in 62 female baboons with a normal pelvis during menstruation, the follicular phase, and the luteal phase. In the second part of this study, 21 baboons were studied during paired laparoscopies in the follicular phase and the luteal phase of the cycle. In the third part of this study, 11 baboons were studied by paired laparoscopies during menses and during the nonmenstrual phase of the cycle. In the fourth part of this study, paired laparoscopies were performed in 7 baboons before and after intrapelvic injection of endometrium. Peritoneal fluid was aspirated and measured in all laparoscopies and assessed for leukocyte concentration. In the third and fourth parts of the study, peritoneal fluid was analyzed for the concentrations of inflammatory cytokines and for the proportions of cells with immunohistochemical staining positive for these cytokines.

RESULTS: During menstruation, in comparison with nonmenstrual phases of the cycle, the leukocyte concentration of the peritoneal fluid was increased significantly, as were the proportions of peritoneal fluid cells with positive staining for tumor necrosis factor α, transforming growth factor β_1, and intercellular adhesion molecule 1 and the peritoneal fluid concentrations of transforming growth factor β_1 and interleukin 6. After intrapelvic injection of endometrium, the peritoneal fluid leukocyte concentration and the proportions of peritoneal fluid cells with positive staining for tumor necrosis factor α, transforming growth factor β_1, CD3, and human leukoctye antigen (DR locus) significantly increased.

CONCLUSION: Subclinical peritoneal inflammation occurs in baboons during menstruation and after intrapelvic injection of endometrium. (Am J Obstet Gynecol 2001;184:917-25.)

Key words: Baboons, cytokines, endometriosis, inflammation, immunology, peritoneal fluid

Sampson[1] hypothesized that endometriosis was caused by intrapelvic transplantation of endometrium shed during retrograde menstruation.

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Several observations in the baboon model for endometriosis[2-4] appear to support this hypothesis. These observations include the increased incidence of retrograde menstruation in baboons with spontaneous endometriosis,[2] cervical occlusion leading to retrograde menstruation and endometriosis,[3] and intrapelvic injection of menstrual endometrium causing experimental endometriosis similar to that observed in spontaneous disease.[4] Studying immune and inflammatory cells and cytokines contained in peritoneal fluid during menstruation may facilitate a better understanding of the pathogenesis of endometriosis. However, only limited data are available in women. The concentration of peritoneal fluid cells has been reported to be higher in 4 fertile, regularly cycling women during menstruation than in 20 women during other phases of the cycle.[5] These limited observations have not been confirmed in larger studies. Furthermore, it is not known whether the inflammatory changes observed in the peritoneal fluid of women with endometriosis are the cause or the consequence of this disease.[6]

Table I. Parts 2, 3, and 4: Study design of paired laparoscopies

	Part 2	*Part 3*	*Part 4*
Baboons			
Total, normal pelvis, endometriosis	21	11	
Normal pelvis	9	4	
Endometriosis	12	7	
Normal pelvis, endometriosis after induction			7
Spontaneous endometriosis (No.)	10 (6 rAFS I, 3 rAFS II, 1 rAFS III)	7 (4 rAFS I, 3 rAFS II)	—
Induced endometriosis (No.)	2 (1 rAFS II, 1 rAFS III)	—	7 (rAFS II-IV)
Weight (kg)	12.1 ± 1.7	13 ± 2.4	12.5 ± 2.5
Duration of captivity (mo, median and range)	18 (6-98)	50 (10-102)	22 (6-141)
Laparoscopies (No.)	42	22	14
Follicular phase (No.)	21	5	3 (after induction)
Luteal phase (No.)	21	6	3 (induction) 4 (after induction)
Menstrual phase (No.)	—	21	4 (induction)
Time between paired laparoscopies (mo, median and range)	7.5 (1-29)*	5 (1-11)†	3 (1.5-3.5)‡

rAFS, Revised American Fertility Society classification.[11]
*Follicular phase versus luteal phase.
†Menstrual phase versus nonmenstrual phase.
‡Induction versus after induction.

It is ethically difficult to address this issue adequately in women because it is impossible to perform paired laparoscopies during menstruation and during the nonmenstrual phase of one of the next cycles in the same woman. Therefore animal models are necessary. In rabbits, induction of endometriosis did not result in an increased total number of peritoneal fluid macrophages.[7] However, for the study of endometriosis, the value of animal models without a menstrual cycle comparable to that in the human being is questionable.[8] Female baboons are similar to women with respect to pelvic anatomy, reproductive physiologic characteristics, and immunologic features and are well-established models for the study of peritoneal endometriosis.[8] In this study we tested the hypothesis that the inflammatory parameters of peritoneal fluid of baboons are affected by both menstruation and intrapelvic injection of autologous endometrium.

Material and methods

Animals and laparoscopies. A retrospective study was performed with the use of the database on all laparoscopies (n = 469) performed in 122 adult female baboons of proven fertility in the wild, weighing 8 to 15 kg, and housed at the Institute of Primate Research, Nairobi, Kenya, for 6 to 15 months between July 1990 and April 1993. The study protocol was reviewed and approved by the Institute of Primate Research Scientific Resources Evaluation and Research Committee.

Perineal staging was used to detect ovulation because perineal inflation and deflation correspond well in the baboon with the follicular and luteal phases of the menstrual cycle, respectively, with ovulation occurring 3 days before perineal deflation.[9] Anesthesia and diagnostic laparoscopy were technically carried out as described previously.[10] Peritoneal fluid was aspirated and its volume was measured immediately after insertion of the laparoscope with a laparoscopic needle from the posterior cul-de-sac, vesicouterine fold, and pararectal gutters before organ manipulation at the beginning of the laparoscopy and before the baboon was placed in the Trendelenburg position. Peritoneal fluid was collected in sterile heparinized tubes and kept at 4°C until processed, which occurred within 2 hours of collection. Only laparoscopies without blood contamination of peritoneal fluid from Veress needle insufflation or trocar manipulation were included.

Endometriosis was staged according to the classification of the revised American Fertility Society,[11] after adaptation for baboon size (Table I). All animals with endometriosis had histologically proven disease (presence of both endometrial glands and stroma).

Effect of cycle phase and menstruation on peritoneal fluid inflammatory parameters (parts 1-3). In the first part of the study, the effect of the menstrual cycle phase on inflammatory parameters of peritoneal fluid (volume, leukocyte concentration) was analyzed. Because we previously reported that both the cycle phase and the presence or absence of endometriosis may affect peritoneal fluid leukocyte concentrations in baboons,[12] only laparoscopies performed in baboons with a normal pelvis (n = 62) and with a recorded peritoneal fluid volume (n = 107) were taken into account for further analysis. These 62 baboons with a normal pelvis had been in captivity for 22 ± 20 months (median, 15 months; range, 6-91 months). Laparoscopies were performed during menses (n = 27), in the follicular phase (n = 21), and in the luteal phase (n = 59).

Because the first part of this study showed no significant differences, possibly as a result of great interindividual variations regarding both volume and leukocyte con-

centration of peritoneal fluid (see Results section), only paired observations (laparoscopy, peritoneal fluid volume, peritoneal fluid leukocyte concentration, peritoneal fluid cytokines) in baboons with or without endometriosis from the database mentioned herein were included in the second, third, and fourth parts of the study (Table I).

In the second part of the study (Table I), the effect of ovulation on inflammatory parameters of peritoneal fluid was analyzed in all 21 baboons (*Papio anubis*) that were identified from the database with a history of paired observations by laparoscopy (to document peritoneal fluid volume and peritoneal fluid leukocyte concentration) during both the follicular phase (n = 21) and the luteal phase (n = 21). These 21 baboons (Table I) included 9 animals with a normal pelvis and 12 primates with endometriosis (10 with spontaneous and 2 with induced endometriosis). In the 2 baboons with induced endometriosis, the first laparoscopy of the 2 paired laparoscopies during the follicular and luteal phases had been carried out at least 3 months after the induction procedure. In these baboons induction of endometriosis had been performed by intraperitoneal injection of menstrual endometrium (obtained on day 2 of the menstrual cycle) in the pelvic cavity, as previously reported.[4] Briefly, a laparotomy was performed (low midline incision) to obtain endometrium because it was impossible to probe the uterus for an endometrial biopsy, even under laparoscopic guidance. For prevention of adhesion formation, a uterine incision was not made; instead, the fundus was penetrated with an 18-gauge needle, followed by a Veress needle. Subsequently, an adapted Novak curette was introduced through the small opening in the fundus. A curettage was performed in each animal, and all endometrial biopsy specimens were pooled together to obtain an appreciable quantity of endometrial tissue. Intraperitoneal seeding was performed to assess the implantation potential of freshly obtained and unmanipulated menstrual endometrium. Therefore the endometrial tissue was immediately aspirated in a 5-mL syringe with assessment of volume and color, fragmented through an 18-gauge needle, and, because it had a pastelike consistency, "seeded" on top of the peritoneum (more specifically, on the uterosacral ligaments, uterovesical fold, posterior uterine peritoneum, cul-de-sac, broad ligament, and ovaries). The places of intraperitoneal seeding were carefully recorded on a pelvic map for each baboon as described previously.[4]

In the second part of the study, laparoscopies performed during the follicular phase had been done either on day 15.9 ± 6.7 (median, day 16; range, days 5-30) of the menstrual cycle and at a median of 9 days (range, 1-51 days) before perineal deflation or at a median of 6 days before ovulation, because ovulation is estimated to occur about 3 days before deflation in baboons, as previously described.[9] Laparoscopies performed during the luteal phase were done at a median of 4 days (range, 1-19 days) after perineal deflation or at a median of 7 days (range, 4-22 days) after ovulation. A recent corpus luteum or signs of ovulation were seen in all laparoscopies performed during the luteal phase within 8 days after perineal deflation (11 days after ovulation) but not in 3 laparoscopies performed 9, 13, and 19 days after perineal deflation (ie, 12, 16, and 22 days after estimated ovulation). In contrast, a recent corpus luteum or signs of ovulation were not seen in any of the laparoscopies performed during the follicular phase.

In the third part of the study, the effect of menstruation on inflammatory parameters of peritoneal fluid was studied in all 11 baboons (*P anubis,* n = 10; *Papio cynocephalus,* n = 1) identified from the database with paired observations (laparoscopy, peritoneal fluid volume, peritoneal fluid leukocyte concentration, peritoneal fluid cytokines) during the menstrual phase and the nonmenstrual phases (follicular phase, n = 5; luteal phase, n = 6; Table I).

Long-term effect of intrapelvic injection of endometrium for the experimental induction of endometriosis on inflammatory parameters of peritoneal fluid (part 4). In the fourth part of this study, peritoneal fluid was analyzed in all 7 adult female baboons (*P anubis*) identified from the database with paired observations (laparoscopy, peritoneal fluid volume, peritoneal fluid leukocyte concentration, peritoneal fluid cytokines) before and after intrapelvic injection of endometrium. These 7 baboons included 2 animals that had been included in part 3 of this study, before the start of part 4, and 1 primate that entered part 3 of this study 3 months after the completion of part 4. At entry into this part of the study, all 7 baboons had a normal pelvis, as confirmed by the first laparoscopy performed in either the follicular phase (n = 2) or the luteal phase (n = 5) of the menstrual cycle.

In 3 baboons that had the first laparoscopy during the luteal phase, experimental induction of endometriosis was performed during this laparoscopy by retroperitoneal injection of luteal endometrium in the pelvic cavity, as previously described.[4] In these baboons endometrium was obtained by transcervical biopsy with an adapted 2-mm Novak curette (curved tip, small opening), placed in a sterile Petri dish, resuspended in 1 to 2 mL sterile 0.9% saline solution, and injected with an 18-gauge needle beneath the peritoneum on the same places as described in part 2 of this study. Each site of retroperitoneal injection was inspected for intraperitoneal leakage for 1 to 2 minutes. The places of retroperitoneal injection and intraperitoneal leakage were carefully recorded on a pelvic map for each baboon, as previously described.[4] Retroperitoneal injection of endometrium has been described in several reports about experimental endometriosis in primates (reviewed in reference 4); however, it was technically difficult and subsequent intraperitoneal leakage of the injected en-

Table II. Antibodies used in this study

Antibody	*Company*	*Specificity*	*Dilution*
CD3	Dako Corporation, Carpinteria, Calif	T lymphocytes	1:50
TIA-1	Biogenex, San Ramon, Calif	Cytotoxic T cells and natural killer cells	1:1000
HLA-DR	Dako	Macrophages, B cells, and activated T cells	1:50
ICAM-1	Boehringer Ingelheim Corporation, Ridgefield, Conn	Intercellular adhesion	1:200
TNF-α	Genzyme Corporation, Cambridge, Mass	Secreted by macrophages, activated T cells, and other cell types	1:500
TGF-β1	Genzyme	Secreted by macrophages, activated T cells, and other cell types	1:50

dometrium was unavoidable. This problem may explain why retroperitoneal injection of endometrium is less successful in inducing endometriosis than intraperitoneal seeding of endometrium, as reported previously.[4] In these 3 baboons with endometriosis induced in the luteal phase, the second laparoscopy was also performed in the luteal phase at a median of 2 months (individual values, 1½, 2, and 3 months, respectively) after the induction procedure.

In the other 4 baboons only screening was performed during the first laparoscopy. A second laparoscopy was performed on the second day of menstruation 7 ± 2 months later, and experimental induction of endometriosis was performed by intraperitoneal injection of menstrual endometrium into the pelvic cavity. In 1 of these 4 baboons the induction was performed as described in part 2 of the study. In the other 3 baboons endometrial tissue was obtained in the same way as in the 3 baboons in which endometriosis was induced with luteal endometrium, and intraperitoneal seeding was performed as described in part 2 of this study. In these 4 baboons that underwent induction during menstruation, a follow-up laparoscopy was performed in either the follicular phase (n = 3) or the luteal phase (n = 1) at a median of 3 months (individual values, 3, 3, 3½, and 3½ months, respectively) after the induction procedure.

The combined data on baboons and laparoscopies in part 4 of this study are presented in Table I.

Peritoneal fluid analysis. The peritoneal fluid sample was measured and centrifuged at 600*g* for 10 minutes, and the supernatant was divided into aliquot portions and stored at –70°C until used. The pellet was resuspended in 1 mL of phosphate-buffered saline solution, and leukocytes were counted on a hemocytometer with trypan blue dye exclusion for viability assessment. A 5-µL portion of each 1-mL suspension was applied separately to each spot of a Teflon-coated 8-spot microscope slide (Roboz Surgical Instrument Company, Rockville, Md). After air-drying, the slides were fixed in acetone for 10 minutes and stored frozen at –70°C until used.

It was not possible to analyze all peritoneal fluid study parameters because of technical difficulties (limited amounts of peritoneal fluid for all determinations). Immunohistochemical analysis of peritoneal fluid cytokines was performed only in the third and the fourth parts of the study.

Peritoneal fluid cell immunohistochemical study. The monoclonal and polyclonal antibodies, their specificities, and the dilutions used are listed in Table II. For negative control, phosphate-buffered saline solution was used in place of a monoclonal antibody on 1 spot of each slide. Leukocyte subpopulations were detected by means of routine immunohistologic techniques with streptavidin-biotin–alkaline phosphatase (Stravigen Supersensitive mouse or rabbit immunodetection system; Biogenex, San Ramon, Calif) that stains positive cells red. Briefly, nonimmune rabbit serum was applied to each slide (10 minutes) for saturation of nonspecific binding sites. Antileukocyte antibodies (Table II) were incubated on individual spots of each slide for 30 minutes at 37°C. Biotinylated secondary antibody was than added (10 minutes), followed by the streptavidin-peroxidase conjugate (5 minutes) and the substrate chromogen (aminoethylcarbazole). After a 5-minute incubation at room temperature, slides were counterstained with hematoxylin for 3 minutes, rinsed with water, and mounted with coverslips by an aqueous mounting solution (Accergel Mounting Medium [Accurate Chemical & Scientific Corporation, Westbury, NY] or glycerol-polyvinyl alcohol aqueous mounting medium; GVA Mounting Solution, Zymed Laboratories Inc, South San Francisco, Calif). Red-brown–stained (positive) and pale blue–counterstained (negative) leukocytes per high-power field were counted on a light microscope fitted with a ×10 10-mm eyepiece reticle. For each determination 200 cells were assessed in representative high-power fields (×40 objective), with data presented as percentages of positive cells (Table I).

Peritoneal fluid cytokines. Frozen aliquots of peritoneal fluid supernatants were thawed for use with enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, Minn) to determine the peritoneal fluid concentration of the cytokines as follows: interleukin 6 (IL-6), lowest limit of sensitivity, 0.7 pg/mL; transforming growth factor β_1 (TGF-β_1), lowest limit of sensitivity, 5 pg/mL; and interleukin 10 (IL-10), lowest limit of sensitivity, 2 pg/mL. The IL-6 cytokine (R&D Systems) had been validated previously for use in peritoneal fluid[13] and in nonhuman primates.[14]

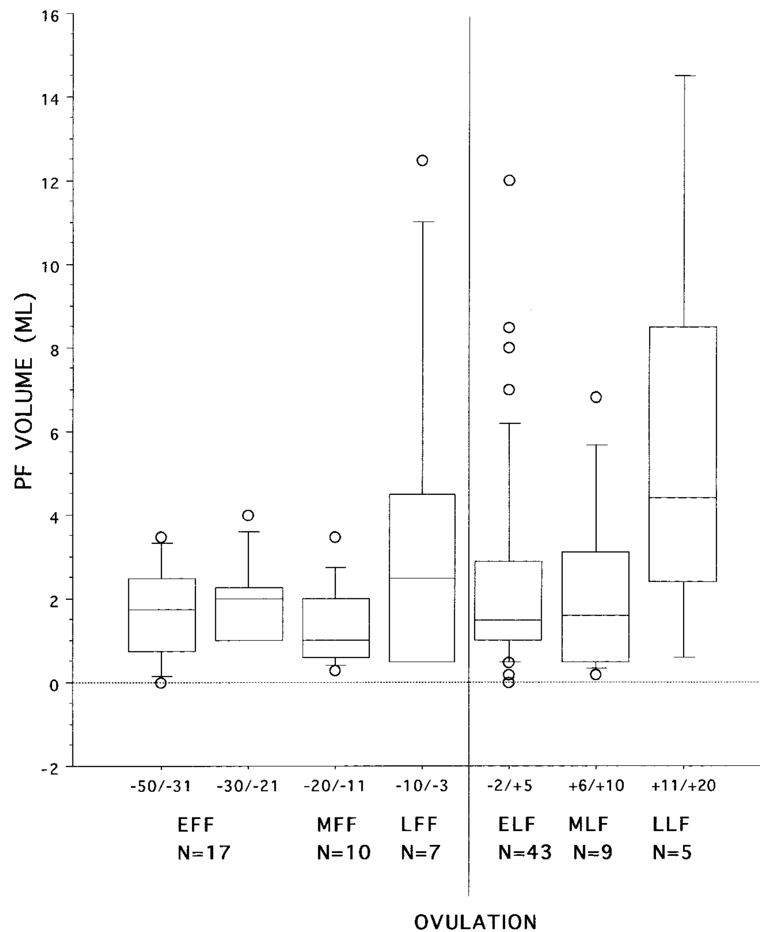

Fig 1. Box plot showing grouping variables with number of days before (*minus signs*) and after (*plus signs*) perineal deflation. Peritoneal fluid *(PF)* volume was not significantly affected by phase of cycle (89 laparoscopies; data grouped around time of ovulation). *Circles,* Values <P10 or >P90. *EFF,* Early follicular phase; *MFF,* midfollicular phase; *LFF,* late follicular phase; *ELF,* early luteal phase; *MLF,* midluteal phase; *LLF,* late luteal phase.

Statistical analysis. Statistical analysis was performed with the Kruskal-Wallis test, the Wilcoxon test for paired samples, the Mann-Whitney test, factorial analysis of variation, and the (un)paired Student t test where appropriate, with $P < .05$ considered significant. Power analysis was performed as published previously.[15]

Results

Effect of cycle phase and menstruation on peritoneal fluid inflammatory parameters. In the first part of the study, peritoneal fluid volume was nonparametrically distributed, showed a high interindividual variation, and was statistically comparable (Kruskal-Wallis test, $P = .2$; Mann-Whitney test, $P = .6$; menstrual phase vs nonmenstrual phase; and Mann-Whitney test, $P = .1$, luteal phase vs menstrual or follicular phase) at laparoscopies performed during menses (n = 27; median, 1.5 mL; range, 0-12.5 mL), in the follicular phase (n = 21; 1.2 mL; range, 0-7 mL), and in the luteal phase (n = 59; median, 2 mL; range, 0-14.5 mL). Similar observations (Kruskal-Wallis test, $P = .4$; Mann-Whitney test, $P = .2$; menstrual phase vs nonmenstrual phase) were made for peritoneal fluid leukocyte concentrations during the menstrual phase (n = 14; median, $3.2 \times 10^3/\mu L$; range, $0.5\text{-}14.4 \times 10^3/\mu L$), the follicular phase (n = 10; median, $2.6 \times 10^3/\mu L$; range, $0.4\text{-}11.2 \times 10^3/\mu L$), and the luteal phase (n = 35; median, $2.3 \times 10^3/\mu L$; range, $0.4\text{-}18.6 \times 10^3/\mu L$). Information about the exact timing of laparoscopies with respect to the time of ovulation was available in 89 cases. Grouping the data of these 89 laparoscopies around ovulation did not result in significant differences in peritoneal fluid volume or peritoneal fluid leukocyte concentration according to the day of the cycle (Spearman correlation: $\rho = 0.085$, $P = .4$, peritoneal fluid volume; $\rho = -0.025$, $P = .8$, peritoneal fluid leukocytes) or between the follicular phase and the luteal phase (Mann-Whitney test, $P = .4$, peritoneal fluid volume), even when data were compared among the early follicular phase, midfollicular phase, late follicular phase, early luteal phase, midluteal phase, and late luteal phase (Fig 1) (Kruskal-Wallis test: $P = .4$, peri-

Table III. Effect of menstruation and of induction of endometriosis (intrapelvic injection of endometrium) on peritoneal fluid inflammatory parameters (medians and ranges) in baboons

	Phase of cycle			Induction	
	Menses (n = 11)	*Follicular (n = 5)*	*Luteal (n = 6)*	*Before (n = 7)*	*After (n = 7)*
Peritoneal fluid volume (mL)	2 (1-4.5)	1 (0.5-8.5)	1.6 (0.5-3)	1 (0.5-8.5)	2 (0.8-2.5)
Leukocytes ($\times 10^3/\mu L$)	3.1 (0.8-14.4)*	0.9 (0.2-1.7)	1.0 (0.1-2.5)	0.9 (0.2-5.2)	4.7 (2-5.4)†
Positive peritoneal fluid cells (%)					
TNF-α	53 (0-87)‡	5 (0-41)	0.5 (0-0.5)	13 (0-41)	66 (53-88)§
TGF-β_1	51 (0-85)‖	1.5 (0-3)	7 (3-32)	0 (0-37)	49 (0-72)¶
ICAM-1	92 (71-98)#	90 (62-97)	80 (66-95)	80 (18-90)	92 (66-99)
CD3	31 (6-90)	11 (1-61)	20 (11-53)	17 (1-38)	73 (8-89)**
TIA-1	4 (0-7)	3 (0-36)	3 (0-58)	6 (0-29)	25 (0-70)
HLA-DR	82 (35-95)	60 (17-91)	70 (20-90)	57 (35-73)	79 (67-99)††
Peritoneal fluid cytokines (pg/mL)					
TGF-β_1	2000 (1300-4000)‡‡	1300 (900-1700)	1400 (1200-1900)	1300 (900-1700)	1100 (800-1500)
IL-6	56 (23-302)§§	8 (2-18)	6 (1-17)	6 (1-18)	6 (2-12)
IL-10	2.3 (2.3-2.5)	2.7	12.8	2 (2-3)	7 (2-14)

* Kruskal-Wallis test, $P = .01$; Wilcoxon test for paired samples, $P = .004$, menses vs nonmenstrual.
† Wilcoxon test for paired samples, $P = .028$.
‡ Kruskal-Wallis test, $P = .05$; Wilcoxon test for paired samples, $P = .08$, menses vs nonmenstrual.
§ Wilcoxon test for paired samples, $P = .07$; Mann-Whitney test, $P = .02$.
‖ Kruskal-Wallis test, $P = .002$; Wilcoxon test for paired samples, $P = .07$, menses vs nonmenstrual.
¶ Wilcoxon test for paired samples, $P = .04$; Mann-Whitney test, $P = .03$.
\# Wilcoxon test for paired samples, $P = .04$, menses vs nonmenstrual.
** Wilcoxon test for paired samples, $P = .07$.
†† Wilcoxon test for paired samples, $P = .03$.
‡‡ Kruskal-Wallis test, $P = .04$; Mann-Whitney test, $P = .02$, menses vs nonmenstrual.
§§ Kruskal-Wallis test, $P = .01$; Mann-Whitney test, $P = .003$, menses vs nonmenstrual.

toneal fluid volume; $P = .7$, peritoneal fluid leukocytes). A significant positive correlation was found between peritoneal fluid volume and peritoneal fluid leukocyte concentration ($\rho = 0.6$; $P < .001$).

In the second part of the study (paired observations only), peritoneal fluid volume was comparable (Wilcoxon test, $P = .6$) at laparoscopies performed in the follicular phase (median, 1 mL; range, 0-10 mL) and in the luteal phase (median, 2 mL; range, 0-6.5 mL). No bias was introduced by including the 3 cases in which laparoscopy was performed 9, 13, and 19 days after perineal deflation, because the peritoneal fluid volume in these baboons was higher in the luteal phase than in the follicular phase. Power analysis was done on 18 paired observations with only the samples from the mid or late follicular phase and the samples from the early or mid luteal phase to prevent potential bias from early follicular or late luteal samples. The differences between the paired peritoneal fluid volume observations approached a normal distribution. Power analysis[15] revealed that the study design allowed the detection of a 60% increase (1-tailed $\alpha = .05$) to a 70% increase (2-tailed $\alpha = .05$) in peritoneal fluid volume during the luteal phase with a power of 80% ($\beta = 20\%$). Similarly, the peritoneal fluid leukocyte concentration was comparable (Wilcoxon test, $P = .9$) at laparoscopies performed in the follicular phase (median, $2.5 \times 10^3/\mu L$; range, $0.8\text{-}17.2 \times 10^3/\mu L$) and in the luteal phase (median, $3 \times 10^3/\mu L$; range, $0.9\text{-}11.5 \times 10^3/\mu L$). Additional analysis of peritoneal fluid volume and peritoneal fluid leukocyte concentration was performed separately in the group with a normal pelvis and in the group with endometriosis, but no significant differences were obtained between the follicular phase and the luteal phase. A significant positive correlation was found between the peritoneal fluid volume and the peritoneal fluid leukocyte concentration ($\rho = 0.6$; $P = .01$).

In the third part of the study (paired observations only), menstruation also had no effect on peritoneal fluid volume (Table III). However, the leukocyte concentration (Table III) was significantly higher in peritoneal fluid obtained during menstruation than in peritoneal fluid obtained during the follicular or luteal phase of the menstrual cycle (Kruskal-Wallis test, $P = .01$; Wilcoxon test for paired samples, $P = .004$; menses vs follicular or luteal stage). Similarly, in peritoneal fluid obtained during menses in comparison with peritoneal fluid obtained during the follicular or luteal phase of the menstrual cycle, a significant increase was observed in the subset of peritoneal fluid cells with positive staining for tumor necrosis factor α (Kruskal-Wallis test, $P = .05$; Wilcoxon test for paired samples, $P = .08$; menses vs follicular or luteal stage), for TGF-β_1 (Kruskal-Wallis test, $P = .002$; Wilcoxon test for paired samples, $P = .07$; menses vs follicular or luteal stage), and for intercellular adhesion molecule 1 (ICAM-1) (Wilcoxon test for paired samples, $P = .04$, menses vs follicular or luteal stage) (Table III). Most of these cells looked like large mononuclear cells, possibly macrophages. The subset of

peritoneal fluid cells with positive staining for CD3, TIA-1, and human leukocyte antigen, DR locus (HLA-DR), were not affected by the menstrual cycle (menses vs follicular or luteal phase; Table III). In peritoneal fluid obtained during the menstrual phase of the cycle, an increased concentration of the cytokines TGF-β (Kruskal-Wallis test, $P = .04$; Mann-Whitney test, $P = .02$) and IL-6 (Kruskal-Wallis test, $P = .01$; Mann-Whitney test, $P = .003$) was observed when compared with measured values obtained from peritoneal fluid aspirated during the follicular and luteal phases of the menstrual cycle (Table III). IL-10 was detectable in only 5 of 19 peritoneal fluid samples (3 of 8 peritoneal fluid samples obtained during menses, 2 of 11 peritoneal fluid samples obtained during the follicular or luteal phase). There were no differences in IL-10 levels observed throughout the cycle.

Long-term effect of intrapelvic injection of endometrium for experimental induction of endometriosis on peritoneal fluid inflammatory parameters (part 4). In the fourth part of the study (paired observations), peritoneal fluid data obtained at the induction laparoscopy during menses (n = 4) were excluded in the analysis to assess a direct effect of intrapelvic injection of endometrium on peritoneal fluid inflammatory parameters, because the results from the third part had shown that menstruation in itself had an effect on peritoneal fluid inflammatory parameters. Therefore peritoneal fluid data obtained during laparoscopy performed during the nonmenstrual phase of the cycle 3 months *after* intrapelvic injection of luteal (n = 3) or menstrual (n = 4) endometrium were compared with peritoneal fluid data obtained during laparoscopies performed before intrapelvic injection of endometrium during the luteal phase (n = 3) in animals subsequently undergoing induction of endometriosis with luteal endometrium and during either the follicular phase (n = 2) or luteal phase (n = 2) in primates subsequently undergoing induction of endometriosis with menstrual endometrium (n = 4).

In peritoneal fluid obtained after intrapelvic injection of endometrium, the leukocyte concentration was significantly higher (Wilcoxon test for paired samples, $P = .028$) than in peritoneal fluid obtained before injection (Table III). Similarly, in peritoneal fluid obtained after intrapelvic injection of endometrium, an increase was observed in the subset of peritoneal fluid cells with positive staining for TNF-α (Wilcoxon test for paired samples, $P = .07$; Mann-Whitney test, $P = .02$), for $TGF\text{-}\beta_1$ (Wilcoxon test for paired samples, $P = .04$; Mann-Whitney test, $P = .03$), for CD3 (Wilcoxon test for paired samples, $P = .07$), and for HLA-DR (Wilcoxon test for paired samples, $P = .03$), in comparison with peritoneal fluid obtained before the injections (Table III). However, no effects of intrapelvic injection of endometrium were noted either on the subset of peritoneal fluid cells with positive staining for TIA-1, ICAM-1, and HLA-DR or on the peritoneal fluid concentrations of cytokines (Table III).

Comment

It is hypothesized that peritoneal fluid in the female pelvis results mostly from exudation of the ovarian capillaries and therefore increases as the vascularity of the dominant ovarian structures increases.[16] Peritoneal fluid is supposed to originate only partially from transudation and exudation from the antrum of the preovulatory follicle or from release of the follicular fluid into the abdomen during ovulation.[16-18] In women the peritoneal fluid volume increases gradually during the preovulatory phase, reaches a maximum in the early luteal phase, and decreases in the late luteal phase of the cycle.[16-18] Most studies in women have reported that the peritoneal fluid volume in the luteal phase is at least twice as high than the peritoneal fluid volume in the follicular phase.[16-18] In contrast, no significant increase in peritoneal fluid volume was found during the luteal phase, in comparison with the follicular phase in this study in baboons, even when laparoscopies were performed during the midfollicular stage (at a median of 6 days before ovulation) and during the midluteal stage (at a median of 7 days after ovulation) in the second part of this study. However, because of the limited sample size, the results of the second part of this study do not exclude a potentially smaller (≤60%-70%) increase in peritoneal fluid volume during the luteal phase in baboons, in comparison with the increase (≥100%) in peritoneal fluid volume during the luteal phase in women. Other potential biases include the facts that cycle definition in baboons is less clear than in women and that the accuracy of the collection of any peritoneal fluid volume of ≤2 mL, especially after postural changes during anesthesia and preparation for surgery, is limited.

The results of this study indicate that the concentration of leukocytes in peritoneal fluid was not affected by the follicular or luteal phase of the cycle, which confirmed our earlier results.[12] In part 1 of our study and in our previous study,[12] the concentration of leukocytes in peritoneal fluid also was not increased during menses in comparison with the nonmenstrual phases of the cycle. However, the high interindividual variability in peritoneal fluid parameters, which has been well documented in both women and baboons, could have introduced considerable bias in these studies. The study design (paired observations) used in part 3 of the current study prevented this bias, even though the number of animals studied was lower than in part 1. In part 3 the concentration of peritoneal fluid leukocytes was increased 3-fold during menses over the concentrations observed at other time points during the menstrual cycle. This finding is similar to a report in which the peritoneal fluid concentration of leukocytes in women was observed to be increased in 4 women during menses.[5]

In our current study the activity of peritoneal fluid leukocytes was also increased during menses, as suggested by the increased subsets of peritoneal fluid cells with positive staining for TNF-α, TGF-β, and ICAM-1 and by the increased peritoneal fluid concentration of IL-6 and TGF-β. This is the first report on cytokine levels in peritoneal fluid of baboons during menses. The cytokines TNF-α, IL-6, TGF-β, and IL-10 were assessed in this study because they are involved in inflammation and have been implicated in endometriosis.

The results of the fourth part of this study suggest that experimental induction of endometriosis causes an increase in peritoneal inflammatory parameters, including the concentration of leukocytes and the proportion of peritoneal fluid cells with positive staining for HLA-DR, TNF-α, and TGF-β. In contrast, in an earlier study the leukocyte concentration in peritoneal fluid of baboons with induced endometriosis was found to be comparable with the leukocyte concentration of peritoneal fluid in baboons with a normal pelvis.[12] This difference may be explained by the fact that the previous study design[12] was cross-sectional and that assessment of peritoneal fluid had been performed at least 7 months after the induction; this time interval may have been too long to discern any significant effects of intrapelvic injection on inflammatory parameters of peritoneal fluid. In the current study each baboon served as its own control (paired laparoscopy before and after induction), and the second laparoscopy was performed only 3 months after the first one. These data, taken together with our previous report,[12] suggest that a significant effect of intrapelvic (intraperitoneal or retroperitoneal) endometrial injection on inflammatory parameters of peritoneal fluid can be measured only during the first months after induction. It is possible that intrapelvic injection of endometrium results in the simultaneous events of both subclinical pelvic inflammation and the establishment of endometriotic implants. Alternatively, intrapelvic injection of endometrium may result, first, in subclinical pelvic inflammation and, second, in the establishment of endometriotic lesions or vice versa.

In part 4 of this study the number of baboons studied was too low to compare the effect of luteal versus menstrual endometrium and the effect of intraperitoneal versus retroperitoneal injection on inflammatory parameters of peritoneal fluid. However, it is possible that the inflammatory parameters of peritoneal fluid after experimental induction of endometriosis can be influenced independently by the type of endometrium (menstrual phase or luteal phase) or by the injection technique used (intraperitoneal or retroperitoneal injection). This hypothesis is supported by a previous study in baboons,[4] which reported that endometriosis was induced more efficiently by injection with menstrual endometrium than with luteal endometrium, as shown by the higher number and larger surface area of endometriotic lesions and by the more advanced stages of endometriosis. There were also significantly more subtle red and more typical black, puckered lesions, occupying a significantly larger surface area after injection with menstrual endometrium than after luteal endometrium.[4] Furthermore, intraperitoneal injection of menstrual endometrium was more effective than retroperitoneal injection, as evidenced by significantly more lesions and a larger surface area of endometriosis.[4] Considerable intraperitoneal leakage was noted at most sites of induction during and immediately after the retroperitoneal injection. The majority of endometriotic lesions and most of the endometriosis surface were actually observed outside the site of retroperitoneal injection.[4] It can be questioned whether the procedure of laparoscopy itself may have contributed to the peritoneal inflammation described in part 4 of this study. Indeed, we recently reported that diagnostic laparoscopy can cause peritoneal inflammation in baboons at the second of 2 paired laparoscopies performed within 3 or 4 days after the first laparoscopy.[19] However, no signs of peritoneal inflammation were observed when the second of 2 paired laparoscopies was performed 1 month after the first laparoscopy.[19] On the basis of these observations, it seems unlikely that the first laparoscopy procedure in the fourth part of this study contributed significantly to the peritoneal inflammation observed at the second laparoscopy, because the second laparoscopy was performed much later, at a median of 3 months (range, 1.5-3.5 months) after the initial experimental induction of endometriosis.

In conclusion, this study in baboons suggests that both menstruation and intrapelvic injection of endometrium are associated with signs of subclinical pelvic inflammation.

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