

Suppression by developing ovarian follicles of the low-dose endotoxin-induced glomerular inflammatory reaction in the pregnant rat

Gerard A. Schuiling, PhD, Neeltje Valkhof, AS, and Maria M. Faas, PhD

Groningen, The Netherlands

OBJECTIVE: In the current study the role of developing ovarian follicles in the control of the endotoxin-induced pregnancy-specific inflammatory reaction was evaluated.

STUDY DESIGN: Follicular development was induced in pregnant rats ($n = 20$) by means of daily intraperitoneal injections of follicle-stimulating hormone from day 11 of pregnancy until the end of the experiment. Control pregnant rats ($n = 20$) received daily sodium chloride injections. All pregnant rats were infused for 1 hour with either 2 mL endotoxin solution ($1.0 \mu\text{g}/\text{kg}$ body weight) or 2 mL sodium chloride solution on day 14 and killed 4 hours or 3 days later. At death, the left kidneys were snap-frozen and immunohistologically stained for the presence of polymorphonuclear leukocytes and monocytes.

RESULTS: The results show that in control pregnant rats endotoxin significantly increased glomerular polymorphonuclear leukocyte and monocyte numbers at both 4 hours and 3 days after endotoxin infusion. Induction of follicular development did not affect glomerular polymorphonuclear leukocyte number after endotoxin infusion but significantly decreased the number of monocytes in the glomeruli at both 4 hours and 3 days after endotoxin infusion.

CONCLUSION: We conclude that follicles stimulated with follicle-stimulating hormone produce a follicular factor or factors that are able to prevent the endotoxin-induced influx of monocytes into the glomeruli of pregnant rats. It is suggested that these factors play a role in the control of inflammatory processes associated with reproduction, including the disease of pregnancy, preeclampsia. (*Am J Obstet Gynecol* 2000;183:89-93.)

Key words: Pregnancy, endotoxin, ovarian follicles, polymorphonuclear leukocytes, monocytes

Administration to female rats of a single dose ($1.0 \mu\text{g}/\text{kg}$ body weight) of the proinflammatory agent endotoxin causes an influx of inflammatory cells (polymorphonuclear leukocytes and monocytes) into the glomeruli of the kidneys and expression of various adhesion molecules both on the inflammatory cells and on the endothelium.^{1, 2} This inflammatory response, however, is not constant; both intensity and duration vary with the reproductive condition of the animals. Thus in pregnant rats the response is much stronger and lasts much longer than in rats in the follicular phase of the ovulatory cycle.¹ Similarly, rats in the luteal phase of the ovulatory cycle and even long-term ovariectomized rats, irrespective of whether they are treated with estradiol or

progesterone, exhibit a stronger inflammatory response than follicular phase rats,³ although the characteristics of the inflammatory responses of pregnant, luteal phase, and ovariectomized rats are not entirely identical.^{2, 3}

The observation that ovariectomized rats exhibit an inflammatory response to endotoxin more resembling that of pregnant and luteal phase rats than that of follicular phase rats may point to a role of the ovaries in the control of the response. It may be that developing follicles present in the ovaries of follicular phase rats but not in those of pregnant, luteal phase, and ovariectomized rats decrease the sensitivity of the animal for proinflammatory stimuli by producing some factor or factors that inhibit the glomerular inflammatory response induced by endotoxin. This suggestion was tested in this study. Pregnant rats were treated with a highly purified preparation of follicle-stimulating hormone (FSH) to induce follicular development. Parameters of the endotoxin-induced glomerular inflammatory response of these animals were studied and compared with those of animals in which no follicular development was induced. The results show a marked suppression of the inflammatory response, notably of the glomerular influx of monocytes in rats in which follicular development was induced by FSH.

From the Division of Reproductive Biology, Department of Obstetrics and Gynecology, University of Groningen.

Received for publication April 15, 1999; revised November 11, 1999; accepted December 16, 1999.

Reprint requests: Gerard A. Schuiling, PhD, Division of Reproductive Biology, Department of Obstetrics and Gynecology, University of Groningen, PO Box 30.001, 9700 RB Groningen, The Netherlands.

Copyright © 2000 by Mosby, Inc.

0002-9378/2000 \$12.00 + 0 6/1/105196

doi:10.1067/mob.2000.105196

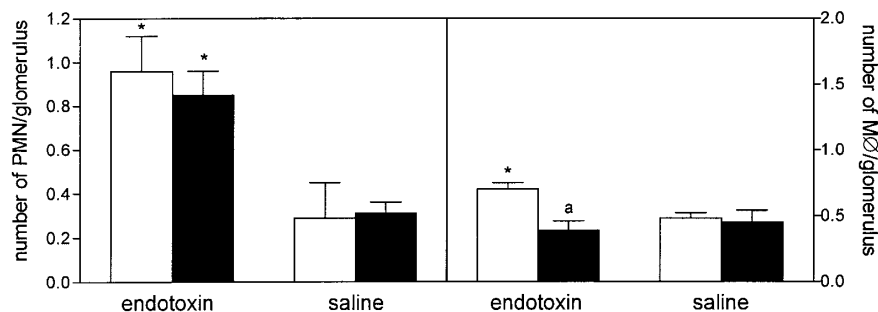


Fig 1. Mean number of polymorphonuclear leukocytes (PMN) (left panel) per glomerulus and mean number of monocytes (MØ) (right panel) per glomerulus 4 hours after infusion of endotoxin (left columns in each panel) or sodium chloride (saline) solution (right columns in each panel) in control pregnant rats (open columns in each panel) and in pregnant rats in which follicular development was induced (solid columns in each panel). Asterisk, Significant increase compared with sodium chloride-infused rats after identical treatment (*t* test; $P < .05$). Letter a, Significant decrease compared with endotoxin-infused control rats (*t* test; $P < .05$).

Material and methods

Experimental animals. Female Wistar rats (Harlan) were kept in a temperature- and light-controlled room (lights on from 6 AM to 6 PM) with free access to food and water. To follow estrus cyclicity, vaginal smears were taken daily until selection for experiments at the age of 3 to 4 months (about 200 g). At proestrus, the rats were housed with a male rat for 1 night. The following day was designated as day 0 of pregnancy when spermatozoa were detected in the smear. On this day rats were equipped with a permanent jugular vein cannula according to the method of Steffens.⁴ Follicular development during pregnancy was induced by daily intraperitoneal injections with 10 IU of FSH (Metrodin; Organon, Oss, The Netherlands) per rat in 0.2 mL sodium chloride solution. Control pregnant rats were injected with sodium chloride solution alone (control rats). Follicular development was assessed by (1) microscopic inspection of the ovaries and (2) the inhibin A levels present in the plasma at the moment of death.⁵ Corpus luteum function was judged on the basis of the plasma progesterone levels at the moment of death. Rats (conscious) were infused for 1 hour with either endotoxin (*Escherichia coli* 0.55:B5; Whittaker MA Bioproducts Inc, Walkersville, Md; 1.0 µg/kg body weight in 2 mL sodium chloride solution) or sodium chloride solution alone (2 mL) through the jugular vein cannula. This protocol (ie, FSH treatment from 3 days before endotoxin infusion) was chosen to allow for follicle development to mimic the situation in follicular phase rats.

Measurement of progesterone and inhibin. Progesterone was measured in duplicate by a radioimmunoassay, as described by de Jong et al^{5a}; the sensitivity of the assay was 0.2 nmol/L, and the interassay and intra-assay variability were each <10%. Inhibin A levels were assayed by Dr F.H. de Jong of Erasmus University, Rotterdam, The Netherlands, with immunoenzymetric assays purchased

from Serotec (Oxford, United Kingdom).⁶ Within-assay variation was 12%.

Demonstration of glomerular inflammation

Immunohistology. Cryostat sections measuring 4 µm were stained according to standard procedures for the presence of polymorphonuclear leukocytes and monocytes by use of monoclonal antibodies against rat polymorphonuclear leukocytes (His48; Pharmingen, San Diego, Calif) and rat monocytes (ED-I; Serotec).² In brief, sections were fixed in acetone and incubated with the first antibody (see description in subsequent text) for 30 minutes. A peroxidase-conjugated second antibody (rabbit antimouse; Dako A/S, Glostrup, Denmark) was used and visualized with hydrogen peroxide and 3-amino-9-ethyl-carbazole (Sigma Chemical Co, St Louis, Mo). Control sections, with either primary or secondary antibody omitted from the staining procedure, were consistently negative.

Evaluation of kidney sections. Kidney sections of each individual animal were scored by light microscopic examination in a double-blind fashion and by 2 independent observers, as described before.² Sections were quantitatively scored by counting the total number of positive cells in 100 glomeruli in 1 section.

Experimental protocol

Experiment 1: Acute glomerular inflammation. FSH treatment was initiated on day 11 of pregnancy (9 AM), and the last injection was given on day 14.

Both control rats ($n = 10$) and rats in which follicular development was induced ($n = 10$) were infused with either endotoxin or sodium chloride solution on the morning of day 14 of pregnancy (10 AM) and were killed 4 hours after the start of the infusion.

Experiment 2: Persistent glomerular inflammation. In this experiment FSH treatment was initiated on day 11 of pregnancy (9 AM), but now the treatment lasted up to and including day 17.

Both control rats ($n = 10$) and rats in which follicular



Fig 2. Mean number of polymorphonuclear leukocytes (PMN) (left panel) per glomerulus and mean number of monocytes (MØ) (right panel) per glomerulus 3 days after infusion of endotoxin (left columns in each panel) or sodium chloride (saline) solution (right columns in each panel) in control pregnant rats (open columns in each panel) and in pregnant rats in which follicular development was induced (solid columns in each panel). Asterisk, Significant increase compared with sodium chloride-infused rats after identical treatment (*t* test; $P < .05$). Letter a, Significant decrease compared with endotoxin-infused control rats (*t* test; $P < .05$).

development was induced ($n = 10$) were infused with either endotoxin or sodium chloride solution on the morning of day 14 of pregnancy (10 AM) and were killed 3 days later (day 17, 10 AM).

At death, specimens of the left kidney, as well as the ovaries, were snap-frozen and prepared for immunohistologic examination and for staining with hematoxylin-eosin stain to check for follicular development, respectively. Blood was collected for assay of progesterone and inhibin A.

Statistics. Results are expressed as mean \pm SEM. To evaluate the effects of endotoxin infusion and follicular development, multiple analysis of variance was used, followed by unpaired *t* tests to evaluate differences between individual groups. Statistical significance was reached at $P < .05$.

Results

Plasma inhibin A and progesterone concentrations

Experiment 1. At the moment of death, 4 hours after the start of the infusion, the plasma inhibin A levels of rats in which follicular development was induced amounted to 248.8 ± 73.9 ng/L and were significantly increased compared with plasma levels of inhibin A in control rats (28.0 ± 5.3 ng/L; multiple analysis of variance followed by *t* test; $P < .05$). No significant differences were observed in plasma progesterone levels between rats in which follicular development was induced and control rats (193.0 ± 26.8 nmol/L and 289.3 ± 48.0 nmol/L, respectively; multiple analysis of variance).

Experiment 2. At the moment of death, 3 days after the infusion, the plasma inhibin A levels of rats in which follicular development was induced and of control rats amounted to 67.8 ± 29.4 and 19.3 ± 3.8 , respectively, with plasma inhibin A levels being significantly increased in rats in which follicular development was induced compared with control rats (multiple analysis of variance followed by *t* test, $P < .05$). Also in this experiment no dif-

ferences in plasma progesterone levels were observed between rats in which follicular development was induced compared with control rats (242.0 ± 15.4 nmol/L and 216.5 ± 33.0 nmol/L, respectively; multiple analysis of variance).

Glomerular inflammation. Control rats exhibited increased numbers of polymorphonuclear leukocytes and monocytes in the glomeruli both 4 hours and 3 days after endotoxin infusion compared with sodium chloride infusion (multiple analysis of variance followed by *t* test, $P < .05$). Figs 1 and 2 (left panels) show that no effect of follicular development was observed on glomerular polymorphonuclear leukocyte numbers: The glomeruli of rats in which follicular development was induced exhibited the same number of polymorphonuclear leukocytes as control rats at both intervals studied (multiple analysis of variance). On the other hand, a significant effect of follicular development could be observed on glomerular monocyte number: glomerular monocyte infiltration in rats in which follicular development was induced was significantly lower at both 4 hours and 3 days after endotoxin infusion compared with control rats (Figs 1 and 2, right panels; multiple analysis of variance followed by *t* test, $P < .05$).

Comment

Both the morphologic characteristics of the ovaries (data not shown) and the inhibin A levels of FSH-treated rats demonstrate that the present FSH treatment effectively induced follicular development in pregnant rats without interfering with the corpus luteum function, as shown by the unchanged plasma progesterone levels. FSH treatment did not interfere with the course of pregnancy because no differences in the total fetal number or number of fetal resorptions were observed between rats treated and not treated with FSH (results not shown). FSH, however, effectively prevented the endotoxin-

induced influx of monocytes into the glomeruli at both 4 hours and 3 days after endotoxin treatment: In pregnant rats with developing follicles, monocyte infiltration at both intervals was comparable with that found in follicular phase rats infused with the same low dose of endotoxin.² The influx of polymorphonuclear leukocytes into the glomeruli, on the other hand, was not affected by the FSH-induced follicular development, probably because of the elevated plasma progesterone levels of the animals.³

The inhibitory effect of FSH on the endotoxin-induced inflammatory response is probably an indirect one. In ovariectomized rats FSH levels are also elevated because of the absence of negative feedback by ovarian hormones, yet in these animals the endotoxin-induced glomerular monocyte infiltration is not suppressed, as it is in FSH-treated pregnant rats.³ We therefore conclude that in pregnant rats FSH treatment probably induced the ovaries to produce some factor or factors inhibiting the inflammatory response, showing that the pregnant rat is a much more complicated experimental model than rats in any other reproductive condition, including the ovariectomized rat. Indeed, of all reproductive conditions studied, many show a pregnancy-like inflammatory reaction,^{1, 3} while only pregnant rats develop a preeclampsia-like disease.^{7, 8} This can be reduced to the presence in pregnant rats of a factor or factors produced by the conceptus and acting on the vascular wall, making these rats more vulnerable to endothelial damage as a result of the inflammatory reaction.^{8, 9} Not only rats¹ but also women¹⁰ may be less vulnerable to proinflammatory stimuli during the follicular phase of the ovulatory cycle than during the luteal phase or other life cycles without ovarian follicles. In women, Cannon et al,¹⁰ studying hormonal influences on stress-induced neutrophil mobilization in health and chronic fatigue syndrome, found a greater mobilization during the luteal phase than during the follicular phase; this greater mobilization correlated with the plasma progesterone levels.^{1, 3}

The current observations concerning the role of the ovaries in control of the endotoxin-induced inflammatory response, together with those cited in the previous paragraphs, raise questions at various levels. At the mechanistic level, for example, one may ask how the endotoxin-induced influx of inflammatory cells into the glomeruli, in particular, that of monocytes, is brought about; how this process is controlled in various reproductive conditions; and which factors, in particular, which ovarian factors, exert this control. This study, however, was not designed to and indeed does not answer this type of question, although suggestions can be made. At another level one may also ask why the inflammatory response to endotoxin varies so strongly with the reproductive condition of the female individual and why, in particular, the ovary is involved in the control of the inflammatory status, rendering individuals in the follicular

phase of the ovulatory cycle relatively insensitive to a proinflammatory stimulus such as endotoxin but causing (pseudo)pregnant individuals to be in a more or less proinflammatory condition.^{7, 11-13} We believe that, although we do not know the answers to these questions, it can be safely assumed that the fact that pregnancy is a proinflammatory condition and the follicular phase of the ovulatory cycle is not in the interest of reproduction.

This consideration may suggest an answer to the question of why the ovaries are involved in the control of the inflammatory reaction. Many key processes in mammalian reproduction appear to be associated with inflammatory phenomena (eg, ovulation¹⁴ but also infiltration by leukocytes of the vaginal epithelium,¹⁵ invasion of the uterine endometrium by immunocompetent cells,¹⁶ implantation of the blastocyst,^{17, 18} and parturition^{19, 20}). These processes occur in an accurately timed sequence, one after another, beginning with follicular development (ie, with follicular hormone production and oocyte maturation) and ending with parturition, with sometimes in between preeclampsia (also an inflammatory phenomenon^{12, 13}), for which the present low-dose endotoxin-treated pregnant rat is a model.⁷ It is extremely important indeed that these processes be very accurately controlled because, in fact, they determine the genetic fate of the individual. It may be suggested here that follicular hormones (some of which may in some species also be produced by the placenta) are part of the instruments by which the maternal genes, present in the oocytes, guard their interests.

Because the follicle should not ovulate during oocyte maturation (ie, should occur only when the oocyte is ready to be fertilized), the production of some anti-inflammatory factor or factors up until ovulation makes perfect sense. Also, the increased sensitivity for proinflammatory stimuli during the luteal phase of the ovulatory cycle and during pregnancy (because of inhibition of follicular development) may be of great biologic importance and may be particularly so in human subjects because in this species the association between implantation and the uterine inflammatory response may be an evolutionary necessity to eliminate unhealthy zygotes,¹² which human subjects abundantly produce.^{21, 22} The other side of the coin, however, is that this inflammatory response, if not adequately controlled, may give rise to preeclampsia.¹² Suppression of the sensitivity of pregnant individuals for proinflammatory stimuli (eg, by means of the present putative follicular factor or factors) may therefore be useful in the treatment of this disease of pregnancy.

This inevitably raises the question of what the nature of the inflammation-modulating factor or factors may be. Among the factors produced by FSH-stimulated ovarian follicles are the inhibins and activins,²⁰ members of the transforming growth factor β family of hormones—growth

factors—cytokines^{23, 24} and well known for their profound effects on immunologic processes.²⁵⁻²⁷ It may be suggested that the factor or factors desensitizing the individual for proinflammatory stimuli such as endotoxin are to be found in this group. In this respect it may be of interest to note that during preeclamptic pregnancies the placenta produces far larger quantities of inhibin and activin than during healthy pregnancies.²⁸ This may possibly reflect an attempt of the fetus to control the maternal inflammatory response, which threatens its life.¹²

We thank Dr F.H. de Jong, Erasmus University, Rotterdam, The Netherlands, for determination of inhibin A.

REFERENCES

1. Faas MM, Bakker WW, Valkhof N, van der Horst MCL, Schuilting GA. Reproductive condition and the low-dose endotoxin-induced inflammatory response in rats. Glomerular influx of inflammatory cells and expression of adhesion molecules. *Biol Reprod* 1997;56:1400-6.
2. Faas MM, Schuilting GA, Bailer JFW, Bakker WW. Glomerular inflammation in pregnant rats after infusion of low dose endotoxin: an immunohistological study in experimental preeclampsia. *Am J Pathol* 1995;147:1510-8.
3. Faas MM, Bakker WW, Valkhof N, Schuilting GA. Effect of estradiol and progesterone on the low-dose endotoxin-induced glomerular inflammatory response on the female rat. *Am J Reprod Immunol* 1999;41:224-31.
4. Steffens AB. A method for frequent sampling of blood and continuous infusion of fluids in the rat without disturbing the animal. *Physiol Behav* 1969;4:833-6.
5. Taya K, Komura H, Watanabe G, Sasamoto S. Peripheral blood levels of immunoreactive inhibin during pseudopregnancy, pregnancy and lactation in the rat. *J Endocrinol* 1989;121:545-52.
- 5a. de Jong FH, Baird DT, van der Molen HJ. Ovarian secretion rates of oestrogens, androgens and progesterone in normal women and in women with persistent ovarian follicles. *Acta Endocrinol (Copenh)* 1974;77:575-87.
6. Schipper I, de Jong FH, Fauser BCJM. Lack of correlation between maximum early follicular phase serum follicle-stimulating hormone concentrations and menstrual cycle characteristics in women under the age of 35 years. *Hum Reprod* 1998;13:1442-8.
7. Faas MM, Schuilting GA, Bailer JFW, Visscher CA, Bakker WW. A new animal model for human pre-eclampsia: ultralow-dose endotoxin infusion in pregnant rats. *Am J Obstet Gynecol* 1994;171:158-64.
8. Faas MM, Schuilting GA, Bailer JFW, Valkhof N, Bakker WW. The increased endotoxin sensitivity of pregnant rats, as reflected by glomerular ecto-ADP-ase activity, is not dependent on the presence of decidual cells. *Placenta* 1996;17:185-8.
9. Faas MM, Bakker WW, Bailer JFW, Schuilting GA. Pregnancy enhances the sensitivity of glomerular ecto-adenosine triphosphate-diphosphohydrolase to products of activated polymorphonuclear leukocytes. *Am J Obstet Gynecol* 1999;180:112-3.
10. Cannon JG, Angel JB, Abad LW, O'Grady J, Lundgren N, Fagioli L, et al. Hormonal influences on stress-induced neutrophil mobilization in health and chronic fatigue syndrome. *J Clin Immunol* 1998;18:291-8.
11. Apitz K. A study on the generalized Shwartzman phenomenon. *J Immunol* 1935;29:255-66.
12. Schuilting GA, Koiter TR, Faas MM. Why pre-eclampsia? *Hum Reprod* 1997;12:2087-92.
13. Sacks GP, Studena K, Sargent IL, Redman CWG. Normal pregnancy and preeclampsia both produce inflammatory changes in peripheral blood leukocytes akin to those of sepsis. *Am J Obstet Gynecol* 1998;179:80-6.
14. Espey LI. Current status of the hypothesis that mammalian ovulation is comparable to an inflammatory reaction. *Biol Reprod* 1994;50:233-8.
15. Koiter TR, Van de Schoot P. Leukocyte invasion of the vaginal epithelium in the absence of bacteria in mice. *Experientia* 1977;33:1149-51.
16. Lea RG, Clark DA. Macrophages and migratory cells in the endometrium relevant to implantation. *Baillieres Clin Obstet Gynaecol* 1991;5:25-59.
17. McMaster MT, Dey SK, Andrews GK. Association of monocytes and neutrophils with early events of blastocysts implantation in mice. *J Reprod Fertil* 1993;99:561-9.
18. Seamark RF, Hadjisavas M, Robertson SA. Influence of the immune system on reproductive function. *Anim Reprod Sci* 1992;28:171-8.
19. Kelly RW. Pregnancy maintenance and parturition: the role of prostaglandins in manipulating the immune and inflammatory response. *Endocr Rev* 1994;15:684-706.
20. Kelly RW. Inflammatory mediators and parturition. *Rev Reprod* 1996;1:89-96.
21. Edwards RG. Causes of early embryonic loss in human pregnancy. *Hum Reprod* 1986;1:185-98.
22. Hertig AT, Rock J, Adams EC, Menkin MC. Thirty-four fertilized human ova, good, bad and indifferent, recovered from 210 women of unknown fertility. *Paediatrics* 1952;23:202-11.
23. Massague J. The transforming growth factor beta family. *Ann Rev Cell Dev Biol* 1990;6:597-641.
24. Kingsley DM. The TGF- β superfamily: new members, new receptors and new genetic tests of function in different organisms. *Genes Dev* 1994;8:133-46.
25. Petraglia F, Sacerdote P, Cossadizza A, Angioni S, Genazzani AD, Franceschi C, et al. Inhibin and activin modulate human monocyte chemotaxis and human lymphocyte interferon-gamma production. *J Clin Endocrinol Metab* 1991;72:496-502.
26. Ohguchi M, Yamato K, Ishihara Y, Koide M, Okahashi N, Noguchi T, et al. Activin A regulates the production of mature interleukin-1 beta and interleukin-1 receptor antagonist in human monocytic cells. *J Interferon Cytokine Res* 1998;18:491-8.
27. Yu EW, Dolter KE, Shao LE, Yu J. Suppression of IL-6 biological activities by activin A and implications for inflammatory arthropathies. *Clin Exp Immunol* 1998;112:126-32.
28. Muttukrishna S, Knight PG, Groome NP, Redman CWG, Ledger WL. Activin A and inhibin A as possible endocrine markers for pre-eclampsia. *Lancet* 1997;349:1285-8.