Effect of Estradiol and Progesterone on the Low-Dose Endotoxin-Induced Glomerular Inflammatory Response of the Female Rat

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PROBLEM: Is the endotoxin-induced glomerular inflammatory response of the female rat under ovarian control?
METHOD OF STUDY: Ovariectomized rats (OVX), with or without progesterone (OVX-P) or estradiol (OVX-E) treatment, as well as rats in the follicular or luteal phase of the ovulatory cycle were infused with endotoxin or saline and sacrificed 3 days later. Cryostat kidney sections were immunohistologically stained for the presence of neutrophils and monocytes (MØ) and the expression of adhesion molecules. RESULTS: After endotoxin, the glomerular number of neutrophils and the number of MAC-1 positive cells were increased in luteal-phase and in OVX-P rats, and the number of glomerular MØ was increased in luteal-phase, OVX, OVX-E, and OVX-P rats. Endotoxin increased ICAM-1 expression in all groups of rats, except in follicular-phase rats. The glomerular number of LFA-1– and VLA-4–positive cells following endotoxin were only increased in OVX rats.
CONCLUSIONS: It is concluded that endotoxin-induced monocyte infiltration and ICAM-1 expression are inhibited by a factor produced during the follicular phase, probably by developing follicles. Infiltration of neutrophils and expression of MAC-1, LFA-1, VLA-4 seem to be under control of progesterone or estradiol.

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INTRODUCTION

The glomerular inflammatory response of the female rat to low-dose endotoxin infusion varies with the phase of the ovulatory cycle. Thus, during the luteal phase, inflammation is observed, which lasts for several days; after 3 days this response is characterized by infiltration of polymorphonuclear (PMN) cells and monocytes (MØ) into the glomeruli, as well as by expression of ICAM-1 on the glomerular endothelium and MAC-1 on the infiltrated

leukocytes.[1] During the follicular phase, however, identical low-dose endotoxin infusion induces a weaker and shorter lasting (1-day) influx of inflammatory cells and expression of adhesion molecules in the glomeruli of the kidneys.[2]

These data may suggest that the endotoxin-induced inflammatory response of the female rat is under the control of hormones associated with reproduction. Thus, the absence of a significant inflammatory response after endotoxin infusion during the follicular phase of the ovulatory cycle, may be due to the relatively high levels (peaking every 4 days of proestrus) of the steroid hormone estradiol (E).[3] Alternatively, the presence of a more intense and persistent inflammatory response after endotoxin infusion during the luteal phase may result from high levels of the steroid hormone progesterone (P), by which this phase is characterized.[3] This hypothesis was tested in the present study. Long-term ovariectomized (OVX) rats, that were or were not treated with either P or E, as well as cyclic rats, either in the follicular or luteal phase of the ovulatory cycle, were infused with low-dose endotoxin, and the inflammatory response of the glomeruli of the kidneys was studied using immunohistology.

MATERIALS AND METHODS

Experimental Animals

Female Wistar rats, bred at the Groningen Central Animal Laboratory, were kept in a temperature- and light-controlled room (lights on from 06.00 to 18.00 hr), with free access to food and water. To follow estrous cyclicity, vaginal smears were taken daily until selection for experiments at the age of 3–4 months (about 200 g). Only rats exhibiting regular 4-day ovulatory cycles were used. As rats do not exhibit a spontaneous luteal phase,[3] a luteal phase (or pseudopregnancy) was induced by electrical stimulation of the cervix uteri at 17:00 hr on proestrus and 15:00 hr on estrus. This latter day was day 0 of the experiment in this group of rats. Rats were ovariectomized according to standard procedures.

P and E were administered to OVX rats by Silastic implants (Dow Corning). The dimensions of the implants containing P (Sigma, St Louis, MO) were length, 4 cm; inner diameter, 3.35 mm; outer diameter, 4.64 mm; and the dimensions of the implants containing E (Sigma) were length, 1 cm; inner diameter, 1.57 mm; outer diameter, 3.18 mm. Control rats received two sham (i.e., empty) implants.

Measurement of Progesterone and Estradiol

P and E were measured using a radioimmunoassay in duplicate. P was measured as described by de Jong et al.[4]; the sensitivity of the assay was 0.2 nmol/L; the inter- and intra-assay variabilities were less than 10%. E was measured as described by Jurjens et al.[5] The sensitivity of the assay was 0.02 nmol/L; the inter- and intra-assay variabilities were less than 8%.

Demonstration of Glomerular Inflammation

Immunohistology. Four-micrometer cryostat sections were stained according to standard procedures for the presence of neutrophils (PMN), MØ, and the adhesion molecules, ICAM-1, VCAM-1 expressed on the endothelium, and LFA-1, MAC-1, and VLA-4 expressed on the leukocytes.[2] In brief, sections were fixed in acetone and after preincubation with either normal rabbit or normal goat serum (5%), they were incubated with the first antibody (see below) for 30 min. A peroxidase-conjugated second antibody (rabbit antimouse; Dako A/S, Glostrup, Denmark) was used and visualized using hydrogen peroxide and 3-amino-9-ethyl-carbazole (Sigma). Control sections, omitting either primary or secondary antibody from the staining procedure, were consistently negative.

Antibodies. All antibodies used in this study have been described before[1]: 1A29 (Genzyme, Cambridge, MA), a mouse immunoglobulin (Ig)G1 monoclonal antibody against rat-ICAM-1; 5F10 (a generous gift from Dr Lobb, Biogen, Cambridge, MA), a mouse IgG2a monoclonal antibody against rat-VCAM-1; WT.1 (Genzyme), a mouse IgG2a monoclonal antibody against rat-LFA-1; WT.5 (Pharmingen, San Diego, CA), a mouse IgA monoclonal antibody against rat-MAC-1α; MRa4 (Pharmingen), a mouse monoclonal IgG2b antibody against rat-VLA-4; ED-1 (Pharmingen), a mouse IgG1 monoclonal antibody against a cytoplasmic antigen of rat macrophages/MØ[6]; and HIS48 (Pharmingen) a mouse IgM monoclonal antibody against a membrane-bound antigen of rat PMN.

Evaluation of Kidney Sections. Kidney sections of each individual animal were scored by light microscopical examination, double blindly, by two independent observers. Sections were scored as described before[1]:

1. sections stained with ED-1, HIS48, α-LFA-1, α-MAC-1, and α-VLA-4 were quantitatively scored by counting the total number of positive cells in 100 glomeruli in 1 section;
2. sections stained with α-ICAM-1 and α-VCAM-1 were semiquantitatively graded by screening a total of 100 glomeruli per section using an arbitrary scale from 1 to 4 (1, no staining; 2, weak staining; 3, moderate staining; and 4, bright staining).

Experimental Protocol

The present experiments included five groups of rats ($n = 10$ in each group):

1. OVX rats (OVX rats);
2. P-treated OVX rats (OVX-P rats);
3. E-treated OVX rats (OVX-E rats);
4. rats in the follicular phase of the ovarian cycle (follicular-phase rats);
5. rats in the luteal phase of the ovarian cycle (luteal-phase rats).

On day 0 (for OVX rats, 3 weeks postovariectomy), rats received a permanent jugular vein cannula under ether anesthesia, according to the method of Steffens.[7] All rats (consciously) were infused for 1 hr with either endotoxin (*Escherichia coli* 0.55:B5, Whittaker MA Bioproducts, Walkersville, MD; 1.0 µg/kg bw in 2 mL saline) or saline alone (2 mL) via the jugular vein cannula on day 5, and sacrificed 3 days later.

In this experimental protocol, follicular-phase rats were cannulated on di-estrus-1, infused on di-estrus-2 in the following cycle, and sacrificed on di-estrus-1 in the subsequent cycle. At the time of cannulation, OVX-P rats received two Silastic implants containing P; OVX-E rats received two E implants; and OVX rats received two empty implants.

At sacrifice, left kidneys were cut transversely at the level of the hilus and 3-mm slices of tissue were snap-frozen in methyl-butane at $-80°C$, and stored at this temperature until further analysis. Blood samples were taken from the individual rats for assay of E and P, except for follicular-phase rats. In these rats, a blood sample was taken just before infusion, because E and P concentrations can vary from day to day.

Statistics

Results are expressed as mean ± standard error of the mean (SEM). To evaluate differences between the five groups after saline or endotoxin infusion, analysis of variance (Kruskall Wallis) was carried out followed by the Dunn's multiple comparisons test (Dunn's). Differences between saline and endotoxin infusion were evaluated using the Mann-Whitney U-test. Differences were considered significant if P was less than 0.05.

RESULTS

Plasma Concentrations of Estradiol and Progesterone

Circulating E was not detected in OVX and OVX-P rats, while E was low but detectable in follicular- and luteal-phase rats (mean 0.05 ± 0.02 and 0.05 ± 0.02 nM, respectively). Plasma E was significantly increased in OVX-E rats as compared with the other

groups (1.16 ± 0.19 nM; Kruskall Wallis followed by Dunn's, $P < 0.05$).

Plasma P was not detected in OVX or OVX-E rats. Plasma P was detectable in follicular-phase rats (41.04 ± 2.39 nM). However, in luteal-phase and OVX-P rats, P was significantly increased as compared with follicular-phase rats (114.95 ± 5.58 and 80.8 ± 5.96 nM, respectively; Kruskall Wallis followed by Dunn's, $P < 0.05$).

Infiltration of Inflammatory Cells into the Glomeruli

Neutrophils. Three days after infusion of endotoxin, the number of PMN cells in the glomeruli of OVX rats did not differ from the glomerular number of PMN cells in saline-infused OVX rats (Fig. 1A). No effect of E treatment of OVX rats was observed on the endotoxin-induced glomerular PMN cell number, while P treatment of OVX rats resulted in significantly increased numbers of PMN cells in the glomeruli of endotoxin-treated OVX rats as compared with saline-treated OVX-P rats (Mann-Whitney). In follicular-phase rats, endotoxin infusion did not affect the number of glomerular PMN cells; in luteal-phase rats endotoxin treatment significantly increased the number of glomerular PMN cells as compared with saline treatment (Mann-Whitney, $P < 0.05$). No significant differences in the number of PMN cells per glomerulus were detected between the saline-infused OVX, OVX-E, and OVX-P, follicular- and luteal-phase rats (Kruskall Wallis).

Monocytes. In OVX rats the number of MØ was significantly increased in endotoxin-infused rats as compared with saline-infused rats (Mann-Whitney, $P < 0.05$; Fig. 1B). No effect of E or P was observed on the glomerular MØ number after endotoxin. The number of glomerular MØ in follicular-phase rats was not affected by endotoxin, but endotoxin infusion did significantly increase the number of glomerular MØ in luteal-phase rats as compared with saline infusion (Mann-Whitney, $P < 0.05$). There was no difference in glomerular MØ number between the five groups of saline-infused rats (Kruskall Wallis).

Expression of Adhesion Molecules

Endothelial Adhesion Molecules (ICAM-1 and VCAM-1). Fig. 1C shows that only low levels of glomerular ICAM-1 expression were detected in the saline-treated rats of all groups, with no significant differences between the groups. In OVX rats, ICAM-1 was significantly up-regulated 3 days after endotoxin infusion as compared with saline infusion (Mann-Whitney, $P < 0.05$). No effect of E or P was observed
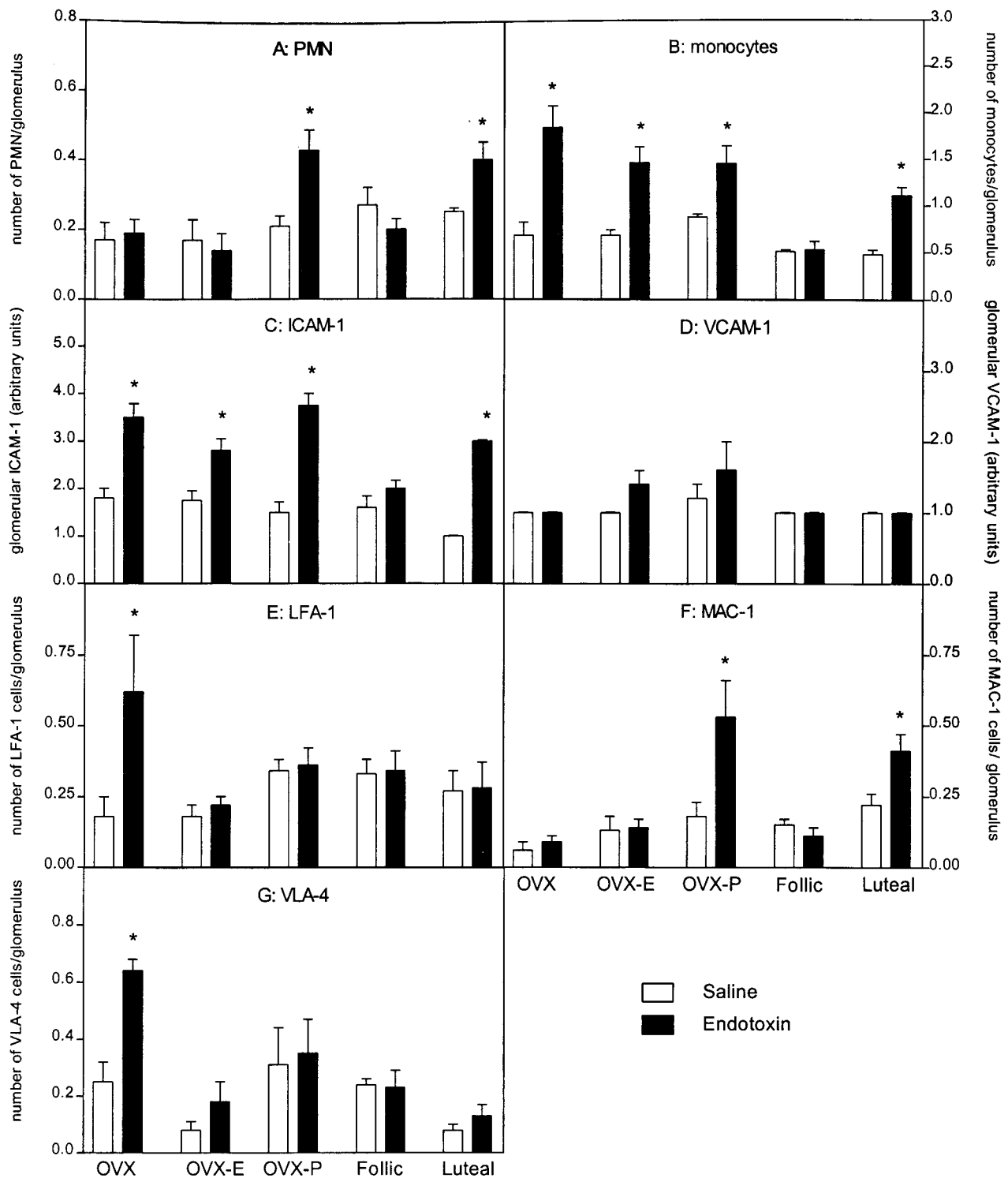
Fig. 1. Mean glomerular number of polymorphonuclear cells (A), monocytes (B), mean glomerular expression of ICAM-1 (C) and VCAM-1 (D), as well as mean glomerular number of LFA-1- (E), MAC-1- (F) and VLA-4-positive cells (G), as detected immunohistologically, 3 days after infusion of saline (open columns) or endotoxin (solid columns) in ovariectomized (OVX) (first set of columns), OVX-estradiol (E) (second set of columns), and OVX-progesterone (P) rats (third set of columns), as well as in follicular-phase rats (Follic; fourth set of columns) and luteal-phase rats (Luteal; last set of columns). Bars represent mean ± SEM. *, significantly increased as compared with saline infusion after identical treatment (Mann-Whitney, P < 0.05).

on this endotoxin-induced up-regulation of glomerular ICAM-1 in OVX rats. Endotoxin had no effect on glomerular ICAM-1 expression in follicular-phase rats, but significantly up-regulated the expression of glomerular ICAM-1 in luteal-phase rats. No significant differences in ICAM-expression after endotoxin were observed between OVX, OVX-E, OVX-P, and luteal-phase rats (Kruskall Wallis, followed by Dunn's).

Only low levels of VCAM-1 expression were observed in saline- and endotoxin-infused rats (Fig. 1D). No differences were detected between infusion-type and treatment group.

Adhesion Molecules on Leukocytes (LFA-1, MAC-1, and VLA-4)

LFA-1: In endotoxin-treated OVX rats, the number of LFA-1 positive cells per glomerulus was significantly increased compared with saline-treated OVX rats (Fig. 1E). Treatment of OVX rats with either E or P, inhibited the endotoxin-induced increase in glomerular number of LFA-1–positive cells; no significant differences were observed in glomerular number of LFA-1–positive cells between endotoxin and saline treatment in OVX-E and OVX-P rats. Also in cyclic rats (both follicular and luteal-phase rats) no effect of endotoxin was observed on the glomerular number of LFA-1–positive cells. No differences between the five groups of rats in the number of glomerular leukocytes expressing LFA-1 were detected following saline infusion (Kruskall Wallis).

MAC-1: Fig. 1F shows that endotoxin had no effect on the glomerular number of MAC-1 positive cells in OVX or OVX-E rats. However, 3 days after infusion of endotoxin, the number of MAC-1–positive cells was significantly increased in OVX-P rats (Mann-Whitney, $P < 0.05$) as compared with 3 days after

infusion of saline. In follicular-phase rats, infusion of endotoxin had no effect on the number of MAC-1–positive cells per glomerular, while in luteal-phase rats the number of MAC-1–positive cells was significantly increased after endotoxin infusion as compared with saline infusion (Mann-Whitney). It can also be seen that there were no differences between the five groups of saline-treated rats with respect to the number of MAC-1–expressing leukocytes in the glomeruli (Kruskall Wallis).

VLA-4: Three days postendotoxin infusion, the number of VLA-4–positive cells was increased in OVX rats (Mann-Whitney, $P < 0.05$) as compared with saline infusion (Fig. 1G). This effect of endotoxin was inhibited by treatment of OVX rats with either E (OVX-E rats) or P (OVX-P rats); no effect of endotoxin on the number of VLA-4–positive cells was found in these two groups. Endotoxin infusion also did not affect the number of VLA-4–positive cells in cyclic rats (follicular- and luteal-phase rats). There were no differences between the five groups of saline-treated rats with respect to the number of VLA-4–expressing leukocytes in the glomeruli (Kruskall Wallis).

Table I summarizes the results of the present study; also our previous results for pregnant rats and rats in the luteal phase with a decidualized uterus (DEC)[1] are incorporated.

DISCUSSION

The present study extends our research into the regulation of the inflammatory reaction during pregnancy,[1,2] and confirms that the inflammatory response following low-dose endotoxin infusion varies with the phase of the ovulatory cycle. Three days after endotoxin infusion, rats in the follicular phase of the

TABLE I. Summary Results of the Effects of Endotoxin Infusion on the Various Parameters of Glomerular Inflammation in Different Reproductive Conditions[a]

	OVX	OVX-E	OVX-P	Luteal phase	Follicular phase	DEC	Pregnant
PMN	=	=	+	+	=	+	+
MØ	+	+	+	+	=	+	+
ICAM-1	+	+	+	+	=	+	+
VCAM-1	=	=	=	=	=	=	+
LFA-1	+	=	=	=	=	=	+
MAC-1	=	=	+	+	=	=	=
VLA-4	+	=	=	=	=	=	+

" *Ovariectomized (OVX), OVX with estradiol (OVX-E) or progesterone (OVX-P), luteal- and follicular-phase rats from the present study, and luteal-phase rats with a decidualized uterus (DEC) and pregnant rats from Faas et al.[1].*

PMN, polymorphonuclear cells; MØ, monocytes.

+, significantly increased 3 days following endotoxin infusion as compared with saline infusion (Mann-Whitney U-test, $P < 0.05$).

=, no differences 3 days following infusion between endotoxin and saline.

ovulatory cycle, exhibiting slightly elevated levels of E at the time of infusion (and increased E levels at the time of sacrifice; results not shown), did not show any sign of glomerular inflammation compared with saline-treated rats (see Table I, showing summary results). On the other hand, rats in the luteal phase of the ovulatory cycle, exhibiting elevated levels of P, showed significant glomerular infiltration of both PMN cells and MØ, as well as expression of the adhesion molecules, ICAM-1, on the endothelium and of its ligand, MAC-1, on the leukocytes. In an earlier study,[1] similar results were explained by the suggestion that the enhanced response of luteal-phase rats (i.e., pseudopregnant rats) as compared with that of follicular-phase rats, is probably caused by the elevated levels of P. This suggestion, however, cannot be maintained as the present results show that OVX rats, which do not have elevated levels of P, also exhibited a significant influx of MØ, but not PMN cells, into the glomeruli, 3 days after treatment with endotoxin.

The fact that after endotoxin infusion, MØ infiltrated into the glomeruli of otherwise untreated OVX rats strongly suggests that during the follicular phase, MØ infiltration is suppressed by some ovarian factor. Apparently, this putative ovarian factor is also absent in luteal-phase rats, as these rats, too, exhibit influx of MØ into the glomeruli after endotoxin treatment. This would suggest that the production of the putative antiinflammatory factor is confined to the follicular phase of the ovulatory cycle and is therefore probably of follicular origin. The factor does not seem to be identical to E, as also in OVX-E rats, there was a significant influx of MØ into the glomeruli. Unlike MØ influx, endotoxin-induced PMN cells influx probably depends on the presence of P, because only P-treated OVX rats exhibited a significant number of PMN cells in the glomeruli. These effects of P on PMN cell infiltration are in line with the results of other authors showing a larger number of PMN cells in inflamed gingiva of P-treated rats[8] or dogs[9] as compared with untreated animals. In cyclic rats (follicular- and luteal-phase rats), therefore, at least two ovarian factors seem to control the influx of inflammatory cells into the glomeruli in response to endotoxin treatment, namely the corpus luteum hormone P[3], which enhances the influx of PMN cells, and a follicular factor, suppressing the influx of MØ and possibly also the influx of PMN cells. It is, however, more likely that the absence of PMN cells in the glomeruli of follicular phase rats should be explained by the suggestion that the levels of P in follicular-phase rats are too low to promote influx of PMN cells.

The different patterns of infiltration of inflammatory cells into the glomeruli of the kidneys after endotoxin infusion in the various reproductive conditions appears to be associated with different patterns of adhesion molecule expression (Table I). Thus, in OVX rats the combination of ICAM-1/LFA-1 probably accounts for the glomerular influx of MØ. Expression of both ICAM-1 and LFA-1, apparently, can occur in the absence of the ovaries; the absence of expression of ICAM-1 in follicular-phase rats, therefore, should probably be explained by the suggestion that in these rats, ICAM-1 is suppressed by an ovarian factor, which may well be the same factor as the one suppressing the influx MØ during the follicular phase, as the expression of ICAM-1 was not affected by E or P. The control of LFA-1 expression after endotoxin seems to be different: in cyclic rats, LFA-1 expression is probably suppressed by both P (during the luteal phase) and E (during the follicular phase), since these steroids have a profound inhibitory effect on LFA-1 expression in OVX rats. The same hormonal control seems to hold for the leukocytic adhesion molecule VLA-4 (Table I).

The fact that, in spite of their elevated levels of P, pregnant rats do express both LFA-1 and VLA-4 (Table I), may suggest that in the pregnant condition, there is some factor that stimulates expression of VLA-4 and LFA-1, overruling the effect of P. This enhancing factor is most likely produced by the conceptus, as VLA-4 and LFA-1 are not expressed in luteal-phase rats with a decidualized endometrium (Table I). Similarly, the conceptus probably also controls the expression of the ligand for VLA-4, VCAM-1, after endotoxin infusion, as expression of this adhesion molecule has only been observed in the glomeruli of pregnant rats (Table I). In luteal-phase rats, glomerular influx of inflammatory cells is associated with expression of ICAM-1 and MAC-1. MAC-1 expression is also observed in OVX-P rats, suggesting that expression of MAC-1 is promoted by P. Still, there are probably more factors involved in the regulation of MAC-1, as, in spite of their elevated P levels, this ICAM-1 ligand is not expressed in luteal-phase rats with a decidualized uterus, nor is it expressed in pregnant rats, which also have a decidualized uterus (Table I). This suggests that MAC-1 is also under the inhibitory control of some factor produced by decidual cells.

The present study shows that in different reproductive conditions, different (combinations of) adhesion molecules regulate the infiltration of inflammatory cells into the glomeruli after endotoxin. The number of inflammatory cells in the glomeruli after endotoxin treatment, however, outnumbered the number of leukocytes expressing either LFA-1, MAC-1 or VLA-4, suggesting that other adhesion molecules, e.g. E-selectin and its ligand Sialyl lewis X, also play a role in

the infiltration of inflammatory cells into the glomeruli following endotoxin infusion.[10] Unfortunately, these adhesion molecules could not be included in the present study; as yet there are no antibodies against them available. Of course, other mechanisms, also, like the production of chemoattractants (i.e. leukotriene B4)[11] by the glomeruli, might play a role in the infiltration of inflammatory cells into the glomeruli[12]; this might be particularly important for PMN cell influx in conditions with elevated P concentrations in which PMN cell influx is enhanced, because P increases the chemoattractive activity upon chemotactic stimuli of these cells.[13]

The present study does not reveal the mechanism by which ovarian (and placental) factors affect the expression of the various adhesion molecules. The fact that leukocytes express E,[14] but not P receptors, may suggest that estradiol modulates the expression of adhesion molecules at the level of these cells. However, we are not aware of data supporting this notion. Endothelial cells express both E and P receptors,[15,16] but the (few) data concerning a direct effect of these hormones on adhesion molecule expression in stimulated endothelial cells are conflicting, and not in agreement with our data; thus, it has been shown that E and P either potentiate[17] or suppress[18] cytokine-induced (mRNA) expression of E-selectin, ICAM-1, and VCAM-1 on endothelial cells *in vitro*. There are, however, also indications that sex hormones may influence endotoxin-induced expression of adhesion molecules by modulating the production of cytokines, some of which, e.g. interleukin-1, have a profound effect on adhesion molecule expression.[19,20] Thus, in humans *in vitro* MØ interleukin-1 production is modulated by physiological levels of P or E: low concentration of gonadal steroids stimulate (< 100 pM), while higher concentrations (> 100 pM) of these steroids inhibit MØ interleukin-1 production.[21]

This study thus demonstrates that the endotoxin-induced inflammatory response of female rats is under the control of a complex endocrine network in which the ovaries play a role. The network includes both E and P, as well as the ovarian, i.e. follicular, factor postulated in this study. This putative factor seems to be produced by developing follicles. It suppresses expression of ICAM-1, resulting in a suppression of inflammatory cell infiltration into the kidneys following endotoxin infusion. In the present experiments we used cryostat kidney sections to evaluate the inflammatory reaction, to compare the results with those in our previous studies.[1,2] However, similar results were obtained in liver sections (to be published separately), indicating that the regulation of the glomerular inflammatory reaction can be extrapolated to other tissues. The influence of ovarian factors on the endotoxin-induced inflammatory reaction may possibly be extrapolated to other inflammatory conditions and other species, in particular humans. In women, inflammatory diseases like rheumatoid arthritis[22] and systemic lupus erythematosus[23] show cyclic rashes, with worsening of symptoms during the luteal-phase of the ovarian cycle. It may be of clinical importance to study the role played by the follicular factor in the regulation of these conditions.

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