Activation of peripheral leukocytes in rat pregnancy and experimental preeclampsia

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OBJECTIVE: The aim of this study was to search for activation markers of peripheral leukocytes in experimental preeclampsia in the rat.

STUDY DESIGN: Experimental preeclampsia was induced in 14-day-pregnant rats by infusion of endotoxin (1.0 µg/kg body weight). For comparison, rats with normal pregnancies that were infused with sodium chloride solution and cyclic rats that were infused with either endotoxin or sodium chloride solution were used. At various points before and after the infusion, blood samples were withdrawn and analyzed by means of whole-blood flow cytometry to evaluate expression of inflammation-associated adhesion molecules (CD11b, CD11a, CD49d, and CD62L) and CD14 on the leukocytes.

RESULTS: Normal pregnancy was associated with increased CD11b (granulocytes and monocytes), CD11a (monocytes and lymphocytes), and CD49d (granulocytes, monocytes, and lymphocytes) expression. In addition to these changes found in normal pregnancy, reduced CD62L and increased CD11a and CD49d expression was found on granulocytes after endotoxin treatment of pregnant rats. No effect of endotoxin was observed in cyclic rats.

CONCLUSION: Leukocytes of rats with experimental preeclampsia and, to a lesser extent, those of rats with normal pregnancies had an activated phenotype. These results are consistent with our previous findings in human subjects and suggest that (experimental) preeclampsia results from a generalized inflammatory response. (Am J Obstet Gynecol 2000;182:351-7.)

Key words: Pregnancy, inflammation, rat, endotoxin, flow cytometry

In our society preeclampsia is the most common and serious antenatal complication of pregnancy characterized by hypertension, proteinuria, and sometimes abnormal fluid retention. It affects about 2% to 3% of all pregnancies.[1] The disease may also be associated with abnormalities of the liver and the central nervous system,[2] as well as with disseminated intravascular coagulation.[3] Many suggestions have been put forward as to the pathogenesis of preeclampsia. Most recently, a generalized inflammatory response has been implicated in its pathogenesis.[4] The fact that circulating leukocytes of preeclamptic patients show an activated phenotype, as characterized by decreased expression of CD62L and an increased production of reactive oxygen species compared with normal pregnancy,[5] supports this hypothesis.

The relevance of activation of the inflammatory system in the pathogenesis of preeclampsia is also consistent with a rat model in which infusion of a very low dose of endotoxin, a potent activator of the inflammatory response, induces a preeclampsia-like syndrome (experimental preeclampsia).[6] This syndrome is characterized by hypertension, proteinuria, and disseminated intravascular coagulation and is very specific for pregnant rats because it cannot be induced in identically treated cyclic rats.[6] Rats with experimental preeclampsia had a pregnancy-specific glomerular inflammatory reaction. They showed a more intense and persistent (until 7 days after infusion) infiltration of (activated) neutrophils and monocytes into the glomeruli than did cyclic rats. There was also more persistent expression of adhesion molecules (ie, intercellular adhesion molecule 1 and vascular cell adhesion molecule 1) on the endothelium and of their respective ligands on the infiltrated leukocytes CD11a and CD49d.[7]

This study was designed to search for peripheral blood markers of the pregnancy-specific inflammatory reaction in experimental preeclampsia in the rat and compare re-

sults with similar human observations. Immunolabeled peripheral blood leukocytes were analyzed by means of flow cytometric examination with antibodies against various inflammation-associated adhesion molecules to establish a phenotype for the leukocytes in experimental preeclampsia. The phenotypes of these leukocytes were compared with those of normal pregnant rats, as well as with those of normal cyclic rats and cyclic endotoxin-treated rats.

Material and methods

Experimental animals. Female Wistar rats (Harlan UK LTD, Oxford, United Kingdom; aged 3-4 months and weighing about 200 g) were kept in a temperature- and light-controlled room (lights on from 6 AM until 6 PM). Daily vaginal smears were taken until selection for experiments. Rats were rendered pregnant by housing them on the night of proestrus with a fertile male for 1 night. The next day, when spermatozoa were detected in the smear, was designated as day 0 of pregnancy. On this day, a permanent jugular vein cannula was inserted after achievement of halothane anesthesia, according to the method of Steffens.[8] Nonpregnant rats were similarly treated, and all rats were allowed to recover from surgery for at least 7 days.

Experimental protocol. Experimental preeclampsia was induced by infusing endotoxin 1.0 μg/kg body weight (*Escherichia coli* 0.55:B5; Wittaker MA Bioproducts Inc, Walkerville, Md) in 2 mL of sodium chloride solution into conscious pregnant rats administered through the jugular vein (infusion rate, 2 mL/h) on day 14 of pregnancy, as described previously.[6] Normal pregnant control rats were infused with 2 mL of sodium chloride solution alone. For comparison, cyclic rats were treated identically and infused with either endotoxin or sodium chloride solution on diestrus 1 or 2 (pilot experiments revealed no differences in response between infusion on diestrus 1 or 2). At various points before and after the infusion, blood samples (100 μL) were withdrawn from the cannula. With pregnant rats, this was on days 7 and 13 before infusion and on days 17 and 21 (ie, 3 days and 7 days after the infusion, respectively). For cyclic rats, blood was sampled 1 day before infusion and 3 and 7 days after infusion. All blood samples were taken between 8 and 12 AM.

Sample processing

Reagents. Hanks' balanced salt solution, sodium azide, bovine serum albumin, and N-[2-hydroxyethyl]piperazine-N´-[2-ethanesulfonic acid] (HEPES) were obtained from Sigma (Poole, Dorset, United Kingdom). LDS-751 was obtained from Molecular Probes (Eugene, Ore). A stock solution of 0.02% LDS-751 in methanol was kept at 4°C in the dark from which, before each experiment, a working solution was freshly prepared (1:100 dilution in 10-mmol/L HEPES-buffered Hanks' balanced salt solution with 5-mmol/L sodium azide) and also kept in the dark.

Antibodies. Fluorescein isothiocyanate–labeled mouse antirat CD14 (clone ED9), mouse antirat CD11b (or MAC-1; clone ED8), mouse antirat CD11a (or LFA-1; clone WT.1), and mouse antirat CD3 (clone IF4) were obtained from Serotec (Oxford, United Kingdom), as were their fluorescein isothiocyanate–labeled isotype controls (mouse immunoglobulin G1 and mouse immunoglobulin G2a). Fluorescein isothiocyanate–labeled mouse antirat CD49d (or very late antigen 4; clone MRα4), hamster antirat CD62L (or L-selectin; clone HRL2), and mouse antirat granulocytes (clone His48) were obtained from Pharmingen (San Diego, Calif), together with the fluorescein isothiocyanate–labeled isotype controls (mouse immunoglobulin G2bk and hamster immunoglobulin G). Antibodies were titrated for flow cytometry and used at saturating doses. The negative controls (isotype controls) were diluted to an identical immunoglobulin concentration.

Sample labeling. Blood samples were labeled according to the method of McCarthy and Macey[9] with minor modifications. Immediately after collection, they were mixed with heparin (1.5 μL heparin [500 IU/mL]/100 μL blood) followed by immediate incubation with LDS-751 working solution (100 μL blood with 100 μL LDS-751) for 17 minutes at 20°C in the dark. The sample was then transferred to ice, and 10-μL aliquots were incubated with 2 μL of the various antibodies and isotype controls. After 10 minutes of incubation on ice in the dark, the samples were diluted with 0.5 mL HEPES-buffered Hanks' balanced salt solution containing 0.5% bovine serum albumin and sodium azide. Samples were maintained on ice in the dark until measured by flow cytometry between 2 and 60 minutes later.

Flow cytometry. Blood cells were analyzed by using the Coulter Epics Elite flow cytometer (with an Argon-ion 488-nm laser). Fluorescein isothiocyanate fluorescence was detected at 575 nm and LDS-751 fluorescence at 630 nm. The fluorescein isothiocyanate fluorescence was calibrated before each experiment with Standard Brite Beads (Coulter, Luton, United Kingdom). Data were acquired while gating on the fluorescence of LDS-751 to exclude the nonnucleated red blood cells. Six thousand signals, largely from granulocytes, monocytes, and lymphocytes, were collected, and the data were saved for later analysis. For every blood sample, secondary gates were set on granulocytes, monocytes, and lymphocytes by using forward-angle and 90° light-scatter characteristics, as well as the aliquots with the specific markers; anti-CD3 was used for lymphocytes, His48 was used for polymorphonuclear neutrophils, and anti-CD14 was used for monocytes. For each population, single-parameter fluorescence histograms were defined, and gates were set so that at least 99% of the cells in any sample were negative with the isotype controls. Mean channel brightness was defined as mean fluorescence intensity of positive cells.

Statistics. To evaluate changes in adhesion-molecule expression during pregnancy, data from rats with normal pregnancies (days 7, 13, 17, and 21) were compared with data from normal cyclic rats by the Kruskal-Wallis test followed by the Dunn test. To evaluate changes in experimental preeclampsia, data of samples from day 17 and day 21 were compared with data of samples taken on day 13 (ie, before infusion of either endotoxin or sodium chloride solution), and paired analysis was done with the Wilcoxon signed-rank test. For cyclic rats data from samples 3 and 7 days after the infusion were compared with data from samples before infusion by the Wilcoxon signed-rank test.

Results

Table I shows the percentage of positive granulocytes, monocytes, and lymphocytes for the various adhesion molecules in cyclic and pregnant rats. For normal pregnancy, there were no differences among days 7, 13, 17, and 21 of pregnancy, and only the data for day 13 are shown. CD11a, CD49d, and CD62L were expressed by all 3 leukocyte types (ie, granulocytes, monocytes, and lymphocytes), whereas, as expected, CD14 and CD11b were expressed only by granulocytes and monocytes. CD11b was expressed by 90% of the granulocyte population, confirming the myelomonocytic lineage of these cells. Moreover, the granulocyte marker (His48), that was used to define the population was expressed by >90% of the cells in this population (results not shown), confirming the relative purity of the population. Greater than 90% of lymphocytes expressed CD3 (results not shown). Also, <10% of the cells expressed the myelomonocytic marker CD11b, and <10% of the cells expressed CD14, the monocyte marker, confirming the relative purity of this population. Monocytes were defined less clearly by forward-angle and 90° light scatter because lymphocytes and monocytes did not form clearly separable populations in terms of these parameters. Of the cells within the assigned gates, about 40% were labeled for CD14, about 35% expressed CD11b, and about 35% were CD3 positive (results not shown).

Expression of the adhesion molecules and CD14 in normal pregnancy. The mean channel brightness values of the positive cells surface-labeled with antibodies against the various adhesion molecules in samples from normal pregnant and cyclic rats are shown in Fig 1 (granulocytes and monocytes) and Fig 2 (lymphocytes). Whereas the percentage of positive cells did not vary, the intensity of the surface expression of the various adhesion molecules (intensity of mean channel brightness) of the positive cells did. The mean channel brightness of α-CD14 (monocytes), α-CD11b (granulocytes and monocytes), α-CD11a (monocytes and lymphocytes), α-CD49d (granulocytes, monocytes, and lymphocytes), and α-CD62L (granulocytes) all significantly increased in pregnant

Table I. Percentage of positively staining leukocytes for α-CD14, α-CD11b, α-CD11a, α-CD49d, and α-CD62L in pregnant (day 13) and cyclic rats

	Pregnant	*Estrus cycle*
Granulocytes		
CD14	81.7 ± 2.4	76.2 ± 3.7
CD11b	89.9 ± 2.1	89.4 ± 2.8
CD11a	99.2 ± 0.3	98.8 ± 0.8
CD49d	70.1 ± 6.8	63.7 ± 8.0
CD62L	89.3 ± 0.6	88.6 ± 2.3
Monocytes		
CD14	42.6 ± 3.4	43.05 ± 2.2
CD11b	36.1 ± 1.7	30.9 ± 2.8
CD11a	89.2 ± 2.4	89.7 ± 1.7
CD49d	87.2 ± 1.2	84.2 ± 4.3
CD62L	81.9 ± 1.5	84.8 ± 0.7
Lymphocytes		
CD14	Negative	Negative
CD11b	Negative	Negative
CD11a	85.5 ± 2.4	87.5 ± 3.0
CD49d	85.0 ± 2.2	84.1 ± 3.8
CD62L	87.2 ± 0.9	86.6 ± 1.5

Leukocyte subgroups were distinguished by forward- and side-scatter characteristics and specific markers (His48 for granulocytes, α-CD14 for monocytes, and α-CD3 for lymphocytes). For each subpopulation, the percentage of positive cells was defined with the use of a gate that included <1 cell labeled with the isotype control. Percentages are given as mean ± SEM.

compared with cyclic rats (Kruskal-Wallis test followed by Dunn test; $P < .05$).

Expression of adhesion molecules and CD14 in endotoxin-treated pregnant rats (rats with experimental preeclampsia) and in endotoxin-treated cyclic rats. The percentage of positive cells for CD14, CD11b, CD11a, CD49d, and CD62L in the separate populations of granulocytes, monocytes, and lymphocytes did not change after endotoxin infusion compared with the preinfusion value in pregnant or cyclic rats (results not shown).

In Fig 3 mean channel brightness is expressed as a percentage of the preinfusion value. The baseline is therefore always 100% on day 13 for pregnant rats or day 0 for cyclic rats (dotted line in each panel). Cyclic rats were completely unaffected by the endotoxin infusion 3 and 7 days afterward (results not shown). Changes associated with endotoxin infusion were only seen in pregnant animals.

In normal pregnant rats infused with sodium chloride solution only, there were transient but significant increases in CD14 (granulocytes and monocytes), CD11a (lymphocytes), and CD62L (granulocytes) on day 17 of pregnancy, which were no longer apparent on day 21 (open bars, Fig 3). Endotoxin infusion was associated with a different pattern of leukocytic adhesion molecule expression in pregnancy. It abrogated the normal increases (day 17) in CD14 on granulocytes or monocytes, in CD11a on lymphocytes, and in CD62L on granulocytes
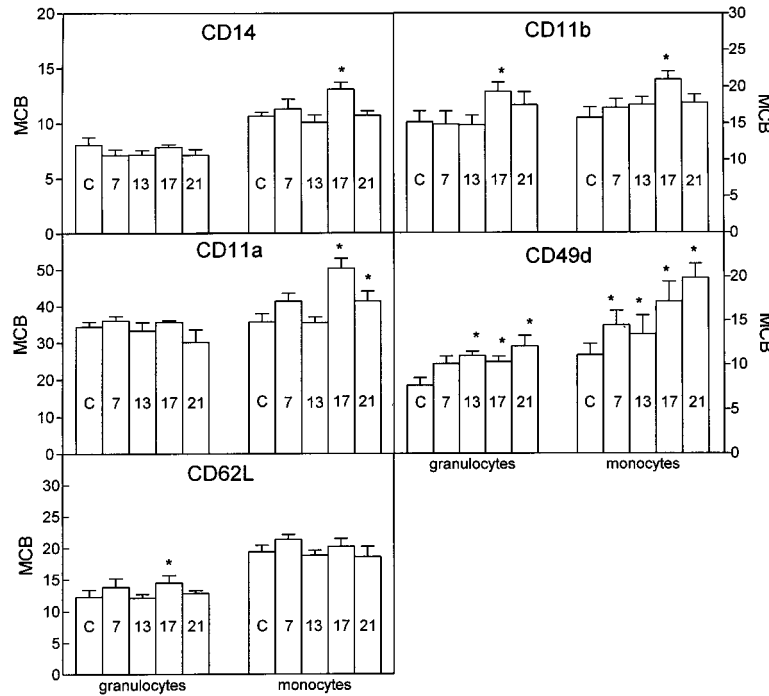
Fig 1. Mean channel brightness *(MCB)* of labeled antibody binding to peripheral granulocytes (*left set of bars* in each panel) and monocytes (*right set of bars* in each panel) in normal cyclic rats (ie, in cyclic rats before infusion *[C]*) and rats with normal pregnancies (days 7, 13, 17, and 21). *Asterisk,* Significant difference from cyclic rats (Kruskal-Wallis test followed by Dunn test, $P < .05$).

Fig 2. Mean channel brightness *(MCB)* of labeled α-CD11a *(left set of bars),* α-CD49d *(middle set of bars),* and α-CD62L *(right set of bars)* to peripheral lymphocytes in normal cyclic rats (ie, in cyclic rats before infusion *[C]*) and rats with normal pregnancies (days 7, 13, 17, and 21). *Asterisk,* Significant difference from cyclic rats (Kruskal-Wallis test followed by Dunn test, $P < .05$).
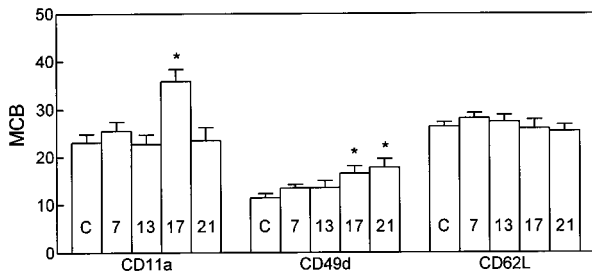
(filled bars, Fig 3). With regard to CD62L on granulocytes, not only was there no increase on day 17 of pregnancy but there was a significant loss compared with the preinfusion baseline. In addition, the granulocytes displayed persistent increases (days 17 and 21) in CD11a and CD49d (filled bars, Fig 3).

There was no change in mean channel brightness of α-CD11b (granulocytes and monocytes), α-CD11a (monocytes), α-CD49d (monocytes and lymphocytes), and α-CD62L (monocytes and lymphocytes) after endotoxin infusion in pregnant rats (results not shown).

Comment

In this study we searched for peripheral blood markers of the pregnancy-specific inflammatory reaction underlying experimental preeclampsia in the rat.[6, 7] We used flow cytometry to study the phenotype of peripheral blood leukocytes. Because the isolation of leukocytes from blood may cause in vitro activation,[10] we analyzed whole blood stained with the vital nucleic acid dye LDS-751 to discriminate anucleated red blood cells from nucleated leukocytes.[9] Phenotypic activation of leukocytes can be identified by a change in adhesion molecule expression, notably by either a rise in CD11b[11] or a decrease in CD62L or both of these[12]; an increase in either CD11a or CD49d or both of them may also indicate an activated leukocyte phenotype (Table II).[13]

In experimental preeclampsia there were sustained increases in CD11a and CD49d expression, as well as a shorter-lived decrease in CD62L expression by granulocytes compared with that found during normal pregnancy. Because leukocyte adhesion molecule expression changes with activation of these cells (Table II), these results are consistent with a generalized activation of granulocytes in experimental preeclampsia. These activation changes were shown to be specific for pregnancy because no activation of granulocytes was observed 3 days after endotoxin infusion in cyclic rats. These results are also consistent with our previous study, in which we evaluated indexes of
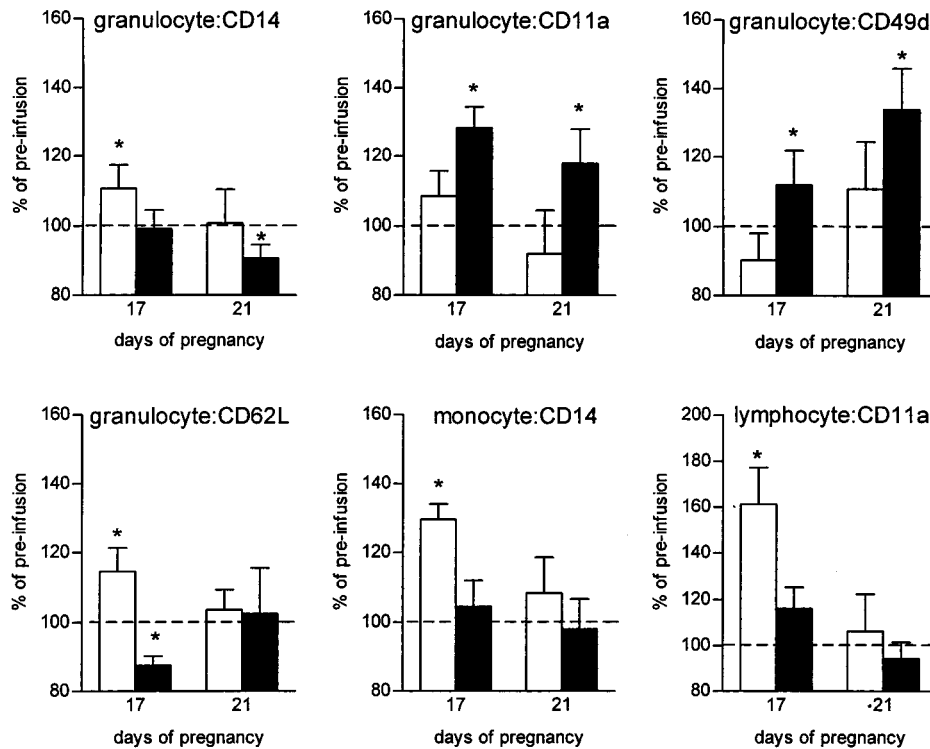
Fig 3. Mean channel brightness (expressed as percentage [mean ± SEM]) of preinfusion value *(dotted line)* of α-CD14, α-CD11a, α-CD49d, and α-CD62L binding to peripheral granulocytes; α-CD14 binding to peripheral monocytes; and α-CD11a binding to peripheral lymphocytes of pregnant rats on days 17 and 21 of pregnancy after infusion of endotoxin *(filled bars)* or sodium chloride solution *(open bars)* on day 14. *Asterisk,* Significant increase or decrease compared with preinfusion value (Wilcoxon test, $P < .05$).

Table II. Expected phenotype of normal and activated leukocytes

	Granulocytes		Monocytes		Lymphocytes	
	Normal	*Activated*	*Normal*	*Activated*	*Normal*	*Activated*
CD11b	+	↑	+	↑	−	−
CD11a	+	↑	+	↑	+	↑
CD49d	+	↑	+	↑	+	↑
CD62L	+	↓	+	↓	+	↓

+, Constitutive expression of adhesion molecule; *↑ or ↓,* increased or decreased expression of adhesion molecule when activated; *−,* negative for adhesion molecule.

glomerular inflammation after the same low-dose endotoxin infusion regimen.[7] The local endotoxin-induced glomerular inflammatory response was characterized by transient (1 day) infiltration of granulocytes and monocytes and up-regulation of leukocytic and endothelial adhesion molecules in cyclic rats, whereas in pregnant rats the response was more persistent and lasted until the end of pregnancy, which is 7 days after the infusion.[7]

The expression of CD11b, CD11a, CD49d, and CD62L by circulating monocytes did not differ in experimental preeclampsia when compared with rats with normal pregnancies. However, CD14 expression, which was increased on monocytes of rats with normal pregnancies compared with levels in cyclic rats, was reduced

on monocytes in rats with experimental preeclampsia compared with rats with normal pregnancies. We have reported similar patterns in women with normal pregnancy or preeclampsia.[5] In nonpregnant patients with sepsis, expression of monocyte CD14 is decreased.[14] The significance of the findings in relation to pregnancy, both in rats and in human subjects, remains to be determined. CD14 is a lipopolysaccharide receptor,[14] and its expression on monocytes changes bimodally in response to its ligand in culture. First it is shed, with loss of surface expression, followed by a more sustained increase.[15]

It is interesting that phagocytes (monocytes and granulocytes) from rats with normal pregnancies already had

an activated phenotype, although not to the degree shown by rats with experimental preeclampsia. When compared with normal cyclic rats, next to the before-mentioned increase in CD14 expression on monocytes, there was increased expression of CD11b (monocytes and granulocytes), CD11a (monocytes), and CD49d (granulocytes and monocytes). Similarly, lymphocytes of rats with normal pregnancies showed an activated phenotype (increased CD11a and CD49d expression). All these changes are suggestive of activation of peripheral leukocytes in normal rat pregnancy (Table II) and are consistent with our previous observations in human pregnancy[5, 16] and thus suggest that pregnancy per se is a proinflammatory condition.

An increased expression of the endotoxin receptor CD14 on monocytes of rats with normal pregnancies might be involved in the well-known increase in the sensitivity to endotoxin during pregnancy.[17] However, the change was only found on day 17, whereas the endotoxin sensitivity is apparent before this late stage of pregnancy.[18, 19] Moreover, if CD14 expression were the main determinant of the pregnancy-related increase in endotoxin sensitivity, it would be expected that low-dose endotoxin infusion would induce a higher tumor necrosis factor α response in pregnant rats than in cyclic rats because CD14 is involved in endotoxin-induced tumor necrosis factor α production.[20] This is, however, not the case,[21] and therefore other factors must be involved.

It is not known what processes induce an activated state of circulating leukocytes in normal pregnancy. The current data may suggest that hormonal factors play a role. Adhesion molecule expression was increased on day 17 of pregnancy but had returned toward a cyclic phenotype on day 21. Similarly, an activated phenotype of granulocytes during experimental preeclampsia was more prominent on day 17 than on day 21. In the pregnant rat luteolysis occurs around day 18 of pregnancy, resulting in decreased progesterone secretion,[22] whereas postpartum surges of luteinizing hormone and follicle-stimulating hormone are generated, resulting in postpartum ovulation.[23] Before this time, either the increased progesterone levels or the absence or presence of ovarian factors or both of these may play a role in either inducing or sustaining the activated leukocytic phenotype of pregnancy. Our recent studies have indeed shown that progesterone and other ovarian factors, as well as trophoblastic factors, play a role in the persistency of glomerular inflammation after low-dose endotoxin infusion.[18, 24]

In conclusion, we show that experimental preeclampsia in the rat is associated with features of a generalized inflammatory response, which is consistent with our observations in women with preeclampsia[5] and our hypothesis about the importance of inflammatory reactions in the genesis of preeclampsia.[4, 16]

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