Glomerular Inflammation in Pregnant Rats after Infusion of Low Dose Endotoxin

An Immunohistological Study in Experimental Pre-Eclampsia

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Increased endotoxin sensitivity during pregnancy occurs in many animals, including rats. The mechanism of this phenomenon is not understood. In the present study it was investigated whether this increased sensitivity is reflected by an altered inflammatory pattern. Inflammatory cell influx, the O_2^--producing potential of these cells, and expression of adhesion molecules was studied in the glomeruli of pregnant and cyclic rats at various intervals after low dose endotoxin infusion. Kidney sections were stained for monocytes and adhesion molecules (ICAM-1, VCAM-1, LFA-1, and VLA-4) using monoclonals, while potentially O_2^--producing neutrophils (ie, activated neutrophils) were quantified using immunohistochemical methods. The results show early glomerular influx of activated neutrophils, maximally 4 hours after endotoxin. Both absolute neutrophil counts and relative numbers of activated neutrophils were significantly increased in pregnant versus cyclic rats. In contrast to cyclic rats, showing transient monocyte influx, in pregnant endotoxin-treated rats monocyte influx reaches a maximum at t = 168 hours. These cell kinetics were paralleled by expression of the various adhesion molecules. It was concluded that pregnancy profoundly influences not only the inflammation kinetics after endotoxin, but also the violence of the reaction, reflected by activated neutrophils. This altered glomerular inflammatory pattern may help to explain why

low dose endotoxin infusion induces pre-eclamptic-like symptoms (such as an intraglomerular prothrombotic microenvironment and proteinuria) exclusively in the pregnant rat. (Am J Pathol 1995, 147:1510–1518)

In 1935 Apitz[1] was the first to observe that pregnant animals are much more sensitive to endotoxin than are nonpregnant animals. This is reflected, eg, by the induction of disseminated intravascular coagulation in pregnant animals after a single endotoxin injection, whereas in nonpregnant animals two injections of endotoxin, with a 24-hour interval, are needed to obtain this effect.[2]

Thus, in a recent animal study we observed disseminated intravascular coagulation in kidney and placenta after a single infusion of a low dose of endotoxin exclusively in pregnant rats, and hypertension and proteinuria also occurred in these rats.[3,4] This endotoxin-induced syndrome bears a strong similarity to pre-eclampsia (PE), a serious complication of human pregnancy.[5] In addition, in glomeruli of pregnant rats, but not in those of cyclic animals, low dose endotoxin infusion decreased the activity of the antithrombotic ectoenzyme ADPase, leading to an increased prothrombotic environment in the glomeruli.[4,6]

An intraglomerular prothrombotic microenvironment may be promoted by inflammatory events such as O_2^- production by infiltrating cells, as it is known that glomerular nucleotidases (including ADPase) are extremely sensitive to oxygen free radicals.[7] Consequently, altered local O_2^- production in preg-

nant *versus* cyclic rats, induced by endotoxin, might play a role in the generation of a proaggregatory environment in the glomerular microvasculature of the kidney. This has led us to the question whether the pregnancy-associated enhanced endotoxin sensitivity is reflected by an altered inflammatory response after endotoxin infusion.

Therefore, in the present study we focused on the kinetics of inflammatory cell infiltration into the glomeruli of pregnant and cyclic rats in association with expression of adhesion molecules at various time points after infusion of endotoxin or saline. Special attention was paid to the cytochemical detection of O_2^--producing potential of infiltrated polymorphonuclear cells (PMNs) in the glomeruli of these rats.

Materials and Methods

Animals

Female Wistar outbred rats, 200 to 220 g, were kept in a temperature- and light-controlled room (lights on from 5:30 AM to 5:30 PM) with free access to food and water. Until selection for experiments estrous cyclicity was followed by taking daily vaginal smears. At the age of 3 to 4 months, rats were rendered pregnant by housing them on pro-estrus with fertile males for one night. The next day, when spermatozoa were detected in the smear, was designated as day 0 of pregnancy.

In cyclic and (day 0) pregnant rats a cannula was surgically inserted into the right jugular vein under ether anesthesia, according to the method of Steffens.[8] This cannula allows stress-free infusion of endotoxin or saline.

As described previously[4] endotoxin (*Escherichia coli*, 0.55:B5, Whittaker MA Bioproducts Inc., Walkerville, MD) (1.0 μg/kg body weight (bw)), was dissolved in 2 ml pyrogen-free saline and infused for 1 hour through the jugular vein cannula, using an infusion pump (infusion rate: 2.0 ml/hour). Control rats received 2.0 ml pyrogen-free saline alone under identical conditions.

Experimental Design

Pregnant rats (day 14; n = 50) and cyclic rats (n = 50) were infused with either saline or endotoxin. Rats were sacrificed at various intervals after the start of the infusion, ie, 1 hour after the start of the infusion ($t = 1$); $t = 4$ hours, $t = 24$ hours, $t = 72$ hours, and $t = 168$ hours (n = 5 in all groups). One group of day 14 pregnant rats (n = 5) and one group

of cyclic rats (n = 5) were sacrificed without receiving infusion ($t = -1$). At sacrifice pregnancy was checked macroscopically, kidneys were taken out, and specimens were snap frozen in precooled methylbutane and stored at $-80°C$ until use.

Immunohistology

Four μm cryostat sections, fixed in acetone (15 minutes, room temperature) were stained according to standard procedures[9] with minor modifications. Briefly, after preincubation with either normal rabbit or normal goat serum (5%) sections were incubated with the first antibody for 30 minutes. Peroxidase-conjugated second antibody (rabbit-anti-mouse or goat-anti-rat (Dako A/S, Glostrup, Denmark)) was used. The reaction product was visualized using 3-amino-9-ethyl-carbazole (Sigma Chemical Co., St. Louis, MO) or 3,3'-diaminobenzidine (Sigma Chemical Co.). Control sections omitting the primary or secondary antibody were consistently negative.

Antibodies

1A29 (Genzyme, Cambridge, MA), a mouse immunoglobulin G1 (IgG1) monoclonal antibody to rat ICAM-1, which has been characterized and used for immunohistology elsewhere[10–12]; 5F10 (a generous gift from Dr. R. Lobb, Biogen, Cambridge, MA), a protein A chromatography-purified mouse IgG2a monoclonal antibody to VCAM-1, which has been used for both blocking adhesion *in vitro*[13] and for immunohistology;[14] WT.1 (Genzyme), a mouse IgG2a monoclonal antibody to rat leukocyte function-associated antigen-1 (LFA-1) α-chain (CD11a), characterized and used for immunohistology elsewhere[11,12,15]; MRα4 (Pharmingen, San Diego, CA), a mouse IgG2b monoclonal antibody to rat very late antigen-4 (VLA-4); and ED-1, a mouse IgG1 monoclonal antibody to a cytoplasmic antigen of macrophages and monocytes, which has been described elsewhere.[9]

Activated PMNs

Activated PMNs present in glomeruli were cytochemically stained for their potential to produce O_2^- by the method of Briggs et al.[16] This staining was combined with standard immunostaining for PMNs, using a mouse IgM monoclonal antibody to a membrane-bound antigen of PMN (HIS48, Pharmingen).[17] Briefly, sections were fixed at 4°C in 1.0% paraformaldehyde in *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (HEPES) buffer (0.1 mol/L HEPES, 1 mmol/L so-

dium azide and 5% sucrose in phosphate buffered saline, pH 7.2). Subsequently, sections were incubated in the HEPES buffer with addition of 4% 3,3′-diamino-benzidine (Sigma Chemical Co.) and 625 μmol/L $MnCl_2$ at 37°C. This cytochemical staining procedure was followed by standard immunohistochemistry with HIS48 as described above, using alkaline phosphatase-conjugated rabbit anti-mouse second antibody (Dako A/S).[18] This double-staining technique enables us to detect in the same section both normal PMNs (showing blue staining at the cell surface) and activated PMNs (showing blue staining as well as brown reaction product in the same cell).

Control sections, which were incubated with superoxide dismutase (Sigma Chemical Co.) and omitting first or second antibody were consistently negative.

Evaluation of Kidney Sections

Kidney sections of each individual animal were scored by light microscopical examination in a double-blind manner, by two independent observers, in the following ways. 1) Sections stained with ED1, α-LFA-1, and α-VLA-4 were quantitatively scored by counting the total number of positive cells in 100 glomeruli. 2) Kidney sections stained for both the presence of PMNs and activated PMNs were quantitatively scored by counting both the total number of PMNs and the total number of double-stained cells per 100 glomeruli. 3) After staining for ICAM-1 and VCAM-1 sections of individual animals were semi-quantitatively graded by scoring 100 glomeruli per section using an arbitrary scale from 1 to 4 (1 = no visible staining, 2 = weak staining, 3 = moderate staining, 4 = bright staining).

Statistics

Results are expressed as mean ± SEM. To compare preinfusion data ($t = -1$) with postinfusion data at the intervals tested analysis of variance (ANOVA) was carried out followed by unpaired Student's t-test as indicated in the Results section. To compared data of pregnant and cyclic rats after identical treatment, unpaired Student's t-tests were carried out. Differences were considered significant when $P < 0.05$.

Results

Intraglomerular Influx of Activated PMNs

By using the double-staining technique (as described in Materials and Methods), we were able to quantify the glomerular influx of PMNs expressed as mean number
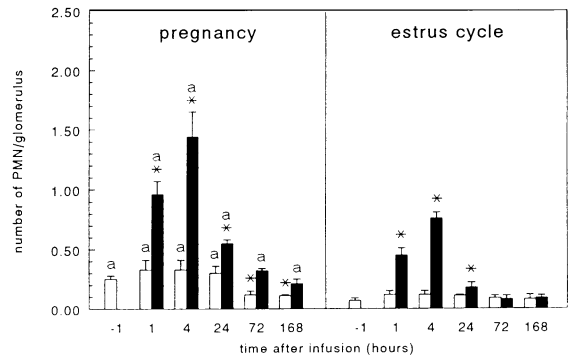

Figure 1. Mean number of PMNs per glomerulus, as detected immunohistochemically with a monoclonal antibody against PMN, in pregnant (left) and cyclic (right) rats before ($t = -1$) and after infusion of 1.0 μg/kg bw endotoxin (solid columns) or saline (open columns). Infusions were started at $t = 0$. Columns represent mean ± SEM for $n = 5$ in all groups. *Significantly increased or decreased as indicated compared with preinfusion values ($t = -1$) ($P < 0.05$, ANOVA followed by unpaired Student's t-test). ^{a}Significantly increased compared with cyclic rats after identical treatment at the same interval ($P < 0.05$, unpaired Student's t-test).

of PMNs per glomerulus (Figure 1) as well as the percentage of these cells having the potential to produce O_2 (Figure 2) in the same section.

It can be seen from Figure 1 that in pregnant rats significantly increased mean numbers of PMNs were present 1, 4, and 24 hours after infusion of endotoxin, as compared with preinfusion values, although to a significant lesser extent the same pattern can be observed in cyclic endotoxin-treated rats.
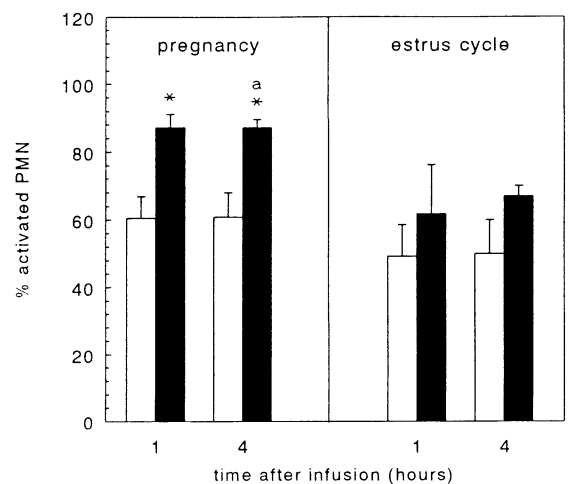

Figure 2. Mean percentage of potential oxygen free radical (O_2) producing PMNs as detected by immunocytochemical double staining in the glomeruli of pregnant (left) and cyclic (right) rats after infusion of 1.0 μg/kg bw endotoxin (solid columns) or saline (open columns) 1 and 4 hours after the start of the infusion. Columns represent mean ± SEM, for $n = 5$ in all groups. *Significantly increased as compared with saline-infused rats at the same interval ($P < 0.05$, ANOVA followed by unpaired Student's t-test). ^{a}Significantly increased as compared with cyclic rats after identical treatment at the same interval ($P < 0.05$, unpaired Student's t-test).
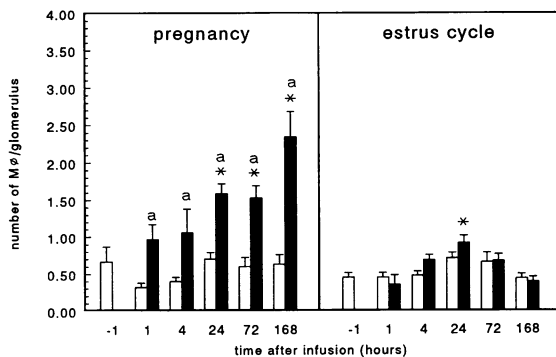
Figure 3. *Mean number of macrophages/monocytes (Mφ) per glomerulus, as detected immunohistochemically with a monoclonal antibody against Mφ, in pregnant (left) and cyclic (right) rats before (t = −1) and after infusion of 1.0 μg/kg bw endotoxin (solid columns) or saline (open columns). Infusions were started at t = 0. Columns represent mean ± SEM, for n = 5 in all groups. *Significantly increased as compared with preinfusion values (t = −1) (P < 0.05, ANOVA followed by unpaired Student's t-test). ᵃSignificantly increased as compared with cyclic rats after identical treatment at the same interval (P < 0.05, unpaired Student's t-test).*

After saline infusion in pregnant animals, no significant increase in PMN influx is shown as compared with the preinfusion value (t = −1); in contrast, 72 and 168 hours after saline infusion a significant decrease in glomerular PMN number was observed. In cyclic animals no significant alterations after saline infusion are seen as compared with the preinfusion level. It further appears from Figure 1 that before (t = −1) and 1, 4 and 24 hours after saline infusion, the level of PMN infiltration in kidneys of pregnant rats is significantly increased as compared with cyclic animals after saline infusion.

The present double-staining method allows the calculation of the relative number of activated PMNs (ie, having the potential to produce O_2^-) versus non-activated PMNs in glomeruli of pregnant and control animals (Figure 2). It can be seen that endotoxin infusion resulted exclusively in pregnant rats in a significant relative increase in activated PMNs as compared with saline-infused pregnant rats (1 and 4 hours after infusion). At the other intervals (t = 24, t = 72, and t = 168 hours) (results not shown) no significant differences were observed between pregnant and cyclic rats, between saline and endotoxin infusion, or between time intervals (percentage of activated PMNs of cyclic rats, 66 ± 6%; of pregnant rats, 74 ± 5%, mean ± SEM).

Influx of Mφ

Infusion of endotoxin in pregnant rats induced, in addition to an early glomerular influx of activated PMNs, a significant glomerular influx of Mφ, although in later stages (Figure 3). The number of glomerular

Mφ after endotoxin increased toward the end of pregnancy and was significantly increased at t = 24, 72, and 168 hours as compared with t = −1. Cyclic rats showed a slight but significant increase in Mφ number 24 hours after infusion of endotoxin as compared with t = −1. The number of Mφ per glomerulus after endotoxin infusion was significantly higher in pregnant rats as compared with cyclic rats. Infusion of saline did not influence the number of Mφ per glomerulus in pregnant or cyclic rats.

Adhesion Molecules

As can be seen from Figure 4, after infusion of endotoxin in pregnant rats, ICAM-1 expression was significantly upregulated 24, 72, and 168 hours after infusion, while in cyclic rats the ICAM-1 expression was significantly upregulated only at 24 hours after infusion. It can also be observed that ICAM-1 was absent or weakly expressed in normal day 14 pregnant or cyclic rats (t = −1) and after infusion of saline in pregnant or in cyclic rats. The photomicrographs (Figure 4, b and c) show representative glomeruli of a pregnant endotoxin-treated rat showing significant staining of reaction product (Figure 4b) and of a pregnant saline-treated rat in which no reaction product can be observed (Figure 4c).

Besides expression of ICAM-1, expression of its ligand on leukocytes (LFA-1) was also seen (Figure 5). The number of LFA-1-positive cells in the glomeruli was significantly increased in pregnant rats after infusion of endotoxin as compared with t = −1 on all intervals. In cyclic rats the number of LFA-1-positive cells was significantly increased after endotoxin only 4 and 24 hours after infusion. It can also be seen that in pregnant endotoxin-treated rats the number of LFA-1-positive cells was significantly increased on all intervals tested as compared with endotoxin-treated cyclic rats. Saline infusion did not significantly change the glomerular number of LFA-1-positive cells in pregnant or cyclic rats.

The expression of glomerular VCAM-1 in pregnant and cyclic rats after infusion of endotoxin or saline is depicted in Figure 6. This figure shows that after infusion of endotoxin in pregnant rats VCAM-1 was significantly upregulated from t = 24 until the end of pregnancy (ie, t = 168 hours). In cyclic rats no significant upregulation of VCAM-1 can be observed. VCAM-1 was absent or weakly expressed in glomeruli in normal day 14 pregnant or cyclic rats (t = −1), and after infusion of saline in pregnant or cyclic rats. The photomicrographs (Figure 6, b and c) show representative glomeruli of a pregnant endotoxin-treated rat, showing significant staining of
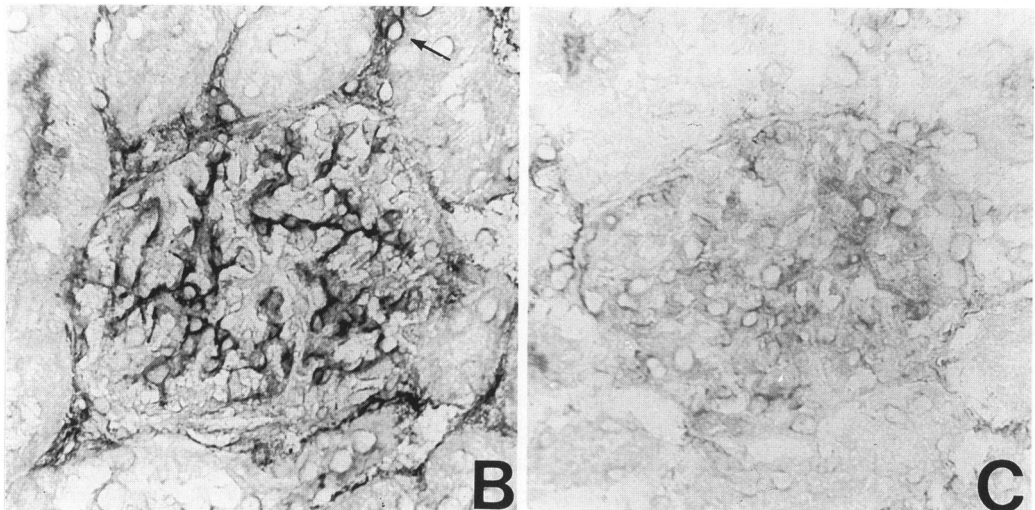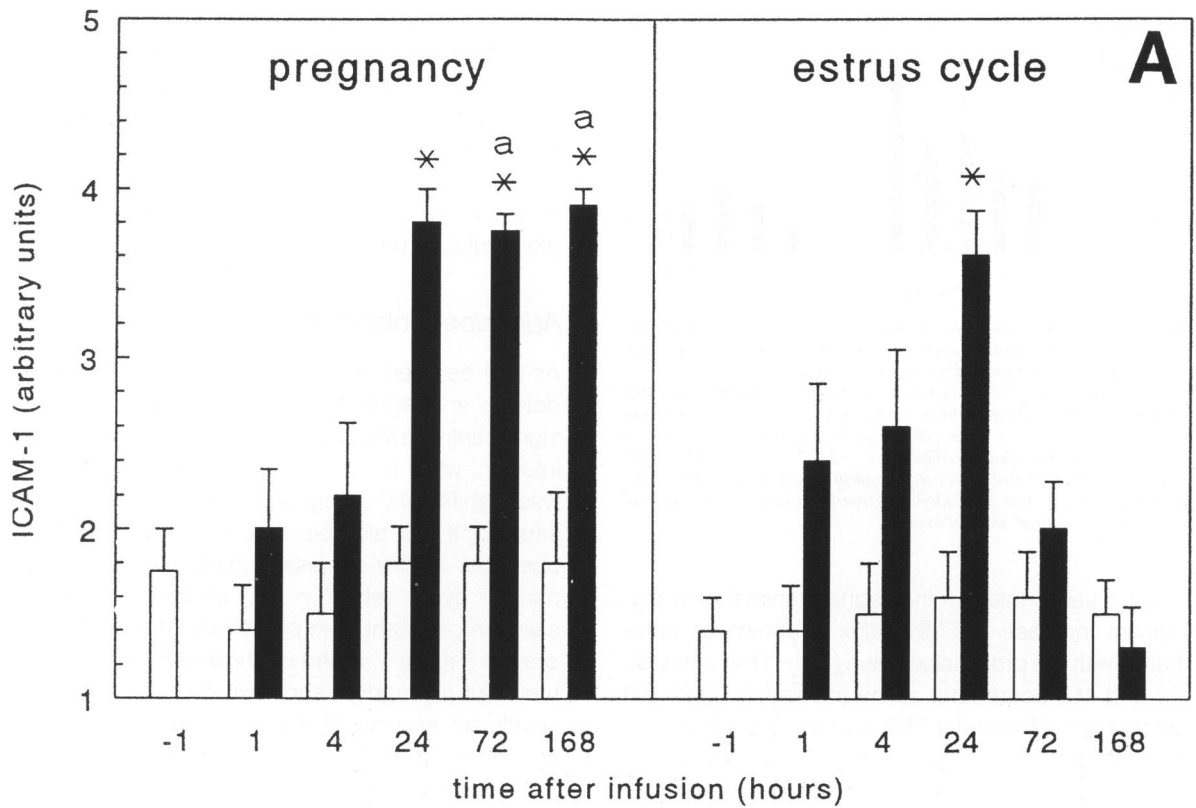
Figure 4. (A) *Mean glomerular ICAM-1, as detected immunohistochemically using anti-ICAM-1, in pregnant* (left) *and cyclic* (right) *rats before* (t = − 1) *and after infusion of 1.0 µg/kg bw endotoxin* (solid columns) *or saline* (open columns). *Infusions were started at t = 0. Columns represent mean ± SEM for n = 5 in all groups. ICAM-1 staining is scored using an arbitrary scale as described in Materials and Methods.* * *Significantly increased as compared with preinfusion values* (P < 0.05, *ANOVA followed by unpaired Student's t-test).* [a]*Significantly increased as compared with cyclic rats after identical treatment at the same interval* (P < 0.05, *unpaired Student's t-test).* (B *and* C) *Light microscopical micrographs of renal glomeruli of pregnant rats stained for ICAM-1. Significant staining of reaction product* (scale 4) *can be observed in the glomerulus of a pregnant rat 24 hours after endotoxin infusion* (B). *Note staining of reaction product predominantly along the glomerular capillary walls; also some interstitial staining* (peritubular capillaries) *can be seen* (arrow). (C) *Glomerulus of a pregnant rat 24 hours after saline infusion; no significant reaction product can be detected* (scale 1). (×350)
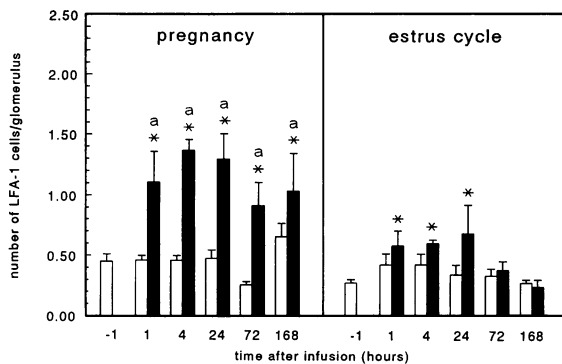
Figure 5. *Mean number of LFA-1-positive cells per glomerulus, as detected immunohistochemically with a monoclonal antibody against LFA-1, in pregnant* (left) *and cyclic* (right) *rats before* ($t = -1$) *and after infusion of 1.0 μg/kg bw endotoxin* (solid columns) *or saline* (open columns). *Infusions were started at* $t = 0$. *Columns represent mean ± SEM, for n = 5 in all groups. *Significantly increased as compared with preinfusion values* ($P < 0.05$, *ANOVA followed by unpaired Student's* t-test). *a Significantly increased as compared with cyclic rats after identical treatment at the same interval* ($P < 0.05$, *unpaired Student's* t-test).

reaction product (Figure 6b), and of a saline-treated pregnant rat, showing no reaction product (Figure 6c).

It can be seen from Figure 7 that after infusion of endotoxin in pregnant rats the number of VLA-4-positive cells was significantly increased 24, 72, and 168 hours as compared with the preinfusion value. The number of VLA-4-positive cells in pregnant endotoxin-treated rats was significantly higher as compared with cyclic endotoxin-treated rats, which did not show increased numbers of VLA-4-positive cells in the glomeruli. Saline infusion did not significantly affect the number of VLA-4-positive cells per glomerulus in pregnant or cyclic rats.

Discussion

In this study we investigated the kinetics of inflammatory cell influx and their potential O_2^- production, as well as the expression of adhesion molecules in the glomeruli of pregnant and cyclic rats after low dose endotoxin or saline infusion.

It is clear from the present data that pregnant endotoxin-treated rats show an altered pattern of glomerular cell influx as compared with cyclic rats after identical treatment. First, significantly increased amounts of infiltrated inflammatory cells were found in kidneys from pregnant *versus* cyclic animals after endotoxin treatment (Figures 1 and 3). Second, the pattern of cell influx, in particular the monocytes and to a lesser extent PMNs, showed a more persistent course as compared with the nonpregnant animals, in which a transient glomerular infiltration pattern could be observed.

In addition, in animals with experimental PE (ie, pregnant endotoxin-treated rats), apart from the increased inflammatory cell influx, significant increase in the percentage of activated PMNs (ie, O_2^--producing potential) occurred 1 and 4 hours after endotoxin infusion as compared with cyclic endotoxin-treated rats (Figure 2). Thus, in pregnancy not only a more persistent but also a more violent inflammatory reaction occurs after endotoxin treatment as compared with the nonpregnant condition.

This altered inflammatory reaction in pregnant animals is reflected by the patterns of adhesion molecule expression in pregnant *versus* cyclic rats after endotoxin infusion.

According to current concepts, adhesion of inflammatory cells to the vessel wall is a necessary step preceding extravasation. This is regulated by a number of adhesion molecules expressed on vascular endothelium (ie, E-selectin, ICAM-1, VCAM-1[19]), and on the surface of inflammatory cells themselves (LFA-1, VLA-4, serving as ligands for ICAM-1 and VCAM-1, respectively[20]). Both ICAM-1 and VCAM-1 show increased expression after 4 hours in glomeruli of endotoxin-treated pregnant rats up to 168 hours after infusion. The significant increase in cells expressing the ligands for these adhesion molecules (LFA-1 and VLA-4, respectively) in the glomeruli of pregnant endotoxin-treated rats is in line with the observed upregulation of adhesion molecules in these animals. Although ICAM-1 is also upregulated in the cyclic endotoxin-treated rat, this upregulation is considerably less than in pregnant rats and declines 24 hours after infusion. Similarly the number of cells expressing the respective ligands is reduced in the cyclic endotoxin-treated rat as compared with the pregnant endotoxin-treated rat.

These data clearly suggest binding of both PMNs as well as Mφ to the glomerular capillary wall in the present study, which is in line with other observations after endotoxin administration *in vivo*.[19,21] The persistent upregulation of VCAM-1 and VLA-4 may underlie the predominant influx of Mφ into the glomeruli in the pregnant endotoxin-treated rats, which together with ICAM-1 and LFA-1 may lead to activation *in situ* of these cells.[22] Since mononuclear cell influx is associated with proteinuria and inflammatory injury in some glomerular diseases,[23,24] it is likely that intraglomerular monocyte influx in the present model, next to activated PMNs, contributes to the proteinuria observed in the pregnant endotoxin-treated rat, although this remains to be confirmed.

The mechanism underlying the striking increase in glomerular adhesion molecules after endotoxin infusion in pregnant rats is not known. Various
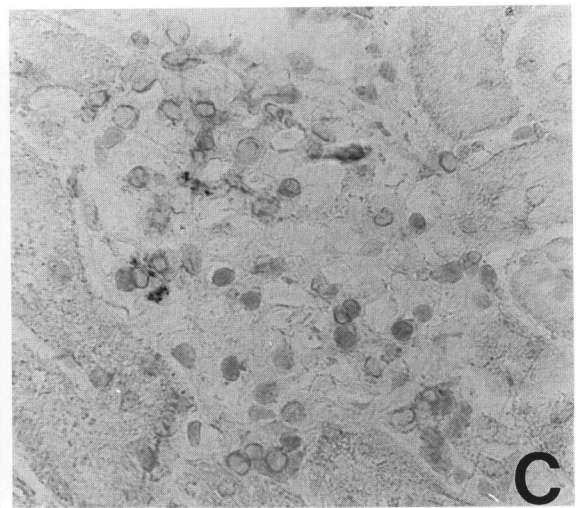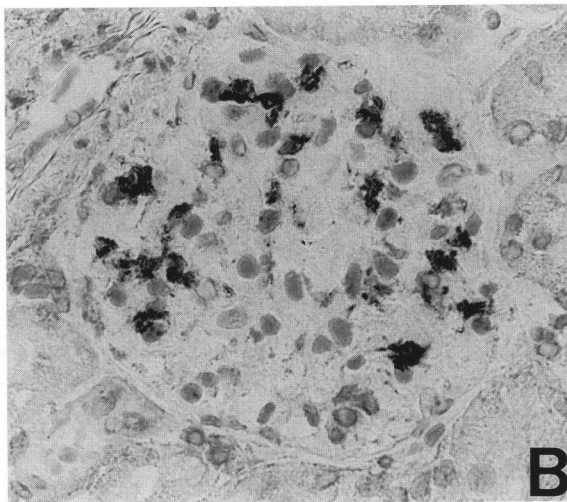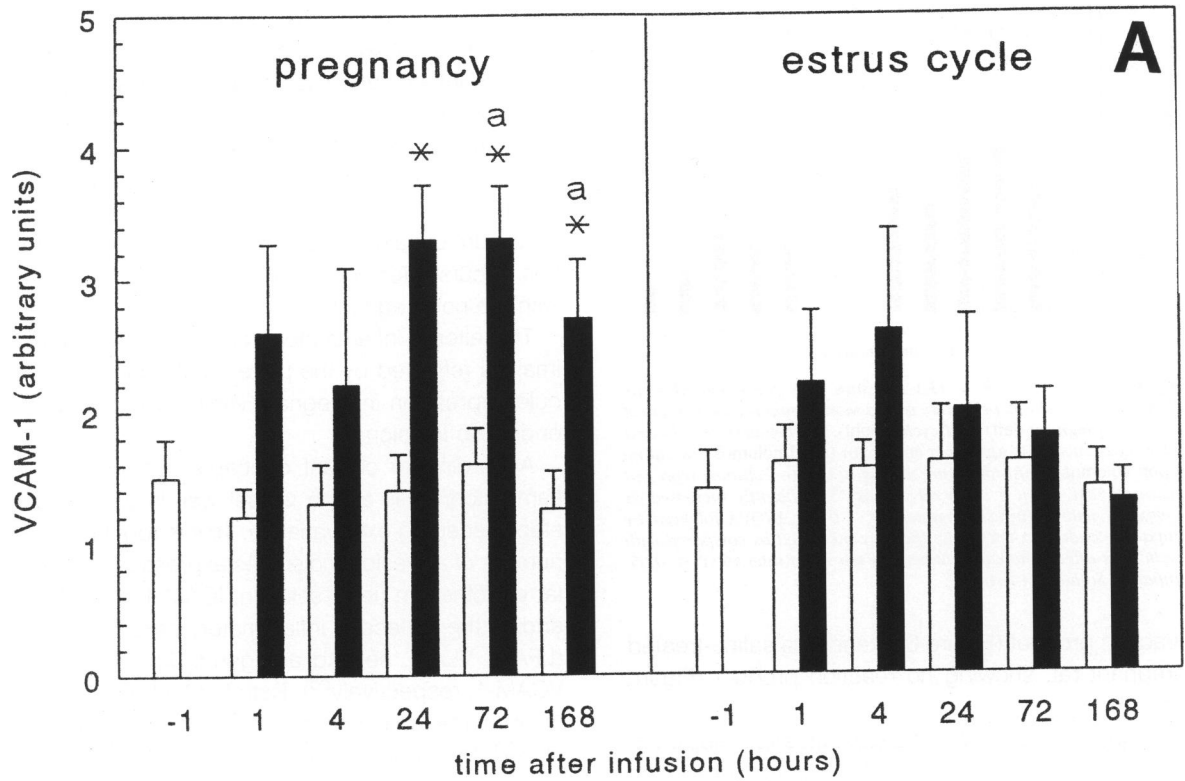
Figure 6. (A): Mean glomerular VCAM-1 staining, as detected immunohistochemically with a monoclonal antibody against VCAM-1, in pregnant (left) and cyclic (right) rats before (t = − 1) and after infusion of 1.0 μg/kg bw endotoxin (solid columns) or saline (open columns). Infusions were started at t = 0. Columns represent mean ± SEM, for n = 5 in all groups. VCAM-1 staining is scored using an arbitrary scale as described in Materials and Methods. *Significantly increased as compared with preinfusion values (P < 0.05, ANOVA followed by unpaired Student's t-test). ᵃSignificantly increased as compared with cyclic rats after identical treatment at the same interval (P < 0.05, unpaired Student's t-test). (B and C) Light microscopical micrographs of renal glomeruli of pregnant rats stained for VCAM-1. (B) Glomerulus of a pregnant rat, 168 hours after endotoxin infusion, showing a mesangial-like staining of reaction product (scale 3). (C): Glomerulus of a pregnant rat 168 hours after saline infusion. No significant reaction product can be observed (scale 1).

stimuli are able to upregulate expression of adhesion molecules including cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1, released by activated Mφ, as well as endotoxin itself.[19,25,26] In this respect it is interesting to note that in vitro 17-β-estradiol and progesterone are able to increase the sensitivity of endothelial cells to TNF-α.[27]
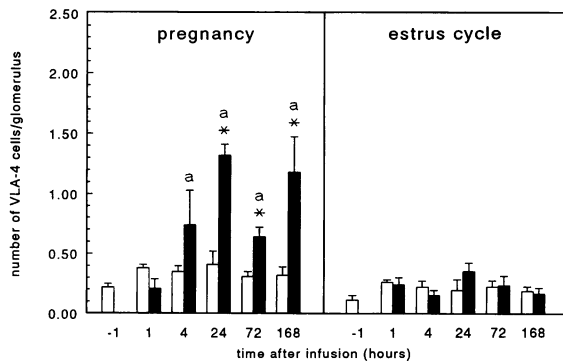
Figure 7. *Mean number of VLA-4-positive cells per glomerulus, as detected immunohistochemically with a monoclonal antibody against VLA-4, in pregnant* (left) *and cyclic* (right) *rats before* (t = −1) *and after infusion of 1.0 μg/kg bw endotoxin* (solid columns) *or saline* (open columns). *Infusions were started at* t = 0. *Columns represent mean ± SEM for n = 5 in all groups.* *Significantly increased as compared with preinfusion values* (P < 0.05, ANOVA followed by unpaired Student's t-test). ᵃ*Significantly increased as compared with cyclic rats after identical treatment at the same interval* (P < 0.05, unpaired Student's t-test).

Interestingly, recent concepts considering human PE as an endothelial cell disorder due to toxic oxygen injury[28] may relate to our data. Also in this experimental model for PE, damage of the microvasculature seems to occur, at least in the glomerular microvasculature. It is likely that this glomerular injury is mediated by oxygen free radicals. Glomerular ectonucleotidases, including endothelial ectoenzymes (eg, ADPase), are highly sensitive to toxic oxygen free radicals.[7] It has been shown in the rat kidney that reduction of the activity of these intraglomerular antiinflammatory and antithrombotic ectoenzymes by oxygen free radicals facilitates deposition of fibrinoid thrombi.[4,29] Thus, decreased activity of this glomerular enzyme exclusively in the pregnant endotoxin-treated rat may be related to increased O_2^- production, as reflected by both absolute and relative increase in activated PMNs in the glomeruli of endotoxin-treated pregnant rats. Moreover, O_2^- production of PMNs may vary with the endocrine condition of the animals, as only glomerular ADPase expression of pregnant rats, in contrast to cyclic, pseudopregnant or ovariectomized rats, is affected by the present regimen of endotoxin treatment.[30]

Taking the results together, it can be concluded that low dose endotoxin infusion in the pregnant condition elicits a persistent gradually increasing inflammatory reaction, which may be considered as pregnancy-specific, because in control nonpregnant animals a transient form of endotoxin-induced inflammation is observed. Up until now the nature of the underlying "pregnancy factor" responsible for this typical reaction is obscure, and requires further investigation.

Because to our knowledge immunohistological data of inflammatory parameters in the glomerular microvasculature in human PE are lacking, the question emerges whether similar inflammation kinetics may be observed in human PE. It is tempting to speculate from these results, that in human as in experimental PE, a "pregnancy-specific" inflammatory pattern occurs. Current experiments will hopefully shed more light on the inflammatory aspect of this intriguing disorder.

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