

Tumor necrosis factor- α in response to endotoxin administration in the pregnant guinea pig

Ilse Beckmann, MSc, PhD,^a Felicja Meisel-Mikołajczyk, MD, PhD,^b Fred K. Lotgering, MD, PhD,^a and Henk C.S. Wallenburg, MD, PhD^a

Rotterdam, The Netherlands, and Warsaw, Poland

OBJECTIVE: Our purpose was to test the hypothesis that an intramuscular endotoxin challenge induces production of tumor necrosis factor- α in the pregnant guinea pig and to investigate some of the metabolic effects.

STUDY DESIGN: Twelve randomly selected guinea pigs at 33 days' gestation with a sampling catheter in the carotid artery received an intramuscular injection of a solution of endotoxin isolated from *Bacteroides fragilis* ($n = 6$) or of solvent alone ($n = 6$). Plasma values of tumor necrosis factor- α , hematocrit, and 6-keto-prostaglandin $F_{1\alpha}$ were determined before and several hours after injection.

RESULTS: Tumor necrosis factor- α was detected in five of six guinea pigs, but it could not be demonstrated in five of six placebo animals. The hematocrit was significantly decreased, and prostaglandin $F_{1\alpha}$ significantly increased 24 to 48 hours after endotoxin injection.

CONCLUSION: In pregnant guinea pigs an intramuscular endotoxin challenge induces the release of tumor necrosis factor- α , followed by a reduced hematocrit and an increased prostacyclin concentration. These effects could be involved in the pathogenesis of endotoxin-induced fetal growth retardation. (Am J Obstet Gynecol 1996;175:218-21.)

Key words: Tumor necrosis factor- α , *Bacteroides fragilis* endotoxin, pregnant guinea pig, prostacyclin

In previous studies we showed in the pregnant guinea pig that repeated administration of *Bacteroides fragilis* endotoxin causes fetal growth retardation associated with fetal hypoglycemia, fetal and maternal hypertriglyceridemia, and a marked increase in maternal plasma prostacyclin (PGI_2) concentrations.^{1, 2} Similar biochemical changes have been reported to be induced by tumor necrosis factor- α (TNF- α) in animal experiments and in man. This potent and multifunctional cytokine, first described as a product of activated macrophages,³ was shown to be produced by many types of cells in female reproductive organs and tissues.⁴ It has a wide range of effects on cellular functions and interferes with carbohydrate—and fat metabolism⁵ and deoxyribonucleic acid—synthesis,⁶ induces anemia,⁷ and enhances prostaglandin synthesis.⁸ Our observations led us to speculate that TNF- α could be involved in the pathophysiologic mecha-

nisms triggered by bacterial endotoxin leading to fetal growth retardation.

The current study was designed to test the hypothesis that administration of *B. fragilis* endotoxin to the pregnant guinea pig induces formation of TNF- α associated with changes in the metabolism of PGI_2 , as previously observed in endotoxin-induced fetal growth retardation.

Material and methods

Experiments were performed in 12 randomly selected albino Dunkin-Hartley guinea pigs at 30 day's gestation. The first day of gestation was defined as the second day of the opening of the vaginal membrane. The animals were kept in individual cages in a controlled environment (19° C, 50% humidity, light-dark cycle 13:11 hours) and were fed commercial guinea pig pellets, hay, and water ad libitum. Vitamin C was added to the drinking water twice weekly. The guidelines for the care and use of guinea pigs approved by the Erasmus University animal experiments committee were followed.

Experimental protocol. On day 30 of gestation a polyethylene catheter was introduced into a carotid artery with the animal under general anesthesia with intramuscular ketamine hydrochloride (15 mg/kg body weight) and xylazine hydrochloride (2 mg/kg). After recovery, as judged by restored weight gain 3 or 4 days after surgery, an arterial sample was taken from all animals for the determination of anti-*B. fragilis* antibodies. The animals

From the Institute of Obstetrics and Gynecology, Erasmus University School of Medicine and Health Sciences,^a and the Department of Clinical Bacteriology, Institute of Biostructure, Medical Academy.^b

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Reprint requests: Henk C.S. Wallenburg, MD, PhD, Institute of Obstetrics and Gynecology, Erasmus University School of Medicine and Health Sciences, EE 2283, P. O. Box 1738, 3000 DR Rotterdam, The Netherlands.

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were then randomly divided into two groups of six, a placebo group (median weight 801 gm [range 695 to 875 gm]) and an endotoxin group (median weight 814 gm [range 636 to 880 gm]). At 0 hours the animals of the endotoxin group were given an intramuscular injection of 100 µg/kg endotoxin dissolved in 100 µl of sterile phosphate-buffered saline solution at pH 7.4 and mixed with 100 µl of complete Freund's adjuvant. The endotoxin was extracted from *B. fragilis* IPL E 323 and isolated as described previously.² The placebo group received an intramuscular injection of 100 µl of phosphate-buffered saline solution mixed with 100 µl of complete Freund's adjuvant. Blood samples (1 ml) for determination of TNF-α and hematocrit were taken from the arterial catheter into heparinized tubes immediately before (sample 0) and 4, 7, 9, 11, 24, and 48 hours after the start of the experiment. For the determination of prostaglandin F_{1α} (PGF_{1α}), the stable metabolite of PGI₂, blood samples (1.5 ml) were collected at 0, 11, 24, and 48 hours into cooled plastic tubes containing 10 µl of heparin and 25 µl of indomethacin (0.1% in phosphate buffer, pH 7.4). The samples were immediately centrifuged for 10 minutes at 1500g and 0° C and the supernatants stored at -80° C until analysis. After each sampling of heparinized blood without indomethacin, the remaining erythrocytes were resuspended in saline solution and returned to the animals through the arterial catheter.

Stimulation of TNF-α release in human and guinea pig whole blood. An additional experiment was done to compare the time course of release of TNF-α by human and guinea pig monocytes in vitro after stimulation with *B. fragilis* and *Escherichia coli* lipopolysaccharide. Blood samples from a healthy nonpregnant female human donor and from a healthy pregnant guinea pig were collected in commercial vacuum tubes with ethylenediaminetetraacetic acid (Becton-Dickinson, Rutherford, N.J.) and 0.5 ml blood was transferred to 6-well tissue culture plates (Costar, Cambridge, Mass.) and diluted 1:10 with RPMI 1640 culture medium 25 mmol/L HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) (Serlab, Drawley Down, United Kingdom) complemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 4 mmol/L L-glutamine, and 10% fetal calf serum (Sebak GmbH, Aidenbach, Germany). The samples were stimulated with 25 µl of phosphate-buffered saline solution (controls), 10 µl of lipopolysaccharide *E. coli* 0111 B4 (Sigma, St. Louis) (1 mg/ml phosphate-buffered saline solution, or 25 µl of lipopolysaccharide *B. fragilis* (1 mg/ml phosphate-buffered saline solution and incubated at 37° C for 48 hours in a humidified, 5% carbon dioxide atmosphere. Samples (1 ml) were taken after 0, 6, 24, and 48 hours of incubation and centrifuged for 10 minutes at 1500g and 4° C, and the supernatants were stored at -80° C until analysis.

Analytic procedures. Antibody titers against *B. fragilis*

endotoxin were determined in all 0-samples by means of a hemagglutination test on microtiter plates, with endotoxin-coated sheep erythrocytes as antigens, as described previously.¹

TNF-α in plasma and blood culture supernatants was assessed by bioassay⁹ with the TNF-α-sensitive murine fibrosarcoma WEHI 164 cell line. The WEHI cell line and human recombinant TNF-α were provided by Dr. R. Marquet, Erasmus University, Rotterdam. Cell death was measured after 20 hours of incubation at 37° C and 5% carbon dioxide by the colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide, Sigma) assay.¹⁰ A standard titration curve prepared with human recombinant TNF-α was used to calculate TNF-α values from measured cytotoxicity. Neutralization of cytotoxicity in supernatants of human blood cultures was performed with a polyclonal rabbit antihuman recombinant TNF-α antibody (Genzyme, Cambridge, Mass.). The detection limit of the assay was 6 pg/ml.

Hematocrit values were determined by the microcapillary technique. Plasma concentrations of 6-keto-PGF_{1α} were determined with a commercial radioimmunoassay (E.I. Du Pont de Nemours-NEN Research Products, Boston) as described previously.²

Statistical analysis. Friedman's two-way analysis of variance and Wilcoxon's rank-sum and rank-sign tests were used, as appropriate, to evaluate the differences between the continuous variables within and between groups. A value of $p < 0.05$ was chosen to represent statistical significance.

Results

Before the start of the experiments, on gestational day 33 or 34, hemagglutination titers against *B. fragilis* endotoxin were <1:10 in all animals.

Bioactive TNF-α was detected in plasma of five of the six animals in the endotoxin group 9 hours after endotoxin administration, with a significant rise to a mean level of 400 pg/ml at 11 hours, followed by a significant fall to levels below the detection limit at 48 hours; one guinea pig showed weak cytotoxicity at 24 hours only. In the placebo group five of six animals did not have any cytotoxicity at all within 48 hours; one animal reacted with low cytotoxicity at 11 and 24 hours. The results for all animals are presented in Fig. 1, A.

In the endotoxin group hematocrit values were significantly ($p < 0.03$) reduced by 33% and 30% at 24 and 48 hours, respectively, but no significant changes in hematocrit values were observed in the placebo group during the course of the experiment (Figure 1, B).

Levels of 6-keto-PGF_{1α} in the endotoxin-treated guinea pigs showed a significant rise after 11 hours, whereas no significant changes were observed in the placebo group (Fig. 1, C).

The results of the experiments with *B. fragilis* and *E. coli*

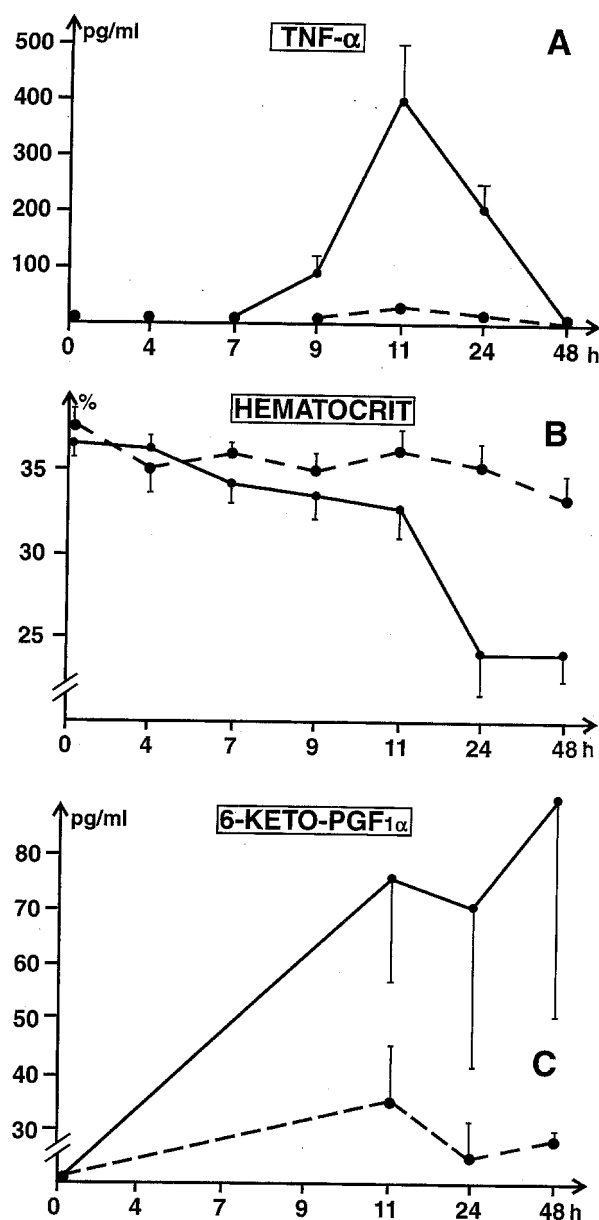


Fig. 1. Mean (\pm SEM) values of TNF- α (A), hematocrit (B), and 6-keto-PGF_{1 α} (C) in plasma of guinea pigs after intramuscular administration of *B. fragilis* endotoxin or placebo. Solid lines, Endotoxin group; dashed lines, placebo group.

endotoxin to stimulate release of TNF- α in guinea pig and human monocytes are shown in Fig. 2. Cytotoxicity in guinea pig samples followed a course similar to that in human blood. In human samples cytotoxicity was completely neutralized with anti-TNF- α antibody. Maximum secretion of TNF- α after *B. fragilis* endotoxin stimulation was reached in both cultures after 24 hours whereas *E. coli* lipopolysaccharide stimulated TNF- α release in both cultures, with maximal response after 6 hours. TNF- α peak levels in supernatants of human and guinea pig blood cultures stimulated with *E. coli* lipopolysaccharide were higher than those by stimulation with *B. fragilis* endotoxin.

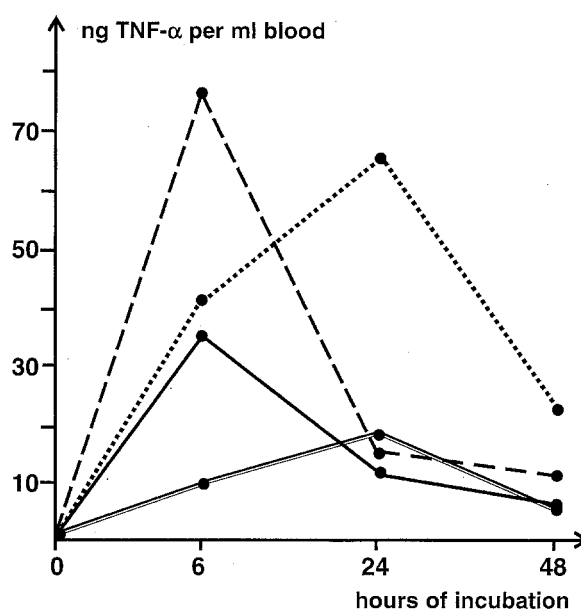


Fig. 2. Release of TNF- α from monocytes in full blood cultures after stimulation with lipopolysaccharide *E. coli* 0111 B4 and lipopolysaccharide *B. fragilis* for 6, 24, and 48 hours. Solid line, Human blood stimulated with lipopolysaccharide *E. coli*; double solid line, human blood stimulated with lipopolysaccharide *B. fragilis*; dashed line, guinea pig blood stimulated with lipopolysaccharide *E. coli*; dotted line, blood stimulated with lipopolysaccharide *B. fragilis*.

Comment

We chose the sensitive and specific WEH1 bioassay for analysis of TNF- α rather than an immunoassay. Depending on the choice of the antibody, immunoassays detect various TNF- α monomers and polymers, TNF- α degradation products, or TNF- α /sTNF- α -receptor complexes, which are biologically inactive.¹¹

In plasma of pregnant guinea pigs we detected TNF- α 9 hours after administration of *B. fragilis* endotoxin, with peak values at 11 hours and a return to baseline levels after >24 hours. No reports of comparable experiments could be found in the literature. Zuckerman and Bendele¹² found that intraperitoneal administration of *E. coli* lipopolysaccharide (2 mg/kg body weight) in nonpregnant guinea pigs caused a rapid rise of serum TNF- α levels, with peak values between <0.2 and 180 ng/ml 2 hours after injection and a return to base level after 6 hours. The differences in the time course and peak values of TNF- α between that study and our experiments in pregnant guinea pigs may be explained by the low concentration of injected lipopolysaccharide (100 μ g/kg body weight) in our experiments and, in particular, by the intramuscular administration of the endotoxin, with a much slower uptake into the maternal circulation than after intraperitoneal or intravenous injection.

Also, the bacterial origin of the endotoxin may influence the host's immunologic and pathophysiologic response. Most studies involving the release of TNF- α after injection of endotoxins in mice used intravenously or in-

traperitoneally administered lipopolysaccharide from gram-negative aerobes, primarily Enterobacteriaceae such as *E. coli* or *Salmonella* species. In our model of endotoxin-induced fetal growth retardation² in the pregnant guinea pig we used the purified lipopolysaccharide of *B. fragilis* because *Bacteroides* species are frequently involved in intrauterine infections during pregnancy.¹³ There is evidence that the endotoxin of *B. fragilis* is biologically less active than are the endotoxins isolated from Enterobacteriaceae.¹⁴ This may also explain the difference in peak levels of TNF- α stimulated by *E. coli* and *B. fragilis* endotoxin as observed by Zuckerman and Bendele¹² and in the stimulation experiments shown in Fig. 2.

The neutralization of cytotoxicity in human blood supernatants by anti hu-re TNF- α antibody identified the cytotoxic agent as TNF- α . The lack of an anti-guinea pig TNF- α antibody and the low and irreproducible cross-reactivity of antihuman TNF- α antiserum with guinea pig TNF- α precluded the exact identification of the cytotoxicity in guinea pig plasma. However, the similar time course of TNF- α release in human and guinea pig blood cultures after stimulation with *B. fragilis* and *E. coli* lipopolysaccharide suggests that the released cytotoxic factor in guinea pig blood was indeed TNF- α . This assumption is supported by the fact that the WEHI cell line used in our experiments to detect cytotoxicity in guinea pig plasma is specifically sensitive to TNF- α and insensitive to other cytokines such as interleukin-1 α , interleukin-1 β , interleukin-6, and interferon gamma.¹⁵

The observation of reduced hematocrit values in endotoxin-treated guinea pigs 24 to 48 hours after endotoxin administration (Fig. 1, B) may be explained by the capacity of endotoxin or TNF- α to induce anemia in vivo by reducing the life span of circulating red blood cells and interfering with erythropoiesis.^{7, 16} Microscopic blood loss in urine or stool from TNF- α cytotoxicity¹⁷ could be involved, but this was not determined in our experiments.

Our observation of a significant increase in circulating PGF_{1 α} 11 and 24 hours after endotoxin injection is in agreement with results of studies in humans in whom TNF- α was triggered by endotoxin and was shown to cause increased levels of circulating PGI₂.⁸ An increased production of PGI₂ was also observed after stimulation of guinea pig macrophages with endotoxin in vitro.¹⁸

In human pregnancy elevated levels of bioactive TNF- α in midtrimester amniotic fluid were associated with subsequent small-for-gestational-age births and various pathophysiologic mechanisms affecting the fetus, placenta, or both, by which this multifunctional cytokine may mediate impaired fetal growth have been suggested.¹⁹ The results of our experimental study in the pregnant guinea pig support the concept that the release of bacterial endotoxin in subclinical infection may induce abnormal activation of the immune system with release of cytokines, which may not only contribute to the occurrence of preterm labor¹³ but may also in some cases lead to impairment of fetal growth.

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