Enhanced tumor necrosis factor- α -specific and decreased interleukin-10-specific immune responses to LPS during the third trimester of pregnancy in mice

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Abstract

It is increasingly apparent that there is a bidirectional interaction between the maternal immune system and the reproductive system during pregnancy. Pregnancy is associated with a suppression of maternal specific immune responses, which process underlies the protection of fetal tissues expressing paternally inherited alloantigens. However, recent evidence indicates that the suppression of specific, lymphocyte-mediated immune responses during pregnancy is accompanied by activation of the non-specific arm of the maternal immune response. In the present study, we have investigated the effect of pregnancy on the non-specific immune response induced by bacterial lipopolysaccharide (LPS, endotoxin) in mice. Pregnancy enhanced the LPS-induced production of proinflammatory cytokines, including tumor necrosis factor- α , interleukin (IL)-6, and interferon- γ . On the other hand,

LPS-induced levels of the anti-inflammatory cytokine IL-10 were suppressed in pregnant mice. These alterations in cytokine production correlated with an increased susceptibility for endotoxemic mortality in the pregnant mice. Although adrenergic receptors are important regulators of cytokine production in non-pregnant mice, the α_2 - and the β -adrenoceptor-mediated modulation of cytokine production ceases to operate during pregnancy associated with severe endotoxemia. These data may explain how excessive activation of the non-specific immune responses during pregnancy can contribute to the increased severity of some maternal diseases, including septic shock, and can be an important pathophysiological factor in disseminated intravascular coagulation or preeclampsia.

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Introduction

In his classic 1953 paper, Medawar proposed the concept of the 'fetal allograft' to explain the immune relationship between mother and fetus. In this model, immunological interaction between mother and fetus is suppressed, through either lack of fetal-antigen presentation to maternal lymphocytes or maternal lymphocyte suppression.

Subsequently, this view of a generalized lymphocyte anergy during pregnancy became more refined, as it was suggested that maternal immune responses would be biased from a T helper (Th)1-type cellular response to a Th2-type, less damaging humoral response (Wegmann *et al.* 1993). These observations are consistent with clinical evidence that pregnant women with rheumatoid arthritis (a Th1-type autoimmune disease) experience a temporary remission of their symptoms, whereas the severity of

systemic lupus erythematosus (a mainly Th2-driven disease) tends to be exacerbated during pregnancy (Ostensen 1999).

Although such a decrease in lymphocyte responsiveness explains some of the clinical findings observed in pregnancy, there are a number of observations suggesting that the model of a shifted Th1/Th2 response does not fully explain the substantial changes in maternal immune system function. For example, if a decrease in Th1-type immune responses is the dominant feature of the pregnant immune system, then it would be expected that pregnant women would be more susceptible to infection with a variety of intra- and extracellular pathogens. However, this is not the case, because despite the decreased production of Th1 cytokines, pregnant women are relatively resistant to overwhelming infection (Sacks *et al.* 1999). Furthermore, in the experimental Shwartzmann reaction, pregnant animals, far from being immunosuppressed, are

uniquely primed to respond, often fatally, to just a single injection of endotoxin (Mori 1981).

To explain these phenomena, Sacks *et al.* (1999) proposed a new paradigm for the maternal immune response. According to this paradigm, the specific, lymphocyte-mediated arm of the immune response is accompanied, and perhaps compensated, by the activation of the non-specific immune system, in which the monocyte/macrophage and granulocyte rather than the lymphocyte assumes a central role in immunological adaptation. In support of this hypothesis, there is a plethora of data demonstrating that the maternal non-specific immune system is activated during pregnancy. For instance, in normal pregnancy, circulating monocytes and granulocytes in whole blood have activated phenotypes, in some ways comparable with changes observed in systemic inflammatory states. These inflammatory changes consist of an increase in the numbers and phagocytotic capacity of monocytes and granulocytes (Koumandakis *et al.* 1986), enhanced expression of CD11b, CD14 and CD64 (Sacks *et al.* 1998, Faas *et al.* 2000) and an increase in intracellular reactive oxygen species (Sacks *et al.* 1998, Faas *et al.* 2000). Importantly, the increased expression of CD11b, CD14 and intracellular reactive oxygen species is even more pronounced in preeclampsia than in normal pregnancy (Redman *et al.* 1999).

Based on the above evidence documenting that various inflammatory functions of the innate immune system are upregulated during pregnancy, we hypothesized that pregnancy would increase systemic levels of inflammatory cytokines derived from cells of the innate immune system. The data presented in this paper demonstrate that in an endotoxemic model of the systemic inflammatory response of the innate immune system, the production of monocyte/macrophage/neutrophil-derived proinflammatory cytokines, including tumor necrosis factor (TNF)- α and interleukin (IL)-6, as well as interferon (IFN)- γ , is greatly increased in pregnant mice. On the other hand, in contrast to the generally held view that pregnancy is characterized by increased IL-10 production (Elenkov *et al.* 1997), we show that the levels of IL-10 are substantially blunted in endotoxemic pregnant mice. Finally, we provide evidence that not only the production of inflammatory cytokines is altered in pregnant mice as compared with non-pregnant mice, but that some of the endogenous regulatory mechanisms provided by the sympathetic nervous system that control cytokine production in non-pregnant mice are lost in pregnant mice.

Materials and Methods

Animals

Swiss mice (20–30 g) were purchased from Charles River Laboratories (Budapest, Hungary) and kept in the Animal Care Unit for 7 days before use. Animals received food and

water freely, and lighting was maintained on a 12 h cycle. The female mice were caged for 24 h with the male mice and then checked for the presence of a vaginal plug. The pregnant group was composed of those female mice that were positive for the presence of a vaginal plug, while the others formed the control, non-pregnant group. In this respect, the assignment of the animals was not random. The experiments were carried out on 17–18 day pregnant mice. These animals are referred to in the text as pregnant mice in the third trimester; 7–8 day pregnant mice were taken as pregnant animals in the first trimester. The animal experiments described in this article were performed with adherence to National Institutes of Health guidelines on the use of experimental animals. Approval of the Animal Use Committee of the Institute of Experimental Medicine, Hungarian Academy of Sciences was obtained prior to initiating the experiments.

Experimental design for plasma cytokine measurements

Animals were injected i.p. with drug vehicle (sterile physiological saline) or drugs in a volume of 0.1 ml/10 g body weight. Thirty minutes later, they were challenged with 10 or 50 mg/kg of bacterial lipopolysaccharide (LPS) administered i.p. Animals were killed 90 min or 4 h after LPS treatment, if not otherwise indicated. The 90 min and 4 h time points represent the peak response of the production of TNF- α and IL-10 respectively in response to a single dose of LPS (Elenkov *et al.* 1995, Haskó *et al.* 1995, 1996, 1998). Propranolol (10 mg/kg), a β -adrenoceptor-blocking drug, or CH-38083 (10 mg/kg), a selective α_2 -adrenoceptor-blocking drug (Vizi *et al.* 1986), was injected 30 min before LPS. Blood was collected in ice-cold Eppendorf tubes containing heparin and centrifuged for 10 min at 4 °C. The plasma was stored at –70 °C until assayed.

Cytokine assays

Plasma levels of cytokines (TNF- α , IL-6, IFN- γ , IL-10 and IL-1 α) were determined using a solid-phase enzyme immunoassay, employing the multiple antibody sandwich principle that specifically detects murine cytokines (Genzyme Corp., Boston, MA, USA). The standard error of the mean (S.E.M.) of duplicate samples for cytokine readings was $\pm 5\%$. Absorbance was recalculated as concentration (ng/ml) using a standard curve by Microplate Manager/PC Data Analysis Software (Bio-Rad, Richmond, CA, USA). Plates were read at 450 nm by a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Detection limits were 15 pg/ml TNF- α , 10 pg/ml IL-6, 5 pg/ml IFN- γ and 15 pg/ml IL-10. Assays were performed according to the manufacturer's instructions and as described in our previous studies (Haskó *et al.* 1996, 2000).

Endotoxin-induced lethality studies

Pregnant or non-pregnant mice were subjected to an i.p. LPS challenge (50 mg/kg). Survival was recorded 24, 48, 72, 96 h and 2 weeks after the LPS injection.

Drugs

LPS from *Escherichia coli* 055:B5, purchased from Sigma Chemical Co. (St Louis, MO, USA), was dissolved in distilled water; the mixture was sonicated for at least 3 min in a sonication bath, and aliquots were stored at -40°C until use. After they were thawed, appropriate dilutions were made in saline in polypropylene tubes. CH-38083 (7,8-(methylenedioxy)-14 α -hydroxy-alloberbane HCl) was obtained from Chinoin (Budapest, Hungary). Propranolol, isoproterenol and EDTA dipotassium salt were purchased from Research Biochemical International (Natick, MA, USA). Drugs were dissolved in saline on the day of experiment and used immediately.

Statistical evaluation

Values in the figures and text are expressed as mean $\pm$ s.e.m. of n observations. Statistical analysis of the data was performed by the Student's unpaired t -test or the Mann-Whitney test, as appropriate. $P < 0.05$ was considered statistically significant.

Results

Effect of LPS on plasma level of cytokines in control and pregnant mice

i.p. injection of 50 mg/kg LPS resulted in appearance of increased plasma levels of TNF- α and IL-10 in non-pregnant mice at 90 min after the LPS challenge (Fig. 1). Although levels of both cytokines were not detected in both pregnant and non-pregnant mice challenged with the vehicle for LPS (physiological saline; data not shown), pregnant mice produced significantly more TNF- α and significantly less IL-10 than non-pregnant mice in response to LPS as determined 90 min after LPS exposure (Fig. 1A and B). At 4 h after LPS, the suppressive effect of pregnancy on IL-10 production was even more pronounced (Fig. 1C), whereas TNF- α levels were not detected in either group (not shown). In addition, following LPS challenge, the plasma levels of IL-6 and IFN- γ were much higher in pregnant mice than in control animals (Table 1). In contrast, no difference was seen between non-pregnant and pregnant animals when IL-1 α plasma levels were assayed (Table 1). Finally, the TNF- α response to LPS was significantly higher in pregnant mice than in their controls

Figure 1 Plasma levels of TNF- α (A) and IL-10 (B and C) in non-pregnant and pregnant mice in response to LPS administered at a dose of 50 mg/kg (i.p.). The plasma was collected 90 min (A and B) or 4 h (C) after LPS injection. Note that TNF- α response to LPS was significantly higher and that of IL-10 was significantly lower. ** $P < 0.01$, *** $P < 0.001$. $n = 8-10$ per group.

even in the first trimester (Fig. 2). Taken together, pregnancy augments the production of the proinflammatory cytokines TNF- α , IL-6 and IFN- γ , but suppresses the production of the anti-inflammatory cytokine IL-10.

Table 1 Changes in the LPS-evoked (50 mg/kg) response of inflammatory mediator levels in pregnancy (third trimester). Plasma levels (pg/ml ± s.d.) were assayed 240 min (IL-1α and IFN-γ) and 90 min (IL-6) after LPS injection. Number of mice in brackets

	IL-1-α	IL-6	IFN-γ
Non-pregnant	70.3 ± 35.9 (8)	42 300 ± 12 700 (10)	104.9 ± 83.0 (10)
Pregnant	89.4 ± 43.2 (8)	113 270 ± 4073 (10)*	182.8 ± 89.6 (10)*

*P<0.01.

Figure 2 Changes in plasma levels of TNF-α following i.p. administration of LPS at a dose of 10 mg/kg in the third trimester. First trimester: 7–8 days of pregnancy. Third trimester: 17–18 days of pregnancy. *n*=8–10 per group. **P*<0.05.

Pregnancy increases mortality in LPS-treated mice

Because pregnancy skewed the cytokine response towards a proinflammatory profile, we hypothesized that pregnancy would increase LPS-induced lethality. Figure 3

Figure 3 Pregnancy increases mortality in LPS-treated mice. Pregnant and non-pregnant mice were treated with 120 mg/kg LPS and observed for 2 weeks after LPS injection. There were no changes in lethality in either group after 96 h. *n*=16 per group. ***P*<0.01, ****P*<0.001.

shows that in agreement with this prediction, pregnancy enhanced the mortality rate in mice treated with 50 mg/kg LPS.

Effect of selective block of α₂- and β-adrenoceptors and activation of β-adrenoceptors on LPS-induced cytokine production in non-pregnant and pregnant mice

Because in previous studies (Elenkov *et al.* 1995, 2000, Vizi *et al.* 1995, Haskó & Szabó 1998, Szelényi *et al.* 2000) we showed that sympathetic outflow modulates LPS-induced cytokine production in non-pregnant mice via α₂- and β-adrenoceptor-mediated mechanisms, in the present study we tested whether such a modulation is operative in pregnant mice.

First, we used the selective α₂-adrenoceptor antagonist CH-38083 (Vizi *et al.* 1986) to study the extent of immunomodulation by α₂-adrenoceptors in non-pregnant and pregnant mice. CH-38083 (10 mg/kg, i.p.) injected 30 min before the LPS challenge (10 mg/kg) caused a significant suppression of TNF-α plasma level in both the non-pregnant and pregnant mice (Fig. 4). In addition, blockade of α₂-adrenoceptors by CH-38083 significantly enhanced the plasma level of IL-10 in non-pregnant but not in pregnant mice (Table 2). The β-adrenoceptor antagonist propranolol, administered at the dose of 10 mg/kg 30 min prior to the administration of LPS, completely antagonized the effect of LPS to increase the IL-10 level in the plasma (Table 2) in both non-pregnant and pregnant mice.

This was not the case when LPS was administered at a high dose mimicking severe toxemia (Table 2). The blockade of β-adrenoceptors by propranolol significantly increased the TNF-α level produced by LPS in the plasma from 4.8 ± 1.1 to 9.9 ± 1.2 ng/ml (*n*=6, *P*<0.05). This effect was also seen in pregnant mice (Fig. 4). In pregnant animals, when LPS was administered at a high dose (50 mg/kg), both propranolol and CH-38083 failed to exert their effects: e.g. IL-10 plasma levels were not reduced or increased respectively (Table 2).

Discussion

In the early 1990s, it was proposed that the immune suppression observed in pregnancy does not affect all

Figure 4 Boosting sympathetic outflow by blocking negative feedback modulation (CH-38083, 10 mg/kg) significantly reduces the TNF- α response to LPS (10 mg/kg, i.p.) in non-pregnant mice, but in the third trimester of pregnant mice it was significantly less effective. CH-38083, a selective α_2 -adrenoceptor blocker, a drug able to block the negative feedback modulation of NE release from the sympathetic neurons, was injected i.p. 30 min prior to LPS administration. $n=6-10$ mice per group. Propranolol (10 mg/kg, i.p.), a β -adrenoceptor blocker administered 30 min earlier, significantly enhanced plasma TNF- α levels in response to LPS (10 mg/kg, i.p.) in both non-pregnant and pregnant animals. There is a significant difference between the two values. $n=6-10$ mice per group. * $P<0.05$, ** $P<0.01$ *** $P<0.001$.

Table 2 Modulation of the LPS-evoked IL-10 levels in pregnancy (third trimester) by adrenergic drugs (10 mg/kg). Values are percent of controls $\pm$ S.E.M. IL-10 levels were 4.2 ± 0.2 and 0.6 ± 0.1 ng/ml in non-pregnant and pregnant mice ($P<0.05$). Number of mice in brackets

Pregnancy		Control (a)	Propranolol (b)	CH-38083 (c)	Significance
LPS (mg/kg) 10	Non-pregnant	100 $\pm$ 13.5 (6)	7.9 $\pm$ 2.0 (6)	272.9 $\pm$ 26.1 (8)	a:b= $P<0.05$ a:c= $P<0.05$
	Pregnant	100 $\pm$ 63.8 (6)	10.6 $\pm$ 6.6 (6)	321.9 $\pm$ 148.9 (8)	a:b= $P<0.05$ a:c=n.s.
50	Non-pregnant	100 $\pm$ 10.3 (6)	17.2 $\pm$ 2.1 (8)	268.8 $\pm$ 56.0 (8)	a:b= $P<0.05$ a:c= $P<0.05$
	Pregnant	100 $\pm$ 27.5 (6)	114.0 $\pm$ 9.3 (8)*	96.4 $\pm$ 20.4 (8)*	a:b=n.s. a:c=n.s.

* $P<0.05$ between non-pregnant and pregnant animals.

lymphocyte functions (Wegmann *et al.* 1993). Rather, there seems to be a shift from a Th1-type cell-mediated immune response towards a Th2-type, antibody-mediated response. According to this hypothesis, the production of Th1-type cytokines (IFN- γ , IL-2 and TNF- α) would be decreased, while the production of Th2 cytokines (IL-10, IL-4 and IL-5) would be increased in pregnant women. Our data obtained in this study demonstrate that systemic stimulation of the innate immune system with LPS results in an inverse cytokine profile, with increased TNF- α and IFN- γ , and decreased IL-10 level production. While our data seem to contradict the notion that pregnancy is characterized by enhanced IL-10 and decreased TNF- α and IFN- γ production, this is not the case. That is because the concept of Th2 cytokine overproduction and deficient Th1-type cytokine production in pregnancy is mainly applicable to cells of the specific immune system (Sacks

et al. 1999). Furthermore, such a shift towards the Th2 direction is most clearly manifest at the level of the maternal-fetal interface of normal pregnancy (Lin *et al.* 1993). However, in our model, we induced systemic production of cytokines by cells of the innate immune system, whereas in most earlier studies, the focus was on cytokines produced by T lymphocytes. Our results regarding the excessive production of proinflammatory cytokines are in good agreement with those that found that maternal innate (non-specific) immune responses are increased during pregnancy (Sacks *et al.* 1999, 2000). Furthermore, because we observed an overproduction of most proinflammatory cytokines, but a substantial decrease in IL-10 production, we propose that the concept of a general activation of the innate immune system needs to be further refined. That is, it appears that placental factors induce a change in the phenotype of the innate immune

system from an anti-inflammatory to a proinflammatory one. Since IL-10 is one of the most important anti-inflammatory factors that can downregulate most pro-inflammatory processes, it is plausible that the decrease of IL-10 production is central to the increased activity of innate immune system during pregnancy.

An important aspect of the excessive monocyte/macrophage activation observed during pregnancy is the recently proposed notion that preeclampsia is a generalized intravascular, inflammatory response, resulting from the extreme activation of the non-specific immune system (Redman *et al.* 1999). This notion is supported by substantial evidence demonstrating that monocytes and granulocytes are activated during preeclampsia. For example, the increased expression of CD11b, CD14 and intracellular reactive oxygen species is even more pronounced in preeclampsia than in normal pregnancy (Redman *et al.* 1999). There is also increased release of TNF- α and IL-6 into the circulation (Vince *et al.* 1995). For these reasons, it was suggested that preexisting or concurrent infections could be an important precipitating factor in the induction of preeclampsia (Redman *et al.* 1999). This is consistent with an important rat model of preeclampsia, in which a single microdose of endotoxin triggers a preeclampsia-like state with hypertension and proteinuria that persists until the end of pregnancy (Faas *et al.* 1994). It is also noteworthy that the generalized Schwartzman reaction, which is induced by endotoxin, shares a number of pathophysiological phenomena with preeclampsia (McKay *et al.* 1953). These include disseminated intravascular coagulation and renal cortical necrosis (Beller & Schoendorf 1972, Faas *et al.* 1994). The fact that similar to our observation in endotoxemia, there is a deficiency in IL-10 production and increase in TNF- α and IL-6 formation in preeclampsia (Hennessy *et al.* 1999) further substantiates the notion that preeclampsia arises when a universal maternal inflammatory response to pregnancy decompensates (Redman *et al.* 1999).

It is known that several factors can influence the development of inflammatory responses both *in vitro* and *in vivo*. Strong evidence is available that the effect of the central nervous system on immune responses is mediated by norepinephrine (NE) released from noradrenergic varicosities via activation of α_{2B} - and β_2 -adrenoceptors (Severn *et al.* 1992, Spengler *et al.* 1994, Elenkov *et al.* 1995, 1996, Vizi *et al.* 1995, Fessler *et al.* 1996, Straub *et al.* 1996, 2000, Suberville *et al.* 1996, van der Poll *et al.* 1996, Elenkov *et al.* 2000, Miller *et al.* 2000). Attention has been focused recently on α - and β -receptor-mediated modulation by endogenous NE of pro- and anti-inflammatory cytokine production (Haskó *et al.* 1995, Fessler *et al.* 1996). For example, the β -adrenoceptor antagonist propranolol, removing the stimulatory effect of endogenously released NE on β_2 -adrenoceptors expressed on cytokine-producing cells, increases the TNF- α (Elenkov *et al.* 1995) and inhibits the IL-10 production

normally induced by LPS (Szelényi *et al.* 2000). On the other hand, when the sympathetic outflow, i.e. the release of endogenous NE, is increased by CH-38083, LPS-induced TNF- α levels are augmented and IL-10 levels are reduced (Szelényi *et al.* 2000). It was surprising to learn in this study that in pregnant animals, the modulation of both IL-10 and TNF- α production by endogenous NE via α_2 - or β -adrenoceptors does not operate. However, with the exception of α_2 -adrenoceptor-mediated modulation of IL-10 production, the effect of this pregnancy-induced unresponsiveness to endogenous NE was only apparent when a high dose of LPS was used. A possible explanation for this phenomenon is that the excessive release of endogenous NE caused by a high dose of LPS administration plus the effect of pregnancy result in a downregulation of both α_2 - and β -adrenoceptors that are the targets of the cytokine-modulatory effect of endogenous NE.

In conclusion, the present study was carried out to test whether pregnancy alters the production of cytokines to a systemic endotoxin challenge. The results of this study demonstrate that there is a substantial change in the cytokine profile in pregnant animals, which consists of an increase in proinflammatory cytokine production and decrease of the production of IL-10. Consistent with such a proinflammatory phenotype of cytokine production, pregnancy enhances LPS-induced mortality, which may have important implications for septic pregnant women. Further studies will be required to elucidate the causative factors and mechanisms responsible for the proinflammatory phenotype of the innate immune response in pregnancy.

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