Cytokine expression in the uterus of mice with pregnancy loss: effect of maternal immunopotential with GM-CSF

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It is believed that failure of the maternal immune system to actively support embryonic development, through production of the appropriate cytokine network, might be responsible for embryonic death. Thus, the aim of this study was to evaluate the possible involvement of cytokines such as tumour necrosis factor α (TNF- α) and transforming growth factor β 2 (TGF- β 2), which are crucial for normal embryonic development, in the early stages of mechanisms that mediate induced pregnancy loss. The early stages of the resorption process induced by lipopolysaccharide (LPS) were characterized by blood accumulation in the vicinity of the embryo, preceding any visible embryonic damage. At that time, immunohistochemical analysis revealed an increased expression of TNF- α in the primary and secondary

decidua, which was reduced as the resorption process was completed. In contrast, TGF- β 2 expression was decreased in the primary and secondary decidua, as well as in the glandular epithelium, at all the times assessed. Maternal immunopotential with granulocyte macrophage-colony stimulating factor (GM-CSF), which controls maternal immune activities supporting normal embryonic development, decreased the resorption rate in LPS-treated mice while normalizing the expression of TNF- α and TGF- β 2 in the uterus of these animals throughout the ongoing resorption process. These results indicate a possible role for maternal immunopotential with GM-CSF in the mechanisms mediating the early stages of pregnancy loss, possibly via modulation of TNF- α and TGF- β 2 activity.

Introduction

Spontaneous abortions are among the most common complications of pregnancy and about 20% of pregnancies end in abortion during the first trimester (Clark and Chaouat, 1989). However, the mechanisms mediating the survival of the fetus or those leading to pregnancy loss are far from understood. It is believed that failure of the maternal immune system to actively support pregnancy, for example, by production of the appropriate cytokine network, may be one of the pathways responsible for embryonic death (Clark and Chaouat, 1989; Wegmann, 1990).

One such cytokine is tumour necrosis factor α (TNF- α), a multifunctional cytokine that plays a prominent role in immune and host defence responses, as well as in the regulation of cellular proliferation, differentiation and death (Beutler and Cerami, 1989; Vassalli, 1992). TNF- α may also be involved in the regulation of reproductive processes, including blastocyst growth and implantation, as well as normal placental growth and function (Hunt *et al.*, 1990; Tartakovsky and Ben-Yair, 1991; Terranova *et al.*, 1995). In addition, data obtained in several studies indicate that abnormal TNF- α production might be associated with

pregnancy failure. TNF- α concentration was increased significantly in the amniotic fluid of women suffering from uterine infections and its increased production correlates with the incidence of pre-term labour (Romero *et al.*, 1989). In addition, administration of TNF- α to pregnant mice resulted in pregnancy loss or retardation of embryonic growth (Chaouat *et al.*, 1991; Heyborne *et al.*, 1992).

Another cytokine that may be involved in embryonic development is transforming growth factor β 2 (TGF- β 2), which has major effects on cell growth and differentiation, as well as on tissue remodelling and repair (Barnard *et al.*, 1990; Roberts *et al.*, 1990). TGF- β 2 is expressed in murine uterus throughout gestation (Cheng *et al.*, 1993). In addition, a factor resembling TGF- β 2 in structure and bioactivity, which was detected in murine decidua and has immunosuppressive properties, may be involved in protection of the embryo from maternal immune effector cells (Clark *et al.*, 1990). The requirement for TGF- β 2 for embryonic survival was validated further by experiments demonstrating an abortifacient effect of anti TGF- β 2 antibodies, whereas women suffering from recurrent spontaneous abortions had a deficiency in decidual immunosuppressor cells producing TGF- β 2 (Clark *et al.*, 1992; Lea *et al.*, 1995).

The correlation between increased TNF- α expression or TGF- β 2 deficiency and pregnancy failure might indicate a possible association between normalization of the expression of both cytokines at the fetomaternal interface

and an improved reproductive performance. Such an improvement is achieved by potentiation of the maternal immune system (immunopotentiality), either specifically with allogeneic leucocytes carrying the paternal major histocompatibility complex (MHC) haplotype (Coulam and Coulam, 1992) or nonspecifically with xenogeneic leucocytes (Torchinsky *et al.*, 1995), complete Freund's adjuvant (Toder *et al.*, 1990) or certain cytokines that are capable of controlling fetal survival (Tartakovsky and Ben-Yair, 1991). Among others, granulocyte macrophage-colony stimulating factor (GM-CSF) was shown to support placental growth and function, as well as to decrease pregnancy loss (Wegmann *et al.*, 1989; Chaouat *et al.*, 1990). In addition, Savion *et al.* (1999) demonstrated the ability of maternal immunopotentiality with GM-CSF to affect embryonic growth and morphogenesis and, thus, to protect the embryo from the detrimental effects of embryotoxic agents. The exact mechanisms by which maternal immunopotentiality might affect embryonic survival and, thus, lead to an improved reproductive performance, are not yet fully understood. However, it can be assumed that an appropriate modulation of the cytokine network at the fetomaternal interface might support embryonic survival. Therefore, the aim of the present study was to evaluate possible changes in the expression of TNF- α or TGF- β 2 in the uterus of lipopolysaccharide (LPS)-treated mice in association with the early stages of the LPS-induced resorption process. In addition, the ability of maternal immunopotentiality with GM-CSF to affect the resorption rate, as well as the altered expression of TNF- α or TGF- β 2 in the uterus of LPS-treated mice, was assessed.

Materials and Methods

Animals

Eight- to twelve-week-old C3H male and female mice were purchased from the Tel Aviv University animal facility and kept under standard conditions. Females were caged with males for 3 h (from 07:00 h to 10:00 h) and the presence of a vaginal plug was determined as day 1 of pregnancy.

Induction and evaluation of pregnancy loss

Pregnancy loss was induced in C3H mice by LPS (Sigma, St Louis, MO), which was injected i.p. at 0.75 μ g (0.2 ml PBS)⁻¹ per mouse on day 9 of pregnancy. Uteri from mice in the non-treated ($n = 10$ – 12) and LPS-treated ($n = 16$ – 18) groups were collected at 3, 6, 24 and 48 h after LPS treatment (days 9 and 10 of pregnancy), and the numbers of implantation sites, resorbing embryos and live embryos were recorded. Resorbing embryos were identified by their smaller size and dark red colour, resulting from the massive haemorrhage and clotting of blood that accompany the resorption process, compared with their healthy counterparts, which were pink and larger in size. The percentage of resorbing embryos was calculated by dividing

the number of resorbing embryos by the number of implantation sites per mouse. The resorption process was investigated further in paraformaldehyde-fixed (4%, w/v) paraffin wax-embedded tissue sections (7 μ m thickness), which were stained with haematoxylin and eosin and examined under a light microscope, as described by Savion *et al.* (2002).

Immunopotentiality

Immunopotentiality of the mice by murine recombinant GM-CSF (Peprtech, Rocky Hill, NJ) was performed twice, as described by Savion *et al.* (1999). In brief, under nembutal anaesthesia (40 mg kg⁻¹), mice ($n = 16$ – 18) were injected with GM-CSF into the uterus on day 1 of pregnancy at 10 U (0.04 ml PBS)⁻¹ per horn, followed by a second injection of 20 U (0.03 ml PBS)⁻¹ per mouse into the tail vein on day 8 of pregnancy. Mice injected accordingly with PBS only were used as controls.

Immunohistochemistry

Tissue sections were deparaffinized and treated with 1.5 mg hyaluronidase ml⁻¹ (Sigma) in PBS, pH 6.5, for 1 h at 37°C. Endogenous peroxidase activity was inhibited by incubating the sections for 15 min in 0.3% (v/v) hydrogen peroxide and non-specific binding sites were blocked by incubation in 10% fetal calf serum (FCS) in PBS containing 0.5% (v/v) Tween-20 (PBST) for 30 min at 37°C. The sections were incubated overnight at 4°C with the primary antibodies diluted in 10% FCS in PBST (rabbit anti-mouse TNF- α : 10 μ g ml⁻¹; Endogen, Woburn, MA; and rabbit anti-mouse TGF- β 2: 2 μ g ml⁻¹; Santa Cruz Biotechnologies, Santa Cruz, CA). Non-immune rabbit serum used at the same dilution as the primary antibodies was used as a negative control. The slides were washed in PBST and incubated for 30 min at room temperature with biotinylated goat anti-rabbit IgG (Jackson Immunoresearch laboratories, West Grove, PA) at 2 μ g ml⁻¹, followed by incubation for 20 min in streptavidin-conjugated horseradish peroxidase (streptavidin-HRP; Zymed Laboratories, San Francisco, CA) in PBST at 10 μ g ml⁻¹. TNF- α - or TGF- β 2-positive cells were visualized by incubating the sections for 5 min in the dark with 0.2 mg diaminobenzidine ml⁻¹ (Sigma) and 0.05% (v/v) hydrogen peroxide, and the positive staining was amplified by a 10 min incubation at room temperature in 8% (w/v) CuSO₄. The sections were counterstained with 0.1% (v/v) Meyer's haematoxylin, mounted in 59% (w/v) glycerol–0.9% (w/v) gelatin solution and examined under a light microscope.

Densitometry

Densitometric analysis of the immunohistochemical staining was performed by an Olympus Cue-2 Image Analysis System (Lake Success, NY) using software (Cue-2 Densitometry, version 4.0) developed by the Galai Corporation (Migdal Ha-Emek), as described by Fisher *et al.*

(1998). For each experimental group, three or four implantation sites from different litters were collected and two or three sections from each implantation site were analysed, giving a total of 6–12 sections per group. Three or four fields within each area were measured for each section and the results of all sections from each experimental group were averaged. Final values of absorbance measured for the various areas of the uterus were calculated by subtraction of values determined in sections from the same experimental group, which were treated with non-immune rabbit serum instead of the primary antibodies, or processed in their absence.

Statistical analysis

Statistical analysis of reproductive performance was performed on a litter basis. The GT2 method for multiple comparisons (Sokol and Rohlf, 1995) was used for statistical analysis of all data obtained. The two-tailed level of significance of differences was $P < 0.05$.

Results

Characterization of the resorption process

A mouse model in which pregnancy loss was induced by injecting C3H mice with LPS at 0.75 µg per mouse was used throughout the study. At 3 h after LPS treatment, most of the embryos appeared morphologically intact and only 14% resorbing embryos could be detected in the LPS-treated mice compared with about 11% in the non-treated mice (Fig. 1). At 6 h after LPS injection, 43% resorbing embryos were detected in the LPS-treated mice compared with about 8% in the non-treated mice (Fig. 1). The percentage of resorbing embryos had increased significantly by 24 h after LPS treatment, reaching about 70%, compared with 12% in the non-treated mice (Fig. 1), whereas by 48 h after treatment, the resorption process was complete and all the embryos were fully resorbed (data not shown). In accordance with our previous study (Savion *et al.*, 2002), the resorption process was accompanied by accumulation of blood in the vicinity of the embryo, which became visible as early as 3 h after LPS treatment in > 90% of the embryos and preceded any morphological embryonic damage. As the resorption process continued, 100% of the embryos showed massive morphological damage, further blood accumulation and large infiltrates of leucocytes, and were resorbed fully by 48 h after LPS treatment (Savion *et al.*, 2002).

Effect of maternal immunopotentiality on the LPS-induced resorption process

The effect of maternal immunopotentiality with GM-CSF on the LPS-induced resorption process was tested at various times after LPS treatment. At 3 h after LPS treatment, the percentage of resorbing embryos collected from

Fig. 1. Percentage of resorbing embryos (mean $\pm$ SE) in non-treated ($\square$; $n = 10$ – 12), lipopolysaccharide (LPS)-treated ($\square$; $n = 16$ – 18) or immunopotentialized and LPS-treated ($\blacksquare$; $n = 16$ – 18) mice, at various times after LPS treatment. Data are presented as 95% limits for the mean calculated by the GT2 method. Means with limits that do not overlap are significantly different.

immunopotentialized and LPS-treated females did not differ significantly from that found in mice treated with LPS only (Fig. 1). At 6 h as well as at 24 h after treatment, a decreased percentage of resorbing embryos was detected after GM-CSF immunopotentialization, which was significant ($P < 0.05$) at 24 h after LPS treatment. In parallel, maternal immunopotentialization also decreased the percentage of embryos that demonstrated blood accumulation in their vicinity; however, the differences were not statistically significant (data not shown).

TNF- α expression in the uterus of LPS-treated mice

Expression of TNF- α in the uterus of LPS-treated mice was analysed at various time points after treatment to establish a possible role for TNF- α in pregnancy loss. Immunohistochemical analysis revealed that TNF- α was expressed in the primary decidua (the immediate surrounding of the embryo), as well as in the secondary decidua (distant to the embryos) (Figs 2a and 3a, respectively). LPS treatment resulted in increased expression of TNF- α in the primary and secondary decidua at 3 h (Figs 2b, 3b and 4) and 6 h (data not shown and Fig. 4) after treatment, which was found by densitometric analysis to be significant ($P < 0.05$) for both the primary and secondary decidua (data not shown and Fig. 4, respectively). However, at 24 h after LPS treatment, when the resorption process was almost complete, the expression of TNF- α that was increased in the early stages of the resorption process

Fig. 2. Immunohistochemical analysis of tumour necrosis factor α (TNF- α)-positive cells in the primary decidua (PD) collected from (a) non-treated, (b) lipopolysaccharide (LPS)-treated or (c) immunopotentiated and LPS-treated mice, at 3 h after treatment. (d) Negative control: primary decidua from non-treated mice stained with non-immune rabbit serum. Scale bars represent 50 μ m.

was decreased in the primary (data not shown), as well as in the secondary (Fig. 4), decidua. No differences in expression of TNF- α were observed in the uteri of non-treated animals at the times tested (Fig. 4).

TGF- β 2 expression in the uterus of LPS-treated mice

Expression of TGF- β 2 in the uterus of LPS-treated mice was examined to investigate the possibility that changes in its expression might correlate with the ongoing resorption process. TGF- β 2 was expressed in the uteri of non-treated mice, especially in the primary (Fig. 5a) and secondary (data not shown) decidua, as well as in the glandular epithelium (Figs 6a and 7). Expression of the cytokine did not change significantly at the times tested (Fig. 7). LPS treatment initiated the resorption process and also resulted in a clear decrease in TGF- β 2 expression in the primary (Fig. 5b) and secondary (data not shown) decidua, as well as in the glandular epithelium (Fig. 6b). The decrease in TGF- β 2 expression was detected at all the times tested (Fig. 7) and was significant ($P < 0.05$) for the

glandular epithelium (Fig. 7) and the primary decidua (data not shown).

Changes in uterine TNF- α and TGF- β 2 expression after maternal immunopotentiality with GM-CSF

As maternal immunopotentiality with GM-CSF reduced the rate of resorption, it was investigated further whether this effect might be associated with normalization of the expression of TNF- α and TGF- β 2 that was altered by LPS treatment. Indeed, the increased TNF- α expression demonstrated in the primary and secondary decidua at 3 and 6 h after LPS treatment was decreased after maternal immunopotentiality (Figs 2c, 3c and data not shown, respectively), and was significant ($P < 0.05$) for the secondary decidua at 6 h after LPS treatment (Fig. 4). Furthermore, the TNF- α expression that was decreased 24 h after LPS treatment, was increased by maternal immunopotentiality in both the primary and secondary decidua, reaching nearly the level demonstrated in the uteri of non-treated animals (data not shown and Fig. 4, respectively). In

Fig. 3. Immunohistochemical analysis of tumour necrosis factor α (TNF- α)-positive cells in the secondary decidua (SD) collected from (a) non-treated, (b) lipopolysaccharide (LPS)-treated or (c) immunopotentiated and LPS-treated mice, at 3 h after treatment. (d) Negative control: secondary decidua from non-treated mice stained with non-immune rabbit serum. Scale bars represent 100 μ m.

contrast, the TGF- β 2 expression that was reduced by LPS treatment at all the times tested was increased significantly ($P < 0.05$) by maternal immunopotentiality, almost to the level in the uteri of non-treated mice (Fig. 7). This normalizing effect of maternal immunopotentiality by GM-CSF was evident in the primary decidua (Fig. 5c) and in the glandular epithelium (Figs 6c and 7), as well as in the secondary decidua (data not shown).

Discussion

Cytokines are important regulators of different cellular processes including cell growth, differentiation and death (Robertson *et al.*, 1994; Lea *et al.*, 1999). TNF- α and TGF- β 2 are involved in the regulation of placental growth and function, as well as in embryonic development and survival (Lea *et al.*, 1992; Wride and Semders, 1995; Hunt *et al.*, 1996; Godkin and Dore, 1998). As the role of these cytokines in the mechanisms mediating pregnancy loss is not well understood, the aim of the present study was to characterize the patterns of TNF- α and TGF- β 2 expression

in the uterus of C3H mice and to correlate them with the ongoing resorption process induced in these mice by LPS. This model has already been used in various studies for the investigation of induced pregnancy loss; however, the abortifacient effect of LPS has always been demonstrated at the endpoint of the resorption process, when most of the embryos are fully resorbed (Chaouat *et al.*, 1990; Gendron *et al.*, 1990; Chaouat, 1994; Baines *et al.*, 1996). Therefore, we tried to follow up possible changes in TNF- α and TGF- β 2 expression associated with the initial stages of the LPS-induced resorption process. In accordance with our previous study (Savion *et al.*, 2002), the early stages of the resorption process were characterized by blood accumulation in the vicinity of the embryo and infiltration of leucocytes, while most of the embryos showed no visible damage. At that time, an increase in the expression of TNF- α was detected in the uterus of these animals, whereas at 24 h after treatment, when the resorption process was almost complete and most of the embryos were almost fully resorbed, the expression of TNF- α was decreased. An increase in TNF- α expression has also been demonstrated

Fig. 4. Densitometric analysis of tumour necrosis factor α (TNF- α) expression (mean absorbance $\pm$ SD) in the secondary decidua collected from non-treated (□), lipopolysaccharide (LPS)-treated (▨) or immuno-potentiated and LPS-treated (■) mice, at various times after treatment. For each experimental group, three or four implantation sites from different litters were collected and two or three sections from each implantation site were analysed (three or four fields within each measured area) and averaged. Data are presented as 95% limits for the mean calculated by the GT2 method. Means with limits that do not overlap are significantly different.

in other models of pregnancy loss, mainly in the CBA/JxDBA/2J combination without further treatment (Tangri and Raghupathy, 1993) or after exposure to stress (Arck *et al.*, 1995). Reznikov *et al.* (1999) demonstrated increased TNF- α expression in the uterus of mice that received an intrauterine inoculation of a high dose of LPS on days 14–15 of pregnancy. However, in these studies, TNF- α expression was assessed at the end of the resorption process, whereas in the present study, increased TNF- α expression is demonstrated in association with the initial stages of the resorption process, preceding any visible damage to the embryo itself. Our results support those of Gendron *et al.* (1990), who demonstrated increased TNF- α expression in the amniotic fluid of mice injected with a high dose of LPS on day 12 of pregnancy, at 2 h after treatment. The results of the present study are also in agreement with two other studies performed in our laboratory in which increased expression of TNF- α mRNA and protein preceded the final stages of the resorption process, induced by either cyclophosphamide treatment (Gorivodsky *et al.*, 1998) or maternal diabetes (Fein *et al.*, 2001). It should be noted that the increase in TNF- α expression in the uterus of LPS-treated mice precedes the apoptotic process that occurs in association with the ongoing resorption (Savion *et al.*, 2002). Moreover, the decreased TNF- α expression noted at the peak of the resorption process, which coincided with

the peak of the apoptotic process (Savion *et al.*, 2002), concurs with the known role of this cytokine in the induction and regulation of apoptosis (Wride and Semders, 1995; Hunt *et al.*, 1996). These data are supported by another study performed in our laboratory, in which increased TNF- α expression was demonstrated in embryonic organs after exposure to cyclophosphamide, which preceded the apoptotic process and the appearance of malformations induced by cyclophosphamide treatment in these organs (Ivnitsky *et al.*, 1998). Together, these results implicate TNF- α in initiation of the LPS-induced resorption process.

In parallel, possible changes in TGF- β 2 expression in the uterus of LPS-treated mice that might be associated with the early stages of the resorption process were evaluated. Indeed, a decreased expression of TGF- β 2 in the decidua at the early as well as the late stages of the LPS-induced resorption process was observed. Our results are supported by other studies showing that decidual TGF- β 2 is either absent or is present at a low level in mice undergoing fetal resorptions and in women suffering from recurrent abortions (Lea *et al.*, 1995; Clark *et al.*, 1997; Raghupathy, 1997). Most of the work in mice is performed in the CBA/JxDBA/2J model without further treatment or after exposure to stress, as well as in CB-17 SCID mice. In these studies, the high resorption rate was accompanied by increased TNF- α as well as decreased TGF- β 2 expression

Fig. 5. Immunohistochemical analysis of transforming growth factor $\beta 2$ (TGF- $\beta 2$)-positive cells in the primary decidua (PD) collected from (a) non-treated, (b) lipopolysaccharide (LPS)-treated or (c) immunopotentiated and LPS-treated mice, at 6 h after treatment. (d) Negative control: primary decidua from non-treated mice stained with non-immune rabbit serum. Scale bars represent 50 μ m.

in the decidua of these animals (Clark *et al.*, 1994; Arck *et al.*, 1995). However, in these studies TGF- $\beta 2$ expression was assessed at the late stages of the resorption process, unlike in the present study, in which the decrease in TGF- $\beta 2$ expression preceded the peak of the process, when the embryos show normal morphology except for some blood accumulation in their vicinity. Our data also concur with the known role of TGF- $\beta 2$ as an immunosuppressive factor protecting the embryo from attack by maternal natural killer and cytotoxic T cells, which are involved in resorption processes leading to embryo death (Clark *et al.*, 1992, 1997; Godkin and Dore, 1998). In addition, there is evidence that TGF- $\beta 2$ can deviate maternal immune responses toward the production of Th2 cytokines that support pregnancy, possibly via regulation or suppression of the Th1 cytokines that are detrimental for pregnancy (Maeda and Shiraishi, 1996; Arck *et al.*, 1997; Raghupathy, 1997; Clark *et al.*, 1999). Therefore, the decrease in decidual TGF- $\beta 2$ expression in the initial stages of the resorption process might be implicated as one of the events responsible for the LPS-induced embryonic death.

There is evidence to indicate that the involvement of the maternal immune system in the normal course of pregnancy might be extended further toward the protection of the embryo from embryotoxic factors (Gorivodsky *et al.*, 1998; Clark *et al.*, 1999; Gorivodsky *et al.*, 1999). In the present study, we attempted to achieve this protective effect by potentiation of the maternal immune system with GM-CSF, which has protective and therapeutic properties (Talmadge *et al.*, 1989; Neta, 1990; Neta *et al.*, 1998) and exerts a direct effect on trophoblast or decidual cells, as well as acting as a powerful mediator of the immune activities that support normal embryonic development (Wegmann *et al.*, 1989; Chaouat *et al.*, 1990). Indeed, maternal immunopotential with GM-CSF decreased the resorption rate at 6 and 24 h after LPS treatment, as well as the percentage of embryos showing blood accumulation in their vicinity, as compared with the non-immunopotentiated and LPS-treated group. These results are in agreement with other studies demonstrating the ability of GM-CSF to protect the embryo from the embryotoxic effects of cyclophosphamide or to reduce the resorption rate in the CBA/JxDBA/2J

Fig. 6. Immunohistochemical analysis of transforming growth factor $\beta 2$ (TGF- $\beta 2$)-positive cells in the glandular epithelium (GE) collected from (a) non-treated, (b) lipopolysaccharide (LPS)-treated or (c) immunopotentiated and LPS-treated mice, at 6 h after treatment. (d) Negative control: glandular epithelium from non-treated mice stained with non-immune rabbit serum. Scale bars represent 50 μm .

combination (Chaouat *et al.*, 1990; Chaouat, 1994; Clark *et al.*, 1994; Savion *et al.*, 1999). To understand the mechanisms by which GM-CSF immunopotentiality might affect the resorption rate, we tested the possibility that alteration of TNF- α and TGF- $\beta 2$ expression in the uterus of LPS-treated mice might be associated with such an effect. Indeed, it was demonstrated that maternal immunopotentiality with GM-CSF partially normalized the expression of both cytokines in the uterus of LPS-treated mice. Thus, in immunopotentiated and LPS-treated mice, TNF- α expression was reduced in the initial stages of the resorption and increased later, as the process was completed. In addition, TGF- $\beta 2$ expression was significantly increased in GM-CSF-immunopotentiated and LPS-treated mice at all the times tested, as compared with mice treated with LPS only. These results are in agreement with those of Arck *et al.* (1995) in the CBA/JxDBA/2J combination, demonstrating a decrease in TNF- α as well as an increase in TGF- $\beta 2$ expression in decidual supernatants collected from Balb/c-immunopotentiated CBA/J mice after exposure to stress. However, both cytokines were assessed at the end of the

resorption process, while in the present study the normalization of TNF- α and TGF- $\beta 2$ expression by GM-CSF preceded any visible damage to the embryo, occurring at the initial stages of the resorption process. Several studies performed in our laboratory also demonstrate that the ability of maternal immunopotentiality to reduce the resorption rate in cyclophosphamide-treated or diabetic mice is accompanied by normalization of TNF- α and TGF- $\beta 2$ expression in the uterus of these animals (Gorivodsky *et al.*, 1998, 1999; Fein *et al.*, 2001, 2002). The mechanisms by which GM-CSF alters TNF- α and TGF- $\beta 2$ expression may depend on the cells expressing these cytokines in the decidua. This heterogeneous population includes, in addition to decidual cells, macrophages and neutrophils, as well as natural killer and T cells. Thus, GM-CSF was shown to regulate the proliferation and function of macrophages and neutrophils, either by downregulation of TNF- α production by these cells (Clark and Chaouat, 1989; Robertson *et al.*, 1994) or by maintenance of the immunosuppressive phenotype of macrophages through upregulation of their TGF- $\beta 2$ production (Robertson *et al.*, 1994). GM-CSF was

Fig. 7. Densitometric analysis of transforming growth factor β 2 (TGF- β 2) expression (mean absorbance $\pm$ SD) in the glandular epithelium collected from non-treated (□), lipopolysaccharide (LPS)-treated (▨) or immunopotentiated and LPS-treated (■) mice at various times after treatment. For each experimental group, three or four implantation sites from different litters were collected and two or three sections from each implantation site were analysed (three or four fields within each measured area) and averaged. Data are presented as 95% limits for the mean calculated by the GT2 method. Means with limits that do not overlap are significantly different.

also shown to prevent resorptions and to enhance trophoblast growth indirectly by downregulation of the cytotoxic activity of natural killer cells that might be mediated by their production of TNF- α . This protective function of GM-CSF may be realized through activation of CD8-positive suppressor T cells, possibly via their production of TGF- β 2 (Clark *et al.*, 1999). Another possibility that cannot be excluded is a direct effect of GM-CSF on ectoplacental cone trophoblast cells, residing in close proximity to the embryo on days 9–10 of pregnancy, that, in turn, might affect the expression of either TNF- α or TGF- β 2 by neighbouring decidual cells (Chaouat *et al.*, 1990). Together, these data indicate that potentiation of the uterine local immune microenvironment by GM-CSF towards production of pregnancy-promoting cytokines such as TGF- β 2 and downregulation of TNF- α , which is involved in pregnancy loss, might serve as key events in the mechanisms mediating the beneficial effects of maternal immunopotential on fetal survival and pregnancy outcome.

In conclusion, the results presented in the present study indicate a possible correlation between the altered decidual expression of TNF- α and TGF- β 2, and the LPS-induced resorption process. More than that, our data implicate maternal immunopotential by GM-CSF as a mechanism for protecting the embryo from LPS-induced pregnancy loss, possibly via modulation of TNF- α and TGF- β 2 activity.

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