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Immunodystrophic Events in Spontaneous Abortions

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The stimulated $CD8^+$ cells secrete high levels of IL-2, TNFα and Ifnτ—all of which are known to be detrimental to placental survival and/or function. TNFα has been reported to induce contraction of smooth muscle and hence may cause fetal expulsion due to uterine contraction or may cause necrosis of implantation sites or implants; additionally TNFα could act by thrombosing blood supply to the conceptus as it is known to do for tumors. Cells bearing NK markers, located in the decidua, have been shown to be closely associated with fetal loss in the CBA × DBA/2 combination. Similarly, the presence of cytotoxic T lymphocytes in resorbing placentas has been demonstrated. It is suggested that these NK-like cells and CTLs may cause fetal loss by getting converted in the presence of TNFα and IL-2 into LAK cells which are capable of killing normally lysis-resistant trophoblast cells. Once the placenta is damaged, fetal demise and resorption may follow shortly thereafter. This model still needs to be confirmed and experiments designed to fill the gaps in the model are currently underway in our laboratory.

Key Words: INF_α, Placental survival, Abortion, IL-2

Introduction

Considering that about 30% of implanted pregnancies in humans and cattle end in abortions, it is clear that pregnancy is not as successful as one might imagine. About 15% of pregnancies diagnosed by hCG assays abort within 14 days of fertilisation and another 15% of pregnancies abort between 4 and 14 weeks (Clark 1989). It is estimated that a proportion of abortions may be due to anatomical and chromosomal problems, but chromosomal analyses of abortus material from habitual aborters show a lower incidence of abnormalities than do abortus tissues from sporadic aborters. In the last decade or so several tantalizing leads have led to the current opinion that most spontaneous abortions represent loss of normal healthy fetuses and that immunological aetiologies may play significant roles in abortions (Clark 1989, Chaouat 1987). This initially stemmed from studies which suggested that recurrent spontaneous abortions can be prevented by immunological treatment consisting of immunisation of aborters with lymphocytes from their husbands (Mowbray 1985). However, some studies have reported high rates of success even in placebo-treated women or in women after psychotherapeutic treatment, bringing into question the validity of immunological events leading to the success of pregnancy after immunization (Hill 1990).

The major convincing leads to an understanding of the interactions between

the immune system and the conceptus have actually come from studies on animal models of spontaneous abortions.

Murine Model of Immunologically-mediated Spontaneous Abortion

The availability of the CBAxDBA/2 murine model of immunologically-mediated spontaneous abortions has provided scope for investigating interactions between the maternal immune system, lymphoid cells and cytokines on the one hand and the fetoplacental unit on the other. In 1980 David Clark first reported that CBA/J females (H-2^k) mated to DBA/2 males (H-2^d) suffered from high rates of spontaneous abortions as compared to CBA/J females mated with males of other strains and also H-2^k males other than DBA/2 (Clark et al. 1980). Resorption rates as high as 45-50% were recorded, and matings in the reverse direction were not susceptible to fetal resorption, with normal rates of resorption in the range of 5-7%.

Several experimental lines of evidence point to an immunological aetiology of fetal resorptions in this system (Kiger et al. 1985) demonstrated that immunisation of CBA/J females with BALB/c cells prior to mating with DBA/2 reduces the resorption rate to normal levels. More arguments in favour of an immunologic basis for increased resorption rates in this mating combination came from findings of Gendron et al. (Gendron & Baines 1988) who demonstrated a histological correlation between fetal resorption and an increase in the appearance of cells bearing NK markers in the vicinity of the resorbing fetus. Modulation of the activity of NK cells had a direct effect on the frequency of embryo loss (de Fougerolles & Baines 1987) confirming their importance as effectors in fetal loss. The most conclusive evidence for an immunologic aetiology in this model of pregnancy failure is that protection against increased abortion by alloimmunisation can be adoptively transferred to virgin CBA/J females by sensitised T cells obtained from alloimmunised mice (Chaouat et al. 1991).

It should be pointed out that while murine models have been available for several years now, relatively little is known about all the immunoregulatory events leading to fetal resorptions in this system and complete information on the immunological sequelae and processes resulting in detrimental effects on pregnancy in this system is lacking.

Immunotrophism and Immunodystrophism in Pregnancy

Observations on the involvement of maternal T cells and cytokines in pregnancy led in Immunotrophism Hypothesis, which suggests that maternal lymphoid cells recognize fetally-derived antigens and secrete cytokines which may help fetoplacental survival (Wegmann 1984). The role of cytokines in promoting placental growth and survival was first described by Wegmann and colleaguest (Athanassakis et al. 1987). They reported that murine placental cells exposed to cytokine-rich cell culture supernatants proliferated better and had greater phagocytic activity than in the absence of cytokines. It was later found that these cells proliferate primarily in response to members of the CSF family, i.e., GM-CSF, IL-3, CSF.

Cytokines which are basically immune response mediators are predominantly secreted by T cells and have been shown to affect reproductive processess including embryo development and trophoblast proliferation *in vitro* and *in vivo*. Certain cytokines enhance fetal growth and survival whereas others can compromise pregnancy (Chaouat et al. 1989, Hill 1991). This leads to the emergence of the concept that there is a delicate balance of cytokines during pregnancy, a disturbance in which could result in fetal loss. A number of recent studies

have localised the production of growth-enhancing cytokines to non-immune components of the reproductive tract including the uterine epithelium and trophoblast tissue (Robertson et al. 1982). It has been suggested therefore that maternal T cells may interact with these tissues via a cytokine regulatory network, but the pathways of these interactions are not yet clear.

A corollary to the immunotrophism model which by definition consists of regulatory mechanisms that are *beneficial* to pregnancy, is the concept of immunodystrophism. Is it possible that under certain circumstances, maternal immune responses can be *detrimental* to pregnancy? In other words, can placental immunostimulation of the maternal immune system result in responses, conceivably mediated by detrimental cytokines, which are deleterious to placental function and fetal well-being.

Immunodystrophic Mechanisms CBAxDBA/2 Pregnancies

Keeping in mind that there are beneficial cytokines which can enhance fetal growth and survival and deleterious cytokines which can compromise pregnancy by damaging the feto-placental unit, we attempted to elucidate some of the immunological mechanisms that may explain fetal resorptions in the resorption-prone CBAxDBA/2 model. When viewed from the context of the immunotrophism and immunodystrophism, it is conceivable that CBA females respond differently to DBA/2 antigens and BALB/c antigens both qualitatively and quantitatively. With a view to understanding the differences between the manner in which CBA lymphoid cells respond to stimuli by CBA × DBA/2 placental cells versus CBA × BALB/c placental cells, we set up Mixed Leucocyte-Placenta Reactions (MLPR) in which responder maternal strain lymphoid cells are allowed to react to stimuli provided by irradiated stimulator placental cells.

In MLPR, we found that placental cells of the "normal" combination (CBA × BALB/c) stimulate maternal lymph node cells to a significantly higher extent than do placentas from the abortion-prone (CBA × DBA/2) combination (table 1, figure 1). When viewed from the perspective of the immunodystrophism model one might extend these data to infer that pregnancy may be compromised due to inappropriate responses by maternal cells towards fetal antigens.

The key mediators in the immunotrophism and immunodystrophism model are cytokines; we therefore went on to analyse cytokine production in MLPR. TNFα levels

Table 1 *Stimulation indices of Mixed Lymphocyte Placental Reactions in abortion-prone I (CBA anti-(CBA × DBA/2) and normal (CBA anti-(CBA × BALB/c) combinations. Results from three representative experiments are depicted*

MLPR Strain Combination	Stimulatio Index		
	Expt. 1	Expt. 2	Expt. 3
CBA anti-(CBA × DBA/2)	6.1	7.2	7.1
CBA anti-(CBA × BALB/c)	14.1	16.3	15.9

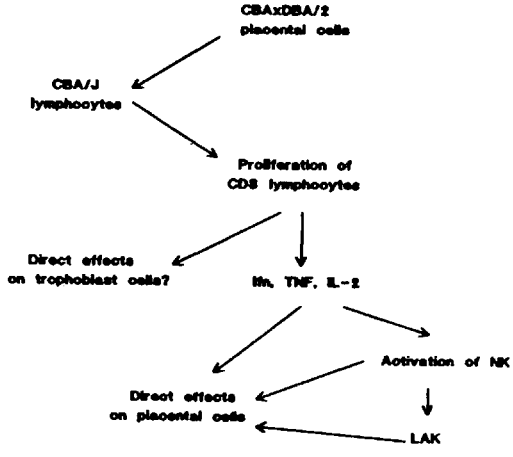

Figure 1: Proposed sequelae of events leading to fetal resorption upon adoptive transfer of MLPR-stimulated cells

were assayed by the ability of the MLPR supernatants to lyse LM cells in a bioassay and also by ELISA. IFNτ levels were estimated by a bioassay employing the WEHI 279 cell line and by an ELISA. IL-2 levels were measured by adding supernatants to the IL-2 sensitive cell line, CTLL, and by ELISA. CBA × BALB/c placental cells stimulated greater proliferation of CBA/J lymphocytes than did CBA × DBA/2 placentas. The levels of TNFα, IFNτ, and IL-2 produced in the CBA/J anti-CBA × DBA/2 MLPR was significantly higher than those produced in the CBA anti-CBA × BALB/c MLPR (table 2). Thus there is clearly a significantly higher production of TNFα, IFNτ and IL-2 in MLPR supernatants from the abortionprone combination than in MLPR supernatants from the normal combination. However, cytokine analyses of supernatants from one way Mixed Lymphocyte Reactions (MLR) from the abortion-prone and normal combinations showed no significant differences in cytokine profile indicating that the phenomenon is restricted to MLPR (mixed leucocyte-placenta reaction).

Incidentally, IL-3, IL-10 and GM-CSF levels were the same in the two sets of MLPR. Interestingly enough, IL-5 levels were three-fold higher in the supernatants from CBA × BALB/c MLPR as compared to the CBA × DBA/2 combination (Tangri et al. 1994). This observation ties in with Wegmann's contention that in normal pregnancy the maternal immune system preferentially generates a TH^2 like response Wegmann et al. 1984.

Coming back to the three cytokines, IL-2, IFNτ, TNFα, which we may collectively term the "Bad Troika", these are the three cytokines which have been very convincingly implicated as having deleterious effects on the outcome of pregnancy, and are thus prime candidates for the mediators of immunodystrophic effects on the conceptus (Chaouat et al. 1991). TNFα has been shown to inhibit embryo development (Warner & Libby 1989). IFNτ also has direct cytocidal effects on embryo development and trophoblast proliferation *in vitro* as well as indirect effects such as the activation of macrophages and induction of NK activity (Parant 1990). IL-2 also stimulates NK cells which may affect fetal survival. The injection of small amounts of IL-2, IFNτ and TNFα into pregnant mice leads to fetal resorptions in a variety of abortion-prone and non-abortion-prone mating combinations (Chaouat et al.).

Our data from the MLPR experiments become particularly relevant when we take into consideration our observations on the production of cytokines at the maternal-fetal interface, i.e., in the placenta. We studied mRNA expression of these cytokines in the placentas of normal and abortion-prone mating combinations, as the placental trophoblast is the only fetally-derived tissue which is in direct and continuous contact with maternal tissues. Using dot hybridisation studies we found that the expression of cytokines TNFα, IFNτ and IL-2 was significantly enhanced in placentas from CBA × DBA/2 pregnancies as compared to those from CBA × BALB/c pregnancies (Tangri & Raghupathy 1993). We performed Northern hybridizations to analyse TNFα, IFNτ and IL-2 transcripts and to examine whether they are different in any way from their counterparts in lymphocytes. Northern hybridisation

Table 2 *Levels of cytokines TNFα, Ifnτ and IL-2 in supernatants of MLPR in abortion-prone (CBA anti-(CBA × DBA/2) and normal (CBA anti-(CBA × BALB/c) combinations.*

	CBA anti-(CBA × DBA 2)	CBA anti-(CBA × BALB c)
TNFα (ng ml)	9.7 ± 0.9	0.9 ± 0.2
IFNτ (U ml)	98.5 ± 11.0	1.8 ± 1.8
IL-2 (U ml)	177.0 ± 8.0	72.5 ± 5.0

studies reveal that the mRNA transcripts for TNFα, IFNτ and IL-2 are identical in size to transcripts for these cytokines found on lymphoid tissues (Tangri & Raghupathy 1993).

What kinds of cells are stimulated by placental cells in MLPR? To characterize the nature of the stimulating antigen and phenotype of the responding CBA lymphocytes stimulated by CBA × DBA/2 placental cells, we added monoclonal anti-CD4, anti-CD8, anti-MHC Class I or anti-MHC Class II antibodies to MLPR. Antibodies to CD8 and MHC Class I antigens inhibited responses in MLPR by 85-90% whereas antibodies to CD4 determinants brought about an inhibition of only 11% (Tangri et al. 1994). On the contrary, MLR (as opposed to MLPR) consists primarily of a $CD4^+$ response and the stimulating MHC molecule is primarily Class II. This suggests that the interactions taking place in MLR are predominantly CD4-Class II-based whereas MLPR are paternally-derived MHC Class I molecules or possibly maternal Class I molecules associated with antigenic peptide(s); this observation ties in with our earlier demonstration the murine placentas express MHC Class I antigens but are lacking in Class II molecules (Raghupathy et al. 1981). The marked inhibition of MLPR by anti-CD8 antibodies and mild inhibition by anti-CD4 antibodies implies that the responder maternal phenotype is primarily $CD8^+$.

It is relevant to point out that $CD8^+$ T cell clones have been shown to secrete IFNτ, TNFα and IL-2 (Bloom et al. 1992), a profile which is reflected by the cytokine profile in the MLPR described above. Indeed our studies have shown that addition of anti-CD8 antibodies inhibited TNFα levels by 40%, IFNτ levels by 80% and IL-2 were produced by $CD8^+$ cells and other cells as well. Bloom and colleagues (Bloom et al. 1992) have reported that one of the subsets of $CD8^+$ has a cytokine profile that is remarkably like the one we have described; this subset secretes high levels of IFNτ, TNFα and IL-2 and low levels of IL-4. We can thus visualize a situation wherein $CD8^+$ maternal cells respond to alloantigenic (paternal) Class I MHC antigens on placental cells or maternal Class I on placental cells presenting antigenic peptides. We may thus speculate that the cytokines TNFα, IFNτ and IL-2 are produced by $CD8^+$ cells and these in turn induce infiltrating NK-like cells to become LAKs which damage the placenta and in turn the fetus.

We took this line of experimental inquiry one crucial step further. Are the MLPR-stimulated cells capable of manifesting any deleterious effects on pregnancy? We then went on to address the question of whether the immunostimulation pattern seen *in vitro* MLPR have any relevance to the outcome of pregnancy; we adoptive-normal combinations into normal pregnant animals. We found that MLPR-stimulated cells from the resorption-prone combination could adoptively transfer the abortive effect to normal pregnant mice; MLPR-stimulated cells from normal mating combinations had no adverse effect on pregnancy as observed in (table 3). This implies that differences in immune responses mounted during MLPR can affect pregnancy. It is indeed interesting that the CBA/J cells that bring about this abortifacient effect are stimulated by

Table 3 *Adoptive transfer of MLPR-stimulated cells into pregnant CBA × CBA mice. CBA cells stimulated separately by CBA × DBA/2 placentas and by CBA × BALB/c placentas in MLPR were injected into pregnant CBA × CBA mice on days 5 and 7 of gestation. Resorptions were recorded on day* 14

MLPR-stimulated cells transferred	Resorption Rate (%)
None	8±1.6
CBA cells stimulated by CBA × DBA/2 placentas	67±7.2
CBA cells stimulated by CBA × BALB/c placentas	12±2.6

CBA × DBA/2 placentas, yet their effects are manifested in CBA × CBA pregnancies. Since CBA/J stimulated by CBA × BALB/c placentas do not induce such effector cells, we may conclude that the stimulation is specific while the effects are non-specific.

If Wegmann et al. (1984) are indeed correct in stating that *normal* pregnancy is a TH2-biased phenomenon steering clear of a TH1-like phenomenon, we might add an important corollary; based on our demonstration of increased production of TH1 cytokines in resorption-prone pregnancies and the fact that the three cytokines TNFα, IL-2 and IFNτ are detrimental to pregnancy, we speculate that abnormal pregnancy is a TH1-like phenomenon at least in terms of cytokine profiles.

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