Resorption of CBA/J × DBA/2 mouse conceptuses in CBA/J uteri correlates with failure of the feto-placental unit to suppress natural killer cell activity*

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Summary. Lipid extraction was used to study the natural killer (NK) suppressive activity of individual feto-placental units. Normal pregnancies showed a lipophilic NK cell suppressive factor that was gestational day specific. Feto-placental units from CBA/J × DBA/2 pregnancies were deficient in the NK cell suppressive factor when compared to normal CBA/J × BALB/c pregnancies. The frequency of non-suppressive feto-placental units from CBA/J × DBA/2 pregnancies correlated with the frequency of feto-placental units infiltrated with NK cells and the frequency of spontaneous resorption. Our results implicate a deficiency of NK suppressive activity in the feto-placental unit as a contributing factor in spontaneous fetal resorption.

Keywords: pregnancy; resorption; natural killer cells; immunosuppression; mouse

Introduction

In viviparous pregnancy, the apposition of allogeneic fetal tissue with the maternal immune system evinces the need for local uterine immunosuppression (Beer & Billingham, 1974; Slapsys & Clark, 1982; Hsi *et al.*, 1984; Redman *et al.*, 1984; Wells *et al.*, 1984; Nakayama *et al.*, 1985; Croy *et al.*, 1987). Most allogeneic pregnancies progress to term with no evidence of rejection. In mice, spontaneous fetal resorption frequencies are below 10% per pregnancy (Rugh & Wohlfromm, 1967). A significantly increased murine fetal resorption frequency of approximately 30% is observed in CBA/J females mated with DBA/2 males (Clark *et al.*, 1987). The reasons for the increased fetal resorption from this mating are unclear although a deficiency of non-T suppressor cells has been implicated (Clark *et al.*, 1986a).

Spontaneous fetal resorption in CBA/J × DBA/2 pregnancies may involve natural killer (NK) cells (Gendron & Baines, 1988). We have observed NK-like cells in the junction between the decidua and ectoplacental cone of resorbing feto-placental units as early as Day 6 of gestation. Cytotoxic T lymphocytes are not involved in this process (Gendron & Baines, 1988). Augmentation of the NK activity by polyinosinic cytidilic acid (POLY I:C) significantly increased the resorption frequency whereas abrogation of the NK activity by anti-asialo-GM1 antiserum (aAGM1) significantly decreased the resorption frequency (deFougerolles & Baines, 1987). We have therefore argued that regulation of uterine NK activity may play an important role in preventing cellular immune reactions against the fetal and trophoblast tissue of the developing feto-placental unit.

Natural killer cell activity can be inhibited by arachidonic acid metabolites (Brunda *et al.*, 1980; Ramstedt *et al.*, 1985). It has been suggested that NK cell activity is regulated *in vivo* through production of prostaglandins (PG) by monocytes and macrophages (Koren *et al.*, 1981).

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Prostaglandins are produced by the pregnant human, rat and rabbit uterus during the peri-implantation period (Pakrasi *et al.*, 1985; Malathy *et al.*, 1986; Parr *et al.*, 1988; Smith & Kelly, 1988). Mouse decidual NK activity can be inhibited by decidual cell-derived PGE-2, and this suppression of decidual NK activity can be reversed by treatment of the mixed population of cells with indomethacin (Lala *et al.*, 1986). Inhibition by indomethacin of PG synthesis in pregnant mice induces partial or complete litter resorption (Lala *et al.*, 1986). Furthermore, the suppressive mechanism in normal human decidual tissue involves PGE-mediated suppression of lymphokine-activated killer (LAK) cell activity (Parhar *et al.*, 1988a, b). These findings suggest that arachidonic acid metabolites may be intimately involved in the regulation of NK activity in the feto-placental unit.

We have suggested that a failure in decidual PG production in the CBA/J $\times$ DBA/2 pregnancy leads to NK cell activation and resorption. Here we present evidence for the deficiency of a lipophilic NK suppressive factor in spontaneous fetal resorption.

Materials and Methods

Animals and tissue processing. CBA/J female mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). CFW Swiss Webster female mice, BALB/c male mice and DBA/2 male mice were purchased from Charles River (St Constant, Quebec, Canada). All animals were 6–8 weeks old. Animals were given free access to chow and water and housed in an animal care facility maintained at 25°C with a 12-h light/dark cycle. Pregnancies were obtained by caging 5 CBA/J or CFW females with either 1 DBA/2 or 1 BALB/c male overnight (the major MHC complex of BALB/c is identical to that of DBA/2; Kiger *et al.*, 1985). The morning of detection of a vaginal plug was designated as Day 1 of gestation. Animals were killed by cervical dislocation on Day 9 of gestation, uteri were removed quickly and frozen immediately on solid CO₂. Individual feto-placental units were separated by cutting through the areas between implantation sites with a razor blade while the tissue remained on the solid CO₂. Frozen feto-placental units were then weighed individually and stored at –80°C.

Pregnant uteri from CFW $\times$ DBA/J pregnancies were processed as described above but feto-placental units from each pregnant uterus were combined. This was necessary to allow for the analysis of immunosuppressive activity as a function of gestational day in normal pregnancies.

Non-pregnant uteri from 2 CBA/J females were removed, frozen and cut into fragments of various sizes (23.4–33.7 mg) and processed as described below for individual feto-placental units.

To examine the role of cyclo-oxygenase products on the putative NK suppressive activity, 4 CBA/J ($\times$ DBA/2) pregnant females were injected (i.p.) with 1 μ g acetyl salicylic acid (Sigma, St Louis, MO, USA) dissolved in phosphate-buffered saline (PBS), 18 h before tissue collection; 4 control (CBA/J $\times$ DBA/2) pregnant females received PBS (i.p.) only. This dose of acetyl salicylic acid has been shown to restore NK activity in tumour-bearing mice which have increased PG production (Brunda *et al.*, 1980).

Identification and processing of feto-placental units containing asialo GM1 positive cellular infiltrates. An indirect immunoperoxidase staining procedure was utilized on frozen sections of CBA/J $\times$ DBA/J feto-placental units at Day 9 of gestation. Feto-placental units were then sorted into normal or presumptively resorbing groups according to the observed NK cell density (Gendron & Baines, 1988). The tissue blocks were placed on solid CO₂ and the infiltrated feto-placental units were separated from those with no infiltration. All infiltrated feto-placental units were pooled in one group and an equal number of normal feto-placental units was pooled in a separate group. Pooled tissue in each group was weighed, processed and extracted as described below.

Tissue extraction. The prostaglandin–thromboxane lipophilic fraction was extracted from the tissue by methods described previously (Powell, 1982). Weighed, frozen tissue was homogenized at 4°C for 30 sec by hand in 0.5 ml PBS, pH 7.4, containing 15% ethanol. The homogenates were centrifuged at 400 g for 10 min at 4°C. The supernatants were collected and adjusted to a pH of 3.5 with 1 N-HCl. The supernatants were applied to SEP-PAK C18 columns (Millipore, Bedford, MA, USA) which had been pre-wet with absolute ethanol and distilled water. Following washes with 20 ml of 15% ethanol and 20 ml absolute petroleum ether, the columns were eluted with 10 ml absolute methyl formate. The methyl formate eluants were dried under a stream of nitrogen and reconstituted in PBS, pH 7.4, containing 1% ethanol. Reconstitution volume for all tissues was adjusted according to the initial frozen weight (0.01 ml per mg tissue). Extracts were apportioned to freezer tubes, purged with nitrogen and stored at –80°C until assayed. Extracts were assayed within 3 days.

Cytotoxicity assay and inhibitory units. Tissue extracts were tested for NK suppressive activity by serial dilution. Suppressive activity was assessed by the degree to which each extract inhibited lysis by virgin female CBA/J spleen cells (effector cells) of YAC-1 target cells labelled with chromium 51 (Pross & Baines, 1979). Assays were carried out in 96-well V-bottom plates (Flow Laboratories, McLean, VA, USA) in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Flow Laboratories, McLean, VA, USA). Effector cells (2×10^6 cells per well),

prepared as whole spleen cell suspensions from 2–3 spleens of CBA/J virgins, were added to wells in which fetoplacental unit extracts had been diluted serially from 1/2 to 1/32. Chromium 51-labelled target cells (4×10^4 per well) were then added to all wells to yield a constant effector cell:target cell ratio of 50:1 and a final well volume of 200 μ l. Control wells contained blank extracts (all extraction steps without tissue). Plates were centrifuged at 200 g for 5 min, incubated at 37°C for 4 h, recentrifuged and 150 μ l supernatant were harvested from each well. Supernatants were counted in a Beckman Gamma counter and the percentage of cell mediated lysis (% CML) was calculated according to the following formula:

$$\% \text{ CML} = \frac{(\text{c.p.m. test} - \text{c.p.m. spontaneous})}{(\text{c.p.m. max.} - \text{c.p.m. spontaneous})} \times 100\%$$

Maximum release (c.p.m. max.) was determined by counting aliquants of resuspended target cells alone while spontaneous release (c.p.m. spontaneous) was determined by counting samples of supernatant medium from wells containing target cells only. Samples of experimental wells were counted to measure c.p.m. test. Assays of each extract were carried out in triplicate. Control % CML at an effector:target ratio of 50:1 was determined by averaging the values of % CML from wells without extract. Suppressive activity of the fetoplacental unit extracts was determined by constructing linear dilution curves of % CML vs extract dilution. Inhibitory units (IU) were defined as the titre of extract yielding 50% of control cytotoxicity, and were normalized to 100 mg tissue.

Data analysis. Analysis of variance (ANOVA) was used to distinguish significant differences in the gestational stage specificity of NK suppressive activity in normal pregnancy. Data from experiments on NK suppressive activity in individual fetoplacental units from CBA/J $\times$ DBA/2 and CBA/J $\times$ BALB/c pregnancies were analysed by Mann–Whitney Wilcoxon non-parametric rank tests. Frequencies of non-suppressive fetoplacental units, NK-infiltrated fetoplacental units and resorption rate were analysed by χ^2 population analysis. Data from experiments on the effect of acetyl salicylic acid on NK suppressive activity were analysed by Student's t test. Significance was assessed at the $P \leq 0.05$ level.

Results

Gestation day specificity of NK suppressive activity in normal pregnancy

The formate-eluted fraction from pooled fetoplacental units of CFW $\times$ DBA/J pregnant uteri (normal pregnancy) showed NK inhibitory activity. The inhibitory activity showed a dependence on day of gestation (Fig. 1). NK inhibitory activity increased sharply between Days 7 and 8 of gestation. Maximal NK inhibitory activity occurred at Day 8. The activity then declined through Day 9, and reached negligible levels by Day 10 of gestation. The mean resorption frequency for 9 CFW $\times$ DBA/2 pregnancies was $2.2 \pm 1.47\%$ when assessed on Day 12 of gestation.

Fig. 1. Differential expression of NK suppressive activity (mean $\pm$ s.e.) as a function of day of gestation in normal CFW $\times$ DBA/2 pregnancies. Between 4 and 7 animals per day of gestation were analysed by extracting whole uteri. Data were analysed by ANOVA. Data points with different letter designations differ significantly.

Inhibition of NK activity by CBA/J × DBA/2 and CBA/J × BALB/c fetoplacental units

The mean inhibitory activity of extracts from CBA/J × DBA/2 fetoplacental units was significantly lower than the mean inhibitory activity for CBA/J × BALB/c fetoplacental units (Table 1). When fetoplacental units containing no inhibitory activity were excluded from the analysis, the mean inhibitory activity of CBA/J × DBA/2 fetoplacental units was still significantly less than that of the CBA/J × BALB/c fetoplacental units (Table 1). The frequency of occurrence of fetoplacental units with no inhibitory activity was significantly lower for CBA/J × BALB/c pregnancies compared to the CBA/J × DBA/J pregnancies (Table 2).

Table 1. NK inhibitory activity of CBA/J × DBA/2 and CBA/J × BALB/c pregnancies

Mating	No. of implantations	Inhibitory units/100 mg	
		All fetoplacental units	Suppressive fetoplacental units
CBA/J × BALB/c	5.8 ± 0.2	210.21 ± 21.4	217.71 ± 20.8
CBA/J × DBA/2	8.6 ± 1.1	91.30 ± 13.4*	130.86 ± 13.9*

Values are mean ± s.e.m. for 5 individuals at Day 9 of gestation.

*Significantly different when compared to control (CBA/J × BALB/c).

Table 2. Correlation between resorption, NK suppression deficiency and NK infiltration in mouse pregnancies

Mating	% resorption at Day 12	% infiltrated fetoplacental units at Day 9	% non-suppressive fetoplacental units at Day 9
CBA/J × BALB/c	4.50 ± 4.5 (4, 23)	5.00 ± 5.0 (5, 33)	5.3 ± 5.3 (5, 29)
CBA/J × DBA/2	28.54 ± 3.0* (8, 81)	30.16 ± 7.3* (6, 44)	29.0 ± 2.3* (5, 43)

Values are mean ± s.e.m. for the no. of animals and individual fetoplacental units indicated in parentheses.

* $P < 0.05$ compared to CBA/J × BALB/c values (χ^2).

Figure 2 illustrates the differences in the NK inhibitory effect observed for extracts from individual fetoplacental units between a CBA/J × DBA/2 and a CBA/J × BALB/c pregnancy. The fetoplacental units from the CBA/J × DBA/2 pregnancy were less inhibitory for NK activity than were units from the CBA/J × BALB/c pregnancy. Furthermore, 3 out of the 9 fetoplacental units in the CBA/J × DBA/2 pregnancy contained no NK inhibitory activity. All fetoplacental units from the CBA/J × BALB/c pregnancy showed inhibitory activity.

When non-pregnant CBA/J uteri were analysed, only 1 of 11 extracted tissue fragments inhibited NK activity, and the level of inhibition (20 IU) was low. Analysis of extracts from 2 whole non-pregnant CBA/J uteri in separate experiments also showed no inhibition of NK activity. No inhibition of NK activity was observed in any of the experiments for blank extracts.

Correlation between NK suppressive activity, resorption rates and asialo GM1-positive cellular infiltrates

The frequency of fetoplacental units with no inhibitory activity in the CBA/J × DBA/J pregnancies was similar to the resorption frequency observed at Day 11 of gestation (Table 2).

Fig. 2. Representative data showing the NK inhibitory activity of individual fetoplacental units from single pregnancies. (a) NK inhibitory activity of all (6) individual units from a CBA/J × BALB/c pregnancy at Day 9 of gestation. (b) NK inhibitory activity of all (9) individual units from a CBA/J × DBA/2 pregnancy at Day 9 of gestation.

Furthermore, the frequency of fetoplacental units with no inhibitory activity was similar to that of units infiltrated by asialo GM1 positive NK cells at Day 9 of gestation (Table 2). In the CBA/J × BALB/c pregnancies, the frequency of non-inhibitory fetoplacental units, the resorption frequency (at Day 11), and the frequency of fetoplacental units with asialo GM1-positive cellular infiltrates were similar and <10% (Table 2).

In the 6 CBA/J × DBA/J pregnancies assessed at Day 9 of gestation, 30% of the total number of implantation sites contained elevated numbers of asialo GM1-positive cells and were thus designated as presumptively resorbing. When assessed as a pooled group, these NK-infiltrated fetoplacental units contained considerably less NK suppressive activity (37 IU/100 mg tissue; 12 units) than presumptively normal fetoplacental units with low numbers of NK cells at Day 9 (90 IU/100 mg tissue; 12 units).

Effect of a cyclo-oxygenase inhibitor on fetoplacental NK suppressive activity

Treatment of CBA/J × DBA/2 pregnant females with acetyl salicylic acid resulted in a significant decrease of the NK suppressive activity (23.3×12.5 IU/100 mg) compared to CBA/J × DBA/2 pregnant females treated with PBS (88 ± 20.0 IU/100 mg).

Discussion

These results demonstrate that the deficiency of a lipophilic factor in the fetoplacental unit may predispose CBA/J × DBA/2 pregnancies to increased resorption. Our analysis of normal CFW × DBA/J pregnancies reveals that the NK suppressive activity is gestational day specific. The peak in NK suppressive activity in normal pregnancies occurs at Day 8–9 of gestation. The NK suppressive activity reaches negligible levels by Day 10–11 of gestation. The demonstration of differential expression of class I MHC genes during mid-gestation in the mouse (Hedley *et al.*, 1989)

shows that antigenic presentation in the feto-placental unit may be tightly stage-specific. The early peak in NK suppressive activity during normal pregnancy may therefore correspond to a time of gestation during which fetally derived antigenic stimulation may be maximal. Indeed, the formation of the ectoplacental cone by invasion into the decidua occurs between Days 6 and 8 (Theiler, 1983). Other groups have reported immunosuppressive uterine compounds of various molecular weights (Davies & Browne, 1985; Daya *et al.*, 1987; Clark *et al.*, 1988; Rolfe *et al.*, 1988). We were able to abrogate a substantial portion of the NK suppressive activity of CBA/J $\times$ DBA/2 extracts by pretreatment of the pregnant animals with acetyl salicylic acid. This suggests that the suppressive activity may be a product of the cyclo-oxygenase pathway. We have preliminary evidence that NK suppressive factors in normal pregnant uterine tissue can be separated into 2 distinct fractions of small and large molecular weight compounds.

Natural killer cell activity present at high levels in normal early mouse decidua (Days 6–8) declines rapidly with gestation and is not associated with resorption (Gambel *et al.*, 1985; Croy *et al.*, 1985). The deficiency of NK suppression in the CBA/J $\times$ DBA/2 pregnancy which we observe at Day 9 may permit decidual NK activity to increase rather than decline. Our previous results implicate NK phenotype cells in the induction of resorption between Days 6 and 10 in the CBA/J $\times$ DBA/2 pregnancy (deFougerolles & Baines, 1987; Gendron & Baines, 1988). The present results suggest that deficient NK suppressive activity precedes or accompanies the infiltration of NK cells and subsequent spontaneous resorption of a predictable percentage of feto-placental units. Our data suggest that a predictable proportion of CBA/J $\times$ DBA/2 feto-placental units cannot provide normal levels of NK suppressive activity. Previous studies on xenogeneic embryo transplants have revealed that suppressor cell recruitment depends upon the type of trophoblast-decidual combination and that failure of the transplant occurs due to a lack of sufficient immunosuppressive activity with subsequent infiltration of maternal lymphocytes (Clark *et al.*, 1986b).

There is good evidence for the role of arachidonic acid metabolites in the suppression of NK activity (Brunda *et al.*, 1980; Koren *et al.*, 1981; Rola-Pleszczynski *et al.*, 1982, 1983, 1985; Ramstedt *et al.*, 1985). Recent studies of the modulation of decidual lymphokine-activated killer (LAK) cell activity and IL-2 receptor expression by prostaglandins demonstrate the importance of these compounds in regulating the local immune response within the feto-placental unit (Parhar *et al.*, 1989). It is therefore likely that the compounds within the feto-placental unit regulating NK activity are also prostaglandins. Experiments using specific inhibitors of lipoxygenase, cyclo-oxygenase and arachidonate metabolites during early gestation are underway and should clarify the mechanisms regulating NK activity in the spontaneous resorption model.

Our results implicate a deficiency in feto-placental suppression of NK activity as a predisposing factor for spontaneous resorption in the CBA/J $\times$ DBA/2 mouse pregnancy. We propose that the absence or deficiency of NK suppressive factors, possibly the E series prostaglandins, is associated with regulation of NK activity and subsequent feto-placental resorption.

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