Thrombocytopenia is an initial maternal response to fertilization in mice

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Summary. There was an increase in weight of the spleens of pregnant and pseudopregnant mice in the first week after mating, but the increase occurred on Day 4 in pregnant mice and Day 2 in pseudopregnant mice. The retardation of the presumed hormonally induced increase in spleen weight during pregnancy corresponded with a significant reduction in the splenic platelet pool. This response by the spleen to early pregnancy suggested that platelets were being supplied to the vascular pool. There was a significant reduction in the platelet count by 10:30 h on the day of mating in pregnant mice and persisted until Day 7 of pregnancy, then returning to normal levels. This response did not occur in pseudopregnant mice. The decrease in platelet count was dependent upon the presence of fertilized eggs. It did not occur in mice sterilized by bilateral ligations of the oviducts and mated with fertile males. Thrombocytopenia did occur within 3 h of transfer of fertilized eggs to pseudopregnant recipients and the magnitude of the response was significantly correlated ($b = -0.86$) with the number of embryos present in the reproductive tract. An initial systemic response to pregnancy in mice was therefore an increased vascular demand for blood platelets, resulting in a significant reduction in the splenic and peripheral blood platelet concentration.

Introduction

An important question in reproductive biology is whether maternal recognition of pregnancy occurs before implantation. Presently, the earliest evidence of maternal response to pregnancy is the maintenance of the corpus luteum by the production of chorionic gonadotrophin following implantation (see Sauer, 1979, for review). There have been equivocal reports of alterations to the maternal lymphocytes before implantation (Morton, Hegh & Clunie, 1976) but the nature and biological significance of these changes remain ill-defined (Whyte & Heap, 1983).

The present study was designed to determine whether changes to basic aspects of maternal physiology occurred as a consequence of fertilization and preimplantation embryonic development.

Materials and Methods

The experiments were carried out in two parts: (1) spleen weight, morphology and cellular composition during the first week of pregnancy and pseudopregnancy were compared, and (2) the peripheral blood count was monitored for the first week of pregnancy and pseudopregnancy and the effects of the presence of the embryo in the reproductive tract were determined.

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Experiment 1

Female mice of the Quackenbush strain, 8 weeks of age and weighing 29.5–30.5 g, were placed with fertile or vasectomized males. They were examined each morning for the presence of copulation plugs (Day 1). The animals were killed by cervical dislocation at 12:30 h on Days 1–7 and their spleens removed, lightly blotted and all fat removed. The reproductive tract of each animal, Days 1–5, was flushed to detect the presence of embryos and thereby confirm the state of pregnancy. The spleens were weighed and placed into Bouin’s fixative, for later histology, or into Alsever’s solution, for determination of the cellular composition.

Histology. The spleens from Day-2 pregnant and pseudopregnant mice were fixed in Bouin’s fixative for 24 h and then in 70% ethanol. After dehydration, embedding in paraffin wax, sectioning at 7 µm and staining with haematoxylin and eosin, the slides were examined for morphological differences. Transverse sections were taken from half way along the length of the spleen and care was taken to view comparable sections from the two groups of mice.

Cellular composition. Each spleen was placed into 2 ml of cold (4°C) Alsever’s solution and finely minced with scissors. The minced spleen was then transferred to a siliconized (30 sec in 1% v/v Prosil-28: PCR Inc., Gainesville, Fl, U.S.A.) glass homogenizer (Kontes Glass Co., Sydney, Australia), and gently ground until free cells were liberated from the reticular meshwork. The homogenate was diluted to 10 ml with Alsever’s solution.

Cell counts. Cell counts were performed with a Neubauer haemocytometer and phase-contrast microscopy. Total cell counts were performed on cells in Alsever’s solution. Erythrocytes could be distinguished from nucleated cells by their morphology and size. Platelets were recognized by their small size, characteristic morphology and highly refractive nature (Brecker & Cronkite, 1950).

Statistical analysis. The results were analysed by analysis of variance, and multiple comparisons of means were performed by the Newman–Keul’s multiple range test (Zar, 1974).

Experiment 2

Quackenbush-strain mice, 7 weeks old and weighing 25 g, were used. To determine the normal blood platelet count, a group of 18 non-pregnant females at random stages of the oestrous cycle were bled and platelet counts performed. Other females were mated (Day 1) with intact or vasectomized males to obtain pregnant and pseudopregnant animals.

Blood was collected, under light ether anaesthesia, from the orbital plexus at 8:30, 10:30, 12:30, 14:30 and 16:30 h on Day 1 and at about 16:00 h on Days 2–7. Blood was diluted in ammonium oxalate solution and platelet counts performed, or placed into microhaematocrit tubes and the haematocrit determined. Blood was collected by piercing the periorbital plexus with a siliconized (30 sec in 1% (v/v) Prosil-28) glass Pasteur pipette. Blood was allowed to flow up the pipette by capillarity until it reaches a 200 µl calibration mark. The blood was mixed immediately with 5 µl 5% (w/v) EDTA (tetra sodium salt, Sigma Chemical Co., St Louis, MO, U.S.A.).

Platelet counts. Platelet counts were performed after a 1:200 dilution of whole blood in 1%(w/v) ammonium oxalate (Aust. Government Stores, Sydney, Australia) after the method of Brecker & Cronkite (1950). Blood was thoroughly mixed with the solution and left for 5 min, during which time all erythrocytes lysed.

Both sides of a Neubauer haemocytometer were filled, and it was placed in a Petri dish containing a moist filter paper for 15 min before counting. Counts were performed by using phase-contrast microscopy. Platelets appeared as small round dark bodies sometimes showing dendritic processes, and could be easily distinguished from erythrocytes and leucocytes. All platelet counts were performed in duplicate.

Bilateral tubal ligation. Females (3 months old) were anaesthetized with pentobarbitone sodium and the oviduct was exteriorized, taking care not to rupture any major blood vessels. The oviduct was ligated with a 4/0 silk suture. The same procedure was followed for the ligation of the contralateral oviduct. Other animals were treated similarly except that the oviducts were not...
ligated. These acted as controls. The reproductive tract and ovary were immediately replaced into the abdominal cavity. The animals were allowed to recover for 8 days and were then placed with fertile males. Each animal was bled at 16:00 h on the day after mating.

**Embryo transfer.** Using Dulbecco’s phosphate-buffered saline (pH 7.4) containing 3 mg bovine serum albumin/ml (Commonwealth Serum Laboratories, Melbourne, Australia), eggs from Day-1 pregnant and pseudopregnant mice were flushed from the oviducts at 14:00 h. Pseudopregnant mice were anaesthetized on the day of mating (Day 1) and their reproductive tract exposed. Care was taken to cause minimal bleeding. Each mouse received 16 fertilized or unfertilized eggs transferred to the ovarian bursa; 3 mice received 5 µl Dulbecco’s phosphate-buffered saline (sham operation). The animals were bled 2, 3, 4 and 5 h after transfer, and platelet counts were performed.

**Correlation of platelet count with embryo number.** At 6 weeks of age females were placed with fertile male mice until mating occurred. These females had just reached sexual maturity, and showed considerable variability in the number of eggs produced. On the day after mating a platelet count was performed. The animals were immediately killed, the oviducts were flushed and the number of embryos present recorded.

**Statistical analysis.** Analyses of platelet counts throughout the 1st week of pregnancy and pseudopregnancy and after transfer of eggs to pseudopregnant recipients were carried out by analysis of variance. Using the error mean square from the analysis of variance, the Newman–Keul’s test was used for comparing means. The peripheral blood platelet counts of females with bilateral tubal ligations or sham operations were analysed by Student’s t test.

**Results**

**Experiment 1**

The spleen weight of pregnant and pseudopregnant mice increased during the week after mating (Text-fig. 1), but the rate of increase in pregnant mice was significantly ($P < 0.05$) slower than in pseudopregnant mice up to Day 4. Analysis of the cell population of the spleens of pseudopregnant and pregnant mice was performed over the 4 days after mating to determine the cause of this retardation of spleen weight gain. The splenic platelet population suffered an immediate and large reduction, being reduced by 50% on Days 2 and 3 of pregnancy (Text-fig. 2).

![Spleen weight](image)

**Text-fig. 1.** The weight (mg) of the spleen of 30 g Quackenbush mice at 16:00 h on various days of pregnancy (○) and pseudopregnancy (●). Each point represents the mean ± s.e.m. of 10 mice. All points showing different letters are significantly different ($P < 0.05$), based on the Newman–Keul’s multiple comparisons test.
Text-fig. 2. The total number ($\times 10^6$) of platelets (a), erythrocytes (b) and nucleated cells (c) liberated from homogenized spleens on Days 1–4 of pregnancy (○) and pseudopregnancy (●). Each point represents the mean ± s.e.m. of 10 mice. All points showing different letters are significantly different ($P < 0.05$), based on the Newman–Keul's multiple comparisons test.

There was also a reduction in the splenic erythrocyte and nucleated cell population (Text-fig. 2), but this reduction was small compared to the reduction in the spleen platelet concentration.

Histological sections from the spleens of Day-2 pregnant and pseudopregnant mice are shown (Pl. 1, Figs 1 & 3). When comparable sections of the red pulp were examined from pregnant and pseudopregnant mice there was an obvious marked reduction in the concentration of megakaryocytes, the platelet precursor cells (Pl. 1, Figs 2 & 4). This observation was consistent in 12 mice studied.

**Experiment 2**

The peripheral blood count of non-pregnant, virgin female mice was $1526 \times 10^3$/mm$^3$ (range $1330–1800 \times 10^3$/mm$^3$, $N = 18$). Although the count was higher than found in previous studies (Copley & Robb, 1942) this was likely to be due to strain variation and/or different counting methodology.

The platelet count of pseudopregnant animals lay in the same range as that for non-pregnant mice (Text-fig. 3). A decrease occurred in pregnant animals, being significant ($P < 0.05$) by 10:30 h on the first day of pregnancy, with a further reduction by 14 : 30 h ($P < 0.01$) to reach about 70% of the level found in pseudopregnant animals. This reduction persisted for the first week of pregnancy. On Day 7 the platelet count had returned to the value obtained in pseudopregnant animals.

To examine whether the reduced platelet count was a consequence of fertilization rather than insemination of spermatozoa, platelet counts were performed on mated females with bilaterally ligated oviducts, and on Day-1 pseudopregnant females that had received fertilized eggs by embryo transfer. After mating, there was no significant decrease ($P > 0.05$) in the mean ± s.e.m. blood platelet count at 16:00 h of animals with bilaterally ligated oviducts ($1578 \pm 106 \times 10^3$/mm$^3$
Transverse sections of the red pulp of spleens from Day-2 ‘pseudopregnant’ (Figs 1 & 2) and ‘pregnant’ (Figs 3 & 4) mice. The ‘pregnant’ red pulp contains markedly fewer megakaryocytes (M) than a comparable area in the spleen of pseudopregnant mice.

Bars = 64 µm (Figs 1 & 3); 32 µm (Figs 2 & 4).

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Text-fig. 3. Peripheral blood platelet count in pseudopregnant (●) and pregnant (○) mice. Each point represents the mean ± s.e.m. of 5 mice. The results for Day-1 animals and Days 2–7 were analysed separately and for each set of data points showing different letters are significantly different (P < 0.05), based on the Newman-Keul’s multiple comparisons test. For haematocrit values (△) on Days 1–7 of pregnancy, each point is the mean of 2 animals.

Text-fig. 4. The peripheral blood platelet count after transfer of fertilized eggs (○), unfertilized eggs (●) and collection medium (△) to the reproductive tract of Day-1 pseudopregnant mice. Each point represents the mean ± s.e.m. of 3 mice. All points showing different letters are significantly different (P < 0.05), based on the Newman-Keul’s multiple comparisons test.

and 1535 ± 70 × 10³/mm³ before and after mating, respectively, compared with 1493 ± 98 × 10³ and 1088 ± 48 × 10³/mm³ in the sham-operated animals, P < 0.001). A reduced count did occur, however, after the transfer of fertilized eggs to pseudopregnant mice, reaching a minimum 4 h after transfer (Text-fig. 4).

Platelet counts were performed on young females which showed considerable variation in the number of eggs ovulated and fertilized. There was a significant (P < 0.002) correlation between the number of embryos present in the reproductive tract and the degree to which thrombocytopenia occurred (Text-fig. 5).
**Discussion**

A significant reduction in the absolute numbers of platelets below the normal level is termed thrombocytopenia (Osol, 1972). After fertilization there was a reduction in both the spleen (Text-fig. 2) and peripheral blood platelet count (Text-fig. 3). There was only a slight decrease in the plasma volume following mating (Text-fig. 3). Therefore, the decrease in the platelet count was not due to a reduction in concentration through dilution, but rather to a reduction in the absolute number of platelets. These results therefore represent a true thrombocytopenia and the phenomenon will be referred to as early pregnancy-associated thrombocytopenia.

Previous studies of the effects of pregnancy on the spleen of mice have invariably been on the post-implantation stages of pregnancy. The spleen increased in size by Day 6 of pregnancy and reached a maximum on Day 12 (Sasaki & Ito, 1980). This was caused by an increased volume of both the red and white pulp (Sasaki & Ito, 1980). Within the white pulp there was an increase in the number of plasma cells, while enlargement of the red pulp was due to an enhancement of the erythropoietic activity of the spleen (Sasaki, Matsumura & Ito, 1981). These changes in spleen physiology were caused by oestrogen (Sasaki & Ito, 1981).

This study confirms the findings of Sasaki & Ito (1980) in that the spleen weight significantly increased by Day 6 of pregnancy, but it also occurred in pseudopregnant mice. The weight gain occurred on Days 2–3 of pseudopregnancy but was retarded until Days 4–5 during pregnancy (Text-fig. 1). A consequence of the preimplantation phase of pregnancy was therefore a transient inhibition of the hormonally induced increase in spleen weight. This retardation of increased spleen weight coincided with a dramatic reduction in the splenic platelet pool (Text-fig. 2) and a reduction in the number of megakaryocytes in the red pulp (Pl. 1, Figs 2 & 4).

A major function of the spleen is a reservoir of blood (Weiss, 1972). In response to vascular demand, the spleen may undergo contraction resulting in the expulsion of considerable numbers of cells into the circulation (Weiss, 1972; Guyton, 1976). After haemorrhage or intravascular coagulation splenic contraction occurs and results in the diminution of the splenic platelet and megakaryocyte pool (Grayson & Mendel, 1965). The provision of these stored platelets to replace those consumed is an important homeostatic mechanism. If the expulsion of splenic platelets during early pregnancy occurred independently of vascular demand then it would be expected that, after the reduction in the splenic platelet pool, the peripheral platelet count would increase. This was not the case; pregnancy resulted in significant thrombocytopenia. It is therefore proposed that the retardation in the increase of spleen weight was due to splenic contraction. This contraction was sufficient to counteract the hormonally induced increase in spleen weight that occurred in pseudopregnancy. The splenic contraction resulted in a marked reduction in the splenic platelet...
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pool apparently due to an increased vascular demand for blood platelets. The provision of the splenic platelets was not sufficient to meet this demand and there was a consequent reduction in the peripheral blood platelet count also.

Most previous studies of the blood platelet count in pregnancy have been performed on women after implantation. There is much controversy regarding the results. There are claims of a decrease in platelet count (Sharper, Kear & MacIntosh, 1968), an increase (Rebadi, 1907; Mor, Yang, Schwarz & Jones, 1960) and no change (Talbert & Langdell, 1964; Todd, Thompson & Bowie, 1965). Some workers have claimed that there was a slight decrease in platelet numbers during the third trimester of pregnancy because of haemodilution or placental trapping of platelets (Sejeny, Eastham & Baker, 1975; Pitkin & Witte, 1979). O'Brien (1976) reported that, while there was a mild decrease in platelet count in late pregnancy, the total platelet mass per ml blood remained approximately constant. There have been no reports in the literature of the thrombocytopenia in early pregnancy described for mice in this study.

Early pregnancy-associated thrombocytopenia was a direct consequence of the presence of fertilized eggs. It did not occur in pseudopregnancy or in mated females with bilaterally ligated oviducts. Therefore, the response was not due to coitus or to the presence of spermatozoa. Thrombocytopenia did, however, occur in pseudopregnant mice after transfer of fertilized, but not unfertilized, eggs to the reproductive tract (Text-fig. 4). The correlation of the degree of early pregnancy-associated thrombocytopenia with the number of embryos present in the oviduct on Day 2 of pregnancy (Text-fig. 5), adds support to the contention that this phenomenon was an embryo-dependent event. The results suggest that early pregnancy-associated thrombocytopenia can be considered as an initial expression of maternal recognition of pregnancy before implantation. On Day 7 of pregnancy the blood platelet numbers returned to the values found in pseudopregnancy. This increase in normal levels might be due to (1) a discontinuation of the demand for platelets, (2) an increased production of platelets, compensating for platelet loss, or (3) a combination of these events.

Intravascular activation of platelets results in the formation of small aggregates which are subsequently trapped by the microvasculature resulting in thrombocytopenia (Camussi, Tetta & Bussolino, 1983). Upon activation, platelets release a vast range of biologically active molecules, these include (i) the biogenic amines, histamine and serotonin which result in vasopermeability and vasodilation, (ii) derivatives of arachidonic acid including the prostaglandins, (iii) growth factors which stimulate growth of connective tissue and transformed cell lines, (iv) factors which have an important role in the cellular attachment and adhesion, and (v) a number of factors of unknown function (see Willis, 1978, for review). The release of the factors as a consequence of early pregnancy-associated thrombocytopenia presumably has significant local and systemic effects. It is conceivable that they may act on the reproductive tract to provide a more suitable environment for embryonic growth and development and/or have a role in uterine preparation for implantation. There is some preliminary evidence to suggest that platelets have a role in the establishment of early pregnancy. Gasic & Gasic (1970) showed, in mice, that depletion of blood platelets, following intravenous injection of antiplatelet serum on Days 4 and 7 of pregnancy, resulted in a 50% reduction in the number of successful pregnancies. Such treatment does not result in platelet activation and the accompanying release of important biologically active molecules but does lead to their destruction by the reticuloendothelial system. Metcalf & Metcalf (1972) showed that phytohaemagglutinin (PHA) stimulation of leucocytes was significantly different in blood collected from early pregnant women when compared with that collected from non-pregnant women and men. They showed that the difference in leucocyte activity was dependent upon a change in the platelets at this time. They were not able to demonstrate the nature of this change although the role of platelets as modulators of the immune response is well established (Willis, 1978). Thus, early pregnancy-associated thrombocytopenia was an initial maternal response to pregnancy and it appeared to be necessary for the establishment of pregnancy (Gasic & Gasic, 1970).

These results show for the first time in any species that physiologically meaningful responses
occur as a response to fertilization and the preimplantation phase of pregnancy. The responses can be easily monitored throughout early pregnancy, and as such provide promise for (1) possible methods of early pregnancy detection, (2) means of monitoring embryo viability, particularly following embryo transfer, and (3) a means of examining the interaction of the embryo and mother before implantation in situ.

References


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