Failure to detect platelet-activating factor using the splenectomized mouse bioassay

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Summary. The ability of purified preparations of platelet-activating factor (PAF), from three different suppliers, to induce thrombocytopaenia in mice after splenectomy and to activate mouse platelets *in vitro* was examined. Although the PAF preparations were potent activators of horse and cow platelets *in vitro*, injections of up to 1 µg PAF failed to elicit thrombocytopaenic responses in either CD1 or Swiss Webster random-bred mice. However, when thrombin was injected into Swiss Webster mice, a dose-dependent decrease in the concentration of platelets was observed. Furthermore, isolated platelets from these strains and from 3 inbred lines (C3H/He, BALB/c, C57BL/6) of mice, were not aggregated by PAF *in vitro* but were sensitive to adenosine diphosphate and thrombin. No change in circulating platelet concentrations was observed over the initial 7 days of gestation in intact Swiss Webster and C57BL/6 or splenectomized C57BL/6 mice, suggesting either an absence of PAF production during early pregnancy in these strains or insensitivity of their platelets to PAF. These results suggest that many mouse strains are unsuitable for the bioassay of PAF.

Keywords: platelet-activating factor; thrombocytopaenia; aggregometry; bioassay; embryonic viability; mouse

Introduction

In mice, a thrombocytopaenic response (i.e. decrease in the concentration of circulating blood platelets) has been reported to occur as soon as 6 h after fertilization and to persist throughout the first week of pregnancy (O'Neill, 1985a). A similar thrombocytopaenic response can be induced in nonpregnant mice by intraperitoneal administration of platelet-activating factor (PAF-acether; O'Neill, 1985c). Both mouse and human preimplantation embryos between the zygote and the blastocyst stages are reported to release a potent platelet-activating factor (O'Neill *et al.*, 1985b; Spinks & O'Neill, 1987; O'Neill *et al.*, 1987), which has similar biochemical and biological properties to the ether phospholipid PAF-acether (O'Neill, 1985b).

Platelet-activating factor has been quantitated in a bioassay using splenectomized mice of the Quackenbush strain (O'Neill *et al.*, 1985a; 1987). Removal of the spleen is necessary because this organ acts as a reservoir for platelets and can release them into the circulation by splenic contraction. This can mask reductions in the circulating platelet population and result in false negative responses in the bioassay (O'Neill, 1985c).

Studies using antagonists of PAF have demonstrated the importance of embryo-derived PAF in the establishment of pregnancy in mice (Spinks & O'Neill, 1987, 1988). In humans, the PAF production from in-vitro fertilized embryos, measured with the bioassay using Quackenbush mice, can be used to determine embryo viability prior to embryo transfer and to monitor embryo survival (O'Neill & Saunders, 1984; O'Neill *et al.*, 1985b). Such a quantitative method for the evaluation of embryonic viability would be of great benefit to the cattle embryo transfer industry, since the rate of loss of morphologically normal embryos following transfer is often >40% (Betteridge, 1986).

However, since the Quackenbush mouse strain is not readily available in North America, and in the light of evidence concerning the insensitivity of platelets from some mouse strains to PAF (Namm *et al.*, 1982), the present study was undertaken to determine whether PAF detection is possible using several more readily available strains of laboratory mice.

Materials and Methods

Experimental animals

Mice. Two random-bred mouse strains, Swiss Webster and CD1, and three inbred mouse strains, C3H/He, C57BL/6 and BALB/c, were obtained from Charles River Laboratories (St Constant, Quebec, Canada) at 4–6 weeks of age. All were housed at 21°C under a 12-h light/12-h dark cycle for at least 2 weeks before use. Food and water were provided *ad libitum*. Pregnancies were established in either Swiss Webster or C57BL/6 mice by placing 2 females with one male of the same genotype. The morning of vaginal plug detection was referred to as Day 0 following removal from the mating cage. Mice in which no vaginal plug was detected were considered unmated and not pregnant, unless upon autopsy their uteri were inflamed or contained foetuses.

Cows and horses. Mature standard-bred mares from the Ontario Veterinary College teaching program and normal, reproductively sound, Holstein heifers from a research embryo donor herd were used as blood donors in this study.

Anaesthetics and surgical procedures

Methoxyflurane inhalation (Metofane; Pitman-Moore Ltd, Washington Crossing, NJ, USA) was used to induce light anaesthesia in mice; 1 ml of metofane was poured onto an absorbent pad in the bottom of a 1.5-litre jar and the mouse was placed on a suspended wire platform within the jar for 1.5–2 min. The mice regained consciousness within 5 min of treatment.

Deep anaesthesia was achieved with an intraperitoneal injection of 0.2–0.5 ml of a 1.25% (w/v) tribromoethanol solution (Aldrich Chemical Company, Milwaukee, WI, USA). The mice remained unconscious for 0.5–1.0 h.

For splenectomy, a left paramedial dorsal incision was performed on deeply anaesthetized 8–10-week-old mice. The spleen was drawn from the abdominal cavity with fine blunt forceps. The splenic blood vessels were electrocauterized and the organ was dissected free of adipose tissue and removed. The incision was sutured with 4-0 silk and the mice were placed under a heat lamp for recovery before being returned to normal housing conditions. Splenectomized mice were used in experiments 8–16 days after surgery.

Blood collection

Mice. Mice were lightly anaesthetized and blood was drawn from the retro-orbital plexus using 20- μ l capillary tubes for the determination of platelet concentrations, 100- μ l heparinized capillary tubes for haematocrit measurement, or siliconized Pasteur pipettes for collection of 0.5–1 ml volumes of blood. Blood collected by Pasteur pipette was prepared for platelet isolation by placing it into a 10 $\times$ 75 mm siliconized glass tube containing sodium citrate (12mM final concentration).

Cows and horses. Blood was collected from the jugular vein through a 14-gauge needle into a 50-ml sterile syringe containing sodium citrate (13mM final concentration). The blood was then placed into siliconized glass 5-ml conical tubes for platelet isolation.

Platelet isolation

Platelet-rich plasma (PRP). Mouse, cow or horse blood containing sodium citrate was centrifuged at 200 *g* for 15 min at room temperature to collect plasma which was rich in suspended platelets.

Platelet-poor plasma (PPP). Following the removal of PRP, the blood was centrifuged at 2000 *g* for a further 15 min. The resulting plasma was free of platelets as determined by microscopic examination.

Washed platelets (WP). Some horse platelets were washed free of plasma and resuspended in Tyrode's buffer, pH 7.4 (Fisher Scientific, Fair Lawn, NJ, USA) as described by Suquet & Leid (1983).

Agents of platelet activation

Platelet-activating factor (PAF). Purified preparations of PAF were obtained from three suppliers: 1-*o*-alkyl-2-acetyl-sn-glycerol-3-phosphoryl-choline from Sigma, St Louis, MO, USA; 1-*o*-hexadecyl-2-acetyl-sn-glycero-3-

phosphocholine from Boehringer-Mannheim, Dorval, Quebec, Canada and L- α -lecithin, β -acetyl, γ -*o*-alkyl from Calbiochem, San Diego, CA, USA. All PAF preparations were dissolved in ethanol, according to the manufacturers' instructions, and then diluted in Dulbecco's phosphate-buffered saline (PBS; Gibco Laboratories, Grand Island, NY, USA) supplemented with 0.25 g bovine serum albumin/100 ml (BSA; Sigma, St Louis, MO, USA; O'Neill, 1985b).

Adenosine diphosphate (ADP). ADP (Sigma, St Louis, MO, USA) was dissolved in a Tyrode's-buffered BSA solution, pH 7.4, for use *in vitro*.

Thrombin. Thrombin (Parke Davis, Scarborough, Ontario, Canada) was prepared for in-vitro and in-vivo use in a Tyrode's-buffered BSA solution, pH 7.4.

Platelet counting

Peripheral blood platelet concentrations were determined on retro-orbital blood samples diluted 1:100 with 70mM ammonium oxalate (Unopette system; Becton-Dickinson, Rutherford, NJ, USA). After thorough mixing with the diluent, the blood was allowed to stand for 5 min, then an aliquant was loaded onto a Bright Line haemocytometer (American Optical, Buffalo, NY, USA). The haemocytometer was placed in a humidified chamber and the platelets were given 15–20 min to settle prior to counting (Brecher & Cronkite, 1950). Platelets appeared as highly refractile small, round to oval, dark bodies with light centres and were counted under phase-contrast microscopic illumination (Carl Zeiss, Don Mills, Ontario, Canada). All platelet counts were performed at least in duplicate and expressed in terms of the concentration of platelets in whole blood.

Experiment 1: determination of variation in platelet concentrations

Blood taken from both retro-orbital plexuses of one splenectomized female C57BL/6 mouse was loaded into 7 haemocytometers and platelet concentrations were determined for each replicate.

Experiment 2: determination of circulating platelet concentrations during pregnancy

Ten pregnant Swiss Webster and 10 C57BL/6 female mice were used to measure circulating platelet concentrations during early pregnancy. For each mouse, blood samples for the determination of platelet concentrations were taken on Days 0, 1, 4, and 7 following removal from the mating cage. The mice were killed on Day 7 and their reproductive tracts were examined for the presence of embryos. Ten nonpregnant female mice of each strain were bled at the same time as the pregnant mice to provide control samples.

The effect of splenectomy on the detection of thrombocytopaenia during early pregnancy was studied in a similar manner, using 3 pregnant and 3 nonpregnant splenectomized female C57BL/6 mice.

Experiment 3a: evaluation of the splenectomized mouse bioassay

Thirty Swiss Webster and 30 CD1 female mice were prepared for use in the PAF bioassay as described by O'Neill *et al.* (1985a). Briefly, a pre-injection control platelet concentration was determined for each splenectomized mouse. Syringes containing 200 μ l of control vehicle (PBS-BSA) or PAF (2.3, 4.6 or 9.2 μ M) were prepared and coded so that their contents remained unknown until the end of the experiment. The content of each syringe was injected i.p. into an individual splenectomized mouse and a blood sample was obtained 30 min later. Each treatment was performed on 3 mice and the thrombocytopaenic effect was calculated for each animal by dividing post-injection by pre-injection platelet concentration and multiplying by 100.

Experiment 3b: estimation of changes in total numbers of circulating platelets during the splenectomized mouse bioassay

Splenectomized Swiss Webster and CD1 mice (10 of each strain) were used to measure platelet concentrations and haematocrits in sequential samples taken before and 30 min after 200- μ l i.p. injections of either control vehicle or PAF (9.2 μ M; Boehringer-Mannheim). Each treatment was performed on 5 mice of each strain. The haematocrits were used to estimate changes in blood volume and to adjust the post-injection platelet concentration accordingly, so that changes in total numbers of circulating platelets could also be estimated. To do so, the post-injection platelet count was multiplied by the ratio of the pre- to post-injection haematocrits. The percentage change in total platelet numbers was then calculated by dividing corrected post- by pre-injection counts and multiplying by 100.

Experiment 4: aggregometric assay of the PAF preparations

A platelet aggregation profiler (Model PAP-2A; Johns Scientific, Toronto, Ontario, Canada) was employed to measure changes in light transmission through a platelet sample during platelet aggregation. The aggregation profiler

was adjusted to 100% transmission with a sample of PPP. A 450- μ l sample of PRP containing 200×10^9 horse or cow platelets/l was then placed into the test chamber to set the 0% transmission reading. A 50- μ l aliquant of PAF ranging from 2nM to 8pM in final concentrations was then added to the PRP sample. The profiler recorded increases in the percentage of light transmitted as platelet aggregation occurred. Fresh dilutions of PAF were prepared and placed either directly into siliconized 12×75 mm glass tubes or loaded into 1-ml disposable syringes and held for 2 h at room temperature. A disposable syringe was used as a container for PAF to assess whether PAF was adsorbed onto plastic, thereby interfering with i.p. delivery to mice (see Exp. 3). To increase the sensitivity of this assay, in some experiments washed horse platelets replaced horse PRP, and Tyrode's buffer replaced PPP. The lowest concentrations of each PAF to cause irreversible and reversible platelet aggregation were recorded.

Experiment 5: induction of thrombocytopenia in mice by thrombin

Twelve female Swiss Webster mice were bled for determination of control platelet concentrations. The mice were randomly assigned to 4 groups and injected into the lateral tail vein with 0, 1, 5 or 20 iu thrombin in 0.5 ml Tyrode's-BSA diluent. After 30 min, blood was sampled for a second platelet count which was expressed as a percentage of the control platelet concentration. The syringes were coded so that their contents were unknown until the completion of the experiment.

Experiment 6: determination of mouse platelet sensitivity to PAF, ADP and thrombin

The sensitivity of platelets from 5 mouse strains (Swiss Webster, CD1, C3H/He, C57BL/6, BALB/c) to PAF, ADP and thrombin was examined using a modified macroscopic haemagglutination procedure (Reisner *et al.*, 1978). For each strain, blood samples from 5 mice were pooled and PRP was prepared. Aliquants of 100 μ l of PRP (400×10^9 platelets/l) were placed on a mirrored plate. To each aliquant, a 10- μ l sample of control buffer (either PBS-BSA or Tyrode's buffer-BSA), thrombin (0.2 iu), ADP (0.1 mM) or one of the three forms of PAF (0.2 mM and 3.7 mM) was added. The droplets were mixed by gently tilting the plate back and forth continuously for 2 min and then detection of platelet clumping was recorded visually as either positive or negative. Each treatment was performed in duplicate.

Statistical analysis

A one-way analysis of variance was used to analyse the effect of PAF, thrombin or ADP injected into splenectomized mice. A two-way analysis of variance was used to analyse peripheral blood platelet concentrations in either intact or splenectomized mice, during the course of early pregnancy. Where appropriate, individual group means were compared using the Student–Newman–Keuls' test.

Results

Determination of variation in platelet concentration (Exp. 1)

Concentrations of platelets in blood samples obtained from each retro-orbital plexus of the same splenectomized mouse over 7 replicate samples ranged from 1450×10^9 /l to 1515×10^9 /l (mean 1487×10^9 /l) and from 1455×10^9 /l to 1515×10^9 /l (mean 1489×10^9 /l), respectively. The coefficients of variation within each sample population were 1.6 and 1.8%, respectively.

Determination of circulating platelet concentration during pregnancy (Exp. 2)

Blood samples from intact pregnant Swiss Webster and C57BL/6 mice during the first 7 days of pregnancy, or nonpregnant mice of the same strains sampled concurrently, did not show significant differences in platelet concentration (Fig. 1). There was a trend, although not significant, for the platelet concentrations in pregnant animals to be lower than in nonpregnant animals in both Swiss Webster and C57BL/6 ($P \leq 0.11$, $P \leq 0.12$, respectively). The mean number of embryos observed in the reproductive tracts of pregnant mice was 11.2 for Swiss Webster and 8.2 for C57BL/6.

When splenectomized C57BL/6 mice were examined over the same period, significant changes in platelet concentrations, with respect to time, were not detected. Furthermore, although differences in the concentrations in pregnant and nonpregnant mice on any day were not significant, those in pregnant animals tended to be higher than in nonpregnant animals ($P \leq 0.14$; Fig. 2). The mean number of embryos observed in the reproductive tracts of pregnant mice was 7.3.

Fig. 1. Peripheral blood platelet concentrations in pregnant (▨) and non-pregnant (□) Swiss Webster (a) and C57BL/6 (b) mice during early pregnancy. Each bar represents the mean $\pm$ s.e.m. of the data from 10 mice.

Fig. 2. Peripheral blood platelet concentrations in pregnant (▨) and nonpregnant (□) splenectomized C57BL/6 mice during early pregnancy. Each bar represents the mean $\pm$ s.e.m. of the data from 3 mice.

Evaluation of the splenectomized mouse bioassay (Exp. 3a)

In the splenectomized mouse bioassay, none of the 3 PAF preparations at any concentration tested (2.3, 4.6 and 9.2 μM) induced a significant change in platelet concentrations with respect to either control or pre-injection concentrations in either strain of mouse used (Fig. 3). The pre-injection platelet concentrations of splenectomized Swiss Webster and CD1 mice ranged from $1430 \times 10^9/l$ to $1595 \times 10^9/l$ and from $1463 \times 10^9/l$ to $1650 \times 10^9/l$, respectively.

Estimation of changes in total numbers of circulating platelets during the splenectomized mouse bioassay (Exp. 3b)

PAF treatment resulted in significant haemoconcentration in both Swiss Webster ($P < 0.05$) and CD1 mice ($P < 0.01$) and resulted in significant increases ($P < 0.01$) in platelet concentrations in both strains of mouse (Table 1). The control injections had no significant effect on either mouse strain. However, when post-injection concentrations were corrected for the change in blood volume, following PAF administration, no significant changes in platelet concentrations were observed.

Fig. 3. Alterations in blood platelet concentration in splenectomized (a) Swiss Webster and (b) CD1 mice following the i.p. administration of 0.0 μM (□), 2.3 μM (▨), 4.6 μM (▩) and 9.2 μM (■) platelet-activating factor (PAF) from 3 sources. Each bar is the mean $\pm$ s.e.m. of the data from 3 mice. PBS-BSA = phosphate-buffered saline-bovine serum albumin.

Aggregometric assay of the PAF preparations (Exp. 4)

Aggregation of cow and horse (unwashed and washed) platelets occurred in the aggregometry assay using all 3 PAF preparations tested (Table 2). Cow platelets were the least sensitive to all 3 PAF preparations, while washed horse platelets were the most sensitive. The PAF obtained from Boehringer-Mannheim was consistently more potent than the other 2 preparations of PAF in all aggregometry assays.

PAF from Calbiochem which had been loaded into a syringe caused aggregation of unwashed horse platelets. The platelet aggregation response was irreversible at a final PAF concentration of 2.0 nM, reversible at 1.0 and 0.5 nM and was not detected at 0.3 nM. PAF prepared at the same time but not loaded into a syringe produced identical results.

Induction of thrombocytopenia in mice by thrombin (Exp. 5)

Thrombin induced a significant ($P \leq 0.01$), dose-dependent reduction in the concentration of peripheral blood platelets following intravenous injection into intact Swiss Webster mice (Fig. 4). The mean ($\pm$ s.e.m.) pre-injection platelet concentration was $1329 \pm 29 \times 10^9/l$.

Determination of mouse platelet sensitivity to PAF, ADP and thrombin (Exp. 6)

Similar results were obtained for all mouse strains tested (Table 3). None of the three preparations of PAF at either concentration aggregated isolated platelets. Both thrombin and ADP produced large platelet aggregates, while buffer controls had no effect on the platelets in the assay (Fig. 5, Table 3).

Discussion

The splenectomized mouse bioassay for the detection of PAF was developed using the Quackenbush strain of mouse (O'Neill *et al.*, 1985a). Our study attempted to reproduce the bioassay using more-common mouse strains. However, three purified sources of PAF which were shown to be potent in-vitro aggregators of cow and horse platelets were unable to induce a thrombocytopenic response in splenectomized mice of either the CD1 or Swiss Webster strain when given in doses comparable to those given to Quackenbush mice (O'Neill, 1985c). It was considered

Table 1. Alterations in blood platelet concentrations and haematocrits of splenectomized mice following the i.p. administration of platelet-activating factor (PAF)

Mouse strain	n	PAF dose (μ M)	Haematocrit				Platelet concentrations			
			Before (B) injection (l/l)	After (A) injection (l/l)	A/B (%)	Before (B) injection (10^{-9} /l)	After (A) injection (10^{-9} /l)	A/B (%)	After (C) injection (10^{-9} /l)	Corrected values C/B (%)
Swiss Webster	5	0	0.493 $\pm$ 0.007	0.473 $\pm$ 0.008	95.9 $\pm$ 0.8	1641 $\pm$ 43	1597 $\pm$ 49	97.3 $\pm$ 1.3	1665 $\pm$ 55	101.4 $\pm$ 1.3
Swiss Webster	5	9.2	0.479 $\pm$ 0.005	0.523 $\pm$ 0.018	109.2 $\pm$ 3.5 ^a	1597 $\pm$ 106	1826 $\pm$ 98	114.8 $\pm$ 4.9 ^a	1668 $\pm$ 80	104.6 $\pm$ 3.4
CD1	5	0	0.454 $\pm$ 0.004	0.452 $\pm$ 0.004	99.6 $\pm$ 0.3	1578 $\pm$ 41	1579 $\pm$ 28	100.1 $\pm$ 0.9	1585 $\pm$ 28	100.5 $\pm$ 0.9
CD1	5	9.2	0.449 $\pm$ 0.012	0.506 $\pm$ 0.007	113.1 $\pm$ 3.5 ^a	1630 $\pm$ 57	1816 $\pm$ 35	111.8 $\pm$ 3.3 ^a	1612 $\pm$ 60	98.9 $\pm$ 0.5

^aValue is significantly different ($P < 0.01$) from its corresponding value.Data are means $\pm$ s.e.m.

Table 2. Reactivity of isolated cow and horse platelets during aggregometry using three preparations of platelet-activating factor (PAF)

Final concentration of PAF	Source of platelets							
	Cow				Horse (washed)			
	Source of PAF				Horse			
	Sigma	Boehringer-Mannheim	Calbiochem	Sigma	Boehringer-Mannheim	Calbiochem	Sigma	Boehringer-Mannheim
2.0 nM	+	+	+	+	+	+	ND	ND
1.0 nM	R	+	+	+	+	+	ND	ND
0.5 nM	R	+	R	R	+	R	ND	ND
0.3 nM	—	R	—	R	+	R	+	+
0.1 nM	—	—	—	—	R	—	+	+
63.0 pM	ND	—	ND	—	R	—	+	+
31.0 pM	ND	ND	ND	ND	—	ND	R	R
16.0 pM	ND	ND	ND	ND	—	ND	—	—
8.0 pM	ND	ND	ND	ND	ND	ND	—	—

Each column of data represents the results from one experiment.
 (+) Irreversible platelet aggregation, (R) reversible platelet aggregation, (—) no response, (ND) not determined.

Fig. 4. Alterations in blood platelet concentration in intact Swiss Webster mice following the i.v. injection of thrombin. Each bar represents the mean $\pm$ s.e.m. of the data from 3 mice.

Table 3. Reactivity of mouse platelet-rich plasma to several platelet-aggregating agents

Treatment (10 μ l)	Mouse strain				
	Swiss Webster	CD1	C57BL/6	C3H/He	Balb/c
Control					
Phosphate-buffered saline	---	---	---	---	---
-bovine serum albumin (BSA)	---	---	---	---	---
Tyrod's-BSA	---	---	---	---	---
Adenosine diphosphate (0.1mM)	+	+	+	+	+
Thrombin (0.2 iu)	+	+	+	+	+
PAF					
Sigma (0.2mM)	---	---	---	---	---
(3.7mM)	---	---	---	---	---
Boehringer-Mannheim (0.2mM)	---	---	---	---	---
(3.7mM)	---	---	---	---	---
Calbiochem (0.2mM)	---	---	---	---	---
(3.7mM)	---	---	---	---	---

(+) Platelet aggregation, (---) no response.

that this could have been due to either genetic differences between mice or a lack of technical competence in platelet counting. Since Quackenbush mice were not available in this laboratory, the genetic differences which might exist between this mouse strain and strains used in this study could not be examined. However, accuracy in platelet counting, the activity of the PAF preparations in other assays and the thrombocytopaenic response of the mice to agents other than PAF were each evaluated.

To rule out technical problems associated with the bioassay, the collection procedures for platelets were standardized, since variability in peripheral blood platelet concentrations $>5\%$ is detrimental to the bioassay (O'Neill *et al.*, 1987). Blood was collected in the same way from all mice from the retro-orbital plexus, and was not allowed to remain in ammonium oxalate for >5 min prior to loading onto a haemocytometer. Platelet counting was performed by the same individual to eliminate observer variation. Replicate efficiency was acceptable, since the coefficient of variation for platelet concentrations from 2 successive blood samples taken from one animal and

Fig. 5. Photomicrograph of isolated Swiss Webster mouse platelets (magnification $\times 60$) following the addition of (a) phosphate-buffered saline-bovine serum albumin, (b) 0.1mM adenosine-diphosphate, (c) 0.2 iu thrombin, (d) 0.2mM Sigma platelet-activating factor (PAF), (e) 0.2mM Boehringer-Mannheim-PAF and (f) 0.2mM Calbiochem-PAF.

loaded into 7 haemocytometers was $<2\%$ in both cases. Also, the range of platelet concentrations reported in this study was $958\text{--}1650 \times 10^9/\text{l}$ compared with the published range of $1330\text{--}1800 \times 10^9/\text{l}$, in different strains of mice (O'Neill, 1985a).

The method of preparing the purified PAF for injection was identical to that described by O'Neill, (1985c) using BSA as a carrier molecule for PAF in PBS. Also, all PAF preparations used in the bioassay were shown to be potent in-vitro aggregators of platelets from other species. Finally, a thrombocytopenic response was readily detected in Swiss Webster mice injected with thrombin in the present study. This suggests that the negative results obtained in the bioassay were caused by a lack of effect of PAF in the mouse strains used and were not due either to technical difficulties in platelet counting or to an inability of the mice to exhibit thrombocytopenia. Experiment 3b was performed in response to a suggestion that thrombocytopenic responses could be masked by concomitant haemoconcentration in the strains of mice that we examined. PAF did, indeed, cause haemoconcentration in both the Swiss Webster and CD1 mice in this experiment. Injections of PAF also increased platelet concentrations to a much larger extent than had been observed in Exp. 3a. The reason for this is unknown, but it may be significant that Exp. 3b was carried out 2 years after Exp. 3a and outbred strains of mice were used. However, after correcting for the haemoconcentration, it was evident that PAF treatment had not reduced the total number of platelets in the circulation, implying that platelet activation had not occurred. Thus, although estimates of changes in blood volumes were not performed in the other experiments of this study, it seems unlikely that they could account for the lack of thrombocytopenic responses. It should also be stressed that the criterion of response in the bioassay has been reported to be a change in platelet concentration, not in total numbers of circulating platelets.

Conflicting results have recently been reported using the splenectomized mouse bioassay. Keefer *et al.* (1989) suggested that thrombocytopenia can only be detected at a very low and narrow dose range of PAF (0.75–5.0 ng) in their particular mouse strain, which is 50–300 times lower than the lowest dose reported by O'Neill (1985c). Other researchers using a Swiss mouse strain have reported that only doses of $\geq 1\mu\text{g}$ PAF have the ability to elicit a thrombocytopenic

response when injected i.p. (Amiel *et al.*, 1989). It is therefore possible that for each of the 5 mouse strains studied the PAF concentrations used were outside the effective dose range.

Both O'Neill (1985c) and Roberts *et al.* (1987) have demonstrated thrombocytopaenia in pregnant C57BL/6 mice. This is contradictory to our findings and the discrepancy is not due to fewer embryos in the reproductive tract of the mice in our study. One explanation for these conflicting data could be that the inbred line (C57BL/6; no source listed) used by O'Neill and by Roberts *et al.* (at the same institution) had undergone genetic drifting resulting in subline divergence (Bailey, 1982).

Another explanation for the failure to observe thrombocytopaenia in either Swiss Webster or C57BL/6 mice during early pregnancy could be the insensitivity of rodent platelets to PAF, as has been described in CD1 mice (Namm *et al.*, 1982) and rats (Inarrea *et al.*, 1984). The present study confirmed PAF insensitivity of isolated CD1 platelets and demonstrated similar PAF insensitivity in platelets isolated from Swiss Webster, BALB/c, C3H/He and C57BL/6 mice using the macroscopic technique of direct observation of platelet aggregation on mirrored plates. Although isolated platelets from all strains of mice tested were insensitive to PAF, they retained their responsiveness to the known platelet-aggregating factors ADP and thrombin, indicating successful platelet isolation procedures.

It has been suggested that a physiological role for the release of PAF from early embryos is to enhance blastocyst growth due to the release of factors from platelets. This is based on observations that blastocyst outgrowth, the in-vitro equivalent to nidation, occurs only in the presence of whole serum and not with serum derived from platelet-depleted plasma (O'Neill, 1985a; O'Neill *et al.*, 1989). This conclusion is not supported by evidence showing that the products of activated platelets are detrimental to the development of ICR mouse embryos *in vitro* (Jinno *et al.*, 1987).

The difficulties of obtaining a mouse strain in which the platelets are sensitive to PAF, combined with the reported low sensitivity of the bioassay (O'Neill *et al.*, 1985a; O'Neill, 1987), make the splenectomized mouse bioassay largely unsuitable for the study of PAF during early pregnancy. Also, recent studies by Amiel *et al.* (1989) cast some doubt on the ability to forecast embryo viability using the measurement of PAF. In addition to finding embryo-derived PAF production in unfertilized oocytes, they question whether embryo-derived PAF is indeed homologous with PAF-acether as claimed by Collier *et al.* (1988), as no PAF activity was detected in the very sensitive aggregometry assay, using culture media which had tested positive for embryo-derived PAF using the mouse bioassay. Milligan & Finn (1990) have recently reported that PAF failed to induce decidualization in mice and, contrary to Spinks & O'Neill (1988), showed that the PAF antagonist (SRI 63-441) was unable to inhibit implantation. Since this study was completed, Smal *et al.* (1990) have reported their inability to detect PAF production by 2-cell embryos, compact morulae, or blastocysts from C57BL/6J $\times$ CBA/H mice using either aggregometry or a highly sensitive radioimmunoassay for PAF.

The contradictions among these published reports, together with the present observations of a lack of sensitivity of platelets from several strains of mice to PAF, lead us to conclude that any critical role for PAF in early pregnancy may be less universal and/or more complex than suggested by earlier studies.

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