Platelet-activating factor and serum components from oestrous mice co-operate to mimic the activity of 'early pregnancy factor' in the rosette inhibition assay

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Summary. When male mouse spleen cells were incubated with a combination of platelet activating factor (PAF, 1-0-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) and sera from female mice in oestrus, the cells displayed a markedly increased rosette inhibition titre (RIT) when subsequently tested in the rosette inhibition assay. Neither PAF nor oestrous mouse sera alone could induce this effect, the combined action was required. Lyso-PAF could not substitute for the PAF, nor could male mouse sera nor the sera from females in dioestrus or metoestrus substitute for the oestrous mouse serum requirement. Pro-oestrous mouse sera could replace oestrous mouse sera but were less effective in their dose-responses. Studies on the mechanism of action of the PAF and oestrous mouse serum components suggested that the PAF stimulated the production and release of soluble factors (termed S2 factors) which by themselves could induce increased RIT values when applied to fresh spleen cells. The PAF-stimulated cell populations were rendered refractory to the action of these S2 factors and did not display increased RIT values, unless oestrous mouse serum was added. This serum acted to reverse the refractory state, allowing the S2 factors to exert their effect, and so cells treated with PAF and oestrous mouse serum displayed increased RIT values.

Keywords: early pregnancy factor; platelet activating factor; rosette inhibition assay; EPF; PAF; mouse

Introduction

An understanding of the mechanisms of the early maternal recognition of pregnancy in the preimplantation period has been a long-sought goal in the study of reproductive physiology. Only two very early phenomena indicative of the maternal recognition of fertilization have been discovered. One of these is the 'early pregnancy factor' phenomenon of Morton *et al.* (1976) which is concerned with the detection of a lymphocyte-modifying activity in maternal serum within hours of fertilization. This serum activity is detected in the rosette inhibition assay. When lymphocytes are exposed to heterologous red blood cells in the presence of a complement source, a small sub-population of the lymphocytes binds red blood cells to form rosettes. The ability of the lymphocytes to form these rosettes can be inhibited in a dose-dependent manner by anti-lymphocyte serum (ALS), and so for a given ALS, a rosette inhibition titre (RIT) may be defined (Bach & Antoine, 1968; Morton *et al.*, 1975). When lymphocytes are incubated in pregnancy sera before testing in this assay, an increased rosette inhibition titre is observed. This ability of pregnancy sera to cause an increase in the RIT of normal lymphocytes is generally referred to as 'early pregnancy factor' (EPF) activity (Morton *et al.*, 1976), although it must be emphasized that the basis for the expression of this activity may well be multi-factorial (Clarke & Wilson, 1982, 1985). This activity can be detected within hours of fertilization and persists in the maternal serum for at least the first two-thirds of pregnancy (Morton *et al.*, 1976, 1977, 1983). In the preimplantation period the expression of serum 'EPF'

activity appears to require the continued production of a signal from the zygote or embryo (Cavanagh *et al.*, 1982).

The other early pregnancy phenomenon is the more recently described (O'Neill 1985a, b, c; O'Neill *et al.*, 1985) maternal thrombocytopenia response as a consequence of the release of an embryo-derived platelet activating factor, which has the chemical, biochemical and physiological properties of the well characterized PAF-acether (1-0-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; Snyder, 1985).

We now have evidence that these two early pregnancy phenomena are probably linked. Orozco *et al.* (1986) found that when synthetic PAF-acether was injected into mature female mice in oestrus, it rapidly induced the expression of 'EPF' activity in the serum, a result suggesting that embryo-derived PAF may be the signal required to induce 'EPF' activity expression during the peri-implantation stages of pregnancy. In the present report we extend these studies on the link between PAF and 'EPF' activity expression.

Materials and Methods

PAF. Synthetic platelet activating factor (1-0-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) was obtained from Boehringer (Mannheim, FRG): 10 mg were dissolved in 2 ml absolute ethanol and aliquants were stored at -30°C . For testing, dilutions of this stock were made in phosphate-buffered saline (PBS; 0.02 M-sodium phosphate, 0.9%, NaCl, pH 7.4) containing 0.2 mg bovine serum albumin/ml (BSA, fraction V; Sigma Chemical Co., St Louis, MO, USA).

[^3H]PAF ([1-0- ^3H]alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, a mixture of C-16 and C-18 alkyl ethers, sp. act. 120 Ci/mmol) was obtained from Amersham (Amersham, Bucks, UK) Lyso-PAF (1-0-alkyl-*sn*-3-phosphocholine) was obtained from Sigma and prepared as described for PAF.

Collection of sera. Blood was collected from male and female mice and the serum separated as described previously (Orozco *et al.*, 1986). All animals were mature Quackenbush mice, 6–12 weeks old. Stages of the oestrous cycle and times of pregnancy were identified as described by Orozco *et al.* (1986).

Spleen cell preparation. Cell suspensions, adjusted to 15×10^6 cells/ml, were prepared from the spleens of mature male Quackenbush mice as described previously (Orozco *et al.*, 1986).

Cell incubations. For the standard cell incubation before testing in the rosette inhibition assay, 1 ml aliquants of cell suspension were centrifuged at 400 *g* for 5 min and the supernatant aspirated. The cell pellets were resuspended in PBS to give a final volume of 200 μl after addition of test samples. PAF and sera were added as 5 μl aliquants with these samples being serially diluted in PBS/BSA (0.2 mg/ml) when required. S1 and S2 (see text) were added as 10 μl aliquants, with samples having been serially diluted in PBS/BSA when required (see text). After incubation at 37°C for 30 min, the cells were sedimented and then washed twice with 2 ml Hanks balanced salt solution (HBSS, Gibco, Grand Island, NY, USA). After the final centrifugation the cells were resuspended in a final volume of 1.5 ml and dispensed into the ALS dilutions as described for the rosette inhibition assay below. Modifications of this cell incubation protocol were used in certain experiments as documented in the 'Results'.

Rosette inhibition assay. The rosette inhibition titre of the cells was determined using the improved and modified assay previously described (Orozco *et al.*, 1986). All materials used in the assay were as described in that paper. In brief, for each assay, ALS was diluted to 1 in 10^6 with HBSS. This 10^{-6} dilution was then used to prepare 8 tubes containing 240 μl of the following dilutions of ALS: 1 in 10^6 , 1 in 4×10^6 , 1 in 16×10^6 , 1 in 64×10^6 , 1 in 10^9 , 1 in 16×10^9 , 1 in 256×10^9 , 1 in 4×10^{12} . Two control tubes containing 240 μl HBSS were also included in each assay. Aliquants (100 μl) of spleen cell suspension and 50 μl of 1 in 60 diluted human serum (as a complement source) were added to each of the tubes which were then incubated at 37°C for 80 min. The preparation of ALS dilutions and the dispensing of cells and human serum were all performed with a Hamilton Microlab M-diluter-dispenser which has an accuracy and reproducibility of $\pm 1\%$.

After incubation for 80 min the tubes were placed on the bench and 100 μl of a suspension of human red blood cells ($10^8/\text{ml}$ in HBSS) were added to each tube of a given assay and the tubes were immediately centrifuged at 200 *g* for 5 min. The pellets were gently resuspended by mixing end over end on a Matburn wheel for 5 min. The suspensions were then spread on double-chamber Neubauer haemocytometers and the numbers of rosettes in the entire field in both chambers were counted using a Visopan projection microscope. The average number of rosettes in each of the ALS dilutions was recorded as a percentage of the mean number in the control tubes containing no ALS. The rosette inhibition titre (RIT) was recorded as the highest ALS dilution in which the number of rosettes formed was $<75\%$ of the number in the control tubes in the absence of ALS. In keeping with past practice, the RIT has been expressed as the log 2 [reciprocal dilution of $\text{ALS} \times 10^{-3}$] (Rolfé *et al.*, 1984). Thus, if rosette formation is inhibited to an ALS dilution of 1 in 4×10^6 , the RIT is expressed as 12.

During the course of these studies 120 control assays were performed in which spleen cells were incubated in PBS alone; of these, 112 yielded RIT values of 12 and 8 RIT values of 14 giving a mean control RIT of 12.1 ± 0.4 . Consequently the observation of an RIT > 16 was considered to be significantly higher than the control and was taken as indicating expression of activity.

Metabolism of [^3H]PAF by mouse serum. [^3H]PAF (0.12 μCi , 1 pmol) was incubated with 5 μl mouse serum sample at 37°C for 60 min in a final volume of 200 μl PBS. Control incubation of [^3H]PAF in the absence of serum was performed in the presence of PBS/BSA (0.2 mg/ml). At the end of the incubation period the reaction was terminated by the addition of 19 volumes of chloroform:methanol (2:1 v/v) and taken for lipid extraction and analysis by t.l.c. as described below.

Metabolism of [^3H]PAF by mouse spleen cells. Aliquants of [^3H]PAF (0.12 μCi , 1 pmol) were incubated with mouse spleen cells (15×10^6 cells) in 200 μl PBS for 30 min at 37°C . The cells were sedimented by centrifugation giving a cell pellet (P1) and a supernatant (S1). Samples of these were taken for liquid scintillation counting to determine total ^3H distribution, while other samples were subjected to lipid extraction and analysis by t.l.c. as described below. Other samples of cell pellets (P1) obtained after incubation with [^3H]PAF were washed twice with PBS, then resuspended in 200 μl PBS for a further incubation at 37°C for 30 min. At the end of this second incubation, cells were again sedimented by centrifugation, giving a cell pellet designated P2 and a supernatant S2. Total ^3H distribution was again determined by liquid scintillation counting and other samples were subjected to lipid extraction and analyses by t.l.c. as described below.

Lipid extraction and analysis. Lipids were extracted by the method of Folch *et al.* (1957). The chloroform phase was removed and evaporated to dryness under O_2 -free N_2 . The lipid extracts were dissolved in a minimal volume (usually 20 μl) of chloroform:methanol (2:1 v/v). Samples of the lipid extracts were taken for liquid scintillation counting to determine total ^3H , while other samples were fractionated by t.l.c. (silica gel plates; Merck, Darmstadt, West Germany) with a solvent of chloroform:methanol/ NH_3 (70:35:7 by vol.) as described by Touqui *et al.* (1985). The chromatograms were divided into 5 mm bands and the radioactivity associated with each band was determined by liquid scintillation counting. The positions of PAF, lyso-PAF and phosphatidylcholine were determined by parallel chromatograms using these standard compounds. The recovery of ^3H in the total lipid extracts ranged from 80 to 95%, while recovery of radioactivity from the t.l.c. plates was in the range of 68 to 75%.

Results

PAF plus sera from mice in oestrus mimic 'EPF' activity

Table 1 presents the rosette inhibition titres obtained with mouse spleen cells following 30-min incubations at 37°C with PAF-acether, lyso-PAF and various types of mouse sera either individually or in combination. Cells incubated with PAF or lyso-PAF (5 nM) alone showed no change in RIT when compared to control cells incubated in the absence of these substances. Similarly, when cells were incubated with sera from males or from females at any stage of the oestrous cycle there was no change in RIT from that observed with controls. On the other hand when the cells were incubated with a mixture of PAF and serum from oestrous or pro-oestrous mice, activity expression was observed, as the cells exhibited a markedly enhanced RIT when tested in the rosette inhibition assay (Table 1). This enhanced titre or activity expression was comparable to that seen for incubation of cells with pregnancy sera alone, which has previously been defined as 'EPF' activity. In contrast to the ability of mixtures of PAF and oestrous or pro-oestrous mouse sera to induce activity expression, incubation of cells with mixtures of PAF with male, dioestrous or metoestrous mouse sera failed to induce activity expression. Also, whereas mixtures of PAF with serum from oestrous or pro-oestrous mice induced activity expression, mixtures of these same sera with lyso-PAF were without effect in the assay (Table 1).

While the results with the PAF/serum mixtures presented in Table 1 were obtained by the simultaneous addition of PAF and sera to the cells at the start of the 30-min incubation period, identical results were obtained even when the PAF was mixed with the serum and incubated for up to 1 h before addition to the cells. To determine whether variations in PAF-acetyl hydrolase activity in the different types of sera could contribute to the results observed in Table 1, 5 nM- ^3H]PAF was incubated with 5 μl of 10^{-1} dilutions of the various sera for 1 h at 37°C , after which

Table 1. RIT of male mouse spleen cells following incubation with PAF, lyso-PAF or serum samples alone or in combination

Sample	RIT	Activity expression
PAF	12 (10)	—
Lyso-PAF	12 (5)	—
Male mouse serum	12 (10)	—
Dioestrous mouse serum	12 (4)	—
Pro-oestrous mouse serum	12 (4)	—
Oestrous mouse serum	12 (4)	—
Metooestrous mouse serum	12 (4)	—
PAF + male mouse serum	12 (3)	—
PAF + dioestrous mouse serum	14 (3)	—
PAF + pro-oestrous mouse serum	28 (4)	+
PAF + oestrous mouse serum	28 (4)	+
PAF + metooestrous mouse serum	12 (4)	—
Lyso-PAF + pro-oestrous mouse serum	12 (2)	—
Lyso-PAF + oestrous mouse serum	12 (3)	—
Pregnancy serum, Day 2	28 (4)	+

PAF and lyso-PAF were tested at 5 nM initial concentration. Sera were tested at 0.5 μ l. Number in parentheses for test with sera indicate number of different sera tested in duplicate with different spleen cell preparations. In tests with PAF or lyso-PAF alone it indicates the number of determinations using different spleen cell preparations.

time the [^{3} H]PAF metabolites were extracted and analysed on t.l.c. The results revealed that there was considerable conversion of [^{3} H]PAF on incubation with sera, with the major product being lyso-PAF. However, even after the 1-h incubation a significant amount of unhydrolysed PAF-acether remained.

In all cases approximately 20–25% remained unhydrolysed and no significant differences were observed between any of the sera and so variations in acetyl hydrolase activity cannot account for the sera specificity seen in the results in Table 1. In experiments presented there, the minimum PAF concentration during the incubation should have been in excess of 1 nM (also see below). That such a concentration is sufficient to induce activity expression when applied in combination with the appropriate serum components is illustrated by the PAF dose–response curve in Fig. 1. To determine the minimum initial concentration of PAF required for activity expression, cells were incubated with 5 μ l of 10^{-1} oestrous mouse serum and serial 10-fold dilutions of PAF ranging from 50 nM to 0.5 pM initial concentration. The minimum initial concentration of PAF required in combination with oestrous mouse serum to induce activity expression consistently was in the range of 50 to 500 pM (Fig. 1). In all mixtures in the experiments reported in Table 1 therefore a sufficient initial concentration of PAF would have been present.

The dose–response of the oestrus-dependent serum component(s) in inducing activity expression was determined by incubating spleen cells with 5 nM-PAF and 5 μ l of 1 in 100 serial dilutions of sera. Typical results obtained with three different oestrous and pro-oestrous mouse sera are shown in Fig. 2. Oestrous mouse sera were effective down to a dilution of 10^{-5} while the pro-oestrous mouse sera were less effective by a factor of approximately 10–100-fold.

Fig. 1. Effect of the initial concentration of PAF on the RIT of male mouse spleen cells when applied in combination with a fixed amount of serum (0.5 μ l) from a female mouse in oestrus. Results (mean $\pm$ s.d.) are from 4 independent experiments with different spleen cell preparations.

Fig. 2. Effect of different doses of sera from oestrous and pro-oestrous mice (5 μ l of indicated dilutions) on the RIT of male mouse spleen cells when applied in combination with a fixed amount of PAF (5 nM). Data (mean $\pm$ s.d.) were obtained with 3 different sera of each type tested on 3 different spleen cell preparations.

Mode of action of PAF and serum component(s)

To investigate further the modes of action of both the PAF and the oestrous mouse serum component(s) in inducing activity expression we examined the effect of the sequential addition of PAF and oestrous mouse serum to the cells and also the kinetics of the cell responses to these substances. When cells were first incubated with oestrous mouse serum (0.5 μ l in 200 μ l PBS, 37°C for 30 min), washed, then incubated for a further 30 min with PAF (5 nM), before being washed and analysed in the rosette inhibition assay, no activity expression was observed (RIT = 12; 9 experiments). On the other hand, if the spleen cells were first incubated with PAF (5 nM), washed, and then incubated with oestrous mouse serum (0.5 μ l), then activity expression was observed as these cells displayed an increased RIT of 28 (9 experiments).

The observation that the PAF and oestrous mouse serum incubations could be conducted sequentially allowed the design of experiments to test the kinetics of the cell responses in terms of

RIT to each of these substances. To determine the kinetics of PAF action, samples of spleen cells were incubated at 37°C for 30 min with PAF (at 5 nM final concentration) being added at various times (30, 20, 15, 10, 5 and 1 min) before the end of this incubation period. The cells were then washed and incubated for a further 30 min in the presence of 0.5 µl oestrous mouse serum, before determination of their RIT in the rosette inhibition assay. The results of 4 independent experiments using spleen cells from 4 different mice on different days are shown in Fig. 3(a). While the different cell preparations showed some variation in their response times, it is evident that an incubation of at least 10 min with PAF was required before an elevated RIT could be observed following subsequent incubation with oestrous mouse serum. In all cases a PAF incubation period of 20 min was sufficient to achieve expression of the maximum RIT. To examine the time course of oestrous mouse serum action, cells were incubated for 30 min with PAF (5 nM), washed, then incubated for a further 30 min at 37°C with oestrous mouse serum (0.5 µl) being added at various times (30, 20, 10, 5, 3, 2 and 1 min) before the end of this incubation period. The cells were then washed as usual and their RIT values determined. The results of 4 experiments with 4 different cell preparations from different animals are shown in Fig. 3(b). In contrast to the action of PAF for which exposure for 10–20 min was required, it is evident that only a very brief period (2–3 min) of exposure of PAF-primed cells to oestrous mouse serum was required to produce a maximally elevated RIT.

Fig. 3. Time course of action of PAF and oestrous mouse serum. (a) RIT of male mouse spleen cells incubated with PAF (5 nM) for the times indicated, washed, then incubated with serum (0.5 µl) from an oestrous mouse for a further 30 min before estimation of RIT. (b) RIT of male mouse spleen cells incubated with PAF (5 nM) for 30 min, washed, then with serum (0.5 µl) from a mouse in oestrus for the times indicated times before estimation of RIT. Values are mean $\pm$ s.d. of 4 independent experiments using different spleen cell preparations.

PAF-stimulated cells release factors active in the rosette inhibition assay

These observations, the ability of PAF to act before the addition of oestrous mouse serum, and the relatively long exposure time required in the action of PAF, suggested that PAF may act by

stimulating the production/release of secondary factors which in turn require the co-operation of component(s) in oestrous mouse serum to induce an increased RIT. To examine further this possibility, the following experiments were performed. Cells (15×10^6 in 200 μ l PBS) were incubated at 37°C for 30 min with 5 nM-PAF and then centrifuged giving a supernatant (S1) and a cell pellet (P1). Aliquants of this supernatant (S1) were added to fresh spleen cells in the presence (0.5 μ l) or absence of oestrous mouse serum and, after a 30 min incubation, the RIT of these cells was determined. The cell pellet (P1) was resuspended and washed twice with PBS before being resuspended in 200 μ l, and then subjected to a second incubation at 37°C for 30 min. Following this incubation the cells were again sedimented by centrifugation giving a second supernatant (S2) and cell pellet fraction (P2). Aliquants of this second supernatant (S2) resulting from the incubation of PAF stimulated cells in PBS were added to fresh cells in the presence (0.5 μ l) or absence of oestrous mouse serum to test for the ability of this supernatant (S2) to induce an increased rosette inhibition titre. To follow the fate of the added PAF acether throughout this protocol, parallel experiments were performed using [^{3} H]PAF (5 nM) and the resulting supernatants (S1 and S2) and cell pellet fractions (P1 and P2) were analysed for total ^{3} H distribution and samples were taken for lipid extraction and fractionation by t.l.c. to determine the fate of the added [^{3} H]PAF in each fraction.

Consideration of the data in Fig. 4 shows that the supernatant (S1) obtained from incubation of the cells with PAF could not induce any increase in the RIT when it alone was added to fresh spleen cells. If, however, this supernatant was added to such cells in combination with a sample of oestrous mouse serum, then an elevated RIT was observed. Indeed, the dose-response data provided in Fig. 4 reveal that this supernatant was very effective with as little as 0.1 μ l being capable of inducing an elevated RIT when assayed in combination with oestrous mouse serum. This supernatant might be expected to contain some residual of the PAF-acether added to the cell incubation and, if so, the presence of this PAF-acether in S1 in sufficient concentration might account for the ability of this fraction to induce an increased RIT when applied to fresh cells in combination with oestrous mouse serum. To examine this possibility, parallel experiments were conducted in which the cells were incubated with 5 nM-[^{3} H]PAF-acether. Subsequent lipid extraction and analysis allowed quantitation of the uptake and metabolism of the added [^{3} H]PAF and to estimate the amount of exogenously added PAF remaining as PAF-acether in the supernatant fraction S1. The results of these experiments, shown in Table 2, revealed the active uptake and metabolism of PAF-acether during the 30-min incubation period. Approximately 40% of the radioactive label was found associated with the cell pellet (P1), and t.l.c. analyses revealed that most of this (69%) was present as alkyl-acyl phosphorylcholine, 24%, as PAF-acether and the remainder (5%) as lyso-PAF. In the supernatant fraction S1, which contained 60%, of the original label, approximately 49% was present in PAF-acether, 5%, as lyso-PAF and the rest (46%) as the alkyl-acyl derivative. From the data it can be calculated that about 30% of the originally added PAF-acether was still present as PAF-acether in this supernatant and, as the initial PAF concentration in the incubation mixtures was 5 nM, the estimated concentration of PAF-acether in S1 was ~ 1.5 nM, emphasizing that the presence of PAF could possibly account for some of the results observed with this fraction (see 'Discussion').

After the first incubation with PAF to generate the supernatant fraction S1, the PAF-treated cells were washed to remove residual exogenous PAF and then incubated for a further 30 min in PBS, followed by centrifugation to yield a second supernatant fraction S2. The data in Fig. 4 show that this supernatant fraction (S2) derived from the incubation of PAF-stimulated cells could induce an increase in the RIT of fresh cells, when applied to those cells either alone or in combination with oestrous mouse serum. The dose-response data reveal that the S2 fraction was just as effective by itself as it was when applied in combination with oestrous mouse serum. Moreover, S2 was extremely potent with a dose equivalent to only 10^{-5} – 10^{-4} μ l capable of inducing an increase in RIT.

Consideration of the [^{3} H]PAF data reveals an active metabolism and release of cell-associated PAF and its metabolites during this second incubation period. As noted above, 40% of the original

Fig. 4. Effect of different doses of S1 and S2 on the RIT of male mouse spleen cells when tested in the presence (0.5 μ l) or absence of serum from an oestrous mouse (OS). Dose-responses were obtained by testing 10 μ l samples of 10-fold serial dilutions prepared in PBS/BSA (0.2 mg/ml). Results are means of 4–6 experiments.

Table 2. Distribution of ^3H label and its association with PAF metabolites after incubation of [^3H]PAF with mouse spleen cells

Fraction	% Total ^3H recovered in fraction	% Total counts in fraction associated with		
		Lyso-PAF	PAF	Alkylacyl PC
S1	60 $\pm$ 8	4.7 $\pm$ 0.6	48.6 $\pm$ 5.5	46.7 $\pm$ 5.8
P1	40 $\pm$ 8	4.7 $\pm$ 0.8	24.3 $\pm$ 3.8	68.7 $\pm$ 9.0
S2	17 $\pm$ 4	6.8	43.2	50.0
P2	23 $\pm$ 4	3.0	32.1	64.9

Data are mean $\pm$ s.d. from triplicate experiments except for metabolite distribution in S2 and P2 which are the means from duplicate experiments.

^3H label was associated with the cells used in the second incubation. After this incubation 42% of the label (corresponding to 17% of the originally added label) was found to be released from the cells and present in the S2 fraction (Table 2). Lipid extraction and analysis revealed that 43% of this was present as PAF-acether, 50% as the alkyl-acyl derivative and the remainder of lyso-PAF. It may therefore be estimated that approximately 7% of the original [^3H]PAF was present in S2 as PAF-acether, giving a concentration of exogenously added PAF remaining in this fraction of only about 350 pM, indicating that PAF-acether could not have contributed to the activity expression displayed by this fraction. The data suggest that PAF has stimulated the release of soluble factors (termed S2 factors) which by themselves cause an increased RIT when applied to fresh spleen cells.

The PAF-stimulated cells did not themselves display elevated RIT values unless they were incubated with oestrous mouse serum before assay (Table 1). Even after washing and a second incubation in PBS, during which period these cells produce soluble (S2) factors capable of increasing the RIT values of fresh cells, these PAF-stimulated cells (designated P2s in the above protocol) still failed to display an increased RIT (RIT = 12, 6 experiments) unless they were first treated with oestrous mouse serum (RIT = 28, 6 experiments). Thus, although PAF-stimulated cells were

capable of producing active S2 factors they themselves were rendered refractory to the action of the S2 factors they produced. Oestrous mouse sera would seem to act to reverse this PAF-induced refractory state.

Discussion

In our previous studies we observed (Orozco *et al.*, 1986) that sera taken from female mice in oestrus injected 1 h earlier with PAF had the ability to induce the expression of an elevated RIT when applied to male mouse spleen cells. The ability of these sera to induce an elevated RIT was comparable to that of pregnancy sera, an ability previously ascribed to the presence in these sera of a so-called 'early pregnancy factor'. In the present studies we have demonstrated that this same effect, the induction of an elevated RIT, can be achieved completely *in vitro* by incubation of the male mouse spleen cells with a combination of PAF-acether and sera from mice in oestrus. Neither PAF nor oestrous mouse serum alone is capable of inducing an increased RIT, the combination is required. Lyso-PAF cannot substitute for PAF, nor can dioestrous, metoestrous or male mouse sera substitute for the oestrous mouse serum requirement. Sera from pro-oestrous mice can replace those from oestrous mice, but appear to be at least an order of magnitude less effective. As PAF acetyl-hydrolase activity (Cabot *et al.*, 1984) was found to be comparable in all these sera, oestrous cycle-dependent changes in this activity were excluded as contributing to the observed serum specificities. One possibility was that all sera contain components with the potential to interact with PAF-stimulated cells to induce elevated RIT values, but some may have been unable to display this potential due to the simultaneous presence of high PAF acetyl hydrolase activity which destroyed the PAF before it could act. Differences in acetyl hydrolase activity, for example low activity in oestrous mouse sera, high activity in the others, could have accounted for the results in Table 1. However, as we demonstrated that there were no significant differences in PAF acetyl hydrolase activities between the sera, and that a sufficient concentration of PAF was present in all experiments, we can exclude this possibility. Rather we can conclude that there is a specific serum component or combination of components which displays oestrous cycle-dependent changes and which co-operates with the PAF stimulus in inducing the expression of increased RIT values.

The results allow us to draw some conclusions as to the mode of action of PAF and the oestrous mouse serum component(s). The addition of PAF to mouse spleen cells has a number of basic consequences relevant to the phenomenon under discussion. Firstly, PAF acts to induce the production and release of soluble factors, which we have termed S2 factors, which are by themselves capable of inducing the expression of elevated RIT values if applied to fresh (non-PAF-treated) mouse spleen cells. Secondly, the PAF-treated cells which produce these S2 factors are rendered refractory to their action and so do not display increased RIT values when tested in the rosette inhibition assay; and thirdly, PAF acts to allow the spleen cell population to interact effectively with the oestrous mouse serum component(s), an interaction which reverses and/or prevents the PAF-induced refractory state and so the PAF-treated spleen cell population in the presence of the oestrous mouse serum component(s) may respond to the PAF-induced S2 factors and display an elevated RIT.

This latter conclusion derives from the observations that treatment of cells with oestrous mouse serum followed by washing then addition of PAF was not effective in inducing the expression of elevated RIT values. However, the reverse order of addition, PAF first, followed by washing, then addition of oestrous mouse serum was effective. The ability of PAF to induce this receptive state was time dependent, requiring an exposure of the cells to PAF for at least 10–20 min (see Fig. 3a). This relatively long induction period suggests that the action of PAF in inducing the receptive state may not be direct. On the other hand the effective interaction of the oestrous mouse serum component(s) with the PAF-primed cells was very rapid requiring only 2–3 min (see Fig. 3b), suggestive of a direct action.

The conclusion that PAF induces the production and release of soluble factors which themselves are active in the rosette inhibition assay when tested on fresh spleen cells, derives from the experimental results presented in Fig. 4 and Table 2. In these experiments the incubation of washed, PAF-stimulated cells in PBS for 30 min resulted in the production of a soluble fraction (S2) which was extremely potent in its ability to induce increased RIT values when applied to fresh spleen cells either in the presence of oestrous mouse serum or, most significantly, in the absence of oestrous mouse serum. Consideration of the [^3H]PAF data, and the fact that PAF alone has never been found capable of inducing elevated RIT values, led us to conclude that PAF-acether does not contribute to the activity of the S2 fraction whether it is tested in the presence or in absence of oestrous mouse serum. Instead, the ability of this fraction to induce elevated RIT values is due to the presence of soluble factors which we have termed S2 factors, which are released from the PAF-stimulated cells. The potency of these S2 factors is obviously an important fact to be borne in mind in studies aimed at their identification (Clarke *et al.*, 1990).

While PAF-stimulated cells produce these active S2 factors, they themselves are rendered refractory to the action of the S2 factors unless oestrous mouse serum is added. The induction of this refractory state could be a direct action of PAF or caused by PAF-induced secondary factors. The inability of the S1 fraction to induce elevated RIT values when applied to fresh cells in the absence of oestrous mouse serum could be due to the lack of a sufficient concentration of active S2 factors produced within this initial 30-min incubation period or, more likely, to the presence of residual PAF acether and/or PAF-induced inhibitory factors in sufficient concentration to induce the refractory state. The quantitative analysis of PAF metabolites in S1 (Table 2) did reveal a significant residual concentration of PAF-acether estimated to be ~ 1.5 nM, and so the concentration range of PAF-acether in the assays in which S1 displayed activity with oestrous mouse serum (the 0.1–100 μl doses, Fig. 4) would have been 0.75 pM to 750 pM. Only at the highest dose (see Fig. 2) would PAF have been in sufficient concentration to induce activity expression in the presence of oestrous mouse serum or, conversely the refractory state in the absence of oestrous mouse serum. Consequently, the S1 fraction may well contain active S2 factors but their action, in the absence of oestrous mouse serum, is counteracted by the simultaneous presence of PAF and/or PAF-induced inhibitory factors which induce a refractory state in the fresh cell population to which the S1 sample is applied.

PAF is a potent, multifunctional stimulus with profound effects on many cell types apart from platelets. On platelets it causes aggregation and degranulation, stimulating arachidonic acid metabolism and thromboxane release (Shaw *et al.*, 1981; Snyder, 1985). PAF also stimulates the release of eicosanoids from several other haemopoietic cell types, including neutrophils (Chilton *et al.*, 1982; Roubin *et al.*, 1983) and macrophages (Hartung, 1983). On neutrophils it may also cause degranulation, and on blood vessel cell walls (Emeis & Kluft, 1985) PAF can induce the release of t-plasminogen activator. While lymphocytes are the major cell type present in the spleen cell preparation used in the present studies, the preparation is by no means homogeneous, with many other haemopoietic cell types also present. Consequently, the potential for PAF to induce the production of a variety of mediators exists in the present system, and the data presented suggest that the various actions of PAF identified in these studies may well be mediated by secondary factors, quite possibly different factors for each of the basic actions we have described.

Clearly these factors need to be identified, particularly the PAF-induced S2 factors which are by themselves capable of inducing elevated RIT values. In this respect they mimic the ability of sera from pregnant animals. The continued study of these PAF-induced factors could well shed considerable light on the mechanism of action of pregnancy sera in inducing elevated RIT values and hence the resolution of the factors responsible for 'EPF' activity.

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