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Endogenous corticosteroids mediate the neutrophilia caused by platelet-activating factor in the mouse

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Abstract

Platelet-activating factor (PAF; 100 ng i.v.) transiently modified the number of circulating neutrophils in the mouse, inducing a fast neutropenia (2 min) followed by a late onset neutrophilia (2 h). The potential involvement in PAF-induced neutrophilia of granulocytotic agents such as interleukin-1 and tumor necrosis factor-α could be excluded on the basis of the ineffectiveness of interleukin-1 receptor antagonist and of a specific monoclonal antibody anti-murine tumor necrosis factor-α. PAF granulocytosis was preceded by a significant rise in plasma corticosterone at 20 min. The involvement of endogenous corticosteroids was confirmed by the experiments with adrenalectomized mice and in animals pretreated with the steroid antagonist RU486 (11β-(4-dimethyl amino-phenyl) 17β-hydroxy, 17α(prop-1-ynyl) estra 4,9-dien-3-one), where PAF-induced neutrophilia was greatly reduced ($\sim$ 50%). Moreover, sustained increase in plasma corticosterone by administration of adrenocorticotropic hormone was paralleled by an intense neutrophilia. We show evidence that endogenous corticosterone acts through the glucocorticoid-inducible protein lipocortin 1.

Keywords: Lipocortin 1; RU486; Interleukin-8; Adrenalectomy; PAF (platelet-activating factor)

1. Introduction

Changes in the number of circulating polymorphonuclear leukocytes have long be used as a parameter for neutrophil activation in vivo both in experimental animals and in humans. Most of the experiments performed with animals have been carried out in the rabbit, and have shown that following intravenous (i.v.) infusion of a variety of chemotactic agents a transient quick neutropenia occurs (O'Flaherty et al., 1977, 1978; Camussi et al., 1981; Marleau et al., 1993; Van Zee et al., 1992; Ley et al., 1993; Jagels and Hugli, 1992). Amongst others, such a property has been described for the following neutrophil activators: the tripeptide formyl-Met-Leu-Phe (fMLP) (Jagels and Hugli, 1992; O'Flaherty et al., 1977, 1978), the chemokine interleukin-8 (Van Zee et al., 1992; Hechtman et al., 1991; Ley et al., 1993), the lipid mediators platelet-activating factor (PAF) (Jagels and Hugli, 1992; Camussi et al., 1981) and leukotriene B_4 (Jagels and Hugli, 1992; Marleau et al., 1993). In all cases the transient reduction in the number of circulating neutrophils is followed by a marked neutrophilia which therefore seems to be a common feature for these agents (Jagels and Hugli, 1992; Ley et al., 1993). This may suggest that a common mechanism(s) and/or mediator(s) may underlie this neutrophilia.

Interleukin-1 and the other macrophage-derived cytokine with overlapping properties, tumor necrosis factor-α, are known to cause a monophasic neutrophilia in the rat which peaks between 90 and 240 min (Ulich et al., 1987). Glucocorticoid hormones, like corticosterone, and their releasing agent adrenocorticotropic hormone (ACTH), are other endogenous mediators reported to affect the number of circulating neutrophils (Dougherty and White, 1943, 1944; Goldstein et al., 1992; Butterfield and Gleich, 1989; Bishop et al., 1968). Interestingly, interleukin-1-and tumor necrosis factor-α-induced neutrophilia is not modified in

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adrenalectomized rats thus indicating that endogenous adrenal corticosteroids do not have a role in this effect of the cytokines (Ulich et al., 1987).

Lipocortin 1 is another endogenous protein which, in contrast to pro-inflammatory cytokines, is positively modulated by both exogenous and endogenous glucocorticoid hormones (Flower and Rothwell, 1994; Vishwanath et al., 1992). Lipocortin 1 down-regulates leukocyte function both in vitro and in vivo (Stevens et al., 1988; Perretti and Flower, 1993). We have recently reported that systemic treatment of mice with human recombinant lipocortin 1 greatly affects neutrophil migration to the site of inflammation (Perretti and Flower, 1993), and that induction of endogenous lipocortin 1 mediates the acute effect of systemic corticosteroids on leukocyte recruitment (Perretti and Flower, 1993; Perretti et al., 1994). Overall these studies point to lipocortin 1 as a negative modulator of neutrophil movement, though the exact mechanism is still unclear.

This study aimed to evaluate the effect of chemotactic agents on the number of circulating neutrophils in the mouse. By using experimental tools which selectively prevented the action and/or release of endogenous mediators, such as interleukin-1 receptor antagonist for interleukin-1, a monoclonal antibody to murine tumor necrosis factor-α to block the effect of this cytokine, and adrenalectomy or RU486 to abolish endogenous corticosterone or its action, we have investigated the possibility that a common mediator could be involved in the effect of different chemoattractants. The main observation is that PAF-induced neutrophilia is partially mediated by the release of endogenous corticosterone.

2. Materials and methods

2.1. Animals

Normal or adrenalectomized male Swiss Albino mice (28–30 g, Tuck, Essex, UK) were used for all experiments. Normal mice were maintained on a standard chow pellet diet and tap water ad libitum. Adrenalectomised mice also received the standard diet but were given drinking water containing 0.9% sodium chloride with 1% glucose. All animals were housed for 1 week prior to experimentation. The effectiveness of adrenal gland removal was confirmed by measuring plasma corticosterone levels.

Male C_3H/HeJ were obtained from Harlan-Olac (Bicester, Oxon, UK) and housed as described above.

2.2. Experimental procedure

Normal mice were treated with a single bolus dose of either PAF, fMLP, or interleukin-8 i.v. in 100 μl bovine serum albumin 0.1% in sterile phosphate buffered solution (PBS). Control mice received PBS with bovine serum albumin 0.1% alone. C_3H/HeJ mice received a single i.v. bolus dose of PAF or vehicle. The chemoattractant doses used throughout this study were chosen because they produced a comparable and significant neutropenia as found in preliminary dose-response experiments. At various times after treatment mice were bled by cardiac puncture using heparinised (20 μl of heparin 2000 U/ml) syringes under halothane anaesthesia and then killed. Previous experiments indicated that this procedure minimised the stress experienced by the animals without affecting the profile of circulating leukocytes and allowed reliable measurements of basal plasma corticosterone (Perretti et al., 1993a). Total white cell counts were determined using a Coulter counter (Coulter Electronics, Luton, UK). Differential counts were obtained from blood smears stained with Turk's solution (crystal violet 0.01% w/v in acetic acid 3% v/v) and the total number of polymorphonuclear leukocytes in each blood sample was then calculated.

Adrenalectomized mice were treated with PAF, fMLP or PBS + bovine serum albumin 0.1% in order to investigate the effect of adrenalectomy on PAF and fMLP-induced neutrophilia. In this case blood was taken by cardiac puncture 2 h after treatment for determination of the circulating polymorphonuclear leukocytes and plasma corticosterone levels.

The effect of several immunological and pharmacological treatments on PAF-induced neutrophilia was also evaluated. The steroid antagonist, RU486, was administered following an established protocol (Peers et al., 1988). Briefly, animals were dosed by oral gavage 20 h and 2 h prior to challenge with PAF or ACTH (see below) at the dose of 20 mg/kg. In other cases, normal mice were pre-treated s.c. with a monoclonal antibody raised against murine tumor necrosis factor-α (Sheehan et al., 1989) 20 h prior to the administration of PAF. This antibody and the dose used have been previously validated to be effective in vitro and in vivo against both murine and rat tumor necrosis factor-α (Sheehan et al., 1989; Perretti et al., 1993b; Thiemermann et al., 1993). Rat interleukin-1 receptor antagonist was co-administered i.v. with PAF. We used the highest possible dose of 10 mg/kg (Meyers et al., 1993). The β-blocker propranolol was given i.v. 5 min prior to PAF at the effective dose of 0.1 mg/kg (Altenburg et al., 1994). In all cases blood was taken 2 h after treatment with PAF for determination of polymorphonuclear leukocyte number.

In other experiments, mice were challenged s.c. either with corticosterone or with ACTH. Blood samples were taken at 2 h and 4 h time points and the number of circulating neutrophils was determined as described.

The potential role of endogenous lipocortin 1 in PAF-and ACTH-induced neutrophilia was investigated. Mice were passively immunised with a specific anti-lipocortin 1 sheep serum (50 μl s.c.) according to the time and dose protocol previously reported (Perretti and Flower, 1993; Perretti et al., 1994). Control animals received an identical volume of non-immune sheep serum. Vehicle, PAF or ACTH were administered 20 h after the passive immunisation of animals and the neutrophilic response assessed as described above.

2.3. Radioimmunoassay for corticosterone measurements

Following total and differential (polymorphonuclear and mononuclear leukocyte) white blood cell counts, blood samples were centrifuged at 3000 × *g* for 10 min in a Bench Eppendorf centrifuge. Plasma (supernatant fraction) was stored at −20°C for no longer than 2 weeks before corticosterone measurements. Plasma corticosterone levels in samples prepared from normal or adrenalectomized mice were assayed with a commercially available radioimmunoassay kit according to the manufacturer's instructions (ICN-Biomedical, UK). For adrenalectomized mice a corticosterone value ≥ 20 ng/ml was considered sufficient for discarding the sample (3 out of 27 mice).

2.4. Materials

PAF (C_{16} form: $C_{26}H_{54}NO_7P$), fMLP, corticosterone, non-immune sheep serum, propranolol, low endotoxin content PBS and bovine serum albumin were purchased from Sigma, Poole, UK. Human recombinant interleukin-8 (monocyte-derived 72 amino acid form) was a generous gift of Dr. I. Lindley (Sandoz Forschungsinstitut, Vienna, Austria) and rat interleukin-1 receptor antagonist was a gift from Dr. R.C. Newton (DuPont-Merck, Wilmington, DE, USA). ACTH (Synacthen) was from Ciba Laboratoires, Horsham, UK. Hamster anti-murine tumor necrosis factor-α monoclonal antibody was from Genzyme, West Malling, UK. RU486 (11β-(4-dimethyl amino-phenyl) 17β-hydroxy, 17α(prop-1-ynyl) estra 4,9-dien-3-one; Mifepristone or RU38486) was a kind gift of Roussel-Uclaf, Romainville, France.

2.5. Data and statistics

Data (mean ± S.E.) are expressed as absolute numbers (circulating polymorphonuclear leukocytes or corticosterone) except for Fig. 1: in this case blood polymorphonuclear leukocytes in the experimental groups are expressed as percentage of the values measured in intact mice. Statistical differences were calculated on absolute numbers either by one-way analysis of variance followed by Bonferroni test for intergroup comparisons, or by unpaired Student's *t*-test when only two groups were compared. A P value < 0.05 was taken as significant.

3. Results

3.1. Kinetics of circulating polymorphonuclear leukocytes in the mouse

The number of circulating leukocytes found for intact mice was $4.90 \pm 0.16 \times 10^6$ per ml of blood, 13.1% of which were polymorphonuclear leukocytes ($0.64 \pm 0.03 \times 10^6$ per ml blood, $n = 100$). Treatment of mice with selected doses of PAF (100 ng i.v.), fMLP (10 ng i.v.) or interleukin-8 (600 ng i.v.) resulted in a marked quick neutropenia maximal at 2 min (results as $\times 10^6$ polymorphonuclear leukocytes per ml blood): 0.29 ± 0.03, $n = 16$, $P < 0.01$; 0.43 ± 0.08, $n = 9$, $P < 0.05$; 0.15 ± 0.03, $n = 16$, $P < 0.01$, for PAF, fMLP and interleukin-8, respectively (Fig. 1). This fast disappearance of circulating neutrophils was resolved by 20 min especially for the mice treated with interleukin-8, which showed a significant neutrophilia at this time-point (Fig. 1). For the other chemoattractants the neutrophilic response appeared later and was substantial for PAF (approximatively 4-fold with respect to intact mice and 2-fold when compared to vehicle-treated group) (Fig. 1). fMLP produced only a mild neutrophilia above that caused by the vehicle alone (Fig. 1). For that concerning the total number of circulating

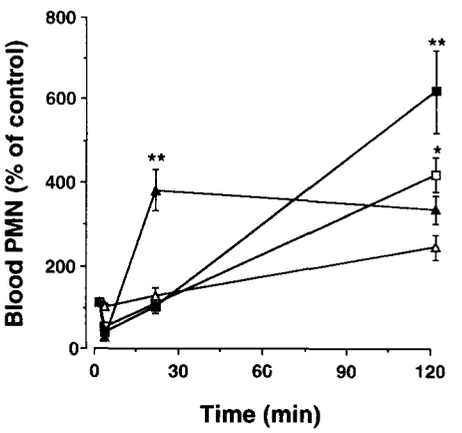

Fig. 1. Kinetics of circulating neutrophils in the mouse following challenge with chemoattractants. Mice received 100 μl i.v. of PBS supplemented with bovine serum albumin (0.1% w/w) alone (vehicle, open triangles) or containing 100 ng PAF (closed squares), 10 ng fMLP (open squares) or 600 ng interleukin-8 (closed triangles). At different times animals were bled and the number of circulating polymorphonuclear leukocytes measured. Values (mean ± S.E., $n = 12$) are reported as percentage calculated from absolute numbers of neutrophils in treated mice over absolute numbers of neutrophils in intact mice. $^{**}P < 0.01$ vs. vehicle-treated group.

leukocytes (polymorphonuclear and mononuclear cells) no difference was observed between vehicle-, PAF-or fMLP-treated mice at 2 h time-point (results as $\times 10^6$ cells per ml blood): 6.34 ± 0.32, $n = 56$, 6.02 ± 0.38, $n = 37$, and 6.40 ± 0.73, $n = 13$, respectively. In the case of interleukin-8 the granulocytosis was resolved by this time-point and, indeed, the total number of white blood cells ($4.47 \pm 0.40 \times 10^6$ cells per ml blood, $n = 8$) was similar to that measured in intact mice (see above).

PAF also induced a significant 2 h-granulocytosis in endotoxin-resistant C_3H/HeJ mice (results as $\times 10^6$ polymorphonuclear leukocytes per ml blood): 1.19 ± 0.08 for intact mice, $n = 4$, 2.03 ± 0.38 in vehicle-treated mice, $n = 5$ (not significant vs. intact mice), and 4.89 ± 0.77 in PAF (100 ng)-treated animals, $n = 6$ ($P < 0.01$ and $P < 0.05$ vs. intact and vehicle-treated mice, respectively).

3.2. Effect of cytokine inhibition on PAF-induced neutrophilia

The potential role of pro-inflammatory cytokines with the property of raising the number of circulating neutrophils was investigated by means of selective agents. Intravenous treatment with interleukin-1 receptor antagonist (10 mg/kg i.v.) did not modify PAF-induced neutrophilia: an increase in circulating polymorphonuclear leukocytes of $0.98 \pm 0.26 \times 10^6$ per ml and of $1.34 \pm 0.14 \times 10^6$ per ml in the absence and presence of treatment with interleukin-1 receptor antagonist ($n = 5$, not significant) was measured. Similarly, pretreatment of mice with a monoclonal antibody raised against murine tumor necrosis factor-α (20 mg/kg) did not significantly modify the neutrophilia caused by platelet activating factor (with an increase of $0.99 \pm 0.28 \times 10^6$ neutrophils per ml of blood, $n = 4$, above the control figure).

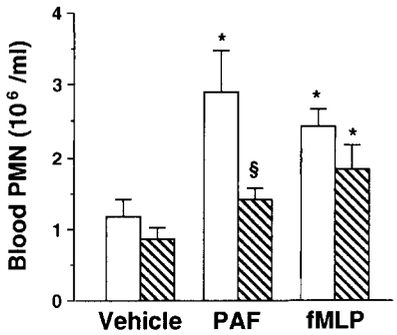

Fig. 2. Effect of adrenalectomy on PAF-and fMLP-induced 2 h neutrophilia. Either intact (open bars) or adrenalectomized (hatched bars) mice received 100 μl i.v. of PBS (supplemented with 0.1% bovine serum albumin) with or without 100 ng PAF or 10 ng fMLP at time 0. Two hours later mice were bled and the number of circulating polymorphonuclear leukocytes (PMN) measured. Values are mean ± S.E. of $n = 5$ for normal mice or $n = 6$–11 for ADX mice. $^*P < 0.05$ vs. respective vehicle group and $^§P < 0.05$ vs. PAF in intact mice.

3.3. Endogenous corticosterone in PAF-induced neutrophilia

Plasma corticosterone levels were measured in intact mice and in mice following treatment with vehicle or chemoattractants. Changes in circulating corticosterone were seen following the injection of all agents after 2 min; however, levels of corticosterone had returned to that of untreated mice by 2 h (Table 1). In the case of the PAF group, there was a consistent and significant increase of corticosterone values above that for vehicle-treated mice at 20 min. No significant changes in circulating corticosterone levels were observed in mice treated with interleukin-8 or fMLP.

Table 1
Plasma corticosterone (CCS) levels after challenge with various chemoattractants

Treatment (i.v.)	Time post-injection (min)		
	2	20	120
Vehicle	107 ± 30 (7)	169 ± 12 (19)	111 ± 12 (23)
PAF 100 ng	156 ± 40 (8)	283 ± 18 (11) [a,b]	111 ± 22 (14)
fMLP 10 ng	76 ± 17 (12)	201 ± 21 (13)	127 ± 20 (16)
Interleukin-8 600 ng	81 ± 23 (8)	136 ± 20 (4)	111 ± 19 (7)

Mice were treated at time 0 with 100 μl of vehicle (PBS with 0.1% bovine serum albumin) alone or containing the reported dose of chemoattractants. At the indicated times animals were bled and plasma prepared for CCS measurements. Values represent ng/ml of CCS and are mean ± S.E. with the number of mice per group shown in brackets. The level of CCS for untreated mice was 61 ± 6 ng/ml ($n = 27$). [a] $P < 0.01$ vs. vehicle-treated group; [b] $P < 0.05$ vs. respective 2 min time-point.

Table 2
Effect of treatment with propranolol on PAF-induced neutrophilia in the mouse

Pretreatment	Treatment	Circulating PMN ($\times 10^6$ per ml of blood)	%
Saline	Vehicle	1.28 ± 0.23	100
Saline	PAF	2.14 ± 0.34 [a]	196
Propranolol	Vehicle	0.61 ± 0.08 [c]	100
Propranolol	PAF	1.30 ± 0.20 [b]	213

Mice received either saline (100 μl i.v.) or propanolol (0.1 mg/kg i.v.) 5 min prior to a second intravenous challenge with PAF (100 ng/100 μl PBS supplemented with 0.1% bovine serum albumin) or with vehicle alone. The number of circulating polymorphonuclear leukocytes (PMN) was quantified 2 h later. Intact mice had $0.52 \pm 0.08 \times 10^6$ PMN per ml of blood ($n = 4$). Values are mean ± S.E. of 5–7 mice per group. [a] $P < 0.05$ and [b] $P < 0.01$ vs. appropriate vehicle-treated group; [c] $P < 0.05$ vs. vehicle-treated group in saline-pretreated mice.

3.4. Effect of adrenalectomy on PAF-induced neutrophilia

The number of circulating polymorphonuclear leukocyte was not different in untreated adrenalectomized mice ($0.70 \pm 0.10 \times 10^6$ neutrophil per ml blood, $n = 6$) when compared to normal animals (see above). The involvement of endogenous corticosterone in PAF-induced neutrophilia was further investigated in adrenalectomized mice by evaluating the effect of this lipid mediator on the circulating polymorphonuclear leukocyte level at 2 h. fMLP was also tested for comparative purposes. Fig. 2 shows that adrenalectomy significantly reduced PAF-induced neutrophilia. Both the vehicle and fMLP caused a comparable neutrophilic response both in normal and adrenalectomized mice (Fig. 2).

3.5. Effect of RU486 on PAF-induced neutrophilia

Treatment of mice with RU486 (20 mg/kg p.o. 20 h and 2 h prior to the specific challenge) did not modify the basal values of circulating polymorphonuclear leukocytes (results as $\times 10^6$ neutrophils per ml blood): 0.62 ± 0.07 in intact mice, $n = 11$, and 0.53 ± 0.06 in RU486-treated mice, $n = 10$ (not significant). However, in the animals pretreated with the steroid antagonist, PAF failed to induce a significant 2 h neutrophilia (Fig. 3A).

3.6. Effect of propranolol on PAF-induced neutrophilia

The role of endogenous adrenaline in the PAF response was investigated with the β-antagonist propranolol. Table 2 reports that treatment of mice with propranolol (0.1 mg/kg) affected that component of the neutrophilic response to PAF which was caused by the vehicle and did not modify PAF-induced neutrophilia per se.

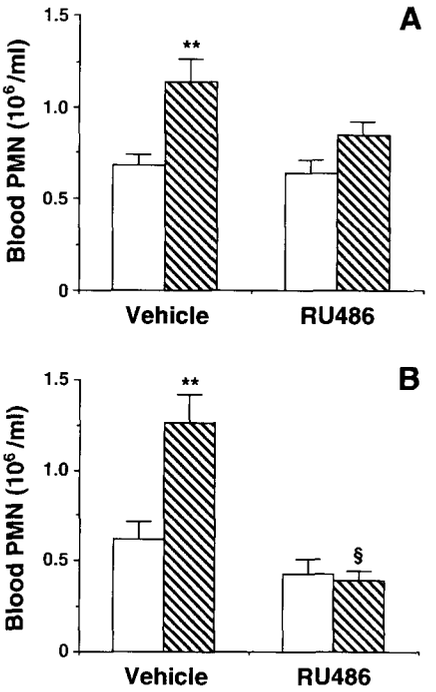

Fig. 3. Effect of treatment with RU486 on PAF-and ACTH-induced neutrophilia. Panel A: Either water (10 ml/kg p.o.) or RU486 (20 mg/kg p.o.) was given to mice 20 h and 2 h prior to challenge with 100 μl i.v. of PBS (supplemented with 0.1% bovine serum albumin) with (hatched bars) or without (open bars) 100 ng PAF at time 0. Two hours later mice were bled and the number of circulating polymorphonuclear leukocytes (PMN) measured. Panel B: Either water (10 ml/kg p.o.) or RU486 (20 mg/kg p.o.) was given to mice 20 h and 2 h prior to challenge with 100 μl s.c. of PBS (open bars) or of ACTH (5 μg, hatched bars) at time 0. Four hours later mice were bled and the number of circulating polymorphonuclear leukocytes (PMN) measured. Values are mean ± S.E. of $n = 9$–12 mice for PAF experiments and $n = 6$ animals for the experiment with ACTH. $^{**}P < 0.01$ vs. respective vehicle-treated group.

3.7. Effect of exogenous corticosterone and ACTH on circulating neutrophils

Acute treatment with high doses of corticosterone did not change the number of circulating polymor-

Table 3
Effect of subcutaneous corticosterone (CCS) and ACTH on circulating polymorphonuclear leukocytes (PMN) in the mouse

Treatment (100 μl)	Time (h)	Circulating ($\times 10^6$ per ml of blood)	PMN (%)	No. of mice
Vehicle	2	0.49 ± 0.05	100	12
CCS 10 μg	2	0.77 ± 0.21	157	11
CCS 100 μg	2	0.50 ± 0.08	102	8
ACTH 5 μg	2	0.44 ± 0.04	90	5
ACTH 20 μg	2	0.96 ± 0.22 [a]	196	5
Vehicle	4	0.39 ± 0.05	100	21
CCS 10 μg	4	0.54 ± 0.08	138	10
CCS 100 μg	4	0.36 ± 0.03	92	6
ACTH 5 μg	4	1.38 ± 0.29 [b]	354	8
ACTH 20 μg	4	2.22 ± 0.30 [b]	569	11

Mice were treated at time 0 with 100 μl of vehicle alone (sterile PBS) or containing the reported dose of CCS or ACTH. At the indicated times animals were bled and the number of circulating PMN calculated. Values are mean ± S.E. for the number of mice shown for each group. [a] $P < 0.05$ or [b] $P < 0.01$ vs. respective vehicle-treated group.

phonuclear leukocytes significantly above control values when measured at 2 h or 4 h time-points (Table 3). Neither dose of corticosterone, however, was sufficient to significantly elevate blood levels of this hormone as assessed at the 2 h time-point: 54 ± 10 ng/ml, $n = 6$, in intact mice; 79 ± 18 ng/ml, $n = 6$, and 56 ± 10 ng/ml in mice which received 10 μg s.c. or 100 μg s.c. of corticosterone at time 0. In contrast to exogenously administered corticosterone, ACTH caused an intense 4 h neutrophilia both at 5 μg and 20 μg doses; only the highest tested dose of 20 μg caused a neutrophilia at the 2 h time-point (Table 3). The increase in the number of circulating neutrophils caused by ACTH

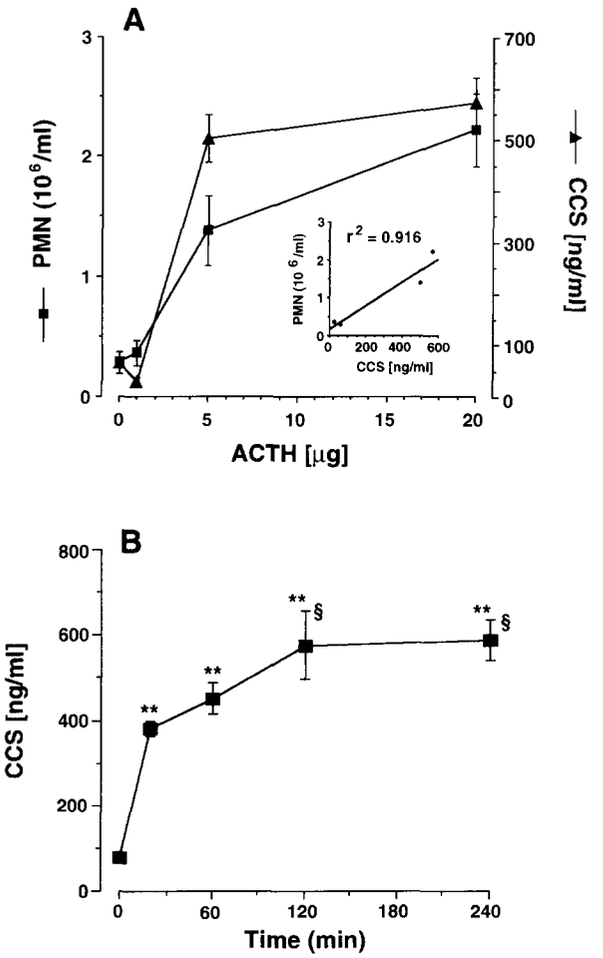

Fig. 4. Effect of ACTH on circulating polymorphonuclear leukocytes (PMN) and corticosterone (CSS). Panel A: Mice (6–11 per group) received either PBS (100 μl s.c.) or the reported doses of ACTH (s.c.) at time 0. Animals were bled 4 h later and circulating PMN and CCS measured as detailed in Materials and methods. Values (mean $\pm$S.E.) for 5 and 20 μg doses are significantly higher than those measured at time 0. Inset: Correlation between the two parameters and ACTH doses. Panel B: Mice ($n = 6$ per group) received 20 μg ACTH at time 0 and were bled at different time points for CCS measurement. Values are mean $\pm$ S.E. All points are significantly higher ($^{**}P < 0.01$) than time 0 values, and concentrations at 2 h and 4 h are significantly higher ($^{\S}P < 0.05$) than at 20 min.

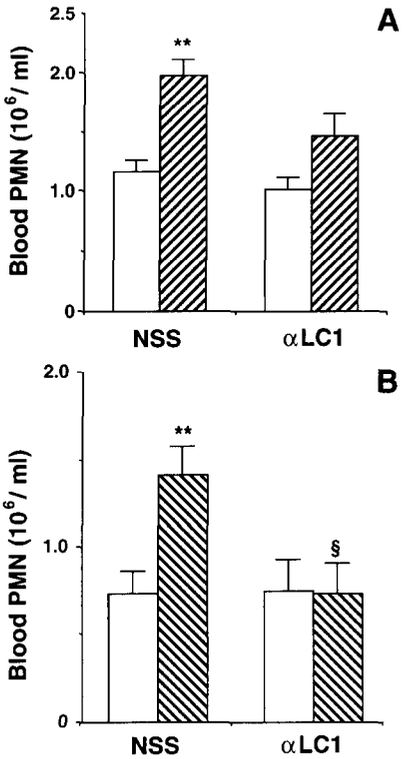

Fig. 5. Passive immunisation against reduces PAF-and ACTH-induced neutrophilia. Panel A: Mice (12 per group) received 50 μl s.c. of either non-immune (NSS) or anti-lipocortin 1 (αLC1) sheep serum 20 h prior to challenge with 100 μl i.v. of PBS (supplemented with bovine serum albumin 0.1%) with (hatched bars) or without (open bars) 100 ng PAF at time 0. Two hours later mice were bled and the number of circulating polymorphonuclear leukocytes (PMN) measured. Panel B: Mice (6 per group) received 50 μl s.c. of either non-immune (NSS) or anti-lipocortin 1 (αLC1) sheep serum 20 h prior to challenge with 100 μl s.c. of PBS (open bars) or of ACTH (5 μg, hatched bars) at time 0. Four hours later mice were bled and the number of circulating PMN measured. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. respective vehicle-treated group. $^{\S}P < 0.05$ vs. ACTH in NSS-treated mice.

was abolished in mice treated with RU486 (Fig. 3B). ACTH granulocytosis was accompanied by an increase in plasma corticosterone (Fig. 4A). Vehicle-treated mice had a plasma corticosterone level of 64 ± 20 ng/ml, $n = 11$, at 4 h which was not modified by the low dose of 1 μg ACTH, but was maximally increased by a dose of 20 μg ($P < 0.01$). A correlation coefficient of 0.916 between the two parameters in response to ACTH treatment was calculated ($P < 0.01$). The time-course for corticosterone release was then studied selecting the 20 μg ACTH dose. A marked increase in corticosterone was observed as early as 20 min after ACTH administration (Fig. 4B).

3.8. Effect of anti-lipocortin 1 serum on PAF- and ACTH-induced neutrophilia

Treatment of mice with either non-immune or the specific 'anti-lipocortin 1 sheep serum did not modify

the number of circulating polymorphonuclear leukocytes at the 20 h time-point (results as $\times 10^6$ neutrophils per ml blood): 0.84 ± 0.09 for vehicle-treated mice and 0.59 ± 0.26 or 0.75 ± 0.11 for non-immune or anti-lipocortin 1 sheep serum, respectively ($n = 4$ in all cases, not significant). Passive immunisation of mice with the anti-lipocortin 1 polyclonal serum, however, significantly attenuated PAF-induced 2 h neutrophilia (Fig. 5A) and abrogated ACTH-induced 4 h neutrophilia (Fig. 5B). Passive immunisation against lipocortin 1 did not significantly affect the rise in corticosterone induced by ACTH 5 μg at 4 h time-point: 137 ± 27 ng/ml plasma corticosterone ($n = 6$) and 506 ± 62 ng/ml ($n = 6$, $P < 0.01$) for PBS and ACTH in the mice pretreated with non-immune sheep serum, respectively, and 95 ± 21 ng/ml ($n = 6$) and 437 ± 121 ng/ml ($n = 6$, $P < 0.01$) for PBS and ACTH in anti-lipocortin 1-pretreated mice, respectively.

4. Discussion

The present study shows that endogenous corticosteroids are involved in the neutrophilic response observed after i.v. treatment with PAF in mice. This conclusion is based upon the rise in endogenous plasma corticosterone which preceded PAF granulocytosis and on the lack of this cellular response in adrenalectomized mice. Moreover, PAF-induced neutrophilia was significantly attenuated when blockade of the steroid receptor was achieved with RU486. Finally, an agent which causes a sustained corticosterone release like ACTH also induced an intense increase in circulating polymorphonuclear leukocytes.

Intravenous treatment of mice with vehicle alone caused a mild alteration of circulating polymorphonuclear leukocyte number. This phenomenon, not reported in the rabbit (Jagels and Hugli, 1992; Marleau et al., 1993; Ulich et al., 1987), may be related to the small animal species used throughout this study. Intravenous injections of vehicle can modify blood pressure and increase the number of circulating polymorphonuclear leukocytes by altering cardiac output (MacNee and Selby, 1990). Thus chemoattractant-induced granulocytosis was more reliably compared to vehicle-treated animals rather than to intact mice.

Systemic administration of chemoattractants altered the kinetics of circulating neutrophils by causing a fast neutropenia followed by a more long lasting neutrophilia. This phenomenon has been described in rabbits and rats (Camussi et al., 1981; Hechtman et al., 1991; Ley et al., 1993; Jagels and Hugli, 1992), and the present study extends these observations to the mouse. Whereas the fast transient neutropenia is attributed to a transient increase in polymorphonuclear leukocyte adherence to the endothelium and/or to a trapping of deformed cells within the microcirculation (Lo et al., 1989; Worthen et al., 1989), less is known about the neutrophilic response. Treatment of mice with PAF and fMLP caused a significant 2 h neutrophilia. The neutrophilia which followed treatment with interleukin-8 was more rapid in onset, the number of polymorphonuclear leukocytes being significantly higher at 20 min. A similar finding has also been reported in the rabbit (Jagels and Hugli, 1992). This difference in kinetics may suggest that there is not a common mechanism underlying the granulocytosis caused by structurally unrelated neutrophil activators. Numerous studies have focussed on interleukin-8-induced granulocytosis (Hechtman et al., 1991; Van Zee et al., 1992; Jagels and Hugli, 1992) and it has been recently demonstrated that this effect of the chemokine is mainly due to polymorphonuclear leukocyte demargination rather than to the release of cell precursors from the bone marrow (Ley et al., 1993). It has yet to be shown whether the same explanation may apply to other chemoattractants.

Intravenous administration of PAF induced a transient neutropenia which peaked at 2 min, resolved by 20 min and was then followed by an intense neutrophilia at 2 h. It is already known that this neutrophilia is unaffected by blocking arachidonic acid metabolism or novel DNA-dependent RNA synthesis (Jagels and Hugli, 1992). The granulocytosis induced by PAF was preceded by a significant rise in circulating corticosterone at 20 min. This is in keeping with the ability of PAF to induce corticosterone release from perfused adrenal glands (Aikawa et al., 1991). At concentrations equivalent to those reached in our in vivo experiments (approximately 30 nM assuming a total blood volume of 5 ml) PAF caused a fast (5–10 min) release of corticosterone from in situ perfused dog adrenal glands. PAF effect appeared to be direct on the adrenal cells and not secondary to changes in renal artery pressure (Aikawa et al., 1991). On this basis, and the fact that corticosterone is well known to represent the major glucocorticoid detectable in mouse plasma (Spackman and Riley, 1978), the effect of exogenously administered corticosterone upon the number of circulating polymorphonuclear leukocytes was tested. Corticosterone, however, failed to induce granulocytosis. This was not due to the steroid used, as a single administration of dexamethasone was equally ineffective in altering the profile of circulating neutrophils (J.G.H., unpublished data). It is of interest that the occurrence of granulocytosis after treatment with glucocorticoids has been differently reported, as in the case of dexamethasone in the rabbit (Ulich et al., 1987; Kajita and Hugli, 1990). We propose that a single bolus administration of corticosterone does not induce a level of circulating hormone sustained enough to cause granulocytosis. In keeping with this, a recent study

failed to observe the expected changes in the number of circulating leukocytes after prednisolone administration in the rat (Wald and Jusko, 1994), and the authors suggested that adverse pharmacokinetics could be the cause. This hypothesis is reinforced by our data obtained with ACTH. A single injection of this hormone increased endogenous corticosterone levels in a long lasting way and also caused a profound rise in the number of circulating polymorphonuclear leukocyte which was slow in onset. These two parameters were significantly correlated to the dose of ACTH administered. Interestingly, the dose of ACTH which caused a marked 2 h-neutrophilia (20 μg) also produced a rise in circulating corticosterone at 20 min similar to that observed after treatment with PAF. Administration of ACTH provokes only minimal changes in the level of other hormones, either directly (such as androgens released from the adrenal cortex) or indirectly (via corticosterone, e.g. neuropeptides released from the pituitary) (Buckingham et al., 1992). However, the major modifications in concentration occur for corticosterone, which in turn mediates most of ACTH actions (Buckingham et al., 1992).

Besides evaluating the effect of a mimicking agent such as ACTH, a role for endogenous corticosterone in PAF-induced neutrophilia is confirmed by the experiments where an experimental or chemical adrenalectomy was achieved. The neutrophilic response to PAF was significantly inhibited in adrenalectomized mice. Moreover, the effect of adrenalectomy appeared to be specific because neither the mild effect of vehicle nor fMLP-induced neutrophilia were significantly affected. Differently from PAF, the granulocytosis which follows treatment with interleukin-1 or tumor necrosis factor-α is unaffected by adrenalectomy (Ulich et al., 1987) and, in keeping with this observation, we found that the interleukin-1 receptor antagonist and a monoclonal antibody raised against murine tumor necrosis factor-α (Sheehan et al., 1989) did not modify PAF response. Chemical adrenalectomy was achieved by treatment of mice with the steroid antagonist RU486. A protocol described to be highly effective in vivo (Peers et al., 1988; Perretti et al., 1993b), with more than 80% occupancy of steroid receptors (Fan et al., 1994), was used with consequent significant reduction of PAF-induced neutrophilia. It should be pointed out that not only PAF failed to significantly increase the number of circulating polymorphonuclear leukocytes both in adrenalectomized mice and in those pre-treated with RU486, but that these treatments affected PAF response to a similar extent, i.e. $\approx$ 50–60%. This indicates that other mediator(s) apart from endogenous corticosterone may have a role in this action of PAF. The occurrence of endogenously released PAF following exogenous administration of this agent may also contribute to the neutrophilia. On the contrary, ACTH-induced granulocytosis was abolished both in adrenalectomized and RU486-pretreated mice: as expected the cellular response to this hormone was entirely due to endogenous corticosterone.

An involvement of endogenous adrenaline in PAF-induced neutrophilia has been recently proposed in a study performed in the rat (Altenburg et al., 1994): on the basis of the abrogating effect of propranolol our data in the mouse complement this study and point to a role for adrenal corticosterone besides adrenaline in modulating the cell response to PAF. However, here we showed that propranolol is not specific in its action since it affects both PAF- and vehicle-induced neutrophilia, whereas adrenalectomy selectively affected the action of the lipid mediator. From all these data it is likely that endogenous corticosterone, rather than adrenaline, has a prominent role in mediating PAF neutrophilia. However, in view of the lack of effect of corticosterone on its own to cause neutrophilia, the possibility that this glucocorticoid homones acts as a permissive agent, as reported for the acute phase response in vivo (Fantuzzi and Ghezzi, 1993), cannot be excluded.

Some of the anti-inflammatory effects that glucocorticoid hormones exert are mediated by the steroid-inducible protein lipocortin 1 (Flower and Rothwell, 1994). Passive immunisation of mice with a specific anti-lipocortin 1 polyclonal serum prevented the inhibition caused by systemic treatment with dexamethasone on neutrophil trafficking in vivo (Perretti and Flower, 1993; Perretti et al., 1994). We suggested that lipocortin 1, which binds to murine polymorphonuclear leukocytes in vitro and in vivo (Perretti et al., 1993a), mediates this effect possibly by modulating leukocyte/endothelium interaction (Perretti and Flower, 1993). This possibility is further supported by the data obtained in the present study. The granulocytosis which followed ACTH administration was abrogated by the anti-lipocortin 1 antibody, which also significantly attenuated the effect of PAF. In contrast, ACTH-induced elevation of endogenous corticosterone was not affected by the specific anti-lipocortin 1 serum. This gives some indications of the site of lipocortin 1 action. If ACTH, similarly to interleukin-8 (Ley et al., 1993), induces neutrophilia by cell demargination it is conceivable that endogenous lipocortin 1 may interfere with the interaction between polymorphonuclear leukocytes and endothelium mediating in this way the increase number of circulating neutrophils.

In conclusion, this investigation has shown that PAF-induced neutrophilia is mediated by endogenous glucocorticoid hormones, likely to be corticosterone since it represents the major glucocorticoid detectable in mouse plasma (Spackman and Riley, 1978). PAF induces corticosterone release by directly stimulating the adrenal glands (Aikawa et al., 1991) and this stimu-

lation also occurs following single i.v. bolus injection. This glucocorticoid hormone may not only control PAF synthesis in a negative feed-back way (Parente and Flower, 1985), but also contribute to the existence of a positive loop which profoundly affects granulocyte kinetics.

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