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Comparison of the reversed passive Arthus and local Shwartzman reactions of rabbit skin: effects of the long-acting PAF antagonist UK-74,505

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1 By using the selective, potent and long acting platelet-activating factor (PAF) antagonist, UK-74,505, we investigated the role of PAF in a local Shwartzman reaction (LSR) and a reversed passive Arthus (RPA) reaction in rabbit skin. For comparison, we also studied the effect of the PAF antagonist on neutrophil aggregation *in vitro* and on acute inflammatory responses induced by intradermally (i.d.) injected lipopolysaccharide (LPS), PAF, bradykinin and zymosan-activated plasma.

2 Neutrophil aggregation was assessed photometrically. Haemorrhage, oedema formation, platelet deposition and neutrophil accumulation were quantified in rabbit skin by measuring the accumulation of i.v. injected ^{51}Cr-labelled red blood cells (RBC), ^{125}I-labelled human serum albumin, ^{111}In-labelled platelets and ^{111}In-labelled neutrophils respectively.

3 UK-74,505 inhibited *in vitro* neutrophil aggregation induced by PAF but not by leukotriene B_4. When injected i.v. into rabbits UK-74,505 suppressed oedema formation in response to i.d. PAF for up to 4 h but had no effect on oedema induced by bradykinin or zymosan-activated plasma.

4 Oedema formation, but not neutrophil accumulation, produced during the RPA reaction was significantly inhibited by i.v. UK-74,505. The PAF antagonist also suppressed ^{111}In-platelet but not ^{111}In-neutrophil accumulation in response to i.d. LPS. UK-74,505 did not affect haemorrhage or oedema formation produced during the LPS-mediated LSR.

5 The results demonstrate that PAF is an important mediator of oedema formation, but not neutrophil accumulation, in the immune-complex mediated RPA reaction in rabbit skin. PAF also appears to be required for platelet, but not neutrophil, accumulation in response to locally injected LPS. Our studies do not suggest a role for PAF in the LPS-mediated LSR.

Keywords: Platelet-activating factor; Shwartzman reaction; Arthus reaction; lipopolysaccharide; neutrophils; platelets; haemorrhage; oedema

Introduction

The local Shwartzman reaction (LSR) and reversed passive Arthus (RPA) reactions of rabbit skin have a strikingly similar gross pathology (localised inflammatory, haemorrhagic necrosis) but appear to be regulated by disparate mechanisms (Arthus, 1903; Shwartzman, 1928; Williams *et al*., 1986; Brozna, 1990; Rossi *et al*., 1992; Norman *et al*., 1994; 1996). Traditionally, the LSR is produced by two injections of lipopolysaccharide (LPS), i.d. priming followed 18–24 h later by i.v. challenge. Inflammatory changes such as increased blood flow, oedema formation, leucocyte and platelet accumulation, and altered adhesion molecule expression occur in response to the priming injection of LPS while systemic challenge initiates events that lead to the intravascular coagulation and haemorrhagic necrosis at primed sites that characterize this reaction (Shwartzman, 1928; Movat *et al*., 1980; Argenbright & Barton, 1992; Norman *et al*., 1996). It is believed that the LSR models disseminated intravascular coagulation (DIC) and associated multiple organ failure that can occur during sepsis (Brozna, 1990).

The dermal Arthus reaction, also a haemorrhagic lesion, develops in the skin of sensitized animals after locally injected antigen forms immune complexes with circulating antibody. The related reversed passive Arthus reaction is produced when locally injected antibody forms immune complexes with i.v. injected antigen. Immune complex formation is important in many conditions including immune complex glomerulonephritis, serum sickness and farmer's lung where inflammation and damage to tissues occur by mechanisms similar to those seen in the RPA reaction.

Immune complexes and LPS both initiate the generation of various inflammatory mediators including complement fragments, platelet-activating factor (PAF), tumour necrosis factor-α, interleukin-1 and interleukin-8 which can influence the accumulation and activation state of inflammatory cells including neutrophils (Movat *et al*., 1980; Beaubien *et al*., 1990; Collins *et al*., 1991; von Uexkull *et al*., 1992; Nourshargh *et al*., 1994; Rossi & Hellewell, 1994) and platelets (Movat *et al*., 1980; Pons *et al*., 1992; Rossi *et al*., 1993).

PAF induces oedema formation and accumulation of platelets in rabbit skin (Pons *et al*., 1992; Rossi *et al*., 1992; 1993). Studies with PAF antagonists have implicated PAF as an important mediator of immune-complex mediated inflammation (Deacon *et al*., 1986; Williams *et al*., 1986; Issekutz & Szejda, 1986; Hellewell & Williams, 1986; 1989; Jancar *et al*., 1988; Warren *et al*., 1989; Pons *et al*., 1992; Rossi *et al*., 1992; 1993; Travares de Lima *et al*., 1992; Teixeira *et al*., 1994). In particular, PAF antagonists administered intradermally or intravenously reduce oedema formation and platelet deposition in the RPA reaction in rabbit skin (Hellewell & Williams, 1986; 1989; Williams *et al*., 1986; Pons *et al*., 1992; Rossi *et al*., 1992; 1993). The PAF antagonist, L-659,989 has also been shown to reduce neutrophil accumulation in an RPA reaction of rabbit skin (Hellewell, 1990).

A number of lines of evidence implicate PAF as an important mediator of responses to LPS. Thus, PAF, injected i.v., reproduces many of the features of clinical or experimental

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septic shock (Anderson *et al*., 1991; Qian *et al*., 1994). PAF antagonists protect against LPS or bacteria-induced shock in different animal models (Terashita *et al*., 1983; Etienne *et al*., 1985; Toyofuku *et al*., 1986; Casals-Stenzel, 1987; Chang *et al*., 1987; Fletcher *et al*., 1989; Kuipers *et al*., 1994) and elevated PAF concentrations have been detected in the plasma of sepsis patients (Bussolino *et al*., 1987). Furthermore, a recent phase II clinical trial indicated improved organ failure statistics in patients treated with a PAF antagonist (Froon *et al*., 1996). The contribution of PAF to local responses to LPS such as the LSR is less well understood.

UK-74,505, a long acting and selective PAF antagonist (Cooper *et al*., 1990; Parry *et al*., 1994) has been used to demonstrate a role for PAF in oedema formation and platelet accumulation in the RPA reaction in the rabbit (Pons *et al*., 1992; Rossi *et al*., 1993). Here we use UK-74,505 to extend our earlier observations regarding the RPA reaction and to study the role of PAF in inflammatory and haemorrhagic reactions induced by bacterial LPS.

Methods

Animals

New Zealand White rabbits (2.5–3.5 kg) were bought from Regal, Sussex, U.K. All procedures described were approved by the Home Office.

Isolation, and radiolabelling of rabbit neutrophils

Neutrophils were isolated from rabbit blood as previously described (Haslett *et al*., 1987; Norman *et al*., 1996). Briefly, citrated blood collected from the carotid artery of a donor rabbit was centrifuged at 300 *g* for 20 min. Platelet-rich plasma (PRP) was removed, under-layered with 3 ml 90% Percoll (Norman *et al*., 1996) and centrifuged at 2000 *g* for 20 min to produce platelet-poor plasma (PPP). Following 30 min dextran sedimentation of the pellet produced by the first spin, leukocyte-rich supernatant was removed and centrifuged at 275 *g* for 6 min. The resultant pellet was re-suspended in PPP, layered onto discontinuous Percoll-plasma gradients and centrifuged for 40 min at 260 *g*. The neutrophil-rich band was collected and contaminating red blood cells (RBC) removed by hypotonic lysis. Unlabelled neutrophils were used in aggregation assays. For radiolabelling, rabbit neutrophils (>98% pure) were suspended in 1–2 ml of PPP and incubated with approximately 100 μCi $^{111}InCl_3$ chelated to 2-mercaptopyridine-N-oxide (400 μg ml^{-1}) for 15 min at room temperature. Cells were washed in PPP to remove unbound radiolabel and suspended in PPP. Each recipient rabbit received 5×10^7 neutrophils in 3 ml PPP through a marginal ear vein.

Measurement of neutrophil aggregation

Rabbit neutrophils (10^7 cell ml^{-1}) were kept at room temperature in Ca^{2+}/Mg^{2+} free buffer (composition in mM: NaCl 138, KCl 2.7, Na_2HPO_4 8.1, KH_2PO_4 1.5 and HEPES 10). Twenty minutes before use, cells were warmed to 37°C and $CaCl_2$ and $MgCl_2$ (final concentrations 1.0 mM and 0.7 mM, respectively) were added. Aggregation was assessed photometrically in a dual channel aggregometer (Chronolog 440 VS). Aliquots of neutrophil suspension (250 μl) were incubated for 5 min at 37°C with continuous stirring at 700 r.p.m. and then stimulated with either PAF or leukotriene B_4 (LTB_4). Aggregation of platelets resulted in increased light transmission through the suspension. Responses which were allowed to develop for 3 min are expressed as a percentage of maximal aggregation induced by 10^{-6} M phorbol 12-myristate 13-acetate (PMA; Teixeira *et al*., 1995). Buffer alone was used as a reference. Where the effect of UK-74,505 on responses to LTB_4 and PAF was studied, antagonist was added to the neutrophil suspension 2 min before agonist stimulation.

Preparation and radiolabelling of rabbit platelets

Citrated blood collected from the carotid artery of a donor rabbit was centrifuged at 300 *g* for 20 min. PRP was removed, under-layered with 90% Percoll and centrifuged at 2000 *g* for 20 min to produce PPP and a layer of platelets at the plasma-Percoll interface. The platelets were harvested, suspended in 1–2 ml of PPP and incubated with 100 μCi $^{111}InCl_3$ chelated to 2-mercaptopyridine-N-oxide (400 μg ml^{-1}) for 15 min at room temperature. Cells were washed three times in PPP to remove unbound radiolabel, and finally suspended in PPP at approximately 0.33×10^9 platelets ml^{-1} (concentration determined with a coulter counter model ZM, Coulter Electronics Ltd, Luton, Bedfordshire). Recipient rabbits received 3 ml platelet suspension (i.e. 10^9 platelets) via a marginal ear vein.

Preparation and radiolabelling of RBC

Blood from a donor rabbit was centrifuged at 250 *g* for 20 min. One millilitre of RBC for each recipient rabbit was collected from the resultant pellet. $Na_2{}^{51}CrO_4$ (0.5 mCi ml^{-1} RBC) was added to the RBC and incubated for 30 min at room temperature. After being labelled, cells were washed in fresh Tyrode solution to remove unbound ^{51}Cr and suspended in 3 ml Tyrode per recipient rabbit.

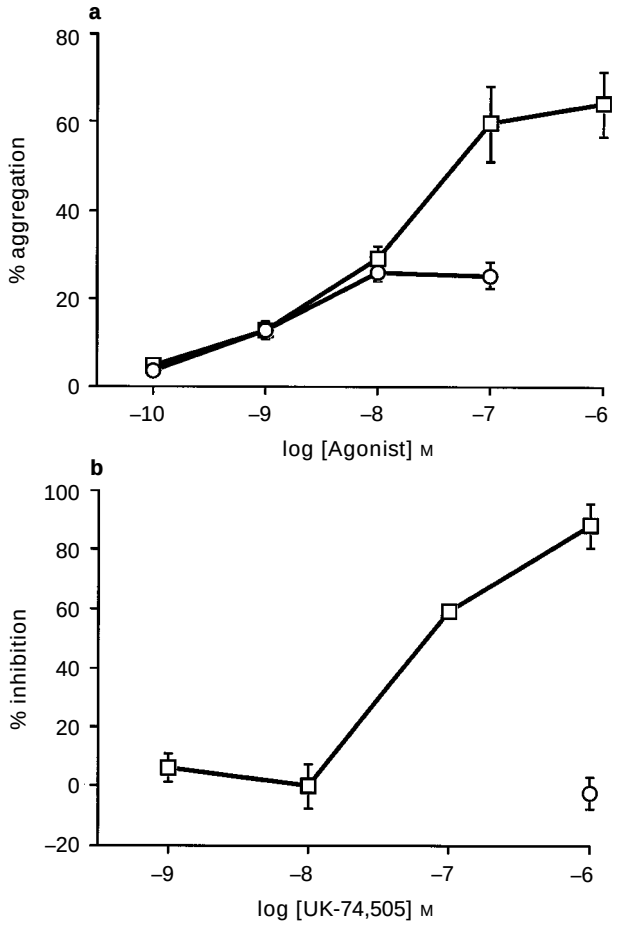

Figure 1 Effect of UK-74,505 on aggregation of rabbit neutrophils induced by PAF (□) and LTB_4 (○). (a) Concentration-dependent agonist-induced aggregation of rabbit neutrophils. Results are expressed as a percentage of maximal aggregation and presented as mean, with vertical lines showing s.e.mean, of $n = 5$ preparations. (b) Effect of UK-74,505 on rabbit neutrophil aggregation induced by PAF (10^{-7} M) and LTB_4 (10^{-7} M). UK-74,505 was added to neutrophil suspensions 2 min before addition of agonist. Results are expressed as % inhibition of aggregation induced by 10^{-7} M PAF and presented as mean and s.e.mean (vertical lines) of $n = 3$ preparations.

The RPA reaction and acute (4 h) inflammatory reactions in rabbit skin

Rabbits were anaesthetized with sodium pentobarbitone (Sagatal, 15 mg kg^{-1}, i.v.) and the dorsal skin was shaved and marked out with 6 replicates of 12–16 treatment sites according to a balanced site plan. ^{111}In-neutrophils and [^{125}I]-human serum albumin were injected i.v. as markers of neutrophil accumulation and plasma protein leakage. UK-74,505 or vehicle was injected i.v. and allowed 10 min to circulate. LPS, Arthus antiserum (anti-bovine-γ-globulin, BGG), PAF plus prostaglandin E_2 (PGE_2), bradykinin (BK) plus PGE_2 and zymosan-activated plasma (ZAP) were injected i.d. in 0.1 ml volumes. To initiate RPA reactions, the antigen BGG was injected i.v. at 5 mg kg^{-1}. Blood samples were collected into heparin 4 h after i.v. BGG and animals killed by an anaesthetic overdose. The dorsal skin was removed and skin sites excised with a 17 mm diameter punch. Radioactivity in skin sites was assessed by an automatic γ-counter. Where neutrophil accumulation was measured the ^{111}In-count per neutrophil was determined, and used to express accumulated ^{111}In in skin sites as number of radiolabelled neutrophils. Where platelet accumulation was measured the ^{111}In-count per platelet was determined, and used to express accumulated ^{111}In in skin sites as number of radiolabelled platelets. Human serum albumin accumulation was expressed as μl plasma per skin site by dividing skin sample ^{125}I counts by ^{125}I counts in 1 μl of plasma.

The local Shwartzman reaction

Rabbits were anaesthetized with intramuscular ketamine (30–35 mg kg^{-1}) and xylazine (2–3 mg kg^{-1}). The dorsal skin was shaved and marked out with 6 replicates of 5 treatment sites according to a balanced site plan. Vehicle saline and LPS from *Salmonella typhosa* were injected at concentrations giving 0, 1, 3.16, 10 and 31.6 μg per skin site. ^{51}Cr-labelled RBC and [^{125}I]-human serum albumin were injected i.v. 20 h after priming. LSR were challenged as described (Norman *et al*., 1996) with 100 μg LPS from *S. typhosa* given intravenously at 20 and 22 h. At 24 h (4 h after first challenge), blood samples were collected into heparin by cardiac puncture and animals killed by an overdose of sodium pentobarbitone. The dorsal skin was removed and skin sites excised with a 17 mm diameter punch. Radioactivity in skin sites was assessed in an automatic γ counter. Accumulation of ^{51}Cr-labelled RBC was expressed as μl blood per skin site by dividing skin sample ^{51}Cr-counts by ^{51}Cr-counts in 1 μl whole blood. Human serum albumin accumulation was expressed as μl plasma per skin site by dividing skin sample ^{125}I-counts by ^{125}I-counts in 1 μl of plasma.

Materials

Arthus antiserum and zymosan-activated plasma (ZAP) were prepared as described by Rossi *et al*. (1992). The following were purchased from commercial sources; Sagatal (pentobarbitone; sodium BP 60 mg ml^{-1}) and Expiral (pentobarbitone; sodium BP 200 mg ml^{-1}), May & Baker Ltd. (Dagenham, Essex); ketamine (Vetalar; Parke-Davis & Co., Pontypool, Gwent); xylazine (Rompum; Bayer UK Ltd., Bury St. Edmunds, Suffolk); [^{125}I]-human serum albumin (20 mg albumin ml^{-1} sterile isotonic saline, 50 μCi ml^{-1}), $^{111}InCl_3$ (2 mCi in 0.2 ml sterile pyrogen free 0.04 N HCl) and $Na_2{}^{51}CrO_4$ (5 mCi in 5 ml sterile isotonic saline) Amersham International plc (Amersham, Buckinghamshire); Percoll and dextran (Pharmacia Ltd, Milton Keynes, Buckinghamshire); Viaflex (sterile, pyrogen-free isotonic saline solution; Baxter Healthcare Ltd., Thetford, Norfolk); sterile, pyrogen free water was purchased from Phoenix Pharmaceuticals Ltd. (Gloucester); LPS (*Salmonella typhosa*, prepared by phenol extraction), PGE_2, zymosan, bovine-γ-globulin (BGG), Freund's complete adjuvant and Freund's incomplete adjuvant were bought from Sigma Chemical company (Poole, Dorset); PAF (Bachem, Saffron Walden, Essex); Diff-Quik stain (BDH, Poole, Dorset). UK-74,505 [4-(2-chlorophenyl)-1,4-dihydro-3-ethoxycarbonyl-6-methyl-2-[4-(2-methylimidazo[4,5-c]pryid-1-yl)phenyl]-5-[N-(2-pyridyl)carbamoyl)pyridine], initially dissolved in 0.1 N HCl and diluted in saline, was a gift from Dr M.J. Parry (Pfizer Central Research, Sandwich, Kent).

Statistical analysis

Data are presented as mean ± s.e.mean for the number of animals indicated. Data were analysed by two way analysis of variance. Significant differences ($^*P<0.05$, $^{**}P<0.01$) from the appropriate controls were determined by the Newman-Keuls procedure which allows for multiple comparisons.

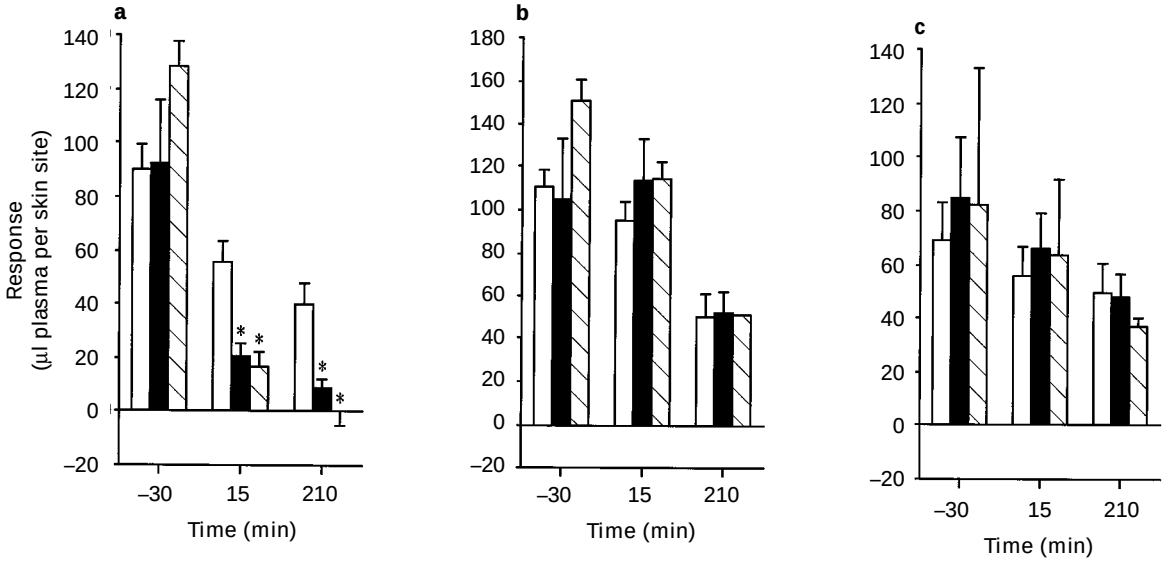

Figure 2 *In vivo* time course of UK-74,505 vs platelet-activating factor (PAF, a), bradykinin (BK, b) and zymosan-activated plasma (ZAP, c) in rabbit skin. Vehicle (open columns) or UK-74,505, 0.5 mg kg^{-1} (solid columns) or 2 mg kg^{-1} (hatched columns), was injected i.v. at time zero. PAF (10^{-9} mol per site), BK (10^{-10} mol per site) and undiluted ZAP, all in the presence of PGE_2 (3×10^{-10} mol site) were injected i.d. at time points 30 min before, 15 min after and 210 min after UK-74,505. Animals were killed 4 h after UK-74,505 and plasma protein leakage determined as detailed in the Methods section. Results are expressed as μl plasma per skin site and presented as mean ± s.e.mean of $n=4$ experiments after subtraction of response to saline. Significant differences from vehicle controls are indicated by $^*P<0.05$.

Results

Effect of UK-74,505 on neutrophil aggregation in vitro

Washed rabbit neutrophils when appropriately stirred and exposed to PAF or LTB_4 undergo a concentration-dependent homotypic aggregation, as assessed by increased light transmission (Figure 1a). To confirm the *in vitro* selectivity and potency of UK-74,505 we examined the effect of this compound on rabbit neutrophil aggregation induced by these lipid mediators. Figure 1b shows that neutrophil aggregation induced by 10^{-7} M PAF was competitively inhibited by UK-74,505. The highest concentration of UK-74,505 used (10^{-6} M) did not effect LTB_4-induced aggregation.

Effect of UK-74,505 on oedema formation in response to mediators (4 h time course)

We next examined the specificity and duration of action of UK-74,505 *in vivo*. Figure 2 shows a time-course of systemic UK-74,505 (0.5 and 2 mg kg^{-1}) against oedema responses to i.d. injected PAK, BK and ZAP. These mediators were injected i.d. together with PGE_2 to potentiate oedema formation, at time points either 30 min before, 15 min after or 210 min after injection of either vehicle or UK-74,505 0.5 or 2 mg kg^{-1}. Responses to mediators injected 30 min before antagonist were not affected by UK-74,505 (0.5 or 2 mg kg^{-1}) (oedema in response to the mediators used develops rapidly and is maximal by 30 min). Oedema formation in response to $PAF + PGE_2$ injected either 15 min or 210 min after antagonist was greatly reduced by UK-74,505 (0.5 or 2 mg kg^{-1}). In contrast, responses to $BK + PGE_2$ and $ZAP + PGE_2$ injected either 15 min or 210 min after antagonist were not affected by UK-74,505 at either dose. Thus at the doses studied, UK-74,505 is selective for PAF and active for at least 4 h in rabbits.

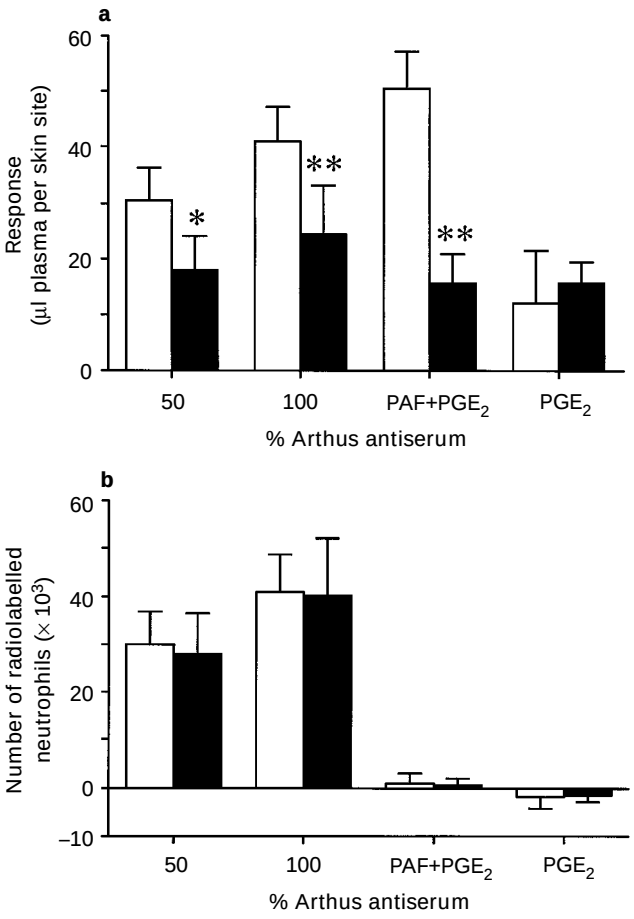

Figure 3 Effect of UK-74,505 on neutrophil accumulation and oedema formation in the RPA reaction in rabbit skin. Rabbits received either vehicle (open columns) or UK-74,505 (2 mg kg^{-1}, solid columns) i.v. Arthus antiserum and PAF (10^{-9} mol per site) + PGE_2 (3×10^{-10} mol per site) were injected i.d. RPA reactions were initiated by injecting the antigen BGG i.v. (5 mg kg^{-1}). Animals were killed 4 h after BGG, and plasma protein leakage (a) and neutrophil accumulation (b) measured as detailed in the Methods section. Results are expressed as μl plasma per skin site and number of radiolabelled neutrophils per skin site and presented as mean ± s.e.mean after subtraction of control saline values in $n = 5-6$ experiments. Significant differences from control values are indicated by $^*P < 0.05$, $^{**}P < 0.01$.

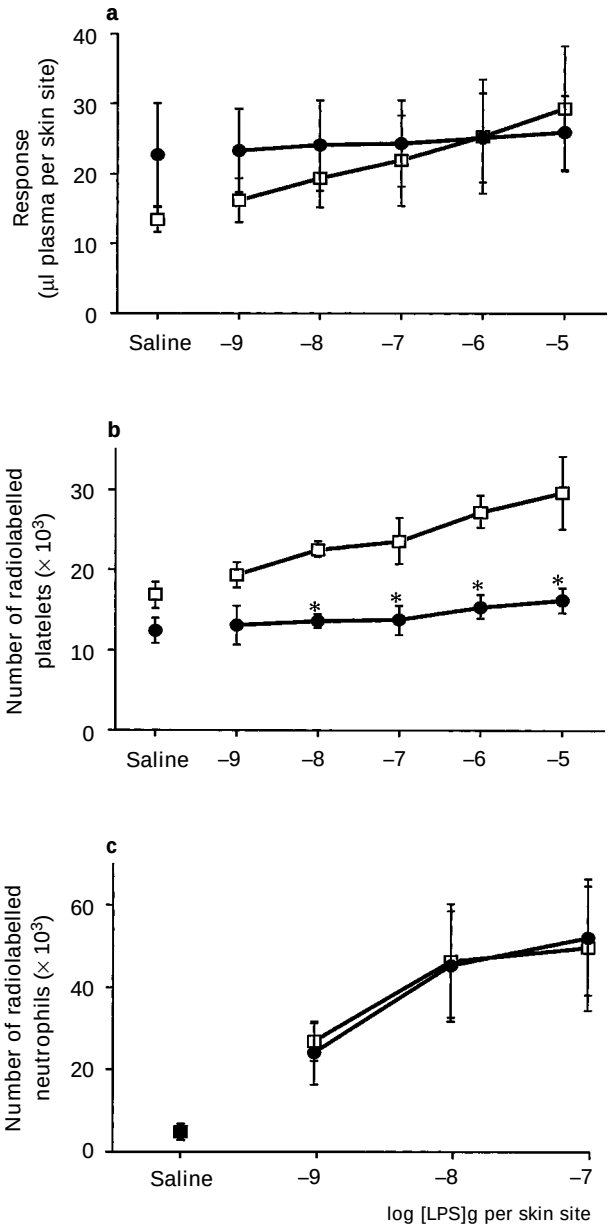

Figure 4 Effect of UK-74,505 on oedema formation (a), platelet deposition (b) and neutrophil accumulation (c) in response to LPS in rabbit skin. Rabbits received either vehicle (□) or UK-74,505 (2 mg kg^{-1}, ●) i.v.. After 10 min, LPS was injected i.d. at the doses indicated. Animals were killed 4 h after i.d. LPS, and plasma protein leakage, platelet deposition and neutrophil accumulation measured as detailed in the Methods section. Results are expressed as μl plasma per skin site and the number of platelets or neutrophils per skin site and presented as mean, with vertical lines showing s.e.mean, of $n = 4$ experiments. Significant differences from control values are indicated by $^*P < 0.05$.

Effect of UK-74,505 on neutrophil accumulation in the RPA reaction

The effect of UK-74,505 on neutrophil accumulation and oedema formation in the RPA reaction determined after a 4 h period in rabbit skin is shown in Figure 3. Vehicle or UK-74,505 (2 mg kg^{-1}) were given i.v., followed by i.d., injections of Arthus antiserum. The antigen BGG (5 mg kg^{-1}) was given i.v. to initiate RPA reactions. Skin sites injected with 50 and 100% antiserum exhibited a dose-dependent oedema formation (upper panel) which was significantly inhibited by UK-74,505 at 2 mg kg^{-1}. The marked oedema induced by PAF + PGE_2 was reduced to levels induced by PGE_2 alone in the presence of UK-74,505 (2 mg kg^{-1}). In contrast, at the same reaction sites where oedema formation was reduced, UK-74,505 (2 mg kg^{-1}) had no effect on radiolabelled neutrophil accumulation induced by the RPA reaction. PAF + PGE_2 and PGE_2 alone produced no significant neutrophil accumulation.

Effect of UK-74,505 on platelet and neutrophil accumulation in response to LPS in rabbit skin

Platelet accumulation has been shown to be a feature of the response to i.d. LPS and, therefore, may be important for LSR priming. The role of PAF in LPS-induced platelet accumulation was investigated by use of i.v. UK-74,505. Figure 4 shows the effect of 2 mg kg^{-1} UK-74,505 on oedema formation, platelet and neutrophil accumulation induced by LPS. The slight oedema formation induced by i.d. LPS in rabbit skin was not affected by UK-74,505 (Figure 4a). I.d. LPS caused a dose-dependent accumulation of platelets which was significantly inhibited by UK-74,505 (2 mg kg^{-1}) (Figure 4b). Figure 4c shows the effect of UK-74,505 on neutrophil accumulation in response to i.d. LPS. Dose-dependent neutrophil accumulation of a magnitude similar to that seen in the RPA reaction was produced by i.d. LPS. This neutrophil accumulation was not affected by UK-74,505 (2 mg kg^{-1}).

Effect of UK-74,505 on LSR priming and challenge

Figure 5 shows the effect of UK-74,505 on LSR priming. Rabbits were given either vehicle or UK-74,505 (2 mg kg^{-1}, i.v.) 10 min before i.d. priming doses of LPS. Injections of 100 μg LPS were given i.v. at 20 and 22 h after priming to challenge the LSR. Haemorrhage (upper panel) and oedema formation (lower panel) were seen at primed skin sites 4 h after the first challenge (i.e. 24 h after priming). The magnitude of these responses was dependent upon the priming dose of LPS. Haemorrhage and plasma leakage in the LSR were not affected by UK-74,505 given at the time of priming.

The effect of UK-74,505 given immediately before LSR challenge is shown in Figure 6. Rabbits were primed with in-

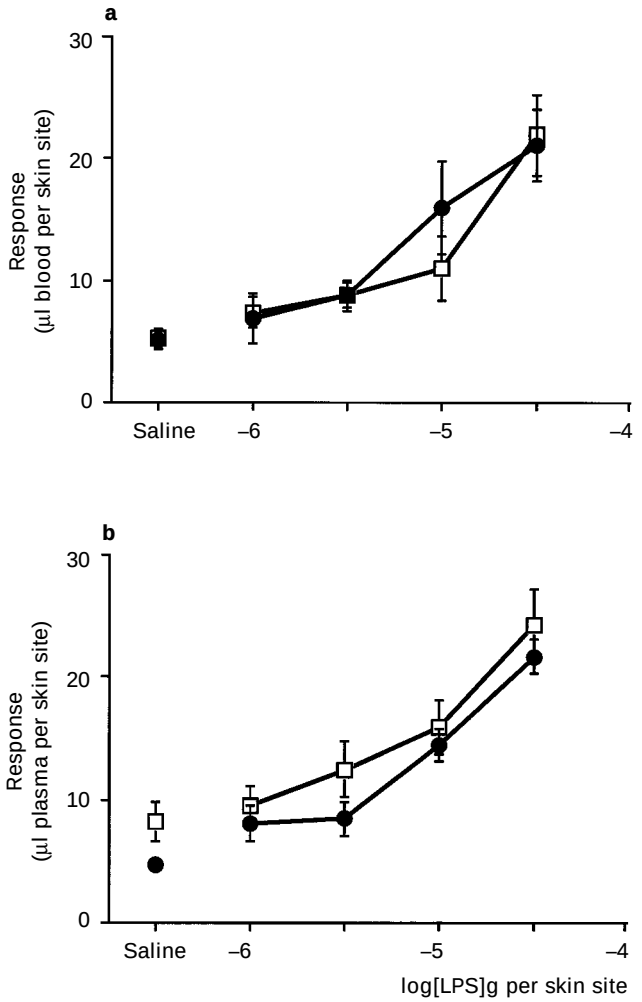

Figure 5 Effect of UK-74,505 on the priming of the local Shwartzman reaction. Rabbits received either vehicle (□) or UK-74,505 (2 mg kg^{-1}; ●) i.v. After 10 min, priming doses of LPS were injected i.d. LSR challenge (100 μg LPS, i.v.) was given at 20 and 22 h. Animals were killed 4 h after i.d. injections (4 h after 1st challenge), and haemorrhage and plasma protein leakage measured as detailed in the Methods section. Results are expressed as μl blood per skin site and μl plasma per skin site and presented as mean, with vertical lines showing s.e.mean, of $n = 4$ experiments.

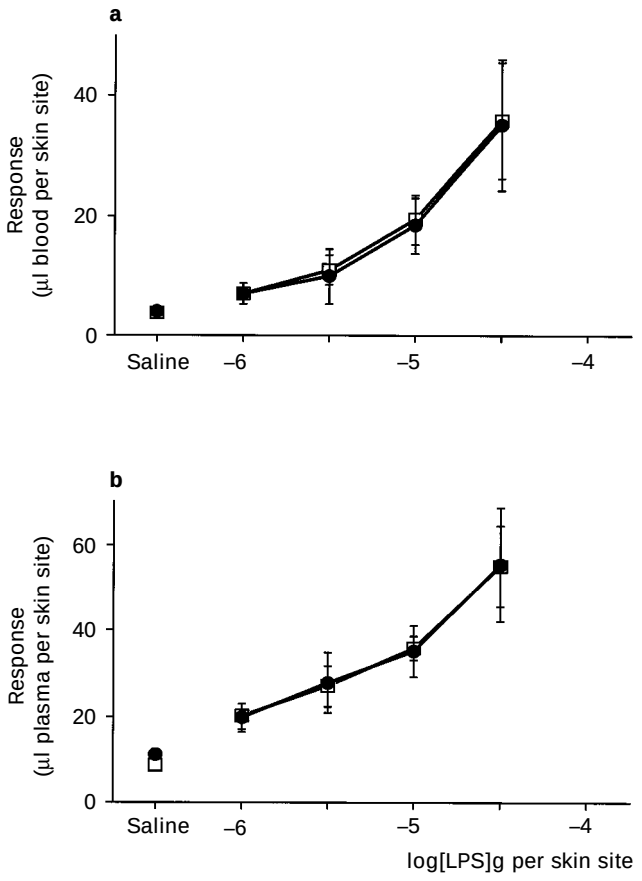

Figure 6 Effect of UK-74,505 on the local Shwartzman reaction (LSR) challenge. Priming doses of LPS were injected i.d. After 20 h either vehicle (□) or UK-74,505 (2 mg kg^{-1}; ●) was given i.v. followed 10 min later by the first LSR challenge (100 μg LPS, i.v.). A second challenge (100 μg LPS, i.v.) was given at 22 h. Animals were killed 24 h after i.d. injections (4 h after first challenge), and haemorrhage and plasma protein leakage measured as detailed in the Methods section. Results are expressed as μl blood per skin site and μl plasma per skin site and presented as mean, with vertical lines showing s.e.mean, of $n = 4$ experiments.

creasing doses of LPS. After 20 h either vehicle or UK-74,505 was given i.v., followed 10 min later by LSR challenge as described above. Haemorrhage and oedema formation produced at appropriately primed skin sites were not affected by UK-74,505 given i.v. before challenge.

Discussion

The results presented in this study demonstrate that neutrophil accumulation in the RPA reaction does not appear to be PAF-dependent. This differs from our earlier findings regarding the PAF-dependence of platelet accumulation in this response (Pons *et al*., 1992; Rossi *et al*., 1993). Similarly, we found that platelet accumulation in response to LPS in rabbit skin is PAF-dependent, whereas neutrophil accumulation is not. Our finding that UK-74,505 does not affect the LSR suggests that PAF is not an important mediator of this response.

A number of studies have implicated PAF as an important mediator of the RPA reaction. PAF antagonists administered locally or systemically have been shown to suppress oedema formation induced in an RPA reaction in the skin of rats and rabbits (Deacon *et al*., 1986; Williams *et al*., 1986; Hellewell & Williams, 1986; 1989; Rossi *et al*., 1992) and we demonstrated previously that platelet accumulation in the RPA reaction was also PAF-dependent (Pons *et al*., 1993; Rossi *et al*., 1993). Whilst PAF antagonism has been widely studied as a possible treatment for septic shock, the role of PAF in local inflammatory responses to LPS and in the LSR has not been addressed. In this study we used the potent and long-acting PAF antagonist UK-74,505 (Cooper *et al*., 1990; Parry *et al*., 1994) to extend our observations concerning the role of PAF in the RPA reaction and to determine what role, if any, PAF has in responses to LPS and in the LSR in rabbit skin.

In a previous study (Pons *et al*., 1992) we showed that UK-74,505 is a long-acting, potent and selective blocker of rabbit platelet PAF receptors both *in vitro* and *in vivo*. We also showed that UK-74,505 inhibits platelet accumulation and oedema formation in the RPA reaction and in certain other reactions of rabbit skin. Here, we have demonstrated that neutrophil aggregation induced by PAF, but not by LTB_4, is competitively inhibited by UK-74,505, demonstrating that UK-74,505 is also an effective and selective blocker of neutrophil PAF receptors *in vitro*. By studying oedema formation in response to mediators (BK, ZAP and PAF) given at various times after injection of UK-74,505 we showed that UK-74,505 is selective for PAF and active *in vivo* for at least 4 h after its injection at the doses employed (0.5 mg kg^{-1} or 2 mg kg^{-1}). PAF injected into rabbit skin in the presence of PGE_2 caused a marked oedema formation which was reduced to levels produced by PGE_2 alone in the presence of UK-74,505. In contrast, PAF caused little or no neutrophil accumulation in rabbit skin. It is important to note, however, that i.d. injected PAF may be rapidly metabolised and that the lack of effect may be due to failure to reach the correct site of action rather than lack of bioactivity *per se*. In agreement with our previous study (Pons *et al*., 1992), oedema formation in the RPA reaction was significantly reduced by UK-74,505. However, at the same reaction sites were oedema formation in the RPA reaction was inhibited, no effect on neutrophil accumulation was observed. This finding is not consistent with data from a previous study showing that a PAF antagonist, L-659,989, reduced neutrophil accumulation in the RPA reaction in rabbit skin (Hellewell, 1990). The disparity between our results and those previously obtained may reflect differences in the selectivity of the antagonists used.

Platelet accumulation has been shown to be a component of the acute inflammatory response to killed *E.Coli* (Jeynes *et al*., 1989) and to LPS (Movat *et al*., 1980). Mechanisms underlying this platelet accumulation have not been determined. LPS injected into rabbit skin caused a dose-dependent platelet accumulation which was almost completely inhibited by UK-74,505 indicating an important role for PAF in this response. This contrasts with observations made for the RPA reaction where UK-74,505 gave significant but only partial inhibition of platelet accumulation (Pons *et al*., 1992). LPS Induced little oedema formation in rabbit skin, suggesting that LPS-induced PAF is, in some way, able to produce platelet-accumulation without affecting plasma permeability. This phenomenon might relate to the magnitude or location of PAF generation. Previously, we hypothesized that qualitative differences in PAF antagonist sensitivity between RPA reactions and responses to i.d injected immune complexes or zymosan might be due to such a phenomenon (Pons *et al*., 1992).

Having determined that PAF is an important mediator of platelet accumulation in response to LPS, UK-74,505 was next used to investigate the role of PAF in the neutrophil accumulation associated with this response. LPS caused a marked accumulation of neutrophils which was not affected by treatment with UK-74,505 suggesting that PAF is not an important mediator of LPS-induced neutrophil accumulation in rabbit skin. It should be stressed that the above experiments tell us little about the behaviour of neutrophils once they have accumulated, only that they do accumulate. PAF may still be an important mediator of neutrophil responses in the above reactions, possibly mediating their activation. Recent experiments, in which intravital microscopy was used to visualise the circulation of rat mesentery, demonstrated that UK-74,505 inhibited extravasation of leucocytes induced by intraperitoneal interleukin-1 (Nourshargh *et al*., 1994). Such a role for PAF in RPA reactions and in responses to LPS is not precluded by our experiments, as measurement of ^{111}In-labelled neutrophil accumulation into skin sites does not distinguish between cells migrated into tissue and cells remaining adherent in blood vessels.

The possible role of PAF in either LSR priming or events that follow challenge in this reaction is not clear. Evidence that PAF may be an important mediator of some of the events associated with septic shock suggests that LPS-stimulated PAF generation may also be important for the development of the LSR. However, UK-74,505 which has been shown to be a potent and selective inhibitor of a number of PAF-dependent responses did not affect haemorrhage or plasma protein leakage in the LSR when given i.v. either immediately before priming injections of LPS, or 10 min before LSR challenge. The observation that UK-74,505 inhibits platelet accumulation in response to i.d. LPS, but not the LSR when given before priming injections suggests that LSR priming is both PAF- and platelet-independent. It is possible that platelet accumulation and other PAF-dependent responses to i.d. LPS are merely delayed by UK-74,505 and that platelet accumulation and priming continue when the antagonist is finally cleared. We consider this possibility unlikely, as the Shwartzman reaction is highly time-dependent and thus delaying the underlying controlling events would be expected to have profound effects on the haemorrhagic necrosis seen after challenge. The role of platelets in the LSR is a subject of controversy, with some studies showing that their presence is an absolute requirement for the Shwartzman reaction to occur (Margaretten & McKay, 1969), or that, conversely, platelet depletion has no effect on the reaction (Levin & Cluff, 1965). Our findings support the latter view. That UK-74,505 injected before LSR challenge had no effect on the subsequent appearance of haemorrhagic lesions at primed sites suggests that the events that follow LSR challenge occur independently of the high levels of circulating PAF found to result from i.v. injection of LPS (Doebber *et al*., 1985; Chang *et al*., 1987).

In summary, our studies indicate that platelet accumulation in the RPA reaction and in response to i.d. LPS may occur through similar PAF-dependent mechanisms, while neutrophil accumulation in these responses occurs independently of PAF. In spite of our finding that platelet accumulation in response to i.d. LPS is PAF dependent, our studies have not indicated a role for PAF in the LSR. The pathology associated with severe, systemic bacterial infection is highly complex, and to be clinically useful a treatment needs to address as many of its

features as possible. If the LSR is, as it is believed, a good model of the disseminated intravascular coagulation and associated organ damage that often accompany severe bacterial infection, then our results indicate that PAF antagonists might fail to address these important disease features.

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