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Effect of soluble P55 tumour-necrosis factor binding fusion protein on the local Shwartzman and Arthus reactions

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1 In this study, the effects of a protein synthesis inhibitor, cycloheximide, and a soluble tumour necrosis factor (TNF) binding/IgG fusion protein, p55-sf2, on the priming and challenge stages of the local Shwartzman reaction (LSR) were assessed and compared with their effects on the acute inflammatory response induced by recombinant human tumour necrosis factor-α (rhTNF), lipopolysaccharide (LPS) and a reversed passive Arthus (RPA) reaction in rabbit skin.

2 The LSR was induced in skin by giving an intradermal (i.d.) priming injection of LPS followed by two i.v. challenge injections 20 h and 22 h later. Accumulation of ^{51}Cr-labelled red blood cells and [^{125}I]-albumin were measured at 24 h as markers of haemorrhage and oedema formation, respectively.

3 The RPA reaction was induced in the rabbit by giving i.d. injections of Arthus anti-serum (anti-bovine-γ-globulin, BGG) followed 5 min later by an i.v. injection of the antigen (BGG). Oedema formation and the accumulation of ^{111}In-labelled neutrophils produced in the RPA reaction and in response to i.d. injection of rhTNF and LPS were measured over the 4 h period after inducing the responses.

4 A single local injection of cycloheximide (10 μg/site) did not inhibit neutrophil accumulation or oedema formation produced by 100% Arthus anti-sera. Although LPS injected i.d. induced a marked dose-dependent neutrophil accumulation, there was little associated plasma leakage. Cycloheximide (10 μg/site) did not significantly inhibit the neutrophil accumulation induced by LPS (0.1 μg/site). In the LSR, priming i.d. injections of LPS caused a dose-dependent increase in haemorrhage and plasma leakage at skin sites after challenge with LPS (two injections of 100 μg, i.v.). Co-injection of a single dose of cycloheximide (10 μg/site) with LPS (30 μg/site) caused a marked reduction in the amount of haemorrhage. Local cycloheximide (10 μg/site) administered immediately before LSR challenge did not affect the responses produced in the LSR.

5 Neutrophil accumulation induced by TNF (0.17 μg/site) was abolished by co-administration of p55-sf2 (3 μg/site) whereas neutrophil accumulation induced by i.d. LPS and produced in the RPA reaction was not affected. In the LSR, haemorrhage and oedema formation were inhibited by p55-sf2 (3 μg/site) when it was administered i.d. with the LPS priming injection, but not when given i.d. immediately before LSR challenge.

6 These data suggest that the acute neutrophil accumulation produced in the RPA reaction and in response to i.d. LPS may not be dependent on local protein synthesis or TNF production. On the other hand, haemorrhage appears to be dependent on local protein synthesis during the priming phase but not during the challenge stage of the LSR. Importantly, haemorrhage and plasma leakage appear to be dependent on local TNF generation during the priming phase but not during the challenge stage of the LSR. Thus TNF appears to play a key role in the LSR in rabbit skin.

Keywords: Shwartzman and Arthus reactions; lipopolysaccharide; tumour necrosis factor; TNF binding fusion protein; haemorrhage; oedema formation; neutrophil accumulation

Introduction

In 1928 Shwartzman reported a haemorrhagic and inflammatory response in rabbit skin induced by an intradermal (i.d.) priming injection followed 24 h later by an i.v. challenge injection of *Bacillus typhosus* culture filtrate (Shwartzman, 1928). The activity in the filtrate was subsequently identified as lipopolysaccharide (LPS). Before challenge with i.v. LPS, the LPS primed skin sites are characterized by leucocyte accumulation and erythema whereas approximately 4-6 h after i.v. LPS challenge, intravascular coagulation and haemorrhagic necrosis occur at the primed skin sites, the hallmarks of the local Shwartzman reaction (LSR) (Brozna, 1990). When an animal receives two i.v. injections of LPS spaced 18-24 h apart, a generalized Shwartzman reaction occurs resulting in a disseminated intravascular coagulation usually followed by a major organ failure (Brozna, 1990). The Shwartzman reaction therefore is often regarded as a model of disseminated intravascular coagulation and the associated organ damage that occurs in sepsis, certain complications of pregnancy and skin diseases (Hjort & Rapaport, 1965; Brozna, 1990). The mechanisms underlying the Shwartzman reaction are unknown but may be due to the ability of LPS to produce and liberate the pleiotropic cytokine tumour necrosis factor-α (TNF) from a number of cell types. TNF, as well as exhibiting tumour necrotising activity, also plays important roles in many inflammatory, haemorrhagic and immunological reactions (Old, 1985; Vassalli, 1992; Tracey & Cerami, 1994). Consequently, TNF has been implicated as a key mediator of clinically relevant diseases such as septic shock (Bates *et al.*, 1994) and rheumatoid arthritis (Maini & Feldmann, 1993). Some evi-

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dence also indicates that TNF may play a fundamental role in the pathogenesis of the LSR; for example, exogenous TNF injected directly into rabbit skin causes neutrophil accumulation (Rampart *et al.*, 1989), a combination of TNF and interleukin (IL)-1 can reportedly prime skin sites for an LSR (Movat *et al.*, 1987) and anti-TNF antibodies can block a TNF-induced Shwartzman-like reaction (Grau *et al.*, 1991). Despite these observations there is no direct evidence that TNF is involved in the LSR.

In this study, we compared the LSR with another inflammatory and haemorrhagic lesion; the reversed passive Arthus (RPA) reaction. Like the LSR, the mechanisms underlying the Arthus reaction are only partly understood. The RPA reaction and the LSR have, however, been demonstrated to be complement- and neutrophil-dependent (Stetson & Good, 1951; Humphrey, 1955; Cochrane & Janoff, 1974; Yeh *et al.*, 1991; Rossi *et al.*, 1992). Furthermore, the Arthus reaction (Mulligan *et al.*, 1992; 1993; Noonan *et al.*, 1993; Norman *et al.*, 1994; Teixeira *et al.*, 1994) and the LSR (Argenbright & Barton 1991; 1992) are inhibited by i.v. administration of mAbs directed against the leucocyte adhesion molecule CD18 and the predominantly endothelial cell adhesion molecule, intercellular adhesion molecule-1 (ICAM-1). Lipids such as the vasodilator prostaglandins (Williams *et al.*, 1986) and the direct-action oedematogen platelet-activating factor (PAF) (see Rossi & Hellewell, 1994) also contribute to the inflammatory response associated with Arthus reactions. However, a possible role for TNF in the Arthus reaction has not been fully investigated although neutrophil accumulation produced in an RPA reaction in rat (Mulligan & Ward, 1992) and rabbit (Issekutz *et al.*, 1992) skin is not reduced by antibodies directed against TNF.

TNF exerts its plethora of biological actions by interacting with two receptor subtypes; TNF-R1 (p55) and TNF-R2 (p75) (Tartaglia & Goeddel, 1992; Vandenabeele *et al.*, 1995). Naturally occurring TNF inhibitors have been detected in body fluids of patients with cancer, fever, sepsis or arthritis (Englemann *et al.*, 1990; Gatanage *et al.*, 1990; Kohno *et al.*, 1990; Cope *et al.*, 1992; Van Zee *et al.*, 1992; Steiner *et al.*, 1995). Cloning of TNF receptors revealed these inhibitors to be the extracellular domains of the two TNF receptor subtypes (Gray *et al.*, 1990; Seckinger *et al.*, 1990; Baker *et al.*, 1994). These soluble TNF-binding proteins, when separately fused to IgG have both proved to be very effective anti-TNF agents (Seckinger *et al.*, 1990; Baker *et al.*, 1994; Weg *et al.*, 1995) and to protect against lethal endotoxaemia (Lesslauer *et al.*, 1991; Mohler *et al.*, 1993). This paper describes the effects of a p55 bivalent TNF binding IgG fusion protein (p55-sf2) on the LSR in the rabbit. In addition, since some responses induced by LPS and TNF have been shown to be dependent on newly synthesized proteins (Cybulsky *et al.*, 1989; Winn *et al.*, 1993), we used the protein synthesis inhibitor, cycloheximide, to investigate the reliance of the LSR on *de-novo* protein biosynthesis. For comparison, the effects of p55-sf2 and cycloheximide on the inflammatory response induced by the RPA reaction and to i.d. injection of LPS were also investigated.

Methods

Animals

New Zealand White rabbits (2.5–3.5 kg) were purchased from Regal, Sussex, England.

Preparation and radiolabelling of rabbit neutrophils

Rabbit neutrophils were isolated from citrated rabbit blood as previously described (Haslett *et al.*, 1987; Pons *et al.*, 1994). Briefly, rabbits were anaesthetized with Sagatal (30 mg kg^{-1}, i.v.) and blood was collected from the carotid artery via an Abbocath catheter (16 gauge, Venisystems) into tri-sodium citrate (0.38% w/v) and centrifuged (250 *g* for 20 min). The rabbits were then killed by anaesthetic overdose. Platelet-rich plasma was removed, under-layered with 3 ml of 90% Percoll and centrifuged (2000 *g* for 20 min) to produce platelet poor plasma (PPP). This latter step allows the centrifuged platelets to settle onto the Percoll layer thereby reducing the possibility of the platelets liberating their contents into the PPP. Following a 30 min dextran sedimentation of red blood cells (RBC) from the lower buffy coat produced by the first spin, the leucocyte-rich supernatant was removed and centrifuged (275 *g* for 6 min). The pellet was re-suspended in PPP and layered onto discontinuous Percoll-plasma gradients (43% Percoll in PPP over 53% Percoll in PPP) which were centrifuged (260 *g* for 10 min). The neutrophil-rich band was collected and contaminating RBC removed by hypotonic lysis. Approximately 5×10^7 neutrophil (>98% pure) were re-suspended in PPP (1–2 ml) and incubated with approximately 100 μCi $^{111}InCl_3$ chelated to 2-mercaptopyridine-N-oxide (400 $\mu g\ ml^{-1}$) for 15 min at room temperature. Cells were washed three times in PPP to remove unbound radiolabel, and re-suspended in PPP. Recipient rabbits received 5×10^7 neutrophils (approximately 5–30 $\mu Ci\ kg^{-1}$) suspended in 3 ml PPP into an ear vein via a butterfly cannula (21 gauge).

Preparation and radiolabelling of RBC

Rabbit blood was collected either by carotid artery cannulation (as above) or by central ear artery cannulation where blood (10 ml) was collect via an Abbocath catheter (19 gauge, Venisystems) into tri-sodium citrate (0.38% w/v) and centrifuged (250 *g* for 20 min). RBC (1 ml volume per recipient rabbit) were collected from the bottom of the resultant pellet and incubated (30 min; room temperature) with $Na_2{}^{51}CrO_4$ (0.5–1.5 ml, 500–1500 μCi; in saline). Subsequently, Tyrode solution was added to increase the volume to 10 ml and the cell suspensions centrifuged (250 *g* for 10 min). RBC were washed three times and finally re-suspended in a volume of 3 ml (approximately 50–150 $\mu Ci\ kg^{-1}$) per recipient rabbit. RBC were administered into an ear vein via a butterfly cannula (21 gauge).

Preparation of Arthus antiserum

Arthus antiserum, anti bovine-γ-globulin (anti-BGG) was raised in rabbits. Subcutaneous injections (4×0.25 ml) of BGG (2 mg ml^{-1} in saline) emulsified with an equal volume of Freund's complete adjuvant were administered. This was followed 14 days later by booster injections (4×0.25 ml) of the same concentration of BGG in Freund's incomplete adjuvant. At day 28 a subcutaneous injection of alum-precipitated BGG (300 μg/rabbit) was given. Blood was collected by carotid cannulation on day 38. The serum from five rabbits was pooled, heat inactivated at 56°C for 30 min and stored in aliquots at −20°C (Rossi *et al.*, 1992).

Acute (4 h) inflammatory reactions in rabbit skin

Rabbits were anaesthetized with Sagatal (30 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-labelled neutrophils (approximately 5 to 30 $\mu Ci\ kg^{-1}$, prepared as described above) and ^{125}I-labelled human serum albumin (5 $\mu Ci\ kg^{-1}$) were injected i.v. as markers of neutrophil accumulation and plasma protein leakage respectively. LPS, TNF, and Arthus antiserum (anti-BGG) alone, or in the presence of cycloheximide (10 μg/site) or p55-sf2 (3 μg/site), 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 sites excised with a 17 mm diameter punch. Radioactivity in sites, blood and plasma samples was counted with a 5 head gamma counter (Canberra Packard Ltd.,

Pangbourne, Berks) with automatic spill-over and cross-talk correction for ^{125}I and ^{111}In. The ^{111}In-count per neutrophil was determined, and used to express accumulated ^{111}In in sites as number of radiolabelled neutrophils. Human serum albumin accumulation was expressed as μl plasma/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 ketamine (30–35 mg kg^{-1}) and xylazine (2–3 mg kg^{-1}) intramuscularly. The dorsal skin was shaved and marked out with 6 replicates of 9 treatment sites according to a balanced site plan. LPS from *Salmonella typhosa* was prepared in sterile isotonic saline. When investigating LSR priming, cycloheximide (10 μg/site) or p55-sf2 were co-injected with LPS. When investigating events that follow LSR challenge, cycloheximide (10 μg/site) and p55-sf2 were injected immediately before i.v. LPS at sites injected with LPS 20 h previously. ^{51}Cr-labelled RBC (approximately 50 to 150 $\mu Ci\ kg^{-1}$, prepared as described above) and ^{125}I-labelled human serum albumin (5 $\mu Ci\ kg^{-1}$) were injected i.v. 20 h after priming into re-anaesthetized (as above) rabbits. Initially, zymosan (10 mg kg^{-1}, i.v.) was given after radiolabelled tracers to challenge the Shwartzman reaction. In later experiments, 2 challenges of 100 μg LPS from *S. typhosa* were given 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 sites excised with a 17 mm diameter punch. Radioactivity in sites, blood and plasma samples was counted with a gamma counter having automatic spill-over and cross-talk correction for ^{125}I and ^{51}Cr. Accumulation of ^{51}Cr-labelled RBC was standardized by dividing skin sample ^{51}Cr-counts by ^{51}Cr-counts in 1 μl whole blood and expressing results as equivalent to μl blood/site. Human serum albumin accumulation was expressed as μl plasma/site by dividing skin sample ^{125}I-counts by ^{125}I-counts of 1 μl of plasma.

Materials

The following were obtained 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 (Rompun), Bayer UK Ltd, Bury St. Edmunds, Suffolk; ^{125}I-human serum albumin (20 mg albumin per ml sterile isotonic saline, 50 $\mu Ci\ ml^{-1}$), $^{111}InCl_3$ (2000 μCi in 0.2 ml sterile pyrogen free 0.04 N HCl), and $Na_2{}^{51}CrO_4$ (5000 μCi 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, Pheonix Pharmaceuticals Ltd., Gloucester; LPS (*Salmonella typhosa*, prepared by phenol extraction), zymosan, bovine-γ-globulin (BGG), cycloheximide, human IgG, Freund's complete adjuvant and Freund's incomplete adjuvant, Sigma Chemical company, Poole, Dorset; Diff-Quik stain, BDH, Poole, Dorset. Recombinant human TNF-α (TNF-α) with a specific activity of $6.1 \times 10^{10}\ \mu g^{-1}$ protein was a gift from Dr S. Foster, Zeneca Pharmaceuticals, Alderley Park, Cheshire, England. Humanized p55 TNF binding protein (p55-sf2) was a gift from Drs B. Scallon and J. Ghrayeb at Centocor, Inc., Malvern, PA, U.S.A.

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 multiple comparisons.

Results

Effect of several challenge strategies on the LSR

Zymosan i.v. challenge gave no visible Shwartzman reactions and measurement of RBC accumulation and plasma protein leakage gave values slightly less than those seen in sham (saline)-challenged animals. Consequently, we investigated the effect of several challenge strategies on RBC accumulation and plasma protein leakage in the LSR. Figure 1 shows the effect of several challenges on RBC accumulation (Figure 1a) and plasma protein leakage (Figure 1b) measured at sites primed with LPS (30 μg) 4 h after the indicated challenge. Like zymosan (10 mg kg^{-1}, i.v.), LPS (100 or 200 μg, i.v.) did not produce reactions that were measurably greater than responses at sham challenged sites. Animals that received 2 injections of LPS (100 μg, i.v.) given at 20 and 22 h after priming or with LPS (100 μg, i.v.) at 20 h followed by zymosan (10 mg kg^{-1}, i.v.) at 22 h produced Shwartzman reactions that were clearly visible 2 h after the second challenge and were measurably greater than responses seen in sham challenged animals. In all subsequent LSR experiments, animals were challenged with 2 injections of LPS (100 μg, i.v.) spaced 2 h apart.

Effect of cycloheximide on responses to exogenous LPS

The importance of *de-novo* protein synthesis on the 4 h reaction to LPS was assessed by use of cycloheximide. LPS injected into rabbit skin at doses between 1 and 100 ng/site produced dose-dependent neutrophil accumulation. Little leakage of plasma protein was observed with these doses of LPS (data not shown). There was no significant reduction in the neutrophil accumulation at sites injected with LPS (100 ng) when cycloheximide was co-injected (Figure 2a).

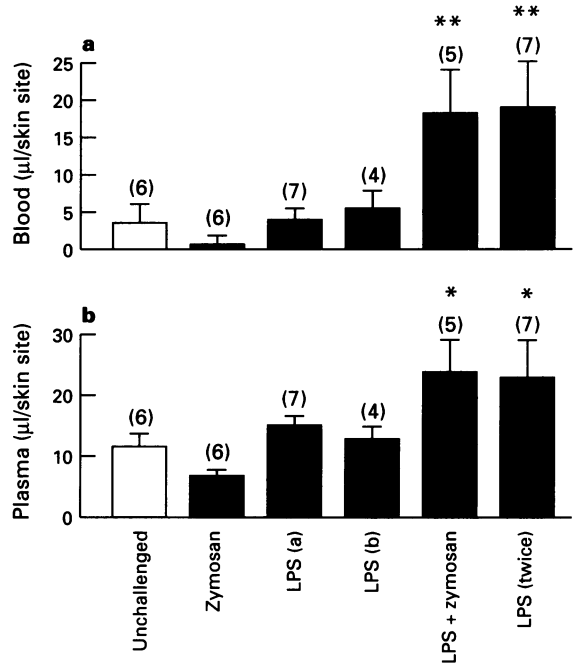

Figure 1 Haemorrhage (μl blood/site; a) and plasma protein leakage (μl plasma/site; b) assessed over a 4 h period during the LSR. Rabbits were challenged i.v. as indicated on the horizontal axis 20 h after i.d. LPS (30 μg). The animals were challenged with zymosan (10 mg kg^{-1}), LPS (a; 100 μg), LPS (b; 200 μg), LPS + Zymosan indicates challenge with LPS (100 μg) at 20 h followed 2 h later by zymosan (10 mg kg^{-1}) and LPS (twice) indicates i.v. injections of LPS (100 μg) at 20 and 22 h. Results are expressed as mean ± s.e.mean of the indicated number of experiments. Significant difference from control is indicated by $*P < 0.05$ and $**P < 0.01$.

Effect of cycloheximide on responses produced in the RPA reaction

The pronounced neutrophil accumulation measured in the RPA reaction (Figure 2b) was associated with marked leakage of plasma proteins (data not shown). Cycloheximide did not affect either neutrophil accumulation or plasma protein leakage seen in the RPA reaction produced at sites injected with 100% Arthus antiserum.

Effect of cycloheximide on the LSR

Haemorrhage and plasma leakage, the magnitudes of which were dependent on the priming dose of i.d. LPS, were seen at sites after two challenges with 100 μg LPS i.v. Co-injection of cycloheximide at 10 μg/site with the highest (30 μg) priming dose of LPS caused a marked and significant reduction in the amount of haemorrhage seen 4 h after the first challenge (Figure 3a). Local cycloheximide did not significantly reduce the plasma leakage in the LSR when it was administered with the priming dose of LPS (Figure 3b). The effect of local cycloheximide administered immediately before LSR challenge is shown in Figure 4. Rabbit skin sites were primed for the LSR with doses of LPS between 1 μg and 30 μg/site. Twenty h after priming, cycloheximide (10 μg/site) or vehicle saline was injected at sites primed with 30 μg LPS. The LSR was then challenged with two doses of 100 μg LPS. Haemorrhage (Figure 4a) and plasma protein leakage (Figure 4b) measured 4 h after the first Shwartzman challenge were not affected by cycloheximide treatment immediately before challenge.

Effect of p55-sf2 on responses to exogenous TNF

Recombinant human TNF injected into rabbit skin at 1.7 ng and 17 ng/site caused a slight increase in the number of neutrophils measured in sites after 4 h. A more marked neutrophil accumulation was seen at sites injected with TNF (170 ng/site) was markedly reduced by p55-sf2 (3 μg/site) (Figure 5a). Plasma protein leakage in response to TNF was not different to that produced by saline (Figure 5b). When injected alone, p55-sf2 gave values for neutrophil accumulation and plasma protein leakage that were not different from those given by vehicle saline.

Effect of p55-sf2 on responses to exogenous LPS

The effect of p55-sf2 on neutrophil accumulation in response to LPS is shown in Figure 6a. LPS again caused a pronounced and dose-dependent neutrophil accumulation. The responses induced by LPS (30 μg/site) was not affected by p55-sf2 (3 μg/site).

Effect of p55-sf2 on responses produced in the RPA reaction

The effect of p55-sf2 on neutrophil accumulation in the RPA reaction is shown in Figure 6b. As in earlier experiments marked neutrophil accumulation was seen in the RPA reaction in rabbit skin, which was associated with plasma protein leakage. The RPA reaction was not affected by p55-sf2.

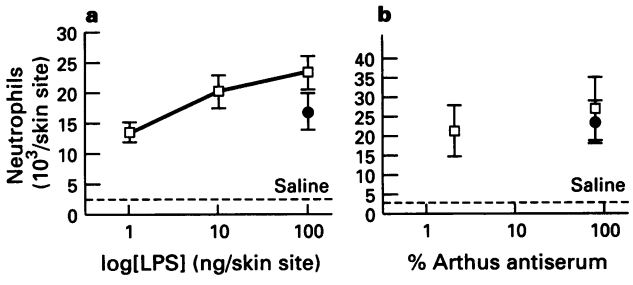

Figure 2 Effect of local cycloheximide on ^{111}In-neutrophil accumulation measured in response to LPS (a) and in the RPA reaction (b) in rabbit skin assessed over a 4 h period. LPS or Arthus antiserum were i.d. injected either alone (□) or in the presence of cycloheximide (●, 10 μg/site). Results are expressed as the mean ± s.e.mean of $n = 7$ experiments for LPS and $n = 4$ experiments for RPA reactions.

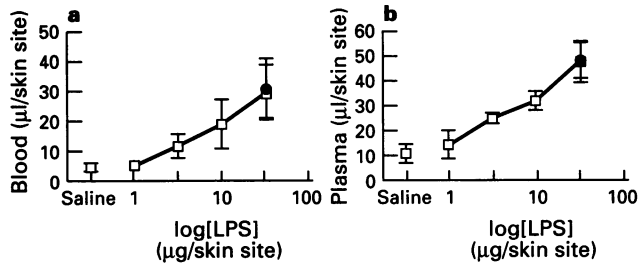

Figure 4 Effect of cycloheximide (given locally at the challenge phase) on haemorrhage (μl blood/site; a) and plasma protein leakage (μl plasma/site; b) assessed over a 4 h period during the LSR. Priming doses of LPS were injected i.d. After 20 h, either saline (□) or cycloheximide (●, 10 μg/site) were injected into primed sites. Animals were challenged at 20 and 22 h with 100 μg LPS i.v. Results are expressed as mean ± s.e.mean of $n = 6$ experiments.

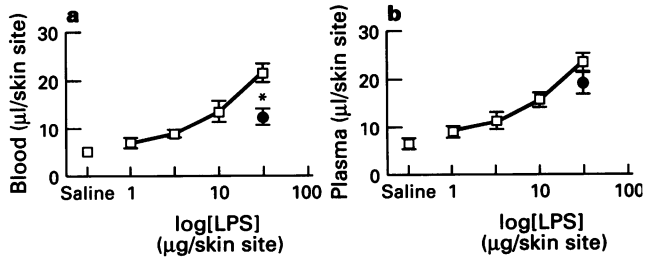

Figure 3 Effect of cycloheximide (given locally at the priming phase) on haemorrhage (μl blood/site; a) and plasma protein leakage (μl plasma/site; b) assessed over a 4 h period during the LSR. Priming doses of LPS were injected i.d. either alone (□) or in the presence of cycloheximide (●, 10 μg/site). Animals were challenged at 20 and 22 h with 100 μg LPS i.v. Results are expressed as mean ± s.e.mean of $n = 6$ experiments. Significant difference from control is indicated by $^*P < 0.05$.

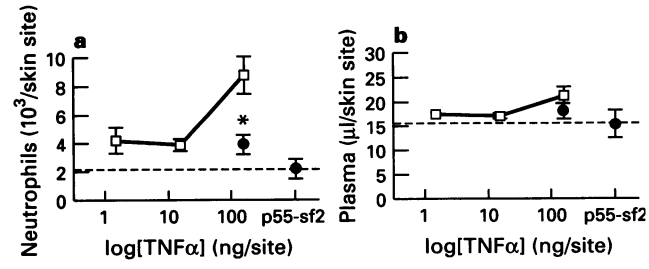

Figure 5 Effect of p55-sf2 on neutrophil accumulation (a) and plasma protein leakage (b) induced by TNF in rabbit skin assessed over a 4 h period. Recombinant human TNF was injected i.d. either alone (□) or in the presence of p55-sf2 (●, 3 μg/site). Results are expressed as mean ± s.e.mean of $n = 6$ experiments. Significant difference from control is indicated by $^*P < 0.05$.

Effect of p55-sf2 on the LSR

Skin sites were primed with doses of LPS between 1 and 30 μg in the presence of either control human IgG or p55-sf2. The magnitude of the haemorrhage (Figure 7a) and plasma protein leakage (Figure 7b) recorded after i.v. LPS challenge was dependent on the priming dose of LPS. The responses produced at LSR sites primed with 10 μg and 30 μg LPS were markedly and significantly reduced when p55-sf2 (3 μg/site) was co-administered with LPS.

In a further set of experiments rabbit skin sites were primed for the LSR with doses of LPS between 1 and 30 μg/site. Twenty hours after priming, p55-sf2 (3 μg/site) or human IgG was injected at sites primed with 10 μg and 30 μg LPS. The animals were then challenged i.v. with two doses of 100 μg LPS. Haemorrhage (Figure 8a) and plasma protein leakage (Figure 8b) measured 4 h after the first LPS challenge were not affected by p55-sf2 treatment given i.d. immediately before challenge.

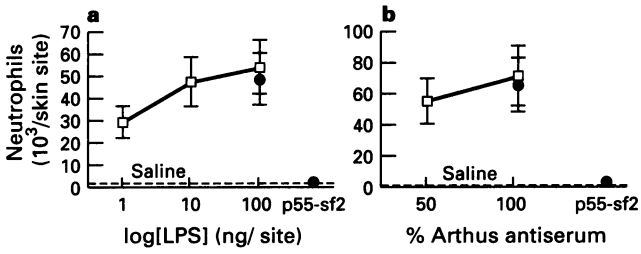

Figure 6 Effect of p55-sf2 on ^{111}In-neutrophil accumulation measured in response to LPS (a) and in the RPA reaction (b) in rabbit skin assessed over a 4 h period. LPS or Arthus antiserum was injected either alone (□) or in the presence of p55-sf2 (●, 3 μg/site). Results are expressed as the mean ± s.e.mean of $n=6$ experiments.

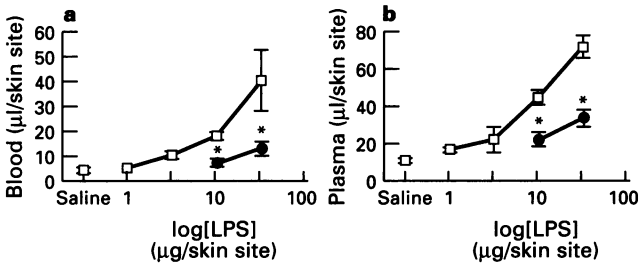

Figure 7 Effect of p55-sf2 (given locally together with priming dose) on haemorrhage (μl blood/site; a) and plasma protein leakage (μl plasma/site; b) assessed over a 4 h period during the LSR. Priming doses of LPS were injected i.d. together with either control human IgG (□, 3 μg/site) or in the presence of p55-sf2 (●, 3 μg/site). Animals were challenged at 20 and 22 h with 100 μg LPS i.v. Results are expressed as mean ± s.e.mean of $n=4$ experiments. Significant difference from control is indicated by $^*P<0.05$.

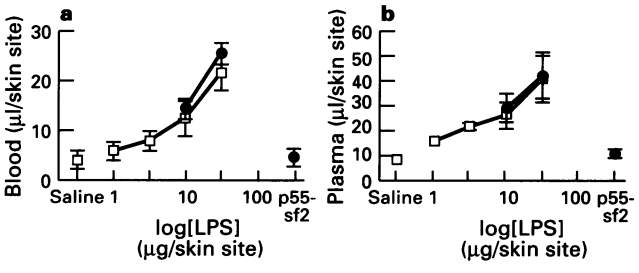

Figure 8 Effect of p55-sf2 (given locally at the challenge phase) on haemorrhage (μl blood/site; a) and plasma protein leakage (μl plasma/site; b) assessed over a 4 h period during the LSR. Priming doses of LPS were injected i.d. After 20 h, either control human IgG (□, 3 μg/site) or p55-sf2 (●, 3 μg/site) were injected into primed sites. Animals were challenged at 20 and 22 h with 100 μg LPS i.v. Results are expressed as mean ± s.e.mean of $n=3$ experiments.

Discussion

In this study we have shown that haemorrhage and plasma leakage induced in a LSR are dependent on TNF generation and that the haemorrhage produced in the LSR is also dependent on local protein synthesis. It appears that local protein synthesis and TNF generation occur during the early priming phase of the LSR but that these processes are not essential during the later challenge stage of the reaction. In addition, the acute inflammation produced during an RPA reaction and in response to intradermal LPS may occur without requirement for new protein synthesis or TNF generation. We have also described a new method for challenging the LSR that produces the marked haemorrhagic lesions characteristic of the reaction. Initially, we found the LSR rather difficult to induce reproducibly when rabbits were challenged with single injections of zymosan and LPS and therefore tried a number of challenge strategies. The present data show that two i.v. challenge injections of LPS spaced 2 h apart, or an injection of LPS followed 2 h later by zymosan, produces visible and measurable reactions in most (approximately 80%), but not all, animals. While challenge with complement activating agents, such as zymosan, has been previously reported to produce Shwartzman-like reactions in approximately primed animals (Movat & Burrowes, 1984) this did not occur in our experiments. The reason for this discrepancy is not clear but one could speculate that in our animal model we failed to achieve sufficient levels of circulating zymosan particles to induce the LSR. Despite these differences we used the relatively reliable double LPS challenge throughout our study.

The mechanisms underlying LSR priming have proved to be an enigma for many years. LPS, the agent used to prime as well as challenge the LSR, stimulates the production of a host of mediators including lipids (e.g., prostaglandins, PAF, leukotriene B_4) and cytokines (e.g., TNF, interleukin-1 (IL-1), IL-8) both *in vivo* and from different isolated cell types. LPS also augments the expression of adhesion molecules such as ICAM-1 on vascular endothelial cells primarily by a protein synthesis dependent mechanism (Rietschel *et al.*, 1994). Cycloheximide (a reversible blocker of RNA translation) when co-injected with priming doses of LPS caused a significant reduction in the amount of haemorrhage with only slight reduction in plasma protein leakage measured after LSR challenge. On the other hand, cycloheximide did not affect haemorrhage or oedema formation measured in the LSR when injected at primed sites immediately before challenge, indicating that LSR priming is dependent on local protein synthesis, while events that follow LSR challenge may not. Interestingly, cycloheximide did not significantly reduce neutrophil accumulation in response to LPS (single intradermal dose) in rabbit skin. This may indicate that neutrophil accumulation in response to LPS is mediated largely via the release of mediators that are either pre-formed or are formed independently of protein synthesis. Although we previously reported that the dose of cycloheximide used in this study effectively blocked neutrophil accumulation (also measured over 4 h) induced by IL-1 (Rampart & Williams, 1988), we did not investigate the effect of cycloheximide on LPS-stimulated neutrophil accumulation. However, Cybulsky *et al.* (1989) did report an inhibition of neutrophil emigration induced by IL-1, TNF and LPS by cycloheximide, actinomycin D and puromycin. The reasons for these apparently opposing observations are unclear but may depend on the time and frequency of administration of i.d. protein synthesis inhibitors and the period over which neutrophil accumulation is measured. For example, Cybulsky *et al.* (1989) injected cycloheximide at $t=-2$ h (same time as the LPS injection) and at $t=-1.5$ h, injected ^{51}Cr-neutrophils at $t=-0.5$ h and measured neutrophil accumulation at $t=0$. Therefore, a double

dose of cycloheximide (due to its short action) may be required in order to inhibit the LPS response. Furthermore, the dose of LPS and cycloheximide used by Cybulsky *et al.* (1989) were higher and lower, respectively, than the doses used in this study. Therefore, although the dose of cycloheximide used in this study inhibited the LSR and inhibited IL-1-induced neutrophil accumulation (Rampart & Williams, 1988; Von Uexkull *et al.*, 1992) it may have been insufficient to inhibit LPS-induced neutrophil accumulation; a possibility requiring further investigation. Interestingly, Cybulsky *et al.* (1989) used LPS derived from *E. coli* whereas we used LPS derived from *Salmonella typhosa*; another possible explanation for the divergent results. Nevertheless, our data do show that cycloheximide (at the same dose which did not affect LPS-induced neutrophil accumulation) co-injected with the priming dose of LPS inhibited the haemorrhage produced in the LSR suggesting that protein synthesis is important at an early point during the priming stage.

A possible role for TNF in the pathogenesis of the LSR had been suggested by Movat *et al.* (1987) who showed in the rabbit that i.d. injection of a combination of TNF (1–10 μg/site) and IL-1 (0.5–4 μg/site) could synergistically prime skin sites for an LSR thereby replacing the need for the i.d. priming injection of LPS. In addition, studies showing that a Shwartzman-like reaction produced by subcutaneous injection of TNF in mice was effectively blocked using anti-TNF antibodies also indicate that TNF may be important in the LSR (Grau *et al.*, 1991). Direct evidence implicating TNF in the rabbit LSR has been lacking. Hence, using the potent neutralising divalent soluble TNF binding protein fused to human IgG (p55-sf2) we investigated the requirement for TNF in LSR priming, the events that follow LSR challenge, and for comparison, the acute inflammatory responses to LPS and the RPA reaction. Neutrophil accumulation in response to TNF was greatly reduced by p55-sf2, supporting other reports that this molecule is an effective anti-TNF agent (Seckinger *et al.*, 1990; Baker *et al.*, 1994). Co-injection of p55-sf2 with priming doses of LPS caused a significant reduction in the haemorrhage and plasma protein leakage seen in the LSR following challenge. Events that follow LSR challenge were not affected by p55-sf2 injected into primed sites at $t=20$ h. These results indicate that LSR priming is dependent on locally acting TNF while the events that follow LSR challenge are not.

Since TNF is one of the earliest mediators detected after LPS administration to experimental animals (Waage, 1990) our results suggest that the inhibitory effect of cycloheximide on the LSR may result from an inhibition of TNF generation. These findings are consistent with the observations demonstrating a role for ICAM-1 in the LSR. Argenbright & Barton (1991; 1992) demonstrated that by injecting anti-ICAM-1 and anti-CD18 mAbs, immediately before challenge, the haemorrhage produced in the LSR was dramatically inhibited. Since LPS is a potent stimulus for the up-regulation of endothelial cell expression of ICAM-1 (Pohlman *et al.*, 1986) it has been proposed that increased expression of ICAM-1 is critical for preparation of LSR sites (Argenbright & Barton, 1991; 1992). Systemic challenge with LPS then results in augmented expression/avidity of CD18 on circulating neutrophils causing them to accumulate preferentially at skin sites expressing increased levels of ICAM-1 where they adhere/aggregate and cause haemorrhagic necrosis. Our findings that LSR priming requires protein synthesis and TNF are consistent with the above theory. TNF can stimulate increased expression of ICAM-1 on endothelial cells (Pohlman *et al.*, 1986), and cycloheximide could effect this increased expression either directly by inhibiting ICAM-1 expression or indirectly via inhibition of TNF generation.

Neutrophil accumulation in response to LPS was not affected by local administration of p55-sf2. Thus, although LPS is a potent stimulus for TNF generation (Abe *et al.*, 1985; Beutler *et al.*, 1985), and TNF can cause neutrophil accumulation in rabbit skin (Rampart *et al.*, 1989), it appears that TNF may not play a major role in the acute neutrophil accumulation induced by intradermal injection of LPS in rabbit skin. Similarly Cybulsky *et al.* (1988) found a role for IL-1 but not TNF in LPS-induced neutrophil accumulation in rabbit skin. Issekutz *et al.* (1992), using a monoclonal antibody to rabbit TNF, partially inhibited (by only 27%) the LPS-induced neutrophil recruitment in rabbit skin. Taken together these observations suggest that if TNF plays a role in the acute inflammatory response induced by LPS it is minor. Alternatively, it is possible that the amount of TNF generated by LPS (and by the RPA reaction) is too high to be effectively mopped up by p55-sf2 since neutrophil accumulation induced by these acute inflammatory reactions was greater than the p55-sf2 blockable TNF-induced neutrophil recruitment. Neutrophil accumulation and plasma protein leakage in the RPA reaction were similarly unaffected by p55-sf2. This finding concurs with those of Issekutz *et al.* (1992) and Mulligan & Ward (1992) who showed that anti-TNF antibodies did not affect the RPA reaction induced in rabbit and rat skin respectively. Thus, we and others have not found a role for TNF in dermal Arthus reactions. TNF has however been shown to participate in LPS (Ulich *et al.*, 1991; 1993; 1994; Horgan *et al.*, 1993; Denis *et al.*, 1994; Conroy *et al.*, 1995) and immune-complex mediated lung injury (Warren *et al.*, 1989; Mulligan & Ward, 1992). The reasons why these acute lung responses, in comparison to dermal reactions, may be more susceptible to inhibition by TNF blocking agents are not known but may reflect differences in respective microvasculatures (MacNee & Selby, 1993; Rossi & Hellewell, 1994); an area requiring further investigation. Interestingly, Weg *et al.* (1995) reported that systemic administration of p55sf2 inhibited LPS-induced eosinophil accumulation in guinea-pig skin. Whether neutrophil accumulation induced by LPS in rabbit skin can also be inhibited by i.v. administration of the TNF binding fusion protein remains to be determined.

Thus, although we have clearly demonstrated a role for TNF and protein synthesis in the LSR, and others have shown that circulating neutrophils, complement activation and expression of adhesion molecules (CD18 and ICAM-1) are all clearly important for the development of haemorrhagic Shwartzman reactions, other mediators or events may also be required. Indeed, it has been reported that IL-1 can substitute, albeit at high doses, for both the priming and challenge doses of LPS (Beck *et al.*, 1986). In addition, Ozmen *et al.* (1994) reported that IL-12 and interferon-γ, as well as TNF, are key cytokines mediating a mouse generalized Shwartzman reaction. Whether these cytokines also play important roles in the rabbit LSR is unknown and therefore awaits further investigation. Nevertheless, from this study, we propose that in the rabbit LSR, the priming injection of LPS stimulates the generation of TNF which in turn up-regulates and/or causes the expression of adhesion molecules (e.g. ICAMs) on vascular endothelial cells. These two processes, we believe, are either dependently or independently regulated by local *de novo* synthesis of proteins. That priming requires time (18–24 h) to occur supports this proposal. The challenge dose(s) of LPS then up-regulate CD18 adhesion molecule expression on circulating neutrophils. The neutrophils then adhere, via CD18 binding to its counter ligands (e.g. ICAMs), to vascular endothelial cells resulting ultimately in the vascular damage and haemorrhage associated with the localized Shwartzman reaction.

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