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Effect of dexamethasone on neutrophil accumulation and oedema formation in rabbit skin: an investigation of site of action

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1 The anti-inflammatory actions of dexamethasone on vascular and leukocyte responses in rabbit skin were investigated.

2 Neutrophil accumulation and oedema formation were simultaneously measured as the local accumulation of i.v. administered ^{111}In-labelled neutrophils and ^{125}I-labelled albumin. Systemically administered dexamethasone (3 mg kg^{-1}) inhibited neutrophil accumulation induced by i.d. zymosan activated plasma (ZAP), N-formyl-methionyl-leucyl-phenylalanine (FMLP) and leukotriene B_4 (LTB_4) when co-injected with prostaglandin E_2 (PGE_2). Dexamethasone also inhibited oedema formation elicited by these stimuli and the responses induced by i.d. platelet activating factor (PAF) + PGE_2 and bradykinin (BK) + PGE_2.

3 Intradermal dexamethasone (2×10^{-10} mol per site) but not indomethacin (10^{-8} mol per site) inhibited oedema formation induced by i.d. ZAP + PGE_2 and BK + PGE_2. This inhibitory effect of dexamethasone was significant only with pretreatment periods of 4 h, shorter pretreatment periods resulting in greatly reduced effects. Intradermal dexamethasone had no effect on neutrophil accumulation induced by ZAP + PGE_2.

4 Intradermal dexamethasone (2×10^{-10} mol per site) had no effect on increase in blood flow induced by PGE_2 as measured by ^{133}Xenon clearance.

5 The accumulation of neutrophils isolated from donor rabbits pretreated with i.v. saline or dexamethasone (3 mg kg^{-1}) was investigated in untreated recipient rabbits. The accumulation of neutrophils, induced by ZAP + PGE_2, FMLP + PGE_2 and LTB_4 + PGE_2, from dexamethasone-pretreated donors was significantly smaller than the accumulation of neutrophils from saline-pretreated donors.

6 The results of this study suggest that dexamethasone can have a direct effect on vascular endothelial cells resulting in an inhibition of oedema formation.

7 Neutrophil accumulation can be inhibited by an effect of dexamethasone on the neutrophil itself or on the vascular endothelium. These results indicate that at least part of the inhibitory effect is on the circulating neutrophil induced by dexamethasone or a dexamethasone-induced product.

Keywords: Dexamethasone; inflammation; vascular permeability; neutrophil accumulation; oedema; rabbit skin

Introduction

The glucocorticosteroids are potent anti-inflammatory drugs, widely used clinically against inflammatory disease states and able to suppress inflammatory signs and symptoms in many animal models. No single mechanistic explanation could suffice to account for all the biological effects of glucocorticosteroids. Numerous anti-inflammatory actions have been attributed to these drugs, including the suppression of inflammatory mediator generation, as reviewed by Williams & Yarwood (1990). The inhibitory effect of steroids on the generation of pro-inflammatory arachidonic acid metabolites such as prostaglandins, leukotrienes and platelet-activating factor (PAF) has been most extensively investigated (reviewed in Flower, 1988). This effect of glucocorticosteroids is believed to be mediated by the steroid-induced family of proteins known as lipocortins (Flower, 1988) or annexins (Crumpton & Dedman, 1992) which inhibit phospholipase A_2, the enzyme activity responsible for release of arachidonic acid (Blackwell *et al.*, 1980; Hirata *et al.*, 1980; Cloix *et al.*, 1983; Di Rosa *et al.*, 1984). Although the exact mechanism whereby these proteins inhibit phospholipase A_2 awaits resolution there is growing evidence supporting lipocortin 1 (annexin 1) as the candidate mediating steroid inhibition of eicosanoid synthesis. Human recombinant lipocortin 1 has been shown to inhibit the formation of arachidonate metabolites *in vitro* (Cirino *et al.*, 1987; Cirino & Flower, 1987a,b) and to mimic the suppressive action of glucocorticosteroids *in vivo* in models where lipid mediators play a major role (Cirino *et al.*, 1989).

In addition to suppression of mediator generation, glucocorticosteroids also inhibit inflammatory reactions *in vivo* in models unlikely to be mediated by eicosanoid synthesis. Tsurufuji *et al.* (1979) have shown that dexamethasone can inhibit oedema formation induced by chemical mediators including bradykinin (BK) and 5-hydroxytryptamine (5-HT) in a model of mouse foot pad oedema. The oedema responses were not affected by the cyclo-oxygenase inhibitor, indomethacin, suggesting that they are independent of local prostaglandin production and thus the inhibitory effect of dexamethasone is unlikely to be due to phospholipase A_2 inhibition. However, the anti-inflammatory effect of dexamethasone was completely blocked by either actinomycin D or cycloheximide, indicating a requirement for the synthesis of an endogenous anti-inflammatory protein (Tsurufuji *et al.*, 1979). Mediators such as BK and 5-HT increase microvascular permeability by a direct action on endothelial cells, causing cell contraction associated with opening of inter-endothelial cell junctions (Majno *et al.*, 1969). It is therefore suggested that dexamethasone or a steroid-induced protein(s) may inhibit oedema formation by a direct effect on the

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endothelium, rendering it less responsive to the action of mediators of increased microvascular permeability such as 5-HT (Tsurufuji *et al.*, 1979). These observations have been supported by other authors using different animal species and an array of inflammatory mediators. Glucocorticosteroids inhibit histamine-induced oedema in rat skin and mouse ear (Church & Miller, 1978) and plasma protein leakage induced by exogenous histamine, BK, PAF and leukotriene C_4 (LTC_4) in the hamster cheek pouch (Bjork *et al.*, 1985; Svensjo & Roempke, 1985).

Chemotactic mediators such as the complement fragment C5a, the arachidonate lipoxygenase product, LTB_4 and the bacterial peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP) induce oedema formation in a neutrophil-dependent manner (Wedmore & Williams, 1981). Glucocorticosteroids have been shown to inhibit neutrophil-dependent oedema induced by exogenous LTB_4, FMLP and the complement fragment C5a in rabbit skin (Foster & McCormick, 1985; Griffiths & Blackham, 1988; Yarwood *et al.*, 1988; 1989; Peers & Flower, 1991). Foster & McCormick (1985) also showed that dexamethasone inhibited the local accumulation of neutrophils in response to LTB_4.

In this study we have investigated further the eicosanoid independent effects of steroids on vascular responses in the rabbit microcirculation. For this purpose we have studied the effects of dexamethasone on oedema response in rabbit skin induced by i.d. neutrophil-dependent and direct-acting mediators. In addition we have studied the effect of dexamethasone on mediator-induced accumulation of ^{111}In-labelled neutrophils in rabbit skin. By using protocols where we have treated either the neutrophil donor rabbit or the recipient rabbit with dexamethasone we have investigated the possible sites of action of steroids in inhibiting plasma protein leakage and neutrophil accumulation *in vivo*.

Methods

Animals

Male, specific pathogen-free New Zealand White rabbits (2–3 kg) were purchased from Froxfield Farm, Froxfield, Hampshire and Hacking and Churchill, Huntingdon, Cambridgeshire.

Measurement of blood flow

Blood flow was measured in the clipped dorsal skin of rabbits by use of a multiple site ^{133}Xe clearance technique (Williams, 1979). The effect of dexamethasone on blood flow induced by PGE_2 was assessed. The vasodilator was mixed with a solution of ^{133}Xe in saline (5–10 μCi/injection) and rapidly injected i.d. (0.1 ml volumes) at 10^{-11} and 3×10^{-10} mol per site, with ^{133}Xe in saline as control. Intradermal injections were given in random-block order according to a predetermined balanced site plan with 6 replicates for each treatment per animal. After 15 min animals were killed with an overdose of i.v. sodium pentobarbitone, the dorsal skin excised and injected sites removed with a 17 mm diameter punch. Skin samples and 0.1 ml aliquots of injection fluids, under paraffin oil in sealed tubes, were counted immediately in an automatic gamma-counter (LKB Wallac 1260 Multigamma II). The change in local blood flow was calculated as the percentage increase above saline controls from the equation: 100 (In^{133}Xe count of saline-injected skin – In^{133}Xe count of agent-injected skin)/(In^{133}Xe count of 0.1 ml injection fluid – In^{133}Xe count of saline-injected skin). The effect of dexamethasone on blood flow was assessed by pretreating skin sites locally with dexamethasone (80 ng per site, equivalent to 2×10^{-10} mol per site) or saline as a control for 4 h prior to re-injection of the sites with doses of PGE_2 or saline mixed with ^{133}Xe as described above.

Preparation of ^{111}In-labelled neutrophils

Rabbit neutrophils were isolated and ^{111}In-labelled according to the procedure previously described (Rampart & Williams, 1988; Nourshargh *et al.*, 1989). Briefly, donor rabbits were anaesthetized with i.v. Sagatal (30 mg kg^{-1}) and bled via a carotid cannula into acid citrate dextrose. Neutrophils (>90% pure) were separated at room temperature by centrifugation over a two layer discontinuous percoll plasma gradient (50% and 70%), after an initial red blood cell sedimentation with 3% hydroxyethyl starch. Neutrophils (approximately 3.5×10^7 cells in 1–2 ml volume) were incubated with $^{111}InCl_3$ (50–100 μCi in 10–15 μl) chelated to 2-mercaptopyridine-N-oxide (40 μg ml^{-1}) for 15 min at room temperature. Labelled leucocytes were then washed twice and finally resuspended in autologous cell free citrated plasma.

Measurement of ^{111}In-neutrophil accumulation and plasma protein exudation in rabbit skin

Rabbits were anaesthetized with i.v. Sagatal (30 mg kg^{-1}) with further maintenance doses given as necessary. Neutrophil infiltration and oedema formation in the rabbit back skin were measured simultaneously as the local accumulation of i.v. injected ^{111}In-neutrophils and ^{125}I-labelled human serum albumin (5 μCi kg^{-1}, mixed with 2 ml of a 2.5% Evans blue dye solution) as previously described (Rampart & Williams, 1988; Nourshargh *et al.*, 1989). Agents under investigation were injected intradermally in 0.1 ml volumes into the clipped dorsal skin according to a balanced site injection plan each treatment having six replicates. All mediators were coinjected with a fixed potentiating dose of PGE_2 (3×10^{-10} mol per site) or AA (3×10^{-9} mol per site) where indicated. After 30 min, a blood sample was taken by cardiac puncture into heparin (10 u ml^{-1} final concentration), animals were killed by an overdose of anaesthetic and the dorsal skin was removed. Injection sites were excised with a 17 mm diameter punch and skin, blood and plasma samples were counted in a gamma counter with automatic spill over and cross-talk correction. Plasma exudation was expressed as μl of plasma/skin site by dividing the skin sample ^{125}I counts by the ^{125}I counts in 1 μl of plasma. Cell accumulation was expressed as the number of ^{111}In-labelled neutrophils per site by dividing skin sample ^{111}In-counts by ^{111}In-counts per neutrophil. All numbers were corrected for 5×10^7 ^{111}In-neutrophils injected into each recipient animal.

In experiments where the effect of local dexamethasone was investigated, skin sites were pretreated i.d. with dexamethasone (2×10^{-10} mol per site) or indomethacin (10^{-8} mol per site) for the times indicated before the intravenous administration of ^{111}In-cells and ^{125}I-albumin. Test agents were then injected i.d. into the pretreated sites. Control sites were pretreated intradermally with saline. The accumulation of labels in skin sites was then measured over a 30 min *in vivo* test period as described above.

The experiments shown in Figures 1 and 2 were designed to evaluate the effect of systemic treatment of recipient rabbits with dexamethasone. Washed labelled neutrophils were divided equally for i.v. injection into two recipient animals, one test and one control rabbit. Test recipient animals were treated systemically with dexamethasone (3 mg kg^{-1}, i.v.) 4 h before i.v. injection of labelled cells and [^{125}I]-albumin. Control animals received an equal volume of saline.

The experiments shown in Figures 6 and 7 were designed to investigate the effect of dexamethasone specifically on the neutrophil *in vivo*. Pairs of neutrophil donor rabbits were treated i.v. with dexamethasone (3 mg kg^{-1}) or an equal volume of saline; 4 h later blood from each pair was collected, neutrophils isolated and ^{111}In-labelled in parallel as described above. ^{111}In-labelled cells from treated and control donor animals were then injected i.v. into two untreated recipient animals, together with [^{125}I]-albumin. Cell numbers were adjusted if necessary so that each pair of recipient

animals received the same number of labelled neutrophils. Neutrophil accumulation and oedema formation were measured in skin sites over 30 min as before. The procedures for each pair of saline and dexamethasone-treated rabbits were carried out in parallel.

Materials

BK, FMLP, prostaglandin E_2 (PGE_2), arachidonic acid (AA), zymosan, indomethacin and 2-mercaptopyridine-N-oxide were purchased from Sigma Chemical Co, Poole, Dorset. PAF was from Bachem, Saffron Walden, Essex. Evans blue dye was from British Drug Houses, Poole, Dorset. Sagatal (pentobarbitone sodium, 60 mg ml^{-1}) was from May and Baker, Dagenham, Essex. Percoll was from Pharmacia Fine Chemicals, Uppsala, Sweden. Hespan (6% hydroxy-ethyl starch in 0.9% NaCl) was from American Hospital Supply, Didcot, Oxfordshire. Steriflex (Sterile, pyrogen free isotonic saline solution) was from The Boots Co. Plc, Nottingham. $^{111}InCl_3$ (2 mCi in 0.2 ml sterile, pyrogen-free 0.04 N hydrochloric acid), ^{125}I-labelled human serum albumin (20 mg albumin per ml of sterile isotonic saline, 50 $\mu Ci\ ml^{-1}$) and $^{133}Xenon$ (10 mCi in 3 ml sterile pyrogen-free saline) were purchased from Amersham International Plc, Amersham, Buckinghamshire. Dexamethasone phosphate (8 mg dexamethasone phosphate in 2 ml ampoules) was from David Bull Laboratories, Warwick. We are grateful to Dr S. Foster of Imperial Chemical Industries, Macclesfield, Cheshire for the generous gift of leukotriene B_4 (LTB_4).

Zymosan-activated plasma (ZAP) as a source of C5a des Arg was prepared by incubating heparinized (10 u ml^{-1}) rabbit plasma with zymosan (1 mg ml^{-1}) for 30 min at 37°C. Zymosan was removed by centrifugation (2 × 10 min, 2500 *g*) and activated plasma stored in aliqots at − 25°C. The C5a des Arg content of activated plasma was approximately 5×10^{-7} M as measured by radioimmunoassay (Jose *et al.*, 1983).

Stock solutions were prepared as follows and kept at − 25°C: PGE_2 and BK 1 mg ml^{-1} in ethanol; arachidonic acid 10 mg ml^{-1} in ethanol; FMLP 0.219 mg ml^{-1} in ethanol; PAF 1 mg ml^{-1} in chloroform/methanol (4:1); LTB_4 0.1 mg ml^{-1} in methanol. For arachidonic acid an intermediate dilution (0.1 mg ml^{-1}) was made in 1.4% sodium bicarbonate solution. Indomethacin (3 mg ml^{-1}) was made up each day in 1.4% sodium bicarbonate solution. ZAP was used undiluted (100%) or diluted 1:10 (10%) in heparinized rabbit plasma. All working solutions were freshly prepared on the day of the experiment in sterile pyrogen-free 0.9% saline.

Statistical analysis

Results are presented as mean values ± s.e.mean for the number of rabbits indicated; one datum unit being the mean of six replicates in each rabbit. Statistical significance of results was evaluated by 2 way analysis of variance. A P value <0.05 was considered statistically significant. Calculations were performed using data after subtraction of the background count for each animal (i.e. i.d. saline).

Results

Effect of i.v. dexamethasone on ^{111}In-labelled neutrophil accumulation and oedema formation in rabbit skin

Figure 1 shows that over a 30 min period bradykinin (10^{-10} mol per site) and FMLP (5×10^{-11} mol per site) injected alone induced a significant but small increase in plasma leakage when compared with control saline injected sites. As previously documented, coinjection of the vasodilator substance PGE_2 (3×10^{-10} mol per site) resulted in a

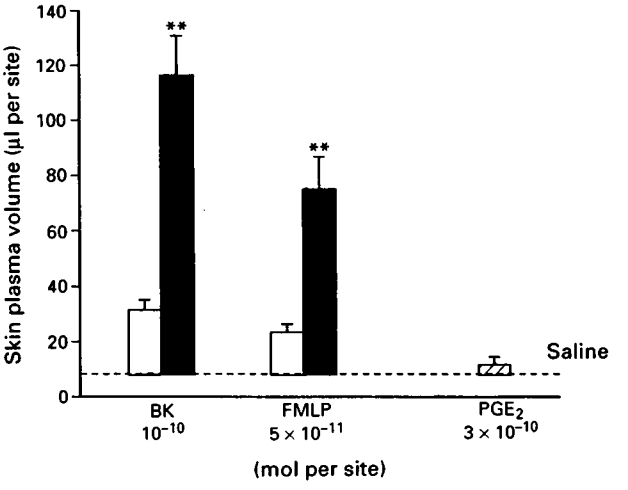

Figure 1 Oedema formation in rabbit skin induced by i.d. injection of bradykinin (BK, 10^{-10} mol per site) and N-formyl-methionyl-leucyl-phenylalanine (FMLP, 5×10^{-11} mol per site) measured over 30 min, showing synergism with prostaglandin E_2 (PGE_2). Open columns represent responses in the absence, and solid columns represent responses in the presence of a vasodilator dose of prostaglandin E_2 (3×10^{-10} mol per site). The hatched column represents the response to PGE_2 alone. The dashed line represents the response to i.d. saline. Each column represents the mean ± s.e.mean from $n = 6$ rabbits. Significant difference between the responses in the presence and absence of PGE_2: $^{**}P<0.01$.

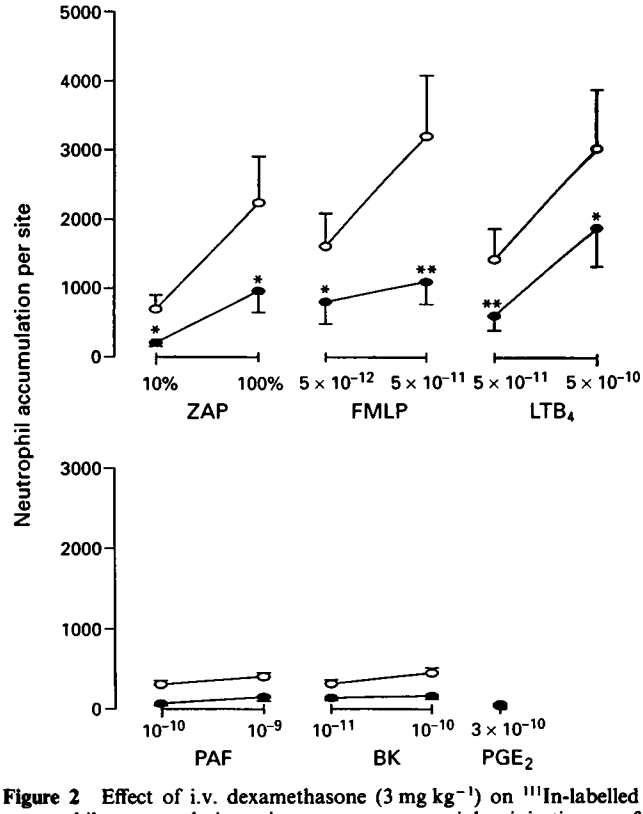

Figure 2 Effect of i.v. dexamethasone (3 mg kg^{-1}) on ^{111}In-labelled neutrophil accumulation in response to i.d. injections of inflammatory mediators. Concentrations are given as mol per site for N-formyl-methionyl-leucyl-phenylalanine (FMLP), leukotriene B_4 (LTB_4), platelet activating factor (PAF) and bradykinin (BK). Zymosan activated plasma (ZAP) was used undiluted (100%) or as a 1:10 dilution in heparinized rabbit plasma (10%). All stimuli were coinjected with a fixed potentiating dose of prostaglandin E_2 (PGE_2, 3×10^{-10} mol per site). ^{111}In-neutrophil accumulation was measured in recipient animals given i.v. dexamethasone (●) or saline (○) 4 h prior to i.v. ^{111}In-neutrophils and i.d. mediators. Responses are corrected for the low levels in saline-injected sites. Each point represents the mean ± s.e.mean for $n = 9$ pairs of rabbits. Significant difference from control: $^{*}P<0.05$, and $^{**}P<0.01$.

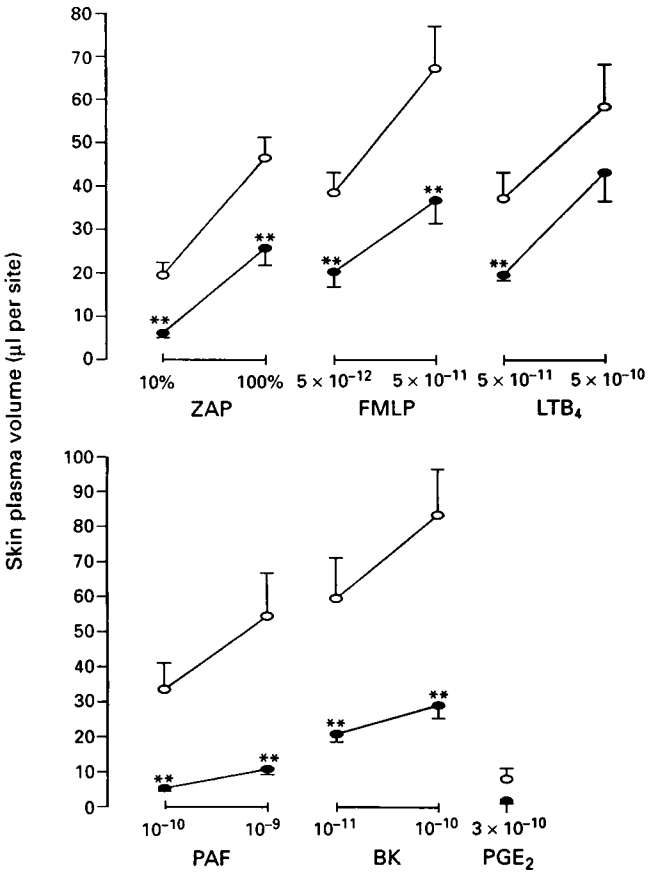

Figure 3 Effect of i.v. dexamethasone (3 mg kg^{-1}) on plasma protein extravasation in response to inflammatory mediators: oedema responses measured in the same recipient animals as in Figure 2. Concentrations are given as mol per site for N-formyl-methionyl-leucyl-phenylalanine (FMLP), leukotriene B_4 (LTB_4), PAF and bradykinin (BK). Zymosan activated plasma (ZAP) was used undiluted (100%) or as a 1:10 dilution in heparanized rabbit plasma (10%). Oedema formation was measured in dexamethasone pretreated recipient animals (●) and recipient animals pretreated with an equal volume of saline (○). Responses are corrected for the low levels in saline-injected sites. Each point represents the mean ± s.e.mean for $n = 9$ pairs of rabbits. Significant difference from control: $^{**}P < 0.01$.

large potentiation of oedema responses when compared with those induced by the same doses of bradykinin and FMLP (Figure 1) (Williams & Morley, 1973; Williams & Peck, 1977; Wedmore & Williams, 1981). PGE_2 alone (3×10^{-10} mol per site) did not induce a significant oedema response (Figure 1). Vasodilator prostaglandins have also been documented to potentiate ^{111}In-neutrophil accumulation in response to i.d. chemoattractants in rabbit skin (Movat *et al.*, 1984). Given that the glucocorticosteroids are known to inhibit the generation of mediators including prostaglandins (reviewed in Flower, 1988), all experiments were designed so that mediators were coinjected with a maximally potentiating dose of the vasodilator, PGE_2. This ensured that the effect of any endogenous vasodilator prostaglandin generation in skin sites was overridden; thus eliminating the possibility of a glucocorticosteroid effect being due to inhibition of prostaglandin synthesis.

For the experiments shown in Figures 2 and 3 recipient animals were treated i.v. with either dexamethasone (3 mg kg^{-1}) or saline (1 mg kg^{-1}) 4 h before i.v. administration of ^{111}In-labelled neutrophils, ^{125}I-labelled albumin and i.d. inflammatory mediators mixed with PGE_2. The accumulation of labels was measured over 30 min. Figure 2 shows that in control recipient animals there was a dose-related increase in ^{111}In-neutrophil accumulation in response to the three chemattractants, ZAP + PGE_2, FMLP + PGE_2 and LTB_4 + PGE_2. Systemic pretreatment of recipient animals with dexamethasone caused a significantly reduced accumulation of ^{111}In-neutrophils in response to all doses of chemoattractants tested. This inhibitory effect of dexamethasone was not due to a difference in the number of ^{111}In-neutrophils circulating in the rabbits as there was no statistical difference between the percentage of labelled leucocytes circulating in control and dexamethasone-treated recipient rabbits at the end of the *in vivo* test period (data not shown). The neutrophil-independent mediators PAF and BK did not, as expected, induce a significant accumulation of ^{111}In-neutrophils in either group of recipient animals (Figure 2).

The simultaneous measurement of plasma protein leakage illustrated in Figure 3 shows that oedema responses to both neutrophil-dependent (except 5×10^{-10} mol per site LTB_4) and neutrophil-independent mediators were significantly suppressed in dexamethasone-pretreated recipient animals. Intradermal injection of PGE_2 alone did not induce significant neutrophil accumulation (Figure 2) or oedema formation (Figure 3).

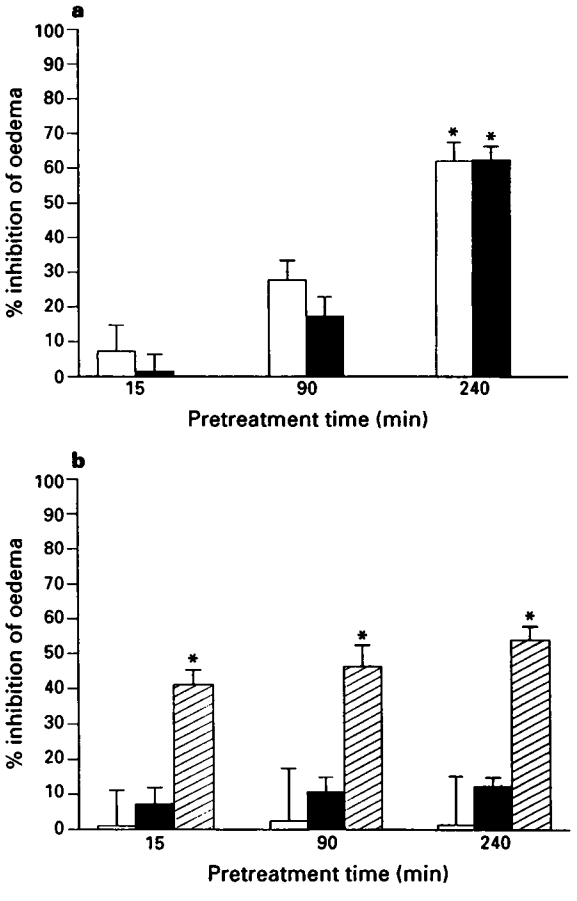

Figure 4 Effect of local i.d. pretreatment with dexamethasone (a) and indomethacin (b) on oedema formation induced by i.d. bradykinin (BK) + prostaglandin E_2 (PGE_2) (open columns), zymosan activated plasma (ZAP) + PGE_2 (solid columns) and BK + arachidonic acid (AA, hatched columns in b). Skin sites were pretreated locally with either dexamethasone (2×10^{-10} mol per site), indomethacin (10^{-8} mol per site) or saline for the times indicated prior to i.v. ^{125}I-albumin and ^{111}In-neutrophils. Pretreated sites were then reinjected with mixtures of mediators under test and the response allowed to proceed for 30 min as described in Methods. Concentrations of mediators used were as follows; BK (10^{-10} mol per site), ZAP (100%), PGE_2 (3×10^{-10} mol per site), AA (3×10^{-9} mol per site). For each time point, responses in drug pretreated sites were calculated as a percentage of those obtained in saline pretreated sites in the same rabbit. Calculations were performed using data after subtraction of the relevant background response for each animal. Responses are mean ± s.e.mean for $n = 4$–7 rabbits. Significant inhibition, $^{*}P < 0.05$.

Effect of local dexamethasone on oedema formation and ^{111}In-neutrophil accumulation in rabbit skin

As shown in Figure 4a, local pretreatment of skin sites with dexamethasone (2×10^{-10} mol per site) caused a time-dependent inhibition of oedema formation induced by both bradykinin (a direct-acting mediator of increased microvascular permeability) and ZAP used as a source of C5a des Arg (a neutrophil-dependent mediator) when coinjected with PGE_2: i.e. after 240 min dexamethasone pretreatment the inhibition was $62.0 \pm 5.4\%$ and $62.4 \pm 3.8\%$ for BK + PGE_2 and ZAP + PGE_2 respectively. This inhibition was considerably greater than that observed with 15 min and 90 min pretreatments which did not achieve significance. In contrast, pretreatment of skin sites with the cyclo-oxygenase inhibitor, indomethacin (10^{-8} mol per site) had no significant effect on oedema responses to bradykinin or ZAP when coinjected with PGE_2 (Figure 4b). However, indomethacin pretreatment did inhibit the potentiation of oedema produced by coinjection of BK with the prostaglandin precursor arachidonic acid and was thus evidently blocking cyclo-oxygenase at all pretreatment times tested (Figure 4b). The effect of local indomethacin was remarkably persistent; inhibition was not significantly different for the 15 min and 240 min pretreatment times. Like its metabolite PGE_2, arachidonic acid was a poor inducer of plasma protein leakage when injected alone (data not shown).

In some animals ^{111}In-neutrophil accumulation was simultaneously monitored. In contrast to the effect on neutrophil-dependent oedema formation, local dexamethasone pretreatment did not significantly inhibit ^{111}In-neutrophil accumulation induced by ZAP + PGE_2, even after 240 min pretreatment. Similarly, indomethacin had no significant effect on cell accumulation at any pretreatment time. Responses to ZAP + PGE_2 expressed as number of ^{111}In-neutrophils per site were as follows: in sites pretreated for 15 min with saline 1799 ± 359, with dexamethasone 1724 ± 336, with indomethacin 2116 ± 488; in skin sites pretreated for 240 min with saline 1804 ± 384, with dexamethasone 1451 ± 363, with indomethacin 2258 ± 372 (results are mean ± s.e.mean for $n = 4$ rabbits; all data subtracted for saline background levels).

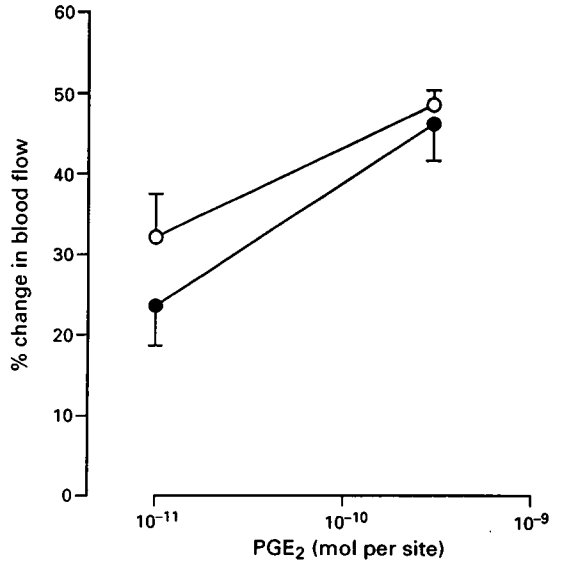

Figure 5 Effect of local pretreatment with dexamethasone (2×10^{-10} mol per site for 4 h) on blood flow induced by prostaglandin E_2 (PGE_2) in rabbit skin. Sites were pretreated locally with dexamethasone (2×10^{-10} mol per site, ●) or saline (○) for 4 h prior to reinjection with doses of PGE_2 shown (mol per site) mixed with ^{133}Xe. The washout of ^{133}Xe was measured over 15 min. The increase in blood flow was calculated as a percentage of saline-injected sites. Results are expressed as the mean of $n = 5$ experiments ± s.e.mean.

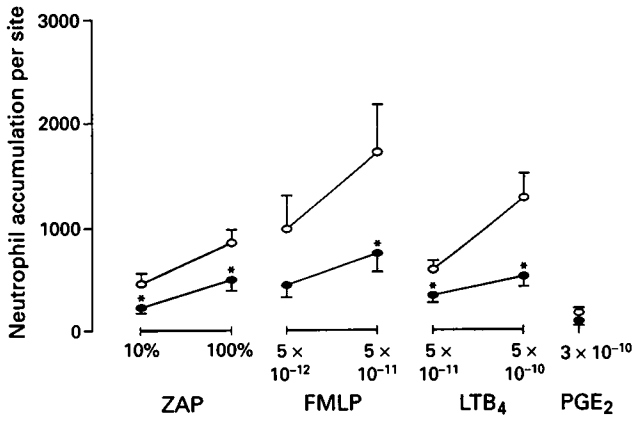

Figure 6 Accumulation of neutrophils isolated from saline (○) or dexamethasone (3 mg kg^{-1}, ●)-pretreated donor rabbits in untreated recipient animals. ^{111}In-neutrophil accumulation in recipient rabbits was induced by i.d. injections of doses of stimuli shown as mol per site for N-formyl-methionyl-leucyl-phenylalanine (FMLP) and leukotriene B_4 (LTB_4) and 100% and 10% zymosan activated plasma (ZAP), all mixed with a fixed potentiating dose of prostaglandin E_2 (PGE_2) (3×10^{-10} mol per site). Responses are corrected for the low levels in saline injected sites. Each point represents the mean ± s.e.mean for $n = 9$ pairs of rabbits. Significant difference from control, $^*P < 0.05$.

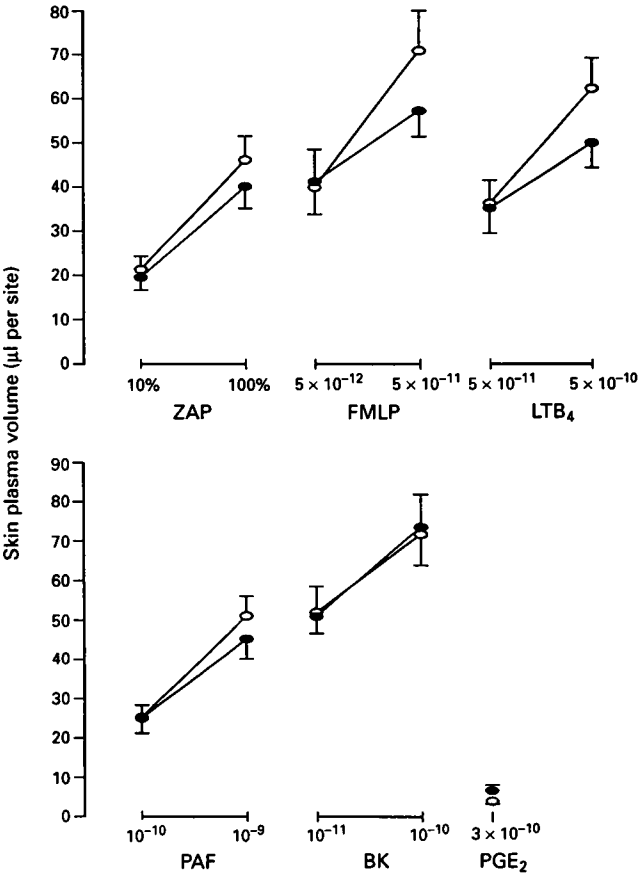

Figure 7 Oedema formation in untreated rabbits receiving labelled-neutrophils isolated from saline (○) or dexamethasone (3 mg kg^{-1}, ●)-pretreated donor rabbits. Oedema responses were measured in the same recipient animals as in Figure 6. Responses are corrected for the low levels in saline-injected sites. Each point represents the mean ± s.e.mean for $n = 9$ pairs of rabbits.

Effect of local dexamethasone on blood flow in rabbit skin

Figure 5 shows the results of experiments designed to determine whether the effects of locally administered dexamethasone on oedema formation (Figure 4) was due to local vasoconstriction. In these experiments we investigated the effect of 4 h pretreatment with dexamethasone (2×10^{-10} mol per site) or saline on vasodilatation induced by PGE_2. Dexamethasone had no significant effect on the dose-related increase in blood flow induced by prostaglandin E_2 (Figure 5). Further, dexamethasone had no significant effect on blood flow in saline-injected sites.

Effect of pretreating neutrophil donor rabbits with dexamethasone

The experiments shown in Figures 6 and 7 were designed to investigate a possible effect of steroid, or a steroid-induced product on the neutrophil *in vivo*. Figure 6 shows that ^{111}In-neutrophils from control, saline-treated donor animals accumulated in recipients in response to the three neutrophil-dependent mediators ZAP, FMLP and LTB_4 when co-injected with PGE_2. Treatment of neutrophil donor animals with dexamethasone significantly inhibited the accumulation of ^{111}In-neutrophils induced by all (except 5×10^{-12} mol per site FMLP) doses of chemoattractants in recipients. At the end of the 30 min accumulation period the percentage of circulating ^{111}In-neutrophils did not differ significantly between the two groups and therefore this did not account for the differences in cell accumulation observed.

Figure 7 shows oedema responses measured in the same recipient animals as in Figure 6. There was no significant difference in responses to direct-acting or neutrophil-dependent mediators between the two groups of recipient rabbits. This is presumably because the ^{111}In-labelled neutrophils account for approximately 3% of the total circulating neutrophils in recipients and therefore do not make a major functional contribution to oedema formation.

Discussion

Glucocorticosteroids are potent anti-inflammatory drugs capable of inhibiting oedema formation and neutrophil accumulation *in vivo*. The precise mechanism by which steroids induce these inhibitory activities is unknown although the possible sites of action are (1) the generation of inflammatory mediators, (2) the microvascular endothelial cell or (3) the neutrophil. In the present study, we have investigated the site of action of dexamethasone in inhibiting mediator-induced oedema formation and neutrophil accumulation in rabbit skin *in vivo*. The results suggest glucocorticosteroids can suppress oedema formation by an endothelial-associated effect and neutrophil accumulation by an effect on the neutrophil itself as well as a possible effect on the endothelium.

Increased microvascular permeability can be induced in a specific and complex manner by a host of inflammatory mediators. These mediators can be divided into two broad categories, depending on whether their effects are mediated by neutrophils (Wedmore & Williams, 1981). In this study we have shown that systemically administered dexamethasone inhibited neutrophil-dependent and independent oedema formation induced by exogenously administered inflammatory mediators. These results are in agreement with previous reports (Foster & McCormick, 1985; Griffiths & Blackham, 1988; Peers & Flower, 1991). In addition, we have shown that dexamethasone when administered i.v. inhibited neutrophil accumulation induced by C5a des Arg, FMLP and LTB_4. These findings clearly demonstrate the anti-inflammatory properties of dexamethasone but fail to indicate its mechanism of action. In this regard, it has been shown that the anti-inflammatory effects of glucocorticosteroids are triggered by a mechanism requiring receptors and synthesis of new RNA and protein (Tsurufuji *et al.*, 1979; Peers & Flower, 1991). To investigate further its site of action we studied the effect of locally administered dexamethasone on inflammatory responses and investigated the *in vivo* responsiveness of neutrophils isolated from dexamethasone-treated donor animals.

Locally administered dexamethasone inhibited oedema formation induced by neutrophil-dependent and direct-acting inflammatory mediators whilst having no effect on neutrophil accumulation. These results indicate that dexamethasone when applied locally can inhibit oedema formation by acting on endothelial cells whereas it has to be administered systemically in order to affect neutrophils. Studies where locally applied dexamethasone inhibited leucocyte accumulation most probably reflect an inhibition of mediator generation (Tsurufuji *et al.*, 1984). In our studies the use of preformed chemoattractants eliminates the occurrence of such a mechanism of action. The inhibitory effect of dexamethasone on oedema formation was time-dependent; inhibition was seen with a 4 h pretreatment but no significant effect was seen at 15 or 90 min. Previous studies have shown that the effect of glucocorticosteroids on oedema formation is protein synthesis-dependent suggesting that dexamethasone can induce the synthesis of a protein that inhibits microvascular responses by an action on the endothelium (Tsurufuji *et al.*, 1979; Peers & Flower, 1991). The identity of this inhibitory protein is yet to be determined. Lipocortin 1 is thought not to be involved as, although it has anti-inflammatory properties in some *in vivo* models (Cirino *et al.*, 1989; Errasfa & Russo-Marie, 1989; Perretti & Flower, 1992), it is not effective against preformed mediators in oedema assays and evidence that an inhibitory protein exists which is distinct from lipocortin has been obtained in rats (Di Rosa *et al.*, 1985). The anti-inflammatory protein activity has been detected in peritoneal lavage fluid from glucocorticosteroid-treated rats (Di Rosa *et al.*, 1985). This activity, which inhibited dextran-induced oedema in the rat paw ('vasocortin'), by chromatography eluted at approximately 100 kD whilst the phospholipase-inhibitory activity ('lipocortin') appeared at 40 kD (Carnuccio *et al.*, 1987). The inhibitory effect of local dexamethasone on oedema formation may be mediated by this or other newly synthetized proteins. However, the precise mechanisms underlying the effect of glucocorticosteroid-induced proteins on the endothelium remain to be determined.

Topically applied glucocorticosteroids have vasoconstrictor properties which may account for some of their anti-inflammatory actions (Reid & Brookes, 1968; Kaidbey & Kligman, 1974; Marks & Sawyer, 1986). It has been suggested that these effects may be ascribed to inhibition of the induction of nitric oxide (NO) synthase (Radomski *et al.*, 1990). Thus, the anti-oedema actions of glucocorticosteroids could be due to an inhibition of vasodilatation induced by stimulated NO release. Although the mechanism of action of glucocorticosteroids under certain inflammatory conditions may be partly mediated by an interference with NO production, such a mechanism appears an unlikely explanation for the results obtained in the present study. In our experiments, a short *in vivo* period was used for measurement during which dexamethasone did not alter increased blood flow induced by PGE_2. The induction of NO synthase in endotoxin- and cytokine-activated cultured endothelial cells is a slow and protein-synthesis dependent process with a lag period of 2 h (Radomski *et al.*, 1990).

Since systemically administered, but not locally administered, dexamethasone inhibited neutrophil accumulation, experiments were carried out to investigate the effect of dexamethasone on the neutrophil itself. For these studies we examined the *in vivo* accumulation of neutrophils isolated from steroid-treated donor rabbits. The results demonstrated that pretreatment of the neutrophil donor with dex-

amethasone resulted in an inhibition of ^{111}In-neutrophil accumulation in untreated recipient animals. These findings suggest that dexamethasone, or dexamethasone-induced products, can suppress neutrophil accumulation *in vivo* by an effect on the neutrophil itself, in addition to a possible effect on the endothelium. Adherence of neutrophils to vascular endothelium is the initial response during the extravascular accumulation of neutrophils at sites of inflammation (for review see: Nourshargh & Williams, 1990) and may be a target response for dexamethasone. In man, glucocorticosteroids induce an increase in circulating neutrophils that may be accounted for by reduced neutrophil adherence (Athens *et al.*, 1961), and there are numerous reports on the inhibitory effects of these drugs on other neutrophil responses *in vitro* (Ward, 1966; Majeski & Alexander, 1976; Weissmann *et al.*, 1976) although many of these effects may be related to the high doses employed. Other studies have, however, failed to detect such inhibitory effects. Neutrophils cultured with dexamethasone for 24 h responded normally with respect to chemotaxis and adherence to cultured endothelial cells (Schleimer *et al.*, 1989). More recently dexamethasone has been shown to act on neutrophils to inhibit their adhesion to thrombin and histamine activated endothelial cells (Watanabe *et al.*, 1991) whilst having no effect on neutrophil adhesion to endotoxin-stimulated endothelial cells in the presence or absence of FMLP (Forsyth & Talbot, 1992). In the latter study, dexamethasone was also without an effect on increased expression of the adhesion molecules CD11b/CD18 on the neutrophil and E-selectin and ICAM-1 on endothelial cells. Since thrombin and histamine have been shown to upregulate the expression of the adhesion molecule P-selectin on cultured endothelial cells (McEver *et al.*, 1989; Lorant *et al.*,1991), the study of Watanabe and colleagues (1991) suggests dexamethasone may modulate the expression of the ligand for endothelial P-selectin on the neutrophil. Such an effect has yet to be investigated. Although adhesion of neutrophils to venular endothelial cells is a prerequisite for neutrophil emigration, the effect of glucocorticosteroids on neutrophil adhesion, which *in vivo* may be mediated by a steroid-induced plasma factor (MacGregor, 1976), is contentious. It is possible that dexamethasone suppresses neutrophil accumulation by inhibiting the neutrophil response at another step such as the passage of the neutrophil across the perivascular basement membrane as has been reported (Katori *et al.*, 1990). Although the precise mechanism by which glucocorticosteroids inhibit neutrophil accumulation is still unknown our results clearly demonstrate that dexamethasone or a dexamethasone-induced factor exert an inhibitory effect on circulating neutrophils.

Glucocorticosteroids can influence the development of an inflammatory reaction by inhibiting the generation of inflammatory mediators and activation of pro-inflammatory cells. Since these are amongst the most widely used pharmacological agents in the treatment of both acute and chronic inflammatory conditions, a better understanding of their mechanism of action could lead to the development of novel anti-inflammatory agents lacking the deleterious side effects of the parent compounds. In this study, we have demonstrated that dexamethasone can suppress neutrophil accumulation *in vivo* by an effect on the neutrophil itself, in addition to a possible effect on the endothelium which also modulates oedema formation.

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