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Priming and induction of eosinophil trafficking in guinea-pig cutaneous inflammation by tumour necrosis factor α

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1 Tissue eosinophilia is a hallmark of allergic and parasitic diseases. Priming mechanisms may play an important role in mediating the process of eosinophil accumulation in these conditions. We have previously shown that blockade of tumour necrosis factor α (TNFα) inhibited the capacity of lipopolysaccharide to prime skin sites for chemoattractant-induced eosinophil recruitment. The present study was carried out to investigate the capacity of TNFα to prime an inflammatory site for enhanced eosinophil accumulation.

2 Initial experiments investigated the capacity of TNFα itself to induce eosinophil accumulation. Intradermal injection of murine TNFα (10–300 ng per site) in the guinea-pig induced significant accumulation of ^{111}In-eosinophils. Kinetic studies showed the response to be delayed in onset and inhibited by cycloheximide, consistent with a dependency on protein synthesis. Trafficking of ^{111}In-eosinophils to sites treated for 2 h with TNFα (10–100 ng per site) was inhibited by monoclonal antibodies (mAbs) against β_2 or α_4 integrins.

3 Intradermal injection of a low dose (3 ng) of TNFα (which by itself had no significant effect on eosinophil trafficking) prior to chemoattractants or antigen in sensitized skin sites, induced significant priming of eosinophil accumulation. Recruitment of both ^{111}In-eosinophils and endogenous eosinophils was enhanced. Trafficking to TNFα-primed responses was dependent on protein synthesis and β_2 integrins. In contrast, the α_4 integrin mAb failed to inhibit the TNFα primed response.

4 Thus, TNFα can induce and also prime eosinophil recruitment in guinea-pig skin. Our results provide further evidence that this cytokine may be an important mediator of allergic- or parasite-induced eosinophilic inflammation.

Keywords: Eosinophils; TNFα; inflammation; priming; adhesion molecules

Introduction

Tissue eosinophilia is a characteristic feature of allergic diseases and a number of parasitic infections (Butterfield & Leiferman, 1993). During the latter, eosinophils accumulate around the migrating larvae onto which they release toxic oxygen radicals and basic proteins, such as major basic protein; these eosinophil-derived products are thought to damage the parasite and prevent further infection (McLaren, 1980). However, in allergic diseases, the inappropriate secretion of the same products onto normal lung leads to tissue damage (Weller, 1997). For example, in asthma, eosinophil-induced damage to airway epithelial cells is considered to play an important role in the pathophysiology of the disease (Djukanovic *et al*., 1990) and hence there is considerable interest in the development of therapies which modulate the recruitment and/or activation of eosinophils in allergic diseases (Teixeira *et al*., 1995). A better understanding of the mechanisms governing eosinophil recruitment into sites of inflammation *in vivo* is essential if such therapies are to be developed.

The i.d. injection of chemoattractants (e.g. PAF, LTB_4) or antigen into sensitized skin sites in the guinea-pig induces a dose-dependent recruitment of ^{111}In-labelled eosinophils (Faccioli *et al*., 1991; Teixeira & Hellewell, 1994). In this model, ^{111}In-eosinophil recruitment is dependent on the β_2 integrins CD11/CD18, as assessed using the monoclonal antibody (mAb) 6.5E (Teixeira *et al*., 1994), and on selectins, as assessed using the polysaccharide fucoidan (Teixeira & Hellewell, 1997). Recently, we have shown that a low dose of lipopolysaccharide (LPS), which had no effect on eosinophil recruitment alone, significantly primed skin sites for further eosinophil recruitment in response to various chemoattractants and in a passive cutaneous allergic (PCA) reaction (Macari *et al*., 1996). The priming effects of LPS appeared to be mediated by tumour necrosis factor α (TNFα), since a soluble p55 TNFα receptor-IgG construct inhibited the primed responses (Macari *et al*., 1996). This was an important finding, inasmuch as the ability of cytokines to prime cellular responses may underlie an important mechanism regulating leukocyte, in particular eosinophil function *in vivo* (Hallett & Lloyds, 1995; Koenderman *et al*., 1996). Increased concentrations of TNFα have been measured in bronchoalveolar lavage fluid recovered from asthmatics (Broide *et al*., 1992; Cembrzynska-Nowak *et al*, 1993) suggesting this cytokine may contribute to airways inflammation. In the rat, i.d. injection of human TNFα induced the accumulation of radiolabelled eosinophils by a mechanism that was independent of PAF and IL-8 but dependent, in part, on α_4 integrins and VCAM-1 (Sanz *et al*., 1997). Moreover, inhalation of guinea-pig TNFα induced the recruitment of eosinophils to the airways of guinea-pig lungs (White *et al*., 1997) and endogenous TNFα has been implicated as a mediator of experimental allergic airways inflammation (Lukacs *et al*, 1995; Renzetti *et al*., 1996; Zuany-Amorim *et al*, 1995).

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Possible effects of TNFα on priming an inflammatory site for enhanced eosinophil accumulation have not been investigated. Therefore, the present study was carried out to investigate whether i.d. injection of low doses of TNFα would prime skin sites for the recruitment of eosinophils induced by chemoattractants and in an allergic inflammatory response. Initial studies were performed to assess the capacity of a range of doses of TNFα to induce eosinophil recruitment to determine an appropriate dose to use in the priming experiments. We also assessed the role of protein synthesis, the β_2 integrin CD18 and the α_4 integrin VLA-4 in mediating the trafficking of eosinophils to skin sites treated with TNFα.

Methods

Induction, purification and radiolabelling of guinea-pig eosinophils

The method is described in detail elsewhere (Faccioli *et al*., 1991). Briefly, female guinea-pigs (Harlan; 500–800 g) were treated with neat horse serum (1 ml i.p.) every other day for 2 weeks and the cells collected by peritoneal lavage with heparinized saline (10 iu/ml) 2 days after the last injection. The cells obtained were layered onto a discontinuous Percoll-Hanks' balanced salt solution (HBSS; calcium- and magnesium-free) gradient followed by centrifugation ($1500 \times g$, 25 min at 20°C). Eosinophils (>95% pure) were collected from the 1.090/1.095 and 1.095/1.100 g ml^{-1} density interfaces. The purified eosinophils were radiolabelled by incubation with $^{111}InCl_3$ (100 μCi in 10 μl) chelated to 2-mercaptopyridine-N-oxide (merc, 40 μg in 0.1 ml of 50 mM PBS, pH 7.4) for 15 min at room temperature. The cells were then washed twice in HBSS (calcium- and magnesium-free) containing 10% guinea-pig platelet-poor plasma and resuspended at a final concentration of 10^7 cells ml^{-1} prior to injection.

Preparation of passive cutaneous anaphylaxis sera and reactions

Details of the preparation of sera are described elsewhere (Weg *et al*., 1991). Briefly, male guinea-pigs (180–200 g; Harlan, Oxon) were immunized with bovine gamma globulin (BGG) in Freund's Complete Adjuvant followed by a boost in Freund's Incomplete Adjuvant on day 21, and serum collected on day 28. Recipient animals received an i.d. injection of 50 μl of a 1/50 dilution of the anti-BGG serum followed 16–20 h later by the injection of antigen (BGG, 1 μg per site).

Measurement of eosinophil accumulation in guinea-pig skin

^{111}In-labelled eosinophils (2.5×10^6 cells per animal) were injected i.v. into recipient guinea-pigs (Harlan; 350–450 g) sedated with Hypnorm (0.2 ml i.m.). Dorsal skin sites were treated by i.d. injection of TNFα or saline vehicle in 0.1 ml volumes either as a pretreatment before, or immediately after, i.v. injection of radiolabelled eosinophils. Zymosan activated plasma (ZAP), PAF, LTB_4 or antigen were injected i.d. in 0.1 ml volumes immediately after the i.v. injection of labelled cells. In experiments investigating TNFα-induced priming, i.d. injection of inflammatory mediators or antigen were superimposed on TNFα- saline-pretreated sites. Each animal received a duplicate of each treatment following a randomized injection plan and the inflammatory response was assessed after a 1–4 h measurement period, depending on the individual experimental protocol. At this time, blood was obtained by cardiac puncture, for determination of the percentage circulating ^{111}In-eosinophils, and the animals killed by an overdose of sodium pentobarbitone. The dorsal skin was removed, cleaned of excess blood and the skin sites punched out with a 17 mm punch. The samples were counted in an automatic 10-head gamma-counter (Canberra Packard Ltd, Pangbourne, Berks, U.K.). The number of eosinophils accumulating is expressed as ^{111}In-labelled eosinophils per skin site.

Histology

Skin assays were performed as described above with the exception that no i.v. injection of cells was given. Each skin site was bisected and one half from each duplicate fixed immediately for 16 h in 10% neutral buffered formalin and processed to paraffin wax. Three 5 μm thick step sections were taken from each block with 100 μm intervals between each section. Sections were stained with hematoxylin and eosin, and further sections stained with carbol chromotrope (Raymond Lamb, London, U.K.). Eosinophils stained with carbol chromotrope were counted in the hypodermis. The area of the hypodermis counted was calculated as the length (mm) of muscle layer × width of hypodermis (determined as the mean of five equidistant measurements). Eosinophil number was expressed as the mean number of cells per mm^2 of the three sections and represented the sum of the cells both within and outside blood vessels.

Reagents

Murine TNFα and human TNFα were purchased from R&D Systems Europe Ltd (Abingdon, Oxfordshire, U.K.) and Boehringer-Mannheim Ltd (Lewes, Sussex, U.K.), respectively, Zymosan, dimethyl sulphoxide (DMSO) and cyclohexamide were purchased from Sigma Chemical Company (Poole, Dorset, U.K.). Leukotriene B_4 (LTB_4) was purchased from Cascade (Reading, Berkshire, U.K.) and C16 PAF from Bachem Ltd (Saffron Walden, Essex, U.K.). HBSS, HEPES and horse serum were purchased from Life Technologies Limited (Paisley, Scotland). Percoll was from Pharmacia (Milton Keynes, Bucks, U.K.). Sodium pentobarbitone (Sagatal; 60 mg ml^{-1}) was purchased from May and Baker (Dagenham, Essex, U.K.) and Hypnorm (0.315 mg ml^{-1} fentanyl, citrate and 10 mg ml^{-1} fluanisone) was from Janssen Pharmaceuticals Ltd (Grove, Oxford, U.K.). $^{111}InCl_3$ was purchased from Amersham International plc (Amersham, U.K.).

$F(ab')_2$ fragments of anti-β_2 integrin mAb 6.5E (murine IgG_1) (Andrew *et al*., 1993; Teixeira *et al*., 1994) and the murine myeloma protein MOPC-21 (IgG_1) were gifts from Dr M. Robinson (Celltech, Slough, U.K.). The anti-α_4 integrin mAb 2B4 (murine IgG_1) (Needham *et al*., 1994) was a gift from Dr R. Pigott (British Biotechnology, Oxford, U.K.). We have previously demonstrated inhibitory functions of 6.5E and 2B4 on guinea-pig β_2 and α_4 integrins, respectively (Teixeira *et al*., 1994, 1996).

ZAP, used as a source of guinea-pig C5a des Arg, was prepared by incubating guinea-pig heparinized (10 iu ml^{-1}) plasma with zymosan (5 mg ml^{-1}) for 30 min at 37°C followed by removal of zymosan by centrifugation. PAF and LTB_4 were dissolved in 100% ethanol and further diluted in saline.

Statistical analysis

Statistical analysis was carried out using two-way analysis of variance (ANOVA) on normally-distributed data. P values were assigned by either Bonferroni selected comparison or Dunnett multiple comparison *post-hoc* test. Results were presented as the mean ± s.e.mean for the number of animals indicated and were considered significant when $P<0.05$.

Results

Characteristics of TNFα-induced ^{111}In-eosinophil trafficking in guinea-pig skin

The aim of these experiments was to compare the capacity of murine (m) and human (h) TNFα to induce eosinophil trafficking in guinea-pig skin, to determine the kinetics of the response and define a threshold dose of TNFα that would be used in the priming experiments. Figure 1a compares the dose-response relationship of ^{111}In-eosinophil accumulation induced over 4 h after i.d. injection of mTNFα and hTNFα.

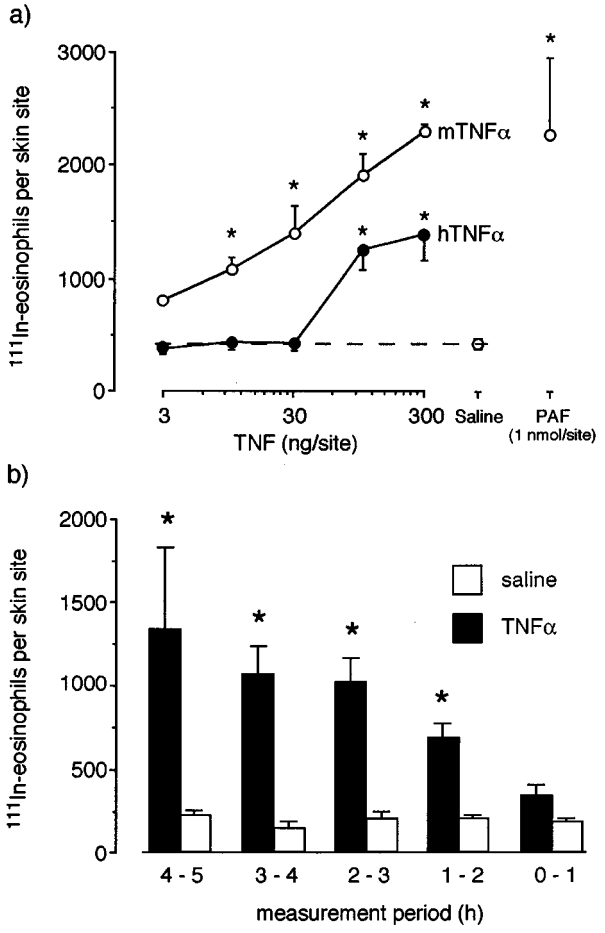

Figure 1 Dose-response relationship and kinetics of TNFα-induced eosinophil accumulation in guinea-pig skin. (a) ^{111}In-eosinophils were injected i.v. followed immediately by i.d. injection of either 3–300 ng per site of either mTNFα or hTNFα, PAF (1 nmol per site) or saline. The ^{111}In-eosinophil accumulation per skin site was assessed 4 h later. (b) mTNFα (100 ng per site) or saline were injected i.d. up to 4 h before i.v. injection of ^{111}In-eosinophils. Accumulation of radiolabelled eosinophils was assessed 1 h later. Results are expressed as means ± s.e.mean of four animals. Statistically significant increases above the saline value are shown.

The response to i.d. injection of PAF (1 nmol per site) is shown for comparison. Significant ^{111}In-eosinophil accumulation above saline background occurred at doses equal to or greater than 10 ng mTNFα/site. In comparison, 100 ng hTNFα per site was required for significant ^{111}In-eosinophil accumulation above saline background. Thus, although both forms of TNFα were effective inducers of ^{111}In-eosinophil accumulation in guinea-pig skin, mTNFα was more potent than hTNFα and subsequent experiments therefore used this form.

The kinetics of ^{111}In-eosinophil accumulation induced by TNFα (100 ng per site) are shown in Figure 1b. Skin sites were injected with TNFα 4, 3, 2 and 1 h before and just after (time 0) an i.v. injection of ^{111}In-eosinophils and cell trafficking was assessed for a period of 1 h. Recruitment of ^{111}In-eosinophils and cell trafficking was assessed for a period of 1 h. Recruitment of ^{111}In-eosinophils was slow in onset with significant trafficking above saline background seen 1–2 h after TNFα pretreatment and reaching a peak 2–3 h later (Figure 1b). Subsequent investigation of TNFα-induced ^{111}In-eosinophil accumulation was based on a 2 h pretreatment time.

Effect of systemic treatment with cycloheximide, and anti-β_2 integrin mAb or an anti-α_4 integrin mAb on TNFα-induced eosinophil recruitment

The delayed onset of action of TNFα to induce ^{111}In-eosinophil accumulation is consistent with induction of protein synthesis and upregulation of molecules that could mediate the recruitment process. To investigate this possibility, TNFα-induced ^{111}In-eosinophil accumulation was tested in the skin of guinea-pigs treated systemically with the protein synthesis inhibitor, cycloheximide. Animals were pretreated with a single subcutaneous injection of cycloheximide (30 mg kg^{-1}) or saline 1 h prior to pretreatment of skin sites with TNFα. Basal ^{111}In-eosinophil recruitment in response to i.d. injection of saline was not significantly different between the two groups of animals (Figure 2a). Similarly, ^{111}In-eosinophil recruitment induced by LTB_4 was unaffected (Figure 2a). In contrast, systemic treatment with cycloheximide effectively inhibited the recruitment of ^{111}In-eosinophils over a 2–3 h measurement period induced by increasing concentrations of TNFα (Figure 2a). Cycloheximide had no significant effect on the number of circulating ^{111}In-eosinophils at 1 h after their i.v. injection (data not shown).

In order to determine the role of β_2 and α_4 integrins in TNFα-induced ^{111}In-eosinophil trafficking, animals were pretreated with an anti-β_2 integrin mAb (6.5E, 2.5 mg kg^{-1}) or an anti-α_4 integrin mAb (2B4, 3.0 mg kg^{-1}). These responses were compared to control animals treated with 3 mg kg^{-1} of the isotype matched murine mAb MOPC-21. Skin sites were treated with TNFα (10–100 ng per site) 2 h prior to i.v. injection of mAbs and ^{111}In-eosinophils. LTB_4 (0.1 nmol per site) and saline were then injected i.d. and responses measured after 1 h. As shown in Figure 2b, pretreatment with 6.5E virtually abolished ^{111}In-eosinophil recruitment induced by TNFα and LTB_4. The anti-α_4 integrin mAb 2B4 inhibited TNFα-induced ^{111}In-eosinophil recruitment by up to 60% while, in contrast, LTB_4-induced ^{111}In-eosinophil accumulation was not significantly altered (Figure 2b). Neither 6.5E or 2B4 had any significant effect on the number of circulating ^{111}In-eosinophils at 1 h (data not shown). These data suggest that the delayed accumulation of ^{111}In-eosinophils in response to TNFα is associated with, in part, upregulation of a ligand for α_4 integrins.

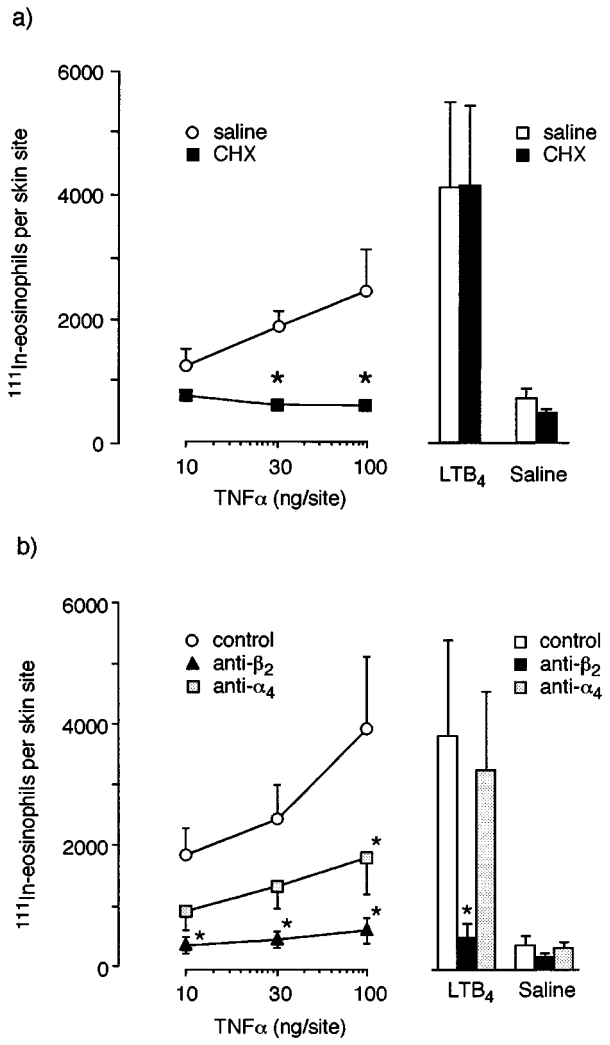

Figure 2 Effect of systemic treatment with cycloheximide and anti-β_2 integrin or anti-α_4 integrin mAbs on TNFα-induced eosinophil recruitment. (a) Animals were pretreated with either cycloheximide (CHX; 30 mg kg^{-1} s.c.) or saline 1 h prior to i.d. injection of TNFα (10–100 ng per site). After 2 h, animals were given an i.v. injection of ^{111}In-eosinophils and i.d. injection of saline or LTB$_4$ (0.1 nmol per site) in naive sites. The ^{111}In-eosinophil accumulation per skin site was assessed 1 h later. (b) Animals were pretreated with i.d. injection of TNFα (10–100 ng per site) 2 h prior to i.v. injection of ^{111}In-eosinophils and further i.d. injection of saline or LTB$_4$ (0.1 nmol per site) in naive sites. Approximately 15 min before the i.v. injection of ^{111}In-eosinophils, animals were treated with an i.v. injection of either control mAb (MOPC-21, 3 mg kg^{-1}), anti-β_2 integrin mAb (6.5E, 2.5 mg kg^{-1}) or anti-α_4 integrin (2B4, 3 mg kg^{-1}). The ^{111}In-eosinophil accumulation per skin site was assessed 1 h after injection of cells. Results are expressed as means±s.e.mean of four to five animals for each treatment group. Statistically significant differences from values in saline or control animals are shown.

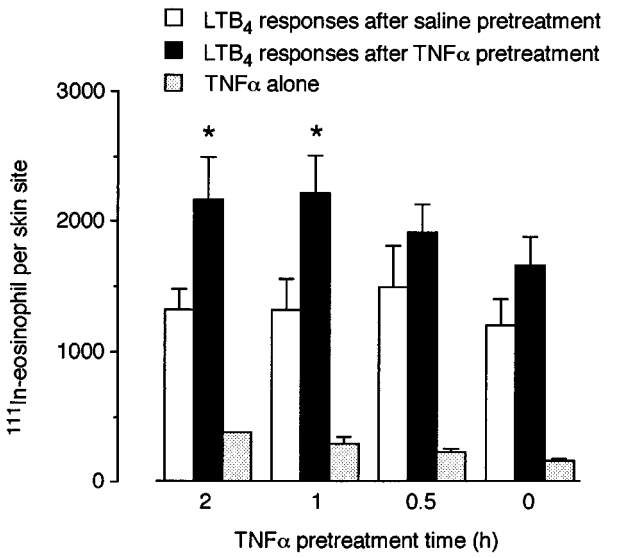

Figure 3 Priming of LTB$_4$-induced eosinophil accumulation by TNFα pretreatment. Skin sites were pretreated with an i.d. injection of either 3 ng TNFα per site or saline 0.5, 1 or 2 h prior to or just after (0 h pretreatment) the i.v. injection of ^{111}In-eosinophils. After the i.v. injection of cells, animals received a further i.d. injection of LTB$_4$ (0.1 nmol per site) in pretreated sites. The ^{111}In-eosinophil accumulation per skin site was assessed 1 h later. The effect of mTNFα pretreatment alone is also shown. Results are expressed as means±s.e.mean of 12 animals. Statistical significance for TNFα-pretreatment-induced increase from saline pretreatment at each time point is shown.

Priming effect of TNFα

Having established the characteristics of TNFα-induced ^{111}In-eosinophil accumulation, we assessed whether pretreatment of skin sites with TNFα could prime for the ^{111}In-eosinophil accumulation induced by other mediators.

Skin sites were pretreated with a low dose of TNFα at 2, 1 and 0.5 h before i.v. injection of ^{111}In-eosinophils and further i.d. injection of LTB$_4$ (0.1 nmol per site) superimposed on the pretreated sites. The dose of 3 ng TNFα per site was chosen as the pretreatment dose since this proved to be close to the threshold for ^{111}In-eosinophil accumulation (Figure 1a). As shown in Figure 3, significant enhancement of LTB$_4$-induced ^{111}In-eosinophil accumulation (approximately 2 fold) occurred with a 1 h pretreatment. This enhancement remained significant after a 2 h pretreatment time (Figure 3) and even after 4 h (data not shown). In contrast, there was no significant enhanced ^{111}In-eosinophil recruitment with co-injection or after a 30 min pretreatment with TNFα (Figure 3). The ^{111}In-eosinophil accumulation induced by TNFα alone at each pretreatment time was negligible compared to the enhancement of LTB$_4$-induced responses (Figure 3). This would suggest TNFα priming of LTB$_4$-induced ^{111}In-eosinophil accumulation to have a similar onset time to LPS-priming (Macari *et al*., 1996), but different in that TNFα-priming is a more protracted phenomenon, since significant LPS-priming was not observed after a 2 h pretreatment.

As observed with priming by LPS, TNFα-priming was not exclusive to LTB$_4$-induced ^{111}In-eosinophil recruitment. As shown in Figure 4, the ^{111}In-eosinophil accumulation induced by PAF, ZAP and the PCA reaction was enhanced 3-, 1.5- and 2 fold, respectively, after a 1 h pretreatment of skin sites with TNFα (3 ng per site).

TNFα priming of endogenous eosinophil accumulation

Next, we investigated whether TNFα would also prime for the recruitment of endogenous eosinophils, as shown to occur with eosinophils derived from the peritoneal cavity. Histological sections of skin sites were obtained and the mean number of carbol chromotrope-stained eosinophils in the hypodermis determined (as described in Methods). As shown in Figure 5, 1 h pretreatment with 3 ng TNFα did not induce accumulation of endogenous eosinophils to the hypodermis greater than that of the 1 h saline pretreatment. In contrast, a significant 3 fold

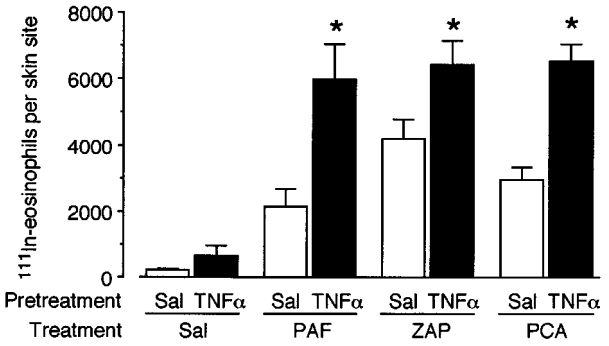

Figure 4 Priming by TNFα of eosinophil recruitment induced by PAF, ZAP and in a PCA reaction. Skin sites were pretreated with an i.d. injection of either 3 ng mTNFα per site or saline 1 h prior to i.v. injection of ^{111}In-eosinophils. PAF (1 nmol per site), ZAP (30% in saline), antigen (1 μg BGG in antiserum treated sites) in the PCA reaction or saline were then injected i.d. in the pretreated sites. The ^{111}In-eosinophil accumulation per skin site was assessed 1 h later. Results are expressed as means ± s.e.mean of four animals. Statistically significant difference between responses in mTNFα-pretreated compared to saline-pretreated sites is shown.

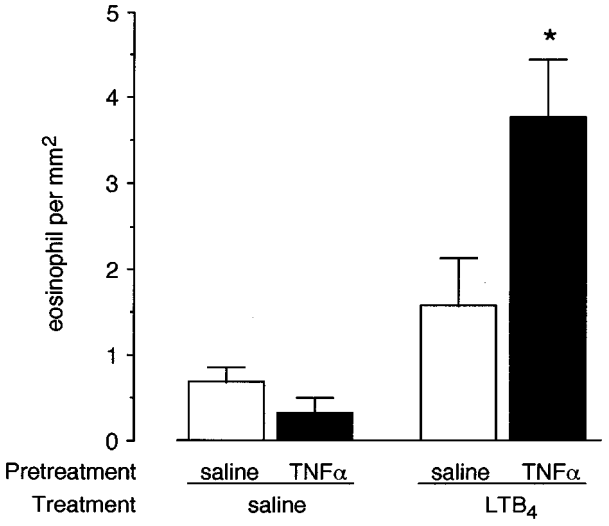

Figure 5 Priming by TNFα or LTB_4-induced accumulation of endogenous eosinophils, as assessed by histological staining. Skin sites were pretreated with i.d. injection of TNFα (3 ng per site) or saline 1 h prior to further i.d. injection of LTB_4 (0.1 nmol per site) or saline in pretreated sites. One hour later, skin sites were punched out and prepared for histological analysis (see Methods). Carbol chromotrope-stained eosinophils within the hypodermis were counted in three sections per skin site, expressed as eosinophils per mm^2 and the mean taken. Results are expressed as the means ± s.e.mean of three animals. Statistically significant difference between responses after mTNFα pretreatment compared to pretreatment with saline is shown.

enhancement of LTB_4-induced endogenous eosinophil accumulation occurred in TNFα-pretreated sites compared to saline pretreated (Figure 5). The majority of cells counted in each section were in the interstitium and not within blood vessels (not shown). Thus, TNFα priming of eosinophil accumulation also occurs with endogenous eosinophils.

Effect of systemic treatment with cycloheximide on TNFα priming of eosinophil accumulation

Figure 6 shows the effect of cycloheximide (30 mg kg^{-1}, s.c.) on the ability of TNFα to prime skin sites for enhanced

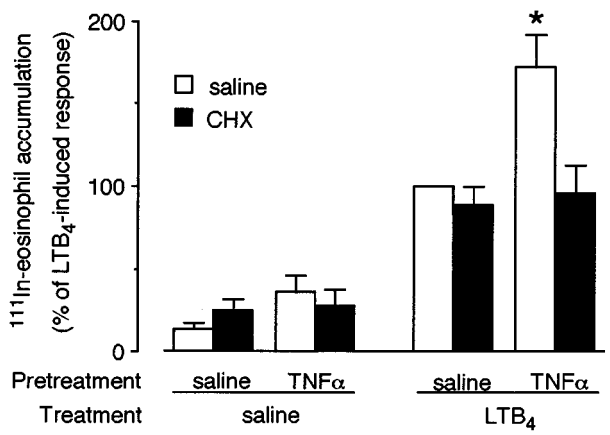

Figure 6 Effect of systemic treatment with cycloheximide on TNFα priming of LTB_4-induced eosinophil accumulation. Animals were pretreated with either cycloheximide (CHX, 30 mg kg^{-1} s.c.) or saline 1 h prior to i.d. injection of either TNFα (3 ng per site) or saline. This was followed 1 h later by the i.v. injection of ^{111}In-eosinophils and the further i.d. injection of saline or LTB_4 (0.1 nmol per site) in pretreated sites. The ^{111}In-eosinophil accumulation per skin site was assessed 1 h later. Results are expressed as a percentage of the LTB_4-induced response in each animal; values shown are means ± s.e.mean of four animals. Statistically significant enhancement of LTB_4-induced responses by mTNFα pretreatment is shown.

eosinophil recruitment. Cycloheximide pretreatment had no effect on the eosinophil accumulation induced by LTB_4 alone. However, in contrast to control animals, no enhancement of the LTB_4-induced response was seen after 1 h TNFα pretreatment in animals pretreated systemically with cycloheximide (Figure 6). These results suggest the priming by TNFα pretreatment to be protein synthesis-dependent.

Effect of anti-β_2 integrin or anti-α integrin mAbs on eosinophil accumulation into TNFα primed skin sites

Figure 7 shows the effect of i.v. treatment of animals with anti-β_2 integrin 6.5E (2.5 mg kg^{-1}) and anti-α_4 integrin 2B4 (3 mg kg^{-1}) on the LTB_4-induced accumulation of ^{111}In-eosinophils in TNFα-primed sites. Both the unprimed and TNFα-primed eosinophil accumulation responses were totally inhibited by 6.5E (Figure 7a). In contrast, and as previously observed (Figure 2b), pretreatment with 2B4 had no significant effect on the recruitment of ^{111}In-eosinophils induced by LTB_4 (Figure 7b). Moreover, treatment of animals with 2B4 did not have any significant effect on the ability of ^{111}In-eosinophils to traffic to TNFα-primed sites injected with LTD_4 (Figure 7b).

Discussion

We have recently shown that the local administration of a low dose of LPS primed skin sites for the recruitment of eosinophils (Macari *et al*., 1996). The LPS-priming was inhibited by a TNFR (p55)-IgG fusion protein, suggesting an important role for TNFα in mediating this phenomenon (Macari *et al*., 1996). Recently, there has been considerable interest in the involvement of TNFα in inflammatory conditions, such as allergic diseases and parasitic infections, where eosinophils are thought to play a major pathophysiological role. For example, the levels of TNFα are elevated in tissue and serum of patients with allergic asthma and inhibition/blockade of TNFα is associated with suppression

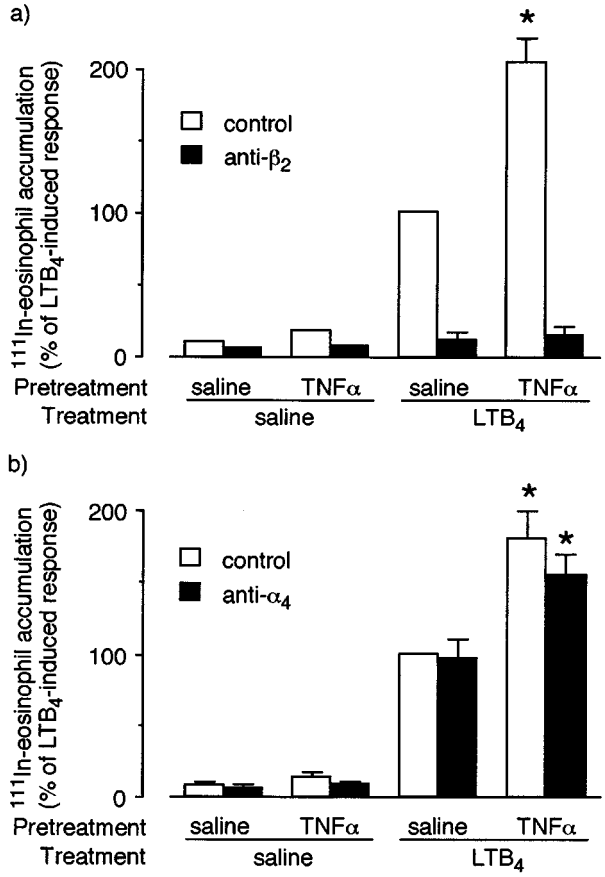

Figure 7 Effect of (a) anti-β_2 integrin and (b) anti-α4 integrin mAbs on eosinophil accumulation into TNFα-primed skin sites. Animals were pretreated with i.d. injection of either TNFα (3 ng per site) or saline 1 h prior to the i.v. injection of ^{111}In-eosinophils and further i.d. injection of LTB_4 (0.1 nmol per site) in pretreated sites. Approximately 15 min before the i.v injection of ^{111}In-eosinophils, animals were treated with an i.v. injection of either control mAb (MOPC21, 3 mg kg^{-1}), anti-β_2 integrin mAb (6.5E, 2.5 mg kg^{-1}) or anti-α_4 integrin (2B4, 3 mg kg^{-1}). The ^{111}In-eosinophil accumulation per skin site was assessed 1 h after injection of cells. Results are expressed as a percentage of the LTB_4-induced response in each animal; values shown are means ± s.e.mean of five animals. Statistically significant difference between responses in anti-β_2 integrin or anti-α_4 integrin mAb-treated animals compared to MOPC-21-treated animals is shown.

of eosinophil migration and amelioration of lung function in animal models (Lukacs *et al*., 1995; Renzetti *et al*., 1996; Shah *et al*., 1995; Zuany-Amorim *et al*., 1995). Therefore, in the present study, we investigated the ability of TNFα to prime for eosinophil recruitment *in vivo*.

In the first part of the study we examined the eosinophil-recruiting activity of TNFα in order to determine a dose that could be used in the priming studies. Intradermal injection of recombinant murine or human TNFα induced a dose-dependent recruitment of ^{111}In-eosinophils to skin sites. The response was delayed in onset, showing a requirement for a 1 h pretreatment with mTNFα for significant ^{111}In-eosinophil accumulation, and protracted, since the eosinophil accumulation remained significant 4 h after mTNFα injection. These delayed kinetics suggested an indirect effect of TNFα *via* local synthesis of proteins, rather than a direct effect on eosinophils, was mediating eosinophil recruitment into skin sites. This possibility was confirmed by the pretreatment of animals with cycloheximide prior to the i.d. injection of TNFα. Thus, despite inducing a small degree of chemokinetic motility of eosinophil *in vitro* (Nagata *et al*., 1993), the recruitment of eosinophils induced by i.d. injection of TNFα *in vivo* as indirect and dependent on the local synthesis of protein. Sanz *et al*. (1997) showed that hTNF-induced trafficking of eosinophils in rat skin was delayed although the effects of a protein synthesis inhibitor were not examined.

Treatment of endothelial cells with TNFα induces the expression of newly-synthesized cell adhesion molecules, including ICAM-1 and VCAM-1 (Pober & Cotran, 1990; Blease *et al*., 1998). To investigate whether an increase in the local expression of CAMs was important for the capacity of TNFα to induce eosinophil recruitment, animals were pretreated with the anti-α_4 integrin mAb 2B4 (Needham *et al*., 1994) or an anti-β_2 integrin mAb 6.5E (Andrew *et al*., 1993). Treatment with 2B4 had no effect on the eosinophil recruitment in response to i.d. injection of LTB_4. In contrast, TNFα-induced eosinophil recruitment was inhibited by up to 60% in animals which received 2B4, suggesting that TNFα was inducing the local expression of a ligand for α_4 integrins. Although we have not identified the ligand involved, VCAM-1 is a distinct possibility. TNFα has been shown to induce the expression of VCAM-1 *in vitro* and *in vivo* (Harrison *et al*., 1997) and VCAM-1 can mediate binding of eosinophils to activated endothelial cells (Bochner *et al*., 1991). Sanz *et al*. (1997) showed upregulation of VCAM-1 in rat skin 4 h after i.d. injection of hTNF and an anti-rat VCAM-1 mAb partially reduced trafficking of ^{111}In-labelled rat eosinophils to these sites. Unfortunately, there are no tools available to investigate the role of VCAM-1 in guinea-pigs *in vivo*.

In contrast to the anti-α_4 integrin mAb, the anti-β_2 integrin mAb abrogated eosinophil recruitment induced by both LTB_4 and TNFα. This is in agreement with our previous studies demonstrating a role for CD11/CD18 in the recruitment of eosinophils to sites of allergic and non-allergic inflammation in guinea-pig skin (Teixeira *et al*., 1994). It is interesting to note that, despite a role for α_4 integrins in mediating TNFα-induced eosinophil recruitment, TNFα-induced responses were still fully inhibited by 6.5E. These results support a fundamental role for β_2 integrins in the recruitment of eosinophils to sites of acute inflammation *in vivo*. However, in sites where a α_4 integrin ligand is induced (e.g. after TNFα injection), β_2 integrins are still essential but not entirely sufficient for mediating eosinophil recruitment. The latter situation could explain the observations made after antigen challenge in sensitized animals where anti-TNFα or anti-VCAM-1 antibodies block the recruitment of eosinophils to the lung (Chin *et al*., 1997; Renzetti *et al*., 1996; Zuany-Armorim *et al*, 1995).

Leukocytes require activation by chemoattractants prior to their firm adhesion to endothelial cells and subsequent migration into the tissue (Springer, 1994). Thus, it is likely that TNFα is inducing the generation of a chemoattractant locally which is in turn inducing the recruitment of eosinophils We have excluded a role for PAF by using a PAF receptor antagonist (data not shown). The finding that TNFα-induced eosinophil recruitment is protein-synthesis dependent raises the possibility that, in addition to newly-synthesized adhesion molecules, TNFα is also inducing the generation of protein chemoattractants. Of particular interest are C-C chemokines, such as eotaxin, which are effective activators of eosinophil recruitment *in vivo* (Teixeira *et al*., 1997).

Because of our earlier study (Macari *et al*., 1996), our main aim was to test whether the i.d. injection of TNFα would also prime skin sites for eosinophil recruitment. For these studies, we chose a threshold dose of TNFα (3 ng per site) which had little effect when injected alone but significantly augmented

eosinophil recruitment in response to i.d. injection of LTB_4, PAF, C5a and in an allergic reaction. More importantly priming occurred not only with ^{111}In-labelled eosinophils, but also when endogenous eosinophils were quantified using histological techniques. Thus, our results demonstrate that, like LPS, low doses of TNFα prime skin sites for increased eosinophil recruitment.

The precise mechanisms for TNFα-induced priming are unknown. Priming of the eosinophil directly is a possibility; TNFα primes the eosinophil for ionophore- and FMLP-stimulated release of LTC_4 (Roubin *et al*., 1987; Takafuji *et al*., 1992), and primes C5a-stimulated eosinophil adhesion to cultured human microvascular endothelium (Burke-Gaffney *et al*., 1997). However, if this is the mechanism, it would have to be a local priming rather than the generalized priming of circulating eosinophils shown to occur with other cytokines in atopics (Moser *et al*., 1992; Warringa *et al*., 1992), since the priming phenomenon observed in the present study was localized to pretreated skin sites. Another possible mechanism of enhanced recruitment by TNFα is the increased expression of adhesion molecules on cutaneous microvascular endothelium. This possibility is in agreement with the ability of the protein synthesis inhibitor cycloheximide to abrogate eosinophil trafficking to the TNFα primed site. In an attempt to address this possibility, the effect of mAbs against the α_4 and β_2 integrins on the TNFα-primed ^{111}In-eosinophil accumulation was investigated. Blockade of β_2 integrin totally inhibited both unprimed and primed responses demonstrating the essential role of this integrin in mediating eosinophil recruitment regardless of the conditions. In contrast, the anti-α_4 integrin mAb had no effect on eosinophil recruitment in TNFα-primed sites. Thus, although an α_4 integrin ligand appears to be expressed in response to high dose TNFα, priming of eosinophil recruitment by a low dose of TNFα is α_4 integrin-independent. In addition to the integrins, selectins play an important role in mediating eosinophil recruitment *in vivo* (Henriques *et al*., 1996; Teixeira & Hellewell, 1997) and it is thus possible that the expression of a TNFα-induced selectin(s) or selectin ligand(s) could account for the priming of eosinophil recruitment observed. However, it is not possible to test this directly in the guinea-pig due to a lack of specific tools. The local induction of protein chemoattractants, such as eotaxin, in response to a low dose of TNFα and synergism with LTB_4 to mediate trafficking of ^{111}In-eosinophils is another possible explanation for these data.

Finally, the capacity of a low dose of TNFα to prime for eosinophil recruitment has similarities to the priming effects induced by a low dose of LPS (Macari *et al*., 1996). Priming induced by both agents is delayed in action although the priming induced by TNFα is more protracted. This discrepancy in duration of action also occurs between eosinophil recruitment induced by high doses of LPS and TNFα (Macari *et al*., 1996). One possibility to explain this discrepancy is that LPS, in addition to induction of TNFα, could be inducing molecules including IL-10, TGFβ or TNF binding proteins which are known to counteract the biological effects of TNFα (Mohler *et al*., 1993; Tracey & Cerami, 1993). Alternatively, clearance of exogenously administered mTNFα may be less rapid than endogenously generated guinea-pig TNFα.

In conclusion, this study has shown TNFα to induce and prime for eosinophil accumulation *in vivo*. Both phenomena are protracted responses, dependent on *de novo* protein synthesis and β_2 integrin-mediated adhesion. As TNFα is released at sites of allergic- and parasitic-induced inflammation, and blocking TNFα modulates the eosinophilic response in these sites, the induction and priming of eosinophil recruitment by TNFα may constitute an important component of the inflammatory response in eosinophilic diseases.

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