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α_4 integrin-dependent eosinophil recruitment in allergic but not non-allergic inflammation

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1 Although anti-α_4 integrin mAbs reduce eosinophil accumulation in several models of allergic inflammation, it is not clear whether this occurs *via* a direct action to block eosinophil α_4 integrins or indirectly on another cell type. The role of α_4 integrins on the accumulation of ^{111}In-labelled eosinophils in allergic and non-allergic inflammation in guinea-pig skin was therefore investigated.

2 Intradermal injection of antigen in sensitized skin sites induced accumulation of ^{111}In-eosinophils that was reduced up to 70% by two anti-α_4 integrin mAbs. In contrast, accumulation of ^{111}In-eosinophils to intradermal chemoattractants was unaffected by the same mAbs.

3 Accumulation of ^{111}In-eosinophils in allergic and non-allergic conditions was partly inhibited by a low dose of an anti-β_2 integrin mAb. In combination with anti-α_4 integrin mAb, responses were not further reduced suggesting that these adhesion pathways are not additive or synergic.

4 Pretreating skin sites with antiserum or contaminating LPS did not reveal an α_4 integrin dependent pathway for chemoattractant-induced ^{111}In-eosinophil accumulation. These data suggest that α_4 integrins are involved in the response to antigen in sensitized skin sites.

5 Pretreating ^{111}In-eosinophil with α_4 integrin mAb blocked their adhesion to fibronectin *in vitro* but did not inhibit their accumulation in allergic inflammation suggesting that the blocking effect *in vivo* was eosinophil independent.

6 These data support the concept that targeting α_4 integrins on cells other than eosinophils could control eosinophil accumulation and have therapeutic potential in allergic diseases such as asthma and atopic dermatitis.

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Abbreviations: AA, arachidonic acid; LPS, lipopolysaccharide; mAb, monoclonal antibody; OA, ovalbumin; PCA, passive cutaneous anaphylaxis; TNFα, tumour necrosis factor α; VCAM-1, vascular cell adhesion molecule-1; ZAP, zymosan-activated plasma

Introduction

Eosinophils are considered to have important effector functions in chronic allergic diseases such as asthma, rhinitis, dermatitis and conjunctivitis (Weller *et al*., 1996). They are often the predominant leukocyte type in these diseases and, through secretion of a cocktail of lipid and protein mediators, are thought to modulate bronchial smooth muscle tone in the airways, cause oedema formation and influence the function of other cells (Martin *et al*., 1996). Cationic proteins (e.g. major basic protein and eosinophil-cationic protein), released from eosinophil granules upon activation, are important for host defence against parasites. Misdirected release of these proteins in allergic inflammation damages host epithelial cells and contributes to disease pathology (Montefort *et al*., 1992; Wardlaw *et al*., 1988). Controlling accumulation and activation of eosinophils may offer therapeutic benefit in allergic diseases. A detailed understanding of the mechanisms underlying eosinophil accumulation *in vivo* is therefore essential to the development of new and safe therapeutic strategies based on reduced recruitment of these cells (Teixeira *et al*., 1995).

Integrins are a family of adhesion molecules comprising α and β chain heterodimers. Integrins expressed on eosinophils include the β_1 (CD29) subunit with α_2, α_4, α_5 or α_6 (CD49b, d, e or f) subunits, and the β_2 (CD18) subunit with α_L, α_M, α_X or α_d subunits (CD11a, b, c or d) (Giembycz & Lindsay, 1999). Our previous work has investigated the adhesion mechanisms involved in the recruitment of radiolabelled eosinophil from blood to sites of cutaneous inflammation in guinea-pigs and mice. We demonstrated contributions of endothelial selectins (Teixeira & Hellewell, 1997) and β_2 integrins (Teixeira *et al*., 1994) to eosinophil accumulation in response to exogenously applied chemoattractants and to mediators generated endogenously at sites of allergic inflammation. α_4 integrins expressed constitutively on eosinophils mediates their adhesion to vascular cell adhesion molecule-1 (VCAM-1) on cytokine activated endothelial cells *in vitro* (Schleimer *et al*., 1992; Weller *et al*., 1991). The contribution of α_4 integrins to eosinophil accumulation *in vivo* is less clear. Blocking monoclonal antibodies (mAbs) to

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α_4 integrins reduce eosinophil accumulation in a number of models of airways inflammation (Chin *et al*., 1997; Fryer *et al*., 1997; Henderson *et al*., 1997; Milne *et al*., 1995; Nakajima *et al*., 1994; Pretolani *et al*., 1994). Common to these studies was the prolonged time-course required for eosinophil recruitment, which usually occurred concomitantly or subsequent to accumulation of other cell types (e.g. lymphocytes, monocytes, macrophages) which are themselves inhibited by anti-α_4 integrin mAbs. Thus, it is not clear from such studies whether pretreatment with α_4 integrins inhibited eosinophil recruitment directly or indirectly by inhibiting the function and/or recruitment of another cell types. This issue was addressed by Henderson *et al*. (1997) who found that intranasal administration of an anti-α_4 integrin mAb could attenuate eosinophil accumulation to the airways without an effect on circulating cells. They concluded that the major effect of α_4 integrins was on intrapulmonary cells.

Using radiolabelled eosinophils, Weg *et al*. (1993) reported that the anti-α_4 integrin mAb HP1/2 could inhibit by approximately 50% chemoattractant (e.g. C5a, LTB_4)-induced recruitment of these cells to guinea-pig skin. Given that the accumulation of eosinophils to chemoattractant occurs rapidly, i.e. within 30 min (Teixeira *et al*., 1997), the effect of HP1/2 is intriguing in view of an absence or very low expression of constitutively expressed VCAM-1 in the cutaneous microcirculation (Harrison *et al*., 1997; Davies *et al*., 1999). In contrast, using another anti-α_4 integrin mAb 2B4 (Needham *et al*., 1994), we found that chemoattractant-induced recruitment of ^{111}In-eosinophils was independent of this integrin (Macari *et al*., 1998). Using mAb 2B4, an α_4 integrin-dependent component to eosinophil recruitment was revealed only when skin sites were treated for at least 2 h with tumour necrosis factor α (TNFα), presumably upregulating VCAM-1.

In view of the importance of eosinophils in allergic inflammation, the aim of the present study was to assess the role of α_4 integrins to the recruitment of ^{111}In-eosinophils in an allergen-induced cutaneous model. In this model, accumulation of ^{111}In-eosinophils to chemoattractants intradermally injected or generated in response to allergen occurs rapidly. In addition, and in contrast to models of eosinophil recruitment in allergen-induced airways inflammation, there is no systemic sensitization as skin sites are sensitized passively. These facets of our model allow effects of anti-α_4 integrin mAbs on eosinophil recruitment to be examined directly. We used two anti-α_4 integrin mAbs: 2B4 (Needham *et al*., 1994) which was raised against human α_4 integrin and cross reacts with guinea-pig eosinophils and blocks adhesion to fibronectin (Teixeira *et al*., 1996), and Max68P, also directed against human α_4 integrins, which we generated ourselves and showed to bind and block guinea-pig α_4 integrins.

Methods

Preparation of zymosan-activated plasma

Zymosan-activated plasma (ZAP) was used as a source of guinea-pig C5a des arg. ZAP was prepared by incubating heparinized (10 iu/ml) plasma obtained from naïve guinea-pigs (Harlan, Bicester, U.K., 350–400 g) with zymosan (5 mg ml^{-1}) for 30 min at 37°C. Zymosan was then removed by centrifugation (2×10 min at 3000 *g*). The activated plasma was desalted using a PD10 Sephadex G25M column and stored in aliquots at −20°C.

Preparation of passive cutaneous anaphylaxis sera and reactions

Details of the preparation of anti-ovalbumin (OA) sera are described elsewhere (Milne & Piper, 1995). Briefly, male guinea-pigs (Harlan, 350–400 g) were immunized on day 0 with 1 mg of ovalbumin and 1 mg of aluminium hydroxide in 1 ml saline (i.p.) and *Bordetella pertussis* vaccine (0.25 ml i.p.). On day 7, the animals were given a booster injection of 0.1 mg ovalbumin and 0.1 mg of aluminium hydroxide in 0.1 ml saline (i.p.). On day 14, the animals were bled, serum prepared and pooled and stored at −20°C. For the skin assays, recipient animals received an injection of 50 μl of a 1 in 30 dilution of the anti-serum i.d., followed 16–20 h later by the i.d. injection of antigen (OA, 3 to 30 μg per site). Preliminary studies showed these doses of antigen and the concentration of antiserum to induce optimal passive cutaneous anaphylactic (PCA) reactions in recipient naïve guinea-pigs. Most of the anti-OA anaphylactic antibodies appeared to be of the IgG_1 subtype as assessed by the short fixation time (4–24 h) and resistance to heat (56°C, 30 min) (data not shown). The endotoxin concentration of a 1/30 dilution of this antiserum was 0.25 ng ml^{-1} (QCL1000, BioWhittaker, Inc., Walkersville, MD, U.S.A.), equivalent to 0.0125 ng per 50 μl injected i.d.

Measurement of ^{111}In-eosinophil recruitment in guinea-pig skin

Eosinophils were purified from the peritoneal cavity of horse serum-treated guinea-pigs and radiolabelled as previously described (Teixeira & Hellewell, 1994). The radiolabelled cells were then injected i.v. (2.5×10^6 cells per animal) into recipient guinea-pigs (350–400 g) which were sedated with Hypnorm (0.15 ml i.m.). After 5 min, duplicate i.d. injections of inflammatory stimuli or antigen were given in 0.1 ml volumes into the shaved dorsal skin following a randomized injection plan. ^{111}In-labelled eosinophil accumulation was assessed 2 h after i.d. injections of inflammatory mediators or antigen. At this time, a blood sample was obtained by cardiac puncture, the animals were sacrificed with an overdose of sodium pentobarbitone, the dorsal skin was removed, cleaned free of excess blood and the sites punched out with a 17 mm punch. The samples were counted in an automatic 10-head gamma-counter (Canberra Packard Ltd., Panbourne, Berks, U.K.). Eosinophil numbers in the skin sites were expressed as the number of ^{111}In-eosinophil per skin site (Teixeira & Hellewell, 1994).

Monoclonal antibodies

The following mAbs were utilized: anti-α_4 integrin 2B4 (mouse IgG_1) (Needham *et al*., 1994), anti-CD18 6.5E (mouse IgG_1) (Andrew *et al*., 1993) and murine myeloma protein MOPC21 (IgG_1) which was used as isotype matched control antibody.

To generate a new anti-α_4 integrin mAb, mice were immunized i.p. with the human T cell leukemia cell line Jurkat J6. After boosting, spleen cells were fused to mouse myeloma cells. Hybridoma supernatants were screened for their ability to block J6 adhesion to fibronectin. Mab Max68P (IgG_1) was identified by this means, was subcloned and purified. This mAb binds to recombinant α_4 integrins ($\alpha_4\beta_1$ and $\alpha_4\beta_7$) (data not shown).

Functional activity of anti-α_4 integrin mAbs in vitro

The wells of a microtitre plate were coated with 0.125 μg of the recombinant Hep II/III CS fragment of fibronectin (H/120; a gift from Prof. M. Humphries, University of Manchester, U.K.). Adhesion of ^{111}In-labelled guinea-pig eosinophils was performed as described previously (Teixeira *et al*., 1996).

Experimental protocol

For systemic treatment of guinea-pigs, the mAbs were given at 3 mg kg^{-1} i.v. 15 min prior to the injection of the ^{111}In-eosinophils. This dose of 6.5E has been shown to be maximally effective at inhibiting eosinophil and neutrophil recruitment in the guinea-pig in earlier studies (Teixeira *et al*., 1994) and the α_4 integrin mAbs were shown to cause saturation of circulating leukocytes at this dose (data not shown). Five minutes after the i.v. injection of ^{111}In-eosinophils, inflammatory stimuli or antigen, in sites previously sensitized with antiserum, were injected intradermally. Stimuli used were zymosan activated plasma (ZAP, a source of guinea-pig C5a des Arg), PAF and high dose arachidonic acid (AA); the latter is converted to LTB_4 in guinea-pig skin (Teixeira & Hellewell, 1994).

In some experiments, in order to assess the potential role of skin sensitization in the effects of the anti-α_4 integrin mAbs, sites were sensitized as for the PCA reaction but inflammatory mediators (PAF, ZAP, AA), and not antigen, were injected 16–20 h later and accumulation of ^{111}In-eosinophils measured. Similarly, in order to assess the potential role of contaminating LPS in the antiserum, skin sites were pretreated for 16–20 h with a concentration of LPS similar to that found in the anti-OA antisera (i.e. 0.0125 ng per site). PAF, ZAP or AA were then injected into the LPS-treated or control sites and ^{111}In-eosinophil accumulation measured.

In experiments designed to assess specifically the role of α_4 integrins on the eosinophil, ^{111}In-eosinophils (1×10^7 ml^{-1}) were pretreated *in vitro* with a saturating concentration of 2B4 (50 μg ml^{-1}) or MOPC21 (50 μg ml^{-1}) for 15 min at room temperature. The cells were then washed and injected intravenously. As above, inflammatory stimuli and antigen were applied 5 min after eosinophil injection and eosinophil accumulation in skin sites were assessed after 2 h.

Flow cytometric analysis of 2B4 and Max68P binding to guinea-pig eosinophils

Purified eosinophils were incubated with saturating concentrations of MOPC21 (50 μg ml^{-1}), 2B4 (50 μg ml^{-1}) or Max68P (50 μg ml^{-1}) for 30 min at 4°C. The cells were then washed twice with PBS, goat anti-mouse IgG antibody conjugated with FITC was added and the cells were incubated for 30 min at 4°C. Cell preparations were then washed twice and fluorescence determined on FACScan flow cytometer (Becton Dickinson, Oxford, U.K.) and analysed using CELLQuest software.

Reagents

The following compounds were purchased from Sigma Chemical Company (Poole, Dorset, U.K.): goat anti-mouse IgG-FITC, arachidonic acid, lipopolysaccharide (0111:B4), ovalbumin and zymosan. PBS, Hanks solutions, HEPES buffer and horse serum were purchased from Life Technologies Ltd (Paisley, Scotland). Percoll was purchased from Pharmacia (Milton Keynes, Bucks., U.K.) and PAF (C16) from Bachem (Saffron Walden, Essex, U.K.) $^{111}InCl_3$ was from Amersham International plc (Amersham, Bucks., U.K.).

Statistics

Data are presented as mean ± s.e.mean and were analysed by using two-way analysis of variance (ANOVA) on normally distributed data. P values were assigned using Newman–Keuls procedure and values of $P<0.05$ were considered statistically significant. Percentage inhibition was calculated subtracting background values.

Results

Figure 1 shows flow cytometric analyhsis of guinea-pig eosinophils in the presence of saturating concentrations of 2B4 of Max68P. The mean fluorescence intensity (MFI) of 2B4- and Max68P-labelled eosinophils was 10–20 times greater than the MFI in MOPC21 (control)-labelled eosinophils. Binding of Max68P was slightly less than 2B4, perhaps a reflection of binding to a different epitope, although this did not appear to markedly affect its inhibitory activity *in vivo* (see below). Pretreatment of eosinophils with PMA (10^{-7} M) did not alter binding of 2B4 or Max68P to purified eosinophils.

Effect of anti-α_4 integrin mAbs on ^{111}In-eosinophil recruitment in guinea-pig skin

The i.d. injection of antigen (3–30 μg OA per site) in sites previously sensitized for 16–20 h with anti-OA antiserum (PCA reaction) induced a dose-dependent accumulation of ^{111}In-eosinophils (Figure 2). ^{111}In-eosinophil accumulation did not occur after injection of antigen in non-sensitized sites (data not shown). When used at the dose of 3 mg kg^{-1}, 2B4 virtually abolished ^{111}In-eosinophil recruitment in the PCA reaction (Figure 2). This inhibitory effect was not limited to this mAb this administration of Max68P (3 mg kg^{-1}) also resulted in a similar inhibition of ^{111}In-eosinophil accumulation in the PCA reaction (Figure 2). Neither mAb reduced the number of circulating ^{111}In-eosinophils thus eliminating a potential explanation for the reduction in ^{111}In-eosinophil recruitment.

In marked contrast to effects in the PCA reaction, neither mAb had any effect on the ^{111}In-eosinophil accumulation induced by ZAP or AA in the same animals (Figures 3a,b).

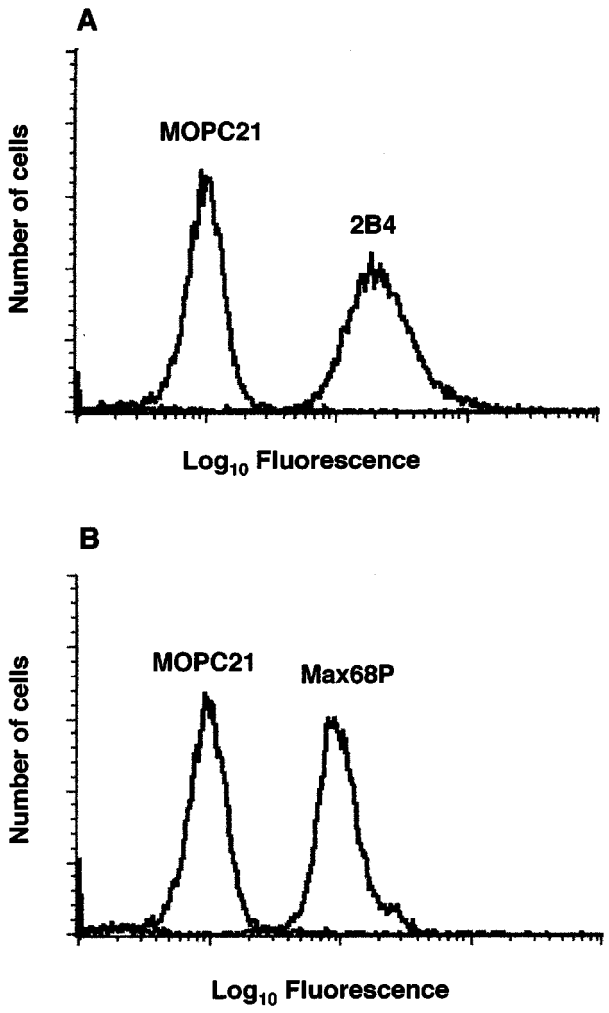

Figure 1 Flow cytometric analysis of the binding of the anti-α_4 integrin mAbs 2B4 (A) and Max 68P (B) to guinea-pig eosinophils. The isotype matched myeloma protein MOPC21 was used for control binding.

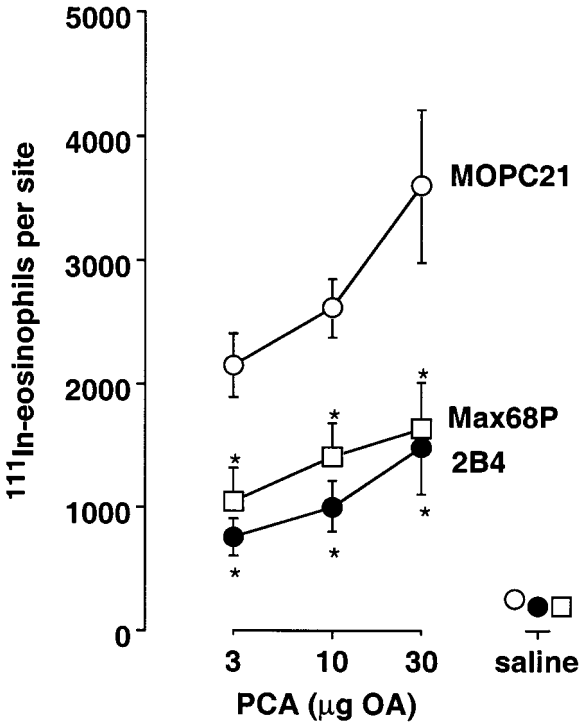

Figure 2 Effect of anti-α_4 integrin mAbs on allergen-induced recruitment of ^{111}In-eosinophils in guinea-pig skin. 2B4, Max68P or MOPC21 as a control were administered (all at 3 mg kg^{-1}) i.v. 15 min before i.v. injection of ^{111}In-eosinophil and i.d. injection of antigen (3, 10 and 30 μg OA, shown as PCA). Sensitized skin sites also received an i.d. injection of saline (shown as saline). Accumulation of ^{111}In-eosinophil in sites was assessed after 2 h. Values are mean$\pm$s.e.mean of experiments in six animals with each mAb. *$P<0.05$ compared to responses in MOPC21 treated animals.

Similarly, responses induced by PAF were not significantly altered by pretreatment with 2B4 (e.g. PAF, 1.0 nmol per site; control, 2186$\pm$905; 2B4, 1798$\pm$659 ^{111}In-eosinophils per site, $n=5$). These results demonstrate the ability of two different anti-α_4 integrin mAbs to suppress ^{111}In-eosinophil recruitment in allergic but not non-allergic inflammation.

Effect of combination treatment with anti-α_4 and anti-β_2 integrin mAbs

We have previously shown that the anti-β_2 integrin mAb, 6.5E, effectively suppresses ^{111}In-eosinophil accumulation in both mediator-induced and allergic inflammation in guinea-pig skin when used at a dose of 2.5 mg kg^{-1} (Macari *et al*., 1998; Teixeira *et al*., 1994). In this series of experiments, we used a dose of 6.5E (0.25 mg kg^{-1}) which caused only partial inhibition of ^{111}In-eosinophil accumulation to assess whether anti-α_4 and anti-β_2 integrin mAbs had additive effects. As shown in Figure 4a, ^{111}In-eosinophil accumulation in the PCA reaction was inhibited by both 6.5E and 2B4 but there was no further inhibition when both mAbs were used together. 6.5E caused partial inhibition of ^{111}In-eosinophil accumulation induced by ZAP, but this was not further inhibited by co-treatment with 2B4 (Figure 4b). These results suggest that β_2 integrins are sufficient to allow ^{111}In-eosinophil accumulation in non-allergic inflammation, while in allergic inflammation eosinophil recruitment uses either β_2 or α_4 integrin-dependent pathways.

Mechanisms underlying the inhibitory effects of anti-α_4 integrin mAbs on eosinophil recruitment in the PCA reaction

Although LPS is weak at inducing VCAM-1 expression on endothelial cells (Blease *et al*., 1998), it was possible that the low level of LPS detected in the PCA antiserum could induce upregulation, over the 16 h sensitization period, of an α_4 integrin ligand such as VCAM-1, and explain the capacity of 2B4 to inhibit allergic but not non-allergic inflammation. The level of LPS in the stock of antiserum was found to be 0.25 ng ml^{-1}. We have previously shown that this concentration of LPS does not induce any ^{111}In-eosinophil accumulation when injected i.d. and does not prime the site for further ^{111}In-eosinophil accumulation using pretreatment times of up to 4 h (Macari *et al*., 1996). In the present study we pretreated skin sites with LPS (50 μl of 0.25 ng ml^{-1}; equivalent to 0.0125 ng). To mimic the sensitization period

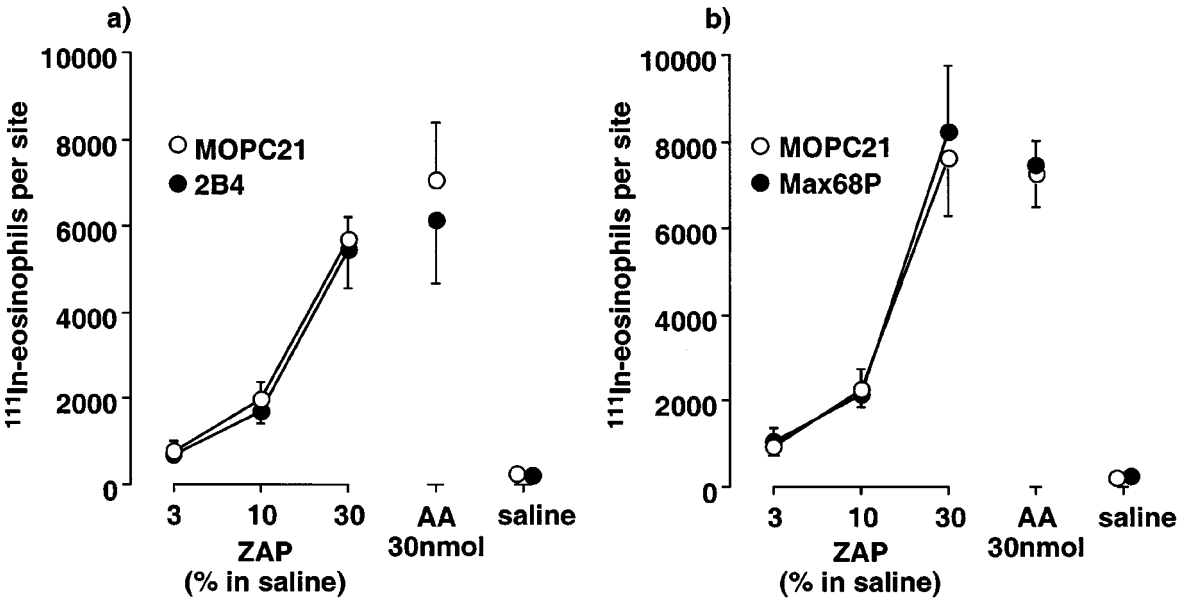

Figure 3 Effect of anti-α_4 integrin mAbs on recruitment of ^{111}In-eosinophil induced by ZAP or arachidonic acid (AA) in guinea-pig skin. 2B4 (a) or Max68P (b), and MOPC21 as a control, were administered (all at 3 mg kg^{-1}) i.v. 15 min before i.v. injection of ^{111}In-eosinophil and i.d. injection ZAP (3, 10 and 30% dilution in saline) or AA (30 nmol). Accumulation of ^{111}In-eosinophils in sites was assessed after 2 h. Values are mean$\pm$s.e.mean of experiments in six animals with each mAb.

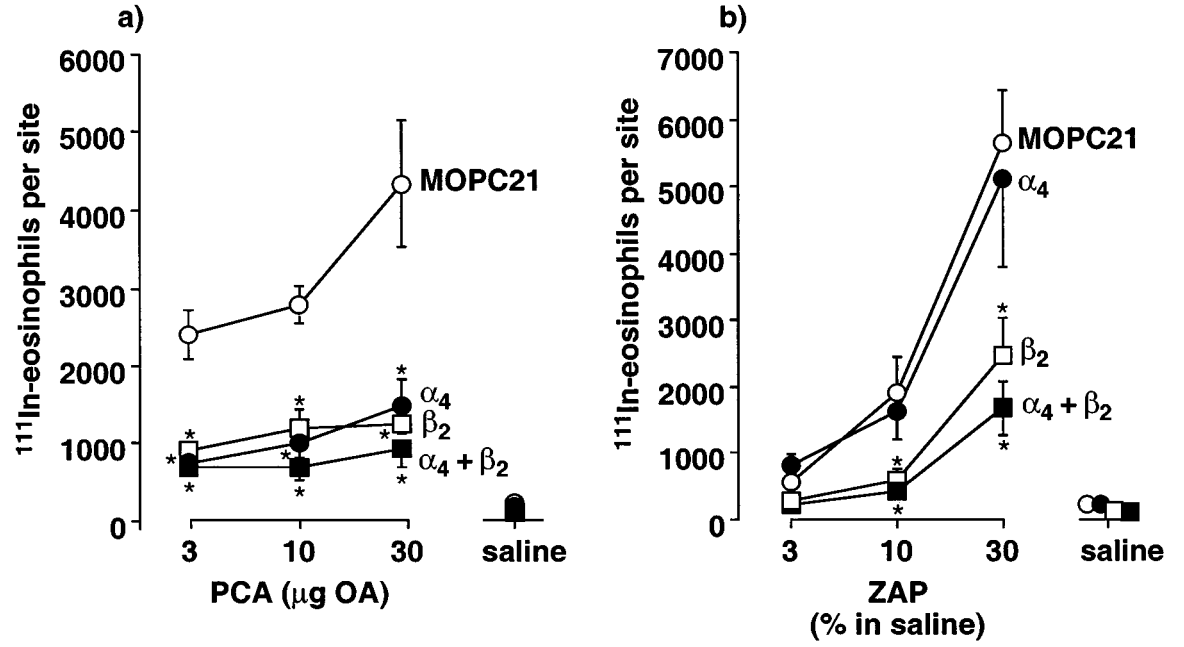

Figure 4 Effect of combined treatment with anti-α_4 and β_2 integrin mAbs on (a) allergen-induced and (b) ZAP-induced ^{111}In-eosinophil accumulation in guinea-pig skin. Approximately 15 min before the i.v. injection of ^{111}In-eosinophils, animals were treated with an i.v. injection of either control mAb (MOPC21, 3.25 mg kg^{-1}), anti-α_4 integrin (2B4, 3 mg kg^{-1}), anti-β_2 integrin mAb (6.5E, 0.25 mg kg^{-1}) or α_4 and β_2 mAbs in combination. Allergen (OA, 3–30 μg), ZAP (3–30%) or saline were injected i.d. and the ^{111}In-eosinophil accumulation per skin site was assessed 2 h later. Results are expressed as mean$\pm$s.e.mean of five animals for each treatment group. *$P<0.05$ compared to responses in MOPC21 treated animals.

for antiserum, a 16 h pretreatment time was used. Despite marked inhibition of eosinophil recruitment in the PCA reaction by 2B4, pretreatment with LPS did not render the responses to ZAP and PAF susceptible to inhibition by 2B4 (Figure 5).

In the same experiment we examined whether injection of antiserum itself modified the local skin site to make it liable to inhibition by 2B4. As shown in Figure 5, injection of antiserum 16 h prior to PAF or ZAP had no effect on ^{111}In-eosinophil recruitment induced by these mediators and did

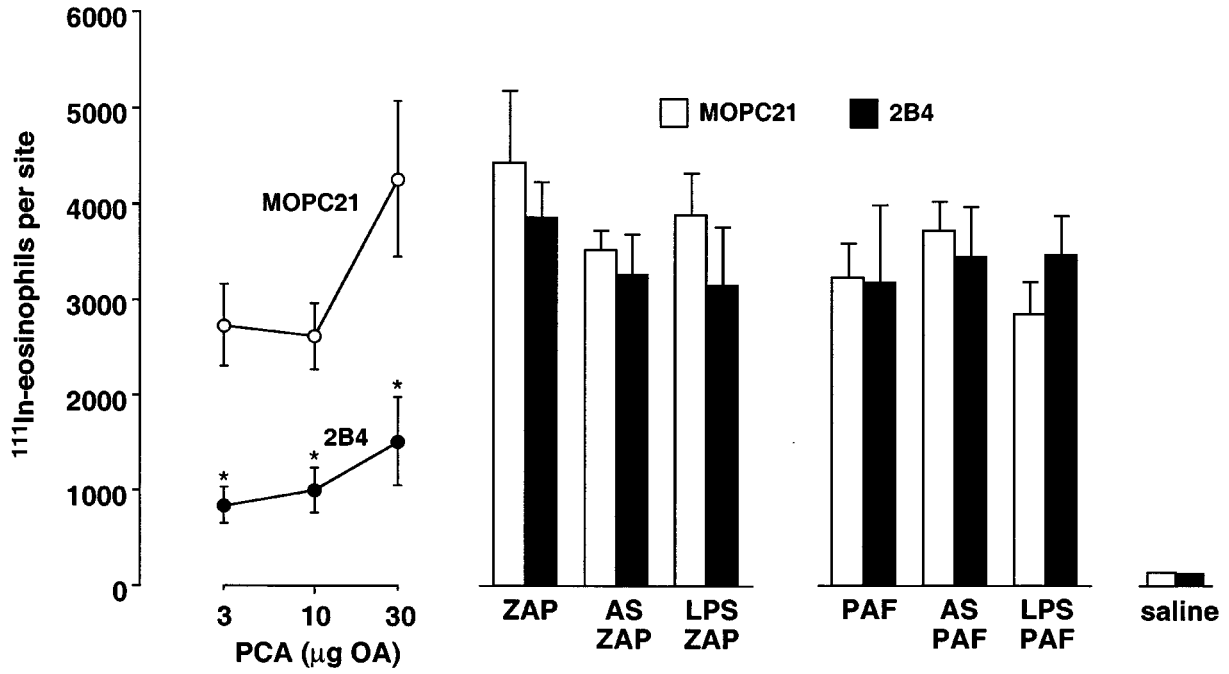

Figure 5 Effect of pretreating skin sites with antiserum or LPS on the capacity of anti-α_4 integrin mAb 2B4 to module ZAP- and PAF-induced ^{111}In-eosinophil accumulation in guinea-pig skin. Skin sites were pretreated for 16–20 h with antiserum (AS, 50 μl of 1 in 30 dilution) or LPS (0.0125 ng) followed by i.d. injection of OA (to induce the PCA reaction), ZAP (10% in saline) or PAF (1 nmol). 2B4 or MOPC21 were administered (3 mg kg^{-1}) i.v. 15 min before i.v. injection of ^{111}In-eosinophil. Accumulation of ^{111}In-eosinophils in sites was assessed after 2 h. Values are mean$\pm$s.e.mean of experiments in four animals per group. *$P<0.05$ compared to responses in MOPC21 treated animals.

not make the responses sensitive to inhibition by 2B4. These data suggest that injection of antiserum alone does not induce an α_4 integrin-dependent component to the local inflammatory response.

To examine whether 2B4 was acting on the eosinophil surface to inhibit the PCA reaction, eosinophils were pretreated with a saturating concentration of 2B4 (50 μg ml^{-1}), washed and injected i.v. Trafficking of ^{111}In-eosinophil accumulation was measured in skin sites 2 h after injection of antigen. As seen in Figure 6, *in vitro* incubation of ^{111}In-eosinophil with 2B4 failed to inhibit their accumulation in inflammatory lesions induced by PAF, ZAP or in the PCA reaction. In contrast, the capacity of the same ^{111}In-eosinophil to bind to the fibronectin fragment FN40 was abolished: BSA blocked well, 3.0$\pm$1.1% total cells added; FN40+control eosinophils, 7.2$\pm$0.5%; FN40+2B4-treated eosinophils, 3.2$\pm$1.3%, $n=4$). Binding of 2B4 to eosinophils was measured by flow cytometry before and after 2 h incubation at 37°C in HBSS containing BSA. Control IgG binding (MFI) was 8, 2B4 before incubation 53 and 2B4 after incubation 49 (means of duplicate experiments). Thus, under these conditions the anti-α_4 integrin mAb 2B4 remains bound to the eosinophil surface for at least 2 h.

Discussion

The major finding of this study was that two different anti-α_4 integrin mAbs inhibited the recruitment of ^{111}In-eosinophil to sites of allergic inflammation in guinea-pig skin yet in the same animals there was no effect on ^{111}In-eosinophil accumulation induced by PAF, ZAP and high dose AA.

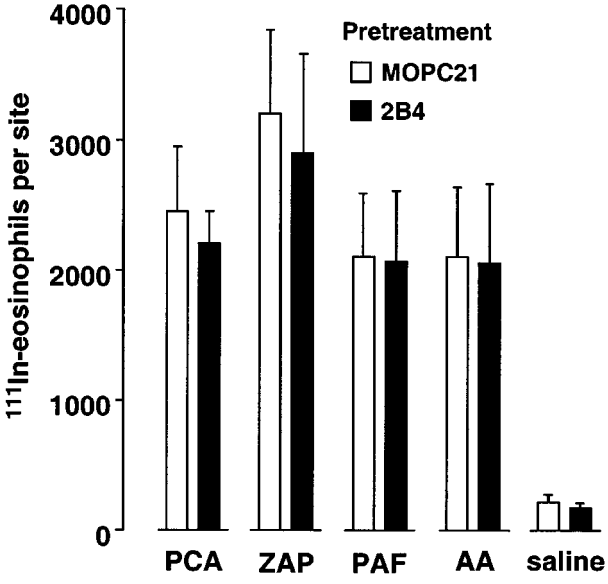

Figure 6 Effect of pretreatment of ^{111}In-eosinophil with anti-α_4 integrin mAb 2B4 on their capacity to migrate to inflammatory sites in guinea-pig skin. Radiolabelled eosinophil were incubated with MOPC21 or 2B4 (both at 50 μg ml^{-1}), washed and infused into separate recipient guinea-pigs. Sensitized skin sites were injected with antigen (30 μg OA, shown as PCA), ZAP (10% in saline) or PAF (1 nmol) and accumulation of ^{111}In-eosinophil in sites assessed after 2 h. Values are mean$\pm$s.e.mean of six experiments.

Further experiments were then designed to investigate the reasons for the specificity of the inhibitory actions of the anti-α_4 integrin mAbs on allergic reactions only. The first step was

to measure the content of LPS of the antiserum used in this series of experiments. This was important since LPS, *via* the release of cytokines such as TNFα, may induce the upregulation of CAMs on the surface of endothelial cells (Blease *et al*., 1998) and thus facilitate the recruitment of eosinophils and at low doses to prime for inflammatory mediator-induced eosinophil accumulation (Macari *et al*., 1998; 1996). However, the effects of LPS on guinea-pig skin are short-lived (around 1–2 h) and occur at concentrations of LPS much higher than those measured in our antiserum stock (Macari *et al*., 1996). When given as a 16 h pretreatment in the same skin sites as PAF or ZAP, i.d. injection of LPS had no effect on the inflammatory response induced by these mediators. Furthermore, treatment of the animals with the anti-α_4 integrin mAb (2B4) had no effect on PAF- or ZAP-induced ^{111}In-eosinophil accumulation in the presence or absence of LPS. Similarly, pretreatment of skin sites with antiserum did not modify the inflammatory responses induced by PAF or ZAP or make them inhibitable by 2B4. This was in marked contrast to the inhibitory effects of intravenously injected 2B4 on ^{111}In-eosinophil accumulation in the PCA reaction in the same animals. Together, these results demonstrate that the antiserum itself or its LPS content could not account for the inhibitory effects of 2B4 on the PCA reaction. Clearly, injection of antigen in sites previously sensitized with an IgG_1-rich antiserum was necessary and sufficient to induce an α_4 integrin-dependent component for the acute recruitment of eosinophils in the skin of guinea-pigs.

The possible role of the eosinophil as the cellular target of the anti-α_4 integrin mAbs used was examined by pretreating these cells with a saturating concentration of 2B4 prior to their i.v. injection. Under these circumstances, 2B4 pretreatment had no effect on ^{111}In-eosinophil accumulation in allergic inflammation. In contrast, the capacity of these eosinophil to adhere to FN fragments *in vitro* was abrogated. These results suggest that the eosinophil is not a target for the inhibitory effects of systemically administered anti-α_4 integrin mAbs. Furthermore, since skin sites were passively sensitized, they suggest that these mAbs are acting at the level of the skin site to inhibit ^{111}In-eosinophil recruitment in allergic inflammation.

One possible target is mast cells and a recent study in rats demonstrated that i.v. administration of an anti-α_4 integrin mAb inhibited the early airway response to allergen and was associated with reduced levels of histamine and tryptase in lavage fluid (Hogo *et al*., 1998). These data suggest that the anti-α_4 integrin mAb inhibited the early responses *via* inhibition of mast cell activation. Studies of mast cells *in vitro* have demonstrated surface expression of β_1 integrins, including $\alpha_4\beta_1$, that mediate adhesion to matrix proteins and facilitate mast cell activation (Columbo *et al*., 1995; Yasuda *et al*., 1995). In view of the assumed role of the mast cell in fixation of antibody and release of mast cell contents in the PCA reaction, this cell represents a potentially important target for α_4 integrin mAbs in the present study, although it will be necessary to address this formally in other studies. Mast cells, *via* the release of TNFα might also induce the local expression of an α_4 integrin ligand, possibly VCAM-1 or a CSI-containing fibronectin variant; human umbilical vein endothelial cells stimulated with inflammatory cytokines express this fibronectin splice variant (Elices *et al*., 1994). Moreover, we have shown that i.d. injection of TNFα in guinea-pig skin induces the expression of an α_4 integrin ligand after 2 h such that trafficking of ^{111}In-eosinophils is reduced by 2B4 (Macari *et al*., 1998). However, upregulation of α_4 integrin ligand expression would have to occur rapidly after antigen exposure which is at variance with the known kinetics of ligand expression *in vitro* (Blease *et al*., 1998; Elices *et al*., 1994) and *in vivo* (Harrison *et al*., 1997).

A role for α_4 integrins on cells other than eosinophils, but that nevertheless triggers eosinophil accumulation, is the conclusion of other studies. In a mouse model, Henderson *et al*. (1997) demonstrated that intranasal anti-α_4 integrin mAb inhibited eosinophil accumulation and airway dysfunction *via* an action on airway cells with a macrophage or dendritic cell phenotype. In addition, inhalation of a small molecule peptide inhibitor of α_4 integrins reduced airways inflammation and dysfunction in sensitized and challenged sheep which is consistent with a local role for α_4 integrins in the lung (Abraham *et al*., 1997).

One alternative possibility is that the chemoattractants generated in the PCA reaction differ from the stimuli used in this study (PAF, ZAP, AA) and they induce a rapid upregulation of eosinophil α_4 integrins and an endothelial ligand. We have previously shown that endogenous generation of LTB_4 accounts for approximately 60% of the chemoattractant activity for eosinophils in the PCA reaction (Teixeira & Hellewell, 1994). To account for this endogenously released LTB_4, we compared the PCA reaction to LTB_4 released in response to i.d. injection of high dose AA. AA-induced eosinophil recruitment was unaltered by pretreatment with 2B4 or Max 68P. Another possibility is that there is low basal expression of VCAM-1 on endothelial cells of post-capillary venules in guinea-pig skin as reported for rats (Davies *et al*., 1999). There is a small possibility, therefore, that chemoattractants released in the PCA reaction, but not injected chemoattractants, could upregulate eosinophil α_4 integrin expression/function resulting in the observed inhibitory effect of α_4 integrin mAbs.

A few studies have evaluated the ability of anti-β_2 and anti-α_4 integrin mAbs to synergize at inhibiting allergen-induced eosinophil recruitment *in vivo* (Nakajima *et al*., 1994; Laberge *et al*., 1995). In the mouse trachea, an anti-β_2 mAb had no effect on antigen-induced eosinophil infiltration when used alone, but significantly enhanced the inhibitory effects of an anti-α_4 mAb (Nakajima *et al*., 1994). In contrast in a rat model of lung inflammation, an anti-α_4 mAb had no effect on eosinophil influx and did not increase the ability of an anti-β_2 mAb to inhibit the number of eosinophil in bronchoalveolar lavage or in tissue (Laberge *et al*., 1995). We have shown previously that eosinophil trafficking in our guinea-pig skin model is abrogated by the anti-β_2 integrin mAb 6.5E (Macari *et al*., 1998; Teixeira *et al*., 1994). In the present study, we wished to determine whether the effect of a lower dose of this mAb to cause a partial inhibition of eosinophil trafficking could be increased in the additional presence of an anti-α_4 integrin mAb. These experiments showed, however, that eosinophil recruitment in response to non-allergic inflammatory stimuli was β_2 integrin-dependent and there was no further inhibition when an anti-α_4 mAb was used. Allergen-induced inflammation was inhibited by anti-β_2 and anti-α_4 mAbs alone but there was no additional effect when both mAbs were used together. This last piece of data suggests

that these two adhesion pathways, β_2-dependent and α_4-dependent, are positioned in series and do not represent redundancy of the system; one possibility is that the α_4-dependent pathway is important for the adequate release of mediators from mast cells and the β_2 pathway is responsible for the adhesion of eosinophils to endothelial cells.

Finally, our results differ from those of Weg *et al*. (1993) who showed the anti-α_4 mAb HP1/2 to inhibit eosinophil recruitment in guinea-pig skin regardless of the inflammatory stimuli used. The reasons for this discrepancy are not immediately apparent but could lie in the difference between HP1/2 and both the anti-α_4 integrin mAbs used in this study.

In conclusion, we showed two anti-α_4 integrin mAbs to be effective inhibitors of eosinophil influx in a passive allergic reaction in guinea-pig. There appears to be no role for the eosinophil as a cellular target for the action of anti-α_4 integrin-based strategies in response to direct-acting eosinophil chemoattractants in our model of acute inflammation. These data support the concept that targeting α_4 integrins on cells other than eosinophils could control eosinophil accumulation and have therapeutic potential in allergic diseases such as asthma and atopic dermatitis.

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