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The effect of the selectin binding polysaccharide fucoidin on eosinophil recruitment *in vivo*

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1 In order to accumulate at sites of inflammation, leukocytes initially roll on endothelial cells of post-capillary venules before becoming firmly attached. This process of rolling is mediated by selectins which bind to carbohydrate counter-ligands present on the surface of both leukocytes and endothelial cells. The polysaccharide fucoidin has been previously shown to inhibit leukocyte rolling in the mesenteric circulation and to reduce neutrophil accumulation in the skin and meninges in experimental inflammation.

2 In the present study we have assessed the effects of fucoidin on eosinophil function *in vitro* and eosinophil accumulation at sites of inflammation in guinea-pig skin.

3 At concentrations of up to 1200 μg ml^{-1}, fucoidin inhibited phorbol myristate acetate (PMA)-induced eosinophil homotypic aggregation by up to 60% but had no inhibitory effect on PMA-induced eosinophil adhesion to serum-coated plates.

4 Fucoidin effectively reduced the binding of the anti-L-selectin mAb MEL-14 to guinea-pig eosinophils. Binding of a P-selectin-IgG chimera to eosinophils was also partially inhibited by fucoidin, but binding of an anti-CD18 or an anti-VLA-4 mAb were unaffected.

5 When given systemically to guinea-pigs, fucoidin suppressed ^{111}In-labelled eosinophil recruitment to sites of allergic inflammation. ^{111}In-labelled eosinophil accumulation induced by platelet-activating factor (PAF) and zymosan-activated plasma (as a source of C5a des Arg) was also inhibited.

6 These results demonstrate a role for fucoidin-sensitive selectins in mediating eosinophil recruitment *in vivo*.

Keywords: Adhesion molecules; cell trafficking; eosinophils; inflammation; fucoidin

Introduction

In response to extravascuar injurious stimuli, circulating leukocytes have to interact with endothelial cells before leaving blood vessels and entering the interstitium. The current paradigm for the accumulation of leukocytes into tissues of the systemic circulation predicts there to be at least three stages of leukocyte/endothelial cell interaction (Carlos & Harlan, 1994; Springer, 1994). Initially, the circulating leukocytes are captured and roll on the endothelial cells of post-capillary venules, a process mediated by selectins present on the leukocytes (L-selectin) and endothelial cells (P- and E-selectin) and their carbohydrate ligands (eg. PSGL-1, ESL-1 and CD34) (Sako *et al*., 1993; Carlos & Harlan, 1994; Varki, 1994; Steegmaler *et al*., 1995). The β_1 integrin VLA-4 may also play a role in mediating the rolling of VLA-4-positive cells *in vivo* (Sriramarao *et al*., 1994; Johnston *et al*., 1996). The rolling leukocyte can then be activated by chemoattractants (eg. C5a, interleukin-8, platelet-activating factor (PAF)) and this leads to up-regulation and increased avidity of β_2 integrins (CD11/CD18 family) present on the leukocyte surface (Springer, 1994). Together with VLA-4, CD11/CD18 mediates the firm adhesion of activated leukocytes to endothelial cells by binding to ligands including ICAM-1 and VCAM-1 (Carlos & Harlan, 1994). The leukocytes are then able to migrate to the interstitium, a process that appears to involve PECAM-1 (CD31) in addition to ICAM-1 (Carlos & Harlan, 1994). Although the cell adhesion molecules which mediate neutrophil, monocyte and lymphocyte migration into tissues have received considerable attention (Carlos & Harlan, 1994; Springer, 1994) there is much less known about the role of these molecules in mediating eosinophil accumulation *in vivo*.

In chronic allergic diseases such as asthma and atopic dermatitis, eosinophils and/or their secretory products (eg. major basic protein) are found in elevated numbers or increased amounts of tissues (Butterfield & Leiferman, 1993). There, eosinophils are not only capable of releasing cationic proteins, but also lipid mediators, cytokines and oxygen radicals which have potential damaging effects on tissue cells (eg. epithelial cells) (Weller, 1991; Kita, 1996). Thus, therapies which inhibit eosinophil accumulation may be of benefit in the treatment of diseases where these cells are thought to play a major pathophysiological role (Teixeira *et al*., 1995b).

In guinea-pig skin, after the intradermal (i.d.) injection of inflammatory mediators (eg. PAF) or in a passive cutaneous anaphylactic (PCA, type I hypersensitivity) reaction, intravenously-injected radiolabelled eosinophils accumulate rapidly into the injected skin sites (Teixeira *et al*., 1993; Weg *et al*., 1994). Using mAbs directed against cell adhesion molecules, our group has previously shown that radiolabelled eosinophil accumulation in this model is dependent on both CD18 and, to a lesser extent, VLA-4 present on the eosinophil surface (Weg *et al*., 1993; Teixeira *et al*., 1994). In the absence of blocking mAbs specific for guinea-pig selectins, we have, in the present study, used fucoidin to assess the role of selectins in mediating eosinophil accumulation in guinea-pig skin. Fucoidin is an algal polymer of fucose-4-sulphate which binds to and inhibits both L- and P-selectin but has little if any inhibitory activity on E-selectin (Skinner *et al*., 1991; Varki, 1994; Yoshida *et al*., 1994). Accordingly, fucoidin has been shown to inhibit human neutrophil homotypic aggregation (Rochon *et al*., 1993), an L-selectin-dependent functional response (Simon *et al*., 1992; 1993). Moreover, systemic administration of fucoidin to experimental animals inhibited basal and stimulated leukocyte rolling in post-capillary venules (Lindbom *et al*., 1992; Ley *et al*., 1993; Kubes *et al*., 1995) and neutrophil accumulation at sites of inflammation in the skin and central

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nervous system of rabbits (Granert *et al*., 1994). Fucoidin has also been shown to inhibit thioglycollate- and antigen-induced recruitment of neutrophils into mouse peritoneum (Bartlett *et al*., 1994).

In this study initial experiments were carried out to evaluate the ability of fucoidin to interfere with guinea-pig eosinophil L-selectin- and CD18-dependent function *in vitro*, as well as potential effects on eosinophil P-selectin ligand and VLA-4. The effects of systemic treatment with fucoidin on eosinophil recruitment to sites of inflammation in guinea-pig skin were then investigated.

Methods

Induction, purification and radiolabelling of guinea-pig eosinophils

Eosinophils were harvested, purified (>95% eosinophils by Kimura stain), radiolabelled with ^{111}In as previously described (Faccioloi *et al*., 1991; Teixeira *et al*., 1994) and were injected, i.v. (2.5×10^6 ^{111}In-eosinophils per animal) into recipient guinea-pigs (350–400 g, Harlan, Bicester) sedated with Hypnorm (Janssen Pharmaceuticals, Oxford; 0.15 ml, i.m.). After 5 min, inflammatory stimuli were injected i.d. in 0.1 ml volumes into the shaved dorsal skin; antigen (BGG) was injected i.d. into sites that had received anti-BGG antiserum 24 h earlier. Each animal received a duplicate of each treatment following a randomized injection plan and ^{111}In-labelled eosinophil accumulation was assessed after 2 h. This time point was chosen based on previous experiments showing that the majority of the ^{111}In-eosinophil accumulation induced by ZAP, PAF and in a PCA reaction occurs during the first 90 min and is complete by 120 min after i.d. injection (Faccioli *et al*., 1991; Weg *et al*., 1994). At this time, a blood sample was obtained by cardiac puncture, the animals were killed by 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 for ^{111}In counts in an automatic 10-head gamma-counter (Canberra Packard Ltd, Panbourne, Berks). Eosinophil numbers in the skin sites are expressed as the number of ^{111}In-eosinophils per skin site.

Systemic treatment with fucoidin was carried out by giving the drug i.v. at the dose of 10 mg kg^{-1}, 15 min before the i.v. injection of the radiolabelled cells. A similar dose of fucoidin has been previously found to be effective at inhibiting leukocyte rolling and neutrophil accumulation *in vivo* (Lindbom *et al*., 1992; Ley *et al*., 1993; Granert *et al*., 1994). When used at a lower dose (3 mg kg^{-1}), fucoidin caused inconsistent inhibition of eosinophil recruitment and further experiments were not carried out with this dose (data not shown).

In some experiments, ^{11}In-eosinophils were pretreated with MEL-14 or rat IgG (300 μg ml^{-1}) for 10 min at room temperature before the final wash. The radiolabelled cells were then washed with excess buffer (300 *g*, 7 min) and resuspended finally at 10^7 ^{111}In-eosinophils ml^{-1}. The radiolabelled eosinophils were then injected i.v. and experiments carried out as outlined above.

Adhesion of eosinophils to serum-coated plastic

The wells of 96-well tissue culture plates (Nunc, Life Technologies) were coated with serum proteins by incubation for at least 2 h at 37°C with RPMI containing 10% FCS. Plates were washed twice with PBS before the addition of 100 μl of ^{111}In-labelled guinea-pig eosinophils (10^6 ml^{-1}) in PBS containing 1.0 mM Ca^{2+}, 0.7 mM Mg^{2+} and 0.25% BSA (w/v). This was followed by mAb (final concentration 80 μg ml^{-1}) or fucoidin (400 and 1200 μg ml^{-1}) and 5 min later the cells were stimulated with PMA (10^{-7}M). After incubation for 30 min at 37°C, the medium was removed and the plate washed 3 times with PBS. Adherent leukocytes were solubilized with neat formic acid and an aliquot counted for radioactivity. The numbers of adhered leukocytes are expressed as the percentage of the total cells added. Experiments were carried out with six replicates on cells from each animal.

Eosinophil aggregation

This was carried out as described previously (Teixeira *et al*., 1995a; 1996). Briefly, guinea-pig eosinophils were purified as described above and resuspended (5×10^6 cells ml^{-1}) in calcium- and magnesium-free PBS containing 10 mM glucose. Calcium and magnesium chloride (final concentrations 1.0 mM and 0.7 mM, respectively) were then added and the cells kept on ice. Ten minutes before use, 300 μl aliquots of the cell suspension were dispensed into siliconized cuvettes and warmed to 37°C with stirring at 700 r.p.m.. The cuvettes were placed into a dual channel platelet aggregometer (Chronolog 440 VS) linked to a dual pen recorder (Chronolog 707) and then activated with PMA 10^{-7} M. The reference cuvette contained buffer alone. Fucoidin (400 and 1200 μg ml^{-1}) and 6.5E (80 μg ml^{-1}) were added 2 min before PMA. MEL-14 or rat IgG (300 μg ml^{-1}) was added 3 min before PAF (10^{-7} M) or C5a (10^{-7} M). Responses were allowed to develop for at least 5 min and the results are expressed as the percentage of maximal aggregation induced by PMA 10^{-6} M (Teixeira *et al*., 1995a). In some experiments, eosinophils were initially pretreated with MEL-14 or rat IgG (300 μg ml^{-1}) for 10 min at room temperature. Excess aggregation buffer was added, the cells were washed (300 *g*, 7 min) and resuspended finally at 5×10^6 eosinophils ml^{-1}. Aggregation experiments were then carried out as described.

Flow cytometric analysis of MEL-14 and P-selectin-IgG binding to guinea-pig eosinophils

Purified eosinophils (5×10^4 cells in 0.5 ml PBS) were incubated with saturating concentrations of MEL-14 (300 μg ml^{-1}), rat IgG (300 μg ml^{-1}), MOPC21 (50 μg ml^{-1}), 2B4 (50 μg ml^{-1}) or 6.5E (10 μg ml^{-1}) for 15 min at 4°C. The cells were then washed twice with PBS, goat anti-mouse (for cells treated with MOPC21, 2B4 and 6.5E) or anti-rat (for cells treated with rat IgG and MEL-14) IgG antibody conjugated with FITC was added (10 μl in 0.5 ml of cell suspension) and the cells were incubated for 15 min at 4°C. For the experiments with P-selectin-IgG and TNFR-IgG, purified eosinophils (5×10^4 cells in 0.5 ml PBS) were incubated with saturating concentrations (50 μg ml^{-1}) of each human IgG fusion protein for 30 min at 4°C. The effect of removing negatively charged sialic acid residues on P-selctin-IgG binding was determined by treating eosinophils with neuraminidase (0.1 u ml^{-1}) for 30 min at 37°C (Sako *et al*., 1993). The effects of EDTA (5 mM) and the anti-P-selectin mAb AK4 (50 μg ml^{-1}) on P-selectin-IgG binding were also assessed. The cells were then washed twice with PBS, FITC-conjugated goat anti-human IgG antibody was added (10 μl in 0.5 ml of cell suspension) followed by incubation for 15 min at 4°C. All cell preparations were then washed twice and fluorescence determined on FACScan flow cytometer (Becton Dickinson, Oxford) and analysed using CELLQuest software.

Materials

The following reagents were purchased from Sigma Chemical Co (Poole): anti-human, anti-rat and anti-mouse IgG-FITC, bovine gamma-globulin (BGG), BSA, formic acid, fucoidin, D-glucose, purified rat IgG, phorbol myristate acetate (PMA) and zymosan. PBS (calcium- and magnesium-free, pH 7.4), HBSS, RPMI and FCS were from Life Technologies (Paisley). PAF (C16) was purchased from Bachem (Saffron Walden). $^{111}InCl_3$ was purchased from Amersham International plc (Little Chalfont). The anti-CD18 monoclonal antibody (mAb) 6.5E (mouse IgG_1) (Andrew *et al*., 1993) and control myeloma IgG_1 (MOPC21) were a gift from Dr M. Robinson (Cellech, Slough). The anti-VLA-4 mAb 2B4 (mouse IgG_1) (Needham

et al., 1994) was a gift from Dr R. Pigott (British Biotech Pharmaceuticals, Oxford). The anti-L-selectin mAb-producing cell line, MEL-14 (rat IgG_{2b}), was purchased from ATCC (Rockville, MD, U.S.A.), grown in a hollow fibre bioreactor and mAb purified by ammonium sulphate precipitation. The anti-human P-selectin mAb AK4 (mouse IgG_1) was obtained from Pharmingen (Cambridge Bioscience, Cambridge). The P-selectin-human IgG fusion protein (P-selectin-IgG) was a gift from Dr S. Watson, Genetech, San Francisco U.S.A. and the human TNFα p55 receptor-IgG fusion protein (TNFR-IgG) was a gift from Prof. M. Feldmann, The Charing Cross Sunley Research Centre, London.

Zymosan-activated plasma (ZAP) was used as a source of guinea-pig C5a des Arg and was prepared as previously described (Teixeira *et al*., 1994). Details of the preparation of IgG_1-rich anti-BGG anti-sera and doses of BGG for the PCA reaction are described elsewhere (Weg *et al*., 1994).

Fucoidin was prepared daily by dissolving in the appropriate assay buffer or saline (for systemic use) and was filtered (0.2 μm) before use.

Statistical analysis

Data were analysed by the statistical programme INSTAT (Graphpad Software V2.03). For the *in vitro* experiments, one-way analysis of variance (ANOVA) was carried out. Two-way ANOVA was used for the *in vivo* data. *P* values were assigned by the Student Newman Keul's post-test and results were considered significant when $P<0.05$. Data are shown as the mean $\pm$ s.e.mean of *n* experiments.

Results

Effects of fucoidin on eosinophil function in vitro

A typical aggregation trace of eosinophils stimulated with PMA is shown in Figure 1a. In the presence of fucoidin (1200 μg ml^{-1}) the peak aggregation response was inhibited and the onset of the response was delayed (Figure 1a); these data are consistent with our previous findings (Teixeira *et al*., 1996). In a series of experiments, at concentrations of 400 and 1200 μg ml^{-1}, fucoidin inhibited PMA-induced eosinophil aggregation by 35% and 52%, respectively (Figure 1b). The effect of fucoidin was specific since a control carbohydrate, dermatan sulphate, was not inhibitory at the same concentration (Teixeira *et al*., 1996). As previously shown (Teixeira *et al*., 1995a; 1996), the anti-CD18 mAb 6.5E also partially inhibited by 43% PMA-induced eosinophil homotypic aggregation (Figure 1b). This was not due to insufficient mAb because at the concentration used (80 μg ml^{-1}), 6.5E completely suppressed eosinophil adherence to serum-coated plates (data not shown), consistent with earlier findings (Teixeira *et al*., 1994). In contrast to the anti-CD18 mAb, fucoidin had no inhibitory effect on eosinophil adhesion (data not shown). These data suggest that fucoidin binds to guinea-pig eosinophils and inhibits eosinophil aggregation (a L-selectin-dependent functional response; Teixeira *et al*., 1996) but does not inhibit eosinophil adhesion to serum-coated plastic (a CD18 dependent functional response; Teixeira *et al*., 1994).

We have previously shown the anti-murine L-selectin mAb, MEL-14, concentration-dependently binds to guinea-pig eosinophils (Teixeira *et al*., 1996). Saturation of binding sites, as assessed by FACS analysis, occurred at around 300 μg ml^{-1} of MEL-14 (Teixeira *et al*., 1996). Binding of MEL-14 was reduced by 70% when eosinophils were treated with PMA (10^{-8} M, 10 min) consistent with shedding of L-selectin (data not shown). Because MEL-14 is a functional blocker in the guinea-pig (Teixeira *et al*., 1996) and appears to bind to the functional lectin domain of L-selectin (Bowen *et al*., 1990; Lasky, 1995), we tested whether fucoidin could inhibit MEL-14 binding to eosinophils. As shown in Figure 2a, fucoidin (1200 μg ml^{-1}) effectively reversed the binding of MEL-14 to eosinophils. In contrast, the same concentration of fucoidin had no effect on the binding of an anti-CD18 (6.5E) or an anti-VLA-4 (2B4) mAb to these cells (Figure 2b and c).

Because fucoidin has been previously shown to bind and inhibit P-selectin in addition to L-selectin (Skinner *et al*., 1991; Varki, 1994; Yoshida *et al*., 1994), we tested whether guinea-pig eosinophils possessed a ligand for P-selectin and whether fucoidin could interfere with binding to this ligand. Initial experiments demonstrated that P-selectin-IgG concentration-dependently bound to guinea-pig eosinophils with saturation occurring at around 50 μg ml^{-1} (data not shown) and that this binding could be reduced $>70\%$ by eosinophil pretreatment with neuraminidase (Figure 3a), consistent with studies of human PSGL-1 (Sako *et al*., 1993). Further evidence for specific binding was indicated by experiments showing a marked reduction in binding in the presence of 5 mM EDTA (data not shown) and the anti-P-selectin mAb AK4 (Figure 3b). As shown in Figure 3c, fucoidin (1200 μg ml^{-1}) partially reversed P-selectin-IgG binding. These data demonstrate that guinea-

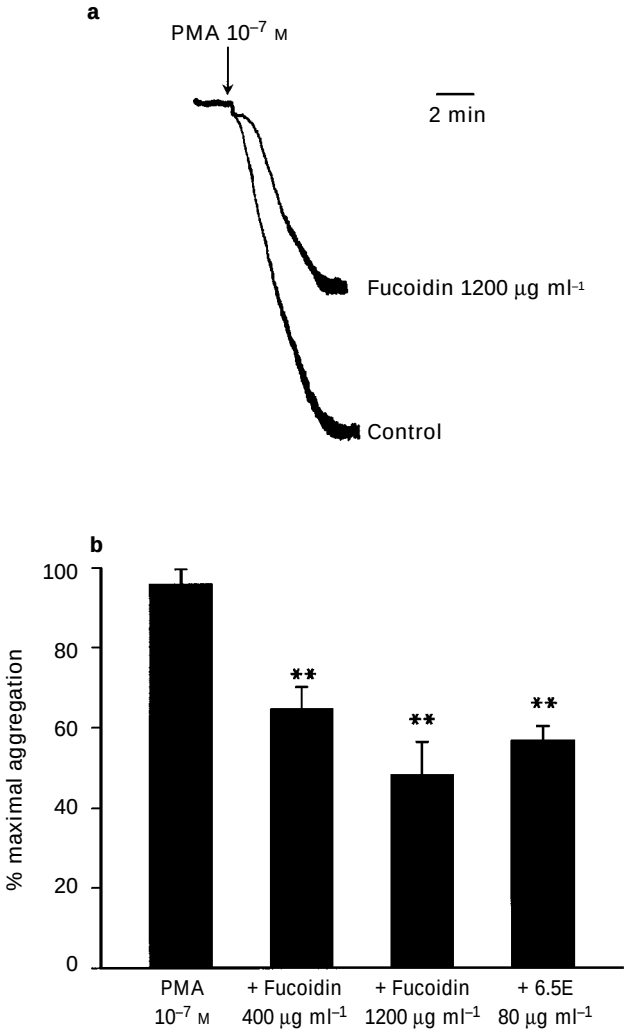

Figure 1 Effect of fucoidin on eosinophil homotypic aggregation. (a) Trace of a typical experiment demonstrating the inhibitory effects of fucoidin (1200 μg ml^{-1}) on eosinophil homotypic aggregation induced by PMA (10^{-7} M). (b) Eosinophils were pretreated for 2 min with fucoidin (400 and 1200 μg ml^{-1}) or an anti-CD18 mAb (6.5E, 80 μg ml^{-1}) and then activated with PMA (10^{-7} M). Aggregation was quantified as changes in light transmission in a platelet aggregometer. Data are shown as the % of maximal aggregation (in the presence of PMA 10^{-6} M) and are the mean $\pm$ s.e.mean of 4–5 experiments. $^{**}P<0.01$ when compared to responses in the presence of PMA alone.

pig eosinophils possess a sialylated P-selectin ligand (possibly PSGL-1) and that fucoidin partly blocks the interaction of human P-selectin with this ligand.

Effects of fucoidin on eosinophil recruitment in vivo

The effect of systemic treatment with fucoidin on ^{111}In-eosinophil accumulation *in vivo* was evaluated in guinea-pig skin. When given 15 min before the i.v. injection of the radiolabelled cells, fucoidin (10 mg kg^{-1}) inhibited ^{111}In-eosinophil accumulation induced by 10, 30 and 100% ZAP by 77, 66 and 60%, respectively (Figure 4a). Accumulation of ^{111}In-eosinophils in the PCA reaction (0.01 to 1.0 μg of BGG per site) was inhibited by up to 73% by fucoidin (Figure 4b). In addition, fucoidin inhibited by 77% ($P<0.05$) PAF (10^{-9} mol per site)-induced accumulation of ^{111}In-eosinophils (control response:

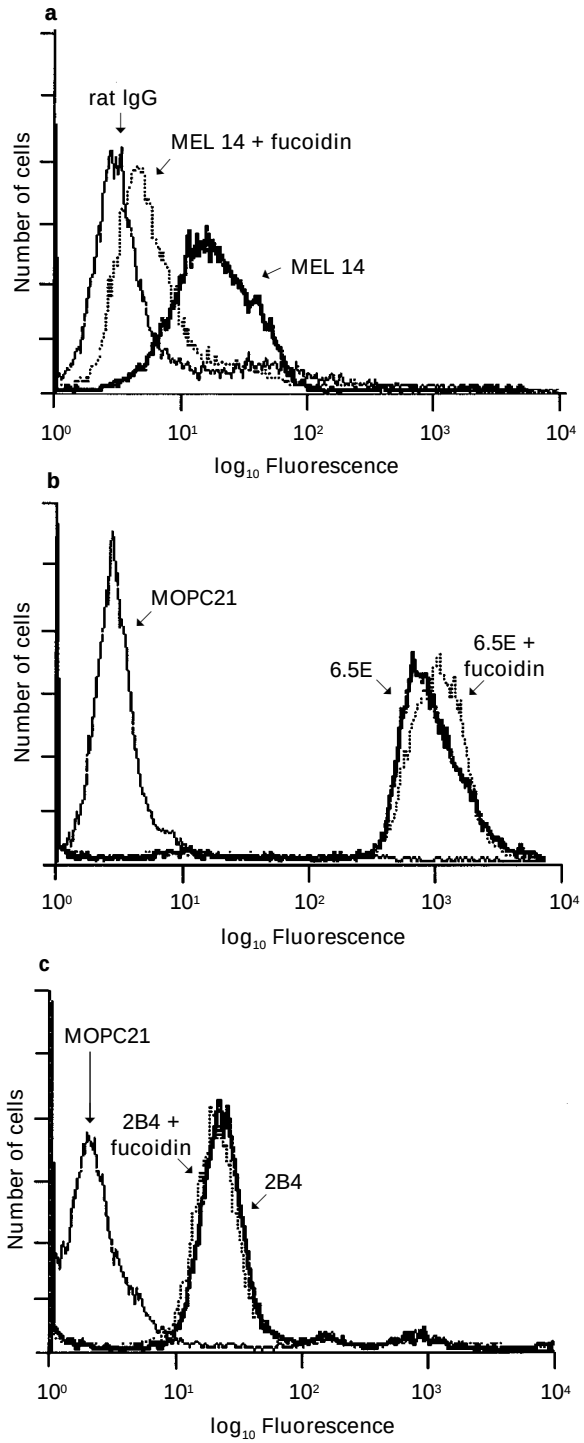

Figure 2 Inhibition of MEL-14 binding to guinea-pig eosinophils by fucoidin, as assessed by flow cytometry. Eosinophils were incubated with saline or fucoidin (1200 μg ml^{-1}) before the binding of (a) the anti-L-selectin mAb MEL-14, (b) the anti-CD18 mAb 6.5E and (c) the anti-α4 mAb 2B4 were assessed. Control binding of rat IgG and MOPC21 is also shown. Data are representative of 2 experiments.

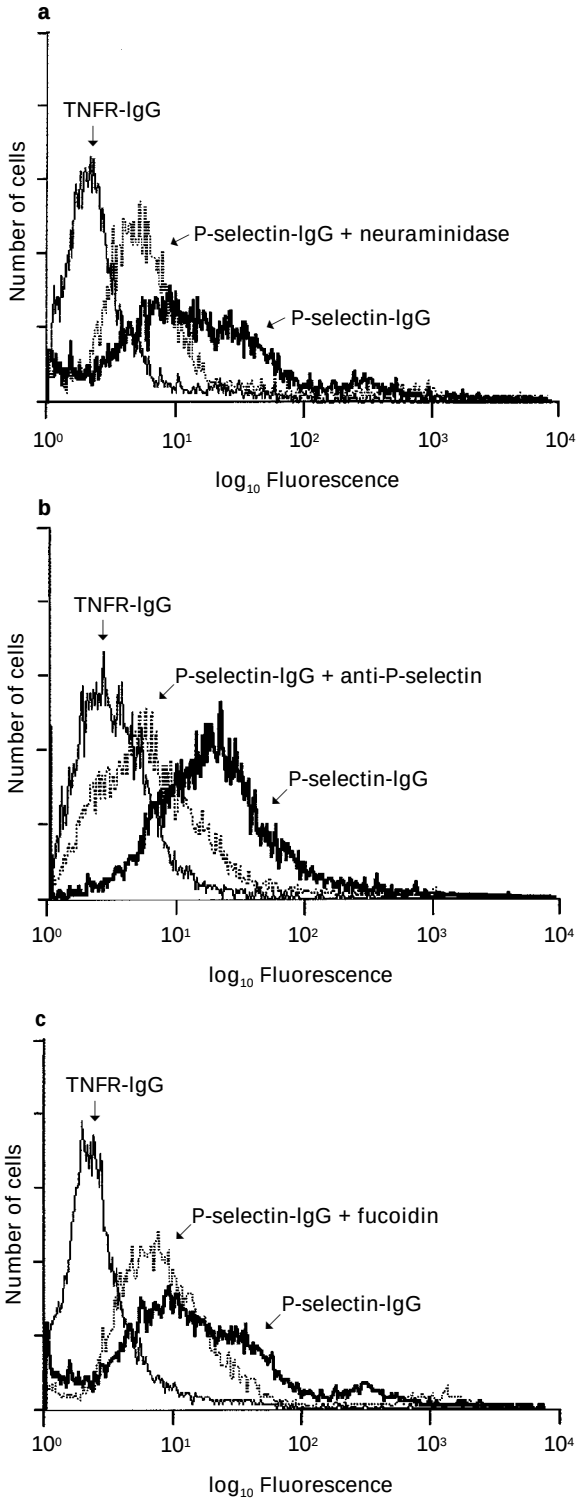

Figure 3 Effect of neuraminidase, a P-selectin mAb and fucoidin on P-selectin-IgG binding to guinea-pig eosinophils as assessed by flow cytometry. Eosinophils were incubated with (a) saline or neuraminidase (0.1 u ml^{-1}) and (c) saline or fucoidin (1200 μg ml^{-1}) before the binding of P-selectin-IgG was assessed. In (b) the anti-P-selectin mAb (AK4, 50 μg ml^{-1}) was incubated with P-selectin-IgG before incubation with eosinophils. Binding of TNFR-IgG was used as a control. Data are representative of 1–3 experiments.

1969 ± 565 ^{111}In-eosinophils per site). Fucoidin had no effect on the levels of circulating ^{111}In-eosinophils measured at two hours after their i.v. injection (10.2 ± 1.8% and 11.0 ± 3.0% of injected dose circulating in saline- and fucoidin-treated animals, respectively, $n = 5$) suggesting that clearance of radiolabelled eosinophils was not the mechanism of its inhibitory effect *in vivo*.

Effects of MEL-14 on eosinophil aggregation and eosinophil recruitment in vivo

When used at a saturating concentration (300 μg ml^{-1}), the anti-L-selectin mAb MEL-14 completely suppressed eosinophil aggregation induced by 10^{-7} M C5a (Teixeira *et al.*, 1996). Similarly, MEL-14 (300 μg ml^{-1}) abrogated eosinophil aggregation induced by PAF (10^{-7} M) (Figure 5a). *In vivo* experiments were designed to assess whether MEL-14 pretreatment of eosinophils would affect recruitment of these cells to sites of inflammation in guinea-pig skin. Under similar experimental conditions, the anti-VLA-4 mAb HP1/2 (50 μg ml^{-1}) was shown to inhibit significantly eosinophil accumulation in guinea-pig skin (Weg *et al.*, 1993). Thus, ^{111}In-eosinophils were pretreated with MEL-14 (300 μg ml^{-1}), the cells were washed and injected i.v. into guinea-pigs. However, at this pretreatment dose, MEL-14 had no effect on ^{111}In-eosinophil recruitment induced by PAF, ZAP or in a PCA reaction (Table 1). We then tested whether MEL-14 was still completely inhibiting eosinophil aggregation after the cells were washed. Figure 5b shows that MEL-14 was considerably less effective as an inhibitor of aggregation when the excess mAb was removed; eosinophil aggregation induced by PAF

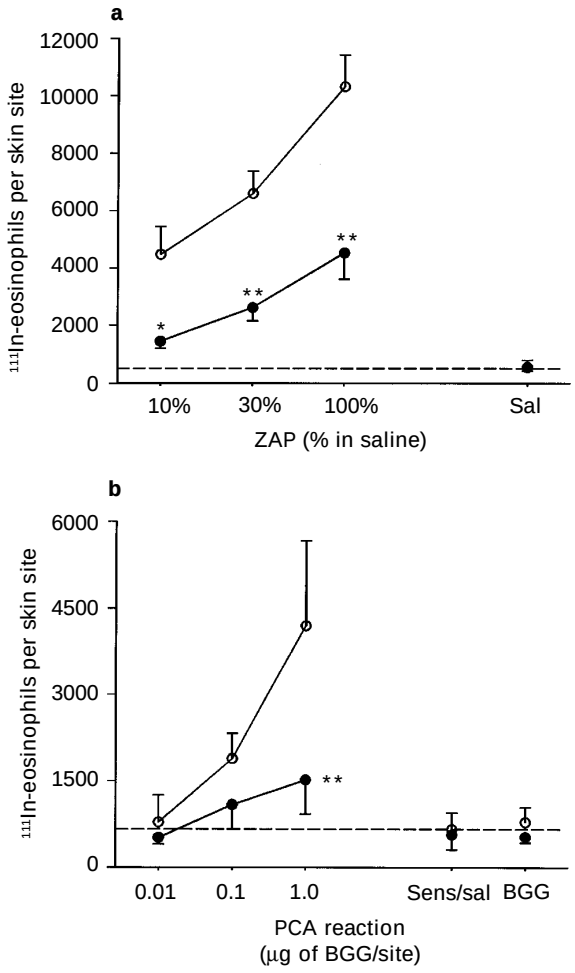

Figure 4 Effect of systemic treatment with fucoidin on ^{111}In-eosinophil accumulation induced by (a) zymosan-activated plasma (ZAP) and (b) the PCA reaction. Fucoidin (10 mg kg^{-1}, i.v.) (●) or saline (○) was given 15 min before the i.v. administration of the ^{111}In-labelled eosinophils. ZAP (10 to 100% dilution in saline) or antigen (BGG, 0.01 to 1.0 μg per site) in sites previously sensitized with an IgG$_1$-rich antiserum (shown as PCA reaction) were given i.d. and ^{111}In-eosinophil accumulation assessed after 2 h. The line across the graphs represent background values obtained in sensitized sites injected with saline. Also shown is the response to BGG (1 μg) in naive skin sites. Results are shown as the number of ^{111}In-eosinophils per skin site and are the mean ± s.e.mean (vertical lines) of 5 pairs of animals. $^*P < 0.05$ and $^{**}P < 0.01$ when compared to responses in control animals.

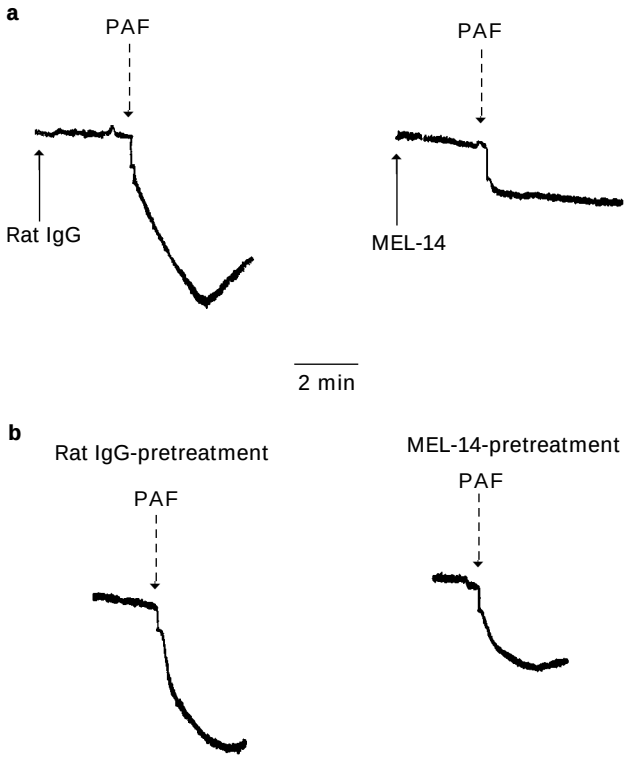

Figure 5 Effect of MEL-14 on eosinophil homotypic aggregation. (a) Eosinophils were pretreated with rat IgG or MEL-14 (both 300 μg ml^{-1}) before addition of PAF. The aggregation response to PAF was almost abolished by MEL-14. (b) Eosinophils were treated with rat IgG or MEL-14 (300 μg ml^{-1}) and then washed before assessment of PAF-induced aggregation. Under these conditions MEL-14 was clearly less effective as an inhibitor of aggregation. Data are representative of 2 experiments.

Table 1 Effect of *in vitro* pretreatment with rat IgG or MEL-14 on ^{111}In-eosinophil accumulation in guinea-pig skin induced by PAF, ZAP and the PCA reaction

	^{111}*In-eosinophils per skin site*	
	Rat IgG pretreatment	*MEL-14 pretreatment*
PAF	1941 ± 422	1775 ± 180
ZAP	6278 ± 786	5927 ± 962
PCA	1993 ± 388	1999 ± 388
Saline	172 ± 24	147 ± 15

^{111}In-eosinophils were incubated with rat IgG or MEL-14 (both 300 μg ml^{-1}) before washing and i.v. infusion into recipient guinea-pigs (see Methods). PAF (10^{-10} mol per site), ZAP (30% dilution in saline), antigen (3 μg of BGG per site) in sites previously sensitized with antiserum (PCA), and saline were given i.d. and ^{111}In-eosinophil accumulation assessed after two hours. Data shown are means ± s.e.mean. There was no significant difference between the treatment groups ($n = 4$ pairs)

was inhibited by only 43% (Figure 5b) and eosinophil aggregation induced by C5a by 25% (data not shown). Flow cytometry showed that the amount of MEL-14 bound to the surface was reduced after washing and further MEL-14 binding could be detected in the presence of excess MEL-14. These results suggest that although MEL-14 is an effective blocker of eosinophil function *in vitro*, it has low affinity for guinea-pig L-selectin. The high concentration of MEL-14 needed to achieve saturation (Teixeira *et al*., 1996) is in accordance with this suggestion.

Discussion

Eosinophils are thought to play a major role in the pathophysiology of allergic diseases. For example, in asthmatic patients the numbers of eosinophils in the bronchoalveolar lavage fluid are elevated and correlate with the degree of lung dysfunction (reviewed in Corrigan & Kay, 1992). In addition, eosinophil secretory products, such as major basic protein, are elevated in the bronchoalveolar lavage fluid and can damage epithelial cells at concentrations relevant to those found *in vivo* (Djukanovic *et al*., 1990), Eosinophil-derived basic proteins are present at sites of chronic allergic cutaneous disease in man (Leiferman, 1991). Moreover, eosinophils also produce cytokines and lipid mediators that could exacerbate the inflammatory process (Weller, 1991; Kita, 1996). It is thus possible that therapeutic strategies aimed at inhibiting eosinophil accumulation *in vivo* will be of benefit in the treatment of allergic diseases (Teixeira *et al*., 1995b).

There are a number of studies which have evaluated the adhesion pathways used by eosinophils to accumulate at sites of inflammation *in vivo*. Whereas there is evidence to suggest that both CD18 and VLA-4 are important for eosinophil recruitment in several animal models (eg. Weg *et al*., 1993; Milne & Piper, 1994; Pretolani *et al*., 1994; Teixeira *et al*., 1994), there is very little information regarding the role of selectins. In monkeys, an anti-E-selectin mAb had no inhibitory effect on eosinophil recruitment into the lung after antigen challenge (Wegner *et al*., 1991). In contrast, by use of a xenogenic system, Sriramarao *et al*. (1994) found that both L-selectin and VLA-4 partly supported human eosinophil rolling at physiological shear rates in rabbit post-capillary venules. Similarly, *ex vivo* studies have suggested that eosinophil adhesion to nasal polyp endothelium is P-selectin-dependent (Wardlaw *et al*., 1994) and *in vitro* experiments have shown that L- and E-selectin are important for eosinophil adhesion to cytokine activated endothelial cells (Weller *et al*., 1991; Knol *et al*., 1994). Thus, the limited available data suggest that selectins support eosinophil adhesion *in vitro* and rolling *in vivo* but there are no studies to date assessing what role selectins play in eosinophil recruitment into tissue.

We have used the algal polysaccharide fucoidin in our experiments. Fucoidin has been shown to bind to both L- and P-selectin and effectively inhibit L- and P-selectin-dependent function *in vitro* (Skinner *et al*., 1991; Varki, 1994; Yoshida *et al*., 1994). For example, mediator-induced neutrophil homotypic aggregation, an L-selectin-dependent functional response, is concentration-dependently inhibited by pretreatment with fucoidin (Rochon *et al*., 1993). Similarly fucoidin also inhibits P-selectin binding to human neutrophils or HL60 cells (Skinner *et al*., 1991). In contrast, fucoidin does not seem to bind to and inhibit E-selectin (Mebius & Watson, 1993; Varki, 1994). The ability of fucoidin to inhibit L- and P-selectin function is probably due to the high amount of fucosylated sites present on the molecule which may mimic the selectin ligands (Patankar *et al*., 1993; Varki, 1994).

In our studies, fucoidin inhibited PMA-induced eosinophil homotypic aggregation by up to 60% but, at a similar concentration, it had no inhibitory effect on eosinophil adhesion to serum-coated plates. As we have previously shown (Teixeira *et al*., 1996), 6.5E partially inhibited aggregation at a concentration which abrogated eosinophil adherence to serum-coated plates. These results support the notion that eosinophil adherence to serum-coated plastic under static conditions is a CD18-dependent process (Harlan *et al*., 1985) whereas eosinophil homotypic aggregation is dependent on both CD18 and a fucoidin-inhibitable component, possibly L-selectin (Teixeira *et al*., 1995a; 1996). The ability of fucoidin to inhibit the binding of MEL-14 to guinea-pig eosinophils provides additional evidence to suggest that fucoidin binds to L-selectin in this species. Alternatively, fucoidin may induce shedding of L-selectin from the eosinophil surface. While we cannot distinguish between these possibilities, less L-selectin would be available for adhesion on the eosinophil whatever the mechanism. In contrast, we found fucoidin to be less effective as an inhibitor of P-selectin binding to guinea-pig eosinophils. While it does not prove that fucoidin binds guinea-pig P-selectin, the data suggests that guinea-pig eosinophils possess a neuraminidase-sensitive P-selectin ligand whose recognition of human P-selectin is partly reduced by fucoidin. Additional experimentation with guinea-pig-specific reagents will be required to pursue these observations. That human eosinophils have been shown to express a P-selectin ligand with characteristics of PSGL-1 (Symon *et al*., 1996) suggest that further studies would be worthwhile.

When tested *in vivo* at a dose (10 mg kg^{-1}) previously shown to diminish leukocyte rolling under different experimental conditions, fucoidin inhibited the accumulation of radiolabelled eosinophils into skin sites injected with ZAP and PAF and in a passive cutaneous anaphylactic reaction. The inhibition of the recruitment of cells to the skin was independent of an effect of fucoidin on circulating radiolabelled eosinophils, since these were not altered by systemic administration of the drug. Since fucoidin has been previously shown to inhibit leukocyte rolling and neutrophil recruitment *in vivo*, it is likely that it was inhibiting radiolabelled eosinophil accumulation in guinea-pig skin in a similar manner. Based on a blood volume of 70 ml kg^{-1}, we calculate the plasma concentration of fucoidin in these animals to be approximately 300 μg ml^{-1}. Our previous studies (Teixeira *et al*., 1996) show this concentration to be around the IC_{50} for inhibition of C5a-induced eosinophil aggregation. In addition, selectin inhibitors tend to be more effective under shear conditions, specifically those found in the vasculature (Lasky, 1995).

In order to test directly whether inhibition of L-selectin was important for the *in vivo* inhibitory effects of fucoidin, we investigated the effects of MEL-14 pretreatment on eosinophil recruitment in guinea-pig skin. Our approach was to pretreat eosinophils *in vitro* with MEL-14 before i.v. infusion since this has proved successful when studying other leukocyte adhesion molecules (Nourshargh *et al*., 1989; Weg *et al*., 1993) and has the advantage that less antibody is used than with systemic treatment; we were unable to carry out systemic treatment with the MEL-14 because of the large quanity of mAb that would be required to achieve an effective inhibitory concentration *in vivo* (approximately 24 mg kg^{-1}). However, because of its presumed low affinity, most of the inhibitory effects of MEL-14 on eosinophil aggregation were lost when the excess of the mAb was removed and, under these circumstances, MEL-14 had no inhibitory effect on eosinophil recruitment *in vivo*. Thus, additional experiments with appropriate tools (ie. anti-guinea-pig selectin mAbs) are necessary to identify whether inhibition of P- or L-selectin or both are important for the inhibitory effects of fucoidin *in vivo*. Our flow cytometry analysis data suggest that fucoidin is a more effective inhibitor of eosinophil L-selectin indicating that this molecule may be the more important for eosinophil recruitment in guinea-pig skin. However, studies in the cat mesentery suggested that fucoidin inhibited a rolling pathway independent of L- and P-selectin (Kubes *et al*., 1995), although this requires further investigation.

In conclusion, our studies support a role for selectins in mediating the recruitment of eosinophils into sites of acute cutaneous allergic and mediator-induced inflammation. Fucoidin also inhibits neutrophil accumulation in rabbit central

nervous system (Granert *et al*., 1994), mouse peritoneum (Bartlett *et al*., 1994) and guinea-pig skin (data not shown). However, subtle differences in the ligands for P- and E-selectin on the surface of eosinophls and neutrophils (Wein *et al*., 1995) may allow the development of carbohydrate-targeted drugs which specifically inhibit the recruitment of one cell type but not the other, although this may not be neccessary if the predominant chemoattractants generated at the inflammatory site are eosinophil specific (eg. eotaxin). Such carbohydrate-targeted therapies may be useful for the treatment of allergic diseases where eosinophils are thought to play an important pathophysiological role.

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