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Alteration of neutrophil trafficking by a lipocortin 1 N-terminus peptide

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Abstract

Sialidase, fucoidin and a peptide corresponding to most of lipocortin 1 N-terminus, termed LC1-(Ac2–26)-peptide, induced an intense 2 h neutrophilia whereas a monoclonal antibody to murine CD11b induced an effect by 1 h. The neutropenic response stimulated by platelet-activating factor (PAF) was significantly reduced in the presence of sialidase, fucoidin, LC1-(Ac2–26)-peptide and monoclonal antibody anti-CD11b. Neutrophil migration into a 6-day-old mouse air-pouch induced by interleukin-1 was inhibited by all the pharmacological agents. In vitro, PAF up-regulated CD11b expression on the neutrophil surface but neither human or mouse LC1-(Ac2–26)-peptide inhibited this response. CD11b up-regulation on neutrophils occurred after PAF administration in vivo and was maximal at 2 min. LC1-(Ac2–26)-peptide mimics the action of agents interfering with leucocyte rolling and adhesion in vivo, however, does not inhibit CD11b up-regulation in vitro suggesting other phenomena are important in the activity of this peptide.

Keywords: Sialidase; Fucoidin; β_2-Integrin; CD11b; Interleukin-8; Interleukin-1

1. Introduction

Lipocortin 1 is a glucocorticoid-inducible protein which mediates several anti-inflammatory actions exerted by these potent hormones (Flower and Rothwell, 1994). Indeed, we have recently shown that endogenous and exogenous lipocortin 1 plays a role in the inhibitory effect that glucocorticoid hormones have on neutrophil migration. In these studies dexamethasone inhibited neutrophil accumulation into a 6-day-old air-pouch induced by local administration of the pro-inflammatory cytokine interleukin-1 (Perretti and Flower, 1993). Its effect was mimicked by exogenously administered lipocortin 1 and abrogated by passive immunisation of animals with a panel of specific antibodies raised against this protein. More recently the ability of a N-terminus peptide of lipocortin 1, peptide acetyl-2–26 (thereafter referred to as LC1-(Ac2–26)-peptide), to mimic some of lipocortin 1 actions has been reported. In particular, intravenous treatment with LC1-(Ac2–26)-peptide resulted in a dose-dependent inhibition of neutrophil migration elicited by interleukin-1 (Cirino et al., 1993), interleukin-8 (Perretti et al., 1993b), and substance P (Perretti, 1994). This lack of specificity with respect to the stimulus applied seems to indicate that LC1-(Ac2–26)-peptide, and possibly its parent protein lipocortin 1, inhibits an important step of neutrophil activation which may be fundamental for in vivo migration (Perretti et al., 1993b; Perretti, 1994).

The number of polymorphonuclear leucocytes within the blood compartment at any particular moment is the overall result of an equilibrium between free circulating polymorphonuclear leucocytes and those which are sequestered: each of these pools represents approximately 50% of total polymorphonuclear leucocytes (MacNee and Selby, 1993). Leucocyte sequestration is due to several phenomena such as true margination (this term refers correctly to neutrophils, located in the postcapillary venules, which roll slowly along vessel walls), mechanical trapping in the capillaries of the pulmonary vascular bed, adhesion to the endothelium, and slow moving through the microvasculature (for a review see MacNee and Selby, 1993). Whereas polymorphonuclear leucocyte trapping in the lung microcirculation appears to be due to the difference in size between leucocytes ($\varnothing \approx 8$ μm) and capillaries of this

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vascular bed ($\varnothing \approx 5$ μm) (MacNee and Selby, 1993; Worthen et al., 1989), leucocyte margination and adhesion to the endothelium are active phenomena mediated by a panel of different adhesion molecules. Experiments performed using the intravital microscopy technique have shown not only that leucocyte rolling is sustained by selectins while adhesion to the vessel wall is mediated by β_2-integrins, but also that the first event is an essential requisite for the second one to occur (Von Andrian et al., 1991).

Amongst neutrophil membrane adhesion proteins, a central role is played by L-selectin and by the CD11/CD18 complex (Springer, 1994). L-Selectin (and E-selectin or P-selectin for that concerning the endothelial wall) mediates leucocyte rolling which requires the involvement of carbohydrate counterparts. Agents like sialidase, which remove carbohydrate moieties protruding from the cell membrane, and fucoidin, which specifically blocks this interaction, have been reported to prevent leucocyte rolling (Ley et al., 1991) in this way interfering with cell homing to specific tissue sites (Rosen et al., 1989; Simmons and Cattle, 1992). The CD11/C18 complex (β_2-integrins) consists of three components each with a unique α-subunit, CD11a, CD11b, CD11c, and a common β-chain CD18 (Albelda and Buck, 1990). Expression of CD11b/CD18 (Mac-1, CR3) on the neutrophil surface is increased following stimulation by a variety of chemotactic agents. As well as being important for appropriate leucocyte-leucocyte and leucocyte-endothelial interactions, this response also acts as an index of neutrophil activation both in vitro and in vivo (Shalit et al., 1988; Witthaut et al., 1994).

The aim of this study was to further investigate the effect of LC1-(Ac2–26)-peptide on polymorphonuclear leucocyte functions. In vivo, the peptide was compared with compounds known to alter specific phenomena, such as sialidase and fucoidin, which block leucocyte rolling, and a monoclonal antibody raised against murine CD11b which blocks adhesion (Rosen and Gordon, 1990). The effect of these agents was analysed in three different in vivo experimental conditions which reflected a different state of activation of polymorphonuclear leucocyte and/or endothelium: (i) physiological interaction, by simply measuring the number of circulating polymorphonuclear leucocytes; (ii) neutrophil activation, by evaluating their effect upon chemoattractant-induced neutropenia; (iii) endothelial activation, by measuring polymorphonuclear leucocyte infiltration into a 6-day-old murine air-pouch in response to interleukin-1β, a process which is primarily due to endothelial activation through the type I receptor of this cytokine (Perretti et al., 1993c). Finally, measurement of CD11b/CD18 neutrophil surface expression, an indicator of neutrophil activation, was used to determine more precisely the mechanisms underlying the inhibitiory activity of the LC1-(Ac2–26)-peptide.

2. Materials and methods

2.1. Animals

Male Swiss Albino mice (28–32 g) were purchased from Tuck (Essex, UK) and kept on a standard chow pellet diet and tap water ad libitum. For air-pouch experiments mice with weights between 22–25 g at day 0, first day of air injection, were used reaching 28–32 g by day 6 of the experiment.

2.2. Neutrophil kinetics in the blood

All compounds were dissolved in phosphate-buffered solution containing 0.1% bovine serum albumin. Treatments were given intravenously (i.v.) at selected times prior to blood collection. Mice were bled by cardiac puncture using heparinised (20 μl of heparin 2000 U/ml) syringes under halothane anaesthesia. This procedure minimised the stress experienced by the animals without affecting the profile of circulating leucocytes. Total white cell counts were determined using a Coulter counter (Coulter Electronics, Luton, UK). Differential counts were obtained from blood smears stained with Turk's solution (crystal violet 0.01% w/v in acetic acid 3% v/v) and the total number of polymorphonuclear leucocytes in each blood sample was then calculated. In some cases neutrophil activation was achieved by injecting either platelet-activating factor (PAF) or interleukin-8 prior to blood collection.

2.3. Model of inflammation

Polymorphonuclear leucocyte accumulation into a 6-day-old murine air-pouch in response to murine interleukin-1β was evaluated as recently described (Perretti and Flower, 1993). Briefly, air-pouches were formed by s.c. injection, in the neck region, of 2.5 ml of air on day 0 and day 3. On day 6, 5 ng of murine recombinant interleukin-1β was dissolved in 0.5 ml of carboxymethylcellulose (0.5% in phosphate-buffered solution) and injected into the air-pouches. LC1-(Ac2–26)-peptide was given i.v. 20 min prior to challenge with the cytokine, whereas monoclonal antibody CD11b was administered 16 h before, as previously reported (Rosen and Gordon, 1987; Perretti et al., 1993b). Sialidase and fucoidin were given i.v. 2 h before interleukin-1β. Air-pouches were washed thoroughly with 2 ml of phosphate-buffered solution containing heparin (50 U/ml) 4 h following challenge with the cytokine, and cells counted after staining in Turk's solution using a Neubauer haematocytometer. The number of poly-

morphonuclear leucocytes recovered from each pouch was then calculated.

2.4. CD11b up-regulation on murine leucocytes

The expression of the CD11b antigen on the membrane of murine leucocytes was measured by flow cytometric analysis using a whole blood technique.

In vitro

Blood was pooled from at least 3–4 mice. Aliquots (200 μl) were incubated with either PAF, interleukin-8 or vehicle for 15 min at room temperature. Previously, this adhesion molecule has been shown to reach maximal levels of expression 10–15 min after PAF and interleukin-8 stimulation, thus a 15 min time-point was chosen to assess changes in vitro (Shalit et al., 1988; Roberts et al., 1993). In some experiments blood was preincubated with LC1-(Ac2–26)-peptide (100 μg/ml) or vehicle for 10 min prior to the addition of PAF (10^{-7} M). Aliquots were then washed twice with phosphate-buffered solution and stained at 4° C with a specific rat anti-murine CD11b monoclonal antibody (clone 5C6) (Rosen and Gordon, 1987) for 60 min. After two washes, cells were then stained with fluorescein isothiocyanate-conjugated anti-rat immunoglobulin G antibody for 5 min at room temperature. After two washes in phosphate-buffered solution red blood cell lysis was performed with Immuno-Lyse for 1 min. For storage purposes, cells were fixed with an equal volume of 2% paraformaldehyde in phosphate-buffered solution. Flow cytometry was performed using a FACScan analyser (Becton-Dickinson, Cowley, UK) with air-cooled 100 mW argon ion laser tuned to 488 nm and Consort 32 computer running Lysis II software. Three distinct cell populations (lymphocytes, monocytes and polymorphonuclear leucocytes) were discriminated using forward and side scatter characteristics. The rat anti-murine CD11b antibody recognised the CD11b antigen on neutrophils and monocytes, leucocyte populations known to express this adhesion molecule on their surface. As expected CD11b was not detected on lymphocytes. The number of molecules of endogenous CD11b per cell was estimated by median fluorescence intensity in the FL1 channel (wavelength of 540 nm) with reference to microbeads labelled with standard numbers of molecules of fluorescein-isothiocyanate (Flow Cytometry Standards Corp., NC, USA) (Le Bouteiller et al., 1983).

Ex vivo

Mice (at least 3 per treatment group) received either a bolus dose i.v. of PAF (100 ng) or vehicle (phosphate-buffered solution containing 0.1% bovine serum albumin, 100 μl) at selected times prior to blood collection by cardiac puncture. Blood samples for each treatment were pooled. Total leucocyte and differential counts were obtained as previously described, for determination of total polymorphonuclear leucocyte number. Aliquots (200 μl) of blood were stained with specific antibody at 4° C and then prepared for flow cytometric analysis as before.

2.5. Materials

Murine recombinant interleukin-1β was a generous gift of Dr R.C. Newton (DuPont-Merck, Wilmington, DE, USA). Human recombinant interleukin-8 was generously supplied by Dr I. Lindley (Sandoz Forschungsinstitut, Vienna, Austria). Fucoidin (Fucoidan; *Fucus vesiculosus*, Cat. No. F-5631), sialidase (*V. cholerae*, type III, Cat. No. N-7885), platelet-activating factor (PAF; C16 form, Cat. No. P-4904), rat immunoglobulin G and low endotoxin bovine serum albumin were obtained from Sigma Chemical Co. (Poole, UK). Rat anti-mouse CD11b monoclonal antibody (clone 5C6) was either a generous gift of Dr D. Bloxham (Celltech, Slough, UK) or purchased from Serotec (Oxford, UK). Fluorescein-isothiocyanate anti-rat immunoglobulin G antibody was also purchased from Serotec (Oxford, UK). Human LC1-(Ac2–26)-peptide (amino acid sequence: *N*-acetyl-AMVSEFLKQAWFIENEEQEYVQTVK; molecular weight = 3 kDa) and mouse LC1-(Ac2–26)-peptide (amino acid sequence: *N*-acetyl-AMVSEFLKQARFLENQEQEYVQAVK) were generously supplied by Dr M. Toda (ONO Pharmaceuticals Co., Osaka, Japan). Lipocortin 5-(204–212)-peptide (nonapeptide 204–212 of lipocortin 5, amino acid sequence: SHLRKVFDK) was purchased from Proteins and Peptide Research (Reading, UK).

2.6. Statistics

Statistical differences were calculated on original values using analysis of variance followed by the Bonferroni test for intergroup comparisons, or by the unpaired Student's *t*-test when only two groups were compared.

3. Results

3.1. Drug effect on the number of circulating neutrophils

Intravenous administration of sialidase (0.01 mU) resulted in a late-onset neutrophilia, with the number of circulating polymorphonuclear leucocytes unmodified at 1 h and almost 3 times that measured in vehicle-treated mice at the 2 h time-point (Fig. 1). A higher dose of the enzyme (0.1 mU) was found to be toxic causing a marked neutropenia at 1 h, thus mask-

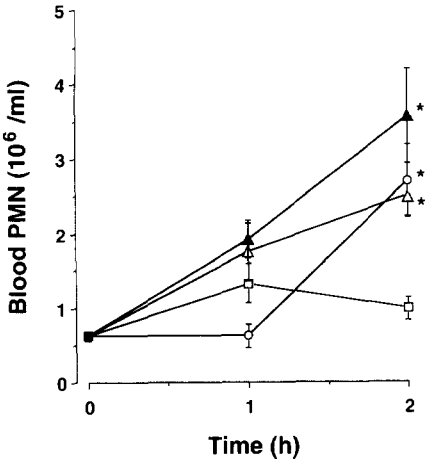

Fig. 1. Effect of sialidase and fucoidin on the number of circulating neutrophils. Mice were treated intravenously with 100 μl of phosphate-buffered solution supplemented with bovine serum albumin (0.1% w/w; vehicle group; open squares), sialidase (0.01 mU per mouse, open circles) or fucoidin (0.03 mg per mouse, open triangles and 0.3 mg per mouse, closed triangles). Animals were bled 1 h or 2 h later and the number of circulating polymorphonuclear leucocytes (PMN) quantified. Values are mean ± S.E. of $n = 5$–12 mice. * $P <$ 0.05 vs. vehicle group.

ing the 2 h neutrophilia (not shown). Fucoidin mimicked sialidase effect (Fig. 1). Blocking of endogenous CD11b by means of a specific monoclonal antibody resulted in a significant neutrophilia present at 1 h and persisting up to 2 h (Fig. 2). Intravenous treatment with the lipocortin 1 human N-terminus peptide, LC1-(Ac2–26)-peptide, produced a significant increase in the number of circulating polymorphonuclear leucocytes at 2 h. Administration of lipocortin 5-(204–212)-peptide, a control peptide used for comparative purposes, was without effect (Fig. 2).

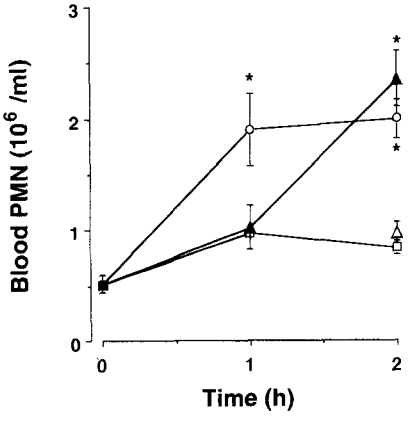

Fig. 2. Effect of monoclonal antibody anti-CD11b and the peptide fragments, LC1-(Ac2–26)-peptide and lipocortin 5-(204–212)-peptide, on the number of circulating neutrophils. Animals were treated intravenously with 100 μl of phosphate-buffered solution supplemented with bovine serum albumin (0.1% w/w; vehicle group; open squares), monoclonal antibody anti-CD11b (500 μg per mouse, open circles), human LC1-(Ac2–26)-peptide (200 μg per mouse, closed triangles) or lipocortin 5-(204–212)-peptide (200 μg per mouse, open triangle) reported. Mice were bled 1 h or 2 h later and the number of circulating polymorphonuclear leucocytes (PMN) evaluated. Values are mean ± S.E. of $n = 5$–10 mice. * $P < 0.05$ vs. vehicle group.

3.2. Drug effect on PAF- and interleukin-8-induced neutropenia

Both PAF and interleukin-8 caused a marked reduction in the number of circulating neutrophils 2 min following intravenous administration (Fig. 3). Doses which induced a similar degree of neutropenia, 100 ng for PAF and 300 ng for interleukin-8, were selected from these dose-response curves and used in subsequent experiments. PAF-induced neutropenia was significantly attenuated when mice were pretreated with sialidase: from 70% reduction to 25% reduction ($P <$ 0.05) (Fig. 4). A similar effect, though to a lesser extent, was again obtained with fucoidin. In the case of interleukin-8, both sialidase and fucoidin were less active and inhibited the neutropenic response to a similar degree. PAF-induced neutropenia was significantly attenuated in mice pretreated with the specific monoclonal antibody CD11b whereas the lipid mediator was fully active in those animals pretreated with a similar dose of non-immune rat immunoglobulin G (Fig. 5A). A similar feature was again obtained with LC1-(Ac2–26)-peptide which abrogated PAF action (Fig. 5B).

3.3. Drug effect on neutrophil accumulation

Injection of murine interleukin-1β into preformed subcutaneous air-pouches induced an intense accumu-

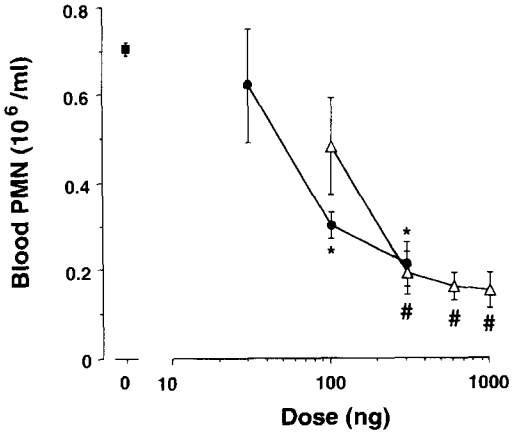

Fig. 3. Change in circulating polymorphonuclear leucocyte kinetic profile following treatment with interleukin-8 and PAF. Mice were treated intravenously with 100 μl of phosphate-buffered solution supplemented with bovine serum albumin (0.1% w/w; vehicle group; closed square) or varying doses of PAF (closed circles) or interleukin-8 (open triangles). Two minutes later mice were bled and the number of circulating polymorphonuclear leucocytes (PMN) measured. Values are mean ± S.E. of $n = 5$–16 mice. * $P < 0.05$ PAF vs. vehicle group. # $P < 0.05$ interleukin-8 vs. vehicle group.

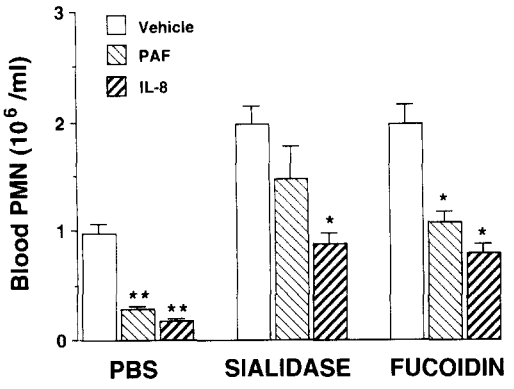

Fig. 4. Effect of sialidase and fucoidin on chemoattractant-induced 2 min neutropenia. Animals were pretreated intravenously with 100 μl of phosphate-buffered solution supplemented with bovine serum albumin (0.1% w/w), sialidase (0.01 mU per mouse) or fucoidin (0.3 mg per mouse) 2 h prior to receiving vehicle (open bars), 100 ng PAF (thin hatched bars) or 300 ng interleukin-8 (thick hatched bars). Mice were bled 2 min later and the number of polymorphonuclear leucocytes (PMN) measured. Values are mean ± S.E. of $n = 10–22$ mice. * $P < 0.05$ and ** $P < 0.01$ PAF or IL-8 group vs. appropriate vehicle group.

lation of polymorphonuclear leucocytes which was slightly different in distinct experiments, though always high above the cell infiltration measured with the vehicle, carboxymethylcellulose. For this reason data are reported in Table 1 with reference to the cellular values obtained in separate experiments for vehicle alone and interleukin-1β. In all cases the pharmacological treatment with sialidase, fucoidin, monoclonal antibody anti-CD11b and LC1-(Ac2–26)-peptide resulted in a significant inhibition of cell migration in response to the cytokine. The dose of sialidase which modified the number of circulating polymorphonuclear leucocytes in the absence of any toxic effect (0.01 mU) reduced interleukin-1β-induced neutrophil migration by almost 50%. A similar degree of inhibition was reached with fucoidin and LC1-(Ac2–26)-peptide, whereas monoclonal antibody anti-CD11b practically abrogated the cell response to the cytokine (Table 1).

3.4. Leucocyte CD11b / CD18 surface expression

In vitro

PAF up-regulated polymorphonuclear leucocyte surface expression of CD11b in vitro in a dose-dependent manner (Table 2). PAF (10^{-7} M)-induced up-regulation of CD11b expression increased significantly from 9207 ± 700 CD11b molecules per cell to 15926 ± 1231 CD11b molecules per cell (cumulative data from 12 experiments, $P < 0.01$). The chemokine interleukin-8 also induced a dose-dependent up-regulation of CD11b on the neutrophil membrane (Table 2), a detectable increase being seen even at the lowest dose used of 10 ng/ml. Only PAF was found to stimulate a significant increase in CD11b surface expression on monocytes in vitro (from 15433 ± 1270 CD11b molecules per cell, $n = 11$ experiments, to 21079 ± 1446 CD11b molecules per cell, $n = 11$ experiments, $P < 0.05$). Interleukin-8, however, was not active on this cell type (17348 ± 1457 CD11b molecules per cell, and 18630 ± 322 CD11b molecules per cell, for vehicle and interleukin-8, respectively; mean ± S.E.M. of $n = 4$ experiments, not significant).

Incubation of murine polymorphonuclear leucocytes with either human or mouse LC1-(Ac2–26)-peptide did not modify PAF-induced up-regulation of CD11b on neutrophils; basal expression was 12053 ± 500 CD11b molecules per cell, up to 18080 ± 772 CD11b molecules per cell after 10^{-7} M PAF and 18523 ± 867 CD11b molecules per cell and 18216 ± 1589 CD11b molecules per cell for human or mouse LC1-(Ac2–26)-peptide (100 μg/ml), respectively.

Ex vivo

Circulating neutrophils from untreated mice expressed 8326 ± 1347 CD11b molecules per cell, $n = 7$,

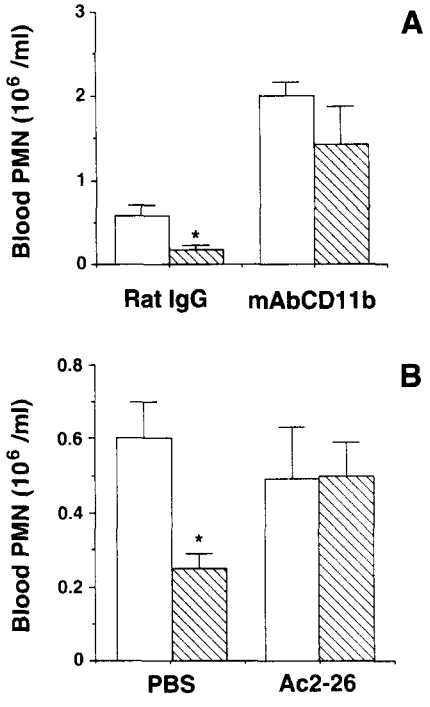

Fig. 5. Effect of monoclonal antibody anti-CD11b and lipocortin 1-(Ac2–26)-peptide on PAF-induced neutropenia. Panel A: Mice were pretreated i.v. with either 500 μg rat immunoglobulin G (Rat IgG) or monoclonal antibody anti-CD11b (mAbCD11b) 2 h prior to receiving 100 μl i.v. of phosphate-buffered solution supplemented with bovine serum albumin (0.1% w/w; vehicle group; open bars) or 100 ng PAF (hatched bars). Animals were bled 2 min later and the number of polymorphonuclear leucocytes (PMN) measured. Panel B: Human LC1-(Ac2–26)-peptide (Ac2–26: 200 μg per mouse) or phosphate-buffered solution (PBS; 100 μl) supplemented with bovine serum albumin (0.1% w/w) was given i.v. 10 min prior to the administration of PAF (100 ng; hatched bars) or vehicle (open bars). Mice were bled 2 min later and the number of polymorphonuclear leucocytes (PMN) measured. Values are mean ± S.E. of $n = 8–12$ mice. * $P < 0.05$ PAF vs. appropriate vehicle group.

Table 1
Drug modulation of interleukin-1β-induced polymorphonuclear leucocyte (PMN) migration

Treatment		PMN (10^6 per mouse)	Net migration (10^6 per mouse)	% Inhibition
Vehicle		1.75 ± 0.13	–	–
Interleukin-1β		9.95 ± 1.23	8.20	0
+ Sialidase	0.1 mU	1.85 ± 0.62 [a]	0.10	98
	0.01 mU	6.08 ± 0.51 [a]	4.33	47
Vehicle		0.80 ± 0.50	–	–
Interleukin-1β		6.65 ± 1.14	5.85	0
+ Fucoidin	0.3 mg	2.10 ± 0.30 [a]	1.30	78
	0.03 mg	3.40 ± 0.90 [a]	2.60	56
Vehicle		0.12 ± 0.05	–	–
Interleukin-1β		4.43 ± 0.54	4.31	0
+ mAbCD11b	500 μg	0.55 ± 0.17 [a]	0.43	90
Vehicle		1.50 ± 0.22	–	–
Interleukin-1β		12.85 ± 1.53	11.35	0
+ LC1-(Ac2–26)-peptide	200 μg	5.61 ± 2.52 [a]	4.21	67

Treatments were as follows: sialidase, fucoidin and monoclonal antibody anti-CD11b (mAbCD11b) were given i.v. 2 h prior to, whereas human lipocortin 1-derived peptide, LC1-(Ac2–26)-peptide, was given i.v. 20 min prior to, the local injection of 0.5 ml carboxymethylcellulose containing 5 ng of murine interleukin-1β into 6-day-old mouse air-pouches. Appropriate controls received either phosphate-buffered solution (100 μl i.v.) or carboxymethylcellulose alone into the air-pouch (vehicle group). Mice were killed 4 h after cytokine challenge and migrated PMN quantified following specific staining in Turk's solution. Values are mean ± S.E. of 5–6 mice per group. [a] $P < 0.05$ vs. respective interleukin-1β group.

as determined ex vivo. Intravenous treatment of mice with PAF (100 ng per mouse) induced a time-dependent up-regulation of CD11b on the neutrophil surface (Fig. 6). A significant increase above that for vehicle-treated animals was observed 2 min after PAF treatment, persisting at 20 min but returning to control levels by 1 h. Monocytes demonstrated higher surface CD11b expression than polymorphonuclear leucocytes prior to i.v. administration of PAF (16 236 ± 1242 CD11b molecules per cell, $n = 11$). Following administration of the lipid mediator, CD11b on monocytes was maximally up-regulated at 2 min (30 164 ± 5320 CD11b molecules per cell, $n = 7$ vs. 14 475 ± 1570 CD11b molecules per cell, $n = 6$, $P < 0.05$, at 2 min after PAF or vehicle respectively).

4. Discussion

This study has compared the effect of a pharmacologically active lipocortin 1 fragment, LC1-(Ac2–26)-peptide, to that of agents known to interfere with

Table 2
Induction of polymorphonuclear leucocyte (PMN) CD11b surface expression by PAF and interleukin-8 in vitro

Treatment	Fluorescein isothyocinate-equivalent CD11b molecules per PMN	% Induction
Vehicle	8842 ± 362	–
PAF		
10^{-9} M	8223 ± 17	0
10^{-8} M	8972 ± 323	1
10^{-7} M	11 985 ± 650 [a]	36
10^{-6} M	14 216 ± 460 [a]	61
Interleukin-8		
10 ng/ml	12 428 ± 144 [a]	41
100 ng/ml	14 141 ± 602 [a]	60
500 ng/ml	15 560 ± 241 [a]	76

Aliquots of blood (200 μl) were incubated with PAF, interleukin-8 or vehicle for 15 min at room temperature, stained with specific rat anti-murine CD11b and then prepared for flow cytometric analysis as described in Materials and methods. Values are mean ± S.E.M. of 7 experiments for basal expression (vehicle group) and 3 experiments for PAF or interleukin-8 stimulation. [a] $P < 0.05$ vs. vehicle group.

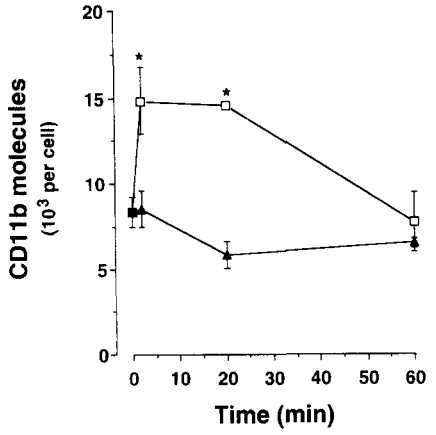

Fig. 6. Changes in CD11b expression on the surface of circulating polymorphonuclear leucocytes induced by PAF in vivo. PAF (100 ng; open squares) or phosphate-buffered solution supplemented with bovine serum albumin (0.1% w/w; vehicle group; closed triangles) was administered i.v. and mice were bled at various intervals after treatment. Aliquots (200 μl) of blood were stained with rat anti-murine CD11b and then prepared for flow cytometric analysis as detailed in Materials and methods. Values are mean ± S.E.M. of $n = 3$–11 experiments. * $P < 0.05$ PAF vs. vehicle group.

specific processes involved in the kinetics of circulating polymorphonuclear leucocytes in the mouse. The effect on specific phenomena such as CD11b/CD18 expression, chemoattractant-induced neutropenia and neutrophil migration into a site of acute inflammation was evaluated.

In physiological conditions a portion of circulating neutrophils is sequestered within the microcirculation as a consequence of slow rolling on the endothelium wall of post-capillary venules and of adhesion to the same site (MacNee and Selby, 1993). Sialidase is an enzyme widely used to remove carbohydrate moieties which are the counter-receptor for the selectins, proteins strictly involved in the rolling phenomenon (Rosen et al., 1989; Lasky, 1992). Interference with this process can also be obtained with fucoidin, a carbohydrate shown to directly block leucocyte rolling (Lindbom et al., 1992). Both agents induced a dose-dependent neutrophilia. A similar finding was obtained with a specific anti-CD11b monoclonal antibody (Rosen and Gordon, 1987): together these observations confirm that leucocyte rolling and adhesion, likely to occur at the level of the post-capillary venule, account for polymorphonuclear leucocyte sequestration and therefore for the endogenous control on the number of circulating cells. Similar data were obtained with human LC1-(Ac2–26)-peptide which caused a late-onset neutrophilia. A control peptide was found to be inactive.

Neutrophil activation was achieved by injection of chemoattractants. Both PAF and interleukin-8 caused a profound 2 min neutropenia. Pretreatment of mice with sialidase and to a lesser extent fucoidin inhibited the neutropenia caused by intravenous injection of chemoattractants. Both agents were more effective on PAF- rather than interleukin-8-induced neutropenia and therefore the effect of the other treatments was assessed only against PAF. Again, monoclonal antibody anti-CD11b and LC1-(Ac2–26)-peptide had a comparable action abrogating the effect of the lipid mediator. A couple of conclusions can now be drawn: (i) leucocyte rolling is also necessary for the neutropenia caused by the chemoattractants which is also dependent upon neutrophil adhesion, therefore these two processes are sequentially related and this has relevance not only to the migration to the site of inflammation (Von Andrian et al., 1992); (ii) LC1-(Ac2–26)-peptide is a potent inhibitor of PAF-induced neutropenia and also of that induced by FMLP (Perretti et al., 1993b), thus suggesting that the action of LC1-(Ac2–26)-peptide is irrespective of the stimulus applied. Since human recombinant lipocortin 1 inhibits FMLP-induced neutropenia, these data reiterate LC1-(Ac2–26)-peptide as an anti-inflammatory agent which mimics the effect of the parent protein.

Endothelial activation is the primary event switched on by interleukin-1 to attract neutrophils into an inflammatory site. In the mouse air-pouch, we have previously shown that interleukin-1 acts through a type I receptor and provided evidence for a role of endogenous PAF and neuropeptides (Perretti and Flower, 1993; Perretti et al., 1993a,c). LC1-(Ac2–26)-peptide was confirmed to have anti-inflammatory activity in the mouse air-pouch model and similarly monoclonal antibody anti-CD11b was highly effective in blocking cell recruitment (Perretti et al., 1993b). Neutrophil migration induced by interleukin-1 was also greatly reduced by sialidase and fucoidin. Though expected on the basis that leucocyte rolling is required to achieve firm adhesion (Von Andrian et al., 1991), these are the first data to report such an effect for sialidase and fucoidin on interleukin-1-induced neutrophil influx in vivo.

Qualitative and quantitative functional alteration in the β_2-integrin CD11b/CD18 on the neutrophil surface is induced following stimulation by a variety of chemotactic factors (Van Zee et al., 1991; Zimmerman et al., 1992). Qualitative functional alterations in the constitutively expressed integrin, possibly involving receptor aggregation or conformational changes, increase the affinity of the adhesion molecule for its counter-receptor (Naccaché et al., 1994). Such changes are believed to be the primary requirement for adherence of polymorphonuclear leucocytes, via this integrin, to endothelial cells (Springer, 1994). A quantitative up-regulation of CD11b/CD18 has been demonstrated to result from mobilisation of certain intracellular pools, a mechanism believed to be dependent on tyrosine phosphorylation (Borregaard et al., 1994; Naccaché et al., 1994). Previously, an increase in the number of surface

integrins has been used as a marker of neutrophil activation in vitro and also in vivo (Shalit et al., 1988; Witthaut et al., 1994); however, the functional importance of this change in vivo has yet to be established. Our results demonstrate a quantitative increase in CD11b expression on the neutrophil and monocyte surface in vitro after stimulation with PAF: the response was confined to neutrophils only in the case of interleukin-8. Relevantly, a time-dependent change in CD11b induced by PAF was observed in vivo. Activation was transient as the increased expression had returned to control levels by 1 h. The neutropenia seen at 2 min was associated with a maximal increase in CD11b on the neutrophil surface, reflecting rapid polymorphonuclear leucocyte activation by the lipid mediator. This phenomenon explains the anti-neutropenic action of the monoclonal antibody anti-CD11b, as this agent abrogated PAF-induced reduction in circulating polymorphonuclear leucocytes. On the contrary, the effect of LC1-(Ac2–26)-peptide cannot be explained in this way. Neither the human nor the mouse derived peptides affected the stimulated recruitment of the adhesion molecule to the polymorphonuclear leucocyte surface. This would suggest that a quantative change in CD11b expression on polymorphonuclear leucocytes does not play a role in either the anti-neutropenic or the anti-inflammatory activity of LC1-(Ac2–26)-peptide observed in vivo. In keeping with this, LC1-(Ac2–26)-peptide and monoclonal antibody anti-CD11b had an additive rather than mutually exclusive anti-inflammatory action in vivo (Perretti et al., 1993b). The possibility that the peptide may alter functional changes in surface CD11b (Springer, 1994) cannot be excluded. Similarly, in view of the comparable biological activity of human LC1-(Ac2–26)-peptide to that of agents known to alter selectin function, this lipocortin 1-derived peptide may have an effect on the selectin group of adhesion molecules. Further direct visualisation of leucocyte behaviour in the post-capillary venule by intravital microscopy is needed to pin-point the exact effect of LC1-(Ac2–26)-peptide on the interaction between leucocytes and the endothelium.

In conclusion, this study examines the mechanism of activity of the anti-inflammatory LC1-(Ac2–26)-peptide and demonstrates the involvement of leucocyte rolling and adhesion in physiological polymorphonuclear leucocyte sequestration. The actions of the peptide observed in vivo correlated well with those of monoclonal antibody anti-CD11b in the same models. LC1-(Ac2–26)-peptide, however, was found to have no effect on neutrophil CD11b up-regulation in vitro. It may be that the action of the peptide in vivo depends on interference with more than one step in the adhesion cascade, possibly affecting functional rather than quantative changes in CD11b expression.

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