British Journal of Pharmacology (1997) 120, 1075–1082

Inhibition of neutrophil and monocyte recruitment by endogenous and exogenous lipocortin 1

Stephen J. Getting, Roderick J. Flower & [1]Mauro Perretti

Department of Biochemical Pharmacology, The William Harvey Research Institute, Charterhouse Square, London EC1M 6BQ

1 The role played by endogenous lipocortin 1 in the anti-migratory action exerted by dexamethasone (Dex) on monocyte recruitment in an *in vivo* model of acute inflammation was investigated by use of several neutralizing polyclonal antibodies raised against lipocortin 1 or a lipocortin 1-derived N-terminus peptide (peptide Ac2-26). The efficacy of peptide Ac2-26 in inhibiting monocyte and polymorphonuclear leucocyte (PMN) recruitment was also tested.

2 Intraperitoneal (i.p.) injection of zymosan A (1 mg) produced a time-dependent cell accumulation into mouse peritoneal cavities which followed a typical profile of acute inflammation: PMN influx was maximal at 4 h post-zymosan (between 15 and 20×10^6 cells per mouse), and this was followed by an accumulation of monocytes which peaked at the 24 h time-point (between 10 and 15×10^6 cells per mouse).

3 Dex administration to mice reduced zymosan-induced 4 h PMN infiltration and 24 h monocyte accumulation with similar efficacy: approximately 50% of inhibition of recruitment of both cell types was achieved at the dose of 30 μg per mouse ($\sim$1 mg kg^{-1}, subcutaneously (s.c.)). Maximal inhibitions of 64% and 67% on PMN and monocyte recruitment, respectively, were measured after a dose of 100 μg per mouse ($\sim$3 mg kg^{-1}, s.c.).

4 Dex (30 μg s.c.) inhibited monocyte (53%) and PMN (69%) accumulation in response to zymosan application in mice which had been treated with a non-immune sheep serum (50 μl s.c.). In contrast, the steroid was no longer active in reducing cell accumulation in mice which had been passively immunized against full length human recombinant lipocortin 1 (serum LCS3), or against lipocortin 1 N-terminus peptide.

5 Treatment of mice with vinblastine (1 mg kg^{-1}, intravenously (i.v.)) produced a remarkable leucopenia as assessed 24 h after administration. This was accompanied by a 60% reduction in 4 h-PMN influx, and by a 27% reduction in 24 h-monocyte accumulation, measured after zymosan administration. The inhibitory effect of Dex on monocyte recruitment was not significantly modified in vinblastine-treated mice, with 36% and 57% of inhibition calculated at the dose of 30 μg Dex, and 70% and 60% of inhibition at 100 μg Dex, in vehicle- and vinblastine-treated mice, respectively.

6 Treatment of mice with peptide Ac2-26 dose-dependently attenuated PMN influx at 4 h post-zymosan with a significant effect at 100 μg per mouse (45% of inhibition, $n=9$, $P<0.05$) and a maximal effect of 61% inhibition at the highest dose tested of 200 μg s.c. ($n=14$, $P<0.05$). No effect of peptide Ac2-26 (200 μg s.c.) was seen on zymosan-induced 24 h monocyte recruitment. In contrast, administration of 200 μg peptide Ac2-26 every 6 h was effective in reducing the number of monocytes harvested from the inflamed peritoneal cavities at 24 h post-zymosan: $9.40 \pm 0.58 \times 10^6$ monocytes per mouse ($n=13$) and 5.74 ± 0.34 monocytes per mouse ($n=14$) in vehicle- and peptide Ac2-26-treated mice, respectively ($P<0.05$).

7 Finally, peptide Ac2-26 produced a concentration-dependent inhibition of the rate of phagocytosis of mouse resident peritoneal macrophages as measured by flow cytometry, with a maximal reduction of 34% at the highest concentration tested of 100 μg ml^{-1} ($n=8$ experiments performed in duplicate; $P<0.05$).

8 In conclusion, this study suggests that *in vivo* monocyte recruitment during acute inflammation is, at least in part, under the negative modulatory control of endogenous lipocortin 1 (as seen after administration of Dex by using the specific antisera) and exogenous lipocortin 1 mimetics (as observed with peptide Ac2-26). In addition to the neutrophil, we can now propose that the monocyte also can be a target for the *in vivo* anti-inflammatory action of lipocortin 1.

Keywords: Annexin I; inflammation; dexamethasone; extravasation; macrophage

Introduction

The abundant distribution of the glucocorticoid-regulated protein lipocortin 1 (LC1; also termed annexin I) in specific subsets of human and animal leucocytes may give some clue to the role played by this protein in the physio-pathology of leucocyte behaviour. Polymorphonuclear leucocytes (PMN) and monocytes contain large amounts of the protein, whereas much lower amounts are found in lymphocytes (LY) (for a review see Flower and Rothwell, 1994). In addition, the notion that differentiated cells contain more LC1 than the un-differentiated cells (Solito *et al*., 1991) is mirrored by the higher amounts found in peritoneal macrophages (Mø) compared to circulating monocytes (De Caterina *et al*., 1993; Perretti & Flower, 1996).

We have recently demonstrated a novel biological function of LC1, the negative modulation of neutrophil extravasation during the host inflammatory response. Both exogenously administered human full length LC1 and an LC1-derived N-terminus peptide, human Ac2-26, inhibited PMN accumulation into murine 6-day-old air-pouches in response to local challenge with several inflammatory stimuli (Perretti & Flower,

[1] Author for correspondence.

1993; Perretti *et al*., 1993; Perretti, 1994). In addition, passive immunization of mice with specific anti-LC1 antisera abrogated the anti-migratory action of systemically, but not locally, injected dexamethasone (Dex) (Perretti & Flower, 1993). Taken together these data suggested that the steroid acted to inhibit neutrophil migration by increasing the amount of available LC1 which can participate in this reacion. Our recent studies have substantiated this assumption, since we were able to show that (i) immunoreactive LC1-like levels were elevated both in mouse PMN and monocytes after intravenous (i.v.) treatment of animals with Dex (Perretti & Flower, 1996); (ii) PMN emigration through the endothelium was the actual step of the extravasation process inhibited by Dex in a LC1-dependent way (Mancuso *et al*., 1995), and finally, (iii) an extensive externalization of LC1 on the cell surface of neutrophils adherent to endothelial monolayers was observed (Perretti *et al*., 1996b). The latter observation supports the passive immunization data showing that anti-LC1 antisera reverses the effects of Dex in some models of inflammation.

In the present study we have focussed our attention on monocyte extravasation to determine whether a similar situation obtains in this cell type. In particular we have evaluated (i) the effect of Dex on accumulation of this cell type in the zymosan peritonitis model; (ii) the role played by endogenous LC1 using three different anti-LC1 antisera including two raised against the pharmacophore, peptide Ac2-26; (iii) the effect of peptide Ac2-26 itself. As a positive control, we have also monitored the effect of all these agents on PMN accumulation in the peritoneal cavity in response to the same inflammatory stimulus.

Methods

Animals

Male Swiss Albino mice (25–30 g body weight) were purchased from Interfauna (CFLP strain; Huntingdon, Cambridgeshire) or from Bantin & Kingman (T.O. strain; Hull, Humberside), and maintained on a standard chow pellet diet with tap water *ad libitum* and a 12:00 h light/dark cycle. Animals were used 3–4 days after the arrival.

Sheep sera

Several different sheep anti-LC1 anti-sera were used in the study: a polyclonal sheep antiserum raised against the intact full length human recombinant protein designated LCS3; two distinct polyclonal sheep antisera raised against the anti-inflammatory LC1-derived peptide Ac2-26 (Perretti *et al*., 1993), termed LCPS1 and LCPS2, and as a control, a non-immune sheep serum referred to as NSS. Although LCS3 was raised against the human protein it also recognizes murine LC1 (Perretti & Flower, 1993; 1996). Similarly, the LCPS1 polyclonal antibody recognises murine LC1 in flow cytometry preparations (Perretti & Flower, 1996).

Zymosan peritonitis

The peritonitis was induced by injection of 1 mg zymosan in 0.5 ml phosphate-buffered solution (0.1 M, pH 7.4; PBS) as previously found (Perretti *et al*., 1992). At different time-points, animals were killed by CO_2 exposure, peritoneal cavities washed with 3 ml of PBS containing 3 mM ethylenediaminetetracetic acid (EDTA) and 25 u ml^{-1} heparin. Aliquots of the lavage fluids were then stained with Turk's solution (0.01% crystal violet in 3% acetic acid) and differential cell counts performed with a Neubauer hematocytometer and a light microscope (Olympus B061). Whereas LY and PMN were easily identified, the distinction between monocytes and macrophages was not so clear, especially in view of the ongoing phagocytosis, and data are therefore presented as combined monocytes and macrophages (mono-Mø). We have confirmed elsewhere the monocytic nature of the cellular infiltrate at later time-points by detection of the expression of F4/80 antigen using flow cytometry (Ajuebor *et al*., unpublished observations). Data are presented as 10^6 cells per mouse.

Lavage fluids were centrifuged at 400 *g* for 10 min and supernatants stored at −20°C before the evaluation of the β-glucuronidase activity (see below).

The large predominance of neutrophils in the PMN population in 4 h-lavage fluids was confirmed in cytospin preparations stained with May-Grunwald and Giemsa, confirming that >98% of PMN were neutrophils (data not shown).

Drug treatment

The glucocorticoid Dex was given sub-cutaneously (s.c.) in doses of 10 μg, 30 μg or 100 μg per mouse (ranging approximately from 0.3 to 3 mg kg^{-1}) 1 h before zymosan. The doses were chosen because they are in the range of those shown to raise LC1-like immunoreactivity in mouse circulating monocytes and PMN (Perretti & Flower, 1996).

In our first study with LCS3 we found that the anti-LC1 titre in the plasma was maximal at 24 h following a single s.c. injection of 50 μl of serum (Perretti & Flower, 1993). On this basis the anti-LC1 antisera or NSS was given s.c. at the dose of 50 μl per mouse 24 h before the administration of Dex.

Peptide Ac2-26 was dissolved in sterile PBS (at 2 mg ml^{-1}) and given s.c. 30 min before zymosan administration. Whereas the peptide was found to be active after a single injection on PMN accumulation, repeated administration was necessary to observe an effect on monocyte accumulation as this takes place over a longer period of time and in preliminary experiments radio-iodinated preparations of Ac2-26 given i.v. had an approximate half-life of 30–45 min (Perretti & Flower, unpublished data) indicating a relatively rapid clearance of the peptide from mouse blood. To detect an effect on monocyte recruitment, peptide Ac2-26 was therefore given 4 times within the 24 h post-zymosan, i.e. at -30 min, 6 h, 12 h and 18 h post-zymosan. Control animals were treated with an equal volume of sterile PBS.

The effect of a marked reduction in circulating and elicited PMN on the influx of monocytes was evaluated by use of the cytotoxic vinblastin. The dose was chosen on the basis of preliminary experiments in which mice received 0.1–1.0 mg kg^{-1}, i.v., of the drug and peripheral blood leucocytes were analysed at different time-points. At the highest dose of 1 mg kg^{-1} vinblastin caused a prolonged leucopenia lasting for 3 days, with 80% and 72% reduction in PMN numbers at 24 h and 72 h, respectively ($n=4$ mice). This dose and a pretreatment time of 24 h were chosen for subsequent experiments.

In all cases peritoneal cavities were washed 4 h or 24 h after challenge with zymosan to assess PMN or monocyte accumulation, respectively.

β-Glucuronidase activity

β-glucuronidase activity in the supernatants was measured according to a published protocol (Iwamura *et al*., 1993). Briefly, 250 μl of cell-free lavage fluids were incubated with the substrate phenolphthalein-β-glucuronic acid (1 mM) in 0.5 ml total volume and kept in a water bath at 37°C with gentle shaking for 18 h. Reactions were stopped by addition of 1 ml of ice cold glycine buffer (200 mM) in 200 mM NaCl (pH 10.4). Absorbance values, measured at 550 nm by a 96-well plate multi-reader, were transformed into u ml^{-1} of lavage fluid, by use of standard curve constructed with 0–2,000 u β-glucuronidase. Data are presented as u per $\times 10^6$ cells per mouse.

Mø phagocytosis

Peritoneal Mø were prepared from untreated mice by peritoneal cavity lavage and counted as described above; 5.0×10^6 cells (>80% Mø) were incubated in RPMI-1640 supplemented

with 2% foetal calf serum with different concentrations of peptide Ac2-26 in a total volume of 1 ml at 37°C for 15 min. Cells were then diluted to 1.0×10^6 ml^{-1} in Krebs solution before the addition of 10 μl of immuno-complexes Red Oxyburst. Uptake of these complexes by the peritoneal Mø was monitored in real time by use of a FACScan (Becton Dickenson, Oxford, UK), which allowed not only the identification of the Mø population by forward and side scatter, but also the quantification of the fluorescence acquired in the FL-3 channel during the 200 s of reaction. Cumulative changes of fluorescence at constant time interval were then constructed and the area under the curve measured.

Materials

Dexamethasone-21-phosphate was obtained from David Ball Laboratories (Warwick, U.K.) as a sterile 4 mg ml^{-1} solution. Zymosan type A, NSS, PBS, EDTA, phenolphthalein-β-glucuronic acid, glycine buffer, β-glucuronidase and all other chemicals were obtained from Sigma Chemical Co. (Poole, U.K.). The phagocytotic stimulus Red Oxyburst was obtained from Molecular Probes (Eugene, U.S.A.).

Peptide Ac2-26 (acetyl-AMVSEFLKQAWFIENEEQEY-VVQTVK) was prepared by The Advance Biotechnology Centre (The Charing Cross and Westminster Medical School, London) by use of solid phase step-wise synthesis. Purity was more than 90% as assessed by h.p.l.c. and capillary electrophoresis (data furnished by the manufacturer).

Statistics

Statistical differences were calculated on original data by analysis of variance followed by Bonferroni test for intergroup comparisons (Berry & Lindgren, 1990), or by unpaired Student's t test (two-tailed) when only two groups were compared. A threshold value of $P < 0.05$ was taken as significant.

Results

Cell influx into the inflamed peritoneal cavity

Local administration of zymosan produced a time-dependent cell accumulation into the peritoneal cavity which followed a typical profile of acute inflammation. PMN were the first cell type to arrive into the cavity with a rate of influx of $\sim 6.0 \times 10^6$ PMN per h during the first 4 h (Figure 1). At 24 h post-zymosan, PMN influx was followed by infiltration of monocytes. The monocyte population increased steadily between 4 and 24 h (Figure 1) and high counts were still found 48 h post-challenge with zymosan. Very few resident lymphocytes could be counted in the lavage fluids, with no changes within the 48 h under observation.

β-Glucuronidase activity was detected and measured in the cell-free lavage fluids and data are presented in Table 1.

Modulation by Dex and endogenous LC1 of leucocyte accumulation

Treatment of mice with Dex, 1 h before zymosan, resulted in a dose-dependent reduction in the extent of monocyte accumulation as assessed at the 24 h time-point (Figure 2a). The steroid produced 49% and 64% inhibition of cell influx at doses of 30 and 100 μg per mouse, respectively.

The dose of 30 μg, which gave approximately 50% reduction in mono-Mø accumulation, was selected for the next set of experiments, in which the effect on the steroid on passive immunisation of mice against LC1 was evaluated. Dex produced 53% inhibition in NSS-pretreated mice. However, it was no longer able to reduce the influx of this cell type in mice which had been pretreated with LCS3, LCPS1 or LCPS2 (Figure 2b).

The effect of these agents on PMN infiltration was also evaluated both to extend previous observations to the zymosan peritonitis model and to establish a parameter which could be monitored as a positive control. Figure 3a shows the dose-dependence of the inhibition by Dex of PMN accumulation into the mouse peritoneal cavity 4 h post-challenge with zymosan, with a calculated 51% and 67% reduction in cell numbers at 30 μg and 100 μg doses of Dex, respectively.

Passive immunization of mice with the three anti-LC1 polyclonal antibodies was again effective, almost abrogating the action of systemic Dex. The steroid, administered at a dose of 30 μg per mouse, produced 69% inhibition of PMN extravasation in NSS-pretreated mice, whereas it was inactive (inhibition ranging from 5–10%, not significant) in the animals pretreated with LCS3, LCPS1 or LCPS2 (Figure 3b).

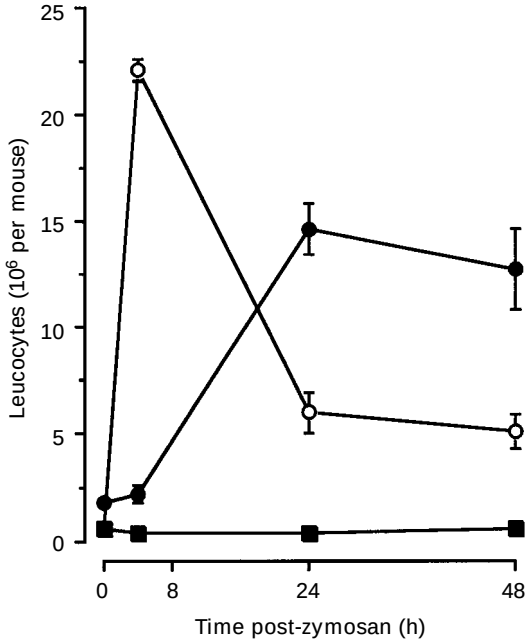

Figure 1 Time-course of cell accumulation in the zymosan peritonitis model. Mice were left untreated (time 0) or received 1 mg i.p. of zymosan (in 0.5 ml sterile PBS). At different time-points peritoneal cavities were washed and differential cell counts measured in the lavage fluids as described in the Methods section. Values (mean ± s.e.mean, $n = 12$ mice per group) refer to the number of PMN (○), monocytes and macrophages (mono-Mø, ●) or lymphocytes (■) recovered in the lavage fluids.

Table 1 Lack of effect of Dex, endogenous LC1 and peptide Ac2-26 on the release of β-glucuronidase activity during zymosan peritonitis

Treatment (−1 h or −30 min)	*β-Glucuronidase activity (u per 10^6 cells)* 4 h	24 h
PBS	121 ± 10 (5)	320 ± 33 (5)
Dex	265 ± 60 (6)	525 ± 84 (6)
PBS	276 ± 32 (4)	188 ± 19 (13)
Peptide Ac2-26	427 ± 100 (4)	ND
Peptide Ac2-26#	ND	193 ± 25 (14)

Mice were injected with PBS (100 μl s.c.), Dex (30 μg s.c., −1 h) or peptide Ac2-26 (200 μg s.c., −30 min) before i.p. challenge with zymosan (1 mg in 0.5 ml PBS). Lavage fluids collected were collected at the reported time. β-Glucuronidase activity in the cell-free fluids was measured as cleavage of the substrate phenolphthalein-β-glucuronic acid as described in the Methods section, and u normalized for the number of leucoctyes found in each cavity. Values are mean ± s.e.mean of (n) mice per group. #Denotes repeated injections of peptide Ac2-26 (see Figure 4b); ND, not done.

Treatment of mice with Dex did not modify the β-glucuronidase activity measured in the cell-free lavage fluids either at 4 h or at 24 h post-zymosan injection (Table 1).

Effect of peptide Ac2-26 on PMN and monocyte extravasation

The efficacy of LCPS1 and LCPS2 in the previous experiments prompted us to test the biologically active LC1 peptide against which these antisera were raised. A single s.c. injection of the LC1 pharmacophore, peptide Ac2-26, produced a dose-dependent reduction of the 4 h-PMN accumulation into the peritoneal cavity in response to zymosan (Figure 4a). A significant inhibition was attained at the dose of 100 μg per mouse (45% inhibition) with a maximal effect at the highest dose tested of 200 μg per mouse (61% inhibition, $P<0.05$).

A single injection of peptide Ac2-26 (200 μg s.c.) did not modify the extent of monocyte extravasation measured at 24 h, such that $10.9\pm0.83\times10^6$ mono-Mø were seen at this time-point in the vehicle-treated group, and $11.2\pm0.72\times10^6$

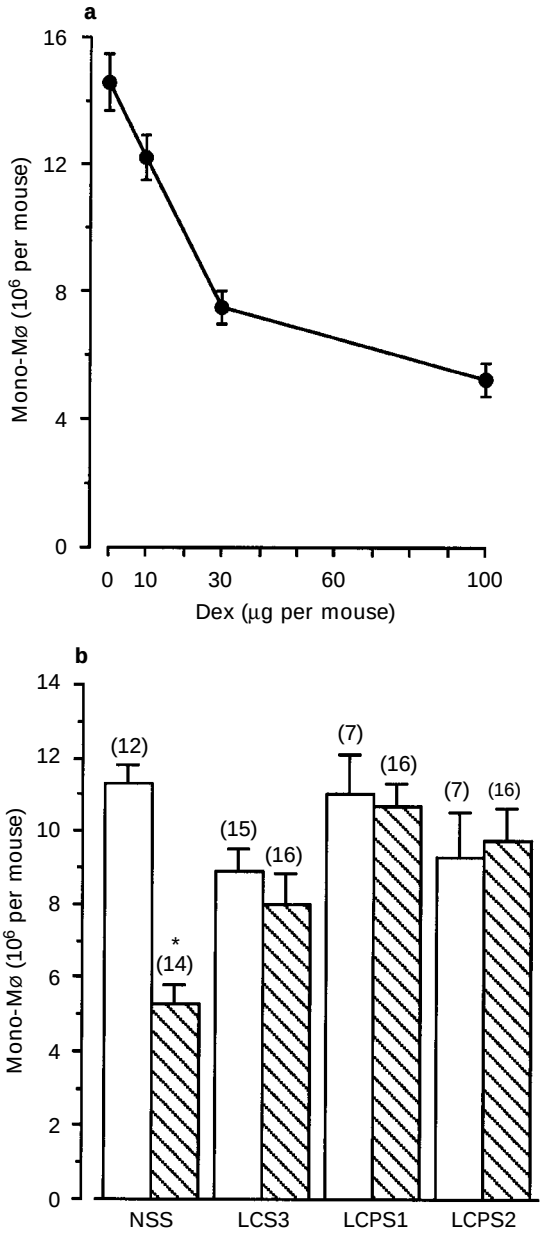

Figure 2 Effect of dexamethasone (Dex) and anti-LC1 sheep sera on monocyte accumulation in the zymosan peritonitis model. (a) Mice received 100 μl s.c. of sterile PBS alone (dose 0) or containing different doses of Dex 1 h before 1 mg i.p. zymosan (in 0.5 ml sterile PBS). Monocyte influx was measured 24 h later following differential cell counts as described in the Methods section. Data are mean$\pm$s.e.mean of $n=12$ mice per group. *$P<0.05$ vs dose 0 group. (b) Mice received 50 μl s.c. of NSS, LCS3, LCPS1 or LCPS2 24 h before the injection of PBS (100 μl s.c., open columns) or Dex (30 μg s.c., hatched columns). Zymosan (1 mg i.p. in 0.5 ml PBS) was given 1 h later and cell influx measured at the 24 h time-point. Data are mean$\pm$s.e.mean of (n) mice per group. *$P<0.05$ vs respective PBS group.

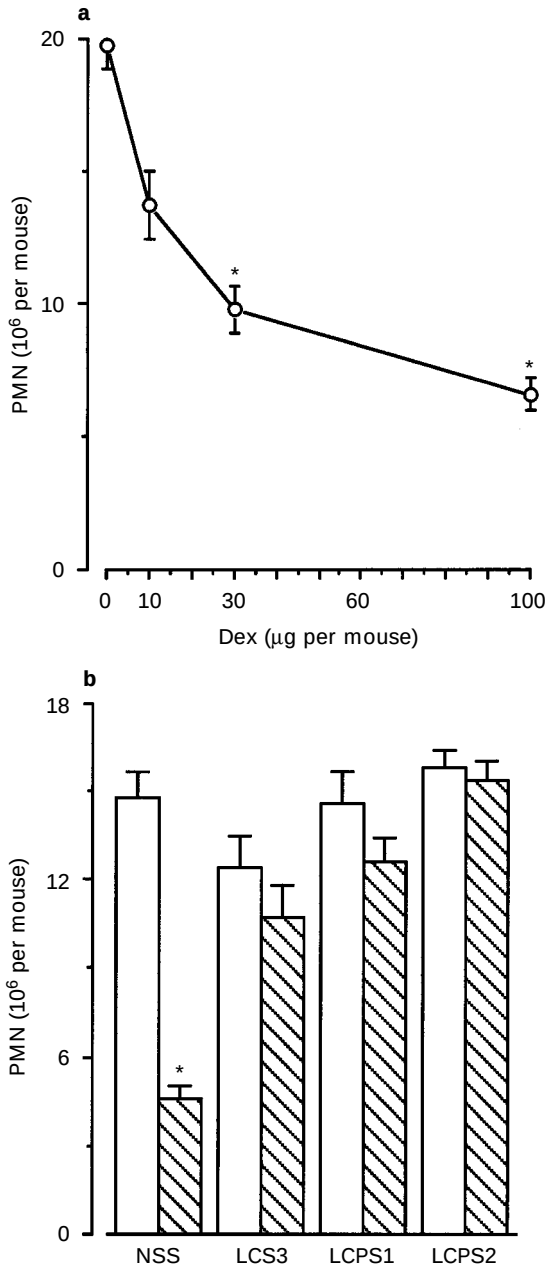

Figure 3 Effect of dexamethasone (Dex) and anti-LC1 sera on PMN accumulation in the zymosan peritonitis model. (a) Mice received 100 μl s.c. of sterile PBS alone (dose 0) or containing different doses of Dex 1 h before 1 mg i.p. zymosan (in 0.5 ml sterile PBS). PMN influx was measured 4 h later following differential cell counts as described in the Methods section. Data are mean$\pm$s.e.mean of $n=12$ mice per group. *$P<0.05$ vs dose 0 group. (b) Mice received 50 μl s.c. of NSS, LCS3, LCPS1 or LCPS2 24 h before the injection of PBS (100 μl s.c., open columns) or Dex (30 μg s.c., hatched columns). Zymosan (1 mg i.p. in 0.5 ml PBS) was given 1 h later and cell influx measured at the 4 h time-point. Data are mean$\pm$s.e.mean of $n=5-6$ mice per group. *$P<0.05$ vs respective PBS group.

mono-Mø were recovered from the peritoneal cavities of mice treated with the peptide Ac2-26 (values are mean ± s.e.mean of 7–8 mice per group).

In contrast to the previous experiment, we found that repeated administrations of peptide Ac2-26 produced a significant reduction in monocyte infiltration into the inflamed cavities. Figure 4b illustrates these data with approximately 40% inhibition of mono-Mø recruitment. No effect was seen on the other cell types which were only present in small numbers in the cavity at this time-point.

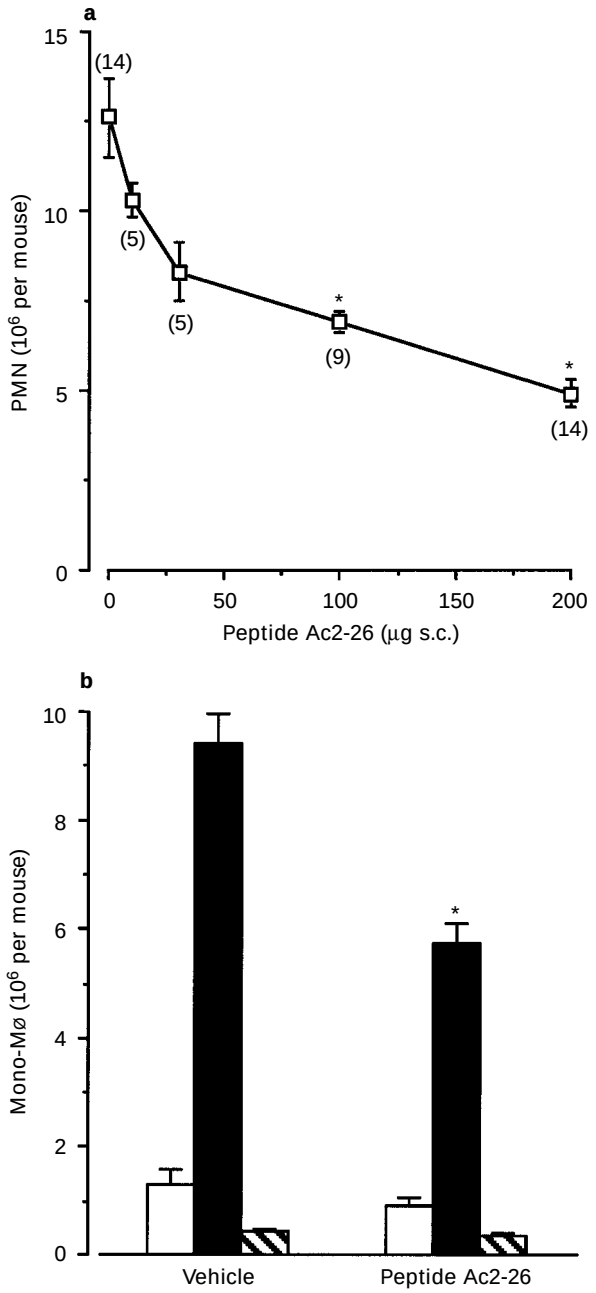

Figure 4 Peptide Ac2-26 inhibits cell accumulation in the zymosan peritonitis model. (a) Mice received 100 μl s.c. of sterile PBS alone or supplemented with different doses of peptide Ac2-26 30 min before the i.p. administration of 1 mg zymosan. PMN influx was measured 4 h later. Data are mean ± s.e.mean of (*n*) mice per group. **P* < 0.05 vs PBS group (dose 0). (b) Mice received 4 h injections of sterile PBS (100 μl s.c.; *n* = 13) alone or containing 200 μg peptide Ac2-26 (*n* = 14) at time 0, 6, 12 and 18 h. Zymosan (1 mg) was given i.p. at time 0, and cell accumulation into the peritoneal cavities was measured 24 h later. Values (mean ± s.e.mean) refer to the number of PMN (open columns), mono-Mø (solid columns) or LY (hatched columns) recovered in the lavage fluids. **P* < 0.05 vs respective value in the PBS group.

Similar to Dex, treatment of mice with peptide Ac2-26 did not affect leucocyte activation as assessed in terms of β-glucuronidase activity released in the exudates (Table 1).

Effect of vinblastine on the inhibition by dexamethasone of monocyte extravasation

Administration of vinblastine (1 mg kg^{-1}, i.v.) to mice significantly reduced the number of circulating PMN and mononuclear cells 24 h later when the inflammatory response was induced (Table 2). This was reflected in a remarkable reduction in PMN accumulation in response to zymosan at the 4 h time-point, such that $11.5 \pm 0.87 \times 10^6$ PMN and $4.58 \pm 0.12 \times 10^6$ PMN were found, respectively, in control mice and in those treated with the cytotoxic drug after zymosan challenge ($n = 4$; $P < 0.05$).

The effect of Dex on mono-Mø numbers recovered 24 h post-challenge with zymosan was then evaluated in these experimental conditions. Figure 5 illustrates the data from these experiments, showing that a similar degree of reduction in mono-Mø accumulation was exerted by two doses of the steroid in control mice and in those treated with vinblastine: 36% and 57% of inhibition for the dose of 30 μg, and 70% and 60% of inhibition at 100 μg Dex, in vehicle- and vinblastine-treated mice, respectively (Figure 5).

Table 2 Effect of vinblastine treatment of mice on peripheral blood leucocyte counts

Vinblastine (mg kg^{-1} i.v.)	*PMN* (10^6 ml^{-1})	*Monocytes* (10^6 ml^{-1})	*Lymphocytes* (10^6 ml^{-1})
0	1.67 ± 0.13 (6)	1.25 ± 0.11 (6)	3.65 ± 0.62 (6)
1.0	0.38 ± 0.06 (8)	0.44 ± 0.08 (8)	1.44 ± 0.22 (8)

Mice were treated with vinblastine (in 100 μl sterile PBS) and blood was collected by cardiac puncture 24 h later. Peripheral blood leucocytes were counted following staining in Turk's solution. Values are mean ± s.e. of (*n*) mice per group. **P* < 0.05 vs vehicle-treated animals.

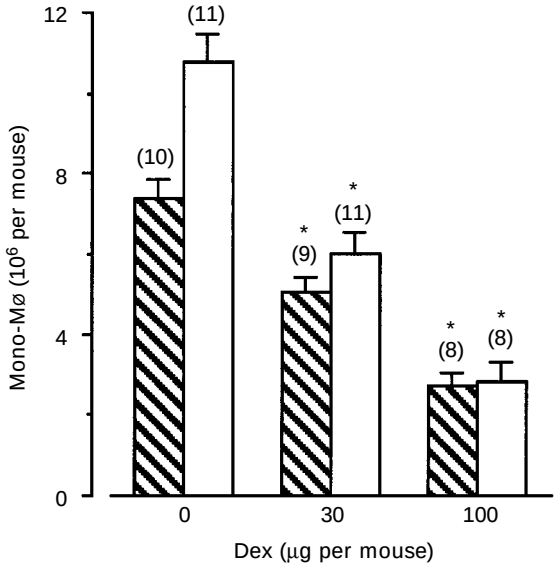

Figure 5 Lack of effect of vinblastine treatment on dexamethasone (Dex)-induced inhibition of monocyte influx in the zymosan peritonitis model. Mice were injected with 1 mg kg^{-1} i.v., vinblastine (hatched columns) or sterile PBS (100 μl i.v.; open columns) 24 h before the administration of Dex (given s.c.) or of the vehicle (100 μl s.c. PBS). Zymosan (1 mg in 0.5 ml PBS) was given i.p. 1 h later and cell influx evaluated at the 24 h time-point. Values are mean ± s.e.mean of (*n*) mice per group. **P* < 0.05 vs PBS group (dose 0).

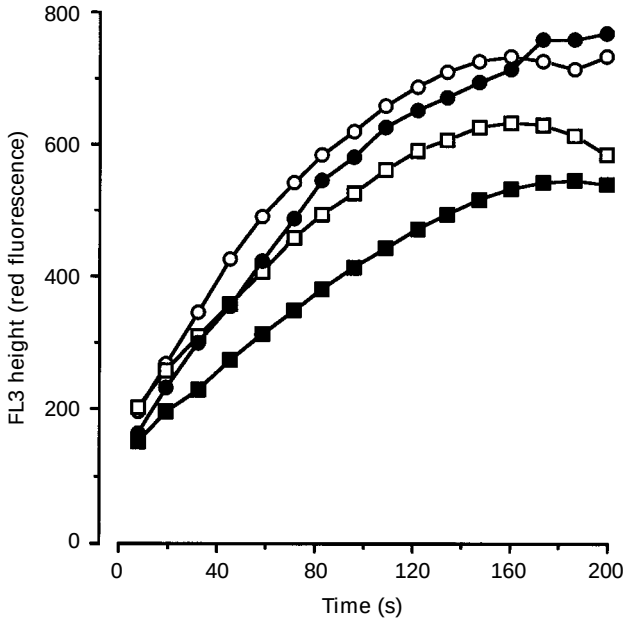

Figure 6 Peptide Ac2-26 inhibits macrophage (Mø) phagocytosis measured *in vitro*. Displayed is a representative graph illustrating cumulative increases in the fluorescence measured in the FL3 channel by flow cytometry following addition of Red Oxyburst to mouse peritoneal Mø. Different concentrations of peptide Ac2-26 (10 μg ml^{-1}, ●; 30 μg ml^{-1}, □; 100 μg ml^{-1}, ■) or vehicle alone (○) were incubated with 5×10^6 mouse peritoneal Mø for 15 min at 37°C before the addition of the immunocomplexes Red Oxyburst and flow cytometric analysis as described in the Methods section. Cumulative data for this set of experiments are presented in Table 3.

Table 3 Concentration-dependent inhibition of mouse peritoneal macrophage phagocytosis by peptide Ac2-26

Treatment	*Phagocytosis* (cm^2)	n	*Inhibition* (%)
None	50±1	8	0
Peptide Ac2-26 10 μg ml^{-1}	43±1	2	14
Peptide Ac2-26 30 μg ml^{-1}	38±3*	4	24
Peptide Ac2-26 100 μg ml^{-1}	33±3*	8	34

Mouse peritoneal macrophages (5×10^6) were incubated with the concentrations of peptide Ac2-26 shown before the addition of the Red Oxyburst immuno-complexes and assessment of phagocytosis by FACS analysis as described in the Methods section. Data are mean±s.e.mean of *n* experiments performed in duplicate.
*$P<0.05$ vs control phagocytosis.

Effect of peptide Ac2-26 on Mø phagocytosis

Finally, the effect of *in vitro* addition of peptide Ac2-26 on mouse peritoneal Mø activation was assessed by measuring cell phagocytosis. Figure 6 shows a representative experiment in which the peptide reduced the extent of immuno-complex internalisation in a dose-dependent manner. The cumulative data are summarized in Table 3 and show that a significant reduction was achieved with a peptide concentration of 30 μg ml^{-1}, whereas the maximal effect was seen at the highest concentration tested of 100 μg ml^{-1} (~33% of inhibition, Table 3).

Discussion

In this study we found that passive immunisation of mice with anti-LC1 antisera was effective in reducing the inhibition of monocyte extravasation exerted by Dex. This observation was substantiated by the reduction in monocyte accumulation obtained following repeated administration of the LC1-derived N-terminus peptide Ac2-26.

We have previously performed a series of studies showing that the circulating neutrophil is a prominent target for LC1 action, and that its elicitation could be inhibited by systemic administration of Dex in an LC1-dependent way (Perretti & Flower, 1993; Mancuso *et al*., 1995). Here, we addressed our attention to the process of monocyte extravasation which is also very sensitive to corticosteroids. This cell type was also chosen because it contains high LC1 levels (Goulding *et al*., 1990; Perretti & Flower, 1996) and also displays a selective LC1 binding capacity (Goulding *et al*., 1996).

An intense cell accumulation typical of an acute inflammatory response was measured in peritoneal cavities inflamed with zymosan, with PMN influx peaking at 4 h, and a maximal accumulation of monocytes at 24 h post-challenge. This model appeared to be highly sensitive to Dex: a single injection of the steroid dose-dependently inhibited 4 h-PMN influx and monocyte accumulation at 24 h to a similar extent. In contrast to this potent effect on cell extravasation, Dex did not affect the degree of cell activation as assessed by the release of β-glucuronidase activity in the lavage fluids. We also failed to find a positive effect of systemic Dex on the release of elastase from elicited neutrophils in a related model of leucocyte extravasation (Klemm *et al*., 1995). Since β-glucuronidase is present both in PMN and monocytes, as well as Mø, it appears that the enzymatic release process from all these cell types is insensitive to corticosteroid administration. Contrasting data have been generated under experimental conditions *in vitro* (Tapper, 1996), although a selective effect of corticosteroids on the release of specific enzymes has long been known (Webb, 1979).

Passive immunization of mice with three distinct anti-LC1 antisera was able to prevent the anti-migratory action of 30 μg kg^{-1} Dex on monocyte extravasation. This strongly suggests an involvement of endogenous LC1 in controlling monocyte extravasation. Circulating monocytes, as well as Mø (Russo-Marie, 1992), are not only abundant sources of the protein (Blackwell *et al*., 1980; Goulding *et al*., 1990; Coméra & Russo-Marie, 1995), but are also targets of LC1 action, as in the case of inhibition of superoxide generation (Maridonneau-Parini *et al*., 1989) or prostaglandin and cytokine production (Sudlow *et al*., 1996).

The efficacy of these anti-LC1 sera and of the protocol of administration was validated by assessing their action on PMN accumulation into the peritoneal cavity. Similarly to other models such as the murine air-pouch inflamed with zymosan (Perretti *et al*., 1996a) and the thioglycollate-induced mouse peritonitis (Miotla *et al*., 1995), the effect of systemic Dex on the rapid influx of neutrophils was abrogated in the animals passively immunized against LC1 or its N-terminus region.

Since the rapid elicitation of neutrophils may contribute to the initiation of the host inflammatory response by production of pro-inflammatory cytokines and chemokines (Cassatella, 1995), we questioned whether the neutralizing effect of the anti-LC1 sera on Dex-induced monocyte accumulation could be secondary to the effect on PMN recruitment. One way to answer this question was to show that the relatively late monocyte infiltration was independent of the rapid early accumulation of neutrophils in this experimental model. To this purpose, we tested the effect of Dex in control and vinblastine-treated mice. Administration of the cytotoxic drug provoked a non-specific leucopenia at 24 h which greatly affected PMN accumulation at 4 h post-zymosan (>60% of inhibition). Monocyte influx, measured at 24 h post-zymosan, was also reduced, though to a lesser extent (~30% of inhibition), by vinblastine, probably due to the reduction in circulating monocytes. However, the inhibitory action of Dex on the accumulation of this cell type was mildly affected or unaffected by treatment of mice with the cytotoxic. This indicates that a substantial reduction in PMN accumulation has little

effect on the subsequent monocyte recruitment or to its sensitivity to steroid treatment. It is therefore conceivable that the neutralizing action of the anti-LC1 antibodies on Dex-induced reduction in monocyte elicitation is subsequent to an alteration of the process(es) underlying the extravasation of this specific leucocyte.

We have recently proposed that LC1 is an important constituent of an endogenous inhibitory feed-back mechanism operating in human neutrophils. Following adhesion to endothelial monolayers, intracellular LC1 is externalised on the cell surface of the adherent neutrophil to inhibit its transmigration through the endothelial cells (Perretti *et al*., 1996b). Whether this autocrine negative modulatory role of leucocyte-derived LC1 applies to monocytes as well, is at the moment, a matter of speculation. However, in preliminary experiments we have also detected LC1 externalization in adherent human monocytes (though to a lesser extent than in neutrophils isolated from the same donors; Perretti & Flower, unpublished data): if confirmed, this observation will represent an additional dimension to the data generated in the present study. Anti-LC1 sera will be able to re-establish monocyte extravasation because they may remove the physiological brake exerted by the externalized endogenous LC1; in addition, the negative action of the protein will be particularly effective after Dex treatment, since its cell-associated levels will be elevated by administration of the steroid. Further studies will be required to confirm and clarify this speculation.

The effectiveness of LCPS1 and LCPS2 antisera in reversing the anti-migratory action of Dex both on PMN and monocyte elicitation prompted the testing of peptide Ac2-26. Confirming previous studies, s.c. treatment of mice with peptide Ac2-26 resulted in a dose-dependent inhibition of PMN accumulation into the inflamed cavity, with an approximate ED_{50} of 100 μg (33.5 nmol) per mouse. A similar value (80 μg per mouse) was calculated previously in a model of cell accumulation stimulated by the pro-inflammatory cytokine interleukin-1 (Perretti *et al*., 1993). Once the efficacy of peptide Ac2-26 in attenuating PMN extravasation was established, its effect upon monocyte recruitment was assessed. However, due to the profile of accumulation of this cell type we found that repeated administration of peptide Ac2-26 was required to achieve a significant inhibitory effect. The relatively short-half life of peptide Ac2-26 in the mouse plasma justifies the necessity of using this repeated dosage regime.

A final comment is required in relation to the degree of inhibition obtained with peptide Ac2-26 on PMN or monocyte accumulation. The superior efficacy of the peptide on PMN influx (60% inhibition compared to the 40% after only 4 injections, on monocyte influx) may be an indication of the different degree of relevance of the 'LC1 pathway' on the emigration process of the two cell types: the differences in the degree of mobilization of LC1 in monocytes and neutrophils taken from the same donor (see above) fits well with the data generated with peptide Ac2-26. Further studies will clarify this point.

Finally, since the resident Mø has long been identified as a target for glucocorticoid action (Russo-Marie, 1992), and its involvement in the inflammatory response activated by zymosan (a phagocytic stimulus) is very well established, the effect of the LC1 pharmacophore, peptide Ac2-26, on a parameter of Mø activation was evaluated. *In vitro* incubation of peptide Ac2-26 resulted in a concentration-dependent inhibition of Mø phagocytosis. The maximal degree of inhibition was around 30%, and such an effect could only partially explain the *in vivo* efficacy previously discussed. Nonetheless, an effect on Mø activation may represent another site of action for endogenous LC1 and Dex which may contribute to their overall anti-inflammatory effect in more complex scenarios.

Inhibition of Mø phagocytosis is a novel activity of LC1-derived peptides described here for the first time, and this parameter of leucocyte activation could be useful to investigate the biological function(s) of this class of anti-inflammatory agents.

This work was supported by an endowment made to the William Harvey Research Institute by ONO Pharmaceutical Co. (Osaka, Japan). R.J.F. is a Principal Research Fellow of the Wellcome Trust. We wish to thank Dr J.D. Croxtall for the supply of LCS3 serum, Dr N.J. Goulding for help with the phagocytosis assay and Mr A. Mustafa for technical help.

References

BERRY, D.A. & LINDGREN, B.W. (1990). *Statistics: Theory and Methods*, p.650, Pacific Grove, CA: Brooks/Cole Publishing Company.

BLACKWELL, G.J., CARNUCCIO, R., DI ROSA, M., FLOWER, R.J., PARENTE, L. & PERSICO, P. (1980). Macrocortin: a polypeptide causing the anti-phospholipase effect of glucocorticoids. *Nature*, **287,** 147–149.

CASSATELLA, M.A. (1995). The production of cytokines by polymorphonuclear neutrophils. *Immunol. Today*, **16,** 21–26.

COMÉRA, C. & RUSSO-MARIE, F. (1995). Glucocorticoid-induced annexin I secretion by monocytes and peritoneal leukocytes. *Br. J. Pharmacol.*, **115,** 1043–1047.

DE CATERINA, R., SICARI, R., GIANNESSI, D., PAGGIARO, P.L., PAOLETTI, P., LAZZERINI, G., BERNINI, W., SOLITO, E. & PARENTE, L. (1993). Macrophage-specific eicosanoid synthesis inhibition and lipocortin-1 induction by glucocorticoids. *J. Appl. Physiol.*, **75,** 2368–2375.

FLOWER, R.J. & ROTHWELL, N.J. (1994). Lipocortin-1: cellular mechanisms and clinical relevance. *Trends Pharmacol. Sci.*, **15,** 71–76.

GOULDING, N.J., GODOLPHIN, J.L., SHARLAND, P.R., PEERS, S.H., SAMPSON, M., MADDISON, P.J. & FLOWER, R.J. (1990). Anti-inflammatory lipocortin 1 production by peripheral blood leucocytes in response to hydrocortisone. *Lancet*, **335,** 1416–1418.

GOULDING, N.J., PAN, L., WARDWELL, K., GUYRE, V.C. & GUYRE, P.M. (1996). Evidence for specific annexin I-binding proteins on human monocytes. *Biochem. J.*, **316,** 593–597.

IWAMURA, H., MOORE, A. & WILLOUGHBY, D.A. (1993). Interaction between neutrophil-derived elastase and reactive oxygen species in cartilage degradation. *Biochim. Biophys. Acta*, **1156,** 295–301.

KLEMM, P., HARRIS, H.J. & PERRETTI, M. (1995). Effect of rolipram in a murine model of acute inflammation: comparison with the corticoid dexamethasone. *Eur. J. Pharmacol.*, **281,** 69–74.

MANCUSO, F., FLOWER, R.J. & PERRETTI, M. (1995). Leukocyte transmigration, but not rolling or adhesion, is selectively inhibited by dexamethasone in the hamster post-capillary venule. Involvement of endogenous lipocortin 1. *J. Immunol.*, **155,** 377–386.

MARIDONNEAU-PARINI, I., ERRASFA, M. & RUSSO-MARIE, F. (1989). Inhibition of O_2^- generation by dexamethasone is mimicked by lipocortin 1 in alveolar macrophages. *J. Clin. Invest.*, **83,** 1936–1940.

MIOTLA, J.M., PERRETTI, M., FLOWER, R.J., JEFFERY, P.K. & HELLEWELL, P.G. (1995). Suppresion of experimental acute lung injury in the mouse by dexamethasone and the role of lipocortin-1. *Br. J. Pharmacol.*, **114,** 62P.

PERRETTI, M. (1994). Lipocortin-derived peptides. *Biochem. Pharmacol.*, **47,** 931–938.

PERRETTI, M., AHLUWALIA, A., HARRIS, J.G., GOULDING, N.J. & FLOWER, R.J. (1993). Lipocortin-1 fragments inhibit neutrophil accumulation and neutrophil-dependent edema in the mouse: a qualitative comparison with an anti-CD11b monoclonal antibody. *J. Immunol.*, **151,** 4306–4314.

PERRETTI, M., AHLUWALIA, A., HARRIS, J.G., HARRIS, H.J., WHELLER, S.K. & FLOWER, R.J. (1996a). Acute inflammatory response in the mouse: exacerbation by immunoneutralization of lipocortin 1. *Br. J. Pharmacol.*, **117,** 1145–1154.

PERRETTI, M., CROXTALL, J.D., WHELLER, S.K., GOULDING, N.J., HANNON, R. & FLOWER, R.J. (1996b). Mobilisation of lipocortin 1 in adherent human polymorphonuclear leukocytes negatively modulates their transmigration. *Nature Med.*, **2,** 1259–1262.

PERRETTI, M. & FLOWER, R.J. (1993). Modulation of IL-1-induced neutrophil migration by dexamethasone and lipocortin 1. *J. Immunol.*, **150,** 992–999.

PERRETTI, M. & FLOWER, R.J. (1996). Measurement of lipocortin 1 levels in murine peripheral blood leukocytes by flow cytometry: modulation by glucocorticoids and inflammation. *Br. J. Pharmacol.*, **118,** 605–610.

PERRETTI, M., SOLITO, E. & PARENTE, L. (1992). Evidence that endogenous interleukin-1 is involved in leukocyte migration in acute experimental inflammation in rats and mice. *Agents Actions*, **35,** 71–78.

RUSSO-MARIE, F. (1992). Macrophages and the glucocorticoids. *J. Neuroimmunol.*, **40,** 281–296.

SOLITO, E., RAUGEI, G., MELLI, M. & PARENTE, L. (1991). Dexamethasone induces the expression of the mRNA of lipocortin 1 and 2 and the release of lipocortin 1 and 5 in differentiated, but not undifferentiated U-937 cells. *FEBS Lett.*, **291,** 238–244.

SUDLOW, A.W., CAREY, F., FORDER, R. & ROTHWELL, N.J. (1996). The role of lipocortin-1 in dexamethasone-induced suppression of PGE_2 and TNFα release from human peripheral blood mononuclear cells. *Br. J. Pharmacol.*, **117,** 1449–1456.

TAPPER, H. (1996). The secretion of preformed granules by macrophages and neutrophils. *J. Leukoc. Biol.*, **59,** 613–622.

WEBB, Z. (1979). Biochemical actions of glucocorticoids on macrophage in culture. Specific inhibition of elastase, collagenase, and plasminogen activator secretion and effects on other metabolic functions. *J. Exp. Med.*, **147,** 1695–1711.

(Received October 21, 1996
Accepted December 13, 1996)