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Measurement of lipocortin 1 levels in murine peripheral blood leukocytes by flow cytometry: modulation by glucocorticoids and inflammation

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1 Lipocortin 1 (LC1) immunoreactivity in murine peripheral blood leukocytes was quantified by use of a flow cytometric technique associated with a permeabilisation protocol with saponin. Using specific antisera raised against the whole protein or against its N-terminus peptide, cell-associated LC1-like immunoreactivity was easily detected in circulating neutrophils and monocytes, whereas very low levels were found in lymphocytes. Of the total protein measured 17.6% and 36% were associated with the external plasma membrane in neutrophils and monocytes, as assessed in the absence of cell permeabilisation, whereas no signal was detected on lymphocyte plasma membrane.

2 Treatment of mice with dexamethasone (Dex; 0.5-5 μg per mouse corresponding to $\sim$0.015-1.5 mg kg^{-1}) increased LC1 levels in neutrophils and monocytes. The 2-3 fold increase in LC1 levels was time-dependent with a peak at 2 h. Treatment of mice with the steroid antagonist, RU486 (two doses of 20 mg kg^{-1} orally) decreased LC1-like immunoreactivity in all three types of circulating leukocytes by $\geqslant$50%.

3 Extravasation of blood neutrophils into inflamed tissue sites resulted in a consistent reduction ($\geqslant$50%) in LC1 levels compared with circulating neutrophils. A high LC1-like immunoreactivity was also measured in resident macrophages, of which approximately one third was membrane-associated. Induction of an acute inflammatory response in the murine peritoneal cavity did not modify total LC1 levels measured in macrophages, but reduced membrane-associated LC1 to a significant extent, i.e. up to 70%.

4 In conclusion, flow cytometric analysis is a rapid and convenient method for detecting and measuring LC1 in murine leukocytes. We confirmed that LC1 protein expression is controlled by exogenous and endogenous glucocorticoids. Amongst other factor(s) influencing protein concentrations, extravasation was found to be associated with a reduced LC1 expression in the emigrated cells.

Keywords: Monocyte; macrophage; dexamethasone; RU486; annexin I; neutrophil; zymosan; emigration

Introduction

Several of the anti-inflammatory actions exerted by the potent glucocorticoids are mediated by the endogenous protein lipocortin 1 (LC1; also called annexin I) (Flower, 1988). In line with this we have recently reported that the inhibitory effect on the recruitment of polymorphonuclear leukocytes (PMN) observed following systemic (intravenous, i.v.) treatment of mice with dexamethasone (Dex) is mediated by endogenous LC1 (Perretti & Flower, 1993). In these experiments local injection of interleukin-1, or interleukin-8, into murine air-pouches provoked a marked PMN accumulation which was attenuated by treatment of mice with Dex: in both cases, passive immunisation of animals with anti-LC1 antibodies abrogated the reduction caused by the corticosteroid (Perretti & Flower, 1993; Perretti *et al.*, 1994).

This anti-migratory property of LC1 and its role as a mediator of the effect of Dex substantiates studies performed by other groups who also reported an inhibitory effect of systemic Dex on the PMN accumulation caused by direct-acting agents, such as the formylated tripeptide, formyl-Met-Leu-Phe (Oda & Katori, 1992; Yarwood *et al.*, 1993), an effect which could not be explained on the basis of an interference with the production of pro-inflammatory cytokines (Schleimer *et al.*, 1989). The role played by LC1 in PMN recruitment has been confirmed further by a recent study of intravital microscopy in which the selective effect of Dex on leukocyte emigration through the post-capillary venules of the hamster cheek pouch microcirculation was abrogated in animals pretreated with neutralizing anti-LC1 antisera (Mancuso *et al.*, 1995).

To complement these studies it was important to confirm that i.v. challenge with anti-inflammatory doses of Dex actually increased endogenous LC1 levels in circulating leukocytes in these species. Other methods for measuring LC1 (e.g. western blotting and ELISA) have been used with success (Goulding *et al.*, 1990; Peers *et al.*, 1993) but there are disadvantages to these techniques in that cell populations have to be separated prior to analysis and LC1 has to be extracted from the different cellular compartments prior to assay. Furthermore, whilst ELISA is sensitive and leads itself to quantitation, western blotting is at best only a semi-quantitative technique.

Flow cytometry has been successfully employed to detect LC1 translocation to the membrane of transformed U937 cells (Solito *et al.*, 1994) and to measure the levels of the protein in human leukocytes (Morand *et al.*, 1995). Here, we have modified a protocol used for detection of LC1 binding to a mixed population of mouse peripheral blood leukocytes (PBL) (Perretti *et al.*, 1993), to quantify the levels of immunoreactive LC1 in these cells and investigated the modulation exerted by Dex. We report that both endogenous and exogenous glucocorticoids positively regulate the extent of LC1 immunoreactivity in mouse PBL and describe for the first time that cell elicitation during acute inflammation causes a marked reduction in LC1 levels in the emigrated cells.

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Methods

Animals

Male Swiss Albino mice (28–32 g body weight; Tuck, Essex, UK) were used for all experiments. Mice were maintained on a standard chow pellet diet with tap water *ad libitum*.

Peripheral blood leukocyte (PBL) preparation

Blood was collected by cardiac puncture into heparinized syringes under brief halothane (3% in a mixture of oxygen and nitrous oxide, 1 $lmin^{-1}$ for both gases) anaesthesia, and diluted (1:2 with sterile PBS) before being layered onto a ficoll-metrizoate gradient (Ferrante & Thong, 1978). Four ml of diluted blood was added to 3 ml of FM-85 gradient and centrifuged at 400 *g* for 30 min at room temperature. Cells were washed twice in PBS and contaminating erythrocytes were removed by hypotonic lysis. Following this procedure it was possible to prepare a mixed population of mouse PBL consisting of approximately 60–70% lymphocytes, 10–15% monocytes and 20–25% PMN. Cell vailability ($\geqslant$ 95%) was always checked by the trypan blue exclusion test.

Measurement of LC1 levels in PBL

Cells ($0.5-1 \times 10^6$ cells) were added to 96-well plates in 20 μl of RPMI-1640 supplemented with bovine serum albumin (BSA, 0.2% w:v) and calcium (1.3 mM). To measure total LC1 levels, cells were fixed by addition of 20 μl of 2% paraformaldehyde and incubated for 30 min at 4°C. Then three washes (of 100, 200 and 200 μl successively) in PBS supplemented with $CaCl_2$ (1.5 mM) and saponin (0.015%, from *Saponaria* species; Sigma), termed PBCS, were performed. After two washes in the PBCS, non-specific binding sites were blocked by adding 20 μl of homologous plasma (1:10 final dilution) and 20 μl of several sheep sera (see below) added. After 1 h on ice, cells were washed 3 times with PBCS and then incubated with 40 μl of a specific $F(ab')_2$ fragment of donkey anti-sheep immunoglobulin G (IgG) conjugated to fluorescein isothiocyanate (FITC; stock solution 1:30 in PBCS; Sigma) and incubated for a further 45 min. Then, two other washes and fixation with an equal volume of 2% paraformaldehyde were performed. Flow cytometric (FACS) analysis was usually carried out within two-three days during which time the samples remained stable.

For FACS analysis of the samples a FACScan II analyser (Becton Dickinson, Mountain View, CA, U.S.A.) equipped with air-cooled 100 mV argon ion laser tuned to 488 nm and a Consort 32 computer running Lysis II software was used. Lymphocyte, monocyte and PMN populations were discriminated by use of forward and side scatter characteristics. Each population was gated according to its position in the screen and the fluorescence estimated as median fluorescence intensity (MFI) units in the FL1 channel. The number of LC1 molecules was then calculated by the MFI values using reference microbeads labelled with standard numbers of FITC molecules (Flow Cytometry Standards Corp) according to the method of Le Bouteiller *et al.* (1983).

In preliminary experiments we tested a polyclonal sheep serum raised against human LC1, termed LCS3 (Perretti & Flower, 1993), as well as other sera raised against the LC1 N-terminus peptide acetyl-2-26, termed LCPS1 and LCPS2. There was no difference between the different batches of non-immune sheep serum, termed NSS, which was always used as a negative control. Nevertheless, the latter sheep sera gave a small signal above FITC alone indicating that there was a non-specific binding of sheep immunoglobulins to some cellular structure, and both controls were used in all experiments. All sera were used at final dilutions of 1:75, 1:150 and 1:300. Figure 1 illustrates the results of a representative experiment where the fluorescence associated with cells treated with two specific anti-LC1 antibodies, LCS3 and LCPS1, was higher than that obtained with NSS. Another anti-LC1 antiserum, LCPS2, whilst satisfactory in ELISA and western blot assays, was not particularly useful under these experimental conditions and was not used further. The difference between the apparent number of LC1-like immunoreactive molecules detected with the specific antisera and those measured with the NSS was taken as the actual level of cell-associated LC1.

The procedure described above detected an immunoreactivity which corresponded to cell-associated LC1 (or total, i.e. intracellular + membrane bound LC1-like immunoreactivity). In some experiments, membrane LC1 alone was measured: the procedure used was identical to that described above except for two precautions: (i) a calcium ion concentration of 1 mM was maintained in the media used for preparing the leukocytes to avoid the detachment of LC1 bound to the membrane (Browning *et al.*, 1990), and (ii) cells were stained with the antibodies without previous fixation and permeabilisation with saponin, the latter reagent being omitted from all the media used.

In initial experiments we validated the permeabilisation protocol using two monoclonal antibodies (1:200 final dilution) specific for two intracellular proteins, actin and vimentin (raised against the human antigens and purchased from Sigma). The protocol was similar to the one described above except that detection was achieved with FITC-conjugated anti-mouse IgG. Fluorescence in the FL1 channel was measured and MFI units converted to number of molecules per cell. No signal above FITC control was measured in the absence of saponin permeabilisation. The values obtained for intracellular staining for actin and vimentin in our experimental conditions are given in Table 1.

LC1 levels in inflammatory cells

LC1 levels were measured in cells taken from an inflammatory site using air-pouches inflamed with interleukin-1, interleukin-8 or zymosan (Perretti & Flower, 1993; Perretti *et al.*, 1994), or from peritoneal cavities inflamed with zymosan (Perretti *et al.*, 1992). Briefly, air-pouches were formed on the back of mice by air injection (2.5 ml) on day 0 and day 3. On day 6 the animals received a local injection of 5 ng of murine recombinant interleukin-1β, 1 μg human interleukin-8, or 1 mg zymosan. Four hours later animals were bled by cardiac puncture and

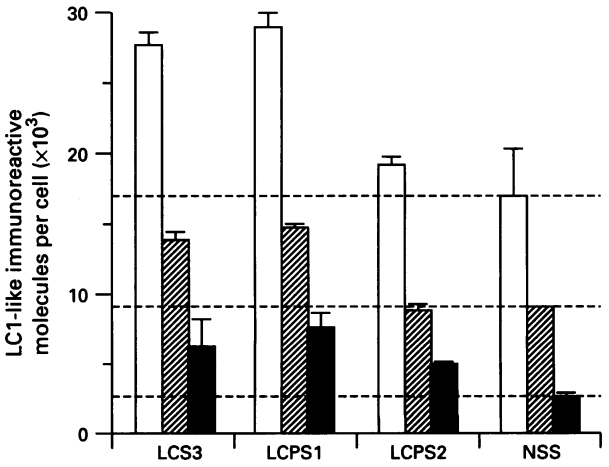

Figure 1 Detection of LC1-like immunoreactivity in mouse circulating PMN. Cells were stained with different dilutions (open columns, 1:75; hatched columns, 1:150; solid columns, 1:300) of sheep serum raised against LC1, LCS3, or of two sheep sera raised against peptide Ac2-26 which corresponds to most of the LC1 N-terminus region (LCPS1 and LCPS2), or of a non-immune sheep serum used as a negative control (NSS). Data are mean ± s.e.mean of a single experiment performed in triplicate, representative of two other experiments with similar results.

PBL prepared as described above. Air-pouches were washed with 2 ml PBS and recovered cells (≥90% neutrophils) washed twice and used in the LC1 assay as described above.

Mouse peritonitis was induced by intraperitoneal injection of 1 mg zymosan in 0.5 ml sterile saline. At different times after zymosan injection, peritoneal cavities were washed with 4 ml PBS and cells washed twice prior to use for the quantification of LC1 levels. When comparisons between circulating and elicited PMN were made, mice were bled by cardiac puncture and PBL prepared as above. When the effect of inflammation on LC1 immunoreactivity associated with macrophages (Mϕ) was studied, peritoneal cavities of untreated mice were washed and cells (≥80% Mϕ) used for the *in vitro* assay.

Drug Treatment

Dex (sodium phosphate salt, David Ball Laboratories, Warwick, UK) was diluted in sterile saline and given i.v. in a volume of 100 μl. The doses used were those previously shown to be anti-inflammatory in the mouse (Perretti & Flower, 1993; Perretti *et al.*, 1994). RU486 (mifepristone) was the kind gift of Rousell-Uclaf, Romainville, France. The drug was sonicated in distilled water (2 mg ml^{-1}) and given orally using a dose (20 mg kg^{-1}) and a protocol (24 h and 2 h prior to cell collection or to Dex injection) previously validated both in terms of receptor occupancy (Fan *et al.*, 1994) and of reversal of the anti-inflammatory effect of several corticosteroids (Peers *et al.*, 1988). In all cases control mice received vehicle.

Materials

Zymosan (type A from Sigma) was prepared by boiling for 30 min in PBS, extensively washed and stored at −20°C prior to use. Murine recombinant interleukin-1β was a generous gift of Dr R.C. Newton (Du Pont-Merck, Wilmington, DE, U.S.A.). Human recombinant interleukin-8 was generously supplied by Dr I. Lindley (Sandozforschunginstitute, Wien, Austria). Both cytokines were diluted in PBS+0.1% BSA, divided into aliquots and stored at −80°C. Fresh aliquots were used for each experiment. All other materials were from Sigma.

Statistics

Statistical differences were analysed by analysis of variance followed by the Bonferroni test for post-hoc intergroup comparisons. A P value less than 0.05 was taken as significant.

Results

Detection of LC1-like immunoreactivity in mouse PBL

The cumulative data for cell-associated LC1-like immunoreactivity in mouse PBL in 10–14 experiments were as follows (× 10^3 LC1-like molecules per cell; mean±s.e.mean): 28±4 in PMN, 35±5 in monocytes and 8±3 in lymphocytes. Omission of the fixation and permeabilisation steps allowed the quantification of membrane-associated LC1. The cumulative data for this set of experiments are given in Table 2. In five distinct experiments using LCS3, membrane-associated LC1 represented between 10–30% of the total amount in circulating PMN and monocytes, whereas a signal was never detected on the plasma membrane of circulating lymphocytes (Table 2).

Table 1 Validation of the permeabilisation protocol in murine peripheral blood leukocytes

Monoclonal antibody	*Cell type (no. of molecules per cell, × 10^3)* PMN	Monocyte	Lymphocyte
Actin			
with saponin	77±4	65±4	43±3
without saponin	<1	<1	ND
Vimentin			
with saponin	33±4	59±4	33±2
without saponin	<0.5	<0.5	<0.5

Cells were left untreated or permeabilised with saponin (0.015% final concentration w:v) and stained either with an anti-actin or an anti-vimentin monoclonal antibody (1/200 final dilution). Values (mean±s.e.mean, n = 3) represent the number of molecule of each antigen as converted from the MFI units.ND: not detectable.

Effect of endogenous and exogenous glucocorticoids on LC1 levels in mouse PBL

The effect of systemic treatment with Dex on total LC1 levels in PBL was evaluated. Dex induced a dose-dependent increase in cell-associated LC1 in monocytes with as little as 0.5 μg per mouse (Figure 2). A significant increase was also detected in PMN with the 5 μg dose. High LC1 levels were also measured

Table 2 LC1 levels detected in untreated mouse peripheral blood leukocytes in the presence or absence of cell permeabilisation with saponin

Cell type	*LC1-like immunoreactive molecules (× 10^3)* *without saponin (membrane LC1)*	*with saponin (total LC1)*
PMN	8±1	44±4
Monocyte	13±1	35±5
Lymphocyte	0	5±1

A mixed population of untreated murine peripheral blood leukocytes was treated with media without or supplemented with saponin (0.015% final concentration, w:v). Cells were stained with LCS3, specific anti-LC1 sheep serum, and NSS, to have the control background (1:75 final dilution in both cases). Samples were analysed by FACS, thus allowing the identification of each specific cell population, based on their forward and side scatter characteristics. Values are mean±s.e.mean of 5 experiments.

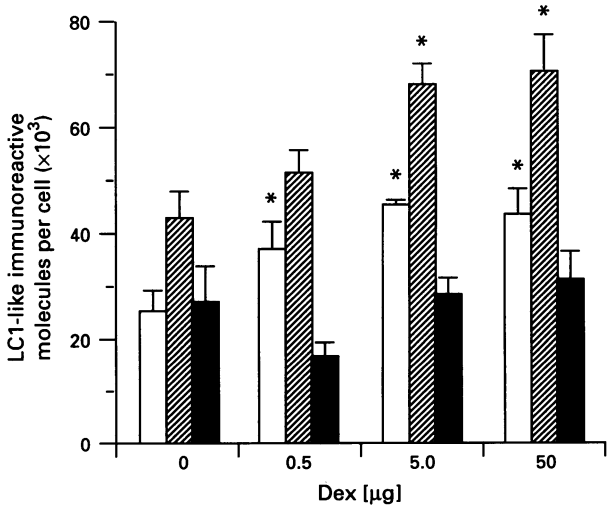

Figure 2 Increase of total LC1-like immunoreactivity in mouse PBL following systemic treatment with dexamethasone (Dex). A mixed PBL population was prepared from blood taken 2 h after treatment of mice with PBS (100 μl i.v.) or with reported doses of Dex. LC1 levels were measured in permeabilised monocytes (open columns), PMN (hatched columns) and lymphocytes (solid columns) as net values following staining with a LCS3 and NSS serum (1:75 dilution). Values are mean±s.e.mean, n=6–8. *P<0.05 vs. respective control value (dose 0).

after treatment with the highest dose tested of 50 μg per mouse. In these particular experiments, a consistent immunoreactivity above the NSS value was measured in lymphocytes; however, this was not significantly modified by treatment with any dose of Dex (Figure 2). Treatment with the anti-inflammatory steroid caused changes in the amount of immunoreactive LC1 with an apparent peak at the 2 h time-point. This is illustrated in Figure 3, where the effect of 50 μg dose on LC1 levels in circulating PMN is shown. Levels peaked within 2 h, then declined by 4 h and were back to basal values by 20 h. A similar profile was also shown by monocytes whereas no time-dependent changes were observed in lymphocytes (data not shown). Evaluation of membrane-associated LC1 levels in PMN showed an increase following 50 μg i.v. Dex at the 2 h time-point (mean ± s.e.mean, $n=5$): from $6.8 \pm 1.1 \times 10^3$ to $13.4 \pm 1 \times 10^3$ LC1-like molecules per cell ($P<0.05$).

The potential effect of endogenous steroids on the measurement of LC1-like immunoreactivity was investigated by treating mice with the steroid antagonist, RU486. Figure 4 shows a reduction in LC1 levels both in circulating PMN and monocytes by more than 50% after treatment of animals with RU486.

LC1 levels in leukocytes taken from an inflammatory site

We compared the levels of LC1-like immunoreactivity in elicited PMN with the same cell type taken from the blood of the same animals. Approximately 50% reduction in total LC1 was measured in PMN collected from air-pouches 4 h after treatment with interleukin-1β, in comparison to the amounts found in blood PMN taken from the same animals ($\times 10^3$ LC1-like molecules per cell; mean ± s.e.mean of 3 experiments): 31 ± 7 in peripheral blood PMN down to 15 ± 4 in interleukin-1β-elicited PMN ($P<0.01$).

Immunoreactive LC1 on the PMN plasma membrane was also measured on circulating and elicited PMN. A substantial reduction was again found if cells were collected from the site of inflammation (air-pouch inflamed with interleukin-1β): $8 \pm 1 \times 10^3$ LC1-like immunoreactive molecules on the membrane of circulating PMN down to $2.5 \pm 0.3 \times 10^3$ LC1-like immunoreactive molecules on the membrane of PMN from inflamed tissue of the same animal (mean ± s.e.mean, $n=4$). In the same experiments total LC1 levels were also reduced, from $48 \pm 3 \times 10^3$ LC1-like immunoreactive molecules in circulating PMN to $21 \pm 3 \times 10^3$ LC1-like immunoreactive molecules in PMN from inflamed tissue ($P<0.01$ in both cases).

The marked reduction in LC1 immunoreactivity observed during acute inflammation was a general feature which appeared not to be related either to the stimulus used for cell elicitation or to the site where the inflammation was caused. Table 3 reports the cumulative data of a series of experiments in which air-pouches were inflamed either with interleukin-8 or with a non-specific stimulus like zymosan. In addition, the reduction in LC1 levels measured in PMN migrated into the peritoneal cavity after challenge with zymosan are also reported in relation to the different time-points analysed (Table 3).

When resident peritoneal Mϕ were stained with LCS3 and NSS, a high number of LC1-like immunoreactive molecules was found (mean ± s.e.mean, $n=6$), i.e. $242 \pm 33 \times 10^3$ cell-associated and $87 \pm 20 \times 10^3$ membrane-associated LC1 molecules per cell.

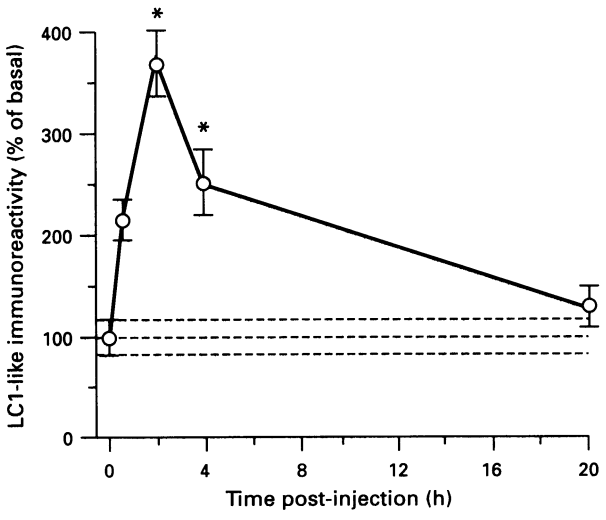

Figure 3 Timecourse of dexamethasone (Dex)-induced increase in total LC1 levels in mouse circulating PMN. Mice received 100 μl of PBS alone or containing 50 μg Dex and blood was taken at different time-points. LC1 levels were measured in permeabilised PMN as net values following staining with a LCS3 and NSS serum (1:75 dilution). Data have been normalised taking LC1 levels measured after PBS treatment as 100% for each time-point (dashed lines) (mean ± s.e.mean, $n=4$ experiments). *$P<0.05$ as calculated from original values.

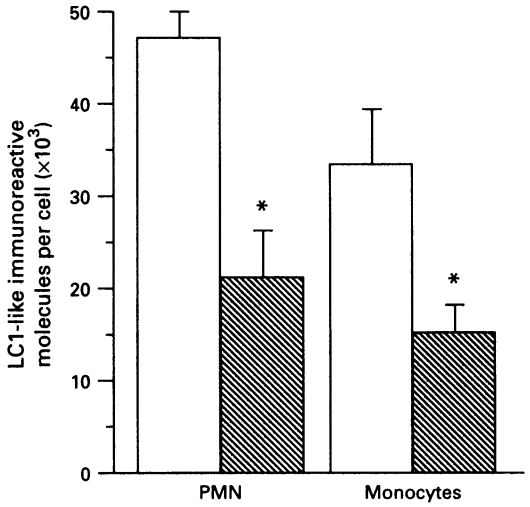

Figure 4 Effect of RU486 (hatched columns) on total LC1 levels in murine circulating PMN and monocytes. Mice were treated either with RU486 (20 mg kg^{-1} orally) or with vehicle (10 ml kg^{-1} orally) 24 h and 2 h prior to blood collection. Cell-associated LC1 levels were measured following staining of permeabilised cells with LCS3 and NSS sera (1:75 final dilution in both cases), the difference of the two stainings giving the LC1 values. Data are mean ± s.e.mean of 3 experiments. *$P<0.05$ vs. respective control value (open columns).

Table 3 Reduction of total immunoreactive LC1 levels in elicited PMN

Stimulus	*Tisssue site*	*Time-point*	*Reduction (%)*	*Number of experiments*
Interleukin-8	air-pouch	4 h	90	1
Zymosan	air-pouch	4 h	51	1
Zymosan	peritoneal	4 h	79	1
	cavity	24 h	87	2
		48 h	67	2

Elicited PMN were recovered from air-pouches inflamed with human interleukin-8 (1 μg in 0.5 ml CMC), or with zymosan (1 mg in 0.5 ml saline), or from peritoneal cavities inflamed with zymosan (1 mg in 1 ml saline). Blood was collected by cardiac puncture from the same animals and a mixed leukocyte population prepared by gradient centrifugation. In all cases, cells were permeabilised with saponin (0.015% final concentration, w:v) and stained with the polyclonal LCS3 and NSS (1:75 final dilution). LC1 levels in blood PMN were $34 \pm 5 \times 10^3$ molecules per cell (mean ± s.e.mean of 5 experiments).

The effect of inflammation on LC1 levels associated with peritoneal Mϕ was also investigated by means of the zymosan peritonitis model. Figure 5 shows that no changes in cell-associated LC1 in peritoneal Mϕ were detected at different time-points after zymosan administration. However, a consistent reduction in membrane-associated LC1 was observed as early as 4 h after injection of the inflammogen (Figure 5).

Discussion

A cornerstone in LC1 biology is its link to glucocorticoid hormones which can regulate its metabolism in patho-physiological situations. Glucocorticoid-dependent induction of LC1 has been demonstrated in cell lines (Solito *et al.*, 1991) as well as in primary cell cultures such as in rat peritoneal macrophages (Peers *et al.*, 1993), in human monocytes and macrophages (Goulding *et al.*, 1990; Ambrose & Hunninghake, 1990a,b; De Caterina *et al.*, 1993). Physiological concentrations of endogenous steroids are sufficient to control basal LC1 levels, as demonstrated by the reduction of LC1 mRNA and protein in cells and organs taken from adrenalectomised rats (Solito *et al.*, 1990; Vishwanath *et al.*, 1992; 1993). On the basis of all these data it was important to measure LC1 levels in murine PBL and assess its modulation by corticosteroids, so that we could relate it to the role of the protein in inflammation. Investigation of this important relationship was greatly facilitated by application of the FACS technique employed to measure LC1 binding to animal leukocytes. Four main advantages are offered by the FACS analysis: (i) it was not necessary to isolate a single population of leukocytes before measurement; (ii) it was possible to distinguish between total (cell-associated) and membrane-associated LC1 by adding or removing saponin from the media; (iii) data could be quantified quite precisely and statistical analysis applied; (iv) no extraction was required prior to analysis.

Reproducible LC1 levels were measured in circulating murine PMN and monocytes, with much a lower and variable signal observed in lymphocytes. It is noteworthy that both the polyclonal serum raised against LC1 whole molecule (LCS3) and a polyclonal serum raised against LC1 whole molecule (LCS3) and a polyclonal serum raised against the anti-inflammatory N-terminus peptide (LCPS1) were both successfully used to detect a positive signal. In addition, between 20–30% of the total immunoreactivity in PMN and monocytes was detected in the absence of cell permeabilisation. These data are novel in large part and a proper comparison can be made only with the single study in which LC1 levels in human blood leukocytes were measured. Goulding *et al.* (1990) found a similar distribution between the membrane-bound, EDTA-detachable, and cell-associated LC1 in human blood leukocytes, as measured with an enzyme-linked immunoassay technique.

More recently, the modulation of LC1 levels in rat peritoneal macrophages by endogenous and exogenous steroids has been reported (Peers *et al.*, 1993). In keeping with these data, we found that mouse peritoneal Mϕ had higher LC1 levels than circulating PMN and monocytes, and, significantly, a high portion of the total immunoreactivity (36%) was detectable in the absence of cell permeabilisation. A study in human subjects has also confirmed that resident (alveolar) macrophages contained higher LC1 levels in comparison to those found in circulating monocytes taken from the same patients (De Caterina *et al.*, 1993). All these indications support the concept that mature cells have much more LC1 than undifferentiated cells, as initially demonstrated in the leukæmic U937 cell line (Solito *et al.*, 1991), and confirm the peritoneal Mø as a major source of LC1.

The LC1-like immunoreactivity detected in PBL by FACS analysis was under the control of exogenous and endogenous glucocorticoids. When mice were treated with a dose of Dex known to be anti-inflammatory through a LC1-dependent mechanism (Perretti & Flower, 1993) a significant increase in cell-associated LC1 was observed both in PMN and monocytes. Overall the data obtained substantiated several aspects of the interrelationship between LC1 and glucocorticoids: the effect of the Dex was both dose-dependent and time-dependent, with a dose of 5 μg per mouse, which inhibited PMN recruitment *via* endogenous LC1, being sufficient to increase LC1 levels in circulating PMN within 2 h (Perretti & Flower, 1993; Perretti *et al.*, 1994).

When mice received the glucocorticoid antagonist, RU486, according to a protocol which produces well validated changes in receptor occupancy (Fan *et al.*, 1994) and reverses the anti-inflammatory effect of several corticosteroids (Peers *et al.*, 1988), a significant decrease in LC1 levels was obtained both in monocytes and PMN. The reduction in cell-associated LC1 measured was around 50% and such an effect is consistent with that reported in adrenalectomized rats (Solito *et al.*, 1990; Vishwanath *et al.*, 1992). Overall these data indicate that endogenous corticosterone controls LC1 levels in PBL. It is also evident that other factor(s) are involved in view of the remaining 50% of the control values detected after RU486 treatment (this study) or surgical adrenalectomy (Vishwanath *et al.*, 1992).

An important part of this project was the measurement of LC1 in elicited PMN. Elicited PMN exhibited a significant reduction in cell-associated LC1. This finding parallels data obtained in experiments where LC1 binding to elicited PMN was measured (Perretti *et al.*, 1993), and suggests that down regulation of LC1 function is a general characteristic of emigrated neutrophils. Indeed, a consistent reduction in immunoreactive LC1 associated with elicited PMN in comparison to the levels measured in circulating PMN taken from the same animals was observed irrespective of the stimulus applied to cause cell infiltration (interleukin-1, interleukin-8 or zymosan) as well as of the site infiltrated (air-pouch or peritoneal cavity). It has been known for a long time that elicited PMN are activated in comparison with circulating PMN, in terms of enzymatic release, adhesion molecule ex-

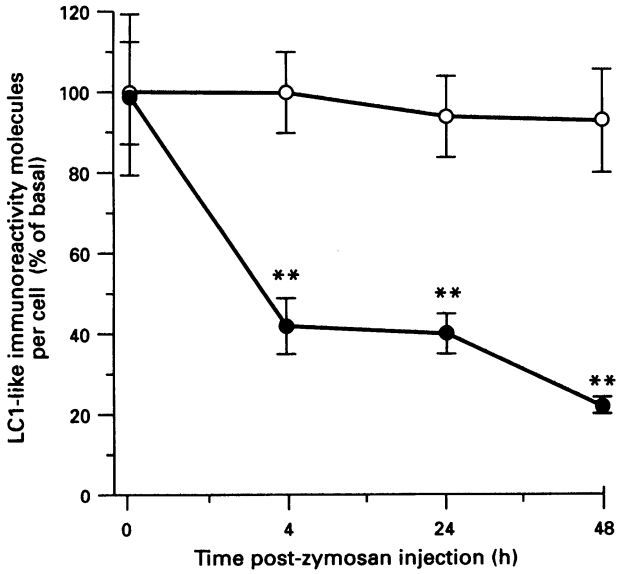

Figure 5 Distinct effect of inflammation on LC1 pools in peritoneal macrophages. Mice were injected with zymosan (1 mg in 0.5 ml saline) into the peritoneal cavity and cells were recovered at different time-points. Control mice were untreated (time 0). Cell-associated (○) and membrane-associated (●) LC1 levels were measured in macrophages following staining with LCS3 and NSS serum (1:75 final dilution in both cases), the difference of the two stainings giving the LC1 values. Values (mean ± s.e.mean of 5 experiments with 5 mice per group) are expressed as % of LC1 levels in untreated mice: $243 \pm 33 \times 10^3$ cell-associated and $87 \pm 20 \times 10^3$ membrane-associated LC1 molecules per cell. $^{**}P < 0.01$ vs. respective time 0, as calculated from original numbers.

pression and superoxide generation (Zimmerli *et al.*, 1986; Kishimoto *et al.*, 1989; Sengeløv *et al.*, 1995). More recent studies have shown that the PMN become actively involved in the synthesis of several pro-inflammatory cytokines when at the site of inflammation (Cassatella, 1995), producing a distinct pattern of release depending on the stimulus used for inducing the migration. It is tempting to propose that PMN extravasation up-regulates specific proinflammatory biochemical events within the cell and, conversely, levels and/or actions of an anti-inflammatory protein such as LC1 are down-regulated.

In conclusion, FACS analysis of LC1-like immunoreactivity in mouse PBL showed a strict dependency upon endogenous and exogenous glucocorticoids. Besides these hormones, other factors and/or events regulate LC1 levels in leukocytes, and some of these may be activated during the acute inflammatory response.

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