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Acute inflammatory response in the mouse: exacerbation by immunoneutralization of lipocortin 1

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1 An immuno-neutralization strategy was employed to investigate the role of endogenous lipocortin 1 (LC1) in acute inflammation in the mouse.

2 Mice were treated subcutaneously with phosphate-buffered solution (PBS), non-immune sheep serum (NSS) or with one of two sheep antisera raised against LC1 (LCS3), or its N-terminal peptide (LCPS1), three times over a period of seven days. Twenty four hours after the last injection several parameters of acute inflammation were measured including zymosan-induced inflammation in 6-day-old air-pouches, zymosan-activated serum (ZAS)-induced oedema in the skin, platelet-activating factor (PAF)-induced neutrophilia and interleukin-1β (IL-1β)-induced corticosterone (CCS) release.

3 At the 4 h time-point of the zymosan inflamed air-pouch model, treatment with LCS3 did not modify the number of polymorphonuclear leucocytes (PMN) recruited: 7.84 ± 1.01 and $7.00 \pm 0.77 \times 10^6$ PMN per mouse for NSS- and LCS3 group, $n=7$. However, several other parameters of cell activation including myeloperoxidase (MPO) and elastase activities were increased (2.2 fold, $P<0.05$, and 6.5 fold, $P<0.05$, respectively) in the lavage fluids of these mice. Similarly, a significant increase in the amount of immunoreactive prostaglandin E_2 (PGE_2; 1.81 fold, $P<0.05$) and IL-1α (2.75 fold, $P<0.05$), but not tumour necrosis factor-α (TNF-α), was also observed in LCS3-treated mice.

4 The recruitment of PMN into the zymosan inflamed air-pouches by 24 h had declined substantially ($4.13 \pm 0.61 \times 10^6$ PMN per mouse, $n=12$) in the NSS-treated mice, whereas high values were still measured in those treated with LCS3 ($9.35 \pm 1.20 \times 10^6$ PMN per mouse, $n=12$, $P<0.05$). A similar effect was also found following sub-chronic treatment of mice with LCPS1: $6.48 \pm 0.10 \times 10^6$ PMN per mouse, vs. 2.77 ± 1.20 and $2.64 \pm 0.49 \times 10^6$ PMN per mouse for PBS- and NSS-treated groups ($n=7$, $P<0.05$). Most markers of inflammation were also increased in the lavage fluids of LCS3-treated mice: MPO and elastase showed a 2.47 fold and 17 fold increase, respectively ($P<0.05$ in both cases); TNF-α showed a 11.1 fold increase ($P<0.05$) whereas the IL-1α levels were not significantly modified. PGE_2 was still detectable in most (5 out of 7) of the mice treated with LCS3 but only in 2 out of 7 of the NSS-treated mice.

5 Intradermal injection of 50% ZAS caused a significant increase in the 2 hoedema formation in the skin of LCS3-treated mice in comparison to PBS- and NSS-treated animals: 16.7 ± 1.5 μl vs. 10.8 ± 1.2 μl and 10.2 ± 1.0 μl, respectively ($n=14$ mice per group, $P<0.05$). ZAS-induced oedema had subsided by 24 h in control animals but a residual significant amount of extravasation was still detectable in LCS3-treated mice: 4.4 ± 0.8 μl ($P<0.05$).

6 A recently described model driven by endogenous glucocorticoids is the blood neutrophilia observed following administration of PAF. In our experimental conditions, a single bolus of PAF (100 ng, i.v.) provoked a marked neutrophilia at 2 h (2.43 and 2.01 fold) in NSS- and PBS-treated mice ($n=11$), respectively, which was significantly attenuated in the animals treated with LCS3: 1.26 fold increase in circulating PMN ($n=11$, $P<0.01$ vs. NSS- and PBS-groups).

7 Intraperitoneal injection of IL-1β (5 $\mu g\ kg^{-1}$) caused a marked increase in circulating plasma CCS by 2 h, to a similar extent in all experimental groups. In contrast, measurement of CCS levels in the plasma of mice bearing air-pouches inflamed with zymosan revealed significant differences between LCS3 and NSS-treated mice at the 4 h time-point: 198 ± 26 ng ml^{-1} vs. 110 ± 31 ng ml^{-1} ($n=8$, $P<0.05$).

8 In conclusion, we found a remarkable exacerbation of the inflammatory process with respect to both humoral and cellular components in mice passively immunised against LC1, suggesting the existence of a negative modulatory role for this protein in the normal regulation of the host defence mechanism.

Keywords: Annexin I; oedema; neutrophil migration; elastase; corticosterone; HPA axis; interleukin-1; tumour necrosis factor; glucocorticoids

Introduction

Lipocortin 1 (LC1; also called annexin I) is a 37 kDa protein whose synthesis, and appearance on the external membrane of cells, is controlled by endogenous and exogenous glucocorticoids (for a recent review see Flower & Rothwell, 1994). The protein is found mainly in certain highly differentiated cell types (Fava *et al.*, 1989) including those intimately involved in the inflammatory response such as monocyte/macrophages and the polymorphonuclear leucocytes (PMN) (Goulding *et al.*, 1990).

Several lines of evidence point to the importance of LC1 as a regulator of inflammation and as a mediator of some anti-inflammatory effects of the glucocorticoids. These hormones

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induce both the mRNA for lipocortin and expression of the lipocortin protein in several cell types both *in vivo* and *in vitro* (Flower & Rothwell, 1994). In some cells an effect of glucocorticoids on the translocation of the protein from the cytoplasm to the membrane has also been documented (Browning *et al.*, 1990; Solito *et al.*, 1994).

In vitro, recombinant LC1 inhibits cell differentiation and proliferation (Violette *et al.*, 1990; Croxtall & Flower, 1992) and eicosanoid release and inhibits the release of several pituitary hormones from the anterior pituitary gland in a manner that is strongly reminiscent of the glucocorticoids themselves (Loxley *et al.*, 1993; Taylor *et al.*, 1993). Furthermore the inhibitory actions of exogenous glucocorticoids in these models is completely overcome in the presence of various anti-LC1 neutralizing antibodies.

Highly purified re-folded full length human recombinant LC1 and its 1-188 fragment exert powerful anti-inflammatory actions in several animal models, inhibiting paw oedema and ischaemic brain damage in the rat (Cirino *et al.*, 1989; Relton *et al.*, 1991) as well as fever in the rat and rabbit (Carey *et al.*, 1990; Davidson *et al.*, 1991). We have recently reported that LC1 potently inhibits PMN accumulation into the site of inflammation, i.e. preformed dorsal mouse air-pouches inflamed with interleukin-1 (Perretti & Flower, 1993). Peptides derived from the N-terminus region of LC1 exhibit similar properties to the parent protein, although with a lower potency, inhibiting PMN elicitation caused by interleukin-1, interleukin-8 or zymosan (Perrett *et al.*, 1993a). Passive immunization of rats and mice with anti-LC1 antibodies severely impairs the ability of exogenous glucocorticoids to develop their full anti-inflammatory and hypothalamus-pituitary-adrenal (HPA) axis feedback action (Perretti & Flower, 1993; Taylor *et al.*, 1995).

Most of the experiments demonstrating the involvement of LC1 in the anti-inflammatory action of exogenous glucocorticoids have been acute in nature, that is to say they have examined LC1-dependent changes in the inflammatory response within hours of giving dexamethasone (see for example Duncan *et al.*, 1993). Few studies have addressed the question of whether synthesis and externalization of LC1, driven by the *endogenous* hormones of the HPA axis itself, is important in regulating inflammation and the inflammatory process *in vivo*. To this end we have designed a series of experiments in which we used an immunoneutralization strategy to test the hypothesis that LC1 is an essential component of the endogenous anti-inflammatory defence mechanisms which are activated by glucocorticoids, released following the HPA axis stimulation associated with the inflammatory response. Our results show, for the first time, that LC1 is important in terminating inflammatory responses which evolve over a period of six or more hours and that this effect depends upon the activity of the HPA axis.

Methods

Animals

Male Swiss Albino mice (20–22 g body weight) were purchased from Interfauna (CFLP strain; Huntingdon, Cambridgeshire) and maintained on a standard chow pellet diet with tap water *ad libitum*. Animals were used at least one week after arrival.

Sheep antisera

Several different sheep antisera were used in the study: a polyclonal sheep antiserum raised against the intact full length human recombinant protein designated *LCS3*; a polyclonal sheep antiserum raised against the anti-inflammatory LC1-derived peptide corresponding to most of the N-terminus region of LC1 (Perretti *et al.*, 1993a), termed *LCPS1*; a non-immune sheep serum referred to as *NSS* (two different batches were used). Although LCS3 was raised against the human protein it also recognises LC1 in several animal species such as mouse (Perretti & Flower, 1993), rat (Taylor *et al.*, 1995) and hamster (Mancuso *et al.*, 1995). The LCPS1 polyclonal antibody was raised against the peptide human Ac2-26, and recognises murine LC1 in flow cytometry (Perretti *et al.*, 1995b) as well as in Western blotting analysis.

Protocol for the passive immunisation of mice

In our first study with LCS3 we found that following a single s.c. injection of 50 μl of serum, the anti-LC1 titre rose in the plasma within the first 24 h and remained high for at least 3 days (Perretti & Flower, 1993). On this basis we decided to administer the anti-LC1 antisera or the NSS or phosphate-buffered solution (PBS, containing 10 mM NaH_2PO_4 and K_2HPO_4; 137 mM NaCl and 2.37 mM KCl) every 3 days (see Figure 1) during the experiment, giving a total of three treatments. We kept to a maximum of 3 injections over seven days to reduce the risk of an immune response against the sheep immunoglobulins. Injections were done under brief (2 min) halothane anaesthesia giving 50 μl s.c. (approximate 1.5 ml kg^{-1}) of LCS3, LCPS1, NSS or PBS. One day after the last injection animals were used for the different experimental protocols detailed below and summarized in Figure 1.

Mouse air-pouch model

Air-pouches were formed on the back of the mice by s.c. injection of 2.5 ml of air on day 2 and day 5 (Figure 1). Three days after the last air-injection (6-day-old air-pouches) 1 mg of zymosan (in 0.5 ml of sterile saline) was injected locally. Zymosan was previously boiled for 30 min in PBS, extensively washed in the same medium and stored at −20°C prior to use.

At different times (4 h and 24 h) after the local injection of zymosan, mice were killed by CO_2 exposure and the air-pouches washed with 2 ml of PBS containing ethylenediaminetetracetic acid (EDTA, 3 mM) and heparin (50 u ml^{-1}). Lavage fluids (essentially the entire 2 ml were consistently recovered) were centrifuged at 200 *g* for 10 min at 4°C and cell pellets resuspended in 2 ml of PBS/EDTA + heparin. The number of PMN and mononuclear cells was determined, in a Neubauer haematocytometer, after staining (1 : 10 dilution) with Turk's solution (crystal violet 0.01% w/v in acetic acid 3% v/v). Data are shown as 10^6 cells recovered from each animal.

In the preliminary experiment performed to assess the efficacy of the LCS3 antibody on the inhibitory action of dexamethasone during the acute inflammatory response induced by zymosan, mice (~30 g body weight) were treated with a single injection of LCS3 serum or NSS (50 μl, s.c.) 24 h prior to the systemic challenge with dexamethasone (50 μg per mouse, i.v.) or PBS (100 μl, i.v.). Two hours after the in-

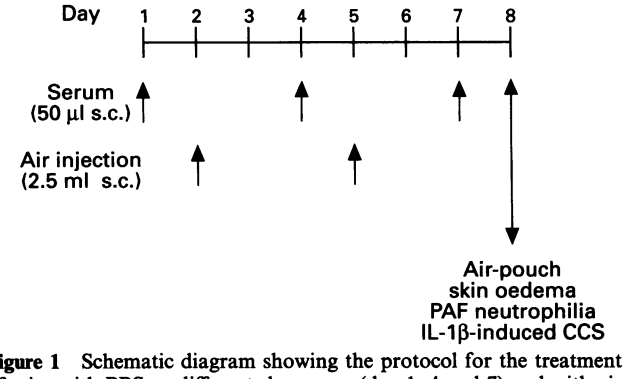

Figure 1 Schematic diagram showing the protocol for the treatment of mice with PBS or different sheep sera (day 1, 4 and 7) and with air to produce dorsal sub-cutaneous air-pouches (day 2 and 5). Experimental procedures were started on day 8.

travenous treatments, zymosan (1 mg in 0.5 ml saline) was injected into the preformed air-pouches and PMN accumulation assessed at the 4 h time-point as described above.

Several parameters related to acute inflammatory reactions were measured in the lavage fluids obtained from the inflamed air-pouches. The concentration of immunoreactive tumour necrosis factor-α (TNF-α) and interleukin-1α (IL-1α) in 50 and 100 μl aliquots respectively of lavage fluids were determined by enzyme-linked immunosorbent assay (ELISA) in 96-well plates. Binding was detected by a peroxidase-conjugated polyclonal anti-mouse TNF-α or anti mouse IL-1α using tetramethylbenzidine as a substrate. Following acidification (sulphuric acid, 0.5 M final concentration), the absorbance of each well was measured at 450 nm (Anthos Labtec Instruments). The ELISA specific for TNF-α does not cross react with murine IL-1α or IL-1β up to 1 μg ml^{-1} and 100 ng ml^{-1}, respectively, or with murine TNF-β (up to 1 μg ml^{-1}). The ELISA specific for IL-1α does not cross react with murine IL-1β or murine TNF-α up to 0.5–1.0 mg ml^{-1}. Intra-assay and inter-assay reproducibilities were 2.0–2.8% and 8.3–8.7%, or 2.9–3.5% and 5.9–7.3% for the TNF-α or IL-1α ELISA, respectively (data furnished by the manufacturer). Data are reported as pg ml^{-1} lavage fluid.

Leukocyte elastase activity released from polymorphonuclear leucocytes in lavage fluids was measured by the methodology already described by Iwamura *et al.* (1993) with slight modifications. Briefly, 50 μl of sample or standard human leucocyte elastase (EC 3.4.21.37; Calbiochem Novabiochem, U.K.) was pipetted in duplicate to a 96-well plate with 50 μl of buffer TBS (0.2 M Tris HCl, 0.15 M NaCl, pH 8.0) and 10 μl of Triton X-100 (0.2% in TBS) added. After mixing for 30 s at room temperature, 10 μl of the specific elastase substrate, methoxy-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide (17.8 mM in dimethylsulphoxide 15% v/v in 200 mM Tris-HCl, pH 8.0) was added. Incubations were carried out for a further 120 min. The concentration of *p*-nitroaniline released was measured spectrophotometrically at 405 nm in a 96-well plate multi-reader (Anthos Reader 2001, Anthos Labtec, Salzburg, Austria). Leucocyte elastase activity is reported as ng leucocyte elastase equivalent per ml of lavage fluid or normalised per 10^6 polymorphonuclear leucocytes.

Myeloperoxidase (MPO) activity released from polymorphonuclear leucocytes in lavage fluids was measured by incubating 50 μl of sample with 25 μl hydrogen peroxide (0.5%) and 25 μl ortho-dianisidine (4.7 mM) for 30 min at 37°C. The reaction was stopped with 50 μl sodium azide (0.4%). Data are reported as mu of absorbance read at 450 nm wavelength in a 96-well plate multi-reader.

The concentration of prostaglandin E_2 (PGE_2) in the lavage fluids was determined by immunoassay (Biotrak, Amersham, Buckinghamshire, UK) performed according to the manufacturer's instructions. This assay has minimal cross-reactivity with other prostanoids: 7% with PGE_1, 4% with $PGF_{2\alpha}$, and 5% with $PGF_{1\alpha}$ (data furnished by the manufacturer). Data are reported as pg ml^{-1} lavage fluid.

Finally, in some cases animals were bled by cardiac puncture, under halothane anaesthesia, and plasma stored at −20°C for measurement of corticosterone (CCS, see below).

Mouse skin oedema model

Formation of local oedema in the skin in response to zymosan-activated serum (ZAS) was measured according to a procedure recently described (Ahluwalia & Perretti, 1994). Briefly, mice were anaesthetized with a cocktail of Hypnorm : Hypnovel : sterile water in a ratio of 1 : 1 : 2 at a dose of 4 ml kg^{-1} given i.p. The backs of the mice were shaved and 2 μCi kg^{-1} of ^{125}I-labelled human serum albumin in 0.5% Evans blue solution was injected i.v. Either saline or ZAS (50% in saline) were injected intradermally in volumes of 50 μl into the dorsal skin. Either 2 h or 24 h later, blood samples were taken by cardiac puncture under halothane anaesthesia and the animals killed by exposure to CO_2. The skin injection sites were punched out with a 6 mm diameter hole punch. The blood samples were centrifuged at 895 *g* for 5 min and the plasma, as well as the skin sites, assessed for radioactivity in a γ-counter.

The volume of extravasated plasma at a site of induced oedema was evaluated by dividing the number of counts per skin site of ^{125}I-labelled albumin by the number of counts obtained in 1 μl of plasma from the same animal. To highlight the modest residual oedema, data for the 24 h time-point are reported as net oedema, the oedema value measured in the site injected with the stimuli being corrected for the value measured in the saline-injected sites.

PAF-induced neutrophilia

Blood neutrophilia in response to platelet-activating factor (PAF) administration to mice is, at least in part, a glucocorticoid-driven phenomenon which also relies upon endogenous LC1 (Harris *et al.*, 1995). According to the previous study, mice were treated with a single bolus dose of PAF (100 ng, i.v.) in 100μl of PBS supplemented with 0.1% bovine serum albumin, or with the vehicle alone, on day 8 of the experimental protocol. Two hours after treatment animals were bled by cardiac puncture using heparinised (20 μl of heparin 2000 u ml^{-1}) syringes under halothane anaesthesia and then killed. Previous experiments indicated that this procedure minimized the stress experienced by the animals without affecting the profile of circulating leucocytes and allowed reliable measurements of basal plasma CCS (Perretti *et al.*, 1993b). Total white cell counts were determined in a Coulter counter (Coulter Electronics, Luton, UK). Differential counts were obtained from blood smears stained with Turk's solution and

Table 1 Basal parameters in the different experimental groups

Sub-chronic treatment	Body weight (g) day 1	Body weight (g) day 8	Plasma CCS (ng ml^{-1})	White blood cells (10^6 ml^{-1}) Ly	Mono	PMN	Total	Anti-LC1 titre (units)
PBS	28±1	33±1	21±5	3.12±0.29	0.05±0.01	0.68±0.41	3.85±0.41	0
NSS	27±1	32±1	98±17	2.93±0.37	0.06±0.01	0.73±0.13	3.72±0.47	8±6
LCS3	27±1	33±1	74±20	2.87±0.38	0.14±0.06	0.78±0.09	3.79±0.44	33,390±2.927
LCPS1	27±1	32±1	25±6	2.80±0.42	0.03±0.02	0.74±0.06	3.57±0.48	5,056±689

Mice were treated with PBS, NSS, LCS3 serum (raised against human recombinant LC1) or with serum LCPS1 (raised against the active LC1 N-terminal fragment) as described in Methods. Body weight values are mean±s.e. of $n=40$ mice per group; plasma corticosterone (CCS) values were taken on day 8 and are mean±s.e.mean of $n=5$, $n=13$, $n=10$ and $n=5$ mice for PBS, NSS, LCS3 and LCPS1 groups, respectively; total and differential white blood cell counts were performed on day 8 and are mean±s.e.mean of $n=5$, $n=13$, $n=13$ and $n=5$ mice per group for PBS, NSS, LCS3 and LCPS1, respectively; the titre of anti-LC1 antibodies in the plasma on day 8 of treated mice is expressed as ELISA units (mean±s.e.mean, $n=10$).

the total number of PMN in each blood sample was then calculated.

In some cases the lymphocytes and monocytes were also monitored to obtain information on the values of circulating leucocytes in basal conditions, i.e. at the end of the passive immunisation protocol (see Table 1).

IL-1β-induced CCS release in the plasma

Administration of rat IL-1β to rats causes a rise in circulating CCS which is abrogated by treatment of animals with dexamethasone, and the effect of the steroid is mediated by endogenous LC1 (Taylor *et al.*, 1995). For this reason, the effect of the sub-chronic treatments on this property of the cytokine was assessed. Murine recombinant IL-1β was given i.p. at the dose of 5 μg kg^{-1} and blood collected 2 h later by cardiac puncture under halothane anaesthesia, this dose and time-point being selected from data provided in previous publications (Besedovsky *et al.*, 1986; Sapolsky *et al.*, 1987).

Radioimmunoassay of CCS levels

Blood samples were centrifuged at 3,000 *g* for 10 min in a bench Eppendorf centrifuge. Plasma (supernatant fraction) was stored at −20°C for no longer than two weeks before CCS measurements. Plasma corticosterone levels in samples prepared from mice after different experimental protocols were assayed with a commercially available radioimmunoassay kit according to the manufacturer's instructions (ICN-Biomedical, U.K.).

ELISA for anti-LC1 titre

The titre of anti-LC1 antibodies in the mouse plasma was determined according to a published methodology with slight modifications (Goulding *et al.*, 1989). Briefly, immuno-plates were coated with 1 μg human recombinant LC1 overnight at 4°C. After blockade of non-specific sites with 1% foetal calf serum, different dilutions (from $1:10^3$ to $1:10^5$) of the samples were added for 1 h at 37°C. Following extensive washing, a donkey anti-sheep IgG conjugated to alkaline phosphatase was incubated for a further 1 h at 37°C. Addition of the substrate disodium-nitrophenyl phosphate caused a coloration quantified at 405 nm. Values are reported as ELISA units as described (Goulding *et al.*, 1989). In these experimental conditions the antisera LCS3 and LCPS1 had a titre of 103,000 and 91,325 respectively, whereas NSS gave a value of 1,140.

Clinical chemistry

In some cases plasma aliquots (100–200 μl) prepared from the blood of the mice at the end of the treatment period were submitted for several biochemical determinations. Analyses were performed by an external laboratory using conventional auto-analyzer technology (VetLab Services, Southwater, Sussex).

Materials

Dexamethasone-21-phosphate was obtained from David Ball Laboratories (Warwick, U.K.) as a sterile 4 mg ml^{-1} solution. The specific ELISAs for murine TNF-α and IL-1α were purchased from Genzyme (MA, U.S.A.). ^{125}I-labelled human serum albumin was obtained from Amersham International. Hypnovel was from Roche Products Ltd, UK and Hypnorm was purchased from Janssen Pharmaceuticals Ltd, UK. Murine recombinant interleukin-1β was a generous gift of Dr R.C. Newton (DuPont-Merck, Wilmington, DE, U.S.A.). Zymosan type A, PAF (C_{16} form: $C_{26}H_{54}NO_7P$), and all other chemicals were obtained from Sigma Chemical Company (Poole, U.K.).

Statistics

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

Results

The three-injection treatment with LCS3, LCP1 or NSS did not affect the normal behaviour or health of the animals. No difference in body weight between these groups and the PBS group was found at the end of the treatment (Table 1). A high anti-LC1 titre was measured in the plasma of the animals treated with the two polyclonal antisera raised against LC1 or its N-terminal peptide, whereas anti-LC1 activity was not significantly different from zero in the plasma of NSS- or PBS-treated mice (Table 1).

Table 2 shows the values obtained for different biochemical parameters in the plasma at the end of the treatment with the different sheep sera or with PBS: overall no major changes were observed between the experimental groups. As expected, higher concentrations of globulin were found in the blood of mice treated with heterologous sera. Treatment with LCS3 produced a consistent increase in the blood glucose concentration, significant against the concentrations in both the PBS- and the NSS-treated mice (Table 2). Less clear were the changes in creatinine, since both the NSS and LCS3 groups were below those in the control mice (PBS group).

Mouse air-pouch model

Since LCS3 has been described as abrogating the anti-migratory effect of dexamethasone in the air-pouch inflamed with interleukin-1 (Perretti & Flower, 1993), we initially tested the activity of this antibody when zymosan was used as an inflammogen. Figure 2 shows the data of this preliminary ex-

Table 2 Biochemical determinations in the plasma following treatment with sheep sera or PBS

Plasma parameter	*Sub-chronic treatment*			
	PBS	*NSS*	*LCS3*	*LCPS1*
Globulin	24.4±0.50 (20)	27.1±0.37 (55)*	27.0±0.66 (58*)	26.5±0.53 (20)*
Ions				
Na^+ (mM)	159.5±0.43 (6)	153.8±1.60 (31)	155.5±1.79 (34)	159.4±1.42 (7)
K^+ (mM)	4.62±0.14 (15)	4.95±0.15 (49)	5.08±0.17 (50)	4.75±0.10 (17)
Ca^{2+} (mM)	1.43±0.05 (20)	1.39±0.05 (45)	1.52±0.05 (47)	1.34±0.10 (20)
PO_4^{3-} (mM)	2.83±0.16 (20)	3.29±0.51 (54)	2.55±0.05 (58)	2.76±0.11 (20)
Urea (mM)	6.62±0.29 (20)	5.72±0.19 (50)	6.13±0.17 (58)	5.97±0.26 (20)
Creatinine (μM)	20.0±1.85 (10)§	15.0±1.15 (35)*	14.6±1.06 (38)*	20.6±2.95 (10)§
Glucose (mM)	9.39±0.33 (19)§	8.85±0.26 (45)*	10.3±0.22 (47)*§	9.37±0.56 (19)§
Cholesterol (mM)	2.43±0.17 (20)	2.48±0.11 (55)	2.47±0.11 (58)	2.44±0.08 (20)

Mice were treated with PBS, NSS, LCS3 serum (raised against full length human recombinant LC1) or with serum LCPS1 (raised against the active LC1 2–26 fragment) as described in Methods. Plasma samples were prepared on day 8 (see Figure 1). Values are mean±s.e.mean of (*n*) animals per group. *$P<0.01$ vs. PBS-group and §$P<0.01$ vs. NSS-group.

periment in which treatment with the steroid resulted in a 66% reduction of the number of PMN recovered at the 4 h time-point in the NSS-pretreated animals ($P<0.01$), whereas only a 33% reduction was measured in the anti-LC1 treated group, which was no longer statistically significant when compared to vehicle-treated group. This experiment validated the use of LCS3 for assessing the involvement of endogenous LC1 in this particular model, the mouse air-pouch inflamed with zymosan.

Local injection of 1 mg zymosan provoked a 4 h recruitment of $\sim 8 \times 10^6$ PMN in the animals sub-chronically treated with NSS, which had subsided to almost 50% at the 24 h time-point (Figure 3). No difference in the cell number was observed in LCS3-treated mice at the 4 h time-point; however, a much larger number of PMN was recovered 24 h after the injection of zymosan. This observation was confirmed, and substantiated, by another experiment in which the effect of the LCPS1 antiserum was evaluated. Between 2 and 4×10^6 PMN were recovered in this experiment in the NSS- and PBS-treated groups 24 h after local administration of zymosan, whereas more than double the number of cells were found in the air-pouches of animals passively immunized with the LCPS1 antiserum (Figure 4).

No significant changes in the influx of mononuclear cells was observed and, for example, 0.53 ± 0.19, 036 ± 0.10, 0.88 ± 0.22 and $0.46 \pm 0.07 \times 10^6$ cells were recovered 24 h after zymosan injection in PBS-, NSS, LCS3- and LCPS1-treated groups, respectively (mean ± s.e.mean of $n=5-7$ mice per group; not significant).

Evaluation of specific enzymatic markers related to PMN activation such as release of MPO and elastase (Sandborg & Smolen, 1988) led to the finding that much higher levels were consistently found in the lavage fluids collected from the animals which received LCS3 antiserum with respect to those receiving NSS or PBS (Table 3). Higher enzyme levels were measured in the fluids collected both at the 4 h and the 24 h time-point. Interestingly, the increase in elastase activity was remarkable and significant even if the extent of PMN recruitment, shown in Figure 3, was also taken into account (Table 3).

High levels of TNF-α and IL-1α were measured in these inflammatory exudates. The amounts of the pro-inflammatory cytokines were transiently increased, being higher at the 4 h than the 24 h time-point (Table 4). No significant difference between the PBS and NSS groups was found at the 24 h time-point; however, significantly higher amounts of TNF-α and IL-1α were measured in the LCS3 group at the 24 h and 4 h time-points, respectively (Table 4). No detectable TNF-α and IL-1α immunoreactivity was found in lavage fluids of air-pouches untreated with zymosan.

The PGE_2 concentration in the lavage fluids was measured only in some animals from the NSS and LCS3 groups. Treatment with the anti-LC1 serum produced higher values at the 4 h time-point (mean ± s.e.mean, $n=5$): $2,120 \pm 290$ pg ml^{-1} for LCS3 vs. $1,170 \pm 210$ ng ml^{-1} for NSS-treated mice ($P<0.05$). Negligible amounts of PGE_2 were found at the 24 h time-point in the NSS group with only 2 out of 7 mice with a PGE_2 value above 0, whereas 5 out of 7 mice had detectable amounts of PGE_2 in the lavage fluids of LCS3-treated mice (760 ± 450 pg ml^{-1}).

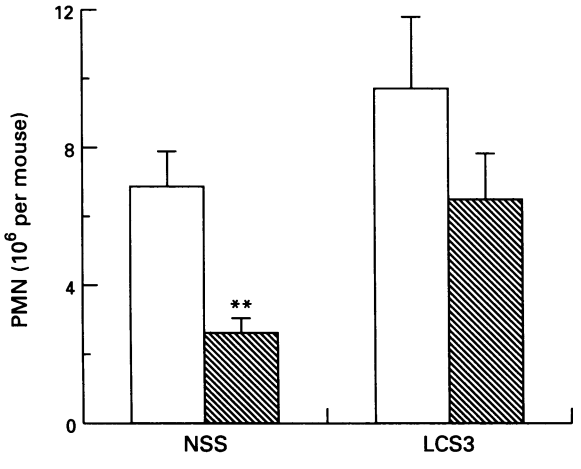

Figure 2 Endogenous LC1 mediates the anti-migratory effect of systemic dexamethasone in the zymosan-inflamed air-pouch model. Mice received NSS or LCS3 (50 μl, i.v.) 24 h prior to intravenous administration of PBS (100 μl, open columns) or dexamethasone (50 μg, hatched columns). Two hours later, 1 mg zymosan (in 0.5 ml saline) was injected into 6-day-old air-pouches and PMN accumulation quantified after 4 h. Values are mean ± s.e.mean of $n=8$ mice per group. $^{**}P<0.01$ vs. appropriate PBS group.

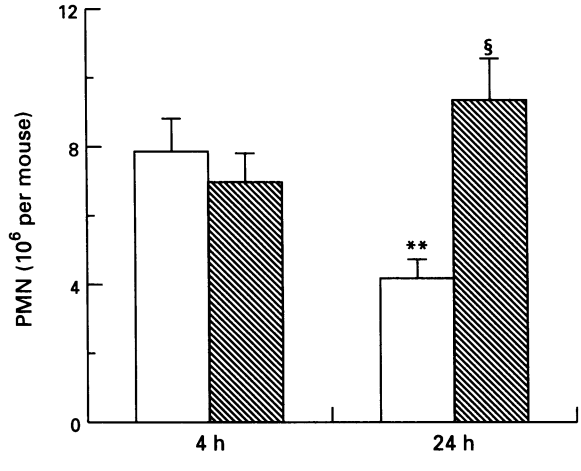

Figure 3 Exacerbation of the PMN recruitment induced by zymosan into dorsal subcutaneous air-pouches in mice passively immunized against LC1. Mice received NSS (open columns) or LCS3 (hatched columns) sera according to the protocol shown in Figure 1. Twenty-four hours after the last injection 1 mg zymosan (in 0.5 ml saline) was injected into the preformed air-pouches and PMN accumulation quantified at 4 h or 24 h time-points. Values are mean ± s.e.mean of $n=7-12$ mice per group. $^{**}P<0.01$ vs. respective 4 h NSS group; § $P<0.01$ vs. 24 h NSS group.

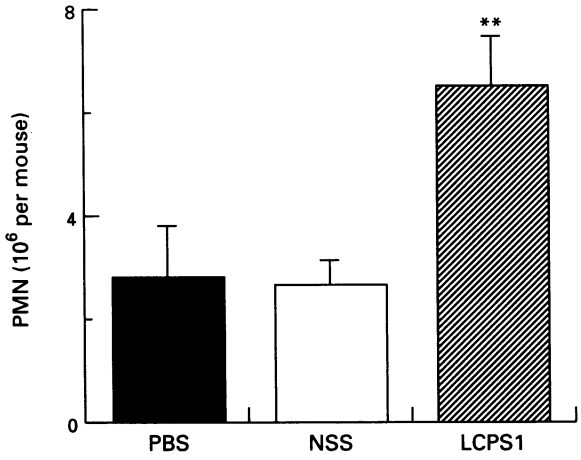

Figure 4 Effect of LCPS1 serum on 24 h PMN recruitment induced by zymosan into dorsal subcutaneous air-pouches. Mice received PBS, NSS or LCPS1 sera as illustrated in Figure 1. Twenty-four hours after the last injection 1 mg zymosan (in 0.5 ml saline) was injected into the preformed air-pouches and PMN accumulation quantified at the 24 h time-point. Values are mean ± s.e.mean of $n=7$ mice per group. $^{**}P<0.01$ vs. NSS or PBS group.

Mouse skin oedema model

Intradermal injection of 50% ZAS resulted in a marked oedema response at 2 h with respect to saline-injected sites. In the LCS3 group the oedema response elicited by ZAS 50% was significantly higher (+50%) than that observed in NSS- or PBS-treated mice (Figure 5).

In view of the prolonged effect of the inflammatory response observed following zymosan injection into the 6-day-old air-pouches, the oedema response in the skin was also measured at a longer time-point (24 h). No significant difference between oedema formation caused by 50% ZAS and saline was observed at this time-point in NSS and PBS groups (Figure 5, inset). In contrast, ZAS-induced oedema was still measurable in the LCS3 group, though corresponding to approximately one third of the response measured after 2 h (Figure 5, inset).

PAF-induced neutrophilia

Sub-chronic treatment with the different sheep sera or with PBS did not alter the profile of white blood cells as assessed on day 8 (Table 1). A similar number of peripheral blood leucocytes was counted in the experimental groups and differential counts for each cell type were comparable.

Intravenous administration of PAF (100 ng) produced a marked neutrophilia at 2 h (+140%) with respect to vehicle injection, both in NSS- and PBS-treated mice (Figure 6). In contrast, this cellular response to PAF was abrogated in the animals which were treated with the LCS3 antiserum (mean ± s.e.mean; PMN per ml of blood): $1.30 \pm 0.27 \times 10^6$ vs. $1.03 \pm 0.17 \times 10^6$ for PAF and vehicle, respectively ($n = 11$, $P < 0.01$).

Plasma CCS in inflammation and post-IL-1β treatment

Basal CCS values after the sub-chronic treatment, on day 8, and before any further experimental intervention are shown in Table 1. Values were within the norm for PBS and LCPS1 groups, whereas an unexpected increase was found in NSS and LCS3 groups. This phenomenon has already been observed by us in the rat and it is due to serum factors other than the IgG fraction (Taylor *et al.*, 1995). Basal CCS values measured in the two latter groups were below those reached during inflammation and/or after IL-1β injection (see below). Nevertheless these data justify the fact that the effect of LCS3 treatment was always compared to that of the NSS throughout the entire study.

Plasma CCS levels during the acute inflammation caused by local injection of zymosan (see above) were measured only at the two time-points used for assessing the inflammatory

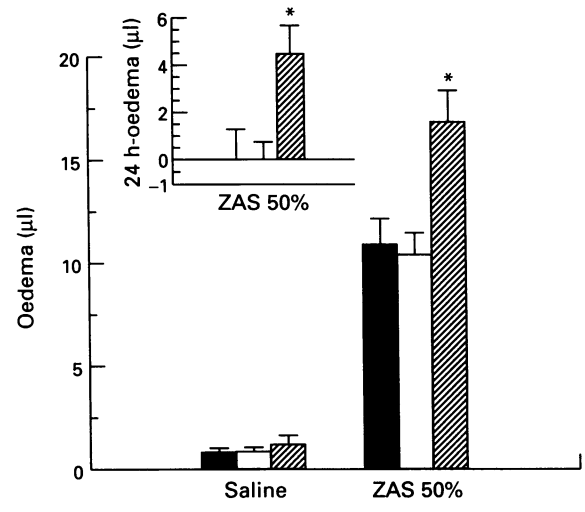

Figure 5 Exacerbation of ZAS-induced skin oedema in mice passively immunized against LC1. Mice received PBS (solid columns), NSS (open columns) or LCS3 (hatched columns) sera according to the protocol shown in Figure 1. Twenty-four hours after the last injection 50 μl of saline alone or containing 50% ZAS were injected intradermally. Oedema was quantified at 2 h. Values are mean ± s.e.mean of $n = 14$ mice per group. *$P < 0.05$ vs. respective 2 h-oedema response in PBS and NSS group. Inset: residual 24 h ZAS (50%) oedema response in LCS3-treated mice. Values (mean ± s.e.mean of $n = 5$ mice) have been corrected for the negligible saline effect. *$P < 0.05$ vs. respective 24 h-oedema response in PBS and NSS group.

Table 3 Levels of myeloperoxidase (MPO) and elastase activities in the lavage fluids of zymosan-inflamed air-pouches

Treatment (sub-chronic)	*MPO activity* (mu) 4 h	24 h	*Elastase activity* (ng per 10^6 PMN) 4 h	24 h
PBS	ND	68 ± 11 (6)	ND	2 ± 2 (6)
NSS	81 ± 4 (8)	68 ± 7 (13)	6 ± 1 (8)	2 ± 1 (8)
LCS3	182 ± 25 (8)*	168 ± 24 (13)*	39 ± 4 (8)*	34 ± 8 (8)*

Mice were treated with PBS, NSS or LCS3 serum as described in Methods. Six-day-old air-pouches were inflamed by local injection of 1 mg zymosan (in 0.5 ml sterile saline). Enzymatic activities in cell-free lavage fluids (collected 4 h or 24 h after zymosan injection) were measured as described. Values are mean ± s.e.mean of (*n*) mice per group; ND = not done. *$P < 0.05$ vs. respective NSS group.

Table 4 Tumour necrosis factor-α (TNF-α) and interleukin-1α (IL-1α) levels in the lavage fluids of zymosan-inflamed air-pouches

Treatment (sub-chronic)	*TNF-α* (pg ml^{-1}) 4 h	24 h	*IL-1α* (pg ml^{-1}) 4 h	24 h
PBS	ND	35 ± 22 (6)	ND	1 ± 1 (6)
NSS	684 ± 177 (8)	49 ± 17 (13)	137 ± 24 (8)	7 ± 7 (13)
LCS3	850 ± 179 (8)	190 ± 22 (13)*	378 ± 56 (8)*	30 ± 21 (8)

Mice were treated with PBS, NSS or LCS3 serum as described in Methods. Six-day-old air-pouches were inflamed by local injection of 1 mg zymosan (in 0.5 ml sterile saline). Cytokine levels in cell-free lavage fluids (collected 4 h or 24 h after zymosan injection) were measured by ELISA. Values are mean ± s.e.mean of (*n*) mice per group. ND = not done. *$P < 0.05$ vs. appropriate NSS group.

parameters. CCS was significantly higher in the LCS3 group 4 h after zymosan injection (Table 5) being increased approximately 2 fold vs. basal values reported in Table 1. No significant difference was evident at the 24 h time-point. This increase in circulating CCS was absent in the NSS-treated mice.

Intraperitoneal injection of murine IL-1β produced a marked increase in plasma CCS at the 2 h time-point. The response to the cytokine was similar in all the experimental groups assessed (Table 5).

Discussion

Evidence from several laboratories, using different types of anti-LC1 neutralizing antibody, have demonstrated the importance of this protein in the action of steroids both *in vivo* and *in vitro*. The design of most of the experiments has been to investigate the role of LC1 as a second messenger of glucocorticoids when these have been given exogenously prior to an inflammatory event. With the exception of the study by Rothwell and colleagues who examined the influence of LC1 in the development of endotoxin resistance in aging mice (Strijbos *et al.*, 1993), the idea that LC1 is crucial to the operation of endogenous anti-inflammatory mechanisms has not received much attention.

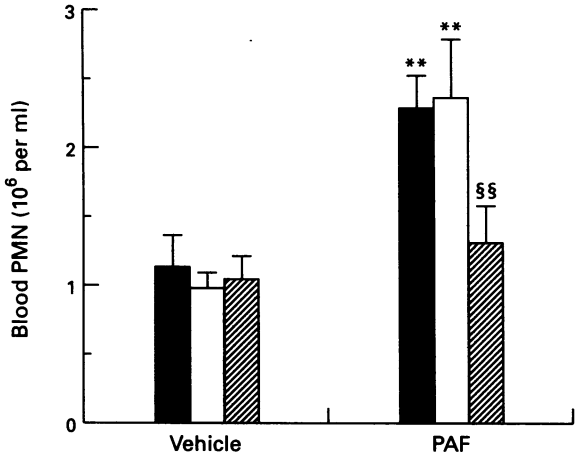

Figure 6 Abrogation of PAF-induced neutrophila in mice passively immunised against LC1. Mice received PBS (solid columns), NSS (open columns) or LCS3 (hatched columns) sera according to the protocol shown in Figure 1. Twnety-four hours after the last injection 100 μl of vehicle (PBS supplemented with 0.1% bovine serum albumin) alone or containing 100 ng PAF were given i.v., and the number of circulating PMN evaluated 2 h later. Values are mean ± s.e.mean of $n=11$ mice per group. **$P<0.01$ vs. vehicle alone; § $P<0.01$ vs. PAF response in PBS and NSS group.

The importance of the HPA axis in the control of the inflammatory response has recently become a subject of intense research interest. In 1986 we observed that the inflammatory response to carrageenin was substantially greater in adrenalectomized animals than in sham-operated controls, that the concentration of lipid mediators such as prostaglandins, leukotrienes and platelet-activating factor in the inflammatory lesion was greatly enhanced and that even the *ex vivo* production of eicosanoids by peritoneal cells was many times that of the control preparations (Flower *et al.*, 1986). These data argued strongly for a tonic inhibitory role of the glucocorticoids and it was speculated at this time that this effect was mediated by the inhibitory action of LC1 on the activity of the enzyme phospholipase A_2. Further studies contributed to this literature with the observation that the concentrations of the cytokine IL-1 were much higher in cells taken from adrenalectomized rats (Perretti *et al.*, 1989). In addition these animals displayed a greatly enhanced sensitivity to endotoxin in response to which higher amounts of pro-inflammatory cytokines were released into the circulation (Perretti *et al.*, 1993b). Several other groups have explored the relationship between adrenalectomy and mediator release: many have confirmed that the tonic action of glucocorticoids, once removed, leads to an exaggeration in prostaglandin turnover and suggested that this occurs by up-regulation of cyclo-oxygenase type 2 (Masferrer *et al.*, 1992; 1994). However this could not explain all the data published in the original study in which it was observed that the levels of all inflammatory lipid mediators were raised following adrenalectomy (Flower *et al.*, 1986).

In the current experiments we sought to contrive a model whereby the sub-chronic effects of passive immunization to LC1 could be observed. Eight days was chosen as a suitable time as this enabled us to prepare animals for the air-pouch experiment during the immunization protocol and reduced the possibility of a primary immune response by the mice which might complicate the interpretation of the results. This assumption seemed justified by our observations comparing both groups of animals which did not differ significantly in their weight or the differential or total leucocyte count. In addition blood chemistry was very similar between the groups. A reduction in glucose and creatinine was seen in the group treated with NSS when compared to the PBS group and there was some evidence suggesting that these changes might be reversed with one or both of the anti-LC1 antisera. It would be premature to suggest that this was indicative of a role for LC1 in the regulation of glucose or nitrogen metabolism although it is certainly a possibility worthy of further investigation.

We checked for the presence of anti-LC1 immunoglobulin in all four groups. Neither of the control groups (PBS- or NSS-treated) tested positive in this assay whereas in the animals which received the anti-LC1 antibodies a very high titre was observed. We establish that this was sufficient to abolish exogenous glucocorticoid action by demonstrating that LCS3 attenuated the inhibitory action of dexamethasone in the zymosan air-pouch model.

In these experimental conditions our data demonstrated that abrogation of the effect of endogenous LC1 produced no

Table 5 Plasma corticosterone (CCS) in inflammation and after murine IL-1β injection

	Plasma CCS (ng ml^{-1})		
Treatment	*Zymosan inflammation*		*Post-IL-1β*
(sub-chronic)	*4 h*	*24 h*	*2 h*
PBS	ND	51 ± 17 (8)	468 ± 65 (5)
NSS	110 ± 31 (8)	105 ± 28 (13)	535 ± 41 (10)
LCS3	198 ± 26 (8)*	137 ± 35 (13)	523 ± 37 (10)

Mice were treated with PBS, NSS or LCS3 serum as described in Methods. Plasma CCS was measured by RIA in 10 μl of plasma prepared from the blood of mice bearing six-day-old air-pouches inflamed by local injection of zymosan (1 mg in 0.5 ml sterile saline), either at the 4 h or 24 h time-points, or from the blood of mice challenged with murine IL-1β (5 μg kg^{-1}, i.p.) 2 h prior to collection. Values are mean ± s.e. of (n) mice per group. ND = not done. *$P<0.05$ vs. appropriate NSS group.

obvious changes in the number of PMN recovered at 4 h. However, there was an increase in most of the biochemical parameters associated with cell activation measured in the lavage fluids, with both MPO and elastase activity being increased at that time-point. In addition, the concentration of IL-1 was also increased. There were, however, gross changes in the numbers of cells migrating at 24 h in the group which had been passively immunized against LC1 and this was again reflected by the increase of MPO and elastase at the 24 h time-point. In addition there was, for the first time, an increase in TNF-α over the control group. It appeared that the inflammatory reaction had subsided in the control animals by 24 h whereas it was still active and more aggressive in the mice immunized against LC1.

No significant alterations in the influx of mononuclear cells was detected; however, in agreement with previous publications from our group and others (Dawson *et al.*, 1991; Klemm *et al.*, 1995), the overall cell accumulation was modest, indicating that this experimental model is not suitable for studying the trafficking of this cell type.

To confirm that these results were not simply a feature of one particular anti-LC1 antibody we repeated the experiment with a separate antibody (LCPS1) and found that this produced qualitatively the same effect. The primary antibody (LCS3) used here was raised against the whole recombinant human molecule whereas the second antibody (LCPS1) was raised against a defined peptide sequence present in the N-terminus region (Perretti, 1994). This experiment also eliminates the possibility that the first antibody was in fact neutralising another member of the annexin family, most of which share strong similarities in the core region but which have distinct N-termini (Barton *et al.*, 1991).

Like the zymosan air-pouch model, the skin oedema test also showed enhanced inflammation in the anti-LC1 antibody treated group, using 50% ZAS as a stimulus. Interestingly, the effect at 24 h was again more marked than the effect at 2 h. Indeed, there was no detectable oedema in the control groups, whereas a moderately strong response was still seen in the LCS3-treated mice at 24 h.

In the neutrophilia experiments, PAF, which acts by liberating CCS directly into the circulation thus promoting a steroid-dependent neutrophilia (Harris *et al.*, 1995), was without effect in LCS3-treated animals.

Finally, during zymosan inflammation animals immunized against LC1 tended to have higher basal CCS levels, at least at the 4 h time-point. This is consistent with an inability of these animals to regulate in a negative feedback fashion the release of ACTH, as this is thought to be an LC1-mediated process (Taylor *et al.*, 1995).

All these data point to an action of LC1 on the inflammatory response during which the HPA axis is activated, leading to the release of endogenous glucocorticoids. It is noteworthy that the biggest differences were seen not at the short time-points (presumably before the HPA axis is fully activated), but only after the inflammatory response had been in progress for some while. The only exception to this was the neutrophilia experiment in which the injection of PAF intravenously causes an immediate release, and therefore action, of corticosterone. The picture which emerges is that the inflammatory response leads to a liberation of cytokines or other factors which increase the circulating CCS levels. This hormone acts in an LC1-dependent fashion to limit the intensity of the inflammatory response, in this case zymosan-induced cell migration and ZAS-induced skin oedema.

There is some evidence of organ specificy in response to glucocorticoids. Miotla *et al.* (1995) observed that the inhibition of glycogen-induced peritonitis in the mouse by dexamethasone was reversed by treatment with LCS3, but that dexamethasone suppression of acute lung injury in the same species was not.

An interesting comparison between the inflammatory response observed following passive immunization of mice against LC1 (this study) and that observed in adrenalectomized rats may be drawn. The correlation is only partial because in adrenalectomized rats the cell recruitment was not only prolonged but also exacerbated, such that many more PMN were collected during the *initial* phase of the inflammatory reaction (Flower *et al.*, 1986). Therefore, removal of endogenous CCS does not exactly duplicate neutralization of endogenous LC1. Perhaps this is to be expected as steroids are more pleiotropic in their actions. Indeed, glucocorticoid hormones tightly regulate the synthesis of pro-inflammatory cytokines, like IL-1, and higher circulating IL-1 levels were detected in adrenalectomized rats (Perretti *et al.*, 1993b) and mice, and a more pronounced release was found from cells taken from these animals (Perretti *et al.*, 1989). Since IL-1 is important in the 4 h cell recruitment caused by zymosan (Perretti *et al.*, 1992) and the control exerted by dexamethasone on IL-1 synthesis is independent of LC1 (Morand *et al.*, 1993), the lack of difference in the number of cells recovered from the air-pouches at 4 h is not surprising. In contrast with the 4 h inflammation, a strong similarity between adrenalectomy and immuno-neutralization of LC1 was found at the 24 h time-point. This suggests that endogenous LC1 plays an important role in attenuating the host inflammatory response, probably by reducing the PMN recruitment to the site of inflammation, i.e. there could be an increase in endogenous CCS in the subsequent phases of the host reaction with an increase in cell-associated LC1 in circulating PMN. In agreement with this hypothesis we have recently shown that the levels of LC1 in circulating mouse leucocytes are partially (~50%) under the control of endogenous CCS (Perretti *et al.*, 1995b).

As in adrenalectomized animals, higher levels of several proinflammatory mediators were found in the lavage fluids of LCS3-treated animals. The increased concentrations of mediators seen in the air-pouch fluid collected from the animals treated with this anti-LC1 antibody may be consequent (i) to the higher number of cells present, or (ii) to a high degree of cell activation. Since elicited PMN are actively involved in the synthesis of proinflammatory cytokines (Cassatella, 1995) we cannot exclude the possibility that the differences seen are secondary, at least in part, to the larger number of cells recovered. On the other hand, higher concentrations of mediators were also found at the 4 h time-point where no difference in cell number was observed between NSS and LCS3 groups.

LC1 has a general inhibitory effect on cell activation: it reduces superoxide generation by PMN and monocytes *in vitro* (Stevens *et al.*, 1988; Maridonneau-Parini *et al.*, 1989); in addition the LC1 N-terminus peptide reduces elastase release from stimulated human PMN (Perretti *et al.*, 1995a). However, it has been reported that LC1 does not directly control IL-1 expression (Morand *et al.*, 1993). To our knowledge the effect of LC1 on TNF-α expression has not been tested. We have recently reported that LC1 can in some way control the expression or activity of inducible nitric oxide synthase so some effect on the pathways leading to cell activation and *de novo* protein synthesis is certainly possible (Wu *et al.*, 1995). Overall it seems plausible that ablation of endogenous LC1 actions by passive immunization leads to a marked long-lasting cell migration, as endogenous LC1 is important in down regulating cell activation. In the absence of passive immunization treatment, the inflammatory response and cell activation are limited because endogenous CCS produces an increase in cell-associated endogenous LC1.

Finally, we cannot exclude the possibility that endogenous LC1 could somehow modulate the removal of PMN from the site of inflammation, and that inhibition of this action could be responsible for the higher number of PMN recovered from the air-pouches at the 24 h time-points in the mice passively immunized against this protein.

The notion that antibodies to LC1 can subvert the correct operation of the endogenous anti-inflammatory defences is an important one because such antibodies have been detected in a variety of human diseases. This was first noted by Hirata and colleagues who observed, using a bioassay technique, that

there was an increased incidence of anti-lipomodulin (since renamed lipocortin) antibodies in patients suffering from rheumatoid arthritis and systemic lupus (Hirata *et al.*, 1981). These observations were confirmed, and greatly extended following the cloning of LC1, by Goulding and his associates who demonstrated the presence of both IgG and IgM autoantibodies to LC1 in rheumatoid arthritis and systemic lupus, but not in conditions such as polymyalgia (Goulding *et al.*, 1989). There have since been reports of the antibodies in other disorders including ulcerative colitis, asthma and psoriasis (Stevens *et al.*, 1993).

Of interest is the aetiology of autoantibody production. In the case of patients with systemic lupus, the autoantibodies appear to be present at every stage of the disease: in some studies there appears to be a correlation between antibody titre and disease index (Goulding *et al.*, 1989) although this was not seen in another study by the same group (Pruzanski *et al.*, 1994). In the rheumatoid patients however it appears that the autoantibodies appear after patients have been treated with oral steroids for a short period of time. If the titre of these autoantibodies becomes excessive, the patients go on to develop a form of steroid resistance, a phenomenon which is easily explained by the relationship observed in this paper.

Taken together with other findings from our group (Taylor *et al.*, 1995) and other laboratories, we conclude that LC1 plays a tonic regulatory role in the physiology of the HPA axis and its effect on the inflammatory response. In addition, the synthesis of LC1 under these circumstances is largely driven by endogenous glucocorticoids. It remains to be seen whether other factors such as cytokines or acute phase proteins can also directly regulate LC1 synthesis in cells under conditions of subchronic inflammation.

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