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# A novel murine model of allergic inflammation to study the effect of dexamethasone on eosinophil recruitment

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**1** We have developed a novel model of allergen-induced eosinophil extravasation into mouse air-pouches following sensitization and challenge with ovalbumin (Ova). This model was used to investigate the mechanism(s) underlying the anti-inflammatory action of the glucocorticoid hormone dexamethasone (Dex).

**2** Injection of 10 $\mu$g Ova into 6-day-old dorsal air-pouches of mice sensitized to the same antigen provoked an intense cell accumulation as early as 6 h post-challenge ($0.08 \pm 0.03$ and $4.0 \pm 1.0 \times 10^5$ leucocytes in saline and Ova-treated air-pouches, respectively), maximal at 24 h ($0.02 \pm 0.01$ and $6.0 \pm 0.8 \times 10^5$ leucocytes in saline and Ova-treated air-pouches, respectively) and persisted up to 48 h. At the 24 h time-point, the cellular infiltrate consisted of 37% eosinophils, 18% neutrophils and 45% mononuclear cells, as assessed by histological examination. The same ratio of eosinophil/neutrophil was obtained by fluorescence-activated cell sorting (FACS) analysis, since 72% of the polymorphonuclear (PMN) population was positive for very-late antigen-4 (VLA-4) expression.

**3** Subcutaneous (s.c.) administration of Dex (50 or 100 $\mu$g per mouse, $-1$ h) inhibited eosinophil accumulation into Ova challenged air-pouches by about 70% ($P<0.05$) and 75% ($P<0.05$), respectively, when compared to controls. Cell accumulation measured at 48 h after Ova injection was also significantly reduced ($-75\%$) by Dex administration at the 24 h time-point ($n=12$, $P<0.05$).

**4** Eosinophil numbers in the bone marrow and blood were quantitated. We found that the sensitization protocol induced a 3 fold increase in eosinophil numbers in the bone marrow (naive mice: $2.7 \pm 0.3 \times 10^5$; sensitized mice: $8.7 \pm 1.7 \times 10^5$, $P<0.05$) and blood (naive mice: $0.5 \pm 0.2 \times 10^5$; sensitized mice: $1.5 \pm 0.3 \times 10^5$, $P<0.01$). However, 24 h following Ova challenge, the eosinophil numbers in the bone marrow had dropped ($3.7 \pm 0.8 \times 10^5$) with no change in the circulating pool, suggesting an equilibrium within the eosinophil pools had been reached.

**5** Dex administration provoked a profound eosinopaenia in the blood of naive ($5.2 \pm 1.5$ to $0.9 \pm 0.6 \times 10^4$) and sensitized mice ($1.5 \pm 0.3$ to $0.08 \pm 0.02 \times 10^5$) at 4 h. This effect was reversed within 24 h. Dex also inhibited the release of eosinophils from the bone marrow in response to Ova challenge.

**6** We show for the first time that eosinophils express the steroid-inducible protein lipocortin 1 (LC1). FACS analysis of eosinophils emigrated into the Ova challenged air-pouches revealed detectable LC1-like immunoreactivity ($373 \times 10^3$). These data were also substantiated by LC1 detection in circulating eosinophils of interleukin-5 transgenic mice (strain: CBA/Ca). However, s.c. injection of Dex (50 $\mu$g) did not alter LC1 levels in blood eosinophils, such that $235 \pm 21 \times 10^3$ LC1-like molecules per cell were measured after vehicle treatment ($n=5$), and $224 \pm 8 \times 10^3$ LC1-like molecules per cell were associated with this cell type 1 h after steroid treatment ($n=5$, not significant). Finally, resident eosinophils (in the pleural cavity) were found to have much higher LC1 levels than that found in the blood circulation (2 fold increase, $P<0.05$).

**7** Passive immunization of mice against LC1 with a validated antiserum (termed LCS3) and protocol failed to modify the anti-migratory activity exerted by Dex towards eosinophil extravasation into Ova-challenged air-pouches. The steroid (50 $\mu$g s.c., $-1$ h) produced a similar degree of inhibition of eosinophil accumulation both in control animals (treated with a non-immune sheep serum) and LCS3-treated mice ($-56\%$ and 59%, respectively, $n=15-21$, not significant).

**8** In conclusion, the air-pouch provides a novel and convenient cavity to study allergen-induced cell recruitment which is sensitive to glucocorticoid hormone treatment. The effect of Dex on eosinophil distribution in these experimental conditions has been studied in detail and we failed to find an important role for endogenous LC1 in these actions of the steroid.

**Keywords:** Ovalbumin; lipocortin 1; dexamethasone; air-pouch; leucocytes

## Introduction

Peripheral blood eosinophilia and localized eosinophil infiltration are characteristic features of a number of allergic diseases including asthma and atopic dermatitis (Martin *et al*., 1996). There is much evidence to suggest that this cell type may be involved in the manifestation of some of the features associated with these disease states. Eosinophils respond to a number of inflammatory stimuli including platelet-activating factor and the recently described eosinophil-specific chemokine, eotaxin (Wardlaw *et al*., 1986; Jose *et al*., 1994). Further, activated eosinophils are able to secrete cytotoxic granule proteins such as major basic protein (Frigas *et al*., 1991), lipid mediators (e.g. platelet-activating factor and leukotriene $C_4$) (Lee *et al*., 1984; Kauffman *et al*., 1987) and cytokines (e.g. interleukin-5 and interleukin-8) (Broide *et al*., 1992; Yousefi *et al*., 1995) which may perpetuate the inflammatory reaction.

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Glucocorticoids are widely used for the treatment of allergic disorders (Schleimer, 1990; Barnes, 1995), and indeed dexamethasone (Dex) is routinely used to characterize *in vivo* models of allergic inflammation. For instance, administration of this steroid to guinea-pigs or rats profoundly affected the infiltration of eosinophils into the trachea following allergen challenge (Barton *et al*., 1991; Elwood *et al*., 1992). In addition, Dex prevented eosinophil accumulation in tissue sites other than within the respiratory tract such as the mouse peritoneal cavity or rat pleural cavity (Spicer *et al*., 1986; Zuany-Amorim *et al*., 1993; Bozza *et al*., 1993). In spite of all these studies, little is known about the mechanism(s) of action of this and other glucocorticoids in inhibiting eosinophil migration.

In the present study, we have attempted to elucidate the mechanism of inhibitory action of Dex on eosinophil migration elicited by antigen challenge in the mouse. For this purpose, we have developed a novel model of ovalbumin (Ova)-induced cell migration into 6 day-old air-pouches of mice sensitized to the antigen. This animal species was chosen as it responded with a profound and reproducible eosinophilic response to Ova challenge and, in contrast to other species such as the guinea-pig (Keightley & Fuller, 1994), is highly sensitive to the actions of Dex. The air-pouch provides a convenient cavity from which to harvest cells. The effect of this steroid on bone marrow, circulating and infiltrated eosinophils was also evaluated with particular attention to the status of the allergic response. Finally, since we have previously demonstrated a role for endogenous lipocortin-1 (LC1) on the inhibition exerted by Dex upon neutrophil migration into murine air-pouches in response to interleukin (IL)-1 or IL-8 injection (Perretti & Flower, 1993; Perretti *et al*., 1994), the potential involvement of this steroid mediator in the current model of allergic inflammation was also investigated.

## Methods

### *Animals*

Female BALB/c mice (18–20 g body weight) were obtained from Tuck (Essex, U.K.). Animals were housed with a 12 h light-dark cycle and were allowed food and water *ad libitum*. On the day of the experiment animal body weight had reached 22–24 g. CBA/Ca mice overexpressing the human IL-5 gene (transgenic line Tg5C1) (Dent *et al*., 1990) were bred at the National Heart & Lung Institute (London, U.K.). Animals were housed with a 12 h light-dark cycle and were allowed food and water *ad libitum*. When used, the animals weighed 25–30 g.

### *Sensitization and challenge protocol*

Animals were given a 0.4 ml s.c. injection of 100 $\mu$g Ova absorbed to 3.3 mg of aluminium hydroxide gel in sterile saline on days 1 and 8. On days 9 and 12 mice were injected with 2.5 ml s.c. of air to initiate the development of the air-pouches as described previously (Perretti & Flower, 1993). On day 15 (6 days after the first air injection) animals were challenged by injection into the air-pouch with 0.4 ml of sterile saline alone or containing 10 $\mu$g Ova.

### *Drug treatments*

Dex was systemically administered 1 h before Ova challenge. In other cases, the steroid was given locally concomitantly with Ova. The anti-LC1 antiserum used is a polyclonal sheep serum raised against human LC1, termed LCS3. This antiserum has been shown previously to reverse the inhibitory actions of the steroid in a murine model of neutrophil migration into air-pouches in response to specific pro-inflammatory cytokines (Perretti & Flower, 1993; Perretti *et al*., 1994). A non-immune sheep serum, termed NSS, was used as a control. Both sheep sera were injected s.c. at a dose of 50 $\mu$l per mouse 24 h before Dex (Perretti & Flower, 1993).

### *Quantification of cell infiltrates in the air-pouches*

At different time-points after challenge with Ova or saline mice were killed by $CO_2$ asphyxiation. Air-pouches were then opened and washed with 1 ml phosphate-buffered saline (pH = 7.4, PBS) containing 3 mM ethylenediaminetetraacetic acid (EDTA). The washes were spun at 300 *g* for 10 min at 4°C. Supernatants were removed and cell pellets resuspended in 0.5 ml PBS-EDTA containing 10% foetal calf serum. An aliquot (10 $\mu$l) was used for total cell counts by use of Kimura stain, whereas differential cell countings were performed on May-Grunwald-Giemsa stained cytospin preparations as described by Das *et al*. (1995).

### *Analysis of blood cell numbers*

Blood was collected into heparinized syringes by cardiac puncture, under halothane anaesthesia. Total leucocyte counts were performed by use of Kimura stain and differential cell counts on blood smears were done following staining with May-Grunwald-Giemsa as described above. Under these conditions it was possible to distinguish eosinophils for neutrophils and mononuclear cells.

### *Quantification of bone marrow eosinophil numbers*

The numbers of eosinophils in the bone marrow of mice was quantitated from bone marrow cell suspensions of femurs. Both femurs from each mouse were isolated and flushed with 10 ml Hank's buffered salt solution containing 0.25% bovine serum albumin (BSA). Samples were spun and cell numbers determined in a Neubauer haemacytometer by use of Kimura's stain. Kimura positive cells were accepted as eosinophils.

### *Fluorescence-activated cell sorter (FACS) analysis on infiltrated leucocytes*

The cellular influx into the air-pouch 24 h after Ova challenge consisted of a mixed population of leucocytes. We wished to differentiate between the mononuclear and PMN populations and, to characterize the PMN population by use of flow cytometry as previously described (Perretti & Flower, 1996). For this purpose we utilized mAbs directed against specific surface antigens present on murine leucocytes. We used an anti-CD3 monoclonal antibody (mAb) (clone KT3, used at a final concentration of 5 $\mu$g ml$^{-1}$), anti-CD11b mAb (clone 5C6, used at a final concentration of 12.5 $\mu$g ml$^{-1}$), anti VLA-4 mAb (clone PS/2, used at a final concentration of 10 $\mu$g ml$^{-1}$) anti-ICAM-1 mAb (clone KAT-1, 20 $\mu$g ml$^{-1}$ final concentration). The washes from six air-pouches collected at 24 h after Ova challenge were pooled, pelleted and resuspended in PBS containing 0.1% BSA (PBS-BSA). The cells were plated in a 96-well plate (Falcon, U.K.) ($0.5 \times 10^6$ cells/well, 20 $\mu$l) and non-specific binding sites were blocked with 20 $\mu$l human IgG (15 mg ml$^{-1}$) before the addition of 20 $\mu$l of the appropriate mAb. Plates were incubated at 4°C for 1 h after which cells were washed, and then stained with 40 $\mu$l of a $F(ab')_2$ fragment of a murine anti-rat IgG conjugated with fluorescein isothiocyanate (FITC) (1 : 50 stock solution). Samples remained stable for this period of time. FACS analysis was performed with a FACScan II analyser (Becton Dickenson, Mountain View, CA) with air-cooled 100 mW argon ion laser tuned to 488 nm and Consort 32 computer running Lysis II software. The value of the mean fluorescence intensity (MFI) was measured at 515 nm with a band width of 10 nm (the FL-1 channel).

### *Effect of Dex on cell-associated LC1 levels in circulating eosinophils*

The effect of Dex on intracellular LC1 levels of circulating eosinophils was measured by use of a protocol previously described (Perretti & Flower, 1996). As the percentage of circulating eosinophils in BALB/c mice is very low, CBA/Ca mice overexpressing the human IL-5 gene were used for these experiments (Dent *et al*., 1990). Mice were injected s.c. with either vehicle or 50 $\mu$g Dex (a dose which inhibits eosinophil migration to Ova challenge in the present study). After 1 h, animals were anaesthetized with sodium pentobarbitone (i.p.) and also injected concomitantly with 50 $\mu$l heparin. Blood samples were collected by cardiac puncture. The red blood cells were lysed by the addition of FACS lysis fluid and kept on ice for 10 min. The samples were then washed two times (310 *g*, 7 min) and the resulting pellets resuspended in 2 ml PBS/BSA containing 1 mM $Ca^{2+}$ (PBC). Cells were fixed for 30 min in an equal volume of 2% paraformaldehyde at 4°C and then plated in a 96-well plate at a density of $1 \times 10^6$ cells per well. The plates were then washed three times in PBC containing 0.02% saponin (from *Saponaria* species, Sigma; PBCS) to achieve cell permeabilization. An excess of blocking human IgG (15 mg $ml^{-1}$, 20 $\mu$l) was then added before 20 $\mu$l of either NSS or LCS3 (1 : 100 final concentration in both cases). After 1 h at 4°C and three washes with PBCS, cells were incubated with 40 $\mu$l of specific $F(ab')_2$ fragment of donkey anti-sheep IgG conjugated with FITC (1 : 50 of stock solution) for 45 min at 4°C. After two more washes with PBCS and one with PBC the samples were analysed by FACS as described above. Different cell populations were discriminated by their forward and side scatter characteristics and the MFI values were calculated in the FL-1 channel. The number of LC1 molecules was then calculated from the MFI values by use of reference microbeads labelled with standard numbers of FITC molecules (Flow Cytometry Standards Corp) as described previously (Perretti & Flower, 1996). The results are presented as the number of LC1-like immunoreactive molecules per cell. Cytospin preparations were also made from the cell samples to determine the percentage of eosinophils within the polymorphonuclear (PMN) cell population. The majority of the PMNs were eosinophils: $96.5 \pm 1.1\%$ (range 89–100%, $n = 10$) and $96.4 \pm 0.69\%$ (range 94–99%, $n = 7$) eosinophils from vehicle and Dex treated animals, respectively.

### *Detection of cell-associated LC1 in cells infiltrating the air-pouches*

The use of the anti-LC1 antiserum (see above) was validated by demonstrating a positive signal for LC1 in migrated eosinophils, by use of a methodology previously applied to mouse peripheral blood leucocytes (Perretti & Flower, 1996). Cells were collected from the air-pouches 24 h after Ova challenge as above, resuspended in PBC, and plated in a 96-well plate at a density of $0.5 \times 10^6$ cells in 100 $\mu$l per well. Cells were then fixed by 30 min incubation with an equal volume of 2% paraformaldehyde at 4°C. The plates were washed with PBCS three times and LC1 staining was carried out as described above.

LC1 levels in pleural cavity cells from CBA/Ca mice were also measured. Untreated CBA/Ca mice were anaesthetized with sodium pentobarbitone (i.p.) containing 50 $\mu$l heparin. The pleural cavities were lavaged with 2 ml PBS and the cells were washed twice. The resulting cell pellets were resuspended in PBC and treated as above to measure cell-associated LC1 levels. The cell population consisted of $58.8 \pm 9.2\%$ eosinophils and the remaining cells were macrophages, as assessed by histological examination.

### *Materials*

Ova, NSS, human IgG, saponin (from *Saponaria* species) and FITC-conjugated donkey anti-sheep IgG were from Sigma (Poole, Dorset, U.K.); aluminium hydroxide gel was from Reheiss (Dublin, Ireland); Dex (sodium phosphate salt) was from David Ball Laboratories (Warwick, U.K.); KT3, 5C6, KAT-1, FITC-conjugated murine anti-rat IgG were from Serotec (Oxford, U.K.); FACS lysis fluid was from Becton & Dickson (San Jose, U.S.A.); sodium pentobarbitone (Sagatal) was from May & Baker (Dagenham, Essex, U.K.); PS/2 was a kind gift of Dr B.A. Wolitzky, Hoffman LaRoche.

### *Statistics*

All results are shown as the mean ± s.e.mean. Statistical differences were analysed by non-parametric tests on raw data. The Mann-Whitney U test was used to analyse differences between two groups and the Kruskal-Wallis test was used if more than two groups were analysed.

## Results

### *Characterization of leucocyte infiltration after Ova challenge in the mouse air-pouch model*

Challenge with 10 $\mu$g Ova in 6 day-old air-pouches of sensitized mice resulted in a time-dependent eosinophil infiltration well above the negligible numbers observed after sterile saline challenge. Influx of this cell type was evident from 6 h after Ova challenge, peaked at 24 h and it was still persistent 48 h post-Ova (Figure 1). There were also increases in the numbers of neutrophils and mononuclear cells which paralleled the eosinophil accumulation (see Table 1 for the 24 h values). The relative proportions of eosinophils, neutrophils and mononuclear cells were approximately 37%, 18% and 45%, respectively at 24 h post-Ova, and 43%, 21% and 36%, respectively, at 48 h post-Ova challenge.

Next, we characterized the profile of surface antigens on the infiltrating leucocytes at 24 h after Ova challenge. Figure 2a shows the side and forward scatter profile of air-pouch cells with the clear demarcation between the two cell populations,

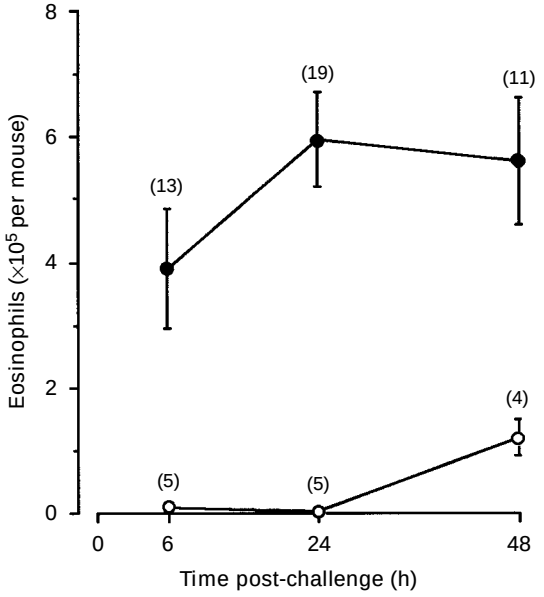


**Figure 1** Time-course of eosinophil infiltration into the air-pouch after ovalbumin (Ova) challenge. Sensitized mice were challenged with either saline (○) or 10 $\mu$g Ova (●) and killed at 6, 24 and 48 h. Results are the mean of *n* mice per group; vertical lines show s.e.mean. Significantly ($P < 0.05$) higher eosinophil numbers were measured after Ova challenge at all time-points.

**Table 1** Number of leucocytes recovered from sensitized murine air-pouches

| | | *Cells* ($\times 10^5$ per mouse) | | |
|---|---|---|---|---|
| *Treatment* | *Total cells* | *Eosinophils* | *Neutrophils* | *Mononuclear cells* |
| Saline ($n=5$) | $2.50 \pm 0.62$ | $0.02 \pm 0.01$ | $0.05 \pm 0.02$ | $2.43 \pm 0.62$ |
| Ova ($n=24$) | $13.78 \pm 1.91$** | $5.12 \pm 0.72$** | $2.49 \pm 0.47$** | $6.24 \pm 0.78$* |

The number of different cell types recovered 24 h after challenge with 10 $\mu$g Ova from 6 day-old air-pouches of sensitized mice is shown, in comparison to those measured in saline-challenged mice. Values are mean $\pm$ s.e.mean of $n$ mice per group. *$P<0.05$ and **$P<0.01$ when compared to saline values.

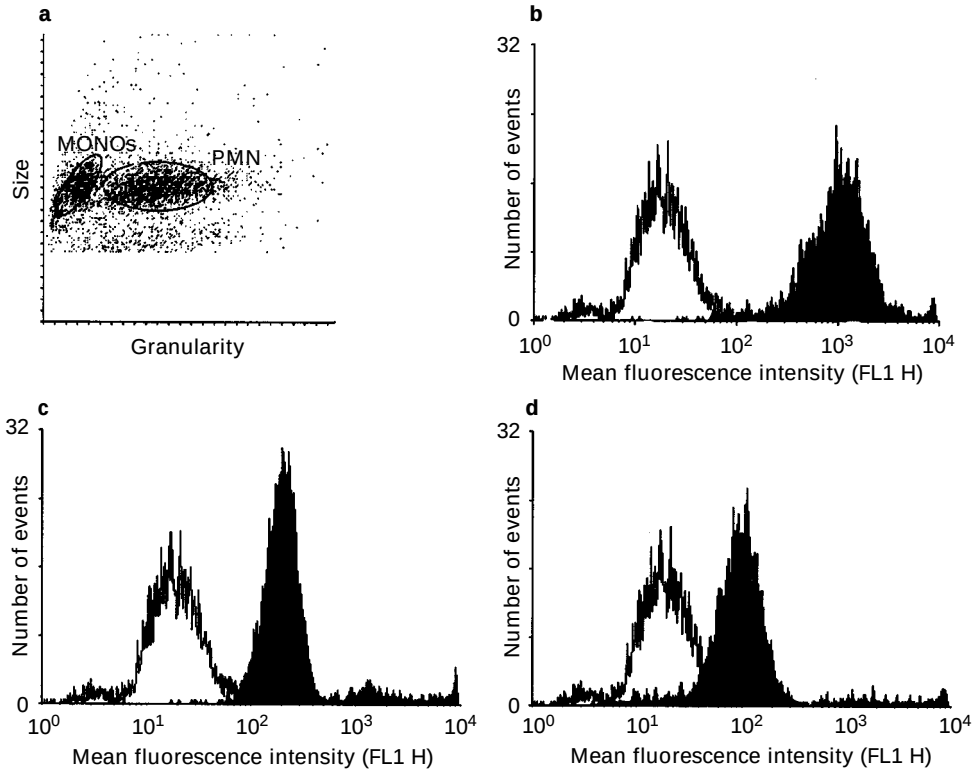


**Figure 2** Flow cytometry analysis of leucocytes collected from air-pouches 24 h post ovalbumin (Ova) challenge. (a) The whole cell population with respect to size and granularity and the two distinct populations of PMN and mononuclear cells (MONOs) are marked. Leukocytes were stained for cell surface antigens (as described in Methods). The cells which were positive for (b) CD11b, (c) ICAM-1 and (d) VLA-4 within the PMN population (open histograms represent the non-specific staining) are shown.

namely mononuclear and PMN leucocytes. The latter population was characterized further and it was found that 99% of the cells were positive for both CD11b and ICAM-1, and 72% of the cells expressed the VLA-4 antigen on their surface (Figure 2b and d). The mononuclear cell population consisted mainly of monocytes and/or macrophages (the proportion of cells positive for CD11b and ICAM-1 were 72 and 42%, respectively), with a small number of T lymphocytes ($\sim$15% as assessed with the anti-CD3 mAb) (data not shown).

The number of circulating eosinophils in the mouse blood was significantly increased after the 2 week sensitization protocol and this number was not further modified following Ova challenge at 24 h (Figure 3a). Sensitization induced a significant increase in bone marrow eosinophils and challenge with Ova provoked a remarkable reduction in this eosinophil pool ($-64$%) at 24 h (Figure 3b).

### *The effect of Dex on eosinophil accumulation and distribution after Ova challenge*

The effect of Dex given s.c. was assessed since this route of administration has been shown to be effective in reducing eosinophil migration over a 24 h protocol in the mouse peritoneum. Figure 4 shows the results of these experiments. Both doses of Dex given by this route were effective in reducing the eosinophilia at 24 h post-Ova. At the highest dose tested, Dex was also effective in attenuating eosinophil infiltration measured at the 48 h time-point, when it was injected 24 h after Ova challenge. This observation suggests that active eosinophil recruitment was still occurring at the 24 h time-point. For all subsequent experiments, the 24 h time-point for eosinophil infiltration after Ova challenge and a Dex dose of 50 $\mu$g s.c. was chosen.

In order to investigate the mechanism of action of Dex in reducing the cell migration in response to Ova challenge, we studied the effect of the steroid on circulating eosinophil numbers in naive and sensitized mice. A difference in circulating eosinophils was again seen between these two groups of animals, confirming the observation illustrated in Figure 3a. In both groups, a dramatic reduction in the number of blood eosinophils was observed 4 h after s.c. treatment with Dex: 83% and 95% reductions were calculated in naive and sensitized mice, respectively (Figure 5). The numbers of all other peripheral blood leucocytes were also reduced, though not as dramatically, by Dex administration. Data are as follows ($\times 10^5$ per mouse): from $5.1 \pm 1.0$ to $1.9 \pm 0.5$ neutrophils in control and Dex-treated naive mice ($n=8-15$; $P<0.05$) and from $7.2 \pm 1.8$ to $1.4 \pm 0.3$ neutrophils in control and Dex-treated sensitized mice ($n=10-15$; $P<0.01$) and from $38.8 \pm 4.9$ to $14.1 \pm 3.7$ mononuclear cells in control and Dex-

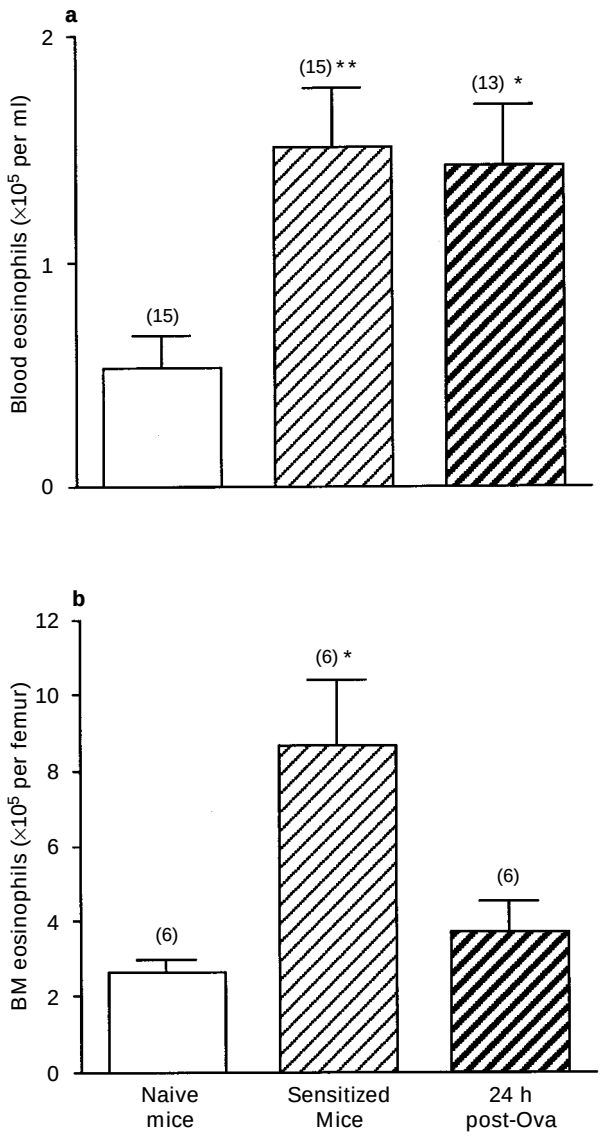


**Figure 3** Effect of sensitization and ovalbumin (Ova) challenge on eosinophil numbers in the blood (a) and in bone marrow (BM; b). Eosinophil numbers in the blood and BM were quantified in naive mice (open columns) and in sensitized mice before (lightly hatched columns) and 24 h after (heavily hatched columns) Ova challenge. Results are the mean ± s.e.mean of $n$ (number in parentheses) mice per group. $^*P<0.05$, $^{**}P<0.01$ when compared to naive mice.

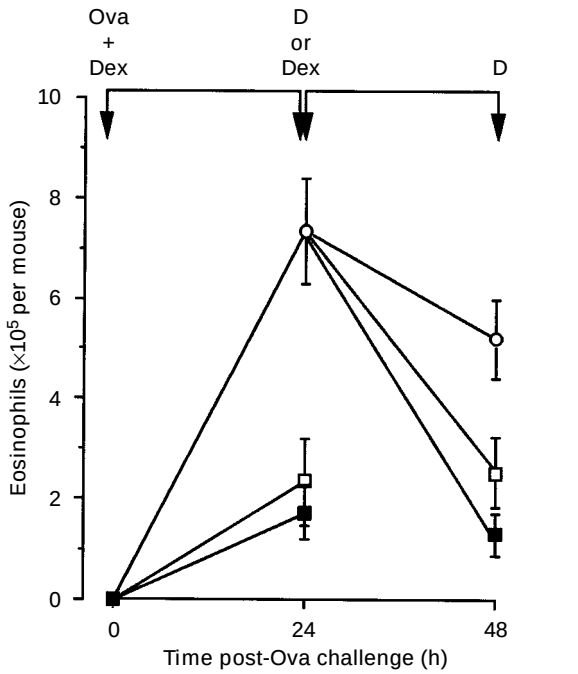


**Figure 4** Time-course of action of dexamethasone (Dex) on eosinophil accumulation into the air-pouch after ovalbumin (Ova) challenge. Sensitized mice were treated s.c. with either 50 $\mu$g (□) or 100 $\mu$g (■) Dex either 1 h before Ova challenge and death (D) at 24 h or at 24 h after Ova challenge and death at 48 h. (○) Animals challenged with Ova only. Results are expressed as the mean of 7–12 mice per group; vertical lines show s.e.mean. $^*P<0.05$, $^{**}P<0.01$ when Dex-treated groups were compared to the control group at the same time-point.

treated naive mice ($P<0.01$) and from $42.2\pm8.2$ to $17.9\pm4.1$ mononuclear cells in control and Dex-treated sensitized mice ($P<0.05$). These actions of Dex on peripheral blood leucocyte numbers were reversible, and the number of circulating eosinophils had returned to pre-Dex levels by 24 h (Figure 5). A similar reversibility was also seen on the other cell types (data not shown).

As a reduction in bone marrow eosinophil numbers were observed 24 h after antigen challenge (Figure 3b), we studied the effect of Dex on bone marrow eosinophil numbers. In mice pretreated with Dex 1 h before Ova challenge, the marked fall in the eosinophil numbers at the 24 h time-point was not seen. The number of bone marrow eosinophils in naive mice was $2.7\pm0.3\times10^5$ and after sensitization the numbers increased to $8.7\pm1.7\times10^5$ (as shown in Figure 3b). However, 24 h after Ova challenge in sensitized mice, the number of bone marrow eosinophils dropped to $3.2\pm0.8\times10^5$ but, when mice were pretreated with Dex the bone marrow eosinophil numbers at 24 h were $6.8\pm1.0\times10^5$ ($n=5$; $P<0.05$ when compared to Ova-challenged mice at 24 h).

### *Role of LC1 in the anti-migratory actions of Dex*

We investigated whether Dex had an effect on circulating eosinophils in the present study. Table 2 shows the immunoreactive LC1 levels in circulating eosinophils of interleukin-5 (IL-5) transgenic mice and the lack of alteration following *in vivo* administration of Dex. It is of interest to note that eosinophils resident in the pleural cavity exhibit much higher levels of LC1 when compared to the levels found in circulating eosinophils (Table 2). In line with this observation, passive immunization of mice with LCS3 failed to modify the anti-migratory action of the steroid. As seen in Figure 6, treatment of animals with Dex affected eosinophil infiltration into the air-pouches to a similar extent in both NSS- and LCS3-pretreated animals. No effect on the numbers of other cell types was seen by passive immunization against LC1 (data not shown).

The possibility that LCS3 was not recognizing eosinophil-associated LC1, in this strain of mice, was excluded by FACS analysis on elicited cells. Following staining of permeabilized eosinophils with LCS3 a net value of $373\times10^3$ LC1-like immunoreactive molecules above that measured with NSS were found associated with this cell type. The LC1-like immunoreactivity values differed between the two strains and this may be due to either strain variations or differences between LC1 levels in blood and elicited cells.

## Discussion

In this study we have established a novel model of allergic inflammation. We used this model to study the mechanism(s) of the anti-inflammatory actions of Dex on the migration of eosinophils into a cavity and whether the actions were mediated through LC1. Our previous work has established that neutrophil migration into the murine air-pouch in response to

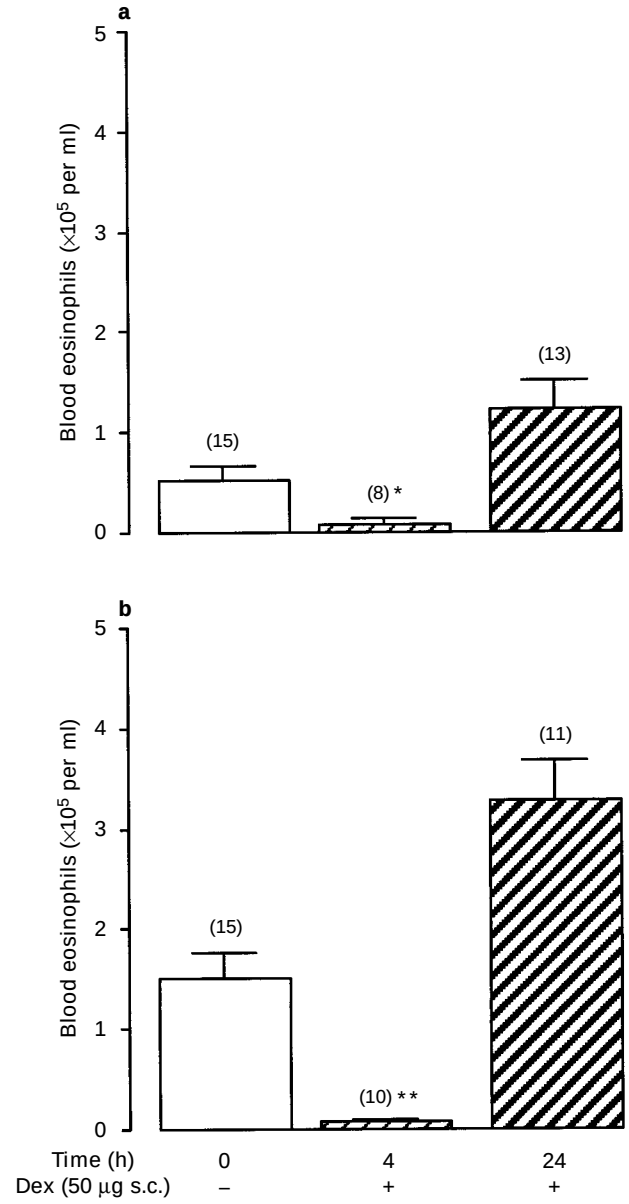


**Figure 5** Effect of dexamethasone (Dex) on blood eosinophil numbers. Naive (a) or sensitized (b) mice were treated with Dex and killed at 0 h (open columns), 4 h (lightly hatched columns) or 24 h (heavily hatched columns) and blood samples collected. Blood eosinophil numbers are shown as the mean ± s.e.mean of $n$ (number in parentheses) mice per group. $^{*}P<0.05$, $^{**}P<0.01$ when compared to 0 h.

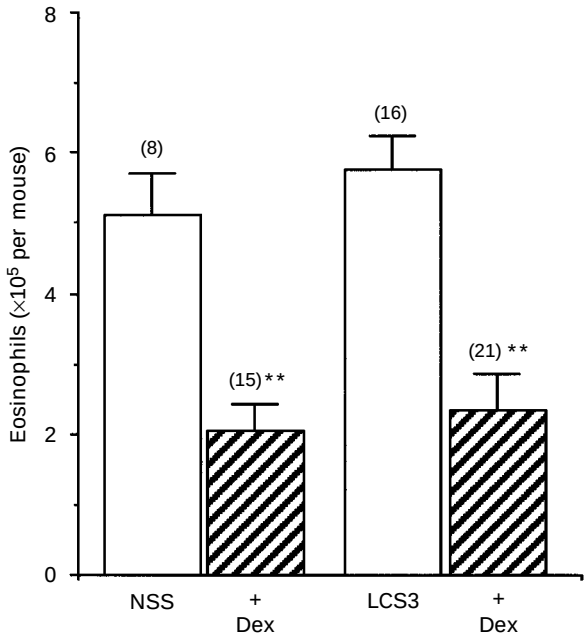


**Figure 6** Effect of LCS3 on dexamethasone (Dex)-induced suppression of eosinophil accumulation in murine air-pouch. Sensitized mice were pretreated either with 50 μl s.c. NSS or LCS3 24 h before the administration of saline (open columns) or Dex (50 μg s.c.; hatched columns). Mice were challenged with ovalbumin (10 μg) 1 h later and killed at the 24 h time-point. Values are mean ± s.e.mean of $n$ (number in parentheses) mice per group. $^{**}P<0.01$ when compared to respective controls.

**Table 2** Lipocortin-1 (LC1) levels in murine eosinophils

| *Cell type* | *Treatment* | *LC1-like immunoreactive molecules per cell* ($\times 10^3$) |
|---|---|---|
| Blood eosinophils | Vehicle | 235 ± 21 ($n=5$) |
| Blood eosinophils | Dex | 224 ± 8 ($n=5$) |
| Blood eosinophils | – | 267 ± 14 ($n=5$) |
| Pleural cavity eosinophils | – | 506 ± 60 ($n=4$)* |

Blood was collected from CBA/Ca mice. In some cases, the mice had been pretreated s.c. with vehicle or 50 μg Dex for 1 h. The pleural cavities of untreated mice were also lavaged. LC1 levels within the eosinophil population were determined by staining with LCS3 and NSS serum, the difference of the two stainings gave the LC1 level.$^{*}P<0.05$ when compared to LC1 levels in blood eosinophils.

different inflammatory mediators is inhibited by Dex and that the glucocorticoid acts via LC1 (Perretti & Flower, 1993; Perretti *et al*., 1993; 1994). Therefore we wished to use the same cavity for the present study. The air-pouch also provides a convenient cavity within which to work. Previous murine models of allergen challenge have targeted either the airways or peritoneal cavity (Zuany-Amorim *et al*., 1993; Kung *et al*., 1994a).

On Ova challenge, we observed a significant cell infiltration from as early as 6 h. The cell infiltrate consisted of eosinophils, neutrophils and mononuclear cells. Although the percentage of each cell type remained the same, the numbers of all cell types increased by 24 h and remained elevated at 48 h. This response differs from many previous findings of allergen challenge in murine models. For example, Zuany-Amorim and co-workers (Zuany-Amorim *et al*., 1993) found an increase in neutrophils at 6 h after challenge with 10 μg Ova in the peritoneal cavity, after which time the neutrophil numbers declined and an eosinophilia was observed. Similar results have been shown when sensitized mice were challenged in the airways (Kung *et al*., 1994a; Yu *et al*., 1996). However, other studies have demonstrated a persistent neutrophil presence for up to 72 h (Kennedy *et al*., 1995). These differences may be due to different sensitization/challenge protocols and mouse strains employed. Further, the air-pouch differs from the peritoneal cavity and airways as it is lined by fibroblasts and macrophages, and granulocytes are present within the lining, whereas few resident cells are found within the actual air-pouch (Edwards *et al*., 1981).

The cellular profile of the infiltrating cells obtained by microscopy was confirmed by FACS analysis showing two distinct cell populations which were characterized further. It is well established that amongst granulocytes, eosinophils, but not neutrophils, express the β1 integrin VLA-4. We found that 72% of the PMN leucocytes were VLA-4 positive. This value correlates well with the percentage of eosinophils within the PMN population as counted by microscopy. The elicited PMN population was also found to express ICAM-1 and CD11b.

ICAM-1 is not expressed on circulating eosinophils (Walker *et al*., 1993). However, on activation (e.g. by cytokines *in vitro* or the process of transmigration *in vitro* and *in vivo*) eosinophils have been shown to express ICAM-1 (Hansel *et al*., 1991; Czech *et al*., 1993). Further, eosinophil and neutrophil extravasation is also associated with enhanced CD11b expression (Walker *et al*., 1993; Mengelers *et al*., 1994). In the mononuclear cell group, 15% were T lymphocytes (CD3 positive) which agrees with the percentages elicited by allergen challenge found in previous studies (Kennedy *et al*., 1995; Corry *et al*., 1996). The remaining cells were likely to be monocytes and macrophages and were CD11b and ICAM-1 positive.

The sensitization protocol induced an increase in bone marrow and circulating eosinophil numbers when compared to non-sensitized animals. The increase in eosinophil numbers could be due to cytokines such as IL-5, released from the Th2 subset of $CD4^+$ T cells, acting on the bone marrow during the sensitization period (Ohkawara *et al*., 1996). On Ova challenge, a reduction in bone marrow eosinophils was observed at 24 h suggesting release of the cells into the circulation. Since no further significant increase in blood eosinophils was observed at 24 h, a dynamic equilibrium may be established between the blood and air-pouch compartments with the bone marrow maintaining the level of eosinophil numbers in the circulation. Indeed, a previous study has shown that following the reduction in bone marrow pool of eosinophils at 24 h following Ova challenge, there was an increase in bone marrow eosinophil numbers at later time-points (Kung *et al*., 1994b). This suggests a dynamic role of the bone marrow in maintaining an equilibrium of eosinophil numbers in the circulation.

Glucocorticoids are potent inhibitors of virtually every type of inflammatory reaction and exert a strong effect on leucocyte recruitment. Dex is routinely used to modulate negatively cell migration into sites of inflammation and this action is accomplished, in part, by a potent effect on the synthesis of pro-inflammatory cytokines and chemokines (Schleimer, 1990) coupled to a reduced degree of leucocyte responsiveness (Mancuso *et al*., 1995). For this purpose, Dex is often used as a positive control drug in models of allergic inflammation. In this study we monitored the efficacy of Dex in inhibiting eosinophil influx. Subcutaneous pretreatment of mice with Dex was effective in modulating the cell influx in response to Ova at 24 h. Dex also inhibited cell migration when it was given 24 h after Ova challenge. This observation suggests that an active recruitment of cells is still occurring at 24 h after Ova challenge to maintain the number of leucocytes at the site of challenge.

The reduction in cell recruitment into the air-pouch by acute Dex treatment appears, in part, to be mediated by reducing the number of circulating cells as well as inhibiting their release from the bone marrow. A number of studies in man have demonstrated that glucocorticoids induce an immediate blood eosinopaenia (Schleimer & Bochner, 1994) which we have also found in the present study. It has been suggested that this action of Dex may be mediated by sequestration of leucocytes to lymphoid organs and inhibit the release of cells from the bone marrow (Schleimer & Bochner, 1994). To support the latter notion, it has been demonstrated that the reduction of eosinophils in the bone marrow 24 h after Ova challenge *in vivo* is inhibited if Dex is administered before allergen challenge (Kung *et al*., 1994b) which we have also confirmed in this study. Many studies have shown that Dex inhibits the stimulated expression and release of IL-5 from inflammatory cells (eg. Corrigan *et al*., 1995) suggesting one mechanism for its effects on the bone marrow. Interestingly, in our present study, Dex reduced the circulating eosinophil numbers to a similar extent (>80%) in both naive and sensitized animals. This would suggest that the action of Dex is through a mechanism common to both immune states and is independent of the effects of sensitization.

Based on our previous work, where we established that neutrophils were an important target for LC1 and that this protein mediated the effects of systemically injected Dex (Perretti & Flower, 1993; Mancuso *et al*., 1995), we investigated whether the eosinophil had a similar function. For the first time we have demonstrated that circulating murine eosinophils do contain LC1 but that systemically administered Dex failed to modify the levels of this protein. To confirm further the lack of involvement of LC1 in the anti-migratory actions of Dex on eosinophils, LCS3 was unable to reverse the inhibition by the glucocorticoid of eosinophil influx in response to Ova challenge. We have also found that resident eosinophils in the pleural cavity have higher basal levels of intracellular LC1 than circulating eosinophils. Previous studies have demonstrated that peritoneal and alveolar macrophages have higher LC1 levels than circulating monocytes (De Caterina *et al*., 1993; Perretti & Flower, 1996) suggesting that the differentiation status of cells is associated with these elevated levels as is the case with other cell types (Solito *et al*., 1991).

In previous studies where the role of LC1 has been established, neutrophil migration in response to a single mediator or irritant was studied over a short time-course. Therefore, in these studies Dex may be acting by inhibiting the release of a small panel of mediators through a mechanistic pathway which involves LC1. In contrast, allergen-induced cell recruitment in sensitized animals is the result of a complex phenomenon which involves a cascade of immunological events over a much longer time-course. All this may have overcome the potential inhibitory action played by LC1. Therefore it is important to test the role of LC1 in a more simple model of eosinophil infiltration before the hypothesis that this mediator is involved in the emigration process of this cell type is discarded.

In this study, we have investigated the mechanism of action of Dex in inhibiting leucocyte migration into the air-pouches of sensitized mice in response to allergen challenge. Dex was found to have an effect on all the compartments of eosinophil distribution studied suggesting the involvement of different mechanistic pathways.

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