

Effect of allergen provocation on inflammatory cell profile and endothelin-like immunoreactivity in guinea-pig airways

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The effect of allergen challenge on the number of leucocytes and the concentration of endothelin 1-like immunoreactivity (ET-LI) in bronchoalveolar lavage fluid (BALF) was investigated in guinea-pigs sensitized to *Ascaris suum*. The animals were twice exposed to allergen aerosol. All animals responded to the second challenge with bronchoconstriction. Twelve hours later, a significant increase in the number of eosinophilic granulocytes in BALF, compared to unsensitized and unprovoked control animals, was noted. Twenty-four hours after provocation, there was also an elevation of ET-LI concentration and content of neutrophils. During the first day post-challenge, the ET-LI values were moderately correlated to the eosinophil levels. One week after challenge, the ET-LI level and the neutrophil count did not differ from corresponding values in control animals whereas the number of eosinophils remained elevated. Pretreatment with dexamethasone before the second allergen challenge did not consistently affect the parameters studied during the first 24 h. Bronchoconstriction induced by carbachol aerosol affected significantly neither the ET-LI concentration nor the number of inflammatory cells in BALF. It is concluded that the allergen-induced inflammation in the guinea-pig airways causes an elevation in the ET-LI concentration in BALF and that this is moderately correlated to the influx of eosinophils during the first 24 h.

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On the basis of several lines of evidence, it has been proposed that endothelin-1 (ET-1) contributes to the manifestations of bronchial asthma; the peptide is found in relatively high concentrations in the lung (14, 23), where it is synthesized not only by endothelial but also by epithelial cells (12), macrophages (8), and mast cells (9). Asthmatics are reported to have elevated levels of ET-LI in the airways (19, 24, 25), and the peptide may be released during asthma attacks (22).

In different experimental systems, exogenous ET induces a variety of effects associated with asthma, such as bronchoconstriction, smooth-muscle proliferation, activation of 15-lipoxygenase, oxygen radical formation in the lung (12), and oedema in lungs and airways due to increased microvascular permeability (10, 13). In ovalbumin-sensitized guinea-pigs, the bronchoconstrictive response to ET is enhanced following allergen challenge (6), possibly indicating

a pronounced role for the peptide in allergic reactions. ET could also have effects on the recruitment of inflammatory cells via the expression of adhesion molecules (20). However, a full understanding of the role of ET in airway disease will not be reached until we have results from experiments with agents that specifically antagonize the formation or effects of endogenous ET.

In previous works, we have found that the ET-LI content increases in lungs of rats subjected to intratracheal instillation of Sephadex beads, and that glucocorticosteroids (GCS) can ameliorate this rise (3). The Sephadex-induced inflammatory reaction is characterized by an eosinophilic peribronchitis/bronchiolitis with a granulomatous alveolitis leading to a long-lasting interstitial lung oedema (3, 5). The main inflammatory processes seem thus to occur in the lung parenchyma in contrast to what is seen in patients with bronchial asthma. We have therefore,

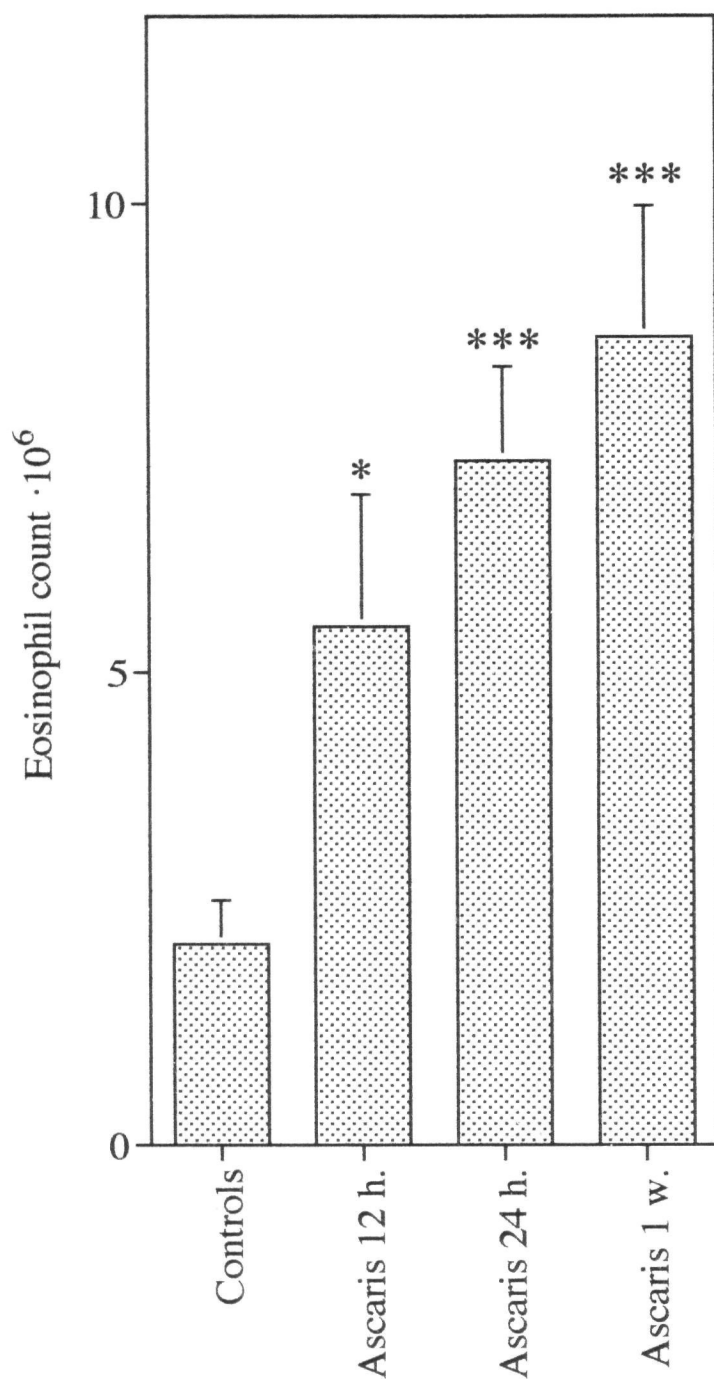


Fig. 1A

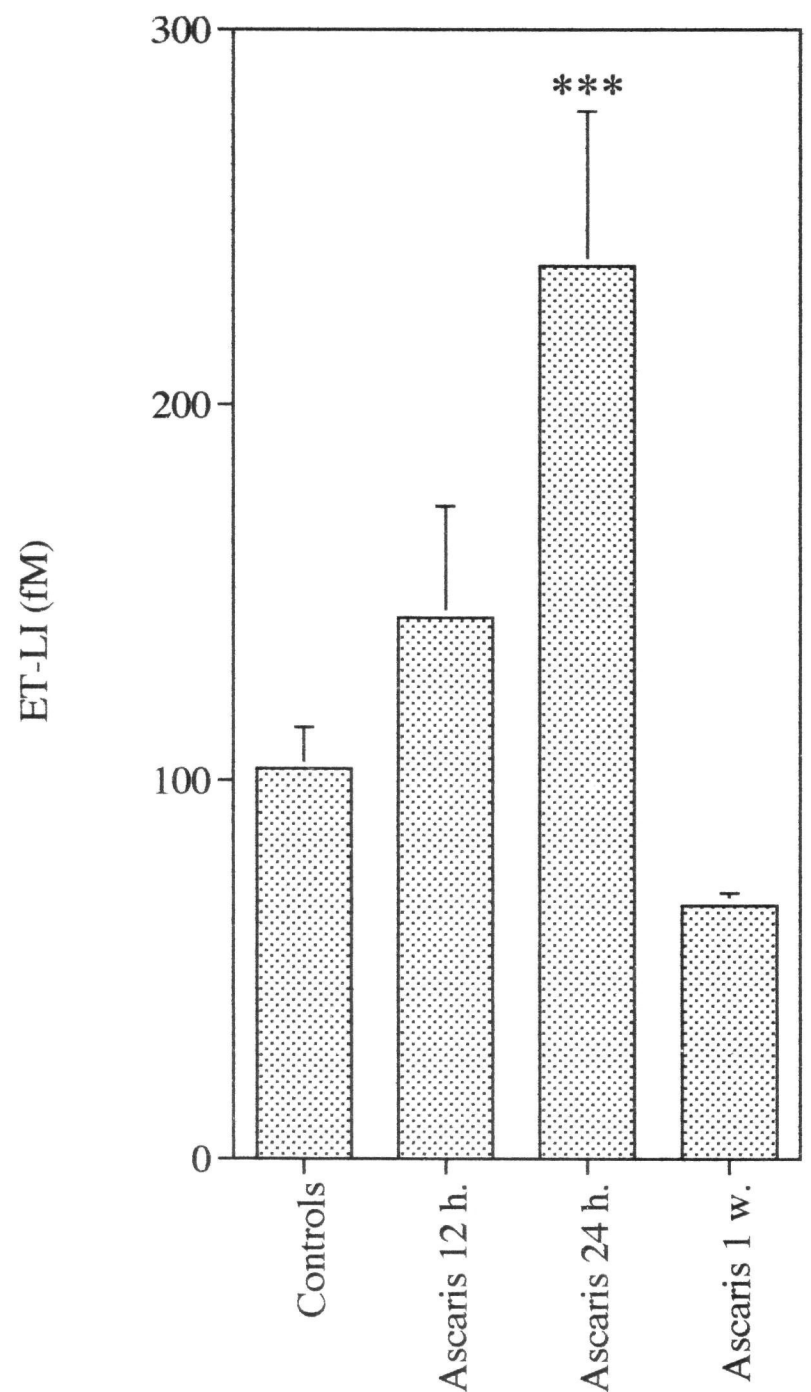


Fig. 1B

in the present study, measured the concentration of ET-LI and the corresponding number of leucocytes in bronchoalveolar lavage fluid (BALF) from allergen-provoked guinea-pigs sensitized to *Ascaris suum* as a model of more relevance to inflammatory processes in the airway mucosa.

Material and methods

Animals, sensitization procedure, A. suum provocation

The experiments were performed on guinea-pigs (Dunkin Hartley, Sahlins, Malmö, Sweden) of either sex. Animals that were to be provoked with allergen were sensitized according to a protocol previously described (18). In brief, animals weighing 150–200 g received an intraperitoneal (i.p.) injection of 4000 PNU *A. suum* extract and 100 mg AL(OH)₃. This was repeated once with 1 week elapsing between the injections. The experiments were per-

formed on three batches of animals supplied by the same breeder. They were treated in the same way, kept in the same room with constant temperature, and allowed food and water *ad libitum*. In each batch, one group of animals was left unsensitized as controls.

Two weeks after the second allergen injection, the guinea-pigs were challenged with nebulized *A. suum* (4000 PNU/ml in saline). The animals were placed in a transparent Perspex box and exposed to an aerosol (generated by a Turbo Turret System 22 nebulizer driven by compressed air; flow 3 l/min, output 0.2 ml/min) of *Ascaris* extract in 40-s intervals with 2 min elapsing between each provocation until bronchoconstriction developed (defined as the development of a slow, laboured breathing with exaggerated abdominal movements). Some animals did not respond to the first aerosol challenge, but all responded with bronchoconstriction after the sec-

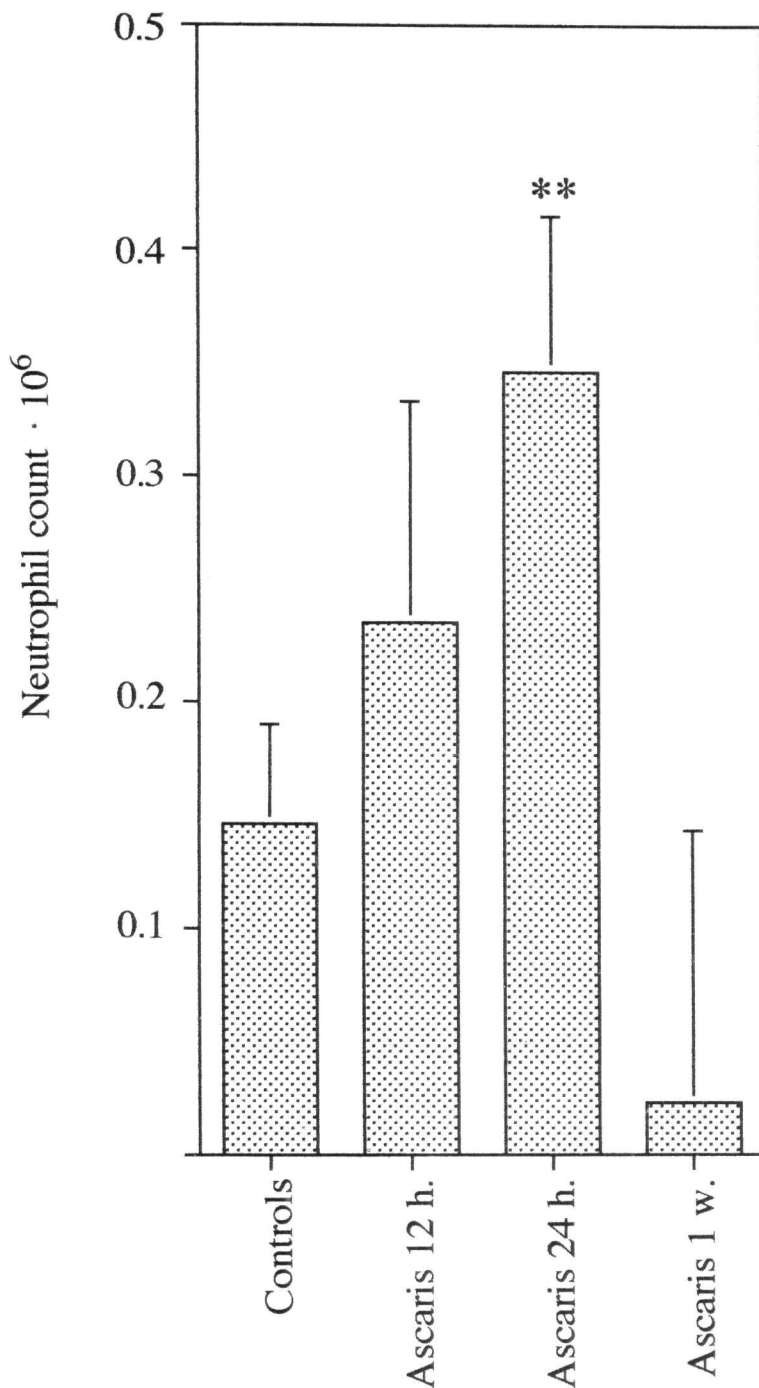


Fig. 1C

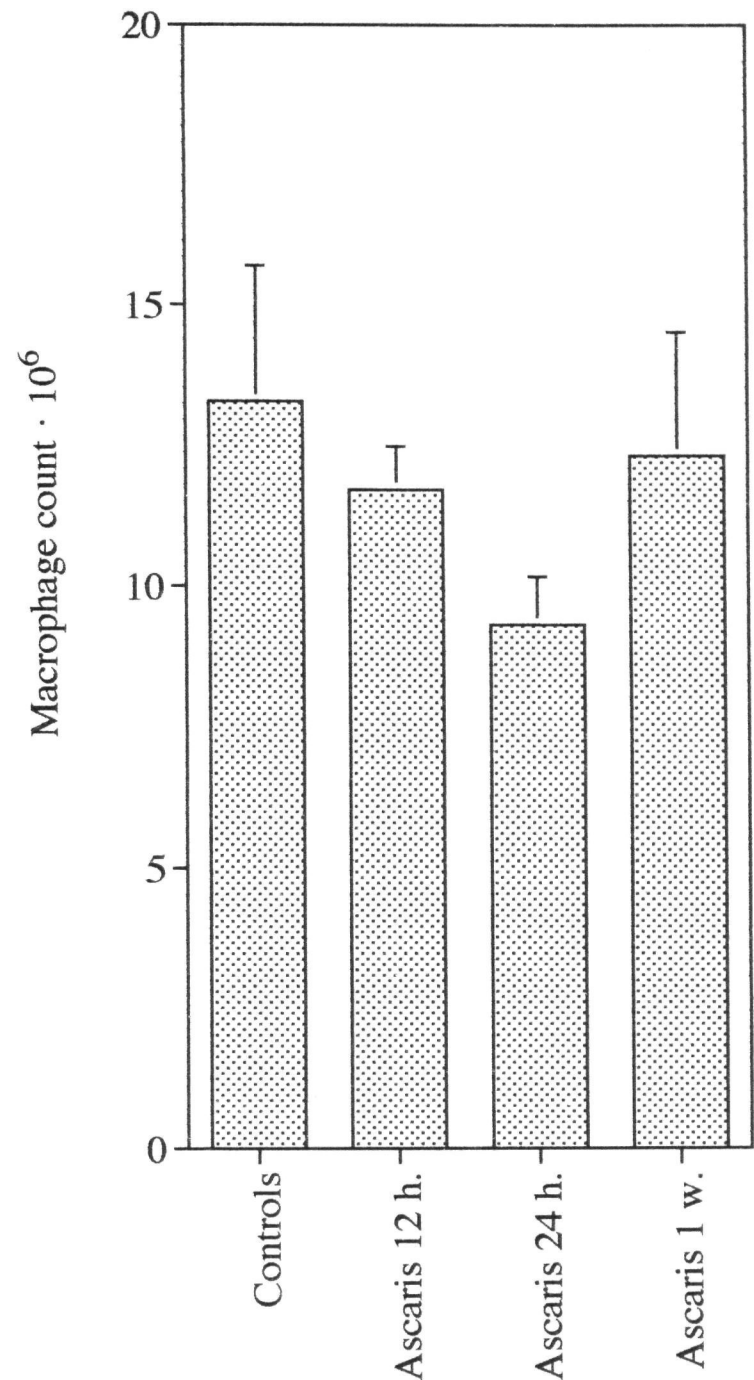


Fig. 1D

ond challenge, which was performed 11 days later. The focus of the study was thus on the responses to the second provocation.

Bronchoalveolar lavage

BAL was performed at 12 h, 24 h, and 1 week after the second exposure to *Ascaris* aerosol. The animals were killed by an overdose of pentobarbital sodium i.p. and were tracheotomized, and a tube, connected to a reservoir containing BALF at 37°C was inserted into the trachea. BALF was composed of sterile PBS with 3.47 g/l xylocaine HCl and 1 µM Phosphoramidon with pH adjusted to 7.4. Xylocaine is routinely used in this type of experiment because it yields a higher recovery of cells (17). Phosphoramidon was added to prevent degradation of ET-LI (29).

The level of the fluid was kept at 10 cm above the trachea at the start of the lavage, and it was allowed to flow into the airways for 3 min. It was then collected and chilled on ice. This procedure was repeated three times for each animal. Finally, the cannula was rinsed with 20 ml of BALF. The collected fluid was then centrifuged at 1000 rpm at 4°C for 10 min. The supernatant was frozen and kept at -70°C until the analysis of ET-LI was performed. The precipitated cells were resuspended in PBS; the concentration of cells counted and the composition of cell types were determined by microscopic examination.

Effect of glucocorticosteroid (GCS)-treatment

In animals lavaged 12 h after provocation, one group was treated with dexamethasone (25 mg kg⁻¹ day⁻¹)

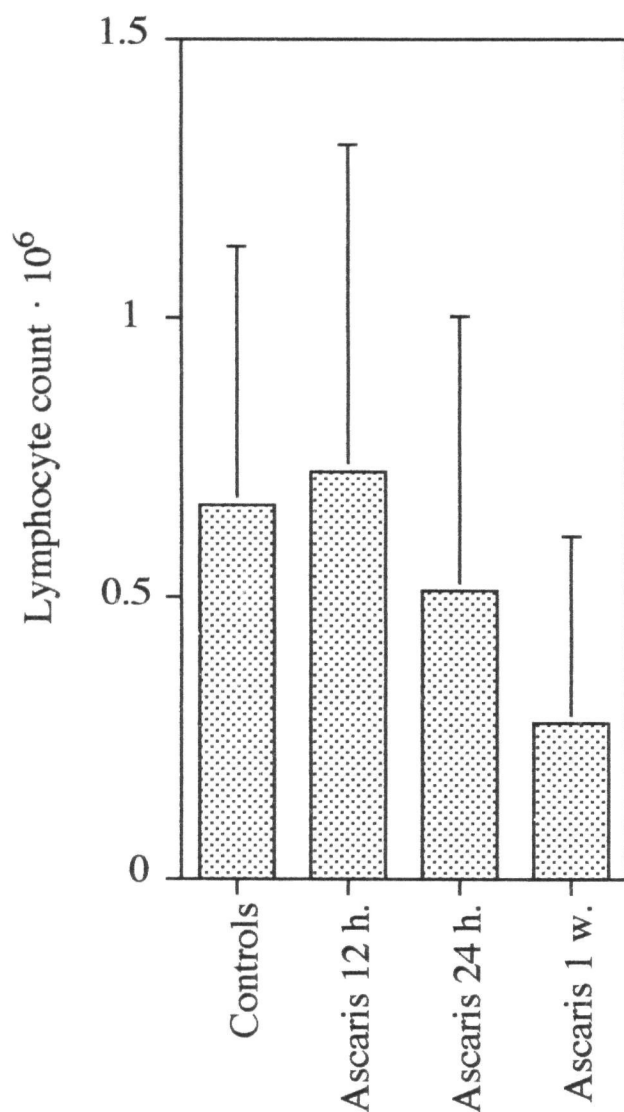


Fig. 1E

Fig. 1A–E. Time course of leucocyte appearance and changes in ET-LI concentration in BALF following *Ascaris suum* inhalation in sensitized guinea-pigs compared to levels in unsensitized control animals. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. For leucocytes, $n = 17, 4, 9$, and 6 in controls, at 12 h , 24 h , and 1 week after challenge, respectively. Corresponding numbers for ET-LI are $n = 17, 3, 5$, and 6 .

s.c. daily for 1 week before challenge, and in those lavaged 24 h after *Ascaris*, one group was pretreated with $17\text{ mg kg}^{-1}\text{ day}^{-1}$ of the steroid for 3 days , in order to determine whether GCS treatment could ameliorate the reaction to *Ascaris*.

Carbachol exposure

One group of unsensitized animals was exposed to carbamylcholine chloride (carbachol) aerosol in order to investigate the effect of bronchoconstriction *per se* on ET-LI concentration and cell content in BALF. The animals were kept in a whole-body plethysmograph constructed according to the principles of Agrawal (1). Computerized real-time-derived values of the specific airway conductance (sGaw) were calculated from measurements of air-flow from the snout and pressure changes in the box (11). Aerosols were generated by pressurized nebulizers (Acorn, MedicAid). Aerosol was introduced through a valve mechanism in the plethysmograph,

enabling aerosol delivery and measurement of sGaw to alternate. The animals were exposed to increasing concentrations of carbachol until a marked reduction in sGaw was noted. Following the last dose of carbachol (usually 1.6 or 3.2 mM), sGaw had decreased by $70 \pm 6\%$. The animals were lavaged as described above 24 h after carbachol provocation.

Analysis of ET-LI

The BAL samples were concentrated and desalted on SepPak[®] cartridges (Waters Chromatography Division, Millipore Corporation, Milford, MA, USA). A volume of 0.3 ml concentrated acetic acid (17.5 M) was added per 10 ml lavage fluid. The SepPak cartridges were then pretreated with 5 ml 4% acetic acid in 86% ethanol, 5 ml methanol, 5 ml distilled water, and finally 4% acetic acid. The acidified sample was then applied to the cartridge. After washing twice with 3 ml distilled water, the ET-LI was eluted with 3 ml 4% acetic acid in 86% ethanol. Before RIA, the samples were evaporated under nitrogen. This procedure gave a recovery of about 80% for ET-1.

ET-LI was analysed with RIA using antiserum E1, which does not recognize big ET-1 (15), and ^{125}I -labeled ET-1 (Amersham) as tracer. ET-LI results from haemolytic BAL-samples were excluded, since it is known that haemoglobin interferes with the E1 assay (16).

Histological examination

Samples were taken from one control animal and from seven animals exposed to allergen for the second time 24 h earlier. The animals were killed by an overdose of pentobarbital sodium i.p. The middle section of the left lung was fixed in neutral formaldehyde solution for 24 h . After dehydration and embedding in paraffin wax (by a standard technique), the samples were cut into $4\text{-}\mu\text{m}$ -thick sections and stained with haematoxylin (Mayer)–eosin for microscopic examination.

Drugs

The following substances were used: Phosphoramidon, dexamethasone, carbachol (Sigma Chemical Co, St Louis, MO, USA), *A. suum* extract ($40\,000\text{ PNU/ml}$) (Artu Biologicals, The Netherlands), pentobarbital sodium (Nord Vacc, Sweden), and xylocaine HCl (Astra, Sweden).

Data analysis, statistical calculations

All data are given as mean $\pm$ SEM. Statistical significance was calculated using the analysis of variance (ANOVA) test followed by the Fisher protected least significant difference (PLSD) when more than

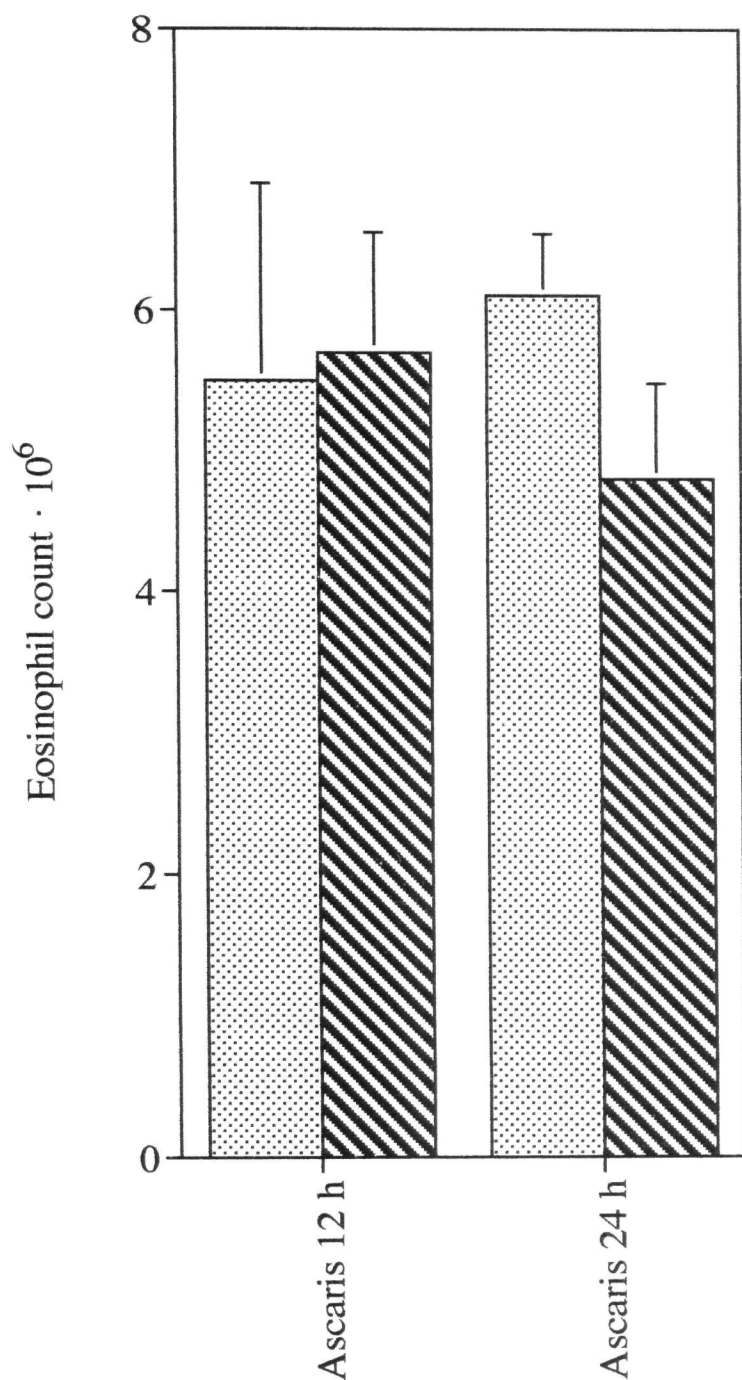


Fig. 2A

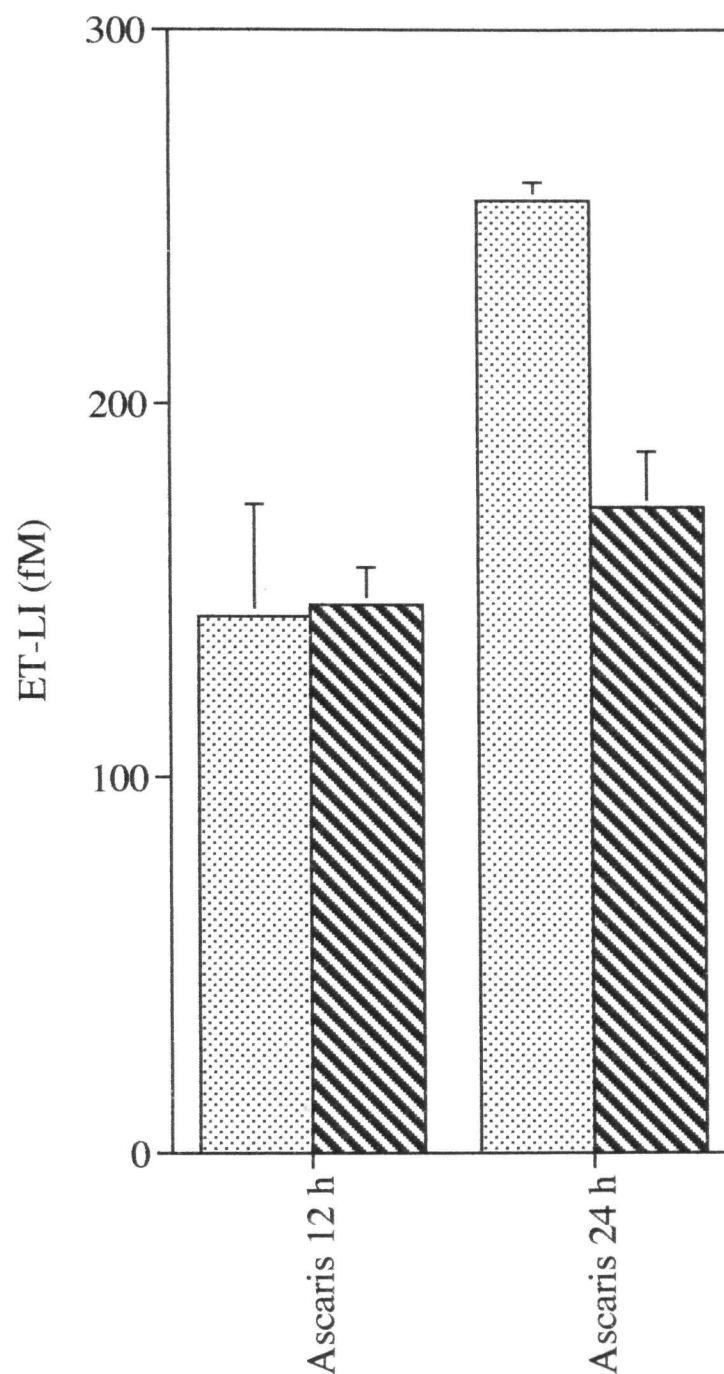


Fig. 2B

two groups were compared. Comparisons between two groups were made using Student's two-tailed *t*-test for unpaired data. All calculations were performed on an Apple computer using StatView 4.0 (Abacus Concepts, Inc, Berkeley, CA, USA) as software. When the effect of GCS treatment was analysed, animals receiving dexamethasone were compared only with litter-mate animals that were challenged with *Ascaris* in the same session. The effect of carbachol was in the same way analysed by comparison with control guinea-pigs and with sensitized, *Ascaris*-challenged animals from the same batch.

Results

Effect of sensitization and Ascaris aerosol

When BAL was performed 12 h after the second *Ascaris* challenge, the number of eosinophils in BALF was markedly elevated, but there was no clear change

in any of the other parameters studied (Fig. 1A). At 24 h after provocation, the mean eosinophil count was further increased, and there was also a marked rise in ET-LI concentration and neutrophil count (Figs. 1B and C). One week after *Ascaris*, neither the ET-LI concentration nor the content of neutrophils differed from the values in control animals (Figs. 1B and C). The number of eosinophils, on the other hand, remained elevated at this point of time (Fig. 1A). The content of macrophages and lymphocytes was not affected by the allergen challenge (Figs. 1D and E). When the ET-LI concentration in BALF and the number of eosinophils were compared in all nonpretreated animals that had been challenged with *Ascaris* and lavaged 12 or 24 h later, and in all control animals, a moderate correlation was found (corr. coeff. = 0.556; *P* = 0.004; *n* = 24), but there was no statistically significant correlation between the number of neutrophils and ET-LI levels. Also when animals pretreated with dexamethasone were included

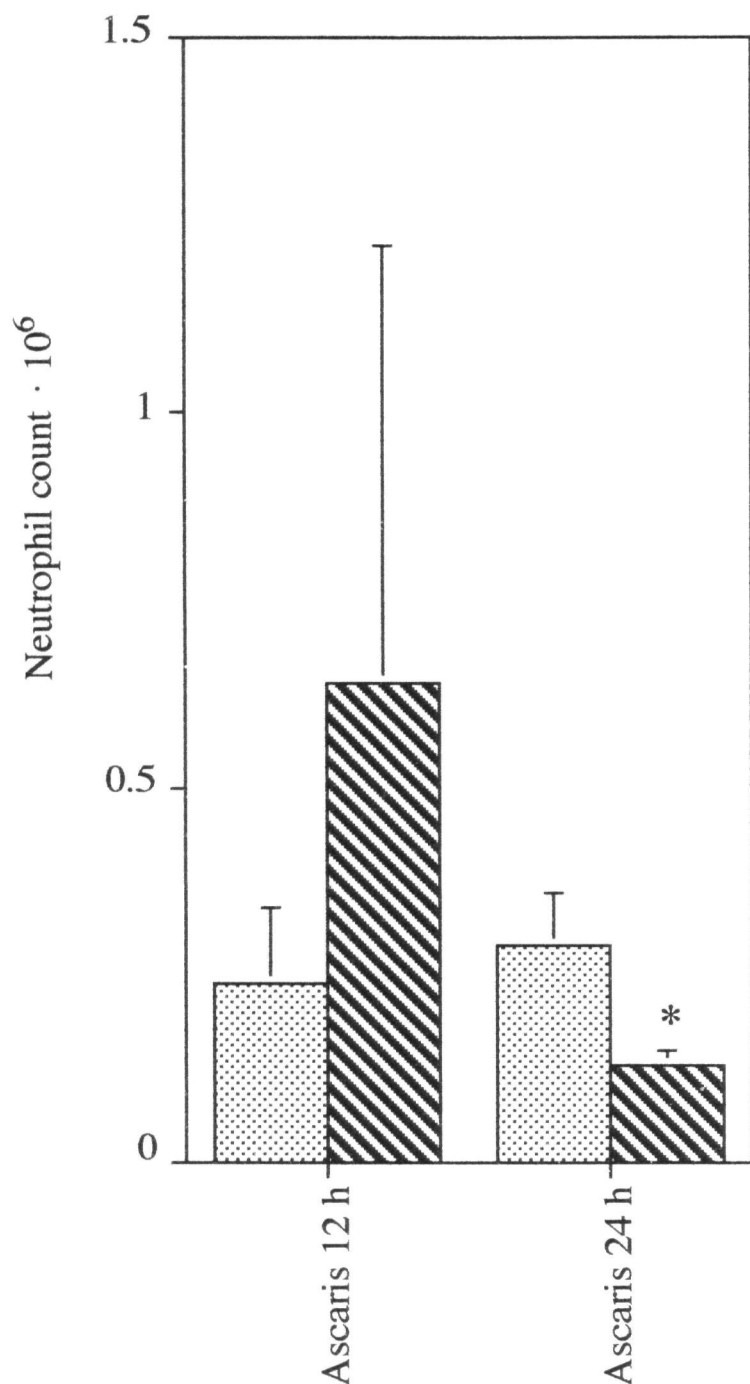


Fig. 2C

Fig. 2. Effect of pretreatment with dexamethasone on levels of eosinophils (A), ET-LI concentration (B), and neutrophils (C), measured 12 and 24 h after provocation with *Ascaris* aerosol without (shaded bars) or with pretreatment with dexamethasone (striped bars). Dexamethasone was given in doses $25 \text{ mg}^{-1} \text{ kg}^{-1} \text{ day}^{-1}$ for 1 week (12 h) or $17 \text{ mg}^{-1} \text{ kg}^{-1} \text{ day}^{-1}$ for 3 days (24 h). Leucocytes 12 h: *Ascaris*, $n = 4$; *Ascaris* plus dexamethasone, $n = 6$; 24 h: *Ascaris*, $n = 7$; *Ascaris* plus dexamethasone, $n = 8$. ET-LI 12 h: *Ascaris*, $n = 3$; *Ascaris* plus dexamethasone, $n = 5$; 24 h: *Ascaris*, $n = 4$; *Ascaris* plus dexamethasone, $n = 6$. * $P < 0.05$.

in the analysis, there was a significant correlation between eosinophil count and ET-LI concentration (corr. coeff. = 0.528; $P = 0.0009$; $n = 35$).

The sensitization and/or the provocations had a retarding effect on the growth of the animals. In the first session, on the day of the second provocation,

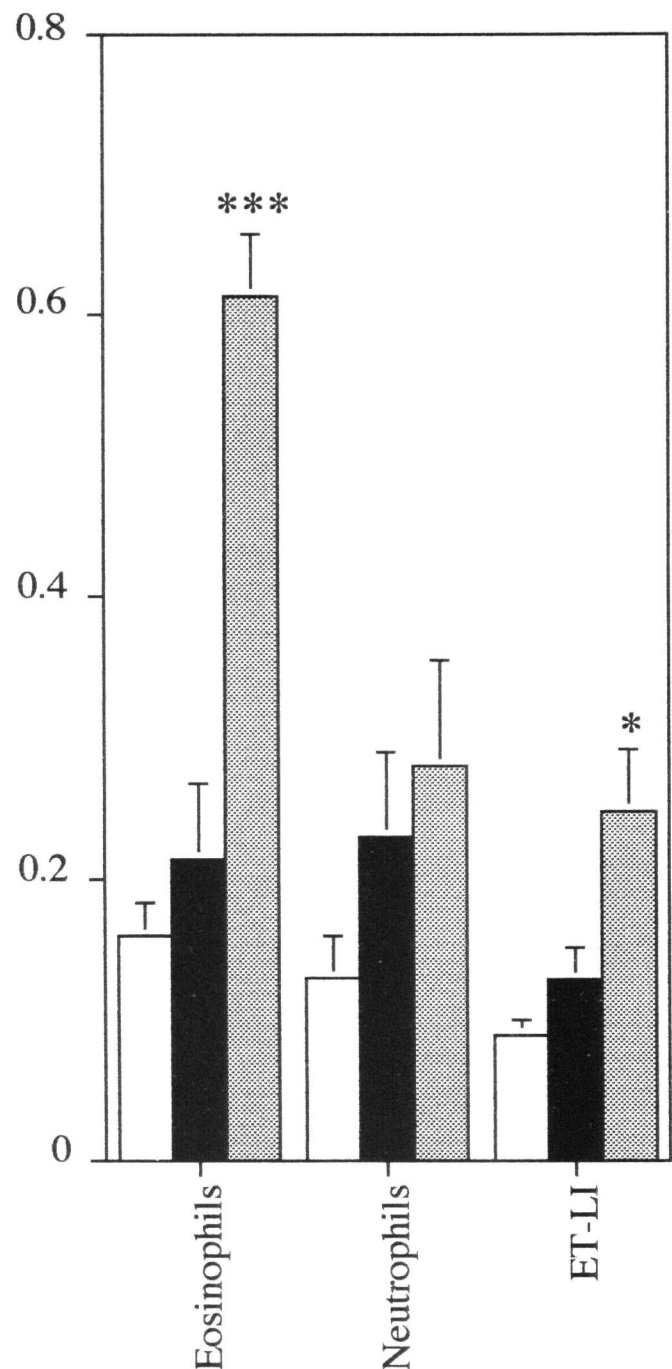


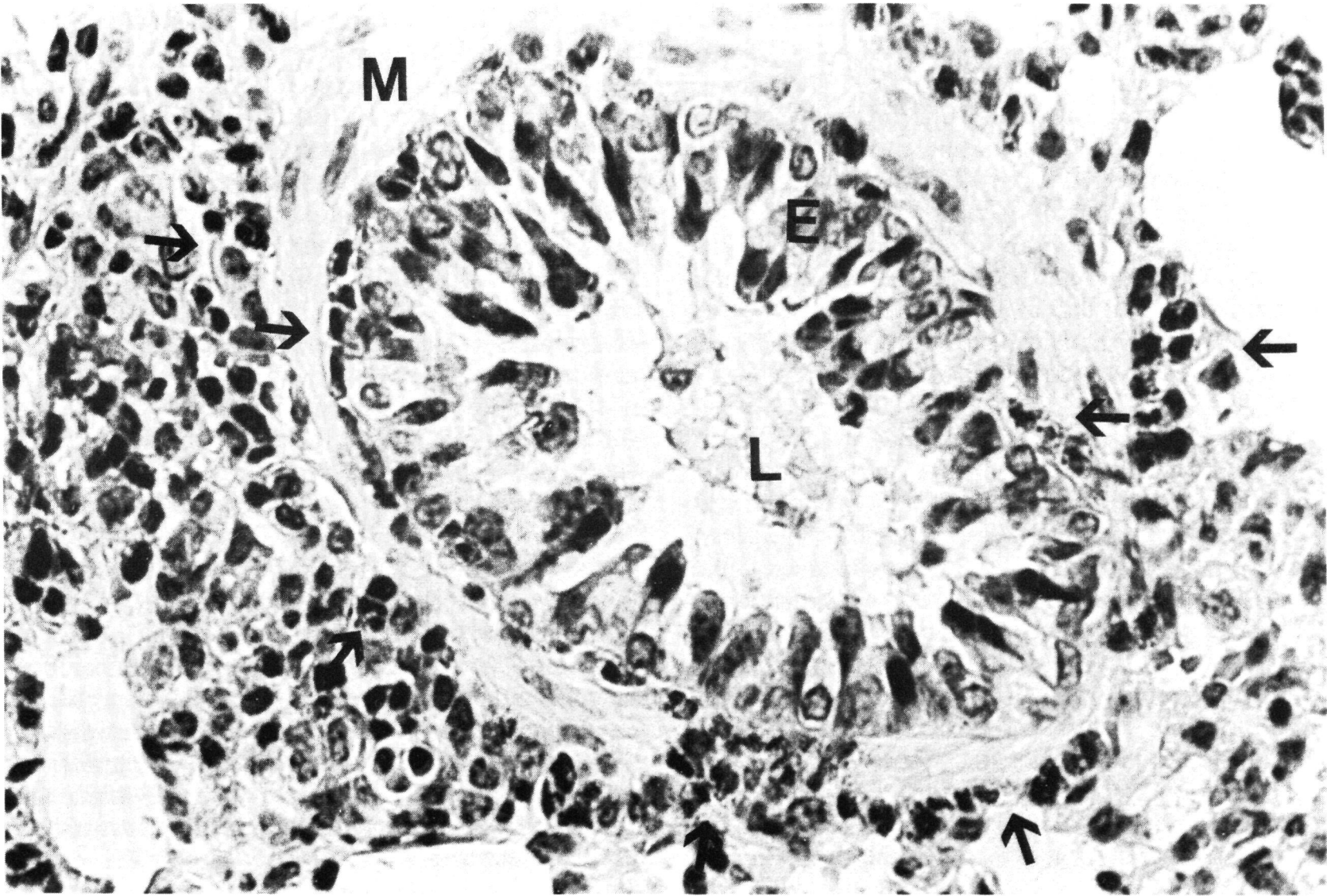
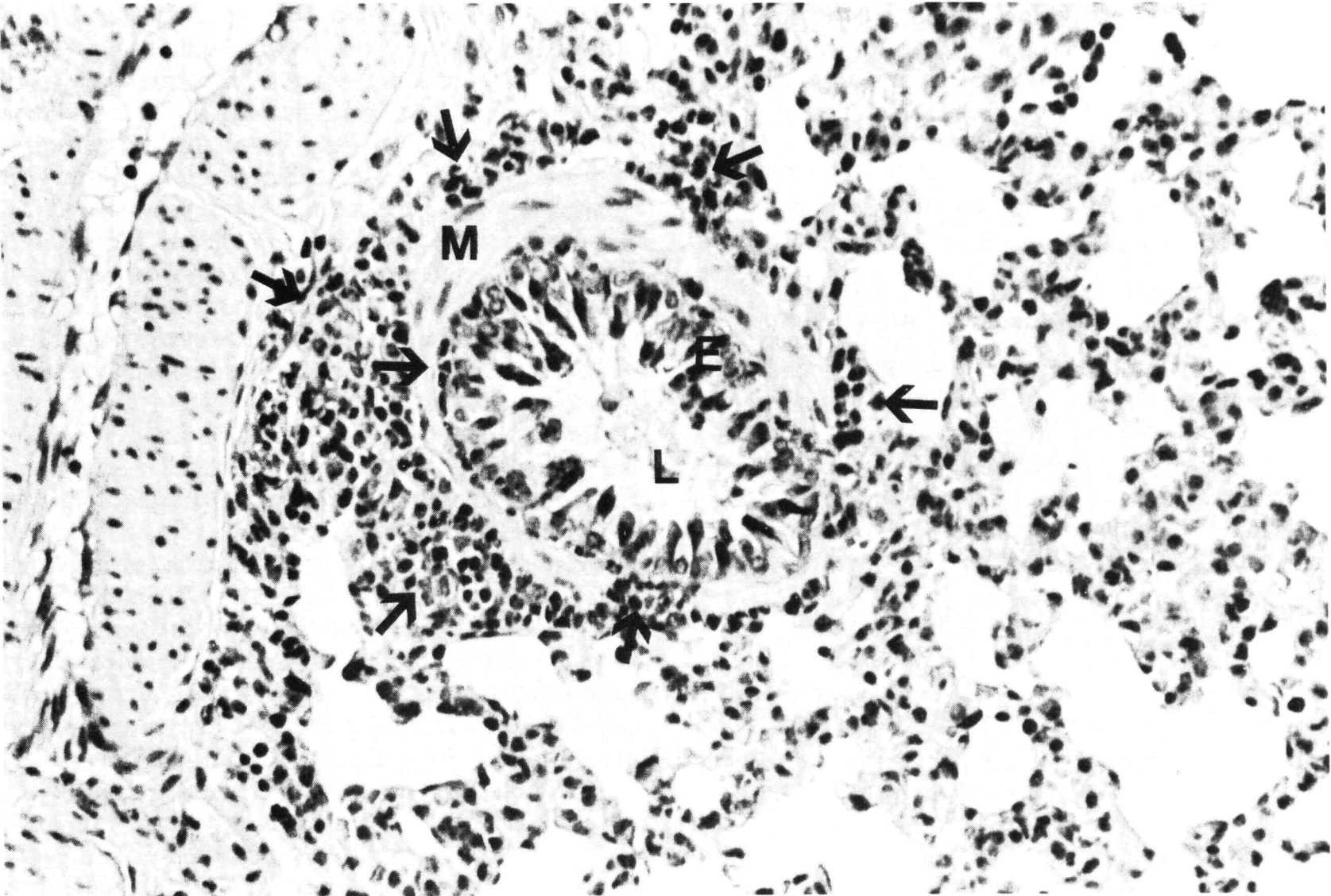
Fig. 3. Levels of eosinophils (10^5), neutrophils (10^6), and ET-LI concentration (pM) in BALF in control animals (open bars) and in animals provoked 24 h earlier with carbachol (filled bars) or with *Ascaris* aerosol (shaded bars). Leucocytes: control animals, $n = 8$; carbachol, $n = 8$; and *Ascaris*, $n = 7$. ET-LI: control animals, $n = 8$; carbachol, $n = 6$; and *Ascaris*, $n = 4$. * $P < 0.05$; *** $P < 0.001$ compared to carbachol-provoked animals.

the sensitized group weighed $491 \pm 18 \text{ g}$, whereas the unsensitized, unprovoked, litter-mate controls weighed $647 \pm 22 \text{ g}$; $P < 0.0001$).

Effect of dexamethasone pretreatment

Pretreatment for 1 week with dexamethasone ($25 \text{ mg} \text{ kg}^{-1} \text{ day}^{-1}$) had no effect on the parameters studied in animals lavaged 12 h after *Ascaris* provocation. At 24 h after challenge, a slightly lower dose of

Fig. 4. Section of left lung in animals challenged twice. Remarkable eosinophil infiltration (arrows) is concentrated around airway, and there are signs of hypertrophied bronchial smooth muscle. (L = bronchial lumen; E = epithelium; M = bronchial smooth muscle.) Haematoxylin-eosin stain: A) $\times 100$; B) $\times 200$.



the steroid given for 3 days attenuated the neutrophil count. The ET-LI level and the number of eosinophils were, however, not significantly affected (Fig. 2A–C). The ET-LI level was still elevated when compared with control animals from the same batch (172 ± 15 fM, $n = 6$ vs 89 ± 11 fM, $n = 8$; $P = 0.0006$). GCS treatment did not significantly affect body weight in the animals (data not shown).

Effect of carbachol provocation

In animals subjected to carbachol-induced bronchoconstriction, the ET-LI concentration was not statistically different from that of the control animals, nor did the levels of inflammatory cells vary significantly. However, the neutrophil count did not differ significantly between carbachol- and *Ascaris*-provoked animals (Fig. 3).

Histological examination

Twenty-four hours after a repeated challenge, there was a massive eosinophil infiltration concentrated at the bronchial wall, and there were intraepithelial eosinophils. There were also signs of an increase in the number of goblet cells and hypertrophy of the bronchial smooth muscle (Fig. 4).

Discussion

The present data show that provocation with *Ascaris* aerosol of sensitized animals induces an influx of eosinophilic leucocytes both peribronchially and into the airway lumen. The rise in eosinophil level in BALF was marked already 12 h after challenge, and, at that time, was the only parameter affected by the provocation. The number of eosinophils remained elevated even 1 week after exposure, possibly indicating an ongoing inflammatory reaction. These results are in agreement with previous findings in guinea-pigs challenged with antigen inhalation (4, 7, 28).

The concentration of ET-LI in BALF was increased 24 h after challenge, as is shown not only by the results from the animals that were provoked with *Ascaris* without pretreatment, but also by the elevation in those pretreated with dexamethasone. There was no clear effect on the ET-LI concentration at 12 h after provocation. In the acute phase, the eosinophil content in the BAL fluid and the ET-LI concentration were moderately correlated, however. This could indicate that the ET-LI rise was secondary to the eosinophil influx or to some factor affecting both parameters.

The elevation in ET-LI levels seemed relatively transient, as it returned to baseline within 1 week after the provocation, in contrast to the eosinophil

count, which remained elevated; hence, it can be concluded that it is the allergen challenge, not the sensitization process, that induces the rise in ET concentration. The time-course of the changes shows a striking similarity to what is seen following Sephadex instillation in the rat, where the lung ET-LI content peaks 24 h after provocation but returns to pre-exposure values within a week (3, unpublished research), despite an ongoing inflammatory reaction. Thus, it could be that, in general, the ET-LI levels are elevated during the first days of a hypersensitivity reaction in the airways, but, without repeated provocation, the release is not sustained.

Increased levels of ET-LI can be caused either by an increased production or a reduced degradation. In bronchial epithelial cells from asthmatic patients, the ET mRNA levels are reported to be increased (27), possibly indicating that there is an increased transcription from the ET gene during airway inflammation which could explain the present findings. However, in the lung, ET is reported to be degraded to a large extent by a neutral endopeptidase (EC 3.4.24.11) (29). Since this enzyme is reported to be downregulated during airway inflammation (21), the possibility of a reduced degradation also exists.

In a previous work, we found that the ET-LI concentration did rise more than 10-fold in BAL from Sephadex-treated rats (2), a rise which could be compared with the twofold elevation seen in the present guinea-pig experiments. In the present experiments, the morphological examination indicates that the inflammatory process is mainly localized peribronchially and in the airway epithelium; this contrasts with the Sephadex model, where there is a more widespread interstitial inflammation (5), which, in turn, could induce a much larger release of ET-LI in the lung. Accordingly, the ET-LI levels in BALF are more elevated in patients with interstitial lung disease than in patients with bronchial asthma (24).

The number of neutrophils was also increased at 24 but not at 12 h after allergen provocation, but the level was much lower than that of eosinophils at both points of time. One week after challenge, the neutrophil count was not different from that in controls, indicating both that the response was of a transient nature and that it was induced by the *Ascaris* exposure, and not by the sensitization process itself. In other studies, marked changes in neutrophil counts have been reported during the first hours after allergen challenge in guinea-pigs; in ovalbumin-sensitized animals, neutrophil infiltration peaked at 6 h post-challenge (4), and Yamada et al. (28) found a prominent increase in neutrophils in BALF measured 4 h after provocation. An early influx of neutrophils thus seems likely although the

changes did not reach statistical significance in the present study, possibly due to the relatively small number of animals tested.

Following carbachol-induced bronchoconstriction, there was no statistically significant increase in any of the parameters studied. A comparison of the effect of carbachol with that of *Ascaris* indicates that the rise in eosinophil and ET-LI levels following allergen challenge was not secondary to the bronchoconstriction *per se*. For neutrophils, the cell count in BALF did not differ markedly between animals exposed to carbachol or allergen; thus, part of the rise in neutrophil count may be an unspecific response with regard to the allergen-induced inflammation. This conclusion is also in line with previous findings in guinea-pigs sensitized to ovalbumin (7).

Pretreatment with dexamethasone did not consistently affect the parameters measured. This is in agreement with results from ovalbumin-sensitized guinea-pigs, where 5 mg kg⁻¹ of budesonide given locally did not significantly reduce the cell content in BALF 20 h after allergen provocation (27). It is still possible, however, that GCSs can modulate the time-course of the cellular infiltration; in ovalbumin-sensitized animals, dexamethasone (25 mg kg⁻¹ day⁻¹) is reported to reduce eosinophil levels in BALF 1 week after challenge. We did not test this possibility in the present study since ET-LI levels at that time had returned to control values. More clear-cut results on the effect of GCS on ET-LI levels are reported from other studies: in the rat lung, both the Sephadex-induced inflammation and the associated increase in ET-LI levels were reduced by budesonide (3), and also in humans the increased ET-LI levels in BALF from asthmatics are reported to be reduced by oral prednisone in addition to bronchodilators (19). Since these drugs in general do not seem to affect the basal levels of ET (26), it could be that the attenuated rise in ET-levels is secondary to inhibition of factors which are released in an inflamed tissue and, in turn, act to increase the ET-LI production (27). These data are in line with the concept that ET-1 is released in the early phase of the airway reaction to allergen and that this release is correlated to the infiltration of eosinophils. The physiological role of ET-1 remains to be established, however.

In conclusion, exposure of sensitized guinea-pigs to *A. suum* aerosol leads to an increase of ET-LI concentration and of the number of eosinophilic and neutrophilic leucocytes in BALF. One week after provocation, the ET-LI value and neutrophil count had returned to pre-exposure values, in contrast to the eosinophil levels, which remained elevated. The present model could be useful for the study of the role of ET-1 in allergic airway inflammation.

Acknowledgments

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