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Adrenalectomy permits a late, local TNF-α release in LPS-challenged rat airways

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Adrenalectomy permits a late, local TNF-α release in LPS-challenged rat airways. A. Miller-Larsson, A. Runström, R. Brattsand. ©ERS Journals Ltd 1999.

ABSTRACT: The normal rise of circulating endogenous glucocorticosteroids (GCS), in response to immunological stimuli, can be impaired in patients with inflammatory diseases. The aim of this study was to investigate whether abolition of the endogenous GCS response promotes local production of the pro-inflammatory cytokine, tumour necrosis factor (TNF)-α, in challenged airways and affects the cellular response in rats.

In adrenalectomized or sham operated rats, the trachea and main bronchi were lavaged at various times after intratracheal instillation of low dose lipopolysaccharide (LPS). TNF-α in lavage fluid and plasma corticosterone were measured, and cells were differentiated.

In adrenalectomized rats, LPS-induced in the airways a biphasic TNF-α release peaking at 2 and 6 h, whereas in sham operated rats the second peak was absent; probably inhibited by the strong rise of plasma corticosterone. The second peak was abolished in adrenalectomized rats by pretreatment with exogenous GCS. The LPS-induced neutrophil influx and a decrease in mononuclear cells were prolonged in adrenalectomized rats.

In conclusion, abolition of the endogenous glucocorticosteroid response promotes the late release of tumour necrosis factor-α in the airways and prolongs the cellular response. This suggests that a normal rise of endogenous glucocorticosteroid after an immunological trigger contributes to a dampening of the late inflammatory activity.
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The normal physiological response to an acute inflammation with systemic involvement, is an increased secretion of endogenous glucocorticosteroids (GCS) into the blood. In turn, the increased level of GCS in blood dampens the inflammatory process. This negative feedback mechanism is probably essential for the normal control of inflammation, preventing it from overshooting and threatening homeostasis [1].

It is possible that this ability to increase the blood concentration of endogenous GCS is impaired in inflammatory diseases. MOHIUDDIN and MARTIN [2] found that the late allergic reaction after bronchial challenge had a shorter latency time and was more severe when the challenge was performed in the evening (at low cortisol level) than in the morning. It was also found that the development of the late reaction after allergen challenge in asthmatics was associated with a low rise of plasma cortisol, and that high plasma cortisol levels at challenge shortened the immediate reaction [3]. It was also shown that immunoglobulin (Ig)E-dependent late phase allergic inflammation in the skin was dampened by high physiological levels of plasma cortisol [4]. KRAFT *et al*. [5] demonstrated a reduced affinity of GCS receptors on T-lymphocytes in nocturnal asthmatics at the time of their symptoms. GUDBJÖRNSSON *et al*. [6] showed an impaired secretion of cortisol in rheumatoid arthritis patients. These results suggest that the level of circulating endogenous GCS plays an essential role in modulation of the inflammatory process. As the key component of the anti-inflammatory action of GCS may be the inhibition of pro-inflammatory cytokines [7], this may suggest that high physiological GCS levels can dampen production of cytokines and hence their cellular effects.

In the present study, the hypothesis that an elimination of the endogenous GCS feedback response to inflammatory stimuli promotes airway inflammation was addressed. Thus, the local airway inflammation induced by intratracheal (*i.t.*) instillation of lipopolysaccharide (LPS) was compared in adrenalectomized and sham operated rats. LPS was selected as the stimulus because it proved in pilot tests to be an effective inducer of tumour necrosis factor (TNF)-α production in the airways. The concentration of TNF-α and the number of inflammatory cells were determined in tracheobronchial lavage performed *ex vivo* at various times after LPS administration. TNF-α is regarded as a central initiator of inflammatory mechanisms, influencing cell traffic and mediating acute and chronic inflammation. TNF-α contributes to the pathophysiology of inflammatory airway diseases such as asthma [8–10] and chronic obstructive pulmonary disease [11, 12]. Airborne LPS, in house and urban dust for example, may contribute to exacerbations of these diseases [13–18].

Materials and methods

Animals

Male Sprague-Dawley rats (175 g) were supplied by Möllegaard Breeding Centre Ltd. (Skensved, Denmark). Rats were housed, six per standard wire-topped cage in rooms with a 12 h artificial light (06:00–18:00 h): 12 h dark cycle, at 20–22°C and 50–60% humidity, with food and water freely available. Seven days before lavage, rats were adrenalectomized or were sham operated under inhaled enflurane (Efrane®; Abbot, Campoverde LT, Italy) delivered *via* vaporizer together with N_2O/O_2. Adrenalectomy was verified at necropsy and by measurement of plasma corticosterone. Adrenalectomized rats were maintained with 0.9% saline as the drinking water. On the day of experiment, the rats weighed 240–280 g. The experimental protocol was approved by the Malmö/Lund ethical committee on animal experiments.

Study design

Adrenalectomized and sham operated rats were instilled *i.t.* either with 100 μg LPS in 50 μL of normal saline (0.9%), or with saline vehicle alone. At 1–48 h after LPS or saline, blood was collected by heart puncture (right ventricle) in anaesthetized animals (intraperitoneal injection of 1.5 mL sodium pentobarbital 60 mg·mL^{-1}, Apoteksbolaget, Umeå, Sweden), and tracheas and extrapulmonary main stem bronchi were immediately lavaged *ex vivo* with phosphate-buffered saline (PBS). Blood serum, blood plasma (heparin) and supernatant of lavage fluid were collected and stored at -70°C for later determination of concentrations of TNF-α and corticosterone (plasma). Cells in resuspended lavage pellets were counted and identified on cytospin preparations.

In the second series, the experiments were repeated with some modifications in neutrophil depleted adrenalectomized rats (see *Neutrophil depletion*), to elucidate the role of neutrophils for the production of TNF-α in the airways.

In the third series of experiments, adrenalectomized rats were injected subcutaneously with the synthetic GCS budesonide (Astra Draco AB, Lund, Sweden) at 1 mg·kg body weight^{-1}, 16 h before LPS *i.t.* instillation, and tracheal lavage was performed 6 h after LPS instillation.

Intratracheal instillation

Rats were lightly anaesthetized for 5 min with inhaled enflurane delivered *via* a vaporizer together with N_2O/O_2 and were placed in the supine position on a board tilted at 30° with head uppermost. LPS (BE Coli 026: B6; Difco Laboratories, Detroit, MI, USA) or saline were instilled *i.t.* through a modified metal cannula (1 mm in diameter) slightly bent at the tip. After instillation, rats were kept on the tilted board until they regained consciousness (~30 s).

Tracheobronchial lavage

Tracheobronchial lavage was performed according to a technique modified from that of Erjefält and Persson [19]. Briefly, after heart puncture of the anaesthetized rat (intraperitoneal injection of 1.5 mL sodium pentobarbital 60 mg·mL^{-1}), the trachea and lungs were taken out *in toto* (the larynx was tied off before excision). After a brief rinse in ice cold saline, the lungs with trachea were placed on a Petri dish kept on ice. Each lobar bronchus was tied off, and a small incision was made directly under the larynx. A plastic tube (PE 50), mounted on a cannula, was gently introduced through a small incision in the left bronchus and secured with a ligature. A syringe filled with 175 μL PBS at room temperature was attached to the cannula, and the trachea and main stem bronchi were carefully lavaged 5-times. Lavage fluid and blood were centrifuged at 4°C for 10 min at $1{,}000 \times g$ (lavage fluid) or $2{,}000 \times g$ (blood).

Tumour necrosis factor-α assay

The TNF-α concentration in lavage fluid and serum was determined by enzyme-linked immunosorbent assay (ELISA) (Cytoscreen^{TM} rat TNF-α kit; BioSource International, Camarillo, CA, USA). Supernatants of the lavage fluid and serum samples were diluted 2-fold (or more if needed) in the dilution buffer provided and assayed in duplicate. The lower limit of detection was 15.6 pg·mL^{-1}. Precision, estimated by coefficient of variation (%CV), was 2.6–2.7% for intra-assay and 3.5–4.3% for interassay variation.

Corticosterone assay

The plasma concentration of corticosterone was measured by radioimmunoassay (RIA) (Rat corticosterone ^{125}I RIA assay system; Amersham International, Amersham, UK). Plasma samples were diluted in the dilution buffer provided: 10-fold for sham operated rats and 5-fold for adrenalectomized rats (the assay requires at least 5-fold dilution of plasma). The lower limit of detection was 0.78 ng·mL^{-1} in diluted samples, *i.e.* 7.8 and 3.9 ng·mL^{-1} in undiluted plasma for sham operated and adrenalectomized rats, respectively. Precision (%CV) for intra-assay was 5% and 4.0–5.9% for interassay variation.

Cell analysis

Cells in the lavage pellet were resuspended in 100 μL sterile cell culture media (RPMI 1640 with 10% foetal calf serum, both from Gibco BRL, Paisley, UK). The total number of cells in the pellets were counted in a Bürker chamber. Differential counting (on 400 cells) was performed on cytospin slides (5×10^4 cells·slide^{-1}) after standard May-Grünwald–Giemsa staining (Sigma Diagnostics, St. Louis, MO, USA). Neutrophils, eosinophils, mononuclear cell fraction (including monocytes/macrophages and mast cells), and lymphocytes separately were differentiated.

Neutrophil depletion

Adrenalectomized rats were rendered neutropenic by *i.v.* administration of rabbit polyclonal anti-rat neutrophil antiserum (AIAD51140, batch G2937; Accurate Chemical and

Scientific Corp., Westbury, NY, USA). Anti-serum was diluted 10-fold in sterile PBS and injected into the tail vein in a dose of 1 mL·200 g body weight^{-1}. The injection was repeated after 7–8 h. Control rats were injected with 10-fold diluted normal rabbit serum (Serotec, Oxford, UK). LPS (100 μg in 50 μL) was instilled *i.t.* ~24 h after the first injection of anti-serum or normal serum. The TNF-α concentration and cell numbers were analysed in lavage fluid collected 6 h after LPS instillation as described above. To control the degree of neutropenia, white blood cells in blood samples (ethylene diaminetetraacetic acid) were counted in an automated haematology analyser (Sysmex K 4500 Vet; TOA Medical Electronics Co., Japan) and differentiated (200 cells) on blood smears stained with standard May-Grünwald–Giemsa staining (Sigma Diagnostics).

Statistical analysis

The results are presented as arithmetic means±SEM. The TNF-α concentration in lavage fluid relates to the 2-fold diluted samples. The TNF-α concentration in serum refers to values in undiluted serum, and plasma corticosterone concentration refers to undiluted plasma. Statistical comparisons were performed with a Student's t-test for independent samples with pooled variance and degrees of freedom calculated by one-way analysis of variance (ANOVA). For the analysis of the number of mononuclear cells, a Student's t-test was also performed on rank transformed data because of the presence of outlying points; this procedure is equivalent to the Wilcoxon–Mann–Whitney test with approximate p-values [20]. Differences were considered significant at $p<0.05$.

Results

Effect of adrenalectomy and intratracheal lipopolysaccharide on plasma corticosterone level

In untreated sham operated rats (*i.e.* not exposed to *i.t.* instillation of LPS or saline), the plasma corticosterone concentration was 102±36 ng·mL^{-1}. As shown in figure 1, plasma corticosterone started to increase more than 2 h after LPS administration and reached a maximum at 4 h after LPS instillation. The peak value at 4 h was 4-fold higher than the corresponding value measured after saline instillation ($p<0.01$). At 6–15 h after LPS, plasma corticosterone was still ~1.5–2-fold higher as compared to untreated and saline-treated rats, but the differences were not statistically significant (data not shown; 24 and 48 h were not tested). Plasma corticosterone after saline instillation was not significantly different at any time point from untreated rats (fig. 1).

In 23 of 28 adrenalectomized rats (untreated or LPS-treated), the corticosterone concentration was below the lower limit of detection (3.9 ng·mL^{-1}). In the remaining five adrenalectomized rats, plasma corticosterone was in the range 12–21 ng·mL^{-1}. Plasma corticosterone in adrenalectomized rats was not increased after LPS *i.t.* instillation (data not shown).

Tumour necrosis factor-α release

Pilot experiments, where tracheobronchial lavage was performed in normal rats 2 h after LPS administration, showed that increasing doses of *i.t.* LPS induced proportional increases in TNF-α release into the airways (5–100 μg tested, data not shown). In untreated rats or rats instilled with saline, the TNF-α concentration in lavage fluid was under or just above the lower limit of detection for the assay (15.6 pg·mL^{-1}), with no difference between sham operated or adrenalectomized rats. No TNF-α was detected in serum in normal, sham operated and adrenalectomized rats after *i.t.* instillation of LPS. In contrast, after intraperitoneal administration of LPS (100 μg in 0.5 mL) the serum concentration of TNF-α was very high (up to 1.5×10^5 pg·mL^{-1} at 2 h after LPS administration; data not shown) whereas no TNF-α was detected in lavage fluid (collected at 2 and 6 h after LPS). This supports the proposition that TNF-α released into the airways after *i.t.* LPS represents local airway production.

Intratracheal instillation of LPS at a dose of 100 μg induced, in the airways of adrenalectomized rats, a biphasic TNF-α release peaking at 2 h and 6 h, and resolving 24–48 h after LPS administration (fig. 2). In sham operated rats, only the first TNF-α peak (at 2 h) was observed, but TNF-α levels remained somewhat increased and returned to basal values first at 24–48 h (fig. 2). The first TNF-α peak did not differ significantly between adrenalectomized and sham operated rats (283±64 and 338±131 pg·mL^{-1}, respectively; $p>0.5$), whereas the second peak in adrenalectomized rats (291±74 pg·mL^{-1}) was 3-fold higher ($p<0.05$) than the TNF-α levels measured at 6 or 8 h in sham operated rats (95.9±19.1 and 103±25 pg·mL^{-1}, respectively). For both sham operated and adrenalectomized rats, the first TNF-α peak differed significantly from the basal value in untreated rats ($p<0.001$ for sham operated rats, and $p<0.01$ for adrenalectomized rats), and from the value at 4 h ($p<0.01$ for sham operated rats, and $p<0.05$ for adrenalectomized rats). In adrenalectomized rats, the second peak also differed significantly from the basal value ($p<0.01$), and the value at 4 h

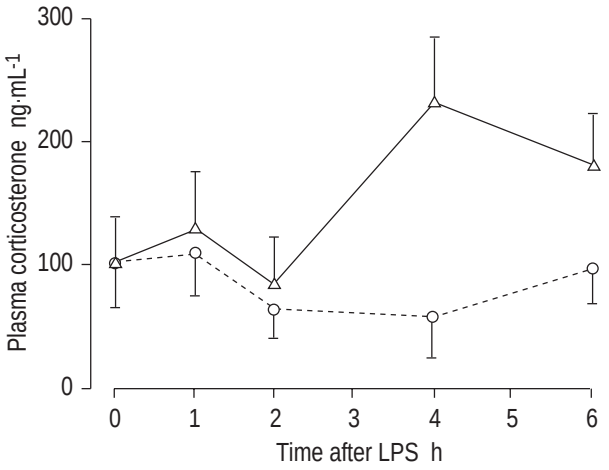

Fig. 1. – Plasma corticosterone (ng·mL^{-1}) in sham operated rats at various times after *i.t.* instillation of lipopolysaccharide (LPS; △) or saline (○). Values at 0 h denote values from untreated rats. Data are mean±SEM (n=6–10). At 4 h after LPS instillation, plasma corticosterone is significantly higher than after saline instillation ($p<0.01$).

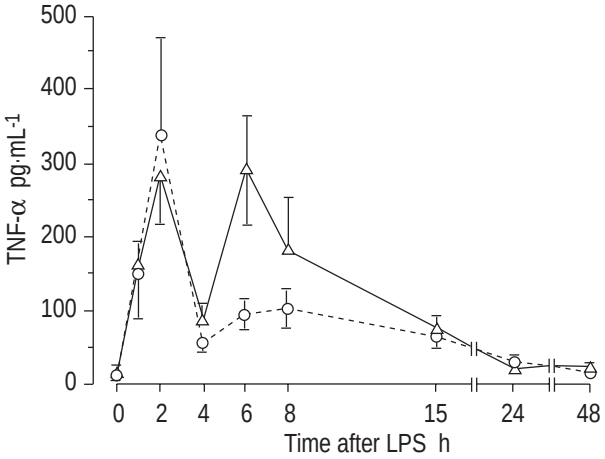

Fig. 2. – Concentration of tumour necrosis factor (TNF)-α in lavage fluid (pg·mL^{-1}) at various times after *i.t.* instillation of lipopolysaccharide (LPS) in adrenalectomized (△) and sham operated (○) rats. Values at 0 h denote values from untreated rats. There is a statistically significant difference between adrenalectomized and sham operated rats in the amplitude of the second peak ($p<0.05$ for adrenalectomized rats at 6 h *versus* sham operated rats at 6 or 8 h). Data are mean±SEM (n=4).

($p<0.02$). In contrast, in sham operated rats, TNF-α levels measured at 6–8 h were not significantly different from the value at 4 h or from the basal levels measured in untreated or saline-treated rats.

Cell influx

In untreated rats, the absolute number of cells in the lavage fluid was $3.52\pm2.14\times10^4$ in sham operated rats and $3.70\pm1.46\times10^4$ in adrenalectomized rats. In untreated rats and saline-treated rats, cells in lavage fluid consisted of 80–90% mononuclear cells, 5–12% neutrophils, only a few lymphocytes, and very occasionally, eosinophils. The total number of cells started to increase 1–2 h after LPS, peaked at 6 h for sham operated rats ($1.32\pm0.15\times10^6$), and at 8 h for adrenalectomized rats ($1.40\pm0.35\times10^6$), and returned to basal levels 48 h after LPS instillation. In both sham operated and adrenalectomized rats, the cell influx induced by LPS consisted of neutrophils, whereas the numbers of other cell types counted were not increased. There was no significant difference between sham operated and adrenalectomized rats in the time course or magnitude of neutrophil influx during the 24 h after LPS administration (fig. 3a). In the 2–15 h period after LPS instillation, neutrophils represented >90% of all cells collected in lavage (fig. 3c).

No influx of mononuclear cells was observed after LPS administration. On the contrary, in the 1–6 h period after LPS administration (during ongoing neutrophil influx), the absolute number of mononuclear cells in the lavage fluid was markedly decreased as compared to untreated rats (fig. 3b). At 2 and 4 h after LPS instillation, this decrease in both sham operated and adrenalectomized rats was statistically significant when analysed with rank-transformed data ($p<0.05$). Although the total cell number returned to nearly basal levels at 48 h after LPS in both sham operated and adrenalectomized rats, neutrophils at that time still comprised >70% of all collected cells in adrenalectomized rats as compared to ~25% in sham operated rats ($p<0.05$; fig. 3c). Thus, the recovery to a proportion

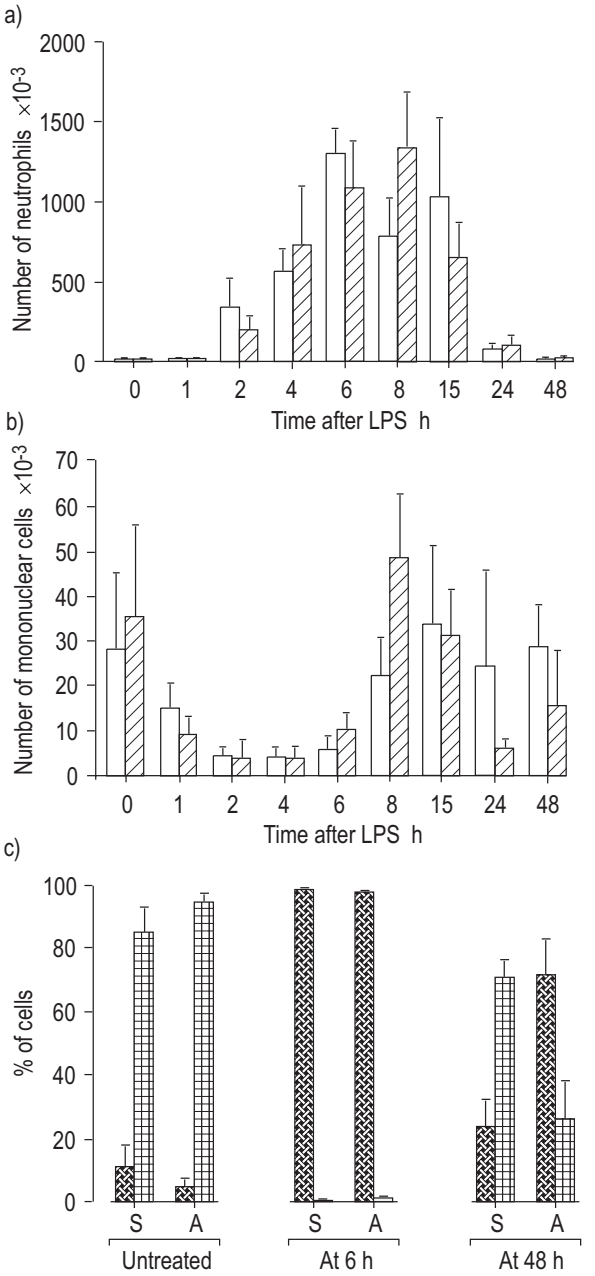

Fig. 3. – Number ($\times10^{-3}$) of neutrophils (a) and mononuclear cells (b) in lavage fluid at various times after lipopolysaccharide (LPS) *i.t.* instillation in adrenalectomized (A; ▨) and sham operated (S; □) rats. In untreated rats (values at 0 h), and 1–24 h after LPS administration, there were no statistically significant differences in the number or percentage of neutrophils or mononuclear cells between adrenalectomized and sham operated rats. At 48 h, the percentage of neutrophils (▩) was significantly higher ($p<0.05$), and the percentage of mononuclear cells (▦) significantly lower ($p<0.05$) in adrenalectomized rats than sham operated rats (c). Data are mean±SEM (n=4).

of 80–90% mononuclear cells in prechallenged rats was delayed in adrenalectomized rats as compared to sham operated rats.

Effect of neutrophil depletion on tumour necrosis factor-α release

Administration of anti-neutrophil anti-serum decreased the number of neutrophils in blood by >95%, and the

number of monocytes by ~80% as compared to rats treated with normal serum ($p<0.001$ for both, data not shown). As a consequence, in anti-serum-treated rats, the number of neutrophils in lavage fluid collected 6 h after LPS decreased by 82% ($p<0.05$; fig. 4a) whereas the 40% decrease of monocytes was not statistically significant ($p>0.2$, data not shown). The significant reduction of neutrophil numbers by 82% did not have any effect on the concentration of TNF-α in the lavage fluid at 6 h after LPS (fig. 4b). Thus, infiltrated neutrophils are not the major determinant of the second TNF-α peak in adrenalectomized rats.

Effect of exogenous glucocorticosteroids

Budesonide administered subcutaneously into adrenalectomized rats at 1 mg·kg of body weight^{-1} 16 h before LPS *i.t.* instillation, decreased the second TNF-α peak by 80% ($p<0.02$; fig. 5) and thereby brought it to the level observed in LPS-challenged sham operated rats. Budesonide pretreatment did not have any significant effect on the number of neutrophils or monocytes/macrophages in lavage fluid (data not shown).

Discussion

This study examined how a normal endogenous GCS feedback response to an inflammatory stimulus modulates the local release of TNF-α in the airways. The selected LPS dose, instilled *i.t.* in 50 μL, resulted in TNF-α release in the airways, but did not raise TNF-α levels in the plasma. The novel finding of this study is that in the airways of adrenalectomized rats, TNF-α is released with a biphasic time pattern, peaking at 2 and 6 h, whereas in sham operated rats only the first peak is observed. Thus, adrenalectomy permits or strongly reinforces the late TNF-α release (fig. 2).

In sham operated rats the level of plasma corticosterone was influenced by the LPS trigger; plasma corticosterone increased 4-fold over control values, and reached a peak at 4 h after LPS *i.t.* instillation (fig. 1). In adrenalectomized rats, LPS instillation had no influence on plasma corticosterone which remained at a level below or slightly above the lower limit of detection for the assay (3.9 ng·mL^{-1}). The corticosterone peak in the sham operated rats preceded by about 2 h the second TNF-α peak in adrenalectomized rats. As the latency time of steroid effects is often 2–3 h and the second peak of TNF-α was not present in sham operated rats, it seems likely that the LPS-induced rise of plasma corticosterone in sham operated rats was responsible for the inhibition of this peak. In contrast, the first TNF-α release was not preceded by a rise of plasma corticosterone and the difference in the basal corticosterone values between sham operated and adrenalectomized rats had obviously no influence on the magnitude of this TNF-α peak.

It can be argued that the induction of the second TNF-α peak in adrenalectomized rats might be attributed not just to removal of the endogenous GCS corticosterone but also to the removal of other adrenomedullary hormones, such as adrenaline. Indeed, it has been shown that TNF-α production can be inhibited *in vitro* by adrenaline and other agents increasing intracellular cyclic adenosine monophosphate (cAMP) levels [21], and *in vivo* by exogenous adrenaline [22]. PETTIPHER *et al.* [23] found in mice that systemic TNF-α production (in serum), induced by high intraperitoneal doses of LPS, was reduced by endogenous adrenaline but not by corticosterone, whereas the local (peritoneal) TNF-α production after a low dose of LPS was not affected by the basal levels of either adrenaline or corticosterone. In the study by PETTIPHER *et al.* [23], only the rapid TNF-α release was investigated (samples were collected 1 h after LPS administration). In the present study, both the early and late local TNF-α release induced by a low, topical dose of LPS were studied. In agreement with PETTIPHER *et al.* [23], basal plasma corticosterone did not affect the first TNF-α peak (no difference between sham operated and adrenalectomized rats; fig. 2). On the other hand, the appearance of the second TNF-α peak in adrenalectomized rats was completely blocked by pretreatment with a synthetic GCS (fig. 5). A subsequent study [24] showed that this second TNF-α peak is more sensitive to GCS action than the first peak, and can be

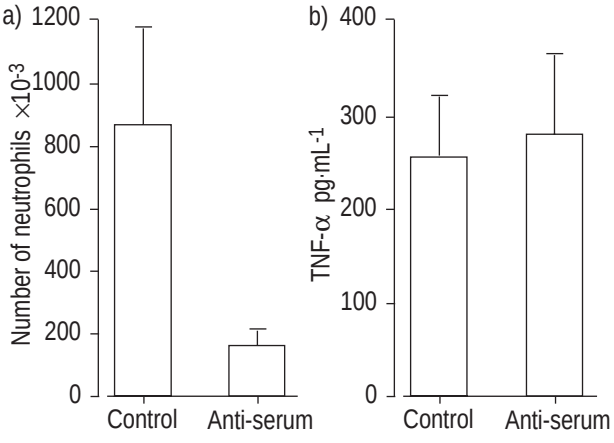

Fig. 4. – Results showing that the significant reduction of neutrophil influx into the trachea (a) had no effect on the magnitude of the second tumour necrosis factor (TNF)-α peak (b) measured 6 h after lipopolysaccharide *i.t.* administration in anti-neutrophil anti-serum treated adrenalectromized rats. Control rats were treated with normal serum. Data are mean±SEM (n=6).

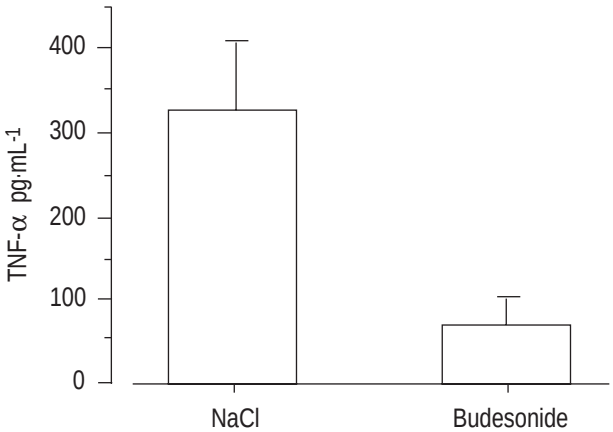

Fig. 5. – Budesonide (1 mg·kg body weight^{-1}) administered subcutaneously 16 h before lipopolysaccharide (LPS) in adrenalectomized rats, inhibited the second tumour necrosis factor (TNF)-α peak (6 h after LPS) by 80% ($p<0.02$) and thereby brought it to the level observed in LPS-challenged sham operated rats. Data are mean±SEM (n=5).

completely abolished even by topical doses of GCS. This suggests that the 4-fold rise of plasma corticosterone in sham operated rats prevents the appearance of the second TNF-α peak seen in adrenalectomized rats. This conclusion is supported by other studies showing that a 2–3-fold rise of endogenous GCS (induced by LPS, or other inflammatory or stress conditions) supresses inflammatory processes [25, 26], including TNF-α production [25]. Whether adrenaline would have some modulatory action on the second TNF-α peak in the present study needs further investigation, but there are data showing that plasma levels of endogenous GCS are more sensitive to LPS than are catecholamine levels; a low LPS dose which was sufficient to induce a 3–4 fold increase of the endogenous GCS plasma concentration, did not enhance adrenaline or noradrenaline levels [27]. Furthermore, other authors report that even the potentiated systemic TNF-α production triggered by high LPS doses is inhibited by exogenous corticosterone or synthetic GCS [28–30].

In both sham operated and adrenalectomized rats, LPS induced the infiltration of neutrophils (fig. 3a) which in adrenalectomized rats coincided with the second TNF-α peak. It was conceivable that the second TNF-α peak was produced in adrenalectomized rats by infiltrated, activated neutrophils, as neutrophils are reported to be a rich source of TNF-α messenger ribonucleic acid (mRNA) and TNF-α protein in LPS-challenged airways and lung. XING and coworkers [31, 32] found that although TNF-α protein and mRNA were localized in alveolar macrophages at 1–3 h after *i.t.* instillation of LPS, they were mainly localized in neutrophils at 6–12 h after LPS. In the present study, however, neutrophils were not responsible for the late TNF-α production in the airways as a >80% reduction of neutrophil influx into the airways (fig. 4a) did not decrease the magnitude of the second TNF-α peak in neutrophil depleted adrenalectomized rats (fig. 4b).

Along with the induction of neutrophil influx, LPS reduced the number of mononuclear cells in the period between 1 h and 6 h (fig. 3b). The reason for this reduction could be the LPS-induced apoptosis of macrophages which has been shown to occur in rodent peritoneal macrophages [33, 34], and human alveolar macrophages [35]. Macrophages present in the bronchi and trachea are mostly of alveolar origin, and are probably translocated into conducting airways by the mucociliary escalator [36]. The temporary impairment of this transport by LPS could be another reason for the observed transient reduction of mononuclear cells. This impairment is caused by increased mucus viscosity and adhesiveness due to the loss of water [37]. Endotoxin and bacteria were shown to increase fluid transport across the alveolar epithelium [38, 39] in part by a TNF-α-dependent mechanism [39].

The biphasic pattern of the mononuclear cell presence (fig. 3b) might explain the biphasic TNF-α release as monocytes and macrophages are known to rapidly express TNF-α mRNA and synthesize TNF-α protein upon stimulation [31, 32, 40–43]. In this case, the potentiation of the second TNF-α peak in adrenalectomized rats would reflect the higher activation of the newly infiltrated macrophages in adrenalectomized rats than in sham operated rats. Besides macrophages, it seems likely that mast cells were the next most important contributors to the early release of TNF-α. Mast cells are unique because, as well as synthesizing TNF-α *de novo*, they contain preformed TNF-α in their granules and are able to release it immediately after an appropriate stimulation [40–42]. ZHANG *et al.* [41] presented evidence that mast cells are critical in the early TNF-α release. Furthermore, not only inflammatory cells, but also bronchial epithelial cells may have contributed to TNF-α release, as epithelial cells in the respiratory tract have been shown to produce TNF-α *in vivo* [44–46].

The two phases of TNF-α release, described in the present model, resemble temporally the early and late inflammatory reaction observed after bronchial challenge with allergen. The immediate allergic reaction is believed to be due to the release of preformed and newly synthesized mediators derived from mast cells *via* stimulation by IgE antibodies. The immediate response is more resistant to exogenous GCS than the late response, and in humans, this has been attributed to an insensitivity of lung mast cells to steroids [47]. This is consistent with the concept that mast cells may have contributed largely to the first TNF-α peak, and explains the high resistance of this peak in the present model to the inhibitory action of endogenous GCS. In a subsequent study [24], it was observed that the difference between the early and late TNF-α peaks in the susceptibility to GCS is also valid for exogenous GCS. This shows that GCS are more potent inhibitors of *de novo* cytokine synthesis in recruited and activated cells (during the late phase) than of the release of preformed cytokines from the resident cells (during the early phase). It is possible that the early TNF-α release makes an essential contribution to the neutrophil influx [41, 48, 49], in which case the lack of inhibition of the first TNF-α peak by endogenous corticosterone in sham operated rats may explain the lack of influence on the neutrophil influx. Alternatively, the neutrophil influx may be initiated mainly by other cytokines/chemokines which share low sensitivity to endogenous GCS with the first phase of TNF-α release.

To the best of the authors′ knowledge, the present study is the first showing the effect of adrenalectomy on the local tumour necrosis factor-α production in the airways. The abolished endogenous glucocorticosteroid feedback response in adrenalectomized rats promoted the lipopolysaccharide-induced late tumour necrosis factor-α release (but not early release) and somewhat prolonged the cellular response. These findings, together with human results on a negative correlation between plasma cortisol level and the extent of the late inflammatory response in the airways, suggest that the normal rise of circulating cortisol is a key factor for dampening the late inflammatory reaction. Further studies are warranted to answer the questions as to what extent this feedback system may be deficient in inflammatory airway diseases, and whether tumour necrosis factor-α is a key mediator during the late as well as the early phase of the inflammatory reaction.

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