Interference of a neutrophil recruitment inhibitory factor upon the accumulation of inflammatory cells and airway hyperreactivity in sensitized guinea-pigs after intranasal antigen challenge

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1 A neutrophil recruitment inhibitory factor (NRIF) recovered from the crude supernatant of lipopolysaccharide (LPS)-stimulated macrophages inhibited neutrophil migration following both intratracheal and intravenous administration of LPS, but did not alter the pattern of leukopenia/leucocytosis induced by intravenous LPS.

2 The correlation between airway infiltration by inflammatory cells and hyperreactivity in lungs from actively sensitized and challenged guinea-pigs was investigated by use of NRIF.

3 Increased eosinophil counts were found in the bronchoalveolar lavage fluid from guinea-pigs sensitized with 10 µg ovalbumin and challenged at day 14 by the intranasal administration of the antigen. The increase was evident 5 h after challenge and persisted at 24 h. Neutrophil numbers were also increased at this time. Pretreatment with NRIF suppressed the leucocyte increase in the bronchoalveolar lavage fluid.

4 Bronchoconstriction and histamine release induced by 3 ng PAF injected into the isolated lungs were increased in challenged guinea-pigs as compared to sensitized but unchallenged controls. Pretreatment of the animals with NRIF did not interfere with this response, but significantly reduced the bronchoconstriction induced by ovalbumin injection.

5 Even though the increased number of inflammatory cells in bronchoalveolar lavage and airway hyperresponsiveness were concomitant, NRIF inhibited cellular infiltration but failed to alter airway hyperreactivity to PAF, demonstrating that these events may occur independently. Conversely, the inhibition of antigen-induced bronchoconstriction by NRIF suggests that this response is dependent upon the emigration of granulocytes.

Keywords: Neutrophil recruitment inhibitory factor; intranasal antigen challenge; cell migration; hyperreactivity; sensitized guinea-pigs

Introduction

Asthma is characterized by a reversible obstruction to airflow and by nonspecific bronchial hyperresponsiveness (Boushey *et al.*, 1980). The mechanisms involved in bronchopulmonary hyperreactivity are not well understood (Boushey *et al.*, 1980), and it has been suggested that inflammation of the bronchial mucosa and submucosa is a key factor for the enhancement of reactivity (Payne & Cheng, 1990). Active sensitization of guinea-pigs followed by a booster injection of antigen leads to *ex vivo* bronchopulmonary hyperresponsiveness to different agonists, including platelet-activating factor (PAF). Such hyperresponsiveness appears to depend on lung infiltration by inflammatory cells, mainly eosinophils (Pretolani *et al.*, 1988).

Leucocytes and particularly neutrophils are implicated in lung injury during shock, as indicated by depletion studies (Worthen *et al.*, 1986; Chang *et al.*, 1987). We reported that the intravenous administration to rats of supernatants from lipopolysaccharide (LPS)-stimulated macrophages inhibited neutrophil migration into the inflamed peritoneal cavity (Cunha *et al.*, 1989). In contrast, the same supernatant did not inhibit mononuclear cell migration to rat peritoneal cavities induced by thioglycollate (Tavares *et al.*, 1989). The substance responsible for this activity, which was recovered from macrophage conditioned medium, was referred to as

neutrophil recruitment inhibitory factor (NRIF) (Tamashiro *et al.*, 1992). Inhibition was not accounted for by the presence of known cytokines such as interleukin-1 (IL-1), IL-8 or tumour necrosis factor- α (TNF- α) (Tamashiro *et al.*, 1992). In addition, the factor was not active against eosinophil recruitment into the rat peritoneal cavity (Oliveira *et al.*, unpublished).

In the present work we first investigated whether intravenous administration of NRIF, obtained from rat macrophages, was able to inhibit the neutrophil emigration to the inflammatory exudate induced by intratracheal or intravenous administration of LPS in the guinea-pig. Because of its effectiveness, we used NRIF as a tool to correlate airway infiltration by inflammatory cells, as assessed by bronchoalveolar lavage, with bronchial hyperreactivity to spasmogens and with histamine and thromboxane B₂ release in lungs from sensitized guinea-pigs after intranasal challenge with antigen. This new model of challenge of actively sensitized guinea-pigs is very convenient, since it requires only 1 day for the expression of hyperresponsiveness.

Methods

Sensitization procedure

Male Hartley guinea-pigs (Elevages Lebau, France, 400–750 g) were actively sensitized at day 0 by a subcutaneous (s.c.)

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injection of 0.5 ml of 0.9% NaCl solution (saline), containing 10 µg of ovalbumin (OA) dispersed in 1 mg Al(OH)₃ (modified from Andersson, 1980). The animals were challenged at day 14 by the intranasal (i.n.) administration of 300 µl of sterile saline containing OA (1 mg). All guinea-pigs were pretreated 1 h before challenge, with an intraperitoneal (i.p.) injection of 500 µg kg⁻¹ of the antihistamine mepyramine, to prevent early bronchoconstriction and death. One group of animals received intravenous (i.v.) injection of NRIF (S-300 P2 fraction, 0.2 ml, equivalent to material released from approximately 5×10^6 macrophages) (Tamashiro *et al.*, 1992), into the exposed saphenous vein, 15 min before and 5 h after the challenge. Controls were actively sensitized guinea-pigs that received saline either i.v. (in place of NRIF) or i.n. (in place of OA). The animals were used 5, 24 or 96 h after challenge.

In vivo preparation

Non-immunized guinea-pigs were anaesthetized with sodium pentobarbitone (30 mg kg⁻¹, i.p.) and the tracheae were cannulated. Spontaneous breathing was suppressed with pancuronium (2 mg, i.v.). A jugular vein was catheterized for i.v. injections and one carotid artery was cannulated for blood sampling.

In a first series of experiments, guinea-pigs received 1 mg of LPS diluted in saline (0.1 ml) by the intratracheal (i.t.) route, at $t = 0$. One group of animals was pretreated with NRIF (0.2 ml, i.v.) 15 min before LPS administration. Controls received saline by i.v. or i.t. routes. Bronchoalveolar lavages were performed 3 h after LPS administration.

In a second series of experiments, animals received an i.v. injection of LPS (100 µg kg⁻¹ in 0.2 ml) 4 h after anaesthesia. The experimental group received NRIF (0.2 ml, i.v.) 15 min before LPS injection, whereas controls received saline i.v. Blood samples were collected from the carotid artery before injections and 10, 20, 30, 60, 90, 120, 150 and 180 min after LPS, for total leucocyte counts.

In a separate experiment, non-anaesthetized guinea-pigs were injected i.v. with 0.2 ml of saline containing LPS (100 µg kg⁻¹). Experimental groups received NRIF (0.2 ml, i.v.) 15 min before LPS injection and controls received saline. Bronchoalveolar lavages and total leucocyte counts in blood were performed 24 h after LPS injection. In this case, blood was obtained from superficial ear veins of non-anaesthetized guinea-pigs, before bronchoalveolar lavage.

Blood cell counts

Blood samples (40 µl) were collected from the carotid artery of superficial ear veins and mixed with 10 ml of a balanced electrolyte solution (Isoton II). Erythrocytes were then lysed with Zapoglobin and the total leucocyte counts assessed with a Coulter ZBI. Blood samples were obtained as described above.

Neutrophil recruitment inhibitory factor production

The method for obtaining crude macrophage supernatants containing NRIF was described in detail by Cunha *et al.* (1989). Briefly, rat macrophages were harvested from peritoneal cavities elicited 4 days earlier with 10 ml of 3% thioglycollate (w/v) and incubated in plastic tissue culture dishes for 1 h at 37°C, in an atmosphere of air containing 5% CO₂. The adherent monolayers (95% macrophages) were washed three times with phosphate-buffered saline (PBS, pH 7.4) and incubated with LPS (10 µg ml⁻¹ in RPMI) for 15 min at 37°C. The cells were again washed three times with PBS followed by a final incubation with 5 ml of LPS-free medium, for 1 h at 37°C. The cell-free incubation fluids were subsequently ultrafiltered through a YM-10 membrane (Amicon, Corp, Lexington, MA, U.S.A.). The final volume (corresponding to 5% of the original volume) was filtered through

0.22 µm membrane (Millipore, Bedford, MA, U.S.A.). According to the procedure described by Tamashiro *et al.* (1992), 1 ml samples of this material, released by 2.5×10^8 adherent cells, were dialysed against 0.5 M NaCl solution buffered with 0.02 M phosphate pH 7.4 and chromatographed on Sephacryl S-300 column (2.6 × 70 cm, Pharmacia, Uppsala, Sweden). The S-300 column was calibrated with a molecular weight standard kit (Pierce Chemical Co., Rockford, IL, U.S.A.). The P2 fraction, containing the original inhibitory activity (Tamashiro *et al.*, 1992), desalted on a PD10 column (Pharmacia) was used for the inhibitory assays, and is designated as NRIF.

Sampling of bronchoalveolar cells

The cell composition of the bronchoalveolar lavage fluid from actively sensitized unchallenged guinea-pigs was compared to that of fluids collected 5, 24 and 96 h following challenge with OA. Since marked effects were noted after 24 h, this interval was used to study the effect of NRIF upon the influx of inflammatory cells into the bronchoalveolar lavage. To do so, 5 ml of sterile saline was instilled, at room temperature, through a polyethylene cannula introduced into the trachea, and recovered immediately. This was followed by nine repeated lavages with 5 ml aliquots of sterile saline until almost all of the 50 ml administered was recovered. Total leucocyte counts were evaluated with a Malassez haemocytometer and cell viability was estimated with the trypan blue exclusion test. The suspension containing 120,000 cells ml⁻¹ was then centrifuged in a Shandon-Elliott cytometer and the cells were counted and differentiated by May-Grunwald-Giemsa staining (Diff-Quick, American Scientific Products, McGraw Park, IL, U.S.A.). Results are expressed as leucocyte numbers × 10⁶ ml⁻¹ of bronchoalveolar lavage fluid.

Lung perfusion

For this experiment guinea-pigs were used 24 h after intranasal antigen challenge. Guinea-pigs were anaesthetized with sodium pentobarbitone (30 mg kg⁻¹, i.p.), tracheae were cannulated and the animals ventilated (60 strokes min⁻¹, 10 ml kg⁻¹) with a Palmer miniature respiratory pump. A thoracotomy was performed and the lungs were removed as described by Lefort *et al.* (1984), placed in a plastic chamber, ventilated (60 strokes min⁻¹, 10 ml kg⁻¹) and perfused intra-arterially (10 ml min⁻¹, 37°C) with a gassed (95% O₂/5% CO₂) Krebs solution containing 0.25% (wt/vol) bovine serum albumin (BSA) and 2 µM mepyramine, to abolish the histamine component of the bronchoconstrictor effect of PAF in lungs from actively sensitized guinea-pigs (Pretolani *et al.*, 1988). After a 10 min equilibration period, increasing doses of PAF (3 and 30 ng in 100 µl of saline containing 0.1% BSA) were sequentially injected into the pulmonary artery of each perfused lung, at 10 min intervals. Bronchoconstrictor responses were recorded by a pressure transducer located between the trachea and the respiratory pump, and expressed as cm² of area of the tracing of the paper record over the base line. Before the end of each experiment, 10 µg OA was administered to assess the anaphylactic response in lungs from the sensitized animals. One minute fractions of each lung effluent were collected before, during the first 3 min and between the 3rd and 6th min after the successive injections of the two doses of PAF and OA, for the determination of thromboxane B₂ (TxB₂) (Sors *et al.*, 1978) and histamine (Lebel, 1983). Results were expressed as histamine or TxB₂ released (ng ml⁻¹).

Evaluation of thromboxane and histamine in the lung effluent

Before storage at -20°C, aliquots (1 ml) of the lung perfusate were maintained at room temperature for 60 min to

allow the conversion of TxA_2 into its stable metabolite TxB_2 . Under such conditions, the conversion of TxA_2 in TxB_2 approaches 100% in 30 min (Charpentier *et al.*, 1986). To perform the radioimmunoassay, 100 μl of each sample were incubated overnight at 4°C with ^{125}I -labelled TxB_2 and anti- TxB_2 antiserum, in a phosphate buffer (10 mM, pH 7.4) containing bovine gamma-globulins (0.3% w/v). The next day, bound and free ligand were separated by the addition of a solution of polyethyleneglycol 6000 (30% in distilled water), followed by centrifugation for 10 min at 1500 g and at 4°C. The supernatants were decanted and radioactivity present in the pellet, which corresponds to the bound fraction, was counted for 1 min in a gamma counter. The monoclonal antibodies employed were less than 0.02% crossreactive with prostaglandin D_2 (PGD_2), PGE_2 , $\text{PGF}_{2\alpha}$, 6-keto- $\text{PGF}_{1\alpha}$ and arachidonic acid for the anti- TxB_2 antiserum. The sensitivity of the assay was approximately 2 pg of immunoreactive TxB_2 , in 0.1 ml sample.

For the histamine assay, 1 ml-aliquots of the collected fractions were mixed with 1 ml of 0.8 N perchloric acid solution. After centrifugation for 10 min at 1200 g and 4°C, the supernatants were stored at 4°C. An automatic spectrofluorimetric assay for histamine was performed according to Shore *et al.* (1959), as modified by Lebel (1983).

Materials

The composition of the Krebs solution was (in mM): NaCl 118, KCl 4.7, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 2.5, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.2, NaHCO_3 25 and glucose 5.6. The drugs used were: chicken ovalbumin (Miles, Naperville, IL, U.S.A.); endotoxin (*E. coli* O₁₁₁: B₄ lipopolysaccharide) from Sigma Chemical Company; PAF-acether (1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocoline, obtained from Bachem, Switzerland); perchloric acid (Merck, Darmstadt, Germany); mepyramine maleate (Rhône-Poulenc, Vitry/Seine, France); pentobarbitone (sodium pentobarbital, Clin-Midy, Montpellier, France); zapoglobin and isoton II (Coultronics, France); pancuronium (Pavulon, Organon, France); $\text{Al}(\text{OH})_3$; BSA fraction V (Sigma); RPMI 1640 culture medium (Flow Laboratories, McLean, VA, U.S.A.); thioglycollate medium (Difco). The antibodies and radio-labelled ligand for radioimmunoassay of TxB_2 were obtained from the UR1A, Institut Pasteur-INSERM U207, France.

Data analysis

A one way analysis of variance (ANOVA) was used to compare the changes between different treatments. If significance was determined, individual comparisons were subsequently tested with Dunnett's *t* test for unpaired values (Dunnett, 1964). For the analysis of $\text{T} \times \text{B}_2$ and histamine release in individual comparisons the Mann Whitney test was used, since the observations were drawn from not normally distributed populations (Siegel, 1956). Differences were considered statistically significant when $P < 0.05$.

Results

Interference of NRIF with the neutrophil recruitment into guinea-pig bronchoalveolar lavage fluid induced by the intratracheal or intravenous administration of LPS

The intravenous injection of NRIF inhibited the neutrophil migration to the bronchoalveolar lavage fluid in naive guinea-pigs challenged 3 h after intratracheal LPS (Figure 1a) or 24 h after its intravenous injection (Figure 1b). There was no significant change in the eosinophil counts.

Lack of effect of NRIF on leukopenia or leucocytosis induced by intravenous LPS ($100 \mu\text{g kg}^{-1}$)

Leukopenia induced by intravenous LPS was maximal 30 min after its injection, persisted for 3 h and was not modified by NRIF (Figure 2).

Similarly, pretreatment with NRIF failed to modify leucocytosis induced 24 h after intravenous injection of LPS. Leucocyte counts were: control = $3.9 \pm 0.13 \times 10^3 \mu\text{l}^{-1}$; LPS = $8.3 \pm 1.48 \times 10^3 \mu\text{l}^{-1}$; NRIF + LPS = $9.01 \pm 0.94 \times 10^3 \mu\text{l}^{-1}$ (mean $\pm$ s.e.mean, $n = 5-6$ animals).

Protection by NRIF against leucocyte recruitment induced by the intranasal challenge of sensitized guinea-pigs with ovalbumin

As seen in Figure 3, the number of eosinophils in the bronchoalveolar lavage fluid increased 5 h after the intranasal

Figure 1 Neutrophil recruitment into the bronchoalveolar lavage fluid induced 3 h after the intratracheal (a, 1 mg) or 24 h after the intravenous (b, $100 \mu\text{g kg}^{-1}$) administration of lipopolysaccharide (LPS) (open columns) to normal guinea-pigs. Interference of NRIF i.v. injected 15 min before LPS (hatched columns). Controls (C) received saline. Columns represent the means $\pm$ s.e.mean (vertical bar) of the leucocyte numbers for 5 to 7 animals. * $P < 0.05$ as compared to controls; * $P < 0.05$ as compared to LPS.

Figure 2 Leukopenia induced at different times after i.v. administration of lipopolysaccharide (LPS, $100 \mu\text{g kg}^{-1}$, ○) to normal guinea-pigs. Lack of effect of NRIF i.v. injected 15 min before LPS (■). Controls received saline (□). Blood samples were collected from the carotid artery 4 h after anaesthesia with sodium pentobarbitone. Results are shown as the means $\pm$ s.e.mean (vertical bars) of the leucocyte counts of 4 to 5 animals. ** $P < 0.01$; * $P < 0.05$ as compared to controls.

Figure 3 Leucocyte recruitment into bronchoalveolar lavage fluid at different times (5, 24 and 96 h) after the intranasal challenge with ovalbumin (OA, 1 mg) on the 14th day after sensitization. Controls received saline ($n = 12$). Results represent the means $\pm$ s.e.mean (vertical bars) of the leucocyte numbers in 5 h ($n = 5$), 24 h ($n = 6$) and 96 h ($n = 6$) group of animals. * $P < 0.05$; ** $P < 0.01$ as compared to controls.

challenge of sensitized animals with OA. This augmentation of eosinophil counts persisted after 24 h and was associated with a significant increase in neutrophil numbers. At no time were mononuclear cell counts altered (data not shown).

For the assessment of potential inhibition, the optimal interval of 24 h was used. Pretreatment with NRIF blocked the OA-induced increase in neutrophil and eosinophil counts (Figure 4).

Figure 4 Leucocyte recruitment induced into the bronchoalveolar lavage fluid 24 h after the intranasal challenge with ovalbumin (Ch, 1 mg, $n = 6$). Protection by NRIF i.v. injected 15 min before and 5 h after challenge (hatched columns, $n = 6$). Controls (C) received saline as intranasal challenge ($n = 12$). Columns represent the means $\pm$ s.e.mean (vertical bars) for the leucocyte numbers of the animals. * $P < 0.05$; ** $P < 0.01$ as compared to controls; * $P < 0.05$; * $P < 0.01$ as compared to challenge.

Interference of NRIF with PAF and antigen-induced bronchoconstriction in lungs from sensitized guinea-pigs

Guinea-pigs were anaesthetized and the lungs removed and perfused as described in the Methods section. After a 10 min equilibration period, 3 and 30 ng doses of PAF were sequentially injected, at 10 min intervals, into the pulmonary artery of each perfused lung preparation. At the end of each experiment, 10 μ g OA was administered to assess the anaphylactic response in lungs from sensitized animals. The injection of 3 and 30 ng PAF into lungs from actively sensitized but unchallenged guinea-pigs resulted in dose-dependent bronchoconstriction (Figure 5, solid columns). The first administration of 3 ng PAF to lungs from challenged animals evoked a significant increase in bronchoconstriction, which was similar to that induced by 30 ng of the phospholipid (Figure 5, open columns). After the administration of PAF, an injection of 10 μ g OA produced a further increase of bronchoconstriction, but this did not differ significantly in area from that induced by the highest dose of PAF. No alteration was found in the intensity of bronchoconstriction induced by PAF in guinea-pigs pretreated with NRIF, although a significant inhibition of bronchoconstriction induced by the antigen was observed (Figure 5, hatched columns).

Failure of NRIF to inhibit PAF-induced histamine release from lungs of challenged guinea-pigs

One minute fractions of each lung effluent were collected before, during the first 3 min and between the 3rd and 6th min after the successive injections of the two doses of PAF and OA, for histamine and TxB₂ determination, as described in the Methods section. The basal release of histamine from lungs of challenged guinea-pigs was above that of control unchallenged lungs. Histamine release was enhanced after the injection of 3 ng PAF into lungs from challenged guinea-pigs (Figure 6, open columns). This release reached greater values in the first 3 min after PAF, but the effect persisted at 6 min. Pretreatment with NRIF failed to alter this histamine release (Figure 6, hatched columns), or that induced by 10 μ g OA

Figure 5 Bronchoconstrictor responses induced by the intra-arterial administration of 3 and 30 ng PAF and 10 μ g ovalbumin (OA) into perfused lungs from sensitized and challenged guinea-pigs (Ch, $n = 11$). Controls (C) received saline as a challenge ($n = 9$). NRIF was injected i.v. 15 min before and 5 h after antigen challenge (cross hatched columns, $n = 8$) and experiments were performed 24 h later. Columns represent means $\pm$ s.e.mean (vertical bars) of the bronchoconstrictor effect. * $P < 0.05$ as compared to control; * $P < 0.05$ as compared to challenge.

Figure 6 Release of histamine from isolated lungs of challenged (Ch) guinea-pigs injected with 3 ng PAF. One minute fractions of the lung effluent were collected before, during the first 3 min and between the 3rd and 6th min after injection of PAF, for the determinations of histamine. Controls (C) received saline. Lack of effect of pretreatment with NRIF (cross hatched columns). Experiments were performed 24 h after intranasal challenge. Results are expressed as the mean values $\pm$ s.e. mean (vertical bars) of histamine release for 6 to 8 experiments. * $P < 0.05$ as compared to controls.

(data not shown). Similarly, no statistically significant differences were noted in TxB_2 release from control ($1.5 \pm 0.77 \text{ ng ml}^{-1}$), challenged ($3.4 \pm 1.2 \text{ ng ml}^{-1}$) and NRIF + challenged ($5.33 \pm 2.39 \text{ ng ml}^{-1}$) isolated perfused lungs, during the first 3 min after 3 ng PAF.

Discussion

In the present study, NRIF obtained from rat macrophages was effective in inhibiting neutrophil migration into the bronchoalveolar lavage fluid following the intratracheal or intravenous administration of LPS to naive guinea-pigs. Since NRIF is not species-specific (it also blocks inflammatory neutrophil migration in mice, Tavares-Murta *et al.*, unpublished), it can be used as a tool for correlating the presence of emigrated leucocytes with tissue events. In contrast to the inhibitory effect upon neutrophil recruitment to the inflamed site, NRIF did not alter the biphasic effect of intravenous LPS, i.e. leucopenia and the subsequent increase in leucocyte numbers in blood. Thus, it is not by acting upon the motility of circulating leucocytes (or as an LPS-binding protein) that NRIF inhibits neutrophil migration. It has also been demonstrated that the inhibitory effect cannot be attributed to neutropenia or to hypotension, since crude macrophage supernatants containing NRIF failed to alter either when given intravenously to conscious rats (Cunha *et al.*, 1989). Moreover, NRIF does not modify the *in vitro* chemotactic responses of neutrophils (Tamashiro *et al.*, 1992) or eosinophils (Coëffier *et al.*, unpublished). Alternatively, it has been hypothesized that NRIF reduces neutrophil adhesion to the endothelium (Cunha *et al.*, 1989; Tamashiro *et al.*, 1992). Previous studies have suggested that intercellular adhesion molecule-1 may be pivotal in the pathogenesis of asthma (Wegner *et al.*, 1990). The hypothesis that NRIF may act by reducing the expression of adhesion molecules is attractive, but remains to be further elucidated.

Sensitized animals of different species develop airways obstruction immediately or several hours after immune challenge (Larsen, 1988), as well as increased leucocyte numbers in the bronchoalveolar lavage fluid (Lellouch-Tubiana *et al.*, 1988; Larsen *et al.*, 1991). A delay of 7 days after the booster injection of antigen for the full expression of bronchoconstrictor hyperresponsiveness has been described by Pretolani *et al.* (1988), whereas in the present work the lungs already showed hyperresponsiveness 24 h after challenge. Although we did not study the duration of the increased responsiveness, as compared with other methods, the short period necessary for sensitization makes this procedure suitable for the study of short-acting substances, such as peptides and protein hormones.

We also investigated the cell composition of the bronchoalveolar lavage fluid after immune challenge by the intranasal route. The number of eosinophils was markedly increased 5 and 24 h after challenge, whereas neutrophil counts were also increased, but were delayed, being significant only at 24 h. Others have found even greater neutrophil counts in rats as compared to eosinophils, 24 h after aerosolized antigen (Kips *et al.*, 1992). One possible explanation for the large neutrophilic effect in our experiments involves the direct nasal instillation of the antigen. With a slightly different procedure of active sensitization, antigen challenge also caused a significant early increase in the number of eosinophils, without a change in neutrophil counts (Desquand *et al.*, 1991). Thus, our present results support the concept that the signal for antigen-induced eosinophil and neutrophil recruitment involves different pathways, which are enhanced in sensitized guinea-pigs.

Isolated lungs from challenged guinea-pigs compared with those of control saline-treated animals showed an increased bronchoconstriction and an enhanced histamine release into the perfused fluid after the first PAF injection. A protocol involving the sequential injections of two doses of PAF and a final challenge with OA was used, in order to assess the possible interference of NRIF with submaximal provocations with PAF and with the responses to antigen. Indeed, sensitization followed by a booster injection of antigen was critical for the development of bronchopulmonary hyperresponsiveness in lungs from actively sensitized guinea-pigs (Pretolani *et al.*, 1988). The fact that we did not find an increased bronchoconstriction with the second injection of PAF or with ovalbumin in lungs from challenged animals may result from our experimental conditions, since the effects of PAF were already supramaximal at its lowest dose. In contrast to Pretolani *et al.* (1988), we did not find an enhanced thromboxane B_2 release after PAF, indicating that in this model, bronchoconstriction and release of this mediator occur independently. It is also of interest that lungs from NRIF-pretreated animals were less responsive to challenge with ovalbumin than controls, suggesting that inhibition by NRIF may extend to some effects of antigen, particularly bronchoconstriction.

Simultaneous accumulation of granulocytes, as assessed by bronchoalveolar lavage counts, and hyperreactivity of isolated lungs to PAF may be triggered by independent mechanisms, since NRIF inhibited neutrophil and eosinophil recruitment but failed to modify the enhanced airway reactivity to PAF. In confirmation, Sanjar *et al.* (1990) detected an increased airway content of neutrophils and eosinophils accompanied by enhanced airway reactivity, which peaked 8 to 24 h after exposure of sensitized guinea-pigs to antigen. Although these events were associated, no significant correlation between them was found, as assessed by drug inhibition. Moreover, one group of sensitized animals responded to antigen exposure with severe bronchial eosinophilia unaccompanied by increased airway reactivity. Pretolani *et al.* (unpublished) obtained similar results in guinea-pigs made hyper-eosinophilic with repeated exposure to antigen by aerosol, since bronchoconstriction following intratracheal methacholine was not modified. Finally, Elwood *et al.* (1992) found in-

creased granulocyte and lymphocyte counts in bronchoalveolar lavage fluid, 18 to 24 h after exposure of actively sensitized rats to aerosolized ovalbumin. Although this was accompanied by increased bronchial responsiveness to inhaled acetylcholine, cyclosporin A failed to prevent airway hyperresponsiveness while producing a significant inhibition of the inflammatory cell influx.

NRIF inhibited leucocyte migration into the lungs without interfering with bronchial hyperresponsiveness to PAF, even though it reduced the bronchoconstrictor effect of OA. Thus, recruited cells may contribute to OA-induced bronchoconstriction. In this context, we have already shown that NRIF inhibits carrageenan-induced rat paw oedema, which depends upon migrating cells, but failed to modify oedema induced by dextran, which depends upon resident mastocytes (Tamashiro *et al.*, 1992; Lo *et al.*, 1982). NRIF also blocks antigen-

induced oedema in rats, suggesting that here also migrating leucocytes are involved (Tamashiro *et al.*, 1992).

Bronchial asthma is a multicellular-dependent process. It seems that the immunological stimulus leads to secretion of substances that produce different effects on airway function. This study shows coincidence between the increased number of inflammatory cells in bronchoalveolar lavage fluid and airway hyperresponsiveness, since they occurred following the same stimulus, but the studies using NRIF demonstrated that the two events can be dissociated.

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