

THE effect of antigen challenge on the airway responses to substance P and on the epithelial neutral endopeptidase (NEP) activity was investigated in aerosol sensitized guinea-pigs. *In vivo*, bronchial responses to aerosolized substance P were similar to the responses observed in antigen-challenged guinea-pigs and in the control groups. In contrast, when the guinea-pigs were pretreated with the NEP inhibitor, phosphoramidon, a significant increase in the airway responses to substance P was observed after antigen challenge *in vivo*. However, *in vitro*, the contractile responses of the tracheal smooth muscle to substance P were similar between groups of guinea-pigs, in respect to the presence or absence of the epithelium and/or phosphoramidon. Histological studies showed an accumulation of eosinophils in the tracheal submucosa after antigen challenge and intact epithelial cells. These results show that *in vivo* bronchial hyperresponsiveness to substance P after antigen challenge in the guinea-pig is not associated with increased responses of the smooth muscle to exogenous SP *in vitro*. In addition, the results with phosphoramidon suggest that loss of NEP activity cannot account for the *in vivo* bronchial hyperresponsiveness to substance P presently observed.

**Key words:** Aerosol, Bronchial hyperresponsiveness, Epithelium, Guinea-pig, Neutral endopeptidase, Ovalbumin, Phosphoramidon, Substance P, Trachea

## Bronchial responses to substance P after antigen challenge in the guinea-pig: *in vivo* and *in vitro* studies

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## Introduction

Non-specific increase in airway responsiveness to bronchoconstrictor stimuli, namely bronchial hyperresponsiveness, is generally associated with airway inflammation in asthma. In the airways, substance P and related neurokinins, mediators of the sensory pathway, may be locally released after stimulation of C fibre endings.<sup>1</sup> These neuropeptides induce contraction of the airways in the guinea-pig and in human.<sup>2,3</sup> They also have pro-inflammatory effects in the airways<sup>4,5</sup> and may thus play a role in airway hyperresponsiveness.

These neuropeptides may be degraded by neutral endopeptidase (NEP), an enzyme localized to airway epithelium.<sup>6</sup> Blockade of NEP by phosphoramidon induces increased airway responses to exogenous and endogenous neuropeptides.<sup>7–10</sup> Bronchial hyperresponsiveness to substance P has been reported after viral infection or cigarette smoke.<sup>11,12</sup> Since the bronchoconstrictor responses to substance P were not increased after NEP blockade as compared to controls, hyperresponsiveness was attributed to an alteration in NEP activity.<sup>11,12</sup>

We recently developed an experimental preparation of bronchial hyperresponsiveness in which guinea-pigs are sensitized and challenged with the antigen by aerosol.<sup>13</sup> Enhanced bronchopulmonary

responses to aerosolized and intravenous acetylcholine and 5-hydroxytryptamine are observed 24–72 h after antigen challenge associated with marked airway infiltration with eosinophils.<sup>13</sup> Our purpose was first to examine the bronchial responses to exogenous substance P both *in vivo* and *in vitro* in sensitized and challenged guinea-pigs. We secondly studied the influence of NEP blockade by phosphoramidon and additionally the effect of epithelium removal *in vitro* in tracheal preparations.

## Materials and Methods

Specific pathogen-free male Hartley guinea-pigs (350–400 g) (Charles River, Saint-Aubin-les-Elbeuf, France) were separated into three groups and studied at random: Group A served as control and inhaled saline aerosol twice; Group B was sensitized to ovalbumin (OA) by aerosol and challenged with saline aerosol; Group C was sensitized to and challenged with OA aerosol.

*Sensitization and challenge procedures:* Series of ten guinea-pigs were exposed for 30 min to an aerosol of OA (2 mg/ml in saline, Ultra-Neb 99, DeVilbiss, Sommerset, PA, USA) in a plexiglass chamber (30 × 50 × 30 cm) twice at 48 h intervals, as previously described.<sup>13</sup> 15–20 days later, guinea-pigs were challenged in the plexiglass chamber by

exposure to aerosols of five successive increasing concentrations of OA (0.01, 0.1, 1, 5 and 10 mg/ml) for 15 min each.

*Assessment of airway responsiveness in vivo:* Airway responses to acetylcholine, histamine or substance P were assessed 48 h after challenge with antigen or saline. Guinea-pigs were anaesthetized (urethane, 1.2 g/kg, i.p.). The trachea was cannulated and the lungs were mechanically ventilated with a constant tidal volume (1 ml/100 g body weight) with a respiratory pump (Ugo Basile, Varese, Italy, 60 breaths/min). Spontaneous breathing was abolished with pancuronium bromide (2 mg/kg, i.v.). Both cervical vagi nerves were transected at the level of the neck. Pulmonary inflation pressure (PIP, mmHg) was measured as an index of the intrathoracic airway calibre by a pressure transducer (Gould PE10, Cleveland, OH, USA) connected to a lateral port of the ventilation circuit. After a 10 min equilibration period, a single 1 min aerosol administration of substance P (0.1 or 1 mg/ml) or four successive 1 min aerosol administration at 10 min interval of acetylcholine (50, 100, 200, 500 µg/ml) or histamine (1, 3, 10, 30 µg/ml) were performed. The time course of the bronchial response to substance P was studied for 10 min. The aerosol was generated by an ultrasonic nebulizer ("Pulmosonic" DeVilbiss), permanently connected in series with the afferent limb of the ventilator circuit.<sup>13</sup>

Two different phosphoramidon pretreatments were used: (i) conscious guinea-pigs were exposed for 15 min to an aerosol of phosphoramidon (0.1 mM in saline), 15 to 60 min before substance P (0.1 mg/ml); (ii) phosphoramidon (1 mg/kg) was administered intravenously to anaesthetized guinea-pigs through the jugular vein 5 min before substance P (0.1 mg/ml).

*Organ bath studies:* In separate experiments, guinea-pigs were killed by cervical dislocation. The trachea was dissected out, carefully cleaned of connective tissue and immersed in Krebs' solution bubbled with 95% O<sub>2</sub>-5% CO<sub>2</sub>. The trachea was opened longitudinally through the cartilage and carefully cut into eight strips of four cartilaginous ring segments. De-epithelialization of some ring segments was achieved by gently rubbing the luminal surface with a cotton wool swab.<sup>14</sup> Tracheal strips were suspended in 10 ml organ baths containing Krebs' solution bubbled with 95% O<sub>2</sub>-5% CO<sub>2</sub>, pH 7.4, at 37°C. The tissue was washed three times every 10 min under an initial 0.8 g tension and equilibrated to a final 2 g basal tension.<sup>14</sup> When the enzyme inhibitor phosphoramidon (10 µM) was used, a 30 min preincubation with the tissue was performed before substance P addition in a cumulative fashion (0.01 nM-10 µM). For SP

experiments, three groups of animals were studied. For clarity in Figure 3, results are presented for the two groups of sensitized guinea-pigs (groups B and C), since unsensitized animals (group A) had a similar response to group B. Changes in smooth muscle tension, expressed in mg of contraction, were measured isometrically using Narco F60 force-displacement transducers and recorded on MKIV physiographs (Narco, Houston, TX, USA).

*Histology:* In separate experiments, tracheas from anaesthetized guinea-pigs were dissected out 48 h after antigen or saline exposure and fixed in a buffered 10% formalin solution, pH 7.4, embedded in paraffin. 5 µm sections were stained either with haematoxylin-eosin or with Luna's reagent specific for eosinophil granule content.<sup>15</sup> Light microscopy was carried out with a Leitz Aristoplan microscope (Rueil-Malmaison, France) and a 64× magnification. For each guinea-pig, four to five tracheal sections were chosen at random and eosinophils were counted in a blind fashion.

*Materials:* Ovalbumin (OA, chicken egg, grade V), acetylcholine chloride, histamine dihydrochloride, phosphoramidon [N-(α-rhamno-pyranosyloxy-hydroxyphosphinyl)-L-leucyl-L-tryptophan, NH<sub>4</sub> salt] were from Sigma (St Louis, MO, USA). The other drugs used were urethane (ethylcarbamate, Prolabo, Paris, France), pancuronium bromide (Pavulon<sup>®</sup>, Organon, Fresnes, France), substance P (Novabiochem, Cléry en Vexin, France). Dilutions of all drugs were done extemporaneously in saline (NaCl, 0.9%) (*in vivo* experiments) or in modified Krebs' buffer with the following composition (mM): NaCl, 120; KCl, 5.9; CaCl<sub>2</sub>, 2.5; MgSO<sub>4</sub>, 1.2; glucose, 5.6; KH<sub>2</sub>PO<sub>4</sub>, 1.2; and NaHCO<sub>3</sub>, 25.5 (*in vitro* experiments).

*Expression of the results and statistical analysis:* Results are expressed as means ± SEM. Statistical differences of the dose-response curves (*in vivo* and *in vitro*) or of the time-courses of the response to substance P *in vivo* between the three groups of guinea-pigs were analysed by a two-way analysis of variance. In *in vitro* experiments, EC<sub>50</sub> values for acetylcholine and histamine have been compared by the unpaired *t*-test.

## Results

*Effect of OA challenge on airway responses to acetylcholine and histamine:* *In vivo* no significant difference in the initial pulmonary inflation pressure (PIP) was observed before stimulation with either acetylcholine, histamine or substance P between the different groups of guinea-pigs. Increasing doses of acetylcholine induced a dose-related increase in PIP (Figure 1). The responses observed in non-sensitized or

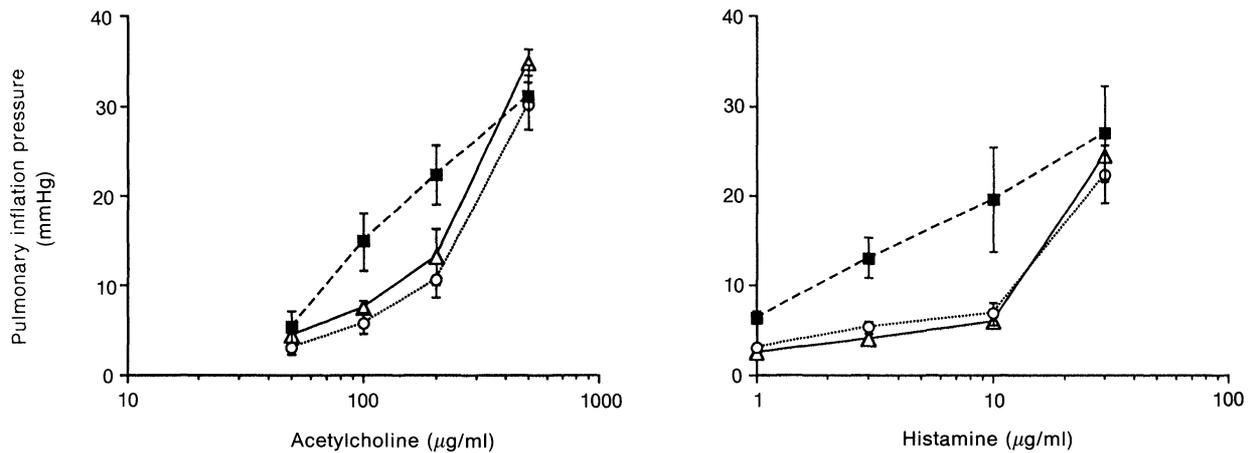


FIG. 1. Bronchopulmonary responses to four successive doses of acetylcholine (left panel) or histamine (right panel) administered by aerosol for 1 min to anaesthetized guinea-pigs from groups A, non-sensitized saline-exposed ( $\Delta$ ); B, ovalbumin sensitized saline exposed ( $\circ$ ); and C, ovalbumin sensitized, ovalbumin exposed ( $\blacksquare$ ) guinea-pigs. Values are means  $\pm$  SEM (bars) of five to six experiments. Significant increases in PIP were observed in group C as compared to groups A and B for both acetylcholine and histamine ( $p < 0.01$ ).

sensitized guinea-pigs challenged with saline, (groups A and B) were similar. By contrast, after OA challenge in sensitized guinea-pigs (group C), the dose—response curve to acetylcholine was significantly shifted to the left ( $p < 0.01$ ). Similarly, increasing doses of histamine induced a greater increase in PIP after OA challenge (group C) as compared to control groups (A and B) ( $p < 0.01$ ) (Figure 1).

*In vitro* in the presence of tracheal epithelium, acetylcholine concentration—response curves were similar for the three groups of guinea-pigs (Table 1). The curves did not reach a plateau even at 10 mM acetylcholine. When the epithelium was removed, the plateau of contraction was observed at 3 mM acetylcholine in all groups. Although maximal contraction was slightly lower than in the presence of epithelium,  $EC_{50}$  values decreased significantly (Table 1).

Histamine concentration—response curves also were not modified by OA sensitization and/or challenge either in the presence or absence of airway epithelium (Table 1).  $EC_{50}$  values decreased significantly when epithelium was removed in all

three groups although maximal contraction (at 0.3 mM with and 0.1 mM without epithelium) was lower.

*Effect of OA challenge on airway responses to substance P: In vivo*, substance P (1 mg/ml) induced a small increase in PIP that did not significantly differ between groups (Figure 2). Pretreatment with phosphoramidon either by inhalation (Figure 2, middle panel) or intravenously (Figure 2, lower panel) markedly enhanced the response to substance P in the three groups of animals and 1/10 of the dose of substance P (0.1 mg/ml) was used. However, the enhancement of the response was significantly greater in OA challenged (group C) than in saline challenged animals (groups A and B) ( $p < 0.01$ , Figure 2).

*In vitro* in the absence of phosphoramidon, concentration—response curves to substance P were similar in sensitized guinea-pigs, whether challenged with OA (group C) or saline (group B) (Figure 3) and in non-sensitized guinea-pigs (group A, not shown). In the absence of epithelium, contractions were of higher amplitude than contractions observed in the presence of the

**Table 1.**  $EC_{50}$  values ( $\mu$ M) and maximal contractions (mg) to acetylcholine (ACh) and histamine (Hist) in trachea of guinea-pigs from groups A (inhaled saline aerosol twice), B (sensitized to OA aerosol and challenged with saline aerosol) and C (sensitized to and challenged with OA aerosol) in the presence (+E) and absence (–E) of the epithelium. Values are means  $\pm$  SEM of five to six experiments. \* indicates  $p < 0.05$  and \*\* $p < 0.02$ . No difference was observed between groups in the presence or absence of epithelium, respectively

Groups	A		B		C	
	+E	–E	+E	–E	+E	–E
ACh $EC_{50}$	8.9 $\pm$ 5.9	1.5 $\pm$ 0.9*	16.2 $\pm$ 10.3	1.7 $\pm$ 0.6**	10.4 $\pm$ 7.5	2.9 $\pm$ 1.6*
ACh Max Cont	2207 $\pm$ 359	1638 $\pm$ 200	2012 $\pm$ 408	1551 $\pm$ 320	2075 $\pm$ 411	1556 $\pm$ 380
Hist $EC_{50}$	7.6 $\pm$ 2.2	3.4 $\pm$ 1.6*	15.1 $\pm$ 6.4	3.8 $\pm$ 1.9*	12.6 $\pm$ 2.9	2.3 $\pm$ 0.7**
Hist Max Cont	2025 $\pm$ 358	1544 $\pm$ 75	2268 $\pm$ 361	1437 $\pm$ 199	2377 $\pm$ 342	1486 $\pm$ 82

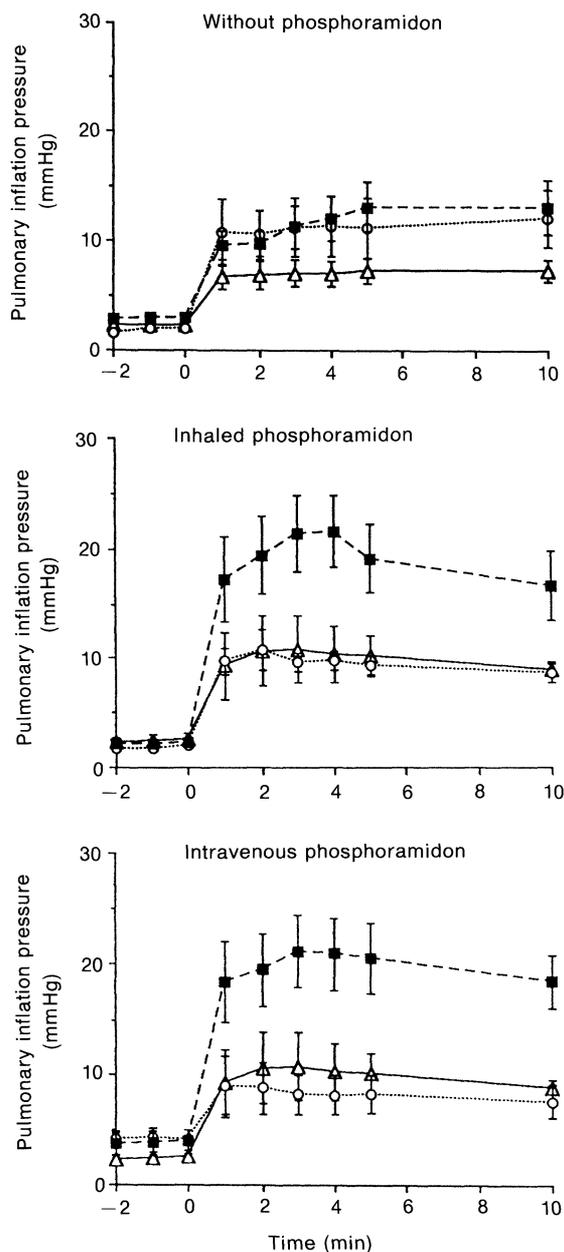


FIG. 2. Time course of the increase in pulmonary inflation pressure to inhaled substance P in guinea-pigs from groups A (1 mg/ml,  $\Delta$ ), B (0.1 mg/ml,  $\circ$ ) and C (0.1 mg/ml,  $\blacksquare$ ): (upper panel) after inhalation of saline, (middle panel) after inhalation of phosphoramidon and (lower panel) after i.v. injection of phosphoramidon. Values are means  $\pm$  SEM (bars) of seven to eight experiments. Significant increases in PIP were observed in group C as compared to groups A and B after pretreatment with phosphoramidon ( $p < 0.01$ ).

epithelial layer and a significant shift to the left was observed (Figure 3). However, a plateau of contraction was not achieved at the highest concentration of substance P used (10  $\mu$ M) neither in the presence nor absence of the epithelium.

Preincubation of tracheal segments with phosphoramidon (10  $\mu$ M) considerably enhanced contraction of the intact trachea to substance P as reflected by an increase in the amplitude of contraction associated with a significant shift to the

left of the concentration—response curves (Figure 3). The increase was similar in sensitized animals (groups B and C, Figure 3) and in non-sensitized guinea-pigs (group A, not shown). However, the plateau of contraction also was not achieved. In epithelium-free trachea, a plateau of contraction was observed at 1  $\mu$ M substance P and, additionally, a significant shift to the left was observed.

**Histology:** There was a marked accumulation of eosinophils in the subepithelial and epithelial layer of tracheas from sensitized animals 48 h after OA challenge (group C) as compared with saline challenge (group B). Numbers of eosinophils in the tracheal submucosa after OA challenge (group C,  $n = 5$ ) were  $677.8 \pm 55.5$  per  $\text{mm}^2$  compared to  $76.4 \pm 18.0$  per  $\text{mm}^2$  in saline exposed guinea-pigs (group B,  $n = 4$ ). Nevertheless, tracheal epithelium appeared normal in guinea-pigs from both groups B and C (Figure 4).

## Discussion

The present study shows that antigen challenge in aerosol sensitized guinea-pigs induces *in vivo* airway hyperresponsiveness to substance P, acetylcholine and histamine, which is not associated with any modification of the smooth muscle response to these agents *in vitro* nor of its modulation by airway epithelium. Forty-eight hours after antigen challenge in sensitized guinea-pigs, a nonspecific bronchial hyperresponsiveness was observed *in vivo* as a leftward shift of the dose—response curves to both acetylcholine and histamine compared to that of control animals. Conversely, in sensitized guinea-pigs challenged with saline, responses were similar to that of control animals, suggesting that bronchial hyperresponsiveness was clearly due to antigen challenge, rather than to sensitization itself.

In phosphoramidon pretreated animals, exposure of sensitized guinea-pigs to antigen also markedly enhanced airway responses to substance P compared with saline challenged and control guinea-pigs. However, in sensitized animals not pretreated with phosphoramidon, no increased response to substance P was observed after antigen challenge. These observations suggest that the exogenous blockade of NEP, i.e., the prevention of substance P degradation, is necessary to visualize bronchial hyperresponsiveness to substance P. This additionally suggests that neutral endopeptidase is still functional in the airways after antigen challenge. This hypothesis is strengthened by our results obtained *in vitro*, where the responses of tracheal segments to cumulative addition of substance P were similar in the three groups of animals irrespective of the experimental conditions used:

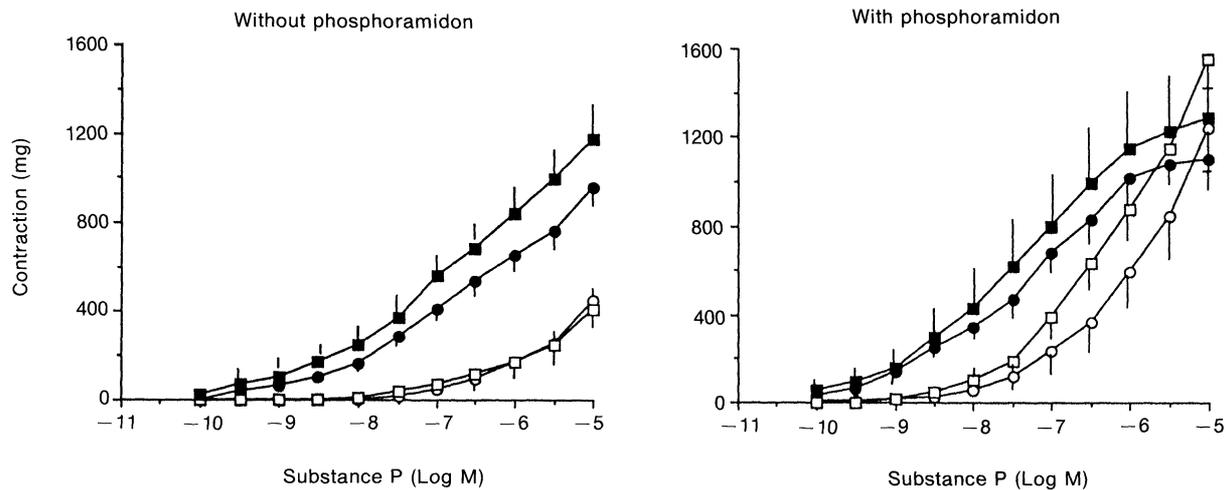


FIG. 3. Concentration-response curves to substance P on guinea-pig trachea from groups B (○, ●) and C (□, ■) in the absence and presence of phosphoramidon and in the absence (closed symbols) and presence (open symbols) of tracheal epithelium. Values are means  $\pm$  SEM of five to six experiments. No difference was observed between groups B and C. However, a significant shift to the left was observed after epithelium removal in the presence or absence of phosphoramidon ( $p < 0.05$ ). Similar curves were obtained from group A.

presence or absence of epithelium, presence or absence of the NEP inhibitor, phosphoramidon. Similarly, no difference was observed between groups in the response to acetylcholine or histamine. The very low contractile response to substance P observed in intact tracheal segments was potentiated to a similar extent by epithelium removal and by pretreatment with phosphoramidon

in the presence of epithelium. Epithelium removal and NEP blockade had a similar effect in the three groups of animals, suggesting that epithelium contains NEP degrading substance P and thus decreases airway contractility. This is in accordance with previous findings in control animals<sup>8,14,16</sup> and leads to the suggestion that epithelial NEP was equally active in the three groups of animals, therefore that NEP was still functional after antigen challenge.

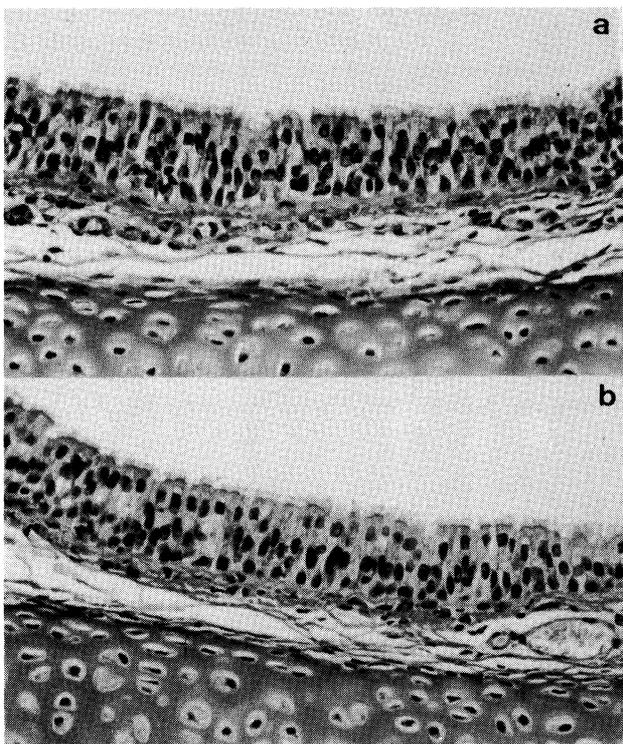


FIG. 4. Photomicrographs of tracheal sections stained with haematoxylin and eosin. Tracheas were obtained 48 h after exposure to (a) saline (group B) or (b) ovalbumin (group C) from aerosol sensitized guinea-pigs. Magnification  $\times 64$ .

Hence, observations that (1) bronchial hyper-responsiveness to substance P was only observed in phosphoramidon pretreated, antigen challenged animals and (2) there was no difference in the responsiveness to substance P *in vitro* after antigen challenge suggest that bronchial hyperresponsiveness exists in sensitized and challenged guinea-pigs in the absence of epithelium alteration or NEP dysfunction. It is noteworthy that both epithelium damage and loss of NEP function have been proposed as factors of bronchial hyperresponsiveness in asthmatic patients<sup>17</sup> in whom epithelium destruction was observed<sup>18</sup> and NEP inactivation was suggested by analogy with its inactivation in experimental animals after viral infection.<sup>11,19</sup> In our study in guinea-pigs sensitized and challenged with antigen, this absence of epithelial dysfunction is in accordance with the histological study of tracheal specimens where no obvious alterations of the epithelial layer could be noted.

The present study also shows that antigen challenge induced a marked accumulation of eosinophils in the tracheal wall, similar to that we previously reported in the lower airways and particularly in the peribronchial area and submucosa.<sup>13</sup> A role for eosinophils and their granule

proteins has been hypothesized as a mechanism involved in bronchial hyperresponsiveness. Eosinophil proteins such as major basic protein (MBP) are toxic for guinea-pig tracheal epithelium<sup>20</sup> *in vitro*. In the present study, airway eosinophilia did not appear to be either directly associated with increased response of tracheal smooth muscle *in vitro* or to epithelium disruption. We may hypothesize that chronic contact with the antigen as occurs in asthmatic airways might induce more profound changes in the airways, associated with more persistent bronchial hyperresponsiveness.

Hence, it seems from our study that some factors necessary for bronchial hyperresponsiveness *in vivo* are absent *in vitro*. A step in the description of such factors would be the determination of the degree of eosinophil activation, which are infiltrated in central and more peripheral airways. However, Iijima *et al.*<sup>21</sup> reported that the eosinophils infiltrating the bronchioli after allergen challenge in the guinea-pig did not appear degranulated. Another explanation could be that alteration of smooth muscle mechanics and/or contractility is not the primary factor of bronchial hyperresponsiveness.

In summary, this study indicates that pathophysiological changes associated with airway hyperresponsiveness induced by antigen challenge in sensitized guinea-pigs do not involve changes in the intrinsic properties of airway smooth muscle. The presence of eosinophils within the bronchial mucosa, although associated with *in vivo* bronchial hyperresponsiveness, is not sufficient to alter morphology of epithelial cells or to induce epithelial NEP dysfunction. There are, of course, several other ways of considering airway hyperresponsiveness, such as modification of the neural influences or of the vascular responsiveness. Such findings in our experimental model may help towards a better understanding of the airway hyperresponsiveness which occurs in asthma.

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