

Bronchial hyperresponsiveness and cellular infiltration in the lung of guinea-pigs sensitized and challenged by aerosol

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Summary

We have studied the development of airway hyperresponsiveness and the pulmonary cell infiltration in a guinea-pig model in which both initial sensitization and subsequent exposure to the antigen were performed by aerosol. Enhanced bronchopulmonary response to aerosol administration of acetylcholine (ACh) and 5-hydroxytryptamine (5-HT) was observed 3–4 hr and 18–24 hr after antigen exposure of sensitized animals. In contrast, when ACh and 5-HT were administered intravenously 3–4 hr after the challenge, no significant alteration of the dose–response curves was observed. However, 18–24 hr after antigen challenge, a marked leftward shift of the dose–response curve was observed on intravenous injection of ACh or 5-HT. The increased bronchial reactivity to aerosolized ACh in sensitized and challenged guinea-pigs reached a maximum by days 2–4, was still significantly increased at day 5 and returned to the basal value by day 8. No further alteration of the dose-related bronchopulmonary response to aerosol or intravenous administration of ACh was recorded 24 hr after a second antigen challenge, performed 8 days after the initial one. The analysis of bronchoalveolar lavage fluids showed a significant increase in the number of polymorphonuclear neutrophils 3–4 hr after the exposure of sensitized animals to the antigen, which was also associated with a significant eosinophilia at 18–24 hr. Histological examination of lung specimens obtained from animals 3–4 hr following challenge demonstrated eosinophil infiltration in the peribronchial regions and bronchial walls, as well as within the epithelium. Furthermore, as compared to time 3–4 hr, less eosinophils in the peribronchial area and submucosa were counted 24 hr after antigen challenge. However, a role of eosinophil-derived products in the development of bronchial hyperresponsiveness in this experimental model remains to be established.

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Introduction

Non-specific increase in airway responsiveness to bronchoconstrictor stimuli, i.e. airway hyperresponsiveness, is considered a central feature of asthma. Indeed, allergen inhalation resulting in early and late asthmatic responses has generally been associated with an increase in airway reactivity [1]. Although clinically well characterized, the mechanisms involved in the development of

airway hyperresponsiveness are poorly determined and the elucidation of the events underlying this phenomenon are prerequisite to understand better the pathological basis of asthma. Various animal models have been developed in which both early and late airway responses associated or not with bronchial hyperresponsiveness after exposure to antigen are observed. Hutson *et al.* [2] have demonstrated that 2 hr after antigen challenge, sensitized guinea-pigs present an early fall in airway conductance followed by a return to the baseline. In addition, bronchopulmonary alterations are also observed 17 and 72 hr after challenge. These changes in

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bronchopulmonary functions are accompanied by an increase in the number of neutrophils in the bronchoalveolar lavage, 6 hr after the antigen challenge and followed by a dramatic influx of eosinophils after 17 hr with a maximum at 72 hr [2]. These changes in the cell composition of bronchoalveolar lavages resemble those occurring after allergen challenge in asthmatic patients [3]. However, the role of the cell infiltration in the lung tissue in the development of the bronchial hyperresponsiveness has not been elucidated.

To assess bronchial reactivity, most investigators have usually administered the spasmogenic substances by the intravenous route [4,5]. This leads to a systemic response where the pulmonary and vascular components are intricately and is thus difficult to dissociate. For these reasons, the direct administration of the antigen in the airways would eliminate, or at least markedly reduce, the systemic effects [reviewed in 6]. Therefore, we developed a model in which guinea-pigs are sensitized and challenged with the antigen by aerosol. In the present study, the bronchopulmonary responses induced by aerosol and intravenous administrations of acetylcholine (ACh) and 5-hydroxytryptamine (5-HT) at various time intervals following antigen challenge without prior pharmacological modulation were compared. In addition, the cell composition of bronchoalveolar lavages and the histological examination of lung specimens from sensitized and challenged animals was analysed.

Materials and methods

Materials

The following drugs were used: ovalbumin (OA, chicken egg, grade 5, Sigma, St Louis, U.S.A.). Urethane (ethylcarbamate, Prolabo, Paris, France), pancuronium bromide (Pavulon, Organon, Fresnes, France), ACh chloride (Sigma), 5-HT chloride (Sigma), EDTA (Sigma). Hanks' balanced salt solution containing 0.35 g/l sodium bicarbonate (Flow laboratories, Irvine, U.K.).

Sensitization procedure

Specific pathogen-free male Hartley guinea-pigs (350–400 g) (Charles River, St-Aubin les Elbeuf, France) were placed twice, after a 48-hr interval, in a plexiglass chamber (30 × 50 × 30 cm) and exposed to aerosols of a 0.9% NaCl solution in saline solution containing 2 mg/ml OA for 30 min, modified from [7]. The aerosol was generated by a Devilbiss ultrasonic nebulizer (Ultra-Neb 99, Sommerset, Pennsylvania, U.S.A.). Under these conditions the aerodynamic diameter of the homogeneated particles averaged 0.5–3 µm.

Antigen exposure

Fifteen to 20 days after the initial sensitization procedure, the guinea-pigs were challenged in the plexiglass chamber, by exposure to five successive solutions of OA of, respectively 10 µg/ml, 100 µg/ml, 1 mg/ml, 5 mg/ml and 10 mg/ml for 15 min each. The aerosol was generated by a Devilbiss ultrasonic nebulizer as described above. These increasing concentrations were used to avoid fatal anaphylactic reactions. Indeed, when the concentration of OA reached 1 mg/ml in the nebulizer, the animals presented labored breathing, coughing and rubbing of the nose. In addition, a few guinea-pigs experienced convulsions, although animal death was never observed. However, 1 hr after the last challenge with the highest concentration of OA, no apparent sign of respiratory failure was observed in the guinea-pigs. As a control group, sensitized guinea-pigs were exposed to an aerosol of the saline solution for 30 min.

Assessment of the in-vivo bronchopulmonary reactivity

In two different sets of experiments the bronchopulmonary reactivity was assessed either 3–4 hr or 18–24 hr after exposure of the animals to the antigen or saline. The guinea-pigs were anaesthetized with urethane (1.2 g/kg, i.p.) and placed in a dorsal recumbent position. The trachea was cannulated and the lungs mechanically ventilated with a constant volume of 1 ml laboratory air/100 g body weight with a respiratory pump (Ugo Basile, Varese, Italy; 60 breaths/min). The right jugular vein was cannulated for intravenous administration of the various drugs. Spontaneous breathing was abolished with pancuronium bromide (2 mg/kg, i.v.) and both cervical vagi nerves were transected at the level of the neck. The initial resistance to inflation was adjusted to 10 cm H₂O according to the method of Konzett and Rössler [8] and the excess air volume was monitored from a lateral port of the ventilator circuit with a bronchospasm transducer (model 7020, Ugo Basile).

After a 10 min equilibration period, four successive 1-min aerosol administrations of ACh (50, 100, 200 and 500 µg/ml) or 5-HT (3, 10, 30 and 60 µg/ml) were performed at 10 min intervals with constant monitoring of the bronchopulmonary response. The aerosol was generated by a Devilbiss 'Pulmosonic' ultrasonic nebulizer permanently connected in series with the afferent limb of the ventilator circuit as modified from [9]. The bronchopulmonary responses are expressed as percentage changes calculated over the 100% obtained by clamping the trachea at the end of the experiment. The percentage of the bronchopulmonary response is used as an index of intrathoracic airway calibre. In parallel experiments, the bronchopulmonary responses induced

by four successive intravenous administrations of ACh (10, 30, 60 and 100 $\mu\text{g/kg}$) or 5-HT (3, 6, 10, and 30 $\mu\text{g/kg}$) were assessed, 3–4 hr and 18–24 hr after the antigen challenge. Similar bronchopulmonary reactivity to ACh and 5-HT was observed when the guinea-pigs were used in the time range of 18 hr or 24 hr after saline or OA challenge exposure (data not shown).

Time-course of the airway hyperresponsiveness to ACh

In this series of experiments, the dose of 100 $\mu\text{g/ml}$ ACh was selected because it induced a submaximal bronchopulmonary response. The response to aerosol administration of the single dose of ACh for 1 min was assessed in different groups of anaesthetized and ventilated guinea-pigs as described above, and 1, 2, 4, 5, 8 and 10 days after the antigen challenge. As a control, the bronchopulmonary response to ACh in a group of guinea-pigs challenged with a saline solution was also analysed throughout the time-course study.

In separate experiments and 8 days after the initial antigen stimulation by aerosol, the animals were placed in the plexiglass chamber and challenged again with the four graded doses of OA. Upon this second stimulation with the antigen, the guinea-pigs presented respiratory symptoms similar to those described above for the initial challenge. The bronchopulmonary response following successive aerosol or intravenous administration of ACh was assessed as described above, 18–24 hr after this second antigen exposure.

Determination of the cell composition of bronchoalveolar lavages

Bronchoalveolar lavages were performed 3–4 hr and 18–24 hr after antigen challenge or saline exposure. Briefly, the animals were anaesthetized with urethane, the trachea cannulated and 5 ml of a warmed (37°C) saline solution containing 2.6 mM EDTA was instilled into the lungs with a 5-ml syringe. The lavage fluid was recovered by gentle aspiration and this procedure was repeated five times. The combined lavage fluids from each animal were adjusted to 30 ml with Hanks' balanced salt solution. After centrifugation at $250 \times g$ for 15 min, the supernatant was discarded and 9 ml of distilled water was added for 30 sec to the cell pellet in order to lyse the erythrocytes. Three millilitres of 0.6 M KCl solution were then added to restore isotonicity and the volume was adjusted to 50 ml with Hanks' solution. After a second centrifugation, the cell pellet was resuspended in 4 ml of the same solution and smears were prepared with a cytocentrifuge (Shandon, Astmoor, U.K.). The smears were stained according to the May-Grunwald Giemsa

procedure. Differential cell counts on at least 200 cells were performed using standard morphological criteria to classify the cells into neutrophils, eosinophils, macrophages and lymphocytes. The cell composition of bronchoalveolar lavage fluids in a control group corresponding to non-sensitized and non-challenged guinea-pigs was also analysed.

Histological techniques

Lungs from anaesthetized guinea-pigs were removed 10 min, 4 hr or 24 hr after antigen or saline exposure. The lungs were washed by injection of 10 ml of a saline solution through the pulmonary artery. The lung tissue was fixed in a buffered 10% formalin solution, embedded in paraffin and 5- μm sections were obtained as previously described [18]. Small bronchial or bronchiolar walls were sectioned normal to the airway axis and stained with Luna's reagent specific for the eosinophil granule content [10]. Histological preparations were observed with a Leitz Aristoplan microscope (Rueil-Malmaison, France) using a $\times 64$ magnification. For each guinea-pig, five to six bronchi or bronchioles were randomly chosen and eosinophils were counted in a blinded fashion using a computerized light analyser (Leitz).

Statistical analysis

All data are expressed as means $\pm$ s.e.m. Statistical differences between the dose-response curves to ACh and 5-HT in the groups of guinea-pigs receiving the various treatment were analysed by two-way analysis of variance. Thus, the whole dose-response curve obtained in these different groups of animals was examined. In the other cases, the data were analysed and compared to the respective control by Student's *t*-test for unpaired data.

Results

Effect of aerosol challenge with the antigen on the responsiveness to ACh and 5-HT

Exposure of anaesthetized guinea-pigs to four successive aerosols of ACh (50, 100, 200 and 500 $\mu\text{g/ml}$) induced dose-related bronchopulmonary responses (Fig. 1). When the animals were previously exposed to OA, the dose-response curve to ACh was significantly shifted to the left, such enhanced response being observed at both time points studied, i.e. 3–4 hr and 18–24 hr (Fig. 1). In another series of experiments, similar results were obtained when dose-related bronchopulmonary responses were induced with four successive aerosol administrations of 5-HT (3, 10, 30 and 60 $\mu\text{g/ml}$) (Fig. 2). With this agonist, an even more dramatic leftward shift of the dose-response curve was observed, as compared to the

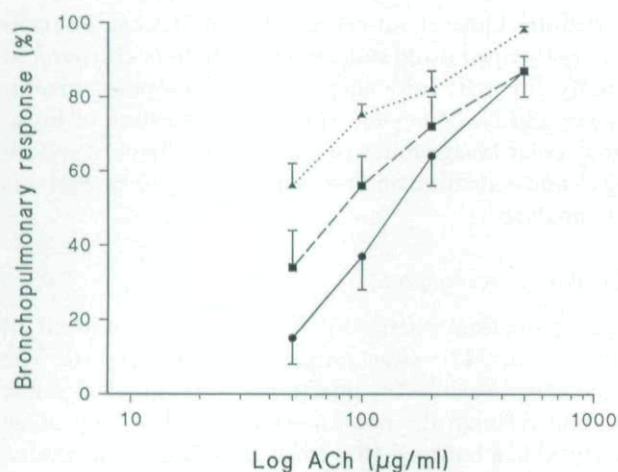


Fig. 1. Dose-related bronchopulmonary response induced by four successive administrations of ACh (50, 100, 200 and 500 µg/ml) by aerosol for 1 min to anaesthetized sensitized guinea-pigs. Bronchopulmonary responses were assessed 3–4 hr (■, $n=7$) and 18–24 hr (▲, $n=6$) after antigen (OA) challenge. The data were compared by two-way analysis of variance to the control obtained by exposure of the guinea-pigs to a saline solution (●, $n=9$). The results are expressed as means $\pm$ s.e.m. of percentages of the maximal bronchopulmonary response obtained by total clamping of the ventilation system. Statistical differences between saline-exposed and OA-challenged guinea-pigs reached levels of $P < 0.05$ and $P < 0.001$ at 3–4 hr and 18–24 hr, respectively.

one noted with ACh, both after 3–4 hr and 18–24 hr following the exposure of the animals to the antigen (Fig. 2). In contrast, the dose–response curves established with ACh or 5-HT administered by aerosol were not significantly altered 3–4 hr or 18–24 hr after aerosol administration of saline to sensitized guinea-pigs. As more than four intravenous or aerosol administrations of ACh to guinea-pigs gave inconsistent results (possibly due to lung function alterations), only the concentration range giving a linear response in saline-exposed animals was investigated in the antigen-challenged ones.

In parallel experiments, the dose-related bronchopulmonary responses following intravenous administrations of ACh (10, 30, 60 and 100 µg/kg) (Fig. 3) or 5-HT (3, 6, 10 and 30 µg/kg) (Fig. 4) were analysed. In contrast to the responses elicited by aerosol challenge, no significant alteration of the dose–response curves of both agonists was recorded 3–4 hr after antigen exposure (Figs 3 and 4). A marked and significant leftward shift of the dose–response curve to intravenous administration of ACh (Fig. 3) or 5-HT (Fig. 4) was, however, observed 18–24 hr after the exposure of animals to the antigen. The dose–response curves established with intravenous ACh or

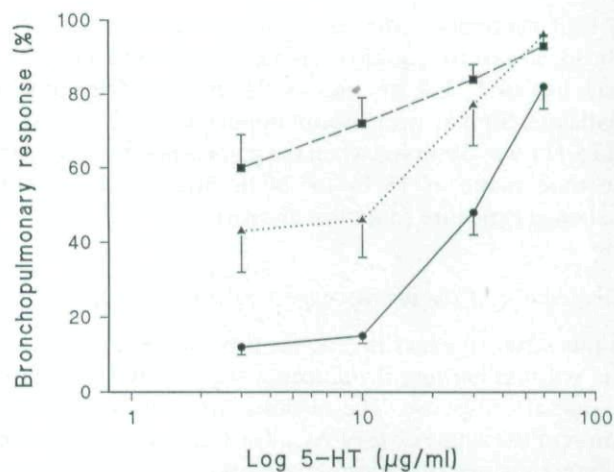


Fig. 2. Dose-related bronchopulmonary response induced by four successive administrations of 5-HT (3, 10, 30 and 60 µg/ml) by aerosol for 1 min, to anaesthetized sensitized guinea-pigs. Bronchopulmonary responses were assessed 3–4 hr (■, $n=9$) and 18–24 hr (▲, $n=6$) after antigen (OA) challenge. The data were compared by two-way analysis of variance to the control obtained by exposure of the guinea-pigs to a saline solution (●, $n=14$). The results are expressed as means $\pm$ s.e.m. of percentages of the maximal bronchopulmonary response obtained by total clamping of the ventilation system. Statistical differences between saline-exposed and OA-challenged guinea-pigs reached levels of $P < 0.001$, at 3–4 hr and 18–24 hr.

5-HT prior to, and 3–4 hr and 18–24 hr after aerosol administration of saline to sensitized guinea-pigs were similar.

Time-course of bronchial hyperresponsiveness to ACh administration

The time-course of the alterations of the bronchopulmonary response induced upon inhalation of a single dose of ACh (100 µg/ml) following aerosol challenge with OA or saline solution was investigated. As presented in Fig. 5, no difference of the bronchopulmonary response following ACh administration was observed in saline-exposed guinea-pigs at all time points of the kinetics study. In contrast, the bronchopulmonary response following aerosolized ACh in antigen-exposed guinea-pigs reached a maximum by days 2–4 and was still significantly increased at day 5, as compared to the value obtained with saline-challenged guinea-pigs. At days 8 and 10, the bronchopulmonary response of antigen-challenged sensitized guinea-pigs to ACh was comparable to values obtained in saline-exposed ones (Fig. 5). Also, no alterations of the dose-related bronchopulmonary response to intravenous ACh was observed 8 days after the exposure of the animals to the antigen (Fig. 6a).

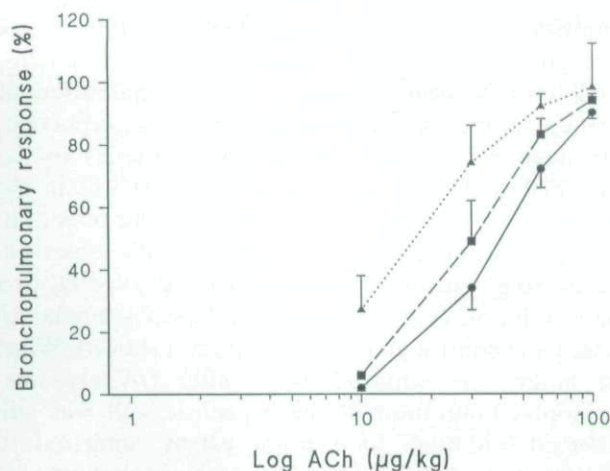


Fig. 3. Dose-related bronchopulmonary response induced by four successive intravenous administrations of ACh (10, 30, 60 and 100 $\mu\text{g/kg}$) in anaesthetized sensitized guinea-pigs. Bronchopulmonary responses were assessed 3–4 hr (■, $n=6$) and 18–24 hr (▲, $n=5$) after antigen (OA) challenge. The data were compared by two-way analysis of variance to the control obtained by exposure of the guinea-pigs to a saline solution (●, $n=12$). The results are expressed as means $\pm$ s.e.m. of percentages of the maximal bronchopulmonary response obtained by total clamping of the ventilation system. Statistical differences between saline-exposed and OA-challenged guinea-pigs reached a level of $P < 0.01$, at 18–24 hr. No statistical difference was recorded at 3–4 hr.

These results demonstrate that the increase in bronchopulmonary responsiveness induced by aerosol challenge of actively sensitized guinea-pigs is non-specific and of limited duration. The possibility that a second antigen challenge, performed 8 days after the initial one could induce an increase in the ACh-induced bronchopulmonary response was investigated. No further alteration of the dose-related bronchopulmonary responses to intravenous (Fig. 6a) or aerosol (Fig. 6b) administration of ACh was recorded 24 hr after this second antigen challenge.

Determination of the cell composition of bronchoalveolar lavages

Exposure of sensitized guinea-pigs to a saline solution did not significantly modify the total cell number, or those of polymorphonuclear neutrophils, eosinophils, macrophages and lymphocytes in the bronchoalveolar lavage fluid (Table 1). The total number of recovered cells in bronchoalveolar lavage fluids was already increased after 3–4 hr, but only significantly 18–24 hr after antigen challenge by aerosol. However, 3–4 hr after exposure of the animals to the antigen, a significant increase in the number of neutrophils was observed (Table 1). This

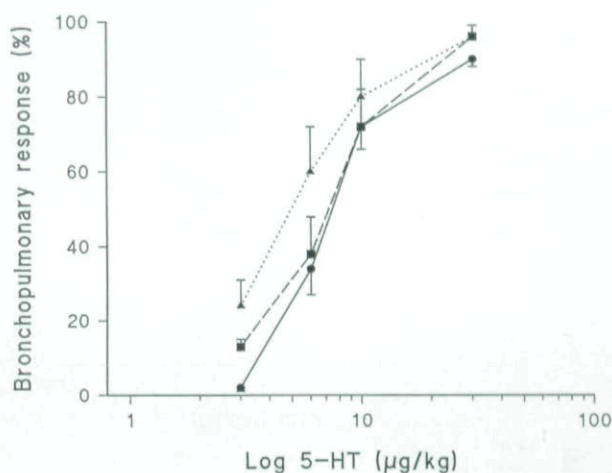


Fig. 4. Dose-related bronchopulmonary response induced by four successive intravenous administrations of 5-HT (3, 6, 10 and 30 $\mu\text{g/kg}$) in anaesthetized sensitized guinea-pigs. Bronchopulmonary responses were assessed 3–4 hr (■, $n=6$) and 18–24 hr (▲, $n=7$) after antigen (OA) challenge. The data were compared by two-way analysis of variance to the control obtained by exposure of the guinea-pigs to a saline solution (●, $n=6$). The results are expressed as means $\pm$ s.e.m. of percentages of the maximal bronchopulmonary response obtained by total clamping of the ventilation system. Statistical differences between saline-exposed and OA-challenged guinea-pigs reached a level of $P < 0.01$, at 18–24 hr. No statistical difference was recorded at 3–4 hr.

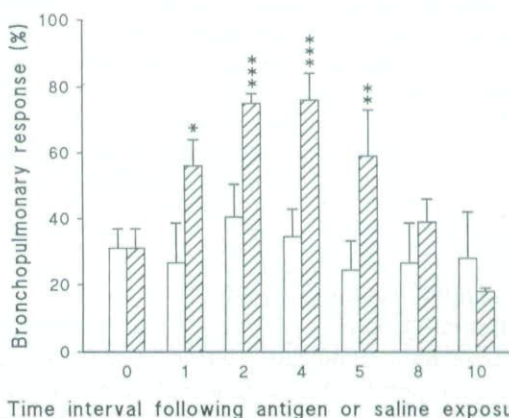


Fig. 5. Time-course of the bronchopulmonary response induced by administration by aerosol for 1 min of acetylcholine (ACh, 100 $\mu\text{g/ml}$) in anaesthetized sensitized guinea-pigs, challenged by saline (□) or OA exposure (■). Significant increases of the bronchopulmonary response were observed until 5 days after antigen exposure, as analysed by Student's *t*-test compared to the saline-exposed guinea-pigs. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ ($n=5-6$ in each group).

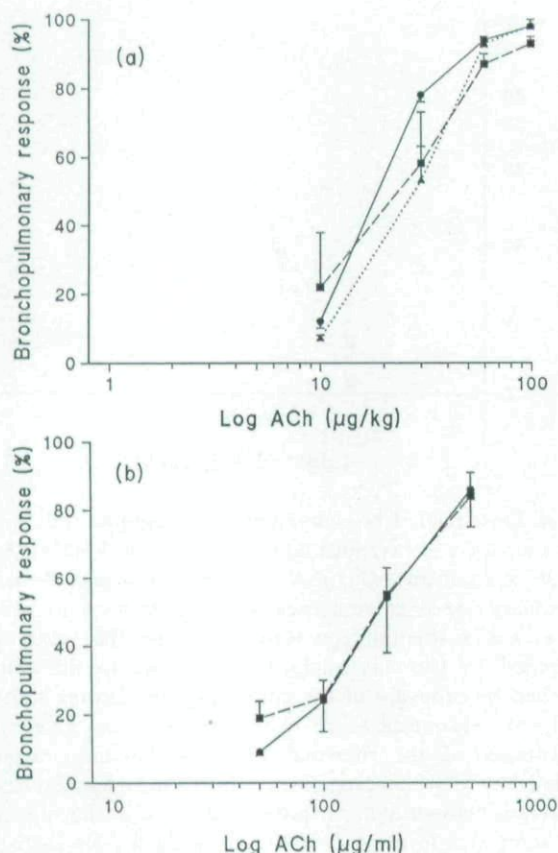


Fig. 6. Dose related bronchopulmonary response induced by four successive intravenous (a) or aerosol (b) administrations of acetylcholine (ACh) in anaesthetized sensitized guinea-pigs. The dose-response curves to ACh established 10 days after the first antigen exposure (■) (a) or 18–24 hr after the second antigen exposure (▲) (a and b), were not statistically different from the dose-response curve obtained by exposure of the guinea-pigs to a saline solution (●), as analysed by two-way analysis of variance. The results are expressed as means $\pm$ s.e.m. of the bronchopulmonary response as percentages of the maximal response obtained by total clamping of the ventilation system ($n=5-7$).

marked neutrophilia in the lavage fluid was still observed 18–24 hr after antigen exposure but was accompanied at this latter time by a significant 10-fold increase in the number of eosinophils. No significant change in the absolute numbers of recovered macrophages and lymphocytes was noted 3–4 hr and 18–24 hr after antigen challenge (Table 1).

Histological examination of cell accumulation in the lung

Ten minutes after OA exposure, no marked cell accumulation was observed. Indeed, as detected after coloration with Luna's stain, eosinophils were present in small

numbers, mostly in the peribronchial area (Fig. 7a). In contrast, 4 hr after antigen exposure of sensitized animals, small bronchi demonstrated a marked infiltration of eosinophils and unstained cells (mostly polymorphonuclear neutrophils upon morphological criteria) in the bronchial walls and the submucosa, as well as in the peribronchial regions (Fig. 7b). The presence of eosinophil-rich mucus in the bronchial lumen was also observed. Lungs from aerosol sensitized guinea-pigs exposed to a saline solution (Fig. 7c) were histologically similar to those from control guinea-pigs (data not shown). When the lungs were removed 24 hr after OA exposure, eosinophil infiltration in the bronchial wall was still observed, although to a lower extent compared to infiltration at 3–4 hr (Fig. 7d). As compared to lung preparations removed 4 hr after OA exposure, fewer eosinophils were observed in the peribronchial regions and in the submucosa. Eosinophil numbers in the peribronchial area 4 hr after antigen challenge by aerosol were 1264 ± 173.7 cell/mm² ($n=5$), compared to 189.8 ± 34.8 cell/mm² ($n=5$) in saline-exposed guinea-pigs. Twenty-four hours after antigen challenge, eosinophil numbers in the peribronchial regions were 861.1 ± 132 cell/mm² ($n=6$), compared to 210.2 ± 40.6 cell/mm² ($n=6$) in saline-exposed guinea-pigs.

Discussion

We established a model to study bronchial hyperresponsiveness involving actively sensitized guinea-pigs challenged with the antigen by aerosol. This protocol was developed because it allows pulmonary reactions without animal death. Indeed, despite the fact that guinea-pigs developed labored breathing during the antigen exposure, only a few of them experienced convulsions. To prevent mortality, Hutson *et al.* [2] have developed an experimental model in which guinea-pigs sensitized by aerosol are treated with an anti-histamine H₁-receptor antagonist prior to the challenge with the antigen. Histamine is, however, the major mediator in the lung involved in anaphylactic reaction in guinea-pigs [11,12] and consequently may modulate the release of secondary mediators implicated in airway hyperresponsiveness [5]. Although bronchial hyperresponsiveness may be evoked despite anti-histamine treatment, we chose to develop an experimental model in which pharmacological manoeuvres were avoided. The data reported here demonstrate that the successive administration of increasing doses of the antigen allowed the induction of homogeneous reactions in the absence of pharmacological modulation.

In this guinea-pig model, antigen challenge induces after 3–4 hr, and to a higher extent after 18–24 hr, an airway hyperresponsiveness, as demonstrated by a left-

Table 1. Total and differential cell counts ($n \times 10^6$ cells) in bronchoalveolar lavage from non-sensitized and saline-exposed (Control), sensitized and saline-exposed (Saline) and sensitized and OA-exposed guinea-pigs

Treatment	Total cell	Neutrophil	Eosinophil	Macrophage	Lymphocyte
Control	11.7 ± 1.6	0.1 ± 0.05	0.6 ± 0.2	10.5 ± 1.3	0.3 ± 0.2
3–4 hr after antigen or saline exposure					
Saline	12.2 ± 1.3	0.6 ± 0.2	1 ± 0.2	10.1 ± 1.2	0.4 ± 0.1
OA	15.2 ± 2.1	$5.4 \pm 0.8^{***}$	0.9 ± 0.3	8.2 ± 1.2	0.4 ± 0.03
18–24 hr after antigen or saline exposure					
Saline	10.1 ± 0.4	0.2 ± 0.03	0.9 ± 0.1	8.7 ± 0.4	0.2 ± 0.02
OA	$19.2 \pm 2.9^*$	$2.5 \pm 0.2^{***}$	$6.3 \pm 2.1^*$	9.8 ± 1	0.3 ± 0.1

Bronchoalveolar lavages were performed 3–4 hr and 18–24 hr after the exposure of animals to the antigen. The comparison of cell numbers between bronchoalveolar lavage fluid obtained from OA-exposed guinea-pigs and saline-exposed guinea-pigs were analysed by Student's *t*-test ($n=4$). * $P < 0.05$; *** $P < 0.001$.

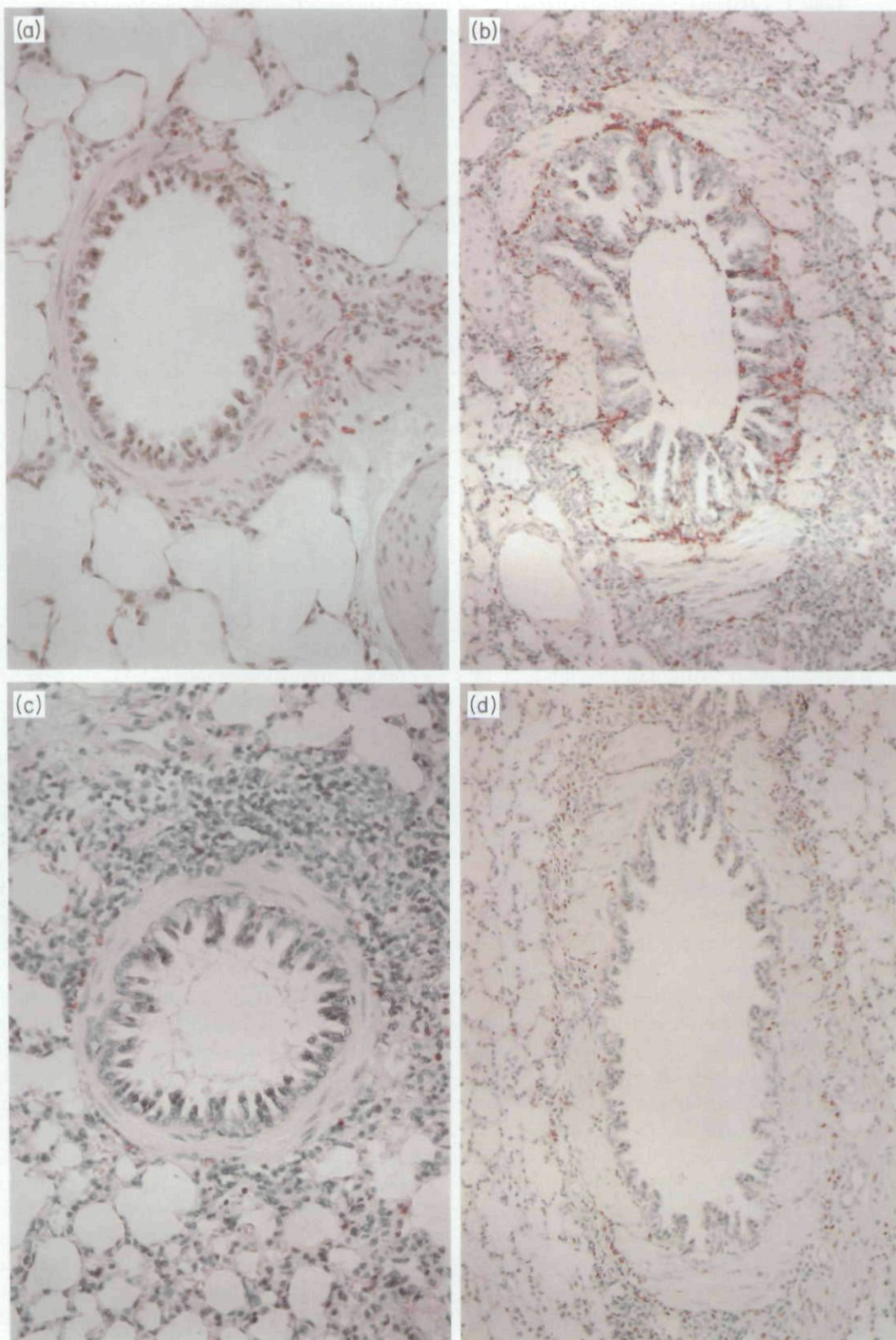
ward shift of the dose-related bronchopulmonary responses curve to aerosol ACh. Although this increase in bronchial reactivity is observed with either aerosolized ACh or 5-HT, as compared to control values higher alterations are noted with the latter agonist and at both time points. When ACh or 5-HT are injected intravenously, alterations of the dose-related bronchopulmonary responses are only observed 18–24 hr after antigen challenge. The increase in pulmonary reactivity observed under these conditions, and underlined by two different spasmogenic agents, is consistent with a non-specific effect, characteristic of the airway hyperresponsiveness in humans [6]. Similar observations were reported for the increased airway responsiveness when ACh was administered either by the intravenous or aerosol route after cigarette smoke exposure [13].

The observed differences in bronchopulmonary reactivity may be due to a bronchial inflammatory reaction induced by the aerosolized antigen. Indeed, in aerosol-sensitized guinea-pigs, no change in blood pressure and circulatory blood cell counts were recorded after antigen challenge by aerosol, a result in contrast to the data obtained when systemic sensitization and intravenous challenge of the animals is performed [7]. Thus, successive antigen aerosols might induce a bronchial reaction leading to hyperresponsiveness restricted to the airways. One explanation for this hyperresponsiveness may be an altered permeability of the airway wall. Indeed airway permeability is increased after exposure to cigarette

smoke [13,14] or antigen challenge [15], possibly allowing a greater proportion of inhaled ACh or 5-HT to reach the smooth muscle.

As allergen challenge induces complex processes in the lung that are considered to be of particular importance in the inflammatory events underlying hyperresponsiveness [16,17], the time-course of airway hyperresponsiveness was investigated. An increased reactivity to ACh was observed for 5 days after antigen challenge which returned to pre-challenge values by day 10. When guinea-pigs exhibiting normal bronchopulmonary reactivity were exposed to the antigen again, no further alteration in the bronchial response to either aerosol or systemic administrations of ACh was noted. These guinea-pigs, however, presented respiratory difficulties during the second antigen exposure, as in the case of the initial challenge. Furthermore, in separate experiments we observed that during the second antigen provocation these guinea-pigs developed a bronchopulmonary response of similar magnitude to that noted during the initial challenge or that of animals exposed to saline and used 10 days after the initial exposure (data not shown). This lack of change in bronchopulmonary reactivity to aerosolized ACh following a second antigen challenge is presently unexplained and remains to be investigated.

The relationship between the development of airway hyperresponsiveness and the late phase response is unclear. In both asthmatic patients [16] and sensitized sheep [18], late-phase responses are usually resolved by 24 hr. In the



guinea-pig, pulmonary function returns to nearly normal between 24 and 48 hr [2], but with a new decrease in airway conductance at 72 hr, suggesting that different mechanisms are operational in this species. Therefore, the severity and duration of bronchial hyperresponsiveness and of the late-phase response are likely to depend on the degree of sensitization of the animal, the amount of antigen presented to the airways, and finally the severity of the anaphylactic response.

Asthmatic reactions in allergic patients have been associated with a bronchoalveolar eosinophilia following allergen inhalation [19,20]. In the guinea-pig model reported here, only a significant neutrophilia in bronchoalveolar lavage fluids is observed 3–4 hr after antigen exposure. This increase in neutrophil counts is followed by a marked eosinophilia 18–24 hr after antigen exposure, an observation similar to that reported by Hutson *et al.* [2]. In human asthma, Metzger *et al.* [3] have also shown that the late airway response is accompanied by an influx of neutrophils followed by eosinophils. In contrast, compared to lungs from saline- or OA-exposed guinea-pigs removed 10 min after the challenge, a marked cell accumulation, principally eosinophils, was observed upon microscopic observation of lung tissue, both after 3–4 hr and 18–24 hr. Furthermore, as compared to time 3–4 hr, less eosinophils in the peribronchial area and submucosa are counted 24 hr after antigen challenge. A marked cell invasion of the bronchial submucosa including eosinophils, mast cells and macrophages has been described 1 hr after OA aerosol in sensitized guinea-pigs [21]. However, marked differences between the alveolar space (as reflected by the cell composition of the bronchoalveolar lavage fluids) and the lung tissue (as assessed by histological techniques) may exist, leading to invalid conclusions if one takes into account only the former. The kinetics of the passage of the neutrophils and the eosinophils through the airway walls probably explains the late appearance of the latter cell type in the bronchoalveolar lavage fluids.

Eosinophil migration through the bronchus and up to the lumen does not appear to be necessary for the development of bronchial hyperresponsiveness because no increase in eosinophil number in bronchoalveolar lavage fluids is observed 3–4 hr after antigen challenge.

After this time period, however, numerous eosinophils are observed upon histological examination of the lungs. In this regard, it would be relevant to distinguish between eosinophil migration and activation because the latter process is probably relevant to the development of bronchial hyperresponsiveness. Indeed, Iijima *et al.* [22] have reported that the eosinophils infiltrating around the bronchioli 7 hr after allergen challenge in the guinea-pig did not appear degranulated. Thus, in the initiation of bronchial hyperresponsiveness, mediator release from resident leucocytes, including eosinophils, may be as important as the subsequent recruitment of circulating cells.

Although the mechanisms underlying the pathogenesis of airway hyperresponsiveness are not elucidated at this time, a major role for eosinophils and their granule proteins is suspected [23]. In the present study, airway eosinophilia and increased bronchial reactivity did not appear to be associated with detectable disruption of epithelial cell integrity. Electron microscopy observations of the lungs that are currently in progress might help to elaborate a final statement regarding possible alterations of the epithelial layer.

In summary, we studied the development of bronchial hyperresponsiveness in guinea-pigs sensitized and challenged with the antigen by aerosol, a process that is associated with an increase of polymorphonuclear cell numbers in lung tissues and bronchoalveolar lavage fluids, although exhibiting distinct kinetics. In this experimental model, the histological changes were not correlated with the cell composition in the lavage fluids, suggesting a dynamic process of eosinophil infiltration (and probably activation) and passage through the bronchial walls. Finally, the dissection of the relationship between cell infiltration in the lung and alterations in bronchopulmonary functions may allow a better understanding of the complex mechanisms implicated in the immunological processes associated with hyperresponsiveness.

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Fig. 7. (a) Photomicrograph of a lung obtained 10 min after OA exposure from aerosol-sensitized guinea-pig. Luna stain. Magnification $\times 64$. (b) Photomicrograph of a lung obtained 4 hr after OA exposure from aerosol-sensitized guinea-pig, showing a marked eosinophil infiltration through the bronchial wall to the airway lumen. Luna stain. Magnification $\times 64$. (c) Photomicrograph of a lung obtained 4 hr after saline exposure from aerosol-sensitized guinea-pig. Luna stain. Magnification $\times 64$. (d) Photomicrograph of a lung obtained 24 hr after OA exposure from aerosol-sensitized guinea-pig. Eosinophil accumulation in the bronchial wall was also observed, although as compared to histological preparations of lungs removed 4 hr after OA exposure, less eosinophils in the peribronchial regions were observed. Luna stain. Magnification $\times 64$.

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