

Optimisation of the sensitisation conditions for an ovalbumin challenge model of asthma

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Abstract

Antigen inhalation in patients with atopic asthma results in an early asthmatic response (EAR), accompanied by a late asthmatic response (LAR) in 60% of patients, airway hyperresponsiveness (AHR) and inflammatory cell infiltration to the lungs. An ideal animal model of asthma should, therefore, provide at least these 4 features consistently and reproducibly. The aim of this study was to optimise the ovalbumin (OA) sensitisation conditions, for achieving EAR, LAR, AHR and cell influx, in a guinea-pig model of asthma. Animals were sensitised with 10 µg or 100 µg OA, as either a single or booster (day 1 and day 5) injection. Airway responses to inhaled OA (10 µg, 1 h) of actively sensitised, conscious guinea pigs were determined by whole body plethysmography as the change in specific airways conductance (sG_{aw}) over a 12 h period and at 24 h. Bronchoconstriction by inhaled histamine (1 mM) was used to investigate AHR, and inflammatory cell influx was determined by bronchoalveolar lavage (BAL), both at 24 h post-challenge.

A single sensitisation with 10 µg OA did not reveal an EAR, LAR or AHR following exposure to OA. However, total and differential cell counts (eosinophils and macrophages) were significantly greater 24 h post-challenge, when compared to saline-challenged sensitised animals. The addition of a booster injection of 10 µg revealed an EAR, but no LAR or AHR after ovalbumin inhalation. However, there was a significant cell influx. Sensitisation with 100 µg OA (single and booster injections) revealed all four parameters of the asthmatic response (EAR, LAR, AHR and cell influx). The incorporation of the booster sensitisation injection resulted in a prolongation of the LAR, and the AHR was more pronounced and cell influx increased significantly, when compared to all other sensitisation protocols. Thus, sensitisation with 100 µg OA (with a booster injection) can reveal an EAR, LAR, AHR and cell influx following inhalation exposure to OA (10 µg). Cellular infiltration to the lung may be a poor marker of the asthmatic response, as a threshold level of cell influx (eosinophils) appears to be required in order to elicit the LAR and AHR. There was an association between the LAR and AHR.

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1. Introduction

A single inhalation of antigen in individuals with atopic asthma results in an early phase bronchoconstrictor response followed by a late phase bronchoconstrictor response [1,2]. The early asthmatic response (EAR) occurs immediately after challenge and lasts up

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to 2 h [3,4]. This is followed by a late asthmatic response (LAR), 3 to 10 h after challenge [3,4]. The late reaction is accompanied by an increase in the responsiveness of the airways to a wide variety of stimuli, as reflected by airway hyperresponsiveness (AHR) to inhaled histamine and methacholine [5].

In asthmatic patients with allergen-induced late phase reactions, Durham and Kay [6] found that blood eosinophil counts were significantly elevated 24 h following antigen challenge. They suggested that there might be a direct association between eosinophils and airway reactivity in patients who develop late phase asthmatic reactions. It is thought that eosinophilic infiltration of the airways is correlated with the severity of the disease [7,8].

Guinea pigs sensitised to ovalbumin (OA) and then challenged with an aerosol of OA are often used as an animal model for examining lung function and cell infiltration after antigen exposure. The detection of both early and late phase bronchoconstrictor responses in guinea pigs has rarely been achieved in anaesthetised animals [9] and it is usually necessary to monitor airway function at regular intervals over a period of 24 h in conscious animals. Various techniques for measuring airway function have been developed based on whole body plethysmography [10–13]. Only three groups have demonstrated both early and late phases of bronchoconstriction after allergen challenge of guinea pigs [14–18]. Hutson et al. [15] and Church et al. [16] obtained both phases of equal size when using a macroshock inhalation of OA (2% for 5 min), protection from fatal anaphylaxis being provided by mepyramine cover. Danahay and Broadley [17] showed a late phase between 17 and 24 h after inhalation, which is much later than in humans. However, the appearance of the late phase has proved elusive and controversial and several groups have failed to demonstrate it even under a wide range of OA sensitisation and challenge conditions [19–21].

The aim of this study was to optimise the sensitisation protocol, in order to achieve the early and late airway function changes that mimic the human asthmatic condition together with AHR and cellular infiltration to the lungs, following sensitisation and exposure of guinea pigs to ovalbumin.

2. Methods

2.1. Animal husbandry

Male Dunkin–Hartley guinea pigs (300–350 g) were used. Animal welfare and experimental procedures were undertaken in accordance with the Animal Scientific Procedures Act 1986 under Home Office personal and project licenses. Sensitised or non-sensitised guinea pigs were used.

2.2. Sensitisation

All guinea pigs were actively sensitised by intraperitoneal (i.p.) injection of ovalbumin (OA, 10 µg or 100 µg per animal) and aluminium hydroxide (100 mg) in 1 ml of normal saline, in divided doses bilaterally. This was administered as either a single injection on day 1, or with the addition of a booster injection of the same solution on day 5. All procedures commenced 14–21 days after the first sensitisation injection. Non-boosted animals did not receive any injection on day 5. The selected sensitisation doses were based on previous levels used in this laboratory [17,18,22].

2.3. Ovalbumin exposure

Sensitised animals were placed in a stainless steel circular metal chamber (70 cm diameter, 16 cm depth) and exposed to a nebulised solution of OA (10 µg in saline), generated by a Wright nebuliser supplied with air at a pressure of 20 psi at 0.3 ml min⁻¹. Animals were exposed for 1 h, after the chamber was saturated with nebulised OA. Respiratory function was measured before, and at intervals up to 12 h and at 24 h post challenge. Control groups of animals sensitised by the same protocols were exposed to aerosolised saline.

2.4. Non-invasive measurement of specific airways conductance

Airway function was recorded as specific airway conductance (sG_{aw}) using whole body plethysmography in unanaesthetised, spontaneously breathing guinea pigs as described previously [22]. Pressure transducers (UP1, Lectromed, Welwyn Garden City, UK) attached to the pneumotachograph and plethysmograph chamber, respectively, measured changes in respiratory flow and box pressure, by means of an AcqKnowledge® software Biopac® data acquisition system [22], which replaced the original oscilloscope and angle resolver as used by Griffiths-Johnson et al. [11].

Guinea pigs are obligatory nose breathers [23] and it is possible that changes in sG_{aw} may also reflect nasal resistance, for example, arising from vascular changes in the nasal mucosa. The nose represents a major contributor in allergen-induced alterations of airways resistance in guinea pigs [24]. However, nasal involvement in responses to aerosols of histamine, carbachol and ovalbumin delivered to the nose has been measured by plethysmography in sensitised guinea pigs. They had no effect on nasal conductance even at doses 100 times higher than that required to decrease lower airway conductance [23]. sG_{aw} is a superior measure of airway function to airway resistance (R_{aw}), which is artificially influenced by changes in lung volume occurring when the animal is stressed by administration of a spasmogen.

2.5. Airway reactivity to histamine and cell counts

Basal specific airway conductance (sG_{aw}) was recorded before challenge with histamine. Histamine was delivered by a Wright nebuliser (1 mM in saline, nose only, for 20 s) into a plastic pipe (diameter 40 mm). Nose only inhalation was

achieved by inserting the guinea pigs snout through a diaphragm in the wall of the pipe and into the aerosol stream. Reactivity was assessed 24 h before and 24 h after OA or saline exposures. sG_{aw} was measured at 0, 5 and 10 min after histamine exposure. This dose was selected because it produces a threshold bronchoconstriction and, therefore, permits mea-

surement of bronchial hyperresponsiveness as an enhanced response. A higher concentration of histamine (3 mM) causes a substantial bronchoconstriction in this model. Control experiments have shown that there is no loss of responsiveness on repeating this challenge 24 h after a saline exposure [25].

Within 20 min of assessing airway reactivity to histamine at 24 h after OA or saline challenge, guinea pigs were overdosed with pentobarbitone sodium (Euthatal® 400 mg kg⁻¹ i.p.). A polypropylene cannula was inserted into the trachea and normal saline solution (1 ml 100 g⁻¹ guinea-pig weight) was passed into the lungs using a 5 ml syringe and was recovered 3 min later. This procedure was repeated and the total cell count (cells ml⁻¹) in the pooled lavage fluid was determined using a Neubauer haemocytometer. Differential cell counts were made after samples of the BAL fluid (100 µl) were centrifuged using a Shandon Cytospin (Cytospin3, Thermo Electro Bioscience Technologies UK, Runcorn, Cheshire, UK), onto glass microscope slides, at 1000 r.p.m. for 7 min. The resulting cytospin smear was differentially stained with 1.5% Leishman's stain (in 100% methanol), and a minimum of 500 cells was counted and the proportion of macrophages, eosinophils and neutrophils was calculated.

2.6. Data analysis

To reduce intersubject variability, specific airways conductance (sG_{aw}) is expressed as the percentage change from the basal value taken immediately before an exposure. The mean values ($\pm$ S.E.M.) for n animals are represented at each time point. Because the peak of the LAR occurred at different times in each animal, the LAR was also expressed as the mean peak response occurring between 6 and 12 h after OA challenge and shown separately on the right of each panel in Fig. 1. Responses to histamine before and after OA exposures were compared at the mean peak change in sG_{aw} by paired Student's t -tests. BAL cell counts ($\pm$ S.E.M.) were compared using analysis of variance, followed by an unpaired Student's t -test. From the mean time courses for changes in airway function following OA challenge, the peaks of the EAR and LAR (both as the peak of the mean time course and as the mean of the peak responses) were compared using analysis of variance (ANOVA), followed by the Bonferroni multiple comparisons test. Differences were considered statistically significant when $P < 0.05$.

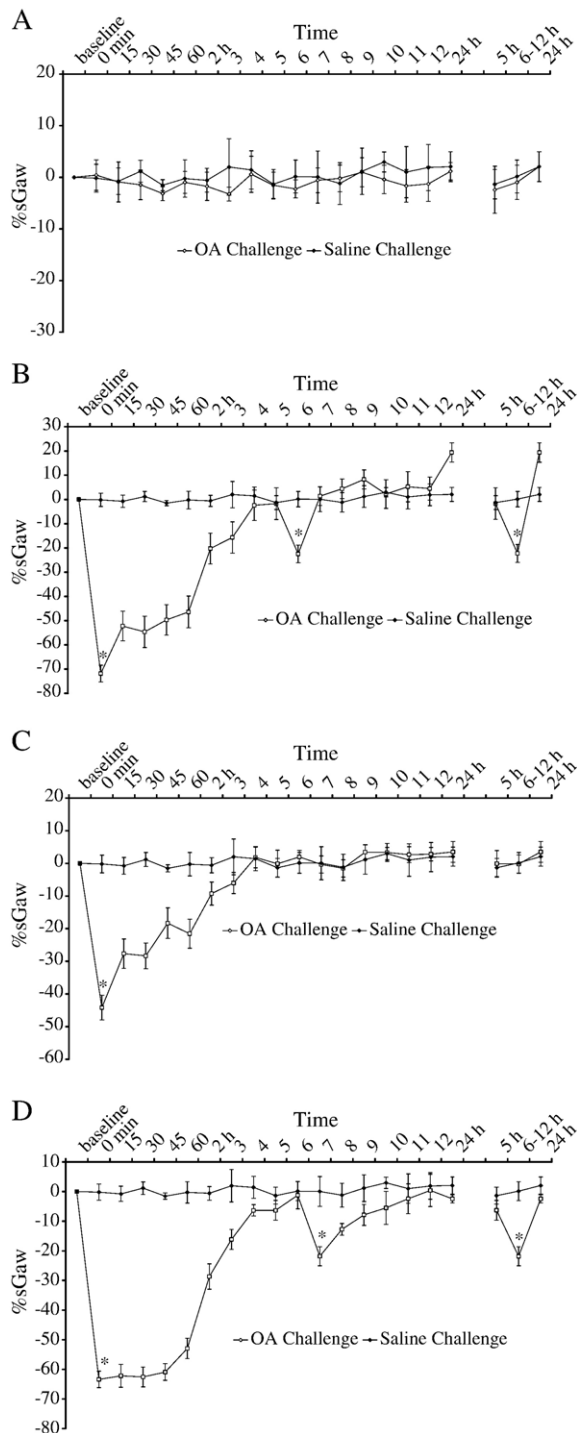


Fig. 1. Mean ($n=6$) time courses for the changes in specific airways conductance (sG_{aw}) following OA exposure (10 µg ml⁻¹) of conscious guinea pigs sensitised with (A) single injection of 10 µg OA; (B) single injection of 100 µg OA; (C) two injections of 10 µg OA on days 1 and 5 and (D) two injections of 100 µg OA on days 1 and 5. Mean changes in sG_{aw} are expressed as the mean $\pm$ S.E.M. percentage change from the pre-inhalation basal value. Negative values indicate bronchoconstriction. The LAR is also represented on the right of each graph as the mean peak fall in sG_{aw} occurring in each animal between 6 and 12 h. * represents a significant difference ($P < 0.05$) of the peak fall in sG_{aw} compared with saline challenged animals sensitised with two injections of 100 µg OA on days 1 and 5.

2.7. Materials

Ovalbumin (OA) was purchased from BDH Laboratory Supplies (Poole, Dorset, UK), aluminium hydroxide from Prolabo (Paris, France) and histamine diphosphate salt from Sigma (Poole, Dorset, UK). OA, aluminium hydroxide and histamine were all dissolved in 0.9% saline (Baxter, Thetford, UK).

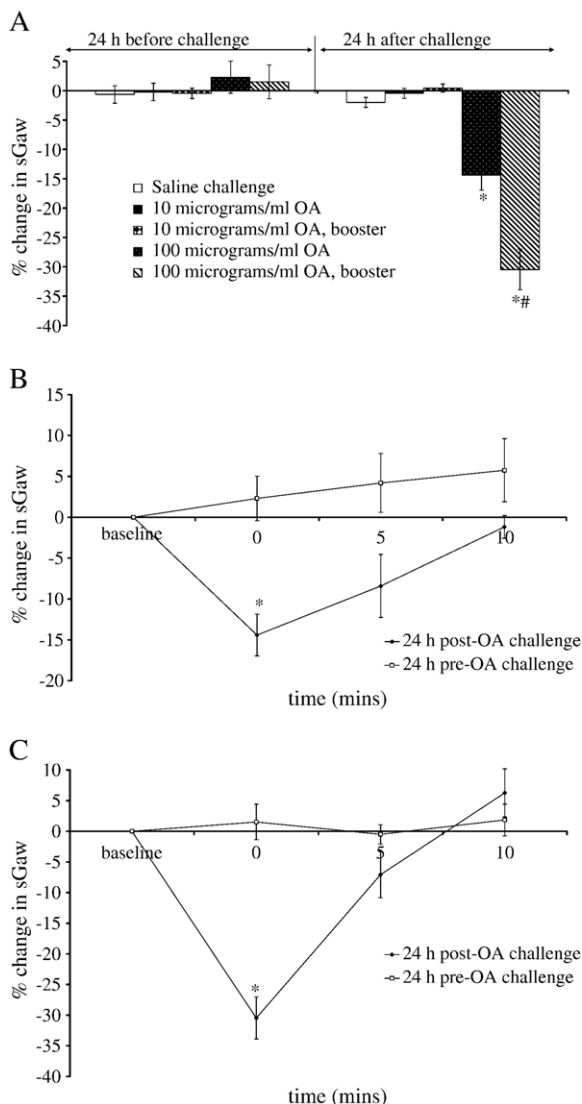


Fig. 2. Responsiveness of the airways to histamine (1 mM for 20 s), 24 h before and 24 h after OA challenges ($10 \mu\text{g ml}^{-1}$) in guinea pigs sensitised with 10 μg or 100 μg OA on day 1, or on days 1 and 5. Each point represents the mean ($n=6$) $\pm$ S.E.M. change in sG_{aw} expressed as a percentage of the baseline value. Negative values indicate bronchoconstriction. (A) Peak changes in sG_{aw} after histamine inhalation. Time course for changes in sG_{aw} before (○) and after (●) OA challenges in guinea pigs sensitised with 100 μg OA in either single (B) or two (C) doses. * represents a significant difference ($P<0.05$) compared with 24 h before exposure to OA or saline and # represents a significant difference ($P<0.05$) when compared with a single 100 μg sensitisation injection.

3. Results

3.1. Effect of OA sensitisation on airway function

Fig. 1 represents mean time courses over 24 h for the changes in sG_{aw} following inhalation exposure to OA (10 μg) or saline of conscious OA-sensitised guinea pigs sensitised with either a single injection of 10 μg OA, a single injection of 100 μg OA, 10 μg OA injection on days 1 and 5, or 100 μg OA injection on days 1 and 5.

Following sensitisation with a single dose of 10 μg OA (Fig. 1A), no early or late bronchoconstrictor response to inhaled ovalbumin was evident, as there was no significant reduction in sG_{aw} . Sensitisation with a single higher dose of 100 μg OA (Fig. 1B) was able to reveal an early bronchoconstrictor response that peaked immediately after exposure, with a maximum reduction in sG_{aw} of $-71.9 \pm 3.5\%$ ($P<0.05$, $n=6$); bronchoconstriction was no longer evident 4 h after exposure. Each animal displayed a late phase response occurring at 6 h, with a mean fall in sG_{aw} of $-22.3 \pm 3.7\%$ ($P<0.05$), which resolved after 1 h. Sensitisation with two doses of 10 μg OA revealed an early phase bronchoconstrictor response, which peaked within the first 15 s with a maximum fall in sG_{aw} of $-44.2 \pm 3.8\%$ ($P<0.05$, Fig. 1C). No animal displayed a late phase response between 6 and 12 h.

Sensitisation with two doses of 100 μg OA was able to reveal an early bronchoconstrictor response, which again peaked within the first 15 s after exposure, with a maximum reduction in sG_{aw} of $-63.4 \pm 2.8\%$ ($P<0.05$), and resolved after 6 h (Fig. 1D). Each animal displayed a late phase bronchoconstriction 7 h after challenge, with a significant late phase peak fall in sG_{aw} of $-22 \pm 3.2\%$, which resolved after 3 h.

3.2. Effect of OA sensitisation on airway reactivity

For all OA sensitisation procedures used, no animal displayed a significant bronchoconstrictor response to histamine (1 mM) 24 h before exposure to OA or saline (Fig. 2). This was therefore regarded as a threshold dose of histamine. 24 h after OA challenge, the animals sensitised with a single dose of 10 μg OA did not produce a bronchoconstrictor response to inhaled histamine, and were therefore not airway hyperresponsive (Fig. 2A). Animals sensitised with a single injection of 100 μg OA displayed AHR to inhaled histamine 24 h after OA challenge, with a significant peak fall in sG_{aw} of $-14.4 \pm 2.6\%$ after challenge, that resolved within 10 min of exposure (Fig. 2B). Sensitisation with 10 μg OA, followed by a booster injection also revealed AHR to inhaled histamine 24 h after OA exposure (Fig. 2A).

Animals sensitised with two doses of 100 μg OA on day 1 and day 5 (booster protocol) displayed AHR to histamine 24 h after exposure to OA, with a significant fall in sG_{aw} of $-30.5 \pm 3.4\%$, immediately after challenge (Fig. 2C). This was significantly greater than after any other challenge.

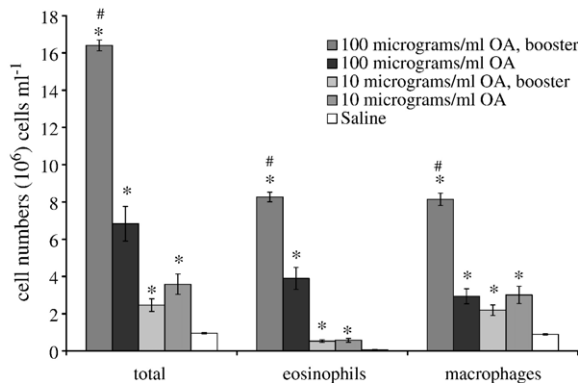


Fig. 3. Total cell count, macrophage and eosinophil counts from bronchoalveolar lavage fluid of sensitised guinea pigs 24 h after exposure to saline or OA inhalation ($10 \mu\text{g ml}^{-1}$). Guinea pigs were sensitised with 10 or 100 μg on day 1 or on days 1 and 5. Results are expressed as the mean $\pm$ S.E.M. numbers of cells ($\times 10^6$) ml^{-1} ($n=6$). * $P<0.05$ represents significant differences compared with saline-challenged animals (sensitised with two injections of 100 μg OA on days 1 and 5), # $P<0.05$ represents significant differences compared with OA-challenged animals sensitised using all other protocols.

3.3. Effect of OA sensitisation on total and differential cell counts

24 h after exposure to OA, all sensitised animals showed a significant increase in total cell numbers in BAL fluid, above that seen following exposure to saline (Fig. 3). Eosinophil and macrophage infiltration was also significantly increased in all sensitised guinea pigs, when compared to saline challenged animals. Animals sensitised using 100 μg OA, with the booster injection on day 5, displayed significantly greater total and differential cell counts, when compared to all other groups (Fig. 3). Neutrophils or lymphocytes were not seen in any of the samples of BAL fluid.

4. Discussion

In this study, we have shown that the demonstration of both immediate and late phase bronchoconstrictor responses, AHR and inflammatory cell infiltration, in sensitised guinea pigs following allergen challenge, depends upon the sensitisation protocol.

Guinea pigs sensitised using a single injection of 10 μg OA were unable to produce an EAR and LAR, or AHR to inhaled histamine following inhalation of OA. Total cell counts, eosinophil and macrophage numbers were, however, significantly increased 24 h post-OA challenge, when compared to saline-challenged animals.

The incorporation of a booster injection 5 days after the initial injection of 10 μg of OA was able to reveal an EAR that peaked immediately after challenge. However, no LAR was evident 6–10 h after challenge, and AHR

to inhaled histamine was absent. Total and differential (eosinophils and macrophages) cell counts were significantly elevated when compared to saline-challenged guinea pigs.

Animals sensitised using 100 μg OA (both single and booster protocols) were able to produce all four parameters studied; EAR and LAR, AHR to inhaled histamine and cellular infiltration into the airways. The incorporation of the booster injection on day 5 of the procedure resulted in the prolongation of the LAR from 1 h following the single injection, to a 2 h response following the booster injection. However, the peak reduction in sG_{aw} of the LAR was not significantly different for the two groups. AHR to inhaled histamine was also more pronounced in the group sensitised using the booster injection, as a greater bronchoconstrictor response was evident. Total and differential (eosinophils and macrophages) cell counts were significantly increased in the booster-injected group, in the case of 100 μg dose, when compared to the single injection sensitisation protocol. A previous study has also shown that a low sensitising dose of OA (1 μg) can induce eosinophilia without AHR but a larger dose can also induce AHR [26].

It can therefore be seen from the results presented here that sensitisation using 100 μg of OA, with the addition of a booster injection on day 5, produced an EAR and LAR, a more pronounced bronchoconstrictor response to inhaled histamine, and a significant increase in total and differential (eosinophilia and macrophages), when compared to all other sensitisation procedures investigated, and saline-challenged animals.

The antigen challenge of sensitised animals initiates the release of pharmacological mediators including histamine, leukotrienes, prostaglandins and PAF, possibly from mast cells and macrophages, which act on smooth muscle to cause bronchospasm [27–29]. This is seen as the early phase bronchoconstrictor response. Allergen can also activate T cells which in turn release cytokines, for example, IL-3, IL-5 and IL-8, GM-CSF (granulocyte-macrophage colony stimulating factor) TNF- α (tumour necrosis factor) and eotaxin, causing the migration of eosinophils into the airways and the subsequent activation of these cells [30]. Degranulation of eosinophils within the mucosa leads to the release of granules containing major basic protein, eosinophil cationic protein and eosinophil peroxidase. This can lead to tissue damage and is thought to play an important role in the underlying airway hyperreactivity that is a hallmark of asthma [31]. The release of PAF, leukotrienes and basic proteins by eosinophils [32,33], which cause bronchial inflammation, could also be associated with the late phase bronchoconstrictor response [6].

Eosinophils were found to accumulate in the airways in the present study, as measured by their increased appearance in the BAL fluid at 24 h after OA exposure. It has been suggested that eosinophils and their mediators are involved in the development of the LAR after allergen challenge [8,34,35]. Significant eosinophilia was found in the BAL fluid of patients who developed a late phase response, when compared to patients who did not produce a late response, 6 h after exposure to allergen [36]. However, this has not been confirmed in the present study, since animals that did not produce a late asthmatic response, for example animals sensitised using 10 µg OA in both protocols (single and booster injections), did produce significant eosinophilia in the BAL, 24 h after OA challenge. In the present study, we have only measured inflammatory cells in the lavage fluid at 24 h after OA challenge and it is possible that cellular levels at the time of the LAR are different. Also, cells in the lung tissue are a better marker than lavage fluid levels. However, the present results show that lower levels of sensitisation were able to produce significant cell influx to the lungs, without the other responses (i.e. EAR, LAR and AHR). Cellular infiltration into the BAL fluid alone may not therefore be required for the other important parameters of the asthmatic response to appear. We can, therefore, conclude that cellular infiltration alone may be poor index of the asthmatic response in animal models, as a threshold level of cell influx may be required in order to elicit the other parameters of the asthmatic response investigated. The question remains whether a higher level of eosinophilia is required for induction of the functional changes of the airways and whether eosinophils are a causative factor or whether AHR, EAR and LAR are independent of the eosinophils. Until the precise mechanism for these functional changes are identified, it will not be possible to assign an eosinophil-related mediator as a causative factor. Previous studies have also dissociated the AHR from eosinophilia in guinea pigs since an anti-IL-5 antibody could block eosinophilia but not the AHR [37]. Similarly, in asthmatic patients, the IL-5 antibody reduced blood eosinophilia but not the AHR or LAR [38]. In contrast, the anti-IL-5 antibody has been shown to inhibit both the LAR and eosinophilia [39]. Thus, the strength of evidence suggests that IL-5 is required for eosinophilia but by blocking this, the AHR and LAR remain, which indicates that they have an additional origin. In contrast to the present study, low doses of house dust mite aerosol could induce AHR in mice without eosinophil influx [40].

The neutrophil dominates most acute inflammatory reactions and, although it is less conspicuous than the eosinophil in the airway wall of asthmatic subjects, it is an

extremely potent cell, capable of producing prostaglandins and thromboxane, leukotriene B₄ and PAF [41]. In this study, neutrophilia was not significant 24 h after OA exposure, when compared to saline-challenged animals. Hutson et al. [15] found that after depletion of neutrophils by injection of a polyclonal antibody against guinea-pig neutrophils, animals still produced a normal late phase response following OA challenge. This was cited as evidence that neutrophils do not participate in the late asthmatic response. This investigation has also confirmed that this polymorphonuclear leukocyte does not play a crucial role in the pathophysiologic processes that give rise to the late phase response. However, it has also been suggested that neutrophils may play a role in the asthmatic response, as neutrophil levels were significantly raised in BAL fluid 1 h after allergen challenge in sensitised guinea pigs [22]. Raised neutrophil levels subsided within 12 h, which may explain why neutrophilia was not evident 24 h after challenge in this study.

AHR is probably acquired during life as a result of airway reactions to various stimuli, although genetic factors such as atopy are likely to predispose the person to develop hyperresponsiveness. It has been shown that allergen-induced AHR occurs in association with the LAR [42]. This has been confirmed in this study as animals that developed a LAR were hyperresponsive to inhaled histamine, while animals that did not reveal a late response did not respond to histamine. We have also been able to show that the more prolonged the LAR, the greater the bronchoconstrictor response to inhaled histamine. For example, the group of animals that revealed a late asthmatic response that resolved within 1 h had a maximum reduction in sG_{aw} of $-14.4 \pm 2.6\%$ immediately after exposure to inhaled histamine. Animals whose late phase lasted for 3 h had a maximum reduction in sG_{aw} of $-30.5 \pm 3.4\%$ immediately after exposure to 1 mM histamine. This association between LAR and AHR does not always occur in clinical practice as there are examples of asthmatics with no AHR 24 h following a LAR, although this was attributed to an already raised responsive state prior to the allergen challenge [43]. In another study, however, those patients failing to demonstrate a LAR also failed to exhibit AHR, but other patients with a LAR had AHR at 7 and 30 h but not prior to the LAR [44].

In summary, the present study has demonstrated that sensitisation using 100 µg OA as a booster injection, followed by a 10 µg OA challenge 12–16 days later produces EAR and LAR, cellular infiltration to the airways and AHR to inhaled histamine. We have also confirmed that AHR is associated with the LAR following allergen challenge in sensitised guinea pigs. Cellular infiltration to the lungs, however, is not a sufficient index

of the allergic response in the airways, as a threshold level of cell influx may be needed in order to elicit the other parameters of asthma (EAR, LAR and AHR). We therefore suggest that eosinophilia alone is not a sufficient marker of the LAR, unless the threshold level of cell influx needed to elicit the other parameters of asthma is established.

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