Airway hyperresponsiveness is associated with activated CD4\(^+\) T cells in the airways


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First published May 29, 2009; doi:10.1152/ajplung.00053.2009.— It is widely accepted that atopic asthma depends on an allergic response in the airway, yet the immune mechanisms that underlie the development of airway hyperresponsiveness (AHR) are poorly understood. Mouse models of asthma have been developed to study the pathobiology of this disease, but there is considerable strain variation in the induction of allergic disease and AHR. The aim of this study was to compare the development of AHR in BALB/c, 129/Sv, and C57BL/6 mice after sensitization and challenge with ovalbumin (OVA). AHR to methacholine was measured using a modification of the forced oscillation technique in anesthetized, tracheostomized mice to distinguish between airway and parenchymal responses. Whereas all strains showed signs of allergic sensitization, BALB/c was the only strain to develop AHR, which was associated with the highest number of activated (CD69\(^+\)) CD4\(^+\) T cells in the airway wall and the highest levels of circulating OVA-specific IgG1. AHR did not correlate with total or antigen-specific IgE. We assessed the relative contribution of CD4\(^+\) T cells and specific IgG1 to the development of AHR in BALB/c mice using adoptive transfer of OVA-specific CD4\(^+\) T cells from DO11.10 mice. AHR developed in these mice in a progressive fashion following multiple OVA challenges. There was no evidence that antigen-specific antibody had a synergistic effect in this model, and we concluded that the number of antigen-specific T cells activated and recruited to the airway wall was crucial for development of AHR.

Asthma is a chronic allergic lung disease characterized by airway hyperresponsiveness (AHR) to bronchoconstricting agents (7). In recent years, mouse models of asthma have become increasingly popular as tools for understanding the pathobiology of this disease (11, 12, 28).

Induction of allergic lung responses in mouse models of asthma typically involves systemic immunization with antigen in conjunction with a T helper (Th)-2 skewing adjuvant (48) followed by antigen challenge via the airways. Inbred strains of mice are known to differ in their capacity to exhibit AHR in response to these protocols (31, 32, 40, 41, 47). When the antigen ovalbumin (OVA) is used, BALB/c and AKR (8) mice develop AHR, whereas others, such as the C57BL/6 and 129/Sv strains, are hyporesponsive (8, 41). Although this strain-dependent responsiveness has been linked to differences in IgE production and eosinophil recruitment to the lung (8, 39, 41), such links are tenuous as these surrogate immunological markers are often discordant with AHR (13, 47).

In recent years, considerable attention has been paid to the role of thymus-derived lymphocytes or T cells in asthma. Studies have shown increased numbers of lymphocytes with a Th-2 cytokine phenotype in the bronchoalveolar lavage (BAL) of asthmatics compared with healthy controls (38), and T cell peptide-induced AHR in human asthmatics is associated with the induction of local CD4\(^+\) T cells (2). Studies in mice have demonstrated the attenuation of lung responsiveness in experimental asthma following the depletion of CD4\(^+\) T cells (16, 29). Similarly, transfer of primed wild-type (18, 19, 22) or antigen-specific transgenic T cells (3, 10, 25) results in the induction of allergic responses and AHR. However, the majority of these studies have used either an inappropriate measure for AHR known as enhanced pause (Peng; Refs. 10, 25), which has been widely criticized as a proxy measure of airway resistance (6), or global measures of lung resistance (3, 10). Thus the role of CD4\(^+\) T cells in the induction of increased responsiveness of the airways (rather than the lung as a whole) to bronchoconstricting agents, a cardinal feature of asthma, remains to be determined.

The primary aim of this study was to determine whether a strain-dependent association exists between the presence of activated CD4\(^+\) T cells in the airways and the development of AHR in an OVA sensitization/challenge model to test our underlying hypothesis that recruitment of activated CD4\(^+\) T cells to the airway wall is the primary determinant for development of AHR. We found that AHR in three commonly used strains of inbred mice (BALB/c, 129/Sv, and C57BL/6) was correlated with the number of CD4\(^+\)CD69\(^+\) T cells in the airway wall but was also associated with systemic production of OVA-specific IgG1, raising the possibility that antigen-specific antibody could also be playing a role. To test this further, we employed models of allergen-specific T cell adoptive transfer and passive antibody sensitization of AHR-susceptible BALB/c mice to demonstrate that genetic susceptibility to AHR development is primarily CD4\(^+\) T cell-dependent and can develop independently of elevated levels of allergen-specific antibodies.

**MATERIALS AND METHODS**

*Animals.* Eight-week-old specific pathogen-free female BALB/c (H-2\(^d\)) and C57BL/6 (H-2\(^b\)) mice were purchased from Animal Resource Center (ARC, Murdoch, Western Australia). 129/Sv (H-2b)
and BALB/c DO11.10 mice were obtained from breeding colonies of these strains at the Telethon Institute for Child Health Research. All experiments were approved by the Institutional Animal Ethics and Experimentation Committee and conformed to the guidelines of the National Health and Medical Research Council of Australia.

Experimental protocol. BALB/c, C57BL/6, and 129/Sv mice were sensitized by intraperitoneal injection with 20 μg of chicken egg OVA (Sigma) in 200 μl of aluminum hydroxide (alum; Serva) on days 0 and 14. On day 21, mice were challenged with aerosolized 1% OVA or saline (control). Inflammatory cells, serum antibodies, T cell number, and AHR were assessed on day 24 (24 h postchallenge). This protocol is a well-established model of allergic asthma disease in BALB/c mice that demonstrates a number of features of atopic asthma including AHR, eosinophilia, and antibody production (48).

AHR. Respiratory system input impedance (Zrs) was measured using the low-frequency forced oscillation technique (LFOT) as described previously (48). Briefly, mice were anesthetized, tracheostomized, and ventilated (flexiVent; SCIREQ) at 450 beats/min with a tidal volume of 8 ml/kg and 2 cmH2O PEEP. This ventilation regime allows for the measurement of LFOT without the need for paralysis. Lung volume history was standardized before measurement of lung mechanics. Zrs was measured during 16-s periods of apnea using a signal containing 19 mutually prime sinusoidal frequencies ranging from 0.25 to 19.625 Hz. A four-parameter model with constant-phase resistance (R) from 0.25 to 19.625 Hz. A four-parameter model with constant-phase allows for the measurement of LFOT without the need for paralysis.

Inflammatory cells and serum antibodies. BAL was collected and processed for cell counts as described previously (48). Blood was collected via cardiac puncture and sera prepared for analysis of total IgE and OVA-specific IgG1 by ELISAs. ELISA plates (Nunc MaxiSorp) were coated overnight with 2 μg/ml purified anti-mouse IgE (Pharmingen) or 10 μg/ml OVA for IgE and IgG1 analysis, respectively. Plates were washed with 0.05% Tween 20 (Sigma) in PBS between each step. Plates were blocked with 1% BSA in PBS. Five LFOT measurements were made after each dose of MCh, and the maximum response recorded for Rsw, G, and H from these 5 measurements was used for construction of dose-response curves and comparison of responses between groups.

Activated CD4^+ T cells in the airways. Groups of 7 mice were euthanized by an overdose of xylazine/ketamine. Main conducting airways (trachea) were isolated, sliced with a scalpel blade, diced, and digested to prepare single-cell suspensions as described previously (43). T cells were identified using MAbs against CD3ε, CD4, and CD8α (clones 145-2C11, RM4-5, and 53-6.7, respectively; BD Biosciences), and activation was measured using a MAb against CD69 (H1.2F3; BD Biosciences). CD69 is an early activation marker that is expressed by CD4^+ T cells and has been used a reliable marker for T cell activation by inhaled OVA in both the lung-draining lymph nodes and airway tissue (43, 44). All MAbs were incubated for 30 min on ice using previously optimized dilutions. Samples were collected using a 4-color FACSCalibur or a 6-color LSR II (BD Biosciences) and analyzed using FlowJo software (Tree Star, Stanford, CA). These experiments were repeated twice (2 replicates) on separate groups of mice (n = 7 in each group) for each treatment and strain.

Adoptive T cell transfer and passive serum sensitization. We (46) have previously established model of T cell transfer that does not rely on the presence of antibody-inducing adjuvants and results in the trafficking of antigen-specific T cells to the airways of recipient mice. Peripheral lymph nodes (PLNs) were collected from 8- to 12-wk-old naïve BALB/c DO11.10 mice that express a transgenic T cell receptor (TCR) specific for the H-2d-restricted immunodominant peptide of OVA and pooled, and a single cell suspension was created by mixing them with a scalpel and forcing the mixed PLNs through a metal sieve. Cells were washed twice with GKN buffer (11 mM D-glucose, 5.5 mM KCl, 137 mM NaCl, 25 mM Na2HPO4, and 5.5 mM NaH2PO4·2H2O) supplemented with 0.2% BSA and then twice with PBS. 1 × 10^7 Cells in 200 μl in saline were injected intravenously into naïve recipient BALB/c mice. Recipients were immunized 2-3 days later with 100 μg of OVA in 50 μl of saline intranasally or 50 μl of saline intranasally under light halothane anesthesia. Two weeks later, all mice were challenged with 25 μg of OVA (in 50 μl of saline) intranasally 1, 3, or 5 times corresponding to days 14-16, 19, and 20 after transfer. We (46) have shown previously that the adoptively transferred CD4^+ T cells expressing the transgenic TCR for OVA are the only cells activated by intranasal OVA administration in this model. AHR was assessed 24 h (1× OVA intranasally) or 48 h (3 or 5× OVA intranasally) after challenge. These time points were chosen to coincide with the peak of AHR shown in our (44, 48) previous studies on single and multiple OVA challenges to maximize our ability to detect AHR in this model.

For passive serum sensitization, BALB/c mice were injected intravenously with 200 μl of sera from OVA-sensitized and -challenged BALB/c mice. Injected sera contained high titers of OVA-specific IgG1 and IgE as determined using the methods described above. Passively sensitized mice were challenged with OVA intranasally 12 h after injection, and AHR was assessed 24 h later.

Statistical analyses. Between strain and treatment comparisons were made using ANOVA and Holm-Sidak post hoc tests. All statistics were produced with SigmaStat 3.5 (Systat Software).

RESULTS

AHR. BALB/c mice developed AHR as indicated by increased responses to 30 mg/ml MCh in Rsw (P = 0.02) and G (P = 0.04) but not H (P = 0.33) compared with controls (Fig. 1). C57BL/6 mice showed increased responses to MCh in the tissues (G, P < 0.001; H, P < 0.001) but not Rsw (P = 0.10) (Fig. 1). 129/Sv mice showed no increased responses (Rsw, P = 0.60; G, P = 0.68; H, P = 0.10; Fig. 1).

BAL inflammatory cells. 129/Sv mice were the only strain to show a significant increase in total cell count (TCC) following OVA challenge (BALB/c, P = 0.15; 129/Sv, P < 0.001; C57BL/6, P = 0.16) compared with controls (Fig. 2, inset). There were no differences in TCC between strains in control mice (Fig. 2, inset).
OVA challenge elicited higher numbers of eosinophils in the BAL of 129/Sv mice compared with OVA-challenged BALB/c (P < 0.001) and C57BL/6 (P < 0.001) mice (Fig. 2). OVA challenge increased the numbers of eosinophils (P < 0.001) and neutrophils (P < 0.001) in the BAL of all strains compared with controls [BALB/c eosinophils not detected, neutrophils 1,295(1,687) cells/ml; 129/Sv eosinophils 1,639(1,379) cells/ml, neutrophils 1,196(1,038) cells/ml; C57BL/6 eosinophils 14(38) cells/ml, neutrophils 598(653) cells/ml], however, there was no difference between strains (P = 0.35) in the number of neutrophils following OVA challenge (Fig. 2). Lymphocytes were rarely detected in the BAL samples from these mice, and there was no significant difference in the number of lymphocytes between strains or between OVA-challenged and control mice (data not shown).

Serum OVA-specific IgE and IgG1. OVA-challenged 129/Sv mice had higher total (Fig. 3A) and OVA-specific (Fig. 3B) IgE than BALB/c (total, P < 0.001; OVA-specific, P < 0.001) and C57BL/6 mice (total, P < 0.001; OVA-specific, P = 0.002). Control and OVA-challenged C57BL/6 mice developed moderate levels of total and OVA-specific IgE, whereas BALB/c mice showed low to undetectable levels (Fig. 3, A and B). OVA challenge failed to boost serum IgE levels in any strain (total, P = 0.39; OVA-specific, P = 0.77; Fig. 3, A and B). In contrast, high levels of OVA-specific IgG1 were detected in sensitized control and OVA-challenged BALB/c mice (vs. 129/Sv, P = 0.02; vs. C57BL/6, P < 0.001), whereas both C57BL/6 and 129/Sv developed low levels of IgG1 (P = 0.24; Fig. 3C). OVA challenge did not boost OVA-specific IgG1 in any strain (P = 0.42; Fig. 3C).

Number of activated CD4+ T cells in the main conducting airways. Total numbers of CD69+ (activated) CD4+ T cells in tracheal digests were high in sensitized control and OVA-challenged BALB/c mice (Fig. 4). In contrast, OVA-sensitized...
129/Sv mice showed low numbers of activated CD4+ T cells in the airway wall, whereas these cells were not detected in the airways of sensitized C57BL/6 mice (Fig. 4). The total number of cells in the tracheal digests was consistent between strains and treatments so the percentage of activated CD4+ T cells showed the same pattern as the absolute numbers [BALB/c saline (3.8, 3.1%) OVA (2.9, 2.3%); 129/Sv saline (0.3, 1.3%) OVA (0.5, 0.4%); C57BL/6 saline (0.1, 0.8%) OVA (0.2, 0.1%)]. Therefore, the presence of elevated numbers of activated CD4+ T cells in airway wall of allergen-sensitized mice was positively correlated with susceptibility to AHR.

The role of OVA-specific T cells and IgG1 in the induction of AHR in BALB/c mice. Although all strains showed features of allergic sensitization, AHR only developed in the BALB/c strain that had the highest levels of OVA-specific IgG1 and numbers of CD4+ CD69+ T cells in the trachea. AHR did not appear to depend on IgE or eosinophils, since BALB/c mice only developed moderate levels of eosinophilia and had low levels of total and OVA-specific IgE compared with the other strains. Thus the susceptibility of BALB/c mice to AHR was related to the enhanced number of activated CD4+ T cells recruited to the airways and/or increased levels of OVA-specific IgG1.

To examine the role of allergen-specific CD4+ T cells in the development of BALB/c AHR, we took advantage of an adoptive transfer model utilizing OVA-specific CD4+ T cells from BALB/c DO11.10 mice that express a transgenic TCR that recognizes the major epitope of OVA (46). This immunization protocol does not induce high levels of specific antibody (M. E. Wikstrom and P. A. Stumbles, unpublished observations).

There was no difference in $R_{aw}$ between OVA-immunized and unimmunized controls at 30 mg/ml MCh ($P = 0.97$) 24 h after 1 ($P = 0.97$) or 3 OVA challenges ($P = 0.60$) (Fig. 5A). In contrast, OVA-immunized T cell recipient mice challenged five times had increased responses in $R_{aw}$ to 30 mg/ml MCh ($P = 0.049$) compared with unimmunized mice (Fig. 5A).

We confirmed that the sera of OVA-immunized mice contained very low levels of OVA-specific IgG1 (Fig. 5B), and although there was an increase after five OVA challenges, up to 30 ng/ml, they remained very low compared with levels in the sera of alum-sensitized BALB/c mice (Fig. 3B). However, it was still possible that this IgG1 contributed to the development of AHR, so we repeated the adoptive transfer experiment, adding an intravenous injection of OVA-immune sera (containing high levels of OVA-specific IgG1 as per Fig. 3B) either alone (data not shown) or together with the transgenic T cells 24 h before OVA challenge. When the airway response to MCh was measured, there was no difference in $R_{aw}$ to 30 mg/ml MCh for mice passively sensitized with OVA-immune sera and DO11.10 T cells or control sera and DO11.10 T cells (Fig. 5C). Therefore, OVA-specific antibody alone or in combination
with antigen-specific T cells did not act to increase the responsiveness of the airways to a single OVA challenge.

**DISCUSSION**

Airway inflammation and AHR are cardinal features of asthma (9). Although many studies have identified underlying genetic factors as contributors to AHR (15, 35, 42), the genetic regulation of cellular recruitment to the airways and the contribution of this to the development of AHR is unclear. To investigate this, we compared the susceptibility of three strains of inbred mice to AHR following allergen sensitization and challenge. Consistent with previous reports, BALB/c mice were susceptible to the development of AHR (13, 47), whereas the C57BL/6 (13, 47) and 129/Sv strains (27) were resistant.

The mechanisms underlying AHR are still poorly understood. Previous use of the term AHR is confounded by the fact that most of methods used are not specific to the airways. A number of studies have used Penh (20), which has been widely criticized as a proxy for the measurement of airway resistance (1, 6, 33). Those studies that have used more invasive, and thus more accurate (5), techniques have typically measured global changes in lung resistance. As such, studies purporting to measure AHR have not necessarily been measuring airway responses. In this study, we have been able to demonstrate that BALB/c mice had hyperresponsiveness in the airways. We observed a significant increase in tissue responses in the C57BL/6 strain, in the absence of any hyperresponsiveness of the airways. Since techniques that measure total lung resistance cannot distinguish between airway and parenchymal responses, it is entirely possible that the AHR observed in C57BL/6 mice in previous studies (41) was due to changes in the lung periphery rather than the conducting airways.

The susceptibility of BALB/c mice to the development of AHR correlated with high levels of circulating IgG1, consistent with the Th-2-biased responses of this strain. In stark contrast, AHR was dissociated from IgE as the 129/Sv strain showed the highest levels of total and antigen-specific IgE but no evidence of AHR. This is consistent with our (48) previous study showing no association between AHR and levels of IgE. A study by Oshiba and colleagues (37) reported that passive sensitization with anti-OVA IgE followed by challenge induces AHR in vitro in BALB/c mice. However, the same study also showed that anti-OVA IgG1 could induce increased airway responses (37). Whether these results have any relevance to AHR as measured in vivo has yet to be determined. Regardless, our results do not allow us to rule out a role for IgG1 in the development of AHR. Indeed, the absence of high levels of

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Fig. 4. Numbers of CD4<sup>+</sup>CD69<sup>+</sup> T cells in airway tissue of sensitized and challenged mice. Single cell suspensions were prepared from tracheal samples taken from OVA-sensitized BALB/c, 129/Sv, and C57BL/6 mice 24 h after challenge with saline (control) or OVA, stained with MAbs to CD4 and CD69, and analyzed by flow cytometry. Data are expressed as total number of CD4<sup>+</sup>CD69<sup>+</sup> T cells in each tracheal digest, where total cell recoveries were similar for each mouse strain and treatment group. Each data point represents a pooled sample of tracheas from separate experiments (n = 7 in each group).

Fig. 5. Maximum responses to 30 mg/ml MCh (A) and serum OVA-specific IgG<sub>1</sub> levels (B) for naïve BALB/c mice that received intravenous DO11.10 CD4<sup>+</sup> T cells, have been immunized with intranasal OVA (black bars) or saline (control; white bars), and were challenged 2 wk later 1, 3, or 5 times (C) for naïve BALB/c mice that received intravenous DO11.10 CD4<sup>+</sup> T cells and have been immunized with intranasal OVA followed by high titer OVA-specific IgG<sub>1</sub> (black bar) or control sera (white bar) 2 wk later and a single intranasal OVA challenge. All data are means (SD); n = 5–9.
specific antibody in our adoptive transfer model may explain why AHR was not observed after a single OVA challenge. To test this, we administered OVA-immune serum before challenge and found no AHR indicating that, under these conditions, specific antibody could not induce AHR. However, we do not know whether a single injection of sera achieved sufficiently high levels of antibody, although we (44) have shown previously that this dose increases OVA uptake in the airways of naïve BALB/c mice, leading to an increase in T cell activation.

There did not appear to be any association between the number of eosinophils in the BAL and AHR in the strains of mice examined here. The role of eosinophils in the development of AHR remains controversial (26, 36). Some studies have purported to show a dependence of AHR on eosinophils (8, 14, 41), whereas others have argued otherwise (17, 47), although, for the reasons already mentioned, conclusions regarding these inflammatory cells and their role in the induction of airway responsiveness may have been obscured by the use of Penh and/or global measures of lung resistance. A recent study has even suggested that eosinophils are essential in the recruitment of effector T cells to the airway (24), however, although we cannot rule out a role for eosinophils in the induction of AHR, it was clear from our results that a marked eosinophilic response (e.g., 129/Sv) alone was not sufficient to induce AHR.

A consistent finding in the current study was the presence of elevated numbers of activated CD4+ T cells in the airway wall of OVA-sensitized BALB/c mice, which correlated closely with AHR susceptibility. This suggested that recruitment of antigen-specific T cells to the airways was a key predictor of strain susceptibility to AHR, although a proinflammatory role for allergen-specific IgG1 could not be ruled out. To test this, OVA-specific transgenic CD4+ T cells were adoptively transferred into wild-type BALB/c mice in which circulating IgG1 could not be detected at the time of T cell transfer. AHR could be induced after OVA challenges, indicating that antigen-specific CD4+ T cells alone were sufficient to induce AHR on a genetically permissive background. In recent times, it has become clear that T lymphocytes are critical in the pathobiology of allergic asthma. For example, asthma has increased numbers of CD4+ T cells in bronchial biopsies (4) and lavage samples (45) compared with healthy subjects, although work in animals has demonstrated that depletion of CD4+ T cells prevents the induction of lung responses (16, 29). We selected an adoptive transfer model involving OVA-specific CD4+ T cells from DO11.10 TCR transgenic mice where a strong local T cell response can be induced in the respiratory tract in the absence of high levels of circulating specific antibody. We (46) have shown previously that OVA-specific CD4+ T cells are efficiently activated and recruited to the respiratory tract in this model. We demonstrate here that AHR can be produced in the absence of high levels of specific antibody. The question arises, however, as to why multiple aerosols were required to produce AHR compared with a single aerosol in BALB/c mice sensitized with OVA/alum. We believe the difference lies in the sensitizing protocols; in the former, two doses of OVA were administered in alum, whereas in the latter a single dose of OVA in saline was used. Alum produces a stronger and longer lasting T cell response compared with antigen alone (26). Thus we hypothesize that although a single dose of OVA alone failed to generate sufficient numbers of activated CD4+ T cells in the airways, multiple challenges were presumably required to recruit antigen-specific effector T cells.

T cell homing and recruitment have been studied intensively leading to the identification of specific molecules involved in directing T cells to specific tissues. For example, T cells expressing chemokine receptor 7 (CCR7) will home to lymph nodes where chemokine ligand (CCL) 19 and CCL21 are produced in high concentrations, whereas CCR9 will direct them to the small intestine. CCR4, CCR8, the lipid prostaglandin D2 chemoattractant receptor CRTH2, and the leukotriene B4 receptor BLT1 have all been implicated in T cell recruitment to lung tissue (34), although studies on specific homing to the main conducting airways have yet to be published. This distinction is important since the large airways and lung parenchyma are supplied by separate circulatory systems (23). We need to learn more about the mechanisms that recruit activated T cells to the airway mucosa if we are to fully understand the role of T cells in the development of AHR.

We have shown that susceptibility to AHR between three mouse strains is most strongly associated with the number of activated CD4+ T cells recruited to the airway wall. These cells are well-placed to direct the local tissue response to inhaled allergen and the development of AHR. This may proceed via distinct yet converging pathways utilizing IgE, IgG1, and/or eosinophils, explaining why each of these mechanisms has been associated with AHR in humans and experimental animal models.

GRANTS

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REFERENCES


