Chronic Inhaled Ovalbumin Exposure Induces Antigen-Dependent but Not Antigen-Specific Inhalational Tolerance in a Murine Model of Allergic Airway Disease

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Sensitized mice acutely challenged with inhaled ovalbumin (OVA) develop allergic airway inflammation, characterized by OVA-specific IgE production, airway eosinophilia, increased pulmonary B and T lymphocytes, and airway hyperreactivity. In this study, a chronic exposure model was developed and two distinct patterns of response were observed. Discontinuous inhalational exposure to OVA (6 weeks) produced airway inflammation and hyperreactivity that were similar to acute (10 days) responses. Continuous inhalational exposure to OVA (6 or 11 weeks) resulted in attenuation of airway eosinophilia and hyperresponsiveness without reduction of OVA-specific IgE and IgG₁ levels. The inhibition of airway inflammation was dependent on continuous exposure to antigen, because continuously exposed mice with attenuated inflammatory responses redeveloped allergic airway disease if the OVA aerosols were interrupted and then restarted (11-week-discontinuous). Inhalational tolerance induced by continuous OVA exposure demonstrated bystander suppression of cockroach allergen-mediated airway eosinophilia. These findings may be attributed to changes in production of the anti-inflammatory cytokine interleukin-10 during continuous OVA aerosol exposure. The symptomatic and asymptomatic allergic responses in human asthmatics could be explained by similar variable or discontinuous exposures to aeroantigens. (*Am J Pathol* 2004, 164:295–304)

asthma, has risen dramatically in the developed world. This disturbing trend is documented best for asthma.^{1,2} A wealth of clinical and experimental data suggests that allergic asthma is due to an aberrant lung immune response mediated through T-helper type 2 cells (Th2 cells) and associated cytokine-signaling pathways. The normal lung is able to distinguish between airborne antigens associated with infectious agents, to which an immunological response is generated, and harmless inhaled antigens, which are usually ignored. In the asthmatic lung, some of these normally harmless antigens activate specific Th2 cells and elicit an inflammatory response characterized by Th2 cytokine production, eosinophilic airway inflammation, airway hyperresponsiveness, and bronchoconstriction. These pulmonary responses may be accompanied by systemic allergic sensitization, manifested by elevated titers of antigen-specific IgE. The mechanisms that control CD4⁺ T lymphocyte polarization to allergenic Th2 phenotypes are incompletely understood but seem to involve genetic predispositions, local factors such as pre-existing cytokine concentrations and inflammation, and antigenic factors (ie, potency, dose, and duration of exposure).

Several investigators have used mouse models to investigate the mechanisms of inhalational tolerance to antigens^{3,4} or of allergic airway sensitization.^{5–10} However, these responses have typically been assessed in isolation from each other. We have recently demonstrated that C57BL/6J mice undergo a biphasic response to aerosolized ovalbumin (OVA) exposure.¹¹ After initial immunization and daily aerosol challenge with OVA for 3 to 10 days, mice develop allergic airway disease, characterized by antigen-specific IgE production, airway eosinophilia, increased pulmonary B and T lymphocytes, and airway hyperresponsiveness. In contrast, 6-week-continuous daily aerosol challenge with OVA results in attenuation of these allergic responses. The purpose of

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Throughout the past 40 years, the prevalence of allergic disease, including atopic dermatitis, hay fever, and

Figure 1. Sensitization and aerosol challenge protocols. Mice were sensitized to OVA by three weekly intraperitoneal injections of 25 μ g of OVA in alum. One week later, all mice were exposed to OVA aerosols (1% OVA in saline for 1 hour) daily for 10 days (**heavily shaded blocks**). Thereafter, 6-week-continuous mice were exposed to OVA 5 days a week for 4 weeks (**lightly shaded blocks**). Six-week-discontinuous mice were not exposed to OVA aerosols during this period (**open blocks**). Mice were then exposed to daily OVA aerosols for 10 days (**heavily shaded blocks**) and were sacrificed (S) 24 hours after the last aerosol. Additional 6-week-continuous mice were sensitized to cockroach antigen (CRA) during their continuous OVA aerosol exposure period and then were challenged with nasal CRA 14 days later in lieu of the final 10-day OVA period. In the 11-week studies, discontinuous mice were not exposed to OVA aerosols for 4 weeks after their initial exposure regimens, before being challenged with 10-day OVA aerosols. Penh (P) measurements were taken after 3 days of final aerosol exposure.

the present study was to begin to investigate the mechanism(s) underlying this local airway unresponsiveness, and whether the blunted responses were because of either classical low-dose tolerance, which is dependent on continuous antigen exposure,^{12,13} or to the presence of immunosuppressive TCR $\gamma\delta$ lymphocytes.³ Our results support a low-dose tolerance mechanism, because interruption of continuous antigen exposure with subsequent re-exposure resulted in the re-establishment of allergic airway disease. The lack of responsiveness in continuously exposed animals represented local inhalational tolerance not reflected in systemic OVA-specific antibody levels.

Materials and Methods

Animals

Male and female C57BL/6J and C57BL/6J-*Tcrd*^{tm1Mom} (TCR $\delta^{-/-}$) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed conventionally in an animal room with ambient temperature 22 to 24°C and a daily light-dark cycle (0600 to 1800 light). Chow and water were supplied *ad libitum*. Mice, weighing 18 to 30 g, were studied at 1.5 to 6 months of age. Animal welfare was in accordance with Institutional and Office of Laboratory Animal Welfare Guidelines.

OVA Exposure Protocol

Mice were sensitized to OVA as previously described (Figure 1).¹¹ Briefly, they were given three weekly intraperitoneal injections of a suspension containing 25 μ g of OVA (grade V; Sigma Chemical Co., St. Louis, MO) and 2 mg of aluminum hydroxide (alum) in 0.5 ml of saline. One week after the last injection the mice were exposed to 1% aerosolized OVA according to one of five protocols: 1)

wild-type and TCR $\delta^{-/-}$ mice exposed to OVA 1 hour/day for 10 days (acute); 2) wild-type and TCR $\delta^{-/-}$ mice exposed to OVA 1 hour/day for 10 days followed by 1 hour/day, 5 days/week for 4 weeks and then 1 hour/day for 10 days (6-week-continuous); 3) wild-type and TCR $\delta^{-/-}$ mice exposed to OVA 1 hour/day for 10 days followed by no aerosol exposures for 4 weeks and then aerosol exposures of 1 hour/day for 10 days (6-week-discontinuous); 4) wild-type mice treated according to the continuous protocol above, then exposed to aerosolized OVA 5 days/week for an additional 4-week period, followed by 1 hour/day for 7 to 10 days (11-week-continuous); and 5) wild-type mice treated according to the continuous protocol above, then had OVA aerosols stopped and then resumed 4 weeks later at 1 hour/day for 7 to 10 days (11-week-discontinuous).

Additional studies were performed in discontinuous exposed mice that received hamster anti-TCR $\gamma\delta$ (GL3) [0.5 mg/200 μ l of phosphate-buffered saline (PBS) i.p.] at days -3 and 3 of the final 10-day aerosol exposure.¹⁴

The OVA aerosols were generated by a Lovelace nebulizer (In-Tox Products, Albuquerque, NM) into a 7.6-L inhalation exposure chamber to which mice placed in plastic restraint tubes (Research and Consulting Co., AG, Basel, Switzerland) were attached. Chamber airflow was 10 L/minute. As previously reported, this aerosol system generated OVA particles of 1.4- μ mol/L mass median aerodynamic diameter and 1.6- μ m geometric standard deviations.¹¹ Exposure to the 1% OVA aerosol for 1 hour resulted in an average inhaled OVA dose of 80 μ g/mouse.¹¹ Twenty-four hours after the final aerosol exposure, the mice were sacrificed by ketamine/xylazine overdose and exsanguination, and analyses of bronchoalveolar lavage (BAL), lung tissue, and blood samples were performed.

Cockroach Exposure Protocol

Naive adult C57BL/6J mice were also sensitized to cockroach allergen (CRA), by modification of the protocol of Campbell and colleagues.¹⁵ Mixed CRA (0.1 mg/ml) was purchased from Hollister-Stier (lot no. 6585GV; Spokane, WA). The allergen preparation was washed in 15 ml of PBS in Centrifuplus YM-3 concentrators (Millipore, Bedford, MA) spun at 3000 rpm for 2 hours to remove the 0.4% phenol. On day 0, mice received a 100- μ l intraperitoneal and a 100- μ l subcutaneous injections of 1:1 CRA (10 μ g) and Incomplete Freund's Adjuvant (Sigma, St. Louis, MO). On day 14, mice inhaled 1 μ g of CRA via intranasal administration of 50- μ l saline solution, under brief ether anesthesia. Animals were studied on day 21, 7 days after the intranasal challenge. In addition, we applied the CRA exposure protocol to OVA-tolerant mice, to determine whether their inhalational tolerance was antigen (OVA)-specific (Figure 1).

BAL Cellular Analysis

Twenty-four hours after the final OVA aerosol challenge, the lungs from each animal were lavaged *in situ* with five 1-ml aliquots of sterile saline. Total leukocytes were counted with a hemocytometer using trypan blue dye exclusion as a measure of viability. Leukocyte differen-

tials were determined in BAL fluid using cytocentrifuged preparations stained with May-Grünwald/Giemsa. Further characterization of the lymphocyte population was performed by fluorescence flow cytometry using appropriately diluted monoclonal antibodies against the following antigens: CD45 (clone 30-F11), TCR β (H57.597), TCR δ (GL3), CD3 ϵ (500A2), CD4 (RM4-5), CD8 (53-6.7), CD19 (1D3), B220 (RA3-6B2) (BD PharMingen, San Diego, CA), or CD4 (GK1.5) (BD Collaborative Technologies, Bedford, MA). These monoclonal antibodies were conjugated with biotin, phycoerythrin, fluorescein isothiocyanate, or allophycocyanine. Biotin-conjugated antibodies were detected with streptavidin-Cy5 (Jackson ImmunoResearch Laboratories, West Grove, PA) or streptavidin phycoerythrin-Cy7 (Caltag Laboratories, San Francisco, CA). For fluorescence flow cytometry, BAL cells were washed in PBS containing 0.2% bovine serum albumin and 0.1% NaN₃. Aliquots containing 10⁴ to 10⁵ cells were incubated with 100 μ l of appropriately diluted antibodies for 30 minutes at 4°C. After staining, the cells were washed twice with the above PBS solution, and relative fluorescence intensities were determined on a 4-decade log scale by flow cytometric analysis using a FACScalibur (Becton Dickinson, San Jose, CA).

BAL Cytokine Analysis

BAL fluid was recovered from 10-day-acute, 6-week-continuous, and 6-week-discontinuous mice after 10 days of OVA aerosol challenge. The BAL fluid was concentrated 10-fold using an Amicon (Beverly, MA) Centrplus YM-10 filtration device. Samples were examined in duplicate by enzyme-linked immunosorbent assay for the presence of interleukin (IL)-5, IL-10, interferon (IFN)- γ , and transforming growth factor (TGF)- β (Pierce Endogen, Rockford, IL) and IL-13 (R&D Systems, Minneapolis, MN), according to the manufacturers' directions. The limits of detection for IL-5, IL-10, IFN- γ , and TGF- β were 5, 1.5, 10, and 32 pg/ml, respectively.

Histology

After sacrifice, unmanipulated lungs from separate animals ($n = 4$ to 6 in each group) not subjected to BAL were removed, fixed with 10% buffered formalin, and processed in a standard manner. Tissue sections were stained with hematoxylin and eosin¹⁶ and periodic acid-Schiff. The whole lungs were fixed and sections from all five lobes were examined in their entirety in a blinded manner by Drs. Schramm and Thrall.

OVA-Specific Serum IgE and IgG₁ Levels

OVA-specific IgE and IgG₁ levels in venous blood samples were measured by enzyme-linked immunosorbent assay using isotype-specific capture monoclonal antibodies.¹⁷ IgE or IgG₁ were captured from serum (diluted 2- to 320,000-fold) using Immulon 2 microtiter plates (Dynatech Laboratories, Chantilly, VA) coated with anti-mouse IgE (R35-72) or anti-mouse IgG₁ (A85-3) (ob-

tained from BD PharMingen, San Diego, CA) at 2 μ g/ml in 0.1 mol/L carbonate, pH 9.5. Detection was with an OVA-digoxigenin conjugate followed by horseradish peroxidase-conjugated anti-digoxigenin, essentially as described.⁴ Development was with the TMB microwell peroxidase substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

Airway Hyperreactivity

Airway responses to methacholine were assessed by whole-body barometric plethysmography, as previously described.¹⁸ Mice were studied 12 hours after the third OVA aerosol in acute animals. In chronic 6-week and 11-week animals, Penh measurements were taken after 3 days of OVA aerosols during the final exposure periods (Figure 1). Mice were placed in the main chamber of a whole-body plethysmograph (Buxco Electronics, Troy, NY) and exposed for 2 minutes to aerosolized saline or increasing concentrations of methacholine from 3 to 300 mg/ml. Respiratory system variables including tidal volume, respiratory frequency, inspiratory and expiratory times, and changes in box pressure were recorded before and during aerosolization and for 4 minutes after each exposure. The maximal enhanced pause (Penh) value response to methacholine was recorded at each dose. Because plateau responses often were not obtained, a conventional half-maximal methacholine concentration could not be calculated. In its place, we calculated the interpolated concentration of methacholine needed to increase the Penh value to 2 U (approximately fivefold increase greater than baseline). The Penh-2 value was selected as the portion of the dose-response curve in which greatest changes in sensitivity would be manifested. Changes in Penh-2 values were used to characterize the development of airway hyperreactivity.¹⁸

Statistical Analysis

Statistical comparisons between groups were made with analysis of variance using StatView 4.5 (Abacus Concepts, Inc., Berkeley, CA). Dose-response data and serum IgE levels were compared by repeated measures analysis of variance. Changes in Penh-2 values before and after aerosol exposure were made by paired *t*-tests and were compared between groups by repeated measures analysis of variance.

Results

BAL Leukocytosis and Eosinophilia, as Elicited with Acute OVA Aerosol Exposure, Was Ablated with Continuous but Re-Established with Discontinuous OVA Aerosol Exposure

Mice immunized with three weekly injections of intraperitoneal OVA/alum and then challenged with 10 days of OVA aerosols (acute group; $n = 10$) displayed significant increases in the numbers of BAL macrophages, eosino-

Table 1. Comparison of BAL Leukocyte Counts and Differentials in Naive, Acute, Continuous, and Discontinuous Mice

Exposure protocol	WBC $\times 10^4$	% Macrophages	% Eosinophils	% Lymphocytes
Naive	3.7 $\pm$ 0.6	>98%	<1%	<1%
10-day acute	147.2 $\pm$ 10.0	8 $\pm$ 2%	73 $\pm$ 2%	19 $\pm$ 3%
10-day acute TCR $\delta^{-/-}$	15.4 $\pm$ 2.4	25 $\pm$ 3%	40 $\pm$ 3%	35 $\pm$ 4%
6-week continuous	35.4 $\pm$ 5.0*	77 $\pm$ 9%*	5 $\pm$ 1%*	18 $\pm$ 2%
6-week continuous TCR $\delta^{-/-}$	7.9 $\pm$ 2.2	65 $\pm$ 15%	7 $\pm$ 1%	28 $\pm$ 5%
11-week continuous	14.3 $\pm$ 1.8†	81 $\pm$ 5%†	5 $\pm$ 2%†	14 $\pm$ 4%
6-week discontinuous	128.8 $\pm$ 23.2	17 $\pm$ 3%	69 $\pm$ 4%	14 $\pm$ 2%
6-week discontinuous TCR $\delta^{-/-}$	14.9 $\pm$ 5.2	42 $\pm$ 12%	42 $\pm$ 7%	16 $\pm$ 4%
11-week discontinuous	107.7 $\pm$ 38.4	41 $\pm$ 8%	50 $\pm$ 11%	9 $\pm$ 2%

Mice were sensitized and challenged with ovalbumin as shown in Figure 1. Relative to naive unexposed animals, acute and 6-week-discontinuous mice demonstrated similar increases in airway leukocytes and eosinophils. Acute and 6-week-discontinuous TCR $\delta^{-/-}$ mice had moderately attenuated eosinophilic airway inflammation, relative to their wild-type counterparts. In contrast, 6-week-continuous wild-type and TCR $\delta^{-/-}$ mice had markedly less airway leukocytosis and eosinophilia, as compared to acute or 6-week-discontinuous animals. Eleven-week-continuous mice also showed minimal eosinophilic inflammation, but 11-week-discontinuous mice demonstrated a strong eosinophilic airway inflammatory response, similar to 10-day-acute and 6-week-discontinuous animals. All exposed animals demonstrated BAL lymphocytosis relative to controls. Data represent mean $\pm$ SE values; *, $P < 0.05$ between 6-week-continuous and 6-week-discontinuous or naive groups; †, $P < 0.05$ between 11-week-continuous and 11-week-discontinuous groups.

phils, and lymphocytes compared to naive, unexposed mice (Table 1). As previously reported,¹¹ BAL cells began to increase by day 3 of aerosol challenge and peaked at 7 to 10 days of continued exposure. Total BAL leukocytes increased 40-fold with acute OVA challenges, and the ratios of cell types changed dramatically. In the unexposed mice, more than 98% of the BAL cells were macrophages, as compared to 8 $\pm$ 2% in challenged mice that received 10 days of OVA aerosol. Eosinophils and lymphocytes increased from less than 1% in unexposed mice to 73 $\pm$ 2% and 19 $\pm$ 3%, respectively, in 10-day-challenged animals ($P < 0.001$ versus naive controls).

Mice in the 6-week-continuous group ($n = 20$) had more total BAL leukocytes as compared to naive mice but much less than the acute animals ($P < 0.01$ compared to each other group). Relative to naive controls, the 6-week-continuous mice continued to demonstrate BAL lymphocytosis ($P < 0.0001$) and eosinophilia ($P = 0.017$); however, the eosinophilia was far less than in the acutely challenged animals ($P < 0.0001$). In contrast, mice immunized with three weekly intraperitoneal injections of OVA/alum and then exposed to a discontinuous or interrupted OVA aerosol schedule (discontinuous group; $n = 20$) developed an intense BAL leukocytosis, eosinophilia, and lymphocytosis similar to the acutely challenged animals (Table 1). After 4 weeks of no exposure to OVA, the airway inflammatory response was restored in its entirety, suggesting that immunological memory of OVA-specific CD4⁺ cells was established and maintained. Whereas the allergic inflammatory response was re-established in the discontinuously exposed animals, mice continuously exposed to aerosolized OVA appeared to suppress their airway eosinophilia and to exhibit an apparent inhalational tolerance to the antigen.

The Relative Distributions of BAL T Lymphocytes Did Not Differ between the Continuous and Discontinuous Animals

Continuously and discontinuously exposed mice demonstrated increased absolute levels of BAL lymphocytes

(6.5 $\pm$ 1.2 and 15.8 $\pm$ 4.5 $\times 10^4$ cells) relative to naive mice. Although the trend was higher in the discontinuous versus continuous mice, the difference was not significant ($P = 0.06$). Moreover, the distributions of lymphocyte subsets were strikingly similar between the two groups (Table 2). TCR $\alpha\beta$ T cells accounted for 67.1 $\pm$ 2.8% of lymphocytes in the continuous group and 59.1 $\pm$ 3.6% in discontinuous animals ($n = 8$ in each group). The percentages of CD4⁺ T lymphocytes and CD8⁺ T lymphocytes were also similar between the two groups (Table 2). However, it was notable that the relative size of T lymphocytes (as indicated by the forward scatter profile measured by flow cytometry in paired samples) was significantly reduced after continuous versus discontinuous OVA exposure ($P = 0.009$ for CD4⁺ cells and $P = 0.028$ for CD8⁺ cells, Table 2). In comparison to similar percentages and numbers of T-cell subtypes, the number of B lymphocytes present in BAL fluid was significantly less in continuous versus discontinuous mice (1.5 $\pm$ 0.3 versus 11.6 $\pm$ 3.3 $\times 10^4$, $P = 0.002$). The reduction of B lymphocytes in BAL from continuously exposed animals is consistent with their apparent inhalational tolerance to OVA; however, this tolerance was not associated with changes in T lymphocyte numbers or CD4:CD8 ratios.

Table 2. Comparison of BAL Lymphocyte Differentials in 6-Week-Continuous and 6-Week-Discontinuous Mice

Parameter	6-week-continuous	6-week-discontinuous
% TCR $\alpha\beta$ cells	67.1 $\pm$ 2.8%	59.1 $\pm$ 3.6%
% CD4 ⁺ cells	40.0 $\pm$ 1.1%	32.6 $\pm$ 3.2%
CD4 ⁺ cell size	359 $\pm$ 1	401 $\pm$ 9†
% CD8 ⁺ cells	29.0 $\pm$ 2.6	29.5 $\pm$ 6.7%
CD8 ⁺ cell size	368 $\pm$ 6	405 $\pm$ 10*
% B cells	4.2 $\pm$ 0.8	9.0 $\pm$ 2.6%*

Mice were sensitized and challenged with OVA as shown in Figure 1. T-lymphocyte distributions did not vary between the 6-week-continuous and 6-week-discontinuous groups. The relative sizes of CD4⁺ and CD8⁺ T lymphocytes (as indicated by the forward scatter profile measured by flow cytometry in paired samples) were greater in the discontinuous than continuous mice. In contrast to T cells, the relative proportion of B lymphocytes was increased in discontinuous versus continuous mice. Data represent mean $\pm$ SE values; *, $P < 0.05$ and †, $P < 0.01$ between the groups.

Figure 2. Comparison of methacholine reactivity in naive and acute OVA-challenged mice. **Left:** Acute mice were sensitized with three weekly injections of OVA/alum and then challenged with three daily OVA aerosols. Airway responsiveness to methacholine was measured before OVA aerosols (open symbols) and 12 hours after the third OVA aerosol (filled symbols). The Penh response to methacholine was potentiated after OVA aerosol challenge in acute sensitized mice. **Right:** Airway responsiveness was also assessed in naive, unexposed mice on the same days. Baseline responses (open symbols) were similar to the acute animals' baselines, and the Penh response did not change after 3 days in naive unchallenged mice (filled symbols). Data represent mean $\pm$ SE levels of Penh responses in naive and acute mice ($n = 5$ in each group); *, $P < 0.02$ from baseline response.

Continuous OVA-Challenged Mice Showed Less Histological and Biochemical Evidence of Lung Injury than Acute or Discontinuous Mice

As we have reported previously,¹¹ no histological evidence of lung damage was found in unexposed naive animals. In contrast, the acute OVA-challenged mice developed dense peribronchial inflammation consisting primarily of lymphoplasmacytic cells and eosinophils. There were also areas of perivascular inflammation and slight peribronchial muscle hypertrophy in the acute animals. The continuous mice had qualitatively less peribronchial lymphoplasmacytic inflammation, mild peribronchial muscle hypertrophy, and slight to mild bronchial lining cell hyperplasia.¹¹ Eosinophils were notably absent in lungs from these chronic stage animals. Discontinuous mice demonstrated histopathology similar to the acute animals, with qualitatively similar inflammatory findings (data not shown).

The Airway Hyperresponsiveness to Methacholine Induced by Acute OVA Aerosol Challenges Resolved in the Continuous OVA-Challenged Mice but Was Re-Established in the Discontinuous Mice

Aerosolized methacholine elicited dose-dependent increases in enhanced pause time (Penh) in conscious, unrestrained naive mice throughout the dose range of 3 to 300 mg/ml (Figure 2). This response was significantly potentiated by acute OVA challenge ($P = 0.0006$ by repeated measures analysis of variance). Before OVA aerosol challenges, baseline Penh measurements did not vary between unchallenged and challenged, naive and acute mice; however, responses at 10 to 300 mg/ml of methacholine were significantly enhanced in the acute-challenged mice, consistent with hyperresponsiveness to the agent. This leftward shift in the dose-response relationship was reflected in the concentration of methacholine

Figure 3. Comparison of airway sensitivity in naive and 10-day-acute, 6-week-continuous, and 6-week-discontinuous OVA-challenged mice. Airway sensitivity to methacholine was determined from Penh measurements at baseline (B) and 12 hours after OVA aerosol challenges (C), as described in the text, with control measurements taken simultaneously in naive mice. Airway sensitivity was defined as the calculated concentration of methacholine associated with a Penh value of 2 units. Naive and continuous mice showed no changes in airway sensitivity between the two measurements. In contrast, acute and discontinuous mice demonstrated increased muscarinic sensitivity after OVA aerosol challenge (ie, decreased concentration of methacholine eliciting the Penh response). **Circles and dashed lines,** paired responses in individual animals; bars, mean values; *, $P < 0.03$ by paired t -test versus baseline.

line eliciting a Penh value of 2 U (Penh-2 concentration). Relative to baseline assessments, the Penh-2 concentration did not change with repeated testing in naive animals ($P = 0.79$ by paired t -test) but halved after three daily OVA aerosol challenges in acute mice ($P = 0.030$, Figure 3). Discontinuous mice demonstrated enhanced Penh responses and sensitivity similar to the acute animals ($P = 0.014$ versus their baseline before OVA aerosol challenges, Figure 3), but the continuous mice no longer demonstrated muscarinic hyperreactivity ($P = 0.88$ versus their baseline before aerosol OVA challenges). Thus, the pattern of airway hyperresponsiveness paralleled that of airway eosinophilia in these animals, with reproduction of acute eosinophilia and hyperresponsiveness in discontinuous mice and loss of these responses in continuous mice.

BAL Cytokine Profiles Differed in 6-Week-Continuous versus 6-Week-Discontinuous Mice

To investigate whether changes in eosinophilic airway inflammation were accompanied by differences in local cytokine production, we compared BAL levels of the Th2 cytokines IL-13 and IL-5 along with levels of the Th1 cytokine IFN- γ in BAL fluid from 10-day-acute, 6-week-continuous, and 6-week-discontinuous mice. Inflammatory cytokines were below detectable levels in BAL supernatants from naive, unexposed mice; however, concentrations of the Th2 cytokines IL-13 and IL-5 were similarly elevated in the 10-day-acute and 6-week-discontinuous mice (Figure 4). The 6-week-continuous mice had even higher IL-5 levels ($P = 0.018$ by analysis of variance) but significantly decreased IL-13 levels ($P = 0.038$) compared to the other animals (Figure 4). BAL concentrations of IFN- γ did not differ between the groups of mice and were not statistically different from zero in any group (data not shown).

We also assessed BAL levels and tissue expression of the immunoregulatory cytokines TGF- β and IL-10. TGF- β was detected in BAL fluid from naive mice, and its BAL

Figure 4. Comparison of BAL cytokine concentrations in naive, 10-day-acute, 6-week-continuous, and 6-week-discontinuous mice. Mice were challenged with OVA aerosols acutely (acute, striped bars), continuously (cont, shaded bars), and discontinuously (disc, filled bars). BAL fluid was assayed for cytokine concentrations by enzyme-linked immunosorbent assay, as described in the text, and compared with BAL levels from naive mice (open bars). BAL levels of IL-13 were unmeasurable in naive mice and were less elevated in continuous than in acute or discontinuous mice. BAL levels of IL-5 were unmeasurable in naive mice and were most elevated in the continuous animals. BAL levels of TGF- β increased with acute OVA aerosol challenges, returned to baseline in continuous mice, and were again elevated in discontinuous animals. BAL levels of IL-10 were similar in naive, acute, and discontinuous mice but were significantly elevated in continuous animals. Data represent mean $\pm$ SE measurements; *, $P < 0.02$ versus acute and discontinuous mice by analysis of variance; **, $P < 0.05$ versus naive and continuous mice by analysis of variance; $n = 5$ to 6 animals in each group.

concentration increased steadily through the 10 days of acute OVA aerosol exposure to twice baseline levels (Figure 4, $P < 0.0001$ by analysis of variance). BAL TGF- β levels returned to baseline with 6 weeks of continuous OVA aerosol exposures; however, BAL TGF- β was elevated again in 6-week-discontinuous mice after seven late daily OVA aerosol exposures ($P = 0.004$ versus 6-week-continuous), to a level similar to that seen during the acute exposure period. The same BAL fluid used for TGF- β measurements was also assayed for IL-10 levels. BAL IL-10 levels were unchanged during the acute OVA aerosol exposure, either in the 10-day-acute or in the 6-week-discontinuous animals (Figure 4). However, IL-10 was significantly elevated in BAL fluid from 6-week-continuous mice ($P = 0.015$ by analysis of variance).

Serum OVA-Specific IgE and IgG₁ Levels Did Not Differ between 6-Week-Continuous and 6-Week-Discontinuous Mice

In contrast to striking differences in airway and lung inflammation, airway hyperreactivity, and BAL cytokine levels, 6-week-continuous and 6-week-discontinuous mice demonstrated similar levels of OVA-specific IgE and IgG₁ levels in their serum. As expected, no OVA-specific antibodies were detected in sera from naive mice. When acute mice were studied 4 weeks after their initial OVA aerosol exposures, OVA-specific IgE levels were barely detectable at 27.6 ± 11.9 ng/ml ($n = 5$). In comparison, OVA-specific IgE was markedly elevated in both continuous and discontinuous mice (994 ± 193 ng/ml and

1492 ± 330 ng/ml, respectively). Although the levels tended to be greater in the discontinuous animals, the values were not statistically different ($P = 0.21$, $n = 13$ in each group). OVA-specific IgG₁ levels were also similar in continuous (1063 ± 288 μ g/ml) and discontinuous mice (1312 ± 330 μ g/ml). These findings suggest that systemic allergic responses were not attenuated during the continuous OVA aerosol exposure period, even though local airway inflammatory and hyperreactive responses were ablated.

Local Inhalational Tolerance with Chronic Antigen Administration Was Not Dependent on the Presence of TCR $\gamma\delta$ Cells

Similar to what we have shown previously,¹⁴ airway eosinophilia was dramatically decreased in OVA-sensitized and -challenged acute mice lacking TCR $\gamma\delta$ cells (Table 1, $n = 6$). Continuous antigen exposure still induced local inhalational tolerance in TCR $\delta^{-/-}$ animals, whereas discontinuous exposure in TCR $\delta^{-/-}$ animals reproduced the attenuated initial acute response (Table 1, $n = 8$ in each group). In addition, local inhalational tolerance could not be broken by the intraperitoneal administration of anti-TCR $\gamma\delta$ antibodies before the final week of OVA aerosols in continuous mice (data not shown), even though it markedly reduced the airway eosinophilia in discontinuous mice in a similar degree to its effect in acute animals.¹⁴

Inhalational Tolerance Required Continuous Exposure to the Antigen

One potential mechanism of the apparent inhalation tolerance in continuous mice was clonal deletion of responsive T cells. If deletion of OVA-responding T-cell clones occurred during the 6-week-continuous exposure, one would expect tolerance to persist after antigen exposure had ceased. To investigate this possibility, we extended our experiments for an additional 5 weeks in a separate series of mice. Mice were initially treated according to the 6-week-continuous protocol and then either continuously exposed to OVA aerosols for 5 more weeks (11-week-continuous) or removed from aerosol exposures for 4 weeks and finally re-exposed for 1 week (11-week-discontinuous). As before, 6-week-discontinuous mice showed robust airway eosinophilia and lymphocytosis. These inflammatory responses were markedly blunted in the 6-week-continuous mice and remained attenuated in the 11-week-continuous mice (Table 1, $n = 5$). In contrast, the 11-week-discontinuous mice—who had previously been exposed to the tolerizing 6 weeks of OVA aerosols—redeveloped strong airway eosinophilia (Table 1, $n = 5$). The absolute number of eosinophils and their percentage of BAL leukocytes were similar in the 11-week-discontinuous and the 6-week-discontinuous animals (72.8 ± 53.4 versus $89.0 \pm 21.7 \times 10^4$ cells, and $50.0 \pm 10.8\%$ versus $60.9 \pm 4.2\%$, respectively). Muscarinic airway hyperreactivity in the 11-week animals paral-

Figure 5. Comparison of BAL leukocytes in 7-day CRA-challenged mice that were naive to OVA or subjected to 6 weeks of continuous OVA aerosol exposure. Mice were sensitized and challenged with CRA, as described in the text. One group of mice was naive to OVA; the other group had been subjected to the 6-week-continuous OVA exposure protocol before CRA administration. These mice continued to receive OVA aerosols during the CRA sensitization period. Note that although both groups of mice showed increased numbers of BAL macrophages, lymphocytes, and neutrophils relative to naive animals, the airway eosinophilic response to 7-day-CRA exposure was ablated in 6-week-continuous OVA-exposed mice. Data represent mean numbers of macrophages (shaded bars), eosinophils (filled bars), and lymphocytes (striped bars); $n = 4$ to 6 in each group.

leled the BAL eosinophilia. The 11-week-continuous mice demonstrated similar reactivity to methacholine as they did before any OVA aerosol exposures (Penh-2 methacholine concentration: 13.2 ± 1.2 mg/ml versus 15.8 ± 1.3 mg/ml at baseline; $P = 0.33$). In contrast, the 11-week-discontinuous mice developed a 2.6-fold increase in airway reactivity (Penh-2 concentration: 7.4 ± 1.1 versus 19.6 ± 4.1 mg/ml; $P = 0.04$), similar to what was observed in acute and 6-week-discontinuous animals. Thus, the interruption of OVA aerosol exposures once airway tolerance had developed allowed the mice to redevelop susceptibility to allergic airway disease on re-exposure to inhaled antigen.

Inhalational Tolerance Exerted Bystander Suppression of CRA-Induced Allergic Airway Disease

In additional studies, we adapted a cockroach model of allergic airway disease, whereby mice are sensitized to CRA by sequential intraperitoneal, subcutaneous, and intranasal administration of antigen. Acutely CRA-sensitized animals developed a mixed inflammatory response, with airway neutrophilia and moderate airway eosinophilia (Figure 5). We then superimposed this CRA model into 6-week-continuous, OVA-tolerant mice. Although both groups of mice demonstrated increased numbers of BAL macrophages, lymphocytes, and neutrophils relative to naive animals, the airway eosinophilic response to 7-day CRA exposure was ablated in 6-week-continuous OVA-exposed mice. Thus, inhalational tolerance induced

by chronic OVA aerosol exposure was able to exert bystander suppression of an allergic response to an unrelated antigen.

Discussion

The OVA model of allergic airway disease has been extensively characterized and studied in the acute stage. Asthma, however, is a chronic disease with recurring exposures and exacerbations. Few previous animal models have addressed the clinically relevant patterns of chronic or intermittent/discontinuous antigen exposure. In a murine model, we previously studied the natural progression of allergic airway disease, from acute exposure with inflammatory and hyperreactive airway disease to chronic exposure with inhalational tolerance characterized by diminished inflammation and normal airway responsiveness.¹¹ In comparison to acutely exposed animals, 6-week-continuous mice showed minimal airway eosinophilia, diminished airway B-cell lymphocytosis, less pulmonary inflammation, no airway hyperreactivity, and altered cytokine profiles; however, their systemic levels of allergen-specific IgE and IgG₁ were unabated. In an attempt to mimic the clinical setting of intermittent exposure, we altered the continuous antigen exposure model by interrupting aerosol antigen exposure for a 4-week period before reintroducing aerosol challenges. These discontinuous mice demonstrated re-establishment of allergic airway inflammation. Their responses were similar to those seen in acutely exposed mice, in terms of airway eosinophil and lymphocyte counts and distributions, peribronchial inflammation, airway hyperreactivity to methacholine, and increased Th2 cytokine production.

The refractoriness to airway inflammation that developed in continuous animals was clearly different from the inhalational tolerance demonstrated in other models. Our continuous mice maintained elevated OVA-specific IgE levels through the 6 weeks of exposure, despite the attenuation of airway inflammatory responses. To date, other animal studies of oral and respiratory tolerance have required that antigen exposure precede the development of the Th2 allergic response for tolerance to occur.^{3,4} In these models, the administration of antigen after initiation of the Th2 response potentiates the inflammatory reaction. Our study is the first to describe the development of inhalational tolerance in asthmatic mice in the presence of a dominant Th2 response. The mechanisms underlying the inhibition of systemic IgE responses by inhalational tolerance are unclear, because the process has been found to be both dependent^{3,19} and independent⁴ of IFN- γ , CD8+ lymphocytes, and TCR $\gamma\delta$ cells. In this study, differences in BAL IFN- γ concentrations or CD8+ T-cell distributions between continuous and discontinuous mice were not observed. In addition, the absence of TCR $\gamma\delta$ lymphocytes had no effect on the development of apparent tolerance in the continuous mice, regardless of whether the TCR $\gamma\delta$ cells were absent throughout the exposure in TCR $\delta^{-/-}$ mice or if they were depleted with monoclonal antibodies before

the final week of aerosol OVA challenges. Rather, the depletion of TCR $\gamma\delta$ cells had an inhibitory effect in our discontinuous model, similar to its effect in the acute stages of allergic airway inflammation.^{14,20}

The maintenance of elevated serum IgE levels in the absence of airway inflammatory responses in continuous mice suggested that our inhalational tolerance was because of local mechanisms rather than systemic lymphocyte clonal deletion or anergy. If chronic OVA aerosol exposure had induced tolerance via clonal deletion or development of a dominant, antigen-specific, inhibitory T-cell population, tolerance should have been sustained with the absence of antigen or recalled with re-exposure. To test this potential mechanism, we extended our protocol for an additional 5 weeks of either continuous or discontinuous antigen exposure. Mice continuously exposed to OVA aerosols for 11 weeks demonstrated the same tolerance as their 6-week-continuous counterparts. Interrupting the OVA aerosol challenges for 4 weeks after establishment of the 6-week tolerant stage followed by re-exposure to OVA aerosols resulted in development of allergic airway disease to an extent similar to that observed in acute and 6-week-discontinuous animals. It has been shown that intravenous administration of OVA peptide 323-339 results in initial brief proliferation of antigen-specific CD4⁺ T cells followed by clonal deletion and functional inactivation of the remaining OVA-specific CD4⁺ T cells.^{21,22} These cells retain their unresponsive status only as long as some peptide persists *in vivo*, implying that some TCR-transduced signal is required to maintain tolerance. A similar mechanism could account for the dependence of continuous antigen exposure to maintain the local inhalational tolerance in our model; however, we would have expected such a mechanism to also suppress systemic OVA-specific IgE production, as observed in the above studies but not in our model.

We did not detect a shift in T-cell phenotypes from a CD4⁺ to a CD8⁺ predominance, nor did we observe a systematic switch from a Th2 to a Th1 cytokine profile in BAL fluid. Indeed, levels of the eosinophil recruiting cytokine IL-5 were paradoxically highest in our continuously exposed animals. This suggests that the block to airway inflammation in these animals was at a local effector level, downstream from IL-5 expression. The elevated levels of IL-13 in the acute and discontinuous mice and its reduction in continuous mice paralleled the patterns of airway responsiveness in the animals and are consistent with the identification of IL-13 as a potent regulator of allergen-induced airway hyperreactivity.^{23,24} That immune deviation is not mediating our inhalation tolerance is also supported by our previous observations in mice infected with murine cytomegalovirus. When murine cytomegalovirus infection induces a Th1 response before development of a Th2 response in our model, 10-day-acute allergic airway disease is suppressed. In contrast, when generation of a Th1 response is initiated after the Th2 response is established, airway eosinophilia is not diminished.¹⁸

Thus, we were unable to detect changes in Th1/Th2 cytokine profiles, CD8⁺ lymphocytes, or TCR $\gamma\delta$ cells in our discontinuous/continuous aerosol exposure model. Similar results have been reported in a murine model of

nasal tolerance, in which intranasal exposure to OVA in BALB/c mice elicits unresponsiveness to subsequent immunogenic challenges. This unresponsiveness is independent of regulatory CD8⁺ T cells or inhibitory cytokines, but is mediated by functionally impaired antigen-specific CD4⁺ T cells.²⁵ The establishment of CD4⁺ T-cell unresponsiveness after intranasal antigen administration is dependent on the presence and activation of B cells.²⁶ B lymphocytes may take up OVA and present it in a tolerogenic manner to CD4⁺ T cells, or they may interact with dendritic cells in a manner that promotes a tolerogenic DC-T cell interaction. It is noteworthy that B cells, although more prevalent in BALs of 6-week-discontinuous animals, were still present in the 6-week-continuous mice. The continued production of IgE in the continuous mice demonstrated that B cells remained active in these animals despite the attenuation of other inflammatory responses. B-cell tolerance is also not induced in the nasal tolerance model.²⁶ TCR $\alpha\beta$ and CD4⁺ T lymphocytes were present in similar proportions in our 6-week-continuous and discontinuous animals; however, their significantly smaller size in the continuous mice suggested that they were less activated than in acute or discontinuous animals.^{27,28}

T-cell activation in continuous mice may have been suppressed by the local production of anti-inflammatory cytokines, such as TGF- β and IL-10. Airway TGF- β levels increased during the 10-day-acute OVA aerosol period and again during the acute OVA re-exposure in 6-week-discontinuous animals, possibly as part of a homeostatic mechanism that limits airway inflammation.²⁹ BAL IL-10 protein levels were significantly elevated in 6-week-continuous mice, suggesting that IL-10 secretion may represent an inhibitory mechanism that down-regulated the inflammatory response in these animals. In this regard, specific populations of regulatory T lymphocytes have been shown to synthesize mainly IL-10 (Tr1 cells).³⁰ The OVA-specific local expression of IL-10 and/or regulatory T cells could explain two key facets of our model: the inhibition of local airway responses but not systemic IgE levels in 6-week- or 11-week-continuous mice; and the bystander suppression of CRA-induced airway eosinophilia in 6-week OVA-continuous animals.^{31,32}

It is also possible that the inhibition of local inflammatory responses was not because of an OVA-specific immunological mechanism but, instead, was dependent on chronic airway exposure to contaminating endotoxin in the OVA aerosols. Recent epidemiological studies have implicated endotoxin as a protective factor against the development of asthma.³³⁻³⁵ Independent analysis (Aldevron, Fargo, ND) of our grade V OVA demonstrated that the 1% solution contained 0.6 to 4.1 $\mu\text{g}/\text{ml}$ of lipopolysaccharide (LPS) endotoxin. Based on the ratio of our calculated OVA inhalation dose to aerosol concentration (80 $\mu\text{g}/10 \text{ mg}/\text{ml}$), we estimate that the mice could have inhaled between 5 and 35 ng/day of endotoxin. The intranasal administration of 1000-fold higher endotoxin doses (10 $\mu\text{g}/\text{day}$ LPS) before OVA aerosol challenges attenuates but does not ablate airway eosinophilia in OVA-sensitized BALB/c mice, and it does not inhibit the development of muscarinic airway hyperreactivity.³⁶ In

contrast, exposure of OVA-sensitized and inhaled OVA-challenged rats to 50 $\mu\text{g}/\text{ml}$ of LPS aerosols suppresses the development of airway hyperreactivity and airway eosinophilia.³⁷ OVA-challenged, LPS-exposed rats have marked airway neutrophilia and enhanced lung parenchymal eosinophilia.³⁷ Airway neutrophilia is a prominent finding after inhaled LPS exposure in mice^{38,39} but has not been observed in our continuous model.¹¹ Thus, it seems unlikely that the low-level LPS exposure could induce the inhalational tolerance observed in our continuous animals. Nevertheless, the possibility that low doses of endotoxin may contribute to the mechanism(s) eliciting tolerance to chronic OVA exposures remains an important issue, which is being addressed in ongoing investigations.

In summary, our inhalational tolerance was an active mechanism dependent on continuous exposure to the antigen, such that interruption of continuous antigen exposure prevented the development of tolerance in 6-week animals and broke tolerance in the 11-week animals. This process appeared to be a local phenomenon, inhibiting airway inflammatory but not systemic IgE responses to antigen exposure. Alternately, because the source of systemic IgE could be OVA-specific IgE-producing plasma cells in the lung, our continuous animals may have exhibited a split tolerance, with inactivation of some functions (ie, airway eosinophilia, IL-13 cytokine production, and hyperresponsiveness) but not others (IL-5 cytokine or IgE production). Nevertheless, the mechanism of tolerance was generalized enough to exhibit bystander tolerance for an unrelated antigen. Such tolerance may have occurred by the functional inactivation of antigen-specific CD4⁺ T cells by locally produced anti-inflammatory cytokines (eg, IL-10) and/or regulatory T cells.

Most human patients sensitized to an inhalational antigen remain sensitized for many years, often for life. The present study suggests that the persistence of allergic responses in humans may be related to the variable or discontinuous exposures they have to these antigens. Insights into the mechanisms of airway sensitization and tolerance gained with further use of this murine model may lead to the development of novel treatment strategies for the prevention or eradication of allergic airway disease and asthma.

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