Oral and Nasal Sensitization Promote Distinct Immune Responses and Lung Reactivity in a Mouse Model of Peanut Allergy

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Despite structural and functional differences between the initial sites of contact with allergens in the gastrointestinal and nasal tracts, few animal models have examined the influence of the mucosal routes of sensitization on host reactivity to food or environmental antigens. We compared the oral and nasal routes of peanut sensitization for the development of a mouse model of allergy. Mice were sensitized by administration of peanut proteins in the presence of cholera toxin as adjuvant. Antibody and cytokine responses were characterized, as well as airway reactivity to nasal challenge with peanut or unrelated antigens. Oral sensitization promoted higher levels of IgE, but lower IgG responses, than nasal sensitization. Both orally and nasally sensitized mice experienced airway hyperreactivity on nasal peanut challenge. The peanut challenge also induced lung eosinophilia and type 2 helper T-cell-type cytokines in orally sensitized mice. In contrast, peanut challenge in nasally sensitized mice promoted neutrophilia and higher levels of lung MAC-1⁺ I-A^b low cells and inflammatory cytokines. In addition, nasal but not oral, sensitization promoted lung inflammatory responses to unrelated antigens. In summary, both oral and nasal peanut sensitization prime mice for airway hyperreactivity, but the initial mucosal route of sensitization influences the nature of lung inflammatory responses to peanut and unrelated allergens. (*Am J Pathol* 2005, 167:1621–1630)

The prevalence of peanut allergy has doubled in the last decade, and it now affects more than 3 million individuals in the United States.¹ This health care problem is further enhanced by potential cross-reactive allergens. Thus, clinical symptoms were reported in peanut allergic patients who had ingested food of the same botanical family^{2–4} or even taxonomically unrelated products.⁵ Allergic respiratory symptoms have also been described in peanut-allergic patients after inhalation of airborne peanut particles in school⁵ or on airline flights.^{6,7} In this regard, food allergens are now well recognized to play a significant role as aeroallergens in the etiology of asthmatic symptoms in individuals with food allergies.⁸

Sensitization to food allergens such as peanut generally occurs in the gastrointestinal (GI) tract. However, it could also occur as a consequence of direct or cross-sensitization by inhalational exposure to peanut or cross-reactive environmental antigens. For example, peanut allergy is frequently associated with pollen allergy,^{9–12} and peanut allergens share sequence homologies with environmental antigens.¹³ A study on children with a history of at least one acute allergic reaction showed that initial reactions to peanut occurred at 24 months of age, with the large majority resulting from a first oral exposure.⁵ Because IgE-mediated allergic reactions require prior exposure to the allergen, one cannot rule out earlier sensitization through inhalation of airborne peanut particles. In addition, the presence of cross-reactive IgE to pollen and peanut antigens in pollen-allergic patients¹⁴ and the reports that these individuals can develop positive skin tests to peanut^{15,16} suggest that allergic symptoms to peanut may also be caused by respiratory sensitization with cross-reactive allergens. Structural and functional differences have been described between the gut-associated lymphoid tissues and the nasopharyngeal-associated lymphoid tissues¹⁷ that are the first sites

Supported by National Institutes of Health grants AI 18958 and DC 04976 and by the French Ministry of Education.

Accepted for publication August 12, 2005.

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of contact with ingested and inhaled antigens, respectively. But it remains unclear how priming through each site could influence subsequent allergic or inflammatory reactions.

It is widely accepted that IgE and cytokines produced by Type 2 helper T (Th2) cells play a pivotal role in allergic manifestations.^{18,19} However, recent studies suggest that a larger number of parameters contribute to allergic responses. For example, in addition to IgE, antibodies (Abs) of the IgG isotype could exert a regulatory effect on allergic reactions;²⁰ however, underlying mechanisms are still poorly understood.²¹ Th1 cells that were believed to only protect against allergic reactions by attenuating the activity of Th2 cells²² now appear to also support Th2 cell-induced allergic asthma.^{23–25} In addition, Th1 cells have been shown to recruit and activate neutrophils for subsequent airway hyperreactivity (AHR).²⁶ The route of allergen sensitization may influence the pattern of Ab and T-cell responses and, therefore, the nature of potential adverse reactions. This increasing complexity of mechanisms underlying allergic and non-allergic inflammatory responses further limits our understanding of adverse effects that occur in individuals with allergies.

Peanut allergy has been mostly investigated in animal models sensitized by the subcutaneous,²⁷ the intraperitoneal,^{28,29} or the oral route^{30–32} and challenged by the oral route.^{27,28,30,31} The nasal route has been less extensively investigated. Furthermore, to our knowledge no study has compared inflammatory lung reactions to unrelated food or respiratory antigens in animal models sensitized by the oral and nasal routes. We compared Ab and T-cell responses induced by oral or nasal sensitization with whole-peanut protein extract (PPE) and cholera toxin (CT) as adjuvant. We then examined the influence of these responses on airway reactivity to nasal challenge with PPE or unrelated antigens. Our data show that the initial mucosal route of peanut sensitization affects the nature of the immune response and the lung reactivity to peanut but also to unrelated antigens.

Materials and Methods

Mice

Female C57BL/6 mice were obtained from the Frederick Cancer Research Facility (National Cancer Institute, Frederick, MD). Mice were maintained in horizontal laminar flow cabinets and were free of microbial pathogens as determined by plasma Ab screening and tissue histopathology performed on sentinel mice. All mice received sterile food and water *ad libitum*. Studies were performed in accordance with institutional guidelines to avoid pain and distress.

Mucosal Sensitization

Whole-peanut protein extracts (PPE) were obtained as previously described by ammonium bicarbonate treat-

ment of defatted peanut extracts.² Mice 8 to 12 weeks of age were sensitized on days 0 and 7 with whole PPE and CT as adjuvant. Anesthetized mice were nasally administered 100 μ g of PPE and 1 μ g of CT in a total volume of 10 μ l with 5 μ l placed into each nare. This volume of the nasal vaccine is retained in the nasal cavity after nasal administration to anesthetized mice.³³ For sensitization by the oral route, mice were deprived of food for 2 hours and then orally treated with 250 μ l of sodium bicarbonate as previously described.³⁴ Oral sensitization consisted of intragastric administration of 1 mg of PPE plus 15 μ g of CT in 250 μ l of phosphate-buffered saline (PBS). Other doses of CT (5 μ g nasal or 60 μ g oral) and PPE (25, 50, or 200 μ g nasal or 2 mg oral) were tested in separate experiments. Some experiments included mice given ovalbumin (OVA) (Sigma Chemical, Saint Louis, MO) as antigen instead of PPE. In these experiments, mice were then either nasally administered 100 μ g of OVA plus 1 μ g of CT or given 1 mg of OVA plus 15 μ g of CT by the oral route. Plasma samples were collected 1 week after each sensitization, on days 7 and 14, for analysis of peanut-specific Ab responses.

Nasal Challenge with Peanut and Unrelated Proteins

Mice nasally or orally sensitized to peanut were nasally challenged on days 15 and 16 with 200 μ g of PPE in a total volume of 100 μ l. More specifically, anesthetized mice were given 25 μ l of PPE per nare, four times at 2- to 3-minute intervals. For analysis of lung responses to unrelated proteins, mice were challenged with 200 μ g of OVA or 40 μ g of *Dermatophagoides farinae* (Der f) protein extract (Greer Laboratories, Lenoir, NC) instead of PPE.

Plasma Antibody Responses

Plasma levels of peanut-specific Abs were measured by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well microplates (Falcon) were coated with 50 μ g/ml PPE in PBS and incubated overnight at 4°C. After blocking with PBS-1% bovine serum albumin, serial dilutions of plasma samples were added and incubated overnight at 4°C. Peanut-specific IgG Abs were detected using 0.3 μ g/ml of horseradish peroxidase (HRP)-labeled goat anti-mouse γ -heavy chain-specific Abs (Southern Biotechnology Associates, Birmingham, AL). Biotin-conjugated rat anti-mouse γ I (clone A85-1), γ 2a (clone R19-15), γ 2b (clone R12-3), or γ 3 (clone R40-82) heavy chain mAbs (BD PharMingen, San Diego, CA) were used at 0.5 μ g/ml; and streptavidin-HRP (BD PharMingen) was diluted at 1:2000 for the detection of peanut-specific IgG subclasses. The colorimetric reaction was developed by the addition of 2,2'-azino-bis(3)-ethylbenzylthiazoline-6-sulfonic acid substrate (Sigma) and H₂O₂. Endpoint titers were expressed as the log₂ of plasma dilution giving an optical density at 415 nm of ≥ 0.1 above those obtained with control plasma. To determine the potential of plasma of peanut-sensitized mice to react with irrelevant pro-

tein antigens, plasma samples were added to ELISA plates coated with OVA (1 mg/ml) or Der f protein extract (10 μ g/ml).

The removal of IgG has been shown to improve the detection of IgE Abs.³⁵ Thus, dilutions of plasma samples were first depleted of IgG by overnight incubation at 4°C in protein G-coated 96-well plates (Reacti-Bind plates; Pierce, Rockford, IL). Total and antigen-specific IgE levels were then analyzed by ELISA. For detection of antigen-specific IgE Abs, IgG-depleted samples were added to ELISA plates coated with PPE (50 μ g/ml, 100 μ l/well). The IgE were detected with 0.5 μ g/ml biotin-conjugated rat anti-mouse IgE (clone R35-118; BD PharMingen) followed by streptavidin-HRP (1:2000). The levels of total IgE Abs were determined using capture and detection antibodies, as well as IgE standard, from the BD OptEIA Set mouse IgE kit (BD Biosciences, San Diego, CA).

Airway Hyperreactivity

Enhanced pause (Penh), an index that reflects changes in amplitude of pressure wave form and expiratory time, was measured 6 hours after the last nasal peanut challenge in mice placed in a barometric plethysmograph according to a previously described method.³⁶ Doses of metacholine (0, 10, and 20 mg/ml) were administered by nebulization. For each dose, Penh were measured every minute over 7 minutes. Controls included sham-sensitized and sham-challenged mice.

Histology and Determination of Lung Inflammation Scores

Lungs were fixed in 10% buffered formaldehyde, paraffin-embedded, and cut into sections of 5 μ m thickness. The sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin for the evaluation of inflammation. The presence of eosinophils in tissue sections was determined by the cyanide-resistant peroxidase activity as previously described.³⁷ Briefly, lung sections were incubated for 1 minute at room temperature in 10 mmol/L KCN, pH 6.5. Slides were then rinsed in PBS and incubated for 15 minutes with the peroxidase substrate 3,3'-diaminobenzidine (Vector Laboratories, Burlingame, CA). After washes in PBS, tissue sections were counterstained with hematoxylin. The eosinophils, which express a cyanide-resistant peroxidase activity, appeared as containing dark brown granules, and their frequency was estimated by microscopic observation at $\times 200$ magnification. The neutrophils, which do not express a cyanide-resistant peroxidase, were segregated based on their characteristic morphology.

For quantification of lung inflammation, the slides were coded, and peribronchial and perivascular inflammation was scored in a blinded fashion by two independent investigators. A value of 1 was given when slides showed no sign of inflammation. Slides were graded from 2 to 4 when bronchi were surrounded by a thin layer of inflammatory cells (2, few bronchi; 3, more bronchi; and 4, most

bronchi). They were graded from 5 to 7 according to the number of bronchi that were surrounded by a thick layer of inflammatory cells (5, few bronchi; 6, more bronchi; and 7, most bronchi). Finally, slides were graded 8 or 9 when inflammation spread into the interstitial area (8, severe; and 9, extreme).

Flow Cytometry

Whole-lung tissue was dissociated by digestion with 1 mg/ml collagenase type V (Sigma) in RPMI-1640 (Cellgro Mediatech, Washington, DC), supplemented with 10 mmol/L HEPES, 2 mmol/L L-glutamine, 5×10^{-5} mol/L 2-mercaptoethanol, 100 U/ml penicillin, and 100 μ g/ml streptomycin (supplemented RPMI) to obtain single cell preparations. Mononuclear cells were collected at the 20 to 75% interface of discontinuous Percoll gradient and stained with anti-CD3 (clone 145-2C11), anti-CD4 (clone GK1.5), anti-B220 (clone RA3-6B2), anti-CD11c (clone HL3), anti-MAC-1 (clone M1/70), or anti-MHC class II Abs (I-A^b, clone AF6-120.1) (BD PharMingen). After washes and fixation, samples were analyzed by flow cytometry.

Purification of CD4⁺ T Cells

Whole-lung tissue was dissociated by digestion with collagenase as described above. Mononuclear cells were collected and washed in supplemented RPMI. The CD4⁺ T cells were purified using the automated magnetic cell sorting (autoMACS) according to the protocol provided by the manufacturer (Miltenyi Biotech, Auburn, CA). Briefly, single cell suspensions were incubated with a biotinylated anti-CD4 mAb (BD PharMingen) for 30 minutes at 4°C and washed in PBS containing 2 mmol/L EDTA and 0.5% bovine serum albumin. Streptavidin-conjugated MicroBeads (Miltenyi Biotech) were then added to cells. After a 30-minute incubation at 4°C, cells were washed, and CD4⁺ T cells were purified by positive selection using autoMACS.

Quantification of Cytokine and Chemokine mRNA by Real-Time PCR

Lung tissue was dissociated as described above; mononuclear cells were collected and washed in supplemented RPMI; and RNA was isolated using STAT-60 (Tel-Test, Friendswood, TX). The reverse transcription was performed with superscript II reverse transcriptase, dNTPs, and poly(dT) oligos. The real-time PCR (Lightcycler; Roche, Indianapolis, IN) was performed with primers generated with Oligo software (Plymouth, MN) and the SYBR green detection system according to the manufacturer. Results are expressed as crossing point (CP), defined as the cycle at which the fluorescence rises appreciably above the background fluorescence as determined by the Second Derivative Maximum Method (Roche Molecular Biochemicals LightCycler Software). The formula $20 - (CP_{\text{cytokine}} - CP_{\beta\text{-actin}})$ was used to

represent the logarithm of the relative mRNA levels of a given cytokine. This formula allows the normalization of all results against β -actin levels to correct for differences in cDNA concentration between the starting templates. Differences of crossing points above two cycles were considered significant.

Bronchoalveolar Lavage and Cytospin

Bronchoalveolar lavage fluids (BALF) were obtained via cannulation of the exposed trachea, by infusion of 0.6 ml of supplemented RPMI through a 22-gauge catheter into the lungs, followed by aspiration of this fluid into a syringe. A volume of 0.4 ml of fluid was consistently recovered. Aliquots were centrifuged, and supernatants were collected and stored at -70°C until analyzed. Cell pellets were subjected to cytospin, and the slides were stained with Giemsa (Sigma).

Cytokine ELISA

Cytokines were measured in the supernatants of BALFs by ELISA. Nunc MaxiSorp Immunoplates (Nunc, Naperville, IL) were coated with anti-mouse tumor necrosis factor- α (TNF- α) (clone MP6-XT22), interferon (IFN)- γ (clone R4-6A2), interleukin-4 (IL-4) (clone BVD4-1D11), IL-5 (clone TRFK5), IL-6 (clone MP5-20F3), or IL-10 (clone JES5-2A5) mAbs (BD PharMingen) or IL-13 (R&D Systems, Minneapolis, MN) in 0.1 mol/L sodium bicarbonate buffer (pH 9.5) and incubated overnight at 4°C . After blocking with PBS-3% bovine serum albumin, cytokine standards and serial dilutions of supernatants of BALFs were added in duplicates. The plates were incubated with biotinylated anti-mouse TNF- α (clone MP6-XT3), IFN- γ (clone XMG-1.2), IL-4 (clone BVD6-24G2), IL-5 (clone TRFK4), IL-6 (clone MP5-32C11), IL-10 (clone JES5-16E3) (BD PharMingen), or IL-13 mAbs (R&D Systems), followed by HRP-labeled goat anti-biotin Ab (Vector Laboratories). The colorimetric reaction was developed with the addition of 2,2'-azino-bis(3)-ethylbenzylthiazoline-6-sulfonic acid substrate and H_2O_2 . Standard curves were generated using murine rIFN- γ , rIL-5, rIL-6, rIL-10 (Genzyme, Cambridge, MA), rIL-4 (Endogen Corp., Boston, MA), rTNF- α , and rIL-13 (R&D Systems). The ELISAs were capable of detecting 3 pg/ml IL-4; 5 pg/ml IL-6; 10 pg/ml IL-5; 20 pg/ml IFN- γ , TNF- α , and IL-10; and 30 pg/ml IL-13. A quantikine ELISA kit (R&D Systems) was used for detection of IL-1 β .

Statistics

The results are reported as the mean $\pm$ 1 SD. Statistical significance ($P < 0.05$) was determined by Student's *t*-test and by the Mann-Whitney *U*-test of unpaired samples. The results were analyzed using the InStat statistical software (San Diego, CA) for Apple computers.

Figure 1. Plasma antibody responses in mice orally or nasal sensitized to peanut. Mice were orally (□) or nasally (■) sensitized by administration of PPE and CT on days 0 and 7. Plasma samples were collected on day 14, and Ab responses were analyzed by ELISA. Results are expressed as mean log₂ titers or mean mg/ml $\pm$ 1 SD and are from nine separate experiments with five mice per group. * $P < 0.01$.

Results

Oral Sensitization with PPE and CT Induces Higher IgE but Lower IgG Antibody Responses Than Nasal Sensitization

Although mucosal surfaces of the GI and respiratory tracts are considered the primary sites of sensitization to food antigens, it remains unclear how peanut priming through each site could influence subsequent allergic or inflammatory reactions. We first compared the plasma levels of peanut-specific Ab responses in mice that received whole PPE and CT as adjuvant by the oral and the nasal route. Both mucosal routes of sensitization promoted peanut-specific plasma IgG Abs, but higher levels of IgG responses were measured in mice sensitized by the nasal route (Figure 1). In addition, nasal and oral sensitization also induced different patterns of peanut-specific IgG subclass responses with a lower IgG1-to-IgG2a ratio in nasally sensitized mice (1.3 ± 0.1 vs. 1.8 ± 0.3 , $P < 0.01$) (Figure 1).

In contrast with plasma IgG responses, higher levels of peanut-specific IgE Ab responses were measured in orally sensitized mice (Figure 1). The difference in the levels of IgE responses between mice sensitized by the oral and nasal routes was maintained when lower or higher doses of antigen or adjuvant were used (data not shown). In addition, no difference was seen in antigen-specific IgE Ab responses between mice that were given OVA (1 mg orally or 100 mg nasally) instead of PPE (data not shown). Taken together, these results suggested that oral sensitization with PPE favors IgE Ab responses, whereas nasal sensitization more effectively primes for IgG Abs.

Both Orally and Nasally Sensitized Mice Experience AHR after Nasal Peanut Challenge

The Penh values of mice orally or nasally sensitized with PPE were measured 6 hours after nasal challenge to determine whether these routes of mucosal sensitization primed for different AHR responses. Despite the difference in IgG and IgE Ab responses, both orally and nasally sensitized mice exhibited similar baseline AHR 6 hours after the last nasal peanut challenge with Penh

Figure 2. Airway hyperreactivity after nasal challenge of orally or nasally sensitized mice. Baseline Penh was measured 6 hours after the last peanut challenge in mice sensitized to peanut by the oral or nasal route. Penh values of AHR to metacholine were measured every minute over 7 minutes. Controls included sham-sensitized and sham-challenged mice. Values are expressed as means $\pm$ SD of four mice.

values of 1.1 ± 0.31 and 1.36 ± 0.72 , respectively (Figure 2). These Penh values were significantly higher than those of control sham-sensitized mice (0.57 ± 0.06). In addition, both orally and nasally sensitized mice showed a similar increase of Penh responses to metacholine challenge (Figure 2). Although the difference of Penh values failed to reach statistical difference, nasally sensitized mice consistently exhibited higher Penh than their counterparts sensitized by the oral route.

Nasal Sensitization Promotes Higher Lung Inflammatory Responses

Lungs of orally sensitized mice showed no sign of inflammation when analyzed 10 days after the last administration of PPE and CT (mean score 1; Figure 3, A and C). In contrast, nasally sensitized mice exhibited a moderate inflammation (mean score 3.3; Figure 3, A and C). Furthermore, the cell density was higher in BALF of nasally sensitized mice ($\approx 20 \times 10^4$ cells/ml) when compared with orally sensitized mice ($\approx 6 \times 10^4$) (Figure 3A). Nasal peanut challenge induced a massive recruitment of polymorphonuclear cells in the BALF of both groups of mice (Figure 3, B and C). The cell density in BALFs of nasally sensitized mice were more than 10-fold higher (400×10^4 cells/ml) than that seen in BALFs of orally sensitized mice (30×10^4 cells/ml) (Figure 3B). In addition, lung inflammation was significantly higher in nasally sensitized mice than in those orally sensitized (mean inflammation scores of 7.3 and 5.1, respectively) (Figure 3, B and C). Lung inflammation was not seen after nasal peanut challenge of control naïve mice or mice given CT only by either the oral or nasal route (not shown).

Tissue sections were also subjected to the cyanide-resistant peroxidase staining to further characterize the

recruitment of eosinophils in the lungs after the nasal peanut challenge. Eosinophils were not detected in the lungs of control PBS-challenged or unchallenged mice (results not shown). Significant lung eosinophilia was observed in the lung of mice sensitized by the oral route (Figure 3D). In contrast, nasal peanut challenge promoted only rare eosinophils but significant neutrophilia in the lung of nasally sensitized mice (Figure 3D).

Nasal Peanut Challenge Induces Higher Th2-Type Cytokine Responses in the Lungs of Orally Sensitized Mice

Cytokine-specific mRNA responses were analyzed on whole-lung tissues before and after nasal challenge with PPE. Before the nasal challenge, lung tissues of nasally and orally sensitized mice exhibited similar levels of both Th1 and Th2 cytokine mRNA, including IL-1 β mRNA followed by CCL-11 and IL-17 and lower levels of IL-5, IL-13, IFN- γ , and IL-4 (Figure 4A). Nasal peanut challenge significantly increased IL-4, IFN- γ , and CCL-11 mRNA in the lungs of mice sensitized by either the oral or nasal routes (Figure 4A). Interestingly, the increase of mRNA for Th2-associated cytokines IL-4 and CCL-11 was higher in orally sensitized mice (oral-to-nasal CP ratio of 2.33 and 1.3, respectively). On the other hand, mRNA levels of the Th1-associated cytokine IL-17 were higher in nasally sensitized mice (nasal-to-oral CP ratio of 2.27).

We next analyzed the contribution of CD4 $^{+}$ T cells to lung cytokine and chemokine responses after nasal peanut challenge. Purified lung CD4 $^{+}$ T cells from nasally sensitized mice expressed significantly higher mRNA levels of the Th1-associated cytokine IFN- γ (Figure 4B). On the other hand, higher mRNA levels of the Th2-associated cytokines IL-4 and CCL-11 were seen in lung CD4 $^{+}$ T cells from orally sensitized mice (Figure 4B).

Cytokine responses to nasal peanut challenge were also analyzed at the protein levels in BALFs. Before challenge, the BALFs of mice orally or nasally sensitized with PPE showed low and similar levels of IL-4 and IL-6 (Figure 4C). Nasal peanut challenge significantly increased IL-4 secretion in BALFs of orally sensitized mice but not in samples from those nasally sensitized. In contrast, only nasally sensitized mice showed significantly increased IL-1 β and IL-6 secretion in BALFs on nasal peanut challenge (Figure 4C).

Nasal Peanut Challenge Induces a Massive Recruitment of MAC-1 $^{+}$ I-A ^{b} low Cells in the Lungs of Nasally Sensitized Mice

Because IL-1 and IL-6 responses after nasal peanut challenge were much higher in BALFs of nasally sensitized mice, we more carefully examined the phenotype of cells that contributed to these responses. Before the nasal peanut challenge, the lungs of orally and

Figure 3. Cell recruitment in lung and BALFs of mice orally or nasally sensitized to peanut. Mice were given PPE and CT as adjuvant on days 0 and 7 by the nasal (5 μ l per nostril) or oral (250 μ l) route. **A:** Lung tissue (**top**) and BALFs (**bottom**) were collected 10 days after the last sensitization and before challenge. **B:** Lungs and BALFs were collected on day 17 after nasal peanut challenges on days 15 and 16. Histology sections were stained with hematoxylin and eosin (magnification $\times 40$), and cytopsin preparations of BALFs were stained with Giemsa (magnification $\times 200$). The BALFs from nasally sensitized mice depicted in this panel were diluted 10-fold before the cytopsin. **C:** Lung inflammation scores on histology sections. The density of perivascular and peribronchial infiltrates was determined in a blinded fashion on a subjective 9-point scale (1, minimal infiltrate; 9, massive infiltrate). Boxes represent mice given peanut proteins orally, whereas ovals represent nasal administration. **D:** Lung eosinophilia after nasal challenge of mice orally or nasally sensitized with PPE. The eosinophils were visualized by peroxidase staining of histological sections pretreated with KCN (magnification: top, $\times 100$ and bottom, $\times 200$).

nasally sensitized mice exhibited the same frequency (ie, 9%) of CD11b⁺ (MAC-1) in the lungs (Figure 5A). Nasal challenge induced macrophage recruitment in the lungs, and their percentage rose to ~30% and ~60% in orally and nasally sensitized mice, respectively (Figure 5B). We also observed a difference in the phenotype of CD11b⁺ cells recruited in the lung after the nasal challenge. Thus, whereas one-third of CD11b⁺ cells recruited in the lung of orally sensitized mice expressed high levels of MHC class II molecules (I-A^b), only a small fraction (one-tenth) of those recruited in the lung of nasally sensitized mice expressed this phenotype associated with activated macrophages (Figure 5B).

Lung Inflammatory Responses of Mice Mucosally Sensitized with Peanut after Nasal Challenge with Unrelated Antigens

Peanut allergy is frequently associated with pollen allergy,^{9–12} and peanut allergens could share sequence homology with environmental antigens.¹³ Therefore, we next investigated whether the change of airway environment induced by oral versus nasal sensitization to peanut would influence inflammatory responses to unrelated food or environmental proteins. Interestingly, mice sensitized to peanut by the oral and nasal route differentially reacted to nasal challenge with unrelated proteins. Thus, low levels of lung inflammation (ie, mean inflammation

Figure 4. Cytokine responses in lungs and BALFs of orally or nasally sensitized mice. **A:** Relative cytokine mRNA expression in whole-lung tissue before (dotted pattern) or after (open or solid pattern) nasal peanut challenge. **B:** Relative cytokine mRNA expression by CD4⁺ lung T cells collected after nasal peanut challenge of mice sensitized by the oral (□) or nasal (■) routes. All of the results were normalized against β -actin expression to correct for differences in cDNA concentration of the starting template and are expressed as $20 - (C_{\text{cytokine}} - C_{\beta\text{-actin}})$. Differences of crossing points above two cycles are considered significant. The results are representative of three separate experiments. **C:** Cytokine secretion in BALFs collected before (dotted pattern) or after (open or solid pattern) nasal peanut challenge. Cytokine levels were determined by ELISA. The levels of IL-5, IL-10, IL-13, TNF- α , and IFN- γ were below detection limit (ND).

score = 2) were seen when orally sensitized mice were challenged with OVA or Der f proteins. On the other hand, nasal challenge with the unrelated food or environmental antigens induced high lung inflammatory responses (ie, mean inflammation score = 4) in mice previously sensitized to peanut by the nasal route (Figure 6).

Discussion

Food allergies have been mostly investigated by sensitization and challenge of animal models via the subcuta-

Figure 5. The frequency of MAC-1⁺ cells in the lung of mice mucosally sensitized to peanut. Orally or nasally sensitized mice were either challenged or not challenged with PPE on days 15 and 16. Lung tissues were collected on day 17 and digested with collagenase. After purification on a discontinuous Percoll gradient, the cells were collected at the 20 to 75% interface and analyzed by flow cytometry.

Figure 6. Lung inflammatory responses to nasal challenge with unrelated antigens. Mice were nasally or orally sensitized by administration of PPE and CT as adjuvant on days 0 and 7 and nasally challenged with OVA or the respiratory antigen Der f on days 15 and 16. Lung tissues were collected at day 17, and inflammation was examined on sections stained with hematoxylin and eosin (magnification $\times 100$).

neous, parenteral, or oral routes.^{28,38–41} Although peanut allergy has been thus far studied on animal models after oral sensitization or challenge,^{31,42} the possibility of allergic reaction after airborne sensitization and/or challenge is now being considered.⁴³ The mucosal tissues of the GI tract are not readily accessible, making difficult the investigation of mechanisms governing adverse mucosal reactions to food and environmental antigens/allergens. Animal models could be developed to address mucosal mechanisms of allergy by taking advantage of the greater accessibility of the mucosal tissues of the respiratory tract. However, it is now well established that distinct mechanisms govern the homing of effector cells induced in the nasopharyngeal and GI tissues.⁴⁴ To date, no animal model has addressed how oral and nasal sensitization to peanut affects allergic manifestations and, more specifically, the cellular and molecular events after nasal challenge with peanut or unrelated antigens. We hypothesized that mucosal sensitization in the presence of CT, a mucosal adjuvant that promotes IgE Ab and Th2-type responses,^{34,45,46} could aid the development of mouse models of peanut allergy by either oral or nasal sensitization. We found that oral sensitization of mice induced higher levels of peanut-specific plasma IgE Ab responses and higher lung eosinophilia after nasal challenge with peanut. In contrast, nasal sensitization led to higher levels of peanut-specific plasma IgG Ab responses and increased lung inflammation with a massive recruitment of macrophages after nasal challenge with peanut. More importantly, nasal but not oral peanut sensitization favored inflammatory responses to nasal challenge with unrelated antigens.

Vaccine studies in humans and animal models have shown that the oral and nasal routes of priming promote distinct profiles of IgG and IgA Ab responses in the respiratory and genito-urinary tract.^{41,47–49} Few studies have directly compared the profile of serum Ab responses after oral and nasal sensitization to the same

antigen/allergen. Our finding that oral delivery of PPE and CT promotes higher levels of peanut-specific IgE responses than nasal delivery is consistent with earlier reports suggesting that Peyer's patches of the GI tract are preferred sites of IgE Ab responses.^{50,51} High IgE responses have been reported after nasal administration of mice with several protein antigens in the presence of CT.^{34,52-54} High levels of antigen-specific IgE Abs were also measured when OVA was used instead of PPE in our nasal sensitization studies (data not shown). Peanut contains a large number of proteins, and the biological activity of most of them has not been carefully investigated. Thus, unique biological activity of some peanut proteins may counteract the Th2-inducing effect of the CT adjuvant, as suggested by others.³² In addition, different subsets of antigen-presenting cells are present in the inductive site of the GI- and nasopharyngeal-associated lymphoid tissues¹⁷ and their respective contribution to IgG and IgE responses remain to be elucidated. Together, these results suggest that oral sensitization with whole PPE and CT is more effective at promoting systemic IgE Abs and Th2-associated responses than when this regimen is delivered by the nasal route.

Antibody and lung inflammatory responses are under the control of cytokines and chemokines in mice⁵⁵ as well as in humans.^{21,56} For example, the Th2-type cytokines IL-4⁵⁷⁻⁵⁹ and IL-13 enhance IgE responses,⁶⁰ an effect antagonized by IFN- γ .⁶¹ A number of studies have demonstrated that IL-5 and CCL-11 favor mucosal (ie, respiratory and GI) eosinophilic inflammations.⁶²⁻⁶⁴ IL-1, IL-6, and IFN- γ are known for their role in inflammation,⁶⁵ and IL-17 is a Th1 cytokine involved in the recruitment of neutrophils into the airways.^{66,67} The signs of mild lung inflammation, which were noticeable in nasally sensitized mice before any challenge, were unlikely due to the diffusion of PPE and CT into the lungs. In fact, these observations were made 10 days after sensitization with a small volume of solution that most likely did not diffuse outside the nasal cavity.³³ Furthermore, lung and BALF proinflammatory cytokine responses of mice sensitized orally and nasally were not different before challenge. The evaluation of other parameters such as chemokines may be needed to explain the higher number of cells in the lungs of mice sensitized by the nasal route.

Mice sensitized both orally and nasally to peanut experienced AHR on nasal peanut challenge. Despite recent arguments about the limitation of the whole-body plethysmography as a reliable method for evaluation of lung functions,⁶⁸⁻⁷⁰ our data show similar alteration of Penh in orally and nasally sensitized mice. However, significant differences were seen in terms of cells infiltrating airway tissues and cytokine responses in the two groups. Thus, nasal challenge significantly increased lung IL-4 and CCL-11 mRNA levels and IL-4 secretion in the BALFs of orally sensitized mice. These findings were consistent with the higher lung eosinophilia seen in these mice and with the previously reported role of IgE for eosinophil-mediated airway hyperactivity.^{71,72} Allergic asthma is described as an atopic disease that involves IgE, Th2 responses, and eosinophilic airway inflammation, resulting in enhanced bronchial reactivity.¹⁹ Our

results suggest that nasal peanut challenge of mice sensitized by the oral route can lead to lung inflammatory responses that resemble allergic asthma. In contrast with mice sensitized orally, those sensitized by the nasal route showed increased IL-17 and IFN- γ mRNA levels in the whole-lung samples and in infiltrating CD4⁺ T cells, respectively. These mice also showed increased IL-1 and IL-6 secretion in the BALFs after nasal peanut challenge. Unlike orally sensitized mice, no significant lung eosinophilia was seen after nasal challenge of those nasally sensitized. Our results are consistent with studies suggesting that IgG antibodies counter allergen-triggered eosinophil recruitment.⁷³ There is now evidence that asthma can also take place in the absence of IgE and eosinophilia. As seen after nasal peanut challenge of mice sensitized by the nasal route, this other form of asthma is characterized by a massive infiltration of neutrophils in the lung.^{66,74} It has also been shown that IL-1, IL-6, and IL-17 secreted by epithelial cells and macrophages play a major role in neutrophil-associated asthma.^{75,76} In this regard, the leading cellular event after nasal challenge of mice sensitized by the nasal route is a massive recruitment of macrophages in the lung. Taken together, these results suggest that IgE-mediated asthma is induced after nasal challenge of mice orally sensitized with PPE, whereas non-IgE-mediated asthma occurred in those sensitized by the nasal route.

As indicated earlier, peanut allergy is frequently associated with pollen allergy,⁹⁻¹² and peanut allergens could share sequence homology with environmental antigens.¹³ A recent report showed that Th2-type responses induced in the GI tract can influence immunophysiological responses in distant noninflamed mucosal tissue and regulate airway responsiveness.⁷⁷ Therefore, it was important to determine whether the immune status resulting from oral or nasal sensitization with peanut affected airway responses to unrelated antigens. Interestingly, only mice sensitized to peanut by the nasal route exhibited lung inflammatory responses to nasal challenges with unrelated antigens, including OVA, without known similarities with peanut proteins. This finding strongly suggests that the cytokine environment and subsequent innate responses dictate the potential of nonspecific airway inflammation of mice sensitized by the nasal route. Of interest, these results are consistent with the greater level of inflammatory cytokine responses in the lungs (ie, IL-17 and IFN- γ mRNA) and BALFs (ie, IL-6) in these mice. Our separate studies suggest that the dose of peanut used for the nasal sensitization does not explain the tendency of nasally sensitized mice to develop nonspecific inflammatory responses. It has been reported that Der f protein extracts stimulate macrophages and inflammatory responses.⁷⁸ Our results suggest that peanut protein extracts can also stimulate cells in the upper respiratory tract and trigger a state of pro-inflammation. These data indicate that nasal sensitization with peanut leads to a lasting proinflammatory status, which could modify bronchial reactivity and favor nonspecific lung inflammation to unrelated environmental antigens.

In summary, we have shown that peanut sensitization via the oral and nasal routes leads to distinct inflamma-

tory responses to subsequent exposure to peanut in the respiratory tract, with mice sensitized by the oral route more prone to allergic-type responses. Nasal sensitization on the other hand, favors nonallergic inflammation and innate responses to unrelated environmental antigens. These findings have important implication for the development of animal models that more accurately reflect allergic pathologies in humans.

Acknowledgments

We thank Mrs. Annette Pitts for technical assistance with histology.

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