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Journal of Applied Physiology 96:1433-1444, 2004. First published Dec 12, 2003;
doi:10.1152/jappphysiol.01128.2003

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Dust mite-induced asthma in cynomolgus monkeys

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Departments of ¹Physiology, ²Medicine, and ³Comparative Medicine, East Carolina University, Greenville, North Carolina 27858; ⁴LABS of Virginia^c, Yemassee, South Carolina 29945; and ⁵EpiGenesis Pharmaceuticals, Cranbury, New Jersey 08520

Submitted 17 October 2003; accepted in final form 5 December 2003

Van Scott, Michael R., Jerry L. Hooker, David Ehrmann, Yoshimi Shibata, Cynthia Kukoly, Kenneth Salleng, Greg Westergaard, Anthony Sandrasagra, and Jonathan Nyce. Dust mite-induced asthma in cynomolgus monkeys. *J Appl Physiol* 96: 1433–1444, 2004. First published December 12, 2003; 10.1152/jappphysiol.01128.2003.—Animal models exhibiting high homology with humans at the genetic and pathophysiological levels will facilitate identification and validation of gene targets underlying asthma. In the present study, a nonhuman primate model of allergic asthma was developed by sensitizing cynomolgus monkeys to dust mite antigen. Sensitization elevated allergen-specific serum IgE and IgG levels, and peripheral blood mononuclear cells isolated from sensitized animals released IL-4, IL-5, and IL-10, but not IFN- γ . Aerosolized allergen decreased dynamic compliance and induced airway inflammation and hyperresponsiveness to aerosolized histamine. Albuterol and dexamethasone inhibited the airway constriction and allergen-induced inflammation, respectively. Airway wall remodeling that included goblet cell hyperplasia, basement membrane thickening, and smooth muscle hypertrophy was particularly evident in neonatally sensitized animals. In contrast to animals sensitized as adults, neonatally sensitized animals exhibited increased sensitivity to adenosine and larger allergen-induced changes in airway resistance and dynamic compliance. These results demonstrate that sensitization of cynomolgus monkeys with dust mite induces asthmatic symptoms, some of which may be dependent on age at the time of sensitization.

airway hyperresponsiveness; airway remodeling; cytokines; histamine; adenosine; compliance; resistance

ASTHMA IS A COMPLEX INFLAMMATORY disease involving both genetic and environmental factors. Diversity in living conditions, behavior, and timing of exposure to environmental triggers complicate identification of the key genes underlying asthma pathogenesis and definition of critical periods in development that impact the process. Genomic analysis of asthma and validation of the gene targets resulting from such analyses would be facilitated by animal models that replicate the human disease genetically, physiologically, and pathologically. Most animal models of asthma are inadequate for this purpose because of basic differences in gene sequences and species-specific differences in postnatal development of the immune and respiratory systems. Nonhuman primate models reduce these concerns. The present study was undertaken to develop a nonhuman primate model of asthma that would support target identification, target validation, and screening of lead therapeutic candidates.

Historically, nonhuman primate models of asthma and airway hyperresponsiveness have been based on two paradigms:

identification of animals exhibiting natural allergic sensitivity and induction of sensitivity through systemic exposure. Over 25 years ago, Patterson and Harris (15) began studying rhesus monkeys that exhibited natural sensitivity to *Ascaris suum* antigens. In 1983, Richards and colleagues (17, 18) demonstrated the feasibility of inducing sensitivity by infecting non-sensitive animals with *Ascaris suum* ova. This model has been used effectively by the pharmaceutical industry to screen novel candidate asthma drugs, although the allergen is not normally associated with human asthma. In 1997, Yasue and colleagues (26) demonstrated that periodic subcutaneous injection of dust mite allergen in rhesus monkeys elevated serum levels of allergen-specific immunoglobulins and induced cutaneous and conjunctival allergic responses, but the response to aerosolized allergen was not reported. In 2001, Schelegle and colleagues (20) used aerosolized dust mite to induce progressive inflammation, decline in pulmonary function, and structural changes in the airway wall of rhesus monkeys. This model has revealed exciting new information on potential neural adaptation and changes in airway innervation in response to allergen and toxins (3). The model involves a single injection of allergen and adjuvant followed by repeated intranasal and aerosol exposures to allergen. Although this model closely resembles the human disease, it is labor intensive, and the dependence on repeated aerosol challenge limits its practicality for routine drug screening.

Previous work in rabbits has demonstrated that periodic injection of allergen and adjuvant from the time of birth and then challenging with aerosolized allergen will induce pulmonary inflammation, early- and late-phase responses, and airway hyperresponsiveness characteristic of asthma. This technique, which was originally developed using *Alternaria tenuis* (21) and later adapted to house dust mite (11) and ragweed (4), has been useful for testing conventional and genomic-based drugs, such as EPI-2010, a respirable antisense against the adenosine A₁ receptor (13). Limitations in the number of immunologic reagents compatible with this species and genetic homology with humans restrict the use of rabbits in biomedical research, but the approach used to induce asthmatic responses in this species could be applicable to other, more relevant species.

In the present study, cynomolgus monkeys were sensitized to house dust mite, either as adults or from the time of birth, and challenged with aerosolized dust mite. The allergen-induced immune response, pulmonary inflammation, airway dysfunction, and tissue remodeling were subsequently characterized.

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METHODS

Animals

All animals used in this study were born and raised for at least 6 mo at LABS of Virginia, Yemassee, SC. On arrival at East Carolina University, the animals were group housed in the Department of Comparative Medicine. Animal husbandry was conducted under US Department of Agriculture guidelines. All protocols were approved by the Institutional Animal Care and Use Committee of East Carolina University.

Protocols

Three- to 4-yr-old (adult) animals. Seven animals, 3–4 yr old, were evaluated for sensitivity to aerosolized histamine and airway inflammation via differential cell counts of bronchoalveolar lavage (BAL) fluid. The animals were then sensitized by bimonthly subcutaneous injections of allergen adsorbed to alum [312 arbitrary units (AU) of *Dermatophagoides pteronyssinus* (Dp) and *Dermatophagoides farinae* (Df) extract, Greer Laboratories, Lenoir, NC; Imject alum, Pierce, Rockford, IL] for 4 mo. As originally conceived, the protocol called for the animals to receive three aerosol dust mite challenges at 1-mo intervals to establish chronic inflammation before the asthmatic response was characterized. However, the animals exhibited significant distress during the first aerosol dust mite challenge, so the protocol was modified as follows. Six weeks after the first dust mite challenge, the subjects were boosted by subcutaneous injection with allergen extract in alum. Two weeks later, responses to aerosolized histamine were determined, the lungs were lavaged, and differential cell counts of BAL fluid were acquired. Twenty-four hours later, the animals were challenged with aerosolized dust mite, and the early asthmatic response was measured. Forty-eight hours after the dust mite challenge, the histamine challenge and BAL were repeated.

Neonatal animals. Fifteen animals were injected with allergen (312 AU of Dp and Df extract adsorbed to alum) within 24 h of birth, once per week for an additional 4 wk, and then twice per month until 6 mo of age. At 8 mo of age, the subjects were boosted by subcutaneous injection with allergen extract in alum. Two weeks later, responses to aerosolized histamine were determined and the lungs were lavaged for differential cell counts. Twenty-four hours later, the animals were challenged with aerosolized dust mite, and the early asthmatic response was measured. Forty-eight hours after the dust mite challenge, the histamine challenge and BAL were repeated. Eight animals were sham-sensitized by subcutaneous injection of saline on the same schedule used for sensitizing to allergen.

After the initial characterization, the animals were allowed to recuperate for 9 wk before being separated into cohorts for additional characterization of late-phase responses to dust mite and responses to steroid and β -adrenergic agonist therapies. Responsiveness to steroid therapy was assessed by use of a sequential protocol in which all subjects were treated with vehicle in the first leg and dexamethasone in the second leg. This ensured that inflammation and pulmonary function had returned to baseline at the time the protocol began. The subjects were boosted by inhalation of allergen (200 AU/ml for 4 min). Two weeks later, sensitivity to aerosolized histamine was determined and BAL samples were collected. The animals were treated daily for 3 days with vehicle or dexamethasone (1 mg twice/day orally). On the fourth day, the animals received the morning treatment via intramuscular injection, were intubated, and then were challenged with 200 AU/ml (1:50 dilution) of dust mite delivered through a Devilbiss ultrasonic nebulizer for 4 min. Dynamic lung compliance (C_{dyn}) and airway resistance (Raw) were monitored for 6 h. Oral treatments were continued until the morning of the sixth day, when the treatment was given by intramuscular injection. Sensitivity to aerosolized histamine was reassessed and the lungs lavaged. A 3-wk washout period separated the vehicle and dexamethasone legs of the study.

Responsiveness to β -adrenergic receptor agonist therapy was assessed by treating the animals with albuterol (2.5 mg/ml, 2 ml delivered by aerosol) 60 min before provocation with aerosolized methacholine or dust mite.

Pulmonary Function Testing

Subjects were anesthetized with 2.5 mg/kg telazol by intramuscular injection and maintained on propofol delivered by intravenous infusion at 10–15 or 20–25 mg·kg⁻¹·h⁻¹ for dust mite or histamine provocation, respectively. An endotracheal tube and esophageal balloon were inserted. C_{dyn} and Raw were measured by standard computer analyses of the flow and pressure signals, digitalized by a MuMed PR800 physiological recorder (MuMed, London, UK). Saline was delivered via a Devilbiss ultrasonic nebulizer for 2 min, and pulmonary function was monitored for 2 min to establish baseline parameters. Aerosolized histamine or adenosine was delivered for 2 min. The animals were allowed to rest for 1 min, and then the response to the challenge was monitored for 1 min. Cumulative doses were delivered until a 30% decrease in C_{dyn} was observed with adenosine or 50% decrease in C_{dyn} was observed with histamine.

Animals were challenged with aerosolized dust mite for 4 min, and pulmonary function was monitored. Increasing concentrations of allergen were delivered at 15-min intervals until a 50% change in C_{dyn} was observed. Initially, dilutions of 1:500 (20 AU/ml), 1:100 (100 AU/ml), and 1:4 (2,500 AU/ml) were delivered. In later experiments, the 1:4 dilution was replaced with 1:20 and 1:50 dilutions to protect against excessive responses.

After dust mite challenge, pulmonary function was monitored for up to 6 h. The data were expressed as a percent of the saline control period.

Bronchoalveolar Lavage and Differential Cell Counts

After the histamine challenge, a polyethylene catheter (PE 190) was advanced through the endotracheal tube into the lungs and gently wedged into an airway. The segment beyond the tip of the catheter was lavaged with 2.5 ml of sterile saline, which was collected by gentle aspiration. Total cell counts were determined by using a Coulter Counter (Beckman Coulter Instruments, Miami, FL). Cytospin preparations were prepared and stained with DiffQuik (VWR Scientific, South Plainfield, NJ). Differential cell counts were acquired by counting 200 cells per sample.

ELISA for Allergen-Specific IgE and IgG

Ninety-six-well plates were coated with dust mite allergen (10 μ g/ml per well, overnight at 4°C in 0.01 M sodium bicarbonate buffer, pH 9.6). The plates were washed and blocked with buffer containing 10% newborn calf serum. After washing was completed, serum samples (1:5 to 1:100,000 dilution) were added to the wells (100 μ l/well). The plates were incubated overnight at 4°C.

IgG was detected by incubating the plate with 1 μ g/ml horseradish peroxidase-conjugated anti-monkey IgG for 1 h at room temperature [GAMon/IgG(H+L), Nordic Immunological Laboratories, Tilburg, The Netherlands]. IgE was detected by incubating the plates with 1 μ g/ml biotinylated goat anti-human IgE for 1 h at room temperature (Vector Lab, Burlingame, CA). The plates were developed by use of streptavidin-conjugated horseradish peroxidase (BD PharMingen, San Diego, CA) and 3,3',5,5'-tetramethylbenzidine (BD PharMingen). The reaction was stopped by addition of sulfuric acid, and the absorbance at 450 nm was determined.

Cytokine Recall Assay

Lymphocytes were isolated from 1–3 ml of EDTA-treated blood diluted up to fourfold with saline. The diluted blood was fractionated on Ficoll-Isopaque (Histopaque 1.077, Sigma Chemical) by centrifugation at

800 *g* for 30 min. Mononuclear cells were collected from the top of the gradient. When necessary, contaminating red blood cells were lysed by hypotonic shock. The cells were washed three times and resuspended in complete media [RPMI 1640 plus 10% FBS plus antibiotics (100 U/ml penicillin, 100 μ g/ml streptomycin, 1 μ g/ml amphotericin B)] to a density of 1×10^6 cells/ml. Cell suspensions were incubated with either dust mite allergen (10 μ g/ml Dp, Greer Laboratories) or a combination of 1 μ M calcium ionophore (A23187) and 1 μ M phorbol myristic acid (PMA) for 1 day at 37°C, 95% CO₂, and 99% humidity. Supernatants were collected, and concentrations of IL-4, IL-5, IL-10, and IFN- γ were determined by ELISA.

For IL-4 detection, purified mouse anti-human IL-4 monoclonal antibody (BD PharMingen) was used for capturing IL-4, and biotinylated rat anti-human IL-4 (BD PharMingen) was used for detection. Recombinant human IL-4 (BD PharMingen) was used as a standard.

For IL-5 detection, purified rat anti-human IL-5 monoclonal antibody (BD PharMingen) was used for capture, and biotinylated rat anti-human IL-5 (BD PharMingen) was used for detection. Recombinant human IL-5 (BD PharMingen) was used as a standard.

IL-10 was detected by use of purified rat anti-human IL-10 monoclonal antibody (BD PharMingen) for capture and biotinylated rat anti-human IL-10 (BD PharMingen) for detection. Recombinant human IL-10 (BD PharMingen) was used as a standard.

IFN- γ was measured by using murine anti-human IFN- γ monoclonal antibody (MD-1 Biosource) for capture, anti-human IFN- γ monoclonal antibody (DiaPharma Group, West Chester, OH) for detection, and recombinant human IFN- γ as a standard (BD PharMingen).

Pathology

Tissues were fixed in 10% neutral buffered formalin, paraffin embedded, cut into 10- μ m sections, and stained with hematoxylin and eosin, alcian blue and periodic acid-Schiff, and Masson's trichrome to evaluate inflammation, smooth muscle hyperplasia, submucosal fibrosis, and mucous cell hyperplasia, respectively. Tissue sections from the trachea, lungs, and nasal passages were evaluated by a pathologist who was blinded to the subject identification.

Statistics

Means $\pm$ SE are reported. Statistical significance was determined by using Student's *t*-test for paired observations and ANOVA with Fisher's least significant difference post hoc test for comparison of multiple groups ($P \leq 0.05$).

RESULTS

Immunoglobulin Levels After Dust Mite Sensitization

Serum dust mite-specific IgE and IgG levels were determined in four cohorts to assess effectiveness of the sensitization protocols: animals sensitized to allergen starting at 3–4 yr of age, 6- to 8-mo-old animals that had been sham-sensitized or sensitized to allergen from birth, and 82 animals of various ages from the colony where the study subjects originated. For the sham- and dust mite-sensitized animals, blood samples were collected within 2 wk of subcutaneous injection with saline or allergen in adjuvant. Sham- and nonsensitized animals exhibited significantly lower levels of dust mite-specific IgE and IgG than allergen-sensitized animals (Fig. 1, $P < 0.03$). Animals sensitized as neonates exhibited higher levels of dust mite-specific IgG in serum than animals sensitized as adults ($P < 0.001$), but the level of dust mite-specific IgE in serum was not significantly different between these two groups.

Interestingly, the nonsensitized animals, which had been

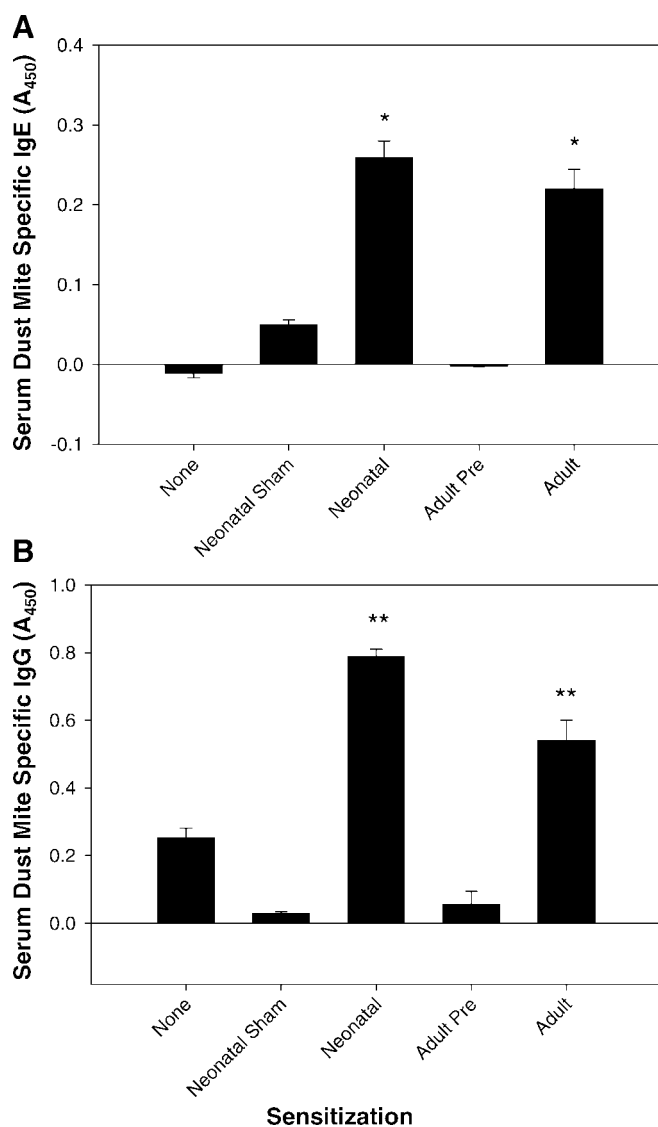


Fig. 1. Serum dust mite-specific immunoglobulin levels. Serum samples drawn from 82 nonsensitized animals of various ages (None), 8 sham-sensitized animals (Neonatal Sham), 12 neonatally sensitized animals (Neonatal), and 7 adult animals before (Adult Pre) and after sensitization (Adult) were analyzed for allergen-specific IgE (A) and total IgG (B) by ELISA. For the dust mite-sensitized groups (Neonatal and Adult), samples were drawn within 2 wk of a subcutaneous boost with dust mite adsorbed to alum. Values are means $\pm$ SE. *Significantly different from nonsensitized, sham-sensitized, and presensitized groups ($P < 0.001$). **Significantly different from all other groups ($P < 0.04$).

raised in outdoor corrals, exhibited higher levels of dust mite-specific IgG than either the sham-sensitized animals or the adult animals before sensitization. In contrast to the former group, both of the latter groups were housed indoors under conditions in which the animals would be expected to have minimal contact with dust mite allergen.

Pulmonary Function Changes in Response to Initial Aerosol Allergen Exposure

Pulmonary sensitivity to allergen was assessed in sham-sensitized and dust mite-sensitized animals. Animals were

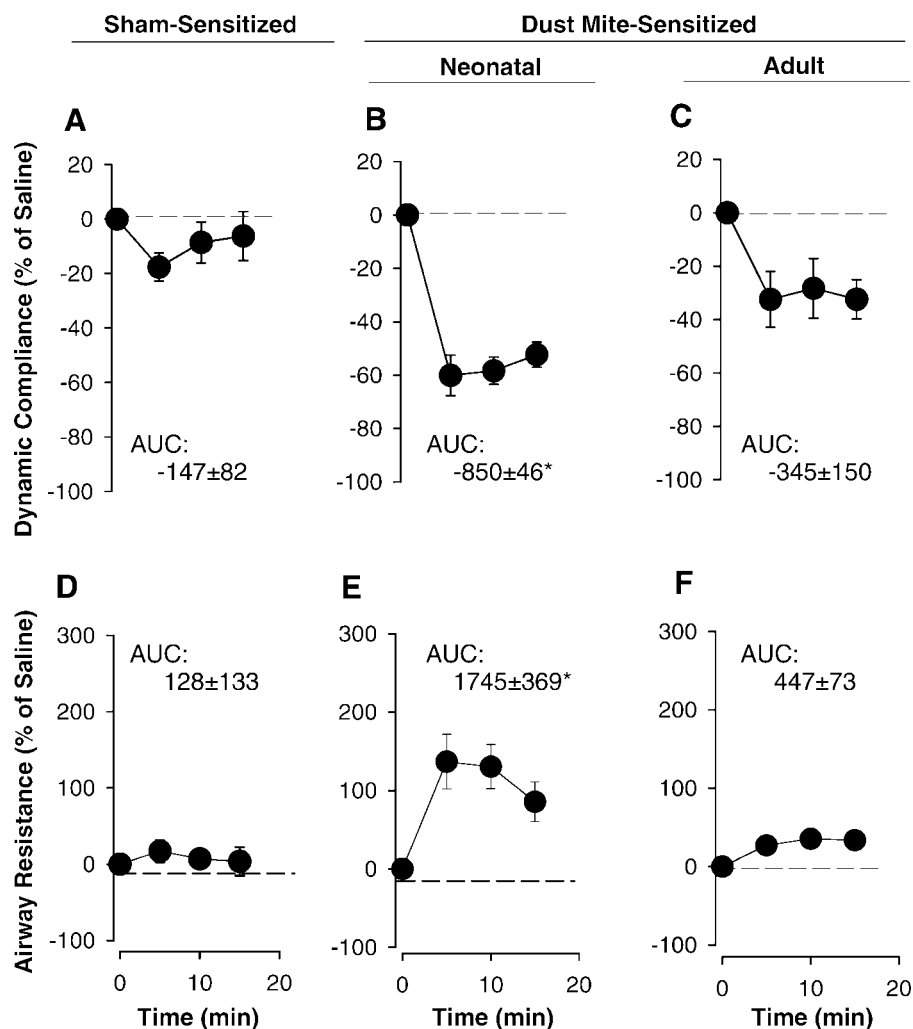


Fig. 2. Changes in dynamic compliance (Cdyn; A–C) and airway resistance (Raw; D–F) during the early-phase response in sham-, neonatally, and adult-sensitized cynomolgus monkeys. A subcutaneous injection of vehicle (sham group) or allergen + alum was given 2 wk before allergen challenge. All animals were challenged with allergen diluted 1:100 in saline from its stock concentration of 10,000 AU/ml. The allergen was delivered over 4 min, and pulmonary function was monitored for 15 min. If less than a 50% decrease in Cdyn was observed, the animals were challenged with a 1:4 dilution of allergen. In the sham-sensitized group (A and D), all animals were administered the 1:4 dilution. In the neonatally sensitized group (B and E), the values represent the mean changes in Cdyn for 6 animals that responded to the 1:100 dilution of dust mite and 9 animals that were administered the 1:4 dilution. For the adult-sensitized group (C and F), the values represent means for 2 animals that responded to the 1:100 dilution of allergen and 4 animals that were administered the 1:4 dilution. AUC, area under the curve. Values are means ± SE. *Different from Sham and Adult groups ($P < 0.007$).

exposed to decreasing dilutions of aerosolized allergen until a 50% decrease in compliance was observed. All sham-sensitized animals were exposed to the lowest dilution (i.e., 1:4 dilution) and exhibited minimal responses in Cdyn and Raw (Fig. 2, A and D). Two adult animals exhibited a 50% decrease in Cdyn after exposure to the 1:100 dilution, and four remaining animals exhibited decreases in Cdyn after exposure to the 1:4 dilution that did not reach the 50% level of inhibition (Fig. 2C). One animal did not complete the protocol because of technical complications. Only a small increase in resistance was observed in the adult animals (Fig. 2F).

In contrast to the sham-sensitized and adult animals, animals sensitized from birth exhibited larger, more consistent changes in both Cdyn and Raw (Fig. 2, B and E). Of the 12 animals entering the protocol, six animals exhibited a >50% drop in Cdyn after exposure to the 1:100 dilution of allergen, and the remaining animals exhibited a >50% drop in Cdyn after exposure to the 1:4 dilution of allergen. Comparison of the area under the curves indicated that the dust mite-induced changes in Cdyn and Raw were significantly greater in the neonatally sensitized group compared with the sham- and adult-sensitized groups ($P < 0.007$).

Responses to Aerosolized Histamine and Adenosine

Responses to aerosolized histamine provocation were measured in sham-sensitized animals, animals sensitized to dust mite as adults, and animals sensitized from birth (Fig. 3). Sham-sensitized animals exhibited a slight decrease in the provocative dose of histamine that caused a 50% decrease in Cdyn (PC_{50}) after exposure to aerosolized dust mite, but the change was not statistically significant. In contrast, animals sensitized to allergen as adults exhibited a statistically significant decrease in the PC_{50} to histamine after allergen challenge ($P = 0.003$). Neonatally sensitized animals exhibited the lowest PC_{50} to histamine before allergen challenge. Challenging with aerosolized dust mite yielded a PC_{50} that was statistically different from the sham-sensitized animals challenged with dust mite ($P = 0.034$) but was not statistically different from the prechallenge PC_{50} recorded for the neonatally sensitized group. These observations indicate that neonatally sensitized cynomolgus monkeys are hyperresponsive to aerosolized histamine, and at least a portion of the responsiveness can be attributed systemic exposure of allergen during the sensitization and priming procedures.

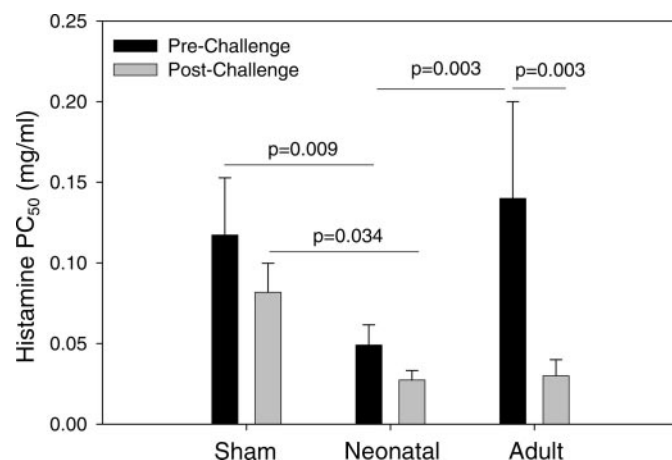


Fig. 3. Responsiveness to histamine before and after the first aerosol dust mite challenge. The provocative dose of histamine that caused a 50% decrease in dynamic compliance (PC₅₀) was determined before the first known exposure to aerosolized dust mite and 48 h after aerosol dust mite challenge in sham- ($n = 8$) and dust mite-sensitized neonatal ($n = 15$) and adult ($n = 6$) cynomolgus monkeys. Values are means $\pm$ SE.

Neonatally sensitized animals exhibited greater sensitivity to adenosine than animals sensitized as adults. Only one of seven adult animals responded to adenosine aerosolized at concentrations ≤ 10 mg/ml. The one adenosine-sensitive animal exhibited a PC₃₀ to adenosine of 0.02 mg/ml. In contrast, 7 of 12 neonatally sensitized animals exhibited sensitivity to aerosolized adenosine (mean PC₃₀ to adenosine = 2.8 ± 1.0 mg/ml, range of PC₃₀ values = 0.01–10 mg/ml, $n = 7$).

Allergen-Induced Airway Inflammation

Sham-sensitized animals exhibited minimal inflammation either before or after aerosol allergen challenge (Table 1). Initial exposure of neonatally sensitized animals to aerosolized dust mite increased the percentage of eosinophils in the BAL fluid from 1 to 6% of the total cells recovered. Rechallenge

with allergen 2 wk after priming with aerosolized allergen (5) further increased the eosinophils in the BAL fluid to 20% of the total recovered cells. Adult animals sensitized to dust mite also exhibited an increase in eosinophils in the BAL fluid to $\sim 5\%$, but rechallenging with aerosolized had minimal effect. These observations indicate that systemic sensitization of cynomolgus monkeys to dust mite induces susceptibility to allergic airway inflammation, and neonatal sensitization is more effective than sensitization of adult animals. However, even in neonatally sensitized animals, repeated allergen challenge may be required to establish peak inflammation.

To verify that 48 h corresponded with the time of peak eosinophilic inflammation, four neonatally sensitized animals were pretreated with the histamine H₁ receptor antagonist diphenhydramine (50 mg/ml, 12.5 mg/animal im) and challenged with allergen. BAL fluid was subsequently collected from one animal at each of the following time points: 6, 24, 48, and 96 h. At the 6-h time point, no eosinophils were observed in the BAL fluid. At 24, 48, and 96 h, the eosinophils accounted for 6, 22.5, and 7.5% of the total cells, respectively. The findings indicated that the 48 h after challenge was an appropriate time point for BAL collection. Furthermore, the observation that the eosinophil counts at the latter three time points were higher than the average of 6.3% recorded in the absence of diphenhydramine indicated that a large early-phase histamine response may oppose the subsequent development of eosinophilic inflammation. In all subsequent experiments, animals were pretreated with diphenhydramine (50 mg/ml, 12.5 mg/animal im) before dust mite challenge to compensate for this possibility.

Early- and Late-Phase Responses With Repeat Allergen Challenges

To better simulate asthma in humans, adult and neonatally sensitized animals were primed by inhalation of allergen in lieu of injection of allergen and adjuvant. Three weeks later, the challenge was repeated, and the early- and late-phase responses

Table 1. Inflammatory cell counts in BALF 48 h after dust mite challenge

	<i>n</i>	Total, $\times 10^4$ /ml	Lymphocytes, $\times 10^4$ /ml	Eosinophils, $\times 10^4$ /ml	Neutrophils, $\times 10^4$ /ml	Macrophages, $\times 10^4$ /ml	Epithelial Cells, $\times 10^4$ /ml
Sham sensitized							
Prechallenge	8	26.5 $\pm$ 3.7	0.9 $\pm$ 0.2	1.1 $\pm$ 0.5	1.0 $\pm$ 0.4	17.3 $\pm$ 2.3	6.3 $\pm$ 1.3
Post-dust mite challenge	8	24.6 $\pm$ 4.1	0.5 $\pm$ 0.1	1.2 $\pm$ 0.6	1.0 $\pm$ 0.2	17.2 $\pm$ 4.0	4.8 $\pm$ 0.8
Neonatally sensitized							
Pre-dust mite challenge	14	59.1 $\pm$ 5.8	0.6 $\pm$ 0.6	0.5 $\pm$ 0.3	0.5 $\pm$ 0.1	39.0 $\pm$ 3.3	18.6 $\pm$ 3.4
Post-dust mite challenge	15	93.1 $\pm$ 13.8	0.4 $\pm$ 0.3	5.9 $\pm$ 3.7*	8.7 $\pm$ 2.6*	48.6 $\pm$ 10.0	29.5 $\pm$ 5.0
Neonatally sensitized (rechallenge)							
Pre-dust mite challenge #2	11	65.4 $\pm$ 11.08	0.2 $\pm$ 0.1	1.8 $\pm$ 0.7	3.5 $\pm$ 1.0	28.5 $\pm$ 7.0	31.5 $\pm$ 4.9
Post-dust mite challenge #2	11	77.5 $\pm$ 12.2	0.2 $\pm$ 0.1	15.3 $\pm$ 2.7*	8.1 $\pm$ 4.7	29.7 $\pm$ 4.4	24.4 $\pm$ 4.3
Adult sensitized							
Pre-dust mite challenge	6	102.2 $\pm$ 21.5	0.3 $\pm$ 0.3	0.3 $\pm$ 0.2	2.5 $\pm$ 0.9	28.4 $\pm$ 6.3	70.8 $\pm$ 19.1
Post-dust mite challenge	6	93.4 $\pm$ 17.01	0.6 $\pm$ 0.6	4.3 $\pm$ 1.7	22.0 $\pm$ 10.3	28.0 $\pm$ 5.5	38.5 $\pm$ 13.4
Adult sensitized (rechallenge)							
Pre-dust mite challenge #2	5	91.8 $\pm$ 16.2	0.0 $\pm$ 0.0	4.4 $\pm$ 2.0	14.4 $\pm$ 2.0	31.1 $\pm$ 6.4	42.0 $\pm$ 12.7
Post-dust mite challenge #2	5	85.2 $\pm$ 16.5	1.1 $\pm$ 1.0	4.4 $\pm$ 0.7	2.4 $\pm$ 1.2	34.0 $\pm$ 9.5	43.3 $\pm$ 11.5

Values are means $\pm$ SE. Differential cell counts were obtained 1 wk before and 48 h after the dust mite challenge in neonatally sensitized monkeys. Animals were boosted with subcutaneous injection of vehicle or dust mite or aerosolized dust mite administered via a face mask as indicated in the first column. Two weeks after the boost, animals were challenged with increasing concentrations of aerosolized dust mite to achieve a 50% decrease in lung compliance. All sham-sensitized animals received the maximum dust mite concentration (1:4 dilution) during the challenge. Cohorts of sensitized animals were rechallenged with aerosolized dust mite 3 to 4 wk after the allergen challenge. BALF, bronchoalveolar lavage fluid. *Different from pre-dust mite challenge, $P < 0.05$.

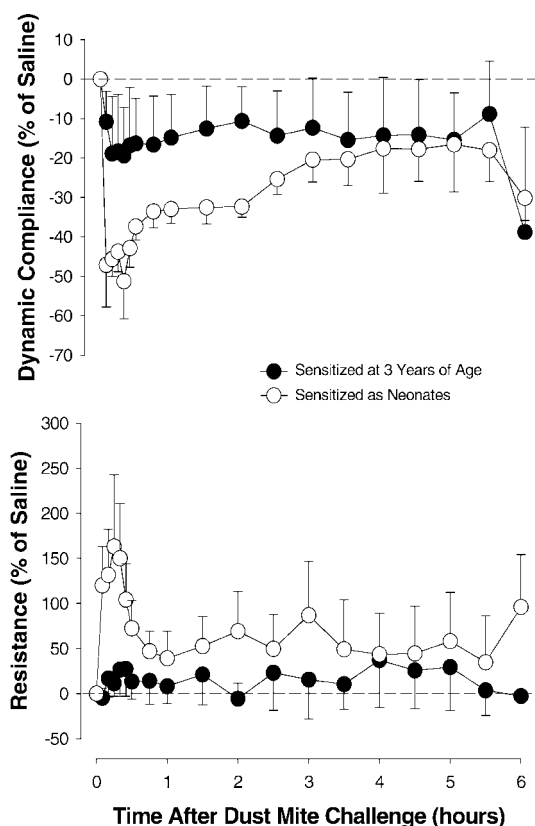


Fig. 4. Early and late responses to allergen after repeat aerosol allergen exposure. Adult and neonatally sensitized animals that had not been exposed to allergen for at least 90 days were primed by inhalation challenge with aerosolized dust mite (1:50 dilution for 4 min). Three weeks later, the challenge was repeated, and the early- and late-phase responses were recorded. Values are means $\pm$ SE for 5 adult animals ($\bullet$) and 10 neonatally sensitized ($\circ$) animals.

were measured. The repeat allergen challenge induced a greater decrease in C_{dyn} and increase in R_{aw} in neonatally sensitized animals than in the adults (Fig. 4). Adult-sensitized animals exhibited minimal change in resistance during the early- and late-phase responses. Both groups exhibited late declines in compliance starting 5 h after allergen challenge. However, only the neonatally sensitized animals exhibited increased resistance late in the protocol.

Pathology

Tissues from three animals were evaluated for histological correlates of asthma. One animal (*animal 1*) had been sensitized as an adult, challenged with aerosolized allergen nine times over the course of 2 yr, and euthanized 2 days after the last allergen challenge. A second adult animal (*animal 2*) was a naive age-matched control. The third animal (*animal 3*) was a 3.5-yr-old female that had been sensitized as a neonate and challenged with aerosolized dust mite eight times over 2 yr, with the last exposure occurring 7 days before tissue collection.

Sections from the naive control animal (*animal 2*) exhibited mild, submucosal tracheal edema and minimal pleocellular tracheitis that was presumed to be a nonspecific effect of inflammation resulting from intubation (Fig. 5). No significant lesions were observed in the airways or nasal passages, al-

though a mild increase in cellularity (primarily mononuclear cells) was observed in alveolar septa.

Animal 1, which was sensitized and challenged as an adult, exhibited moderate, mucous cell hyperplasia in the tracheal and bronchial epithelium (Fig. 5D). In the trachea, moderate, diffuse, submucosal edema was noted, with mild lymphoplasmacytic and eosinophilic tracheitis along with thickening of the basement membrane (Fig. 5B). Prominent submucosal blood vessels containing increased numbers of marginated and circulating eosinophils were also evident. Pulmonary changes consisted of bronchial mucous cell hyperplasia, bronchial and bronchiolar submucosal edema, and mild to moderate lymphoplasmacytic and eosinophilic bronchitis. Minimal peribronchiolar and perivascular lymphoid follicular hyperplasia was observed (Fig. 5F). Within the nasal passages, submucosal vessels contained increased numbers of marginated and circulating eosinophils. Nasal passages exhibited rhinitis (lymphoplasmacytic, eosinophilic, and neutrophilic) and submucosal vascular ectasia (Fig. 5H).

Animal 3 exhibited bronchial mucous cell hyperplasia (Fig. 6A) and eosinophilic bronchitis (Fig. 6B). Bronchioles and terminal bronchioles exhibited hyperplasia of smooth muscle (Fig. 6, C and D).

These results demonstrate that systemic sensitization of either adult or neonatal cynomolgus monkeys followed by exposure to aerosolized dust mite induces immunologic, inflammatory, physiological, and morphological features consistent with early phases of asthma. However, animals sensitized at an early age exhibit more robust functional and morphological responses to aerosolized allergen.

Early and Late Responses to Aerosolized Dust Mite Allergen After 90 Days

To evaluate the longevity of airway sensitization to allergen, four neonatally sensitized animals were allowed to rest for 90 days after the initial provocation with aerosolized dust mite and were then subjected to a second aerosol challenge without another allergen boost. Animals were pretreated with diphenhydramine before allergen challenge as a protective measure. Even in the presence of diphenhydramine, one animal exhibited an excessive early-phase response and had to be resuscitated. The other three animals exhibited early- and late-phase responses. A representative tracing of the response is presented in Fig. 7. These results demonstrate that allergic sensitization and aerosol allergen challenge induce a prolonged sensitivity of the airways to aerosolized allergen, which negates the need for monthly systemic boosts with allergen.

Th2 Cytokine Production

Evidence that systemic sensitization induced a Th2 cytokine profile was obtained by isolating mononuclear cells from peripheral blood of sensitized animals and stimulating in vitro with dust mite allergen or calcium ionophore with PMA. Addition of dust mite to the medium induced IL-5 release (Fig. 8A), but no IL-4 or IFN- γ release was detected. Stimulation with A-23187 and PMA induced release of both IL-4 and IL-5, but not the Th1 cytokine, IFN- γ (Fig. 8B). Interestingly, the cells exhibited a relatively high unstimulated rate of IL-10 release, but the rate of IL-10 release was not increased by

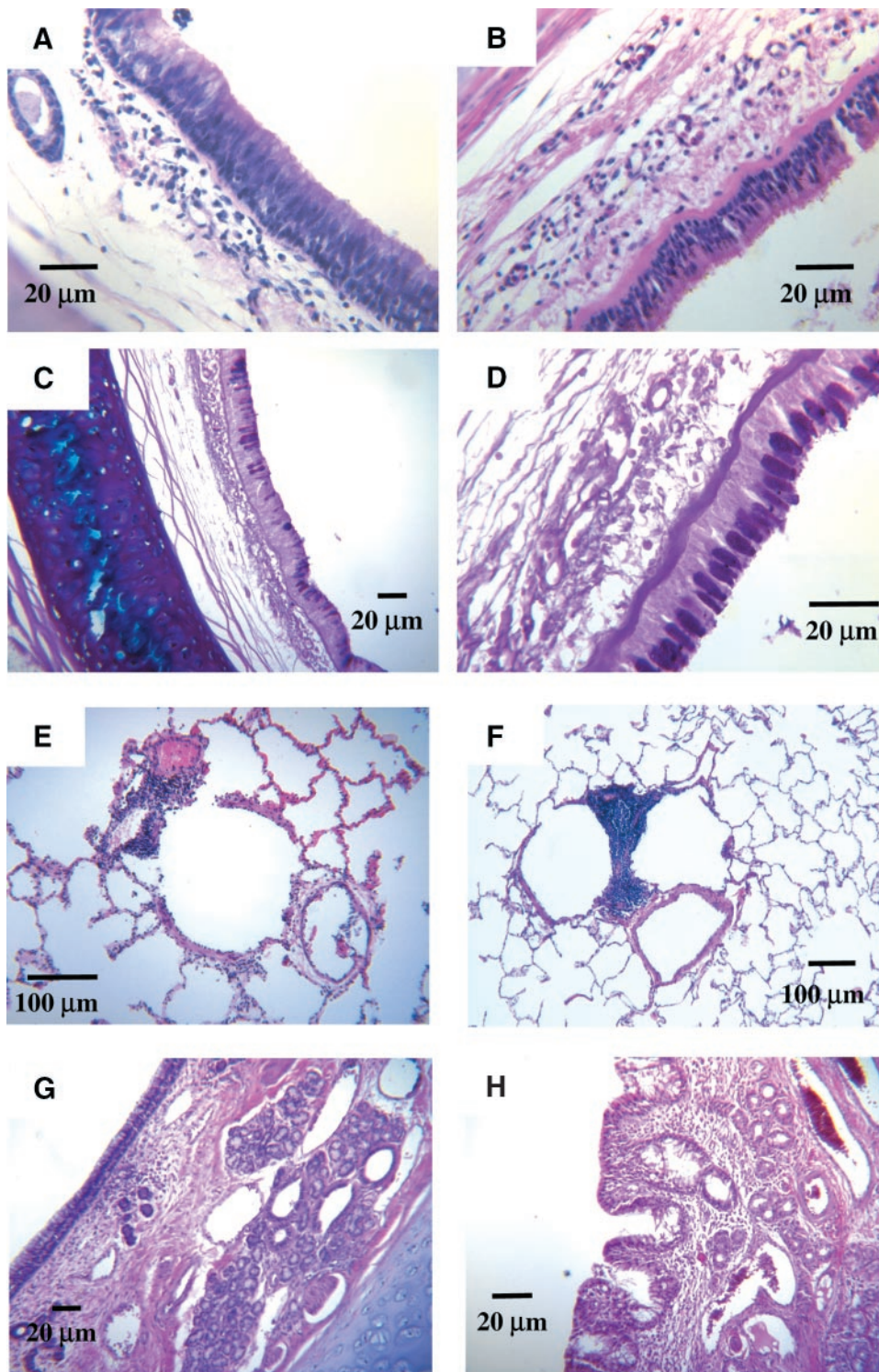


Fig. 5. Histological sections of trachea, lung, and nasal passages from 4- to 5-yr-old cynomolgus monkeys. Tracheal sections are from naive (A and C) and sensitized (B and D) animals stained with hematoxylin and eosin (A and B) and alcian blue/periodic acid-Schiff (C and D). Lung sections are from naive (E) and sensitized (F) animals stained with hematoxylin and eosin. Nasal passages are from naive (G) and sensitized (H) animals stained with hematoxylin and eosin.

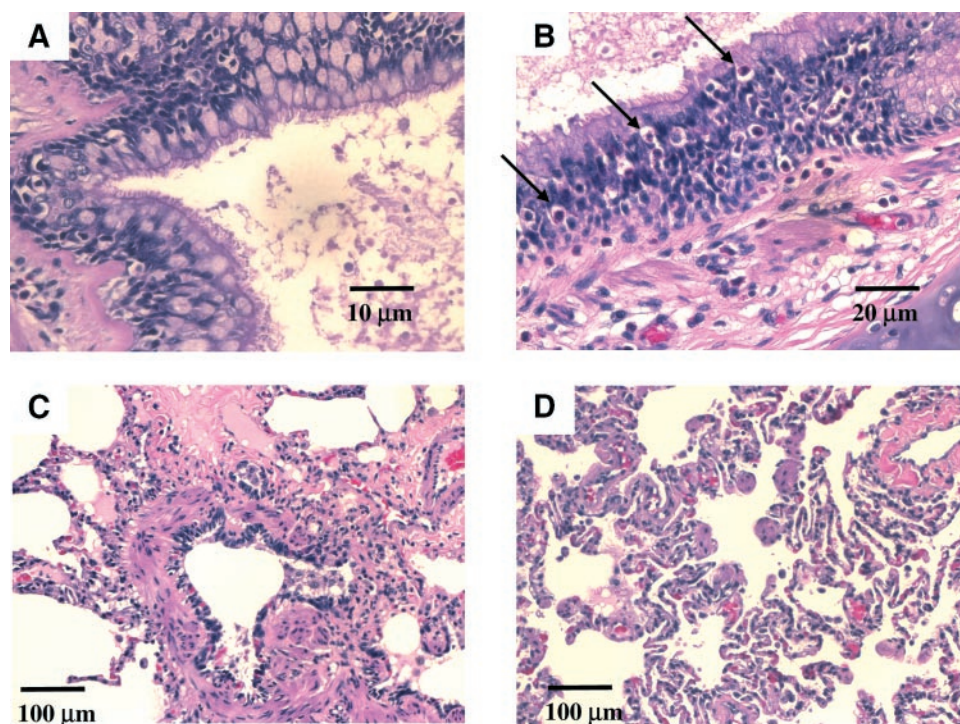
exposure to dust mite and A-23187 + PMA. These results confirmed that subcutaneous injection of dust mite and alum induced a Th2 phenotype in the cynomolgus monkey.

Response to Albuterol and Dexamethasone

The ability of inhaled β -adrenergic agonists to dilate the airways and inhibit allergen-induced airway constriction

was assessed. Responsiveness of the cynomolgus monkey to inhaled β -agonist was confirmed by treating animals with aerosolized albuterol and determining the dose response to inhaled methacholine 60 min later. A log rightward shift in the PC_{40} to methacholine was observed after treatment with albuterol, demonstrating that the animals were responsive to the β -agonist (Fig. 9). The effect of albuterol on the early-

Fig. 6. Histological sections of lungs from a 3.5-yr-old neonatally sensitized cynomolgus monkey exposed to aerosolized dust mite 8 times starting at 1 yr of age. Bronchial sections exhibited goblet cells in the surface epithelium (A) and intraepithelial eosinophils (B, arrows). Sections of bronchioles (C) and distal lung exhibited smooth muscle hyperplasia (D). Arrows indicate intraepithelial eosinophils. Sections were stained with hematoxylin and eosin.

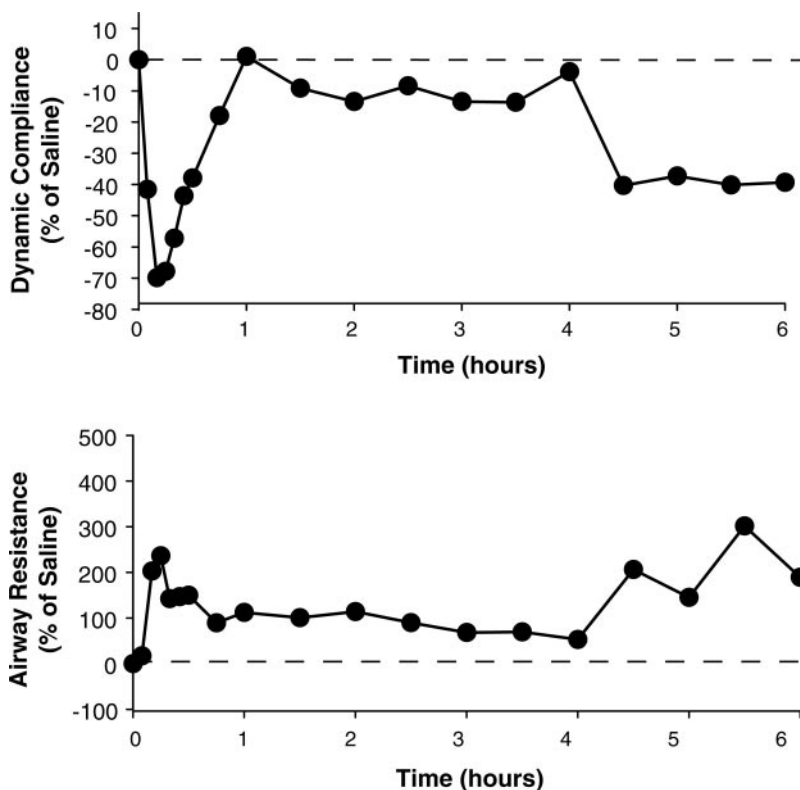


phase dust mite response was then measured in four neonatally sensitized monkeys. Albuterol reduced the maximum change in Cdyn during the early phase by 63%. Raw was also improved, but variability in the control group and small

sample size kept the difference from being statistically significant (Table 2).

Sensitivity to corticosteroid treatment was assessed by using dexamethasone. The protocol was a linear design in which all

Fig. 7. Rechallenge with allergen 3 mo after the initial challenge. A neonatally sensitized animal was rechallenged with aerosolized dust mite at least 90 days after the previous allergen challenge. The animal was pretreated with diphenhydramine (12.5 mg sq) before challenge. Cdyn (top) and Raw (bottom) were monitored for 6 h after the challenge.



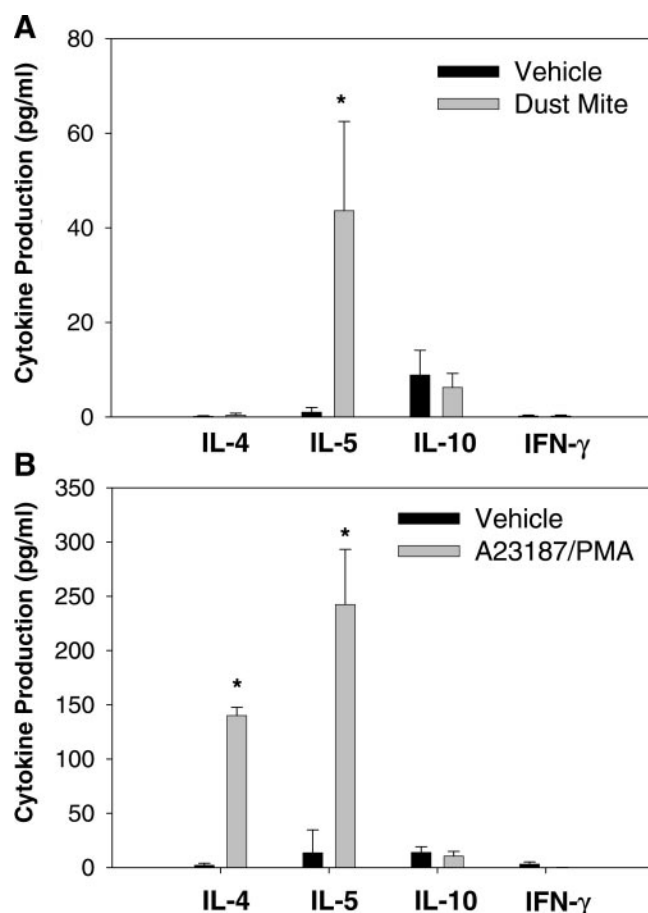
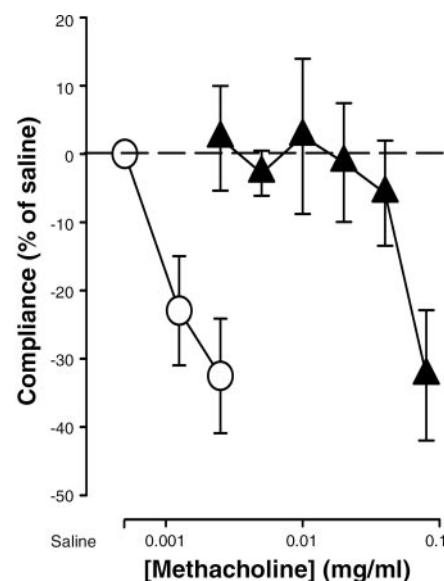


Fig. 8. Th2 cytokine secretion by peripheral blood mononuclear cells in vitro. After sensitization, peripheral blood mononuclear cells were isolated from dust mite-sensitized animals and stimulated in vitro. Cells were stimulated in vitro with dust mite (0.3 μ g/ml) or vehicle for 4 days (A) or 1 μ M calcium ionophore (A-23187) and 2 μ M phorbol myristic acid (PMA) for 1 day (B). The cytokine concentrations in the media were determined by ELISA. Values are means $\pm$ SE; n = 11 animals. *Different from vehicle (P < 0.05).

animals received vehicle first. After a washout period of 3 wk, the animals were treated with dexamethasone (1 mg bid). Dexamethasone was delivered orally, except in the morning on days 3 and 6, when the animals underwent pulmonary function testing and the dexamethasone was delivered intramuscularly. Allergen challenge was performed on day 3 of treatment, and BAL fluid was collected 48 h after the allergen challenge. The treatment inhibited allergen-induced eosinophilia (Table 3) and attenuated the changes in C_{dyn} and Raw induced by inhalation of allergen (Fig. 10). In addition, treatment with dexamethasone appeared to inhibit lymphocytic inflammation, although statistical significance was not demonstrated by ANOVA (Table 3). The repetitive allergen challenge induced a progressive rise in the percentage of lymphocytes in BAL, as indicated by the increase in percentage of lymphocytes between the prevehicle, postvehicle, and predexamethasone time points. This trend was reversed by dexamethasone treatment as indicated by a decrease in the percentage of lymphocytes between the predexamethasone and postdexamethasone time points.

No change in airway hyperresponsiveness to histamine was observed with steroid treatment. The PC₅₀ values before and



Treatment	PC ₄₀	n
Saline	0.01 $\pm$ 0.01	4
Albuterol	0.13 $\pm$ 0.03	4

Fig. 9. Effect of albuterol on C_{dyn}. Neonatally sensitized monkeys were treated with aerosolized saline and 1 h later challenged with aerosolized methacholine until a 40% decrease in compliance was achieved ($\circ$). Twenty-four hours later, the animals were treated with aerosolized albuterol (2.5 mg/ml, 2 ml delivered over 10 min; $\blacktriangle$), and 60 min later the response to methacholine was reassessed. Values are means $\pm$ SE; n = 4. Only doses delivered to all animals are included in the plots.

after vehicle and dexamethasone were 0.014 $\pm$ 0.008, 0.010 $\pm$ 0.003, 0.017 $\pm$ 0.007, and 0.016 $\pm$ 0.004 mg/ml, respectively.

DISCUSSION

The goal of this project was to develop a model of asthma suitable for target gene identification, target validation, and testing of genomic-based drugs. Protocols for efficiently sensitizing animals were adopted and tested in adult and neonatal animals. The animals were then examined for hallmark characteristics of extrinsic asthma and shown to exhibit a Th2 phenotype, eosinophilic inflammation, bronchial hyperresponsiveness, reversible airway obstruction, and airway wall remodeling including basement membrane thickening, goblet

Table 2. Effect of aerosolized albuterol on dust mite-induced early-phase responses

	n	Max Δ C _{dyn} (% of saline)	Max Δ R _{aw} (% of saline)
Control	4	-71 $\pm$ 12	473 $\pm$ 285
Albuterol	4	-26 $\pm$ 13*	34 $\pm$ 30

Values are means $\pm$ SE. Four neonatally sensitized monkeys were challenged with dust mite, and the maximum changes in dynamic compliance (Max Δ C_{dyn}) and resistance (Max Δ R_{aw}) during the early-phase response were determined (Control). Three months later, the animals were treated with albuterol (2.5 mg/ml, 5 mg aerosolized over 10 min), and the dust mite challenge was repeated 1 h later (albuterol). *Different from control, P $\leq$ 0.05.

Table 3. Effect of dexamethasone on BALF differential cell counts

	<i>n</i>	Total, ×10 ⁴ /ml	Lymphocytes, ×10 ⁴ /ml	Eosinophils, ×10 ⁴ /ml	Neutrophils, ×10 ⁴ /ml	Macrophages, ×10 ⁴ /ml	Epithelial Cells, ×10 ⁴ /ml
Vehicle treatment							
Pretreatment	10	63.8±12.09	0.2±0.1	1.8±0.7	3.4±1.1	25.4±7.4	33.1±6.1
Posttreatment/challenge	10	88.7±22.4	7.8±7.6	18.6±5.3*	16.5±9.7	27.9±4.8	18.0±2.1
Dexamethasone treatment							
Pretreatment	10	78.4±8.2	4.0±1.8†	8.4±2.9†	5.0±1.6	29.5±5.0	31.5±4.6
Posttreatment/challenge	10	55.4±7.5	0.4±0.2	1.5±0.4*	7.4±6.0	26.4±4.0	19.7±3.5

Values are means ± SE. Neonatally-sensitized monkeys were boosted with aerosolized allergen. Two weeks later, BALF was obtained and differential cells counts were performed (vehicle treatment/pretreatment). The following week, the animals were treated with vehicle twice daily for 5.5 days. Allergen challenge was performed on day 3. The lungs were lavaged 48 h after allergen challenge (vehicle treatment/posttreatment/challenge). Two weeks after allergen challenge, BALF was again obtained (dexamethasone treatment/pretreatment), and the following week, the animals were treated with dexamethasone (1 mg orally bid). On the morning of the 3rd day, the drug was delivered via intramuscular injection prior to the allergen challenge. The final bronchoalveolar lavage was performed 48 h later (dexamethasone treatment/posttreatment/challenge). *Significantly different from vehicle pretreatment, dexamethasone pretreatment, and dexamethasone posttreatment/challenge ($P < 0.05$). †Significantly different from vehicle pretreatment ($P < 0.05$).

cell hyperplasia, and smooth muscle hyperplasia. Differences were observed between neonatal and adult animals, with the former exhibiting more consistency and robust changes in pulmonary function on exposure to inhaled allergen and airway remodeling.

The choice of allergen, the species used, and the overall approach to establishing the model reflected the stated goal of the research. Dust mite is commonly associated with human

asthma (10) and has been shown to be effective in inducing symptoms of asthma in common laboratory animals, including mice (14, 19), rats (8), rhesus monkeys (20, 26), and rabbits (11). In addition, natural sensitivity to dust mite had been documented in monkeys (7), increasing the relevance of the allergen. Research conducted by Turner and colleagues at Pfizer (23–25), Gundel et al. at Boehringer Ingelheim (6), Mauser et al. at Schering-Plough (9), Bell et al. at Abbott Laboratories (2), and Baugh et al. at Merrell Dow (1) provided a strong precedent for using cynomolgus monkeys for testing novel pulmonary therapeutics, and the cynomolgus monkeys exhibit ~95% homology with humans (27), making this a candidate species suitable for genomic studies. The protocols for sensitizing the adult and neonatal animals were based on the work of Yasue et al. (26) and Metzger (11), who demonstrated the feasibility in rhesus monkeys and rabbits, respectively. The ability to prime an animal systemically and then precipitate pulmonary symptoms at a defined point in time should facilitate chronological studies of gene-expression changes associated with asthma pathogenesis and be suitable for high-throughput screening of targets emerging from genomic studies.

Nonspecific hyperresponsiveness is a key feature of human asthma (10). In this model, both adult and neonatally sensitized animals exhibited hyperresponsiveness to histamine and aeroallergen. Early- and late-phase responses to inhaled allergen were observed in both adult and neonatally sensitized animals, but allergen-induced increase in Raw was more prominent in neonatally sensitized animals (Figs. 2 and 4). Enhanced Raw is often attributed to constriction of the conducting airways, which would indicate greater involvement of the bronchi in animals sensitized from birth. However, this interpretation may not be valid because significant closure of the small airways or changes in tissue mechanics can also be reflected in increase total Raw (22). At present, there is no technique for assessing pulmonary function that definitively differentiates the involvement of proximal and distal regions in the asthmatic response. Forced oscillation is becoming more common (22), but even this technique relies on homogeneity within the lungs, which is not present in asthma. Thus the relative involvement of proximal vs. distal lung regions in the asthmatic response remains unclear. Regardless, the data indi-

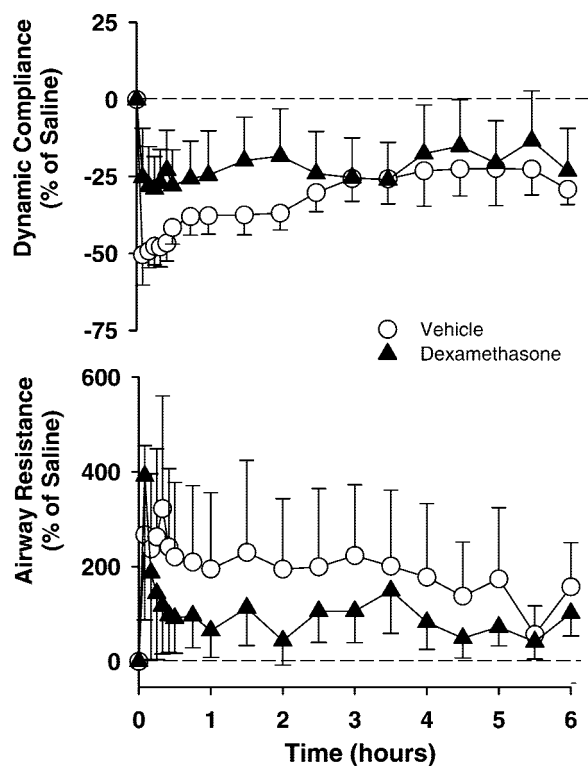


Fig. 10. Effect of dexamethasone on the pulmonary function response to allergen challenge. Neonatally sensitized monkeys were boosted with aerosolized allergen. Three weeks later, the animals were treated with vehicle twice daily for 5.5 days. Allergen challenge was performed on day 3, and pulmonary function was monitored for 6 h (○). The animals recuperated for 3 wk and were treated with dexamethasone (1 mg orally bid). On the morning of the 3rd day, the drug was delivered intramuscularly, the animals were challenged with inhaled allergen, and pulmonary function was monitored for 6 h (▲). Values are means ± SE for 10 animals.

cate that the asthmatic response is accentuated in neonatally sensitized animals.

A key distinguishing clinical feature of asthma is a high degree of reversibility of airway obstruction by bronchodilators and anti-inflammatory agents (10), and this feature was demonstrated in the present model by treating neonatally sensitized animals with albuterol and dexamethasone. Treatment with either of these agents attenuated the pulmonary function changes after inhalation of allergen (Figs. 9 and 10). Ideally, an inhaled corticosteroid would have been used for this purpose. However, animal welfare concerns precluded that experiment because the animals would have been anesthetized twice per day for multiple days for proper dosing of the inhaled steroids.

One benefit of using nonhuman primates as a model of asthma is that the animals are long-lived and thus chronic aspects of the disease can be studied. In the long term, elimination of systemic boosts with allergen would be more representative of the disease in humans. Therefore, boosting with aerosolized allergen was investigated as an alternative to the systemic allergen boost used to sensitize the animals. Both adult and neonatally sensitized animals exhibited some response to the allergen challenge under these conditions, but the younger animals were clearly more responsive, exhibiting nearly a 50% reduction in C_{dyn} and 150% increase in resistance in response to a 1:50 dilution of dust mite allergen (Fig. 4). The cohorts were separated in age by ~3 yr, and thus the differences could have been due to the age difference at the time of challenge. Work with rabbits indicates that age at the time of sensitization determines the degree of severity and the chronicity of the asthmatic response. Sensitization of adult rabbits leads to a more diverse immunoglobulin profile (16, 21), milder allergen-induced pulmonary function changes (11), and lower degree of hyperresponsiveness to histamine (12). The heightened asthmatic responses observed in the neonatally sensitized monkeys throughout the present study are consistent with the differences observed in rabbits sensitized at different ages.

An adult cynomolgus monkey subjected to the systemic sensitization protocol and challenged three to four times per year for 2 yr exhibited histological alterations consistent with a chronic antigenic stimulus and suggestive of early asthma associated with mild chronic antigenic stimulation. A lack of appreciable submucosal mucous glands, mucous plugs in the airways, and smooth muscle hyperplasia precluded diagnosis of fully developed asthma in this animal. In contrast, a neonatally sensitized animal challenged an equivalent number of times over a 2-yr period exhibited significant increases in the amount of bronchial and bronchiolar smooth muscle (Fig. 6). This finding was consistent with the elevated resistance responses to allergen observed in this cohort (Figs. 2 and 4). These findings indicate that the neonatally sensitized animals more closely model the human asthmatic condition than adult-sensitized animals.

There is a possibility that the adult-sensitized animal in the pathology evaluation was exposed to allergen early in life, which initiated remodeling. However, this subject was raised in HEPA-filtered air from 1 yr of age. Even if exposed to an environmental allergen during the first year of life, it is unlikely that the animal would have experienced exposure after that time sufficient to drive the remodeling. This argument is

supported by pathology of one animal that was sensitized as an adult but died prematurely after receiving only one aerosol allergen challenge. That animal did not exhibit basement membrane thickening in the airways at the time of death (data not shown).

This report constitutes the first phase of a longitudinal study of cynomolgus monkeys sensitized to dust mite allergen. The results indicate that sensitization of either adult or neonatal animals establishes an allergic background on which inhaled allergen induces pulmonary responses. Animals sensitized from birth exhibit inflammatory, functional, and morphological features similar to those observed in allergic asthma, including airway eosinophilia, increases in lung compliance and airway resistance, and smooth muscle goblet cell hyperplasia. The model offers control over the sensitization process and initiation of the lung disease and therefore should be useful in genomic studies and drug screening activities.

ACKNOWLEDGMENTS

We thank Brandi Wynne, Emily Cozzi, Sarah Wall, and Joyce Chandler for technical support and Dr. Howard Ball for reviewing the manuscript.

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