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Cockroach Allergens Induce Biphasic Asthma-Like Pulmonary Inflammation in Outbred Mice

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Abstract

Objectives—The aim of this study is to define the kinetics of the pulmonary inflammatory response in cockroach allergen (CRA) sensitized and challenged outbred mice.

Methods—Asthma-like pulmonary inflammation was induced with three pulmonary exposures to CRA, without the use of adjuvants. Mice were sacrificed at multiple time points and asthma-like pulmonary inflammation quantified.

Results—Several pulmonary parameters showed a pronounced biphasic inflammatory response with an early stage (1.5 hours post challenge) and late stage (24 hours). The initial phase was characterized by the production of multiple inflammatory mediators, including CXC chemokines, and the recruitment of neutrophils to the lung. The number of pulmonary eosinophils decreased in the early phase but quickly rebounded. Both the early and late phases had increases in TNF production in addition to airways hyperreactivity. The model also demonstrated early production of mucin with clearance by 12 hours followed by new accumulation of mucin in the pulmonary epithelial cells. Eotaxins within the lung peaked at about 12 hours and the numbers of eosinophils in the lung remained constant throughout the 48 hours of the study.

Conclusions—The pulmonary inflammatory parameters in response to a clinically relevant allergen define a biphasic response. These data may be used to investigate the pathogenesis of the disease and develop targeted therapies for the distinct phases.

Keywords

allergy; asthma; biphasic; chemokines; cockroach; cytokines; inflammation; murine; outbred; pulmonary

Introduction

Asthma is a complex, multifactorial disease characterized by episodes of reversible airways obstruction with chronic inflammatory processes including lung eosinophilia and airways

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Declaration of Interest

The authors have no competing interests to disclose.

remodeling (1, 2). Asthma represents one of the most common chronic health conditions in the developing world and an estimated 30 million Americans are afflicted with this disease (3, 4). Treatment significantly improves the lives of sufferers by offering symptomatic relief, but the underlying causes of asthma remain nebulous.

Since over 90% of asthmatics have been diagnosed with extrinsic or allergic-type disease, increasing focus has been placed on the identification of environmental allergens and the interruption of the allergic inflammatory process (5, 6). Urban dwellers and those of low socioeconomic class are disproportionately represented in new allergic asthma diagnoses (7–10). This observation led to the urban asthma hypothesis, which states that there are agents present in dense population centers that either predispose to the development of allergies or are themselves potent allergens (11–14). A landmark paper published in 1998 identified cockroach proteins in high concentrations in the homes of urban asthmatic children (15). Furthermore animal studies defined that these insect-derived particles were potent allergens (16–18). Cockroach allergens (CRA) thus appeared to be a promising candidate for a ubiquitous, highly potent urban allergen (19).

Human asthmatics typically display a biphasic response to allergen triggers (20, 21), including an acute early inflammatory process driven largely by mast cell degranulation (22) and a later exacerbation caused by the actions of late-arriving eosinophils, allergen-specific lymphocytes, and adaptive immune cells (23–25). Limited previous studies have reported biphasic asthmatic responses in animal models (26–29), which mostly focused on the role of TNF in the development of airways hyperreactivity (AHR) methacholine (Mch).

In this study, the pulmonary inflammatory parameters defining a biphasic asthma-like response in outbred mice are detailed. This inflammation was induced in outbred mice with only pulmonary challenges and did not require the use of adjuvants.

Materials and Methods

Experimental Mice

Female HSD:ICR (CD-1) mice (Harlan, Indianapolis, IN, USA) were obtained at 8 weeks of age from Harlan–Sprague–Dawley and given 1 week to acclimate to the housing facility. The environment was rigorously controlled for day–night light cycle, temperature, and humidity. Mice received daily health checks as well as weekly bedding and total cage changes with periodic inspection by Boston University Laboratory Animal Care Service. All studies were approved by the Institutional Use and Care of Animals Committee.

Allergen Challenge and Asthma Induction

On days 0, 14, and 21, mice received direct hypopharyngeal challenges of 4, 2, and 2 µg of lyophilized whole body German cockroach extract (CRA; Greer, Lenoir, NC, USA) in 50 µL of sterile phosphate-buffered saline (PBS) using our previously described protocol (30–33). Briefly, mice were lightly anesthetized with isoflurane and suspended by the front incisors from an inclined board. Using forceps, the tongue was gently extended and held against the lower mandible. Allergen solution was delivered to the hypopharynx in two separate 25 µL aliquots. Inhalation of the solution was confirmed by inspiratory sounds and previous

radiological studies (data not shown). Within 5 minutes of dosing, all mice resumed normal activity. Various groups of mice were established including naïve, 0- (given CRA challenges on days 0 and 14 and then examined on day 21 when they would have gotten their final allergen challenge), 1.5-, 4-, 12-, 24-, and 48-hour time points where mice received 3 pulmonary CRA challenges on days 0, 14, and 21 and then examined and killed at the stated number of hours post final allergen challenge.

Control Groups

Numerous controls were evaluated to establish the appropriate inflammatory parameters in this model. All sensitization and challenge protocols were delivered using the direct hypopharyngeal challenge described above. These controls include (1) naïve mice (no exposure to CRA or vehicle); (2) CRA/PBS/PBS, sensitized but not challenged (mice were sensitized on day 0 and exposed to the PBS vehicle on days 14 and 21 and evaluated 90 minutes after the last PBS exposure); (3) CRA group received only the CRA without prior CRA sensitization (these mice were studied 90 minutes after challenge); (4) day 0 mice sensitized on day 0, challenged on day 14, and then examined on day 21—included in the graphs as time 0; and (5) asthma, as a positive control, mice, which have had the full asthma induction protocol of sensitization on day 0 and hypopharyngeal challenges on days 14 and 21 and examined and killed at 90 minutes post final challenge.

Assessment of Airways Hyperreactivity

Following final allergen challenge, mice were placed in unrestrained whole body plethysmograph chambers (Buxco, Wilmington, NC, USA) and subjected to increasing doses of nebulized Mch. Each time point represents an independent group of mice that were killed and examined afterwards. Airways parameters were measured for 5 minutes following a 2-minute exposure to nebulizer treatment of PBS and 25 and 50 mg/mL of Mch.

Assessment of Airways Resistance

Assessment of Airways Resistance was conducted as a validation of the relationship of Penh to directly measured airways resistance. Briefly, in a separate group of mice, at the indicated time points following final allergen challenge, mice were anesthetized with phenobarbital, the trachea surgically cannulated, and the mice paralyzed with pancuronium bromide. The mice were mechanically ventilated (SCIREQ, Tempe, AZ) and their airways resistance measured in response to PBS and 25 and 50 mg/mL of Mch nebulizer treatment (30).

Salbutamol Treatment

Two groups of mice were sensitized using the complete model (3 CRA challenges on days 0, 14, and 21 followed by examination and sacrifice). At 1.5 hour post final allergen challenge they were placed in the Buxco chambers and treated with nebulized salbutamol (5 mg/mL) or water for 3 minutes. Following this initial treatment period, the mice were exposed to 25 and then 50 mg/mL of nebulized Mch for 2 minutes with a 5-minute recording period.

Sacrifice and Tissue Collection

Following assessment of airways parameters (Buxco), mice were anesthetized with ketamine/xylazine and sacrificed by exsanguination followed by cervical dislocation. The lungs were lavaged with 2 mL of warm HBSS in 250 μ L aliquots. This bronchoalveolar lavage (BAL) fluid was centrifuged to pellet the cells. The supernatant of the first 1 mL of BAL was reserved for cytokine analysis and the cell pellets from both samples were combined, counted, and placed on slides and a differential of inflammatory cells performed using light microscopy. The right lung of each mouse was homogenized and the lung homogenate (LH) supernatant reserved for cytokine analysis while the pellet was used to assess eosinophil (EPO) and neutrophil (MPO)-specific peroxidase activity. The left lung was fixed in 70% ethanol for histological analysis. In a separate control group of mice, lungs were prepared to be evaluated for alveolitis with a standard insufflation protocol. Briefly, lungs were harvested, the trachea was connected to a cannula connected to a 30 cm column of 10% formalin, and a stopcock was opened to insufflate the lungs and prepare them for histological sectioning without disturbing any cells that might be present in the small airspaces.

Assessment of BAL and LH Cytokines and Chemokines and Serum IgE

Cytokines and chemokines in BAL and LH samples were assessed using sandwich ELISA employing biotin-linked detection antibodies and streptavidin HRP development. Leukotrienes were measured with a kit from Cayman Chemical (Ann Arbor, MI, USA). CRA-specific IgE levels were determined as follows: a 96-well Nunc Maxisorp microtiter plate (Thermo Scientific; Rochester, NY, USA) was coated overnight at 4°C with 50 μ L/well of CRA (Greer Labs; Lenoir, NC, USA) diluted to 5 μ g/mL in PBS. The plate was then washed 5 times with 0.05% Tween in PBS, blocked for 2.5 hours with Blocker Casein (Thermo Scientific; Hudson, NH, USA), and washed again. Mouse plasma samples were diluted 1/100 in dilution buffer (10% Blocker Casein, 0.1% BSA, 0.05% Tween 20 in PBS) and added to CRA-coated plate in triplicate, 50 μ L/well, and incubated overnight at 4 °C. The plate was then washed and 50 μ L of goat anti-mouse IgE-HRP (Bethyl Laboratories; Montgomery, TX, USA) diluted 1:2000 in dilution buffer was added to each well and incubated for 1 hour at room temperature. The wells were washed and 100 μ L TMB (0.01% 3,3',5,5'-tetramethylbenzidine and 0.01% H₂O₂ in sodium acetate buffer pH 6) added per well to incubate for 30 minutes at room temperature. The reaction was stopped by adding 100 μ L 1.5 M H₂SO₄ and absorbance read at 465 and 590 nm. Values are expressed as delta optical density (OD).

Assessment of EPO and MPO

LH cell pellets were treated with cetyltrimethylammonium chloride or hexadecyltrimethylammonium bromide and sonicated to release eosinophil and neutrophil granules, respectively. The activity was measured as previously described (34).

Histological Analysis

Fixed lungs were sent to the Boston University School of Medicine, Department of Pathology Core Services, for histology sectioning and staining. Image analysis for the PAS¹¹

stained area was performed using ImageJ freeware (<http://rsbweb.nih.gov/ij/>). Briefly, whole lung images were captured at 2× magnification. These images were subjected to background subtraction and color deconvolution. The magenta-separated image was converted to black and white image and the total PAS (black)-stained area was calculated. All histology grading was performed by a board-certified pathologist in a blinded fashion using established methods for inflammation scoring (35).

Statistical Analysis

All data are presented as mean ± SEM with the numbers of animals provided in Figures 1–7 and Table 1. Groups of data were analyzed using ANOVA with a Dunnett's or Bonferroni post hoc analysis to compare groups if appropriate. The groups used for comparison are listed in Figures 1–7 and Table 1. All statistical analyses were performed using Graphpad software (GraphPad Software Inc.).

Results

Validation of Asthma Model

We sought to validate the CRA asthma model induced by demonstrating the presence of multiple characteristics of the human allergic asthmatic response (Figure 1, Table 1). First, we needed to determine whether our direct hypopharyngeal instillation of an allergenic fluid would result in specific sensitization (with an IgE response) without causing nonspecific irritant alveolitis. To examine the CRA-specific IgE response mice were challenged with increasing doses of CRA, and plasma IgE specific to CRA was measured after the last challenge. All doses of CRA challenge resulted in a significant increase in CRA-specific IgE compared to naïve mice (Figure 1A). Histology analysis of our asthmatic mice also revealed that there was essentially no alveolitis as a result of CRA challenge.

In humans, the early phase of an asthmatic exacerbation is typically caused by bronchoconstriction and can be alleviated with short-acting β -2 adrenergic agonists such as salbutamol while the late phase is driven by inflammation and can be improved with corticosteroid administration (2, 36) (Figure 1). We sought to demonstrate that this was also true in our model of asthma. In our experiments, at 1.5 hour post final CRA challenge (the early phase), the administration of aerosolized salbutamol completely prevented the increase in Penh in response to 50 mg/mL Mch. In a previously published manuscript, we demonstrated that at 24 hours post final CRA challenge (the late phase) the administration of dexamethasone prior to the final allergen challenge resulted in significantly diminished pulmonary inflammation and AHR in response to Mch (33).

The CRA used in these experiments is a complex mixture containing allergens, endotoxin (LPS), chitin, and numerous structural proteins and proteases. Although the CRA used in these experiments is the same material used for human skin testing and thus a clinically relevant allergen, multiple controls were studied to show that this was an allergic response and not nonspecific inflammation. Additionally, we conducted a series of control experiments to rule out nonspecific inflammatory effects from the PBS vehicle and from the Mch as well as assessing the late inflammatory effects of the second allergen exposure. Five

different groups of mice were examined: (1) naïve mice not exposed to any allergens or vehicle; (2) mice immunized on day 0 and then challenged with the PBS vehicle on days 14 and 21; (3) mice challenged a single time with CRA; (4) mice immunized with CRA on day 0, challenged on day 14, and studied on day 21, without exposure to allergen on day 21; and (5) mice with the full asthma protocol of immunization on day 0 followed by CRA challenge on days 14 and 21. Table 1 provides a listing of the parameters of asthma-like inflammation examined in these groups. These experiments demonstrated that there was no nonspecific inflammatory response to the vehicle (PBS) or to Mch in nonimmunized mice. Additionally, there was no alveolitis observed on histology and a single CRA challenge produced only minimal changes in AHR and pulmonary inflammation. However, there were late inflammatory changes observed following the second CRA challenge. Mice examined 7 days after the second CRA challenge (CRA/CRA day 21) still show ongoing/residual inflammation with pulmonary eosinophils, eotaxin, and airways mucin suggesting that one CRA exposure is sufficient sensitization for future adaptive immune responses. These data demonstrate that this novel model with only pulmonary CRA challenge (i.e., without adjuvants) will induce asthma-like inflammation in an allergen-specific manner.

Kinetics of Pulmonary Inflammatory Cytokines, Chemokines, and Cells

Having established that robust, specific, asthma-like pulmonary CRA sensitization was occurring, we next examined the kinetics of cytokines, chemokines, and pulmonary inflammatory cell recruitment at various time points following final CRA challenge (Figure 2). The neutrophil CXC chemotactic agents KC and MIP-2 were sharply elevated within 1.5 hours following the final allergen challenge in both the BAL fluid and LH supernatant. KC gradually returned to baseline over a period of 24 hours while MIP-2 levels decreased more rapidly reaching prechallenge levels by 4 hours. Accordingly, neutrophils rapidly entered the air spaces reaching their peak coincident with the CXC chemokines at 1.5 hours. The number of neutrophils in the BAL then progressively diminished during the course of the experiment but had yet to reach baseline by 48 hours.

The eosinophil chemotactic agents eotaxin-1 and eotaxin-2 were also sharply elevated during the experimental time frame. Eotaxin-1 peaked shortly after the final allergen challenge at 1.5 hours while eotaxin-2 peaked later at the 12-hour time point (Figure 3). Unlike BAL neutrophil levels, eosinophils followed a complex pattern. BAL eosinophils were already elevated at 0 hours, prior to administration of final CRA challenge (also see Table 1, the CRA/CRA day 21 control group which follows the same time frame). That these cells were already present prior to the final challenge indicated that they had persisted in the lungs since the second allergen challenge 1 week prior to sacrifice. Immediately following the final allergen challenge (i.e., at 1.5 hours) BAL eosinophil levels dropped sharply, before rapidly increasing in numbers to a plateau level which held steady throughout the remainder of the experimental time frame. The sudden decrease in the number of eosinophils observed at 1.5 hours, confirmed in four independent experiments, may have been due to degranulation and apoptosis after allergen exposure (37).

Lymphocytes and their secreted inflammatory mediators play a significant role in the asthmatic response (38, 39) (Figure 4). BAL lymphocytes were elevated at the 0-hour time

point similar to the eosinophils. This again indicated that a long-lived population of lymphocytes persisted in the lungs following the previous CRA challenge. New lymphocytes infiltrated the air spaces 24 hours after final allergen challenge and remained elevated out to 48 hours. The Th2 cytokines IL-4, IL-5, and IL-13 are known to play an important role in asthma (40) and their presence was quantified. IL-4 was elevated at the 0-hour time point (where the mice did not receive a final allergen challenge and had not been exposed to the allergen for 1 week) indicating chronic release of this cytokine in the lung following the previous allergen challenge. Following the final CRA challenge, IL-4 levels rapidly returned to baseline and remained depressed for the remainder of the experiment. IL-5, conversely, was not present at baseline but peaked rapidly after final allergen challenge and remained at an elevated plateau out to 48 hours. IL-13 was elevated compared to naïve mice at 0 hours and remained at this level following final allergen challenge, without deviating, for the entire experimental time frame. This could indicate that IL-13 secretion is established during sensitization, and afterwards it is not significantly effected by subsequent allergen exposures.

There are several other important inflammatory mediators that would be anticipated to be increased in the local environment in asthma-like pulmonary inflammation (Figure 5). The Th1 skewing cytokine IL-12 was measured to determine what, if any, regulatory effects it could be having during the asthma-like inflammation seen in our model. Interestingly, IL-12 followed a similar pattern to IL-4 since it was elevated at 0 hours (e.g., chronically following previous allergen challenge 1 week prior) and rapidly returned to baseline following final challenge. Interferon-gamma was increased in both the BAL and LH following allergen challenge and displayed a biphasic pattern of release. TNF has also been shown to be a potent mediator of several chronic inflammatory conditions including asthma (40, 41). In the LH supernatant TNF peaked shortly after final challenge but then continued to increase until the end of the experimental time frame. In the BAL, TNF peaked rapidly after final CRA challenge, returned to near baseline levels by 12 hours, and reached a second peak at 24 hours displaying a clear biphasic pattern. These TNF data were the first parameters from the current experiments to indicate that a biphasic response to the CRA challenge was occurring.

Biphasic Airways Hyperreactivity

Having established the kinetic profiles of a number of important cytokines, chemokines, and inflammatory cells, respiratory parameters were measured following the induction of CRA asthma. We analyzed enhanced pause (Penh) since it allows the rapid collection of data and has been used in the past to document a biphasic asthmatic response (42) (Figure 6). Penh showed a biphasic time course indicating peak AHR at 1.5 and 24 hours with a brief recovery period at 12 hours. The Penh exacerbations seen at 1.5 and 24 hours were significantly elevated as compared to 12 hours. Our experimental design typically provides the pulmonary challenge in the morning, so mice sacrificed at 12 hours are analyzed in the early evening. This raised the possibility that the 12-hour time point may be affected by normal diurnal variation in hormone and cytokine levels. We controlled for this by sacrificing groups of mice in the morning as well as in the evening (there was no observable difference and the 12-hour data from these experiments were combined).

In order to verify that $Penh$ was related to directly measured airways resistance, we repeated the 1.5-, 12-, and 24-hour time points using mechanical ventilation with forced oscillation (Flexivent, ScireQ, Tempe, AZ) to assess airways resistance (R). A comparison of the resistance (R) values to $Penh$ found that they followed the same biphasic pattern such that they were closely correlated at all time points by Pearson analysis.

Cysteinyl leukotrienes (Cys-LT) in the LH of the experimental animals were measured at 1.5, 12, and 24 hours following final allergen challenge. Cys-LT levels also followed a biphasic pattern with elevated levels at 1.5 and 24 hours compared to 12 hours.

Biphasic Airways Mucin Production

The next experiments examined whether production of mucin by airway epithelial cells also occurred in a biphasic manner (Figure 7). Airway mucin was examined in all of the controls by staining sections with Periodic acid-Schiff (PAS), at multiple time points after the full asthma induction protocol. Table 1 shows that substantial airways mucin remained 7 days after the day 14 pulmonary challenge with CRA indicating an ongoing inflammation/mucin-inciting stimulus. Representative images revealed significant airway epithelial mucin staining at the 1.5- and 24-hour time points with decreased staining at the 12-hour time point. The total lung mucin levels were quantitated using ImageJ software analysis. This analysis revealed that total lung mucin levels were significantly elevated at 1.5 and 24 hours as compared to the 12-hour time point, displaying a clear biphasic pattern.

Discussion

Asthma triggered by allergens in patients may have a biphasic response with early airways narrowing and a late phase characterized by airways hyperresponsiveness and infiltration of eosinophils and mucus hypersecretion (2, 43). In this study we were able to demonstrate a robust, reproducible model of biphasic, asthma-like pulmonary inflammation in outbred mice. Neutrophils, CXC chemokines, and leukotrienes arrived quickly following final allergen challenge. Although we did not measure other neutrophil chemotactic agents such as complement fragments, the CXC chemokines and leukotrienes accurately predict the kinetics of neutrophil attraction. Furthermore, Nagarkar et al. have previously demonstrated that the CXC chemokine receptor CXCR2 is necessary for the development of AHR and pulmonary inflammation (44). Neutrophil infiltration into the lungs is a commonly seen feature of animal models of asthma and is also observed in the most severe and recalcitrant cases of the human disease (45–48). The data suggested that the early exacerbation in pulmonary inflammation was driven by these potent inflammatory cells.

Lung eosinophilia is a hallmark of human allergic asthma and is also present in appropriate animal models of the disease (20, 49–51). In our experiments these inflammatory cells behaved in an interesting fashion. Since they were elevated at the 0-hour time point (i.e., the mice were sacrificed at the time immediately prior to when they would have received their final allergen challenge), the eosinophils had persisted from the previous CRA challenge which occurred 1 week prior to sacrifice. Although eosinophils are generally considered to be short-lived cells similar to neutrophils (52, 53), it has been shown that their life span can be significantly increased in inflammatory environments such as an inflamed lung (54). By

examining the Th2 cytokines, we can see that IL-4 and IL-13 were also increased at 0 hours indicating that they had been tonically elevated following the allergen challenges. Both these cytokines are known to chemoattract eosinophils (55, 56), and IL-13 can prolong the survival of these cells (57). Additionally, IL-13 has been shown to be necessary for eosinophil transmigration into the lung (58). Thus the tonic elevation in the Th2 cytokines provides an explanation for the long-lived eosinophils seen in our experiments. Following final allergen challenge, the BAL eosinophil levels dropped sharply. This sudden decrease is probably due to the activation, degranulation, and death of the old, resident eosinophils which had persisted from the previous allergen challenge. The increase in BAL eosinophils at the 4-hour time point is likely stimulated by the early peak of eotaxin-1 release. Furthermore, the persistence of these cells out to 48 hours is likely maintained by the late-peaking eotaxin-2 and perhaps IL-5.

Airways mucus hypersecretion is also a characteristic feature of human and animal asthma (36, 59). In our model, lung mucus production also followed a biphasic pattern. The significant drop in mucus levels at the 12-hour time point can be explained by two mechanisms, in either isolation or combination. These two main mucus-clearing processes are reabsorption and clearing of the lungs by coughing. The mucus clearing seen at 12 hours is likely a combination of the two. However, to our knowledge, neither of these mechanisms has been rigorously studied or verified in mice.

The asthma model used for these studies does not depend on the use of an adjuvant to induce the pulmonary inflammation. Instead, the lungs were directly exposed to the various components of CRA by placing the solution in the back of the hypopharynx and allowing direct aspiration. This hypopharyngeal delivery method has been used by us (16, 30, 41) and others to deliver allergens (60, 61). The total CRA exposure was only 8 μ g, which is similar to other asthma models without adjuvants that use 6 μ g of ovalbumin (62, 63) or 200 μ g of house dust mite extract (64). However, these previous publications did not perform kinetic studies and therefore did not identify a biphasic response.

In fact, very few research groups have published data on biphasic, asthma-like pulmonary inflammation in animal models. Mizutani et al., Toward et al., and Adachi et al. have demonstrated and characterized an allergen-induced biphasic asthmatic response in guinea pigs; however, this model has the disadvantage of being modeled in relatively large and expensive animals with fewer opportunities for genetic manipulation and less robust supporting literature than similar mouse models (65–67). Meanwhile, Nabe et al. have demonstrated a biphasic asthma model in Balb mice (68). This model employs OVA sensitization by repeated intraperitoneal injection with alum adjuvant. This technique differs from ours in that a systemic sensitization is achieved as opposed to a specific, pulmonary inflammatory response. We feel that our model is more applicable to the human asthmatic population in that exposures are intratracheal only, no adjuvants are used, or, for that matter necessary, the allergen CRA is widely distributed in urban areas and implicated in asthmatic sensitization in children and our mice are genetically heterogeneous outbred animals rather than effective clones (7–10). Furthermore, we have investigated many more time points than other models which typically do not observe beyond 24 hours and in finer temporal resolution.

It is important to continue to research biphasic asthma models in animals for a variety of reasons. Although there are dozens of tested and established treatments for asthma, the disease is merely managed as a chronic condition. Although most individuals can achieve excellent control with scrupulous medication compliance, many patients are not sufficiently concerned or are incapable of effectively managing their medications and thus have frequent exacerbations and corresponding overuse of short-acting agents (1–4). Furthermore, even with proper compliance, some patients display highly recalcitrant disease which is difficult if not impossible to treat in a satisfactory manner resulting in frequent hospitalizations and the use of systemic corticosteroids with their myriad adverse effects (69, 70). It is thus clear that despite decades of research, the underlying mechanisms of the asthmatic process and the variability seen in the human population remain poorly understood. Therefore, animal models with close correspondence to human disease are vital to future efforts to treat and eventually eliminate this disease. Gaining further insight into the cellular, molecular, and kinetic aspects of the asthmatic process will allow the development of new and perhaps counterintuitive treatments. Given the annual cost in health-care dollars and lost school and work hours it is imperative that new and better treatments are identified. This can only be accomplished through more accurate modeling and basic scientific research.

Conclusion

We have demonstrated a CRA-induced biphasic model of asthma-like pulmonary inflammation in outbred mice. This model shows a complex inflammatory response and may be used to investigate the inflammatory changes found in humans, to differentiate the mechanisms of early and late inflammation.

Acknowledgments

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Figure 1.

(A) CRA-specific IgE in mice exposed to different concentrations of CRA allergens. Mice were exposed 3 times to the indicated concentrations of CRA and plasma collected 1 day after the final allergen challenge. * $p < .05$ compared to all other groups by one-way ANOVA and Dunnett's post-test. (B) Salbutamol treatment prevents airway hyperreactivity. * $p < .05$ compared to water. All values in each panel are mean $\pm$ SEM.

Figure 2.

Lung neutrophils and CXC chemokines. Bronchoalveolar lavage and lung homogenate KC (A), MIP- 2 (B), and total BAL neutrophils (C). KC and MIP-2 peaked rapidly (1.5 hour) after the final allergen challenge. BAL neutrophils (C) respond to KC and MIP-2 and quickly peak before returning gradually to near the baseline values. The 0-hour time point is the value from mice that were sensitized on day 0, challenged on day 14, and killed on day 21, prior to the third exposure to CRA. Each value is the mean $\pm$ SEM. ANOVA showed a significant time effect.

Figure 3.

Lung eosinophils and eotaxins. BAL and LH concentrations of eotaxin-1 (A) and eotaxin-2 (B) show an early increase in eotaxin-1 with a later increase in eotaxin-2. Eosinophils are recovered from the BAL throughout the experimental timeframe (C). All values are mean $\pm$ SEM of three independent experiments; $n = 11$ mice. ANOVA showed a significant time effect.

Figure 4.

Lung lymphocytes and Th2 cytokines. BAL lymphocytes (A) and bronchoalveolar lavage (BAL) and lung homogenate IL-4 (B), IL-5 (C), IL-13 (D). Lymphocytes peak late (24 hour) but are present at lower levels at earlier time points. IL-4 and IL-13 were tonically elevated at time 0, that is, after two exposures to CRA but IL-13 remained elevated following final challenge. All values are the mean $\pm$ SEM of three independent experiments; $n = 11$ mice. ANOVA showed a significant time effect on the data.

Figure 5.

IL-12, interferon-gamma (IFN), and TNF. IL-12 was elevated tonically following the previous allergen challenge (1 week prior) and quickly returned to baseline following final CRA challenge. IFN increased in the lung homogenate (LH) over the course of the study. BALTNF shows evidence of a biphasic response with an early (1.5 hour) and late (24 hour) peak. All values are the mean $\pm$ SEM of three independent experiments; $n = 11$ mice. ANOVA showed a significant time effect for IL-12 and IFN. * $p < .004$ for the 1.5- and 24-hour time points by ANOVA with Bonferroni correction compared to the 12-hour time point.

Figure 6.

Kinetics of airways hyperreactivity, airways mucus, and leukotrienes. Enhanced pause (A) at various time points post final challenge. Penh displayed a biphasic pattern with an early peak (1.5 hours) and a late peak (24 hours). Penh was compared to resistance (B) recorded on a mechanical ventilator with forced oscillation at 1.5, 12, and 24 hours post final challenge. Resistance and Penh showed similar patterns at all measured time points. (C) Lung homogenate supernatant Cys-LTs at 1.5, 12, and 24 hours post final challenge. For (A) and (C), values are the mean $\pm$ SEM from 3 independent experiments for $n = 11$ mice. For resistance, values are the mean $\pm$ SEM of 2 independent experiments, $n = 6$ mice.

* $p < .05$ compared to 12Hr timepoint

Figure 7.

(A) Representative images of HE+PAS-stained airways at 20× showed significant airway epithelial mucus production at 1.5 and 24 hours. Total mucus staining (B) was quantitated using ImageJ analysis software. Analysis showed significantly higher mucus production at 1.5, 24, and 48 hours compared to 12 hours by ANOVA with Bonferroni post hoc analysis. (B) Values are the mean ± SEM for $n = 11$ from 3 independent experiments.

* $p < .05$ compared to 12Hr timepoint.

Table 1

Controls for cockroach allergen-induced asthma-like pulmonary inflammation.

Parameter	Naïve	CRA/PBS/PBS	CRA once	CRA/CRA day 21	Asthma model
Penh	0.8 ± 0.1	0.8 ± 0.2	0.8 ± 0.1	1.8 ± 0.5	3.71 ± 0.6
Histology					
PAS staining (×1000)	11 ± 6 ^{**}	28 ± 4 ^{**}	23 ± 7 ^{**}	496 ± 88	334 ± 56
Histology score	0 ± 0	0 ± 0	2 ± 0	No data	5.5 ± 1
Alveolitis score	0 ± 0	0 ± 0	0 ± 0	No data	0 ± 0
Lung neutrophil parameters					
BAL PMNs (×1000)	0.3 ± 0.3	0.5 ± 0.5	126 ± 12	43 ± 11	574 ± 65
BAL CXC chemokines	BDL	BDL	BDL	0.2 ± 0.01	2.7 ± 0.3
LH CXC chemokines	1.6 ± 0.2 [*]	5.1 ± 0.9	12.7 ± 3 [*]	3.8 ± 0.2	6.7 ± 0.3
LH MPO delta blank (×100)	No data	12 ± 3	27 ± 6	14 ± 2	125 ± 9
Lung eosinophil parameters					
BAL eosinophils (×1000)	0.2 ± 0.1	0 ± 0	0 ± 0	278 ± 66 [*]	63 ± 16
BAL eotaxins	BDL	BDL	BDL	3.7 ± 0.2	6.7 ± 0.3
LH eotaxins	0.2 ± 0.1	BDL	0.4 ± 0.1	4.3 ± 0.2	5.8 ± 0.6
LH EPO delta blank (×100)	No data	2 ± 1	3 ± 1	22 ± 5	60 ± 24

Notes: Multiple parameters were evaluated to rule out a nonspecific irritant effect. CXC chemokines indicate the sum of KC and MIP-2; eotaxins is the sum of eotaxin-1 and eotaxin-2. Naïve are mice that were never exposed to allergens or vehicle. CRA/PBS/PBS were sensitized to CRA on day 0 and then challenged with PBS on days 14 and 21. CRA challenge was exposed a single time to CRA via the hypopharyngeal route and pulmonary parameters assayed the same day. CRA/CRA day 21 received two exposures to CRA and studied on day 21, before they received the final CRA exposure. Asthma model are mice with the complete regime of allergen sensitization on day 0 and challenge on days 14 and 21. The data for the CRA/PBS/PBS, CRA once, and asthma model were collected 90 minutes after the last hypopharyngeal challenge. Values are mean ± SEM for 4–8 mice. Parameters listed in bold are those where the asthma group is significantly different from all other groups by ANOVA with Dunnett's post hoc analysis. CRA, cockroach allergen; Penh, enhanced pause; PMN, polymorphonuclear cell; CXC chemokines, KC + MIP-2; eotaxins, eotaxin-1 + eotaxin-2; MPO, myeloperoxidase; EPO, eosinophil peroxidase; BAL, bronchoalveolar lavage fluid; LH, lung homogenate supernatant; BDL, below detection limit.

^{*} $p < .05$ compared to asthma;

^{**} $p < .05$ comparing groups to CRA/CRA day 21 and asthma.