

Treatment of Cockroach Allergen Asthma Model with Imatinib Attenuates Airway Responses

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In the present study it was determined whether a pharmacologic approach to blocking receptor tyrosine kinase-mediated activation during allergic airway responses could be beneficial. To examine these responses, allergic mice were given a single oral dose of imatinib at clinically relevant concentrations, ranging from 0.05 to 50 mg/kg, by oral gavages just before allergen challenge. The reduction in the allergen-induced responses was significant and centered on reducing overall inflammation as well as pulmonary cytokine levels. In particular, the treatment of the mice with imatinib significantly attenuated airway hyperreactivity and peribronchial eosinophil accumulation, and significantly reduced Th2 cytokines, interleukin-4 and interleukin-13. In addition, chemokines previously associated with allergen-induced pulmonary disease, CCL2, CCL5, and CCL6, were significantly reduced in the lungs of the imatinib-treated animals. Together these data demonstrate that the pharmacologic inhibitor imatinib may provide a clinically attractive therapy for allergic, asthmatic responses.

Keywords: asthma; eosinophils; imatinib

Peribronchial leukocyte accumulation is the hallmark of asthma, which affects a significant proportion of the worldwide population (1–6). The major pathophysiologic event that occurs during asthma is airway hyperreactivity (AHR) during the late-phase response. In particular, eosinophils have been reported to be the primary cell associated with induction of bronchial mucosal injury, and are believed to participate in bronchial obstruction and AHR (7–11). Several therapeutic strategies have focused on attenuating airway inflammation, including glucocorticoids and other agents that nonspecifically affect the response. The limited therapeutic options for the treatment of the disease likely reflect the lack of our understanding of the mechanisms that cause airway inflammation and hyperreactivity. A correlation can be drawn between the intensity of the leukocyte infiltration and the severity of the late-phase response. In particular, the activation and degranulation of eosinophils may be one of the causative events related to the exacerbation of airway disease (12–14). The apparent correlation with Th2 type immune responses has closely linked the intensity of the allergic responses with the production of interleukin (IL)-4 and IL-13 within the lungs of individuals with asthma.

The activation of cytokine and growth factor receptors relies on activation of receptor tyrosine kinases for initiation of critical inflammatory and immune processes. Several drug development programs have begun to focus on inhibiting specific receptor tyrosine kinases. Most inhibitors have some nonspecific activity

that makes it difficult to target a specific pathway. However, it appears that some success has been demonstrated for targeting specific classes of tyrosine kinases. The tyrosine kinase inhibitor imatinib mesylate (imatinib), also known as Gleevec, has revolutionized therapy for chronic myeloid leukemia with more than 90% of previously untreated patients in chronic phase achieving a complete hematologic response and more than 70% a complete cytogenetic response after one year of therapy (15–17). Specifically, imatinib was developed to inhibit BCR-ABL kinase activity. However, imatinib has been demonstrated to have significant efficacy in blocking both c-kit ligand, stem cell factor (SCF) and PDGFR- α protein tyrosine kinase activity, whereas it spares most other kinase-activated pathways (18). In addition to being an attractive and well tolerated therapy for a number of cancerous diseases, imatinib mesylate has recently demonstrated efficacy in hypereosinophilic syndrome patients where few other options for therapy exist (19–21). Interestingly, a role for SCF overproduction in the airway during allergen-induced responses has been identified that was associated with alterations of eosinophil accumulation and hyperreactivity (22–25). Although SCF appears to be intimately involved in the initial phases of the response and mast cell activation, the down-stream effects on the late-phase response after blocking SCF function were profound and may be linked to the ability of SCF to directly activate eosinophils (23, 24, 26, 27). A most recent study indicated a significant increase in both SCF and c-kit expression in the airways of individuals with asthma related to the severity of disease (28). Thus, at least one of the targets of imatinib mesylate, SCF/c-kit activation pathway, may be a focus for therapy in asthmatic disease, but other receptor tyrosine kinases may also be inhibited by imatinib mesylate.

METHODS

Sensitization and Induction of the Airway Response

To induce a TH2 type response, the following procedure was established in normal Balb/c mice, as previously described (29, 30). The mice were immunized with 10 μ g of cockroach allergen (Holister-Stier, Spokane, WA) in incomplete Freund's adjuvant on Day 0. On Day 14 the mice were given an intranasal challenge of 10 μ g of cockroach allergen in 10 μ l of diluent to localize the response to the airway. Mice were then rechallenged 6 days later by intratracheal administration of 6 μ g of cockroach allergen in 50 μ l of sterile phosphate-buffered saline (PBS). Naive control animals were also given cockroach allergen intratracheally as a control. The magnitude of leukocyte recruitment in both the vehicle control- and cockroach allergen-challenged mice was examined histologically, and AHR was determined at 24 hours postchallenge.

Enumeration of Peribronchial Eosinophil Accumulation

Morphometric analysis of peribronchial eosinophil accumulation was performed from lungs of mice immunized and challenged with cockroach allergen on lungs preserved with 1 ml of 4% paraformaldehyde at 24 hours postchallenge. The fixed lungs were embedded in paraffin and multiple 50 μ m sections were differentially stained with Wright-giemsa for the identification of eosinophils and viewed at 1,000 $\times$. The individual eosinophils were counted from 50 high-powered fields per lung at each time point using multiple step sections of lung. Only the eosinophils in the peribronchial region were counted, which assured the

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enumeration of only those eosinophils within or immediately adjacent to an airway. The inflammation observed in this model is completely associated with the airway with little or no alveolitis.

Measurement of AHR

AHR was measured as previously described using a direct ventilation mouse plethysmograph (Buxco, Troy, NY), which is specifically designed for low tidal volumes (25, 30). Briefly, the mouse to be tested was anesthetized with sodium pentobarbital and intubated via cannulation of the trachea with an 18-gauge metal tube. The mouse was subsequently ventilated with a Harvard pump ventilator ($V_T = 0.4$ ml, frequency = 120 breaths/minute, positive end-expiratory pressure 2.5–3.0 cm H_2O) and the tail vein was cannulated with a 27-gauge needle for injection of the methacholine challenge. After determining a dose response curve (0.001 to 0.5 mg/kg), an optimal dose was chosen, 0.1 mg/kg of methacholine, which induced minimal AHR in naive control mice but a very significant induction of AHR in allergic animals. After the methacholine challenge, the response was monitored and the peak airway resistance recorded as a measure of AHR.

ELISAs

Cytokines were quantified from homogenized (phosphate-buffered saline [PBS] with 0.05% Triton X-100 nonionic detergent and antiproteases) lung aqueous extracts using a double-ligand ELISA system. The murine ELISAs were set up using standardized antibodies purchased from R&D Systems (Rochester, MN) that detect protein at concentrations above 10 pg/ml, are specific, and do not cross react with any other cytokines.

Statistics

Statistical significance was determined using analysis of variance with p values less than 0.05.

RESULTS

Treatment of Mice with Imatinib Attenuates Cockroach Allergen-induced Pulmonary Disease

Previous studies have demonstrated a role for SCF/c-kit activation pathway in the development of allergen-induced AHR and inflammation (23–25, 31, 32). To further examine the potential for targeting this activation pathway, we treated mice with an inhibitor for c-kit–associated tyrosine kinase, imatinib. Allergen-sensitized mice were treated by oral gavages with 0.2 ml of PBS containing various doses of clinical-grade imatinib covering a 4 $\log_{10}$ dose of inhibitor. Thirty minutes after the oral gavage of PBS or PBS + imatinib, allergic mice were challenged by intratracheal injection with cockroach allergen (6 μ g) suspended in 40 μ l of PBS. As a control, naive mice were also challenged with cockroach allergen. After 24 hours, the mice were examined for induction of AHR after a methacholine challenge (*see METHODS*). The results in Figure 1 depict that treatment of mice with imatinib significantly reduced the induction of AHR at higher doses, 50 and 5 mg/kg, but not at the lower doses, 0.5 and 0.05 mg/kg. The level of airway resistance that was measured by direct ventilation plethysmography in the imatinib-treated group was reduced to nearly the levels observed in the naive control-challenged group.

A significant correlation can often be drawn between the intensity of the pulmonary inflammation and AHR responses in animal models of asthma. Figure 2 depicts that those animals treated with imatinib demonstrated significant reduction in the overall inflammatory response to the cockroach allergen challenge compared with vehicle-treated allergic animals. This was further examined by enumerating the number of eosinophils that accumulated around the airways of the allergen-challenged animals (Figure 3). The imatinib nearly abrogated the presence of eosinophils around the airways and directly correlated with the reduction in airway hyperresponsiveness observed above in the higher doses of imatinib. In contrast, in addition we examined

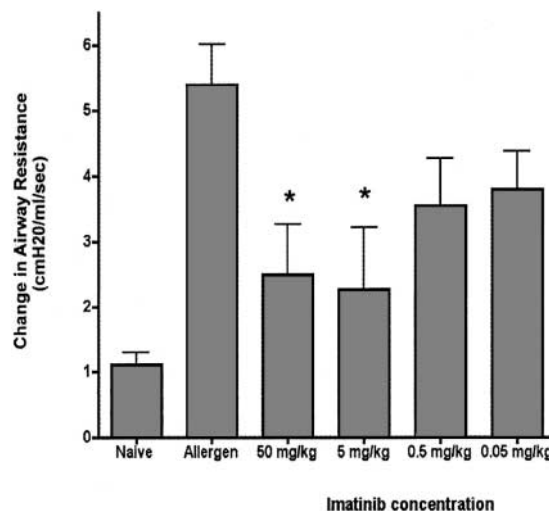


Figure 1. Treatment of allergic mice with imatinib alleviates development of airway hyperreactivity (AHR). Cockroach allergen-sensitized mice were treated with imatinib (0.05 to 50 mg/kg) suspended in phosphate-buffered saline (PBS) by oral gavage 30 minutes before the final allergen challenge. Vehicle control allergic animals were treated with only PBS. Naive animals (Control) were treated only with PBS and challenged with cockroach allergen at the same time as the allergic mice. After 24 hours the animals were anesthetized, trachea, and ventilated in a mouse plethysmograph. After baseline measurements were taken, the mice were challenged with methacholine (100 μ g/kg) via an intravenous instillation. Peak airway resistance changes were taken, and the change in airway resistance was calculated by subtraction of the baseline measurements. Baseline values in all groups were between 0.9 and 1.2 cm H_2O /ml/sec. Data represent the mean $\pm$ SE from 6 mice/group. * $p < 0.5$ compared with cockroach allergen-challenged, PBS-treated mice (Allergen).

the expression of MUC5AC mucin by quantitative polymerase chain reaction and airway goblet cells by periodic acid-Schiff/alcan blue stain, and neither was altered by the treatment of a single dose of imatinib before allergen challenge (data not shown). Thus, there appeared to be an alteration in the inflammation and airway physiology but no decrease in mucus.

Alteration of Pulmonary Cytokine and Chemokine Levels with Imatinib

To further characterize the nature of the response with imatinib treatment of mice, separate studies were designed to examine an oral dose response (0.5 to 50 mg/kg). Whole-lung homogenates were analyzed for cytokine and chemokine levels using specific ELISAs (Figure 4). The data indicate that both IL-4 and IL-13 levels were significantly reduced by treating the mice with all doses of imatinib (Figure 4A). Even at a dose of imatinib that did not alter AHR, 0.5 mg/kg, there was a significant reduction in both IL-4 and IL-13. When a number of allergen response related chemokines were examined, we also found a significant reduction in CCL2, CCL5, and CCL6, especially at the higher doses (Figure 4B). Because we found a significant reduction in several of the cytokines/chemokines measured even at the lower dose used, we performed a separate experiment using an even lower dose of 0.05 mg/kg and found that these mice had no significant reduction in cytokine or chemokine production (data not shown), reflecting the AHR and eosinophil responses observed above. These mediators have all been independently implicated in the progression of allergic responses in several

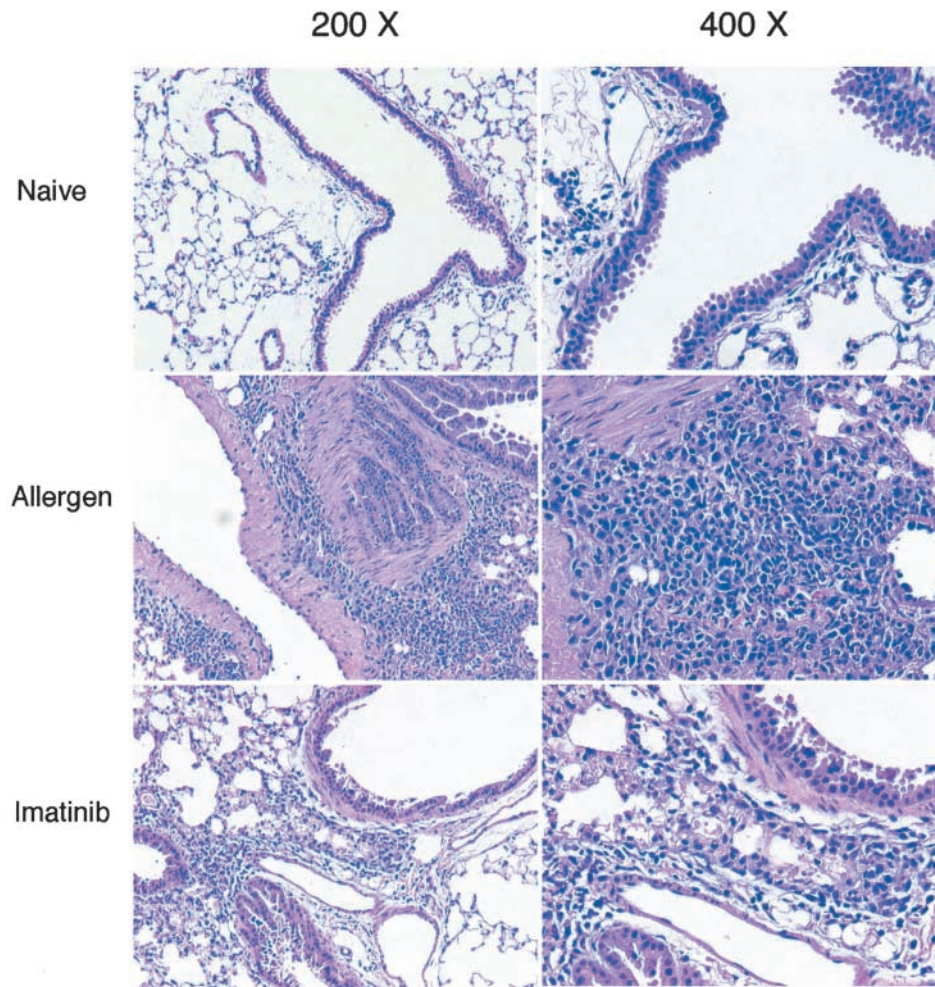


Figure 2. Histological examination of allergen-challenged mice demonstrates reduced inflammation in imatinib-treated animals. Lungs from mice in the experiment described in Figure 1 were inflated and fixed with buffered formalin and processed for histology. Lung sections were stained with hematoxylin and eosin and examined by light microscopy. Representative sections indicate that mice treated with imatinib (50 mg/kg) display significantly less inflammation in the peribronchovascular regions of allergic mice.

different models of airway diseases and relate to the overall inflammation within the lungs. Thus, the overall reduction of inflammation may be a result of inhibition of a number of interrelated responses.

DISCUSSION

The identification of effective therapy for patients with moderate-to-severe asthma has been relegated in recent years to developing more efficient delivery of steroids to the airway (33, 34). These nonspecific compounds decrease the production and release of a wide array of immune/inflammatory mediators and significantly limit the effect of the overall immune response. However, the ability to specifically block certain critical activation pathways utilizing signaling blockades may prove to be beneficial to alleviate long-term chronic responses. In the present studies, on the basis of previously published data from our laboratory and others (23–25, 27, 32), we initiated an analysis of whether we could alleviate responses in our preclinical model of cockroach antigen-induced asthma by blocking receptor tyrosine kinases related to c-kit. Imatinib has been shown to inhibit the c-kit and PDGFR activation pathways as well as arginine kinase pathways, but not other receptor tyrosine kinase pathways examined (18, 35). The data from the present study have been striking, as not only was the development of AHR significantly reduced, but also the inflammatory response was nearly abrogated. In particular, the Th2 cytokines that dominate the allergic airway responses were reduced in the lung postchallenge. Although it

is not clear which specific tyrosine kinase pathways were altered with imatinib, these studies demonstrate that this approach and, more importantly, this drug may provide a viable therapeutic option for blocking certain aspects of asthmatic responses. However, further studies also found that although AHR and inflammation were reduced after a single treatment of allergic mice with imatinib, neither serum IgE levels nor airway mucus expression were reduced, indicating that not all aspects of chronic asthma are alleviated. This latter issue will surely need to be addressed in additional studies focused on longer term treatment with imatinib during the development of allergic airway responses.

A striking aspect of these studies is the reduction in eosinophils within and around the airway. Recent studies in patients with hypereosinophilic syndrome have established a role for imatinib in the reduction of eosinophil numbers and the associated pathophysiology of this often devastating disease (19–21, 36–42). Although it is not completely clear by what mechanism imatinib is operating during hypereosinophilic syndrome, it has had an extremely beneficial effect in a significant number of patients related to a mutation in PDRFR- α (37, 38). The data from the present studies suggest that imatinib may have an overall effect on the immune/inflammatory response during antigen-specific reactions. Others and we have established that inhibition of SCF in the airways of allergic mice can significantly attenuate the inflammatory/immune responses (23–25, 27, 32). Previous data suggest that the role of SCF would be multifactorial by inhibiting not only the local mast cell populations involved early

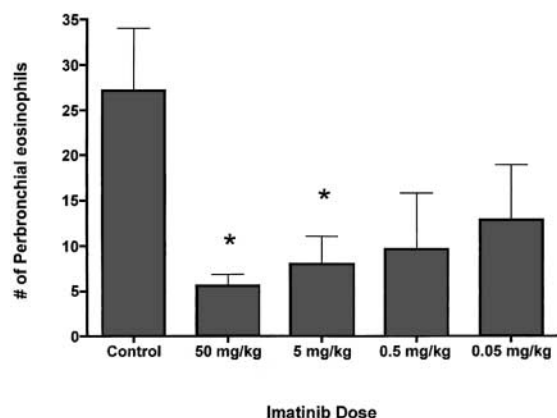


Figure 3. Morphometric enumeration of peribronchial eosinophils demonstrates significant attenuation in imatinib-treated animals (0.05 to 50 mg/kg). As outlined in METHODS, fixed and processed lung sections were examined for eosinophil accumulation around the airways by counting eosinophils directly in the peribronchial areas. Each mouse was assessed in a blinded fashion using randomly selected airways and counting 50 high-power fields (1000 $\times$ magnification). The data depict the mean $\pm$ SE from each group containing 6–10 mice/group. * p < 0.5 compared with cockroach antigen-challenged, vehicle-treated allergic mice.

on during the response but also the recruitment and activation of eosinophils (22, 23, 43–45). Furthermore, the recent identification of increased expression of both SCF and c-kit in airways of individuals with asthma gives additional support for targeting this activation pathway (28). However, because imatinib may also have effects on other receptor tyrosine kinase family members, such as PDGFR, the effects observed in this study likely were an outcome of blocking other pathways as well. The responses are also likely to encompass the alteration of bone marrow-derived cells, especially if imatinib is given long term. We found no alteration of circulating leukocyte numbers (data not shown), but have not performed extensive studies to examine bone marrow or peripheral leukocyte counts in the present studies. However, in these studies imatinib was given a single time just before allergen challenge, and the effects may be centered on the alleviation of the inflammatory responses directly activated within the airways. The reduction of chemokines may have resulted from decreased Th2 cytokines as well as the direct effects of blocking specific signaling processes related to specific receptor tyrosine kinase pathways.

Previous observations in patients treated with imatinib for chronic myeloid leukemia have identified increases in IFN after a 3-month treatment protocol, potentially suggesting an alteration of the overall balance of Th1 and Th2 type responses (46). In contrast, another study that examined T cell responses in patients with chronic myeloid leukemia before and after imatinib treatment found no difference in the Th1/Th2 cytokine levels on polyclonal activation (47). Interestingly, data suggest that imatinib may produce long-term, event-free survival in patients with T cell lymphoid blastic phase (48). Another recent publication has indicated that imatinib treatment affects the development of CD34 $^{+}$ progenitor cells into dendritic cells (49), further supporting a role for imatinib in altering the development of detrimental immune responses. Related to the current study are previous observations where SCF has been specifically blocked in the airway, either by antibody or antisense therapy, and an alteration in Th2 cytokines observed (31, 32). Thus, by blocking

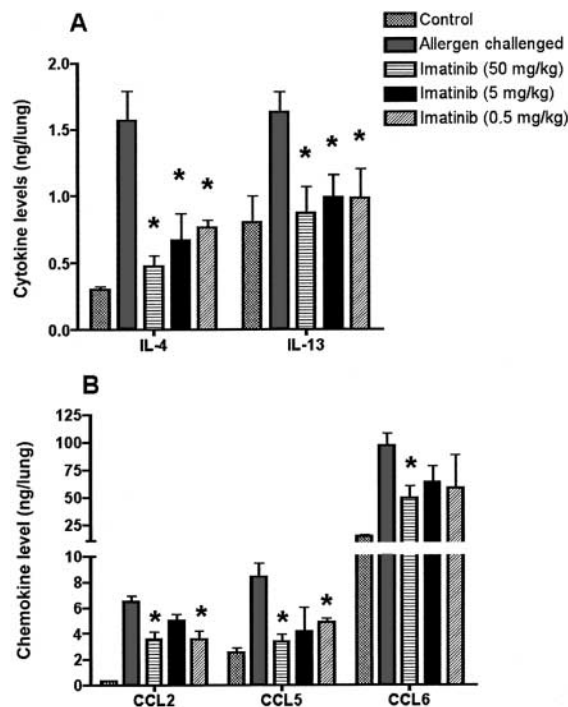


Figure 4. Treatment of allergic mice with imatinib attenuates cytokine (A) and chemokine (B) levels in cockroach antigen-challenged mice. Using multiple doses of imatinib (0.5 to 50 mg/kg), the cytokines in whole lung homogenates demonstrated a significant reduction in the total pulmonary levels of critical activating and chemotactic cytokines. The data depict the mean $\pm$ SE from 5 mice/group. * p < 0.5 compared with cockroach antigen-challenged, vehicle-treated allergic mice. IL = interleukin; CCL = CC motif chemokines.

the initiation of this pathway a significant effect can be observed in the expression of a number of allergen-induced cytokines.

These studies have identified a potential avenue of treatment that centers on blocking certain activation pathways that have previously not been considered for asthma therapy. These results, although striking, deserve additional investigation within preclinical models of allergic airway disease and possibly, subsequent investigation in populations of patients with asthma.

Conflict of Interest Statement: A.A.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; N.W.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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