

Factor B of the alternative complement pathway regulates development of airway hyperresponsiveness and inflammation

Christian Taube*, Joshua M. Thurman[†], Katsuyuki Takeda*, Anthony Joetham*, Nobuaki Miyahara*, Michael C. Carroll[‡], Azzeddine Dakhama*, Patricia C. Giclas*, V. Michael Holers[§], and Erwin W. Gelfand*^{¶1}

*Division of Cell Biology, Department of Pediatrics, National Jewish Medical and Research Center, Denver, CO 80206; Divisions of [†]Nephrology and Hypertension and [§]Rheumatology, Department of Medicine, University of Colorado Health Sciences Center, Denver, CO 80262; and [‡]Department of Pediatrics and Pathology, The CBR Institute for Biomedical Research, Harvard Medical School, Boston, MA 02115

Communicated by Charles A. Dinarello, University of Colorado Health Sciences Center, Denver, CO, March 22, 2006 (received for review November 20, 2005)

Exposure to inhaled allergens leads to increases in airway hyperresponsiveness (AHR) and inflammation, associated with increased levels of biologically active fragments derived from the complement C3 and C5 family of proteins. Further, complement activation during allergen challenge in sensitized animals is necessary for the development of AHR and airway inflammation. To define the complement pathway involved, we studied mice deficient in complement factor 4 (*C4*^{-/-}), a critical component of the classical pathway, or factor B (*fB*^{-/-}), an essential protein in the alternative complement pathway. WT, *C4*^{-/-}, and *fB*^{-/-} mice were sensitized to ovalbumin and subsequently exposed to nebulized ovalbumin (1% in saline) on 3 consecutive days. After allergen sensitization and challenge, *fB*^{-/-} mice demonstrated significantly lower airway responsiveness to methacholine and less airway inflammation. In contrast, *C4*^{-/-} mice showed no reduction in AHR and airway inflammation compared with WT mice. Tissue inflammation, goblet cell hyperplasia, and IL-4, IL-5, and IL-13 levels in BAL fluid were significantly reduced in *fB*^{-/-} mice compared with *C4*^{-/-} and WT mice. The development of AHR and airway inflammation in sensitized *fB*^{-/-} mice could be restored after intranasal administration of purified factor B before the airway challenge. In addition, administration of a neutralizing anti-factor B mAb to sensitized mice before airway challenge reduced the development of AHR and airway inflammation. These results demonstrate that in sensitized hosts complement activation through the alternative pathway after allergen exposure is critical to the development of AHR and airway inflammation.

asthma | allergy | innate immunity | lung

In patients with allergic asthma, exposure to inhaled allergens leads to increases in airway hyperresponsiveness (AHR) and airway inflammation, associated with increased levels of biologically active fragments derived from the complement C3 and C5 family of proteins. The anaphylatoxins C3a (1) and C5a (2) have been found in increased amounts in bronchoalveolar lavage (BAL) fluid of patients with allergic asthma after allergen challenge. These results suggest an allergen-induced mechanism leading to complement activation in the lungs of asthmatics. Animal models have helped to further understand the significance of complement activation in the development of allergic airway disease. Animals deficient in C3 or C3a receptor are protected from the development of allergen-induced airway disease; the presence of C5a may also be protective (1, 3–5). Similarly, prevention of complement activation before allergen exposure of sensitized mice results in less airway dysfunction and inflammation (6, 7).

To date, little is known about which pathway of complement activation follows allergen exposure and may be involved in the development of altered airway function and inflammation. The three pathways of complement activation (classical, alternative, and lectin) converge at the central complement component C3,

and inhibition of C3 activation prevents cleavage into active C3 fragments and largely reduces the downstream activation of C5 and the release of C5-derived activated fragments (8). It has been proposed that allergen–IgG immune-complexes trigger activation of the classical pathway, whereas certain antigens may directly activate C3 via the alternative pathway (9). In addition, neutral tryptases released from mast cells or pulmonary macrophages may directly (proteolytically) cleave C3 or C5 (10, 11). In previous studies, inhibition of complement activation was achieved by inhibitors that indiscriminately target and inhibit the effects of activating any of the three pathways (6, 7).

To define which complement pathway is critical to these allergic responses in the lungs of sensitized and challenged mice, we used mice deficient in complement component 4 (*C4*), which is essential to the activation of the classical (and lectin) pathway (12), and mice deficient in factor B, which is an essential protein in the alternative pathway (13). We show that complement activation through the alternative, but not classical, pathway is critical to the development of allergic airway disease in sensitized mice after allergen exposure. Furthermore, we demonstrate that treatment of sensitized normal mice with a specific inhibitor of factor B before allergen exposure can prevent the development of AHR and airway inflammation.

Results

Complement Activation Occurs After Sensitization and Challenge. To monitor activation of complement, levels of C3a desArg were assessed in BAL fluid. Challenged-only mice showed low levels of C3a desArg (Fig. 1A). In contrast, sensitized mice showed increased levels of C3a desArg in BAL fluid after the first, second, and third challenge, with the highest values at 48 h after the last challenge (Fig. 1A). Interestingly, sensitized and challenged *C4*^{-/-} mice showed similar levels of C3a desArg compared with the sensitized and challenged WT mice, in contrast to sensitized and challenged *fB*^{-/-} mice, which showed lower C3a desArg levels compared with their respective sensitized and challenged WT mice (Fig. 1B). Further evidence for activation of complement after sensitization and challenge in WT but not *fB*^{-/-} mice was obtained by staining for C3 deposition in the lungs as demonstrated in Fig. 5, which is published as supporting information on the PNAS web site.

Complement Activation Through the Alternative Pathway Is Critical to the Development of AHR. To assess the role of the classical and alternative pathways in the development of AHR and airway

Conflict of interest statement: No conflicts declared.

Abbreviations: AHR, airway hyperresponsiveness; anti-fB, anti-factor B; BAL, bronchoalveolar lavage; MCh, methacholine; OVA, ovalbumin.

^{¶1}To whom correspondence should be addressed. E-mail: gelfande@njc.org.

© 2006 by The National Academy of Sciences of the USA

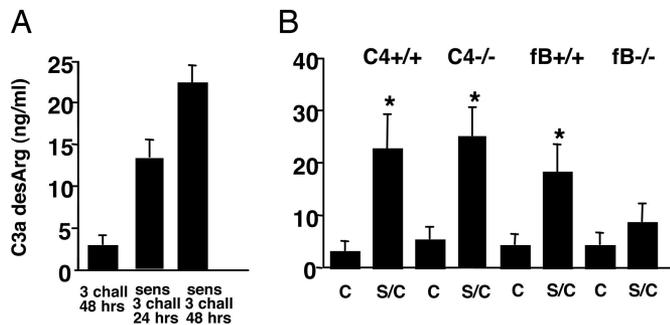


Fig. 1. *fB*^{-/-} mice but not *C4*^{-/-} mice fail to activate complement after sensitization and challenge. (A) Levels of C3a desArg in BAL fluid at 48 h after three OVA challenges or 24 and 48 h after the third airway challenge in sensitized WT mice ($n = 4$ in each group). (B) Levels of C3a desArg were obtained 48 h after the last challenge in challenged-only *C4*^{+/+} and *C4*^{-/-} mice (C , $n = 4$), sensitized and challenged *C4*^{+/+} and *C4*^{-/-} mice (S/C, $n = 8$), challenged-only *fB*^{+/+} and *fB*^{-/-} mice ($n = 8$), and sensitized and challenged *fB*^{+/+} and *fB*^{-/-} mice ($n = 8$). Means \pm SEM are given. *, $P < 0.05$ compared with *C4*^{+/+} C, *C4*^{-/-} C, *fB*^{+/+} C, *fB*^{-/-} C, and *fB*^{-/-} S/C.

inflammation, ovalbumin (OVA)-sensitized and nonsensitized *fB*^{-/-} and *C4*^{-/-} mice and matched control mice (*fB*^{+/+} and *C4*^{+/+}, respectively) were challenged with an aerosol of 1% OVA. Sensitized and challenged *fB*^{+/+} mice showed increased responsiveness to methacholine (MCh) compared with challenged-only *fB*^{+/+} mice (Fig. 2A). Sensitized and challenged *fB*^{-/-} mice demonstrated a significantly ($P < 0.01$) lower response to MCh throughout the dose-response curve compared to the sensitized and challenged *fB*^{+/+} mice. In contrast, sensitized and challenged *C4*^{-/-} mice did not show a decrease in MCh responsiveness compared with sensitized and challenged *C4*^{+/+} mice (Fig. 2B).

Activation of the Alternative Pathway Is Critical for the Development of Airway Inflammation and Regulation of Cytokine Levels in BAL Fluid.

Inflammatory cell accumulation in the BAL fluid and lung tissue was evaluated 48 h after the last airway challenge. Sensitized and challenged *fB*^{+/+} mice showed an increase in total cell counts and eosinophil numbers in BAL fluid compared with challenged-only mice, where few, if any, eosinophils were detected in the BAL fluid (Fig. 2C). Sensitized and challenged *fB*^{-/-} mice showed significantly lower total cell numbers and numbers of eosinophils in the BAL fluid when compared with sensitized and challenged *fB*^{+/+} mice. In contrast, sensitized and challenged *C4*^{-/-} mice ($n = 10$) showed no decrease in BAL total cell

counts (mean \pm SEM; $163 \pm 35 \times 10^3$ cells) or lymphocyte ($28 \pm 9 \times 10^3$ cells) and eosinophil ($98 \pm 23 \times 10^3$ cells) numbers compared with the sensitized and challenged control mice ($n = 10$; 175 ± 53 , 35 ± 12 , and $115 \pm 32 \times 10^3$ cells, respectively). In addition, sensitized and challenged *fB*^{-/-} mice showed markedly reduced peribronchial inflammation and goblet cell metaplasia compared with sensitized and challenged control mice (Table 1 and Fig. 6, which are published as supporting information on the PNAS web site).

Sensitized and challenged *fB*^{-/-} mice also showed reduced levels of IL-4, IL-5, and IL-13 in the BAL fluid. However, levels of IL-12 and IFN- γ were significantly higher in sensitized and challenged *fB*^{-/-} mice compared with sensitized and challenged WT mice and similar to challenged-only mice (Fig. 7, which is published as supporting information on the PNAS web site).

Factor B Deficiency Does Not Affect Serum Levels of Antigen-Specific Antibodies.

In contrast to the reduced levels of T helper 2 cytokines, serum levels of total IgE and OVA-specific IgE and IgG1 were measured 48 h after the last airway challenge. Sensitized and challenged *fB*^{+/+} mice showed increased levels of total IgE and OVA-specific IgE and IgG1 compared with challenged-only control mice (Table 2, which is published as supporting information on the PNAS web site). Similarly, *fB*^{-/-} mice showed increased levels of total IgE and OVA-specific IgE and IgG1, which were not statistically different from sensitized and challenged *fB*^{+/+} mice.

Failure of Development of AHR and Airway Inflammation in Factor B-Deficient Mice Is Not Specific to OVA.

To determine whether the absence of AHR after allergen sensitization and challenge was caused by a specific unresponsiveness to OVA, *fB*^{-/-} and WT mice were sensitized and challenged with ragweed. Ragweed-sensitized and -challenged *fB*^{-/-} mice showed a decrease in responsiveness to MCh, whereas *fB*^{+/+} mice developed a strong response to MCh (Fig. 8A, which is published as supporting information on the PNAS web site). Similarly, airway inflammation and eosinophil numbers in BAL fluid were reduced in ragweed-sensitized and -challenged *fB*^{-/-} mice compared with the *fB*^{+/+} mice (Fig. 8B).

Administration of Factor B Reconstitutes the Ability to Develop AHR and Airway Inflammation in *fB*^{-/-} Mice.

As results in factor B genetically deficient mice may be complicated by the absence of the protein during both the sensitization and challenge phases, we tested the consequences of administering factor B to deficient

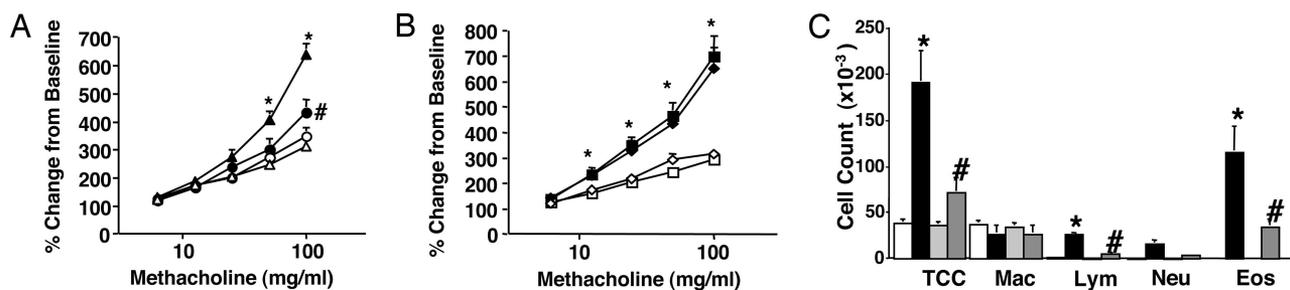


Fig. 2. Airway responsiveness and inflammation in *fB*^{-/-} and *C4*^{-/-} mice. (A) Airway responsiveness (resistance) in *fB*^{-/-} mice. Sensitized and challenged *fB*^{+/+} mice (\blacktriangle , $n = 12$), challenged-only *fB*^{+/+} mice (\triangle , $n = 12$), sensitized and challenged *fB*^{-/-} mice (\bullet , $n = 12$), and challenged-only *fB*^{-/-} mice (\circ , $n = 12$). *, $P < 0.05$ compared with *fB*^{-/-} sensitized and challenged, *fB*^{+/+} challenged only, and *fB*^{-/-} challenged only; #, $P < 0.05$ compared with *fB*^{+/+} challenged only and *fB*^{-/-} challenged only. (B) Airway responsiveness (resistance) in *C4*^{-/-} mice. Sensitized and challenged *C4*^{-/-} mice (\blacklozenge , $n = 8$), sensitized and challenged *C4*^{+/+} mice (\blacksquare , $n = 8$), challenged-only *C4*^{-/-} mice (\diamond , $n = 8$), challenged-only *C4*^{+/+} mice (\square , $n = 8$). *, $P < 0.05$ compared with *C4*^{+/+} challenged only and *C4*^{-/-} challenged only. (C) Airway inflammation in BAL fluid. Challenged-only *fB*^{+/+} mice (white bar, $n = 12$), sensitized and challenged *fB*^{+/+} mice (black bar, $n = 12$), challenged-only *fB*^{-/-} mice (light gray bar, $n = 12$), and sensitized and challenged *fB*^{-/-} mice (dark gray bar, $n = 12$). Results are expressed as mean \pm SEM. *, $P < 0.05$ compared with all other groups; #, $P < 0.05$ compared with *fB*^{+/+} challenged only and *fB*^{-/-} challenged only.

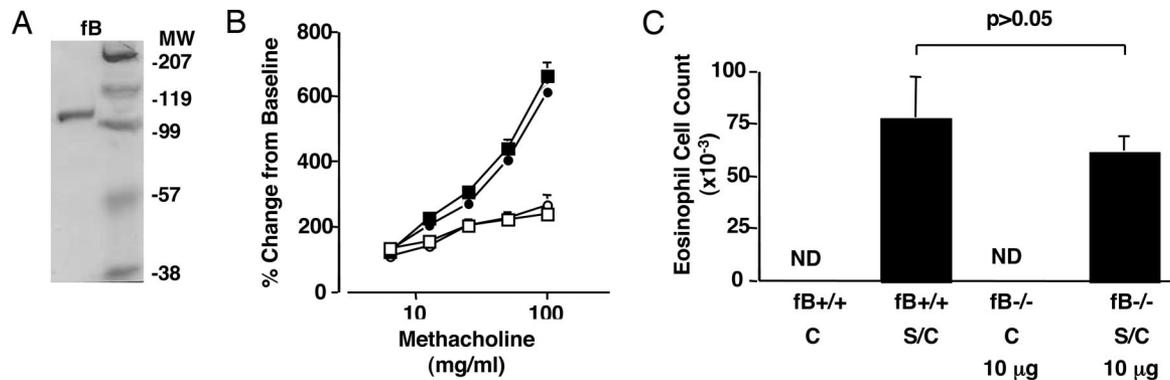


Fig. 3. Administration of purified factor B via the lung reconstitutes the development of AHR and airway inflammation in *fB*^{-/-} mice. (A) Factor B was purified as described in *Materials and Methods*, and purity was confirmed by electrophoresis on a 10% Tris-glycine gel and stained with Coomassie. MW, molecular weight. (B) Factor B was administered intranasally to sensitized *fB*^{-/-} mice before each airway challenge. Sensitized and challenged *fB*^{-/-} mice that received 10 µg of factor B (●, *n* = 10), sensitized and challenged *fB*^{+/+} mice (■, *n* = 10), challenged-only *fB*^{-/-} mice that received 10 µg of factor B (○, *n* = 10), and challenged-only *fB*^{+/+} mice (□, *n* = 10). (C) Eosinophil numbers in BAL fluid. Mean ± SEM are given.

mice. Sensitized and challenged *fB*^{-/-} mice treated with 10 µg of purified factor B (Fig. 3A) before each airway challenge showed a significant increase in response to MCh, similar to the sensitized and challenged *fB*^{+/+} mice (Fig. 3B). In parallel, treatment of sensitized and challenged *fB*^{-/-} mice with 10 µg of purified factor B before each airway challenge increased airway inflammation and eosinophil numbers in BAL fluid, similar to the numbers observed in sensitized and challenged *fB*^{+/+} mice (Fig. 3C).

Treatment with a Factor B-Neutralizing Antibody Inhibits the Development of AHR in Sensitized and Challenged C57BL/6 and C4^{-/-} Mice. To further confirm the results in *fB*^{-/-} mice and extend the findings to normal mice, sensitized C57BL/6 mice were treated with systemically administered or nebulized anti-factor B (anti-*fB*) mAb. Both routes of administration of factor B antibody reduced airway responsiveness to MCh compared with sensitized and challenged mice treated with a control antibody (Fig. 4A). Treatment with either systemic or nebulized antibody also reduced the number of eosinophils in the BAL fluid (Fig. 4B). Additionally, lung tissue inflammation was reduced in

systemic or nebulized anti-*fB* mAb-treated mice compared with the control mice (Fig. 9, which is published as supporting information on the PNAS web site). This reduction in inflammation was also reflected in a reduction of peribronchial eosinophil numbers (Table 1). Numbers of goblet cells were also significantly lower in sensitized and challenged mice treated with nebulized and systemic anti-*fB* mAb compared with control mice (Fig. 9 and Table 1).

As shown in Fig. 4C, treatment of sensitized and challenged *C4*^{-/-} mice with anti-*fB* similarly decreased airway responsiveness and airway inflammation (eosinophil numbers in BAL fluid decreased from $120 \pm 19 \times 10^3$ to $40 \pm 6 \times 10^3$, *n* = 8).

Discussion

A number of studies have suggested that activation of complement occurs after allergen exposure of sensitized hosts (1, 2), and an increasing number of studies using either genetically deficient animals (1, 3, 4, 14) or complement inhibitors (6, 7, 15) have shown that complement activation and generation of complement split products (C3a and C5a) contribute to the development of allergic airway disease. Currently, it is not known which

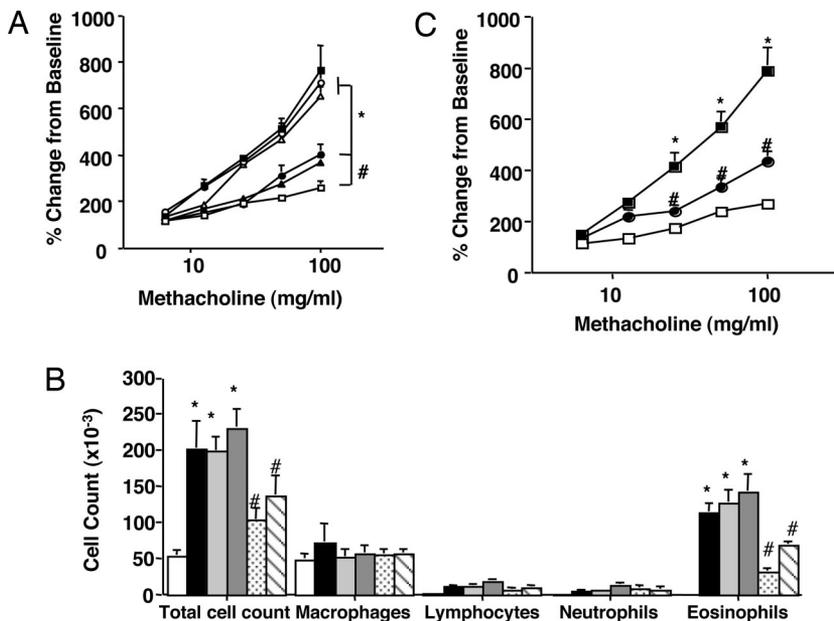


Fig. 4. Administration of a factor B-neutralizing antibody to sensitized and challenged WT and *C4*^{-/-} mice decreases AHR and airway inflammation. (A and B) Airway resistance (A) and cellular composition in BAL fluid (B) were assessed in sensitized and challenged C57BL/6 mice 48 h after the last challenge. Sensitized and challenged mice (■, black bars, *n* = 8), sensitized and challenged mice treated with systemic (△, light gray bars, *n* = 8) or nebulized (○, dark gray bars, *n* = 8) control Ab, challenged-only mice (□, white bars, *n* = 8), sensitized and challenged mice treated with systemic (▲, dotted bars, *n* = 8) or nebulized (●, hatched bars, *n* = 8) anti-*fB* mAb. Mean ± SEM are given. *, *P* < 0.05 compared with challenged, anti-*fB* i.p., and anti-*fB* inhal-treated mice. #, *P* < 0.05 compared with challenged-only mice. (C) Effect of anti-*fB* antibody in *C4*^{-/-} mice. Airway resistance was assessed in sensitized and challenged mice 48 h after the last challenge. Sensitized and challenged *C4*^{-/-} mice (■, *n* = 8), challenged-only *C4*^{-/-} mice (□, *n* = 8), treatment of sensitized and challenged *C4*^{-/-} mice with systemic anti-*fB* mAb (●, *n* = 8). Mean ± SEM are given. *, *P* < 0.05 compared with all other groups; #, *P* < 0.05 compared with challenged-only mice.

pathway of complement dominates after allergen exposure, and all three (classical, alternative, and lectin pathways) could contribute to complement activation after allergen exposure (9). In the present study we confirmed that complement activation after sensitization and challenge does indeed occur as demonstrated by the elevation of C3a desArg levels in BAL fluid and immunostaining for C3 deposition in the lungs. Evidence for complement activation was markedly reduced in sensitized and challenged *fB*^{-/-} mice, supporting the concept that the alternative, but not the classical, pathway is critical to the development of allergic airway disease after allergen exposure of sensitized hosts.

Previously, studies have shown that inhibition of complement activation prevented the development of a late airway response (6), ongoing airway inflammation (7, 15), and development of AHR after allergen (7) or ozone exposure (16). As C4 is an essential component of both classical and lectin pathway activation (12, 17), we used *C4*^{-/-} mice to identify a role for either pathway in the changes observed in sensitized and challenged mice. In the present study, sensitized and challenged *C4*^{-/-} mice developed similar levels of AHR and airway inflammation as the sensitized and challenged *C4*^{+/+} mice. These findings suggest that activation of the classical pathway in this model is not necessary for the development of allergic airway disease. This notion is in line with previous results demonstrating that the development of AHR and airway inflammation in this model of systemic sensitization and allergen exposure is also independent of B cells and antibody production (19).

The alternative pathway components factor B, properdin, and factor D are involved in self-assembly and proteolytic steps that result in the generation of a highly effective C3 convertase enzyme consisting of C3bBbC3b that is stabilized by properdin (20). Activation of the alternative pathway is usually initiated after encounter with bacteria, parasites, viruses, or fungi, IgA Abs, and certain IgL chains (21–23). IgG1 antibody responses to T cell-dependent antigens and sensitivity to endotoxic shock appear normal in *fB*^{-/-} mice (13). Indeed, after sensitization with allergen these mice displayed similar levels of allergen-specific IgE and IgG antibodies compared with the WT mice, indicating that the humoral response to allergen sensitization and challenge remained intact in these mice. On the other hand, *fB*^{-/-} mice showed a marked inability to develop AHR after sensitization and challenge. Furthermore, the influx of inflammatory cells into the airways, inflammatory infiltrates in lung tissue, goblet cell hyperplasia, and T helper 2 cytokine levels in the BAL fluid were decreased in these *fB*^{-/-} mice. These results imply that activation of the alternative pathway is critical for the development of most of the components of allergic airway disease, especially those in the lung. The failure to fully develop AHR and airway inflammation was not caused by a specific unresponsiveness restricted to a single allergen such as OVA. Sensitization and challenge with ragweed similarly failed to induce AHR and airway inflammation in *fB*^{-/-} mice while inducing allergic airway disease in *fB*^{+/+} mice. More likely, inhibition of complement activation through the alternative pathway led to a reduction in inflammatory cells, especially T cell migration into the lung (7), which resulted in reduced levels of IL-4, IL-5, and IL-13 in the lung, cytokines that have been directly linked to the development of AHR, airway inflammation, and goblet cell hyperplasia (14, 24–28). The absence of the generation of C3a and C5a as a consequence of factor B deficiency and their effects on other cell types including eosinophils and airway smooth muscle cells, which are involved in the development of AHR, also could have contributed to the failure to develop airway dysfunction and limited airway inflammation (29–33).

To define whether it is local complement activation in the lung in response to allergen challenge that is critical for the development of airway disease, we administered (intranasally) purified factor B to *fB*^{-/-} mice after sensitization but before

challenge. Administration of 10 μ g of factor B before each challenge was sufficient to fully reconstitute the development of AHR and eosinophil influx into the airways of sensitized *fB*^{-/-} mice. If factor B was given before airway challenge but without previous sensitization of the recipient, AHR or airway inflammatory responses were not observed, indicating that the sensitization phase was needed for the responses to develop on challenge and demonstrating that the sensitization phase in the *fB*^{-/-} mice was indeed intact. These data highlight that local activation of the complement cascade in the lung in a sensitized host is critical to the development of allergic airway disease, as has been suggested in previous studies (7), and defines the unique importance of the alternative pathway.

To further establish a role for activation of the alternative pathway in the development of allergic airway disease, we determined whether inhibition of the alternative pathway is effective in normal mice, using a specific inhibitor of factor B. This mAb binds to the third short consensus repeat domain of factor B and leads to complete inhibition of the activation of the alternative pathway *in vitro* and *in vivo* (34). This antibody also has been shown to effectively protect mice from antiphospholipid-induced fetal injury (34). In the present study, the antibody was administered either systemically or locally into the lung by nebulization, which has been shown to be an effective route for administration of other complement inhibitors (7). Indeed, C57BL/6 mice treated after sensitization but during the challenge phase with either systemic or local anti-fB showed a significant decrease in AHR and an inhibition of airway inflammation and eosinophils in the airways and the lung tissue. In addition, the number of goblet cell was reduced. These results are similar to studies that used complement inhibitors that affect both the classical and alternative pathways and block the development of a late airway response (6) and AHR (7).

The mechanism of activation of the alternative pathway after allergen exposure is unclear. The alternative pathway can be activated on the surface of pathogens that have neutral- or positive-charge characteristics and do not express or contain complement inhibitors. This phenomenon is caused by a process termed “tickover” of C3 that occurs spontaneously, involves the interaction of conformationally altered C3 with factor B, and results in the fixation of active C3b on pathogens or other surfaces (20). Further potential pathways for activation include antibodies that block endogenous regulatory mechanisms (35), reduction (36–40), or dysfunction (41, 42) of regulatory proteins. In addition, the alternative pathway is activated by a mechanism, the “amplification loop,” when C3b that is deposited onto targets via the classical or lectin pathways then binds factor B (20). Interestingly, there have been several recent reports showing a critical role for the alternative pathway in different models of antibody-mediated disease that have previously been associated with classical pathway activation (43–47). In allergic airway disease it has been proposed that antigenic epitopes on the surface of the allergen might directly activate the alternative pathway (9). However, at this time further studies are needed to identify the mechanism(s) underlying alternative pathway activation after allergen challenge of sensitized mice.

In summary, based on the data in *C4*^{-/-} mice, *fB*^{-/-} mice, reconstitution experiments with factor B, and administration of anti-fB, we demonstrate a critical role for factor B and the alternative pathway in the allergen-induced development of AHR, T helper 2 responses in the lung, lung eosinophilia, and goblet cell metaplasia after allergen exposure of the sensitized host. The fact that treatment with a specific inhibitor of an essential component for alternative pathway activation is effective in reducing AHR and airway inflammation, even after sensitization, suggests that targeting the alternative complement pathway might be a potent therapeutic target for patients with allergic asthma and established disease.

Materials and Methods

Animals. Female C57BL/6 mice, 8–12 weeks of age, were obtained from The Jackson Laboratory. As described, factor B heterozygote-deficient mice ($fB+/-$) were intercrossed and then backcrossed for seven generations with C57BL/6 mice to generate an $fB-/-$ strain (13). As a control, mice congenic $fB+/+$ littermates were used. $C4-/-$ mice ($C4-/-$ backcrossed for 17 generations with C57BL/6 mice) (12, 17) were maintained in the animal facility. All experimental animals used in this study were maintained on OVA-free diets and were under a protocol approved by the Institutional Animal Care and Use Committee of the National Jewish Medical and Research Center.

Experimental Protocol. Mice were sensitized by i.p. injection of 20 μ g of OVA (Grade V; Sigma) or ragweed (*Ambrosia artemisiifolia*; Greer Laboratories, Lenoir, NC) suspended in 2.25 mg of aluminum hydroxide (Alum Imject; Pierce) on days 1 and 14 and then challenged via the airways, using nebulized OVA or ragweed (1% in saline), with an ultrasonic nebulizer (DeVilbiss Health Care, Somerset, PA) for 20 min daily on days 27, 28, and 29.

For reconstitution of factor B, 10 μ g of purified factor B (50 μ l in PBS) was administered by intranasal application 1 h before each airway challenge to nonsensitized and sensitized $fB-/-$ mice. This dose was found to be most effective in dose–response experiments. As a control PBS was administered.

In a different study, 2 h before each OVA challenge an inhibitory anti- fB mAb was administered to sensitized mice either by i.p. injection (2 mg per treatment per mouse) or nebulization. For nebulization, four mice were placed in a Plexiglas box, and 0.5 mg of anti- fB (in 5 ml of PBS) was nebulized by using an ultrasonic nebulizer (DeVilbiss Health Care). As a control mouse IgG at the same dose and volume was injected i.p. or nebulized at the same time points. On day 31, AHR was assessed, and animals were killed the same day for the collection of BAL fluid, blood, and lung tissue.

Purification of Factor B. To reconstitute alternative pathway activity in $fB-/-$ mice, mouse complement factor B was purified from normal mouse serum by affinity purification (34). For additional materials and methods see *Supporting Text*, which is published as supporting information on the PNAS web site.

Generation of Anti- fB Antibody. Anti-mouse factor B mAbs were produced as described (34). For additional materials and methods see *Supporting Text*.

Determination of Airway Function. Airway responsiveness was assessed (in a blinded fashion) as a change in airway function after challenge with aerosolized MCh administered for 10 s (60 breaths per min, 500 μ l of tidal volume) in increasing concentrations (6.25, 12.5, 25, 50, and 100 mg/ml). Anesthetized (pentobarbital sodium, i.p., 70–90 mg/kg), tracheostomized (18G cannula) mice were mechanically ventilated (160 breaths per min, tidal volume of 150 μ l, positive end-expiratory pressure of 2–4 cm H_2O), and lung function was assessed (48). Airway resistance (RL) was continuously computed (LABVIEW, National Instruments, Austin, TX) by fitting flow, volume, and pressure

to an equation of motion. Maximum values of RL were taken and expressed as a percentage change from baseline after PBS aerosol.

BAL and Measurement of Cytokines and C3a desArg. After assessment of airway function, lungs were lavaged via the tracheal tube with Hank's balanced salt solution (1×1 ml, $37^\circ C$). The number of BAL cells was counted by using a cell counter (Coulter). Differential cell counts were made from cytocentrifuged preparations, and percentage and absolute numbers of each cell type were calculated. Cytokine levels were assessed by ELISA in BAL fluid (16). IFN- γ , IL-4, IL-5, IL-12 (all Pharmingen), and IL-13 (R & D Systems) ELISAs were performed according to the manufacturers' directions.

Levels of C3a desArg in BAL fluid were measured in non-sensitized and sensitized mice at 24 h after the first or second allergen challenge and at 24 and 48 h after the third and final challenge by ELISA, following the manufacturer's directions (Cedarlane Laboratories).

Histologic and Immunohistochemistry Studies. After obtaining BAL fluid, lungs were inflated through the trachea with 2 ml of 10% formalin and then fixed in the same solution by immersion. Tissue sections were stained with hematoxylin and eosin, periodic acid/Schiff reagent, and immunohistochemically for cells containing eosinophilic major basic protein (MBP), using a rabbit anti-mouse MBP antibody (provided by J. J. Lee, Mayo Clinic, Scottsdale, AZ) (49). Slides were examined in a blinded fashion, and numbers of eosinophils in the peribronchial tissue and goblet cells were analyzed separately by using SCION IMAGE software (version 1.62, developed at the National Institutes of Health, Bethesda and available at <http://rsb.info.nih.gov/nih-image>).

Immunofluorescence Studies. For additional materials and methods see *Supporting Text*.

Measurement of Total IgE and OVA-Specific Antibodies. Serum levels of total IgE and OVA-specific IgE and IgG1 were measured by ELISA as described (18). The OVA-specific antibody titers of samples were related to internal pooled standards, which were arbitrarily assigned to be 500 ELISA units. The total IgE level was calculated by comparison with a known mouse IgE standard (Pharmingen).

Statistical Analysis. ANOVA was used to determine the levels of difference between all groups. Comparisons for all pairs was performed by the Tukey-Kramer honest significant difference test. *P* values for significance were set at 0.05. Values for all measurements were expressed as the mean \pm SEM.

We thank L. N. Cunningham and D. Nabighian (National Jewish Medical and Research Center) for their assistance and Dr. J. J. Lee for providing the anti-major basic protein antibody. This work was supported by National Institutes of Health Grants HL-36577 and HL-61005 (to E.W.G.), Environmental Protection Agency Grant R825702 (to E.W.G.), National Institutes of Health Grant AI-31105 (to V.M.H.), National Institutes of Health Grant K08 DK064790-01 (to J.M.T.), and Deutsche Forschungsgemeinschaft Grant Ta 275/2-1 (to C.T.).

1. Humbles, A. A., Lu, B., Nilsson, C. A., Lilly, C., Israel, E., Fujiwara, Y., Gerard, N. P., & Gerard, C. (2000) *Nature* **406**, 998–1001.
2. Krug, N., Tschernig, T., Erpenbeck, V. J., Hohlfeld, J. M., & Kohl, J. (2001) *Am. J. Respir. Crit. Care Med.* **164**, 1841–1843.
3. Drouin, S. M., Corry, D. B., Hollman, T. J., Kildsgaard, J., & Wetsel, R. A. (2002) *J. Immunol.* **169**, 5926–5933.
4. Bautsch, W., Hoymann, H. G., Zhang, Q., Meier-Wiedenbach, I., Raschke, U., Ames, R. S., Sohns, B., Flemme, N., Meyer zu Vilsendorf, A., Grove, M., et al. (2000) *J. Immunol.* **165**, 5401–5405.
5. Walters, D. M., Breyse, P. N., Schofield, B., & Wills-Karp, M. (2002) *Am. J. Respir. Cell Mol. Biol.* **27**, 413–418.
6. Abe, M., Shibata, K., Akatsu, H., Shimizu, N., Sakata, N., Katsuragi, T., & Okada, H. (2001) *J. Immunol.* **167**, 4651–4660.
7. Taube, C., Rha, Y. H., Takeda, K., Park, J. W., Joetham, A., Balhorn, A., Dakhama, A., Giclas, P. C., Hokers, V. M., & Gelfand, E. W. (2003) *Am. J. Respir. Crit. Care Med.* **168**, 1333–1341.
8. Sahu, A., & Lambris, J. D. (2001) *Immunol. Rev.* **180**, 35–48.
9. Kohl, J. (2001) *Mol. Immunol.* **38**, 175–187.

