Cutting Edge: Guinea Pigs with a Natural C3a-Receptor Defect Exhibit Decreased Bronchoconstriction in Allergic Airway Disease: Evidence for an Involvement of the C3a Anaphylatoxin in the Pathogenesis of Asthma

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References

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Asthma is a major health problem worldwide. The prevalence and severity have been increasing at an alarming rate in recent decades; up to 25% of children under the age of 14 years have asthma (1). The disease is triggered by Ag inhalation and characterized by an early phase of bronchoconstriction, followed by a late-phase airway hyperresponsiveness and excessive mucus production associated with the appearance of eosinophils and T_{H2} lymphocytes in the airways. The early airway response is associated with cross-linking of IgE bound to FcεRI on mast cells inducing degranulation (2). Thus, IgE is considered to be a major trigger of asthma, a view recently supported by the therapeutic value of a humanized mouse anti-IgE mAb (3). However, other mechanisms also contribute to the disease as shown by the strong association of asthma with several genetic susceptibility genes in the human genome, some of which seem to act independently of serum IgE or other measures of atopy (4, 5). The limited knowledge of the pathophysiology of asthma hampers the development of more effective therapies for this disease.

Certain important features of asthma can be modeled in mice, rats, and guinea pigs. OVA-sensitized animals challenged with aerosolized allergen react with an immediate, early-phase bronchoconstriction associated with histamine release and peptidoleukotriene secretion from mast cells, and a late-phase bronchial hyperreactivity associated with a high eosinophil content in the lung, although the molecular details of this association are not well understood. Using such models, allergic airway disease could be demonstrated in B cell deficient mice, clearly showing that non-IgE related mechanisms are operative in these animal models, too (6).

The C3a anaphylatoxin is a potent proinflammatory mediator that is generated in the early phase of an inflammatory reaction by proteolytic cleavage of the complement component C3 (7). It promotes multifarious in vitro reactions that include bronchoconstriction (8, 9), edema formation, as well as attraction and activation of mast cells (10, 11) and eosinophils (12, 13); but it has no effect on neutrophil chemotaxis. C3a binds to a single ligand-specific receptor, the C3aR, which has only recently been cloned from several species including man, mouse, rat, and guinea pig (14–19). The high potency, the generation right at the beginning of an inflammatory reaction and the characteristic activity pattern suggest a central involvement of C3a in the pathophysiology of allergic disease states, like asthma. Indeed, C3a levels are increased in the...
bronchoalveolar lavage fluid of asthma patients (20) where they could be generated by two different routes: immune complex formation involving Abs of the IgM/IgG class (but not IgE), or proteolytic cleavage of complement component C3 by the neutral trypsin, the major protease present in mast cell granules (21). But experimental evidence for such an involvement has not yet been obtained due to the absence of well-characterized C3aR-defective animal models or potent receptor antagonists.

Guinea pigs have been widely used to analyze the early bronchoconstriction phase following allergen inhalation, as these animals are exquisitely sensitive to airway Ag challenge; indeed, an antihistamine has to be administered before allergen challenge to prevent acute fatality. Consequently, these animals have also been used to analyze Ca2+-induced bronchoconstriction, which can be antagonized by cyclooxygenase inhibitors but not antihistamines (8) similar to the situation in humans. Although an inbred guinea pig strain, called C2BB/R, has been described that is nonresponsive to C3a in platelet-based assays (22), the molecular nature of this defect remained obscure and few animal studies have been performed with this strain (23).

Materials and Methods

Materials

Guinea pigs of the inbred strains of C3aR-positive C2BB/R+ and C2BB/R− were obtained from our own breeding stocks and kept in closed colonies. Human C3a was obtained from Advanced Research Technologies (San Diego, CA), and 32P-labeled C3a from NEN (Boston, MA). Human embryonic kidney cells (HEK)293 were cultured as described elsewhere (24).

Molecular analysis of the C3aR locus in strain C2BB/R−

The coding region of the C3aR was amplified from guinea pig genomic DNA by PCR using oligonucleotide primers P1 (5′-GATACCATGTCAGTTGACGTGTTGAGTGTTGCTTGGGACACCTTG-3′, position +5 to +87) and P2 (5′-CTTTGGTGGCTCAACTTGGTACCCATTGC-3′, position +1546 to +1538), and both strands were sequenced. For demonstration of the presence/absence of the point mutation at position +955 within the C3aR coding sequence, a 1152-bp fragment was amplified from genomic DNA by PCR using oligonucleotide primers P3 (5′-GATACCATGTCAGTTGACGTGTTGAGTGTTGCTTGGGACACCTTG-3′, position +1528) and P4 (5′-CCACGAGATAAAAGATGATGCTCACATTTAGTTGGACACCTTGACACCCAAGA-3′, position +1143 to +1124). The fragment was digested with restriction endonucleases SspI and XbaI (New England Biolabs, Beverly, MA) according to the manufacturer’s instructions, and the fragments were visualized after agarose gel electrophoresis.

A polyclonal antiserum against the guinea pig C3aR was obtained by immunizing rabbits with the second extracellular loop of the C3aR fused to glutathione-S-transferase, following the procedures described previously (25). A 1:500 to 1:1000 dilution of the immune serum specifically recognized the guinea pig C3aR (but not the human C5aR) in transiently transfected HEK 293 cells by indirect immunofluorescence or flow cytometry (data not shown). The C→G transition was introduced into position +955 of the guinea pig C3αR sequence by PCR-mediated site-directed mutagenesis, as described previously (24). Flow cytometry and binding assays were performed essentially as described elsewhere (24).

Sensitization and in vivo allergen challenge of guinea pigs

Animals of both sexes with an average weight of 600 ± 50 g (C2BB/R+; 618.4 ± 47.9 g, range 540–680 g, 7 female and 7 male; C2BB/R−; 596.6 ± 54.8 g, range 503–709 g, 13 female and 7 male; at the day of allergen challenge) were used for the experiments. Guinea pigs were sensitized with an i.p. injection of OVA (10 μg plus 20 mg aluminum hydroxide in 0.5 ml saline) on day 0 and 7, followed by an inhalational boost on day 21, and the challenge on day 35. Thirty minutes before inhalational boost/allergen challenge, animals were given 5 mg/kg pyrilamine i.p. in saline. Lung function parameters were measured in four animals simultaneously (26) in whole-body double-chamber plethysmographs equipped with two pneumotachographs and two Validyne DP45–14 transducers each, using a modified method according to Penkoc et al. (27) by means of a Buxco LS-20 system (Buxco Electronics, Sharon, CT). The OVA booster and challenge inhalations (1% in saline) were performed by means of the aerosol generation system Bronchly Type III (developed by the Fraunhofer Institute of Toxicology and Aerosol Research Hannover, Germany; Refs. 26 and 28). Mass median aerodynamic diameter (geometric SD) of OVA was 1.2 (2.5) μm. Exposures were performed under continuous monitoring of the inhalation atmosphere using a gravimetrically calibrated air manule aerosol photometer. After a 5-min adaptation, baseline (3 min), allergen challenge (8 min), and postchallenge values (12 min) of respiratory rate, tidal volume, and specific airway resistance (SRAW) were recorded in spontaneously breathing awake animals. Results are expressed as percent increase above baseline averaged over a 20-min interval period (challenge plus post-challenge) and related to baseline. Therefore, the averaged values were much lower than the respective maximum values but were nearly independent of artifacts and more representative for the early allergic response.

Histocchemistry, eosinophil peroxidase (EPO) measurement and Ab titer

Six hours later, the animals were killed by exsanguination and the pulmonary arteries were flushed with saline containing 10 mM EDTA. The right lobe of the lung was removed, chopped, and processed immediately to determine eosinophil numbers in the lung tissue using the EPO assay as described elsewhere (29). For histocchemistry, the left lobe was either frozen in liquid nitrogen and stored at −70°C, or fixed in formalin. Cryostat sections (7.0 μm) or 2.0 μm sections of paraffin-embedded tissue were stained with hematoxylin-eosin according to standard techniques.

\[
\begin{align*}
\text{C3aR} & \quad \text{ThrHisGluTyrArgThrProArg...} \\
(1+1 \to +942) & \quad \text{ACACATGAAATGCGACACCCAAGA...} \\
\text{SspI} & \quad \text{ThrHisGluTyr...} \\
(1+1 \to +942) & \quad \text{ACACATGAAATGCGACACCCAAGA...} \\
\text{XbaI} & \quad \text{ThrHisGluTyr...} \\
\end{align*}
\]

\[\text{FIGURE 1. A point mutation in the C3aR coding region of strain C2BB/R−. Upper, DNA and protein sequence of wild-type and mutant guinea pig C3aR. The C→T transition is indicated by an arrow; the novel SspI restriction site generated by this exchange is given in italics. Lower, Demonstration of the SspI restriction site in the C3aR coding sequence of strain C2BB/R−, but not C2BB/R+. A 1152-bp PCR fragment of the C3aR coding sequence was amplified from genomic DNA of strain C2BB/R− (lanes 1–3), C2BB/R+ (lanes 4–6), or a plasmid containing the wild-type C3aR (lanes 7–9) and separated by gel electrophoresis either undigested (lanes 1, 4, 7) or digested with SspI (lanes 2, 5, and 8) or XbaI (lanes 3, 6, and 9). The SspI site in strain C2BB/R− is revealed by the size reduction of the PCR fragment (lane 5) while the released 200-bp fragment has diffused of the gel and is no longer visible. S, Size marker.}\]
IgG Ab titers of each animal were determined by ELISA using OVA-coated 96-well plates and goat anti-guinea pig IgG (1:10,000 dilution; Dianova, Hamburg, Germany) according to standard procedures. A threshold OD of 0.15 (2.5-fold buffer value; 1/6 of the total signal) was used for determination of the titer. Results are presented as group mean ± SEM. Untransformed data were analyzed using the Wilcoxon two-sample test (U test of Mann/Whitney); \( p < 0.05 \) was considered statistically significant.

Results and Discussion

**Strain C2BB/R\(^2\) carries a point mutation within the C3aR gene locus**

To elucidate the contribution of the C3a anaphylatoxin to the development of asthma, we first analyzed the genetic defect in the phenotypically C2BB/R\(^2\) inbred guinea pig strain C2BB/R\(^2\) at a molecular level. Previously, we had cloned the coding sequence of the guinea pig C3aR from a genomic DNA library (18). Using primers directed against DNA sequences flanking the C3aR locus, the complete C3aR coding sequence was amplified and sequenced from genomic DNA of strain C2BB/R\(^2\). As shown in Fig. 1A, the C2BB/R\(^2\) strain of guinea pigs harbors a single C→T transition in position +955 of the C3aR that changes a CGA arginine codon into a TGA stop codon. This mutation introduces within the C3aR open reading frame a novel SspI endonuclease restriction site. As shown in Fig. 1B, this provides a convenient genetic assay to screen for the underlying genotype.

More than 40 animals each of strains C2BB/R\(^-\) and C2BB/R\(^+\), the corresponding wild-type strain (22), have been screened for the presence or absence of this restriction site; genotype and phenotype were in complete agreement in all cases. This same test was used to confirm the genetic identity of a given animal in the experimental allergic asthma model described below.

The mutation effectively inactivates the C3aR in strain C2BB/R\(^-\)

The C3aR is a single-copy gene that exhibits a unique feature among all G-protein coupled receptors: an unusually large second extracellular loop of \(>150\) residues. The mutation found in the C3aR coding region of strain C2BB/R\(^-\) is located at the C-terminal end of this loop shortly before the beginning of the transmembrane helix V, thus effectively deleting ~1/3 of the receptor. This would easily explain the complete unresponsiveness of this strain in the platelet C3a-mediated ATP release assay, an exquisitely sensitive and specific assay for anaphylatoxins (18). Interestingly, in guinea pigs, a second C3aR sequence has been described that lacks 35 amino acids in the second extracellular loop. However, this C3aR variant behaved identically with the wild-type receptor with respect to ligand binding and signal transduction properties (19). The mutation...
found in strain C2BB/R− is located just C-terminal to and thus outside the proposed splicing region. Therefore, both putative C3aR isoforms should be affected in the C2BB/R− strain.

To confirm the deleterious consequences of a deletion of the distal 1/3 in the C3aR, we introduced the same point mutation into the wild-type sequence of the guinea pig C3aR by site-directed mutagenesis, and functionally characterized both the wild-type and mutant genes after transient transfection into human HEK 293 cells. Using a polyclonal rabbit antiserum directed against the large second extracellular loop, a residual minor expression of the mutant C3aR on the cell surface was found by flow cytometry (Fig. 2A). However, no binding of radioiodinated C3a could be detected (Fig. 2B), confirming that this stop codon effectively inactivates the receptor. These data show that this guinea pig strain is a good model to analyze C3aR functions in vivo.

Strain C2BB/R− exhibits decreased airways resistance in an OVA-induced asthma model

To analyze the pathophysiological function of C3a in an allergic asthma model, animals of strains C2BB/R+ (wild-type C3aR) and C2BB/R− were sensitized with OVA by intraperitoneal and inhalational immunization. Two weeks after the final boost, lung function parameters of spontaneously breathing animals in response to aerosolized OVA were recorded by whole-body plethysmography.

Both animal groups responded with a bronchoconstriction following OVA antigen inhalation. However, the increase in SRAW was significantly lower by ~30% in the C2BB/R− strain when compared with strain C2BB/R+ harboring a functional C3aR (Fig. 3). This result is also reflected in the respiratory rate, which compensatorily increased in both groups, but which was significantly more pronounced in C2BB/R− animals (39.7% ± 9.2; mean ± SEM) than in the C3aR-negative animals (14.7% ± 21.8; p < 0.01; data not shown). Also, the prechallenge respiratory rate was slightly higher in the C3aR-negative group (142.3 ± 15.1 vs 121.6 ± 9.7 in C2BB/R−, p < 0.01) indicating that these animals took up even slightly more Ag than the C2BB/R+ group; all other lung function parameters were identical among both groups.

Recently, C3aR expression has been reported on stimulated B- and T-lymphocytes (30, 31) where it could be involved in the regulation of Ab generation. To exclude the possibility that the observed difference between the two guinea pig strains is simply due to differences in the complement activating Ig titers, we determined the serum IgG titers of the animals 6 h after allergen challenge; no difference was observed between the groups (C2BB/R+: 2314 ± 4148, n = 7; C2BB/R−: 3671 ± 4644, n = 8), while very little or no IgM could be detected. The same result was obtained with another group of animals where higher Ag concentrations had been used for immunization (3 × 2 mg/kg i.p. at 2-wk intervals; n = 6) (data not shown). Three animals in our experimental series, all of the C2BB/R− strain, did not mount a detectable IgG response at all; however, they all exhibited a comparatively strong bronchoconstriction reaction on allergen challenge (data not shown), indicating that other as yet unknown genetic factors contribute to the bronchoconstriction response, and may even predominate under certain circumstances.

The eosinophil influx in strain C2BB/R− is not decreased 6 h after allergen challenge

The late response in asthma is characterized by an influx of cells, predominately eosinophils, into the lung tissue (strikingly increased already 6 h after allergen challenge) and bronchiolar lavage fluid (after ≥ 24 h) (29). Because C3a is a known eosinophil

FIGURE 4. Peribronchiolar infiltration of eosinophils 6 h after allergen challenge in strain C2BB/R− (a) and C2BB/R+ (b). (hematoxylin and eosin stain)
chemoattractant (12, 13), we analyzed lung sections 6 h after allergen challenge. A strong eosinophil influx into the lung was observed in both animal strains, which was most prominent around the bronchioli (Fig. 4). As a quantitative measure, we determined the EPO content in lung homogenates. No significant difference between the two animal groups was detected at this time point. We even observed a trend toward a higher EPO content in the C3aR−negatives animals (data not shown), which might be explained, however, by the slightly higher Ag uptake in this group (see above).

This result was not completely unexpected, as it is known that C3a alone can only promote adherence of eosinophils to the capillary wall but not their transmigration into the lung tissue (13), but requires other eosinophil chemoattractants, such as eotaxin and IL-5. The concentration of these mediators also raises quickly after allergen challenge (29, 32), parallel to the influx of eosinophils.

In summary, we describe a new animal strain to study the pathophysiological role of the C3a/C3aR system in vivo, i.e., an inbred guinea pig strain with a natural C3aR defect. In a model of OVA-induced allergic airway disease a reduced bronchoconstriction in the defective strain was observed, providing the first experimental evidence of a pathophysiological role of the C3a anaphylatoxin in vivo. While this paper was under consideration, a similar observation was reported by Humbles et al. (33) using a genetically engineered C3aR knockout mouse strain. These authors also observed an effect on bronchoconstriction (reduced late-phase airway hyperresponsiveness) in an OVA-induced asthma model, but no evidence of a pathophysiological role of the C3a anaphylatoxin in vivo. While this paper was under consideration, a similar observation was reported by Humbles et al. (33) using a genetically engineered C3aR knockout mouse strain. These authors also observed an effect on bronchoconstriction (reduced late-phase airway hyperresponsiveness) in an OVA-induced asthma model, but no effect on eosinophil influx, in full agreement with our report. Taken together, these data underscore the importance of the complement-derived anaphylatoxins as proinflammatory mediators in disease pathogenesis and suggest a novel target for drug intervention strategies in human asthma.

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References


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References