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***Aspergillus fumigatus* synergistically enhances mite-induced allergic airway inflammation**

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Summary

Background:

Infection is an important trigger of exacerbation of bronchial asthma. The fact that *Aspergillus fumigatus* (*Af*) causes a distinct clinical syndrome, allergic bronchopulmonary aspergillosis (ABPA), suggests unique immunological properties in allergic asthma. The present study aimed to determine how *Af* enhances preexisting allergic airway inflammation and colonizes the airway in asthma.

Material/Methods:

Six groups of BALB/c mice were prepared: Control; live or dead *Af*infected (Live *Af* or Dead *Af*); *Dermatophagoides farinae* (*Df*) allergen-sensitized (*Df*); and *Df*sensitized plus live or dead *Af*infected (*Df*live *Af* or *Df*dead *Af*). Pulmonary pathology, cytokine profiles and mucous production in the airway were evaluated in these groups.

Results:

Af infection significantly enhanced *Df* allergen-induced eosinophilic inflammation via enhancement of Th2-like response. Live, but not dead, *Af* significantly exacerbated neutrophilic inflammation. Induction of IL-13 and Muc5ac proteins by *Df* allergen sensitization was significantly enhanced by both live and dead *Af* infection, resulting in mucous hypersecretion.

Conclusions:

Collectively, the present study showed that mite allergen sensitization concomitant with *Af* infection enhanced Th2-dominant immune response in the airway, which induced mucous hypersecretion and potentially permitted further colonization by *Af* spores. It is likely that *Af* enhances allergic airway inflammation as an allergen, while it enhances neutrophilic airway inflammation as a pathogen. Future studies on pharmacological intervention in the present murine model utilizing glucocorticoid, and anti-fungal drugs are thus promising.

key words:

***Aspergillus fumigates* • bronchial asthma • *Dermatophagoides farinae* • allergic bronchopulmonary aspergillosis (ABPA)**

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BACKGROUND

Bronchial asthma is an immune-mediated inflammatory disease of the airway, and various inflammatory cells and mediators are involved in its development, maintenance, and exacerbation [1]. Naïve CD4⁺ helper T cells (Th0) differentiate into Th1 (producing IFN- γ and IL-2) and Th2 (producing IL-4 and IL-5) phenotypes [2]. Th1 and Th2 cells counterbalance one another by producing cytokines, and imbalance between Th1 and Th2 cells is an important aspect of the pathophysiology of asthma, in which Th2 cells are dominant [3–7].

Respiratory tract infections, as well as mite allergen sensitization, represent an important risk factor in the development of asthma [6]. In addition, respiratory tract infections are the most frequent cause of asthma exacerbation in both children and adults [7–10]. Although the precise mechanisms of infection-induced asthma exacerbation remain unknown, respiratory infection may enhance Th2-dominant immune responses in the asthmatic airway [11]. Among respiratory pathogens, viruses and atypical pathogens had been exclusively investigated in the development and exacerbation of asthma [10–16]. These pathogens can infect the cytoplasm of respiratory cells in both healthy and asthmatic subjects.

Similarly to viruses and atypical pathogens, *Aspergillus fumigatus* (Af), a ubiquitous fungus, is considered to be an important risk factor in the development and exacerbation of asthma [17]. In marked contrast, Af spores are easily cleared from normal lungs by innate immunity, involving alveolar macrophages [18], and Af typically infects the airway of immuno-compromised neutropenic hosts. However, in a subset of asthmatics, particularly in patients with allergic bronchopulmonary aspergillosis (ABPA), Af spores colonize the bronchial mucous. It is unclear why Af spores are able to colonize the bronchial mucous of ABPA patients, who are not immuno-compromised hosts.

The mouse is an excellent model for studying interactions between Af infection and mite allergen sensitization [19]. Many previous reports used a model that was first sensitized and then challenged with Af protein [19,20]. However, many atopic asthma patients are sensitized to common aeroallergens other than Af, such as mites. It is therefore likely that patients are first sensitized to such aeroallergens *in utero* or in early life, and are then exposed to Af after the establishment of allergic airway inflammation by the mite allergen. Thus, in the present study, preexisting allergic airway inflammation was first developed through the conventional protocol utilizing *Dermatophagoides farinae* (Df) allergen, followed by live and infectable Af exposure. The present study aimed to determine how Af enhances preexisting allergic airway inflammation and colonizes the airway in asthma.

MATERIAL AND METHODS

Preparation of Af conidia

A. fumigatus MF-13 [21], which was isolated from the sputum of a patient with pulmonary aspergilloma, was subcultured on Sabouraud dextrose agar (Becton Dickinson, Cockeysville, MD) at 30°C for 7 days. The conidia were then harvested with sterile saline containing 0.02% Tween 80 (Wako Pure

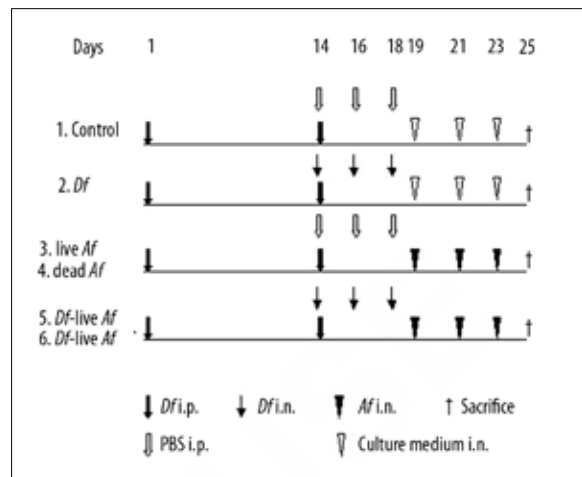


Figure 1. Experimental design. BALB/c mice were each immunized twice intraperitoneally on days 1 and 14 with 0.5 mg of Df precipitated in aluminum hydroxide. Control group: Mice were sham sensitized intranasally (i.n.) with PBS on days 14, 16 and 18 and sham infected i.n. with culture media on days 19, 21 and 23. Df group: After immunization with Df, mice were challenged intranasally (i.n.) with 50 μ g/50 μ L Df allergen on days 14, 16 and 18. Live Af group: 5×10^6 live Af conidia were administered i.n. on days 19, 21 and 23. Dead Af group: 5×10^6 dead Af conidia were administered i.n. on days 19, 21 and 23. Df-live Af group: live Af were administered after Df immunization and Df challenge. Df-dead Af group: dead Af were administered after Df immunization and Df challenge. On day 25, all mice were sacrificed.

Chemical Industries, Tokyo, Japan), counted in a hemocytometer, and diluted with sterile saline for intranasal infection [21]. Dead conidia were prepared by autoclaving live conidia.

Mice

Six groups of mice were prepared in the present study (Figure 1). Four-week-old female BALB/c mice (Charles River Japan, Inc., Yokohama, Japan) were each immunized twice intraperitoneally on days 1 and 14 with 0.5 mg of Df (crude extract of mite: LG-5339; Cosmo Bio, Tokyo, Japan) precipitated in aluminum hydroxide. These mice were then challenged intranasally (i.n.) with 50 μ g/50 μ L Df allergen (crude extract of mite) on days 14, 16 and 18 (Df group) [22]. To the live Af group, 5×10^6 live Af conidia were administered i.n. on days 19, 21 and 23, and the effects of dead Af conidia were similarly evaluated in the dead Af group. The other two groups were the Df-live Af group and Df-dead Af group, which were administered live or dead Af after Df sensitization and Df challenge. Control groups were challenged with PBS after Df sensitization and were then sham infected i.n. with culture media. On day 25, all mice were sacrificed. Bronchoalveolar lavage (BAL) samples and lung tissues were obtained from each group. All procedures were reviewed and approved by the Nagasaki University School of Medicine Committee on Animal Research. All experiments were repeated at least three times.

Bronchoalveolar lavage and lung pathology

Twenty-four hours after final Af conidia or sham infection, mice were sacrificed and BAL was conducted with 1 mL of

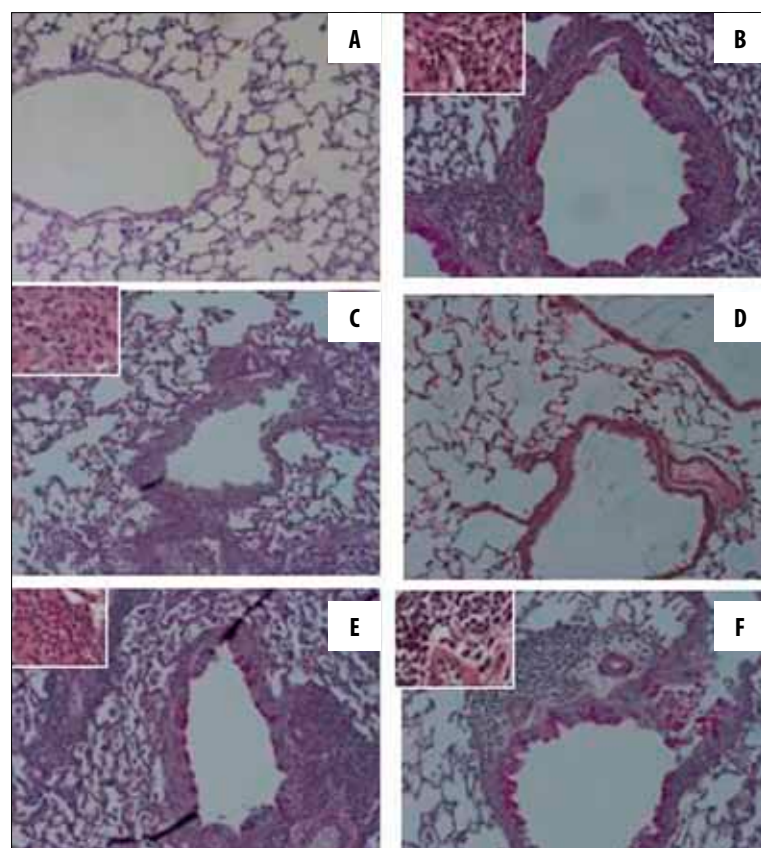


Figure 2. Pulmonary pathologies. Lung tissues were obtained from each group and stained with PAS and HE. Representative PAS stained photomicrographs accompanied by small HE stained photomicrographs from each group (n=6 for each) are shown. (A) Control, (B) *Df* (*Df* allergen-sensitized), (C) Live *Af* (live *Af*-infected), (D) Dead *Af* (dead *Af*-infected), (E) *Df*-live *Af* (*Df*-sensitized plus live *Af*-infected), (F) *Df*-dead *Af* (*Df*-sensitized plus dead *Af*-infected).

PBS in the immediate postmortem period. The obtained BAL samples were centrifuged. Differential cell counts were performed using cytocentrifuged BAL samples stained with May-Grünwald-Giemsa. Formaldehyde fixative was gently infused through the lavage catheter set in the trachea. Resected lungs were fixed for an additional 24 h and embedded in paraffin. Sections (4 μ m) were stained with hematoxylin and eosin (HE), and with periodic acid-Schiff (PAS). Gomori methanamine silver staining was also performed in order to identify *Af* in the lung tissues; fungal spores were confirmed in live *Af* and *Df*-live *Af* mice (data not shown).

Analysis of cytokines concentrations in homogenized lung

Lung homogenates were prepared by homogenizing a freshly excised lung. Concentrations of Th2 cytokines (IL-5 and IL-13), MIP-2 (murine cytokine corresponding to human IL-8) and IFN- γ , a Th1 cytokine, in homogenized lung samples were measured by enzyme-linked immunosorbent assay, according to the manufacturer's directions (Endogen, Woburn, MA). Detection limits for IL-5, IL-13, MIP-2 and IFN- γ were 5, 1.5, 1.5 and 10 pg/mL, respectively.

Western blot analyses for Muc5ac

Western blot analyses for murine Muc5ac were performed as described elsewhere [23]. Lung homogenates were prepared by homogenizing a freshly excised lung. For western blot analysis, proteins in the supernatant of lung homogenates were separated by polyacrylamide gel electrophoresis and were transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). One

hundred micrograms of protein were used for each sample, except for the stomach sample, which contained 20 μ g of protein. Membranes were then blocked with 3% bovine serum albumin in Tris-buffered saline (TBS) and were incubated with HO1 primary antibody. Membranes were washed with 0.05% Tween 20 in TBS and incubated with second antibody for 1 h. After two additional washes, signals were developed with ECL-Plus (Amersham Pharmacia Biotech). HO1 antibody was generated in chickens using mouse gastric muc5ac glycoprotein as an immunogen. Horseradish peroxidase (HRP)-conjugated rabbit anti-chicken immunoglobulin G and HRP-conjugated goat anti-rabbit immunoglobulin G were purchased from Sigma (St. Louis, MO). The signal intensity of each band was subsequently measured by AlphaImager (Alpha Innotech) in order to quantitate glycoprotein levels.

Statistical analysis

Results are expressed as means $\pm$ standard error of mean (SEM). Differences between groups were examined for statistical significance using repeated-measures analysis of variance (ANOVA) with a Bonferroni multiple comparison test. *p* values of <0.05 were considered significant.

RESULTS

Pulmonary inflammation

Figure 2 (A–F) shows representative pulmonary pathology. Goblet cell hyperplasia, hypersecretion and inflammation were noted on PAS staining, and various infiltrating cells were recognized on HE staining. Quantitative analysis of

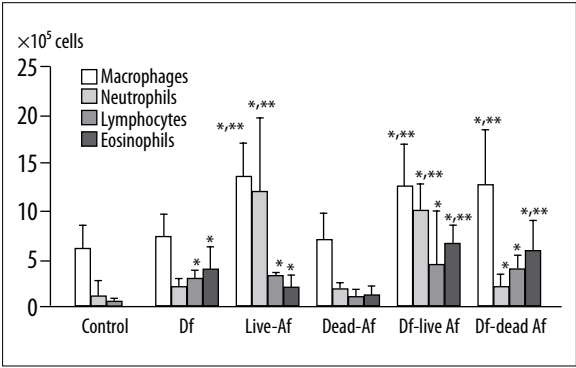


Figure 3. Differential cell counts in BAL samples. Cell counts of BAL samples from each group are shown. Results are expressed as means (n=8 for each group) ±SEM. Values are given in terms of pg/g lung. * $P<0.01$ vs. Control, ** $P<0.05$ vs. Df.

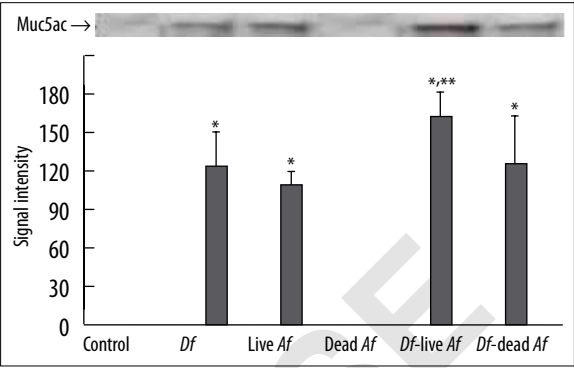


Figure 4. MUC5ac. Expression of MUC5ac in lung homogenates was semi-quantified by western blotting. Bars represent mean absorbance values (n=6) ±SEM. * $p<0.01$ vs. Control and Dead Af mice, ** $p<0.05$ vs. Df and Live Af mice.

Table 1. Cytokine concentrations in homogenized lung.

	IL-5	IL-13	MIP-2	IFN-γ
Control	0.3±0.0	0.2±0.2	0.1±0.2	67.2±5.0
Df	82.5±25.3*	385.0±46.9*	294.4±98.3*	2489.2±962.2*
Live Af	54.1±28.2*	424.3±149.8*	335.2±124.1*	5102.1±1425.2**
Dead Af	1.1±1.2	0.3±0.2	0.3±0.5	63.8±46.2
Df-live Af	155.9±43.5***	506.8±151.0*†	526.5±135.2*†	2404.2±868.4*
Df-dead Af	121.9±33.0***	483.8±51.0*†	286.5±3.2*	1404.2±288.4*

Results are expressed as means (n=8 for each group) ±SEM. Values are given in terms of pg/g lung. * $P<0.01$ vs. Control; ** $P<0.01$ vs. Df.

airway inflammation by BAL is also shown in Figure 3. In comparison with Control mice (A), Df mice showed significantly higher levels of both lymphocytes and eosinophils [(B) and Figure 3]. All cellular components, including eosinophils and neutrophils, were significantly elevated in the lung tissue of live Af mice [(C) and Figure 3]. When dead Af was given to the mice, pulmonary pathology (D) and cellular components of BAL (Figure 3) were similar to those in control mice. In comparison with Df mice, significant increases in both eosinophils and macrophages were seen in the lung tissue of DfAf mice, regardless of whether Af was live or dead [(E, F) and Figure 3]. In contrast, airway neutrophils were significantly elevated in Dflive Af mice, but not in Df-dead Af mice, as compared with Df mice (Figure 3). Taken together, these results indicate that Af differentially regulates allergic airway inflammation depending on infectivity and host status. In healthy hosts, live but not dead Af cause airway inflammation comprising all cellular components. Both live and dead Af enhance airway eosinophilia in allergen-sensitized hosts, while only live Af enhances airway neutrophilia in allergen-sensitized hosts.

Cytokine profile in homogenized lungs

As shown in Table 1, analysis of cytokine concentrations in homogenized lungs revealed that both Dfsensitization and live Af infection induced Th2 cytokines (IL-5 and IL-13), MIP-2 (murine cytokine corresponding to human IL-8) and IFN-γ. No cytokines were enhanced following dead

Af infection alone. In comparison with Df sensitization alone, Dfsensitization plus live Af infection significantly increased Th2 cytokines and MIP-2. Similarly, dead Af significantly increased Th2 cytokines in Df mice, but failed to increase MIP-2.

Hypersecretion

As shown in Figure 2, Df sensitization induced goblet cell hyperplasia, while Af infection alone failed to induce goblet cell hyperplasia. However, DfAf mice exhibited marked increases in goblet cell hyperplasia in their lung tissues, regardless of whether Af was live or dead. Semi-quantitative analysis of MUC5ac protein expression in the supernatant of lung homogenates demonstrated that both Df sensitization and live, but not dead, Af infection significantly increased MUC5ac protein expression (Figure 4). Dflive Af mice showed significant increases in pulmonary MUC5ac protein expression when compared with Df and Af mice.

DISCUSSION

It has long been postulated that the large amounts of viscous sputum produced in the asthmatic airway facilitate Af colonization of the airway. In addition to these simplistic physical mechanisms, the present study found several immunological interactions between Af infection and preexisting allergic airway inflammation by utilizing a novel murine model of allergic asthma and Af infection.

Pathological analysis revealed that *Df* allergen sensitization increased pulmonary eosinophils, while live *Af* infection increased both neutrophils and eosinophils. In *Df* allergen-sensitized mice, live or dead *Af* infection synergistically increased pulmonary eosinophils, which were associated with the cytokine profiles observed in BALF. *Df* allergen sensitization and live *Af* infection induced both Th1-like and Th2-like responses, as well as neutrophilic responses. In *Df* allergen-sensitized mice, both live and dead *Af* infection synergistically enhanced the Th2-like response, which may exacerbate pulmonary eosinophilia.

In accordance with the present results, Kobayashi *et al.* reported that environmental fungi, such as *Alternaria*, potently induce Th2 adjuvant activity [24]. Gao *et al.* also reported that *Af* can exacerbate airway inflammation and remodeling in a rat asthma model [25]. In contrast to their experiments, in our study, mice were sensitized with mite allergen, a representative inhaled allergen, followed by live and dead *Af* infection, which more accurately reflects the actual disease pathology.

The role of cellular immunity in the pathogenesis of ABPA is an area of current research. In addition to eosinophils, neutrophils were also predominant cells in the airway of *Df* allergen-sensitized and live *Af* infected mice. It has been reported that sputum neutrophilia occurs in association with sputum eosinophilia, and that this correlates with the degree of bronchiectasis in patients with ABPA. Neutrophil influx in ABPA may induce lung damage via the release of matrix metalloproteinase-9, potentially leading to bronchiectasis [27]. In the present study, MIP-2, a cytokine involved in neutrophil influx, was also increased following live *Af* infection and may cause pulmonary neutrophilia. The observation that dead *Af* enhanced Th2-associated allergic airway inflammation in a manner similar to live *Af* suggests that a component of *Af* conidia is an allergen, but that metabolic products are not related to these phenomena.

As *Af* was only administered during the challenge phase, it remains uncertain how mite allergen-sensitized mice were able to recognize *Af* as an allergen in the present study. Cross-reactivity between mite and fungal allergens is possible, or structural components of *Af* may be responsible for its highly allergenicity. Bozza *et al.* examined the immune sensing of fungal components [28], while Glaser *et al.* focused on the immunological characterization of cell wall allergens in *Af* [28]. In contrast, neutrophilic airway inflammation, which is potentially irreversible, appears to be associated with live *Af* conidia.

In immunocompetent hosts, *Af* spores are easily cleared from the airway by the mucociliary barrier and mucociliary clearance, along with phagocytosis by alveolar macrophages. The present study examined the mechanisms of *Af* colonization of the asthmatic airway from mucous hypersecretion. PAS staining of the lung tissues demonstrated that *Df* allergen sensitization increased goblet cell hyperplasia, while *Af* infection further increased *Df* allergen-induced goblet cell hyperplasia. These increases were associated with *Af* induced production of two critical molecules in the regulation of airway mucous secretion, IL-13 and Muc5ac [29,30].

Both *Df* sensitization and *Af* infection induced significant increases in IL-13 and Muc5ac, while *Af* infection significantly

enhanced IL-13 and Muc5ac levels in *Df* mice. Interestingly, although only *Af* infection increased IL-13 and Muc5ac, PAS staining failed to confirm increased goblet cell hyperplasia in the lung tissues of *Af* mice. Thus, in addition to IL-13 and Muc5ac, other molecules may be required to elicit goblet cell hyperplasia. Taken together, these results support the proposed mechanism of *Af* colonization of the asthmatic airway; mite allergen sensitization concomitant with *Af* infection enhances Th2-dominant immune response in the airway, which induces mucous hypersecretion and potentially permits further colonization by *Af* spores.

CONCLUSIONS

The present study elucidated the immunological interactions between *Af* infection and preexisting allergic airway inflammation. *Af* infection enhanced not only Th2 responses and airway eosinophilia, but also neutrophil airway inflammation, goblet cell hyperplasia and mucus secretion. Future studies on pharmacological intervention in the present murine model utilizing glucocorticoid, the current recommended therapeutic regimen for ABPA targeting *Af* as an allergen, and anti-fungal drugs, targeting *Af* as a pathogen and under clinical investigation for ABPA [31, 32] or severe asthma with fungal sensitization [33], are thus promising.

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