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Chitin enhances serum IgE in *Aspergillus fumigatus* induced allergy in mice

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ABSTRACT

Aspergillus fumigatus (A. fumigatus) is a ubiquitous fungus that activates, suppresses or modulates the immune response by changing its cell wall structure and by secreting proteases. In this study, we show that chitin acts as an adjuvant in a murine model of *A. fumigatus* protease induced allergy. The mice were immunised intraperitoneally with *A. fumigatus* culture filtrate antigen either with or without chitin and were subsequently challenged with the culture filtrate antigen intranasally. Alum was used as an adjuvant control. Compared to alum, chitin induced a weaker inflammatory response in the lungs, measured as the total cell efflux in BAL, EPO and chitinase production. However, chitin enhanced the total IgE, specific IgE and specific IgG1 production as efficiently as alum. Pre-treatment with chitin but not with alum depressed the concentration of the Th2 cytokines IL-4 and IL-13 in BAL fluid. These results shows that chitin, in spite of a reduction of the Th2 cytokine levels in the lungs, enhanced the total and specific IgE production in *A. fumigatus* culture filtrate induced allergy.

Introduction

Chitin is a biopolymer of N-acetylglucosamine (GlcNAc) found in the cell wall of almost all fungal species. In *A. fumigatus*, the chitin content increases drastically from the conidia to hyphae, thereby providing both a protective and an invasive ability to the fungus (Latge, 2007). In the past decade, the effect of chitin as a direct activator of the immune system and as an adjuvant has been analysed intranasally, orally and intraperitoneally. It is evident that chitin directs the immune responses in different directions depending on the route of administration, but the use of different sources, sizes, and potential contaminations have led to controversies in the literature (Alvarez, 2014; Bueter et al., 2013; Muzzarelli, 2010). When given orally or intranasally in mice, chitin can ameliorate Th2 induced *Dermatophagoides pteronyssinus* and ragweed allergic immune responses (Shibata et al., 2000; Strong et al., 2002), an effect that may be partially explained by the chitin mediated inhibition of T-cell proliferation because the direct intranasal administration of ovalbumin (OVA) and chitin resulted in a diminished antigen-specific T-cell expansion and Th2 polarisation compared to OVA alone (Wagner et al., 2010). In other settings, chitin has been shown to induce innate pro-inflammatory Th2 directed immune responses (Da Silva et al., 2009; Reese et al., 2007), and recently chitin has been shown to dampen Th1/Th17 induced gut inflammation when administered orally in parallel with DSS induced colitis (Azuma et al., 2012; Nagatani et al., 2012).

Alum, which is an insoluble aluminium salt, is known to activate the innate immune system in a manner that ultimately results in a Th2-type immune response, but the mechanisms are incompletely understood (Grun and Maurer, 1989; Kuroda et al., 2013; Marrack et al., 2009).

The development of asthma and allergies are increasing in the developed world, and the exposure to fungal related antigens correlates with the severity of asthma. Up to 20–25% of asthma patients who are referred to specialist treatment have skin-test reactivity to *A. fumigatus* or other fungi (Denning et al., 2006). *A. fumigatus* is a saprophytic mould; in immunocompetent hosts, the inhaled spores are cleared by alveolar macrophage mediated oxidative killing (Philippe et al., 2003), and the host is protected by unnecessary immune activation by the hydrophobin layer. In immune compromised hosts, the spores of *A. fumigatus* germinate, and the fungus starts growing and secreting cytotoxins and proteases into the microenvironment. In parallel, fungal cell wall polysaccharides

Abbreviations: AMCase, acidic mammalian chitinase; *A. fumigatus*, *Aspergillus fumigatus*; BAL, bronchoalveolar lavage; EPO, eosinophil peroxidase assay; 1WCF, one-week culture filtrate antigen; OVA, ovalbumin.

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such as β-glucan and chitin are synthesised during the growth and remodelling of *A. fumigatus* (Latge, 2007). The proteins secreted by the fungus during morphogenesis usually serve as antigens that are capable of activating the innate and adaptive immune system. Repeated exposures to these antigens can result in asthma, characterised by elevated total IgE, *A. fumigatus* specific IgE, specific IgG1, eosinophilia, enhanced Th2 cytokines (IL-4, IL-5 and IL-13) and bronchial hyperresponsiveness (Agarwal et al., 2013). An analysis of dust collected from the homes of asthmatic individuals has revealed elevated levels of chitin, and chitin is also known to be associated with asthma induced eosinophilic allergic inflammation in the lungs of mice exposed to an allergy model (Van Dyken et al., 2011). When chitin is given as an adjuvant, it has enhanced the specific immune response in an ovalbumin allergy model (Da Silva et al., 2010). However, the role of chitin and how it helps to activate the innate and adaptive immune responses along with secretory proteases with regard to fungal allergens is unknown.

In the present study, we have examined the role of chitin particles below 40 μm given along with an *A. fumigatus* one-week culture filtrate antigen (1WCF) in a mouse model and studied the immune response compared to the known adjuvant alum. We found that chitin, when given along with *A. fumigatus* 1WCF, can act as an adjuvant by enhancing the levels of EPO, mirroring the eosinophil infiltration in mouse lungs as well as the total IgE and specific immunoglobulin's compared to the 1WCF administered alone. Surprisingly, we found a reduction of the Th2 cytokines in the BAL fluid compared to both the 1WCF alone and to the 1WCF together with alum, suggesting that chitin modulates the immune response in a different manner compared to the classical adjuvant alum.

Materials and methods

Buffers and reagents

The tris-buffered saline (TBS) was composed of 140 mM NaCl, 10 mM Tris–HCl and 0.02% (w/v) NaN_3 (pH 7.4). The TBS/Tw was composed of TBS containing 0.05% (v/v) Tween 20 (polyoxyethylene sorbitan monolaurate, MERCK-Schuchardt, Hohenbrunn, Germany). The phosphate buffered saline (PBS) was composed of 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4 and 1.5 mM KH_2PO_4 (pH 7.4). The coating buffer was composed of 60 mM Na_2CO_3, 35 mM $NaHCO_3$ and 0.02% (w/v) NaN_3 (pH 9.6). The RPMI-1640 medium, penicillin, streptomycin, glutamine, trypsin-EDTA, Hanks' balanced salt solution (HBSS), foetal bovine serum (FBS), SuperScript® III Reverse Transcriptase and TRIzol were all obtained from Life Technologies Europe BV (Invitrogen), Denmark. The crab shell chitin (C3641) and the Chitinase Assay Kit (CS1030) were obtained from Sigma–Aldrich, Schnelldorf, Germany. The dehydrated sabouraud dextrose broth (SD-broth) powder was obtained from BD-Difco, Albertslund, Denmark. The cell strainers (40 μm) were obtained from BD biosciences, Denmark. The ELISA kits were obtained from eBioscience (AH diagnostics, Aarhus, Denmark), R&D systems, Oxon, United Kingdom and Biolegend (Nordic bio Site ApS, Copenhagen, Denmark). The gene specific primer assays for the cytokines and chemokines were obtained from Applied Biosystem, Nærum, Denmark.

Chitin microparticle preparation

The chitin microparticles were prepared from crab shell chitin as described previously (Strong et al., 2002) by the sonication of a suspension of chitin in sterile, endotoxin-free phosphate-buffered saline (PBS). The particles were collected and filtered through a 40 μm cell strainer and washed with 20% (v/v) ethanol to ensure sterility. Subsequently, the particles were washed with sterile PBS, and their size distribution was examined by flow cytometry and comparison with 1 μm, 10 μm and 48.9 μm-standardised beads (Polysciences, Inc, Eppelheim, Germany). Sterility was confirmed by the absence of colony-forming units after plating on an agar plate. Endotoxin levels were measured by the Limulus amebocyte lysate assay (Lonza Inc.). The endotoxin level was found to be below the limit of detection.

A. fumigatus *allergen extracts*

A. fumigatus allergen extract from a 1-week culture filtrate (1WCF) was prepared as described previously (Strong et al., 2002). After 1 week of growth, the culture broth was filtered through a miracloth to remove the mycelia fragments and was subjected to dialysis with a 10-kDa cut-off. The dialysed proteins were pooled, concentrated, filtered through 0.22 μm and the total protein was quantified using the Bradford method against BSA as the standard and analysed by SDS–PAGE in the reduced and non-reduced states. The 1WCF was further tested for reactivity against human serum from *A. fumigatus* allergic patients obtained from the National Institute of Biological Standards and Control (NIBSC, Hertfordshire, UK) before being stored at −20 °C until use.

Animals

The female C57BL/6 mice (Charles River, UK) were housed under specific-pathogen-free conditions in individual ventilated cages and used at 6–8 weeks of age. The animals had ad libitum access to pelleted food and water. The mice were randomised in various groups before the experiments. The Danish Animal Experiments Ethical Committee approved all the animal experiments.

Sensitisation and challenge

The sensitisation and challenge were performed as described previously (Banerjee et al., 2004) with slight modifications. The female C57BL/6 mice were given 4 intraperitoneal injections of the 1WCF (50 μg) alone or combined with chitin particles (50 μg), alum (2 mg) or its vehicle controls in 100 μl of sterile PBS (Fig. 1A). After the last intraperitoneal dose, all the mice were challenged intranasally with 25 μg of the 1WCF alone. Twenty-four hours after the intranasal challenge, the mice were killed, BAL was performed and the lungs and serum were collected for further analysis.

Bronchoalveolar lavage (BAL) fluid and BAL cell pellet lysate

The animals were sacrificed by CO_2 asphyxiation. Subsequently, a median sternotomy was performed, and the tracheas were exposed by blunt dissection. A 22-gauge catheter was inserted into the trachea, and BAL was performed by instilling 1 ml of PBS into the trachea and gently aspirating back. This procedure was repeated twice, and the samples were pooled. The samples from each animal were centrifuged, and the supernatants were stored at −80 °C until use. The BAL cell pellet was resuspended in 1 ml of PBS and was used for the total cell count determination. The total cell count was determined using a Beckman Coulter Z2 particle count and size analyser. The BAL cells from all the groups were adjusted to same cell concentration (100,000 cells/sample) and were lysed using PBS with 1% triton X-100. After the lysis, the cell lysates were centrifuged, and the supernatants were used for the eosinophil peroxidase assay (EPO) assay.