Characterisation of *Aspergillus niger* and *A. tubingensis* related allergens relevant to asthma

6 months research fellowship

EAACI report

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November 2017

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I. Introduction

About 5% of the general population (1), and as much as 70% of severe asthmatics (2) are sensitised to fungi. Sensitisation to the common fungus *Aspergillus fumigatus,* in particular, has been associated with a decreased lung function in asthmatics (3, 4). We had investigated the lung microbiome by high-throughput sequencing (HTS) to find other fungi, which are associated with asthma and are not commonly found in culture. One observation from this data was that the amounts of fungi of the *Aspergillus* section *Nigri* in the lungs of patients with asthma were significantly higher compared to healthy subjects (paper in preparation). This suggests a contribution of black fungi to the asthma phenotype. Three allergens are listed for *A. niger* (WHO/IUIS, November 2017), which had been discovered in the context of occupational asthma in settings of food processing and animal feedstuffs (5, 6). Although *A. niger* can cause ABPM (7, 8) and had been recovered from patients with moderate-to-severe asthma (3), its contribution to sensitisation and lung damage is unclear. To get a better understanding of this contribution, we investigated the IgE response to the black fungi. Our specific aims were:

- To identify the major IgE binding proteins of *A. niger* and *A. tubingensis* using pooled sera from patients with asthma and fungal sensitisation
- To clone, sequence and produce in a recombinant form the major IgE binding proteins of *A. niger* and *A. tubingensis*
- Use purified recombinant proteins to determine the prevalence of sensitisation to these allergens in sera from patients with asthma

II. Methods

1. Fungal culture

Three well characterised clinical isolates of each *A. niger* and *A. tubingensis*, an *A. fumigatus* clinical isolate and *A. fumigatus* strain NCPF7097 were chosen for protein and RNA extractions from hyphae and spores (H&S) (9, 10). All cultures were grown at 37°C, but culture times differed, ranging between 3 to 14 days. *A. fumigatus* H&S were derived from a liquid culture, whereas *A. niger* and *A. tubingensis* H&S were derived from a potato dextrose agar plate for the spores and liquid Sabouraud dextrose agar, shaking at 200 rpm, for the hyphae.

2. Protein extracts and SDS-PAGE

Fungal proteins were produced either by grinding the fungal material with liquid nitrogen and pestle and mortar, or bead bashing at 4°C using acid-washed glass beads (212-300 µm, Sigma). Five millilitre lysis buffer (0.5X or 1X cOmplete, EDTA-free protease inhibitor cocktail, 150 mM NaCl, 2 mM EDTA, 50 mM $NaHCO_3$, 2 mM PMSF) was applied per gram of material (9, 11). Proteins were retrieved from the supernatant and the concentration was determined by Bradford assay (BioRad), which ranged between 0.21 and 1.26 µg/µl. Four microgram of crude protein extracts or 2 µg, 5 µg and 10 µg of recombinant proteins, alongside PageRuler™ Prestained (ThermoScientific) were used for SDS-PAGE with 12% gels under denaturing conditions. For mass spectrometry, gels were stained with Coomassie brilliant blue. Gels were imaged with FluorChem E (ProteinSimple).

3. Mass fingerprinting

Bands of IgE-reactive proteins were excised and sent for LC-MS/MS analysis either to the VetCore – Facility for Research (Vienna, Austria) or the Protein Nucleic Acid Chemistry Laboratory (PNACL) (University of Leicester, UK). The former used SCIEX ProteinPilot™ software, the latter Mascot and X! Tandem software via Scaffold4 for protein identification.

4. Immunoblotting

For immunoblotting, proteins were transferred from the SDS-PAGE gel onto a 0.2 µm nitrocellulose membrane by tank (wet) transfer at 100 V for 1 h. The transfer efficiency was

analysed by Ponceau red staining of the membrane. After destaining, immunoblotting was performed as described in the Clarity Western ECL Substrate manual (BioRad), using PBST (PBS containing 0.5% Tween 20) for blocking, washing and as diluent for the serum (1:10) and mouse anti-human IgE Fc-HRP (SouthernBiotech) (1:5000). After treatment with SuperSignal West Pico substrate (ThermoScientific) for 5 min, chemiluminescent signals from the membrane were detected by FluorChem E (ProteinSimple). Blots were visualized with AlphaViewSA software (Version 3.4.0.0).

For the inhibition blot, the serum was diluted and then divided into two equal parts, which were incubated spinning, o/n at 4°C. While one part only contained the diluent, the part also contained fungal protein extract or a mixture of recombinant proteins (10x the amount of protein/s per millilitre loaded on a SDS-PAGE gel). Two identical immunoblots were incubated separately with the pre-incubated serum with or without fungal proteins and detection of bound IgE antibodies was performed as described above.

5. RNA extraction

For the RNA extractions fungi were disrupted by bead bashing as described above, and with the addition of RLT/2-mercaptoethanol buffer (RNeasy Plant Mini Kit (Qiagen)/Fisher Scientific). After centrifugation, RNA was obtained from the supernatant using the RNeasy Plant Mini kit (Qiagen) according to the manufacturer's instructions. RNA concentrations measured with Qubit ranged between 2.68 ng/µl and 42 ng/µl. In case of *A. fumigatus* NCPF7097 the concentration could not be measured, because it was too high. The RNA integrity number (RIN), which was determined by the Bioanalyzer 2100, ranged between 5.2 and 7.1 for 5 of seven samples. cDNA was generated using the SuperScript™ II Reverse Transcriptase kit (Invitrogen).

6. Production of recombinant allergens

Specific protein-coding genes were amplified from cDNA using a DNA HF polymerase (ThermoScientific), thereby introducing a 6x His-Tag at the C-terminus of the respective proteins. PCR products were cloned into the bacterial expression vector pET-17b (Novagen) after restriction digest of the PCR products and the vector with *Nde*I and *EcoR*I (ThermoScientific). The ligation reaction was transformed into competent *Escherichia coli*

XL1-Blue. Successful cloning was confirmed by restriction digest analysis of extracted plasmids (Gene JET Plasmid MiniPrep kit, ThermoScientific) and Sanger sequencing.

For protein expression, the generated vectors were transformed into competent BL21 *E. coli* cells. Expression was induced by isopropyl β-D-1 thiogalactopyranoside (IPTG) at an OD_{600} ~0.5. Bacteria were harvested after 2 h of IPTG-induction and proteins were extracted using a native buffer (10 mM Tris, 300 mM NaCl, 0.1% Triton X-100, 20 mM Imidazole, pH 8.0) and homogenized (homogenizer Glas-Col) on ice. The His-tagged proteins were purified using Protino Ni-NTA agarose (Macherey-Nagel) and were finally dialysed against 10 mM sodium phosphate buffer (pH 7.0) o/n at 4°C using a Slide-A-Lyzer cassette (MWCO 3.5). The protein concentration was determined using a Micro BCA Kit (ThermoScientific). The purity of the proteins was analysed by SDS-PAGE, followed by Coomassie staining, and immunoblotting with 1:1000 diluted Penta-His™ antibodies (Qiagen), which were detected by goat anti-mouse IgG HRP antibodies (1:10,000) (Jackson labs). Proteins were aliquoted and stored at -20°C.

III. Summary of the findings

Immunoblots showed that only those patients who were highly sensitised to *A. fumigatus* (≥ 17 kU/L) showed IgE-reactivity towards *Aspergillus* section *Nigri*, though fewer allergens were detected compared to the *A. fumigatus* controls. This could possibly derive from differences in culture times and fungal strains, which have been reported before (9). Proteins were detected at 15, 18, 23, 35, 50 and 100 kDa in *Aspergillus* section *Nigri*. The strongest reactions as judged by band intensities were observed for the proteins with molecular weights of 18, 35 and 50 kDa. Cross-reactivity between the different *Aspergillus* species was indicated by inhibition blots, where *A. fumigatus* proteins could inhibit IgE-reactivity towards *Aspergillus* section *Nigri* allergens when sera were pre-incubated with *A. fumigatus* extracts. However, a degradation of the IgE antibodies by the proteases present in the crude extracts is also possible, emphasizing the value of using recombinant allergens. To identify specific cross-reactive allergens, IgE immunoblots were performed with available recombinant allergens of *A. fumigatus* (Asp f 3, 4 and 6). Asp f 4 could not inhibit IgE-reactivity towards the allergens of the *Aspergillus* section *Nigri*. Asp f 3, a peroxisomal protein (12), and Asp f 6, a manganese superoxide dismutase (MnSOD) (13) (available at the FH Campus Wien), could inhibit IgE-reactivity towards the three cross-reactive 15, 18 and 23 kDa proteins of *A. niger* and *A. tubingensis*. Cross-reactions of these *A. fumigatus* allergens with allergens of other fungal species have been reported before (12, 14). We propose to name these allergens Asp n 3 and Asp t 3 for the peroxisomal proteins, and Asp n 6 and Asp t 6 for the MnSODs of *A. niger* and *A. tubingensis*, respectively. Since Asp f 3 and Asp f 6 had been identified as important cross-reactive allergens, larger amounts of these recombinant proteins were produced. Some impurities were detected most of which showed up at higher loading concentrations and were identified as degradation products by anti-His detection. The impurities should not affect the performance in immunodetection experiments. A dimeric form of Asp f 3 seemed to be present at ~38 kDa, which had already been reported for the related Cand b 2 (12).

Since *A. fumigatus* and *Aspergillus* section *Nigri* are closely related species (15, 16), cross-reactions are likely to occur, though they had rarely been reported between these sections with the serine proteases Asp n 18 and Asp f 18 the only examples clearly identified (17, 18). However, a recombinant form of the protein produced from *A. tubigensis* cDNA was not recognized by IgE antibodies from individual patients.

The peptide mass fingerprinting, performed to identify the remaining *Aspergillus* section *Nigri* allergens, yielded many potential candidates, including known and uncharacterised proteins.

Examples for the 35 kDa allergen are the proteasome subunit alpha type, malate dehydrogenase, and transaldolase. IgE-reactivity to the latter two had been demonstrated in patients with ABPA (19). Both are known allergens in other fungi (allergen.org). The 50 kDa protein was unique to one of the *A. tubingensis* clinical isolates. The mass fingerprinting data are suggestive of uncharacterised proteins and proteins involved in caspofungin resistance. HSP90 or catalase are potential candidates for the 100 kDa allergen because they are known allergens in other fungal species (20-22). Despite these examples of allergen candidates, the mass spectrometry data are difficult to interpret: Proteins have to be considered with bigger or smaller sizes than the respective bands detected because of factors such as glycosylation or partial degradation. Additionally, the method of cutting the gel influences the number and composition of proteins observed. Therefore, the next steps would be to narrow down potential proteins of interest by techniques such as 2D IgE immunoblots gel electrophoresis and immunoprecipitation followed by peptide mass fingerprinting.

In conclusion, several allergens of the *Aspergillus* section *Nigri* were identified with some cross-reactivity to allergens found in *A. fumigatus*. Further characterisation of these allergens is ongoing.

IV. Personal reflection

I am very happy that I was granted the 6 months EAACI research fellowship for my PhD project allowing me to cover my expenses and research. Though I had some experience in protein work during my bachelor and master projects, I was delighted to learn new techniques at the FH Campus Wien, including immunoblotting and production of recombinant allergens, which will be very useful for my future work. The people of the department were very supportive and helpful at any time. I regularly corresponded with Prof Swoboda about the progress of the project and further steps, which will continue after the end of the fellowship. A highlight was the cooperation of the VetCore mass spectrometry facility researchers, who gave me an insight into their work and I am hopeful that the PNACL facility researchers will guide me through their procedures and analysis as well. The only set-back were problems with the mass spectrometer machines in Vienna, which delayed the analysis of potential allergen candidates. Concerning the outcomes of the project to date, not all objectives could be explored. The sensitisation to the *Aspergillus* section *Nigri* was tested with a small cohort of patients with asthma. More patients as well as healthy controls would need to be included in future experiments. It was interesting, though not totally unexpected, to find at least some degree of cross-reactions between the *Aspergillus* section *Nigri* and *A. fumigatus* (Asp f 3 and Asp f 6), which have not been explored in that detail before. Further experiments will be necessary to narrow down the potential allergen candidates such as mass spectrometry of 2D gel electrophoresis gel dots and or 1D gel samples after immunoprecipitation. I am looking forward to work with Prof Cousins in Leicester, who has expertise in this area. The implications to asthma will need to be fully assessed after having tested the reactions of more subjects and having evaluated clinical data in that context. In addition, we would like to explore the IgG reaction to our fungi of interest as well to assess this side of the inflammatory response. I am looking forward to exploring more about the area of allergen research and the immunological responses in my future career.

V. References

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