

Fungal allergens induce cathelicidin LL-37 expression in chronic rhinosinusitis patients in a nasal explant model

Eng Hooi Ooi, M.B.B.S.,* Peter-John Wormald, M.D.,* A. Simon Carney, M.D.,*
Craig Lloyd James, F.R.C.P.A.,# and Lor Wai Tan, Ph.D.* (Australia)

ABSTRACT

Background: Fungus is thought to play an important role in some subgroups of chronic rhinosinusitis (CRS) patients with eosinophilic mucus (EMCRS). The cathelicidin LL-37 is an important innate defense peptide with antimicrobial activity but its responses in CRS and EMCRS patients have not been established. We investigated the innate immune responses of LL-37 in nasal tissue from CRS and EMCRS patients to fungal allergen challenge.

Methods: The levels of LL-37 produced by nasal tissue and secreted in response to fungal allergen challenge were determined by a nasal tissue explant *in vitro* model. LL-37 mRNA and protein levels were quantified by real-time reverse-transcriptase–polymerase chain reaction and immunoassay methods.

Results: LL-37 mRNA expression in CRS, but not EMCRS patients, is significantly upregulated by *Aspergillus* (mean fourfold increase) and *Alternaria* (mean sixfold increase) extracts in a dose-response manner ($p < 0.001$). LL-37 peptide levels in the nasal tissue from CRS patients are increased in response to *Alternaria* ($p < 0.05$). In contrast, with EMCRS patients, the expression of LL-37 peptide in nasal tissue is increased with *Aspergillus* ($p < 0.001$) but is reduced with *Alternaria*. We also observed a trend where levels of secreted LL-37 were decreased with higher doses of *Alternaria* and *Aspergillus* extracts.

Conclusion: LL-37 is significantly up-regulated at the mRNA and protein level in CRS patients in response to fungal allergens. However, EMCRS patients do not show increased LL-37 at either the mRNA or the protein level in response to *Alternaria*.

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Key words: Allergic fungal sinusitis, antimicrobial peptide, cathelicidin LL-37, chronic rhinosinusitis, innate immunity

Allergic fungal sinusitis (AFS) is a severe form of chronic rhinosinusitis (CRS) characterized by clinical and pathological features histologically analogous to allergic bronchopulmonary aspergillosis.¹ Historically, *Aspergillus fumigatus* was implicated in AFS but other species including *Bipolaris* (*Drechslera*) spp *Curvularia lunata*, *Alternaria alternata*, *Cladosporium herbarum*, *Exserohilum rostratum*, and *Penicillium chrysogenum* have been isolated in AFS patients.^{2,3} Other patients classified as eosinophilic mucus CRS (EMCRS) present clinically like AFS with thick EM but may not have fungus identified.^{4,5} The nasal mucosa is continually exposed to inhaled allergenic fungal spores and pollen under normal everyday circumstances both indoors and outdoors.⁶ In the immunocompetent host, fungi are seldom pathogenic because it is contained by the innate immune system. The nasal epithelial and immune cells respond to the presence of fungi by increasing production of cathelicidin antimicrobial peptides (CAMPs) and inflammatory mediators to signal and recruit immune cells.⁷ There is only one human cathelicidin hCAP18 identified stored in neutrophil granules as a propeptide, which undergoes extracellular cleavage releasing its C-terminus, termed LL-37, containing antibacterial activity.⁸ LL-37 is

shown to be expressed by airway epithelium and secreted into airway surface fluid.⁹ Cathelicidins possess antifungal activity *in vitro* and play an important role in the innate mucosal defense against airborne pathogens.¹⁰ The abbreviations CAMP, hCAP18, or LL-37 have been used in the literature to refer to human CAMPs, but for this study, LL-37 refers to human cathelicidin.^{8,9}

The innate immune responses of CRS or EMCRS patients to fungi have not been as well studied to date. In comparison to normal inferior turbinates, nasal polyps express increased LL-37 mRNA as detected by reverse-transcriptase–polymerase chain reaction (RT-PCR) implying chronic inflammation up-regulates LL-37.¹¹ We propose that LL-37 is stimulated in response to fungi as an innate defense mechanism and tested this hypothesis by a series of *in vitro* experiments measuring LL-37 mRNA and protein in nasal tissue and comparing the responses in CRS and EMCRS patients.

MATERIALS AND METHODS

Nasal tissue biopsy specimens were collected from patients undergoing endoscopic sinus surgery for CRS. The diagnosis of CRS was defined as continuous symptoms of rhinosinusitis for at least 12 consecutive weeks according to published criteria.¹² Nasal tissue from six patients with CRS and four patients with thick EM with negative fungi and fungal allergy (EMCRS) were used for these experiments. The EM was confirmed by histology as previously published.³ None of the CRS patients had fungi present by fungal stains (Gomori's methenamine silver) or culture.³ The hospital Ethics of Human Research Committee approved the study, and all patients gave written informed consent. Patients were excluded if they had received systemic or topical corticosteroids, anti-

From the *Department of Surgery–Otorhinolaryngology Head and Neck Surgery, University of Adelaide and Flinders University, Adelaide, Australia, and #Adelaide Pathology Partners, South Australia, Australia
Supported by grants from the Garnett Passe and Rodney Williams Memorial Foundation, Victoria, Australia

Address correspondence and reprint requests to Peter John Wormald, M.D., Department of Surgery–Otorhinolaryngology Head and Neck Surgery, The Queen Elizabeth Hospital, 28 Woodville Road, Woodville South, South Australia 5011, Australia
E-mail address: peterj.wormald@adelaide.edu.au
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biotics, or antifungals at least 1 week before surgery or were immunocompromised with tissue invasive fungal rhinosinusitis.

Specimen Collection and Fungal Challenge Experiments

Immediately after endoscopic removal, tissue specimens from each patient were cut into approximately equal pieces of 5 mm³ in a sterile laminar flow environment and placed in tissue culture plates (Nunc; Roskilde, Denmark) with airway culture medium DMEM/HamsF12 (Sigma, Sydney, Australia) supplemented with 1% fetal calf serum (Gibco, Invitrogen Corp., Grand Island, NY).

Experiment Design

Experiment 1. A time-course experiment to determine whether 24 hours was suitable for examining the effects of fungal allergens on nasal tissue was initially performed. Tissue specimens from CRS patients ($n = 3$) were incubated at 37°C and 5% CO₂ with 1250 ng/μL (final concentration) of the fungal allergens *A. fumigatus* or *Alternaria tenuis* (10 μg/μL; Hollister-Stier Laboratories, Spokane, WA) in duplicate in a final volume of 400 μL of DMEM/HamsF12 culture medium. The tissue was harvested at 4, 16, and 24 hours.

Experiment 2. To examine the effects of different doses of fungal allergens on nasal tissue, we challenged nasal tissue from CRS ($n = 4$) and EMCRS ($n = 4$) patients with 1250, 250, and 50 ng/μL *A. fumigatus* and *A. tenuis* extracts (final concentrations; Hollister-Stier Laboratories) in a final volume of 400 μL of airway culture medium using a nasal tissue explant *in vitro* model. This *in vitro* explant model is different from primary epithelial cell culture and was designed to simulate an *in vivo* response of nasal tissue to the fungal allergens and allow us to detect LL-37 secreted into the culture supernatant. The specimens were incubated at 37°C and 5% CO₂ and the tissue and media were harvested after 24 hours. Untreated tissue specimens with equivalent volumes of culture medium served as the controls for both experiments. Because of the significant quantities of tissue required to conduct the experiments (with replicates for both RT-PCR and enzyme-linked immunosorbent assay [ELISA] and repeated experiments), we were ethically unable to surgically remove the required amount of tissue from normal patients for an experimental normal control group.

The harvested specimens were placed in RNeasy Lysis Buffer (Qiagen, Austin, TX) or snap frozen in liquid nitrogen and stored at -80°C until analysis. The culture supernatant was stored at -80°C until analysis. Each experiment was performed in duplicate and was analyzed by quantitative RT-PCR (qRT-PCR) and immunoassay (ELISA).

qRT-PCR

Total RNA was extracted from the sinus tissue using the RNeasy Mini Kit (Qiagen, Hilden, Germany) with an On-Column Dnase Digestion step with the Rnase-Free Dnase Set (Qiagen) performed according to the manufacturer's protocol. The total RNA concentration was determined by measuring the absorbance at 260 nm using the Bio-Rad SmartSpec 3000 Spectrophotometer (Bio-Rad Laboratories, Hercules, CA). Each sample was measured in duplicate.

First-strand cDNA synthesis was performed using Omniscript Reverse Transcriptase kit (Qiagen) according to the manufacturer's instructions. The no-template control had the Omniscript enzyme omitted from the RT mix. The real-time qPCR assay for LL-37 was performed using the QuantiTect SYBR Green PCR kit (Qiagen) on a MyIQ (Bio-Rad Laboratories) Thermocycler. Primers used for the PCR were purchased from Geneworks (Adelaide, Australia) and sequences are as previously published¹³: LL-37 forward primer, 5'GCAGTCACCAGAGGATTGTGAC3', and reverse primer, 5'CACCGCTTCACCAGCCC3'; hypoxanthine phosphoribosyl transferase (HPRT) forward primer, 5'TGACACTG-GCAAAACAATGCA3', and reverse primer, 5'GGTCCCTTTCACCAGCAAGCT3'. Two microliters of cDNA was used for the PCR assay in a final total volume of 25 μL with 2× QuantiTect SYBR Green PCR Master Mix (Qiagen), 10 nM of fluorescein (Bio-Rad Laboratories), and 0.3 μM of final concentration primers. The PCR assay was performed initially at 95°C for 15 minutes. Forty cycles of amplification were then performed at 95°C for 15 seconds and at 60°C for 1 minute. A melting curve analysis of the products was performed at the end of the PCR assay and single melting peaks were identified for both the target and the reference genes. The PCR products were examined by agarose gel electrophoresis (data not shown) and single bands of the predicted size were identified for both LL-37 (50 bp) and the reference gene HPRT (93 bp) genes. There also were no bands present in the no-template controls. PCR products sequenced were confirmed by searching against all available sequences in the National Center for Biotechnology Information database using the BLAST program (www.ncbi.nlm.gov/BLAST). Ct or cycle threshold is defined as the PCR cycle at which detected fluorescence first rises significantly above the baseline threshold.

The results were analyzed by the comparative Ct ($\Delta\Delta Ct$) method as published by Livak and Schmittgen,¹⁴ which quantifies the relative expression by calculating $2^{-\Delta\Delta Ct}$ to account for the exponential amplification of the PCR, rather than an absolute mRNA value. We calculated $\Delta\Delta Ct = \Delta Ct (\text{disease}) - \Delta Ct (\text{control})$, where $\Delta Ct (\text{disease})$ denotes the fungal allergen specimen and $\Delta Ct (\text{control})$ denotes the untreated specimen. Briefly, this method analyzes the relative change in expression of the target gene relative to a control. To validate the assumption that the amplification efficiencies of the target (LL-37) and reference (HPRT) primers are approximately equal, dilutions of cDNA were made and the target and reference gene were amplified in triplicate by qRT-PCR. The change in Ct, $\Delta Ct = Ct (\text{reference}) - Ct (\text{target})$, was calculated for each cDNA dilution. This ΔCt value was plotted against the log of the total RNA concentrations and the data were fit using least-squares linear regression analysis. It was shown that the slope was ≤ 0.1 in three different patient samples; hence, the efficiencies of the target and reference genes are similar and the $\Delta\Delta Ct$ calculation for the relative quantification of target may be used. The control was included with the treated samples in each PCR run. Triplicate PCRs were run on each sample.

Protein Extraction from Nasal Tissue and Bradford Assay

Total protein was extracted from the specimens by grinding in liquid nitrogen with Cell Lytic-M Mammalian Cell Lysis

Extraction reagent (Sigma) and Protease Inhibitor Cocktail (Sigma). The lysate was intermittently agitated and then centrifuged at $12,000 \times g$ for 15 minutes at 4°C . The resultant supernatant containing protein from tissue homogenates was removed and stored at -80°C until analysis. Total protein concentration from the tissue homogenates and culture supernatant was determined by the mini-Bradford assay protocol.

ELISA

A commercially available sandwich ELISA measuring LL-37 levels with a sensitivity range from 0.14 to 100 ng/mL was performed according to the manufacturer's instructions (Hycult Biotechnology b.v., Uden, The Netherlands). Fifty micrograms of total protein per sample was used for the assay; all assays were performed in duplicate and the results are given as the concentration of LL-37 peptide in nanograms per microliter.

Statistics

One-way ANOVA with Tukey's multiple pair wise comparison posttests were performed on the $2^{-\Delta\Delta\text{Ct}}$ and ELISA data using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA). Values of $p < 0.05$ were considered statistically significant.

RESULTS

LL-37 mRNA Expression to *Aspergillus* and *Alternaria* Extracts Is Significantly Up-Regulated in CRS Patients in a Dose-Response Effect

LL-37 mRNA in nasal tissue from CRS patients was significantly up-regulated by *A. fumigatus* and *A. tenuis* extracts in a dose-dependent manner ($p < 0.0001$, one-way ANOVA) as shown in Fig. 1. The 1250-ng/mL *Alternaria* (mean sixfold increase) had the greater effect on inducing LL-37 mRNA expression and this was statistically significantly >250 ng/ μL of *Alternaria* ($p < 0.01$), 250 ng/ μL of *Aspergillus*, 50 ng/ μL of *Alternaria*, or 50 ng/ μL of *Aspergillus* ($p < 0.001$). The 1250-ng/ μL *Aspergillus* (mean fourfold increase) induced a statistically significant increase in LL-37 mRNA compared with 50 ng/ μL of *Aspergillus* or 50 ng/ μL of *Alternaria* ($p < 0.01$). The results indicate that nasal tissue from CRS patients respond to fungal allergen challenge by increasing LL-37 mRNA expression. By contrast, with the EMCRS patients, there was an up-regulation of LL-37 mRNA levels with increasing doses of fungal allergens but this was not statistically significant ($p = 0.09$, one-way ANOVA; Fig. 2).

LL-37 Protein Levels Are Increased within 24 Hours in Response to *Aspergillus* and *Alternaria* Extracts

The time-course experiments, as shown in Fig. 3, showed the response to fungi with LL-37 protein in nasal tissue increasing within the first 4 to 16 hours in response to *Aspergillus* and *Alternaria* extracts. We chose 1250 ng/ μL fungal extract as a dose high enough to stimulate a response and a 24-hour time course of induction to show the accumulation of LL-37 peptide in the culture supernatant to validate the time period for experiment 2.

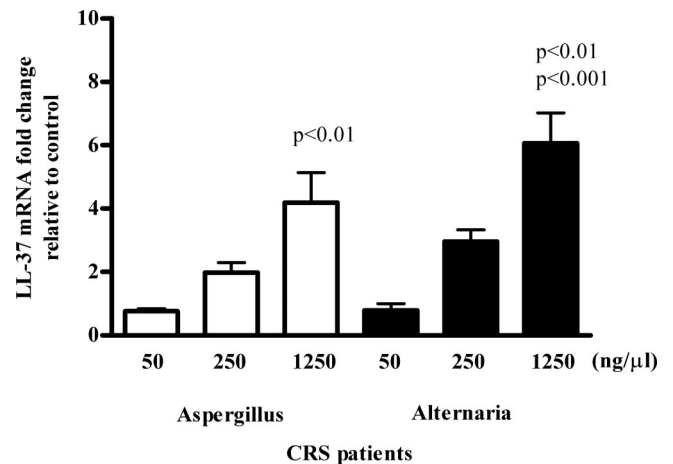


Figure 1. Expression of LL-37 mRNA quantified by qRT-PCR in human nasal tissue from CRS patients challenged with *A. fumigatus* and *A. tenuis*. Levels are normalized to HPRT. Data are expressed as the fold-change in mRNA levels of LL-37 relative to the control tissue (means $\pm$ SEM). Both *Aspergillus* and *Alternaria* showed a dose-dependent increase in nasal tissue LL-37 mRNA expression. The 1250-ng/ μL *Aspergillus* compared with 50 ng/ μL of *Aspergillus* and 50 ng/ μL of *Alternaria* ($p < 0.01$). The 1250-ng/ μL *Alternaria* compared with 250 ng/ μL of *Alternaria* ($p < 0.01$) and 250 ng/ μL of *Aspergillus*, 50 ng/ μL of *Aspergillus*, and 50 ng/ μL of *Alternaria* ($p < 0.001$) by one-way ANOVA and Tukey's multiple comparison test.

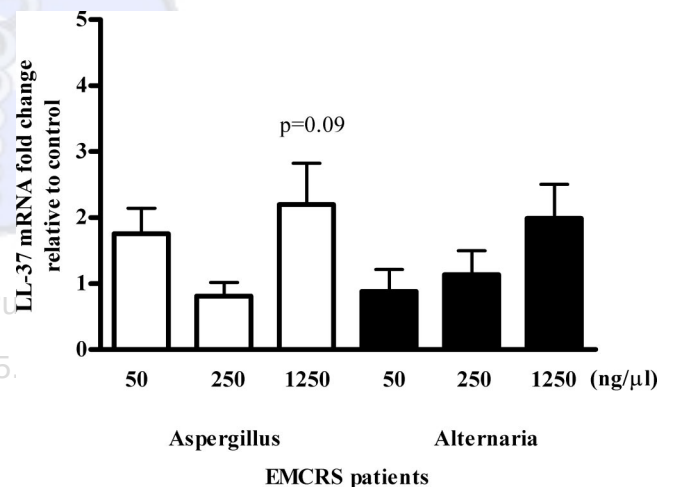


Figure 2. Expression of LL-37 mRNA quantified by qRT-PCR in human nasal tissue from EMCRS patients challenged with *A. fumigatus* and *A. tenuis*. Levels are normalized to HPRT. Data are expressed as the fold-change in mRNA levels of LL-37 relative to the control tissue (means $\pm$ SEM). Both *Aspergillus* and *Alternaria* did not induce statistically significant changes in LL-37 mRNA levels ($p = 0.09$).

LL-37 Protein Levels in Nasal Tissue and Culture Supernatant from CRS and EMCRS Patients

CRS Patients. We detected a significant increase in LL-37 levels in the nasal tissue homogenates from CRS patients challenged with *Alternaria* and *Aspergillus* extracts as shown

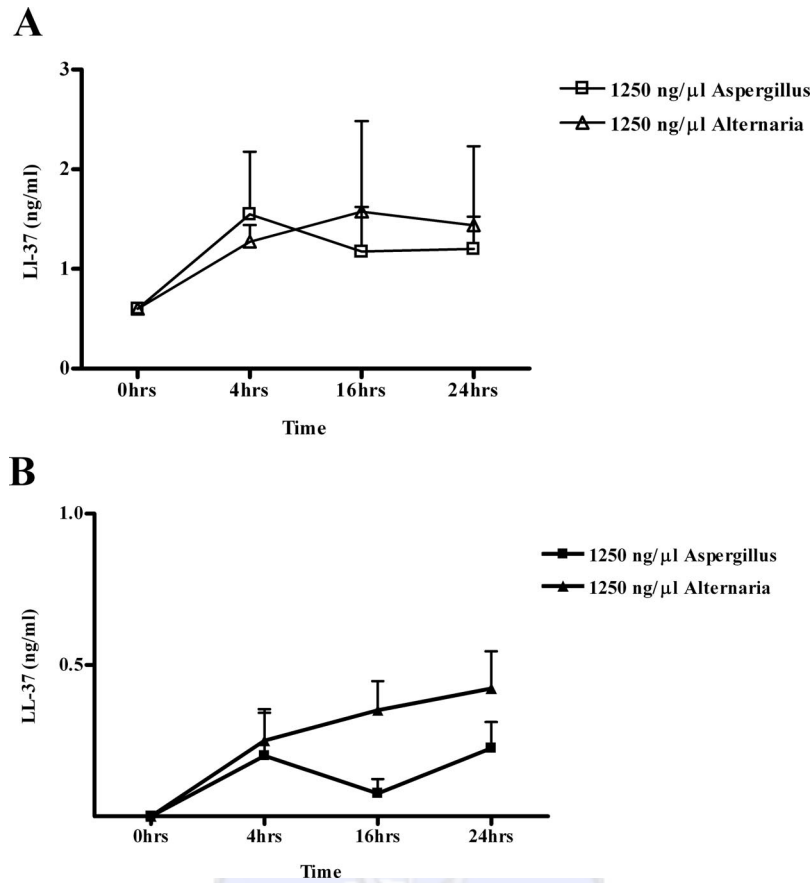


Figure 3. ELISA measuring LL-37 peptide in (A) tissue homogenates and (B) culture supernatant from human nasal tissue challenged with 1250 ng/μL of *A. fumigatus* and *A. tenuis*. Nasal tissue and culture supernatant harvested at 4, 16, and 24 hours after challenging with fungal allergens. ELISA values represented as the mean $\pm$ SEM ng/μL of LL-37 peptide.

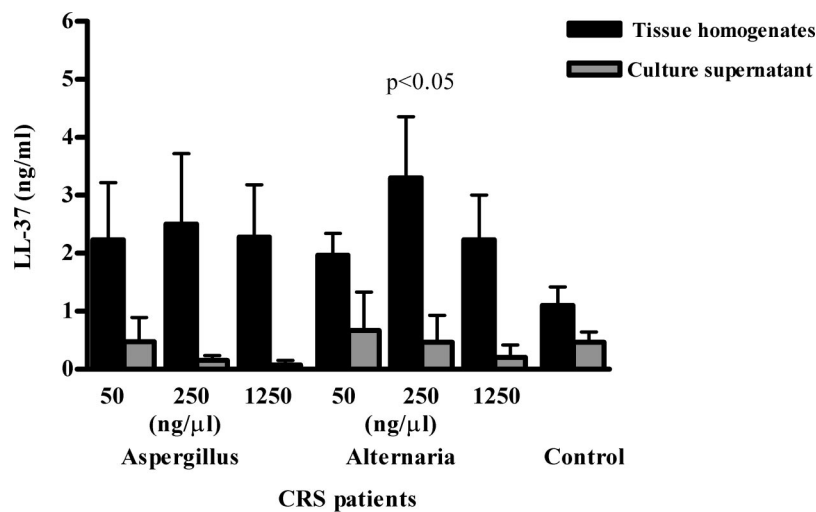


Figure 4. ELISA measuring LL-37 peptide levels in tissue homogenates and culture supernatant from nasal tissue of CRS patients challenged with *A. fumigatus*, *A. tenuis*, and control (no fungi). Values represented as nanograms per milliliter of LL-37 protein (mean $\pm$ SEM). There was a statistically significant difference in LL-37 levels in the tissue homogenates between 250 ng/μL Alternaria and control by one-way ANOVA and Tukey's multiple comparison test ($p < 0.05$).

in Fig. 4 ($p = 0.014$, one-way ANOVA). The 250-ng/ μL *Alternaria* induced an increase in LL-37 levels in the nasal tissue compared with untreated nasal tissue explant controls (mean, 3.3 ng/mL versus 1.1 ng/mL; $p < 0.05$). With 1250 ng/ μL of *Aspergillus* and *Alternaria*, there was an increase in LL-37 levels in the tissue (both mean, 2.2 ng/mL) compared with untreated nasal tissue controls as shown in Fig. 4 but this was not statistically significant. We observed a trend where the amounts of LL-37 protein in the culture supernatant decreased with higher doses of fungal allergens as shown in Fig. 4.

EMCRS Patients. The levels of LL-37 protein in nasal tissue homogenates from EMCRS patients *in vitro* were significantly increased to *Aspergillus* challenge but not to *Alternaria*. The 1250-ng/ μL *Aspergillus* produced the most significant increase in LL-37 protein levels in the nasal tissue ($p = 0.0034$, one-way ANOVA) compared with 1250 ng/ μL of *Alternaria* (mean, 4.8 ng/mL versus 6 ng/mL; $p < 0.05$) and untreated nasal tissue explant controls (mean, 4.8 ng/mL versus 27 ng/mL; $p < 0.001$) and the culture supernatant for all the *Alternaria* treatments and the control culture supernatant ($p < 0.01$) as shown in Fig. 5. There also was a trend for a reduction or inhibition in the amount of LL-37 peptide secreted into the culture supernatant in response to increasing doses of fungal allergens but this was not statistically significant.

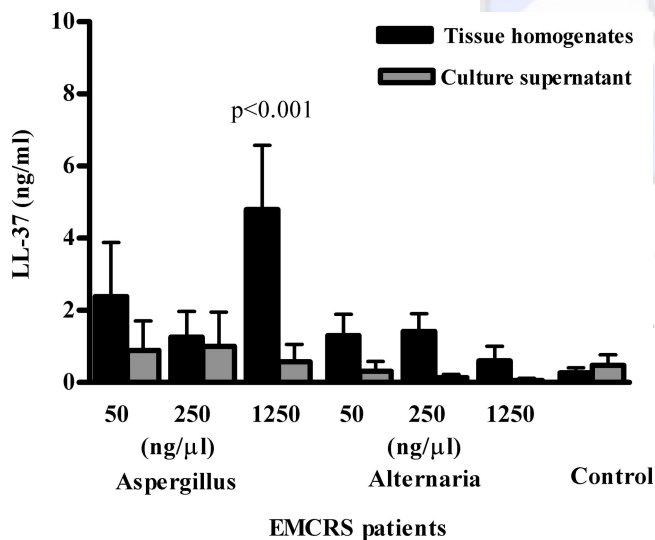


Figure 5. ELISA measuring LL-37 peptide levels in tissue homogenates and culture supernatant from nasal tissue of EMCRS patients challenged with *A. fumigatus*, *A. tenuis*, and control (no fungi). Values represented as nanograms per milliliter of LL-37 peptide (mean $\pm$ SEM). There was a statistically significant difference in LL-37 levels between 1250 ng/ μL of *Aspergillus* tissue homogenates and the control tissue homogenates ($p < 0.001$), the 1250 ng/ μL of *Alternaria* tissue homogenates and *Aspergillus* culture supernatant ($p < 0.05$), the culture supernatant for *Alternaria* and controls ($p < 0.01$) by one-way ANOVA and Tukey's multiple pair-wise comparison test.

DISCUSSION

We investigated the innate immune responses of nasal tissue to common fungi implicated in some patients with CRS in this study. This is the first study, to our knowledge, that shows increased LL-37 mRNA in response to *Aspergillus* and *Alternaria* allergens using an *in vitro* nasal tissue explant model in CRS patients. Using real-time PCR the increased LL-37 mRNA in response to 1250 ng/ μL of *Alternaria* was quantified as a mean sixfold increase and a mean fourfold increase to 1250 ng/ μL of *Aspergillus* in CRS patients with an increase in the tissue levels of LL-37 as detected by ELISA.

LL-37 mRNA also was up-regulated in EMCRS patients but this was not statistically significant. Our results suggest that EMCRS patients are initially able to express LL-37 mRNA constitutively but then are unable to increase LL-37 mRNA levels in response to increasing fungal allergen challenge possibly because of transcriptional interference. Other investigators have reported transient down-regulation of LL-37 in the gut from *Shigella* infection because of interference with transcription of LL-37.¹⁵ Using semiquantitative RT-PCR analysis of gut biopsy specimens from patients with *Shigella* dysentery compared with noninfected controls, it was shown that infection with *Shigella* spp was associated with loss and/or down-regulated expression of LL-37 mRNA from day 1 to day 30 biopsy specimens.¹⁵ However, by day 60, when the patients were clinically recovered, there was LL-37 mRNA detected by RT-PCR. Immunohistochemical staining of the gut biopsy specimens showed loss or barely detectable staining for LL-37 in the surface epithelium and gastric crypts but positive staining returned again at day 60.¹⁵ However, the LL-37 peptide was detected on Western blot at day 11, suggesting that down-regulation at the RNA level is caused by an interference with the transcription of LL-37. The same investigators then incubated the epithelial cell line HT-29 and monocyte cell line U937 with *Shigella* and showed a down-regulation in LL-37 mRNA by RT-PCR in those cell lines infected with *Shigella* compared with uninfected controls.¹⁵

The nasal tissue explant model rather than primary epithelial cell culture allows us the possibility to perform investigations of the responses of whole nasal tissue to fungal allergens. Nasal tissue explants have been used previously to investigate the role of superantigens on glucocorticoid receptor expression¹⁶ and the role of macrolides and corticosteroids on cytokine responses *in vitro*.¹⁷ Challenging nasal tissue from EMCRS patients with the highest dose of *Aspergillus* used in this study produced an increase in tissue LL-37 levels. In contrast, no increased expression of both tissue and secreted LL-37 with *Alternaria* in the EMCRS patients was observed. Possibly different species of fungi may have different pathogenicity to account for the differences in tissue LL-37 levels in the EMCRS group. The differences in pathogenicity with *Alternaria* may be caused by germination of *Alternaria* spores releasing a greater amount of the major allergens, especially Alt a 1.¹⁸ Increases in the airborne concentrations of *Alternaria* spores are shown to be associated with increases in symptoms of airway hyperresponsiveness.¹⁹

Airborne fungi and their spores are ubiquitous and inhaled in substantial amounts on a daily basis.⁶ In fact, fungus can be cultured from the nasal mucus of neonates and infants.²⁰ The presence of antimicrobial peptides at birth in the amniotic

fluid and human *vernix caseosa* displaying antibacterial activity against *Bacillus megaterium* and antifungal activity against *Candida albicans* contribute to eliminating pathogenic fungi.¹⁰

Because of ethical and technical limitations, we can not conclude from this study whether normal controls would have exhibited different LL-37 responses to the fungal allergens than the diseased patients. We observed a trend for LL-37 levels in the culture supernatant to decrease in response to increasing fungal allergens in both CRS and EMCRS but this were not statistically significant. Deficiency in the secretion of LL-37 into airway surface fluid has been implicated in chronic bacterial infections in cystic fibrosis.²¹ We speculate that a similar model may exist where fungal allergens may play a role affecting the secretion of LL-37 and this immune response may be pathogenic in CRS. The biological significance of altered LL-37 responses between different patient groups was not determined in this study but further *in vitro* studies exploring these observations are required.

CONCLUSIONS

In summary, the results from this study establish the response of cathelicidins to fungi at the mRNA level in CRS patients. Other factors are certainly involved in the inflammatory response in CRS patients and abnormal immune responses to fungi may be an important factor in the pathophysiology of CRS. These observations suggest that fungal allergens may possess the capability to induce mucosal innate immune mechanisms in CRS and EMCRS patients.

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