

COMMENTARY

Immunologic response to fungus is not universally associated with chronic rhinosinusitis

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ABSTRACT

In the December 2009 issue of this journal, Orlandi et al presented a study in which peripheral blood mononuclear cells (PBMCs) from chronic rhinosinusitis (CRS) patients (5 from Texas, 5 from Utah) and seven nonhealthy controls were stimulated with fungal extracts. Despite the small numbers, they confirmed important aspects of previous studies: 1) CRS patients' PBMCs react to certain fungal stimuli by producing significantly ($P < 0.05$) higher amounts of interleukin (IL)-5 and IL-13 when compared to controls; 2) CRS patients have an enhanced humoral response (significantly elevated immunoglobulin [Ig] G levels to *Alternaria*); and 3) CRS patients react independently from an IgE-mediated allergy, as evidenced by that fact that nonallergic CRS patients also produced IL-5 in response to fungal stimuli. Unfortunately, the authors chose not to highlight their positive results. They emphasized what they failed to demonstrate, specifically an immune response to fungi above a certain threshold in some patients (Utah) with milder CRS. However, these results are potentially explained by the different methods used, and care should be applied when interpreting their results.

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Recently, Orlandi et al published a study comparing the immune response to fungi in chronic rhinosinusitis (CRS) patients to controls.¹ They also investigated whether this response differs in patients residing in different climates (Texas vs Utah).

Orlandi et al used peripheral blood mononuclear cells (PBMCs) of five CRS patients from Texas who met the diagnostic criteria for allergic fungal rhinosinusitis (AFRS), stimulated them with five different fungal extracts, measured cytokine responses (interleukin [IL]-5 and IL-13), and compared them with five patients and seven controls from Utah, some of whom also had allergic rhinitis.

Although Orlandi et al found very high cytokine responses from Texas patients, there were lesser responses from Utah patients. They also reported cytokine responses in their control population without CRS, although with lesser amounts. The

patients' fungal-specific IgE correlated with the amount of IL-5 produced by the PBMCs, and fungus-specific IgG to *Alternaria* showed significant elevation in the CRS population.

Orlandi et al concluded that the immune response to fungi is not universal and does not correlate with the presence of CRS.

Some of Orlandi's results, and especially their interpretations and conclusions, vary from the original study by Shin et al, which the Orlandi study attempted to reproduce.²

Shin demonstrated that certain fungal extracts induced PBMC cytokine production (IL-5, IL-13) in CRS patients, which is crucial for the eosinophilic inflammation, independently from immunoglobulin E (IgE)-mediated allergy. More importantly, PBMCs from healthy controls did not respond. In addition, Shin found humoral responses to fungi in CRS patients. Fungal-specific immunoglobulin G (IgG) levels were not only elevated compared to healthy controls, but also correlated with the amount of IL-5 produced in the CRS patients. These findings that CRS patients have an exaggerated immune response to certain fungi, especially *Alternaria* antigens, were highlighted by a press release from the National Institutes of Health, which concluded that this "first-of-its-kind" study demonstrated a possible immunological basis for CRS.³ They found it an important starting point to better understand the etiology of CRS.

The methods used by Orlandi et al differ significantly from those of Shin et al, which makes the studies incomparable. They vary in their patient selection criteria, fungal extracts used, and different test settings, and especially in the interpretation of their results. Despite using fewer patients, Orlandi actually succeeded in confirming important elements of the Shin study:

- CRS patients produce significantly higher amounts of IL-5 in response to fungal stimulation when compared to controls.
- CRS patients have an enhanced humoral response (significantly elevated IgG levels to *Alternaria*).
- Fungus-induced IL-5 production is independent from an IgE-mediated allergy (some nonallergic Utah patients produced IL-5).

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Unfortunately, none of these positive findings is mentioned in the abstract or highlighted in the paper. We would like to discuss the difference between the two studies in detail, since each one of them limits the conclusions that can be drawn from Orlandi's paper.

In our view, the quintessence of Orlandi's publication is not whether CRS patients have an immune response to fungi, but whether one actually has a sensitive method to accurately test for it. A meaningful test requires a correct stimulus, which in this case was an *Alternaria* culture extract from Greer (GREER Laboratories, Inc., Lenoir, NC). Fungal extracts are a complex heterogeneous mixture of thousands of proteins. Their composition depends partially on the growth cycle of the live fungus, resulting in striking inhibitory or stimulatory differences for immune responses between the lots.^{4,5} Orlandi described those differences (Table A of their article, online only; available at www.otojournal.org), reflected in Figure 3 of their article,¹ where *Alternaria* extract 1 is shown to induce elevated IL-5 production in CRS patients versus controls; however, using *Alternaria* extract 2 resulted in much higher IL-5 production in both controls and CRS patients. As discussed by Orlandi, other ingredients such as endotoxins or glucan can also either stimulate or inhibit IL-5 production. All lots except one in Orlandi's study contained endotoxins, whereas the Shin study was endotoxin free (Greer lot XDM1-F17-32.8). In subsequent studies at the Mayo Clinic, striking differences were found between other *Alternaria* lots; some were much weaker or failed to produce consistent results with CRS patients' PBMCs. For example, lot F19, used by Orlandi, contained 320 µg total protein and induced a strong IL-5–dependent eosinophilia in mouse airways; however, lot F20 contained 60 µg/mL total protein and had no such effect. The lot in the Shin study was produced in 1995, contained 32.8 µg/mL total protein, and was very distinct in its capability to produce an immune response in CRS patients versus healthy controls.

The lot Shin used was no longer available for the Orlandi study, making valid comparisons impossible and potentially explaining differences in results between Shin and Orlandi. Thus the interpretation should be careful, especially when negative results are used to draw far-reaching conclusions by accepting the "null hypothesis" that the immune response to fungi is not universal. The finding that some of the antigen lots contain proteins that induce cytokine production, while others do not, is now very helpful. We can focus on proteins that are contained in certain lots but not in other lots that do not induce cytokine production. For example, *Alternaria* antigens responsible for the degranulation of eosinophils were found on a narrow 61-kD protein band.⁶ Some extracts do not contain these proteins and therefore have limited capabilities for stimulating eosinophil degranulation.⁶ The ultimate goal is to identify and purify the proteins responsible for the cytokine production, making future studies more comparable.

IL-5 production was measured in both studies because IL-5 is the most important cytokine for the eosinophilic inflammation. It is significantly elevated in CRS compared to healthy controls, and it partially controls eosinophil production, differentiation, activation, and life prolongation.⁷ IL-5 is produced with an IgE-associated mechanism (e.g., allergic rhinitis to fungi), but IL-5 is also produced without (independent from) an IgE-associated mechanism.^{2,7-9} Shin's data suggest that patients with CRS (with and without IgE allergy) respond specifically to *Alternaria* antigens. In addition, some patients produce IgE, further fueling IL-5 production via the IgE-associated mechanism.²

Orlandi states that CRS patients were randomly selected; however, all five Texas patients were preselected fulfilling the diagnostic criteria for AFRS, which is not representative of a randomly selected CRS population, since most patients do not fulfill the criteria for AFRS. By including only AFRS patients (Texas), Orlandi preselected patients with very severe disease, with a very intense eosinophilic inflammation, and (by AFRS definition) with only an IgE-mediated allergy to fungi. The five patients from Utah were selected using different criteria than the Texas patients, thus stacking the deck.

By preselecting the five Texas patients with an IgE-mediated allergy to fungi and very severe disease, Orlandi guaranteed a high IL-5 response to fungal protein antigens. Therefore, by study design, a correlation between fungal-induced IL-5 production and fungal IgE levels will be found. The findings displayed in Figures 1, 2, and 4 as well as the statistical analyses in Table 3 of the Orlandi study are simply a result of patient preselection. The authors admit that "it became apparent that higher values of IL-5 were seen in patients with higher total IgE, many of whom were CRS-Texas patients."

Patients preselected for AFRS have been shown to respond with striking amounts of cytokines to fungal stimuli, far higher than in the Shin study using Midwestern patients.¹⁰ Thus, Orlandi erroneously concludes that they demonstrated a profound effect of fungal-specific IgE on IL-5 production in many of their patients highly allergic to fungi.

One other striking difference between Orlandi and Shin is the fact that Orlandi's control group is not negative. This might be because some controls had elevated IgE (including some to fungi [Table 2 of Orlandi's article]), and would therefore produce IL-5 as part of an IgE-associated allergy, independent from CRS.

Shin's 15 healthy controls did not have elevated specific IgE (no detectable allergies) and did not produce any IL-5 in response to *Alternaria*. The 18 CRS patients produced between 2 and 120 pg/mL of IL-5 after *Alternaria* stimulation (one outlier producing 491 pg/mL), with a mean of 50 pg/mL.

By contrast, Orlandi had to use a threshold of 50 pg/mL of *Alternaria*-induced IL-5 production (Figure B of Orlandi's article).

di's article, online only; available at www.otojournal.org) to call patients positive, because of elevated IL-5 levels in the controls. This resulted in fewer patients classified as positive in their immune response to fungi, which questions the accuracy of the Orlandi title.

Elevated IL-5 in the control group also reduces the likelihood of reaching statistical significance between groups. This is especially the case with small groups of five to 10 patients, where a type I error, namely finding no statistically significant difference because of small sample sizes, is likely. Nevertheless, despite meager numbers, Orlandi demonstrated significantly higher IL-5 (*Alternaria* extract, $P = 0.03$) and IL-13 production (*Penicillium* extract, $P = 0.03$) in CRS patients, and a trend towards elevated IL-13 production with *Alternaria* ($P = 0.09$).

While Orlandi calls it a "potential shortcoming" of the Shin study that patients with allergic rhinitis were excluded in the control group, we call it a necessity. Using healthy controls, Shin ensured that the control group did not produce IL-5 via an IgE-associated pathway, an important design criterion when testing the hypothesis of a nonallergic IL-5 response in CRS patients.

Besides cellular (cytokine) responses to fungi, Shin also found that CRS patients (not healthy controls) have elevated humoral responses (elevated IgG) to various fungi. Those elevated levels correlated with the amount of IL-5 produced. IgG in general is viewed as a marker of exposure, and according to Shin's findings the greater the exposure to fungi (increased IgG), the greater the immune response (increased IL-5). In this way, Shin was the first to link exposure to a fungal organism and disease severity (or reactivity).

Despite the small numbers, Orlandi found significantly elevated ($P = 0.049$) IgG levels to *Alternaria* in CRS patients compared to controls (mainly Texas patients). IgG levels did not correlate with IL-5 levels; Orlandi analyzed the patients and controls together, making it less likely to find a correlation, whereas Shin demonstrated that this correlation only exists in CRS patients.

There is no question that warm and humid climates give different fungal exposure levels to patients. This results in elevated IgG levels, more severe disease, and higher IL-5 production (feeding the eosinophilic inflammation) in patients with CRS, all of which is supported by Orlandi's data.

Whether milder disease in Utah or the use of different stimuli put some patients below a positive threshold for such a complex ex vivo test is unknown.

So, can this study really exclude the possibility that a non-IgE response to fungus is not the universal answer to the question "What causes rhinosinusitis"? Negative data has its limitations. It shows that what one has done did not work, but it cannot answer why it did not work. And it certainly cannot invalidate studies that have worked.

Understanding the pathophysiology of CRS is more controversial than ever before. If we fail to communicate com-

mon observations, such as the similar findings between the Orlandi and the Shin studies, we will not succeed in finding the reason why the eosinophilic inflammation occurs, and continue to call it "multifactorial."

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Author Contributions

Jens U. Ponikau, substantial contributions to conception and design, analysis and interpretation of data, drafting the article and revising it critically for important intellectual content, final approval of the version to be published; **David A. Sherris**, substantial contributions to conception and design, analysis and interpretation of data, revising the article critically for important intellectual content, final approval of the version to be published; **Eugene B. Kern**, substantial contributions to conception and design, analysis and interpretation of data, revising the article critically for important intellectual content, final approval of the version to be published.

Disclosures

Competing interests: **Jens U. Ponikau**, listed as an inventor on patents regarding the antifungal treatment of CRS, asthma, and colitis, the diagnosis of CRS using major basic protein as a marker, the diagnosis of acute rhinosinusitis using neutrophil elastase as a marker, and the detection of fungi in human mucus; consultant: Hartington Pharmaceutical, and Immco Diagnostics (consultant fees are donated to charity); **David A. Sherris**, listed as an inventor on patents regarding the diagnosis of CRS using major basic protein as a marker, the diagnosis of acute rhinosinusitis using neutrophil elastase as a marker, and the detection of fungi in human mucus; **Eugene B. Kern**, listed as an inventor on patents regarding the diagnosis of CRS using major basic protein as a marker.

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