

Letters to the Editor

A novel method for assessing unchallenged levels of mediators in nasal epithelial lining fluid

To the Editor:

There is a need to develop noninvasive methods to sample epithelial lining fluid (ELF) from the respiratory system. The nasal mucosa is easily accessible, and it has long been recognized that there is a strong functional and immunologic relationship between the nose and bronchi.¹

It is possible to obtain samples of ELF by means of nasal lavage,² which has been used to measure inflammatory protein secretion after nasal allergen challenge. However, nasal allergen challenge exaggerates natural exposures, and the unknown dilution factor from nasal lavage is a significant confounder and might dilute the mediators to less than the detection limit of the assay.³ Therefore there is a need to measure mediators in the undisturbed ELF.

We propose a method for sampling nasal ELF using a synthetic absorptive matrix (SAM), separating the fluid by means of centrifugation, and analyzing the sample with a multiplex quantitative protein assay for a panel of inflammatory mediators. The advantage of this technique is that it analyzes undiluted ELF at the tissue interface without need for stimulation of the mucosa.

We conducted a case-control study of children with symptomatic allergic rhinitis and healthy control subjects from the Copenhagen Prospective Study on Asthma in Childhood birth cohort⁴ to assess whether unchallenged levels of nasal mediators could be detected in ELF collected with SAM and whether such levels associated with symptoms of allergic rhinitis.

The study was conducted August to September 2008 and was approved by the Copenhagen Ethics Committee.

Case status was determined based on allergic rhinitis⁵ with troublesome symptoms in the previous 24 hours, and control

status was determined based on the absence of any rhinitis symptom and any allergic sensitization. Ten cases (mean age, 8.3 years [SD, 1.1]; 70% male) and 15 control subjects (mean age, 8.2 years [SD, 1.1]; 73% male) were enrolled. Before investigation, subjects were not allowed to use antihistamines for the last 48 hours and nasal steroids or cromones for 14 days.

Nasal ELF was sampled with a strip of SAM (5 × 25 mm) inserted gently by means of direct vision into the nasal cavity laterally against the anterior portion of the inferior turbinate (see Figs E1 and E2 in this article's Online Repository at www.jacionline.org). A nasal clip was applied, and the SAM was left for absorption for 2 minutes. Thereafter, the SAM strip was removed and immediately stored at -80°C.

After thawing, the SAM was immersed in 300 µL of assay buffer and then placed in the cup of a tube filter within an Eppendorf tube and centrifuged for 5 minutes in a cooled centrifuge at 16,000g. The levels of IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13, IFN-γ, TNF-α, CXCL8 (IL-8), CCL11 (eotaxin-1), CCL26 (eotaxin-3), CXCL10 (IFN-inducible protein 10 [IP-10]), CCL2 (monocyte chemoattractant protein [MCP] 1), CCL13 (MCP-4), CCL22 (macrophage-derived chemokine [MDC]), CCL4 (macrophage inflammatory protein [MIP] 1β), and CCL17 (thymus activation-regulated chemokine [TARC]) in the eluates were analyzed by using the MesoScale Discovery multiplexed array system (MesoScale Discovery, Gaithersburg, Md). The sensitivities for all cytokines were 1 pg/mL or less, and those for chemokines were 1 to 50 pg/mL.

SAM consists of Accuwik Ultra Medium, a synthetic, fibrous, hydroxylated polyester medium that is commercially available and designed for sample collection, storage, and conjugate release. SAM is available in sheets of 8 inches × 10 inches (catalog no. SPR0730; Pall Life Sciences, Ann Arbor, Mich) and is cut into small strips for absorption of nasal ELF. The material is hydrophilic with low biomolecular binding, and

TABLE I. Cytokine and chemokine levels in SAM eluates

Nasal mediator	Patients with allergic rhinitis		Healthy control subjects		Patients with allergic rhinitis vs healthy control subjects P value
	Median	Range	Median	Range	
IL-1β	11.5	0.6-168.0	22.9	3.3-65.7	.45
IL-2	2.8	0.9-6.1	3.3	0.6-5.6	.74
IL-4	0.1	0-0.3	0.1	0.05-0.8	.66
IL-5	2.6	0.4-8.7	1	0.5-2.4	.01
IL-10	8.7	3.9-16.2	8.1	2.9-16.4	.91
IL-12p70	0.9	0.5-1.8	1.3	0.3-2.3	.26
IL-13	1.5	0.7-4.7	1.1	0.5-1.6	.01
IFN-γ	1.4	0-6.1	2	0.003-15.1	.28
TNF-α	6.7	2-11.8	8	2.3-20.7	.66
CXCL8 (IL-8)	3,313.9	1,305-10,634.7	4,764	739.8-14,274.4	.45
CXCL10 (IP-10)	28,650.8	8,492-49,344.6	12,413.9	346.7-39,635.0	.03
CCL2 (MCP-1)	41.2	18.6-107.2	22.2	10.7-86.4	.05
CCL4 (MIP-1β)	56.2	18.8-235.8	17.9	4.2-151.1	.01
CCL11 (eotaxin-1)	152.4	41.7-770.9	71.5	3.3-784.8	.04
CCL13 (MCP-4)	19.2	0-110.6	34	0-66.8	.70
CCL17 (TARC)	31.8	14.6-495.6	7.1	0-41.5	.02
CCL22 (MDC)	12	0-38	15.5	0-64.2	.68
CCL26 (eotaxin-3)	158.7	14.4-1,555	93	0-533	.93

All levels are presented as picograms per milliliter.

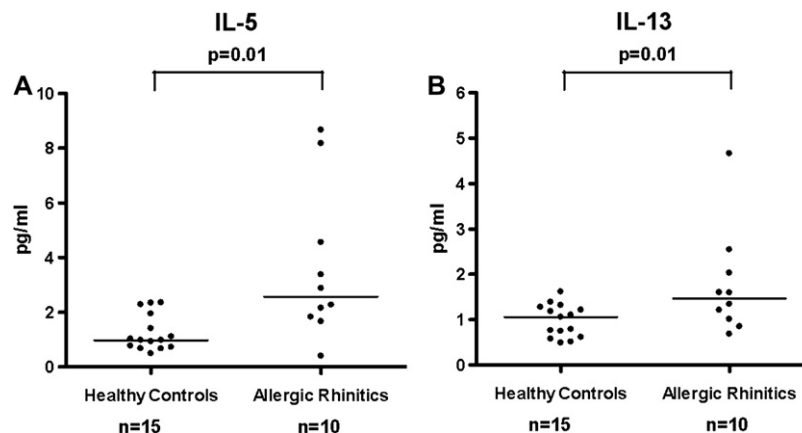


FIG 1. Levels of IL-5 (A) and IL-13 (B) in nasal secretions sampled with SAM in 25 children with allergic rhinitis and healthy control subjects. Horizontal bars represent median values for each group.

protein-containing samples are stable during storage at -80°C . The SAM is highly absorbent, and the fiber surfaces have been modified to enhance water wettability.

Differences in mediator levels in cases versus control subjects were tested by using the nonparametric Wilcoxon rank sum test.

Further details of the methods are outlined in this article's Online Repository at www.jacionline.org.

Unchallenged levels of 12 of the 18 mediators (IL-1 β , IL-2, IL-5, IL-10, IL-12p70, IL-13, TNF- α , CXCL8 [IL-8], CXCL10 [IP-10], CCL2 [MCP-1], CCL4 [MIP-1 β], and CCL11 [eotaxin-1]) were detectable in all samples, and IL-4, IFN- γ , CCL13 (MCP-4), CCL22 (MDC), CCL26 (eotaxin-3) and CCL17 (TARC) were detectable in approximately two thirds of the samples (Table I).

Levels of the cytokines IL-5 and IL-13 (Fig 1), as well as the chemokines CCL11 (eotaxin-1), CCL17 (TARC), CCL2 (MCP-1), CCL4 (MIP-1 β), and CXCL10 (IP-10), were significantly increased in patients with symptomatic allergic rhinitis compared with those seen in healthy control subjects (Table I).

Assay specificity was suggested from the association between levels of IL-5 and IL-13 (β -coefficient, 1.11; 95% CI, 0.7-1.6; $P < .0001$; $r^2 = 0.52$).

Thus we were able to detect nasal mediators in the ELF at undisturbed baseline levels in healthy children. Children with allergic rhinitis had increased levels of key T_H2-derived cytokines (IL-5 and IL-13) and chemokines (CCL11 [eotaxin-1] and CCL17 [TARC]) well known to be associated with eosinophilic and allergic inflammation.¹ These findings provide a completely new insight into the presence of mediators in healthy children and children with symptomatic allergic rhinitis without prior allergen challenge. Purely at a quantitative level, CXCL8 (IL-8) and CXCL10 (IP-10) were detectable in all subjects at levels exceeding 300 pg/mL, suggesting that there might be a physiological/pathophysiological function of these chemokines in ELF.

The major advantage of the SAM method is that it is noninvasive and easy to perform. It enables characterization of undisturbed immune mediator profiles in ELF of the upper airways. It is suitable for use in children, and it enables detection of many biomarkers that might be difficult to detect in the diluted nasal lavage fluid.⁶

In conclusion, the proposed method to measure immune mediator profiles in ELF is a feasible method to assess unchallenged baseline levels of a broad spectrum of nasal mediators in

children with healthy and diseased nasal passages. The technique enables important studies of early-life immune priming, upper airway inflammatory endophenotypes,⁷ infections (eg, H1N1v influenza⁸), pharmacotherapy, and immune therapy and might be used to characterize lower airway immunology by means of bronchoscopic microsampling.⁹

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REFERENCES

- Bousquet J, Van CP, Khaltaev N. Allergic rhinitis and its impact on asthma. *J Allergy Clin Immunol* 2001;108(suppl):S147-334.
- Howarth PH, Persson CG, Meltzer EO, Jacobson MR, Durham SR, Silkoff PE. Objective monitoring of nasal airway inflammation in rhinitis. *J Allergy Clin Immunol* 2005;115(suppl 1):S414-41.
- Bisgaard H, Robinson C, Romeling F, Mygind N, Church M, Holgate ST. Leukotriene C4 and histamine in early allergic reaction in the nose. *Allergy* 1988;43:219-27.

4. Bisgaard H. The Copenhagen Prospective Study on Asthma in Childhood (COP-SAC): design, rationale, and baseline data from a longitudinal birth cohort study. *Ann Allergy Asthma Immunol* 2004;93:381-9.
5. Chawes BL, Kreiner-Moller E, Bisgaard H. Objective assessments of allergic and nonallergic rhinitis in young children. *Allergy* 2009;64:1547-53.
6. Alam R, Sim TC, Hilsmeier K, Grant JA. Development of a new technique for recovery of cytokines from inflammatory sites in situ. *J Immunol Methods* 1992;155:25-9.
7. Anderson GP. Endotyping asthma: new insights into key pathogenic mechanisms in a complex, heterogeneous disease. *Lancet* 2008;372:1107-19.
8. de Jong MD, Simmons CP, Thanh TT, Hien VM, Smith GJ, Chau TN, et al. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. *Nat Med* 2006;12:1203-7.
9. Ishizaka A, Watanabe M, Yamashita T, Ogawa Y, Koh H, Hasegawa N, et al. New bronchoscopic microsample probe to measure the biochemical constituents in epithelial lining fluid of patients with acute respiratory distress syndrome. *Crit Care Med* 2001;29:896-8.

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Invasive fungal disease in autosomal-dominant hyper-IgE syndrome

To the Editor:

Autosomal-dominant hyper-IgE (Job) syndrome (AD-HIES), a result of heterozygous dominant-negative mutations in signal transducer and activator of transcription 3 (*STAT3*), is characterized by staphylococcal skin abscesses, eczematous dermatitis, connective tissue defects, and elevated serum IgE.^{1,2} Recurrent bacterial pneumonias, attributed to dysfunctional *STAT3*, frequently lead to bronchiectasis and formation of pneumatoceles. Fungal pneumonias, particularly aspergillosis, have also been reported in patients diagnosed with AD-HIES, albeit by clinical scoring system, and are a cause of mortality.³ However, the few reports of invasive mycoses in patients with HIES predated the ability to confirm the patients' diagnoses genotypically, potentially admixing heterogeneous phenocopies and precluding comprehensive understanding of fungal disease in AD-HIES. We sought to define the epidemiology of invasive mycoses in patients with confirmed *STAT3*-mutant AD-HIES. Further, because *STAT3* is implicated in multiple hematopoietic and nonhematopoietic processes, and the former are critical to antifungal resistance in other at-risk groups, we evaluated myeloid function related to fungal susceptibility.

Medical records of all patients with the diagnosis of AD-HIES enrolled on protocols of the National Institute of Allergy and Infectious Diseases, National Institutes of Health, and confirmed by *STAT3* sequencing² were reviewed. Patients with clinical, computed tomography, and laboratory evidence of "proven" and "probable" invasive fungal disease, based on European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) consensus definitions,⁴ were identified.

Sixty-four patients with *STAT3* AD-HIES were identified: 23 (36%) had no computed tomography evidence of lung bronchiectasis/cysts/cavities/pneumatoceles, whereas 41 (64%) did. Of those without lung damage, none had concurrent or previous invasive mycoses. Among the 41 patients with parenchymal lung defects, 18 (44%) had at least 1 episode of invasive mold disease, typically involving the respiratory tract. Thus overall, 28.1% of all patients developed an invasive mycosis. The isolated molds were *Aspergillus* spp (n = 16; 83.3%), *Scedosporium apiospermum* (n = 2), and *Histoplasma capsulatum* (n = 2). One patient had aspergillosis and subsequently *S apiospermum*; another had laryngeal histoplasmosis followed by aspergillosis. The other

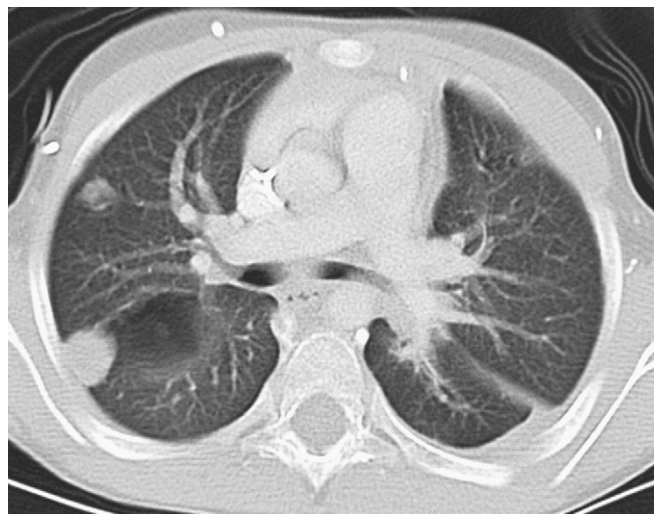


FIG 1. Computed tomography of invasive fungal pneumonia in AD-HIES.

TABLE I. Clinical characteristics of invasive fungal disease in patients with AD-HIES based on location of *STAT3* mutation

	Mutation in DNA-binding domain	Mutation in SH2-binding domain
No. of patients	34	30
Frequency of patients with structural lung disease	23 (67.6%)	18 (60%)
Frequency of patients with invasive fungal disease	10 (29.4%)*	8 (26.7%)*
Median age of onset of first fungal pneumonia (y)	32	30
Mortality as a result of invasive fungal disease	3	0

*All cases occurred in patients with structural lung disease.

case of histoplasmosis manifested as a tongue ulcer with right middle lobe lung involvement necessitating lobectomy. Mold disease did not solely manifest as "fungus balls" restricted to lung cavities but also manifested as consolidations without halo sign (Fig 1); 5 patients had disseminated disease. Two cases caused by the yeast *Cryptococcus* sp presented as an esophageal mass⁵ and meningitis, respectively; both occurred in patients with abnormal lung.

Stratification of patients based on location of *STAT3* mutation (DNA-binding domain vs SH2 domain) was performed to identify any genotype-phenotype correlation in regard to the development of fungal infections (Table I). The occurrences of structural lung disease, fungal disease, median age of onset of first fungal pneumonia, and mortality caused by fungal infection were not statistically different between domains (Fisher exact test). Treatment was variable (systemic antifungal therapy, with/without intracavitary instillation of antifungals or surgical resection). Overall, the mortality rate among those who developed fungal disease was 16.7%.

The integrity of myeloid functions associated with susceptibility to invasive mycoses was investigated. Neutrophils, PBMCs, and plasma were isolated from patients (3 AD-HIES; 3 chronic granulomatous disease [CGD]) and healthy donors (n = 4), and *Aspergillus fumigatus* B-5233, a pulmonary isolate from a leukemia

METHODS

After thawing, the SAM was immersed in 300 μ L of Milliplex assay buffer (catalog no. L-AB; Millipore, Billerica, Mass) and then placed in the cup of a cellulose acetate 0.22- μ m pore size tube filter (Spin-X Centrifuge Tube Filter, catalog no. CLS8161; Sigma-Aldrich, St Louis, Mo) within an Eppendorf tube and centrifuged for 5 minutes in a cooled centrifuge at 16,000g. The levels of IL-1 β , IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13, IFN- γ , TNF- α , CXCL8 (IL-8), CCL11 (eotaxin-1), CCL26 (eotaxin-3), CXCL10 (IP-10), CCL2 (MCP-1), CCL13 (MCP-4), CCL22 (MDC), CCL4 (MIP-1 β), and CCL17 (TARC) in the eluates were analyzed with the MesoScale Discovery multiplexed array system (Human 10-plex T_H1/T_H2 cytokine assay and 9-plex chemokine assay). Assays were conducted according to standard manufacturer's protocols, and samples were read with the Sector Imager 6000. The sensitivities for all cytokines were 1 pg/mL or less, and those for chemokines ranged between 1 and 50 pg/mL.



FIG E1. An SAM strip sized 5 × 25 mm cut from a sheet of 8 inches × 10 inches.



FIG E2. Insertion of an SAM strip into the nasal cavity laterally against the anterior portion of the inferior turbinate.