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Novel antigens of *Mycobacterium immunogenum* relevant in serodiagnosis of occupational hypersensitivity pneumonitis in machinists

Hypersensitivity pneumonitis (HP) in machinists is a chronic interstitial lung disease associated with exposures to in-use metal working fluid, particularly fluid contaminated with nontuberculous mycobacteria species *Mycobacterium immunogenum* (MI).[1–3] In this context, MI also has been shown to induce HP-like pathology in mice.[4] The general assumption has been that preformed antigens (and not a live infection) are responsible for inducing HP. However, a recent clinical study reported culturing of MI from the sputum of a metalworker.[5] Our recent study using a murine alveolar macrophage cell line demonstrated that MI can multiply intracellularly and induce inflammatory mediators implicated in HP.[6] Hence, machinists repeatedly exposed to the inciting mycobacterial antigens, preformed or generated de novo during infection, are at risk for developing HP.

Despite recognition of the mycobacterial etiology, there are no reliable serodiagnostic methods currently available for HP. Detection of serum immunoglobulins (eg, IgG) to the etiologic agent can be useful if their critical antigens are identified. In this direction, our first immuno-proteomic study on MI led to the identification of 33 immunoreactive proteins, including 4 secretory, 6 associated with the cell wall, 11 membranous, and 12 cytosolic.[7] In the present study, we generated soluble recombinant forms of 3 cell wall-associated proteins (arbitrarily designated antigens A through C [Ag A through Ag C]) and 2 secretory proteins (Ag D and Ag E) in *Escherichia coli* Rosetta Blue (DE3) cells (eTables 1 and 2). All 5 recombinant proteins were purified to homogeneity by the nickel and nitrilotriacetic acid method and made free from endotoxin (eFig 1).

Five physician-diagnosed cases of occupational HP representing machine workers (n = 5) from automotive plants exposed to microbial-contaminated machining fluids were included in the study and sampled for their blood sera (8 samples). Unidentified healthy control subjects (n = 8) for the study were volunteer blood donors to the Hoxworth blood center of the University of Cincinnati. All human serum samples were obtained and handled according to the protocol approved by the institutional review board. Detection of antibody response in the human subjects' sera against individual recombinant antigens (Ag A, Ag B, Ag C, Ag D, Ag E) and whole cell lysate of MI (crude antigen) was performed by optimizing a direct enzyme-linked immunosorbent assay method.[8] Individual sera were tested in triplicate and the results, calculated as means of optical density at 450 nm (eFig 2), were further analyzed based on receiver operating characteristics curve analysis using XLSTAT 2014 (Addinsoft, New York, New York). Sensitivity and specificity values were determined for each optical density value and threshold values were selected based on the highest sensitivity with greater than 80% specificity from the software-generated table for each antigen and crude MI lysate. Recombinant MI antigens and the MI lysate were compared by the area under the curve based on receiver operating characteristics curves and sensitivity-vs-specificity plots (eFig 3).

All 5 antigens showed high sensitivity (100%, 95% confidence interval [CI] 0.622–1.00) and specificity (87.5%–100%, 95% CI 0.505–0.0995) for optimal threshold (Table 1). Ag B showed the best response (sensitivity 100%, 95% CI 0.622–1.00; specificity 100%, 95% CI 0.622–1.00), whereas the MI lysate produced the least response (sensitivity 87.5%, 95% CI 0.505–0.995; specificity 87.5%, 95% CI 0.505–0.995) with a high threshold value (1.434).

Area under the curve values for receiver operating characteristics curves of all antigens were highly significant ($P < .0001$) compared with the cutoff area under the curve value (0.5); values for the different antigens, in descending order, were Ag B (1.00), Ag A, Ag C, Ag D, and Ag E (0.984), and MI lysate (0.953; eTable 3).

Optimal threshold values (decided based on highest sensitivity with specificity ≥80%) were determined for each antigen and the MI lysate. Values superior or equal to the threshold values of the patients' sera were considered positive for each antigen. Of the 8 sera (Hp1 through Hp8) from the patients with HP, 6 (Hp1 through Hp6) responded to all 5 antigens based on the threshold values, whereas Hp7 and Hp8 responded to only 4 of the 5 antigens (eTable 4). The entire panel of 5 antigens was used to set a global interpretation of likelihood of the disease (HP) on a scale of 1 to 4 (score 1, excellent [≥4 antigens were positive]; score 2, good [2–3 antigens positive]; score 3, fair [1 antigen positive]; score 4, poor [no antigen positive or all 5 antigens negative]; eTable 4).

Collectively, the results demonstrated that the candidate antigens are highly sensitive (100%) and specific (87.5%–100%) for discriminating patients with HP from healthy unexposed controls compared with the crude antigen (MI lysate), which showed lower sensitivity and specificity (87.5% each). In contrast to our findings, 2 candidate antigens, acyl-coenzyme A dehydrogenase and dihydrolipoyl dehydrogenase, recently tested for serodiagnosis of machinists with HP, produced much lower sensitivity (80% and 70%, respectively).[9]

Candidate antigens evaluated in our study represent proteins that are known to play important roles in mycobacterial physiology and pathogenesis. For example, Ag A, Ag B, and Ag D play important roles in DNA replication and translation, whereas Ag C and Ag E are required for the synthesis of cell wall components and are implicated in mycobacterial pathogenesis.[10]

This study is a significant step toward the development of clinical diagnostics for HP in machinists. The limitations of the study include a small study cohort and no matched control group of exposed subjects with no apparent symptoms, because of the widely recognized practical difficulty of accessing such patients and their matched exposed subjects. With the identified panel of antigens in hand, we are poised to extend the study to a larger cohort of patients with HP and exposed controls as and when these become available. Overall, the ongoing focus is to develop a comprehensive knowledge of the most relevant antigens of the etiologic agent (MI) using cutting-edge immuno-proteomic and immuno-informatics approaches for developing better diagnostics tools to assess the risk of personal exposure

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Table 1
Evaluation of individual recombinant antigens and whole cell lysate of MI on human sera from patients with hypersensitivity pneumonitis and controls using a serodiagnostic assay based on an enzyme-linked immunosorbent assay

Antigen	Threshold value	Sensitivity (95% CI)	Specificity (95% CI)	PPV	NPV	LR^+	LR^-	Accuracy
A	0.329	1.00 (0.622–1.00)	0.875 (0.505–0.995)	0.889	1.00	8.0	0.00	0.938
B	0.249	1.00 (0.622–1.00)	1 (0.622–1)	1	1	+Inf[a]	0.0	1.00
C	0.484	1.00 (0.622–1.00)	0.875 (0.505–0.995)	0.889	1.00	8.0	0.00	0.938
D	0.279	1.00 (0.622–1.00)	0.875 (0.505–0.995)	0.889	1.00	8.0	0.00	0.938
E	0.318	1.00 (0.622–1.00)	0.875 (0.505–0.995)	0.889	1.00	8.0	0.00	0.938
MI lysate	1.434	0.875 (0.505–0.995)	0.875 (0.505–0.995)	0.875	0.875	7.0	0.143	0.875

Abbreviations: CI, confidence interval; LR^-, negative likelihood ratio; LR^+, positive likelihood ratio; MI, *Mycobacterium immunogenum*; NPV, negative predictive value; PPV, positive predictive value.
[a]Plus infinity value for LR^+.

and disease in workers exposed to metal working fluid and to understand the immunologic mechanisms of the disease.

Supplementary Data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.anai.2015.03.005.

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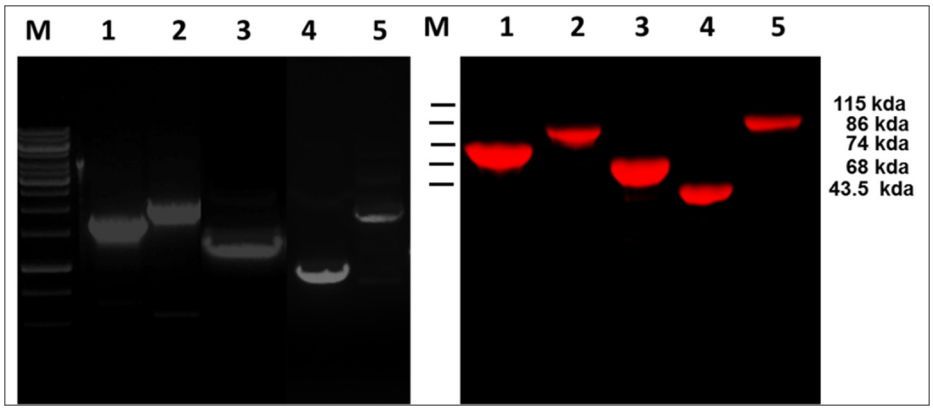
Contemplating the etiology of chronic urticaria and the implications of current guidelines

Chronic urticaria (CU) is a disorder defined as the presence of pruritus and urticaria for 6 weeks or longer.[1] Currently, a cause of CU is identified in only 5% to 20% of patients.[1–3] Although most specialists treating patients with symptoms of chronic urticaria pursue basic laboratory investigations to find an explanation for chronic urticaria, the recent American Board of Internal Medicine and American Academy of Allergy, Asthma, and Immunology Choosing Wisely Guidelines state that clinical history and physical examination are necessary for the diagnosis of CU but that laboratory evaluation has limited diagnostic utility.[4] Therefore, laboratory testing is only recommended when clinically indicated based on the presence of signs or symptoms beyond urticaria and pruritus. We describe one adult patient and one pediatric patient with CU and no other associated symptoms who were found to have autoimmune liver disease on further laboratory evaluation.

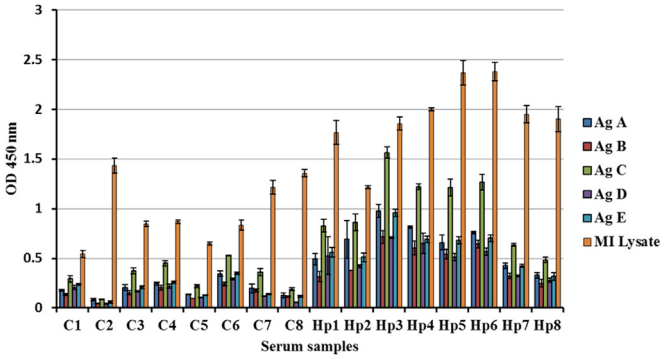
The first patient is a 44-year-old woman with a history of Hashimoto thyroiditis who presenting with daily pruritus and urticaria for 6 weeks. The rash was refractory to treatment with over-the-counter antihistamines but resolved after starting methylprednisolone therapy. Because the rash recurred 1 week after discontinuation of corticosteroid therapy, additional laboratory evaluation was pursued. The patient was prescribed colchicine, 0.6 mg twice daily, because of sedation from both first- and second-generation antihistamines with the plan to titrate the dose to 0.6 mg 3 times daily. The patient experienced complete resolution of her pruritus and urticaria within 1 month.

Initial laboratory workup revealed a normal complete blood cell count with differential, normal erythrocyte sedimentation rate, normal hepatic panel (including γ-glutamyl transpeptidase), and negative basophil histamine release assay. The thyroperoxidase antibody level was 306 IU/mL (reference range, <20 IU/mL), the C-reactive protein level was 0.69 mg/dL (reference range, <0.8 mg/dL), the antinuclear antibody titer result was mildly positive at 1:40, and the antimitochondrial antibody titer result was positive at 1:640. On subsequent testing, the mitochondrial M2 antibody level was significantly elevated at 131 IU/mL (reference

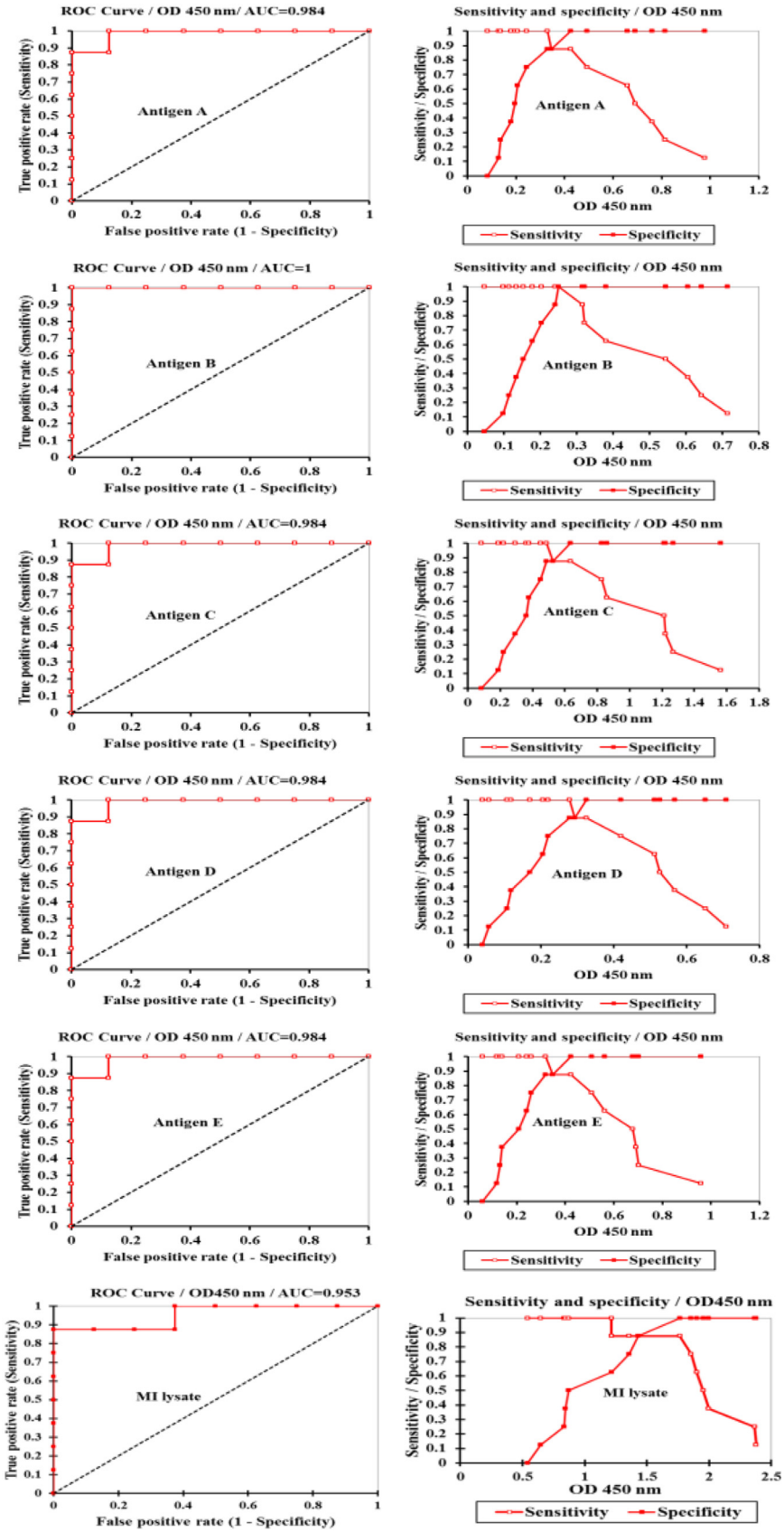
Disclosures: Authors have nothing to disclose.



eFigure 1. Cloning and expression of *Mycobacterium immunogenum* antigens. *(Left)* Genes amplified by polymerase chain reaction. *(Right)* Recombinant antigens purified by nickel and nitrilotriacetic acid. *Lane M,* DNA or protein marker; *lanes 1–5,* antigens A, B, C, D, and E.



eFigure 2. Antibody response of sera from machinist patients with hypersensitivity pneumonitis (Hp1–Hp8) and healthy control subjects (C1–C8) against *Mycobacterium immunogenum* (MI) recombinant antigens and whole cell lysate. Ag, antigen; OD, optical density.



eFigure 3. *(Left)* Receiver operating characteristics (ROC) curves and *(right)* sensitivity-vs-specificity plots for *Mycobacterium immunogenum* (MI) antigens (A–E) and whole cell lysate. Curves and plots were generated using XLSTAT 2014. Ag, antigen; AUC, area under the curve; OD, optical density.

eTable 1
Strains, plasmids, and primers used in this study

Strains	Genotype/phenotype	Source
Escherichia coli TOP 10	F-mcrA D(mrr-hsdRMS-mcrBC) W80lacZDM15 DlacX74 recA1 araD139 D(ara leu) 7697 galU galK rpsL (StrR) endA1 nupG	Invitrogen
Rosetta Blue (DE3)	endA1 hsdR17(rK12-mK12-) supE44 thi-1recA1 gyrA96 relA1 lac F′[proA + B + lacIqZΔM15::Tn10(TcR)] (DE3) pRARE6 (CmR)	Novagen
MI	*Mycobacterium immunogenum* 700506	ATCC
Plasmids		
pET-30a(+)	kanamycin resistance expression vector	Novagen
pHCJ-A	pET-30a containing antigen A	present study
pHCJ-B	pET-30a containing antigen B	present study
pHCJ-C	pET-30a containing antigen C	present study
pHCJ-D	pET-30a containing antigen D	present study
pHCJ-E	pET-30a containing antigen E	present study
Primers	sequences (5′ to 3′)	present study
Ag A FP	ATCGGATCCATGACCGATGGACCGCT	present study
Ag A RP	CGCAAGCTTCTAACCGATGGTGGGGC	present study
Ag B FP	ATCGAGCTCATGTCCGGCGTCACACC	present study
Ag B RP	CGCAAGCTTCTAGCTGTTGTTGGCGGC	present study
Ag C FP	ATAGGATCCGTGGCGGCATCCGGAAC	present study
Ag C RP	CGCAAGCTTTCAATCATCCAGGCTCCCAA	present study
Ag D FP	ATCGAATTCGTGGCGAAGGCGAAGTTC	present study
Ag D RP	CGCAAGCTTTCACTTGATGATCTTGGTGACG	present study
Ag E FP	ATCGAGCTCGTGGTGAGTGCCCAAGATCTG	present study
Ag E RP	CGCAAGCTTTCAGGACAGCAGGGCGG	present study

Abbreviations: Ag, antigen; FP, forward primer; MI, *Mycobacterium immunogenum*; RP, reverse primer.

eTable 2
Mycobacterium immunogenum antigens used in study

Antigen	Gene homolog	Protein name	Gene size (kb)	Cellular localization
A	MAB_0860c	DNA helicase	1.6	cell wall
B	MAB_1310	GTP binding translation, elongation factor	1.974	cell wall
C	MAB_2001	N-acetylmuramoylanyl-D-glutamate-2, 6-diaminopimelate ligase	1.5	cell wall
D	MAB_3848c	elongation factor Tu	1.194	secretory
E	MAB_0578c	trehalose phosphatase	2.5	secretory

eTable 3
AUC values for receiver operating characteristics curves of individual MI recombinant antigens (A through E) and MI whole cell lysate

Antigens	AUC	SE	95% CI lower bound	95% CI upper bound	Comparison of AUC to 0.5 (2-tailed test), *P* value (95% CI)
A	0.984	0.060	0.867	1.000	<.0001 (0.367–0.602)
B	1.00	0.062	0.879	1.000	<.0001 (0.379–0.621)
C	0.984	0.060	0.867	1.000	<.0001 (0.367–0.602)
D	0.984	0.060	0.867	1.000	<.0001 (0.367–0.602)
E	0.984	0.060	0.867	1.000	<.0001 (0.367–0.602)
MI lysate	0.953	0.000	0.953	0.953	not defined

Abbreviations: AUC, area under the curve; CI, confidence interval; MI, *Mycobacterium immunogenum; SE, standard error.*

eTable 4
ELISA index values for individual sera from patients with hypersensitivity pneumonitis against MI recombinant antigens and MI lysate and their comparison with respective threshold values for global interpretation of diagnosis[a]

Antigen	ELISA index (optical density 450 nm) for sera								
	Hp1	Hp2	Hp3	Hp4	Hp5	Hp6	Hp7	Hp8	Threshold value
A	0.494[b]	0.690[b]	0.976[b]	0.813[b]	0.659[b]	0.760[b]	0.425	0.329	0.426
B	0.315[b]	0.379[b]	0.715[b]	0.605[b]	0.545[b]	0.643[b]	0.322[b]	0.249[b]	0.249
C	0.828[b]	0.861[b]	1.564[b]	1.220[b]	1.211[b]	1.268[b]	0.635[b]	0.484[b]	0.484
D	0.527[b]	0.419[b]	0.708[b]	0.651[b]	0.512[b]	0.567[b]	0.324[b]	0.279[b]	0.279
E	0.561[b]	0.510[b]	0.959[b]	0.690[b]	0.679[b]	0.703[b]	0.423[b]	0.318[b]	0.318
MI lysate	1.765[b]	1.215[b]	1.857[b]	1.999[b]	2.368[b]	2.379[b]	1.953[b]	1.900[b]	1.434
Global interpretation	1	1	1	1	1	1	1	1	

Abbreviations: ELISA, enzyme-linked immunosorbent assay; Hp1–8, sera from patients with hypersensitivity pneumonitis; MI, *Mycobacterium immunogenum*.
[a]Threshold values were generated by receiver operating characteristics curve analysis using XLSTAT 2014. Values superior or equal to threshold values were considered positive. Global interpretation for the likelihood of hypersensitivity pneumonitis was made on an arbitrary scale of 1 to 4 for a test involving a panel of 5 recombinant MI antigens: 1 = excellent (≥4 positive antigens), 2 = good (3 positive antigens), 3 = fair (1–2 positive antigens), 4 = poor (all 5 antigens negative).
[b]Values superior or equal to threshold values.