

	E1	
MidLDH Epitope peptide	YDVVVLGAGPGGYVAAIRAAQLGLKTAIVEE-KYWGGVCLNVGCIPSKALLRNAELANVI	59
LcDLDH Epitope peptide	YDVVVIGGGPGGYPAAIKAAQQGLKVCIEKRGSLGGTCLNVGCIPSKAMLNNSHIYNAI	60
	*****:*.***** ***:*** ***: * :*: ***,*****:*,*.:. * *	
	E2 E6 E3 E4	
MidLDH Epitope peptide	IATGSVHTHTLSEALQEAIGMEFAYVLKNGYVDVTIVEFLPRALPNEDIDERMRNTNVP	119
LcDLDH Epitope peptide	IATGSCHAHPTLSEAFKEAIGLELGSVWSRLGAETVVEYLDAGAGMDVDEYQNTSTSG	120
	*****:*****:*****:*. * .. *:***:***: * :*** ..	
	E4 E5	
MidLDH Epitope peptide	IYAIGDVTSLQLAHVAEQPQVASFGLTEEQARA	153
LcDLDH Epitope peptide	VLALGDACGNYELTPVAIHPTAGTIGLTEDQARA	154
	:*:*.. .: :*: ** :* ..:*****:***	

FIG 2. Putative epitope peptides E1, E2, E6, E3, E4, and E5 from *Mycobacterium immunogenum* and *Lichtheimia corymbifera* were joined without amino acid internal sequences to produce 2 artificial recombinant epitope peptides called MiDLDH Epitope peptide and LcDLDH Epitope peptide, respectively.

*Perfect conservation. :Conservative substitution.

epitopes. The amino acid sequences of these 2 proteins were found to be 38% identical and 54% similar, respectively (Fig 1). Based on the hypothesis that highly similar parts of MiDLDH and LcDLDH were likely to be involved in antibodies/antigens reaction, we identified 6 sequences from 8 to 57 amino acids as potentially common epitopes. Then, we produced 2 large artificial recombinant epitope peptides where the 6 selected highly similar MiDLDH and LcDLDH parts were joined without amino acid internal sequences (Fig 2). These 2 large peptides, called MiDLDH Epitope peptide and LcDLDH Epitope peptide, were 54% identical and 66% similar, respectively.

Detection of specific IgG in patients with FLD (n = 12), patients with MOL (n = 14), healthy nonexposed controls (n = 12), and healthy exposed controls (n = 14 for FLD and 12 for MOL) was performed on sera sampled at the time of diagnosis by ELISA tests as previously described,⁶ using MiDLDH, LcDLDH, MiDLDH Epitope peptide, and LcDLDH Epitope peptide. Patient and control groups as well as methods for producing recombinant antigens and ELISA testing are described in this article's Online Repository at www.jacionline.org. Results of ELISA tests were given in the IgG index. The index was calculated as follows: IgG index = optical density of the patient or control sample/optical density of the reference sample.

Antibody level for each antigen in each group is indicated in Fig E1 in this article's Online Repository at www.jacionline.org, and the performance assessed using the receiver operating characteristics curve analysis is reported in Table E1 in this article's Online Repository at www.jacionline.org. The lower performance for discriminating patients with FLD using LcDLDH in the present study may be due to the smaller number of samples tested than in the previous one.⁵

ELISA responses (measured as IgG indexes) using LcDLDH were correlated to those obtained using MiDLDH in patients with MOL; conversely, ELISA responses using MiDLDH were correlated to those obtained using LcDLDH in patients with FLD (correlation coefficient: $r = 0.650$ and 0.904 for FLD and MOL, respectively; Pearson test, $P < .001$). These results attest to a strong cross-reactivity between the 2 orthologous proteins (see Fig E2 in this article's Online Repository at www.jacionline.org). ELISA responses obtained using full-length DLDH were correlated to responses obtained using the corresponding Epitope peptide in each of the diseases (correlation coefficient: $r = 0.954$ for LcDLDH Epitope peptide vs LcDLDH in patients with FLD; $r = 0.725$ for MiDLDH Epitope peptide vs MiDLDH in patients with MOL [see Fig E3 in this article's Online Repository

at www.jacionline.org]; Pearson test, $P < .001$). These results suggest that the Epitope peptides include the parts of MiDLDH or LcDLDH interacting with IgG antibodies in patients with MOL and FLD. Finally, ELISA responses obtained using LcDLDH Epitope peptide and using MiDLDH Epitope peptide were correlated in patients with FLD and in patients with MOL (correlation coefficient $r = 0.84$ and 0.97 in FLD and MOL, respectively; Pearson test, $P < .001$) (see Fig E4 in this article's Online Repository at www.jacionline.org).

In conclusion, we have shown the existence of common antigenic amino acid sequences in orthologous proteins from a fungus of the Mucorales order and a mycobacterium, each acting as a main etiologic agent of a specific form of HP. We accurately identified common epitope peptides in the DLDH antigen. A similar approach could be used to look for additional common epitope peptides in other orthologous antigens such as *M immunogenum* and *L corymbifera* Acyl coA dehydrogenase, which were also found to be antigenic disease-specific proteins in FLD and MOL, respectively.^{4,7} The existence of common epitopes in orthologous immunogenic proteins from microorganisms belonging to very different phylogenetic families explains how, in some cases of HP, more and more etiologic agents have been found for the same disease. This point was referred to as a "Pandora's box" at the time when HP serodiagnosis using immunoprecipitation techniques with crude antigens was initiated.²

Discovering epitopes involved in HP could promote a change in the current classification of HP based on the activity of the patient and on the microorganisms to which he or she is exposed. The result would be a more simple classification related to initial immunological interaction with an epitope peptide. The identification of epitope peptide sequences linked to HP could help us to better understand first interactions with the immune system of patients, and could help in developing new diagnostic and therapeutic strategies. *In vitro* studies of human specific cellular immune response to short peptides corresponding to part of the common epitope sequences are ongoing to complete this work.

Laurence Millon, PharmD, PhD^{a,b}

Benedicte Rognon, PhD^{a,b}

Benoit Valot, PhD^a

Coralie Barrera, PhD^a

Anne Pauline Bellanger, PharmD, PhD^{a,b}

Sandrine Roussel, PhD^{a,b}

Jean-Charles Dalphin, MD, PhD^{a,c}

Michel Monod, PhD^d

Gabriel Reboux, PhD^{a,b}

From ^aCNRS, University of Bourgogne-Franche-Comté, Chrono-Environnement, Besançon, France; ^bthe Department of Parasitology-Mycology, University Hospital of Besançon, Besançon, France; ^cthe Department of Pneumology, University Hospital of Besançon, Besançon, France; and ^dthe Department of Dermatology, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland. E-mail: laurence.millon@univ-fcomte.fr.

This work was supported by the French Ministry of Health (Projet Hospitalier de Recherche Clinique National SOPHIA, 2009-A00546-51).

Disclosure of potential conflict of interest: L. Millon receives grant support from the French Ministry of Health (Projet Hospitalier de Recherche Clinique National SOPHIA, 2009-A00546-51, and Projet Hospitalier de Recherche Clinique National ModiMucor, 2014-A00580-47). M. Monod receives grant support from Fond National Suisse. The rest of the authors declare that they have no relevant conflicts of interest.

REFERENCES

1. Lacasse Y, Selman M, Costabel U, Dalphin JC, Ando M, Morell F, et al. Clinical diagnosis of hypersensitivity pneumonitis. *Am J Respir Crit Care Med* 2003;168:952-8.
2. Pepys J, Ferrand C. Studies in extrinsic allergic respiratory disease: a personal history. *Allergy Proc* 1994;15:103-12.
3. Gari M, Smets P, Pinon JM, Recco P, Sequela JP. Use of immuno-electro-diffusion on cellulose acetate for the research of the precipitating antibodies in the screening of patients suspected of farmer's lung disease. *Mycopathologia* 1982;78:151-3.
4. Roussel S, Rognon B, Barrera C, Reboux G, Salamin K, Grenouillet F, et al. Immuno-reactive proteins from *Mycobacterium immunogenum* useful for serodiagnosis of metalworking fluid hypersensitivity pneumonitis. *Int J Med Microbiol* 2011;301:150-6.
5. Millon L, Rognon B, Barrera C, Valot B, Bellanger A, Quadroni M, et al. Immunoreactive proteins of *Lichtheimia corymbifera* for farmer's lung serodiagnosis. *Mycoses* 2015;58:219.
6. Barrera C, Millon L, Rognon B, Quadroni M, Roussel S, Dalphin JC, et al. Immunoreactive proteins of *Saccharopolyspora rectivirgula* for farmer's lung serodiagnosis. *Proteomics Clin Appl* 2014;8:971-81.
7. Rognon B, Barrera C, Monod M, Valot B, Roussel S, Quadroni M, et al. Identification of antigenic proteins from *Lichtheimia corymbifera* for Farmer's lung disease diagnosis. *Plos one* 2016;11:e0160888.

<http://dx.doi.org/10.1016/j.jaci.2016.06.037>

METHODS

Patients

Briefly, 12 patients with FLD, 14 patients with MOL, 26 healthy exposed controls (12 farmers [HECs/FLD] and 14 machine operators [HECs/MOL]), and 12 healthy nonexposed controls were included in the study.

Patients with FLD were farmers who had been recruited in the pneumology departments of hospitals in Clermont-Ferrand, Saint-Etienne, and Besançon (France) between 2008 and 2013. All patients with FLD were given a diagnosis according to consensual criteria^{E1} and had at least 1 positive serologic test result using electrosyneresis with 1 presumed FLD agent.^{E2}

HEC/FLD subjects were healthy farmers who were exposed to hay in the same geographic areas as patients with FLD. Spirometry, serology, and standardized medical questionnaires were used to ensure that none of the controls presented with any general or respiratory symptoms.

Thirteen patients with MOL and 12 HEC/MOL were employed at the same factory in the city of Lille, in northern France. An additional factory employee with MOL was recruited in Metz, in northeastern France (Department of Moselle). All patients with MOL were diagnosed in the Pneumology Department of the University Hospital of Lille according to previously published criteria.^{E3,E4} HEC/MOL subjects were volunteers exposed to metalworking fluids at the same plant, in the same workshop, and performing the same task as patients with MOL. They were selected and examined by the occupational medical doctor when they were included in the study. All patients had at least 1 positive serologic test result using electrosyneresis with 1 presumed agent of MOL.^{E5}

Nonexposed control subjects (n = 12) were healthy volunteers living in an urban area (Paris). They were recruited during blood donations in a specialized center. A questionnaire was used to establish that control subjects were not exposed to a farming environment, birds, or molds in their dwellings or workplaces. All were nonsmokers.

Clinical and biological data concerning patients and controls are described in previous articles (Tillie-Leblond et al^{E5} for MOL; Millon et al^{E2} for FLD).

The protocol was approved by the local ethics committee. Blood samples were taken for each subject who had given written consent, and centrifuged within 4 hours after sampling. Sera was immediately stored at −80°C.

Other methods

The 2 artificial recombinant proteins made up of the sum of the 6 analogue zones for each of the 2 species were produced using the plasmid pET-11aH6 and the *Escherichia coli* expression system as previously described.^{E6} The first one

was composed of amino acid sequences specific to DLDH from *M immunogenum* (called MiDLDH Epitope peptide) and the second of amino acid sequences specific to DLDH from *L corymbifera* (called LcDLDH Epitope peptide). His-tag recombinant protein production was induced with isopropyl β-D-1-thiogalactopyranoside and the proteins were then purified by affinity chromatography using Ni-NTA resin (Qiagen, Courtaboeuf, France) columns.

ELISA tests were performed with 52 sera (12 FLD, 14 HEC/FLD, 14 MOL, 12 HEC/MOL) to look for IgG against the 2 Epitope peptide recombinant antigens. The ELISA test was also carried out using MiDLDH and LcDLDH previously produced.^{E6,E7} Detection of IgG was also performed in 12 healthy nonexposed controls using LcDLDH.

ELISA tests for specific IgG were then conducted as previously described.^{E8} Serum samples from patients and controls were deposited, in triplicate, in the wells of 96-well plates. Each plate included a reference sample. Results of ELISA tests were given in the IgG index by calculating the ratio of optical density from the patients or control samples divided by the optical density of the reference sample.

REFERENCES

- E1. Lacasse Y, Selman M, Costabel U, Dalphin JC, Ando M, Morell F, et al. Clinical diagnosis of hypersensitivity pneumonitis. *Am J Respir Crit Care Med* 2003;168:952-8.
- E2. Millon L, Roussel S, Rognon B, Quadroni M, Salamin K, Reboux G, et al. Aspergillus species recombinant antigens for serodiagnosis of farmer's lung disease. *J Allergy Clin Immunol* 2012;130:803-5.e6.
- E3. Bourke SJ, Dalphin JC, Boyd G, McSharry C, Baldwin CI, Calvert JE. Hypersensitivity pneumonitis: current concepts. *Eur Respir J Suppl* 2001;32:81s-92s.
- E4. Girard M, Lacasse Y, Cormier Y. Hypersensitivity pneumonitis. *Allergy* 2009;64:322-34.
- E5. Tillie-Leblond I, Grenouillet F, Reboux G, Roussel S, Chouraki B, Lorthois C, et al. Hypersensitivity pneumonitis and metalworking fluids contaminated by mycobacteria. *Eur Respir J* 2011;37:640-7.
- E6. Roussel S, Rognon B, Barrera C, Reboux G, Salamin K, Grenouillet F, et al. Immuno-reactive proteins from *Mycobacterium immunogenum* useful for serodiagnosis of metalworking fluid hypersensitivity pneumonitis. *Int J Med Microbiol* 2011;301:150-6.
- E7. Rognon B, Barrera C, Monod M, Valot B, Roussel S, Quadroni M, et al. Identification of antigenic proteins from *Lichtheimia corymbifera* for Farmer's lung disease diagnosis. *Plos one* 2016;11:e0160888.
- E8. Barrera C, Millon L, Rognon B, Quadroni M, Roussel S, Dalphin JC, et al. Immunoreactive proteins of *Saccharopolyspora rectivirgula* for farmer's lung serodiagnosis. *Proteomics Clin Appl* 2014;8:971-81.

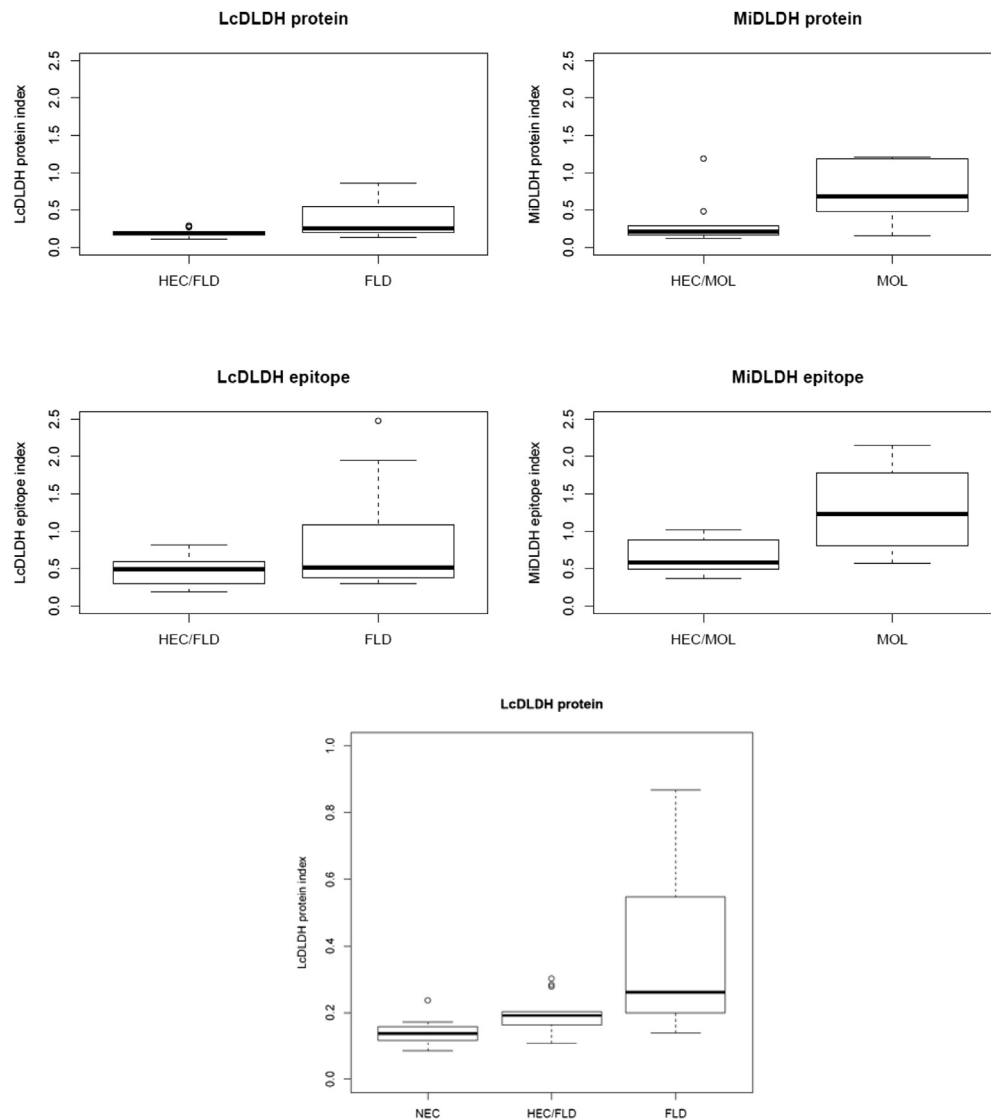


FIG E1. Antibody levels (measured as IgG index) for each group (HEC/FLD, FLD; HEC/MOL, MOL) with each antigen, and for NEC, HEC/FLD, and FLD with the LcDLDH antigen. *NEC*, Nonexposed control.

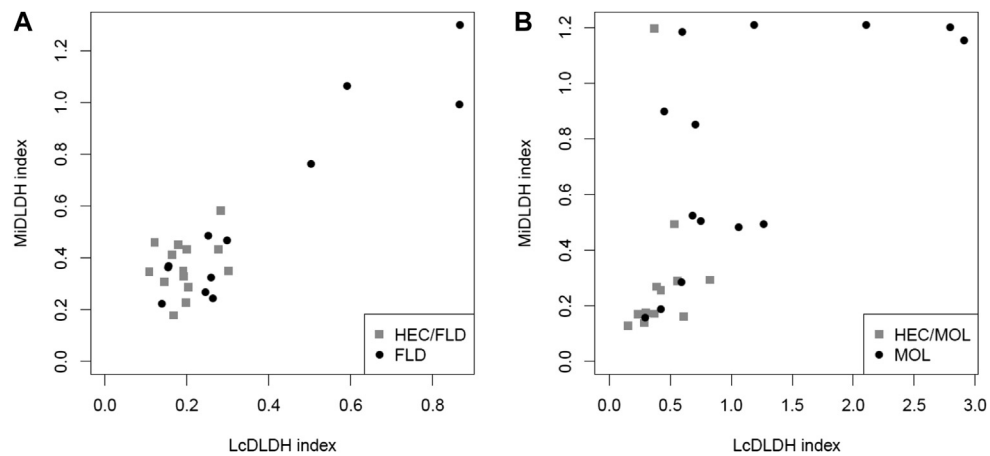


FIG E2. Correlation between ELISA response (measured as IgG index) obtained when using MiDLDH or LcDLDH in patients with FLD and MOL (correlation coefficient: $r = 0.904$ for FLD [A], and $r = 0.65$ for MOL [B]). If we remove the controls to calculate the correlation coefficient, $r = 0.937$ for FLD and $r = 0.636$ for MOL.

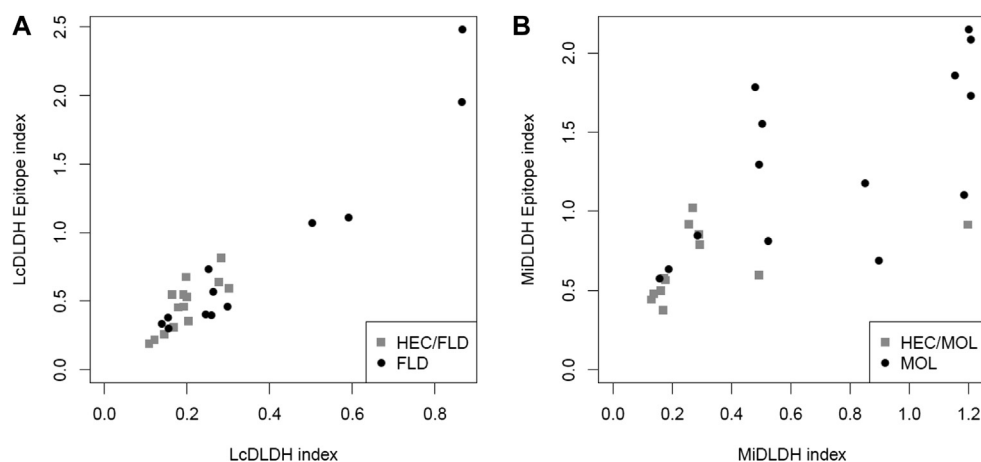


FIG E3. Correlation between ELISA response (measured as IgG index) obtained using full-length DLDH and obtained using the corresponding Epitope peptide in each of the diseases (correlation coefficient: $r = 0.954$ for LcDLDH Epitope peptide vs LcDLDH for FLD [A]; $r = 0.725$ for MiDLDH Epitope peptide vs MiDLDH for MOL [B]). If we remove the controls to calculate the correlation coefficient, $r = 0.964$ for FLD and $r = 0.655$ for MOL.

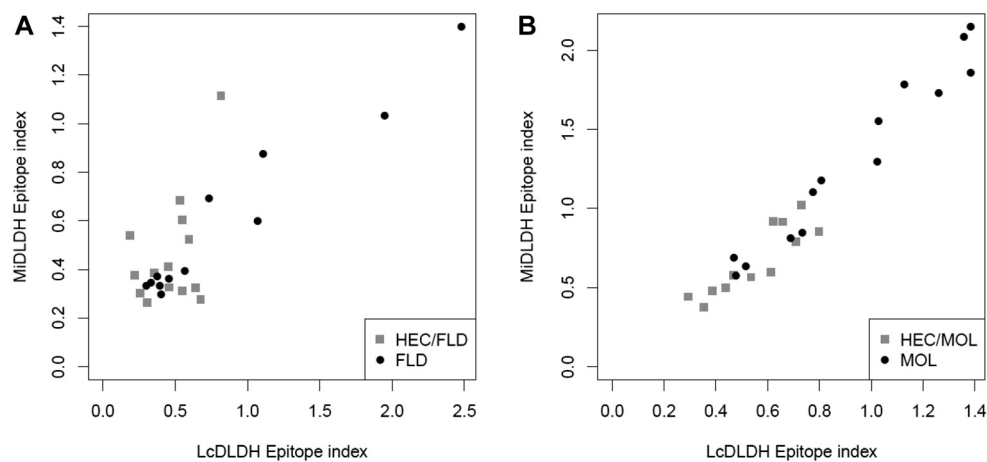


FIG E4. Correlation between ELISA response (measured as IgG index) obtained using MiLDH Epitope peptide or LcDLDH Epitope peptide in patients with FLD and MOL (correlation coefficient: $r = 0.839$ for FLD [A], and $r = 0.975$ for MOL [B]). If we remove the controls to calculate the correlation coefficient, $r = 0.870$ for FLD and $r = 0.978$ for MOL.

TABLE E1. Performance of ELISA test using the different recombinant antigens (receiver operating characteristics curve analysis, AUC)

Antigen	AUC	
	FLD (n = 12) vs HEC/FLD (n = 14)	MOL (n = 14) v HEC/MOL (n = 12)
LcDLDH	0.72	0.85
LcDLDH Epitope peptide	0.64	0.84
MiDLDH	0.63	0.82
MiDLDH Epitope peptide	0.62	0.85

AUC, Area under the curve.