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Research paper

Fungal peptides from pneumonitis hypersensitivity etiologic agents are able to induce specific cellular immune response

Anne-Pauline Bellanger [a,b,*], Thibaud Lignon [b], Yann Godet [c], Bénédicte Rognon [a], Gabriel Reboux [a,b], Houssein Gbaguidi-Haore [d], Christophe Borg [c], Laurence Millon [a,b]

[a] Chrono-Environnement CNRS 6249 Research Team, Franche-Comté University, Besançon, France
[b] Parasitology-Mycology Department, Besançon University Hospital, Besançon, France
[c] INSERM Unit 1098, University of Franche-Comté, Besançon, France
[d] Infection Control Department, Besançon University Hospital, Besançon, France

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ABSTRACT

Purpose: Hypersensitivity pneumonitis (HP) is an immunoallergic disease due to chronic exposure to high quantities of different microorganisms such as *Mycobacterium immunogenum* (Mi), a mycobacterium, and *Lichtheimia corymbifera* (Lc), a filamentous fungus. It has recently been demonstrated that the protein DLDH (dihydrolipoyl dehydrogenase), is common to these microorganisms. This study aimed to investigate the immune potential of overlapping peptide pools covering the MiDLDH and LcDLDH.
Experimental design: A selection of 34 peptides, from the MiDLDH and LcDLDH, able to interact with Major Histocompatibility Complex (MHC) 1 and MHC 2, was obtained using three different epitope prediction websites. By means of ELISPOT assays, we compared the frequency of Interferon gamma (IFNγ) secreting peripheral blood mononuclear cells (PBMC) after stimulation with overlapping peptide pools. Tests were performed using cells from 35 healthy blood donors.
Results: One peptide pool containing five peptides from MiDLDH and able to interact with MHC 2 induced a marked IFNγ specific immune response (Pool F, $p < 0.001$, Wilcoxon signed-rank test).
Conclusion: This study demonstrated that peptides from microorganisms involved in HP were able to induce a high IFNγ specific immune response after stimulation of PBMCs from healthy blood donors which could be useful to develop an effective prevention strategy.

1. Introduction

Hypersensitivity pneumonitis (HP), also known as extrinsic allergic alveolitis, is an inflammatory lung disease associated with a lymphocytic alveolitis, caused by an exacerbated immune response to repeated inhalations of antigens. A wide variety of causative antigens and environmental settings have been described including actinomycetes, bacteria, and filamentous fungi (Lacasse et al., 2012).

The most commonly reported HP form is called Farmer's Lung Disease (FLD) and is associated with farming (moldy grain or hay handling) (Girard et al., 2009). *Lichtheimia corymbifera* (Lc), a filamentous fungus frequently identified in environmental samples, plays an important role in HP development by triggering a marked innate immune response (Bellanger et al., 2010). A novel form of HP among machinists exposed to contaminated metalworking fluids by *Mycobacterium immunogenum* (Mi) has recently emerged and is called Machine Operator's Lung (MOL) (Beckett et al., 2005; Tillie-Leblond et al., 2011).

Previous studies investigating cytokine profiles in broncho-alveolar lavage fluid (BALF) have established that HP is caused by a Th1 type immune response, as specimens obtained from individuals with HP displayed abundant IFNγ, TNFα, IL-8, and IL-12 (Mroz et al., 2008; Yamasaki et al., 1999; Ye et al., 2009). Previous *in vitro* studies also demonstrated that, ultimately, a Th1 response was promoted by dendritic cells of healthy blood donors, regardless of the nature of HP etiologic agent tested (actinomycetes, mold and mycobacteria) (Bellanger et al., 2013). All these results are consistent with the hypothesis that upregulation of Th1 signaling plays a critical role in the pathogenesis of HP.

A strategy consisting in using sera from patients with HP (FLD and MOL) to identify immunogenic proteins from environmental microorganisms has been applied recently and allowed the selection of immune-reactive proteins for *M. immunogenum* (Mi) (Barrera et al., 2014a; Roussel et al., 2011), *S. rectivirgula* (Barrera et al., 2014b), *Aspergillus* species (Millon et al., 2012; Millon et al., 2014) and *L. corymbifera* (Lc) (Rognon et al., 2016). Among the immune-reactive proteins useful for a standardized and sensitive HP serodiagnosis, the protein dihydrolipoyl dehydrogenase (DLDH) was identified and

Abbreviations: HP, hypersensitivity pneumonitis; DLDH, dihydrolipoyl dehydrogenase; Lc, *Lichtheimia corymbifera*; Mi, *Mycobacterium immunogenum*.

* Corresponding author at: Parasitology-Mycology Department, University Hospital Jean Minjoz, 25030 Besançon, France.
E-mail address: apbellanger@chu-besancon.fr (A.-P. Bellanger).

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proved to be suitable to discriminate HP patients from controls for both FLD and MOL. The DLDH plays a role in the decomposition of the dihydrolipoamide deshydrogenase and is a virulence factor in *Mycobacterium tuberculosis* (Chang et al., 2007). This protein of 486 amino-acids (aa) presents the particularity of being common to two major etiologic agents of HP, Mi and Lc, which are from very different phylogenetic families (Millon et al., 2016).

This study aimed to investigate the potential of overlapping peptide pools covering the MiDLDH and LcDLDH to induce specific recall responses and Interferon gamma (IFNγ) immune response after stimulation of PBMC from healthy blood donors.

2. Methods

2.1. Peptide selection

Comparing the DLDH amino-acid (aa) sequence for Mi and Lc made it possible to select six sequences from 8 to 57 aa as potentially common epitopes (Millon et al., 2016). Three disparate websites of epitope prediction were used to test the ligation strength to a defined Major Histocompatibility Complex (MHC) type for each sequence of aa: Syfpeithi, Immuneepitope, and NETMHCII.

Peptides were tested for ligation with HLA-A*01,*02, *03 and B*07 with syfpeithi: all the peptides with a score ≥20 for one of the MHC allele tested were selected; peptides were tested for ligation with HLA-DRB1*01, *07, *11, *13,*15, DRB4*01 and DRB5*01 with syfpeithi, immuneepitope and NetMHCII. Peptides, whose ability to bind with these MHC was supported by 2 out of 3 algorithms, were selected. These MHC alleles were chosen according to their frequencies in the Caucasian population (Gragert et al., 2013). Peptides were synthesized by ProteoGenix SAS (Schiltigheim, France) and dissolved in 10% DMSO (Sigma). Peptide pools A, B, C and F contained peptides from the MiDLDH sequence, and pools D, E and G contained peptides from the LcDLDH sequence. Stimulation experiments were done using peptide pools of 4 to 5 peptides: 5 peptide pools (A to E) were pools of 9 to 10 amino-acid (aa) peptides and 2 peptide pools (F and G) were pools of 15 to 20 aa peptides.

2.2. Cell collection

Stimulation experiments were performed with PBMCs from healthy blood donors from the French Blood Institute of the Bourgogne/Franche-Comte regions (EFS). Peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation using Ficoll-Paque™ Premium (Dutscher, Brumath, France).

2.3. Stimulation protocol

PBMCs were washed, counted and stimulated by peptide pools (4 μg/mL of each peptide for 4 million PBMC). Cells were incubated in RPMI 1640 (Dutscher) supplemented with 8% human serum (EFS) and 5% penicillin streptomycin (Dutscher) for 14 days. Human Interleukin 7 (IL-7) (Peprotech, Neuilly sur Seine, France) was added on day 3 (5 ng/mL) and human IL-2 (Novartis, Switzerland) was added to the culture media on day 6 (20 UI/mL) and day 9 (20 UI/mL). CEF-class I peptide pool classic (CTL, Bonn, Germany) was used as a positive control (2 μg/mL). The CEF peptide pool is made of 23 individual peptides, each corresponding to a defined HLA class-I-restricted T cell epitope from cytomegalovirus, Epstein Barr virus and influenza virus (Flu). The CEF peptide pool stimulates corresponding peptide-specific $CD8^+$ memory T cells to release IFNγ. These peptides have been shown to elicit recall responses in the majority of individuals. This control was appropriate for our aim of investigating if stimulation with the DLDH peptide epitopes were able to induce recall responses in randomly selected human donors. Cells were tested for IFNγ secretion by ELISPOT assay (Diaclone, Besançon, France) on day 14. Recent methodological studies demonstrated that a longer period of *in vitro* stimulation (over 10 days) provided an effective method to enhance the detection of antigen-specific T-cell populations (Chudley et al., 2014; Geyeregger et al., 2013). The *in vitro* stimulation protocol applied here was previously used to investigate antitumor T cell response in immunochemotherapy studies (Godet et al., 2012a, 2012b).

2.4. IFNγ ELISPOT assay

A peptide pool's ability to induce cellular immune recall response was determined by measuring IFNγ upon stimulation of PBMC. For IFNγ ELISPOT assay, 96-well multiscreen filter plates (Diaclone) were coated with 100 μL coating IFNγ antibody (Diaclone) and incubated overnight at 4 °C. PBMC were seeded in triplicate at 1×10^5/well without peptides (negative control) or with peptides (4 μg/mL) or with a mix of PMA (50 ng/mL, Phorbol 12-myristate 13-acetate, Sigma Aldrich, St Quentin Fallavier, France) and Ionomycin (1 μg/mL, Sigma) and incubated at 37 °C/5% CO_2. The PMA/Ionomycin mix was used as positive control for the Elispot assay. Plates were washed three times with PBS/0.05% Tween 20 (Sigma) and incubated 2 h at room temperature (RT) with 100 μL biotinylated detection IFNγ antibody (Diaclone), washed three times and incubated 1 h at RT with 100 μL streptavidin (Diaclone). Spots were developed by adding 100 μL BCIP/NBT (Diaclone) for 15 min. IFNγ spots were counted using an ELISPOT reader (CTL, Bonn, Germany). Fig. 1 presents an example of an ELISPOT assay. The number of spots forming units (SFU)/10^5 PBMCs was calculated from triplicates after subtraction of a negative control. An IFNγ ELISPOT was considered positive for >10 SFU/ 10^5 PBMC as recommended by Alexander et al. (2013).

2.5. Blast analysis

The sequences of each peptide were tested for cross-reactions with other micro-organisms on the NCBI blast (proteins) website. Each sequence was tested on the following address http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins and the micro-organisms having a protein sequence cross-reacting at 100% with the peptide tested were listed.

2.6. Statistical method

For comparisons of paired continuous data (related samples), the Friedman test and the Wilcoxon signed-rank test were performed. In addition, Spearman's rank correlation test was carried out to assess the relationship between these continuous variables. The software package Stata 10 (StataCorp LP, College Station, TX) was used for the statistical analysis. All tests were two-tailed, and p values of <0.05 were considered statistically significant.

3. Results

3.1. Prediction and in silico peptide selection

Aligning amino-acid sequences of MiDLDH and LcDLDH revealed several conserved domains (Millon et al., 2016). Focusing on these domains, a set of twenty-four peptides of 9 to 10 aa was first selected according to their prediction score to bind to HLA-A*01, *02, *03 or B*07, based on a score ≥20 using Syfpeithi (Table 1). These peptides were pooled in 5 groups. Peptide pools A, B and C are composed of HLA class I restricted peptides derived from the MiDLDH. Peptide pools D and E are composed of HLA class I restricted peptides derived from the LcDLDH sequence (Table 1). Peptides B2 and E3 are predicted to be promiscuous with binding scores of 21, 24 and 21, 21 for HLA-A*02 and HLA-A*03 respectively.

A set of ten peptides of 15 to 20 aa was selected according to their prediction score to bind to DRB1*01, *07, *11, *13,*15, DRB4*01 or