Research paper

Positive fungal quantitative PCR and Th17 cytokine detection in bronchoalveolar lavage fluids: Complementary biomarkers of hypersensitivity pneumonitis?

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Interstitial lung disease (ILD) is a large group of diseases, including hypersensitivity pneumonitis (HP), idiopathic pulmonary fibrosis (IPF) and sarcoidosis. It is often difficult to distinguish between ILDs, due to their nonspecific clinical and functional signs (Jeong et al., 2014). However, accurate diagnosis is important because the various ILDs have different prognoses and require specific management.

HP is an immunologically mediated inflammation of the lung parenchyma in response to the repeated inhalation of large amounts of microbial antigens. The disease is characterized by lymphocytic alveolitis and noncaseating granulomas, and it may develop into a chronic form with fibrosis (Girard et al., 2009). HP is classified as a Th1 disease, as TNFα, IL-8 and IL-12 have been detected in the bronchoalveolar lavage fluid (BALF) of HP patients (Mroz et al., 2008; Ye et al., 2009). Recent studies have suggested that, in addition to Th1 factors, a Th17 response may be involved in HP (Joshi et al., 2009; Simonian et al., 2009). The absence of switch to a Th2 response in the immunopathology of HP was recently confirmed (Andrews et al., 2016). The clinical diagnosis of HP is based on the association of clinical and biological markers, evidence of interstitial abnormalities on chest X ray, the presence in the serum of precipitating antibodies against the antigens involved, lymphocytic alveolitis on bronchoalveolar lavage and/or a granulomatous reaction on lung biopsy (Girard et al., 2009).

An imbalance in the production of cytokines/chemokines, with a shift to the Th1 profile, has also been identified as a key feature of sarcoidosis, a multisystem inflammatory disease of unknown etiology characterized by the formation of epithelioid granulomas, with the accumulation of CD4⁺ Th1 lymphocytes and macrophages (Tondell et al., 2014). Recent data demonstrated that Th17 cells were also involved and actively participated in sarcoidosis progression (Facco et al., 2011).

IPF is a devastating disease, and its pathogenic mechanisms remain poorly understood (Schupp et al., 2015; Oruqaj et al., 2015). The immunopathology of IPF have only partially been unraveled, showing macrophage activation and fibrotic foci formation, possibly promoted by TNFα and TGFβ (Schupp et al., 2015; Oruqaj et al., 2015).
Bronchoalveolar lavage fluids (BALF) samples are often collected for the determination of cell and cytokine profiles, for the biological diagnosis of ILD. In this study, we determined the levels of a panel of 10 cytokines/chemokines in the BALF of ILD patients, including IL-8, IL-6, TNFα, IFNγ, IL-1β (Th1 response), IL-4 (Th2 response) and IL-17, IL-21 and IL-23 (Th17 response). We also determined thymic stromal lymphopoietin (TSLP), as this cytokine is produced by epithelial cells during chronic inflammatory processes and its potential role in the pathogenesis of ILD remains largely unexplored (Kuethe et al., 2014). In addition, we carried out QPCR on BALF for 10 microorganisms involved in farm-associated and/or domestic forms of HP. Europium amstelodami, Lichtheimia coryniformis, mesophilic Streptomyces spp., Saccharopolyspora rectivirgula, and Wallemia sebi are microorganisms commonly isolated in hay and considered as playing an important role in farm-associated HP (Peps et al., 1963; Roussel et al., 2005). Alternaria alternata, Aspergillus fumigatus, Aspergillus versicolor, Penicillium chrysogenum, and Stachybotrys chartarum are microorganisms commonly isolated in moisture-damaged dwellings and suspected to play a pivotal role in domestic form of HP (Roussel et al., 2008; Bellanger et al., 2009). QPCR is a highly sensitive technique for the detection of fungi and actinomycetes that is becoming part of the routine workflow in our mycology department (Bellanger et al., 2009; Scherer et al., 2014).

2. Material and methods

2.1. BALF collection

Over a two-year period, all patients undergoing bronchoalveolar lavage for the diagnosis of ILD were asked to provide a residual amount of 2 ml of BALF for the study. This study was approved by the ethics committee of Besançon University Hospital. We included all the BALF samples from patients diagnosed with HP (n = 16). Precipitin analysis showed that nine of the 16 BALF samples collected from HP patients corresponded to farmer’s lung disease (FLD, a farm-associated form of HP), six corresponded to HP caused by domestic exposure to mold and one corresponded to a case of bird breeder’s lung (BBL, avian HP form). All HP were chronic forms and diagnosis was based on clinical course, insidious onset over months, history of antigen exposure, and radiological data following international guidelines (American Thoracic Society et al., 2002). A control group was established, consisting of the BALF from patients diagnosed with two other ILDs: IPF (n = 11) and sarcoidosis (n = 12). Diagnosis was based on clinical, radiological and/or pathological data following international guidelines (American Thoracic Society et al., 2002). These two ILDs were chosen for study because they are common differential diagnoses of HP for which it was possible to obtain homogeneous groups of samples. We checked the profession of each patient noted in their medical files, to identify any farmers.

2.2. Luminex immunoassays

A panel of 10 key cytokines/chemokines were selected for testing, based on the scientific literature concerning ILD immunopathology (Mroz et al., 2008; Ye et al., 2009; Joshi et al., 2009; Simonian et al., 2009; Andrews et al., 2016; Tondell et al., 2014; Facco et al., 2011; Schupp et al., 2015; Oruqaj et al., 2015): IL-8, IL-6, TNFα, IFNγ, IL-1β (Th1 response), IL-4 (Th2 response) and IL-17, IL-21 and IL-23 (Th17 response). The TSLP, unexplored up to now, was also measured in patient’s BALF (Kuethe et al., 2014).

We quantified a panel of chemokines and cytokines with Multiplex Luminex bead technology. This approach was chosen because it can be used to quantify a large number of metabolites simultaneously in samples with a small volume (25 μL) (Moncunill et al., 2014). Another reason for selecting this technique was the lack of ELISA tests for recently discovered cytokines (such as TSLP). Two human cytokine/chemokine panels were used: MPXHCYT0-60K (Milliplex Map, Millipore, Billerica, MA, USA) for the quantification of IL-4, IL-17, IL-8, IL-6, IL-1β, TNFα and IFNγ, and MPXHCYT02MAG-62 K (Milliplex) for the quantification of TSLP, IL-21 and IL-23. Each BALF sample was centrifuged for 5 min at 1200 rpm and the supernatants were collected and used for analysis. Duplicate analyses were carried out for each sample, and the assay was performed according to the kit manufacturer’s instructions. Detection limits were: TSLP = 2.4 pg/ml, IL-21 = 2.2 pg/ml, IL-23 = 1.5 pg/ml, IL-4 = 1.2 pg/ml, IL-17 = 1.8 pg/ml, IL-8 = 1.2 pg/ml, IL-6 = 1.6 pg/ml, IL-1β = 1.6 pg/ml, TNFα = 1.3 pg/ml and IFNγ = 1.6 pg/ml.

2.3. QPCR assays

Europium amstelodami, Lichtheimia coryniformis, mesophilic Streptomyces spp., Saccharopolyspora rectivirgula, and Wallemia sebi are microorganisms commonly isolated in hay and considered as playing an important role in farm-associated HP (Peps et al., 1963; Roussel et al., 2005). Alternaria alternata, Aspergillus fumigatus, A. versicolor, Penicillium chrysogenum, and Stachybotrys chartarum are microorganisms commonly isolated in moisture-damaged dwellings and suspected to play a pivotal role in domestic form of HP (Roussel et al., 2008; Bellanger et al., 2009). DNA was extracted automatically from 1 ml of BALF supernatant, with a Large Volume MagNa Pure Isolation Kit, on a MagNa Pure Compact Apparatus (Roche Diagnostic, Meylan, France). The DNA eluted in a volume of 50 μl.

DNA from eight fungi (Aspergillus fumigatus, A. versicolor, Alternaria alternata, Eurotium amstelodami, Lichtheimia coryniformis, Penicillium chrysogenum, Stachybotrys chartarum, Wallemia sebi) was detected with primers and Taqman™ probes previously described by Haugland et al. (2004). Information about the primers and probes of these eight targets can be obtained from: http://www.epa.gov/nerlcwww/moldtech.htm. DNA from mesophilic Streptomyces spp. was detected by QPCR with the primers described by Rintala and Nevalainen (2006). DNA from Saccharopolyspora rectivirgula was detected by QPCR with primers and a probe designed from the gyrase B gene (gi:558485395) at the Mycology-Parasitology Department of Besançon University Hospital. The primers and the probe were designed with Genscript tools (https://www.genscript.com/sst-bin/app/primer), and specificity was evaluated by comparison with orthologs from Saccharopolyspora eythrae (gi:257720963) and Saccharopolyspora spinosa (gi:326937722). The sequences used were as follows: forward primer, SrF1, 5′-GATCTACGGGACGC TAC-3′; reverse primer, SrR1, 5′-AACCCTGTACCTGAACA-3′, and reverse probe, SrP1, 5′-AATCTCTGTACCGCTGGC-3′. Identical PCR mixtures were prepared for the 10 targets, in a final volume of 20 μl, with the Brilliant III Ultra-fast QPCR master mix (Agilent Technologies, Massy, France), using ROX as a passive reference, and including 5 μl DNA, 1000 nM primers, 200 nM probe and DNA-free water. Reactions were prepared in a 96-well optical reaction plate (Life Technologies, Carlsbad, CA, USA). The amplification protocol was as follows: 3 min at 95 °C, followed by 45 cycles of 15 s at 95 °C for template denaturation and one minute at 60 °C for the annealing of primer and probe and extension. PCR was carried out with the Applied Biosystems 7500 FastSystem (Life Technologies).

2.4. Statistical analysis

Data are presented as means ± standard deviation (SD) or frequency (%) for each ILD group. For group comparisons, we used Kruskal-Wallis tests. Analysis was performed with Systat version 12 software (Systat Software, Inc., San Jose, CA, USA). All statistical tests were two-tailed and p values below 0.05 were considered statistically significant.
3. Results

3.1. Characteristics of BALF

Demographic parameters and BALF cell counts for patients with different ILDs are presented in Table 1. The patients with IPF were significantly older than the other patients (p < 0.005, one-way Kruskal-Wallis test). The HP patients had the highest percentages of lymphocytes in BALF (p < 0.005, one-way Kruskal-Wallis test), consistent with alveolitis lymphocytosis being a major criterion for HP diagnosis (Girard et al., 2009). The HP patients also had lower percentages of macrophages in BALF (p < 0.005, one-way Kruskal-Wallis test) than the patients with sarcoidosis or IPF. The patients with sarcoidosis had the lowest percentages of eosinophils (p < 0.005, one-way Kruskal-Wallis test). For all the ILDs considered, there were more male than female patients (the total population consisted of about 75% men). All the patients with FLD were farmers, the patient with BBL looked after birds and two of the six patients with domestic HP were farmers. None of the patients in the sarcoidosis group was a farmer, whereas one of the patients with IPF reared ducks, chickens and rabbits and was exposed to hay.

3.2. Luminex immunoassays

BALF from HP patients, and especially those of FLD patients, contained significantly higher concentrations of three Th1 factors (IL-8, IL-6 and TNFα) (p < 0.05, one-way Kruskal-Wallis analysis) than BALF from patients with sarcoidosis and IPF (Table 2). No significant difference was observed for IFNγ (2.4 ± 0.4 pg/mL), IL-1β (2.6 ± 0.8 pg/mL), IL-4 (no detection) and TSLP (2.6 ± 0.3 pg/mL).

IL-17 was detected only in BALF from HP patients, specifically BALF from FLD and patients with domestic exposure (p < 0.05, one-way Kruskal-Wallis) (Table 2). In contrast, IL-21 was detected in all group of patients without any significant difference (4.2 ± 1.3 pg/mL).

No statistical comparison between FLD and domestic exposure cases was carried out, due to the small number of samples available. However, levels of IL-23 appeared to be slightly higher in cases of domestic mold exposure and BBL than in FLD patients.

3.3. QPCR assays

The results for fungal DNA detection in BALF from patients with different ILDs are reported in Table 3, as numbers of samples testing positive, for 10 microorganisms (Aspergillus fumigatus, A. versicolor, Alternaria alternata, Eurotium amstelodami, Penicillium chrysogenum, Saccharopolyspora rectivirgula, Stachybotrys chartarum, mesophilic Streptomyces spp., Wallemia sebi). All QPCRs were negative for the group of patients with sarcoidosis. Positive results were obtained for A. fumigatus, A. versicolor and W. sebi in the IPF group, with one to two positive samples per target (Table 3). Positive results were obtained for six of the 10 targets for the HP group (A. fumigatus, A. versicolor, A. alternata, E. amstelodami, mesophilic Streptomyces spp. and W. sebi) (Table 3). The number of positive samples was greatest for the HP group, particularly for E. amstelodami (n = 5) and W. sebi (n = 7) and corresponded to FLD cases (p < 0.05, one-way Kruskal-Wallis analysis).

4. Discussion

Hypersensitivity pneumonitis (HP) is often difficult to diagnose and may be confused with other pulmonary interstitial diseases, such as sarcoidosis. The results of this study suggest that positive fungal QPCR and IL-17 detection may help to improve HP diagnosis.

HP and sarcoidosis are generally considered as Th1 diseases (Mroz et al., 2008; Ye et al., 2009; Tondelli et al., 2014) but recent studies showed for both of them that a Th17 response was also part of the immunopathology (Joshi et al., 2009; Simonian et al., 2009; Facco et al., 2011). These diseases have in common to lead ultimately to formation of granulomas and a fibrotic phase (Girard et al., 2009; Facco et al., 2011). The mechanisms involved in IPF immunopathology remain poorly understood but include macrophage activation, fibrotic foci formation, possibly Th1 promoted (Schupp et al., 2015; Oruqaj et al., 2015). No Th2 switch was described for any of these ILD.

Technical advances have made it possible to test for 10 cytokines/chemokines simultaneously in BALF. Using this approach, we showed that, in addition to Th1 factors, such as IL-8, IL-6 and TNFα, Th17 mediators were detectable in the BALF of HP patients. IL-17 was present at detectable levels only in the BALF from HP patients (all samples from the sarcoidosis and IPF groups tested were negative). Moreover, a comparison of Th17 mediators as a function of the type of HP suggested that...
slightly higher levels of IL-23 were produced in cases of HP due to domestic exposure than in FLD. Luminex assays are useful to screen for large numbers of cytokines and are characterized by very low sensitivity in comparison with ELISA assays (Moncunill et al., 2014). Our results suggest that, in addition to Th1 factors, IL-17 and IL-23 could be useful to better distinguish between ILDs; however, in daily clinical practice, such low levels of IL-17 and IL-23, obtained using Luminex technology, should probably be further confirmed using specific ELISA (Dupuy et al., 2013). In contrast, thymic stromal lymphopoietin (TSLP), a cytokine that can be produced by epithelial cells in chronic inflammatory processes, was not useful as a biomarker specific to HP as it was detected at similar levels in all the ILDs explored (HP, IPF, sarcoidosis); a similar result was reported in 2013 (Willems et al., 2013).

HP due to domestic exposure to common molds has been increasingly reported in recent years, with >30 mold species implicated as causal agents (Jacobs, 2008). Many factors and circumstances within the homes of patients with this form of HP may be involved, but the principal risk factors seem to be contaminated humidifiers, impaired ventilation systems and home reservoirs (Roussel et al., 2005). The fungal species isolated in moisture-damaged buildings may vary from one geographic area to the other, depending on climatic conditions; it was shown previously that the fungal species mostly isolated in eastern France, associated with an impact on health, are, Aspergillus versicolor, A. fumigatus, Alternaria alternata, Penicillium chrysogenum and Stachybotrys chartarum (Roussel et al., 2008; Bellanger et al., 2009; Scherer et al., 2014). A murine model of S. chartarum-induced HP was developed and showed a robust pulmonary expression of IL17 and IL-23 (Bhan et al., 2013). These data are in coherence with our findings. Involvement of the Th17 mediators has previously been demonstrated on validated HP murine models for S. rectivirgula (Joshi et al., 2009; Simonian et al., 2009), the first microorganism recognized as etiologic agent of Farmer Lung Disease (Pepys et al., 1963), and now for S. chartarum (Bhan et al., 2013), one of the more feared microorganism for HP due to domestic exposure, because of its mycotoxin production (Carey et al., 2012). The host immune response induced by chronic exposure to different microorganisms could vary according to the composition of the microorganism’s cell wall but this hypothesis warrants further investigations.

QPCR assays for the detection of multiple microorganisms involved in pulmonary diseases are usually performed on various environmental samples (such as swabs, dust, or even impacted culture media), to assess the level of contamination of a dwelling and to identify more accurately the microorganisms present (Bellanger et al., 2009; Scherer et al., 2014). Knowledge of the specific contaminants present is required to ensure the use of the most appropriate antigens for serodiagnosis and to investigate the possibility of a toxic impact of the mold. For example, S. chartarum has been shown to secrete mycotoxins, such as satratoxin, which can cause pulmonary hemorrhage (Carey et al., 2012). An accurate determination of the contamination source is also required, to guide the choice of the most appropriate remediation measures for the dwelling (Roussel et al., 2005; Méheust et al., 2014). As HP patients chronically inhale large amounts of microbial antigens, we hypothesized that traces of microbial DNA would be directly detectable by QPCR in their BALF. Our findings indicate that fungal QPCR is a potentially valuable tool, particularly for the diagnosis of FLD, because QPCR was frequently positive for E. amstelodami and W. sebi in patients with this condition. Positive fungal QPCR results reflect exposure to high levels of molds in the environment and were sometimes obtained for patients with conditions other than HP, including a farm working with IPP who tested positive for W. sebi. Surprisingly, none of the patients with FLD tested positive for S. rectivirgula, despite the recognition of this actinomycete, in the 1960s, as the main causal agent of FLD (Pepys et al., 1963). This finding may reflect changes in agricultural techniques, modifying the densities of the various etiologic agents. A previous study investigating the concentrations of microorganisms in hay from 42 farms in our area reported densities of E. amstelodami and W. sebi up to 100 times higher than those of S. rectivirgula in contaminated hay (Roussel et al., 2005). The use of DNA extracted from unprocessed BALF rather than from the BALF supernatant would probably increase the amount of microbial DNA detected. However, these preliminary QPCR assays on BALF from HP patients suggest that QPCR targeting multiple microbes is potentially useful for HP diagnosis, particularly in cases of FLD.

5. Conclusions

This study strongly suggests that Th17 cytokine detection and fungal QPCR could be added to the analysis of BALF to improve the biological diagnosis of HP. Both the techniques used, Luminex and QPCR assays, are now easy to incorporate into the routine hospital work flow of most of mycology and immunology departments of university hospitals. The volume of BALF is generally sufficient and the use of 1 to 2 ml for additional biological assays should be feasible.

Funding

This study was supported by the Besançon University Hospital and by the Franche Comte Region (PROT-PHIA study and MP-PHIA study).

Declaration of interest

None to declare.

Acknowledgments

We thank Monique Mourey (Head Nurse, Fibroscopy Department), who played an essential role in the prospective collection of BALF. We thank Lois Rose for her editorial assistance.

References


