New evidence of the involvement of Lichtheimia corymbifera in farmer’s lung disease

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Farmer’s lung disease (FLD) is a form of hypersensitivity pneumonitis resulting from recurrent exposure to moldy plant materials. We investigated and compared the initial response of respiratory epithelium after exposure to extracts of Sacharopolyspora rectivirgula, Lichtheimia corymbifera (formerly Absidia corymbifera), Eurotium amstelodami and Wallemia sebi. The two criteria for selection of these species were their high prevalence in the hay handled by FLD patients and the presence of high levels of specific precipitins to these molds in FLD patients’ sera. Hydrosoluble extracts were prepared from spores and hyphae grown in culture under optimal conditions for each of the four species. Confluent A549 cells were inoculated with one of the four calibrated soluble extracts. Two mediators, one inflammatory (Interleukin (IL)-8) and one allergic (IL-13), were quantified using real-time PCR and ELISA assay, after four exposure periods (30 min, 2 h, 4 h and 8 h). S. rectivirgula and L. corymbifera extracts were the only ones which induced a marked upregulation of IL-8, as shown by both real-time PCR and ELISA assay 8 h after the initial contact. This study adds to the growing body of evidence that L. corymbifera should be recognized as an etiologic agent of FLD along with S. rectivirgula.

Keywords farmer’s lung disease, Lichtheimia corymbifera, airway cells, IL-8

Introduction

Farmer’s lung disease (FLD) is a form of hypersensitivity pneumonitis (extrinsic allergic alveolitis) resulting from recurrent exposure to moldy plant materials during the handling of hay, straw or grains [1]. In the acute form, influenza-like symptoms often predominate, presenting 2–9 h after exposure [2]. The immunologic and allergic mechanisms of FLD are complex and little is known about them. As mentioned by the NHLBI/ORD (National Heart, Lung, and Blood Institute and the Office of Rare Diseases) Conference [3], in vitro cellular models are of particular help in providing a better understanding of these systems.

It is well-known and generally accepted that Sacharopolyspora rectivirgula, a thermophilic actinomycete (synonyms: Faenia rectivirgula, Micropolyspora faeni and Thermomonospora monospora), is a causative agent of FLD [1,4]. Previous experimental studies analyzing the bronchoalveolar lavage (BAL) of mice exposed to S. rectivirgula have demonstrated that the initial response is marked by the presence of neutrophils which were detected in BAL in less than 24 h after exposure. In contrast, lymphocytes were detected approximately 72 h after exposure [5]. It has been suggested that molds, such as Lichtheimia corymbifera (=Absidia corymbifera) [6], Eurotium amstelodami and Wallemia sebi, are also causative agents of FLD because of their prevalence in the hay handled by FLD patients, and the presence of high levels of specific
precipitins to these molds in FLD patients’ sera [7,8]. How-
however, no in vitro or in vivo experiments have yet provided
proof of their involvement in FLD. All the in vitro studies
investigating the immunologic mechanism of FLD have
measured mediators, primarily alveolar macrophages after
stimulation with S. rectivirgula [2]. Only one team studied
the interaction of S. rectivirgula with respiratory epithel-
ium, using A549 cell lines [9]. Respiratory epithelium is,
however, the first tissue encountered by inhaled antigens,
and this first interaction is most probably of singular im-
portance in the development of FLD. To our knowledge, there
are no reports on the initial response of A549 cells to poten-
tial etiologic agents of FLD other than S. rectivirgula.

The aim of our study was to investigate the early inflam-
mathory signals in airway epithelial cells after exposure
to soluble extracts of S. rectivirgula, L. corymbifera,
E. amstelodami and W. sebi. We used real-time quantita-
tive PCR (RT-qPCR) to quantify the expression of two
mediator genes, i.e., Interleukin 8 (IL-8), an inflamma-
tory chemokine whose main role is to attract neutrophils
[10], and IL-13, selected because it is a key effector in the

Materials and methods

Bacterial and fungal strains

We used Lacey’s strain of S. rectivirgula (DSMZ 43113)
and three fungal strains isolated from the hay of FLD-
patients in Franche-Comté, a region in Eastern France,
i.e., L. corymbifera (BCCM/IHEM 3809), E. amstelodami
(BCCM/IHEM 16286) and W. sebi (BCCM/IHEM 16284).
All were cultured for 1 week under the following condi-
tions, (1) at 44°C on R8 medium for S. rectivirgula [12],
(2) at 20°C on Sabouraud dextrose agar (Becton Dickinson
and Company, Le Pont de Claix, France) for W. Sebi
and (3) at 30°C on DG18 (Oxford, Unipath, Basingstoke,
England) for L. corymbifera and E. amstelodami (on
DG18, E. amstelodami produces only ascospores) [13].

Temperatures and media were chosen on the basis of the
optimal growth conditions of each species.

For each species, four culture plates were gently brushed
with a swab and the resulting structures were harvested in
2 ml of sterile water, resulting in milk-like suspensions
(>7 Mc Farland). Each suspension was frozen at −20°C
for one night. The following day, lyophilization was carried
out in a Labconco Apparatus (Labconco, Kansas
City, MO, USA) and the lyophilizate was resuspended in
1 ml of sterile water. After centrifugation for 2 min at
10,000 rpm, the supernatants (the hydrosoluble extracts)
were harvested, and proteins were quantified by the Lowry
technique using the DC protein Assay (Bio-Rad, Marne-La-
Coquette, France). For each of the four species, the extract
was calibrated using the protein contents in the extract
(50 µg of proteins per ml of extract).

Cell lines

The alveolar epithelial cell line A549 was purchased from
the German Collection of Micro-organisms and Cell Cul-
tures (DSMZ, Braunschweig, Germany) and cultured in
Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen,
Cergy Pontoise, France), with 10% heat-inactivated fetal
bovine serum (Biowest, Nuaillé, France) and 25 µg/ml gen-
tamycin (Sigma-Aldrich, St-Quentin Fallavier, France).
The medium was changed every 2 days and cells were confluent
in 5 days. Cell cultures were checked for the presence of
Mycoplasma spp. every month using the Venor GeM Myco-
plasma Detection PCR kit (Biovalley, Marne-La-Vallée,
France). Cells were cultivated in 24-well microtitre plates
(Dutsh, Brumath Cedex, France). Confluent cells (about
5 × 10^6 cells per well) were inoculated with each of the four
calibrated soluble extracts at 1 ml (containing 50 µg) of
extract added to each well. Each experiment included a con-
trol, in which epithelial cells were exposed to culture media
only. Cell exposure was terminated at 30 min, 2, 4 and 8 h.
These experiments were performed at least three times with
careful replication of all conditions cited above.

mRNA quantification

RNA was extracted using the RNA MagNa Pure Compact
Isolation kit in the MagNa Compact (Roche Diagnostics,
Meylan, France). Quantity and quality of RNA were checked
using a spectrophotometer (Nanodrop ND 1000) before
samples were stored at −80°C.

Reverse transcription was carried out in a final volume
of 20 µl as follows; 4 µl (400µg) RNA were added to 2 µl
Dithiothreitol (DTT) 0.1M (Invitrogen, Cergy Pontoise,
France), 0.5 µl Superscript II Rnase H- Reverse Trans-
criptase H- 200U/µl (Invitrogen), 4 µl First Strand Buffer
5X (250 mM Tris-HCl, pH 8.3, 375mM KCl, 15 mM
MgCl₂) (Invitrogen), 2µl dNTP mix 5mM (Amersham
Pharmaccia, Uppsala, Sweden), 6 µl random hexamers
pd(N)₆ 0.5 µg/µl (Amersham Pharmaccia), 0.5 µl RNAsin®
40U/µl (Promega, Madison, WI, USA). Sterile water was
used to dilute the cDNA (1/20), which was then stored at
−20°C until amplification. For each reaction, controls with-
out RNA and without reverse transcriptase were employed.

The primers used are listed in Table 1. Real-time PCR
was carried out in a LightCycler 2.0 (Roche Diagnostics,
Meylan, France) in a 20 µl final volume containing; 2µl
Fast Start SYBR Green Buffer 10X (Fast Start SYBR Green
KitTM, Roche Diagnostics), 3 mM of MgCl₂, 50 µM sense
and antisense primers, 8 µl cDNA (diluted 1/20) and sterile
water. The thermal cycling conditions were denaturation at
the N-fold difference in target gene expression relative to the P0 gene (termed N\textsubscript{target}), were obtained by the formula: 
\[ N_{\text{target}} = 2^{\Delta C_{\text{target}}} \]

The \( \Delta C_t \) sample was determined by subtracting the mean \( C_t \) value of the target gene from the mean \( C_t \) value of the P0 gene. The N\textsubscript{target} values of the samples were subsequently normalized, so that the median N\textsubscript{target} value of the control sample (epithelial cells exposed to culture media only) was 1 [14,15]. To present the results in graph form, the N\textsubscript{target} = 2\( \Delta C_t \) sample was calculated for each experiment (3 replicates of each type of exposure). Each column represents a mean of the 3 replicates of N\textsubscript{target} plus SEM with the control bar is y = 1.

### IL-8 dosage in cell supernatants

In order to determine if synthesized IL-8 was released into cell supernatants exposed to S. rectivirgula and L. corymbifera, IL-8 levels were evaluated by a specific ELISA kit (Thermo Scientific, Rockford, USA), following the manufacturer’s instructions. Each sample was measured in duplicate. The detection limit of the assay was 2 pg/ml.

### Fig. 1

Standardized mRNA expression of the gene encoding IL-8 after exposure of A549 cells to Sacharopolyspora rectivirgula, Lichtheimia corymbifera, Eurotium amstelodami and Wallemia sebi soluble extracts (50 \( \mu \)g/ml) calculated with the \( \Delta \Delta C_t \) method, with P0 as the reference gene. Values were calculated from three separate experiments and gene expression was subsequently normalized against the median values of the controls. The data are presented as means of the N\textsubscript{target} ± SEM; a significant overexpression of mRNA of the gene encoding IL-8 was observed during time: at 4 h (#) for S. rectivirgula (\( P = 0.005 \)) and at 8 h (*) for S. rectivirgula and L. corymbifera (\( P = 0.0005 \) for both species). The control bar is y = 1.

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Statistical analysis

Data are presented as means ± SEM from three separate experiments. Statistical analysis was performed using XLSTAT 2009. Differences were considered statistically significant for $P < 0.05$. Non-parametric tests (a Kruskal-Wallis test followed by a Mann-Whitney test) were used to detect significant variations in the quantification of mRNA and protein levels.

Results

Relative mRNA quantifications

Exposure to $S. \text{rectivirgula}$ and $L. \text{corymbifera}$ soluble extracts induced significant upregulation of the $IL-8$ gene with the intensity and time lapse before appearance of overexpression varying from one agent to another (Fig. 1). Specifically, exposure to $S. \text{rectivirgula}$ was progressive and clear, beginning at 4 h ($N = 3.7$) and continuing to 8 h ($N = 8.1$) (Mann-Whitney test, $P = 0.005$ at 4 h and $P = 0.005$ at 8 h). Exposure to $L. \text{corymbifera}$ was late and abrupt, with $N = 8.75$ at 8 h (Mann-Whitney test, $P = 0.005$). No significant variations were observed with exposure to $E. \text{amstelodami}$ and to $W. \text{sebi}$ during the entire 8 h of the experiment.

Exposure to $L. \text{corymbifera}$ induced significant upregulation of $IL-13$, (Fig. 2), i.e., $N = 3.56$ at 8 h (Mann-Whitney test, $P = 0.005$). No significant variations were observed on exposure to $S. \text{rectivirgula}$, $E. \text{amstelodami}$ or $W. \text{sebi}$ during the entire 8 h of the experiment.

Concentrations of synthesized mediators in cell supernatants

$IL-8$ levels in cell supernatants confirmed the results obtained using RT-qPCR. Concentrations of $IL-8$ in A549
supernatants began to rise 4 h after the initial contact with \textit{S. rectivirgula} (350 pg/ml, Fig. 3). Eight hours after exposure, IL-8 levels were 923 pg/ml and 551 pg/ml in response to \textit{S. rectivirgula} and \textit{L. corymbifera}, respectively (Fig. 3). These levels were significantly higher than those measured in unexposed cell supernatants (Mann-Whitney test, \( P/11005 \leq 0.0004 \) at 4 h and 8 h for \textit{S. rectivirgula} and at 8 h for \textit{L. corymbifera}). In contrast, synthesized IL-13 was not detected in any A549 cell supernatants.

**Discussion**

Using RT-qPCR, we observed a significant increase in IL-8 mRNA amounts in A549 epithelial cells at 8 h, when exposed to \textit{S. rectivirgula} or \textit{L. corymbifera}. This was not observed for the other two suspected etiologic FLD agents tested in this study.

Identification of putative etiological FLD agents is generally performed using serologic and culture techniques. Previous studies have suggested that \textit{L. corymbifera}, \textit{E. amstelodami}, and, to a lesser degree, \textit{W. sebi}, are important etiologic agents of FLD [7,16,17]. The causative role of \textit{L. corymbifera} in FLD, based on serologic data, has been suggested in two studies, one carried out in Finland [18], the other in Eastern France [7]. However, for years, \textit{S. rectivirgula} has been the only pathogen used \textit{in vivo} and \textit{in vitro} to study the pathogenesis of FLD, probably because of its high prevalence in patients’ hay in the 1960s and because it is easier to standardize bacterial inocula than those of fungi.

The overexpression of IL-8 after exposure to \textit{S. rectivirgula}, first described for A549 cells by Gudmundsson \textit{et al.} [9], was confirmed in our study. \textit{L. corymbifera} was the only one to generate IL-8 upregulation levels similar to those of \textit{S. rectivirgula}. This result strongly suggests that \textit{L. corymbifera} plays an important role in the pathogenesis of FLD. However, there is not yet enough evidence to categorize \textit{L. corymbifera} as a recognized cause of FLD [19]. In fact, the categorization as ‘recognized cause of FLD’ would only be justified if \textit{L. corymbifera} induced hypersensitivity pneumonitis after chronic inhalation in a mouse model (as defined by Denis \textit{et al.}) [20]. However, the importance of \textit{L. corymbifera} in FLD was previously suggested by three other studies in which (i) high levels of specific precipitins of \textit{L. corymbifera} were found in FLD patients’ sera [7,18], and (ii) FLD relapses due to high amounts of \textit{L. corymbifera} in FLD patient-handled hay were reported [8]. Thus the present \textit{in vitro} experiment not only adds to already-existing support for the role of \textit{L. corymbifera} in FLD, but also

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**Fig. 3** Concentrations (pg/ml) of synthesized IL-8 in A549 supernatants during time (30 min, 2 h, 4 h and 8 h) after exposure to \textit{Sacharopolyspora rectivirgula}, \textit{Lichtheimia corymbifera} and in unexposed cells. The data are presented as means±SEM; compared to IL-8 levels in supernatants of unexposed cells, higher levels of IL-8 were observed at 4 h and 8 h (*) after exposure to \textit{S. rectivirgula} (\( P = 0.0004 \)) and at 8 h (#) after exposure to \textit{L. corymbifera} (\( P = 0.0004 \)).
demonstrates that it triggers an inflammatory reaction at the epithelial level.

Concomitantly, we do not think that the present study presents sufficient evidence to categorically exclude the etiologic role of W. sebi or E. amstelodami in FLD. Even though two studies using culture and serologic techniques support the involvement of E. amstelodami and W. sebi in FLD [7,16], these two species had only minor effects on the respiratory epithelium in our study. One hypothesis for this is that cross-reactions may have occurred between species when serological tests using fungal antigens were performed [21]. However, the nature of inocula (in our study these were hydrosoluble extracts which were calibrated using their protein contents) could influence the cellular response observed in in vitro models. Nor can we exclude the fact that other immunologic components (for example, polyside chains), not quantified by our calibration, may have interfered. The use of purified extracts (either proteins or polysides) and specific recombinant proteins could help to better define the interaction between mold and epithelial cells.

Exposure to L. corymbifera also induced a significant increase in the concentration of IL-13 mRNA. However, the specific ELISA assay did not detect the protein IL-13 in cell supernatants. This discrepancy could be due to a lack of sensitivity on the part of the ELISA kit or incomplete spatial conformation of the IL-13 protein produced by A549 cells, resulting in absence of recognition by specific IL-13 antibodies.

Environmental surveys and serologic data are essential to identify the putative etiologic agents of FLD and other types of hypersensitivity pneumonitis. Testing their ability to initiate an immune-allergic response in vitro, however, could help to identify the microbial species which are really involved in the development of the disease. The more we know about the intensity and the time lapse before the cytokine response induced by each of the suspected etiologic agents, the more precisely the real responsible microorganisms can be targeted by hay treatment techniques, such as hay-drying systems[22] or salting [23], addition of commercial chemicals [24], and inoculation of bacteria [25]. Prevention measures are indeed of prime importance, because there are no effective treatments for FLD. In our opinion, L. corymbifera should be recognized as an etiologic agent of FLD along with S. rectivirgula.

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