

Characterization of *Micropolyspora faeni* antigens by human antibodies and immunoblot analysis

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IgG, IgM and IgA antibody responses against *Micropolyspora faeni* (*Mf*) antigens were studied by means of immunoblotting experiments using 70 sera derived from three groups of farmers, namely patients with extrinsic allergic alveolitis (EAA) due to thermophilic actinomycetes ($n = 25$), patients without EAA but with hay exposure ($n = 14$), and patients suspected to have EAA ($n = 31$), and 27 sera from two groups of control persons (healthy laboratory workers, $n = 13$; healthy farmers, $n = 14$). Patients with EAA showed IgG, IgM and IgA antibody responses mainly against the antigens with molecular weights (MW) of 11, 12, 25, 35 and 60 kD ("major antigens"), and in addition, but less often, against six antigens with MW in the range of 15 to 62.5 kD ("minor antigens"). The other two groups of patients and also the exposed control persons showed very similar results; however, the antibody response in healthy farmers was substantially weaker in comparison to the three groups of patients and was almost limited to the major antigens with MW 11, 25 and 60 kD. Although patients with proven EAA had higher amounts of antibodies, there was no correlation between this antibody response and the onset of disease. The results indicate the necessity of including at least the major antigens with MW of 11, 25 and 60 kD in all extracts used for *in vitro* diagnosis of *Mf*-induced EAA.

Key words: extrinsic allergic alveolitis; farmer's lung; immunoblotting; major and minor antigens; *Micropolyspora faeni*.

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CLINICAL ASPECTS

The commercial extracts of *Mf* which are used for diagnostic tests differ significantly in their antigenic content, so that the patient's antibody responses in farmer's lung appear to be heterogeneous and, therefore, the disease can not be diagnosed with sufficient certainty in different laboratories. In this study, we characterized three major antigens of *Mf* with MW of 11, 25 and 60 kD which seem to play a significant role in the immune response, so that these antigens must be present in any extract used for the detection of antibodies in patients suspected of having EAA.

Extrinsic allergic alveolitis (EAA) or hypersensitivity pneumonitis is an allergic lung disease resulting from the inhalation and subsequent sensitization and repeated exposure to a variety of dust (7). Sources of antigens are numerous and may include microorganisms

(bacteria, fungi, amoebae), animals and plants. Certain thermophilic and mesophilic actinomycete organisms have been observed to produce disease in such diverse situations as contact with avian excrete, contaminated humidifiers, mushroom picking, processing of

sugar cane, etc. at work or at home (7, 11, 22, 23).

The actinomycetes are often incorrectly identified as fungi because of their similar morphology, but they are in fact true bacteria (Eubacteriales). They grow in decaying biological material such as hay or bagasse, or others at elevated temperatures ranging between 37°C and 60°C. Actinomycete organisms grow particularly well in compost with a neutral pH of 7.0. The higher moisture content of old hay and bagasse (approx. 40%) supports the growth of thermophilic organisms much better than in fresh hay or bagasse (11). Thermophilic actinomycetes may occur ubiquitously in soil, foods, fresh water, the atmosphere and other natural sources. *Micropolyspora faeni* (*Mf*), now designated as *Faenia rectivirgula* (15), is one of the most important sources of actinomycete antigens involved in the pathogenesis of farmer's lung (FL) (11, 14).

Although there are many studies about the detection of specific antibodies against *Mf* to clarify the pathogenesis of FL, using different assays such as enzyme-linked immunosorbent assay (ELISA) or crossed immunoelectrophoresis (CIE) or double immunodiffusion test (2, 9, 12, 13, 17, 20), there is much controversy between different authors as to the characterization of *Mf* antigenic/allergenic components.

Edwards (6) studied the antigenic components of *Mf*, using immunoelectrophoresis. The author identified eight "major" antigens and designated those proteins according to their electrophoretic mobility in major precipitation lines, namely major precipitation lines in regions A, B and C with average molecular weight (MW) of 44, 77, 85 and 101 kilo Dalton (kD).

Kurup (14, 16) described purified fractions of *Mf* as major antigens of MW 16, 29 and 51 kD; Baur (4) reported on about 15 antigens of *Mf* with MW between 16 and 100 kD.

Thus, it is of great importance to establish and characterize extracts of *Mf* by means of a sensitive method in order to study the antibody response in a panel of FL patients. In this study, by testing various commercial ex-

tracts of *Mf*, we observed that these extracts differ markedly in their antigenic content. Based on these results, we tried to ascertain which *Mf* antigens are characteristic for FL patients and must be present in the *Mf* extract being used for FL diagnosis.

MATERIAL AND METHODS

Patients

1) Sera from 25 patients, 19 male, 6 female (age range 34 to 75 years, median 54.5). FL was confirmed by case history, clinical symptoms, typical lung X-ray, positive double immunodiffusion test and positive provocation test with mouldy hay.

2) Sera from 14 patients, 11 males, 3 females (age range 38 to 75 years, median 56.5), without FL (negative provocation test) but with exposure to hay and suffering from other diseases of the respiratory tract.

3) Sera from 31 patients, 19 males, 12 females, with suspected FL. These sera from patients with a typical case history and strong suspicion of FL were sent by chest physicians to the Institute of General and Experimental Pathology for diagnostic tests. Data about results of bronchial provocation and final diagnosis were not available.

4) Sera from 13 healthy laboratory workers, 9 males, 4 females (age range 23 to 60 years., median 41.5), without exposure to hay served as controls. 11 sera were pooled and used as negative control serum pool (N).

5) Sera from 14 healthy farmers, 6 males, 8 females (age range 24 to 61 years, median 42.5), with exposure to hay served as second control group.

Chemical and general reagents

Tris, glycine, acetic acid, methanol, ethanol, glycerol, ammonium carbonate, sodium azide (NaN₃), chloramine-T, dinatriumhydrogenephosphate, natriumdihydrogenephosphate, sodiummetabisulfite, Coomassie Brilliant Blue G

250 and R 250, EDTA (ethylenedinitrilotetra acetic acid dinatriumsalt) and phosphoric acid were obtained from Merck (Darmstadt, W. Germany). Tween 20 (polyoxyethylenesorbitan monolaurate), SDS (sodiumdodecylsulfate), acrylamide and N.N.-methylene-bis-acrylamide were purchased from Sigma (Munich, W. Germany).

Ammoniumpersulfate, 2-mercaptoethanol, N.N.N.N.-tetramethylethylenediamine (TEMED) were obtained from Bio Rad Laboratories (Richmond, CA, USA). Bovine serum albumin (BAS), normal goat serum (NGS) and gelatine were obtained from Janssen (Olen, Belgium). ¹²⁵Iodine, hyperfilm MP (RPN 6) were obtained from Amersham International, UK.

Extracts

Four different commercial extracts of *Mf* which, according to the manufacturer originate from the same species, were investigated:

- 1) *Mf*. from Hollister-Stier, Spokane, Washington, USA
- 2) *Mf*. from ALK, Copenhagen, Denmark
- 3) *Mf*. from Mercia Diagnostics, West Byfleet, UK
- 4) *Mf*. from HAL, Harlem, Holland

Each extract was dialysed against distilled water for 2–3 days and then lyophilised.

SDS-PAGE

Samples were diluted 3:1 in a Laemmli sample buffer (0.0625 M Tris, 2.3 % (w/v) SDS, 5 % (v/v) mercaptoethanol, 10 % (v/v) glycerol, 0.025 % bromphenol blue) and boiled for 4 min.

SDS-PAGE was performed on a vertical slab gel apparatus (Höfer, San Francisco, CA, USA).

10 to 16 % gradient gels, respectively (160 × 180 × 5 mm) with 5 % stacking gels were used. Electrophoresis was carried out in a discontinuous buffer system (0.025 M Tris, 0.2 M glycine) containing 0.1 % (w/v) SDS. For

immunoblotting experiments a one well comb was used. *Mf* extracts were applied in a concentration of 0.3 mg protein (extracts A, B & D) and 0.7 mg protein (extract C)/cm gel. MW were judged, using prestained protein standards (MW 14.3–200 kD) (rainbow markers, Amersham Int., UK), adjusted in comparison to unstained proteins (cytochrome C, carboanhydrase, bovine serum albumin) by use of the Bio-Rad silver stain kit.

Staining of gels was done either for 2 h with Coomassie Brilliant Blue G 250 (CBB), 0.25 % (w/v) in 50 % (v/v) methanol, 15 % (v/v) acetic acid, destaining was done in 10 % (v/v) methanol, 15 % (v/v) acetic acid and 3 % (v/v) glycerol, or silver stained with Bio-Rad silver stain kit.

Protein assay

The protein concentration of lyophilised extracts was determined by the method of Bradford (5).

Immunoblot (Westernblot)

SDS-PAGE was performed as mentioned above, the separated proteins were transferred to nitrocellulose by use of a Hoefer blotting apparatus at 200 mA for 3 h from gradient gels onto nitrocellulose filters (NC) (pore size 0.2 µm, Schleicher & Schüll, Dassel, W. Germany) (26). The transfer was performed in a Tris-glycine medium (25 mM Tris, 192 mM glycine, 20 % (v/v) methanol, pH: 8.3). NC-sheets were cut into 4–5 mm strips, which were treated according to an improved method based on Moereman's immunogold staining, using Tween 20 as blocking reagent in the incubation buffers (3). Per strip, 1 ml incubation buffer was used.

Buffer A: 50 mM natriumphosphate-buffer, pH: 7.5
 0.5 % w/v Tween 20
 0.5 % w/v BSA
 0.05 % w/v sodium azide
 Buffer B: A + 1 % (v/v) normal goat serum

Buffer C: A + 20 mM NaN_3 , 0.4% (w/v) gelatine

Strips were processed by saturation during 30 min at room temperature (RT) in buffer A. Then they were incubated overnight at 4°C with patient serum (for IgG and IgM detection diluted 1:20, for IgA detection diluted 1:4 in buffer B).

For the detection of IgG, IgM and IgA response, a sandwich technique was performed, using an affinity purified rabbit anti-human IgG antiserum, Fc-specific, rabbit anti-human IgA antiserum alpha-chain specific, rabbit anti-human IgM antiserum, Fc- μ fragment-specific (Jackson, MD, USA), which were diluted in buffer B (rabbit anti-human IgG and IgM 1:2000, rabbit anti-human IgA 1:1000). The used dilutions of serum and antiserum were found by titration using a strongly positive patient's serum (as detected by double immunodiffusion tests and ELISA) and antiglobulins in dilutions ranging from 1:4 to 1:128 (patient's serum) and 1:500 to 1:3000 (antiglobulins).

Strips were incubated for 1 h at RT, washed in A for 3 min and incubated for 1 h at RT with a ^{125}I -goat anti-rabbit antibody (300,000 cpm/ml buffer C) (Kirkegaard & Perry Laboratories, London, GB).

Labelling was performed according to the method of McConahey (18), using ^{125}I -sodium-iodide (Amersham Int., UK) for a goat anti-rabbit antibody (Kirkegaard & Perry, London, GB). Control sera were incubated with an anti-*Mf* antibody raised in sheep (Mercia diagnostic, UK). Autoradiography was performed at -70°C for 3-4 days, using intensifying screens in Kodak exposure cassettes.

RESULTS

Characterization of extracts by SDS-PAGE

Four different commercial extracts were investigated for their antigenic content by SDS-PAGE (10-16%).

Extract A shows several antigens of MW between 10 and 70 kD, extract B shows just one

antigen of MW of 60 kD and extract C shows antigens in molecular ranges at about 21.5 and 80-85 kD. Extract D shows several antigens of MW between 10 and 90 kD (Fig. 1). The problem with this extract was that the background was also intense.

Immunoblot (Westernblot)

Primarily, we tested all four extracts with a panel of 10 patients' sera. These tests showed that in contrast to the others, extract A contains many antigens/allergens of different molecular weights (11-70 kD) and is therefore qualified for further investigations.

In three groups of patients we investigated their IgG-, IgM-, and IgA-antibody response against *Mf* antigens by means of immunoblotting. The IgG antibody response in 14 sera from exposed healthy persons was investigated. 13 sera from healthy persons without exposure to hay were also tested and showed almost negative results. These sera were later pooled and used as control serum (N).

IgG-antibody response

64% of patients with EAA and 42.8% of patients without EAA showed IgG antibody response against three antigens with MW of 11, 25 and 60 kD. Both antigens with MW of 11 and 25 kD were recognized by 92%, and the antigens with MW of 12, 35 and 60 kD were recognized by 64%, 64% and 68% respectively, of all investigated patients with EAA. In addition to these antigens, in some cases we observed IgG antibody response against antigens with MW 15, 24, 30, 52.5, 55 and 62.5 kD (Figs. 2 and 3).

These observed antibody patterns did not correlate with disease history, absence or presence of hypersensitivity pneumonitis, and were found within all three groups of patients. The control group of healthy persons with hay exposure also showed a similar binding pattern, although much weaker and in most cases to only one or a few antigens. The control serum

pool (N) showed almost no reactivity (Figs. 4 and 5).

IgM-antibody response

IgM-antibodies were mainly directed against the antigens with MW 11, 25 and 60 kD. 44.3 % of investigated persons showed IgM-antibody response against the antigen with MW 11 kD, 68 % against the antigen with MW 25 kD, and 78.6 % against the antigen with MW 60 kD; the percentage of patients with EAA was 56 %, 88 % and 80 %, respectively. A similar response was also observed in all other examined patients.

IgA-antibody response

IgA-antibody response was most frequently directed against the antigen with MW 25 kD, and less frequently against antigens of 11 and 60 kD, again within all groups of persons.

DISCUSSION

On the basis of our SDS-PAGE investigations, commercial extracts of *Mf* (correctly designated

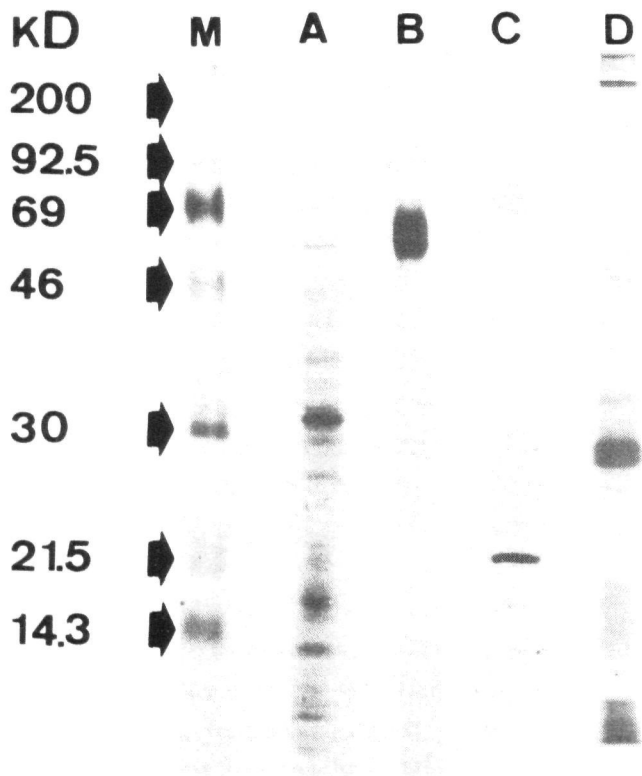


Fig. 1. 10–16 % SDS-PAGE of *Mf*: Four different commercial extracts (A: 0.3; B: 0.3; C: 0.7; D: 0.3 mg protein/cm) were tested (M: gel marker).

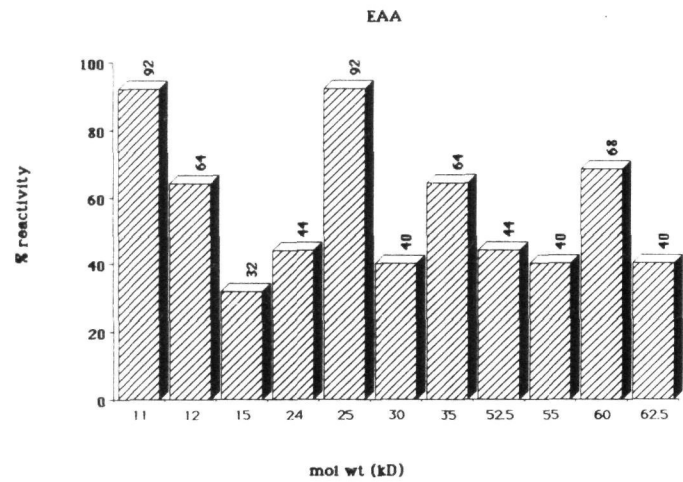


Fig. 2. Percentage of IgG-antibody response of patients with EAA (Gr. 1; *n* = 25) against major and minor *Mf* antigens.

as *Faenia rectivirgula* according to Kurup & Agre (15)) used for the diagnosis of EAA, differ significantly in their antigenic components, and may explain why discordant results from various laboratories were obtained. The quality of patients' antibody responses in FL is heterogeneous and according to published data (10, 14, 16, 27) does not correlate with the disease history. Therefore, the goal of this study was to establish a more sensitive immunological assay, such as immunoblotting, to characterize the important *Mf* antigens and to ascertain whether there are specific *Mf* antigens leading to the onset of EAA.

From our results we observed that the antigens of 11, 12, 25, 35 and 60 kD are recognized

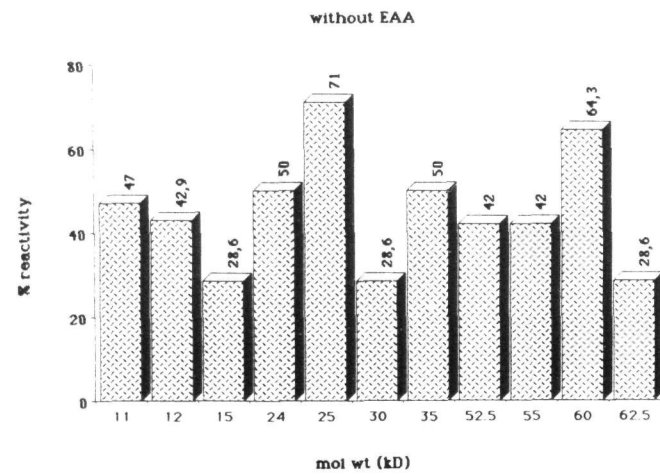


Fig. 3. Percentage of IgG-antibody response of patients without EAA (Gr. 2; *n* = 14) against major and minor *Mf* antigens. These patients were farmers with exposure to hay but showed negative provocation tests.

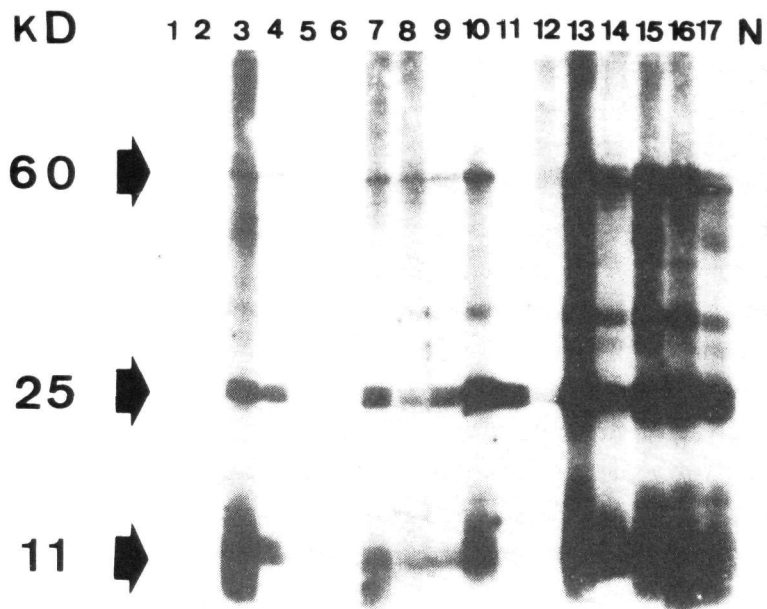


Fig. 4. IgG-antibody response: immunoblot of *Mf* extract with sera from patients with EAA (Gr. 1: 4, 5, 6, 8, 9, 11, 12, 15, 16, 17), patients without EAA, but with exposure to hay (Gr. 2: 1, 2, 3, 7, 10, 13), and a patient with suspected EAA (GR. 3: 14); N = negative control human serum pool.

by more than 50 % of investigated patients from all three groups, and therefore we designated these molecules as major antigens. The antigens with 15, 24, 30, 52.5, 55 and 62.5 kD, which are recognized with less frequency, were designated minor antigens.

It was interesting to find that the major antigens with MW of 11, 25, and 60 kD were recognized by all three immunoglobulin classes investigated (IgG, IgM and IgA), whereas the antigens with MW of 12 and 35 kD and the

minor antigens were recognized predominantly by IgG.

An earlier study (14, 16) has reported on three major *Mf* antigens with MW 16, 29, and 51 kD, and a recent study reports on two major antigens with MW of 28 and 49 kD (1). We also observed antigens with similar MW (15, 30, and 52.5 kD), but regarded these molecules, in contrast to the above mentioned studies, as minor antigens. This discrepancy might be because we examined the sera from a much

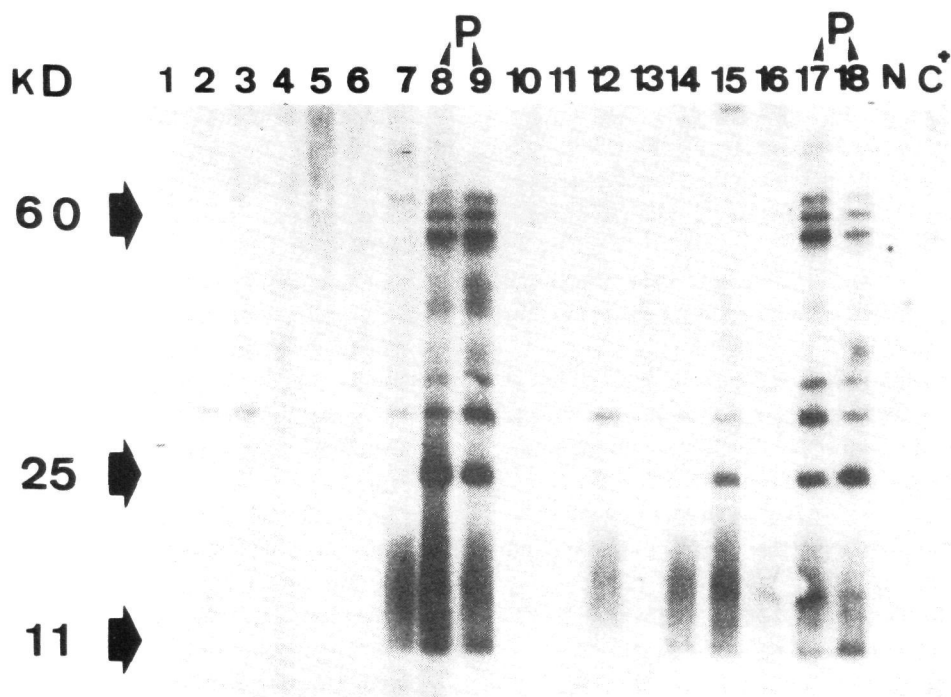


Fig. 5. IgG-antibody response: Immunoblot of *Mf* extract with sera from healthy farmers with hay exposure (1-7, 10-16) in comparison with patient (P) with EAA (N = negative control human serum pool, C = anti-*Mf* antiserum with weak reactivity).

larger number of patients and used a new series of protein markers with MW 14.3, 21.5, 30, 46, 69, 92.5 and 200 kD (see Material and Methods) which allow a more precise characterization of MW of the proteins wanted. Our study shows that all patients with defined EAA have antibodies against the major antigens with MW of 11, 25, and 60 kD, thus these antigens should be present in all *Mf* (*Fr*) extracts used for diagnostic purposes.

Despite these results, we were unable to predict the existence of EAA in the third group of patients, based exclusively on the antibody responses of EAA patients compared with controls. Moreover, it confirms the assumption that other genetic, environmental or immunopathological factors might also be involved in the manifestation of EAA (8, 17, 19, 21, 24, 25, 28). More investigations are necessary to elucidate these factors. However, we consider that characterization of *Mf* extracts and patients' immune responses will facilitate these investigations.

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