

Occurrence of *Aspergillus umbrosus* Antibodies in Danish Farmers

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Antibodies to *Aspergillus umbrosus* were investigated in 181 Danish farmers, 137 farmers' spouses and a control group of 104 male blood donors with no relation to farming. By enzyme-linked immunosorbent assay (ELISA) higher levels of IgG antibodies were found in farmers than in their spouses and in non-farming controls ($P < 0.0001$). By double immunodiffusion precipitating antibodies occurred in 11 farmers, in none of farmers' spouses and in one control subject. Farmers and farmers' spouses who were non-smokers had higher IgG antibody levels than smokers in the same groups ($P < 0.04$). Farmers more than 30 years of age had higher IgG antibody levels than younger farmers ($P < 0.04$). Farmers rearing cattle tended to have higher IgG antibody levels than farmers without cattle. No correlation between antibody levels and respiratory symptoms or lung function parameters (FEV₁ or FVC) could be demonstrated.

Key words: *Aspergillus umbrosus*; enzyme-linked immunosorbent assay; farmer's lung; IgG-antibody; smoking.

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Allergic alveolitis in farmers (farmer's lung) is elicited by inhalation of dust of mouldy hay (11). Thermophilic actinomycetes have been considered the most important antigens in the United Kingdom and North America (5, 11, 16), whereas fungi may play the same role in Finland (14). In accordance with this, Finnish farmers often have antibodies to aspergillus species, especially *A. umbrosus*, in their serum (7, 10). The presence of antibodies indicate exposure to offending antigens and not necessarily disease, since antibodies may be detected both in patients with allergic alveolitis and in healthy controls (7).

To determine whether Danish farmers are also exposed to *A. umbrosus*, sera from farmers and farmers' spouses were investigated for IgG antibodies to *A. umbrosus* by double immunodiffusion and enzyme-linked immunosorbent assay (ELISA). Male blood donors with no relation to farming served as a control group. It was also investigated whether antibody findings were influenced by age, smoking habits or

farming methods, and whether antibodies were related to respiratory disease.

MATERIAL AND METHODS

Subjects

A total of 422 persons were included in the study. They comprised 181 male farmers, 137 farmers' spouses and 104 male non-farming controls. The farmers were members of the Danish Farmers' Association in two districts in the County of Aarhus, Denmark. The group was selected by an agricultural adviser, who was asked to include farms of varying area of land, different livestock, and varying age of farmers. Among those selected, 92 % volunteered for the study. A serum sample was obtained from each subject and was stored at -20°C for up to 1 year. The farmers comprised 181 males aged 16 to 84 years (mean 48 years). 179 worked full-time on the farm, while two also had jobs outside farming. The farmers'

spouses comprised 137 females aged 23 to 71 years (mean 47 years). 49 had full-time jobs outside farming and 88 were housewives and worked for various periods on the farm. The non-farming controls comprised 104 male blood donors aged 20 to 65 years (mean 43 years) from the Blood Bank, University Hospital, Aarhus. They all lived in an urban area and had no relation to farming.

Each person was interviewed about smoking habits, presence of respiratory symptoms and farming methods. When interviewed about smoking habits, each subject stated the number of cigarettes, cigars and grams of smoking tobacco consumed per day. They were classified as non-smokers if they had never smoked, as previous smokers if they had stopped smoking more than 6 months ago, and as smokers if they were presently smoking or if they had smoked within the last 6 months. Cough, expectoration, wheezing and dyspnoea, and their relation to work were recorded. Each symptom was assigned an arbitrary score of 1, except for cough and expectoration lasting more than 3 months within 1 year, which was assigned a score of 2 each. A cumulated symptom score was computed for each individual. Farmers were asked about the kind of livestock, size of herd and area of land, type of crops cultivated, and about storage methods.

Lung function measurement

The forced vital capacity (FVC) and the forced expiratory volume in the first second (FEV₁) were determined by spirometry (Vitalograph, UK). The best of two performances was chosen for calculation of lung function parameters related to age- and sex-dependent normal values. FVC-values 1.16 l and 0.74 l or more and FEV₁-values 1.04 l and 0.66 l or more below expected values were considered abnormal for men and women, respectively (4).

Antigen preparation

An *A. umbrosus* strain, kindly supplied by Dr. M. L. Katila, University of Koupio, Finland, was grown in asparagin-synthetic medium (3),

supplemented with 10 % NaCl, at 25°C for 4 weeks. The culture filtrate was concentrated approximately 20 times and dialysed against Tris-buffer (0.05 M Tris, 0.15 M NaCl, pH 7.4) for 3 days. The dialysate was centrifuged at 15,000 *g* for 40 min, sterile-filtered and stored at -70°C until use. The antigen had a brown colour, which interfered with routine protein assays. The protein concentration was estimated to 0.5 mg/ml by the Biorad method (13), using bovine serum albumin (BSA) as a reference. The same antigen batch was used in the double immunodiffusion test and in the ELISA.

Immunodiffusion

Immunodiffusion was performed in 1 % agarose gels (Litex, Denmark) in 0.08 M barbitalbuffer, pH 8.2 with 0.2 % phenol. Wells containing 15 µl were arranged in a hexagonal pattern 4 mm apart with a central well containing antigen. Sera were investigated undiluted and diluted 1:4. Antigen was used undiluted and diluted 1:8. The wells were filled 3 times with intervals of 24 h. After a total incubation period of 72 h at room temperature, the plates were rinsed in 5 % sodium citrate for 1 h, washed in 0.5 M NaCl overnight, pressed and swelled several times in distilled water, dried and stained with Coomassie Brilliant Blue R-250 (2).

ELISA

The determination of antibodies to *A. umbrosus* by ELISA was carried out in microtiter plates (Immunoplate I, Nunc, Denmark). Fifty µl of antigen diluted 1:100 in 0.05 M carbonate buffer, pH 9.6 were added to the wells for 1 h at 37°C. After a rinse in phosphate-buffered saline (PBS) pH 7.4 with 0.05 % Tween 20 (PBS-Tw), 50 µl of 2 % BSA in PBS were added to each well and left at 4°C overnight. After another rinse in PBS-Tw, 50 µl of serum diluted 1:1000 in PBS-Tw were added to the wells and incubated at 37°C for 2 h. The wells were rinsed with PBS-Tw and 50 µl of an alkaline phosphatase-conjugated swine anti-human IgG conjugate (Orion, Finland) were added and incubated at 4°C overnight. The

conjugate was diluted 1:500 in PBS-Tw. After a wash in PBS-Tw, 50 µl of 4-nitrophenyl phosphate (Sigma, USA) at a concentration of 1 mg/ml in 10 % diethanolamin buffer with 0.5 mmol MgCl₂, 6H₂O, pH 10.0, were added to the wells and incubated at room temperature for 30 min. Reactions were stopped with 100 µl 1 N NaOH, and absorbance of wells was read in absorbance units with a filter photometer at 410 nm (Minireader MR 590, Dynatech, USA). All sera were investigated in duplicate with and without antigen added to the wells. A positive and negative control were included in each experiment. The positive control was a high-titred serum from a healthy farmer and the negative control was PBS-Tw instead of serum.

Antibodies to *A. fumigatus* culture filtrate antigen were determined by ELISA as previously described (12).

RESULTS

Occurrence of *A. umbrosus* antibodies

IgG antibodies to *A. umbrosus* culture filtrate antigen in farmers, their spouses, and non-farming controls, appear from Table 1. The median antibody level was higher in farmers than in spouses and controls (Kruskal-Wallis test, $P < 0.0001$). ELISA antibody values above 0.5 were found in 53 farmers, in 16 spouses and in none of the controls. Precipitating antibodies occurred in 11 farmers, in none

of their spouses and in one blood donor. Thus, *A. umbrosus* antibodies were found more often and at higher levels in farmers than in their spouses and in blood donors with no relation to farming. The median antibody level was higher in 159 farmers over 30 years of age (0.26) than in 22 farmers aged 30 years or less (0.18) (Mann-Whitney test, $P < 0.04$). No correlation between antibody level and age could be demonstrated in farmers' spouses and in controls.

Antibody levels and smoking habits

The proportion of non-smokers was 43 % in farmers and 16 % in blood donors, whereas 40 % of farmers and 62 % of blood donors were smokers. This difference was statistically significant (Chi-square test, $P < 0.0005$). It is seen from Table 2 that in farmers the median antibody level was lower in smokers than in previous smokers and in non-smokers. The difference between these three groups was statistically significant (Kruskal-Wallis test, $P = 0.04$). In farmers' spouses the median antibody level in non-smokers was higher than in previous smokers and in smokers (Kruskal-Wallis test, $P = 0.02$). There was no difference in antibody levels depending on whether the subjects smoked cigarettes, pipe or cigars, and antibody levels could not be related to tobacco consumption. In blood donors it was not possible to demonstrate any correlation between antibody levels and smoking habits.

The elevated antibody levels found in farmers could not be explained by differences in smoking habits. The median antibody levels found in farmers who were smokers, previous smokers or non-smokers, were significantly higher than the levels in the corresponding groups of spouses and blood donors (Table 2).

Antibodies related to farming methods, lung function and respiratory symptoms

Antibody levels were analysed in relation to livestock (Table 3). Cattle breeders tended to have higher antibody levels than farmers without cattle, and the lowest antibody level was

Table 1

Serum IgG antibodies and precipitating antibodies against *A. umbrosus* in farmers, their spouses and blood donors

	No. of subjects	ELISA IgG* antibody activity (median and range)	Precipitating antibodies	
			No. pos.	Titer range
Farmers	181	0.24 (0.00-1.79)	11	1-16
Farmers' spouses	137	0.11 (0.00-1.35)	0	-
Male blood donors	104	0.06 (0.00-0.49)	1	16

* $P < 0.0001$ by comparison of all three groups (Kruskal-Wallis test).

Table 2

Serum IgG antibodies to *A. umbrosus* in farmers, farmers' spouses and non-farming controls determined by ELISA in relation to smoking habits. Results are expressed as median and range (in parentheses). For comparison of three groups (rows) or columns) the *P*-values computed by Kruskal-Wallis test are included

	Smokers	Previous smokers	Non-smokers	Statistics
Farmers (<i>n</i> = 181)	<i>n</i> = 72 0.18 (0.00-1.65)	<i>n</i> = 32 0.37 (0.02-1.79)	<i>n</i> = 77 0.27 (0.02-1.75)	<i>P</i> = 0.04
Farmers' spouses (<i>n</i> = 137)	<i>n</i> = 53 0.07 (0.00-0.91)	<i>n</i> = 17 0.08 (0.00-0.49)	<i>n</i> = 67 0.17 (0.00-1.35)	<i>P</i> = 0.02
Male blood donors (<i>n</i> = 103)*	<i>n</i> = 64 0.06 (0.00-0.49)	<i>n</i> = 22 0.08 (0.01-0.48)	<i>n</i> = 17 0.07 (0.03-0.24)	n.s.
Statistics	<i>P</i> < 0.0001	<i>P</i> < 0.0001	<i>P</i> < 0.0001	

* One blood donor did not state smoking habits.

found in farmers not rearing animals. However, the differences observed did not reach statistical significance. No correlation between antibody levels and size of herd or area of land could be demonstrated, and no relationship was observed between antibody levels and lung function or respiratory symptom scores. No cases of allergic alveolitis were found.

Correlation between A. umbrosus and A. fumigatus antibodies

The occurrence of *A. fumigatus* antibodies in our study groups was reported previously (12). Comparison of antibody activities to the two aspergillus species in individual sera showed that high *A. umbrosus* antibodies (> 0.7) occurred in 16 farmers who had low *A. fumigatus* antibodies (< 0.35) and the reverse was true in one farmer. Thus, some sera contained antibodies predominantly reacting with one of the species.

DISCUSSION

IgG antibodies to *A. umbrosus* culture filtrate antigen were present in both farmers, farmers' spouses and in city dwelling controls. Therefore, sensitization to this antigen seems to occur in both rural and urban environments. The increased levels of antibodies in farmers com-

pared with non-farming controls indicate that farm work may cause heavy exposure to *A. umbrosus* antigens. The tendency towards higher antibody levels in farmers rearing cattle suggests that contact with hay and straw used in cattle breeding may especially result in sensitization to *A. umbrosus*. In farmers' spouses the antibody activity was lower than in farmers, probably because the spouses only occasionally took part in the farm work. The higher

Table 3

Levels of serum IgG antibodies to *A. umbrosus* in farmers in relation to livestock (*n* = number of subjects)

Livestock		Farmers	
Cattle	Pigs	ELISA IgG antibody activity (median and range)	Non-smokers (%)
+	-	<i>n</i> = 19 0.34 (0.01-1.75)	47
+	+	<i>n</i> = 76 0.27 (0.01-1.49)	45
-	+	<i>n</i> = 74 0.19 (0.00-1.79)	40
-	-	<i>n</i> = 12 0.14 (0.00-0.77)	42

antibody levels in older than in younger farmers might be explained by length of time of antigen exposure.

Fungi of the genus *Aspergillus* contain cross-reacting antigens (8). In this study, a small group of sera showed high antibody levels to *A. umbrosus* and low antibody levels to *A. fumigatus*. This might indicate that sensitization had resulted from exposure to *A. umbrosus*.

Smoking has previously been shown to suppress IgG antibody levels to inhaled antigens (1, 9). This is consistent with the present findings in farmers and their spouses, where antibody levels were higher in non-smokers than in smokers. There were more non-smokers among farmers than among city dwelling controls. This has also been found in other studies (15), but it could not explain the increased antibody levels in farmers, as both smoking and non-smoking farmers had higher antibody levels than the corresponding non-farming controls.

Sensitization to *Micropolyspora faeni* in American dairy farmers (6) followed essentially the same pattern as found in this study for *A. umbrosus*. Precipitating antibodies to *M. faeni* were associated with non-smoking, cattle keeping as well as size of herd and area of land. This indicates that farmers may be sensitized to various microbial antigens during their work, but occurrence of IgG antibodies against these antigens has not been related to pulmonary disease apart from allergic alveolitis (6, 11). In this study only a minor part of the farmers and their spouses complained of respiratory symptoms, and their antibody levels did not differ from the levels found in those with none or few respiratory symptoms. Neither was it possible to demonstrate any correlation between lung function parameters and antibody levels. Thus, the presence of IgG antibodies to *A. umbrosus*, at levels demonstrated here, did not seem to result in bronchopulmonary disease.

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