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Circulating IgG antibodies against fungal and actinomycete antigens in the sera of farmer's lung patients from different countries

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Abstract

Sixty-nine farmer's lung patients and 28 normal controls from four countries (Finland, Switzerland, Canada and the United States) were investigated for antibody levels against 13 antigens commonly used for the screening panel for hypersensitivity pneumonitis. Of these antigens, eight were from the Medical College of Wisconsin (United States) and five were from the University of Kuopio (Finland). IgG antibodies against these antigens were studied in 97 sera using a sensitive biotin-avidin-linked enzyme immunoassay. The results indicate that the mean antibody titer against *Micropolyspora faeni* was highest in the United States (U.S.) followed by Finland. Both Finnish and U.S. antigens reacted almost identically against various groups of patients, although the degree of reactivity varied considerably. Higher antibody levels against *Thermoactinomyces vulgaris* were detected in Finnish patients than patients from other countries while patients from all four countries showed elevated levels of antibodies against *T. candidus*. This study demonstrates that antigens from identical species, irrespective of geographic origin, reacted similarly. However, variability between antigens of the same species was still considerably significant. Since the microbiological flora of moldy hay varies widely in different regions, the microbial species associated with the disease at a given geographical area has to be determined before selecting antigens for serological studies. The antigens currently used in various laboratories are crude preparations and need to be purified and standardized for dependable results. Until such antigens are available, all antigenic preparations used in the immunological evaluation of patients should be immunochemically characterized for their reproducibility and reliability although the ultimate goal should be to obtain standardized pure antigens for dependable immunodiagnosis of farmer's lung.

Introduction

Hypersensitivity lung diseases resulting from repeated exposure to antigen-laden environments is one of the most common groups of occupational lung diseases [11]. Hypersensitivity pneumonitis (HP) or extrinsic allergic alveolitis belongs to this group which also includes farmer's lung, bagassosis, mushroom worker's lung, contaminated air-conditioner and humidification system-induced diseases and many others [11]. The diagnosis of these diseases is mainly dependent on clinical, pathological, physiological, radiological and immunological findings and history of exposure to the antigen-laden environments [5]. Clinical symptoms alone are usually noncontributory, as several other lung diseases show similar features. Hence more emphasis has to be placed on immunologic parameters and exposure history. Farmer's lung patients usually show circulating antibodies against specific antigens involved in the disease. However, the presence of antibodies in the sera is not diagnostic in itself because the presence of antibodies is only an indication of exposure to the antigens and not any disease per se [20]. On the other hand it has been

reported that all the patients demonstrate high levels of serum precipitins against the offending antigen(s) [20]. Therefore both clinical and immunological findings would be of great value to the diagnosis of farmer's lung disease.

Demonstration of specific antibodies in the sera is of considerable value to the differential diagnosis of the disease [5, 19]. In addition, this may help in monitoring the course of the disease in patients particularly when exacerbation due to exposure to antigens or remission due to treatment or avoidance of antigens occurs. For the demonstration of the antibodies, dependable antigens and sensitive serological methods are essential. Although several sensitive methods are available, none of them are completely dependable due to the lack of pure and reliable antigens. The currently available antigens are nonstandardized crude extracts derived from fungi or actinomycetes. Most laboratories produce antigenic preparations for their own use while other facilities depend mainly on commercial sources. The chemical and immunological characteristics of these antigens vary greatly from batch to batch and among laboratories. Commercial antigens have been shown to be consistently inferior to the in-house antigens used by academic laboratories [6]. Because of these reasons it is not possible to compare the results of different laboratories while evaluating patients with hypersensitivity lung diseases. Standardized antigens are essential for reliable diagnosis of these diseases.

In the present study we compared antigens from thermophilic actinomycetes and fungi from two laboratories, one in the United States (U.S.) and the other in Finland, for demonstrating specific antibodies in the sera of patients with farmer's lung from four different countries to determine the antibody responses in these patients.

Materials and methods

Antigens

The antigens used in the present study were obtained from either the culture filtrates or sonicated cells of various thermophilic actinomycetes and fungi. These antigenic preparations from the two laboratories were used in the present study irrespective of their quality, reproducibility or method of preparation. The U.S. antigens were culture filtrates while Finnish antigens were sonicates of mycelia. *Micropolyspora faeni* (T-150) was grown in synthetic broth as described before, while *Thermoactinomyces candidus* (T-106), *T. vulgaris* (T-101) and *Saccharomonospora viridis* (T-216) were grown in casein hydrolyzate medium [9, 25]. All organisms were incubated at 50 °C. *M. faeni* was grown in a fermenter with a constant air supply of (8 L/min) and continuous stirring (200 rpm) for 4 to 5 days while the other organisms were grown in stationary cultures for 2 to 3 weeks [9, 14]. The culture fluid was separated from the mycelia by filtration and freeze-dried after extensive dialysis against distilled water at 4 °C. *M. faeni* and *T. vulgaris* were grown on trypticase soy agar at 50 °C by a modified method of Ojanen *et al.* [18] and the growth was scraped out after 5 to 7 days, washed thoroughly and resuspended in phosphate buffered saline (PBS). The suspension was sonicated for 3 minutes [18]. It was then centrifuged and the supernate freeze-dried after extensive dialysis. The antigenicity of the preparations were ascertained by testing the reactivity of these antigens against known positive and negative control sera [17]. These antigens were used for the detection of circulating antibodies in the sera of patients in the present study.

Aspergillus fumigatus and *Penicillium* antigens were obtained by growing the organisms in synthetic broth as described before [12]. The culture filtrates were obtained after 3 to 4 weeks of growth and dialyzed and freeze-dried. The *A. fumigatus, A. umbrosus* and *Penicillium* antigens used at the University of Kuopio were cell sap preparations of the respective growth [18]. The cell saps were obtained by sonication and eventual dialysis and lyophilization.

Characterization of antigens

a. Chemical composition. The protein content of the antigens were determined by the method of Bradford using Coomassie blue dye binding [1],

while carbohydrate content was estimated according to the method of Dubois *et al.* [4].

b. Polyacrylamide gel electrophoresis. The protein profile of the antigen extracts were studied by polyacrylamide gel electrophoresis (PAGE) as described before [13]. Twenty-five μl of an 80 mg/ml antigen was applied on 10% polyacrylamide gel. The gels were stained with Coomassie blue or silver nitrate.

c. Rocket electrophoresis. The immunogenicity and number of reactive components in the antigen preparations were studied by rocket electrophoresis using rabbit antisera raised against various antigens. The method used was the same as described before [13]. After electrophoresis the gels were stained with Coomassie brilliant blue and the number of precipitin arcs and the peak heights were noted and compared.

d. Enzyme-profile of the extract. We have recently demonstrated that the enzyme profile of various antigen preparations is of considerable value for determining the reproducibility of the extracts in antibody detection assays. We used API-ZYM™ strips (Analytab Products, Plainview, NY) to demonstrate the enzyme activity of the preparation. Two drops of a 2 mg/ml solution of the antigen was added to the substrate and incubated for 4 hours at 37 °C in a humidified atmosphere. The enzyme activity was visualized by the addition of reagents A and B supplied with the strips. The reactions were read as '+' or '−' by comparison to a color chart provided by the manufacturer.

Patient sera

Sixty-nine farmer's lung sera and 28 normal control sera were studied. The farmer's lung sera included 22 from Finland, 20 from Switzerland, 17 from Canada and 10 from the U.S. Although the criteria used for the diagnosis of the farmer's lung cases varied considerably among the four countries, all patients included in the present study had clinical symptoms and long-term history of exposure to farming environments. Pulmonary physiology, chest X-ray, lung biopsy and presence of precipitins in the sera were known for only a few patients. Normal controls were healthy laboratory employees from urban environments.

Detection of specific IgG antibody by biotin-avidin-linked immunosorbent assay

The biotin-avidin-linked immunosorbent assay (BALISA) employs the strong binding capacity of avidin to biotin for amplifying the system several-fold greater than the conventional enzyme-linked immunosorbent assay (ELISA). The method used is a modification of the one described before [8, 22]. The procedure is as follows: Polyvinyl round bottom microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with 100 μl of the antigen in carbonate buffer (pH 9.6). All except two *M. faeni* antigens were used at a concentration of 100 ng/ml protein while *M. faeni* was used at 400 ng/ml (U.S.) and at 200 ng/ml (Finland) protein for coating the plates. After overnight adsorption at 4 °C, the wells were washed with PBS containing 0.05% (v/v) Tween-20. Any free sites remaining in the wells were blocked by treating with 1% bovine serum albumin (BSA) in PBS Tween for 2 hours at room temperature. The wells were again washed three times with PBS Tween.

The human sera were diluted 1:1000 in PBS Tween containing 1% BSA and 100 μl were added to the wells. The microtiter plates were then incubated at room temperature for 1 hour. After washing the wells with PBS Tween, 100 μl of a 1:1000 dilution of normal goat serum was added and incubated for 30 minutes. One hundred μl of a 1:1000 dilution of goat anti-human IgG (Miles Laboratories, Elkhart, IN) was added to the washed wells and incubated at room temperature for 1 hour. After washing, the wells were treated with 100 μl of a 1:1000 dilution of normal rabbit serum for 30 minutes. The wells were next treated for 30 minutes at room temperature with a 1:200 dilution of rabbit anti-goat IgG conjugated with biotin (Vector Laboratories, Burlingame, CA). The wells were again washed as before and the avidin and biotinylated peroxidase were added. This reagent

(Vectastain® ABC reagent, Vector Laboratories) was used at a dilution of 1:1 000 and incubated for 30 minutes. After washing the wells, the enzyme substrate o-phenylene diamine hydrochloride (0.06%) and hydrogen peroxide (0.015%) in citrate buffer (0.1 M, pH 4.5) were added. The reaction was stopped after 30 minutes by the addition of 10 µl of 8 N H_2SO_4. The optical density (OD) was read at 492 nm using an ELISA reader. Appropriate controls were included in all plates and the OD of those controls were compared. All dilutions of the reagents were determined by checkerboard titration using positive and negative control sera.

Statistical analysis

IgG values (minus the corresponding control baseline optical density) were summarized by the mean and standard deviation for each study group. Mean values of three or more groups (Tables 3, 4 and 5) were compared by one-way analysis of variance and the least significant difference (LSD) multiple comparison test [23] when the analysis of variance F-test was significant. Mean values of the two groups were compared with t-test (Table 3). Probability levels of 0.05 or smaller were used to indicate statistical significance.

Results

Characterization of antigens

Protein and carbohydrate contents of the antigen preparations included in the study varied considerably (Table 1). Protein contents of the preparations ranged from 5 to 35 percent of the total dry weight whereas carbohydrate content ranged from 2 to 15 percent of the dry weight. Ten percent polyacrylamide gel electrophoresis revealed several Coomassie blue and silver nitrate stainable bands. All three *T. vulgaris* antigens, *S. viridis*, and *T. candidus* failed to demonstrate any protein bands when stained by Coomassie blue, but showed 3 to 10 bands when stained by silver stain. *M. faeni* antigen from Finland revealed over 30 bands with both stains while *M. faeni* antigen from the U.S. showed a considerably fewer number of protein bands by both stains. All four *A. fumigatus* strains had comparable protein components while *A. umbrosus* showed only a few bands.

Table 1. Chemical composition of the antigens used in the study.

Antigen	Protein content % w/w	Carbohydrate content % w/w
Thermophilic actinomycetes		
M. faeni, T-150	35.00	7.60
M. faeni, Finland	22.00	7.00
S. viridis, T-216	5.60	13.00
T. vulgaris, T-101	6.00	7.60
T. vulgaris, III	8.00	15.20
T. vulgaris, Finland	21.00	2.00
T. candidus, T-106	9.60	3.80
Fungal antigens		
A. fumigatus, 507	9.68	6.20
A. fumigatus, 515	8.25	12.97
A. fumigatus, 534	8.12	15.00
A. fumigatus, Finland	9.00	8.70
A. umbrosus, Finland	6.20	5.00
Penicillium notatum, 538	11.00	6.80
Penicillium, Finland	7.40	3.40

Rocket electrophoresis of *A. fumigatus* antigens using hyperimmune rabbit sera showed considerable similarity in the number of precipitin arcs. *A. umbrosus* antigens on the other hand failed to show any precipitin arcs when tested against rabbit anti-*A. fumigatus* serum. The number of precipitin bands produced by the four *A. fumigatus* antigens varied from 8 to 20 against rabbit anti-*Aspergillus* sera when optimal concentrations of antigens and antibodies were used. *M. faeni* antigens showed comparable results in rocket electrophoresis using anti-*M. faeni* serum although PAGE showed considerable differences between the two antigens. Both *T. vulgaris* antigens from Finland were far less reactive than the *T. vulgaris* antigen from the U.S. against rabbit anti-*T. vulgaris* serum when tested by rocket electrophoresis (results not shown).

The enzyme profile of the antigen preparations

Table 2. Enzyme profile of antigens used in this study.

Antigens	Alkaline Phosphatase	Esterase C4	Esterase Lipase C8	Leucine amino Peptidase	Acid Phosphatase	Phosphoamidase	α-galactosidase	β-galactosidase	α-glucosidase	β-glucosidase	N-acetyl β-glucosidase
A. fumigatus 534	–	–	–	+	+	+	+		+	+	+
A. fumigatus 515	+	+	+	+	+	+	+	+	+	+	+
A. fumigatus 507	+	–	–	+	+	+	+	+	+	+	+
A. fumigatus Finland	–	+	+	–	+	+	–	–	–	–	–
A. umbrosus Finland	–	+	+	–	+	+	–	–	–	–	–
Penicillium 538	+	+	+	–	+	+	+	+		+	+
M. faeni T150	+	+	+	–	–	–	–	+	–	–	–
M. faeni Finland	–	+	+	+	–	–	–	–	–	–	–
T. vulgaris T101	–	–	–	–	–	–	–	–	–	+	+
T. vulgaris III	–	–	+	–	–	–	–	–	–	–	–
T. vulgaris Finland	–	–		–	–	–	–	–	–	–	–
T. candidus T106	+	–	+	–	+	+	–	–	–	–	–
S. viridis T216	–	+	+	+	–	–	–	–	–	–	+

used in the study are given in Table 2. Acid phosphatase and phosphoamidase activity was noted in all four strains of *A. fumigatus* and one strain of *A. umbrosus* antigens studied. *A. fumigatus* antigens from the U.S. revealed several more enzymes than the Finnish *A. fumigatus* or *A. umbrosus* antigens. The thermophilic actinomycete antigens exhibited very few enzymes compared to the fungal antigens. The two *T. vulgaris* strains, one from the U.S. and the other from Finland, revealed considerable differences in their enzymatic activity. However, the two preparations from the Finnish *T. vulgaris* strain showed similar enzyme profile with only minor quantitative differences. On the other hand, the U.S. *T. vulgaris* antigen failed to show esterase lipase activity but showed the presence of β-glucosidase and N-acetyl β-glucosidase activities. *T. candidus* showed esterase lipase as in the case of *T. vulgaris* (Finland) in addition to alkaline phosphatase, acid phosphatase and phosphoamidase. All four *A. fumigatus* strains showed considerable diversity in the enzyme contents, although at least two enzymes were common to all strains. The high degree of variability exhibited by these antigens is well-demonstrated by their enzyme activity.

Detection of specific IgG antibodies against various antigens in sera of patients by BALISA

All 97 sera studied showed antibodies against all 14 antigens studied. The results are summarized in Table 2. Although the ELISA results varied extensively (OD readings) within the patient and control groups, patients invariably demonstrated significantly higher OD readings than controls. Against the thermophilic actinomycetes antigens the responses of the patients from different countries varied considerably. For example all three *T. vulgaris* antigens revealed significantly higher antibody levels in the patients from Finland than from other countries (Tables 3 and 4). Similarly *T. candidus* showed higher antibody titers in Finnish

Table 3. IgG antibodies in the sera of farmers from four countries against fourteen antigens detected by BALISA.

Antigens used	Farmer's lung (N = 69)		Normal controls (N = 28)	
	Optical density		Optical density	
	Mean	S.D.	Mean	S.D.
Thermophilic actinomycetes				
M. faeni, T-150	0.593[a]	0.565	0.073[a]	0.055
M. faeni, Finland	0.444[a]	0.490	0.027[a]	0.247
S. viridis, T-216	0.330	0.360	0.155	0.179
T. vulgaris, T-101	0.869	0.664	0.708	0.412
T. vulgaris, III	0.303[a]	0.237	0.121[a]	0.089
T. vulgaris, Finland	0.685[a]	0.628	0.230[a]	0.261
T. candidus, T-106	0.815[a]	0.598	0.161[a]	0.274
Fungal antigens				
A. fumigatus, 507	0.271[b]	0.388	0.030[b]	0.023
A. fumigatus, 515	0.479[c]	0.561	0.199[c]	0.224
A. fumigatus, 534	0.392[a]	0.358	0.128[a]	0.086
A. fumigatus, Finland	0.494[b]	0.443	0.201[b]	0.289
A. umbrosus, Finland	0.506[a]	0.349	0.156[a]	0.171
Penicillium notatum, 538	1.087[a]	0.629	0.568[a]	0.444
Penicillium, Finland	0.675	0.588	0.598	0.484

[a] Statistically significant, $P < 0.001$.
[b] Statistically significant, $P < 0.005$.
[c] Statistically significant, $P < 0.01$.

Table 4. Antibodies against *Micropolyspora faeni, Thermoactinomyces candidus* and *Aspergillus umbrosus* in farmer's lung patient sera measured by BALISA.

Countries of origin of the patient	No.	Antibodies against *M. faeni,* T-150		Antibodies against *M. faeni,* Finland		Antibodies against *T. candidus*		Antibodies against *A. umbrosus*	
		Mean O.D.	S.D.	Mean O.D.	S.D.	Mean O.D.	S.D.	Mean O.D.	S.D.
1. Finland	22	0.621	0.539	0.563	0.454	1.156	0.4195	0.7213	0.3983
2. Switzerland	20	0.402	0.562	0.328	0.394	0.6633	0.678	0.4389	0.1818
3. Canada	17	0.308	0.0925	0.175	0.286	0.599	0.488	0.333	0.2699
4. U.S.A.	10	1.396	0.318	0.893	0.634	0.7356	0.6885	0.4652	0.4149
Significant differences (LSD)		1v4, $P<0.001$ 2v4, $P<0.001$ 3v4, $P<0.001$		1v3, $P<0.01$ 2v4, $P<0.005$ 3v4, $P<0.001$		1v2, $P<0.01$ 1v3, $P<0.005$		1v2, $P<0.01$ 1v3, $P<0.005$ 1v4, $P<0.05$	

O.D. = optical density.
S.D. = standard deviation.

and U.S. patients than others. On the other hand, antibody response to both Finnish and U.S. *M. faeni* antigens was more pronounced in the U.S. patients, followed by the Finnish, than in patients from the other two countries studied. No significant antibody response to any of the antigens tested was observed in patients from Canada and Switzerland. *A. umbrosus*, a common member of the moldy hay flora in Finland, demonstrated significant antibody responses in both patients as well as normal individuals from Finland. The patients from all four countries revealed considerably low levels of antibodies against *S. viridis* and *Aspergillus* species. Finnish patients showed invariably high antibody titers against all three *T. vulgaris* antigens, while patients from other countries showed comparatively low titers to these three antigens (Table 5). High titers of antibodies were also detected in all patient groups against *T. candidus* while antibodies against *A. umbrosus* were detected in high titers among Finnish patients (Tables 3 and 4).

Table 5. Antibodies against various *Thermoactinomyces vulgaris* antigens in the sera of patients with farmer's lung from various countries measured by ELISA.

Country	No.	T.V., III		T.V., T-101		T.V., Finland	
		Mean O.D.	S.D.	Mean O.D.	S.D.	Mean O.D.	S.D.
1. Finland	22	0.566	0.2211	1.5328	0.3936	1.3925	0.4511
2. Switzerland	20	0.1694	0.1326	0.369	0.63	0.4887	0.5136
3. Canada	17	0.1677	0.926	0.4607	0.3405	0.2008	0.1394
4. U.S.A.	10	0.2237	0.943	0.7081	0.562	0.3561	0.2015
Significant differences (LSD)		1v2, $P<0.005$ 1v3, $P<0.005$ 1v4, $P<0.005$		1v2, $P<0.001$ 1v3, $P<0.001$ 1v4, $P<0.001$		1v2, $P<0.001$ 1v3, $P<0.001$ 1v4, $P<0.001$	

T.V. = *Thermoactinomyces vulgaris*
O.D. = optical density
S.D. = standard deviation

Discussion

Farmer's lung is a disabling disease among people engaged in active farming. The disease results from inhalation of dust from moldy hay. The microbiological flora of hay vary from one geographical region to another [19, 20]. It has been reported in the past that *M. faeni* is the causative antigen in farmer's lung disease in England and the U.S. [3, 21]. There were reports on the prevalence of this organism in moldy hay from other parts of the world and its involvement in farmer's lung disease [2, 7, 15, 16, 26]. However, previous studies conducted in Finland showed that *T. vulgaris* and *A. umbrosus* were the most common microorganisms present in moldy hay [24]. This finding was further corroborated by the detection of circulating antibodies against these two antigens in farmer's lung patients [10]. However, the significance of high titers of circulating antibodies against *A. umbrosus* in farmer's lung patients and other exposed individuals is not yet understood. It is also interesting to note that the sera of all 22 patients from Finland exhibited high titers of antibodies against both *T. vulgaris* and *T. candidus*. Previous reports on the microbiological flora of moldy hay from Finland failed to demonstrate the presence of *T. candidus*. We were able to isolate both *T. vulgaris* and *T. candidus* with equal frequency in the hay samples from Finland (unpublished results). Thus the presence of *T. candidus* antibodies in patient sera is in agreement with microbiological results. Patients from Switzerland demonstrated higher antibody levels against *T. candidus* antigens than any of the other antigens tested. However, it has not been established whether *T. candidus* is present in moldy hay in Switzerland. Previous reports indicate that the predominant antibody response in farmer's lung patients in Switzerland was directed against *M. faeni* and *T. vulgaris* [2].

From the results presented above, we could not detect any major differences between antigens from Finland or the U.S. in demonstrating circulating antibodies in farmer's lung patients. The differences which can be seen may be due to the variability of the antigens as evidenced by rocket electrophoresis, polyacrylamide gel electrophoresis, enzyme profile and chemical composition. The preparations of *T. vulgaris* antigens from Finland and the U.S. showed considerable difference in their reactivity against individual patient sera. The *T. vulgaris* antigen from the U.S. showed stronger reactivity among Finnish patients than the two Finnish antigens indicating that local strains may not be essential for antibody detection (Table 5). However, it is essential to determine the predominant species/strains of a geographical region and the role they play in the disease process when selecting the antigen for diagnostic purposes.

The lack of standardized antigens for the immunological evaluation of farmer's lung patients is a limiting factor in the diagnosis of the disease and patient management. This study confirms the diversity in the immunochemical characteristics of the antigens and the variability of these antigens in their reactivity with patient sera [6, 10, 12]. For dependable serological diagnosis of farmer's lung, more pure and standardized antigens are necessary. The information acquired from various immunochemical tests such as PAGE, rocket electrophoresis and enzyme profile indicates the heterogeneity and resulting lack of reproducibility of thermophilic actinomycetes antigens. On the contrary, the *A. fumigatus* antigens showed some similarity in their enzyme profile, PAGE and rocket electrophoretic patterns and reactivity against patient sera. The results thus indicate that enzyme profile may be of considerable value in assuring the quality of antigens employed in the assays until more purified and standardized antigens are universally available. Greater enzyme activity usually correlated with stronger antigenicity. However, no such correlation was detected in the PAGE profile and reactivity of the preparations with patient sera. It is interesting to note that the *M. faeni* antigen from Finland showed several more protein bands in PAGE and was less reactive than the U.S. antigen which showed fewer protein bands. This may be due to the fact that only certain proteins (antigens) are relevant in the antibody detection. This study also indicates the need for understanding significant antigens involved in the disease in a given geographical region, as the microbiological flora differ considerably from place to place depen-

ding on hay-making techniques and climatic conditions. Further collaborative studies in identifying significant antigens from diverse regions and their purification and standardization for reliable serological diagnosis of farmer's lung are in progress.

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