

IgG and IgA subclass antibodies against *Aspergillus umbrosus* in farmer's lung disease

K. KAUKONEN, J. SAVOLAINEN, M. VIANDER*, M. KOTIMAA† and E. O. TERHO

Departments of Clinical Allergology and *Medical Microbiology, University of Turku, Turku, and

†Kuopio Regional Institute of Occupational Health, Kuopio, Finland

Summary

Fifty patients with farmer's lung disease (FL), 20 asymptomatic exposed farmers, and 18 healthy controls were investigated for total IgG and total IgA, as well as subclass antibody levels against *Aspergillus umbrosus*, the most common fungus to which FL patients in Finland have been exposed. Enzyme-linked immunosorbent assay (ELISA) revealed significantly elevated levels of specific total IgG and IgA antibodies in the sera of patients during the acute phase of the disease. Both IgG1 and IgG2 subclass antibodies and both IgA1 and IgA2 subclass antibodies were significantly associated with the disease. The antibody levels decreased during the first 3 months, when mould exposure was avoided and when an oral 6-week steroid treatment was administered. During the next 12 month follow-up, the mean antibody levels continued to decrease, even though re-exposure might have occurred. The results (with sensitivity 94% and specificity 95%) imply that in addition to the measurement of *A. umbrosus*-specific IgG, determination of both *A. umbrosus*-specific IgG1 and IgA might also be useful in the serodiagnosis of acute stage of FL.

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Introduction

Aspergillus umbrosus, a fungus of *A. glaucus* group [1], has been isolated from mouldy hay, and is most frequently associated with farmer's lung disease (FL) among Finnish farmers [2,3]. The diagnosis of FL is based on clinical findings in accordance with internationally accepted diagnostic criteria [4]. The presence of total IgG and IgA antibodies against mannoprotein [5] or crude *A. umbrosus* antigen [6] has been recently reported to be indicative of antigen exposure. The antibodies are not, however, disease-specific. The type of antigen, route and duration of antigen exposure and genetic background of the patient seem to determine the subclass of IgG antibody response [7]. Although *A. umbrosus*-specific IgG3 antibodies have been reported to correlate with decreased pulmonary diffusing capacity [8], no such associations have been reported for other *A. umbrosus*-specific IgG and IgA subclass antibodies.

In the present study we have measured circulating total IgG and total IgA as well as subclass antibodies against *A. umbrosus* during different phases of the disease using the ELISA technique. Further, we have analysed their serological significance in farmer's lung disease.

Material and methods

Diagnostic criteria for FL

The diagnosis of FL was based on the criteria formulated by Terho [4]. The main criteria were exposure to offending antigens, lung infiltration on chest X-ray, and symptoms compatible with FL [9]. The additional criteria were basal crepitant rales, impaired pulmonary capacity, decreased oxygen saturation of the arterial blood during exercise, restrictive ventilation defect in the spirometry, and positive provocation test. The diagnosis was considered to be confirmed if the patient fulfilled all the main criteria and at least two of the additional criteria.

Patient sera

A total of 130 serum samples from 50 clinically confirmed FL patients during the acute phase and at the 3 month

Correspondence: Dr K. Kaukonen, Department of Clinical Allergology, University of Turku, Medicity Research Laboratory, Tykistökatu 6, 20500 Turku, Finland.

follow-up, and from 12 of the 50 patients also at the 6- and at the 12-month follow-ups was studied. Total IgG and IgA as well as subclass antibodies against *A. umbrosus* antigen were determined. Sera from 20 asymptomatic farmers (exposed controls) and 18 healthy non-farmer controls were also investigated. Serum samples, obtained from the Department of Pulmonary Diseases, Kuopio University Hospital, Finland, were stored at -70°C and studied simultaneously. All the patients were admitted to the hospital during the acute phase of the disease and after the diagnostic investigations, a 6-week oral steroid treatment was initiated. After hospitalization, the patients resumed their previous work and were advised to use dust respirators when handling hay or straw.

A. umbrosus cultures

A. umbrosus (strain M 118, isolated from Finnish mouldy hay by M. Kotimaa, PhD, Kuopio Regional Institute of Occupational Health, Kuopio, Finland) was grown on agar plates containing 2.0 g Malt Extract (Oxoid L39), 1.5 g Bacto Agar (Difco 0140-01), and 10.0 g NaCl in 100 ml of distilled water at $+25^{\circ}\text{C}$ for 7 days. Five millilitres of culture medium containing 2.0 g malt extract (Oxoid L39), 1.0 g mycological peptone (Oxoid L40), 4.0 g glucose, and 10 g NaCl in 100 ml of distilled water was added to the sub-cultures, stirred gently and transferred by pipettes to Roux bottles containing 100 ml of the culture medium. The cultures were incubated horizontally at $+25^{\circ}\text{C}$ for 5 days.

Preparation of the crude antigen of *A. umbrosus*

The cultures were washed three times with 0.15 M NaCl, transferred to 0.125 M NH_4HCO_3 , and autoclaved at $+120^{\circ}\text{C}$ for 2 hr. The mycelia were collected by centrifugation in a continuous-flow centrifuge (Sorvall RC5C) at 7000 r.p.m. for 30 min and washed twice with 0.15 M NaCl. The pellet was weighed, ruptured with a hand-held electric mixer (Brown), pressed through an X-Press chamber (Biox Ab, Göteborg, Sweden) six times under pressure of 4000 k.p.m./ cm^2 at -25°C , and sonicated in continuous flow five times for 2 min (Branson Sonifier, U.S.A.). The suspension was centrifuged at 20 000 r.p.m. for 30 min. Thereafter the supernatant was filtered with a 0.2 μm filter, dialysed three times (Amicon YM 1, Diaflo, Danvers, U.S.A.), lyophilized and stored at $+4^{\circ}\text{C}$.

Enzyme-linked immunosorbent assay (ELISA)

Circulating IgG and IgA antibodies to *A. umbrosus* were quantitated by ELISA, as described earlier [5]. Polystyrene microtitre plates (Nunc, Roskilde, Denmark)

were coated with 100 μl of the *A. umbrosus* antigen (at 0.3 mg/ml in PBS, pH 7.3), kept at $+4^{\circ}\text{C}$ overnight and saturated with 100 μl of 1% normal sheep serum in PBS (NSS-PBS) at $+37^{\circ}\text{C}$ for 2 hr. The plates were then incubated with 75 μl of 1:250 diluted sera in NSS-PBS at $+37^{\circ}\text{C}$ for 2 hr. Thereafter, 75 μl of alkaline phosphatase-conjugated swine anti-human IgG and IgA (Orion Diagnostica, Helsinki, Finland), diluted 1:300 and 1:100 in NSS-PBS, respectively, were incubated on the plates at $+37^{\circ}\text{C}$ for 2 hr. Next, 75 μl of *p*-nitrophenylphosphate in diethanolamine-MgCl₂ buffer (1 mg/ml; Orion Diagnostica, Helsinki, Finland) was added and incubated at $+37^{\circ}\text{C}$ for 30 min. The enzyme reaction was stopped with 120 μl of 1 M NaOH. The absorbances were measured with a Multiscan Photometer (Labsystems, Helsinki, Finland) at a wave-length of 405 nm. All the serum samples were tested in duplicate.

The circulating anti-*A. umbrosus* IgG and IgA subclass antibodies were measured using a modified procedure, as reported earlier [10]. The plates (Nunc, Roskilde, Denmark) were coated with 100 μl of the *A. umbrosus* antigen (0.3 mg/ml in PBS, pH 7.3), incubated at $+4^{\circ}\text{C}$ overnight, saturated with 100 μl of 3% bovine serum albumin in PBS (BSA-PBS) at $+37^{\circ}\text{C}$ for 3 hr and then incubated with 75 μl of serum samples (1:100 in 0.3% BSA-PBS) at $+37^{\circ}\text{C}$ for 2 hr. Monoclonal antibodies of the IgG subclasses, IgG1, IgG2, IgG3, and IgG4 (Oxoid, Basingstoke, England; clones NL16, GOM1, ZG4, RJ4 and code Nos M15015, M10015, M08010, M11013, respectively), were diluted at 1:3000 for IgG1 and 1:200 for IgG2, IgG3 and IgG4. A total of 75 μl of the monoclonal antibody dilution was applied to the plates and incubated overnight at room temperature. Thereafter 75 μl (1:3000 in 0.3% BSA-PBS) of alkaline phosphatase-conjugated rabbit anti-goat IgG+IgM (Medac, Hamburg, Germany; code No M30808) were added and incubated at $+37^{\circ}\text{C}$ for 3 hr. Monoclonal antibodies of the IgA subclasses, IgA1 and IgA2 (Becton Dickinson, Mountain View, CA, U.S.A.; code Nos P1259 and P1128, respectively), were diluted at 1:100 in 0.3% BSA-PBS and incubated on the plates at room temperature overnight. Rabbit anti-mouse immunoglobulin (Miles-Yeda, Kiryat Weizmann, Rehovot, Israel) absorbed with human IgG was diluted at 1:1000 and 75 μl were added and incubated for 3 hr at $+37^{\circ}\text{C}$. Next, 75 μl of 1:250 in 0.3% BSA-PBS diluted swine alkaline phosphatase conjugated anti-rabbit IgG (Orion Diagnostica, Helsinki, Finland; No 67850) was allowed to react overnight at room temperature. The enzyme reactions and the absorbance measurements were done as described above for the IgG and IgA determinations. The antibody levels are expressed as the optical density (OD) at 405 nm wavelength.

The sensitivity and specificity for *A. umbrosus* anti-

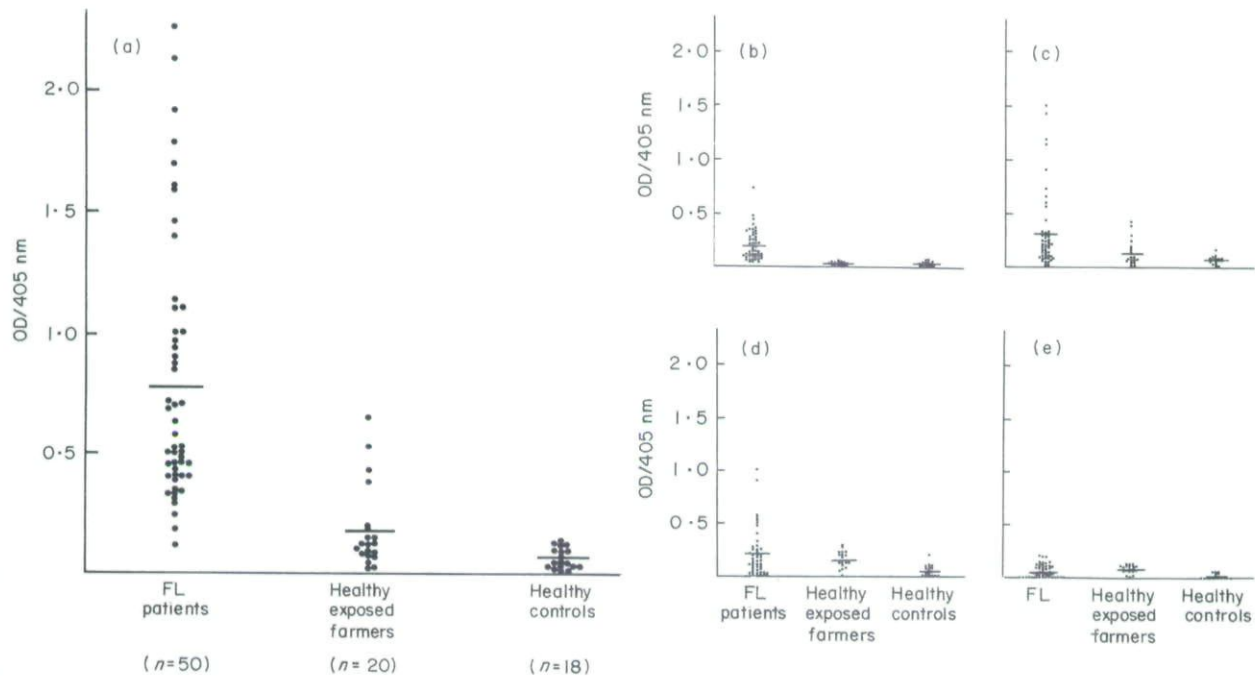


Fig. 1. Levels of a, IgG; b, IgG1; c, IgG2; d, IgG3 and e, IgG4 antibodies against *A. umbrosus* antigen in the sera of FL patients, of healthy exposed farmers, and of healthy controls. Number of individuals (n) in each group are the same for all immunoglobulin classes.

bodies were calculated. Absorbance values for patients and healthy exposed farmers of at least two standard deviations (2 s.d.) higher than the mean absorbance in the healthy control group were considered as positive.

Statistical analysis

ANOVA, Fisher PLSD-test and Scheffe F-test were used for the statistical analyses.

Results

IgG and IgG subclass antibodies

The mean concentrations of *A. umbrosus*-specific total IgG as well as IgG subclass antibodies for each individual in the different groups are presented in Fig. 1. During the acute phase of disease, FL patients had significantly higher levels of *A. umbrosus*-specific total IgG antibodies than the healthy exposed farmers ($P < 0.001$) and the healthy controls ($P < 0.001$). The patients also had significantly higher levels of IgG1 and IgG2 antibodies than the exposed farmers ($P < 0.05$) and the healthy controls ($P < 0.01$), and significantly higher levels of IgG3 antibodies ($P < 0.001$) than the healthy controls. The levels of IgG4 class antibodies were low in each group (absorbances < 0.08); only minor differences were observed. Three months after the acute phase there were no significant changes in the number of *A. umbrosus*-specific IgG subclass antibodies (data not shown).

IgA and IgA subclass antibodies

Significantly higher concentrations of *A. umbrosus*-specific total IgA antibodies in FL patients during the acute phase than in exposed healthy farmers or healthy controls ($P < 0.001$ and $P < 0.01$, respectively) were detected (Fig. 2). Moreover, the *A. umbrosus*-specific IgA1 and IgA2 antibody levels were significantly elevated in FL patients during the acute phase, compared with that observed in the exposed farmers and healthy controls ($P < 0.001$ and $P < 0.001$, respectively for both IgA subclasses). The number of *A. umbrosus*-specific IgA subclass antibodies in FL patients at 3 month follow-up did not significantly differ from that during the acute phase (data not shown).

IgG and IgA class antibodies during 12 month follow-up

During the acute phase of the disease, elevated levels of *A. umbrosus*-specific class IgG and IgA antibodies were observed. We wanted to follow the antibody response more closely during the next 12 months. As can be seen in Figs 3 and 4, a decreasing tendency of all IgG and IgA class antibodies for those 12 patients was noticed. At the end of the follow-up, all 12 patients could still be considered positive for IgG, IgA, IgA1 and IgA2 class antibodies; and eight patients, positive for IgG1 and IgG2 subclass antibodies. The increase of IgG, IgG1, IgG2,

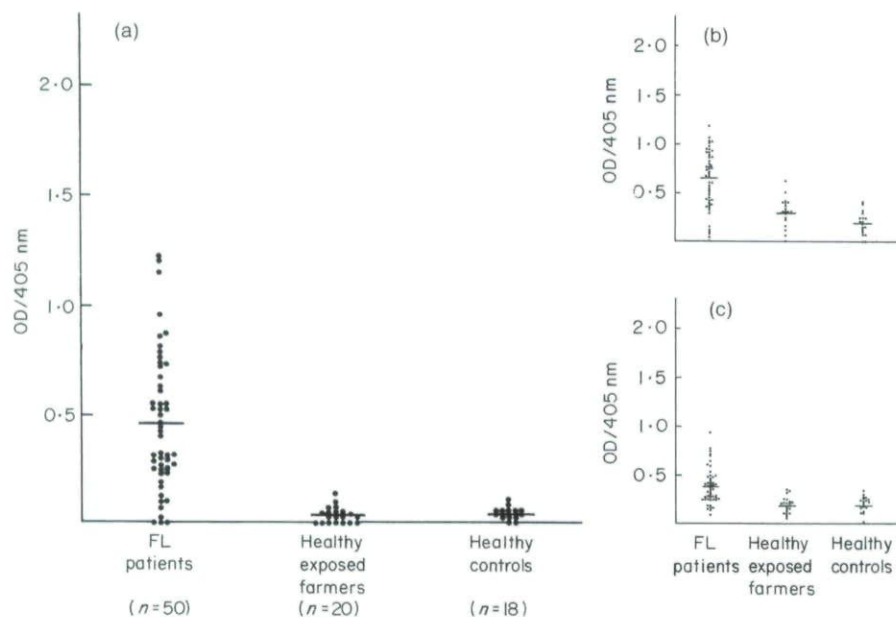


Fig. 2. Levels of a, IgA; b, IgA1, and c, IgA2 antibodies against *A. umbrosus* antigen in the sera of FL patients, of healthy exposed farmers and of healthy controls.

IgA, IgA1 and IgA2 antibodies for patients 2, 3, 5 and 12 could be explained by a history of continuous re-exposure to mouldy hay without the use of a proper dust respirator.

Sensitivity and specificity of the antibody panels

The sensitivity and specificity of *A. umbrosus*-specific antibodies in the serodiagnosis of FL were calculated

separately for the IgG and IgA class antibodies. The sensitivity and specificity for IgG detection were 96% and 80%; for IgG1 82% and 100%; and for IgG2, 58% and 60%, respectively. Both values were under 50% for IgG3 and IgG4. The sensitivity and specificity for total IgA were 86% and 95%; for IgA1, 66% and 90%; and for IgA2, 50% and 100%, respectively. The best combination

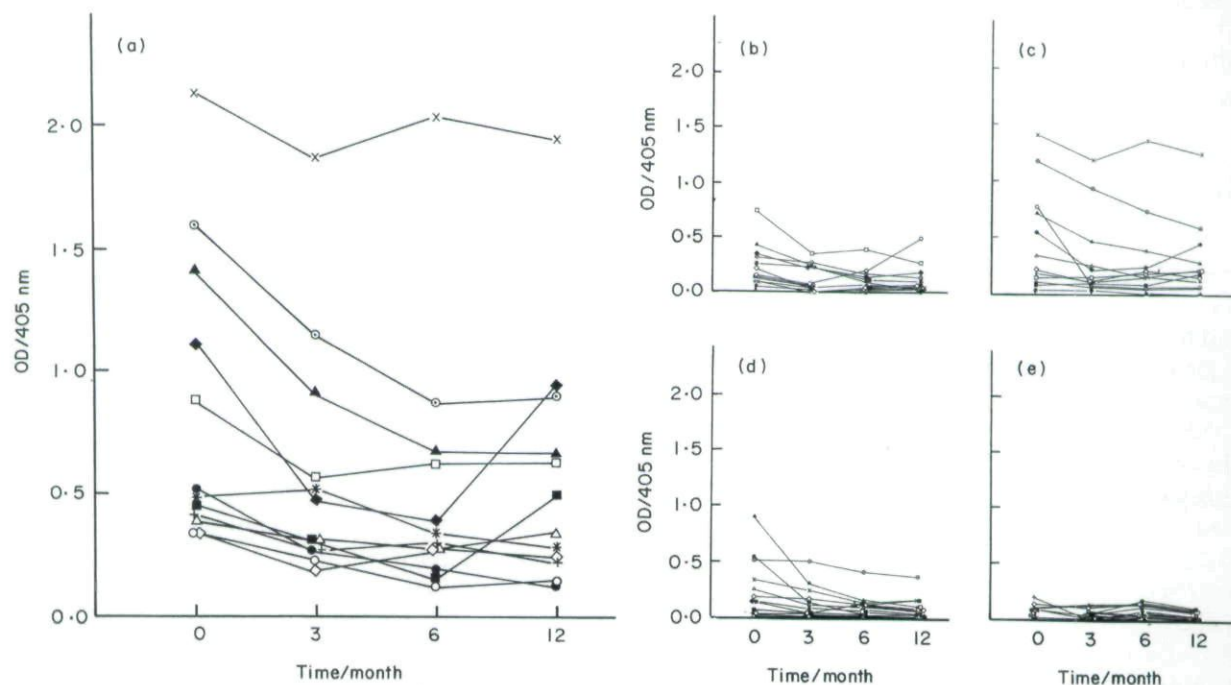


Fig. 3. Levels of a, IgG; b, IgG1; c, IgG2; d, IgG3 and e, IgG4 antibodies against *A. umbrosus* antigen in patient sera during follow-up period. Patients: 1, $\square$; 2, $\blacklozenge$; 3, $\circ$; 4, $\diamond$; 5, $\blacksquare$; 6, $\bullet$; 7, $\blacktriangle$; 8, $\triangle$; 9, $\circ$; 10, $+$; 11, $\times$; 12.

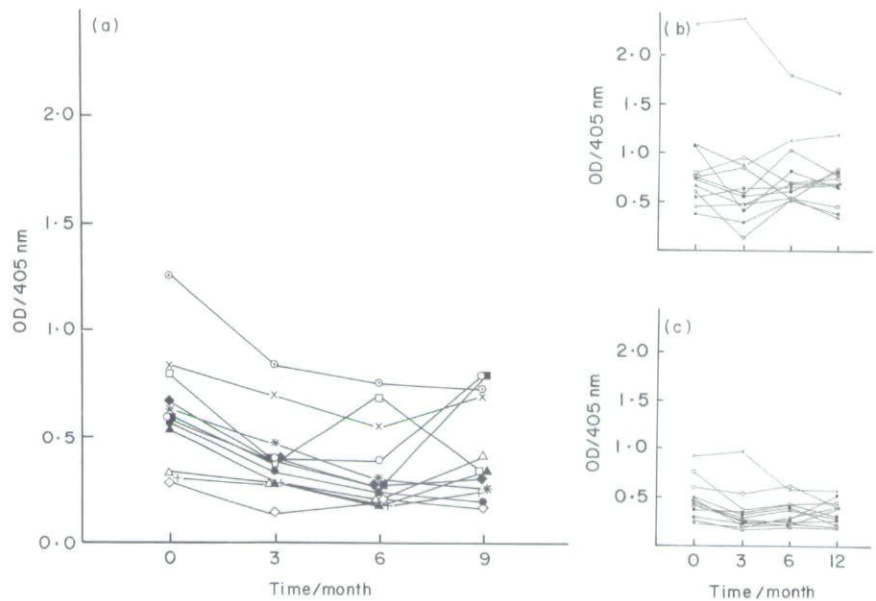


Fig. 4. Levels of a, IgA; b, IgA1 and c, IgA2 antibodies against *A. umbrosus* antigen in patient sera during follow-up period. For symbols please see Fig. 3.

was the measurement of both *A. umbrosus*-specific IgG1 and IgA antibodies, whereupon the sensitivity was 94% and the specificity was 95%.

Discussion

In FL patients, circulating antibodies of several immunoglobulin classes against many environmental microbes can be detected, while in healthy exposed farmers and in controls primarily IgG class antibodies against only one microbe are detected [11,12]. *A. umbrosus*, a fungus of the *A. glaucus* group, is the most common microbe to which FL patients in Finland are exposed. This study confirms previous findings [6] that FL patients have high concentrations of *A. umbrosus*-specific total IgG and IgA antibodies and, further, that the presence of these antibodies in the sera of patients supports the diagnosis. The role of *A. umbrosus*-specific IgG subclass antibodies has been obscure. The data from this study show that patients with FL have significantly higher concentrations of *A. umbrosus*-specific IgG1, IgG2, IgA1 and IgA2 subclass antibodies than healthy exposed farmers and non-farmer healthy controls.

The immune response against polysaccharides primarily consists of IgG2 subclass antibodies, while IgG1 subclass antibodies are suggested to react against protein antigens [13,14]. In our ELISA studies with *Candida albicans*, mannan elicited significantly high concentrations of IgG2 subclass antibodies (data not shown here). Since *A. umbrosus* crude antigen consists mainly of mannan/mannoprotein and galactose [5], we suggest that at least the IgG2 subclass antibodies detect the most antigenically active mannogalactose epitopes. The elevated, antigen-specific IgG1 antibodies in the present

study may represent the antibody response to mannoprotein antigens. *A. fumigatus* and *A. niger*, as we suggest for *A. umbrosus*, consists primarily of mannan, galactose and glucose [15,16]. *A. fumigatus*-specific IgG2 subclass antibody reactivity has been found to be important in the serological diagnosis of FL in Germany [17]. Correspondingly, we feel that in our *A. umbrosus*-exposed patients, IgG2 anti-mannan antibodies may be elicited. In a study that compared IgG3 and IgG4 subclass antibody levels with pulmonary functions of FL patients, *A. umbrosus*-specific IgG3 subclass antibodies correlated inversely with lung function [8].

Very little is known about IgA subclass antibodies in FL disease except that, according to Sennekamp *et al.* [18], IgA class antibodies are more specific for FL than IgG class antibodies. Our results further show that *A. umbrosus*-specific IgA1 and IgA2 antibodies are more specific for FL than *A. umbrosus*-specific IgG subclass antibodies.

A. umbrosus-specific class antibodies decreased during the 12 month follow-up period, except in those patients who did not use an effective dust respirator while handling hay or straw. Possible changes in the avidity (specificity and strength of the binding) of IgG and IgA antibodies against *A. umbrosus* antigen during the follow-up period is an area of interest that certainly warrants further study.

In this study, the sensitivity and specificity of *A. umbrosus*-specific IgG1 and IgA antibodies together in the serodiagnosis of FL were the best seen, and exceeded those of *A. umbrosus*-specific total IgG. Our findings, together with results of other reports, imply that it might be useful to measure antigen-specific total IgG, IgG1 and IgA.

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