Clin Exp Immunol 1994; 95:162–165

Avidity of *Aspergillus umbrosus* IgG antibodies in farmer's lung disease

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(Accepted for publication 15 September 1993)

SUMMARY

Farmer's lung disease (FL), the commonest form of allergic alveolitis caused by repeated inhalation of mouldy hay, is associated with exposure to the fungus *Aspergillus umbrosus* among Finnish farmers. The antigen-binding avidity of *A. umbrosus*-specific IgG antibodies was measured in 12 FL patients in acute phases of initial and recurrent attacks and during 1 year follow up as well as in 12 healthy farmers and five healthy urban controls. The farmers' groups were further divided into two subgroups: subjects with short exposure (< 7 years) and subjects with long exposure (> 25 years). During the first acute phase FL patients with long exposure exhibited a high avidity of *A. umbrosus*-specific IgG antibodies that remained high during the 1 year follow up, although the *A. umbrosus*-specific IgG antibody titre decreased. A re-exposure to mouldy hay leading to a recurrence further enhanced the maturation of the antibody avidity, so that an even higher *A. umbrosus*-specific IgG avidity with a less significant increase of antibody titre occurred than during the first acute attack. Notably higher IgG antibody avidity was observed in FL patients with long exposure than in healthy farmers or in healthy controls.

Keywords allergic alveolitis *Aspergillus umbrosus* IgG avidity farmer's lung disease

INTRODUCTION

Farmer's lung disease (FL), a diffuse interstitial granulomatous allergic lung disease, results from repeated inhalation of organic dusts originating from hay containing microbes such as aspergilli and thermophilic actinomycetes, especially *Faenia rectivirgula* (previously *Micropolyspora faeni*) and *Thermoactinomyces* spp. The fungus *Aspergillus umbrosus* is often associated with the disease among Finnish farmers [1]. In the immunopathogenesis of FL both cell-mediated and immune complex-mediated immune reactions are involved. IgG and IgA antibodies to the offending antigens can usually be demonstrated in the sera of the patients, and both IgG and IgA immune complexes have been found in the bronchoalveolar lavage fluid of the patients [2]. The diagnosis is based on a history of exposure, clinical features, chest x-ray, and pulmonary function tests. The presence of serum IgG antibodies to suspected antigen supports the diagnosis but is not diagnostic alone, since antigen-specific IgG antibodies can also be found in the sera of healthy farmers.

The initial antigen exposure primarily induces a clonal expansion of B cells producing low-avidity antibodies. As a result of maturation of the immune response, continuing antigen exposure leads to selection of B cell clones producing antibodies with higher avidity. The measurement of avidity and functional affinity of polyclonal antibodies to large sized immunologically complex microbial antigens has not succeeded using conventional immunological affinity assays such as the equilibrium dialysis and precipitation tests [3]. Recently, an ELISA assay for avidity measurements, based on a new principle, worked well after vaccination and in the differentiation between previous infections and fresh recurrent infections [4,5]. In a quantitative avidity-ELISA technique introduced for serodiagnosis of rubella infections [4–6], low-avidity IgG antibodies are eluated from the immobilized antigens by urea. Urea disrupts hydrophobic bonds [7] between low-avidity IgG antibodies and antigens, while most of the high-avidity IgG antibodies remain bound and are then measured.

In order to see the avidity pattern in a hypersensitivity disease like FL, we have adapted the urea elution IgG avidity-ELISA for determining the avidity of *A. umbrosus*-specific IgG antibodies. In this study we demonstrate the avidity of *A. umbrosus*-specific IgG antibodies in FL patients in acute phases of initial and recurrent attacks as well as in healthy farmers (exposed controls) and healthy controls.

PATIENTS AND METHODS

Patient sera

The avidity of IgG antibodies to *A. umbrosus* antigen was measured from 58 serum samples. The FL patients ($n = 12$) and healthy exposed farmers ($n = 12$) were further divided into two subgroups according to the length of the mould exposure

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period: exposure for less than 7 years was considered short, and exposure for at least 25 years was considered long. Both among FL patients and healthy farmers there were seven with long and five with short exposure. There was also a subgroup of five patients with long exposure who were later diagnosed as having a recurrence of the disease. A 6-week oral steroid treatment was prescribed after diagnostic investigations, and the patients were encouraged to avoid mouldy hay exposure. The patients' sera were drawn during the acute phase, as well as 3 or 6, and 12 months thereafter. Samples of the group with recurrences were drawn in a similar manner after the initial acute and recurrent phases. There was also a reference group of five non-farming healthy controls.

Crude antigen of A. umbrosus

Aspergillus umbrosus (strain M 118 isolated from Finnish mouldy hay by M. Kotimaa, Kuopio Regional Institute of Occupational Health, Kuopio, Finland) was cultivated and prepared to crude antigen as described earlier [8].

Urea elution IgG avidity-ELISA

The ELISA for the avidity of *A. umbrosus*-specific IgG antibodies was performed as reported earlier for rubella [6]. The microwell plates (Nunc, Roskilde, Denmark) were coated with *A. umbrosus* antigen 0·1 mg/ml in PBS containing 8 M urea, incubated overnight at 4°C, washed twice with PBS-urea, three times with PBS containing 0·05% Tween 20 (PBS-T), and once with deionized water. Serum samples were diluted two-fold from 1:25 to 1:51 200 in PBS-T, and 100 μl were incubated in each well in duplicate for 2 h at 37°C. Thereafter, one well was washed with PBS-T and the other well with PBS-T containing 8 M urea three times, 5 min each. The wells were incubated with alkaline phosphatase-conjugated anti-human IgG (Orion Diagnostica, Espoo, Finland) 1:100 in PBS-T for 1 h at 37°C, washed three times with PBS-T, and the enzyme reaction with *p*-nitrophenyl phosphate in diethanolamine-$MgCl_2$ buffer (2 mg/ml; Orion Diagnostica) was allowed to occur for 30 min at 37°C. The enzyme reaction was stopped with 120 μl of 1 M NaOH and the absorbances were measured at a wavelength of 405 nm with a Multiscan Photometer (Labsystems, Helsinki, Finland). All the tests were made in duplicate.

The avidity was calculated from the two dilution curves obtained for each sample. Background absorbances were reduced before the dilution curves were drawn. End-point titres at $OD_{405} = 0{\cdot}200$ as the cut off were determined, and the avidity ratio (titre (urea+)/titre (urea−)) × 100 was calculated. The end-point titre at the cut-off level from the dilution curve without urea is expressed as the abscissa (serum titre) in Figs 2 and 3.

Statistical analysis

The significance of the differences in avidity and titres of *A. umbrosus*-specific IgG antibodies between the study groups was tested using Student's *t*-test.

RESULTS

According to our pilot study with different urea concentrations the optimal discrimination of antibody avidity was reached using an 8 M urea concentration. Dilution curves for one patient and one exposed healthy farmer with long exposure are shown in Fig. 1. The mean avidities and serum titres for each group are shown in Table 1.

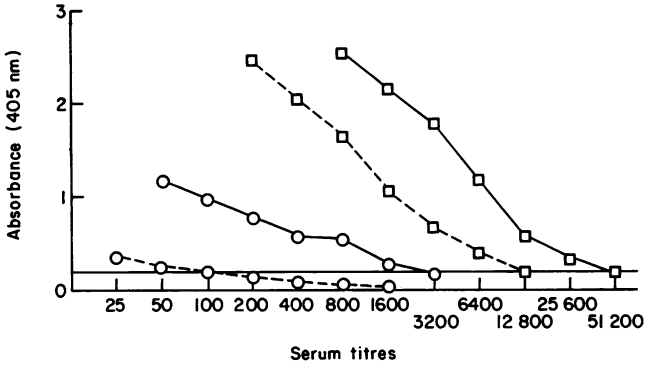

Fig. 1. Effect of 8 M urea on sera from one farmer's lung (FL) patient with long exposure (□) and from one healthy farmer with long exposure (○). The serum dilutions at the cut-off level ($OD_{405} = 0{\cdot}200$) for the curves with urea (——) and without urea (- - - -) were determined and the *Aspergillus umbrosus* IgG avidity for each sample was calculated.

Single IgG avidity

The IgG avidity of *A. umbrosus*-specific antibodies in the acute phase sera was measured in 12 FL patients. The mean avidity was high (39 ± 13%, Fig. 2a) for the seven FL patients with long exposure. The mean serum titre at the cut-off level for those high-avidity antibodies was also high (1:21 000 ± 19 000). On the other hand, both the mean avidity 10 ± 5% ($P < 0{\cdot}001$) and serum titre 1:310 ± 180 ($P < 0{\cdot}05$) were low for the five FL patients with short exposure. Figure 2b shows that the mean avidity was low in both long and short exposure subgroups of healthy farmers: 19 ± 10% ($P < 0{\cdot}01$) and 11 ± 3% ($P < 0{\cdot}001$), respectively. The serum titres were also low: 1:1100 ± 900 ($P = 0{\cdot}052$) and 1:360 ± 220 ($P < 0{\cdot}05$), respectively. The five healthy urban controls showed unmeasureably low *A. umbrosus*-specific IgG antibody avidities and titres (9 ± 6% and 110 ± 100, respectively, Fig. 2b).

IgG avidity during follow up and in recurrences of FL

During the follow up the initially high avidity of *A. umbrosus*-specific IgG antibodies increased slightly (Fig. 3), but the titres of IgG antibodies decreased. In case of a recurrence of the disease, the avidity exceeded that measured during the first acute episode. An explanation for one exception to this could be that the serum sample tested was taken 3 months after the acute phase of the recurrence. The avidity of *A. umbrosus*-specific IgG antibodies among short exposure patients remained initially low during the 3-month follow up (Fig. 3b). The highest concentration of antibodies that combined avidly with *A. umbrosus* antigen was found during the acute phase, whereafter the serum titres decreased. During recurrence the titres of antibodies increased again, but did not reach the level found during the first acute phase.

DISCUSSION

Avidity measurements have been a useful tool in the differential diagnosis of primary viral infections and reinfections [9,10] and in following the maturation of antibodies during the following 6 months after successful rubella vaccinations [4]. In allergic

Table 1. The size, mean age, *Aspergillus umbrosus*-specific IgG avidity and end-point titre (without urea) for each subgroup

	Farmer's lung patients							Healthy exposed farmers		
	Long exposure >25 years			Short exposure <7 years		Recurrence, long exposure		Long exposure, >25 years	Short exposure, <7 years	Healthy controls
n	7			5		5		7	5	5
Mean age, years	50±10			32±6		48±12		49±4	29±3	39±9
Sampling time, months	0	3/6	12	0	3	+0	+12			
Avidity (%)	39±13	40±11	43±13	10±5	10±6	52±8	48±5	19±10	11±3	9±6
Titre (±s.d.)	1:21 000 (19 000)	1:10 100 (9700)	1:7000 (9000)	1:310 (180)	1:170 (140)	1:15 400 (21 500)	1:12 800 (8700)	1:1100 (900)	1:360 (210)	1:110 (100)

The sampling time in months is calculated from the initial acute phase of farmer's lung (FL). The sampling time for acute phase of recurrence and 12 months later are marked with a +. Statistical analysis by Student's *t*-test. NS, Not significant.

Statistical significances:

FL patients, long exposure	Avidity, %	Titre
versus FL patients; short exposure	$P<0.001$	$P<0.05$
versus FL patients; recurrence	$P=0.08$	NS
versus healthy farmers; long exposure	$P<0.01$	$P=0.052$
versus healthy farmers; short exposure	$P<0.001$	$P<0.05$
versus healthy controls	$P<0.001$	$P<0.05$

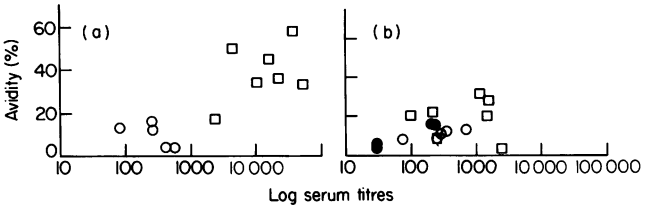

Fig. 2. The avidity of *Aspergillus umbrosus*-specific IgG antibodies in acute phase sera from farmer's lung (FL) patients with long (□) and short (○) exposure (a), and in sera from healthy farmers with long (□) and short (○) exposure and from healthy controls (●) (b).

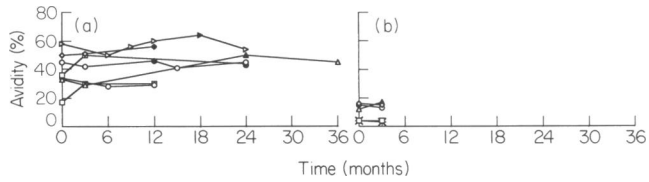

Fig. 3. The avidity of *Aspergillus umbrosus*-specific IgG antibodies in farmer's lung (FL) patients with long (a) and short (b) exposure during the follow up after the initial acute phase.

diseases, the antigen exposure has usually continued for a long time before the disease manifests itself. In FL, there may have been several years exposure before the appearance of acute clinical disease. Also, healthy farmers have antibodies against microbes to which they have been exposed, which means that serodiagnosis alone is not specific enough to distinguish patients from exposed healthy farmers, nor to confirm a recurrence of the disease [11]. This makes the use of avidity determinations in the diagnosis of FL interesting.

Different ideas about the basis of affinity maturation in antibody response have been raised. As early as 1957 a theory of selective lymphocyte multiplication was suggested by Burnet [12]. Later on Weigert *et al.* [13] reported somatic mutations in the variable region of immunoglobulins. Werblin *et al.* [14] thought that the diversity of genetic information required to synthesize all the various antibodies formed during the course of the normal heterogeneous immune response would be present before antigen exposure. In an animal model they found that not until approximately 6 weeks after immunization did rabbits produce antibodies of the highest affinity. Thereafter the changes in affinity are accounted for by shifts in the relative proportions of antibodies of the different affinities already present. After the maximal affinity is reached and the antigen exposure has ceased, the population of low-affinity antibodies remains essentially unaltered, while the number of high-affinity antibodies falls [15]. This decrease in the number of high-affinity antibodies is in accordance with our findings (Table 1). Twelve months after the initial acute phase the mean *A. umbrosus*-specific IgG antibody titre had decreased to one third.

It is known that antigen-driven B cell selection that increases the complementarity of the antigen–antibody binding site maturates the affinity [16]. Somatic hypermutations in immunoglobulin variable region (V) genes occur during the genesis of memory B cells, a population of antigen-induced and -selected cells derived from new or virgin B cells, and not during the conversion of a B lymphocyte into a clone of antibody-forming plasma cells [17,18]. Berek *et al.* [19] found that germinal centres of lymphoid tissues are the sites for these mutations. According to their studies with mice, the mutation and selection process progresses during the second week after immunization, leading to a higher affinity of antibodies.

The immune response to repeated antigenic stimulation differs from that to the initial antigen exposure. An expanded population of responding cells enables a rapid increase of already high-avidity antibodies that form more stable com-

plexes with the antigen [15]. In our study, patients' repeated exposure to mouldy hay, even though they had been advised to avoid it, leading to the recurrence of the disease, seemed to enhance the maturation of avidity and of the population of *A. umbrosus*-specific IgG antibodies. Apparently due to the avidity maturation, a smaller antibody population was needed than during the first acute phase.

The affinity and avidity of an antibody for the antigen markedly influence its biological activity [20]. The synthesis of high-affinity antibodies is generally advantageous for the host, while a continuous low-affinity response may have immunopathological consequences. Such a low-affinity response is found with small-sized soluble antigen–antibody complexes [21]. In FL the formation of small-size complexes seems improbable, since FL patients have the ability to synthesize high-avidity *A. umbrosus*-specific IgG antibodies at least after long exposure.

According to our earlier studies the serologically active component of *A. umbrosus* is a mannose-containing polysaccharide [11]. Polysaccharides, for example mannan of *Candida albicans*, are known to induce antibody synthesis by direct B cell activation without an MHC class II T cell contact [22]. However, in the production of high titres of antigen-specific antibodies with high avidity after long exposure, an MHC II-dependent B cell activation seems likely. The differentiation of T cells into Th1 (producing IL-2 and interferon-gamma (IFN-γ)) and Th2 subsets (producing IL-4 and enhancing IgE and IgG production) has recently been discovered [23]. So far there have been no reports concerning the Th1/Th2 subset pattern in FL. The development of a secondary T cell antibody response strengthening the production of high-avidity antibodies could be a result of a possible Th1/Th2 subset imbalance.

Antigen-specific IgG antibodies with high avidity are related to the acute phase of FL developed after long antigen exposure. At the moment, avidity measurements are a tool of basic research on the immune mechanisms of FL; the diagnostic significance of these measurements in FL awaits to be proved.

ACKNOWLEDGMENTS

The comments of Doctors Klaus Hedman, Jarmo Oksi and O.-P. Lehtonen have been most valuable. We would like to thank Marjut Kotimaa PhD for the M 118 strain, and Mrs Leena Kavén-Honka for skillful technical assistance. This study was supported by The Finnish Anti-Tuberculosis Association, The Viipuri Tuberculosis Foundation, The Tampere Tuberculosis Foundation, The Turku University Foundation, The Sigrid Jusélius Foundation, and The Paulo Foundation.

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