

Development of a sandwich ELISA to detect IgG and IgG sub-class antibodies specific for a major antigen (Ag 7) of *Aspergillus fumigatus*

CHRISTINE HARVEY and JOAN L. LONGBOTTOM

Department of Allergy and Clinical Immunology, Cardiothoracic Institute, Brompton Hospital, London

(Received in original form 22 January 1986 and in revised form 17 February 1986; accepted for publication 28 February 1986)

Summary

A sandwich ELISA has been developed, using an affinity purified monospecific antiserum as a capture antibody, to detect specific IgG and IgG sub-classes to a major antigen (Ag 7) of *Aspergillus fumigatus* in the sera of patients with allergic bronchopulmonary aspergillosis (ABPA). Significantly elevated levels of specific IgG to Ag 7 were detected in 97% of ABPA sera tested, as compared to control sera and to sera from *A. fumigatus* skin-prick test positive individuals. IgG sub-class antibody levels to Ag 7 were also determined in a similar sandwich ELISA, but using specific monoclonal antisera instead of the polyclonal anti-IgG. Both Ag 7 specific IgG1 and IgG4 levels were found to be significantly raised in the ABPA sera compared to controls. It is proposed that this antigen-specific ELISA may provide a more specific diagnostic test for IgG antibody detection in sera of ABPA patients.

Introduction

The enzyme-linked immunosorbent assay (ELISA), which is now being applied to many fields of analysis, is a sensitive solid phase assay for detection of specific antibodies and antigens [1,2]. In the indirect assay for detection of antibodies, the solid phase is coated with antigen and is then incubated with serum. By comparison, the sandwich assay uses a capture antibody coated to the microtitre plate to bind the antigen, and the increased sensitivity and specificity of such assays has recently been recognized for measurement of IgG and IgE antibodies [3-5].

Patients with ABPA have relatively low levels of specific IgG antibodies to *A. fumigatus* and, although precipitin tests have most commonly been used for their detection, indirect ELISAs have been reported from a number of laboratories [6-10]. However, most of the antigenic preparations used have been unpurified [6,7] or

partially purified extracts [8–10]. Crude extracts, by definition, represent a problem in this type of assay system, not only because they are not reproducible extracts, but also all components are present in differing proportions and the individual components are likely to exhibit different binding affinities and kinetics for the microtitre plate. The use of a single component, through a capture antibody, therefore permits greater standardization of the assay system [5].

The aim of this study was to develop such a standardized ELISA in order to measure levels of specific IgG antibodies to a particular characterized major antigen (Ag 7) of *A. fumigatus* in sera from ABPA patients and to confirm the significance of antibodies to this component, and further to determine which, if any, of the specific IgG sub-class antibodies might be significantly raised.

Methods

Sera

Sera included those of a previous investigation collected from thirty patients who fulfilled the clinical and immunological criteria of allergic bronchopulmonary aspergillosis [11], and those from seven individuals who showed positive skin-prick test reactions to *A. fumigatus*, but without other features of ABPA. The third group of eight sera for use as controls were collected from healthy atopic and non-atopic individuals. All sera were stored at -20°C .

Antigen

The culture filtrate from a long-term stationary culture of *A. fumigatus* was precipitated with ammonium sulphate and partially separated by gel filtration on Sephacryl S200 (Pharmacia, Milton Keynes) [12]. An initial peak of optical density in the molecular weight range 150–200 Kd, known to contain Ag 7, was used as the partially purified antigen.

Crossed immunoelectrophoresis (XIE)

This procedure was as described in earlier studies [12, 13] using an intermediate gel, and a reference pooled rabbit antiserum raised to culture filtrate antigens of *A. fumigatus* in the main gel area.

Monospecific antiserum

A monospecific antiserum to Ag 7 was produced as described previously, by immunizing rabbits with the precipitin peak, excised from several identical XIE plates [12].

Affinity purification of monospecific antiserum

The monospecific antiserum raised to Ag 7 was affinity purified to form the capture antibody for the ELISA. The partially purified antigen from Sephacryl S200 separation (30 mg) was covalently bound to CNBr-activated Sepharose (3 g) (Pharmacia) according to manufacturer's instructions. The coupled Sepharose (3 g) was incubated overnight at 4°C with 15 ml of the whole monospecific antiserum, unbound material was removed by filtration on a sintered glass funnel, and the Sepharose was washed with phosphate buffered saline (PBS) until the OD_{280 nm} ≤ 0.001 . Antibody was eluted from the coupled Sepharose by incubation with 1 M propionic acid (10 ml) for 10 min. After centrifugation, the supernatant was

immediately added to an equal volume of 1 M Tris pH 8.4. This was repeated once with 10 ml propionic acid and twice with 10 ml PBS. After extensive dialysis the combined supernatants were freeze-dried. The specificity of this resulting affinity purified antiserum was confirmed by incorporating it in an intermediate gel in XIE.

ELISA

The wells of microtitre plates (Dynatech, Billingshurst, Sussex) were coated by overnight incubation at 4°C with 200 µl of affinity purified monospecific antiserum (5 µg/ml) diluted in 0.05 M carbonate buffer pH 9.6. Plates were washed three times in PBS containing 0.05% Tween 20 (i.e., assay buffer) and then incubated for 2 hr at 4°C with 200 µl partially purified antigen (10 µg/ml) diluted in assay buffer. After washing, plates were incubated with test serum (diluted 1:10 in assay buffer) for 1 hr at 37°C, and unbound serum was washed away as before. Two hundred µl of alkaline phosphatase conjugated anti-human IgG (Miles, Stoke Poges), diluted 1:500 in assay buffer, was then added to the plates which were incubated for 1 hr at 37°C. After further washing, freshly prepared substrate, p-nitrophenol (pNP) (Sigma) at 1 mg/ml in 9.7% diethanolamine pH 9.8 was added to the wells. The reaction was stopped by addition of 50 µl 3 M NaOH and the optical density was measured at 405 nm on an automatic microtitre plate reader (Flow Laboratories, Rickmansworth, Herts.) using an empty microtitre plate as blank. Results are expressed as optical density units at 405 nm. All samples were assayed in triplicate and mean values determined.

IgG sub-class ELISA

The procedure for the Ag 7 specific sub-class ELISA was as for the sandwich ELISA except that, after incubation with test serum, sub-class specific monoclonal IgG1, IgG2, IgG3, IgG4 or total IgG antibody (NL16, GOM1, ZG4, RJ4, 8a4, Unipath, Bedford) was used, diluted 1:1000 in assay buffer, followed by a further incubation for 1 hr at 37°C with alkaline phosphatase conjugated anti-mouse-IgG (Miles) also diluted 1:1000. Substrate was then added, as before, and the optical density units at 405 nm. Results are expressed as optical density units at 405 nm. Statistical analysis of results was performed using the unpaired Student's *t*-test.

Results

Specificity of affinity purified monospecific antiserum

The specificity of the monospecific antiserum, following affinity purification, was demonstrated by crossed immunoelectrophoresis (Fig. 1), by incorporating the monospecific antiserum into an intermediate gel of a reference XIE pattern, using the partially purified antigen fraction from the Sephacryl S200 separation. Only one antigen peak, corresponding to Ag 7, is visible in the intermediate gel containing the monospecific antiserum.

Dilution of sera

Chequerboard titrations were performed to determine optimal concentrations of reagents. Results for dilutions of two individual ABPA sera and a control serum are shown in Fig. 2. At all points less than 5% variation was observed for triplicate values. Both patient samples showed parallel sigmoid curves compared to the minimal values

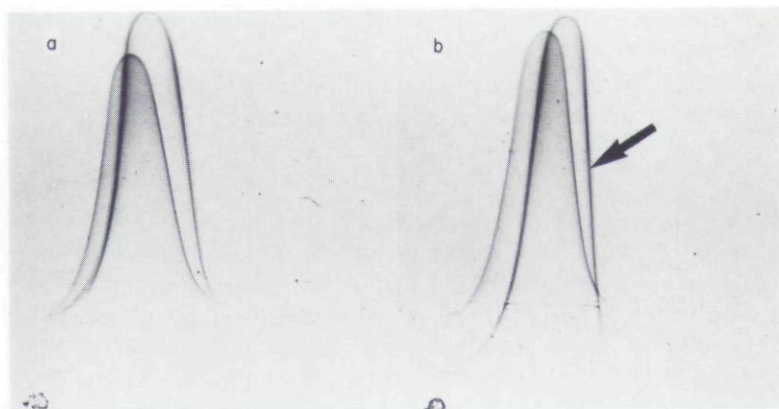


Fig. 1. Crossed immunoelectrophoresis test of partially purified antigen ($3 \mu\text{l}$ at 12 mg/ml) electrophoresed through (a) control and (b) affinity purified monospecific antiserum ($12.5 \mu\text{l/cm}^2$) into reference pooled antiserum ($11 \mu\text{l/cm}^2$). The absorbed Ag 7 peak is indicated (b).

of the control serum. From this, serum dilution of 1:10 (being on the linear part of the graph) was regarded as being optimal for the assay.

Ag 7 sandwich ELISA

Results of Ag 7-specific IgG levels are shown in Fig. 3 for the three groups of sera. Since there is no standard method for expression or calibration of ELISA results, and as most arbitrary units are based on optical density, it was considered that results were adequately represented as optical density readings. The mean values for each group are indicated, and the mean ± 2 s.d. of the control sera is marked. The values for skin-prick test positive sera are not significantly increased over the control sera, but the levels for the ABPA sera are significantly higher ($P < 0.001$)

IgG sub-class antibodies to Ag 7

Results of IgG sub-class levels to Ag 7 are shown in Fig. 4 for twelve ABPA and six normal control sera, as well as total specific IgG levels by the same assay system using an anti-IgG monoclonal followed by an anti-mouse enzyme conjugate. Ag 7-specific IgG1 and IgG4 levels in ABPA sera are significantly raised ($P < 0.001$) compared to the corresponding control sera, as are levels of total specific IgG to Ag 7 ($P < 0.001$), and to a lesser extent IgG2 and IgG3 ($P < 0.01$ and $P < 0.5$ respectively) were also raised.

Discussion

Previously Ag 7 was described as a 'major' antigen of *A. fumigatus* because the majority of ABPA sera were shown to have IgG precipitating antibodies to this component [11]. In this study, by the development of a specific Ag 7 ELISA which will detect both precipitating and non-precipitating IgG antibodies, raised levels of specific IgG to this antigen were detected in 97% (29/30) ABPA sera. The mean value for ABPA sera was significantly greater ($P < 0.001$) than that obtained for the normal control sera and for the *A. fumigatus* skin-prick test positive patients' sera. These results therefore substantiate the significance of antibodies to this component and confirm the definition made in the earlier study of Ag 7 as a major antigen [11].

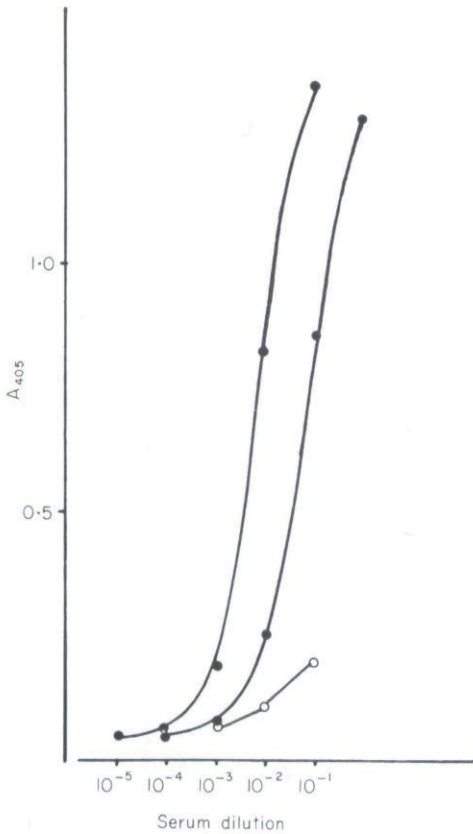


Fig. 2. Dilution curves for two individual ABPA sera and control serum using the Ag 7 specific sandwich ELISA.

Only one ABPA serum had a value below the control (mean + 2 s.d.) level (i.e., false negative?), and although this individual fulfilled all the clinical criteria for ABPA and had high levels of specific IgE antibodies, precipitating (IgG) antibodies to a culture filtrate extract of *A. fumigatus* could not be demonstrated by crossed immunoelectrophoresis. It is also possible, though unlikely, that the human IgG antibodies were to an epitope already covered by the 'capture' antibody or blocked by IgE as this did not occur with any of the other sera.

Ag 7, a high molecular weight acidic glycoprotein with 36 Kd subunits, has been shown to bind both IgG and IgE antibodies in sera from ABPA patients [12]. However, as the concentration of IgE is so much lower than that of IgG, it is unlikely to cause blocking of the antigen in this solid phase ELISA assay.

The specificity of the antiserum which was used as the capture antibody in this ELISA system was unaltered by the affinity purification procedure and its binding capacity for Ag 7 was demonstrated in crossed immunoelectrophoresis. It was seen from this that other components in the partially purified fractions from the gel filtration column did not react with the affinity purified monospecific antiserum.

A parallel ELISA system using sub-class specific monoclonal antibodies was also set up to determine for the ABPA sera in which of the IgG sub-classes, antibodies to Ag 7 were raised over those of the controls. Since the monoclonals have different affinities

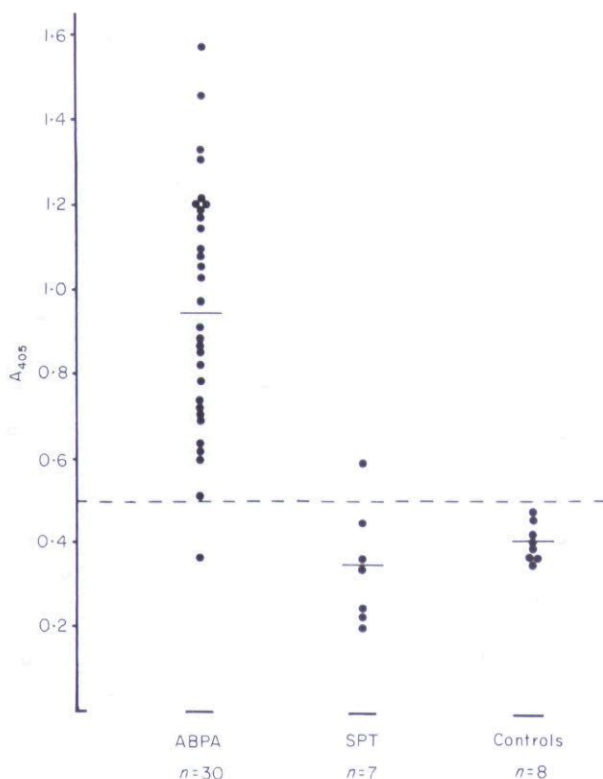


Fig. 3. Ag 7 specific IgG measured by polyclonal sandwich ELISA in sera from thirty ABPA patients, seven positive (*A. fumigatus*) skin prick test individuals and eight normal controls. The mean values for each group and the mean + 2 s.d. of the control sera are indicated.

(manufacturer's information), values for the individual sub-classes cannot be compared with each other. However all sub-classes for the ABPA sera, when compared with their respective control groups, were significantly raised, with IgG1 and IgG4 being the most significantly increased. Total specific IgG levels measured using the monoclonal anti-IgG antibody were comparable to those obtained using the polyclonal antiserum.

It is interesting that that two IgG sub-classes which were significantly raised in the Ag 7 specific ELISA were IgG1 and IgG4. The normal level of IgG4, although approximately 4% of the total IgG, may account for over 50% of the total IgG antibody activity after prolonged immunization of atopic individuals [14]. IgG4, a univalent antibody which is unable to fix complement and may have a protective role, correlates well with specific IgE for individual allergens, and possibly reflects exposure to environmental allergens, though its role in allergic disease is at present unresolved [15]. Sera from ABPA patients are known to contain not only Ag 7 specific IgG, but also specific IgE [11,12] which may be connected with the raised levels of Ag 7 specific IgG4.

Raised levels of Ag 7 specific IgG1 are in direct contrast to those of IgG4, since this subclass constitutes the bulk of IgG (approximately 65%) and is able to fix complement.

The Ag 7 specific IgG subclass results differ from those of total IgG subclasses in

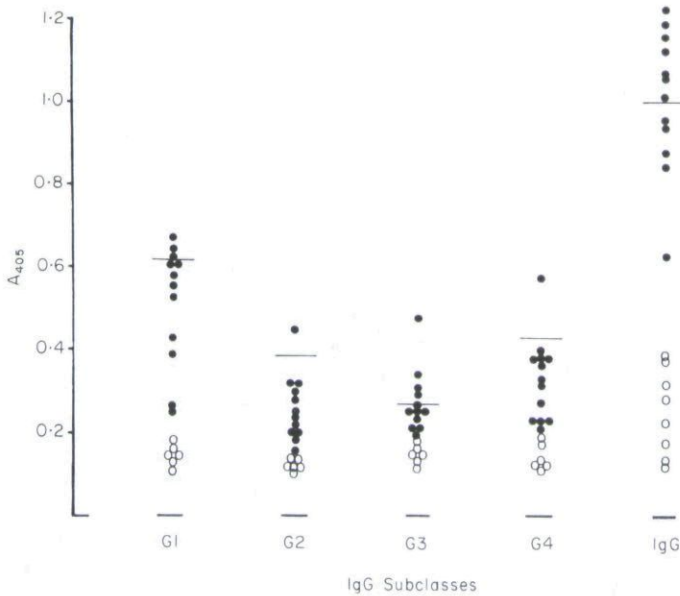


Fig. 4. Distribution of Ag 7 specific IgG sub-class (and total specific IgG) levels, measured by sandwich ELISA (monoclonal), for twelve ABPA sera and six normal control sera, showing the mean values for each sub-class for the ABPA sera. Results for different sub-classes cannot be directly compared due to different affinities of monoclonal antibodies.

ABPA, where all were elevated but IgG1 and IgG3 were found to be more significantly raised [16]. However, ABPA patients have IgG antibodies specific for components of *A. fumigatus* other than Ag 7.

Most studies using ELISA for detection of antibodies to *A. fumigatus* have used crude or partially purified preparations [6–10], however there has been one other report of an *A. fumigatus* ELISA using a single component [17]. The assay was an indirect ELISA rather than a sandwich ELISA with a capture antibody, and the antigen used was the C antigen which is known to possess chymotryptic activity [18]. Although raised levels of IgG to this antigen were detected in most sera from ABPA and aspergilloma patients, overall results did not differentiate these groups completely from control sera. The antigen, in another ELISA which was used to demonstrate high levels of specific IgG in sera from ABPA patients, was a partially purified glycoprotein from a culture filtrate, prepared by its ability to bind to concanavalin A (Con A) [10]. It is likely that Ag 7, also a Con A binding glycoprotein, was contained in this fraction and partially responsible for specificity of the assay.

An ELISA using three partially purified fractions of molecular weights 470, 250 and 25–50 Kd from a somatic extract were all reactive with sera from ABPA patients to differing extents, but levels in test sera overlapped with those of control sera [9]. Similarly, where other extracts have been used as antigens in ELISA systems for antibodies to *A. fumigatus*, values obtained for patients' sera have generally not differentiated well from control values [6–10]. The sensitivity and specificity of the sandwich ELISA assay as described in this paper permit greater standardization than can be achieved using a multicomponent system, and should provide a more specific diagnostic test.

Acknowledgments

We are grateful to Dr D.M. Kemeny for helpful advice in setting up the assay. This study was supported by a project grant from the Medical Research Council.

References

- Engvall E, Perlman P. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry* 1971; 8:871-4.
- Voller A, Bidwell DE, Bartlett A. Enzyme-immunoassays in diagnostic medicine: theory and practice. *Bull WHO* 1979; 53:55.
- Urbanek R, Kemeny DM, Samuel D. Use of the enzyme-linked immunosorbent assay for measurement of allergen-specific antibodies. *J Immunol Methods* 1985; 79:123-31.
- Kemeny DM, Urbanek R, Samuel D. Increased sensitivity and specificity of a sandwich ELISA for measurement of IgE antibodies. *J Immunol Methods* 1985; 78:217-26.
- Kemeny DM, Urbanek R, Samuel D, Richards D. Improved sensitivity and specificity of sandwich, competitive and capture enzyme-linked immunosorbent assay for allergen-specific antibodies. *Int Archs Allergy Appl Immunol* 1985; 77:198-200.
- Khan ZU, Richardson MD, Warnock DW. Evaluation of a rapid enzyme-linked immunosorbent assay for IgG antibodies to *Aspergillus fumigatus* in the serological diagnosis of allergic aspergillosis. *Int Archs Allergy Appl Immunol* 1984; 73:205-11.
- Kauffman HF, Beaumont F, Meurs H, Van Der Heide S, De Vries K. Comparison of antibody measurements against *Aspergillus fumigatus* by means of double diffusion and enzyme-linked immunosorbent assay (ELISA). *J Allergy Clin Immunol* 1983; 72:255-61.
- Wilson EV, Hearn VM. Comparison of partially purified mycelial and culture filtrate antigens on *A. fumigatus* by enzyme-linked immunosorbent assay. *Sabouraudia* 1983; 21:195-203.
- Schonheyder H, Andersen P. IgG antibodies of purified *Aspergillus fumigatus* antigens determined by enzyme-linked immunosorbent assay. *Int Archs Allergy Appl Immunol* 1984; 74:262-9.
- Kurup VP, Ting EY, Fink JN. Immunochemical characterisation of *Aspergillus fumigatus* antigens. *Infect Immunol* 1983; 41:698-701.
- Longbottom JL. Allergic bronchopulmonary aspergillosis: Reactivity of IgE and IgG antibodies with antigenic components of *Aspergillus fumigatus* (IgE/IgG antigen complexes). *J Allergy Clin Immunol* 1983; 76:668-75.
- Harvey C, Longbottom JL. Characterisation of a major antigenic component of *Aspergillus fumigatus*. *Clin Exp Immunol*. in press.
- Longbottom JL. Immunological aspects of infection and allergy due to *Aspergillus* species. *Mykosen* 1978; 1:207-17.
- Aalberse RC, Van Der Gaag R, Van Leeuwen J. Serologic aspects of IgG4 antibodies. I. Prolonged immunisation results in an IgG4-restricted response. *J Immunol* 1983; 130:722-6.
- Bruynzeel PLB, Berrens L. IgE and IgG4 antibodies in specific human allergies. *Int Archs Allergy Appl Immunol* 1979; 58:344-50.
- Stokes TC, Turton CWG, Turner-Warwick M. A study of immunoglobulin G subclasses in patients with allergic bronchopulmonary aspergillosis. *Clin Allergy* 1981; 11:209-15.
- De Magaldi SW, Mackenzie DWR. Specificity of antigens from pathogenic *Aspergillus* species. I. Studies with ELISA and immunofluorescence. *Sabouraudia: J Med Vet Mycol* 1984; 22:381-94.
- Tran van Ky P, Biguet J, Fruit J. Localisation et fréquence des arcs des immunoélectrophorégrammes produits de le serum des malades atteints de mycétomes aspergillaires appliquées contre l'antigène *Aspergillus fumigatus*. *Revue d'Immunologie Paris* 1966; 30:13-20.

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.