

Characterization of a second major antigen Ag 13 (antigen C) of *Aspergillus fumigatus* and investigation of its immunological reactivity

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(Accepted for publication 7 May 1987)

SUMMARY

A second major antigen of *Aspergillus fumigatus* (Ag 13) has been identified and characterized as a 70 kD, heat-labile component which is able to bind to the lectin, concanavalin A. Ag 13 proved to be identical to the previously recognized C antigen, known to possess chymotryptic activity. A monospecific antiserum to Ag 13 has been affinity purified and used to develop a sandwich ELISA for measuring levels of Ag 13 specific IgG antibodies in patients' sera. Although all ABPA sera (25/25) had significantly raised levels, some aspergilloma sera (2/5) had only very low levels; of the control sera, 10/10 fungal atopics were negative, but 5/12 farmer's lung sera had low but positive values of Ag 13 specific IgG, i.e. sensitivity 100%, specificity 80%. Ag 13 is therefore a diagnostically important component of *A. fumigatus* and the antigen-specific ELISA may be of importance for the improved detection of disease-specific antibodies.

Keywords *Aspergillus fumigatus* allergic bronchopulmonary aspergillosis (ABPA) major antigen (Ag 13=C antigen) ELISA

INTRODUCTION

The importance of serology in the diagnosis of aspergillus-related diseases has long been recognized (Longbottom & Pepys, 1964; McCarthy & Pepys, 1971; Patterson & Roberts, 1974; Philpot & Mackenzie, 1976) and there are tests available for the detection of *Aspergillus fumigatus* specific IgG and IgE antibodies (Greenberger & Patterson, 1982; Kauffman *et al.*, 1983; Kurup *et al.*, 1984; Shale & Faux, 1985; Hearn, Donaldson & Healey, 1985).

Patients with allergic bronchopulmonary aspergillosis (ABPA) have relatively low levels of specific IgG precipitating antibodies compared with aspergilloma patients, and these antibodies may pass undetected in some of the less sensitive serological assays.

In order to distinguish these low levels of IgG from non-specific antibodies, attempts have been made to identify the relevant 'disease-related' antigens. Using the technique of self-crossed radioimmuno-electrophoresis, two major antigens, Ag 7 and Ag 13 were identified (Longbottom, 1983); a 'major' antigen being defined as one to which the majority of sera contained antibodies. One of these, Ag 7, has been purified and characterized (Harvey & Longbottom, 1986a) and a sandwich ELISA developed to detect Ag 7 specific IgG antibodies (Harvey & Longbottom, 1986b), was shown to be highly specific for aspergillus-related diseases (Harvey, Shaw & Longbottom, 1987).

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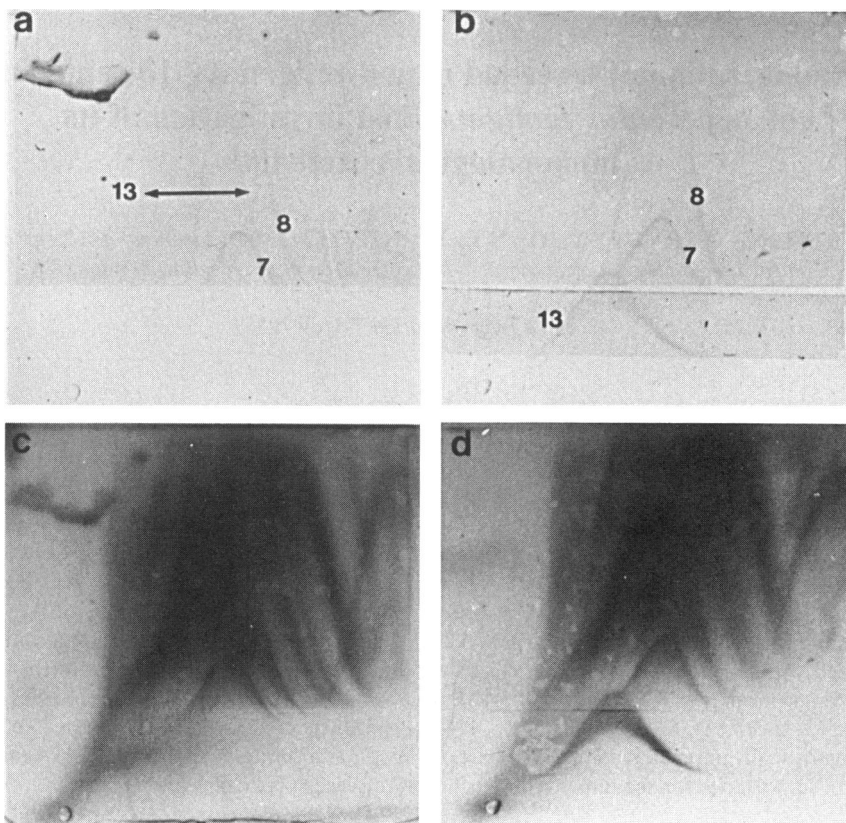


Fig. 1. Self-crossed immunoelectrophoresis of the reference antigen (B27) ($1.5 \mu\text{l}$ at 12 mg/ml) electrophoresed into a standard ABPA serum pool ($23 \mu\text{l/cm}^2$) through an intermediate gel containing antigen C monospecific antiserum ($10 \mu\text{l/cm}^2$) (b) shown with control (a). (c) and (d) are the corresponding autoradiographs developed after 7 days exposure.

This paper reports the identification, characterization and immunological reactivity of the other major antigen, Ag 13. A further aim of the study was to determine whether either of these major antigens was the C antigen which has chymotryptic activity (Tran van Ky, Biguet & Fruit, 1966) and shown to react with 53% of sera from patients with aspergillosis (Dessaint *et al.*, 1976).

MATERIALS AND METHODS

Preparation of antigenic extracts. The fungus (IMI 16030) was grown as long-term stationary cultures (4–5 weeks) on a synthetic asparagine medium (Smith *et al.*, 1948). After removal of the mycelial mat, the culture filtrate was Seitz-filtered, extensively dialysed against distilled water (4°C), concentrated and freeze-dried.

Reference antigen. The 'reference' antigen (B27), also from a long-term stationary culture, was as previously used (Longbottom, 1978; 1983).

Salt precipitation. Crude culture filtrate extract was dissolved in water ($30\text{--}50 \text{ mg/ml}$) and an equal volume of saturated ammonium sulphate was slowly added with stirring. Solid ammonium sulphate was added until saturation. After standing overnight at room temperature, the precipitate was removed by centrifugation and washed twice with saturated ammonium sulphate. The precipitate was resuspended in distilled water, extensively dialysed and freeze-dried.

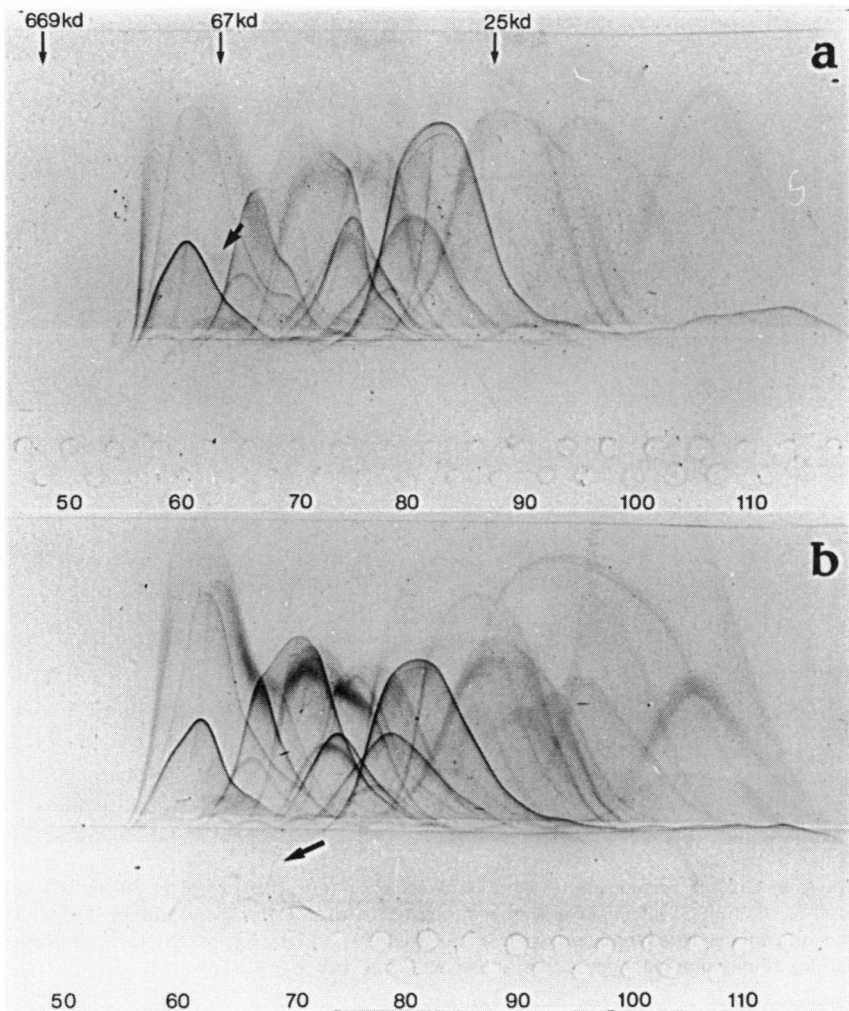


Fig. 2. (a) Fused rocket immunoelectrophoresis profile following Sephacryl S-200 separation of protein-enriched culture filtrate extract. Samples ($4\ \mu\text{l}$) from alternate fractions were allowed to diffuse for 1 h before electrophoresis into rabbit antiserum ($11\ \mu\text{l}/\text{cm}^2$). In (b) an intermediate gel containing anti-Ag 13 monospecific antiserum has absorbed the Ag 13 peak (arrow). The elution positions of molecular weight markers are shown.

Human sera. Sera were obtained from 25 patients with ABPA, five aspergilloma patients, 12 with farmer's lung (precipitin positive to *Micropolyspora faeni*), from 10 individuals with a positive skin prick test to either *A. fumigatus* or *Alternaria alternata*, and also from eight healthy control subjects. All sera were stored at -20°C . A standard ABPA serum pool was prepared as described previously (Longbottom, 1983).

Production of antisera. Antisera were produced using crude culture filtrates as previously described (Harvey & Longbottom, 1986a). Resulting antisera were tested by crossed immunoelectrophoresis, and high titre sera from several bleeds from five hyperimmune rabbits were pooled and used as a reference antiserum.

Monospecific antiserum. Monospecific antiserum to the C antigen was produced as previously described (Harvey & Longbottom, 1986b). Briefly, the antigen was electrophoresed into antiserum to the C antigen (kindly supplied by Drs S. De Magaldi and D. W. R. Mackenzie) and the relevant precipitin arc was used as immunogen. The specificity of the resulting antiserum was examined by

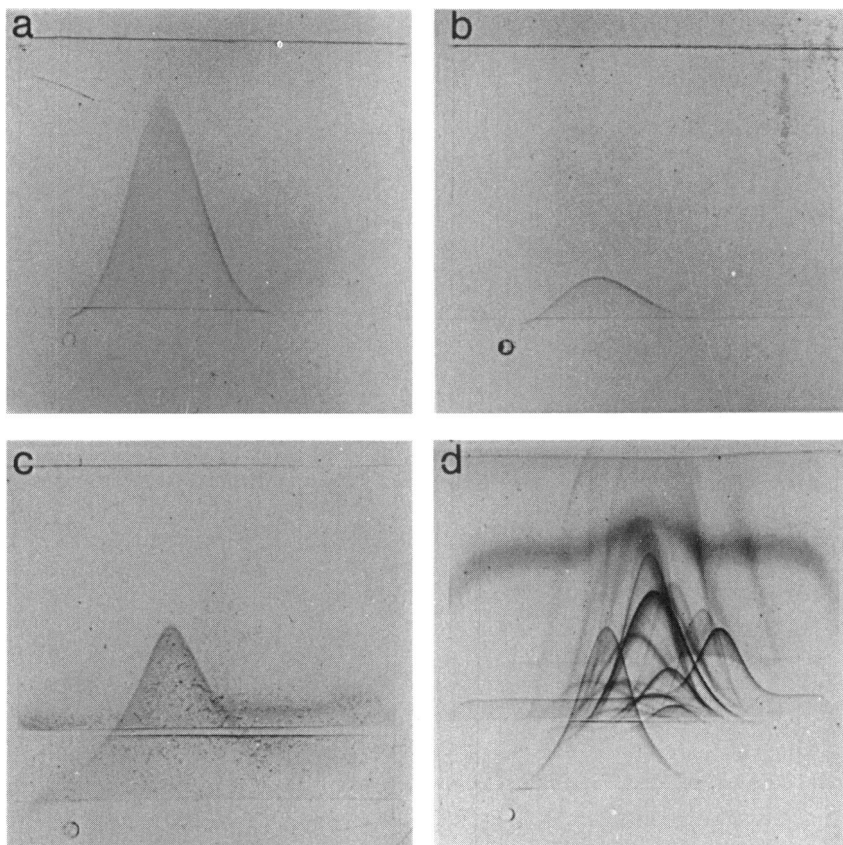


Fig. 3. (a & b) Crossed immunoelectrophoresis of culture filtrate (B27) ($3 \mu\text{l}$ at 30 mg/ml) into Ag 13 monospecific antiserum ($11 \mu\text{l/cm}^2$). The sample was heated to (a) 56°C for 30 min and (b) 75°C for 15 min. (c) XIE through an intermediate gel containing the lectin Con A ($50 \mu\text{l}$ at 2 mg/ml). (d) XIE through an intermediate gel containing affinity-purified monospecific antiserum ($23 \mu\text{l/cm}^2$).

crossed immunoelectrophoresis.

Crossed immunoelectrophoresis (XIE)/self-crossed radio-immunoelectrophoresis (self-XRIE). XIE and self-XRIE were performed as previously described (Longbottom, 1978; 1983). In some tests an intermediate gel containing antiserum or lectin was placed between the sample and the main antibody containing gel of the second dimension. Such tests were compared with controls in which antiserum/lectin was omitted from the intermediate gel.

Fused rocket immunoelectrophoresis (FRIE). Samples from alternate fractions following gel filtration were applied to sample wells in two zig-zag rows approximately 0.5 cm apart, and allowed to diffuse for 1 h before electrophoresis (overnight), 1 V/cm , into antibody-containing gel. After an overnight wash in 0.9% saline, gels were warm air-dried and stained with Coomassie R250.

Sephacryl S200 gel filtration chromatography. One hundred and fifty milligrams of protein-enriched culture filtrate (following salt precipitation) was applied in 5 ml of 0.05 M ammonium hydrogen carbonate to a pre-equilibrated, pre-calibrated Sephacryl S200 column (500 ml). The sample was eluted with a further 500 ml of NH_4HCO_3 at 25 ml/h , and 4 ml fractions were collected.

Affinity purification of anti-Ag 13 monospecific antiserum. The method was as previously described for the antiserum to Ag 7 (Harvey & Longbottom, 1986b). Briefly, partially purified antigen (fractions 65 to 70 from Sephacryl S200 separations), was covalently linked to CNBr-activated Sepharose and then incubated with monospecific antiserum. After removal of unbound material, the antibody was eluted from antigen using 10 ml of 1 M propionic acid. The eluate was

Ag 13 ELISA. The sandwich ELISA was performed essentially as previously described (Harvey & Longbottom, 1986b). Optimal concentrations of reagents were determined by chequerboard titrations. Microtitre plates were coated by overnight incubation (4°C) with 200 µl of affinity purified antibody (10 µg/ml) diluted in 0.05 M carbonate buffer, pH 9.6. After washing with PBS/0.05% Tween 20, i.e. assay buffer, 200 µl crude culture filtrate antigen (20 µg/ml) was applied in assay buffer, and plates were incubated for 2 h at 4°C. Plates were washed and 200 µl serum (diluted 1 in 10 with assay buffer) was added for 1 h at 37°C. Alkaline phosphatase conjugated anti-human IgG (Miles, Stoke Poges) was used at 1 in 1000 dilution with *p*-nitrophenol substrate (1 mg/ml in 9.7% diethanolamine, pH 9.8). Reactions were stopped by addition of 50 µl of 3 M NaOH and the absorbance at 405 nm was determined using an automatic microtitre plate reader (Flow Laboratories, Rickmansworth, Herts.).

RESULTS

Incorporation of the C antigen monospecific antiserum into an intermediate gel in the standard self-XRIE reference pattern of pooled ABPA serum identified the component specifically absorbed as Ag 13 (Fig. 1), previously demonstrated to be a major antigen. Autoradiography indicated that Ag 13 did not bind strongly to specific IgE from ABPA sera, but the degree of radioactive uptake was

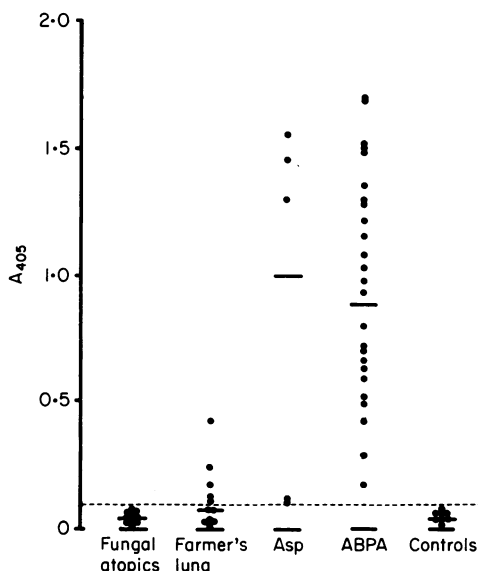


Fig. 4. Antigen 13 ELISA. The results shown are those for the assay with antigen minus those for the assay without antigen. The sera were from 25 patients with allergic bronchopulmonary aspergillosis (ABPA), five aspergilloma patients (Asp), 12 with farmer's lung, from 10 individuals with a positive skin prick test to either *A. fumigatus* or *Alternaria alternata* (fungal atopics), and also from eight healthy control subjects (controls).

difficult to assess, since in Fig. 1c & d, the Ag 13 peak was in a position where some uptake could be caused by co-precipitation of other allergenic molecules in its immuno-precipitate.

Physicochemical characteristics of Ag 13

Molecular weight. The protein-enriched culture filtrate extract was partially separated by Sephacryl S200 gel filtration chromatography and the separation monitored by fused rocket immunoelectrophoresis. Incorporation of the monospecific antiserum to Ag 13 in an intermediate gel (Fig. 2) showed that Ag 13 eluted from gel filtration with a molecular weight of approximately 70 kD.

Binding to Concanavalin A. The lectin, Con A, was incorporated in an intermediate gel in XIE of the culture filtrate antigen into the Ag 13 monospecific antiserum. The Ag 13 peak was absorbed by the lectin (Fig. 3c), indicating that the antigen has terminal residues of either α -D-mannopyranoside or α -D-glucopyranoside.

Heat stability. Samples of *A. fumigatus* antigen (B27) were heated to different temperatures before XIE with the monospecific Ag 13 antiserum. Ag 13 was found to be heat-labile (Figs 3a & b), such that its antigenicity was considerably reduced by heating to 75°C for 15 min and destroyed by heating to 100°C for 2 min.

Affinity purification of monospecific antiserum

Incorporation of affinity purified monospecific antiserum in an intermediate gel in XIE (Fig. 3d) indicated the presence of trace amounts of three antigens causing tailing of their precipitin peaks.

Ag 13 ELISA

Serum samples were assayed simultaneously in the Ag 13 sandwich ELISA and in the control ELISA without addition of culture filtrate antigen to overcome the presence of contaminating antigen in the affinity purified capture antibody. The results, shown in Fig. 4, are expressed as the difference in absorbance between samples tested with and without the antigen. The control limit (i.e. mean + 2 s.d. obtained for control sera) is indicated, as are the mean values for each group. All ABPA sera were higher than the control limit, whereas in the aspergilloma sera, although three gave high values, two were only weakly positive with values just above the control limit. In the other disease groups, all the fungal atopsics were negative but 5/12 of the farmer's lung sera were positive (two weakly).

DISCUSSION

Two antigens of *A. fumigatus*, Ag 7 and Ag 13, have been defined as major antigens for ABPA sera (i.e. more than 50% of ABPA sera have specific IgG) (Longbottom, 1983). Ag 13 has now been characterized as a 70 kD component with terminal residues of either α -D-mannopyranoside or α -D-glucopyranoside. It differs from Ag 7, the 150–200 kD glycoprotein (Harvey & Longbottom, 1986a), in that it is heat-labile and does not bind appreciably to IgE from ABPA sera. Assessment of IgE binding by autoradiography proved rather difficult, however, in the previous study (Longbottom, 1983), although the majority of 32 ABPA sera contained IgG antibody to Ag 13, only four of these also showed IgE binding.

An antigen of *A. fumigatus* with chymotryptic activity hence termed C antigen was described by Tran van Ky *et al.* (1966) and was later shown to react with 53% of aspergilloma sera (Dessaint *et al.*, 1976). It was of interest therefore to determine whether this antigen was one of the recognized major antigens in our system. An anti-C monospecific antiserum has now confirmed the identity of the C antigen as Ag 13 of the ABPA reference pattern. Using the technique of self-XRIE, 75% (24/32) of ABPA sera were previously shown to have precipitating IgG antibodies to Ag 13 (Longbottom, 1983), and now, with the more sensitive Ag 13 sandwich ELISA, 100% (25/25) ABPA sera were found to have Ag 13 specific IgG antibodies—further evidence of the importance of this antigen.

Another report (De Magaldi & Mackenzie, 1984) describes an indirect ELISA for measuring C antigen (Ag 13) specific IgG antibodies. The antigen for this ELISA was affinity purified using a

monospecific antiserum, but its purity was not determined. The majority of both ABPA (12/14) and aspergilloma (15/17) sera were positive in this assay, although the mean for the ABPA sera was higher than for the aspergilloma sera.

The sandwich ELISA uses affinity purified monospecific antiserum as capture antibody and the major problem encountered in this purification was the leaching of three contaminating antigenic components from the solid phase, as demonstrated by the incorporation of the affinity purified antibody in an intermediate gel in XIE. This highlights the need to check affinity purification procedures, even from batch to batch, and not only for antibody preparation as in this case, but also for antigen purification. A proportion of the IgG binding from the ABPA sera was due to this contaminating antigen and, to overcome this problem, each serum sample was tested simultaneously in ELISA with and without the culture filtrate antigen being added and the specific binding to Ag 13 determined as the difference between the two values.

The specificity of the Ag 13 ELISA was examined using sera from other disease groups. Taking as the control limit the mean plus 2 s.d. of control sera values, all the sera from individuals with positive prick test to either *A. fumigatus* or *Alternaria alternata* were negative, but 5/12 farmer's lung sera were positive (two only weakly) in this assay, possibly due to exposure to *A. fumigatus* which, with *M. faeni*, is also likely to be present in mouldy hay.

Surprisingly, as levels of *Aspergillus fumigatus* specific IgG in the sera of aspergilloma patients are generally considerably higher than in ABPA and more easily detectable with relatively insensitive techniques, 2/5 aspergilloma sera gave doubtfully positive values, below the range for ABPA sera. In this respect the Ag 13 specific ELISA differs from the Ag 7 assay, in which not only were all ABPA sera positive, but in addition all aspergilloma sera had high levels of Ag 7 specific IgG (Harvey *et al.*, 1987). Apart from its reduced reactivity with aspergilloma sera, the Ag 13 assay displays a similar sensitivity (100%) but slightly lower specificity (80%) compared with those of the Ag 7 ELISA, (100% and 92.3% respectively). These results confirm the earlier observations of Dessaint *et al.* (1976), Longbottom (1983) and De Magaldi & Mackenzie (1984) which indicated that this Ag 13 (C antigen) is important in the serological diagnosis of ABPA.

Twelve sera tested in the Ag 13 sandwich ELISA had previously been assayed in the Ag 7 specific ELISA (Harvey *et al.*, 1987) and by linear regression analysis a good correlation ($P < 0.01$) was observed between antibody titres to the two antigens. Whilst these limited numbers of ABPA samples were positive in both tests, it may help to measure levels of antibody to both major antigens to reduce the number of false positives and false negatives which, although not experienced here, may occur in a larger population.

We are very grateful to Drs S. W. De Magaldi and D. W. R. Mackenzie for the gift of a monospecific antiserum to the *A. fumigatus* C antigen and we also thank the Photographic Department of the Royal Marsden Hospital. This study was supported by a project grant from the Medical Research Council.

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