

The uptake of *Aspergillus fumigatus* protein by serum IgG antibody from patients with pulmonary aspergillosis

B. JACOBY, JOAN L. LONGBOTTOM and J. PEPYS

Department of Clinical Immunology, Cardiothoracic Institute, Brompton Hospital, London SW3 6HP

Summary

A method is presented for measuring the uptake of *Aspergillus fumigatus* protein by IgG antibodies from human serum. The human IgG is first isolated with an anti-IgG conjugated immunosorbent and then incubated with radio-iodinated *A. fumigatus* protein. A group of twenty-three control sera gave the same background levels as tests without sera. The highest uptake of *A. fumigatus* protein was given by the sera of patients with aspergilloma, and lower values were obtained with six out of thirteen sera from patients with asthma and twenty-five out of twenty-eight sera from patients with asthma and pulmonary eosinophilia, i.e. allergic bronchopulmonary aspergillosis. These results were in agreement with precipitin tests. Inhibition studies with unrelated antigens showed that the reactions were specific for the *A. fumigatus* protein, of which more was bound by test sera than with a crude extract.

Introduction

Fungi of the genus *Aspergillus* can cause several different lung diseases, including bronchial asthma, a form of asthma with pulmonary eosinophilia, termed allergic bronchopulmonary aspergillosis and aspergilloma. The principle causative species is *Aspergillus fumigatus* (Pepys, 1971). A variety of serological tests have been used for the detection of antibodies to *A. fumigatus* including double diffusion, immunoelectrophoresis, complement fixation and a polystyrene tube radioimmunoassay, PTRIA (Patterson & Roberts, 1974). The detection of precipitins in the double diffusion test can be of diagnostic value for allergic bronchopulmonary aspergillosis and aspergilloma. Primary binding tests such as the ammonium sulphate (Farr) test may show antibodies undetected in other tests. For example, the ammonium sulphate test has been used to detect antibodies of a non-precipitating type to mucopolysaccharide components derived from *Aspergillus* spp. (Bardana *et al.*, 1972a, b).

Cross-reactions in serological tests to polysaccharides from different organisms have been reported (Longbottom & Pepys, 1974). Protein components of *A. fumigatus* are preferred for diagnostic tests (Longbottom & Pepys, 1964) but are not suitable for

Correspondence: Professor J. Pepys, Department of Clinical Immunology, Cardiothoracic Institute, Brompton Hospital, London SW3 6HP.

the ammonium sulphate test because the free and the antibody-bound components cannot be separated by precipitation.

A simple primary binding technique (Jacoby & Pepys, 1976) was used in the present study to measure the uptake of *A. fumigatus* protein by IgG antibody. The test consisted of an immunosorbent conjugated with anti-human IgG which is used to extract the IgG from the test serum. This is then incubated with iodinated antigen, the uptake of which is measured.

Materials and methods

Reagents

Phosphate buffered saline (PBS-azide) at pH 7.2 was used with 0.05% w/v sodium azide as preservative. Gelatin was obtained from the British Drug Houses Ltd. The stock solution was prepared by dissolving 1 g gelatin per 30 ml PBS-azide at 60°C. After filtering the solution was subsequently dispensed at 37°C. The preparation of Sepharose anti-IgG and of ^{125}I -subtilisin have been described previously (Jacoby & Pepys, 1976). Bovine serum albumin (fraction V and crystalline grades) were obtained from Sigma. ^{125}I -Sodium iodide (code IMS-30) was from the Radiochemical Centre, Amersham.

Preparation of extracts

Aspergillus fumigatus cultures A (IMI 163683) and B (IMI 16030) were maintained in this laboratory. Extracts from culture B were used except where indicated. Surface cultures were grown on histoplasmin synthetic medium at room temperature for 4–5 weeks. The culture filtrate was dialysed against water and freeze dried. It was referred to in the text as the 'crude extract'. The *A. fumigatus* protein was prepared from the crude extract by saturation with ammonium sulphate, the precipitate so obtained being re-dissolved in and dialysed against water and freeze dried.

The proteins extracted from the two different cultures, A and B, were selected because tests by two-dimensional immunoelectrophoresis (Haslam *et al.*, 1976) had shown that they differed substantially antigenically although the protein contents, i.e. A, 92%; B, 106%, were comparable. These extracts were assayed by the Folin technique (Lowry *et al.*, 1951), with bovine serum albumin as standard and expressed as a percentage of the dry weight. The protein content of the crude culture filtrate A was 35%.

A crude extract of *Candida albicans* (group A, strain B 311), grown on casein hydrolysate medium was prepared in the same way as described above for *A. fumigatus*, whilst the preparation of the protein has been described elsewhere (Longbottom *et al.*, 1976).

Test sera

Sera were obtained from patients at the Brompton Hospital with aspergilloma, allergic bronchopulmonary aspergillosis (abbreviated to allergic aspergillosis) and asthma. Normal sera were obtained from twenty-three healthy blood donors. The survey reported in Fig. 2 was made with the sera of forty-one patients who gave a positive prick reaction to *A. fumigatus*. A diagnosis of allergic aspergillosis was made in twenty-eight of these patients who had asthma and pulmonary eosinophilia with at least one episode of fleeting radiographic shadow, and in the majority, a positive

precipitin test to *A. fumigatus*. Of the remaining thirteen patients with asthma, a diagnosis of allergic aspergillosis was excluded by the absence of fleeting shadows in the chest radiograph. A positive control serum was prepared from the sera of three patients with aspergilloma and is referred to in the text as the 'pooled aspergilloma serum'. Sera were tested for precipitins by the method of counter-immunoelectrophoresis (Malo *et al.*, 1977) using the *A. fumigatus* A and B crude culture filtrate antigens at 30 mg/ml and the results are indicated in Fig. 2.

Method of assay for uptake of ^{125} I-A. fumigatus protein

Modifications were made to the method of Jacoby & Pepys (1976).

Radio-iodination of A. fumigatus protein. Iodination with ^{125} [I]-sodium iodide was by the chloramine T method as outlined by Hunter (1974), using on this occasion a higher ratio of chloramine T to protein, than was used by Jacoby & Pepys (1976), in order to minimize losses. *Aspergillus fumigatus* protein (50 μ g) was iodinated for 4 min in a glass tube in the presence of chloramine T (500 μ g) before the addition of sodium metabisulphite (1200 μ g) to stop the reaction. The reaction products were separated on a 2 $\times$ 20 cm column of Sephadex G-50 (fine grade) which had been treated with 2 ml of the stock gelatin solution. The pooled fractions from the protein peak were stored frozen. From 67 to 70% of the radioactivity was precipitable by 10% w/v trichloroacetic acid whilst with saturated ammonium sulphate 80% of the radioactivity was recovered in the precipitate after centrifugation (1 hr at 4°C, 10 000 g).

Assay. In the first stage of the assay during which patient sera are incubated with the immunosorbent, a variety of diluents were tested as described and finally a gelatin diluent was used (100 μ l of stock solution/tube). Patient serum (0.5 μ l/tube) was for convenience dispensed as a 1+100 dilution in PBS-azide (50 μ l/tube). The pooled aspergilloma serum was tested in quadruplicate and all other sera were tested in duplicate.

In the second stage of the assay, the antigen-binding stage, ^{125} I-A. *fumigatus* protein was added to the tubes in a volume of 10 μ l (protein: 50–60 ng, radioactivity: about 400 000 ct/min). The tubes contained 200 μ l of the stock gelatin solution, dispensed at 37°C. Manipulation of gelatin in the assay did not present any problem provided that normal room temperature was maintained. The tubes were incubated for a longer period of 48 hr before washing.

The uptake of antigen was expressed in ct/min and not as ng protein bound, as in the report by Jacoby & Pepys (1976). Test results were considered positive if the mean value (in ct/min) was >3 s.d. above the mean of the controls. The control tubes, of which there were six, were those from which patient serum was omitted. Duplicates were rejected if the samples differed from the mean by more than 10%. The mean value of the controls varied slightly from test to test and ranged from 800 to 1200 ct/min, whilst the mean value of the pooled aspergilloma serum ranged from 20 000 to 25 000 ct/min. Between-test comparisons were made in relation to the value given by the pooled aspergilloma serum.

Diluents for test sera

The following diluents were tested for their effect on the uptake of ^{125} I-A. *fumigatus* protein by a pooled aspergilloma reference serum; bovine serum albumin, crystalline grade and fraction V in both stages of the assay, and with fraction V in the first stage and crystalline in the second stage of assay, rabbit serum and turkey serum; the

Table 1. The effect of different protein diluents on the uptake of ^{125}I -*A. fumigatus* protein by a pooled reference serum

Protein diluent*	Uptake of radioactivity (ct/min)
Bovine serum albumin, Crystalline grade	21 480
As above, but with bovine serum albumin Fraction V in first stage of assay†	21 807
Bovine serum albumin, Fraction V	17 624
Rabbit serum (a)‡	17 716
Rabbit serum (b)	18 407
Rabbit serum (c)	17 980
Turkey serum	16 836

* 0.1 ml/tube. Bovine serum albumin prepared at 100 mg/ml in PBS-azide.

† Referred to in the text.

‡ Sera a, b and c were from three different rabbits.

latter two from Wellcome Reagents Ltd (Table 1). The highest uptake of labelled antigen was obtained with crystalline grade bovine serum albumin. A more detailed analysis suggested that the crystalline grade albumin was essential only for the second, antigen-binding stage, of the assay since the less pure fraction V could be successfully substituted during the first stage, when patient serum is incubated with the immunosorbent (Table 1). Rabbit serum which was satisfactory for the subtilisin antibody assay (Jacoby & Pepys, 1976) gave lower counts in this assay.

Subsequent tests showed that gelatin was as effective a protein diluent as was crystalline grade albumin and it was used as the protein diluent in the assay.

The three rabbit sera used were examined for the presence of antibody to *A. fumigatus* by indirect radioimmuno-electrophoresis to see if this might explain the lower counts obtained when they were used. After electrophoresis in agar and precipitation with an antiserum to whole rabbit serum, agar plates were incubated with ^{125}I -*A. fumigatus* protein, with washing where appropriate. Autoradiographs showed an arc of radioactivity coincident with the electrophoretic mobility of rabbit IgG, and with one of the rabbit sera there was also another arc in the alpha region which has not been identified. Components of rabbit sera used could therefore bind *A. fumigatus* protein, though this may not have been due exclusively to antibody.

Preliminary assays

Uptake of IgG. Two serum specimens, one the pooled aspergilloma serum and the other a serum from a patient with allergic aspergillosis were used for estimating the capacity of the anti-IgG immunosorbent to bind serum IgG (Fig. 1a). Purified human IgG was prepared from pooled cord serum and radio-iodinated (Jacoby & Pepys,

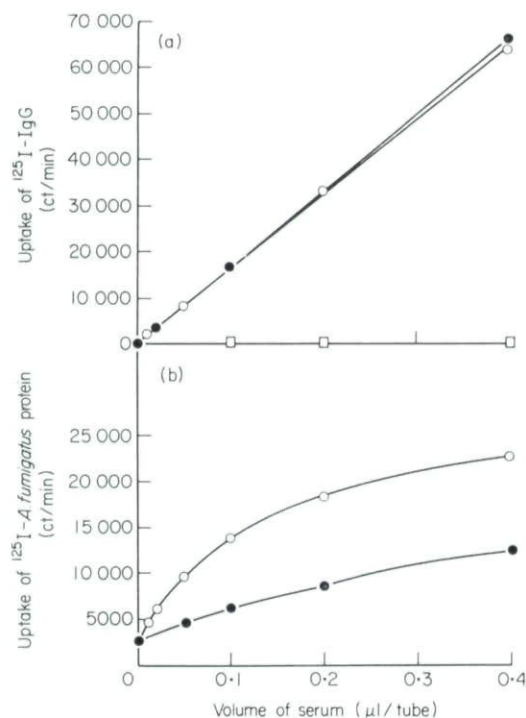


Fig. 1. Titration of patient sera. (a) Titration of immunosorbent by patient sera containing a trace label of ^{125}I -IgG. (b) (i) (Not shown here). Titration of immunosorbent by patient sera, followed by washing. (ii) Shown here. Titration of patient IgG on immunosorbent (from i) by ^{125}I -*A. fumigatus* protein.

The same amount of immunosorbent was used throughout. $\circ$, Pooled reference serum; $\bullet$, serum from patient with allergic aspergillosis; $\square$, control immunosorbent (tested with pooled aspergilloma serum).

Table 2. Ability of human IgG antibodies to discriminate between two different antigens

Serum	Uptake of antigen (ct/min)*	
	^{125}I - <i>A. fumigatus</i> †	^{125}I -subtilisin‡
Pooled reference serum	17 680	-61
Serum from enzyme worker	-250	74 280
Rabbit antiserum to <i>A. fumigatus</i>	420	Not tested
Aspergilloma serum (a)§	Not tested	193
Aspergilloma serum (b)	Not tested	21
Aspergilloma serum (c)	Not tested	15

* After subtraction of background. The negative values are not considered significant.

† Total ct/min added: 360 000.

‡ Total ct/min added: 140 000.

§ Sera a, b and c were from three patients with aspergilloma.

1976). Amounts of this preparation were added to each of two test sera so that 1 μ l of serum would contain 280 000 ct/min or about 5 ng of ^{125} I-IgG. The sera, which had been trace labelled in this way, were dispensed in increasing amounts over the range 0.01–0.04 μ l into tubes containing bovine serum albumin (crystalline grade, 5 mg/tube). The solid phase consisted of Sepharose anti-IgG and, as a control, Sepharose which had been activated and then blocked with 1 M glycine. After overnight incubation and washing the contents of the tubes were counted. The bound radioactivity increased in a linear manner as the volume of serum was increased. There was no significant uptake onto the control gel.

Uptake of ^{125} I-A. fumigatus protein. In a separate test, run concurrently, the ability of the same two serum specimens to bind ^{125} I-*A. fumigatus* protein was examined (Fig. 1b). The sera were tested over the same range of volumes but the trace label of ^{125} I-IgG was omitted. Sera were not tested against the control gel. Neither serum gave a linear response, but the non-linear effect was more pronounced with the pooled aspergilloma serum which also bound more radioactivity. The limiting factor was not

Table 3. Inhibition of the uptake of ^{125} I-*A. fumigatus* protein by a pooled reference serum

Inhibitor	Uptake of ^{125} I- <i>A. fumigatus</i> protein (ct/min)	
	0.025 μ g inhibitor	5 μ g inhibitor
<i>Candida albicans</i> protein	16 254	15 360
<i>Candida albicans</i> culture filtrate	15 847	15 719
<i>A. fumigatus</i> protein	11 359	2 530
None		16 440

the capacity of the immunosorbent to take up IgG from the sera (Fig. 1a) and so the lack of linearity must have been a result of the progressive exhaustion of the ^{125} I-*A. fumigatus* protein. The maximum percentage of radioactivity bound in this test represented only 7% of the radioactivity added.

The uptake of ^{125} I-*A. fumigatus* protein by IgG from the pooled aspergilloma serum was studied in the presence of unlabelled inhibitors (Table 3). A very small degree of inhibition was exhibited by the *Candida albicans* preparations whilst as expected the *A. fumigatus* protein was strongly inhibitory.

A crude extract of an *A. fumigatus* culture filtrate B was iodinated and used in place of the *A. fumigatus* protein B. The preparation was assayed before iodination and contained 35% protein. The specific activity was about 1 μ Ci/ μ g dry weight compared with that of about 10 μ Ci/ μ g protein of the *A. fumigatus* protein. The results of the test were similar to those obtained with the *A. fumigatus* protein except that relative to the controls with no patient serum, all the sera bound about 56% less radioactivity.

Specificity of IgG to A. fumigatus protein. The specificity of the antigen-antibody reaction was examined (Table 2). The uptake of ^{125} I-*A. fumigatus* protein by IgG

from the pooled aspergilloma serum was compared with that from the serum of a worker in the detergent industry with known precipitins to the bacterial enzyme subtilisin. A rabbit antiserum with precipitins against *A. fumigatus* was also tested to see whether any non-specific uptake of antigen would occur. Since the immunosorbent itself contained antibodies of rabbit origin, no uptake of antigen by a rabbit antiserum would be expected. Only the pooled aspergilloma serum gave a positive result. Sera were also tested separately for uptake of ^{125}I -subtilisin (Table 2). Only the control serum for this test, that from an enzyme worker, gave a positive result.

Results

Assay of sera of controls and of patients for uptake of ^{125}I -A. fumigatus protein (Fig. 2)
The sera of twenty-three blood donors all gave negative results. Positive results were given by the sera of twenty-five out of twenty-eight patients with allergic aspergillosis, and by six out of thirteen sera from patients with asthma. Sera from patients with

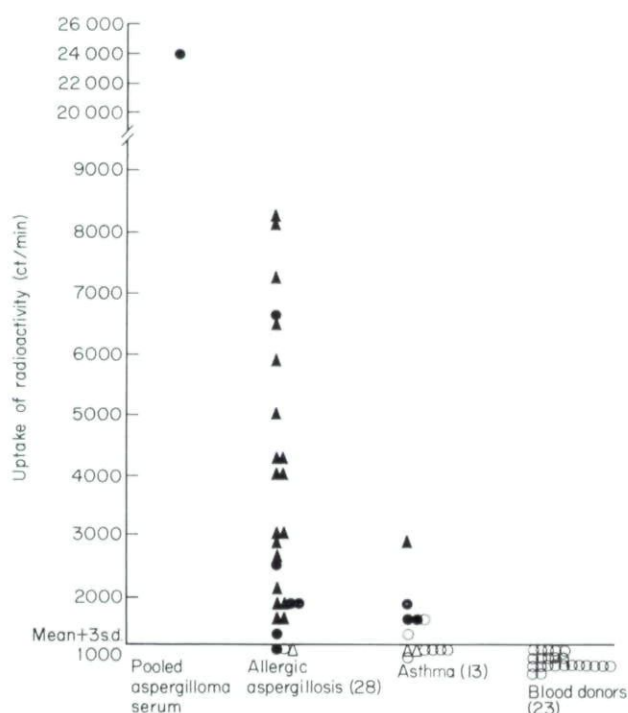


Fig. 2. Uptake of ^{125}I -*A. fumigatus* protein by serum IgG. ▲, △, Patients on steroid therapy; ●, ▲, precipitin positive sera; ○, △, precipitin negative sera.

allergic aspergillosis bound up to nine times the radioactivity of the controls, whereas the pooled aspergilloma serum gave over twenty times the control values. In a preliminary experiment six aspergilloma sera tested separately gave values of 11 400–15 000 ct/min, a serum of a patient whose aspergilloma was removed 1 year previously gave 6750 ct/min, whilst eleven allergic aspergillosis sera ranged from the control + 3 s.d. level of 4000 up to 9600 ct/min.

Of the patients with allergic aspergillosis, positive precipitin reactions were given

by twenty-six out of twenty-eight compared with four out of thirteen patients with asthma and none of the twenty-three control sera.

Effects of different A. fumigatus preparations

A limited study was performed concerning the effect of batch to batch variation between different extracts of *A. fumigatus*. Sera from forty of the forty-one patients with a positive prick reaction to *A. fumigatus* were tested for uptake of iodinated proteins A and B, and the calculated coefficient of correlation between the two sets of results was 0.95.

Discussion

The method presented here for estimating specific IgG antibody, in this case against an *A. fumigatus* protein extract, is simple to perform and economical of serum. The use of an immunosorbent conjugated with anti-human IgG for uptake of the patient's IgG means that on the subsequent addition of iodinated antigen, this is not brought into contact with the patient's whole serum. In this way competition between different classes of serum antibody or unwanted interactions between antigen and serum components other than antibody are minimized. Competition for antibody can interfere with those assays which require an antiglobulin reagent, such as the radio-allergosorbent test (RAST) for IgE and unwanted side reactions between antigen and serum components can complicate both anti-globulin tests and the ammonium sulphate (Farr) test. An interaction between *A. fumigatus* protein and rabbit serum, not involving antibody, is also reported here.

The highest uptake of ^{125}I -*A. fumigatus* protein by IgG was given by sera from patients with aspergilloma. Lower values were given by sera of patients with allergic aspergillosis and negative values by control sera from blood donors. About half of the sera from subjects with only asthma gave positive results. There was good agreement between these results and the precipitin test (Fig. 2). Therefore, these tests for specific IgG antibody using ^{125}I -*A. fumigatus* protein give a clear diagnostic distinction between the different forms of aspergillosis, with negative reactions in controls. This is in contrast to the findings of Bardana *et al.* (1972b) in their primary binding tests with mucopolysaccharide antigens. They showed virtually no distinction between allergic aspergillosis and control sera, presumably because of the wide cross-reactivity of such preparations with extracts from many different sources.

Most of the patients with allergic aspergillosis whose serum showed significant binding of the ^{125}I -*A. fumigatus* protein were receiving steroid therapy (Fig. 2), ranging for a period of 6 months up to 11 years, prior to their serum samples being taken. Therefore it would seem unlikely that steroid therapy would account for the lack of significant binding of the mucopolysaccharide, as postulated by Bardana *et al.* (1972b) in their allergic aspergillosis patients.

The sensitivity of our method is limited by the inability of the human IgG antibodies to bind more than a small proportion of the ^{125}I -*A. fumigatus* protein. This may mean that the bulk of the protein extracted from *A. fumigatus* culture filtrates is not of relevant antigenicity.

The failure of the iodinated antigen to saturate the serum IgG taken up by the anti-IgG immunosorbent (Fig. 1b) might be overcome by reducing the amount of serum used in the test or by increasing the amount of antigen or perhaps by purifying the antigen. The first two alternatives would, however, lower the ratio of sample to

background radioactivity, thus reducing the sensitivity of the test. There was good reproducibility with two different batches of iodinated protein. When iodinated crude culture filtrate was used the results were similar, but the test was less sensitive because much less radioactivity was bound.

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