

Prevalence of precipitating antibodies to different extracts of *Aspergillus fumigatus* in a North American asthmatic population

J.-L. MALO, R. PAQUIN and JOAN L. LONGBOTTOM*

Departments of Respiratory Diseases and Haematology, Sacré-Cœur Hospital, Montreal, Canada, and *Department of Clinical Immunology, Cardiothoracic Institute, London

(Received 9 December 1980; accepted for publication 19 January 1981)

Summary

This study was carried out to find the prevalence of precipitin reactions in the sera of 200 North American asthmatic subjects. Precipitins were detected by the double diffusion technique using different extracts of *Aspergillus fumigatus*, including a reference 'home produced' extract and five commercial extracts from three different suppliers. In addition, antigenicity of these extracts was assessed by crossed immunoelectrophoresis (XIE). Of the sera, 13.5% reacted to the reference extract and from 2.5 to 12% to the different commercial extracts; 22.5% of the sera reacted to at least one extract. No one serum reacted to all the extracts. Two of fifty-one (4%) non-atopic patients with a negative immediate prick test to *A. fumigatus*, six of eighty-seven (6.9%) atopic patients with a negative immediate reaction to *A. fumigatus*, and thirty-seven of sixty two (59%) atopic patients with a positive immediate reaction to *A. fumigatus* had precipitins to at least one of the extracts used, the skin tests being performed using the *A. fumigatus* reference extract. The prevalence of precipitin reactions bore a strong correlation with the antigenicity of the extracts by XIE. The same reference extract was also used for specific IgE measurements (Brompton extract, Malo & Paquin, 1979). It was found that patients with precipitins had significantly ($P < 0.001$) higher specific and total IgE values than patients without precipitins. In the group of patients with positive skin test, those with precipitins had significantly ($P < 0.05$) higher specific IgE values than those without. The authors conclude that different extracts of *A. fumigatus* should be used to assess the presence of precipitins. The antigenicity of these extracts should also be assayed.

Introduction

Recent studies (Schwartz *et al.*, 1978; Malo & Paquin, 1979) have shown that the incidence of immediate sensitivity to *Aspergillus fumigatus* in asthmatics is at least equal in North America to that found in the U.K. These findings do not explain why

Correspondence: Dr J.-L. Malo, Department of Respiratory Diseases, Sacré-Cœur Hospital, 5400 Gouin West, Montreal, Canada H4J 1C5.

allergic bronchopulmonary aspergillosis (ABPA) seems less frequent in North America. Pathogenesis of this disease may be explained by the presence of both type I and type III immunological reactions. We decided to investigate the prevalence of precipitin reactions, reflecting the type III component of the allergic mechanism, in a North American asthmatic population.

As the use of inadequate antigenic extracts has been blamed for lack of recognition of ABPA previously (Hoehne, *et al.*, 1973) and, since the use of several extracts has been recommended to overcome known antigenic variation (Longbottom & Pepys, 1964), we used six different extracts. These included a 'reference' extract prepared by one of us (J.L.L.) which had already been extensively used satisfactorily and five commercially available extracts from three different companies. This was not only in order to assess the reactivity of the sera but also to compare and note the reactivity/antigenicity of some presently available commercial extracts. The antigenicity of all the extracts was tested by a crossed immunoelectrophoresis (XIE) test, and presence of C-substance was examined using a CRP-positive serum.

Patients and methods

Patients

Two hundred asthmatic subjects were studied. The relevant information about the clinical features of these patients and the results of immediate sensitivity to *A. fumigatus* (prick immediate reactions, total IgE, specific IgE to *A. fumigatus*) were described in a previous paper (Malo & Paquin, 1979). These prick tests and specific IgE measurements included the same reference batch of *A. fumigatus* (Brompton extract) as the reference extract used in the present study. At the time of the study, blood was taken and the serum kept at -20°C until the precipitin test was performed.

Methods

A micromethod variant of the double diffusion test (Ouchterlony, 1953; Longbottom & Pepys, 1964) developed by one of us (J.L.L.) was used for the assessment of precipitins. This micromethod is a scaled down version of the standard double diffusion test that has been extensively used and recommended by one of the commercial suppliers (Bencard). This micromethod was found to be just slightly less sensitive than the standard double diffusion test due to a lower serum/antigen ratio. The gel depth is 2 mm, the central well 6 mm in diameter, peripheral wells 2.5 mm with distance between, edge to edge, 3 mm. The neat serum was placed in the central well, approximately 30 to 60 min before putting the antigens in the peripheral wells. Plates were kept at room temperature (20°C) which did not fluctuate significantly. Humidity was kept constant by placing a plastic cover over the plates. Plates were examined at 18, 24, 48 and 72 hr for the presence of precipitins.

XIE was performed using the reference extract and four of the five commercial extracts according to the method previously described by one of us (Longbottom, 1978). The test was carried out on cleaned glass slides (8.2 cm^2) with a 1-mm layer of 1% veronal buffered agarose gel ($\text{pH} = 8.6$) and $3.4\text{ }\mu\text{l}$ of antigen solution was introduced in a single lower lateral well. A potential of $4.5\text{--}5\text{ V/cm}$ was applied for 60–90 min. After removing the slide from the bath, a window of $3.5 \times 5\text{ cm}$ was cut out of the gel in the central area. The buffered agarose (2.4 ml) at 56°C containing $300\text{ }\mu\text{l}$ ($= 15\text{ }\mu\text{l/cm}^2$ of gel) of reference serum was poured into the window. After rotating the slides 90° , a

potential of 1.4 V/cm was applied for 20 hr in the second dimension. The plates were washed, dried and stained with Coomassie (Kenacid) blue G or R 0.5% in 45:10:45 mixture of ethanol:glacial acetic acid:distilled water. The reference serum was a pool of sera from four aspergilloma patients. Each of these sera had given thirty or more peaks when tested individually using a reference batch of antigen.

Testing for C-substance was done using a CRP-positive serum with a method previously described (Longbottom & Pepys, 1964), i.e. a double diffusion test but without citrate buffer in the agar.

Antigenic extracts

Six different antigenic extracts of *A. fumigatus* were used. The reference extract (= Brompton extract, Malo & Paquin, 1979) was prepared by one of us (J.L.L.) from freeze-dried, dialysed culture filtrates of the fungus grown at room temperature for 5 weeks on an histoplasmin synthetic medium (Longbottom & Pepys, 1964). This extract was also used for eliciting the skin test reactions and specific IgE measurements (Malo & Paquin, 1979). Five commercial extracts were also used. These antigens were obtained from two North American companies (Hollister-Stier and Greer Lab.) and one U.K. manufacturer (Bencard) distributing under licence in North America. Greer Lab. supplies in the same kit two different antigens of *Aspergillus* (No. 1, broth antigen; No. 6, cellular antigen). Bencard also provides two extracts in their serodiagnostic kit so as to be complementary to each other (Longbottom, 1978), being prepared from different types (short term deep and long term surface) of fungal culture. Finally, the Hollister-Stier antigen was as supplied in their hypersensitivity pneumonitis test and was prepared at a concentration to give positive precipitin lines with specific antisera. Except for this latter antigen, all the preparations were used at a concentration of 20 mg/ml (i.e. as supplied).

Results

Precipitins

The prevalence of positive precipitins to all the extracts is listed in Table 1. No one serum reacted with all the extracts. Forty-five (22.5%) sera reacted to at least one of the extracts, twenty-nine (14.5%) to at least two, and fifteen (7.5%) to more than two extracts. The overall prevalence varied from 2.5% to 13.5% depending on the extract used. It can be seen that the combined use of the two extracts provided by Bencard and Greer Lab. respectively, yielded an increase in the number of positive reactions. Indeed, in most instances, sera reacting to one extract did not react to the other extract and vice-versa.

Crossed immunoelectrophoresis

The antigenicity of the extracts was assessed by counting the number of peaks demonstrated by XIE. As shown in Table 1, there was a good correlation between the antigenicity of the extracts and the prevalence of precipitin reactions. Two extracts showed more than twenty-five peaks on XIE, two between twenty-one and twenty-five peaks and one between fifteen and twenty peaks. Figure 1 illustrates these peaks for the five extracts used.

Table 1. Prevalence of precipitin reactions and antigenicity of the extracts

Extracts	Prevalence of precipitins		Antigenicity of the extracts by XIE*	C-substance
	Number of sera	%		
Reference	27	13.5	+++	+
Bencard No. 1	5	2.5	+	—
Bencard No. 2	24	12.0	+++	—
Bencard No. 1 and No. 2†	29	14.5		
Greer No. 1	10	5.0	++	—
Greer No. 2	7	3.5	Not done	Not done
Greer No. 1 and No. 2†	16	8.0		
Hollister-Stier	17	8.5	++	+

* XIE = crossed immunoelectrophoresis.

+++ , more than twenty-five peaks; from twenty to twenty-five peaks; + from fifteen to nineteen peaks.

† Number of subjects reacting to one or the other or both of the extracts

Table 2. Prevalence of precipitins in different subgroups of asthmatics

Number of subjects with precipitins against:	Subgroups*				
	Non-atopic <i>A. fumigatus</i> — (n = 51)	Atopic <i>A. fumigatus</i> — (n = 87)	Atopic <i>A. fumigatus</i> + (n = 62)		
			1 mg/ml and 10 mg/ml	10 mg/ml only	Total
Reference extract	0 (0%)	1 (1.2%)	15 (24%)	11 (18%)	26 (42%)
1 Extract	1	6	4	5	9
2 Extracts	1	0	8	5	13
3 Extracts	0	0	7	6	13
4 Extracts	0	0	2	0	2
Total	2 (4%)	6 (6.9%)	21 (34%)	16 (25%)	37 (59%)

* Atopy present if two or more positive immediate prick reactions to routine battery of allergens. *A. fumigatus* —, negative immediate skin reaction to the reference extract of *A. fumigatus*; *A. fumigatus* +, positive immediate skin reaction to the same reference extract of *A. fumigatus*.

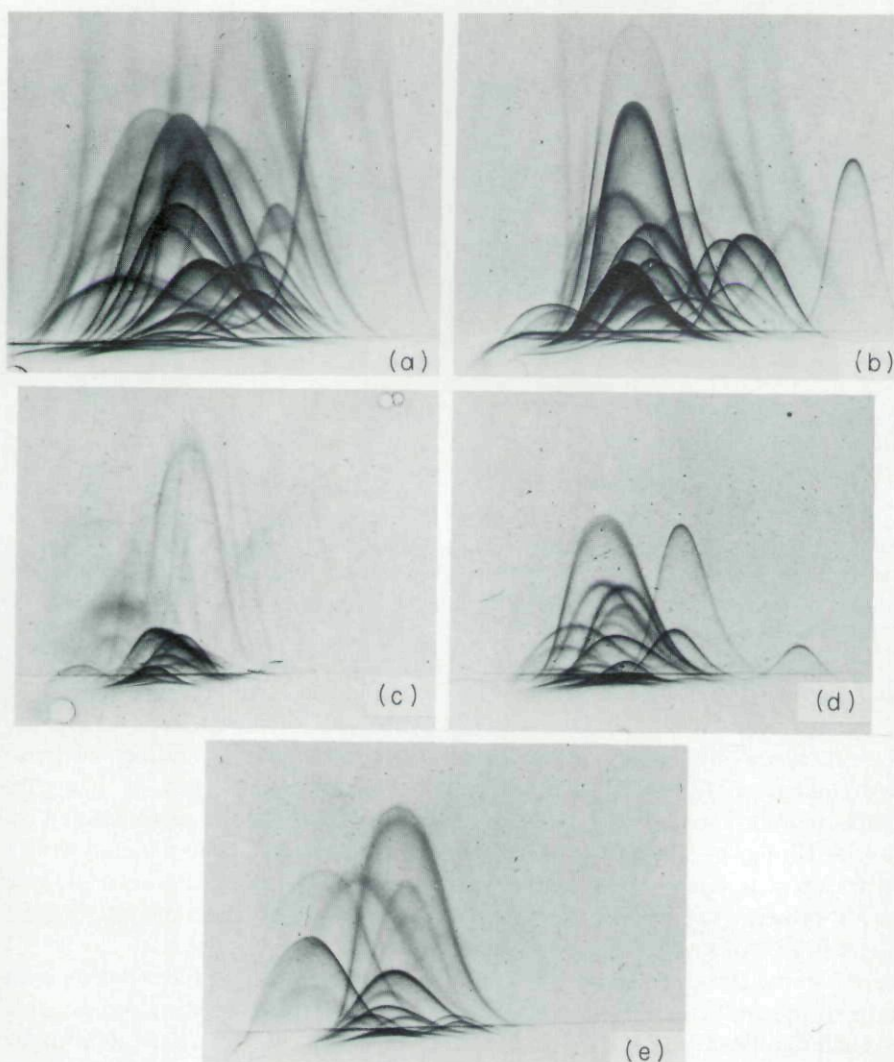


Fig. 1. Crossed immunoelectrophoresis with five different extracts of *A. fumigatus*. (a) Reference extract; more than twenty-five peaks seen; (b) Bencard no. 2; more than twenty-five peaks seen; (c) Bencard no. 1; fifteen to nineteen peaks demonstrated; (d) Greer No. 1; between twenty and twenty-five peaks demonstrated; (e) Hollister-Stier; between twenty and twenty-five peaks seen.

Correlation between the results of immediate sensitivity and precipitins to the same A. fumigatus extract

Among the 200 asthmatic subjects, it was shown (Malo & Paquin, 1979) that there were fifty-one non-atopic patients, eighty-seven atopics (two or more positive immediate prick reactions to a routine battery of common allergens) with a negative immediate skin test to *A. fumigatus* and sixty-two atopics with a positive immediate skin test to *A. fumigatus*. Table 2 gives the prevalence of precipitins in these sub-groups. Only one patient with a negative prick test to *A. fumigatus* had precipitins. Patients with positive precipitins were mainly found in the group with a positive prick test to *A. fumigatus*. On the same table, atopic patients with a positive skin test to *A. fumigatus* are divided in

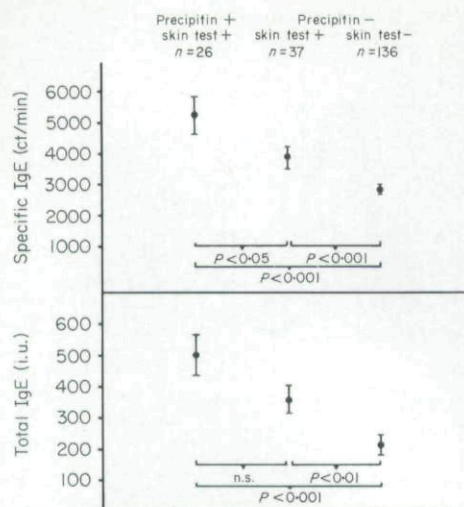


Fig. 2. Mean (± 1 s.e.) values of specific IgE and total IgE in three subgroups of patients: patients with positive precipitin and positive skin test to the reference extract of *A. fumigatus*; patients with negative precipitin but positive skin test; patients with negative precipitin and negative skin test. The level of statistical significance for the between-group comparisons of values obtained by the Student's unpaired *t* test is shown.

those who reacted at a concentration of 1 mg/ml from those who only reacted at 10 mg/ml. As a whole, twenty-six of sixty-two (42%) atopic patients with a positive prick test to *A. fumigatus* also had precipitins to the reference extract. Fifteen of these sixty-two patients (24%) had precipitins and a positive prick test at 1 mg/ml concentration while eleven of sixty-two (18%) besides having precipitins reacted on their skin at 10 mg/ml only. Thirty-seven of sixty-two (59%) atopic patients with a positive prick test to *A. fumigatus* had precipitins to at least one of the extracts. Of these thirty-seven subjects, twenty-one (34% of sixty-two) reacted on their skin at 1 mg/ml while sixteen (25% of sixty-two) had a positive skin test at 10 mg/ml.

Figure 2 shows the mean values (± 1 s.e.) of specific and total IgE in relation with the results of precipitins and skin test. Patients with positive precipitins and positive skin tests had significantly ($P < 0.05$) higher values of specific IgE than patients without precipitins but with positive skin tests. As a whole, patients with positive precipitins had significantly ($P < 0.001$) higher values for specific and total IgE than patients without precipitins.

Discussion

Our study shows that the presence of *A. fumigatus* precipitating antibodies in 22.5% of an asthmatic population is rather higher than that found in most other reports. Prevalences of 9% (Longbottom & Pepys, 1964), 7% (Mearns, Longbottom & Batten, 1967), 13% (Henderson, English & Vecht, 1968), and, more recently, 10.5% (Schwartz *et al.*, 1978) has been found in different British asthmatic populations. Prevalences of 11.5% (Hoehne, Reed & Dickie, 1971), 9% (Hoehne, Reed & Dickie, 1973), and 7.5% (Schwartz *et al.*, 1978) have been found in North American asthmatic adults. These discrepancies cannot be accounted for by non-specific reactions since positive reactions were very rarely found in non-atopics or in atopics with a negative immediate skin test. All the reactions were of the sharply defined protein-antigen/antibody type

(Longbottom & Pepys, 1975). Moreover, the addition of citrate buffer in our gel would have prevented any false positive reactions due to the C-reactive protein, as two of the six antigenic extracts did contain C-substance. These reactions are also unlikely to be due to the cross-reactivity of polysaccharide-type antigenic components with those of other fungi or dermatophytes. If this had been the case, a similar prevalence of such precipitins, characteristically of a broad diffuse nature, would have been expected in atopic patients with or without a positive prick reaction to *A. fumigatus*. These atopic individuals are indeed more likely to be sensitive to several antigens. Only one atopic subject out of eighty-seven with a negative prick reaction to *A. fumigatus* had precipitins to the reference extract. Another explanation would be that these individuals with positive precipitins, of whom the majority also had a positive skin reaction to *A. fumigatus*, were patients with ABPA. At the time of the study, all the patients had a chest radiograph taken and no acute shadows such as described in acute ABPA (McCarthy & Pepys, 1971) were identified. Nevertheless, any previous shadow could not be excluded. Bronchography was not done to detect the presence of bronchiectasis. Even if it had been done, Rosenberg *et al.* (1977) have suggested that in the early stage of ABPA bronchography might be reported as normal. Patients with positive skin tests and precipitins but without other evidence of ABPA have been found by others (Malo *et al.*, 1977) and they may be likely candidates to develop ABPA.

Other explanations for this difference in prevalence of precipitins found in our study could be proposed. The numbers of asthmatics included in the studies by Mearns, Longbottom & Batten (1967) and by Henderson, English & Vecht (1968) are sixty and thirty-nine respectively which are both lower than the number of subjects in our survey. We also used six different extracts of *A. fumigatus*. Only one extract was used by Schwartz *et al.* (1978), two by Longbottom & Pepys (1964) whereas the number of extracts used by Mearns, Longbottom & Batten (1967) and by Henderson, English & Vecht (1968) is not specified. The other studies published by Hoehne and colleagues (Hoehne, Reed & Dickie, 1971; Hoehne, Reed & Dickie, 1973) are comparable to our work as regards the number of antigens used (eight) and the number of adult asthmatics included (222 and 225). These investigators nevertheless 'considered positive (reactions) if it formed at least one band with two or more of the preparations'. Using the same criteria in our study, it can be seen from Table 2 that the prevalence found in our population would then be 14.5%, which is close to the 11.5% and 9% mentioned by these authors.

Positive precipitins were found predominantly in the group of subjects with positive immediate skin reactions to *A. fumigatus* and very rarely in non-atopics or in atopics with a negative immediate skin reaction to *A. fumigatus*. Of atopic patients with a positive prick test to *A. fumigatus* 42% showed precipitins with the reference extract and 59% with at least one of the extracts. These figures could be compared with those of Hoehne, Reed & Dickie (1973) who found precipitins in half of their patients with positive skin tests to *A. fumigatus* and in only 3% of intrinsic asthmatics. Our results are nevertheless very much higher than in the series reported by Schwartz *et al.* (1978) who found figures of 15 and 22% of positive precipitins in atopic patients with a positive skin test to *A. fumigatus* in Cleveland and London respectively. The concentration of *Aspergillus* used by these authors is not specified and they did not use the same *Aspergillus* extract for the skin test and precipitin test.

Our study clearly shows that depending on the antigen used the prevalence of precipitin reactions varied greatly from 2.5% to 13.5%. Such differences could be

correlated to the antigenicity of the extracts used, the extracts giving the highest percentage of precipitins being those with the greatest number of peaks shown on XIE. Other studies (Longbottom & Pepys, 1964; Hoehne, Reed & Dickie, 1971; Hoehne, Reed & Dickie, 1973; Fink *et al.*, 1977) have also shown that the presence of precipitins varies depending on the extract used. Two companies (Bencard and Greer Lab.) provide two different antigens in their diagnostic kits and, if used as such, it is seen from our results that this significantly increases the yield of the test. Since our results also show that the prevalence of precipitins is linked with the antigenicity of the extracts as demonstrated by XIE, it would seem to us informative if the manufacturer could provide details on the antigenicity of their extracts by methods similar to those described here.

We also want to stress that our results on the presence of precipitins hold for specific batches of antigen provided by each company at a specific time. Consequently, these results should not be interpreted as representing the overall antigenicity of all the extracts provided by each manufacturer. From batch to batch, antigenicity may indeed vary greatly and testing with different batches may have yielded considerably different results. Use of the tandem crossed immunoelectrophoresis method of antigen assay (Longbottom, 1978) would minimize such batch to batch variation.

The method used here for assessing precipitins is a scaled down version of the double diffusion technique of Longbottom & Pepys (1964) which is also recommended by one of the manufacturers (Bencard). This micromethod has been extensively used and found to be just slightly less sensitive than the standard double diffusion technique (personal observation J.L.L.). In their diakit, Greer Lab. supply precipitin plates whose characteristics (i.e. gel depth, diameter of the wells, distance between wells) are similar (± 1 mm) to the method used in this study. Hollister-Stier provides plates which are significantly different for thickness of the gel (5–6 mm) and distance between central and peripheral wells (9 mm). There is a possibility that this different technique suggested by the manufacturer could have yielded slightly different results for their antigen.

The patients with precipitins had significantly ($P < 0.001$) higher values for total and specific IgE with the reference extract of *A. fumigatus* than the patients who did not show reactions. In addition, patients with positive skin tests and precipitins to the same extract had significantly ($P < 0.05$) higher values of specific IgE than patients with positive skin test but without precipitins. Such an association between precipitins and reagins has been suggested by Longbottom and Pepys (1964). Correlations between the levels of IgE and the likelihood of developing precipitins have also been shown in ABPA (Malo *et al.*, 1977) where both type I and type III immunological mechanisms are likely to take part.

The aetiological importance of environmental factors in ABPA has been discussed recently (Vernon & Allan, 1980). These authors came to the conclusion that host susceptibility played a greater role than the level of *Aspergillus* spores in the atmosphere. Solomon (1976) found a prevalence of 31% of North American houses with *Aspergillus* spores. They did not find, however, the seasonal variation described in England. Monthly comparisons of levels in different parts of North America and the U.K. may throw new light on the subject.

Acknowledgments

This study was partly supported by the McAbbie Foundation, University of Montreal.

References

- FINK, J.M., BARBORIAK, J.J., KURUP, V.P. & SCRIBNER, G.H. (1977) Variability of extracts used in immunoprecipitin tests. *Journal of Allergy and Clinical Immunology*, **60**, 238.
- HENDERSON, A.H., ENGLISH, M.P. & VECHT, R.J. (1968) Pulmonary aspergillosis. A survey of its occurrence in patients with chronic lung disease and a discussion of the significance of diagnostic tests. *Thorax*, **23**, 513.
- HOEHNE, J., REED, C. & DICKIE, H. (1971) Allergic bronchopulmonary aspergillosis is not rare. *Journal of Laboratory and Clinical Medicine*, **78**, 1007.
- HOEHNE, J.H., REED, C.E. & DICKIE, H.A. (1973) Allergic bronchopulmonary aspergillosis is not rare. *Chest*, **63**, 177.
- Longbottom, J.L. & PEPYS, J. (1964) Pulmonary aspergillosis: diagnostic and immunological significance of antigens and C-substance in *Aspergillus fumigatus*. *Journal of Pathology and Bacteriology*, **88**, 14.
- Longbottom, J.L. & PEPYS, J. (1975) Diagnosis of Fungal Diseases. In: *Clinical Aspects of Immunology* (eds P.G.H. Gell, R.R.A. Coombs and P.J. Lachmann), p. 104. Blackwell Scientific Publications, Oxford.
- Longbottom, J.L. (1978) Immunological aspects of infection and allergy due to *Aspergillus* species. *Mykosen*, Suppl. **1**, 207.
- MALO, J.L. & PAQUIN, R. (1979) Incidence of immediate sensitivity to *Aspergillus fumigatus* in a North American asthmatic population. *Clinical Allergy*, **9**, 377.
- MALO, J.L., Longbottom, J., MITCHELL, J., HAWKINS, R. & PEPYS, J. (1977) Studies in chronic allergic bronchopulmonary aspergillosis. 3. Immunological findings. *Thorax*, **32**, 269.
- MCCARTHY, D.S., SIMON, G. & HARGREAVE, F.E. (1970) The radiological appearances in allergic bronchopulmonary aspergillosis. *Clinical Radiology*, **21**, 366.
- MEARNS, M., Longbottom, J. & BATTEN, J. (1967) Precipitating antibodies to *Aspergillus fumigatus* in cystic fibrosis. *The Lancet*, **i**, 538.
- OUCHTERLONY, O. (1953) Antigen-antibody reactions in gels: types of reactions in coordinated systems of diffusion. *Acta pathologica et microbiologica scandinavia*, **32**, 231.
- ROSENBERG, M., PATTERSON, R., MINTZER, R., COOPER, R.J., ROBERTS, M. & HARRIS, K.E. (1977) Clinical and immunologic criteria for the diagnosis of allergic bronchopulmonary aspergillosis. *Annals of Internal Medicine*, **86**, 405.
- SCHWARTZ, H.J., CITRON, K.M., CHESTER, E.H., KAIMAL, J., BARLOW, P.B., BAUM, G.L. & SCHUYLER, M.R. (1978) A comparison of the prevalence of sensitization to *Aspergillus* antigens among asthmatics in Cleveland and London. *Journal of Allergy and Clinical Immunology*, **62**, 9.
- SOLOMON, W.R. (1976) A volumetric study of winter fungus prevalence in the air of midwestern homes. *Journal of Allergy and Clinical Immunology*, **57**, 46.
- VERNON, D.R.H. & ALLAN, F. (1980) Environmental factors in allergic bronchopulmonary aspergillosis. *Clinical Allergy*, **10**, 217.

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.