

Lymphocyte sensitization to *Aspergillus fumigatus* antigens in pulmonary diseases in man

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Summary

In vitro studies of T-lymphocyte responses using five different batches of *Aspergillus fumigatus* antigens, were undertaken in twelve patients with *A. fumigatus* related lung disease and in three normal controls.

Using a leucocyte migration method, five of the twelve patients showed significant inhibition of leucocyte migration with a migration index of 0.80 or less to *A. fumigatus*, but in only three was this demonstrated with more than one batch of antigen (one patient with aspergilloma and two with bronchopulmonary aspergillosis).

The same antigens were used in lymphocyte transformation tests. Only two patients, one with aspergilloma and one with bronchopulmonary aspergillosis, showed clearly significant transformation although there were several borderline results.

Only three patients had evidence of delayed skin responses to *A. fumigatus* antigens *in vivo*, one with aspergilloma, one with bronchopulmonary aspergillosis and one with atypical bronchopulmonary aspergillosis. Two of these three patients also had one or more positive *in vitro* test results.

Thus T-lymphocyte sensitization to *A. fumigatus* as demonstrated by these *in vitro* methods, although present in occasional patients, was not clearly related to any one particular clinical syndrome in this small group of patients with aspergillus related pulmonary disease. There was, however, one of the three aspergilloma patients with positive lymphocyte transformation to all five batches of antigens and having higher transformation indices than in any other patient. This suggests that lymphocyte studies should be extended in this group.

In contrast to the frequent negative results using *A. fumigatus* antigens, evidence of T-lymphocyte sensitization to either *Candida albicans* or *Mycobacterium tuberculosis* or both, was shown by positive delayed hypersensitivity skin responses and *in vitro* inhibition of leucocyte migration in the majority of the patients, despite which lymphocyte transformation was often negative. The possibility of impaired capacity to transform is supported by the finding of an impaired response to phytohaemagglutinin (PHA) in four of the twelve cases.

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This information, together with other data discussed, helps to complete the contrasting immunological profiles seen in different *Aspergillus fumigatus* related lung diseases.

Introduction

Aspergillus fumigatus affections of the lung in man are of particular interest because of the variety of distinct clinical syndromes which occur.

Aspergillus fumigatus has been reported in some 10% of appropriately cultured random sputum samples (Pepys *et al.*, 1959). As an allergen it may induce immediate asthma and the specificity of this has been confirmed on bronchial challenge tests (Pepys, 1969). 'Late' airways obstruction occurring after an interval of 4-6 hr has also been observed in challenge tests where a dual (immediate and late) response may be found, corresponding in time to a dual skin reaction. *Aspergillus fumigatus* is related to pulmonary eosinophilia (i.e. transient pulmonary infiltrates associated with a blood eosinophilia), especially in cases with extrinsic asthma. It has been implicated in some 80% of cases of pulmonary eosinophilia with asthma seen in the U.K. (McCarthy & Pepys, 1971) and the term bronchopulmonary aspergillosis has been given to this syndrome. In these patients, dual responses can be demonstrated, both on intracutaneous skin testing and on bronchial challenge. IgG precipitins to *A. fumigatus* antigens can be demonstrated in the serum of the majority, although concentration of serum prior to testing is necessary in about 25% of them (Pepys, 1969). High serum levels of total and *A. fumigatus* specific IgE are characteristic (Patterson *et al.*, 1973).

Aspergillus fumigatus may also colonize areas of destroyed lung, especially pre-existing cavities, where it tends to form a mycelial mass or aspergilloma. These cases are characterized by the presence of precipitating antibodies apparently involving a much larger range of antigens than in bronchopulmonary aspergillosis, and numerous lines are easily demonstrable on double diffusion Ouchterlony plates using unconcentrated serum and *A. fumigatus* crude antigens. In general, skin tests are negative in these cases although positive responses, usually of the immediate type, have been found in about 20% of patients with aspergilloma (Longbottom & Pepys, 1964). Occasional patients with aspergilloma appear to have a stronger hypersensitivity component which responds to corticosteroids (Davies & Somner, 1972).

In view of its suitable spore size, its capacity to stimulate precipitating antibody and to induce an apparent Type III skin response, it is perhaps surprising that *A. fumigatus* is rarely if ever associated with extrinsic allergic alveolitis and peripheral granulomata in the lung. Liebow (1973) has, however, recently reported an unusual appearance in the lung periphery associated with *A. fumigatus* which he has termed bronchocentric granulomatosis, and other species of aspergillus, especially *A. clavatus*, have been found causing extrinsic allergic alveolitis when patients are exposed to excessively large amounts of antigens (Riddle *et al.*, 1968).

Where an antigen or group of antigens from a single agent causes several different pulmonary syndromes, it becomes important to know whether this can be accounted for by subtle differences in the antigens or alternatively, whether different patterns of host immunological response can be identified. In particular, we were interested to know whether specific cell-mediated immune responses to *A. fumigatus* antigens or impairment of cell-mediated immunity, play any part in the development of these separate clinical disorders.

In this paper we report studies using five different antigenic preparations of *A. fumigatus* in *in vitro* inhibition of leucocyte migration and lymphocyte transformation tests. In addition, T-lymphocyte function in these patients has been monitored by following their lymphocyte responses to common specific antigens using *C. albicans* and *M. tuberculosis*, and to the non-specific mitogen phytohaemagglutinin (PHA).

Materials

Patients (Table 1)

Twelve patients and three normal controls were studied. In Group 1 were three patients with typical aspergillomas. All had numerous serum precipitins to *A. fumigatus* antigens. Skin prick tests using *A. fumigatus* antigens were negative in all three patients, but weak immediate skin reactions were demonstrated on intracutaneous testing; two showed a dual (i.e. an immediate and 'late' response) response and one of these also showed a delayed reaction.

In Group 2 were six patients with bronchopulmonary aspergillosis showing either fleeting shadows with peripheral eosinophilia or the more chronic radiographic changes associated with bronchiectasis. All had a history of atopic asthma and all gave dual skin responses after prick testing with *A. fumigatus* antigen, and one showed a further response at 36 hr. Serum from four of the six patients showed specific precipitins. In Group 3 were three patients where the clinical features were atypical for bronchopulmonary aspergillosis; all had asthma and a very widespread pattern of peripheral bronchiectasis. None had precipitins using unconcentrated serum although one was later shown to be positive after serum concentration. None had positive prick test responses to *A. fumigatus* but two gave small immediate reactions on intracutaneous testing and one gave an additional reaction at 72 hr.

Antigens and non-specific mitogens

Aspergillus fumigatus antigens. These were obtained from the Bencard-Allergy division of Beecham's and were typical sero-diagnostic antigens.

(a) Culture filtrate antigens. B 27, CO143A and CO143B (antigen 1, antigen 2, antigen 3 respectively) were three different batches of freeze-dried culture filtrate from long term surface cultures of *A. fumigatus* grown to extinction (4-5 weeks) in a simple inorganic salt medium.

(b) Deep culture antigens. CO155A and CO155B (antigen 4, antigen 5 respectively) were two different batches of freeze-dried mycelial extract obtained from rapid three-day deep fermentation cultures of *A. fumigatus* in a simple organic peptone medium.

The five different batches were compared using two-dimensional immunoelectrophoresis (Laurell, 1965) as modified by Longbottom *et al.* (1976) and conventional Ouchterlony immunodiffusion. A standard pool of aspergilloma sera containing a broad spectrum of precipitating antibodies to diverse *A. fumigatus* antigens was used to test each of the five batches of antigens.

Candida albicans antigen. This was the protein fraction precipitated with ammonium sulphate from the culture filtrate of a 4-day shake culture of *C. albicans* strain A, grown in Sabouraud's broth (Faux, 1968).

Mycobacterium tuberculosis antigens. These were obtained from the Central Veterinary Laboratory, Weybridge.

(a) Purified protein derivative of tuberculin (Batch PPD 228) was a neutralized, freeze-dried preservative-free powder.

(b) Mycobacterial mixture was a mixture of heat-killed, freeze-dried strains C,D,T,P, and N.

Phytohaemagglutinin. This was obtained from Wellcome Reagents Ltd. (Batch PHA/676/80/B.) It was highly purified and contained 94 'Wellcome' units of mitogenic activity and 463 'Wellcome' units of haemagglutinating activity in approximately 10 mg of protein. It was stored at -20°C in solutions containing 0.3 mitogenic units and 0.03 mitogenic units per 10 μl of phosphate buffered saline.

Methods

Leucocyte migration test

The test was first described by Søberg & Bendixen (1967) and our own modification has been reported previously in detail (Haslam, Turner-Warwick & Lukoszek, 1975).

For each patient or control study, leucocytes were obtained from 40 ml freshly defibrinated blood by gelatin sedimentation, and each study was completed using all five *A. fumigatus* antigens, the two *M. tuberculosis* antigens, *C. albicans* culture filtrate protein and antigen-free controls. The antigens were dissolved as required in the test culture medium, Eagle's Basal Medium (Wellcome Reagent's Ltd) containing 20% heat-inactivated fetal calf serum. Dose response was followed using concentrations of 300 and 100 $\mu\text{g}/\text{ml}$ for *A. fumigatus* and *M. tuberculosis* antigens, and 100 and 50 $\mu\text{g}/\text{ml}$ for *C. albicans* culture filtrate protein.

Results were expressed as a Migration Index (MI) derived from the area of leucocyte migration in medium containing antigen $\div$ the area of leucocyte migration in the antigen-free control.

Migration Index values of 0.80 or less have been regarded as definitely positive. Results between 0.81 and 0.84 have been regarded as 'probably positive' because the mean MI using five *A. fumigatus* antigens in fifteen tests on three normal controls gave a grouped mean -2 s.d. of 0.84.

In each test two or three doses of antigen were used and the lowest migration index has been tabulated (Tables 1, 2, and 3).

Lymphocyte transformation test

The method used was the whole-blood microculture technique (Junge *et al.*, 1970; Han & Pauly, 1972; Paty & Hughes, 1972) using a modification we have developed and evaluated in clinical and normal control studies over the past 3 years.

For each patient or control study, 40 ml of venous blood was taken, the blood cells sedimented by low speed centrifugation at 250 *g* for 10 min and washed three times in the basic test culture medium (Minimum Essential Medium containing Hank's salts and 25 mM Hepes buffer, Gibco-Biocult, Code 237, and containing 2 mM L-glutamine, 100 units/ml sodium benzylpenicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin). The washed cells were resuspended to the original blood volume, 5 ml set aside for use as the test cells and the remainder resedimented and used to absorb heat inactivated human AB serum, using one part of washed packed blood cells to four parts of serum and absorbing for 30 min at room temperature to remove iso-leucoagglutinins and iso-leucocytotoxins.

Human AB serum (inactivated and absorbed) was the standard 10% serum additive for all test cultures. Human serum was essential in this whole blood system to observe specific antigen transformation. Even in the PHA tests, transformation was approximately ten times higher using human serum than using fetal calf serum. Human AB

Table 1. Skin test results correlated with migration and transformation indices using five antigens of *A. fumigatus*

Aspergillus fumigatus antigens																	
Skin tests					Precipitation peaks		Migration index					Transformation index					
		Immediate		Late	Delayed	1		1	2	3	4	5	1	2	3	4	5
Group 1	Aspergilloma	+	+	+	+	19	0.97	0.80	0.82	0.95	0.86		2.55	2.38	2.32	5.76	3.86
		+	+	+	—	17	0.81	0.80	0.81	0.91	0.87		1.03	0.83	0.85	0.78	0.89
		+	+	—	—	25	0.96	0.91	0.96	1.06	1.01		1.48	1.03	1.14	1.27	1.14
Group 2	Bronchopulmonary asperillosis	+	+	+	+	0	0.91	1.01	0.94	0.98	1.05		1.18	1.32	1.48	1.48	1.19
		+	+	+	—	2	1.03	0.95	0.94	0.97	1.06		1.31	1.57	1.74	1.38	1.44
		+	+	+	—	1	0.72	0.65	0.75	0.92	ND		0.90	0.81	0.80	0.78	ND
		+	+	+	—	6	0.91	0.79	0.72	0.74	0.75		1.07	1.86	0.41	0.98	0.82
		+	+	+	—	2	1.04	1.02	1.03	1.03	1.00		1.24	2.18	2.10	2.54	1.76
		+	+	+	—	0	1.18	1.21	1.18	1.29	1.18		1.15	1.41	1.20	1.19	1.42
		+	+	+	—												
Group 3	'Atypical' bronchopulmonary aspergillosis	+	+	—	—	0	0.87	0.95	0.94	0.91	0.92		0.99	0.88	0.80	0.84	0.75
		—	—	—	—	0	0.93	0.91	0.99	0.97	1.04		0.88	0.69	0.88	0.98	0.88
		+	+	—	+	1	0.93	0.94	0.74	0.92	0.96		1.08	1.91	1.38	1.49	ND
		—	—	—	—	0	0.93	0.96	0.87	0.98	0.95		0.96	1.33	0.72	1.28	1.00
		—	—	—	—	0	1.01	0.99	1.08	1.13	1.06		0.85	0.75	0.82	0.84	0.89
		—	—	—	—	0	0.97	0.92	0.89	0.98	0.98		1.20	0.99	1.04	1.10	1.19
		—	—	—	—		Mean for controls —2 S.D.						Mean for controls +2 S.D.				
							Grouped mean —2 s.d. 0.84					Grouped mean +2 s.d. 1.37					

* Positive results obtained with intracutaneous tests only. --- Probable positive MI 0.84-0.81; TI 1.38-1.99, — Positive MI ≤ 0.8; TI ≥ 2.0.

Table 2. Skin test results using *C. albicans*, correlated with migration and transformation indices in patients with *Aspergillus* related lung diseases

Name	<i>Candida albicans</i> antigen				
	Skin tests			Migration index	Transformation index
	Immediate	Late	Delayed		
Aspergilloma					
K	+	—	+	0.58	2.68
W	ND	ND	ND	0.75	0.90
L	+	—	+	0.88	1.16
Bronchopulmonary Aspergillosis					
B	—	—	+	0.82	1.33
T	+	—	+	0.75	1.57
I	—	—	—	ND	0.62
R	—	—	+	0.70	1.47
Te	—	+	+	ND	1.78
G	—	—	+	1.06	1.28
'Atypical' Bronchopulmonary Aspergillosis					
S	+	—	—	0.88	0.82
C	—	—	+	0.82	0.71
G	—	—	+	0.76	2.22

Table 3. Skin test results using PPD and *M. tuberculosis* antigen correlated with migration and transformation indices in patients with *Aspergillus* related lung diseases

	Mantoux test		Migration index		Transformation index	
	Strength	Result	PPD	Mycobacteria	PPD	Mycobacteria
Aspergilloma						
K	1/1000	+	0.61	0.48	2.80	2.90
W	1/10,000	—	0.83	0.80	1.14	1.00
L	1/1000	+	1.00	0.72	2.79	ND
Bronchopulmonary aspergillosis						
B	1/1000	+	1.00	0.71	1.57	1.89
T	1/1000	—	0.91	0.68	1.33	2.76
I	1/100	+	ND	0.54	0.62	0.60
R	1/1000	+	0.66	0.65	1.26	0.34
Te	1/100	+	ND	ND	3.24	3.78
G	1/1000	—	1.18	0.97	1.85	1.83
'Atypical' bronchopulmonary aspergillosis						
S	1/100	+	0.83	0.78	1.15	1.24
C	1/1000	+	0.76	0.68	1.37	2.76
G	1/1000	+	0.69	0.70	1.85	2.03

serum was used in preference to autologous plasma to rule out interference from autologous serum-derived lymphocyte stimulating or blocking factors, and to eliminate serum variability particularly in quantitative studies with PHA.

Whole blood cultures with specific antigens. Each patient or control study was completed using the five *A. fumigatus* antigens, the two *M. tuberculosis* antigens, the *C. albicans* culture filtrate protein and antigen-free controls. The antigens were dissolved in basic test culture medium (without serum) at double the actual concentration required for the test and allowed to dissolve over the morning. Solutions were sterilized where necessary by graded membrane filtration down to 0.22 μ m. The final test concentrations used were 300, 100 and 10 μ g/ml for *A. fumigatus* and *M. tuberculosis* antigens, and 100 and 10 μ g/ml for *C. albicans* culture filtrate protein.

Test cultures were set up in duplicate for each antigen dose in sterile Falcon polycarbonate tissue culture tubes, 12 $\times$ 75 mm (Falcon Plastics, Code 2005), by adding 1.5 ml of concentrated (2 $\times$) antigen solution and 1.5 ml of test culture medium containing 20% inactivated absorbed human AB serum (a final 3 ml test culture containing the correct antigen dose and a 10% final concentration of AB serum). A duplicate pair of antigen-free controls was prepared using antigen-free basic culture medium.

Finally, 0.1 ml of washed whole blood test cells was added to each tube, the tubes stoppered and incubated at 37°C for a total period of 6 days. On the sixth day, cultures were labelled over the final 6-hr period with 1 μ Ci/ml methyl- H^3 thymidine, specific activity 2 Ci/mM (The Radiochemical Centre, Amersham). The cells were harvested, washed once with 10 ml of cold 3% acetic acid to reduce red cell contamination, and twice more with 10 ml volumes of cold phosphate buffered saline. The amount of cell incorporated radioactivity was determined for each sample by counting in a Packard Tri-Carb Scintillation Counter. Samples were prepared for counting using the H_2O_2 bleaching method recommended by Packard for Instagel scintillator (Packard Information Bulletin 0753-13), cold and dark equilibrated over 24 hr, then counted for 5 min each on the Tritium channel using external standardization. Counts were recorded as net counts per min (c.p.m.) and corrected for quenching. The mean count efficiency was 22%.

Results were expressed as a Transformation Index (TI) derived from the c.p.m. corrected for cells cultured with antigen $\div$ c.p.m. corrected for control cells cultured without antigen. A Transformation Index of 2 or greater was considered indicative of positive lymphocyte sensitization.

Results between 1.37 and 1.99 have been regarded as 'probably positive' because the mean TI using five *A. fumigatus* antigens in fifteen tests on three normal controls gave a grouped mean $\pm$ 2 s.d. of 1.37.

Results have been tabulated using the highest TI observed in each test study with two or three doses of antigen (Tables 1, 2 and 3).

Whole blood cultures with phytohaemagglutinin (PHA). Each patient or control was studied in full dose response using cultures containing 0.1, 0.01, 0.005 and 0.0025 mitogenic units (mu) of PHA per ml. For each study, test cultures were set up by adding 3 ml of basic culture medium containing 10% inactivated absorbed human AB serum to every tube in a set of five pairs of Falcon polycarbonate tissue culture tubes. Using the stock PHA solutions, 10 μ l containing 0.3 mu PHA was added to the first pair of tubes, 10 μ l containing 0.03 mu to the second pair, 5 μ l containing 0.015 mu to the third pair and 2.5 μ l containing 0.0075 mu to the fourth pair (0.1, 0.01, 0.005

and 0.0025 mu/ml respectively). PHA was not added to the fifth pair which served as the mitogen-free control.

Finally, 0.01 ml* of washed whole blood was added to each tube, the tubes stoppered and incubated at 37°C for a total period of 3 days. On the third day cultures were labelled with H³ thymidine over the final 6-hr period, cells harvested and the amount of radioactive uptake determined by scintillation counting as described above.

Results were plotted as PHA dose response curves per 0.1 ml of blood expressing response as log₁₀ c.p.m. corrected in order to make satisfactory comparisons with normal controls.

PHA dose response curves were examined by reference to mean normal population dose response curves prepared using the same technique for 25 male and 11 female normal controls. The normal control range was taken as the mean uptake $\pm$ 2 s.d. (log. c.p.m. corrected) for the total individuals in the male and female groups. PHA dose response curves falling outside the normal control range in at least two PHA doses in the ascending portion of the curve were considered abnormal.

Serological studies

Serum from patients and controls, taken at the time of the lymphocyte sample, was examined by two-dimensional immunoelectrophoresis (Longbottom *et al.*, 1976) using *A. fumigatus* antigen 1.

Skin tests

Aspergillus fumigatus prick tests using antigen 1 were performed using concentrations of 0.1, 1.0 and 10.0 mg/ml. If the weals were less than 3 mm diameter, intradermal tests starting with 0.1 ml of the weakest strength of antigen were used. The diameter of the weal was recorded at 10 min, 6, 24 and 48 hr.

A suspension of heat-killed *C. albicans* diluted w/v 1/1000 was used intradermally in a dose of 0.1 ml and tests were read at similar time intervals over 48 hr.

Intradermal tests using 0.1 ml of PPD at 1/10,000, 1/1,000 and 1/100 dilutions were used. Unfortunately not all patients showing a negative response to the higher dilutions were followed through using the greater strengths.

Results

Aspergillus fumigatus antigens

The five *A. fumigatus* antigens were tested against a standard aspergilloma serum pool previously shown to give multiple precipitin lines to several different batches of *A. fumigatus* antigens.

Two-dimensional immunoelectrophoresis showed that the culture filtrate serodiagnostic antigens 1, 2 and 3 (i.e. those obtained from long-term surface cultures grown to extinction) gave more numerous and stronger peaks of precipitation than the mycelial extract serodiagnostic antigens 4 and 5 obtained from short-term deep cultures; antigen 1 giving the maximum number of precipitation peaks overall (Fig. 1. antigen 1, 28 peaks; antigen 2, 22 peaks; antigen 3, 20 peaks; antigen 4, 16 peaks and antigen 5, 14 peaks).

*Note. We now recommend parallel studies adding a volume of washed whole blood containing 3×10^5 lymphocytes as determined by total and differential white cell counts for quantitative studies with PHA using the whole blood technique.

Aspergillus fumigatus precipitins

The number of precipitin peaks observed in each patient and control serum using the most potent antigen is set out in Table 1.

All three aspergilloma sera gave a large number of arcs ranging from 17 to 25.

These results contrast strikingly with those obtained using sera from patients with

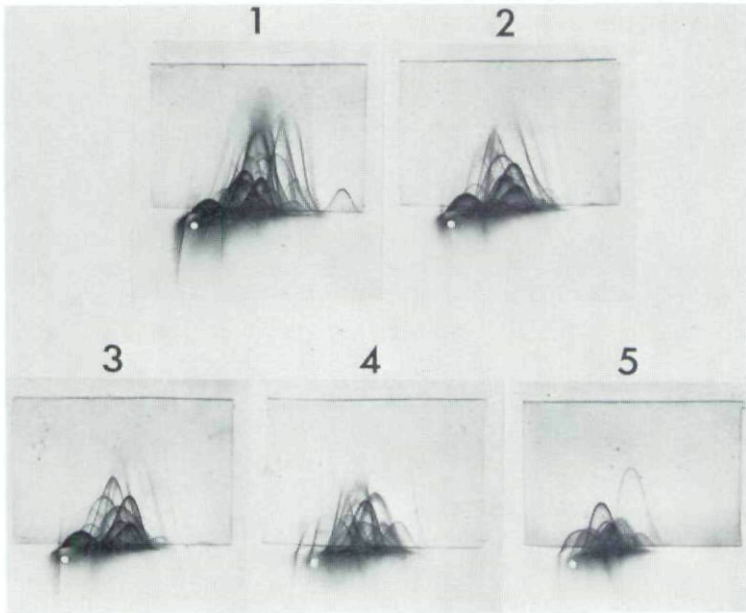


Fig. 1. Two-dimensional immunoelectrophoresis of the five batches of *A. fumigatus* antigens used in the *in vitro* lymphocyte tests.

(1), (2) and (3). Freeze-dried culture filtrate from long-term surface cultures (B 27, CO143A, CO143B respectively).

(4) and (5). Freeze-dried mycelial extract from rapid 3-day deep fermentation cultures (CO155A, CO155B respectively).

First dimension electrophoresis using 3.4 μ l of 60 mg/ml antigen solution. Second dimension electrophoresis using 17 μ l serum/cm² in the agarose gel.

bronchopulmonary aspergillosis, where even after doubling the concentration of serum incorporated in the gel very few precipitin peaks were detected and in only four of the six sera.

A single precipitin peak was given by one other serum from a patient with 'atypical' bronchopulmonary aspergillosis.

Lymphocyte responses to A. fumigatus

Inhibition of leucocyte migration (ILM). The results are shown in Table 1.

Using the criteria for acceptance of positive results described in the Methods section, five of the twelve patients showed definite positive inhibition, with migration indices of 0.80 or less, to at least one of the five *A. fumigatus* antigens.

The different batches of *A. fumigatus* antigens gave variable results, and no single batch identified positive results in all five patients. Further, only two of the five patients were definitely positive with more than one antigen. Two other patients positive to antigen 2 also had border-line positive results with other batches. Forty-nine

of the total sixty tests using the five batches of antigens on the twelve patients were clearly negative.

Culture filtrates from long-term surface cultures grown to extinction, particularly antigens 2 and 3, best demonstrated the positive responses, while mycelial extracts from short-term deep cultures (antigens 4 and 5) identified only one of the five positive patients. Thus, antigens giving the most satisfactory results in two-dimensional immunoelectrophoresis by identifying the maximum number of *A. fumigatus* specific precipitating antibodies, also proved the most satisfactory for inhibition of leucocyte migration.

All five patients with positive inhibition of leucocyte migration had positive immediate skin responses. Four out of five gave 'dual' reactions and two had delayed responses. All showed positive serum precipitins. However, neither positive late skin



Fig. 2. (Patient K with Aspergilloma.) Seventy-two-hour delayed skin test response following intracutaneous injection of 0.1 ml B 27 (antigen 1) 0.1 mg/ml.

responses, nor positive serum precipitins were consistently associated with positive inhibition of leucocyte migration, but two of the three patients with delayed skin reactions showed significant inhibition of migration. The leucocyte migration test was always negative in patients with both negative late and delayed skin responses and in those with negative serum precipitins.

Significant inhibition of leucocyte migration, but only to antigen 2, was seen in two of the three patients with aspergilloma and one of these had shown a typical delayed response to *A. fumigatus* on skin testing (Fig. 2).

Two of the six patients with bronchopulmonary aspergillosis showed positive inhibition and in each case the results were confirmed by more than one batch of antigens (three batches in one case, and four in the other).

There was only a single positive response in one patient in group 3 having 'atypical' bronchopulmonary aspergillosis. This patient had a positive skin reaction appearing at 72 hr which may have indicated a delayed response.

From these results it appears that inhibition of leucocyte migration is occasionally positive in *A. fumigatus* related lung diseases but from this small study does not occur in any one particular type of disease.

Lymphocyte transformation

These results are also summarized in Table 1.

The criteria for acceptance of positive results have been described in the methods section. Only two patients showed consistently positive lymphocyte transformation to *A. fumigatus* antigens with transformation indices of 2.0 or greater. One patient with aspergilloma and a positive delayed skin test responded to all five batches and the other with bronchopulmonary aspergillosis responded to three (batches 2, 3, and 4). Both patients had dual skin responses, one had a delayed response and both had precipitating antibodies.

Only one of the five patients positive in the leucocyte migration tests was also positive in lymphocyte transformation tests, although two others showed border-line positive results.

Occasional border-line positive results were seen in four other patients. Unlike the situation in the leucocyte migration and immunoprecipitation studies, no particular antigen batch could be identified in lymphocyte transformation studies consistently giving more positive results than the others. The mycelial extracts were as good, or even slightly better than the culture filtrates.

Thus, it appears that in general, using the conditions of our tests, convincingly positive lymphocyte transformation is uncommon in aspergillus related lung diseases.

Lymphocyte responses to C. albicans, PPD and M. tuberculosis

Results of delayed hypersensitivity skin reactions to *C. albicans* and PPD in comparison with *in vitro* tests using lymphocyte transformation and inhibition of leucocyte migration are summarized in Tables 2 and 3.

Two patients with aspergilloma were tested with *C. albicans* and both showed positive delayed responses. In one patient the *in vivo* result was confirmed with the *in vitro* tests. Both of these patients also had positive delayed skin reactions to PPD and this too was reflected *in vitro* with a positive migration and transformation index in each case to either PPD or mycobacterial antigen.

In bronchopulmonary aspergillosis five of the six patients gave a normal positive delayed skin response to *C. albicans* and this related to a significantly positive migration index in three of the four patients tested. Lymphocyte transformation using our methods appeared to be less sensitive and no significant transformation was observed.

Positive delayed skin responses to PPD were found in four of the six patients: five of these patients were re-tested *in vitro* by inhibition of leucocyte migration using PPD and mycobacterial antigens, and the combined results confirmed positive skin tests in every instance. Lymphocyte transformation appeared to be rather less sensitive in that only one of the four patients with a positive skin test also gave a significant lymphocyte transformation.

Of the patients with 'atypical' bronchopulmonary aspergillosis all three gave positive delayed skin tests with PPD. All were reflected by a positive migration index

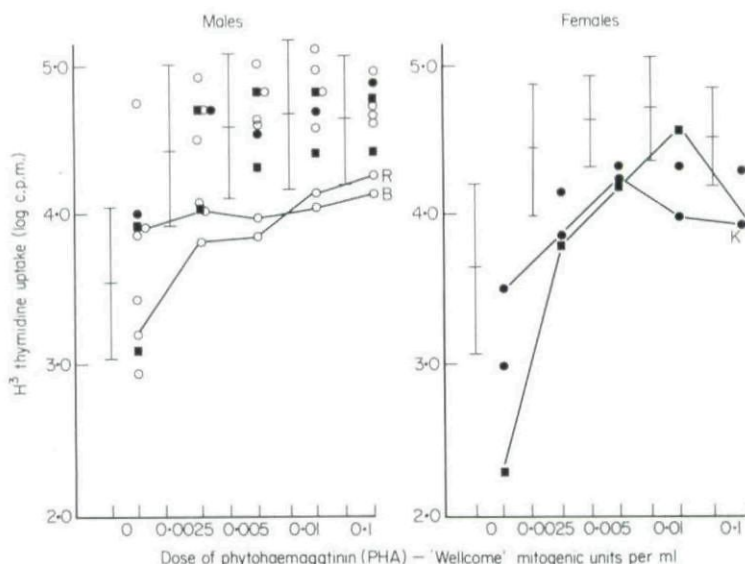


Fig. 3. Dose response curves to phytohaemagglutinin. Males and females have been set out separately because of the different normal values. $\bar{x} \pm 2$ s.d., ■, aspergilloma; ○, bronchopulmonary aspergillosis; ●, atypical patients.

in vitro and two also by a positive transformation index. Two gave positive skin tests to *C. albicans* and one was confirmed using the *in vitro* tests. Once again, lymphocyte transformation was, in general, less sensitive than leucocyte migration.

Thus, the expression of delayed hypersensitivity to common antigens appeared to be normal in our patients, when tested either *in vitro* or *in vivo*.

Responses to phytohaemagglutinin

The dose response curves of lymphocyte transformation with phytohaemagglutinin for males and females are shown in Fig. 3.

Table 4. *In vivo* and *in vitro* responses to *C. albicans* and *M. tuberculosis* in four patients with depressed responses to phytohaemagglutinin

Patient	<i>C. albicans</i>			<i>M. tuberculosis</i>		
	Delayed skin test	MI	TI	Delayed skin test	MI	TI
R	+	0.70	1.47	+	0.65	0.34
B	+	0.82	1.33	+	0.71	1.89
C	+	0.82	0.71	+	0.68	2.76
K	+	0.58	2.86	+	0.48	2.90
(U-D*)	+	0.34	0.74	+	0.68	0.89

* A further patient with aspergilloma showing a marked depression of PHA response. The results in transformation tests to specific antigens remained negative when they were repeated with a 24-hr pulse of methyl- H^3 thymidine.

Eight patients had normal responses but four were slightly depressed. Lymphocyte counts per 0.1 ml of whole blood in two of these four patients were slightly reduced in comparison with the mean counts minus 2 s.d. for the normal controls, but this was not sufficient to account for the impairment in response to PHA.

The responses to specific antigens in skin and *in vitro* tests are set out in Table 4.

Delayed skin test responses to both *C. albicans* and PPD were positive in all four patients and this correlated well with positive results with inhibition of leucocyte migration tests. However, lymphocyte transformation to specific antigens was negative in five of the eight tests.

Discussion

Five different batches of *A. fumigatus* antigens showed quite marked differences in precipitating ability when tested against serum known by previous studies to show multiple precipitation peaks against a range of *A. fumigatus* antigens. It is clear from this study that different results are likely to be obtained even when quite minor differences in batches of antigens from different laboratories are used, and this may account for some of the divergent views on the importance of *A. fumigatus* in pulmonary eosinophilia. These findings also stress once again that it is important to use the same batch of antigens throughout an entire study, that it is important to use the same crude antigen when different immunological tests are being compared, and that it is also perhaps important to test several types of crude antigen to ascertain which is the most appropriate for a particular study. A batch of antigens which is potent in precipitation tests is not necessarily as good in other immunological systems. For this reason it was important in our study to assess the lymphocyte responses to a range of crude antigens prepared by different methods, but having defined precipitating characteristics.

Freeze-dried culture filtrates from long-term surface cultures grown to extinction, which gave the maximum precipitation peaks picked up the majority of positive responses in both *in vitro* lymphocyte tests over the dose range used in the present study, but the mycelial extracts prepared from rapid deep cultures were less satisfactory in the leucocyte migration tests.

Under the conditions of our tests, and in particular using the whole blood method for lymphocyte transformation studies, inhibition of leucocyte migration gave a positive result more frequently than the transformation test. There are a number of possible explanations for these disparate results. It is possible, using the whole blood method, that too few lymphocytes were present to show significant transformation. However, further studies in our laboratory have shown that similar transformation indices are obtained using 10^6 separated lymphocytes as are obtained using whole blood, although the overall scintillation counts are lower using the latter method. We have recently found that a modification of our whole blood method employing a test blood volume containing 3×10^5 lymphocytes, a longer culture period of 162 hr and a longer 24-hr labelling pulse with methyl- H^3 thymidine increases the sensitivity of the whole blood method for the detection of specific antigen transformation, and these modifications might also have improved the transformation test results. The divergent results might suggest that the negative transformation tests reflect absence of T-lymphocyte sensitization while the positive inhibition of leucocyte migration reflects either immune complex triggered liberation of migration inhibition factor, or non-lymphokine inhibition of migration induced by antigen linkage of cells possessing membrane attached cytophilic antibody. Other studies (Assem & Turner-

Warwick, 1976) using a histamine release method to detect cytophilic antibody on peripheral leucocytes following antigen challenge, have demonstrated large amounts of cytophilic antibody in bronchopulmonary aspergillosis but negligible amounts in patients with aspergillomas. Thus if the presence of cytophilic antibody was the explanation of our positive inhibition results, they should have occurred in all of our patients with bronchopulmonary aspergillosis but in none of those with aspergilloma. In fact four out of six patients with bronchopulmonary aspergillosis gave negative results while two of the three tests on aspergilloma patients were positive. These results further suggest that the presence of cytophilic antibody may not give false positive inhibition of leucocyte migration as frequently as previously supposed.

Many other groups have observed inconsistent results when leucocyte migration and transformation methods are compared (Senyk & Hadley, 1973) and it seems likely that the conditions needed for production of migration inhibition factor may be different from those determining transformation.

The main purpose of this study was to assess whether there was a consistent difference in T-lymphocyte sensitization between the different pulmonary syndromes relating to *A. fumigatus*. From these studies clear evidence perhaps reflecting T-lymphocyte sensitization was found in six out of twelve patients, but without any obvious bias towards any particular disease.

The finding of T-lymphocyte sensitization in only four out of the nine patients with typical bronchopulmonary aspergillosis would seem sufficient evidence to confirm that T-lymphocyte sensitization is not an important feature of this disease. It is still possible that it may have a greater contribution in aspergilloma where only three patients were included. Two had positive inhibition of leucocyte migration to antigen 2 and one of these had positive lymphocyte transformation to all five batches with higher transformation indices than in any other patient. More patients must now be studied using the better culture filtrate antigens.

Since T-lymphocyte sensitization appears to be a relatively unimportant feature of *A. fumigatus* related lung diseases it becomes important to exclude the possibility of a depression of T-lymphocyte responses in these individuals.

Our findings show that normal positive delayed skin tests to *C. albicans* occurred in nine of eleven patients tested and positive skin tests to PPD were found in ten of twelve patients tested. A positive migration index correlated with the positive skin test in twelve of sixteen instances but the transformation index was only positive in six of seventeen tests. These results are consistent with those obtained with *A. fumigatus* antigen and suggest that the specific capacity of lymphocytes to transform may be impaired in some cases with aspergillus related lung disease and this feature can also be demonstrated with PHA.

Again it has to be stressed that minor modifications of *in vitro* tests can substantially alter the results. Further tests done recently in an attempt to increase the sensitivity of the transformation response by increasing the culture period and the length of thymidine pulse have increased the transformation index in some instances but have not improved the correlation with either the migration index or skin test responses. With this additional evidence it seems improbable that technical factors alone account for many of the negative transformation results found in our study.

The immunological profiles of *A. fumigatus* related lung disease can now be summarized. In aspergilloma, *A. fumigatus* specific IgG precipitating serum antibodies occur in large amounts but IgE and cytophilic IgG antibodies are rarely present

(Assem & Turner-Warwick, 1976). Evidence of T-lymphocyte sensitization to the antigen was seen in two of three cases studied. By contrast, in bronchopulmonary aspergillosis serum precipitins are more difficult to detect, but cytophilic IgE and IgG antibodies can be easily demonstrated (Assem & Turner-Warwick, 1976). T-lymphocyte sensitization was seen in only four of nine cases studied and thus seems to be unimportant in the disease. T-lymphocyte responses to other antigens causing positive reactions in most normal adults suggest that delayed hypersensitivity skin responses and inhibition of leucocyte migration is normal, but the capacity to transform may be impaired in some instances.

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