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Hypersensitivity studies in asthmatic patients with broncho-pulmonary aspergillosis

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Summary

The serum IgE levels of four patients with allergic broncho-pulmonary aspergillosis ranged from 201 to 16,800 I.U./ml. The corresponding mean level for control subjects was 205 ± 68 I.U./ml. A considerable proportion of the patients' serum IgE was found to be specific reagin directed against extracts of *Aspergillus fumigatus*. Notwithstanding the high levels of reagin, the circulating basophils were relatively insensitive to histamine release with allergen or antiserum to IgE. The effects of steroid therapy may have contributed in part towards this lack of reactivity of the basophils. Two of the patients showed *in vitro* evidence of Type IV in addition to *in vitro* Type I hypersensitivity but did not demonstrate the presence of precipitins to *A. fumigatus* in their serum, one of these previously having a positive precipitin test. Precipitins were present in the sera of the two other patients, both of whom demonstrated *in vivo* Type I hypersensitivity. One of these was tested *in vitro* for Type IV hypersensitivity but was adjudged negative. The sputum of one patient (L.P.) contained fungal mycelia which was conjugated with both IgA and IgE antibodies.

Introduction

Allergic broncho-pulmonary aspergillosis (Hinson, Moon & Plummer, 1952) is the most frequent form of pulmonary eosinophilia among Caucasians in Australia as it is in the United Kingdom (McCarthy & Pepys, 1971). While it is generally associated with asthma (Elder & Smyth, 1967) the incidence of allergic broncho-pulmonary aspergillosis among asthma sufferers in Australia is relatively rare (Munro-Ford, 1966).

The disease is characterized by episodic wheeze associated with transitory pulmonary infiltrations and peripheral blood eosinophilia. It is produced by an immunological response to *Aspergillus fumigatus* and related *Aspergillus* species, with the development of Type I hypersensitivity as evidenced by *in vivo* skin reactions. Type III skin reactions may also be demonstrated and confirmed by the presence of precipitating antibodies in serum, although precipitating antibodies are not invariably found in

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patients with this disease. Diagnosis may be further confirmed by culturing *Aspergillus* from the sputum.

Three patients whose clinical picture fulfils most of the above requirements have been reported in detail in a previous publication (Elder & Smyth, 1967). The present study concerns a more detailed immunological investigation of these and a fourth patient who, while manifesting the above features, had no history of asthma.

Materials and methods

Allergens

The allergens used for all *in vitro* experiments and for PK reactions in monkeys were 'D' strength (0·1 mg N/ml) commercial preparations obtained from the Commonwealth Serum Laboratories, Melbourne, Australia. The concentrations reported refer to the total protein and not to that of specific allergens. *Aspergillus fumigatus* extracts used for prick testing in the patients were Bencard preparations.

In vitro histamine release

In vitro allergic histamine release was determined on $3–8 \times 10^6$ leucocytes suspended in Tris ACM containing 8% homologous AB serum according to the method of May *et al.* (1970). A figure of 30% histamine release (HR) was taken as significant (Melam, Pruzansky & Patterson, 1970) and the results are reported as the minimal concentration of allergen (μg N) which induced 30% HR from the standard suspensions of leucocytes in the reaction mixture. Reverse allergic histamine release was measured as above but using dilutions of sheep anti-human IgE (W.H.O. batch number 8930) in the reaction mixture in lieu of allergen.

Lymphocyte transformation

Leucocytes were separated from 20 ml of blood and cultured *in vitro* for lymphocyte transformation experiments as reported elsewhere (Turner, Alpers & Wight, 1972) with the modification that the culture medium contained 0·035% $NaHCO_3$ plus 10% autologous serum. Antigen induced transformation was accomplished by replacing the PHA-P in the culture medium with dilutions of *Aspergillus fumigatus* extract in the range of $2·5–2·5 \times 10^{-6}$ μg N per reaction vessel. All dilutions of antigen were made in 199 instant tissue culture powder medium (Gibco).

Fluorescent antibody studies on sputum samples

One volume of sputum was mixed with two volumes of N-acetyl cysteine (Mead Johnson, U.S.A.) in 0·2 M sodium phosphate buffer, pH 7·3, and stood for 5 min at room temperature. The mixture, which was viscous but free flowing, was centrifuged at 600 *g* for 15 min and the lower third of the contents of the centrifuge which contained the majority of the cellular elements was retained. This fraction was mixed with a further aliquot of N-acetyl cysteine recentrifuged and the cell suspension harvested. Aliquots of the cell suspensions were smeared on to glass slides and air dried. The slides were gently washed in phosphate buffered saline (PBS) for 5 min and then fixed in 95% ethanol for 30 min. After fixing, the slides were stained for 1 hr with fluoresceine isothiocyanate (FITC) conjugated anti-immunoglobulin sera, washed, counter-stained with 0·1% Evans blue and rewashed. They were then mounted in PBS buffered glycerine and examined under a Reichert Zetopan fluorescence microscope.

Control slides containing sputum cells were stained with non-specific rabbit globulin conjugated with FITC while other slides were stained with Wright stain for histological examination.

Protein and immunological quantitation of sera and sputum
Fifteen millilitres of sputum were diluted with an equal volume of phosphate buffered saline (PBS) and stirred overnight at 4°C. The mixture was then clarified by centrifugation at 3500 rpm and the sediment discarded. The clear supernatant was concentrated to 5 ml by pressure dialysis and then dialysed against PBS. The total protein content of the sputum extracts and serum were determined by the Micro-Kjeldahl method and the immunoglobulins IgG, IgA and IgM by single radial immunodiffusion (Mancini, Carbonara & Heremans, 1965) using Partigen immunodiffusion plates (Behringwerke AG).

Serum IgE quantitation
Serum IgE levels were measured by the radioactive single radial diffusion method of Rowe (1969) using a final concentration of 1/2000 sheep anti-human IgE in agarose gels. The values are reported as International Units (I.U.) per ml with reference to a standard serum (code number 68/341) supplied by Dr D. Rowe (W.H.O. Reference Centre for Immunoglobulins, Lausanne). IgE levels were diluted to lie within our standard range of 93–2800 I.U./ml.

Immunodiffusion
Immunodiffusion was carried out by the method of Ouchterlony (1958) using 1% agarose (Behringwerke batch number 9254). The antigen wells contained 20 μg N/ml *Aspergillus fumigatus*.

Skin reactivities
Skin reactivities were measured directly in the patients by prick testing with Bencard allergen extracts or by PK reactions in monkeys. The results are expressed as arbitrary units ranging from 4+ to 0. Serial dilutions of allergic sera were sometimes used in order to derive a 'PK titre' which is expressed as the minimal dose of serum which would elicit a 1+ reaction in monkey skin when challenged with 'D' strength allergen extracts.

Results

Case histories
Four patients with allergic broncho-pulmonary aspergillosis were investigated in this study. The case histories of three of these, S.B., P.B. and L.P., are reported in detail elsewhere (Elder & Smyth, 1967) as cases 1, 2 and 3 respectively. All these had asthma throughout this period and at the stage of the current study, but the fourth (C.F.) showed no symptoms of asthma although he had had nasal polyps removed in 1968. The following additional clinical details relate to the period March–December 1971 during which the current series of investigations were undertaken.

S.B. showed X-ray shadowing of the lung throughout the period February–April 1971. She was given 40 mg prednisone daily during March, reducing to 30 mg daily

Table 1. Serum IgE levels in patients with allergic broncho-pulmonary aspergillosis and normal control subjects

Group	Patient	Sex	IgE (I.U./ml)
1. Allergic broncho-pulmonary aspergillosis	L.P.	M	16800
	S.B.	F	3350
	C.F.	M	560
	P.B.	F	201
2. Controls (115 subjects)		M and F	205 ± 68

during the first 2 weeks of April and to 10 mg daily by May. Thereafter the dose was gradually reduced to 4 mg daily by November 1971.

P.B. showed slight X-ray shadowing of the lung when examined in August but gave no evidence of shadowing throughout the remainder of 1971. She has been maintained constantly on a daily dose of 5 mg prednisone since April.

L.P. showed one episode of fresh shadowing in March at which stage his daily maintenance dose of 5–10 mg prednisone was increased to 35 mg but rapidly reduced to 10 mg by mid-April. Prednisone was increased again for a few days in May and late July. Since mid-August he has been maintained on a dose of 10–15 mg daily.

C.F. showed evidence of shadowing of the lung in May but has never been given steroids.

Serum IgE levels

The levels of serum IgE in the four patients are compared with the mean and standard deviation of a control group of normal subjects in Table 1. The control group con-

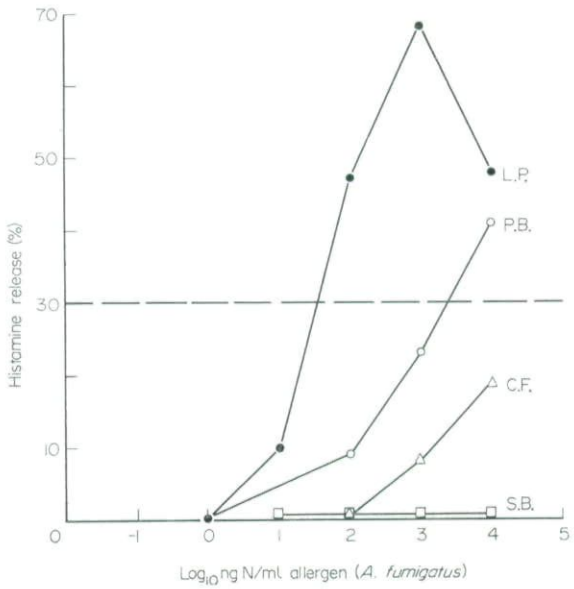

Fig. 1. *In vitro* allergic histamine release from blood leucocytes of the four patients (L.P., P.B., C.F. and S.B.) with allergic broncho-pulmonary aspergillosis. Histamine release figure is a percentage of the total cellular histamine.

sisted of 115 adults of both sexes and varying ages. Three of the four patients with allergic broncho-pulmonary aspergillosis had serum IgE levels elevated above that of the control group. The value of 16,800 I.U./ml for L.P. was in excess of the range (210–6900 I.U./ml) found in another study of a group of forty asthmatic patients with no clinical evidence of allergic broncho-pulmonary aspergillosis (Fitch, Turner & Morton, 1972).

In vitro allergic histamine release

The results of *in vitro* allergic histamine release with the four patients (L.P., P.B., C.F. and S.B.) are shown in Fig. 1. Thirty per cent histamine release occurred at an aspergillus extract concentration of 33 ng/ml for L.P. and $2{\cdot}5 \times 10^3$ ng/ml for P.B. Leucocytes from C.F. failed to release 30% of their histamine over the concentration range tested and those for S.B. did not release any histamine at all. The family of curves in Fig. 2 represent allergic histamine release from leucocytes of four patients (D.F., M.G., L.B. and D.L.) with clinical histories of hay fever resulting from exposure to rye grass (*Lolium perenne*) pollen. These four patients all yielded positive skin reactions following intradermal scratch testing with the allergen. The minimum concentration of allergen required to elicit 30% histamine release is related to the serum IgE level in Table 2 and contrasted with the comparable data obtained from the three aspergillosis patients.

In vitro reverse allergic histamine release

Peripheral leucocytes from the four patients with allergic broncho-pulmonary aspergillosis, L.P., P.B., C.F. and S.B., from the hay fever patient, D.F., and from two control subjects, K.T. and J.B., were incubated *in vitro* with dilutions of sheep antiserum to human IgE over the concentration range of 1/500 to 1/20,000. Histamine

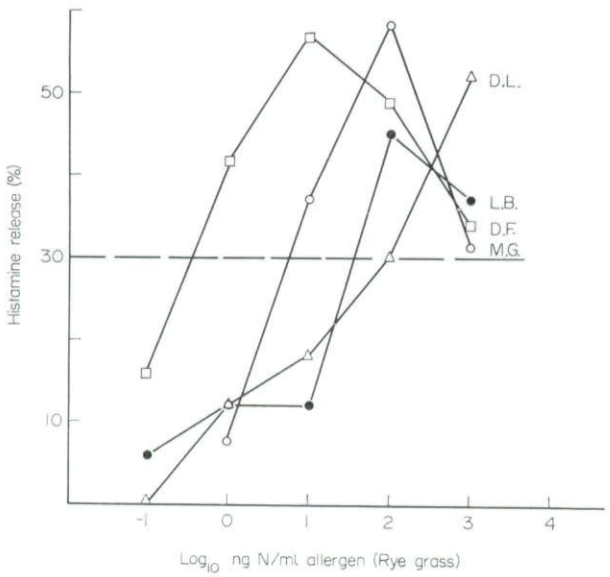

Fig. 2. *In vitro* allergic histamine release from blood leucocytes of four hay fever patients (D.L., L.B., D.F. and M.G.) with clinically defined hypersensitivity to rye grass (*Lolium perenne*) pollen.

Table 2. Allergen induced *in vitro* histamine release

Patient	Serum IgE (I.U./ml)	Allergen conc. (μg N) for 30% histamine release
A. Allergic broncho-pulmonary aspergillosis patients (allergen = *A. fumigatus* extract)		
L.P.	16800	33
P.B.	201	$2{\cdot}5 \times 10^{3}$
C.F.	557	N.A.
S.B.	3350	N.A.
B. Hay fever patients (allergen = rye grass pollen extract)		
D.F.	222	$3{\cdot}6 \times 10^{-1}$
M.G.	341	5·7
L.B.	570	36
D.L.	287	1×10^{2}

N.A. = Not attained, i.e. maximum histamine release was less than 30% of the total.

release occurred with three of the four patients with pulmonary aspergillosis (Fig. 3) but one (S.B.) failed to release histamine in duplicate experiments over the entire range of antiserum tested. Leucocytes from D.F. and the two control subjects all released histamine under these conditions. Thirty per cent histamine release was attained with leucocytes from D.F. and the two control subjects but only with leucocytes from one (C.F.) of the patients with allergic broncho-pulmonary aspergillosis (Table 3).

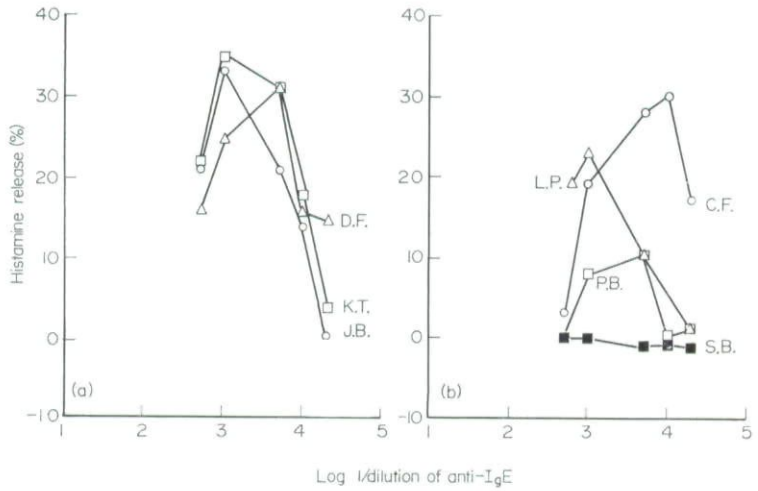

Fig. 3. *In vitro* reverse allergic histamine release in the presence of dilutions of sheep antiserum to human IgE. (a) Histamine release as percentage of total histamine from leucocytes of controls (K.T. and J.B.) and a hay-fever patient (D.F.). (b) Corresponding data from the four patients (L.P., P.B., C.F. and S.B.) with allergic broncho-pulmonary aspergillosis.

Table 3. Reverse allergic histamine release with sheep anti-IgE

Subject	Condition	Dilution of anti-IgE required for 30% histamine release
L.P.	Pulmonary aspergillosis	N.A.
S.B.	Pulmonary aspergillosis	N.A.
C.F.	Pulmonary aspergillosis	1:10000
D.F.	Hay fever	1:5100
K.T.	Control	1:5100
J.H.	Control	1:9600
J.B.	Control	1:1500

N.A. = Not attained, i.e. maximum histamine release was less than 30% of the total.

PK reactions in monkey skin

Skin reactivities in monkeys (Table 4) showed a correlation for the aspergillosis patients between serum IgE levels and PK titre defined as the reciprocal of the limiting dilution of serum which yielded a 1+ reaction following challenge with 'D' strength allergen (0·1 μg N/ml). This correlation is shown graphically in Fig. 4 where the plot of $\log_{10}$ PK titre *v.* $\log_{10}$ serum IgE intercepts the ordinate at the value of the mean IgE level for normal individuals. The PK titres for two normal subjects are also shown in Table 4. Of particular interest is the fact that the serum of D.F., whose circulating leucocytes were particularly sensitive to allergic histamine release with rye grass allergen, gave a PK titre of 100. This contrasts with the corresponding data from L.P. whose serum demonstrated a high PK titre but whose leucocytes were relatively insensitive to allergic histamine release. The high PK titre of L.P.'s serum suggests that a considerable proportion of the serum IgE is specific reagin to aspergillus. Specificity is also indicated by the low PK titres of L.P.'s serum to rye and couch

Table 4. Monkey skin PK titres

Patient	Allergen	PK titre*	Serum IgE (I.U./ml)
L.P.	*Aspergillus fumigatus*	2000	16800
S.B.	*Aspergillus fumigatus*	100	3350
C.F.	*Aspergillus fumigatus*	10	560
P.B.	*Aspergillus fumigatus*	1	201
Control 1	*Aspergillus fumigatus*	0	260
Control 2	*Aspergillus fumigatus*	2	218
D.F.	Rye grass	100	222
L.P.	Rye grass	2	16800
L.P.	Couch grass	1	16800

* Reciprocal dilution of sensitizing sera to yield 1+ skin reaction upon challenge with 'D' strength (0·1 μg N/ml) allergen.

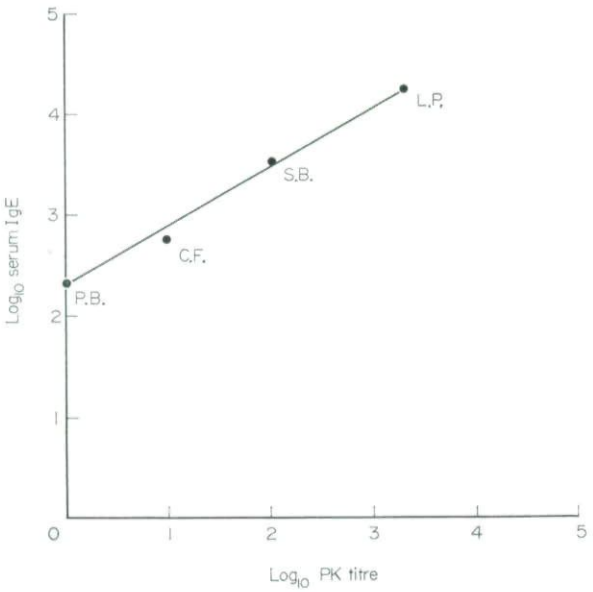

Fig. 4. Relationship between serum IgE and 'PK titre' of sera from the four patients (L.P., S.B., C.F. and P.B.) with allergic broncho-pulmonary aspergillosis.

grass pollens, two allergens which are of major importance in pollinosis in Western Australia.

Lymphocyte transformation

Peripheral lymphocytes from pulmonary aspergillosis patients, L.P., P.B. and C.F., when cultured *in vitro* with 0·1 ml or 0·05 ml phytohaemagglutinin (PHA-P Difco Bacto) per reaction vessel, incorporated ^{3}H-thymidine to an extent comparable with that of normal lymphocytes. In addition the lymphocytes of L.P. and P.B. demonstrated increased uptake of ^{3}H-thymidine when exposed to an extract of *Aspergillus fumigatus* (C.S.L.) over the dose range of $2{\cdot}5$–$2{\cdot}5 \times 10^{-6}$ μg N per reaction vessel. Maximum stimulation ratios (cpm stimulated : cpm unstimulated cultures) occurred at $2{\cdot}5 \times 10^{-4}$ μg N aspergillus extract per reaction vessel for L.P. (stimulation ratio of 1·5) and at $2{\cdot}5 \times 10^{-1}$ μg N aspergillus extract for P.B. (stimulation ratio of 8·6). By contrast the lymphocytes from C.F. failed to demonstrate any increased uptake of ^{3}H-thymidine over the entire concentration range of aspergillus extract used.

Serum precipitating antibodies

Precipitating antibodies to *Aspergillus fumigatus* extract were detected by immunodiffusion in agar gels in the sera of C.F. and S.B. but not in the sera of P.B. and L.P. The serum of L.P., however, contained precipitating antibodies to *Aspergillus fumigatus* when tested in 1967 (Elder & Smyth, 1967). Precipitin bands were detected optimally at a concentration of 20 μg N/ml of aspergillus extract.

Protein levels in sputum and serum

Table 5 reports the total protein concentration, and that of albumin and the immunoglobulins IgA, IgG and IgM in the sputum of L.P. IgE was below the threshold of

Table 5. Protein levels in sputum and serum of patient L.P.

Protein	Sputum		Serum	
	Concentration (μg/ml)	% of total protein	Concentration (mg/ml)	% of total protein
Total protein	2700	100	74	100
Albumin	130	4·9	45	60
IgA	230	8·6	3·2	4·3
IgG	21	0·8	13	18
IgM	1·8	0·1	0·87	1·2
IgE	*	*	0·016	

Sample	Albumin/IgA	Albumin/IgG	Albumin/IgM	Albumin/IgE
Sputum	0·57	6·1	70	—
Serum	14	3·4	52	870

* Insufficient for quantitation.

quantitation in the sputum sample. The results are calculated as concentrations per ml of the original sputum sample. Table 5 also reports the corresponding protein concentration in L.P.'s serum and the ratios of albumin/immunoglobulin in both serum and sputum. Albumin plus the immunoglobulins in sputum account for 15% of the total protein but in serum represent 84% of the total.

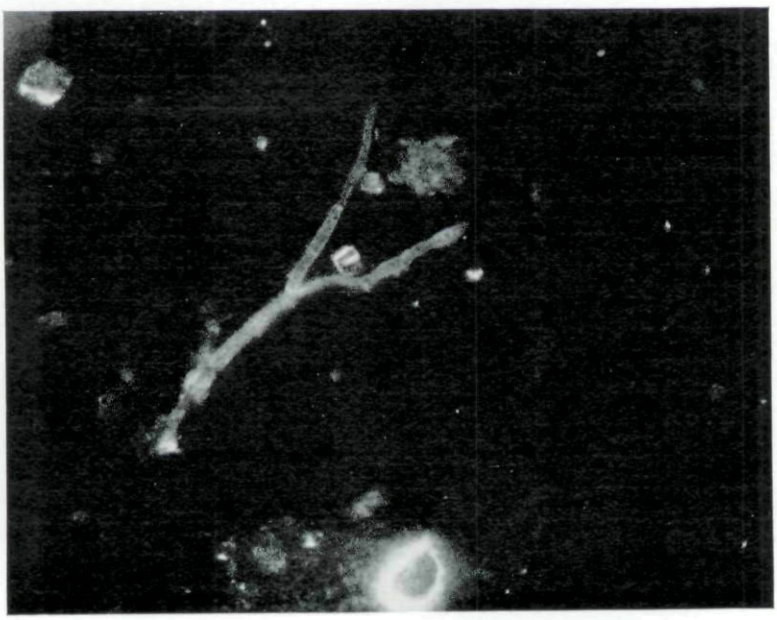

Fig. 5. Fungal mycelia in the sputum of patient L.P. (allergic broncho-pulmonary aspergillosis) fluorescing with FITC conjugated rabbit anti-human IgE. Similar fluorescence occurred with FITC conjugated anti-IgA but not with anti-IgG or anti-IgM.

Fluorescent antibody studies

The sputum of patient L.P. contained polymorphonuclear cells and epithelial cells but was devoid of lymphoid elements. Mycelia were present in all samples examined throughout the course of the investigation. Both *Candida albicans* and *Aspergillus* were grown from the sputum of L.P. Fluorescent antibody studies showed significant amounts of IgE and IgA, some of which was associated with polymorphonuclear cells. Only trace amounts of IgG were detected but IgM was absent. Mycelia present in the sputum were coated with both IgE and IgA (Fig. 5) but not IgG or IgM.

Discussion

Intradermal challenge with *A. fumigatus* elicits in patients with allergic broncho-pulmonary aspergillosis an immediate weal and flare (Type I) response followed at times by an Arthus (Type III) reaction. This sequence of events is characteristic of the disease (McCarthy & Pepys, 1971; Goldstein & Yokoyama, 1970). In the present study lymphocyte transformation experiments with leucocytes from C.F. gave clear evidence of Type IV hypersensitivity which was also indicated to a lesser extent by leucocytes from L.P. The leucocytes from C.F., however, in the presence of *A. fumigatus* extract failed to incorporate ^{3}H-thymidine above the level of non-stimulated cultures. Clearly, therefore, Type IV reactions may also be implicated in some cases of allergic broncho-pulmonary aspergillosis.

Three (L.P., S.B. and C.F.) of the four patients with allergic broncho-pulmonary aspergillosis who are reported in this study had serum IgE levels significantly elevated above the normal value, while the fourth (P.B.) was well within the normal range. The serum IgE of L.P. was approximately three times the upper range of that found for a group of forty asthmatic patients with no clinical history of allergic broncho-pulmonary aspergillosis. The serum IgE levels of the other three patients, however, all fell within the range for asthmatics. All four patients produced typical Type I weal and flare reactions when challenged with *Aspergillus fumigatus* extracts. Skin sensitizing activity in man is generally associated with the IgE class of immunoglobulins (Ishizaka & Ishizaka, 1967) which have been identified as the serum reagins in allergic broncho-pulmonary aspergillosis (Goldstein & Yokoyama, 1970). Monkey skin reactions showed a linear correlation between PCA titre and the level of serum IgE suggesting that in the present group of patients the reagins directed against *Aspergillus fumigatus* belong to the IgE class. Furthermore, they indicate that a high proportion of the serum IgE is specific reagin to aspergillus. This is supported by the fact that the serum of L.P. in particular gave skin reactions within the normal range in monkeys challenged with rye and couch grass pollens. The latter are two of the major pollens associated with seasonal pollinosis in Western Australia.

The sputum of L.P. contained the major immunoglobulins in addition to albumin but combined these accounted for only 19% of the total sputum proteins. IgA was the major immunoglobulin present and the ratio of albumin/IgA in sputum was 25 times less than the corresponding ratio in serum. This trend has been taken to indicate that IgA may be synthesized locally in the respiratory mucosa (Ishizaka & Newcomb, 1970; Hobday, Cake & Turner, 1971). The serum IgE levels of a group of five Singapore patients with pulmonary eosinophilia and associated parasitic infestation were grossly (from 20 to 200 fold) elevated above the normal range (K. J. Turner & M. R. Simons, unpublished observations, 1971). This, together with the above data, suggest that the respiratory mucosa may be a major source of synthesis of both exocrine and

serum IgE. Both *Aspergillus fumigatus* and *Candida* were grown from the sputum of L.P. and fluorescent antibody studies showed the presence of mycelia conjugated with IgA and IgE but not with IgG or IgM. Notwithstanding the presence of antibodies in sputum with specificity towards the mycelia, we were unable in the present study to demonstrate precipitating antibodies to *Aspergillus* in the serum of L.P. This substantiates the claim for local synthesis of IgA and IgE in the respiratory mucosa.

Circulating basophils from only one (L.P.) of the four patients gave any appreciable allergic histamine release when challenged *in vitro* with aspergillus extracts. The magnitude of histamine release, however, was less than could be anticipated in relation to the high levels of specific IgE as indicated by PK reactivities in monkey skin. Reverse allergic histamine release with anti-IgE again indicated that the basophils were relatively insensitive to challenge, notwithstanding normal intracellular levels of histamine. The results were more striking with leucocytes from S.B. which failed to release histamine upon challenge with either *A. fumigatus* or anti-IgE. By contrast, the leucocytes from C.F. released normal levels of histamine upon challenge *in vitro* with anti-IgE but minimal release occurred with *A. fumigatus* extracts. The observation that allergen–IgE antibody reactions and anti-IgE–IgE reactions on the surface of basophils do not necessarily lead to histamine release has also been reported by Ishizaka, Tomioka & Ishizaka (1970) and Lichtenstein, Levy & Ishizaka (1970). The release of histamine is controlled by biochemical factors in a series of reactions following antigen–antibody union on the surface of the target cell. Any alteration in the biochemical mechanisms of allergic histamine release might therefore produce changes in cell reactivity which are not antigen specific. Ishizaka *et al.* (1971) have shown that agents capable of increasing intracellular levels of cyclic adenosine 3′,5′ monophosphate inhibit *in vitro* both antigen induced and reversed type histamine release. Catecholamines also inhibit both reactions without preventing antigen–antibody union (Lichtenstein & Margolis, 1968). Corticosteroids can change the permeability of cell membranes thus reducing the ability of histamine to leave the cell (Dockhorn, 1971) but the protective role of corticosteroids *in vivo* is still not clear. Booij-Noord, Orie & de Vries (1971) reported that prednisolone failed to give any significant protective effect on the immediate bronchial obstruction reaction to inhalation of house dust but had some protective effect on late obstructive reactions. Corticosteroids may have contributed towards the apparent loss in cell reactivity towards allergic histamine release in the present study since L.P., P.B. and S.B. received from 4 to 10 mg prednisone daily at the stage when *in vitro* histamine release experiments were undertaken. Kuman, Newcomb & Hornbrook (1971) found that the level of serum IgE was depressed in children after long-term steroid therapy and suggested that steroid therapy inhibits IgE synthesis. Similar results have been obtained in rats by Taniguchi & Tada (1971). It is doubtful whether steroid therapy inhibited IgE synthesis in the present study, however, as L.P. was receiving 25 mg prednisone daily at the stage when his serum IgE was 16,800 I.U./ml and S.B. 22 mg daily when her serum IgE was 3350 I.U./ml. Clearly, further experimentation will be necessary in order to elucidate the potential role of corticosteroids in IgE synthesis and associated histamine release.

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