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Lymphocyte sensitization to *Aspergillus fumigatus* in allergic bronchopulmonary aspergillosis

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SUMMARY

Peripheral blood mononuclear cell (PBMC) proliferation induced by an extract of *Aspergillus fumigatus* (AF) was examined in patients with allergic bronchopulmonary aspergillosis (ABPA), all of whom had an immediate skin prick test reaction (SPT) and increased RAST binding to AF, and, for comparison, in individuals without immediate SPT reactivity or increased RAST binding to AF. The proliferative responses of PBMC from the ABPA patients were greater than those from the comparison donors. A substantial proportion of the comparison group, however, showed evidence of a specific immune response to AF, with AF-specific IgG measured by ELISA and specific lymphoproliferative responses. AF-responsive T cell lines and T cell clones were established from both ABPA patients and IgE-negative individuals. These clones, of helper/inducer ($CD4^+$) phenotype, showed antigenic specificity and MHC restriction. The stimulating antigen was determined for four of six clones derived from a skin-prick-test-negative individual, and found to be of Mr 18 kD, possibly the major allergen, 'Ag 3'. ABPA patients showed a marked diminution of the proliferative response during disease exacerbation.

Keywords *A. fumigatus* T lymphocyte allergens APBA T cell clones

INTRODUCTION

Aspergillus species are widespread in nature and inhalation of their spores is inevitable. Allergic bronchopulmonary aspergillosis (ABPA), the most common of the allergic bronchopulmonary mycoses, is characterized by a peripheral blood eosinophilia with pulmonary shadowing on chest radiograph and by the development of an immune response to antigens of *Aspergillus fumigatus* (AF). Specific IgE, IgG, IgA and IgM antibodies have been documented (Greenburger, 1984; Ricketti *et al.*, 1984). Animal studies suggest that both IgE and IgG antibodies are implicated in the development of the pulmonary lesions (Slavin *et al.*, 1978).

Previous studies of T lymphocyte responsiveness to AF have found that AF provoked a proliferative response in only a proportion of patients studied (Haslam *et al.*, 1976; Rosenberg *et al.*, 1977; Forman *et al.*, 1978; Goldstein, 1978). The stimulation index was apparently unrelated to disease state (Forman *et al.*, 1978) and to corticosteroid treatment, although the number of patients and controls studied was small. Proliferation has not been reported other than in occasional control individuals.

The pulmonary lesions in ABPA show a dense infiltrate of mononuclear leucocytes as well as eosinophils (Ricketti *et al.*, 1984). The demonstrations that the T lymphocyte product, IL-5, induces proliferation and activation of eosinophils (Lopez *et al.*, 1988) and that eosinophil generation of inflammatory mediators can be triggered by surface bound AF-IgG complexes (Cromwell *et al.*, 1988) led us to investigate the *in vitro* T lymphocyte proliferative responses to AF from ABPA patients and from a comparison group in whom AF extract did not elicit an immediate SPT (AF-IgE negative), and to establish long-term cutures, both T cell lines and clones, from ABPA patients for further functional studies, in particular of specificity and lymphokine production. We also sought to establish AF-responsive T cell lines and clones from AF-IgE negative individuals.

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MATERIALS AND METHODS

Patients

Twenty-one patients attending an out-patient clinic with ABPA were studied (11 male, 10 female; median age 49, range 20–76 years). All had been previously diagnosed by conventional clinical criteria i.e. all patients had previously manifested at least six, but usually seven or eight of the following: asthma, immediate SPT to AF, precipitating antibodies to AF, elevated total serum IgE, history of infiltrates on chest radiograph, blood eosinophilia, central bronchiectasis and elevated IgE and IgG antibodies to AF in serum (Greenberger, 1984; Rosenberg *et al.*,

1977). The diagnostic criteria were reviewed and confirmed, and the disease activity assessed by the clinic physician at the time of study.

The patients were classified into four groups according to their disease activity.

Group 1 comprised 12 patients (15 visits) who had been stable and well for at least 3, and usually more than 6 months prior to the clinic visit. Nine (11 visits) were taking no oral corticosteroid treatment. The remaining three were taking 2·5, 5 and 6 mg/day prednisolone. Group 2 comprised four patients (four visits) and these were seen during a disease exacerbation, defined by infiltrates on the chest radiograph and circulating eosinophilia. None of these patients was taking oral corticosteroid treatment. Group 3 consisted of three patients (four visits) who were seen 2–6 weeks after the commencement of oral corticosteroid treatment (15–30 mg/day prednisolone) for an exacerbation of disease. Group 4 comprised eight patients (10 visits) with increased severity of respiratory symptoms consistent with an exacerbation of asthma or a chest infection, but in whom it was not possible to document a definite exacerbation of ABPA. Several of these patients had commenced corticosteroid and antibiotic therapy shortly before their clinic visits. In others, infiltrates were not seen on the chest radiograph or a chest radiograph was not available. Five patients (six visits) were on oral corticosteroid treatment (5–30 mg prednisolone) when seen.

The median age of the ABPA patients was 47 years, with no significant differences between the four groups. Original diagnostic criteria were no different between the groups.

Eleven patients were seen on more than one occasion; in five disease activity altered between visits. In the comparisons between disease groups such patients have been included in both relevant groups, while for comparison with the AF-IgE negative group only the first proliferative response from each patient has been used in analysis.

The comparison group comprised 23 individuals; 10 were out-patients at the chest clinic and 13 were hospital staff. All these individuals had a negative SPT to AF; 13 were atopic, with a positive SPT to one or more of three common allergens; house dust mite, grass pollen and cat fur. Four were asthmatic. The median age was 37 years, (range 22–65) years. None was taking oral corticosteroid therapy.

Antigens

A. fumigatus (AF) antigen was a culture filtrate extract from a long-term (4–5 week) stationary culture on an asparagine synthetic medium, at 25°C. The culture filtrate was separated from the mycelial mat, filtered, dialysed, and freeze-dried. When characterized by crossed immunoelectrophoresis, using hyperimmune rabbit antiserum as previously described (Longbottom & Austwick, 1986), 25–30 precipitin peaks were visible. The AF preparation was free of mitogenic activity since it did not induce proliferation of T cell clones specific for other allergens, but which were otherwise sensitive to the mitogenic effects of phytohaemagglutinin (PHA) and Concanavalin A (Con A). Moreover, as shown in the Results, AF-induced proliferation was self-MHC-restricted. *Dermatophagoides farinae* and grass pollen antigens were partially purified, biologically standardized, freeze-dried extracts for immunotherapy (Pharmalgen) from Pharmacia, Sweden. *Alternaria alternata* and *Cladosporum herbarum* extracts were obtained from Dome/Hollister-Stier (a division of Miles Laboratories Limited, Slough, UK).

Lymphocyte preparation

Peripheral blood mononuclear cells (PBMC) were obtained from venous blood samples, after removal of the plasma by centrifugation (1100 ***g*** for 15 min). They were separated by density gradient centrifugation over Ficoll-Paque (1100 ***g*** for 20 min; Pharmacia, Bucks., UK) and washed three times in RPMI 1640 (Flow Laboratories, Herts., UK) prior to use.

Proliferation assays

Proliferative assays were performed in 96-well, flat-bottomed microtitre plates (Flow Laboratories), in medium alone and in the presence of a range of concentrations of soluble AF antigen. PBMC (2×10^5/ vell) were cultured in RPMI supplemented with glutamine (2×10^{-5} M), penicillin (50 u/ml), streptomycin (50 μg/ml) (Flow Laboratories) and 10% autologous plasma, for 7 days, (shown in preliminary experiments to be optimal), incubated at 37°C, in a humidified atmosphere containing 5% CO_2. The cultures were pulsed with 0·7 μCi of tritiated thymidine (TRK61; Radiochemicals Inc, Amersham, Bucks., UK) for the final 18 h of culture, harvested onto glass filter paper and counted on a beta scintillation counter (LKB 1217 Rack Beta). Results have been expressed as incremental counts per minute (inc ct/min) i.e. ct/min (antigen stimulated) − ct/min (medium alone), and as a stimulation index (SI), i.e. ct/min (antigen stimulated)/ct/min (medium alone).

Proliferation of T cell lines and clones to soluble antigen was usually performed with 1×10^4 responder cells with 3×10^4 autologous irradiated (3000 rad) PBMC as antigen presenting cells (APC), for 72 h in U-bottomed microtitre plates. For particulate antigen, $3–10 \times 10^4$ responding T cells were cultured with $1–3 \times 10^5$ APC. In some experiments APC were omitted, or allogeneic PBMC were used. All proliferative assays were performed in triplicate.

Preparation of antigen-reactive T cell lines and clones

Antigen-reactive T cell lines and clones were established essentially as described by Lamb *et al.* (1982). Lymphoblasts recovered over Ficoll-Paque following 7 days culture were mass cultured as a cell line, or cloned using limiting dilution, at 0·3 cells/well in Terasaki plates (Miles Scientific Laboratories) in the presence of autologous APC, AF, 10% autologous plasma and a source of interleukin 2 (Lymphocult T, Biotest Folex, Birmingham, UK). They were maintained and expanded by feeding weekly with autologous APC and antigen, and twice weekly with IL-2. Subsequent proliferative assays were performed on cells separated over Ficoll-Paque 8 days after the addition of APC.

Clones were phenotyped with anti-CD3 (UCH-T1), anti-CD4 (T4-407) and anti-CD8 (UCH-T4) monoclonal antibodies (Oxoid) on a FACS 420 (Becton Dickinson).

SDS-polyacrylamide gel electrophoresis and preparation of immunoblots

SDS-PAGE and electrophoretic transfer to nitrocellulose were performed exactly as described previously by Tee, Gordon &

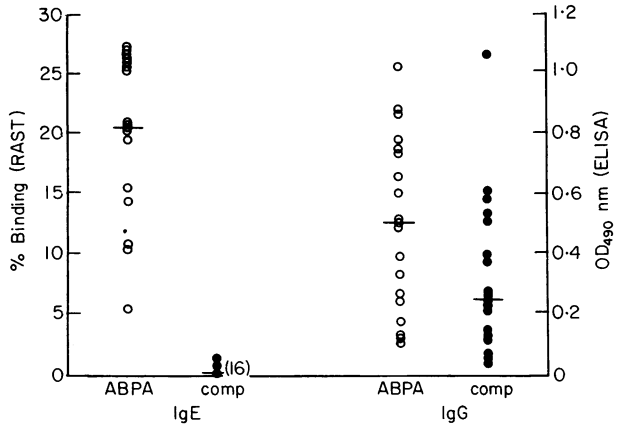

Fig. 1. Serum *A. fumigatus*-specific IgE (RAST binding, neat serum) and IgG (1/800 serum, buffer background of 0·01 substracted) in the ABPA and comparison groups.

Newman Taylor (1987). Blots were washed with 0·3% Tween-20 in PBS, and stained overnight with Auro dye (Janssen Life Sciences Products, Oxon, UK).

Preparation of nitrocellulose particle suspensions

Protein bands were cut from the stained nitrocellulose, dissolved in dimethylsulphoxide (DMSO; Sigma Chemical Co Ltd., Dorset) for 2 h, re-precipitated with carbonate/bicarbonate buffer to form a fine suspension of particles, and washed in RPMI as described by Abou-Zeid *et al.* (1987). Samples were stored at −20°C prior to use.

IgG enzyme-linked immunosorbent assay

One-hundred microlitres AF extract (5 μg/ml in PBS) were incubated overnight (4°C) in a microtitre plate (m 129B Dynatech laboratories), then washed and blocked (1 h, 37°C) with casein buffer at pH 7·6 (Kenna, Major & Williams, 1985). Samples, diluted in casein buffer, were incubated (2 h, 37°C), then the plates were washed four times and incubated with a horseradish-peroxidase-labelled sheep anti-human IgG (Amersham, UK) (1/750, 2 h, 37°C). Ortho-phenyldiamine (OPD;100 μl) was added (15 min at room temperature) then reaction stopped with 50 μl of 2 M H_2SO_4. Optical density was read at 490 nm (MR 700 Dynatech, Sussex UK). ABPA and comparison group sera were run in parallel, together with positive and negative control sera to give consistent interassay values. Inhibition studies confirmed the specificity of antibody binding.

Radio allergo sorbent test (RAST)

The RAST assay and coupling of pre-activated discs with AF extract were performed as described by Tee *et al.*, (1987). Briefly, neat test sera, positive and negative control sera, were incubated overnight with the discs, washed and incubated overnight with 50 μl of 125 I-anti-IgE (Phadebas RAST isotope, Pharmacia). Bound radioactivity was determined using a gamma counter. Results are expressed as percentage binding i.e. ct/min bound/ct/min total × 100.

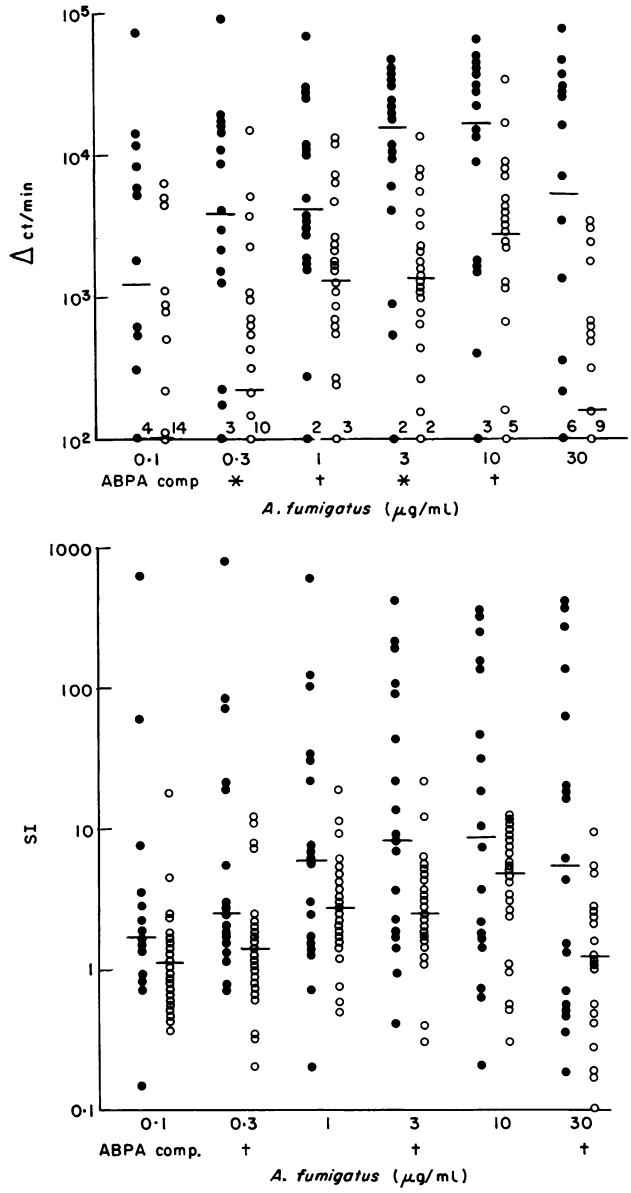

Fig. 2. Proliferative responses from ABPA patients (closed circles) and AF skin-test-negative donors (open circles) were measured in a 7-day proliferation assay, with a range of AF concentrations. Incremental ct/min (Panel a) or stimulation index (Panel b) are shown for each individual. The median value is indicated by the line. $*P<0·005$ $\dagger P<0·05$.

Statistical analysis

A Mann–Whitney two-sample rank procedure was used to compare proliferative responses and antibody titres between the ABPA and comparison groups, and between the different ABPA health groups. Regression analysis was used to compare proliferative responses and antibody titres for the individuals within the ABPA and comparison groups. Fisher's exact test

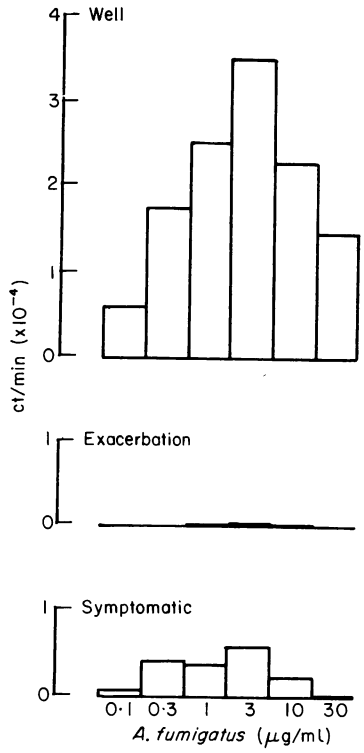

Fig. 3. Median values for PBMC proliferative responses (incremental ct/min) of the ABPA patients, grouped by disease activity, as defined in Materials and Methods, for each stimulating antigen concentration.

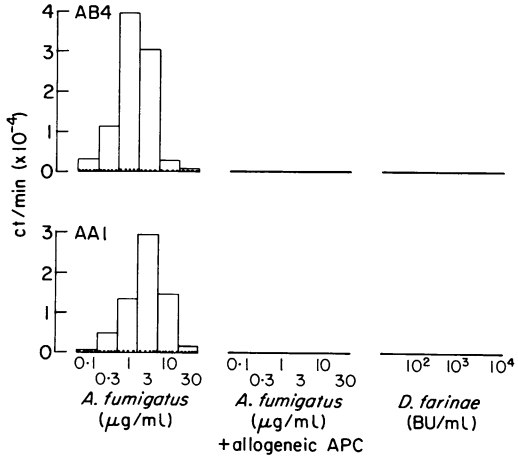

Fig. 4. Mean proliferation (ct/min) for two clones of helper/inducer phenotype; AB4 derived from an AF-IgE-negative donor and AA1 from an ABPA patient. Thymidine incorporation induced by AF stimulation of clones in the presence (——) or absence (---) of autologous APC or in the presence of allogeneic APC (central panel) and by stimulation with *D. farinae*, in the presence of autologous APC (right panels) is shown. Assays were performed in triplicate and the mean standard errors were 5%.

was used to compare the stimulation index values between the ABPA and comparison groups. Significance was taken as $P<0.05$.

RESULTS

Figure 1 shows the AF-specific IgE (RAST percentage binding) and IgG (ELISA OD) titres in the serum of patient ($n=19$) and comparison ($n=18$) groups. Specific IgE was detected in the serum of all ABPA patients, and, at much lower concentrations, in two of the comparison individuals. The difference in RAST binding between the groups was highly significant ($P<0.001$). In contrast, AF-specific IgG was detectable in both ABPA and comparison individuals with the difference between the groups not reaching statistical significance ($0.05<P<0.1$).

Incremental proliferative responses for both ABPA patients and comparison individuals, stimulated by concentrations of AF, from 0·1–30 μg/ml are shown in Fig. 2a. While individual responses were dose-dependent in both groups, the range of responses by individuals in both groups was large. Unstimulated thymidine incorporation was similar in both groups. At each stimulating antigen concentration, however, the median incremental proliferation was higher and the range wider in the ABPA group than the comparison group, the difference being significant ($P<0.05$) from 0·3–10 μg/ml inclusive. Peak responses for the ABPA and comparison group individuals, as a percentage of each group were most commonly at 3 μg/ml (41%; 32%) and 10 μg/ml (29%; 47%) respectively. A diminished response to the highest stimulating concentration (30 μg/ml) was seen in most of the ABPA patients, and in all comparison individuals.

Within the comparison group, the atopic individuals showed significantly higher proliferative responses than the non-atopic individuals in the absence of antigen, and at the single stimulating AF concentration of 10 μg/ml ($P<0.05$). Figure 2b shows the results expressed as a stimulation index (SI). Responses were dose-dependent, and again, the median SI values of the ABPA patients were higher at all antigen concentrations, significantly at the 0·3, 3 and 30 μg/ml stimulating antigen concentrations. The number of ABPA patients and comparison donors with an SI of greater than 2·5 were, however, not significantly different at any stimulating antigen concentration. Three of the ABPA patients and five of the comparison group failed to reach a stimulation index of 2·5 at any stimulating antigen concentration. The proliferative response did not correlate with serum IgE or IgG titres in either the ABPA or comparison group and was not related to corticosteroid treatment, age or length of disease in the ABPA group.

The relationship of disease activity to the proliferative responses for the ABPA patients is shown in Fig. 3. The median values of proliferative responses were higher for the 'well' patients (Group 1 upper panel) than for all patients who were recently or currently ill (Groups 2, 3 and 4 combined) at all concentrations tested, significantly so at 0·3, 1 and 3 μg/ml. For patients with untreated exacerbation (Group 2 middle panel) and symptomatic illness (Group 4 lower panel), although the numbers are small, responses were significantly lower than for the 'well' group at the same concentrations ($P<0.05$). Two individuals were studied during the treatment of an exacerbation: both showed higher responses following 2–4 weeks of treatment than in exacerbation. Two individuals initially stu-

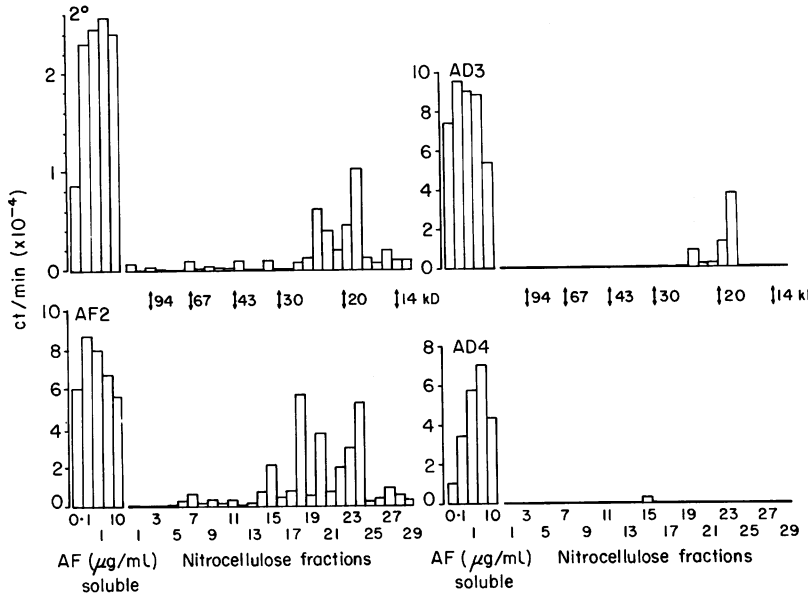

Fig. 5. Incremental proliferation (ct/min) to soluble AF and to fractions separated on the basis of molecular weight (marks shown) of primed lymphocytes (2°, upper left), a T cell line (AF2, lower left), and two typical T cell clones (AD3 upper and AD4 lower right), all derived from an AF-IgE-negative donor.

died when well also showed marked falls in proliferative responses during the subsequent disease exacerbation.

T cell clones were established using AF as stimulating antigen, from both skin-test-positive and negative individuals. All 12 AF-responsive clones characterized functionally showed proliferative responses to the stimulating soluble antigen, and were of helper/inducer phenotype ($CD3^+$, $CD4^+CD8^-$). Figure 4 shows the antigen responses of two representative clones. Both clones AB4 (derived from an AF-IgE negative donor) and AA1 (derived from an ABPA patient) showed dose-dependent proliferative responses to AF, peaking at 1 μg/ml and 3 μg/ml respectively, only in the presence of autologous APC. Neither clone responded to AF alone or in the presence of allogeneic APC or to *D. farinae*. In further experiments (data not shown), neither clone responded to *A. alternata*, *C. herbarum* or grass pollen over a wide concentration range.

The response to nitrocellulose particles of cells re-stimulated after 10 days in primary culture, a T cell line and two T cell clones derived from an AF-IgE-negative donor are shown in Fig. 5. Both the primed lymphocytes and the T cell line AF2 respond to several fractions including those prepared from antigens of Mr 18–19 kD, 24 kD, 28 kD and 33–35 kD. The clone AD3 responded predominantly to the antigen of Mr 18–19 kD. A response was also detectable to the 24 kD fraction. Clone AD4, while responding to soluble antigen, showed only a small response to one fraction (Mr 33–35 kD). The cell lines and clones did not respond to particles in the abscence of autologous APC or in the presence of allogeneic APC. Maximal responses to soluble AF were at 0·3 μg/ml for the cell line and clone AD3, and at 3 μg/ml for the secondary response and clone AD4. Neither of these clones showed a response to soluble *D. farinae* or to *D. farinae* nitrocellulose particles.

The patterns of response of these, and other, clones remained consistent with different batches of nitrocellulose particles. Dose responses were seen with different concentrations of fractions of particular molecular weight, while fractions not stimulating a proliferative response failed to do so when tested over a wide dose range.

DISCUSSION

This study shows that the magnitude of the proliferative response to AF antigens in ABPA patients is dependent on the state of the disease at the time of study, with response in the circulation being largely undetectable during disease exacerbation. We also document for the first time that the circulating cells from a majority of normal subjects also proliferate, although to a lesser extent, to AF antigens.

AF-specific IgE antibodies were demonstrated in all the ABPA patients by both immediate skin reactivity and elevated RAST binding. Specific IgG antibodies were demonstrable by ELISA in most patients, and in some of the AF-IgE negative donors (Fig. 1), as has previously been reported by some, but not all, investigators (Forman *et al*, 1978; Kurup *et al.*, 1986). Disease-related specificity of IgG binding with protein, but not polysaccharide AF fractions was reported by Sepulveda, Longbottom & Pepys (1979), an observation which may suggest that antigenic differences may, in part, account for the differing results. The antigen used in the present investigation was unfractionated, containing both protein and polysaccharide moieties.

AF-specific T cell sensitization has been reported by several groups in a variable proportion of relatively small numbers of patients with ABPA, but although *Aspergillus* spores are widespread, T cell sensitization has not previously been detected other than in occasional control individuals (Goldstein, 1978; Rosenberg *et al.*, 1977; Haslam *et al.*, 1979). In this study circulating PBMC from 15 of 18 patients with ABPA, and from 18 of 23 AF-IgE-negative donors showed dose-dependent proliferation with AF (Fig. 2).

The range of individual responses was wide in each group, the ABPA responses being significantly greater over the concentration range 0·3–10 μg/ml (Fig. 2). The high frequency of response in both groups may have resulted, in part, from using a wide concentration range of an AF extract which had been validated by crossed immunoelectrophoresis.

Standardized or recombinant antigens are not yet available and extracts show considerable variation in their individual antigenic content (Kurup *et al.*, 1986; Longbottom & Austwick 1986). Forman *et al.* (1978) detected stimulation at concentrations of 1–50 μg/ml with 'toxicity' at 100 μg/ml.

The AF extract was not mitogenic since it did not induce proliferation of either T cell clones of other specificities or of AF-specific clones in the absence of APC, or in the presence of allogeneic APC (Fig. 4). Clones did respond to the mitogens PHA and Con A. No correlation of proliferative response with specific antibody titre was detected in either group.

The magnitude of PBMC proliferative responses stimulated by other common inhalant allergens in non-allergic individuals is controversial. Some groups (Buckley *et al.*, 1977; Halvorsten, Bosnes & Thorsby, 1986) working with ragweed AgE and *D. farinae* respectively have found responses of equal magnitude in non-allergic individuals while others (Rawle, Mitchell & Platts-Mills 1984), using DerP1, have found greater responses among IgE-producing individuals. Some of the published differences may be attributable to the use of widely different stimulating antigen concentrations (Rocklin, 1979).

Phillips *et al.* (1987) recently reported that they were unable to induce ragweed-AgE-responsive T cell lines or clones from non-allergic individuals, but did not state whether the non-allergic individuals showed detectable AgE-specific IgG, or provide other evidence of immune recognition of this antigen.

Disease activity was found to influence proliferative response (Fig. 3). A marked diminution of response was noted in patients with a documented disease exacerbation with pulmonary eosinophilia, which is effectively a marker of specific response to AF in the lungs. A less marked, although still significant, diminution was found in patients with increased symptoms, but no definite disease exacerbation (Fig. 3).

Reduced antigen-specific proliferation response might also be due to immunological tolerance, induced by exposure to high antigen concentrations (Lamb *et al.*, 1987), but since the lung, rather than the blood, is the major site of interaction of AF with host cells, increased disease activity may be associated with accumulation of the AF-specific T lymphocytes in the lung, with reduced representation in the circulation. Of interest Rossi, Balbi & Manca (1987) demonstrated PPD-reactive T lymphocytes in the pleural spaces in tuberculous pleurisy whilst the peripheral blood T cells were not reactive.

The specificity of a number of our T cells clones was investigated further (Fig. 5). Of the six AF-responsive clones derived from the AF-IgE-negative donor represented in Fig. 4, four were of the pattern exemplified by AD3, possibly suggesting the presence of a dominant T cell epitope for this individual on the 18–19 kD antigen, probably the component previously defined as a major allergen (Ag3) by Longbottom (1986). Interestingly, three separate T cell lines derived from a single individual over a 12 month period also showed considerable consistency of response. A proportion of clones, for example AD4 (Fig. 5), although responding to soluble antigen, showed only minimal responses to fractionated antigen, suggesting loss of the relevant stimulating epitope during the preparation of the nitrocellulose fractions. The capacity of clones to respond or not, was found to be constant over time, and with several different nitrocellulose preparations. O'Hehir *et al.* (1987) noted a similar effect with *D. farinae*-responsive clones.

The establishment of AF-specific T cell clones has allowed us to begin to seek differences in specificity between clones derived from ABPA patients and AF-IgE negative donors, and of particular interest, to commence functional studies of the influence of these clones on antibody production *in vitro*, seeking correlation of clonal lymphokine production with their effects on *in-vitro* IgG and IgE production.

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