

T Cell Proliferation and Cytokine Secretion to T Cell Epitopes of Asp f 2 in ABPA Patients

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The pathogenesis of allergic bronchopulmonary aspergillosis (ABPA) involves specific cytokines secreted by lymphocytes in response to *Aspergillus fumigatus* (Af) allergens. To gain information about the lymphoproliferative response and cytokine production against a major Af allergen, Asp f 2, we studied Asp-f-2-specific T cell clones (TCCs) from ABPA patients. TCCs were stimulated with rAsp f 2, its deletion mutants, and synthetic peptides to identify the T cell epitope(s) and to understand cytokine production. PBMCs from four of five ABPA patients showed proliferation in response to Asp f 2. Three TCCs from one patient showed higher IL-5 secretion compared to IL-4 and IFN- γ . Two TCCs from the second patient showed a mixed Th1/Th2 response, as evidenced by production of IL-4, IL-5, and IFN- γ . An epitope from the N-terminal region of Asp f 2 induced only IL-5 secretion. High IL-5 secretion might explain the marked eosinophilia observed in ABPA patients. © 2001 Academic Press

Key Words: allergy; *Aspergillus*; ABPA; antigens/peptides/epitopes; Th1/Th2; cytokines.

INTRODUCTION

ABPA is a hypersensitivity disease of the lungs caused by a ubiquitous fungus, *Aspergillus fumigatus* (Af) (1). It is characterized by allergic asthma, pulmonary infiltrates, central bronchiectasis, peripheral blood and pulmonary eosinophilia, immediate skin reactivity to Af, and elevated serum IgE and IgG to Af (2). Accumulating evidence from animal model studies and from limited immunological studies in humans suggests that CD4⁺ T-cell-derived cytokines play a crucial role in the regulation of IgE synthesis (3) and eosinophil maturation, recruitment, and differentiation (4). Allergen-specific Th2 cytokine secreting cells may play an important role in the pathogenesis of allergic bronchopulmonary aspergillosis (ABPA) (5, 6).

Af antigens are diverse in their physiochemical and immunological characteristics (7, 8). A number of Af antigens have been cloned and purified (9–11). Our lab has identified and cloned Asp f 2, a major allergen in Af with 87–96% IgE binding with sera from ABPA patients but not from normal controls (10–12). Lymphoproliferative responses to crude Af extracts and recombinant Af allergens have been reported with T cells from ABPA patients (13, 14). Cytokine profiles of these T cells show a predominant Th2 type of response (13, 15), but subsequent studies have demonstrated that TCCs obtained from ABPA patients were either Th2 or Th0 cells (15, 16).

Different peptides of Af have been shown to induce different cytokines in a murine model (17), indicating that Af-sensitized T cells may recognize multiple epitopes within an allergen. IL-4 is critical for IgE synthesis and IL-5 for eosinophil maturation, survival, and activation. The use of such antigenic peptides, that can directly bind to MHC Class II molecules and are presented to T cells follow the rationale that since B cell epitopes are mostly conformational, small linear peptides should not be able to trigger IgE-mediated responses.

In recent years, T cell epitopes of several major allergens, including grass, bee venom, and house dust mites, have been identified and the T cells made tolerant by immunizing with the dominant peptides of these allergens (18–20).

In the present study deletion mutants of Asp f 2 lacking the IgE binding domains and synthetic peptides were used to determine the T-cell-reactive domain(s) of the molecule. We have also studied the cytokine secretion patterns induced by Asp f 2 as well as deletion fragments and peptides of Asp f 2. We show that a T cell epitope in Asp f 2 is located at the N-terminal of the molecule and is distinct from the IgE binding epitopes. Stimulation of T cells with the epitopic peptide induces marked IL-5 secretion but no detectable IL-4 and IFN- γ secretion.

METHODS

Patients

Patients who fulfilled the criteria for the diagnosis of ABPA as outlined by Patterson *et al.* (21) were selected for the study. Briefly, ABPA was diagnosed by the presence of recurrent wheezing, chest radiographic infiltrates, peripheral blood eosinophilia, immediate skin reactivity to Af, elevated total serum IgE and IgG, and Af-specific serum IgE and IgG. Informed consent was obtained from all subjects according to the institutional human research review committees. Peripheral blood was obtained in heparinized tubes. Blood was fractionated on Histopaque gradients (Sigma, St. Louis, MO) and PBMCs were collected at the interface. Cells were washed thrice in HBSS (GIBCO/BRL, Life Technologies; Baltimore, MD) and resuspended in RPMI 1640 supplemented with antibiotics, glutamine, and 10% fetal bovine serum, henceforth termed complete medium (CM).

Stimulation of PBMCs with rAsp f 2

PBMCs (2×10^5) were stimulated with 5 μ g/ml rAsp f 2 in triplicates in U-bottom microwells (96-well plates) for 5 days (200 μ l final volume). Control wells received medium, but no antigen. Eighteen hours prior to the termination of the cultures, 0.5 μ Ci of tritiated thymidine (^{3} H-TdR) was added to each well. Cells were harvested onto glass fiber filter mats (Skatron; Sterling, VA) and incorporated radioactivity was counted by liquid scintillation. The stimulation index (SI) was calculated as cpm in experimental wells with antigen/cpm in control wells with medium alone.

Recombinant Asp f 2, Fragments, and Synthetic Peptides

Asp f 2 deletion mutants (Fig. 1) were constructed based on IgE binding regions as determined in a previous study (22). Cloning, expression, purification, and reactivity of rAsp f 2 and Asp f 2 constructs with deleted IgE binding domains have been described elsewhere (23). Synthetic 18-mer peptides with 8-amino-acid overlap were obtained from Alpha Diagnostics (San Antonio, Texas).

Generation of T Cell Clones against Asp f 2

T cell clones were obtained by culturing 1×10^6 PBMCs with Asp f 2 in 1 ml CM in 24-well plates for 5 days. Cells were washed and restimulated with 5 μ g/ml of Asp f 2 in the presence of equal numbers of irradiated (8000 rad) autologous, EBV-transformed B cells as antigen presenting cells (APCs) along with 25 U/ml

human IL-2 (Sigma). Thereafter, cells received IL-2 every 3 days and restimulation in the presence of irradiated APCs every 7 or 8 days until the cell numbers expanded.

Cells were cloned by limiting dilution in microwells in the presence of irradiated APCs, IL-2, and Asp f 2. Positive cultures were transferred to 24-well plates. The cells were restimulated as described above until sufficient cells were obtained to test for specificity in proliferation assays.

Proliferation Assay for T Cell Clones (TCCs)

In microwells, TCCs (1×10^5 /well, 200 μ l final reaction volume) were stimulated with 5 μ g/ml of Asp f 2 and deletion fragments and irradiated APCs (2.5×10^4) for 5 days. ^{3} H-TdR (0.5 μ Ci/well) was added 18 h prior to termination of the assay. Cells were harvested and incorporated radioactivity was measured by scintillation counting. The SI was calculated as described above. The cpm values in wells with APCs but no antigen served as background and were subtracted from all other cpm values.

For stimulation with synthetic peptides, irradiated APCs were incubated with 5 μ g/ml of peptides in CM for 4 h and washed. APCs (2.5×10^4) were mixed with TCCs (1×10^5) and incubated as described above for Asp f 2 and deletion fragments.

Cytokine Measurements

TCCs (1×10^6) were incubated with Asp f 2, deletion fragments, and synthetic peptides in the presence of 2.5×10^5 irradiated APCs in 1 ml of CM for 48 h in 24-well plates. As described above, APCs were preincubated with Asp f 2 and Asp f 2 deletion mutants before the addition of TCCs. Culture supernatants were harvested, centrifuged, and stored at -70°C . IL-4 and IL-5 (Th2 cytokines) and IFN- γ (Th1 cytokine) were measured by ELISA. IL-4 and IFN- γ were measured by using DuoSet ELISA kits (R&D Systems, Minneapolis, MN), and IL-5 was measured by OptEIA kit (Pharmingen, San Diego, CA) according to the manufacturer's instructions. All results are expressed in pg/ml in comparison with the standards. The detection level of cytokines was 31.2 pg/ml for IL-4 and IFN- γ and 15.6 pg/ml for IL-5.

Statistical Analysis

Two-tailed unpaired Students *t* test was used to determine the level of significance between proliferative responses in ABPA patients and normal controls.

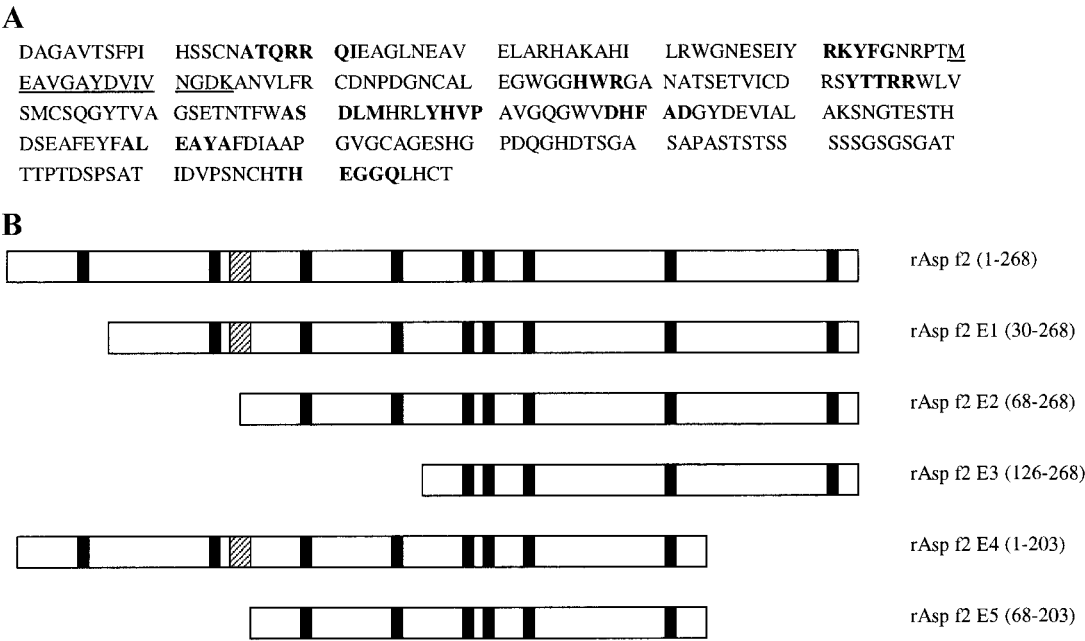


FIG. 1. (A) Amino acid sequence of recombinant Asp f 2. Bold letters represent IgE binding domains. Underlined letters represent a T cell epitope. (B) Schematic representation of six cDNA constructs of Asp f 2. Solid bars represent IgE binding domains. The hatched bar represents a T cell epitope. All six cDNA fragments were expressed in *Escherichia coli* (pET -23⁺ vector).

RESULTS

Proliferation of PBMCs and T Cell Clones against Asp f 2

PBMCs obtained from four of five ABPA patients showed significant proliferation upon stimulation with rAsp f 2 compared to unstimulated controls (Fig. 2). PBMCs from normal controls showed no proliferation in response to Asp f 2 stimulation, indicating Asp f 2 to be a specific and relevant antigen in ABPA. Although a number of clones developed, only a few survived long-term cultures. A total of five Asp-f-2-specific TCCs were obtained, three from one patient (P1) and two from a second patient (P2). Stimulation with rAsp f 2 induced significant proliferation of all five TCCs (Fig. 3).

Proliferation of TCCs against Deletion Mutants

To identify the domain containing the T-cell-reactive epitope(s), TCCs were also stimulated with Asp f 2 deletion mutants E1–E5. The schematic structures of the mutants are shown in Fig. 1. Asp f 2 E1 and E4 were able to induce proliferation of TCCs (Fig. 3). In TCCs from one patient (P1), the proliferation induced by E1 and E4 was similar to that seen with the complete Asp f 2 molecule. This indicates that a putative T-cell-reactive epitope(s) lies in the N-terminal region of rAsp f 2 between amino acids 30 and 68.

Proliferation of TCCs against Synthetic Peptides

In order to characterize more precisely the epitope region, two TCCs from patient P1 were stimulated with 15-mer synthetic peptides containing an 8-amino-acid overlap, as shown in Fig. 4A. Two of these peptides, aa 54–68 and aa 60–74, were able to induce proliferation of both TCCs (Fig. 4B). TCC P1-1E6 was stimulated to the same extent with both the peptides, whereas TCC P1-1D4 showed higher proliferation in response to peptide aa 54–68.

Cytokine Secretion from TCCs

Stimulation of TCCs with Asp f 2 induced the secretion of both Th1 and Th2 cytokines (Fig. 5). Although IFN-γ was produced by all three TCCs from patient P1 the response was predominantly of a Th2 type, with very high secretion of IL-5 but very little IL-4. TCCs from patient P2 showed a mixed response, with secretion of IL-4, IL-5, and IFN-γ.

Two TCCs from patient P1 were studied for cytokine production by stimulation with deletion mutants and T-cell-reactive peptides. In accordance with the results obtained with the proliferation of TCCs with different deletion mutants and peptides, only E1, E4, and peptide aa 60–74 were able to induce cytokine secretion from the TCCs (Fig. 6A). E1 was able to induce a

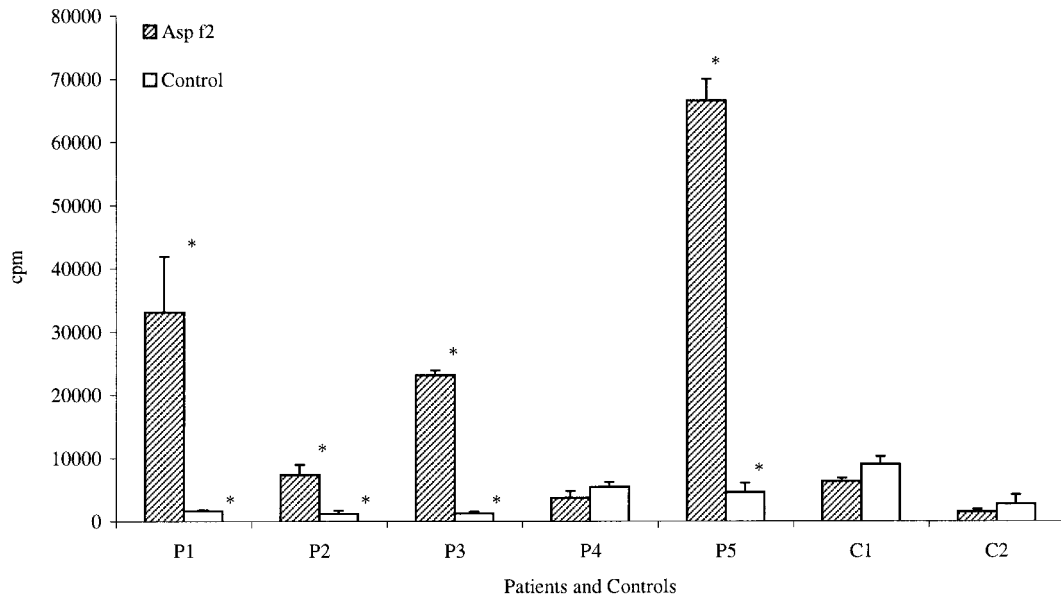


FIG. 2. Proliferation of PBMCs stimulated with rAsp f 2 compared to unstimulated cells from ABPA patients (P1–P5) and healthy controls (C1 and C2). PBMCs were incubated with 5 μ g/ml of rAsp f 2 or in medium alone for 5 days. Error bars represent +SD. An asterisk indicates $P < 0.01$ compared with respective control wells containing medium alone.

predominantly Th2 response with very high IL-5, but no IL-4 compared to IFN- γ secretion in TCC P1-1E6. The deletion mutant E4 showed the same pattern but the synthetic peptide aa 60–74 induced only IL-5 se-

cretion. In TCC P1-1D4, E1 induced a mixed response, with higher IFN- γ secretion than IL-5 (Fig. 6B), but no detectable IL-4. As with P1-1E6, the peptide aa 60–74 induced only IL-5 secretion from P1-1D4.

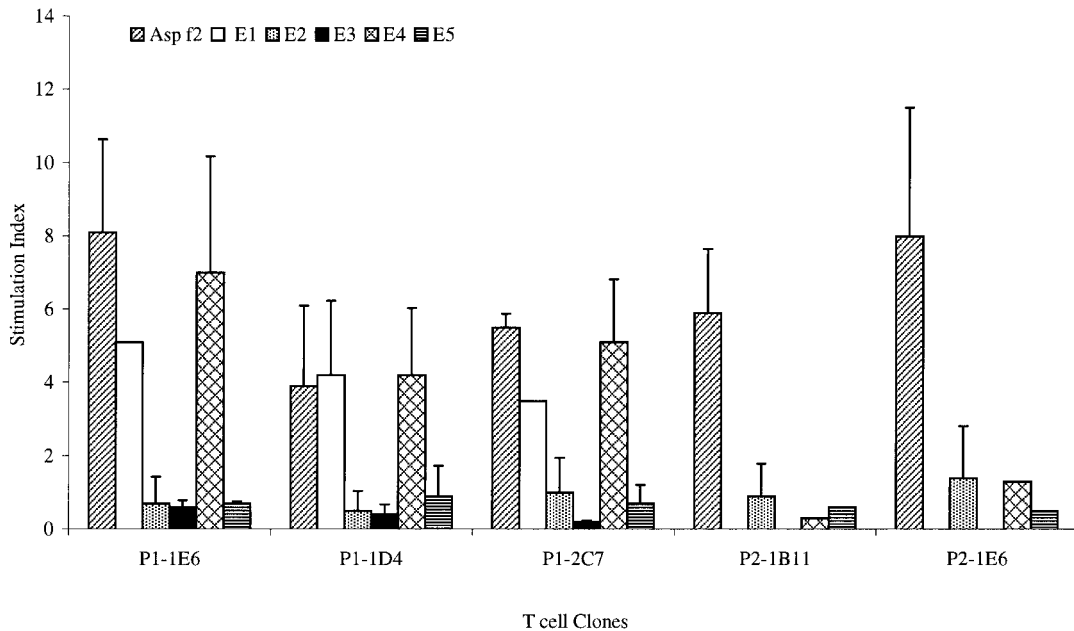


FIG. 3. Stimulation of T cell clones with rAsp f 2 and the deletion mutants. TCCs were stimulated with 5 μ g/ml of each antigen for 5 days. Results for P1 T cell clones are mean values from three independent experiments. Results for P2 T cell clones are mean values from two independent experiments. The stimulation index of deletion mutants with E1 and E3 was not determined for P2 clones. Error bars represent +SD.

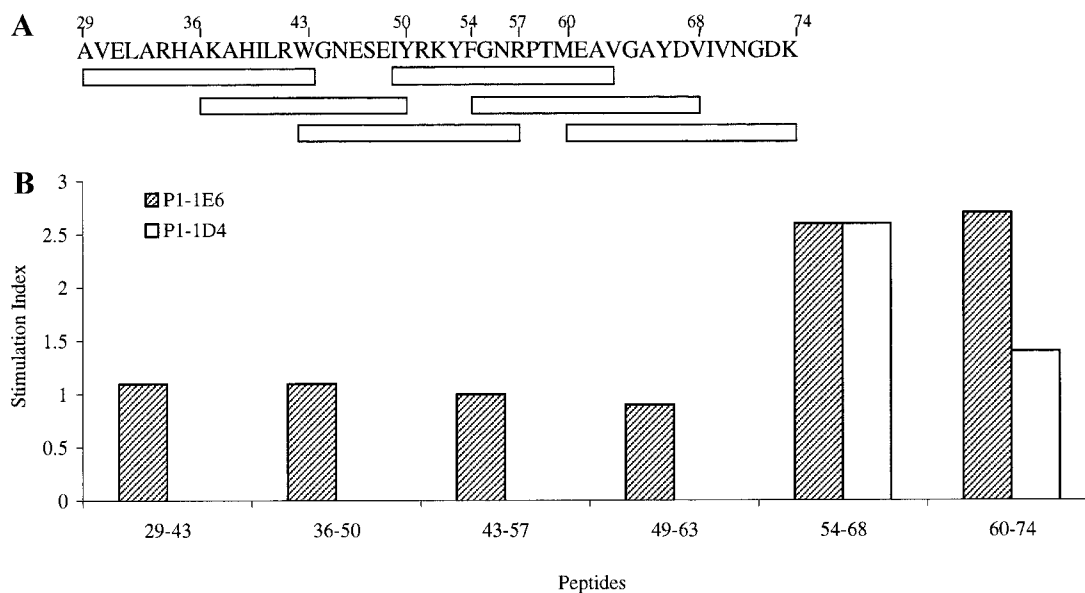


FIG. 4. Epitope mapping using synthetic peptides. (A) Amino acid sequence of the synthetic peptides corresponding to the N-terminal region of Asp f 2 used for epitope mapping. (B) Stimulation of two TCCs of Patient P1 with the synthetic peptides. APCs were preincubated with peptides for 4 h, washed before addition of TCCs, as described under Methods.

DISCUSSION

Activation of antigen-specific CD4⁺ T cell that produce Th2 cytokines (IL-4, IL-5) is thought to play a critical role in the immunopathogenesis of ABPA and probably is a major consideration in the treatment of allergic disease. Therefore, it is crucial to define T cell reactivity and epitope specificity toward a given allergen. This knowledge may lead to our understanding of how to modulate the immune response in a nonpathologic direction.

Increased antigen-specific T cell responses in ABPA patients have been reported using purified antigens (14, 15, 24). Cells from ABPA patients showed significantly higher secretion of IL-4 and IL-5 than those from healthy controls. Although T cells were also found to secrete IFN- γ , the levels of IL-4 and IL-5 were predominant, indicating a Th2 bias of the T cell response (24).

In the present study, Asp f 2 was found to be a specific and relevant allergen and likely to be important in the immunopathogenesis of ABPA. We have used deletion mutants of Asp f 2 to distinguish the T-cell-reactive epitope(s) from the IgE binding domain(s) in the Asp f 2 molecule. We have previously shown that Asp f 2 mutants, E2 and E4 and to a much lesser extent E1, bind IgE from the sera of ABPA patients (22). In this study we found that only E1 and E4 were capable of stimulating the Asp-f-2-specific TCCs. Since E1 has low IgE binding activity, the T cell epitope(s) in the Asp f 2 molecule seems to be distinct from the IgE binding epitope(s).

Proliferation with synthetic peptides identified two T cell epitopes in Asp f 2, between amino acids 54 and 74. It has been demonstrated by others that T cells may respond to different allergenic epitopes and that the majority of the T cells could be specific for an immunodominant epitope (15, 16). In the current experiments, T cell reactivity was restricted to two peptides of Asp f 2, peptides aa 54–68 and aa 60–74. This finding might indicate that there are two distinct epitopes in the region defined by these two peptides.

The cytokine secretion pattern of the TCCs revealed a mixed response. Asp f 2 induced a predominantly Th2 cytokine, with little IFN- γ . The Asp f 2 deletion mutants E1 and E4 induce high levels of IL-5 in one TCC and both IL-5 and IFN- γ in the second TCC but little or no IL-4 in either. This is unlike previous studies with other Af antigens and crude extract preparations where both IL-4 and IL-5 have been detected at significant levels (14, 24, 25). In addition, the peptide aa 60–74 induces only IL-5 and no detectable IL-4 and IFN- γ . Although peptide aa 54–68 induced proliferation of the TCCs, no detectable levels of either Th1 or Th2 cytokines was observed. Therefore, it is likely that peptide aa 60–74 may contain the major T cell epitope of the Asp f 2 molecule. Induction of IFN- γ in addition to IL-5 by Asp f 2 and deletion mutants as opposed to only IL-5 by synthetic peptide might be due to other minor epitopes in the protein molecule.

TCCs against another Af allergen, Asp f 1, have been reported to have a CD4⁺ phenotype, very similar to results obtained by us with Asp f 2 (data not shown) (5,

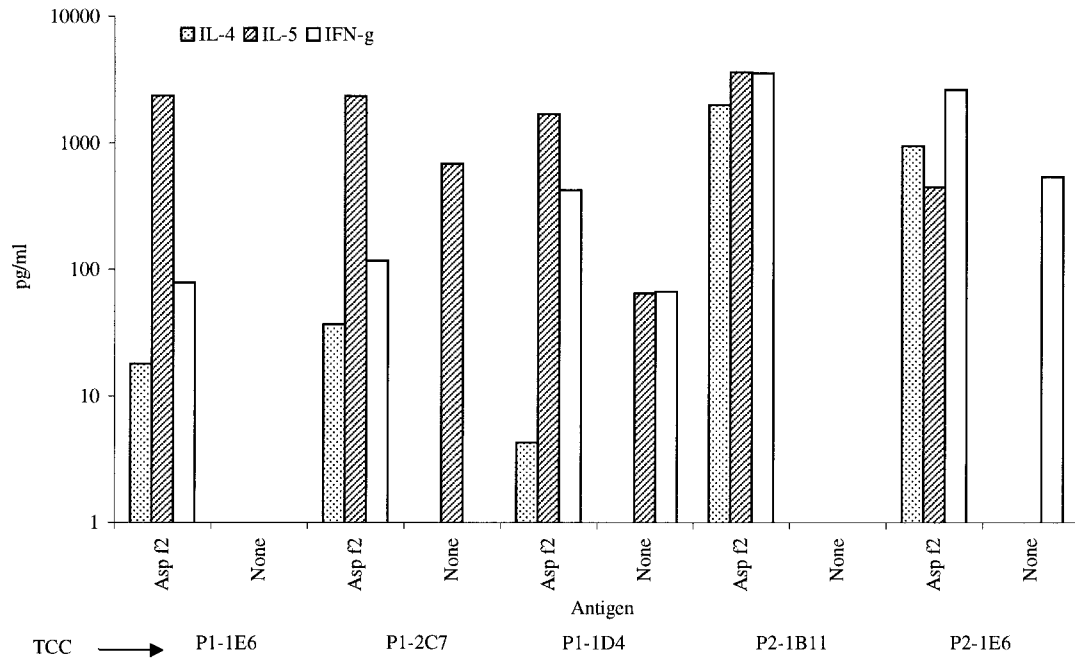


FIG. 5. Secretion of cytokines in the culture supernatants of indicated TCCs from two ABPA patients, P1 and P2; TCCs were stimulated with rAsp f 2 and in medium alone for 48 h and the cytokines determined by ELISA, as described under Methods.

15). Chauhan *et al.* have identified two major and three minor T cell epitopes distributed throughout the Asp f 1 molecule (15). In contrast, the T cell epitope in Asp f

2 is localized within a short stretch of amino acids at the N-terminal region. Earlier studies from our lab indicated that the T cell epitope region reported by

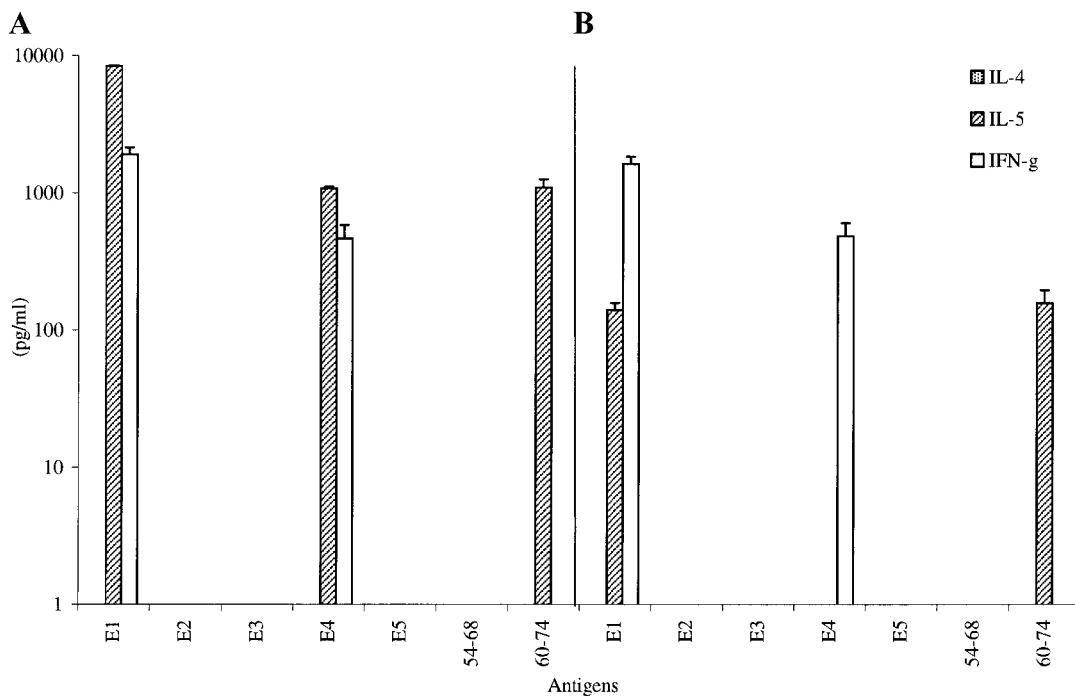


FIG. 6. Secretion of cytokines in the culture supernatants of two TCCs from patient P1 stimulated with Asp f 2 deletion fragments and synthetic peptides for 48 h. (A) P1-1E6, (B) P1-1D4. Error bars represent +SEM.

Chauhan *et al.* (15) might contain an IgE binding domain of the Asp f 1 molecule (17). Previously, we have identified nine epitopes in Asp f 2 recognized by IgE from the sera of ABPA patients (22). The T-cell-reactive peptide sequence(s) in the present study is distinct from the IgE binding epitopes. These results suggest that these peptides could alter T cell responses in the patients without triggering IgE-mediated responses, which could have significant therapeutic implications.

The Asp-f-1-specific TCCs secreted high levels of IL-4 with little or no IFN- γ , whereas Asp-f-2-specific TCCs produce little or no IL-4 and high levels of IL-5 upon stimulation. Both IL-4 and IL-5 are major Th2 cytokines and at times the level of one cytokine may be predominant over the other. Induction of high IL-5 levels from TCCs by Asp f 2 may explain the significant peripheral blood eosinophilia observed in ABPA patients.

Recently it has been demonstrated that antigenic peptides with different affinities for the T cell receptor (TCR) can preferentially induce a Th1 or Th2 response (26). MHC Class II restriction also appears to be important for allergen-specific T cell activation. Different affinity of the antigenic peptides to the presenting MHC Class II allele on the antigen presenting cells could dictate the immunodominance of an epitope (15, 16). Chauhan *et al.* have shown a higher frequency of HLA-DR2/5 subtypes in ABPA patients compared to the normal population, suggesting a role in the pathophysiology of ABPA at the level of T cell activation (16). Genotypic analysis of three ABPA patients in the current study showed the presence of HLA-DR2, but not the HLA-DR5 allele, whereas only one of three normal controls showed the presence of HLA-DR2 (data not shown). More extensive studies will be required to clarify the relationship of MHC Class II alleles and cellular responses.

The findings from this study provide additional insights into the immunologic mechanisms induced by Asp f 2, a major allergen associated with ABPA. Asp-f-2-specific TCCs were characterized by high IL-5 production, which is especially relevant in ABPA. It would be of interest to determine whether non-ABPA asthmatic subjects and cystic fibrosis patients with ABPA demonstrate reactivity toward the T cell epitope of Asp f 2. Alterations in the T cell epitope can presumably induce T cell tolerance or change in the cytokine pattern from Th2 to Th1. The modulation of T cell responses with different peptides of Asp f 2 could provide useful information for immunotherapeutic approaches to the treatment, diagnosis, and management of ABPA.

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