T Cell Epitope-Specific Defects in the Immune Response to Cat Allergen in Patients with Atopic Dermatitis

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Atopic dermatitis (AD) is often associated with high titer IgE antibodies (ab) to allergens, and IL-10-mediated regulation of IFN-γ has been proposed to contribute to this IgE ab production. However, the relevance of IL-10 and IFN-γ to IgE associated with AD has not been examined in the context of an allergen-specific system. Analysis of PBMC responses in vitro showed deficient T cell proliferation to overlapping IL-10- (peptide (P) 2:1) and IFN-γ- (P2:2) inducing chain 2 major epitopes of cat allergen (Fel d 1) in cultures from sensitized AD patients (mean IgE to cat = 20.9 IU/ml). Diminished IFN-γ induction by Fel d 1 and P2:2, along with elevated peptide-induced IL-10 (except for P2:1) was observed in PBMC cultures from AD subjects compared with non-AD (sensitized and non-sensitized) subjects. Neither T cell proliferation nor IFN-γ production to chain 2 epitopes could be restored by anti-IL-10 mAb in cultures from sensitized AD subjects. Moreover, allergen avoidance was associated with a paradoxical decrease in both IL-10 and IFN-γ in peptide-stimulated PBMC from these subjects. Control of IFN-γ production to chain 2 epitopes by IL-10 may be relevant to sensitization status. Development of high titer IgE ab in AD could reflect a failure of this mechanism.

Key words: CD4+ T lymphocytes/downregulation/immune tolerance/interleukin-10/T cell epitopes

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Cytokines elaborated by the Th2 subset of T lymphocytes govern the production of immunoglobulin E (IgE) antibodies (ab) by B lymphocytes and eosinophil recruitment to sites of inflammation, resulting in the development of allergic disease. Enhanced IgE ab production is a notable feature of the chronic inflammatory skin disease, atopic dermatitis (AD) (Ogawa et al, 1971). The presence of high-titer IgE ab to inhalant allergens, elicitation of an eczematous rash after application of allergen in an atopy patch test, and improvement of disease during allergen avoidance suggests an important role for allergen-specific immune responses in the pathogenesis of AD (Mitchell et al, 1982; Chapman et al, 1983; Tan et al, 1996; Scalabrin et al, 1999; Erwin and Platts-Mills, 2002). Despite the evidence of Th2 skewing, as judged by alterations in T helper cell cytokine profiles, immune mechanisms which govern such changes have yet to be defined. It has been proposed that elevated interleukin-10 (IL-10) secretion by monocytes contributes to T helper cell polarizing events in AD (Lester et al, 1995; Ohmen et al, 1995; Aiba et al, 2003). Indeed, diminished interferon (IFN)-γ production by T cells from AD patients does not appear to result from an intrinsic T cell defect since IFN-γ production can be restored by co-stimulation with IL-12 or engagement of CD28 (Jung et al, 1999).

Allergen-reactive T cells derived from patients with AD are predominantly CD4+ T cells which express the skin-homing marker, cutaneous lymphocyte-associated antigen (CLA), suggesting a direct function of these cells in the disease (Santamaria Babi et al, 1995; Jung et al, 2003). Despite this, few studies of AD patients have examined T cell responses in allergen-specific systems. Instead, much work has focused on phenotypic analysis of T cells isolated directly from whole blood, or on cells stimulated in vitro using non-specific stimuli such as phorbol 12-myristate 13-acetate and ionomycin, or anti-CD3 and anti-CD28 mAb (Sato et al, 1998; Aleksza et al, 2002; Kallstrom et al, 2002; Okazaki et al, 2002; Simon et al, 2002). The impetus for such studies may arise from the view that AD reflects an underlying broad-based defect in immune regulation which influences the immune response to a multitude of antigens from diverse sources, including allergens, in a non-specific manner. However, the recent observation that IL-10 secretion by CD8+ T cells specific for the mite allergen, Der p 1, is diminished in severe disease suggests that alterations in allergen-specific T cell- as well as monocyte-derived IL-10 may contribute to disease pathogenesis (Senearvath et al, 2002).

Definition of the modified Th2 response in individuals exposed to high-dose cat allergen, Fel d 1, has facilitated studies on immunologic tolerance induced by natural exposure to an inhalant allergen. High-titer IgG and IgG4 ab to Fel d 1 in the absence of IgE is the hallmark of the modified Th2 response (Platts-Mills et al, 2001). Given the

Abbreviations: AD, atopic dermatitis; IFN, interferon; IgE, immunoglobulin E; IL, interleukin; MHC, major histocompatibility complex; NS, non-sensitized; P, peptide; S, sensitized; TT, tetanus toxoid

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high titers of IgE ab to Fel d 1 in sensitized patients with AD, we theorized that this disease represents a "breakdown of tolerance" which would be evident at the T cell level. Here we show that major epitopes of Fel d 1 which map to the amino-terminal region of chain 2 of the molecule selectively induce high levels of IL-10 or IFN-γ. Activation of T cells which target these epitopes during conventional immuno-therapy suggests that these epitopes fulfill a regulatory role (Reef er et al., in press). Since alterations in IL-10 and IFN-γ production have been implicated in AD, we examined T cell responses to peptides spanning both polypeptide chains of the Fel d 1 molecule in sensitized patients with AD. We report that diminished T cell proliferation to chain 2 major epitopes is specifically associated with sensitization to cats and with disease severity in patients with AD. Furthermore, chain 2 epitopes stimulate an altered pattern of IL-10 production and markedly reduced IFN-γ in PBMC cultures from AD patients. In contrast to patients without AD, T cell responsiveness to chain 2 epitopes is not restored by blocking IL-10 in AD subjects with high-titer IgE ab. These observations are consistent with a role for allergen-specific T cell dysregulation in AD.

Results

Sensitization to cat allergen in patients with AD is associated with high-titer antigen-specific IgE ab. Analysis of serum ab responses to cat showed a significantly decreased prevalence of the modified Th2 response to cat (i.e., IgG3/2 and IgE) among patients with AD (four of 55) compared with subjects without AD (14 of 57, p = 0.013) (Fig 1a). Among those individuals with measurable IgE ab to cat (> 0.35 IU per mL), mean titers of IgE were increased in AD patients (11.2 IU per mL) compared with those without AD (2.3 IU per mL) (p = 0.001). Furthermore, the prevalence of high-titer IgE ab (> 10 IU per mL) was significantly increased in AD (22 of 40) compared with non-AD (five of 24) subjects (p = 0.009) (Fig 1b). All subsequent studies were carried out in nine cat-sensitized patients with AD (mean IgE ab to cat = 20.9 IU per mL) (S + AD), ten non-cat-sensitized patients with AD (NS + AD), fourteen cat-sensitized patients without AD (mean IgE ab to cat = 4.4 IU per mL) (S), and 11 serum ab-negative subjects (Control). Mean levels of total IgE were increased in cat-sensitized patients with AD compared with all other patient groups (p < 0.004). Furthermore, cat-sensitized patients with AD had markedly elevated titers of IgE ab to both cat extract and Fel d 1 compared with cat-sensitized patients without AD (p < 0.01) (Fig 1c, d). Cat-sensitized patients with AD also showed increased IgG ab specific for Fel d 1 compared with their non-sensitized (NS) counterparts (Fig 1d).

Fel d 1 induces an increased ratio of IL-10 to IFN-γ in cultures from AD patients compared with healthy controls. Mean T cell proliferative responses to Fel d 1 in 6-d PBMC cultures were moderately decreased in sensitized AD patients (stimulation index = 9) compared with non-sensitized AD and control subjects (stimulation indices = 16 and 14, respectively) (p > 0.3) (Fig 2a). Analysis of cytokine profiles showed diminished IFN-γ production in both sensitized AD (p = 0.013) and non-sensitized AD (p = 0.027) patients compared with serum ab-negative controls (Fig 2b). As a result, the mean ratio of IL-10 to IFN-γ was enhanced in PBMC derived from sensitized AD (IL-10:IFN-γ = 63) and non-sensitized AD (IL-10:IFN-γ = 25) patients compared with controls (IL-10:IFN-γ = 5) (p < 0.043). The levels of IL-5 and IL-13 were consistently low (i.e., < 30 pg per mL) in all cultures whereas no IL-4 was measurable in any cultures. The endotoxin content of Fel d 1 was high (> 10,000 EU per mg Fel d 1) and attempts to remove endotoxin by conventional methods were unsuccessful (Woodfolk et al., 2000). Thus, IL-10 secretion by monocytes likely contributed to the cytokine profile observed in cultures containing Fel d 1. Subsequent studies used endotoxin-free synthetic peptides derived from Fel d 1 which were designed as optimal major histocompatibility complex (MHC) class III ligands, thereby excluding adjuvant effects of endotoxin on T cell responses in vitro.

Altered T cell responsiveness to chain 2 major epitopes of Fel d 1 is associated with AD. In order to further study the response of AD patients to cat allergen, lymphocytes were cultured in vitro with each of the 20 overlapping peptides spanning Fel d 1. Peptides mapping to the amino-termini of Fel d 1 polypeptide chain 1 (P1:2) and chain 2 (P2:1 and P2:2) stimulated strong T cell proliferative responses in PBMC cultures from sensitized patients without AD (mean standardized index ≥ 2.5) (Fig 3a). No difference in the pattern of T cell epitope recognition was observed between sensitized patients without AD and healthy controls (data not shown). Cultures from sensitized patients with AD showed diminished T cell proliferation to P2:1 and P2:2 compared with non-AD patients (p = 0.003); however, strong proliferation to these epitopes was observed in cultures from AD patients without IgE ab (Fig 3a and b). In addition to an association with IgE ab, decreased proliferation to P2:1 and P2:2 was associated with severe AD (Fig 3c). In contrast to chain 2 peptides, T cell proliferative responses to the chain 1 peptide, P1:2, were diminished in both AD groups (p < 0.05). Thus, T cell responses to Fel d 1 peptides in sensitized and non-sensitized patients with AD were distinguished based on recognition of chain 2, but not chain 1, epitopes.

Peptides 2:1 and 2:2, were identified as promiscuous MHC class II ligands (see Materials and Methods). Analysis of cytokines in PBMC cultures from sensitized subjects without AD showed that production of IL-10 and IFN-γ localized to P2:1 and P2:2, respectively (Fig 4). Cytokine production to chain 2 epitopes was altered in cultures from AD patients; the highest levels of IL-10 were induced by P2:3 (not P2:1) whereas IFN-γ production to P2:2 was markedly reduced regardless of the IgE ab response to Fel d 1 (Fig 5). Levels of IL-10 to all 20 peptides were increased in sensitized AD (26.5 ± 2.3 pg per mL (mean ± SEM)) and non-sensitized AD (26.1 ± 3.9 pg per mL) groups compared with sensitized subjects without AD (17.8 ± 3.2 pg per mL) and controls (12.3 ± 1.7 pg per mL) (p < 0.05). However, analysis of the mean IFN-γ production for all 20 peptides showed no significant difference for AD and non-AD subjects suggesting that deficient IFN-γ production was selective for P2:2. In keeping with altered recognition of chain 2 epitopes, analysis
of the pattern of epitope recognition throughout the whole molecule as judged by the distribution of mean levels of both IL-10 and IFN-γ was significantly different between cat-sensitized AD patients and controls (p < 0.003). Surprisingly, mean levels of IL-5 for all 20 peptides were significantly decreased in both AD groups (2.24 ± 0.1 and 2.09 ± 0.1 pg per mL for sensitized and NS subjects, respectively) compared with sensitized subjects without AD (2.95 ± 0.2 pg per mL) (p < 0.001) (Fig 5).

Blocking IL-10 fails to restore proliferation and IFN-γ responses to chain 2 epitopes in AD patients with high-titer IgE ab to cat We next tested whether blocking IL-10 could restore T cell proliferation and IFN-γ production to chain 2 peptides in cultures from AD patients. Donors selected for study included two non-sensitized and two sensitized AD patients (#1 through #4, Table I). In Fel d 1-stimulated cultures, blocking IL-10 using anti-IL-10 mAb induced increased proliferation and production of IFN-γ in all patients, although the magnitude of the response was diminished in AD patients with high-titer IgE ab to cat. However, there was no effect of anti-IL-10 mAb on either proliferation or IFN-γ levels in cultures stimulated with chain 2 peptides from these patients. This was in contrast to a marked enhancement of T cell reactivity to chain 2 peptides in non-AD patients (Fig 6).

Allergen avoidance is associated with a rapid decrease in IL-10 production and a paradoxical decrease in IFN-γ production in AD patients with high-titer IgE ab to cat In order to examine the association between allergen exposure and Fel d 1-specific T cell responses, cellular responses were monitored in vitro in two cat-sensitized patients with AD receiving a complete allergen avoidance regimen. Both patients had high-titer IgG ab to Fel d 1 (9.968 U per mL (patient #7) and 17.913 U per mL (patient #8)) and high-titer IgE ab to cat (> 100 IU per mL (7) and 61.7 IU per mL (8)) (Table I). Assay of floor dust confirmed the presence of Fel d 1 in each patient’s home (0.56 and
1435 μg per g bedroom floor dust for #7 (no cats in home) and #8 (two cats in home), respectively. Allergen avoidance of 12-d duration was associated with a modest decrease in disease severity score in both patients (55.5–36.5 (#7); 42.5–38 (#8)) without any change in serum IgE or IgG antibodies. The pattern of T cell epitope recognition was highly consistent during the study period. In contrast, production of IL-10 and IFN-γ to chain 1 and chain 2 epitopes was

Figure 2
Fel d 1 induces high IL-10 but diminished IFN-γ in peripheral blood mononuclear cells (PBMC) from atopic dermatitis (AD) patients. PBMC were stimulated with 10 μg per mL Fel d 1 for 6 d. (a) Proliferation was measured by [3H] thymidine incorporation (counts per min) and expressed as mean stimulation index ± SEM. (b) Culture supernatants were assayed for cytokines and values expressed as the mean ± SEM. No Ag, no antigen. (*p < 0.03).

Figure 3
Diminished proliferation to chain 2 major epitopes of Fel d 1 is associated with IgE antibodies to cat and severe disease in patients with atopic dermatitis. Peripheral blood mononuclear cells PBMC were stimulated with each of the 20 Fel d 1 peptides (7.5 μM) for 6 d and proliferation determined by [3H] thymidine incorporation. Each peptide was assayed in 12 replicate wells. Data (cpm) were then log transformed and standardized indexes derived. In (a), the mean standardized index ± SEM for each peptide is shown for [S], [S + AD], and [NS + AD] groups. *p < 0.003 for [S] versus [S + AD] groups. (b) Individual data points for P2:1 and P2:2 within each group. Horizontal bars represent the mean standardized index value for P2:1 and P2:2 in each group. Arrows denote AD patients with severe disease. (*p < 0.05, **p < 0.01). In (c), the mean standardized index ± SEM for each of the 20 Fel d 1 peptides is shown for patients with severe (n = 6), moderate (n = 7), and mild (n = 6) AD. *p < 0.05 for P2:1 and P2:2 (moderate vs severe groups) and for P2:7 (moderate vs mild groups). Dotted lines indicate a mean standardized index value of 1.5. U, unstimulated wells containing no antigen.
with AD might reflect an underlying defect in T cell effector or regulatory function, we analyzed T cell responses to Fel d 1 in patients with AD. Results showed that altered T cell responsiveness to chain 2 IL-10- and IFN-γ-inducing epitopes was a feature of AD. Specifically, PBMC from AD patients with high-titer IgE ab and severe disease showed diminished T cell proliferation to these epitopes. Furthermore, in the context of the whole molecule, T cell responses among sensitized and non-sensitized patients with AD were distinguishable based solely upon differential recognition of P2:1 and P2:2.

Diminished T cell proliferation to chain 2 epitopes in sensitized AD patients suggests that differences in the allergen-specific memory T cell repertoire underlie immune responses associated with different levels of IgE ab production. It is tempting to speculate that alterations in the balance between Th1 and regulatory cytokines resulting from decreased recognition of chain 2 epitopes may contribute to disease pathogenesis in sensitized patients with AD. Whether such differences in the T cell repertoire directly influence IgE ab responses remains to be determined. Although proliferative responses to chain 2 epitopes are diminished, our data suggest that chain 2 epitope-specific T cells are not absent from the repertoire given the ability for P2:1 and P2:2 to induce T cell responses, albeit in an altered fashion, in a minority of sensitized AD patients (e.g., patient #7, Fig 7). It also seems unlikely that anergy resulting from high-dose exposure to cat allergen is a cause since removal of a sensitized patient from an environment containing high levels of Fel d 1 did not restore T cell proliferation to chain 2 epitopes, despite changes in cytokine production (patient #8, Fig 7). Nevertheless, anergy resulting from lack of co-stimulatory signals provided by Langerhans cells or cytokines present during T cell priming cannot be excluded (Van Reijsen et al., 1994; Akdis et al., 1996). We propose that differences in the T cell repertoire associated with AD arise from T cell activation by allergen at anatomic sites (i.e., the skin) which are distinct from those in patients without AD (i.e., the respiratory tract). This could be explained by increased delivery of allergen through the skin coupled with generation of distinct antigenic determinants within resident antigen presenting cells, such as Langerhans cells or inflammatory dendritic epidermal cells (Wollenberg et al., 1996). Selective expansion or inhibition of T cells with different specificities and effector/regulatory functions could then ensue in regional lymph nodes. The observation that T cell proliferation to chain 2 epitopes is diminished in patients with severe AD is consistent with the view that increased access of allergen through barrier defects in the skin may contribute to alterations in the T cell repertoire.

Despite the failure of proliferation to chain 2 epitopes in sensitized patients with AD, the altered pattern of cytokine responses to chain 2 epitopes was consistent among AD patients, regardless of sensitization status. Interestingly, peptide 2:3, not P2:1, induced the highest mean levels of IL-10, whereas the IFN-γ-inducing peptide, P2:2, generally failed to stimulate IFN-γ production in either AD group. Computer analysis of HLA binding motifs identified P2:3 as a promiscuous ligand capable of binding to multiple MHC class II molecules. Thus, the ability for this peptide to induce
**Figure 5**
Altered cytokine responses are specific for chain 2 epitopes of Fel d 1 in patients with AD. PBMC were cultured with Fel d 1 peptides and culture supernatants assayed for cytokine content on day 6. Values represent geometric means with upper 95% confidence intervals for each of the four patient groups. Dotted lines represent limit of sensitivity of the assay. For interleukin (IL)-10 production to P2:3, \( p = 0.027 \) for [S + AD] versus [S]; \( p < 0.01 \) for [NS + AD] versus [S] and controls. For IFN-\( \gamma \) production to P2:2, \( p < 0.01 \) for [S + AD] versus [S] and controls; \( p = 0.028 \) for [NS + AD] versus controls. For IL-13 production to P2:3, \( p < 0.03 \) for [S] versus [S + AD] and [NS + AD]. U, unstimulated wells containing no antigen.

**Table I.** Characteristics of subjects studied in IL-10 blocking experiments (#1 through #6) and those who received an allergy avoidance regimen (#7 and #8)

<table>
<thead>
<tr>
<th>Subject</th>
<th>Group</th>
<th>IgG to Fel d 1 (U per mL)(^{a})</th>
<th>IgE to cat (IU per mL)(^{b})</th>
<th>DRB1(^{c})</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>NS + AD</td>
<td>&lt;140</td>
<td>&lt;0.35</td>
<td></td>
</tr>
<tr>
<td>#2</td>
<td>NS + AD</td>
<td>5,918</td>
<td>&lt;0.35</td>
<td>*0401 *1401</td>
</tr>
<tr>
<td>#3</td>
<td>S + AD</td>
<td>3,297</td>
<td>28.5</td>
<td>*0103 *0404</td>
</tr>
<tr>
<td>#4</td>
<td>S + AD</td>
<td>13,151</td>
<td>38.4</td>
<td>*0301 *1501</td>
</tr>
<tr>
<td>#5</td>
<td>Sensitized</td>
<td>338</td>
<td>0.94</td>
<td>*0404 *1301</td>
</tr>
<tr>
<td>#6</td>
<td>Control</td>
<td>&lt;140</td>
<td>&lt;0.35</td>
<td>*0701 *1101</td>
</tr>
<tr>
<td>#7</td>
<td>S + AD</td>
<td>9,968</td>
<td>&gt;100</td>
<td>*0102 *1101</td>
</tr>
<tr>
<td>#8</td>
<td>S + AD</td>
<td>17,913</td>
<td>61.7</td>
<td>*0404 *0801</td>
</tr>
</tbody>
</table>

\(^{a}\)Measured by antigen binding radioimmunoprecipitation assay.

\(^{b}\)Measured by CAP assay.

\(^{c}\)Alleles highlighted in bold represent those predicted to bind to one or more of the chain 2 peptides, P2:1, P2:2, P2:3, or P2:4, using the computer prediction algorithm, TEPITOPE.

\(^{d}\)ND, not done.

Strong IL-10 production in AD patients with diverse HLA-DR types was not surprising per se. However, given that P2:1 and P2:2 also contained promiscuous epitopes, strong reactivity to P2:3 in the presence of low cytokine responses to P2:1 and P2:2 in AD patients cannot be explained by differences in HLA binding properties. Increased production of IL-10 to all Fel d 1 peptides (with the notable exception of P2:1) was evident in AD patients.
It has been proposed that IL-10 from a variety of cell sources contributes to Th2 skewing in AD (Lester et al., 1995; Ohmen et al., 1995; Aleksza et al., 2002; Aiba et al., 2003). Although diminished production of IFN-γ to Fel d 1 and P2:2 in AD patients would be consistent with this theory, there was no evidence of enhanced IL-5 or IL-13 in cultures from AD patients. Furthermore, blocking IL-10 failed to increase IFN-γ secretion induced in cultures from sensitized AD patients stimulated with pooled chain 2 peptides. Surprisingly, blocking IL-10 did increase T cell responses to whole allergen in cultures from these patients suggesting a selective defect in regulation of the response to chain 2 peptides. This raises the important question of whether IL-10 control of IFN-γ production to chain 2 epitopes is a determinant of sensitization status. During allergen avoidance, levels of peptide-induced IL-10 were significantly diminished for the two cat-sensitized patients studied suggesting that allergen exposure contributes to enhanced epitope-specific IL-10 production; however, these changes were associated with an unexpected decrease in IFN-γ. Taken together, our findings could be interpreted in two ways: (1) responsiveness to IL-10 is abnormal in AD patients, despite the high levels produced (i.e., IL-10 dysregulation); or (2) alternate factors to IL-10 downregulate IFN-γ in AD.

Among non-sensitized AD patients, T cell proliferation and IFN-γ secretion were enhanced in the presence of anti-IL-10 mAb irrespective of whether antigen was present. Furthermore, T cell proliferation and IFN-γ responses in unstimulated cultures were high in the absence of anti-IL-10 mAb suggesting endogenous T cell or monocyte activation. Indeed, spontaneous proliferation and cytokine production by cells derived from AD patients have been reported (Glinski et al., 1995; Poulsen et al., 1995; Akdis et al., 1999). Thus, it could be argued that the effects of blocking IL-10 in these patients are non-specific. This is in contrast to results obtained for patients without AD (#5 and #6, Fig 6) which show a marked increase in proliferation and IFN-γ in the presence of anti-IL-10 mAb for cultures containing Fel d 1 and chain 2 peptides.

In relation to the increased levels of IL-10 in unstimulated cultures derived from AD patients, (Fig 2) it should be stressed that a variety of cell types, including monocytes, may produce IL-10 in our culture system. Monocytes likely contributed to the high levels of IL-10 observed in Fel d 1-stimulated cultures, by virtue of the potent effects of endotoxin on this cell type. Furthermore, it could be argued that the production of high levels of IL-10 in peptide-stimulated cultures in the absence of T cell proliferation (patient #8, Fig 7) reflects IL-10 secretion by non-T cells; however, levels of the Th1 cytokine, IFN-γ, were also measurable in these cultures suggesting that epitope-specific T cells become activated but that cytokine production and proliferation are dissociated (Evavold et al., 1991; Woodfolk et al., 2001). Nevertheless, this still does not exclude secondary effects on in vitro T cell responses resulting from interactions between non-specifically activated antigen presenting cells and T cells. All Fel d 1 peptides were determined to be endotoxin-free; thus, IL-10 induction most likely results from antigenic properties of the
peptides themselves. Although a variety of peptides (e.g., neuropeptides) are known to induce IL-10 production by monocytes, the authors are unaware of a mechanism of direct activation of monocytes by Fel d 1 peptides. Data which support the view that IL-10 in peptide-stimulated cultures results primarily from T cell activation includes: (1) localization of IL-10 induction to chain 2 epitopes containing multiple putative HLA class II ligands; (2) localization of IL-10 induction to chain 2 epitopes which induce strong T cell proliferation; and (3) differences in the pattern of IL-10 responsiveness to chain 2 peptides in patients with and without AD. This latter observation would be difficult to explain if non-specific monocyte-derived IL-10 was the major source of IL-10 in these cultures.

In conclusion, our results support a role for selective defects in the allergen-specific T cell repertoire associated with high-titer IgE ab responses. The focus of this phenomenon on overlapping IL-10- and IFN-γ-inducing peptides of the major cat allergen Fel d 1, implicates changes in epitope-specific Th1 and/or T regulatory cells in this process.

Materials and Methods

Subjects Peripheral blood samples were obtained from 112 subjects, including 56 patients with AD who were diagnosed using the criteria of Hanifin and Rajka (1980). Healthy subjects without AD (n = 57) were recruited by advertisement. Subjects were classified as cat-sensitized with AD (n = 9), non-cat-sensitized with AD (n = 10), cat-sensitized without AD (n = 14), and controls (n = 11). Sensitized patients with AD were classified on the basis of serum IgE ab to cat > 0.7 IU per mL measured by CAP assay (Pharmacia Biotech, Uppsala, Sweden). Sensitized patients without AD were those with both a positive skin prick test (≥ 5 x 5 mm wheal) to cat extract and serum IgE ab to cat > 0.7 IU per mL. Non-cat-sensitized subjects with AD were those with no measurable serum IgE ab to cat (< 0.35 IU per mL) with (n = 3) or without (n = 7) serum IgG ab to Fel d 1 measured by radioimmunoprecipitation assay (Rowntree et al., 1987; Platts-Mills et al., 2001). Patients with no detectable serum IgE ab to cat extract and without IgG ab to Fel d 1 were classified as serum ab-negative controls. Severity of disease was assessed in 19 patients with AD (six severe, seven moderate, six mild) using the SCORAD index (Stalder et al., 1993) which combines objective (extent and intensity of lesions) and subjective (daytime pruritus and sleep loss) criteria. No patients received systemic immunosuppressive drugs within 6 mo of starting the study. Genotyping for the major HLA allele, DRB1, was carried out using PCR-based methods previously described (Woodfolk et al., 2000). Studies on human subjects were approved by the University of Virginia Human Investigation Committee and adhered to Declaration of Helsinki Guidelines.

Reagents Natural Fel d 1 was affinity purified from cat hair (Chapman et al., 1988; De Groot et al., 1988) and labeled with [125I] for use in radioimmunoprecipitation assays. Peptides (14–19 residues in length, n = 20) with a 10 amino acid overlap spanning both polypeptide chains of the Fel d 1 heterodimer (peptides P1:1 through P1:9 for chain 1 and P2:1 through P2:11 for chain 2) were...
synthesized by standard F-moc chemistry (University of Virginia Biomolecular Research Facility) using a Symphony automated peptide synthesizer (50 μM scale; Rainin, Woburn, Massachusetts) and purified to >90% by reverse phase high-performance liquid chromatography. Peptides were determined to be endotoxin free by quantitative chromatic Limulus Amebocyte Lysate Assay (BioWhittaker, Walkersville, Maryland). Fel d 1, tetanus toxoid (TT), and PHA were sterile-filtered prior to use. IL-4 was measured by ELISA (Pelikine, CLB, Amsterdam, The Netherlands). Matched mAbs pairs for measurement of IL-5, IL-10, IL-13, and IFN-γ by ELISA and anti-IL-10 mAb (rat IgG2a kappa) used in blocking studies were obtained from Pierce-Endogen (Woburn, Massachusetts). Rat IgG2a kappa isotype control was obtained from Biosource International (Camarillo, California).

Cell cultures PBMC were isolated from 100 mL of peripheral blood by density gradient centrifugation over Ficoll–Paque (Pharmacia Biotech) and cultured in RPMI 1640 (Life Technologies, Gaithersburg, Maryland) supplemented with 10% FCS. For proliferation assays, PBMC were cultured in the presence of 10 μg per mL Fel d 1 (2 x 10^6 cells in quadruplicate wells) or 7.5 μM Fel d 1-derived peptides (3 x 10^6 cells in 12 replicate wells). TT (10 μg per mL) and PHA (20 μg per mL) in quadruplicate wells were used as positive controls and 60 unstimulated wells (12 wells on each assay plate) were negative controls. Cells were cultured for 6 d at 37°C in 96-well plates, and pulsed with 1 μCi of [3H] thymidine per well during the final 8 h of culture before harvesting and counting (Topcount NXT, Packard Bioscience Co., Downers Grove, Illinois). For cytokine assays, cells were cultured in 24-well plates (4 x 10^6 cells per well in 1 mL volume) in the presence of antigen and culture supernatants were harvested on day 6 and assayed by ELISA for cytokines. Values were interpolated from standard curves to a sensitivity of 0.2 pg per mL for IL-4, 2 pg per mL for IL-5, IFN-γ, and IL-13, and 4 pg per mL for IL-10.

Studies using anti-IL-10 monoclonal antibody Freshly isolated PBMC were stimulated with Fel d 1 (10 μg per mL) or pooled chain 2 peptides (P2:1, P2:2, P2:3, and P2:4; 7.5 μM each) for 7 d, with or without anti-IL-10 mAb (10 μg per mL). Unstimulated cultures and cultures containing an isotype control were included as negative controls. For proliferation assays, cultures incorporated 12 replicate wells (2 x 10^6 cells per well in 96-well plates). For cytokines, assays were carried out in 24-well plates (4 x 10^6 cells per well) and culture supernatants assayed for IFN-γ by ELISA.

Computer prediction of CD4+ T cell epitopes using TEPITOPE The primary amino acid sequence of Fel d 1 chains 1 and 2 was loaded into the HLA-DR ligand prediction software, TEPITOPE (Vaccinome, Kearny, New Jersey), to predict promiscuous HLA-DR ligands (Sturniolo et al, 1999). HLA-DR alleles most frequent in the caucasian population (*0101, *0301, *0401, *0701, *0801, *1101, *1501) were selected and the TEPITOPE prediction threshold was set at 5%. Peptides binding to three or more different allotypes were defined as promiscuous.

Allergen avoidance trial Two cat-allergic patients with severe AD were admitted to the University of Virginia General Clinical Research Center under a protocol that included complete allergen avoidance. T cell responses, serum ab profiles, and disease severity were monitored at 6-d intervals for 12 d.

Statistical analysis Between-group frequencies of serum ab were compared using the Pearson Χ^2-square test and mean serum ab titers were compared between groups using an independent two-sample Student’s t test. Proliferative responses were expressed as mean stimulation indexes (Fel d 1) and mean standardized indexes (peptides) (Woodfolk et al, 2000). Briefly, standardized indexes were derived by log transformation (Ln) of cpm (in order to account for variability among peptide-stimulated replicates) and subtraction of background levels. By this method of analysis, a standardized index value >1.5 represents a stimulation index calculated by traditional methods >8. Initial analysis of the pattern of cytokine induction of Fel d 1 peptides in sensitized subjects without AD was carried out using arithmetic means ± SEM. Between-group cytokine responses to peptides were compared using geometric means with 95% confidence intervals in order to account for the wide variation within each AD group. An independent two-sample Student’s t test was used to analyze proliferation and cytokine responses to Fel d 1 and Fel d 1 peptides. One-way ANOVA was used to compare the mean cytokine level for the sum of all 20 peptides between groups and the distribution of mean levels of cytokine responses to all 20 peptides was compared by Wilcoxon matched-pairs signed-ranks test. Proliferation and cytokine responses before and after allergen avoidance were compared using a paired two-sample Student’s t test.

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