

# Altered T Cell Ligands Derived from a Major House Dust Mite Allergen Enhance IFN- $\gamma$ but Not IL-4 Production by Human CD4<sup>+</sup> T Cells<sup>1</sup>

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Changes in the affinity of the interaction between T cell Ag receptors and their ligands can modulate selected T cell effector functions. Since both allergen-specific Th2 and Th0 cells are present in the peripheral CD4<sup>+</sup> T cell pool of atopic individuals, the potential to inhibit cytokine production by Th2 cells and promote Th1-type cytokines from Th0 cells may contribute to the down-regulation of allergic inflammation. The aim of this study was to investigate the effects of peptide analogues of a dominant T cell epitope of the group II allergen derived from house dust mite, residues 28 to 40, on proliferation and cytokine production by human Th2 and Th0 cells. From both functional competition and proliferation assays, using analogues substituted with alanine or charged amino acids, the influence of different positions in p28–40 on TCR recognition and/or MHC class II binding was determined. For the specific Th0 cells, generally those analogues that lead to a reduction in proliferation also decreased both IL-4 and IFN- $\gamma$  production. However, the p28–40 analogues with alanine residues at positions 34 and 36 altered the IFN- $\gamma$ :IL-4 ratio by selectively enhancing IFN- $\gamma$  secretion. In the case of Th2 cells, stimulation with the peptide analogues induced different patterns of effector function. Selected analogues were capable of inducing IL-4 production in the absence of proliferation, whereas in response to other peptide variants of p28–40, both IL-4 production and proliferation were inhibited. *The Journal of Immunology*, 1996, 157: 2160–2165.

The qualitative nature of allergen-specific immune responses in sensitized individuals, based on the analysis of peripheral T cells (1–3) and clinical biopsies (4), suggests that both Th2 and Th0 cells contribute to the functional phenotype of the peripheral CD4<sup>+</sup> T cell repertoire (5). The functional activity of these T cell subsets is characterized by their cytokine response profiles, with Th2 cells producing IL-4, IL-5, IL-10, and IL-13, and Th0 cells synthesizing IL-2, IFN- $\gamma$ , and TGF- $\beta$  in addition to Th2 cytokines (6–9). The induction of allergen-specific IgE requires the presence of Th2-type cytokines, and it is in part the balance between the agonist and antagonist effects of IL-4 and IFN- $\gamma$  that regulates IgE synthesis (10). Therefore, the potential to inhibit IL-4 production and/or promote IFN- $\gamma$  secretion may contribute in the long term to the down-regulation of IgE and be of clinical benefit to individuals with atopic allergy.

In the presence of costimulatory signals, recognition of peptide fragments of allergen bound to MHC class II molecules by TCR is required for the activation of all CD4<sup>+</sup> T cell subsets (11, 12). However, there is evidence indicating that altering the affinity of the interactions between TCRs and their cognate ligands can lead

to the selective activation of T cell effector functions (13–18). This is illustrated by the finding that murine Th1 cells, when stimulated with an altered T cell ligand, failed to produce cytokines or proliferate, yet retained cytolytic activity and up-regulated their IL-2 receptors (13). Furthermore, stimulation of murine Th2 cells with analogues of an immunogenic peptide in which a secondary TCR contact residue had been altered by conservative amino acid substitutions was sufficient to induce IL-4 production and provide B cell help, but not clonal expansion (14). Peptide analogues with partial agonist activity have been reported that are capable, in the presence of live APC, of inducing anergy in both Th1 and Th2 cells. These cells fail to proliferate when restimulated with their natural ligand, although in the case of Th2 cells, their ability to produce IL-4 is retained (15, 16). Moreover, observations made in vivo in mice revealed that structural differences in antigenic peptides or class II molecules of different MHC haplotypes can influence the development of Th1 or Th2 responses (17). In particular, it appeared that high affinity MHC/peptide interactions with the TCR favored the induction of Th1 responses, while interactions of low affinity resulted in the activation of Th2 cells. In a recent report it has been demonstrated that stimulation of T cells in vitro with an altered peptide ligand derived from the myelin proteolipid protein induces Th2 and Th0 cells, which, when adoptively transferred to mice, confer protection from experimental autoimmune encephalomyelitis (19).

The aim of the experiments reported here was to determine whether peptide analogues of a major human T cell epitope of the group II allergen of house dust mite (HDM)<sup>3</sup> *Der p* II (20), residues 28 to 40, with single amino substitutions introduced at each position can modulate T cell proliferation and cytokine production

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<sup>3</sup> Abbreviations used in this paper: HDM, house dust mite; *Der p* II, the group II allergen of *Dermatophagoides pteronyssinus*; [<sup>3</sup>H]dThd, tritiated thymidine.

Table I. Amino acid sequence of wild type and analogues of p28–40

| 28 | I | H | R | G | K | P | F | Q | L | E | A | 40 |
|----|---|---|---|---|---|---|---|---|---|---|---|----|
| I  | I | H | R | G | K | P | F | Q | L | E | A | V  |
| A  | A |   |   |   |   |   |   |   |   |   |   |    |
|    |   | A |   |   |   |   |   |   |   |   |   |    |
|    |   | E |   |   |   |   |   |   |   |   |   |    |
|    |   | K | A |   |   |   |   |   |   |   |   |    |
|    |   |   | E | A |   |   |   |   |   |   |   |    |
|    |   |   | K | E | A |   |   |   |   |   |   |    |
|    |   |   |   | K | E | A |   |   |   |   |   |    |
|    |   |   |   |   | R | K | A |   |   |   |   |    |
|    |   |   |   |   |   |   | K | A |   |   |   |    |
|    |   |   |   |   |   |   |   |   | A |   |   |    |
|    |   |   |   |   |   |   |   |   |   | A |   |    |
|    |   |   |   |   |   |   |   |   |   |   | A |    |
|    |   |   |   |   |   |   |   |   |   |   |   | A  |

by *Der p* II-specific Th2 and Th0 cells isolated from an atopic individual.

## Materials and Methods

### Antigens

The *Der p* II peptides, residues 81 to 96 (VKGQQYDIKYTNVVPK) and 28 to 40 (IIHRGKPFQLEAV), and analogues of p28–40 were synthesized on a Multipetide Synthesis block BT7400 (Cambridge Research Biochemicals, Northwich, U.K.) using 4-(2',4'-dimethoxyphenyl F-moc aminomethyl)phenoxy resins (Novasyn Chemicals Ltd., Nottingham, U.K.) as previously described (21). Couplings were performed using F-moc side chain protected pentafluorophenyl or oxo-benzotriazine amino acid esters. Full-length peptides were cleaved from the resin and deblocked by treatment with TFA/thioanisole/ethanedithiol (94/5/2.5, v/v/v) for 1 h and then precipitated and washed twice with ether. The precipitates were dissolved in 10% formic acid (v/v) and freeze-dried for 48 h. Each peptide produced a single peak by reverse phase HPLC. The peptide analogues used in this study are listed in Table I.

### Isolation of *Der p* II-reactive, DRB1\*1101-restricted, cloned human Th0 and Th2 cells

Cloned T cells of AC1 (Th0) and AC2 (Th2) were isolated from a HDM atopic individual (DRB1\*0701, DRB1\*1101; DQ2, DQ7 (3); DPB1\*0101/0402) by limiting dilution cloning from a long term T cell line as described previously (20). The T cell clones were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin (Life Technologies, Paisley, Scotland), and 5% inactivated human A<sup>+</sup> serum (National Blood Transfusion Service, Edgware, U.K.) by stimulation with the p28–40 peptide at optimal concentrations and irradiated histocompatible PBMC ( $5 \times 10^5$ /ml; 3000 rad) as a source of APCs together with IL-2 (10%, v/v; Lymphocult T, Biotest Folex, Frankfurt, Germany). In all experiments, T cell clones were rested for 7 to 8 days after the last addition of Ag and APCs.

### T cell proliferation assays

Cloned T cells ( $2 \times 10^4$ /well) were cultured in flat-bottom 96-well microtiter trays (Nunc, Life Technologies) with native peptide or analogues of p28–40 (3 and 30  $\mu$ g/ml) in the presence of APCs. Mitomycin C-treated murine fibroblasts expressing HLA-DRB1\*1101 ( $2 \times 10^4$  cells/well) were used for Ag presentation. Cultures were pulsed with tritiated thymidine (1  $\mu$ Ci/well; [<sup>3</sup>H]dThd; Amersham International, Aylesbury, U.K.) after 48 h and harvested 8 to 16 h later. Proliferation was determined by [<sup>3</sup>H]dThd incorporation by liquid scintillation spectroscopy. The results are expressed as mean counts per minute for triplicate cultures, with an SEM < 20%.

### Cellular competition assays

The binding of native p28–40 peptide and analogues to DRB1\*1101 was assessed by their capacity to inhibit the proliferation of a DRB1\*1101-restricted T cell clone (AC29) reactive with p85–97 (20). Peptide analogues of p28–40 (50  $\mu$ g/ml) together with the peptide p81–96 (5  $\mu$ g/ml) were added to mitomycin C-treated murine fibroblasts expressing DRB1\*1101. To these cultures T cells of clone AC29 were added, and proliferation was measured by [<sup>3</sup>H]dThd incorporation. AC29 cells were

isolated and expanded as described above. The results are expressed as the percentage of inhibition of AC29 proliferation relative to that induced by the native p28–40 peptide.

### Measurement of IFN- $\gamma$ and IL-4

Supernatants were collected at 48 h from T cells stimulated with peptide or cultured in medium alone. Triplicate wells were pooled, and IFN- $\gamma$  and IL-4 were measured by ELISA kits as described previously (22).

## Results

### Effect of stimulation with p28–40 peptide analogues on Ag recognition by specific T cell clones representative of the Th0 and Th2 functional phenotypes

The T cell clones AC1 and AC2, both of which are reactive with residues 28–40 of *Der p* II and restricted by HLA-DRB1\*1101, exhibit the cytokine profiles of Th0 and Th2 cells, respectively (20, 23). Initially, AC1 and AC2 T cells were stimulated with peptide analogues of p28–40 synthesized with alanine substituted sequentially at each position (Table I), and the proliferative responses were determined (Fig. 1, A and B). The antigenicity of the analogues with substitutions at positions 31, 32, and 33 was markedly reduced compared with that of the native peptide for both T cell clones, although minor differences in the pattern of proliferation in response to these analogues were observed. For example, the response of AC1 T cells was more susceptible to the alanine substitution at position 31, whereas A<sup>32</sup> had a greater inhibitory effect on T cell recognition by AC2 T cells. The patterns of responsiveness and effector function were not differentially affected by variations in the concentrations of peptides (data not shown). The presence at alanine at other positions in p28–40 had minimal or no effect on the proliferative responses of either of the T cell clones.

To further examine the relative importance of different residues in p28–40 on T cell recognition, in particular those at positions 31, 32, and 33, peptides with charged amino acids were synthesized (24). Arginine (R) occurs at position 31 in the native peptide, but when replaced with either a conservative (K<sup>31</sup>) or a radical (E<sup>31</sup>) substitution, a reduction in the proliferative response of both AC1 (Fig. 1C) and AC2 (Fig. 1D) was observed. Similarly, both T cell clones responded weakly to the peptide analogue in which glycine at position 32 had been replaced with lysine (K<sup>32</sup>). In contrast, although AC2 (Fig. 1D) failed to recognize the analogue E<sup>32</sup>, the response of AC1 was only partially inhibited (Fig. 1C). The conservative substitution of R<sup>33</sup> for K<sup>33</sup> induced proliferation similar to that of the native peptide, whereas E<sup>33</sup> was only weakly antigenic for both T cell clones. With the exception of E<sup>30</sup>, the introduction of charged residues at positions 30, 34, and 35 markedly reduced T cell proliferation.

The modulatory effect of the analogues on T cell recognition may occur as the result of altering either the binding of the peptide to HLA-DRB1\*1101 or the affinity of the peptide-TCR interaction. To test the first of these possibilities, native peptide and analogues of p28–40 were investigated for the ability to competitively inhibit presentation of p81–96 to the T cell clone AC29 (Fig. 2). If the binding capacity of a *Der p* II 28–40 analogue to DRB1\*1101 is not affected by the introduced substitution, it would be expected to equally inhibit proliferation of AC29 cells to the native 28–40 peptide. Indeed, this was the case with analogues substituted at positions 28, 31, 32, 34, 36, 37, and 40. In contrast, A<sup>29</sup>, E<sup>33</sup>, and A<sup>38</sup> were more efficient at inhibiting the proliferation of AC29 cells, suggesting that they bind with higher affinity to DRB1\*1101 than the native peptide. However, since A<sup>30</sup>, R<sup>33</sup>, and A<sup>35</sup> were less effective than native peptide at inhibiting AC29 proliferation, it is likely that they bind with lower affinity to DRB1\*1101. From the combined results of proliferation and functional competition

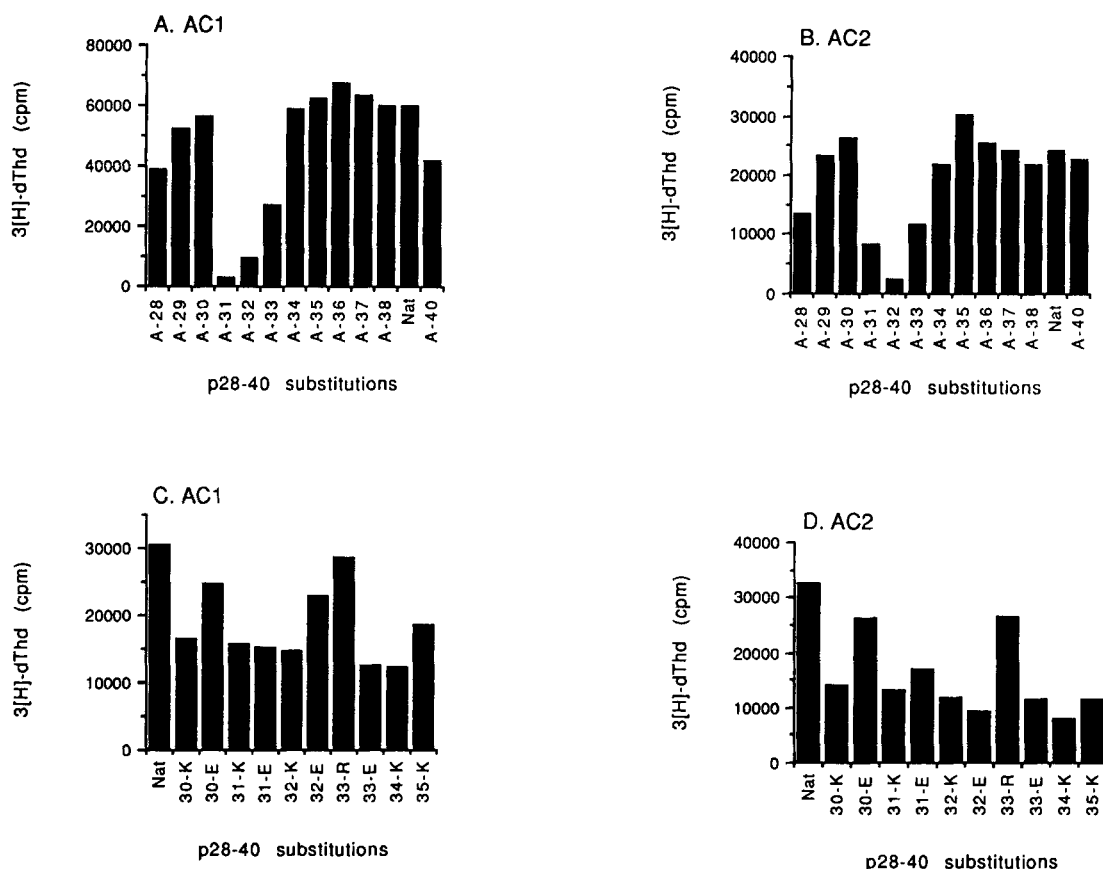


**FIGURE 1.** The proliferative response of *Der p* II (p28–40)-specific Th0 and Th2 cells to peptide analogues of p28–40. T cells of clone AC1 (Th0) and AC2 (Th2) were stimulated with p28–40 peptide analogues in the presence of mitomycin C-treated murine fibroblasts expressing HLA-DRB1\*1101 as a source of APCs. Proliferation was determined by [<sup>3</sup>H]dThd incorporation. Background responses of T cells cultured with APCs in the absence of Ag were <450 cpm.

assays, it appears that amino acids at positions 31 and 32 are critical for T cell recognition by both AC1 and AC2 T cells. In contrast, due to the different capacities of A<sup>33</sup>, E<sup>33</sup>, and R<sup>33</sup> to competitively inhibit AC29 T cell proliferation, position 33 appears to affect T cell recognition as well as binding to MHC class II molecules.

#### *Effect of p28–40 peptide analogues on cytokine production by the T cell clones AC1 (Th0) and AC2 (Th2)*

The p28–40 analogues were investigated for their ability to modulate cytokine production by AC1 (Th0) and AC2 (Th2) cells. In response to stimulation by the p28–40 analogues with alanine at positions 28, 31, 32, 33, and 40, the production of IL-4 by AC1 cells was markedly reduced compared with that elicited by the native peptide (Fig. 3A). Stimulation with A<sup>29</sup> and A<sup>30</sup> also resulted in diminished levels of IL-4 secretion. Although the analogues A<sup>31</sup>, A<sup>32</sup>, and A<sup>33</sup> markedly down-regulated the proliferative response of the Th2 cells (AC2), their inhibitory effects on IL-4 secretion were minimal (Fig. 3B), demonstrating that IL-4 production and proliferation could be dissociated. Background release of IL-4 by both AC1 and AC2 cells in the absence of peptide was <10 pg/ml. The additional analogues with charged amino acid substitutions were also tested for their ability to stimulate IL-4 production by AC1 and AC2 cells. Compared with the native peptide glutamic acid or lysine at positions 31, 32, 34, and 35 as well as E<sup>33</sup> reduced the capacity of AC1 cells to produce IL-4 by 35%

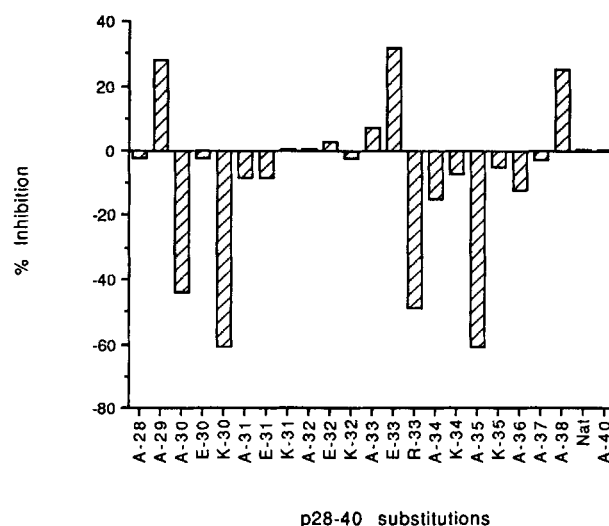


**FIGURE 2.** Assessment of the binding of p28–40 peptide analogues to HLA-DRB1\*1101. Peptide analogues of p28–40 (50  $\mu$ g/ml) were added with the stimulating peptide p81–96 (5  $\mu$ g/ml) to mitomycin C-treated murine fibroblasts expressing DRB1\*1101 ( $2 \times 10^4$ /well). To these and control cultures containing no p28–40-derived peptides, AC29 T cells (p82–96 specific and DRB1\*1101 restricted) were added ( $2 \times 10^4$ /well), and proliferation was determined by [<sup>3</sup>H]dThd incorporation. The results are expressed as the percentage of inhibition relative to that induced by the native p28–40 peptide.

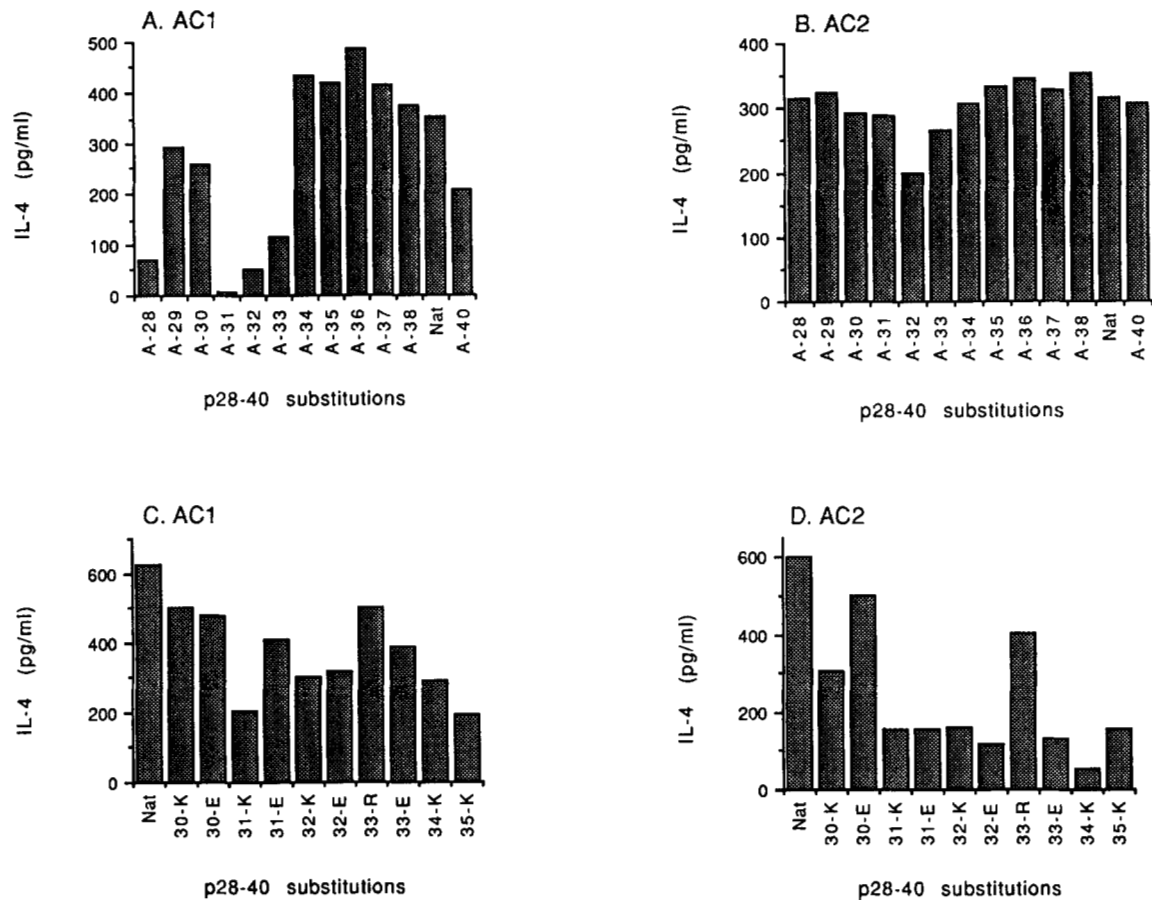


**FIGURE 3.** The effects of peptide analogues on IL-4 production by *Der p* II (p28–40)-specific Th0 and Th2 cells. T cells of clone AC1 (Th0) and AC2 (Th2) were stimulated with p28–40 peptide analogues in the presence of mitomycin C-treated murine fibroblasts expressing HLA-DRB1\*1101 as a source of APCs. Supernatants were collected at 48 h, and the levels of IL-4 were determined by ELISA.

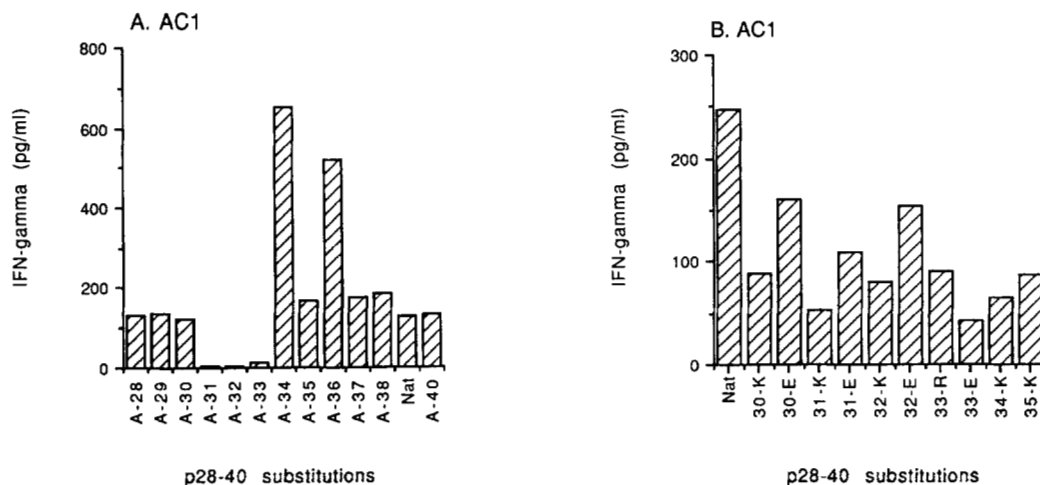


**FIGURE 4.** The effects of peptide analogues on IFN- $\gamma$  production by *Der p* II (p28–40)-specific Th0 cells. T cells of clone AC1 (Th0) were stimulated with p28–40 peptide analogues in the presence of mitomycin C-treated murine fibroblasts expressing HLA-DRB1\*1101 as a source of APCs. Supernatants were collected at 48 h, and the levels of IFN- $\gamma$  were determined by ELISA.

or more (Fig. 3C). The analogues E<sup>30</sup> and K<sup>30</sup> induced IL-4 production by both T cell clones, although for Th2 cells, E<sup>30</sup> was the most potent of these two peptides (Fig. 3, C and D). Analogues with charged amino acids at positions 31, 32, and 33 reduced IL-4 production by AC2 cells, although in the case of R<sup>33</sup>, IL-4 secretion was diminished by only 33% (Fig. 3D).

The ability of p28–40 peptide analogues to stimulate IFN- $\gamma$  production by Th0 cells (AC1) was also investigated. The analogues A<sup>31</sup>, A<sup>32</sup>, and A<sup>33</sup> all failed to induce IFN- $\gamma$  production (Fig. 4A), and similarly, stimulation by peptides with charged residues at these positions was also unable to elicit the release of IFN- $\gamma$ . Substitutions with alanine at positions 28, 29, 30, 35, 37,

38, and 40 induced the same level of IFN- $\gamma$  secretion as the native peptide (Fig. 4A). In contrast, A<sup>34</sup> and A<sup>36</sup> increased IFN- $\gamma$  secretion compared with native p28–40, with enhancements of five- and fourfold, respectively (Fig. 4A). None of the analogues with charged residue substitutions was as potent as the native peptide at inducing IFN- $\gamma$  secretion (Fig. 4B); however, the IFN- $\gamma$  response profile of AC1 cells was similar to that observed for IL-4 in response to stimulation with these analogues. Undetectable levels of IFN- $\gamma$  were produced by AC1 cells cultured with APCs in the absence of peptide.

## Discussion

The potential to modulate the qualitative nature of immune responses with structural analogues of antigenic peptides was investigated in vitro for T cell responses to a major human T cell epitope of the HDM. In atopic disorders it may be possible to achieve clinical improvement by decreasing the ratio of Th2 to Th1 cytokines. This change could be accomplished by either a quantitative reduction in allergen-specific Th2 responses or the selective induction of Th1 cytokines by allergen-reactive Th0 cells, both present in the T cell repertoire of atopic individuals (25). This prompted us to analyze peptide analogues of *Der p* II, residues 28 to 40, for their antigenicity and ability to stimulate cytokine production by DRB1\*1101-restricted T cell clones representative of the Th0 and Th2 functional phenotypes (20).

The substitution of single amino acids with alanine at each position in p28–40 had similar effects on the pattern of proliferation of both Th0- and Th2-specific cells and markedly reduced the response only when introduced at each of three positions (31, 32, and 33). In contrast, analogues containing positively and negatively charged amino acid substitutions had consistently negative effects on Ag recognition, although this was less pronounced when the substituted natural amino acid was of a similar charge. These results taken together with the MHC-peptide binding studies suggest that the residues at positions 31 and 32 are critical for TCR recognition. The capacity of analogues with different amino acid substitutions at position 33 to bind with varying affinities to class II MHC implies that this position makes contact with DRB1\*1101. However, the residue at this position may also contact TCR, since the analogue A<sup>33</sup> appears to bind to DRB1\*1101 with an affinity similar to that of the native peptide, but is less antigenic. These conclusions are consistent with earlier findings suggesting that for both AC1 and AC2 T cells, the peptide core for TCR recognition may be located between residues 28 and 35, whereas sequences 22 to 28 or 35 to 40 may be implicated mainly in binding to HLA-D region molecules (20). Results from the functional competition assays imply that, in addition to residue 33, those amino acids at positions 29, 30, 35, and 38 in p28–40 contribute to peptide binding to DRB1\*1101. These residues either directly contact MHC or influence the binding capacity of the peptide indirectly by altering the conformation of the side chains of adjacent amino acids. For peptides bound to HLA-DRB1\*1101, a potential motif has been suggested, consisting of three main anchoring positions, an aromatic residue at position 1 followed by a hydrophobic and a positively charged amino acid at positions 4 and 6, respectively (26, 27). Assuming that the aromatic residue H<sup>30</sup> in p28–40 corresponds to the relative position 1, the residues implicated in MHC binding found at positions 4 and 6 would be K<sup>33</sup> and F<sup>35</sup>. However, although the residues at these positions appear to contact MHC, their natures would not comply with the proposed binding motif for DRB1\*1101, and this may account for the ability of *Der p* II 28–40 to bind to other class II molecules, including HLA-DQ (20). MHC class II-restricted T cell recognition of peptides not

exhibiting the binding requirements for that particular MHC class II specificity have been described (28).

Rules analogous to those governing MHC class II-peptide binding also appear to influence the outcome of TCR interactions with altered peptide ligands. It has been demonstrated that analogues of an antigenic peptide with the primary TCR contact unaltered but containing selected substitutions at secondary TCR contact residues could act as agonists or partial agonists for specific Th cells (13, 14). Furthermore, unrelated peptides with sequence similarities at these positions were found to be capable of eliciting similar functional activity. Nevertheless, TCR recognition remains a uniquely specific phenomenon, since Th cells with different fine specificities presented a variable pattern of responsiveness to the same ligands (29).

In addition to the effects of alterations in the structure of the p28–40 peptide on Ag recognition, the effects on cytokine production by both Th0 cells and Th2 cells were analyzed. Despite the similarity in the proliferative responses of both subsets of specific Th cells to the p28–40 analogues, the pattern of IL-4 secretion varied markedly. In response to the p28–40 analogues, Th0 cells induced IL-4 production, which generally reflected the pattern of clonal expansion, although minor quantitative differences were noted. In contrast, stimulation of Th2 cells with selected p28–40 analogues induced IL-4 secretion in the absence of proliferation. Although differences in the fine specificity of the two T cell clones may account for this disparity, it cannot be excluded that stimulation with the altered T cell ligands may have different effects on the signaling pathways that induce IL-4 production in both Th0 and Th2 cells. Stimulation of Th0 cells with p28–40 analogues containing alanine at position 34 or 36 resulted in a selective enhancement of IFN- $\gamma$  secretion that was independent of IL-4 production and the degree of proliferation. Previous reports have demonstrated that proliferation and cytokine production in murine Th2 cells have distinct thresholds of induction and can be selectively triggered (16). In particular, IL-4 synthesis appears to be favored by signals provided by less potent TCR-ligand interactions, as in the case of reduced ligand density (30) or by stimulation with peptides of low affinity for TCR or MHC class II (13, 17, 18). Furthermore, IL-4 secretion by Th2 cells seems to have less stringent requirements for coreceptor or accessory molecule-mediated costimulation. It has been observed that murine Th2 cells retained their capacity to secrete IL-4 when stimulated by specific peptide on chemically fixed APCs (16). It appears also that certain anti-CD4 Abs that inhibited Ag-dependent proliferation of p28–40-specific Th2 cells failed to down-regulate IL-4 secretion (23). Moreover, when activated in the absence of costimulatory signals from APCs, Th0 cells adopted the cytokine profile of Th2 cells, implying that the Th2 phenotype correlates with the induction of anergy in Th0 cells (31).

The potential use of altered T cell ligands to modulate the effector function of Th2 cells may be of limited value, since selective inhibition of IL-4 production has not been achieved. However, when *Der p* II-specific Th0 cells were stimulated with p28–40 analogues (A<sup>34</sup> and A<sup>36</sup>), IFN- $\gamma$  production was enhanced fivefold compared with that of the native ligand, whereas IL-4 production remained unchanged. The importance of regulating the balance between Th1- and Th2-derived cytokines was demonstrated in a recent clinical study, in which it was reported that successful desensitization with bee venom was accompanied by alterations in the IFN- $\gamma$ :IL-4 ratios (32). Increased expression of mRNA for Th1-type cytokines at the site of late phase cutaneous reactions was described in successfully treated individuals (4). Therefore, an alternative strategy to beneficially intervene in atopic disorders

may be by targeting Th0 cells in an attempt to alter the IL-4:IFN- $\gamma$  ratio in favor of IFN- $\gamma$ .

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