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Modulation of Th2 Responses by Peptide Analogues in a Murine Model of Allergic Asthma: Amelioration or Deterioration of the Disease Process Depends on the Th1 or Th2 Skewing Characteristics of the Therapeutic Peptide[1]

Edith M. Janssen,*† Antoon J. M. van Oosterhout,† Annemiek J. M. L. van Rensen,‡ Willem van Eden,* Frans P. Nijkamp,† and Marca H. M. Wauben*[2]

Allergen-specific CD4$^+$ Th2 cells play an important role in the immunological processes of allergic asthma. Previously we have shown that, by using the immunodominant epitope OVA$_{323–339}$, peptide immunotherapy in a murine model of OVA induced allergic asthma, stimulated OVA-specific Th2 cells, and deteriorated airway hyperresponsiveness and eosinophilia. In the present study, we defined four modulatory peptide analogues of OVA$_{323–339}$ with comparable MHC class II binding affinity. These peptide analogues were used for immunotherapy by s.c. injection in OVA-sensitized mice before OVA challenge. Compared with vehicle-treated mice, treatment with the Th2-skewing wild-type peptide and a Th2-skewing partial agonistic peptide (335N-A) dramatically increased airway eosinophilia upon OVA challenge. In contrast, treatment with a Th1-skewing peptide analogue (336E-A) resulted in a significant decrease in airway eosinophilia and OVA-specific IL-4 and IL-5 production. Our data show for the first time that a Th1-skewing peptide analogue of a dominant allergen epitope can modulate allergen-specific Th2 effector cells in an allergic response in vivo. Furthermore, these data suggest that the use of Th1-skewing peptides instead of wild-type peptide may improve peptide immunotherapy and may contribute to the development of a successful and safe immunotherapy for allergic patients. *The Journal of Immunology,* **2000, 164: 580–588.**

Nowadays, there is convincing evidence that allergen-specific CD4$^+$ Th2 cells play a key role in allergic asthma. Th2-associated cytokines such as IL-4, IL-5, IL-9, and IL-13 are known to be involved in the development of allergen-specific Th2 cells (1), IgE production (2, 3), airway eosinophilia (4, 5), and airway hyperresponsiveness (6, 7). Consequently, the inhibition or modulation of allergen-specific Th2 cells and their cytokines has become an attractive target for novel therapeutic intervention strategies in allergy.

Previously it has been shown that allergen immunotherapy, by s.c. administration of increasing doses of allergen, can inhibit clinical symptoms and allergen-specific Th2 cytokine production upon challenge with the allergen (8–10). Although this classic form of immunotherapy is beneficial for treatment of rhinitis and insect venom allergy, it is less effective in allergic asthma (11). Furthermore, it has been reported that s.c. administration of allergens can induce severe systemic reactions, due to cross-linking of allergen-specific IgE on mast cells (12, 13). To prevent this cross-linking of IgE during immunotherapy, interest has focused on immunotherapy using small synthetic peptides of immunodominant allergen-derived T cell epitopes. It has been shown in a murine model that s.c. pretreatment with Fel d I peptide can prevent immediate hypersensitivity and airway hyperresponsiveness (14). Moreover, in the first clinical immunotherapy studies using peptides of immunodominant T cell epitopes of the major bee and cat allergens, amelioration of airway symptoms in humans (15, 16), which coincided with a reduced Th2 cytokine production in vitro, has been described (15, 17). However, Fel d I peptide therapy provoked side effects in 65% of the patients directly after injection with peptide (16). Recently, we showed in an experimental model of allergic asthma that s.c. treatment of OVA-sensitized mice with the immunodominant OVA$_{323–339}$ epitope deteriorated both airway function and airway inflammation upon exposure to OVA (18). These findings indicate that immunotherapy using allergen-derived peptides, after allergen sensitization had already occurred, has the potential to activate allergen-specific Th2 cells, leading to an unfavorable enhanced Th2 cell response upon challenge with the allergen.

An alternative and safer approach for peptide immunotherapy could be the development of peptide analogues of the wild-type (WT)[3] epitope bearing T cell modulatory capacities. Several studies have shown that stimulation of T cells with T cell epitope-derived peptide analogues can result in changes in the effector function of T cells, e.g., dissociation of proliferation and cytokine production, shifts in cytokine profile, or induction of anergy (19–21). There is ample evidence that these changes are caused by an altered interaction between the MHC/peptide-TCR complex, which affects the signaling through the TCR (22). Although the exact mode of action is largely unclear and the prediction of

*Institute of Infectious Diseases and Immunology, Department of Immunology, Faculty of Veterinary Medicine, and Departments of †Pharmacology and Pathophysiology and ‡Pharmaceutics, Faculty of Pharmacy, Utrecht University, Utrecht, The Netherlands

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[2] Address correspondence and reprint requests to Dr. M. H. M. Wauben, Institute of Infectious Diseases and Immunology, Department of Immunology, Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80165, 3508 TD Utrecht, The Netherlands. E-mail address: M.Wauben@VET.UU.NL

[3] Abbreviations used in this paper: WT, wild type; BAL, bronchoalveolar lavage; LN, lymph node.

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modulatory peptide analogues is still poor, the use of Th1-skewing or anergy-inducing peptide analogues for immunotherapy in allergic diseases has a great potential. Besides the prevention of cross-linking of IgE on mast cells, peptide therapy with Th1-skewing or anergy-inducing peptides could also prevent the induction of adverse side effects which can occur after WT peptide therapy.

In this paper, we have defined peptide analogues of an allergen-derived immunodominant epitope that modified allergen-specific Th2 cells in vitro as well as a Th2-dominated allergic response in vivo. We used a murine model of allergic asthma in which sensitization of BALB/c mice with OVA before OVA challenge resulted in airway hyperresponsiveness, eosinophilia, OVA-specific IgE, and production of Th2 cytokines upon OVA restimulation in vitro (23, 24). The immunodominant epitope of OVA, $OVA_{323–339}$, is recognized by a population of OVA-specific Th2 cells, which produces large amounts of IL-5 upon stimulation with $OVA_{323–339}$ in vitro and which plays an important role in the development of airway symptoms in vivo (18).

Our present data show that peptide immunotherapy with a Th1-skewing peptide analogue inhibited allergen-specific Th2 responses and airway inflammation, whereas a Th2-skewing peptide analogue, like the WT peptide, aggravated the ongoing allergic immune response.

Materials and Methods

Peptides

$OVA_{323–339}$ WT peptide (ISQAVHAAHAEINEAGR) was obtained from Isogen Bioscience (Maarn, The Netherlands). Peptide analogues of $OVA_{323–339}$ were synthesized as single alanine substitutions of all nonalanines. As control, peptide $HA_{126–138}$ (HNTNGVTAASSHE) was used. Peptide analogues and control peptides were synthesized by automatic multiple peptide synthesis (25). For use in the MHC-peptide binding assay, marker peptide $HA_{126–138}$ was biotinylated during synthesis. Peptides were analyzed, purified via reversed-phase HPLC, and checked by fast atom bombardment mass spectrometry.

Animals

Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments. Specified pathogen-free male BALB/c mice (6–8 wk old) were obtained from the Central Animal Laboratory (Utrecht, The Netherlands) and were housed in macrolon cages and provided with OVA-free food and water ad libitum. $OVA_{323–339}$ TCR transgenic DO11.10 (26) mice were a gift from Prof. L. Adorini (Roche Milano Ricerche, Milan, Italy)

MHC class II binding assay

MHC class II binding assays were performed as described before (27). Briefly, murine MHC class II I-A^d molecules were purified from A20 hybridoma cell lysates through affinity chromatography using the MKD6 Ab (28). Purified MHC molecules were dissolved in PBS containing 0.1% azide and 1% *n*-β-octyl glucopyranoside (Sigma, St. Louis, MO). A total of 3 μM purified MHC class II I-A^d molecules were incubated with 200 nM of biotinylated $HA_{126–138}$ and a dose range of competitor peptide (WT or analogue; 0.5–250 μM) at pH 5 for 48 h at room temperature in the presence of a protease inhibitor mix (final concentration 4.3 μM PMSF, 0.33 μM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, 0.35 μM L-*p*-tosylamina-2-phenylethyl chloromethyl ketone, 10 μM *N*-ethyl maleimide, 2.6 μM ethylene diamine-tetra-acetic acid, 13 μM 1.10 phenanthroline, and 0.73 μM pepstatin A). Samples were analyzed by SDS-PAGE under nonreducing conditions and blotted onto nitrocellulose (Hybond-ECL, Amersham, Bucks, U.K.). Biotinylated peptide was visualized through enhanced chemiluminescence (Western blot ECL kit, Amersham), and IC_{50} values for the binding of biotinylated $HA_{126–138}$ for each peptide were determined.

Cells and culture medium

$OVA_{323–339}$-specific DO11.10 T cells were obtained by negative selection. DO11.10 splenocytes were incubated for 1 h with Dynabeads (Dynal, Oslo, Norway) coupled to Abs to MHC class II I-A^d (MK-D6; Ref. 28), B220 (RA3–6B2; Ref. 29), and FcγRII/III (24G2; Ref. 30) at 4°C. Negative selection was performed by two passes over a magnetic particle concentrator (Dynal). Cells obtained after depletion were shown to be >90% $OVA_{323–339}$-specific T cells as demonstrated by FACS analysis using the clonotype-specific Ab KJ1.26 (31). Viability of the cells was >95%. T cells were cultured in Iscove's medium supplemented with 10% FCS, 2 nM L-glutamine, 100 IU penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME (Iscove's+).

Activation of DO11.10 T cells

To investigate the primary response of T cells toward the different peptides, 1×10^5 freshly isolated DO11.10 T cells were cultured with 2×10^5 irradiated BALB/c splenocytes (APC; 3000 rad) and a dose range of peptide (0.01–10 μg/ml) in 96-well plates. After 24, 48, 72, and 120 h supernatant was collected for cytokine analysis. At the same time points proliferation was determined in parallel cultures by pulsing the cells for another 16 h with [^{3}H]thymidine. Cells were harvested on fiberglass filters, and [^{3}H]thymidine incorporation was determined by liquid scintillation spectroscopy.

To study the capacity of the peptides to skew naive cells toward Th1 or Th2 cells, 4×10^6 freshly isolated DO11.10 T cells were cultured with 10 μg/ml peptide (WT or analogue) in the presence of 4×10^6 APC in a total volume of 2 ml. After overnight culture, cells were washed, dead cells were removed by Ficoll gradient (Lympholyte-M, Cedarlane Laboratories, Hornby, Ontario, Canada), and the remaining cells were cultured in Iscove's+ for either 5 or 7 days. Cells were restimulated in 96-well plates (1×10^5 T cells + 2×10^5 APC/well) with a dose range (0.01–10 μg/ml) of WT peptide. Cytokine production and proliferation were determined after 24, 48, and 72 h of culture.

To determine the modulatory effects of the peptide analogues on differentiated T cells, Th2 cells were generated. DO11.10 T cells were cultured at 4×10^6 per well with 4×10^6 APC and 1 μg/ml WT peptide in a total volume of 2 ml. After 7 days cells were washed, and dead cells were removed by Ficoll gradient. Cells were restimulated with APC and WT peptide under the same conditions used for the initial stimulation. After the third cycle of stimulation, T cells displayed a Th2 phenotype (high IL-4, IL-5, and intermediate IFN-γ production). Subsequently, these Th2 cells were restimulated in 96-well plates (1×10^5 T cells + 2×10^5 APC/well) with a dose range (0.01–10 μg/ml) of WT peptide or peptide analogue, and cytokine production was determined after 48 and 72 h of culture.

Cytokine analysis

IL-2 production was measured using the IL-2-dependent CTLL2 clone (32). A total of 1×10^4 CTLL2 cells were cultured with 100 μl supernatant collected from the DO11.10 T cell cultures. After 24 h of culture, cells were pulsed for 16 h with [^{3}H]thymidine, and proliferation was determined. Levels of IL-4, IL-5, IL-10, and IFN-γ in DO11.10 culture supernatants were determined by capture ELISA (PharMingen, San Diego, CA) as described by the manufacturer. The detection limits of the ELISAs were 16 pg/ml for IL-4 and IL-5, and 100 pg/ml for IL-10 and IFN-γ.

Flow cytometric analysis of T cell activation

For flow cytometric analysis of T cell activation, 1×10^5 freshly isolated DO11.10 T cells were cultured in the presence of 2×10^5 irradiated BALB/c splenocytes (APC) and 1, 5, or 10 μg/ml peptide in 96-well plates. After 24 h incubation, cells were collected and incubated with PBS buffer containing 5% rat serum and 1:500 diluted supernatant of 24G2 hybridoma cells (αFcγRII/III). Subsequently, cells were stained with annexin-V-FITC, Abs against Thy1.2-PE (clone 30-H12; 1:200), CD69-FITC (clone H1.2F3; 1:200), CD80-FITC (clone 1G10; 1:100), CD86-FITC (clone GL1; 1:100), or the relevant isotype controls (PharMingen). Dead cells were excluded using propidium iodide. Cells were analyzed on a FACS scan apparatus using Cell Quest (Becton Dickinson, San Jose, CA).

Preparation of peptide-liposomes

Liposomes were prepared as described by 't Hart et al. (33) with minor modifications. Briefly, phospholipids and cholesterol (egg-yolk phosphatidyl choline (EPC): egg-yolk phospatidyl glycerol (EPG): cholesterol = 10:1:4 molar ratio) were dissolved in a mixture of chloroform and methanol (3:1 v/v) in a round-bottom flask. The solvent was removed through rotary evaporation at 40–50°C under reduced pressure for 1–2 h. Lipid films were subsequently flushed with nitrogen for at least 20 min. The lipids were hydrated with a small volume of the peptide solution (25 mg/ml in PBS) by vigorously shaking at room temperature (initial phospholipid concentrations were >100 mM). Glass beads were added for optimal dispersion of the lipid film. Subsequently, the dispersions were diluted with small aliquots of PBS. The external phase was removed via three rounds of ultracentrifugation (20,000 × *g* for 30 min at room temperature). Final liposome pellets were dispersed in

Table I. *Proliferation and cytokine production by freshly isolated DO11.10 T cells after culture with $OVA_{323–339}$ peptide analogues*[a]

	Proliferation ($\times 10^3$)	IL-2 (U/ml)	IFN-γ (pg/ml)	IL-4 (pg/ml)	IL-5 (pg/ml)
Medium	1.6 ± 0.8	<0.01	ND	ND	ND
WT	128.9 ± 17.9	27.9 ± 5.7	680 ± 81	219 ± 38	673 ± 68
323I-A	134.6 ± 21.9	28.2 ± 6.4	669 ± 73	208 ± 40	676 ± 89
324S-A	128.2 ± 20.3	25.8 ± 5.5	652 ± 74	215 ± 38	679 ± 65
325Q-A	127.9 ± 18.4	27.6 ± 4.0	586 ± 126	217 ± 33	661 ± 126
327V-A	126.3 ± 18.3	28.0 ± 4.8	663 ± 78	211 ± 31	705 ± 103
328H-A	21.4 ± 4.2	1.4 ± 0.7*	157 ± 43*	ND*	ND*
331H-A	1.8 ± 0.7	<0.01*	ND*	ND*	ND*
333E-A	1.8 ± 0.7	<0.01*	ND*	ND*	ND*
334I-A	123.0 ± 22.0	28.6 ± 4.8	700 ± 69	218 ± 28	695 ± 83
335N-A	21.7 ± 8.3	5.0 ± 1.6*	186 ± 35*	88 ± 12*	235 ± 41*
336E-A	201.8 ± 19.8	48.6 ± 5.2*	1093 ± 93*	217 ± 48	664 ± 47
338G-A	123.1 ± 14.1	27.6 ± 4.8	687 ± 62	213 ± 46	684 ± 124
339R-A	127.6 ± 17.0	28.9 ± 5.1	669 ± 69	219 ± 39	693 ± 95

[a] Freshly isolated DO11.10 T cells were cultured with WT peptide or peptide analogue (0.1 μg/ml for proliferation; 1 μg/ml for cytokine production) and irradiated BALB/c splenocytes (APC). After 72 h of culture, proliferation was determined by pulsing the cells with [^{3}H]thymidine for another 16 h. At the same time point, the cytokines in the supernatant were determined in parallel cultures. IFN-γ, IL-4, and IL-5 were detected by ELISA, and IL-2 was detected by the IL-2-dependent CTLL2 line as described in *Materials and Methods*. Results are represented as mean ± SEM of four independent experiments. ND, not detectable.

*, $p < 0.05$ compared with stimulation with WT peptide (Dunnett).

PBS. By preparing the liposomes in this way, high-encapsulation efficiencies of the peptides could be achieved (routinely, 40–50% encapsulation as assayed via HPLC analysis).

Disease induction and treatment protocol

Active sensitization was performed without adjuvant by 7 i.p. injections of 10 μg OVA (grade V, Sigma) in 0.5 ml saline on alternate days (23). Peptide treatment was performed 14 days after the last sensitization by s.c. injection of 150 μl liposomes containing 300 μg of peptide. Starting six days after liposome administration, mice were exposed to OVA (2 mg/ml) or saline aerosol challenge for 5 min on 8 consecutive days. Aerosols were performed in a Plexiglas exposure chamber coupled to a Jet nebulizer (Pari IS-2 Jet nebulizer, Pari Respiratory Equipment, Richmond, VA; particle size 2–3 microns) driven by compressed air at a flow rate of 6 L/min. Aerosols were given in groups of a maximum of six animals.

Determination of OVA-specific Igs

Serum samples were taken 24 h after the last challenge, and OVA-specific IgG1, IgG2a, and IgE were determined by ELISA as described before (18, 34). The detection limits of the ELISA were 0.005 U/ml for IgG1, 0.05 U/ml for IgG2a, and 0.5 U/ml for IgE.

Analysis of bronchoalveolar lavage (BAL) cells

After blood collection, a cannula was placed in the trachea, and lungs were lavaged five times with 1 ml aliquots of pyrogen-free saline warmed to 37°C. The lavage cells were washed with cold PBS, and the cells were resuspended in 150 μl cold PBS. A Burker-Türk chamber was used to count the total number of BAL cells. For differential BAL cell counts, cytospin preparations were made and stained with Diff-Quick (Merz & Dade, Düdingen, Switzerland). Per cytospin, 400 cells were counted and differentiated into mononuclear cells, lymphocytes, neutrophils, and eosinophils by standard morphology.

Cytokine analysis of lung-draining lymph node (LN) cells

Twenty-four hours after the last aerosol, lung-draining LN were collected, and single cell suspensions were made. Cells (2×10^5 cells/well in 96-well plates) were cultured in Iscove's+ in the presence of OVA (10 μg/ml), medium, or control Ag hen egg lysozyme (Sigma; 10 μg/ml). As a positive control, cells were cultured with immobilized CD3 Ab (αCD3; clone 17A2, 50 μg/ml; Ref. 24). After 120 h of culture, supernatants were collected for cytokine analysis.

Statistics

Unless stated otherwise, data are expressed as mean ± SEM and evaluated using two-way ANOVA and then a Dunnett test for comparison between two groups. A p value <0.05 was considered statistically significant.

Results

Selection of T cell modulatory $OVA_{323–339}$ peptide analogues

Twelve peptide analogues were synthesized based on single alanine substitutions of all nonalanine residues in peptide $OVA_{323–339}$ (WT peptide). First, peptide analogues were tested for their ability to induce proliferation and cytokine production in freshly isolated DO11.10 T cells. Peptide analogues 323I-A, 324S-A, 325Q-A, 327V-A, 334I-A, 338G-A, and 339R-A induced T cell proliferation comparable to that of WT peptide (Table I). Peptides 331H-A and 333E-A did not induce proliferation (Table I), and higher concentrations (up to 500 μg/ml) could not restore proliferation (data not shown). Peptides 328H-A and 335N-A induced proliferation, but were 20–50 times less potent than WT peptide. In contrast, peptide 336E-A had a superagonistic effect and was significantly more potent than WT peptide in inducing proliferation (Table I).

Similar results were found for IL-2, IFN-γ, IL-4, and IL-5 production (Table I). Peptide analogues that induced proliferation comparable to that of WT peptide also induced comparable levels of IL-2, IFN-γ, IL-4, and IL-5 (Table I). Corresponding with the lack of proliferation, cytokine production was undetectable after stimulation with peptides 331H-A, and 333E-A. The partial agonistic peptide 328H-A could only induce detectable levels of IL-2 and IFN-γ, which were significantly reduced compared with levels after WT peptide incubation, whereas partial agonistic peptide 335N-A induced significantly reduced levels of IL-2, IFN-γ, IL-4, and IL-5. Interestingly, the superagonistic peptide 336E-A was more potent than the WT peptide for inducing IL-2 and IFN-γ, but both IL-4 and IL-5 levels were comparable with those from WT peptide stimulation. This indicates that the 336E-A peptide was not only more potent in activating DO11.10 T cells, but also induced a shift in the cytokine profile. Proliferation and cytokine data are shown for incubation with respectively, 0.1 and 1 μg/ml peptide. The characteristics of the peptides did not change in the entire dose range (0.01–10 μg/ml) tested (data not shown). Based on these data, peptide analogues that induced changes in T cell proliferation and cytokine production compared with results achieved with WT peptide (peptides analogues 328H-A, 331H-A, 333E-A, 335N-A, and 336E-A) were selected for further study.

Table II. *Relative affinity of $OVA_{323–339}$ and $OVA_{323–339}$ peptide analogues for MHC class II I-A^d binding*[a]

Peptide	Abbreviation	IC_{50} (μM)
ISQAVHAAHAEINEAGR	WT	1–3
ISQAVAAAHAEINEAGR	328H-A	180–246
ISQAVHAAAAEINEAGR	331H-A	1–2
ISQAVHAAHAAINEAGR	333E-A	3–5
ISQAVHAAHAEIAEAGR	335N-A	1–3
ISQAVHAAHAEINAAGR	336E-A	1–4

[a] The relative peptide-MHC binding affinities were determined in a MHC class II I-A^d peptide-binding assay on isolated detergent-solubilized I-A^d molecules. Purified MHC class II I-A^d molecules (3 μM) were incubated with 200 nM biotinylated $HA_{126–138}$ peptide and a dose range of WT peptide or peptide analogues. Peptide binding was analyzed after 48 h, as described in *Materials and Methods*, and IC_{50} values were determined for each peptide (concentration of competitor peptide (μM) resulting in 50% inhibition of the binding of biotinylated $HA_{126–138}$ peptide to MHC class II).

To determine whether the observed modulatory effects on T cell activation were due to altered MHC binding affinity of the peptide analogues rather than altered TCR interaction, the selected peptide analogues 328H-A, 331H-A, 333E-A, 335N-A, and 336E-A were tested for relative MHC class II I-A^d binding affinity. All peptide analogues, except peptide 328H-A, had a similar MHC binding affinity to that of WT peptide ($IC_{50} < 1–5$ μM; Table II). Because 328H-A had a strongly decreased affinity for MHC class II, the final panel of peptide analogues for further study consisted of WT peptide, 331H-A, 333E-A, 335N-A, and 336E-A.

Flow cytometric analysis of peptide analogue-induced T cell activation

To study T cell activation, freshly isolated DO11.10 T cells were cultured overnight with 10 μg/ml peptide (WT or analogue) and

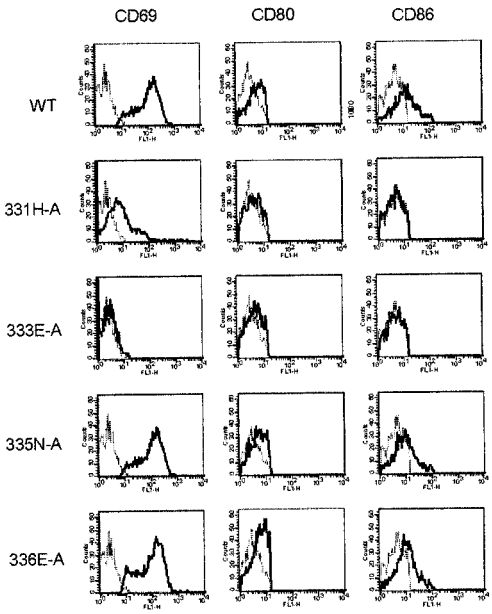

FIGURE 1. Flow cytometric analysis of freshly isolated DO11.10 T cells after stimulation with WT peptide or peptide analogues. DO11.10 T cells were cultured overnight with peptide (10 μg/ml) and APC. FACS analysis was performed using a double staining for Thy1.2-PE and CD69, CD80, or CD86-FITC. Dotted line histograms represent incubation with the nonrelated control peptide $HA_{126–138}$. Solid line histograms represent incubation with WT peptide or peptide analogue. Background staining of isotype controls was comparable to unstained cells (data not shown). The results of a representative experiment are shown. Results were reproduced in five independent experiments.

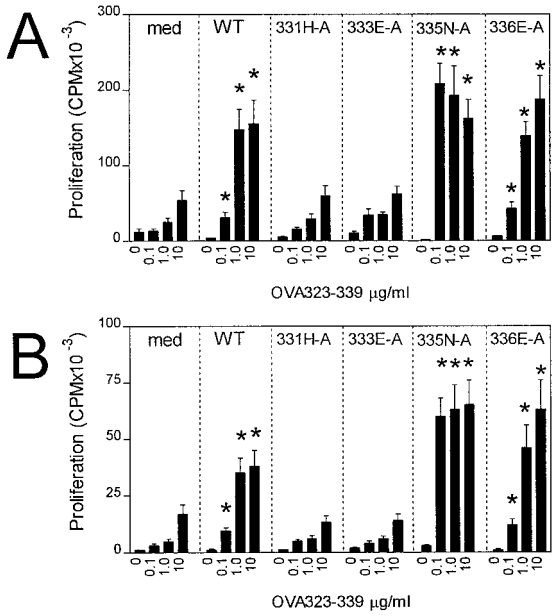

FIGURE 2. Effect of peptide analogue preincubation on proliferation and IL-2 production by DO11.10 T cells upon WT peptide restimulation. Freshly isolated DO11.10 T cells were preincubated with 10 μg/ml peptide (WT or analogues) in the presence of APC, expanded, and after 5 days restimulated with various concentrations of WT peptide and APC. *A*, Proliferation of DO11.10 T cells to WT peptide after preincubation with WT or peptide analogues. Proliferation was assayed by determining [^{3}H]thymidine incorporation and expressed as cpm. Responses to immobilized αCD3 were >40.0 cpm in all conditions (data not shown). *B*, Corresponding IL-2 production of DO11.10 T cells upon restimulation with WT peptide. IL-2 production was assayed using the IL-2-dependent cell line CTLL2. CTLL2 cells were cultured with 100 μl supernatant for 24 h. Proliferation was determined by [^{3}H]thymidine incorporation and expressed as cpm. Data are represented as mean ± SEM of four independent experiments. *, $p < 0.05$ compared with medium preincubation (Dunnett).

APC. T cells were stained with anti-Thy1.2-PE and analyzed for CD69, CD80, CD86, and annexin-V expression using FITC-labeled mAbs.

Compared with a nonrelated control peptide (Fig. 1, dotted-line histograms), incubation with WT peptide resulted in a strong expression of CD69 on T cells. Furthermore, up-regulation of B7.2 (CD86) was seen, whereas B7.1 (CD80) levels remained low. Analysis of the two peptide analogues that did not induce proliferation or cytokine production (Table I) showed that incubation with peptide 331H-A induced an up-regulation of CD69, but B7.1 and B7.2 expression remained low, whereas incubation with peptide 333E-A did not result in any up-regulation of CD69, B7.1, or B7.2 (Fig. 1). Incubation with the partial agonistic peptide 335N-A and the superagonistic peptide 336E-A led to up-regulation of CD69 and B7.2 comparable to that found after incubation with WT peptide. Similar results were obtained after incubation with 1 or 5 μg/ml peptide. Furthermore, none of the tested peptides induced apoptosis as measured by annexin-V/propidium iodide double staining (data not shown).

Analysis of the ability of peptide analogues to skew naive cells toward Th1 or Th2 phenotypes

In subsequent experiments, we tested the ability of the peptide analogues to skew freshly isolated DO11.10 T cells toward a Th1 or Th2 phenotype. T cells were preincubated with 10 μg/ml peptide (WT or analogue) and APC, expanded, and subsequently restimulated with WT peptide. T cells preincubated with medium,

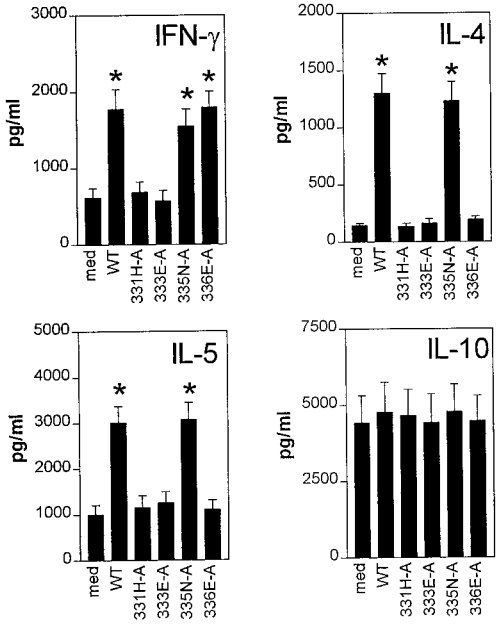

FIGURE 3. Effect of peptide analogue preincubation on cytokine production by DO11.10 T cells upon WT peptide restimulation. Freshly isolated DO11.10 T cells were preincubated with 10 μg/ml peptide (WT or analogues) in the presence of APC, expanded, and after 5 days restimulated with 1 μg/ml WT peptide and APC. IFN-γ, IL-4, IL-5, and IL-10 production was determined in supernatants after 72 h of culture by ELISA. The results are represented as mean ± SEM of three independent experiments. *, $p < 0.05$ compared with preincubation with medium (Dunnett).

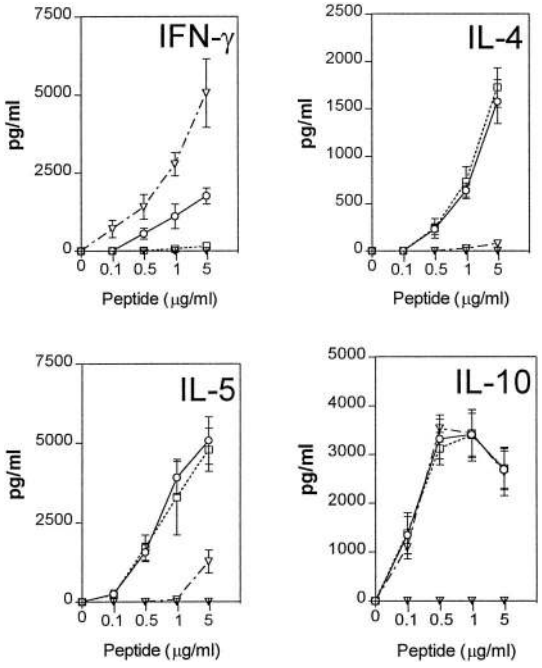

FIGURE 4. Modulatory effects of peptide analogues on polarized Th2 cells. DO11.10 T cells were cultured for three cycles with WT peptide. After the third cycle, T cells displayed a Th2 phenotype (high IL-4 and IL-5, and intermediate IFN-γ). The DO11.10 Th2 cells were then cultured with 1 μg/ml peptide (WT or analogue) and APC. IFN-γ, IL-4, IL-5, and IL-10 in the supernatant were determined by ELISA after a 72-h culture period. The results are represented as mean ± SEM of three independent experiments. *, $p < 0.05$ compared with stimulation with WT peptide (Dunnett). ○, WT; +, 331H-A; ◇, 333E-A; □, 335N-A; ▽, 336E-A

331H-A, or 333E-A showed a comparable dose-dependent proliferation (Fig. 2*A*) and IL-2 production (Fig. 2*B*) upon restimulation with WT peptide. Preincubation of T cells with WT peptide, 335N-A, or 336E-A significantly augmented the proliferation and IL-2 production induced by the WT peptide upon restimulation. Whereas WT peptide and 336E-A preincubation led to a comparable 50- to 100-fold increased response upon WT peptide restimulation, peptide 335N-A preincubation resulted in a >1000-fold increased response upon WT stimulation (Fig. 2, *A* and *B*).

After preincubation with the different peptide analogues, marked differences in IFN-γ, IL-4, IL-5, and IL-10 production were observed upon restimulation with WT peptide. Cells that were preincubated with medium, 331H-A, or 333E-A showed a cytokine profile upon WT peptide restimulation similar to that found in freshly isolated DO11.10 T cells (intermediate levels of IFN-γ and IL-5 and low levels of IL-4; Fig. 3). Preincubation with both WT peptide and partial agonistic peptide 335N-A profoundly increased the production of Th2-associated cytokines IL-4 (10-fold) and IL-5 (3-fold), whereas IFN-γ production was only doubled (Fig. 3). Interestingly, preincubation with the superagonistic peptide 336E-A resulted in a Th1-like cytokine profile. Upon WT peptide restimulation, both IL-4 and IL-5 production were low and comparable to levels produced by naive cells, whereas IFN-γ production was significantly increased. Data are shown for restimulation with 1 μg/ml WT peptide, but the characteristics were essentially the same for the entire dose range (0.1–10 μg/ml) tested (data not shown).

Analysis of the ability of peptide analogues to modulate a Th2 response

Next we studied the effects of the peptide analogues on polarized Th2 cells. Th2 cells were generated by culturing DO11.10 T cells with WT peptide and APC for three stimulation cycles. After the third cycle, T cells displayed a Th2 phenotype (high IL-4 and IL-5, and intermediate IFN-γ production). Restimulation of such Th2 cells with WT peptide resulted in production of high levels of IL-4 and IL-5, and intermediate levels of IFN-γ (Fig. 4). Restimulation with peptide analogues 331H-A and 333E-A did not result in any detectable cytokine production. Interestingly, partial agonistic peptide 335N-A induced high levels of IL-4 and IL-5 (comparable to levels found after WT peptide stimulation), but failed to induce IFN-γ production. In contrast, superagonistic peptide 336E-A did not induce IL-4 and only induced small amounts of IL-5, whereas IFN-γ production was significantly increased (Fig. 4). These data show that the WT peptide and the partial agonistic peptide 335N-A favor a further skewing toward a Th2 cytokine profile, whereas superagonistic peptide 336E-A can modulate already polarized Th2 cells toward a Th1 profile. Data are shown for cell lines obtained after three restimulations with 1 μg/ml peptide, but the characteristics were essentially the same for cell lines that were obtained after restimulations with 5 or 10 μg/ml (data not shown).

Evaluation of peptide administration in vivo by incorporation of peptide in liposomes

To study the effect of peptide administration in a Th2-mediated disease process, we used a murine model of allergic asthma. Because it was expected that, compared with protein, the half lives of peptides were rather low, we compared three different peptide administration protocols.

Previously we showed that two s.c. administrations of 150 μg WT peptide (with a 3-day interval) in OVA-sensitized mice led to a significant increase in airway hyperresponsiveness and eosinophilia upon challenge with OVA (18). Now we compared this former immunotherapy protocol with a single high dose (300 μg)

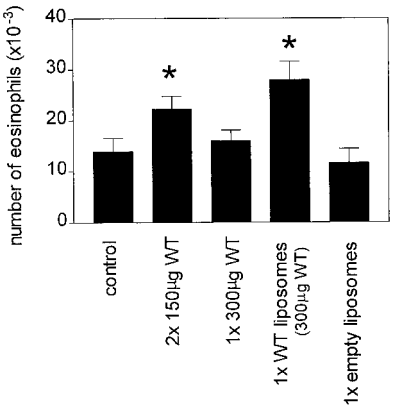

FIGURE 5. Total numbers of eosinophils in the BAL of OVA-challenged mice after different WT peptide treatment strategies. Mice were sensitized with OVA, and 2 wk later they were s.c. treated with WT peptide (1×300 μg, 2×150 μg, or 1×300 μg incorporated in liposomes). In the case of the 2×150 μg treatment, injections were given with a 3-day interval. Six days after the last s.c. administration, mice were challenged with OVA aerosol and the absolute numbers of eosinophils were determined 24 h after the last aerosol, as described in *Materials and Methods*. Data are expressed as mean ± SEM ($n = 5$ mice/group). *, $p < 0.05$ compared with empty-liposome treated mice (Dunnett).

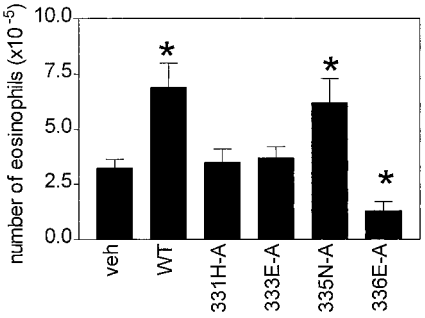

FIGURE 6. Total numbers of eosinophils in the BAL of OVA-challenged mice after peptide analogue therapy. Mice were sensitized with OVA, and 2 wk later they were s.c. treated with peptide liposomes (containing 300 μg peptide, WT or analogue). Six days later, mice were challenged by OVA aerosol, and the absolute number of eosinophils was determined 24 h after the last aerosol, as described in *Materials and Methods*. Data are expressed as mean ± SEM ($n = 8$ mice/group). *, $p < 0.05$ compared with vehicle-treated, OVA-challenged mice (Dunnett).

of WT peptide, or one dose of liposomes containing 300 μg WT peptide, which can act as a depot. Six days after the treatment, mice were challenged with OVA, and infiltration of eosinophils in the airways was determined by BAL. Comparison of the different treatment protocols showed clearly that 2×150 μg WT peptide augmented infiltration of eosinophils more than a single high dose of 300 μg (Fig. 5). However, a single dose of 300 μg WT peptide incorporated in liposomes showed the strongest aggravation of airway inflammation (Fig. 5). Although treatment with the WT peptide induced a nondesirable aggravation of the disease, the liposome-peptide formula appeared to be the most efficient method for peptide administration. Therefore, we selected the liposome-peptide formula for immunotherapy.

Evaluation of peptide analogue immunotherapy in vivo

To determine the effect of the peptide analogues in vivo, we incorporated the peptide analogues in liposomes and administered the liposomes s.c. in OVA-sensitized mice. Six days after treatment, mice were challenged with OVA or saline, and airway eosinophilia, OVA-specific Igs, and OVA-specific T cell responses were measured.

To analyze inflammatory cells in the BAL fluid, BAL was performed 24 h after the last aerosol. In all treatment groups, OVA challenge induced a significant increase of total numbers of cells compared with the saline-challenged groups (Table III). The increase in total number of cells was largely due to the increase of eosinophils, because no significant changes in neutrophils or mononuclear cells were found (Table III). As shown previously, WT peptide treatment resulted in a significant increase in eosinophils in OVA-challenged mice compared with those induced after vehicle treatment (Table III; Fig. 6). Treatment with the Th2-skewing partial agonistic peptide 335N-A also resulted in a significant increase in eosinophil infiltration (Table III; Fig. 6). Interestingly, treatment with the Th1-skewing superagonistic peptide 336E-A led to a significant reduction of eosinophil infiltration compared with vehicle-treated mice (Table III; Fig. 6). Treatment with 331H-A or 333N-A had no effect on eosinophil infiltration in OVA-challenged mice.

To study the effects of the peptide analogue treatment on Ab production, we determined levels of OVA-specific IgG1, IgG2a, and IgE 24 h after the last OVA or saline challenge. Sensitization with OVA and then saline challenge resulted in clearly detectable levels of OVA-specific IgG1 and IgE, whereas OVA-specific

Table III. *Total numbers of mononuclear cells, neutrophils, and eosinophils in the BAL fluid*[a]

Liposome Contents	Challenge	Total nr Cells ($\times 10^5$)	Mononuclear Cells ($\times 10^5$)	Neutrophils ($\times 10^3$)	Eosinophils ($\times 10^5$)
Vehicle	Saline	2.68 ± 0.55	2.68 ± 0.55	0	0
Vehicle	OVA	6.86 ± 0.60*	3.63 ± 0.36	0.23 ± 0.19	3.23 ± 0.24*
WT	Saline	2.93 ± 0.15	2.93 ± 0.15	0	0
WT	OVA	10.31 ± 0.87*	3.55 ± 0.18	0.85 ± 0.85	6.76 ± 0.69*#
331H-A	Saline	2.99 ± 0.78	2.99 ± 0.78	0	0
331H-A	OVA	6.45 ± 0.84*	2.74 ± 0.42	0.81 ± 0.44	3.71 ± 0.42*
333E-A	Saline	2.60 ± 0.61	2.60 ± 0.61	0	0
333E-A	OVA	6.51 ± 0.49*	2.98 ± 0.16	0	3.53 ± 0.33*
335N-A	Saline	3.50 ± 0.17	3.50 ± 0.17	0	0
335N-A	OVA	8.80 ± 1.24*	2.93 ± 0.50	0.31 ± 0.16	5.87 ± 0.74*#
336E-A	Saline	2.47 ± 0.93	2.47 ± 0.93	0	0
336E-A	OVA	5.69 ± 0.65*	4.00 ± 0.51	0	1.69 ± 0.14*#

[a] Total numbers of various cell types in BAL fluid recovered 24 h after the last OVA or saline challenge in OVA-sensitized mice treated with different peptide analogues. Data are expressed as mean ± SEM. $n = 8$/group.

*, $p < 0.05$ compared with corresponding saline-challenged mice.

#, $p < 0.05$ compared with vehicle-treated, OVA-challenged mice (Dunnet).

Table IV. *Levels of OVA-specific Igs in serum*[a]

Liposome Contents	Challenge	OVA-Specific IgE (EU/ml)	OVA-IgG1 (EU/ml)	OVA-Specific IgG2a (EU/ml)
Vehicle	Saline	215 ± 53	331 ± 101	21.1 ± 7.9
Vehicle	OVA	937 ± 259*	963 ± 191*	59.3 ± 11.5
WT	Saline	256 ± 49	334 ± 98	23.2 ± 6.2
WT	OVA	837 ± 90*	732 ± 90*	61.5 ± 9.2
331H-A	Saline	199 ± 79	243 ± 71	25.0 ± 13.1
331H-A	OVA	993 ± 172*	999 ± 172*	61.5 ± 25.4
333E-A	Saline	257 ± 48	261 ± 97	12.3 ± 9.9
333E-A	OVA	733 ± 191*	733 ± 121*	56.0 ± 12.3
335N-A	Saline	238 ± 33	258 ± 66	30.1 ± 12.0
335N-A	OVA	723 ± 142*	623 ± 142*	55.8 ± 16.1
336E-A	Saline	201 ± 52	210 ± 103	10.7 ± 8.3
336E-A	OVA	849 ± 138**	648 ± 130*	55.7 ± 22.1

[a] Serum levels of OVA-specific IgG1, IgG2a, and IgE in experimental units/ml (EU/ml), as determined by ELISA 24 h after the last OVA or saline challenge in OVA-sensitized mice treated with different peptide analogues. Values are expressed as mean ± SEM. $n = 8$/group.

*, $p < 0.05$ compared with corresponding saline-challenged animals (Dunnett).

IgG2a was hardly detectable (Table IV). The production of OVA-specific IgG1 and IgE in these mice indicated that already during the sensitization period OVA-specific Th2 cells and B cells were induced. OVA challenge of sensitized mice induced a significant rise of OVA-specific IgG1 and IgE in all treatment groups (Table IV) compared with corresponding saline-challenged groups. OVA-specific IgG2a was slightly, but not significantly, increased after OVA challenge compared with the results of saline challenge. None of the peptides used for immunotherapy did affect the levels of OVA-specific IgG1, IgG2a, or IgE (Table IV).

To analyze the cytokine production in lung-draining LN cells after peptide analogue treatment, these LN were isolated 24 h after the last OVA or saline challenge, and OVA-specific cytokine production was determined. Lung-draining LN cell cultures from saline-challenged mice showed no cytokine production upon OVA restimulation in vitro, although high responses were found after stimulation with immobilized αCD3 (data not shown). In contrast, LN cell cultures from OVA-challenged mice showed substantial levels of IL-4, IL-5, and IL-10 after in vitro OVA restimulation (Fig. 7). Compared with vehicle treatment, treatment with WT peptide, 331H-A, 333E-A, and 335N-A did not affect OVA-specific IL-4, IL-5, or IL-10 production. Remarkably, treatment with the Th1-skewing peptide 336E-A resulted in a significant reduction of IL-4 and IL-5, but not of IL-10 production (Fig. 7). Interestingly, in none of the groups was IFN-γ production detectable after in vitro restimulation with OVA, but αCD3 induced similar levels of IFN-γ in all groups.

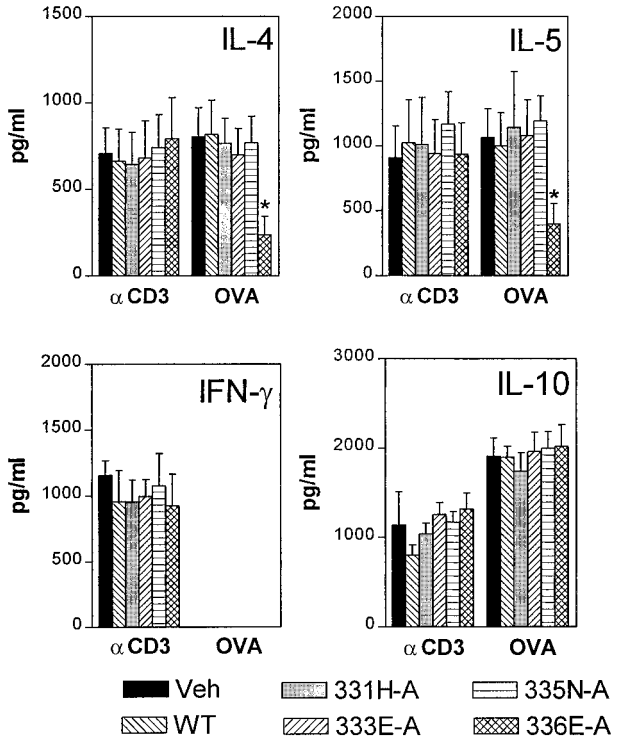

FIGURE 7. OVA-specific cytokine production in vitro by lung-draining LN cells after peptide analogue therapy. LN cells derived from OVA-challenged mice were cultured with αCD3, or OVA (10 μg/ml) for 120 h. IFN-γ, IL-4, IL-5, and IL-10 production in the supernatants was determined by ELISA. Data are expressed as mean ± SEM ($n = 6$ mice/group). One representative experiment of three is shown. *, $p < 0.05$ compared with vehicle-treated, OVA-challenged mice (Dunnett).

Discussion

In this study we defined and used peptide analogues of $OVA_{323–339}$ to interfere in an experimental model of allergic asthma. We showed that immunotherapy using a Th2-skewing peptide analogue deteriorated the disease, whereas a Th1-skewing peptide analogue ameliorated the disease process.

For the definition of modulatory peptide analogues we used different in vitro assays. Based on changes in proliferation, cytokine production, or Th1/2 skewing capacities, four modulatory peptide analogues were selected for in vivo studies. Importantly, the MHC class II binding affinity of these peptide analogues was comparable with that of the $OVA_{323–339}$ WT peptide. Using $OVA_{323–339}$-specific DO11.10 T cells, we showed that the agonistic WT peptide was a strong inducer of Th2 cytokines and favored skewing toward a Th2 phenotype. Peptide analogue 331H-A appeared to be a partial agonist, inducing T cell activation as measured by CD69 upregulation, whereas no proliferation, cytokine production, or anergy was induced in the DO11.10 T cells. Stimulation of DO11.10 T cells with peptide 333E-A did not induce any T cell activation. Also, more conserved substitutions (like 333E-D) failed to induce T cell activation (data not shown), suggesting that position 333E is a primary TCR contact residue in which no substitutions are tolerated. Peptide analogue 335N-A was a partial agonist, inducing cytokines similar to those induced by WT peptide (albeit at lower levels). Comparable with the WT peptide, 335N-A also skewed toward a Th2 phenotype. In contrast, peptide analogue 336E-A was a superagonist and strongly promoted skewing toward a Th1 phenotype. More importantly, peptide analogue 336E-A even induced a Th1 cytokine profile in an already polarized Th2 population, leading to an enhanced IFN-γ production and an almost complete absence of IL-4 and IL-5 production. Although there is a clear shift toward a Th1 profile in this Th2-skewed population, we cannot dissect whether the changes in cytokine pattern resulted from an inhibition of IL-4 and IL-5 in fully polarized Th2 cells, or from an increased IFN-γ production by and proliferation of less polarized cells.

How peptide analogues induce T cell modulatory effects is still poorly understood (35, 36). It has been reported that changes in the MHC class II binding affinity of peptide analogues may lead to altered T cell activation. Several studies have suggested that peptide analogues with low affinity for MHC induce IL-4 production

and Th2 development, whereas peptide analogues with high affinity promote Th1 development (35, 36). However, our data show that this is no general rule because the partial agonistic peptide 328H-A, which has decreased MHC binding affinity compared with the WT peptide, induced Th1 (IL-2 and IFN-γ), but not Th2 cytokines (Table I). Alternatively, modification of TCR contact residues within the peptide has been suggested to affect the dissociation of the TCR from the peptide MHC complex and, consequently, to change the early biochemical events leading to T cell activation (37, 38). In vitro studies have indicated that weak TCR-mediated signaling preferentially induces Th2 differentiation, or anergy, whereas strong TCR-mediated signaling induces Th1 responses (20, 39). To distinguish between modulatory effects due to changes in MHC binding affinity and altered TCR affinity, we selected only peptide analogues with MHC binding affinity comparable with WT peptide. Our findings suggest that peptide analogues 331H-A and 333E-A have a very low affinity, 335N-A has an intermediate affinity, and 336E-A a high affinity for the $OVA_{323–339}$-specific DO11.10 TCR.

Previously it has been reported that position 331H in the $OVA_{323–339}$ peptide is an important TCR contact residue for I-A^d-restricted T cell recognition (40). Our data indicate, besides the 331H TCR contact residue, the presence of at least three other TCR contact residues at positions 333E, 335N, and 336E. Recently, Scott et al. (41) showed, after crystallization of MHC class II I-A^d with $OVA_{323–339}$, that residues 323–333 were involved in MHC binding and that residue 331H protruded toward the TCR. Although 323–333 is probably the most preferable peptide core sequence for MHC binding, they suggested that I-A^d can bind $OVA_{323–339}$ in two alternative alignments due to the minimal side-chain requirements for peptide binding by I-A^d (41). In these two alternative alignments involving residues 325–335 and 328–338, residues 333E and 336E protrude from the MHC toward the TCR, like 331H in the crystallized alignment (41). This may explain why modifications of residues 335 and 336 which, according to the most abundantly present alignment, are not involved in MHC or TCR binding can have such a clear effect on T cell activation. Although we cannot exclude that the two other described alignments are important for recognition of $OVA_{323–339}$ by the DO11.10 TCR, our data suggest that alignment 328–338, containing all four TCR contact residues as defined in this study, would be the most favorable conformation recognized by the DO11.10 TCR.

To study the possibilities of peptide analogue therapy in the experimental asthma model, we treated mice s.c. with peptide analogues after OVA sensitization. Subsequently, mice were challenged with OVA. Our data show that treatment with peptide analogues that were very poor T cell activators (331H-A and 333E-A) had no effect on airway eosinophilia and cytokine production. In contrast, administration of the Th2-skewing WT peptide and the Th2-skewing partial agonistic 335N-A peptide dramatically increased airway eosinophilia upon OVA challenge, indicating that the Th2 response in the lungs was augmented. Treatment with the Th1-skewing 336E-A peptide resulted in a significant decrease in eosinophilia and OVA-specific IL-4 and IL-5 production by the lung-draining LN cells.

In all experimental groups, OVA-specific IgE was significantly increased after OVA challenge compared with the results of saline challenge, but was not affected by peptide therapy, indicating that there is no clear correlation between serum levels of OVA-specific IgE and the degree of airway inflammation. These findings correspond with our previous study (18) and the studies by Korsgren et al. (42) and Mehlhop et al. (43), who demonstrated that airway inflammation can even occur in B cell-deficient and IgE-knockout mice. Although peptide analogue therapy did not clearly affect B cell responses, our data show that T cell responses were modulated. Treatment with Th2-skewing peptides enhanced the Th2-associated allergic response, whereas a Th1-skewing peptide analogue inhibited OVA-specific Th2 cells in vivo. Because the Th1-skewing peptide 336E-A had the capacity to inhibit IL-4 and IL-5 production in polarized Th2 cells in vitro, the observed reduction of OVA-specific Th2 cytokines in vivo after treatment with this peptide analogue may be due to a peptide-induced Th2→Th1 cytokine shift in OVA-specific Th2 cells in vivo. We and others have shown that treatment with the Th1-associated cytokine IFN-γ before and during challenge significantly reduced airway hyperresponsiveness and eosinophil infiltration in murine asthma models (44, 45). These findings suggest that IFN-γ plays an important role in the down-regulation of a Th2-mediated allergic response. However, although peptide 336E-A induced high levels of IFN-γ in vitro, after peptide therapy, no IFN-γ production by lung-draining LN cells or IFN-γ-induced IgG2a could be detected. Alternatively, it is possible that the reduction of Th2 cytokines in vivo was due to the induction of anergy or activation-induced cell death. It has recently been reported that anergy followed by activation-induced cell death can be induced in effector T cells by stimulation with high doses of Ag and by antigenic stimulation that is prolonged beyond an optimal time (46). Increasing the affinity of the MHC peptide complex for the TCR may result in overstimulation of these Th2 cells and eventually may lead to anergy or apoptosis. A third possibility could be the induction of a regulatory T cell population, consisting of either a novel T cell subset or the modulated Th2 cells. Previously it has been shown that IL-10 is a very powerful regulatory cytokine (47) and that successful immunotherapy in humans is associated with increased production of IL-10 by allergen-specific T cells (48). Although our data do not show an increase in OVA-specific IL-10 production, it is possible that the altered IL-4/IL-10 and IL-5/IL-10 ratios after Th1 peptide analogue therapy positively contributed to the reduction of the allergen-specific Th2 response.

In summary, we have shown that a Th1-skewing peptide analogue of a dominant allergen epitope can modulate an allergen-specific polarized Th2 response in vitro. More importantly, we demonstrated for the first time that an in vitro-defined Th1-skewing peptide analogue can modulate an ongoing allergen-specific Th2 response in vivo, thereby inhibiting airway inflammation. Furthermore, the efficacy of peptide immunotherapy was clearly correlated with Th1- or Th2-skewing characteristics of the therapeutic peptide as defined in vitro. These findings indicate that the design of Th1-skewing peptide analogues, instead of using WT peptides, may improve peptide immunotherapy and may contribute to the development of successful and safe allergen-specific immunotherapy for allergic patients.

Acknowledgments

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