

Antagonistic peptides specifically inhibit proliferation, cytokine production, CD40L expression, and help for IgE synthesis by Der p 1-specific human T-cell clones

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Background: Allergic disorders are characterized by IgE antibody responses to a multitude of allergens as a result of the ability of these antibodies to specifically bind to high-affinity IgE receptors on mast cells and basophils. This interaction results in receptor activation and release of soluble mediators such as histamine and leukotrienes, which cause allergic reactions in various target organs. Because the synthesis of IgE is tightly regulated by cytokines and CD40 ligand (L) interactions, CD4⁺ helper T cells are obvious targets, with the aim to modulate allergen-induced IgE responses.

Objectives: Because of the central role of allergen-specific T-helper type 2 (T_{H2}) cells in the pathway leading to IgE synthesis in vitro and in vivo, we have evaluated the possibility of inhibiting allergen-induced activation of these cells by using allergen-derived peptides that have been modified by single amino acid substitutions.

Methods: Three cloned human T_{H2}-like CD4⁺ T-cell lines, specific for Der p 1, the major allergen in house dust, were used in this study. Upon activation with Der p 1 or specific Der p 1-derived wild-type peptides, these T-cell clones produce high levels of IL-4 and IL-5 and low levels of interferon- γ and IL-2, respectively, and furthermore give help to B cells for the production of IgE in vitro. Modified synthetic peptides were generated by the introduction of single amino acid substitutions into two different T-cell activation-inducing epitopes on Der p 1. The effects of these modified peptides were studied in Der p 1-induced proliferation, cytokine production, and in vitro IgE production assays.

Results: Several substituted Der p 1-derived peptides failed to induce T-cell proliferation, in contrast to the native peptides. In addition, some of these peptides acted as antagonists by strongly inhibiting wild-type peptide-induced proliferation as well as the production of interferon- γ , IL-2, IL-4, and IL-5, although the production of the latter two cytokines was less affected than that of interferon- γ , even at a 100-fold molar excess of the antagonistic peptides. In addition, the presence of an excess of each of the antagonistic peptides during the activation of Der p 1-specific T-cell clones prevented induction of CD40L expression, resulting in a failure of these cells to give help to B cells for the production of IgE in vitro, even in the presence of exogenous IL-4.

Conclusions: Substitution of certain amino acid residues in immunogenic Der p 1-derived peptides results in the generation of peptides that fail to induce proliferation of Der p 1-specific T-cell clones. In addition, these modified peptides have strong antagonistic activities on Der p 1-induced proliferation, cytokine production, and CD40L expression by allergen-specific T-cell clones as well as on T cell-mediated IgE production by B cells. These findings suggest that modified peptides interfere with allergen-induced activation of T cells, including the production of cytokines and the expression of surface molecules important for successful T cell-B cell interactions, and may therefore have therapeutic potential by inhibiting the expansion and function of allergen-specific T_{H2} cells. (*J Allergy Clin Immunol* 1998;101:521-30.)

Key words: Der p 1, allergen-specific T lymphocytes, allergen-derived peptides, cytokine production, CD40L, IgE synthesis

Allergic disorders are characterized by the presence of antibodies of the IgE isotype, which in house dust mite-induced atopy are mainly directed to the *Dermaphagoides pteronyssinus* group 1 (Der p 1) and group 2 (Der p 2) allergens.¹⁻³ The synthesis of IgE by B cells in vitro is specifically induced by IL-4 and IL-13,⁴⁻⁶ together with a T cell-B cell contact-mediated signal, accounted for by the interaction of CD40 and CD40 ligand (L).⁷⁻⁹ The pivotal role of CD40L in induction of IgE isotype switching and IgE synthesis has been demonstrated in patients with hyper-IgM syndrome who have mutations in their genes encoding CD40L and, as a result, have no or minimal levels of IgG, IgA, and IgE in their circulation.¹⁰⁻¹³ Although CD40L is expressed on all activated T cells, CD4⁺ T-helper type 2 (T_{H2}) cells are considered to play a pivotal role in house dust mite-induced atopy because of their capacity to produce high levels of IL-4 and IL-13.¹⁴⁻¹⁶ Therefore Der p 1-specific T_{H2} cells are obvious targets for immune intervention in house dust mite-related atopic diseases and prevention of activation, and subsequent cytokine production by these cells may be beneficial to the clinical status of atopic patients.

Allergen-specific CD4⁺ T cells recognize peptide antigens, presented by antigen-presenting cells (APCs) in the context of a major histocompatibility complex (MHC) class II molecule. On the basis of crystallographic studies, it has been demonstrated that all but one of the amino acid side-chains within the peptide-binding groove of the MHC class II protein can interact with the T-cell receptor (TCR) upon contact between

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Abbreviations used

APC:	Antigen-presenting cell
CD40L:	CD40 ligand
EBV-LCL:	Epstein-Barr virus-transformed lymphoblastoid B-cell lines
IFN:	Interferon
mAb:	Monoclonal antibody
MHC:	Major histocompatibility complex
TCR:	T-cell receptor
T _{H2} :	T-helper type 2 (cells)

the CD4⁺ T cell and the APC.^{17,18} Depending on the positions of replaced amino acids, however, the change will affect the interaction of the peptide with the peptide-binding groove of the MHC class II protein, the interaction of the peptide-class II complex with the TCR, or both.

In addition, there is increasing evidence that substitution of certain amino acids of immunogenic peptides will induce qualitative changes in T-cell responses as a result of altered signaling by the TCR upon its interaction with the peptide-MHC complex. Stimulation of mouse T cells with antigenic peptides containing single amino acid substitutions reportedly resulted in TCR antagonism, segregation of proliferative and cytokine responses, or even induction of anergy.¹⁹⁻²⁴ Also, in human systems, stimulation of T-cell clones with altered peptides has been shown to result in changes in T-cell responses. Peptides with a single amino acid substitution, which acted as TCR antagonists, were found to selectively induce the production of transforming growth factor-β1 by myelin basic protein-specific human T-cell clones but were no longer able to induce the production of IL-2, IL-4, IL-10, and interferon (IFN)-γ by these cells.²⁵ Recently, it was reported that stimulation of T-cell clones with altered peptides, derived from dominant epitopes on the house dust mite allergen Der p 2 allergen,²⁶ or the Japanese cedar pollen allergen Cry j 1,²⁷ respectively, resulted in the selective enhancement of IFN-γ but not of IL-4 production or induction of cytokine production in the absence of proliferation.²⁶ The above-mentioned studies show that single amino acid substitutions in T-cell activation-inducing epitopes on major allergens may modulate the cytokine production profile of allergen-specific T cells. However, no information has been provided on the capacity of these peptides to inhibit in vitro IgE synthesis. Moreover, the effects of modified peptides on T cell-B cell-mediated interactions, which are required for productive IgE synthesis, have not been reported yet.

Therefore in this study we have investigated the capacity of peptides, derived from two minimal T-cell activation-inducing epitopes on the major house dust mite allergen Der p 1 and modified by single amino acid substitutions to block the activation of human Der p 1-specific T-cell clones and to inhibit the production of

T_{H2} cytokines, the expression of CD40L and T cell-mediated IgE synthesis by B cells in vitro.

METHODS

Cells

The human CD4⁺ T_{H2}-like, HLA-DR1101-restricted, T-cell clones NP-12 and NP-44, specific for the p94-104 sequence of Der p 1, have been described previously.¹⁵ The CD4⁺ T_{H2} clone AR-142 recognizes the Der p 1-derived peptide p171-182 in the context of HLA-DR1501 (S. Fasler, unpublished results). All Der p 1-specific T-cell clones were generated by stimulation of peripheral blood mononuclear cells, obtained from atopic patients who were allergic to Der p 1, with 1 μg/ml of a lyophilized extract of Der p 1 (ALK, Bornholm, Denmark), followed by cloning by using a limiting dilution method, as has been described.¹⁵ The T-cell clones were cultured at a concentration of 2×10^5 cells/ml in 24-well Linbro plates (Flow, McLean, Va.) after stimulation with a feeder cell mixture consisting of 10^6 cells/ml irradiated (40 Gray) allogeneic peripheral blood mononuclear cells, 10^5 cells/ml of the irradiated (50 Gray) Epstein-Barr virus-transformed lymphoblastoid B cell line (EBV-LCL) JY, and 0.1 μg/ml PHA (Wellcome Diagnostics, Dartford, U.K.), as described previously.²⁸ Three to 4 days after each restimulation with feeder cells, the cultures were expanded in culture medium containing 20 U/ml rIL-2 and 200 U/ml rIL-4 (kindly provided by Dr. G. Zurawski, DNAX Research Institute). All cloned T-cell lines and EBV-LCL were free of mycoplasma and were cultured in Yssel's medium, supplemented with 1% human serum.²⁹ Experiments were carried out in the same culture medium unless indicated otherwise. HLA DR1101-matched monocytes to be used as APC for NP-44 cells (generously provided by Drs. C. Figdor and R. Huybens, Academisch Ziekenhuis Nijmegen, The Netherlands) were isolated from the peripheral blood of a healthy donor by centrifugal elutriation as described previously.³⁰

Proliferation assay

Cellular proliferation was measured with an [³H]TdR assay as has been previously described.¹⁵ In brief, 10^5 T cells were mixed with 5×10^4 irradiated (40 Gy) HLA-matched monocytes or 5×10^4 irradiated (50 Gy) autologous EBV-LCL that had been pulsed for 3 hours with specific peptide at concentrations ranging from 0.1 to 10 μg/ml in a flat-bottomed, 96-well tissue culture plate (Flow Laboratories) in a total volume of 200 μl. After 72 hours of incubation at 37°C, 37 kBq [³H]TdR (Amersham, Arlington Heights, Ill.) was added to each well. Six hours later, the cells were harvested onto glass fiber strips and the amount of incorporated [³H]TdR was measured by liquid scintillation counting. The results are expressed as the mean of triplicate cultures ± the standard deviation. Inhibition of proliferation by substituted peptides was calculated by the following formula:

$$\frac{\text{Proliferation with wild type peptide} - \text{spontaneous proliferation}}{\text{proliferation with antagonistic and wild type peptide} - \text{spontaneous proliferation}} \times 100\%$$

Stimulation of T-cell clones and assays for cytokine production

One million T-cell clones per milliliter were cocultured with 2×10^6 autologous EBV-LCL that had been pulsed for 3 hours with specific peptide (0.1 to 10 μg/ml) in 24-well Linbro tissue culture plates. After 48 hours of activation, supernatants were

TABLE I. Substituted peptides derived from two native Der p 1 sequences

Wild-type	A	V	N	I	V	G	Y	S	N	A	Q	G
	1	1	1	1	1	1	1	1	1	1	1	1
171-182	7	7	7	7	7	7	7	7	7	8	8	8
	1	2	3	4	5	6	7	8	9	0	1	2
Substitution	G	A	A K G W I D S	A	A	A	A	A	A	G	A	A
Effect*	Ac	Ac	An	Ac	No	No	Ac	No	Ac	No	An	Ac
Wild-type	Y	R	Y	V	A	R	E	Q		S	C	R
							1	1		1	1	1
94-104	9	9	9	9	9	9	0	0		0	0	0
	4	5	6	7	8	9	0	1		2	3	4
Substitution	G	A	A	A	G	A	A	A		A	A	A
Effect*	Ac	An	An	Ac	Ac	Ac	An	No		Ac	Ac	Ac

Ac, Activation-inducing; No, nonstimulatory; An, antagonistic.

*Effect of substituted peptide.

harvested and stored at -80°C before testing. Cytokine levels in the supernatants of activated T cells were determined by ELISA, with cytokine-specific antibodies for IL-2, IL-4, IFN- γ , and IL-5, as has been described.³¹ The sensitivity of each assay was approximately 50 pg/ml.

Analysis of CD40L expression by flow cytometry

One million T cell cells per milliliter were stimulated with 2×10^6 irradiated (50 Gy), autologous EBV-LCL that had been pulsed for 3 hours with 1 $\mu\text{g}/\text{ml}$ of specific immunogenic peptide in the presence or absence of a 100-fold molar excess of substituted peptide for 16 hours. To prevent downregulation of CD40L on activated T cells as a result of interaction with CD40 expressed on the APC, 1 $\mu\text{g}/\text{ml}$ of anti-CD40L monoclonal antibody (mAb) was added to the cultures (R. Noelle, personal communication). Methods of immunofluorescence staining, flow cytometry, and data analysis have been described by Lanier and Recktenwald.³² Flow cytometry to analyze the expression of CD40L on activated T-cell clones was performed with a FACS Calibur flow cytometer (Becton Dickinson Co., San Jose, Calif.) and the anti-CD40L mAb LL48 (CD154, kindly provided by Dr. J. Banachereau, Schering-Plough Research Laboratories, Dardilly, France).

Measurement of Ig production

One million T cells per milliliter were stimulated with 2×10^6 irradiated (50 Gy), autologous EBV-LCL (AR-142) or 2.5×10^5 HLA-matched monocytes (NP-44) that had been pulsed for 3 hours with 1 $\mu\text{g}/\text{ml}$ of specific immunogenic peptide in the presence or absence of a 100-fold molar excess of substituted

peptide, and culture supernatants were harvested after 24 hours of culture. The supernatants were added to 10^4 highly purified human B lymphocytes ($> 99\%$ CD20⁺), isolated from normal spleens,³³ which were then stimulated with the anti-CD40 mAb 89³⁴ in 96-well round-bottomed plates (Flow) in Yssel's medium, supplemented with 10% fetal calf serum and 10 $\mu\text{g}/\text{ml}$ ultrapure transferrin (Pierce, Rockford, N.Y.) in a final volume of 200 μl . For T cell-mediated IgE synthesis, T-cell clone NP-44 was activated for 24 hours with HLA-matched monocytes, pulsed for 3 hours with 1 $\mu\text{g}/\text{ml}$ of p94-104 in the presence or absence of a 100-fold molar excess of Y96A, and after washing of the cells, 10^4 T cells were added to 10^4 purified B cells in the presence of exogenous rIL-4 (100 U/ml). Fourteen days later, the supernatants from triplicate cultures of B cells were harvested, pooled, and analyzed for Ig isotype content by ELISA as described previously.³³ The sensitivity of the ELISA was determined with a calibrated standard from Behring (Marburg, Germany) and was 0.2 ng/ml.

Generation of Der p 1-derived peptides

Der p 1-derived peptides, representing minimal T-cell activation-inducing epitopes or including single substitutions, were synthesized on an advanced Chemtech 350 Synthesizer (Advanced Chemtech, Louisville, Ky.). The carboxyl-terminal amino acid coupled to Wang resin was used as the solid phase and the peptides were extended with Fmoc chemistry, according to the manufacturer's instructions. Peptides were characterized by laser desorption mass spectrometry (FinniganMat, San Jose, Calif.), peptide sequencing, and quantitative amino acid analysis and had a purity of approximately 90%.

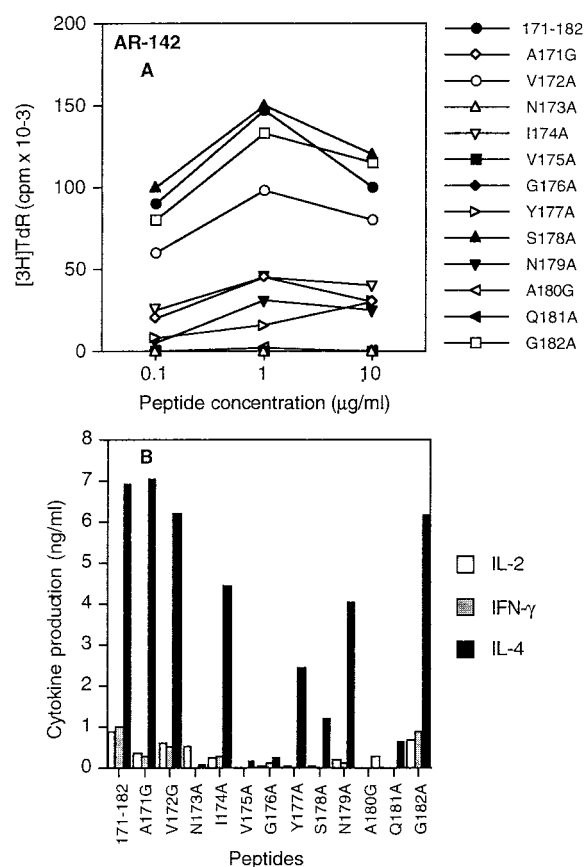


FIG. 1. Effect of amino acid substitutions in peptide p171-182 on proliferation and cytokine production of Der p 1-specific T-cell clone AR-142. T cells were stimulated with various concentrations of wild-type and substituted peptides, in presence of autologous EBV-LCL, and proliferative responses (**A**) were measured after 72 hours as described in Methods section. Production of IL-2, IL-4, and IFN- γ (**B**) was analyzed after incubation of T cells with 1 μ g/ml of peptide and EBV-LCL for 48 hours.

RESULTS

Mutational analysis within minimal T-cell activation-inducing epitopes

The T-cell clones NP-12, NP-44, and AR-142 specifically proliferate and secrete cytokines in response to stimulation with the immunogenic, Der p 1-derived wild-type peptides p94-104 and p171-182, respectively, in the presence of autologous or HLA-matched APCs. To generate modified peptides that could block allergen-specific activation of these T-cell clones, single amino acid substitutions were introduced into the immunogenic peptide sequences (Table I). Stimulation of the T-cell clones with autologous EBV-LCL in the presence of various concentrations of substituted Der p 1-derived peptides resulted in various levels of T-cell proliferation as well as cytokine production. Maximal proliferative responses to wild-type peptide were obtained at approximately 1 μ g/ml of peptide as shown in Fig. 1 and Fig. 2. Peptides derived from p171-182 with substitutions of amino acid residues at positions 173, 175, 176, 180, and

181 with Ala (A) or Gly (G) failed to induce proliferative responses and production of IL-2, IL-4, and IFN- γ by clone AR-142 (Fig. 1). In addition, peptides in which the neutral Asn (N) residue at position 173 was replaced by either basic [Lys:K], hydrophobic [Trp:W, Ile:I], acidic [Asp:D], or hydrophilic residue [Ser:S] also did not induce proliferation and cytokine production of AR-142 (results not shown), indicating that Asn at position 173 is one of the critical residues required for T-cell activation. Similarly, substitutions with Ala or Gly at positions 95, 96, 100, and 101 of peptide 94-104 resulted in modified peptides that were ineffective in inducing proliferation and IL-2, IL-4, and IFN- γ production by T-cell clones NP-12 and NP-44 (Fig. 2). The lack of proliferation-inducing capacity of these peptides was always associated with the inability to induce cytokine production, and no dissociation between proliferation and cytokine production or between the production of IL-2, IL-4, and IFN- γ was observed. Substitutions at amino acid positions other than those mentioned above induced normal or only slightly reduced proliferative responses and cytokine production of the T-cell clones compared with those induced with the native, nonsubstituted, peptides.

Inhibition of proliferation by antagonistic peptides

Next we determined whether the substituted peptides that failed to induce T-cell activation could act as antagonists by inhibiting the wild-type peptide-induced proliferation of the Der p 1-specific T-cell clones. Stimulation of T-cell clone AR-142 with wild-type peptide p171-182 and a 10-fold molar excess of the substituted peptides N173A or Q181A cell clone AR-142 resulted in an inhibition by more than 50% of the proliferation induced by p171-182 only (Fig. 3). Maximal inhibition of proliferative responses of approximately 70% was observed with a 100-fold molar excess of antagonistic peptides. Three nonstimulatory, modified peptides, generated by single amino acid substitutions in the p94-104 peptide, had strong inhibitory activities on the proliferation of T-cell clones NP-12 and NP-44. Substituted peptides R95A, Y96A, and Q101A strongly inhibited the proliferation of T-cell clone NP-12 in a dose-dependent manner. Weaker inhibitory effects of these peptides were observed on proliferative responses of T-cell clone NP-44. Preincubation of the APCs for 6 hours with a 100-fold molar excess of antagonistic peptides, before addition of the wild-type peptide, yielded similar inhibitory effects (S. Fasler, unpublished results).

The substituted peptides that inhibited Der p 1-induced proliferation of T-cell clone AR-142 were not able to block proliferative responses of T-cell clones NP-12 and NP-44 and vice versa, indicating that the inhibitory effect of these peptides was not due to toxic effects. All other substituted peptides with stimulatory activity, listed in Table I, did not affect Der p 1-specific proliferative responses of the T-cell clones at any concentration used (data not shown). Further mutational analysis

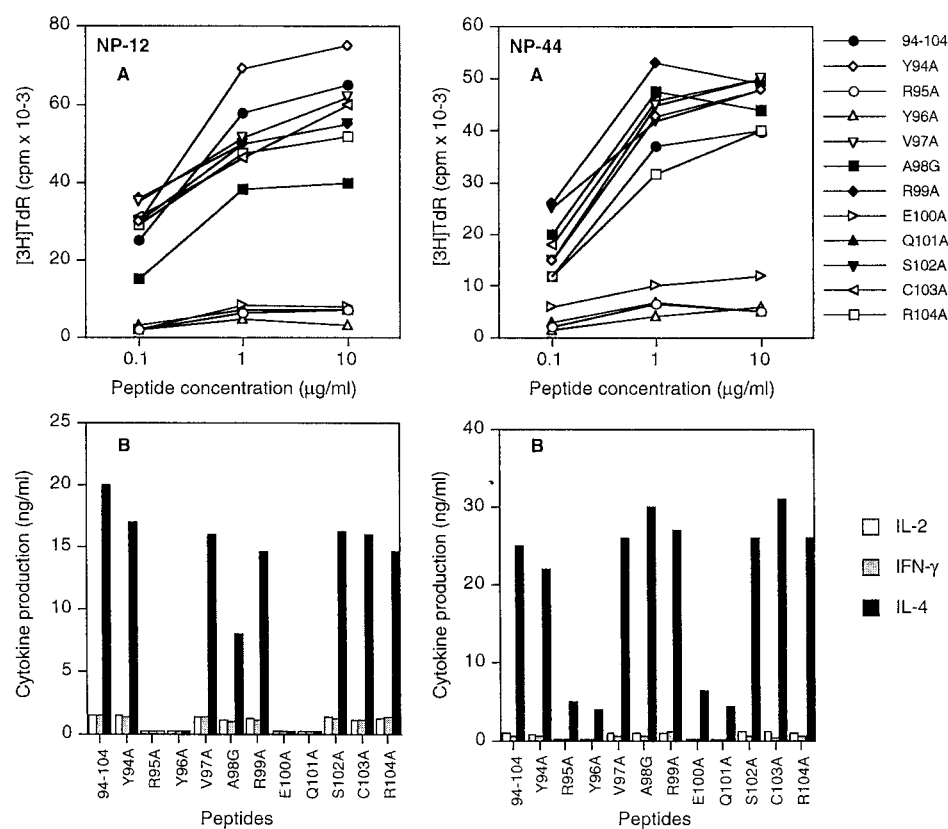


FIG. 2. Effect of amino acid substitutions in peptide p94-104 on proliferation and cytokine production of Der p 1-specific T-cell clones NP-12 and NP-44. Proliferative responses (A) and cytokine production (B) of T-cell clones NP-12 and NP-44 were measured as described in Fig. 1.

of residue 173 in the wild-type peptide p171-182, after substitution of Asn with Lys, Gly, Trp, Ile, Asp, and Ser, produced peptides with varying antagonistic activities. The inhibition of the Der p 1-induced proliferation of T-cell clone AR-142, at a 100-fold molar excess of substituted peptide, ranged from 9% to 78% with decreasing inhibitory activity, in the order Ala, Ser, Gly, Lys, Trp, Ile, and Asp (data not shown).

Inhibition of cytokine production by antagonistic peptides

To further examine the effects of substituted peptides on T-cell effector function, the cytokine production by Der p 1-specific T-cell clones, induced by the wild-type peptides in the presence of an excess amount of modified peptide, was studied. As is shown in Table II, the antagonistic peptides inhibited the proliferative responses of the Der p 1-specific T-cell clones in a dose-dependent fashion with a maximal inhibitory effect at a 100-fold molar excess. Therefore this concentration of peptide was used in further experiments. The antagonistic peptides R95A, Y96A, and E100A, which inhibited the proliferation of T-cell clones NP-12 and NP-44, also strongly blocked the production of IL-2 and IFN-γ at a 100-fold molar excess. Comparable results were

obtained with the antagonistic peptides N173S, Q181A, and N173A, which had strong inhibitory activities on the proliferative responses of T-cell clone AR-142. However, the relatively high production levels of IL-4 and IL-5, in particular those produced by T-cell clones NP-44 and AR-142, were only partially affected (Table II), even in the presence of a 500-fold molar excess of antagonistic peptide (data not shown), suggesting a difference in sensitivity of the T_{H1} versus the T_{H2} cytokines to the inhibitory effects of the antagonistic peptide. However, none of the substituted peptides had a differential effect on the production of the various cytokines; furthermore, antagonistic peptides that inhibited cytokine production also inhibited proliferative responses.

Antagonistic peptides inhibit help for IgE synthesis by B cells by blocking IL-4 production and expression of CD40L

Activated Der p 1-specific T-cell clones give help for IgE synthesis to purified B cells for the synthesis of IgE through their production of IL-4, as well as a signal mediated by the interaction of CD40 and its ligand CD40L. In view of their inhibitory effects on the cytokine production of Der p 1-specific T-cell clones, we investi-

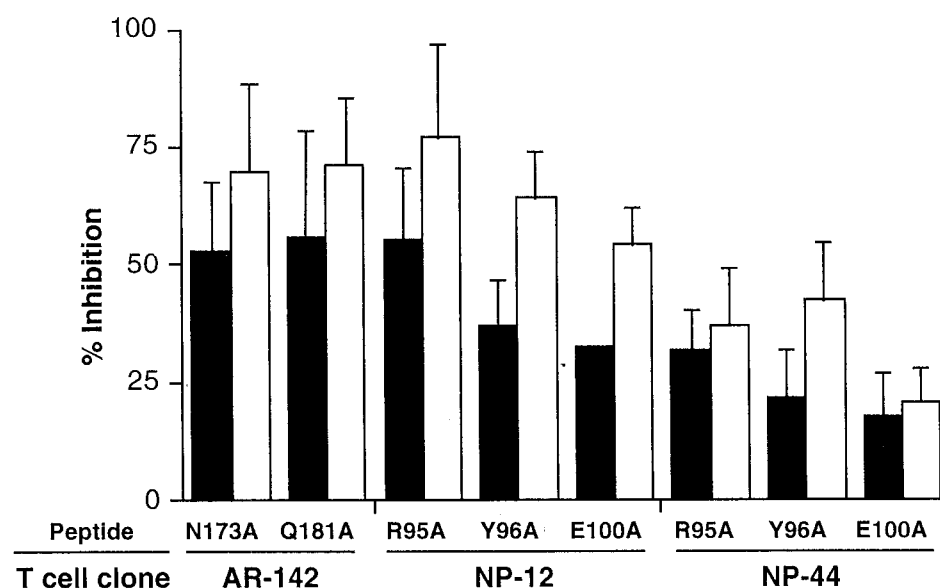


FIG. 3. Inhibition of proliferative responses of Der p 1-specific T-cell clones by antagonistic Der p 1-derived peptides. T-cell clones AR-142, NP-12, and NP-44 were incubated with autologous EBV-LCL in the presence of 0.1 μ g/ml of the native peptides p171-182 or p94-104, respectively, and a 10-fold (filled columns) or 100-fold (open columns) molar excess of antagonistic peptides. Proliferative responses were measured after 72 hours as described in Methods section. Results are expressed as percentage inhibition of native peptide-induced proliferation and represent mean and standard deviation of three independent experiments.

TABLE II. Inhibition of cytokine production of Der p 1-specific T-cell clones by substituted Der p 1-derived peptides

T-cell clone*	Substituted peptide	% Inhibition of cytokine production†			
		IFN- γ	IL-2	IL-4	IL-5
AR-142	Q181A	86	100	51	34
	N173A	93	100	50	37
	N173S	94	100	50	29
	N173G	76	100	44	11
	N173K	68	100	29	4
	N173I	63	89	27	5
	N173W	56	73	28	3
NP-12	N173D	37	12	6	-12
	R95A	44	99	69	81
	Y96A	70	95	75	92
	E100A	90	100	86	99
NP-44	R95A	98	100	46	50
	Y96A	101	100	44	60
	E100A	98	100	43	78

*T cells were stimulated with autologous EBV-LCL, pulsed with 1 μ g/ml of wild-type peptides p171-182 (AR-142) or p94-104 (NP-12 and NP-44), respectively, and a 100-fold molar excess of substituted peptides as described in Methods section.

†Cytokine production was measured after 48 hours of culture as described in Methods section. Values for cytokine production after stimulation with wild-type peptides are similar to those shown in Figs. 1 and 2.

gated whether antagonistic peptides could inhibit T-cell-mediated induction of IgE synthesis by purified B cells in vitro. As demonstrated previously, the addition of culture supernatants of T-cell clone NP-44, activated with

peptide p94-104 and APC, to highly purified B cells resulted in the induction of high levels of polyclonal IgE (Fig. 4).³⁴ Supernatants collected from cultures of NP-44 cells, stimulated with the wild-type peptide p94-104 and a 100-fold molar excess of Y96A, showed a reduction of approximately 50% to induce IgE synthesis in vitro by B cells preactivated with anti-CD40 mAb (Fig. 4, A). Similar results were obtained with T-cell clone AR-142 and N173A (Fig. 4, B), reflecting the level of partial inhibition on the production of IL-4 by these antagonistic peptides. To determine whether such antagonistic peptides could also block IgE synthesis mediated by activated T-cell clones in vitro, purified B cells were cocultured with NP-44 cells, stimulated with p94-104, a 100-fold molar excess of substituted peptide Y96A or combinations of p94-104 and Y96A, and IgE production was analyzed after 12 days of culture. As shown in Fig. 4, C, NP-44 cells, stimulated with p94-104 and excess Y96A, were no longer able to provide help to B cells for the production of IgE, even in the presence of exogenous IL-4. To examine whether the presence of antagonistic peptides can interfere with T cell-B cell-mediated signals leading to IgE synthesis, their effect on the induction of CD40L expression was analyzed. T-cell clones NP-44 and AR-142 expressed significant levels of CD40L, as detected by flow cytometry, 6 hours after stimulation of the cells with APCs and wild-type peptide. As shown in Fig. 5, the presence of a 100-fold molar excess of Y96A or N173A during antigen-specific stimulation of T-cell clones NP-44 and AR-142 strongly inhibited the induction of CD40L expression. Taken together, these results suggest that antagonistic peptides

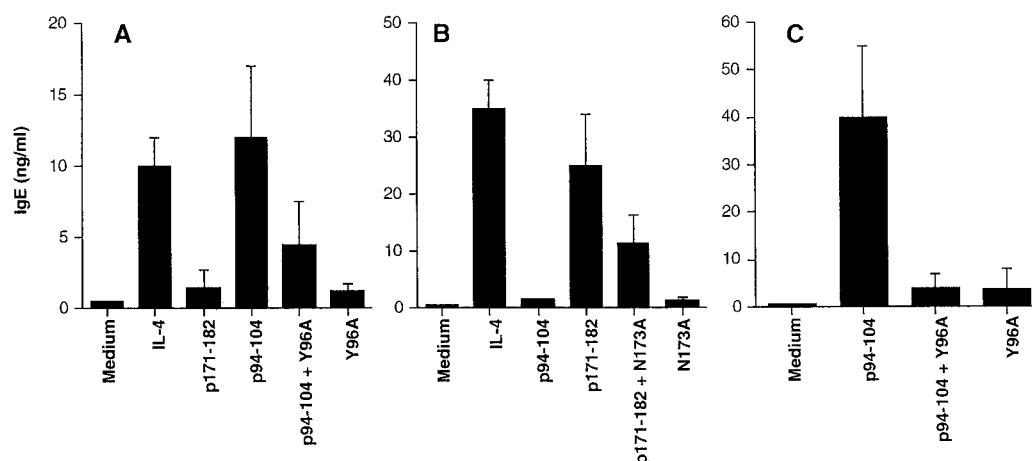


FIG. 4. Inhibition of T-cell help for IgE synthesis by antagonistic peptides. Ten thousand highly purified B cells (> 99%) were incubated with anti-CD40 antibody (1 μ g/ml) and cultured with 25% dilution of culture supernatants from T-cell clones NP-44 (**A**) and AR-142 (**B**) that had been stimulated with wild-type peptide (1 μ g/ml), APCs, and a 100-fold excess of antagonistic peptides for 24 hours as described in Methods section. Alternatively, 10^4 NP-44 cells that had been stimulated as described in **A** were washed twice with medium cultured with highly purified B cells in presence of 100 U/ml rIL-4 (**C**). After 14 days, IgE levels in B-cell cultures were measured by ELISA as described in Methods section.

block Ig isotype switching and subsequent IgE production by B cells *in vitro* by decreasing IL-4 production by Der p 1-specific T-cell clones and by interfering with the expression of the CD40L and possibly other surface molecules on T cells, required for optimal T cell–B cell interaction.

DISCUSSION

In this study we demonstrate that certain modified peptide sequences, derived from native T-cell activation-inducing sequences in the major house dust mite allergen Der p 1, fail to induce proliferative responses and cytokine production of Der p 1-specific T_{H2} -like clones. In addition, when added in excess, they strongly inhibit allergen-induced activation, resulting in the inability of these T-cell clones to give help to B cells for the synthesis of IgE. An overview of the effects of the substituted peptides on T-cell activation is shown in Table I. These peptides were derived from the p94-104 and p171-182 sequences, which are minimal T-cell activation-inducing epitopes on Der p 1 and are recognized in the context of HLA DR11 and HLA-DR15, respectively. Single substitutions of Arg⁹⁵, Tyr⁹⁶, Glu¹⁰⁰, and Gln¹⁰¹ in p94-104 abolished proliferative responses of T-cell clones NP-12 and NP-44. Similarly, stimulation of T-cell clone AR-142 with modified peptide p171-182 containing substitutions at Asn¹⁷³, Val¹⁷⁵, Gly¹⁷⁶, Ser¹⁷⁸, Ala¹⁸⁰, and Gly¹⁸¹ by Ala did not induce a proliferative response when used at similar concentrations as the nonsubstituted peptide. In addition, stimulation of the T-cell clones with these modified peptides also failed to induce the production of cytokines. These results indicate that the above-mentioned residues are critical for recognition of p94-104 and p171-182 by the TCR or may function as anchors involved in binding to agretopes on

HLA DRB1*1101 and DRB1*1501, respectively. The failure of some of the substituted peptides to induce T-cell activation might be due to an impaired interaction of these peptides with the TCR, with HLA-DR molecules, or both. Future studies with MHC-binding analysis may provide an answer to this question.

The Der p 1-specific T-cell clones used in this study have a T_{H2} -like cytokine production profile, secreting high levels of IL-4 and IL-5 and low levels of IFN- γ upon activation. The production of the latter cytokine probably is due to the presence of IL-12 in the cultures, which has been shown to induce the production IFN- γ by polarized T_{H2} cells.^{35,36} Furthermore, IL-2, which is produced by human T_{H1} as well as T_{H2} clones, was produced at detectable levels. Stimulation of the T-cell clones with the antagonistic peptides resulted in a general inhibition of activation and did not induce a segregation of proliferation and cytokine production, as has been reported for murine^{21,23} and certain human T_{H0} clones.²⁵ In addition, incubation of the Der p 1-specific T-cell clones with the modified peptides, at concentrations required for optimal T-cell proliferation, did not induce a state of unresponsiveness as shown previously for altered peptides derived from ovalbumin.³⁷ Our results also partially contrast with those of Tsitoura et al.²⁶ and Ikagawa et al.,²⁷ who reported that stimulation of allergen-specific T-cell clones with altered peptides resulted in enhanced production of IFN- γ but did not affect the production of IL-4. In view of the latter studies showing that single amino substitutions can alter the profile of cytokine production by T-cell clones, the effects of amino acid residues other than the Ala substitutions were studied in the antagonistic peptide N173A. The initial substitution with the neutral amino acid residue, Ala or Ser, produced the most efficient antago-

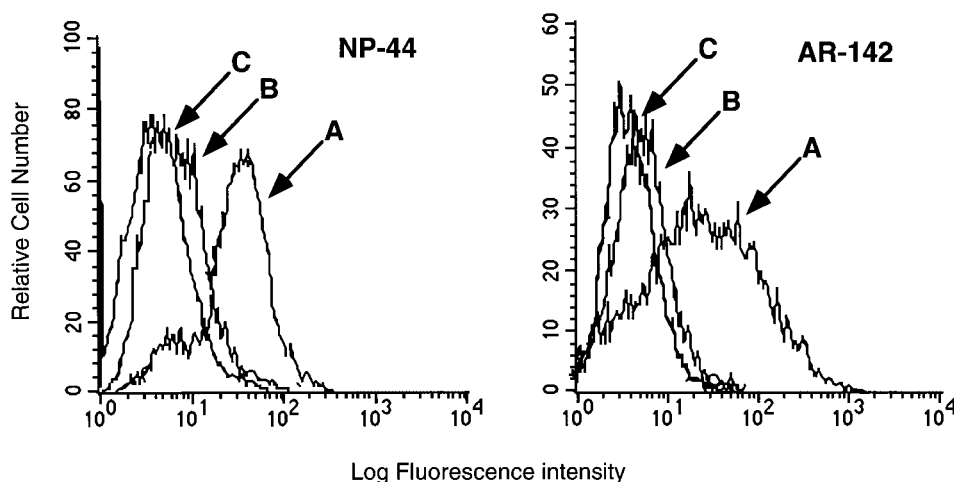


FIG. 5. Inhibition of CD40L expression by antagonistic peptides. NP-44 and AR-142 cells with autologous EBV-LCL and wild-type peptides p94-104 or p171-182, respectively, in absence (A) or presence (B) of a 100-fold molar excess of antagonistic peptide as described in Methods section. CD40L expression was analyzed by flow cytometry after 6 hours of culture. The x- and y-axes represent fluorescence (four-decade log scale) and relative cell number, respectively. CD40L expression on resting, nonstimulated T-cell clones is shown in histogram (C).

nists, whereas substitution with the hydrophobic Ile or acidic Asp was less effective in inhibiting cytokine production (data not shown). However, none of these other substitutions at position 173, albeit resulting in the generation of peptides with antagonistic activity, selectively inhibited cytokine production or proliferation alone or changed the T_{H2} -like cytokine secretion pattern of the T-cell clones.

Among the substituted peptides that failed to induce activation of the Der p 1-specific T-cell clones, three of four derived from p94-104 and two of six derived from p171-182 were found to have strong antagonistic activity (Tables I and II and Fig. 3). Incubation of the T-cell clones in the presence of the nonsubstituted immunogenic peptide in the presence of an excess of these antagonistic peptides resulted in a strong inhibition of proliferation as well as of the production of IL-2 and IFN- γ . Because the proliferative responses of the Der p 1-specific T-cell clones used in this study are strictly dependent on the presence of IL-2 despite their T_{H2} -like phenotype (H. Yssel, unpublished results), the inhibitory effects of the antagonistic peptides are therefore likely to be the result of the strong inhibition of IL-2 production as well as of CD25 expression (data not shown). Interestingly, however, the TCR antagonists had less effect on IL-4 and IL-5 production induced by the nonsubstituted native peptides. For example, peptide analogues N173A and Q181, derived from the p171-182 molecule, strongly inhibited proliferative responses and IL-2 production already at a 10-fold molar excess over the native peptide, whereas the presence of a 100-fold molar excess of these antagonists resulted in the decrease of IL-4 and IL-5 production of less than 50%. Although these results do not show an uncoupling between proliferation and cytokine production, they do not rule out the possibility that

the antagonistic peptides may have differential effects on signal transduction by the TCR. In particular, the low inhibitory effects of the antagonistic peptides on IL-4 production are in line with previously published observations, suggesting that the synthesis of IL-4 is less dependent on costimulatory signals, as demonstrated by the ability of mouse T_{H2} clones to continue to produce IL-4 when stimulated with chemically modified APCs.²¹ Moreover, IL-4 production seems to be favored by signals resulting from less potent TCR-ligand interactions, such as those after stimulation of T cells with peptides that have a low affinity for the TCR or for MHC class II molecules.²¹ In addition, it has been reported that mouse T_{H1} and T_{H2} clones use different T-cell signaling pathways, suggesting that TCR-mediated signaling is likely to differ between IL-4 and IFN- γ production.³⁷ On the other hand, however, because the antagonistic peptides, when used by themselves had an "all or none" effect on the cytokine production profile of the Der p 1-specific T-cell clones, it cannot be excluded that the observed differences in sensitivity between the various cytokines, after stimulation of the cells in the presence of antagonist and native peptide, may be the result of differences in the threshold of the activation signal or signals mediated by the TCR, rather than differences in signaling pathways.

The synthesis of IgE by B cells has been shown to be specifically induced by IL-4 and IL-13 in the presence of a signal provided for by the interaction of CD40, expressed on B cells and CD40L, expressed on activated T cells.⁴⁻⁹ Moreover, IL-4/IL-13-induced IgE synthesis is inhibited by IFN- γ .⁴ Culture supernatants of T-cell clones NP-44 and AR-142 that had been activated with specific peptide in the presence of excess antagonistic peptides Y96A and N173A, respectively, were still able to induce anti-CD40-driven

IgE synthesis by B cells. However, levels of IgE synthesis were inhibited by approximately 50% to 75%, compared with those induced by T-cell supernatants generated after stimulation of the T-cell clones with native peptides alone. These results reflect the partial inhibitory effects on the reduced levels of IL-4 and, most likely, IL-13 production by the T-cell clones. In addition, T-cell clone NP-44, activated in the presence of native peptide p94-104 and excess antagonistic peptide Y96A, was no longer able to give help to B cells for the synthesis of IgE, even in the presence of exogenous IL-4. The inhibitory effects on these antagonistic peptides on T cell-mediated IgE synthesis appear to be the result of their capacity to downregulate or prevent the induction of CD40L expression on the T cells. Because these results underscore the notion that the antagonistic peptides used in this study do not appear to induce uncoupling of TCR-mediated signals leading to proliferation, cytokine synthesis, and expression of cell surface molecules, it is likely that the expression of other cell surface molecules that may be associated with productive T cell-B cell interaction, such as membrane-bound tumor necrosis factor (TNF)- α ,³³ are downregulated as well. Taken together, these results demonstrate that antagonistic Der p 1-derived peptides are able to effectively inhibit T_{H2} cell-mediated IgE synthesis by decreasing the production of the IgE synthesis-inducing cytokine IL-4, as well as by inhibiting the functional expression of cell surface molecules, which are important for productive T cell-B cell interactions.

In conclusion, in this study we describe the generation of antagonistic peptides, after the substitution of single amino acid residues that are effectively interfering with the activation of and help for IgE synthesis by Der p 1-specific T-cell clones. Although the activation of the T-cell clones in this study was carried out with wild-type Der p 1-derived peptides, identical inhibitory effects of the antagonistic peptides were also observed when the T-cell clones had been activated with the native Der p 1 allergen (data not shown). In this respect, these peptides may be of potential interest for therapeutic use. It must be noted, however, that house dust mite extracts consist of a large number of major allergens, each containing multiple T-cell activation-inducing epitopes.^{15, 38, 39} In addition, allergens are recognized in the context of different HLA class II molecules, which hampers efforts to design immunotherapy protocols with the aim of inactivating allergen-specific T cells. It is therefore important to state that despite their effectiveness in inhibiting IgE synthesis in vitro, more information is required to evaluate the potential use of antagonistic allergen-derived peptides in immunotherapy.

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