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Use of Antagonist Peptides to Inhibit In Vitro T Cell Responses to Par j1, the Major Allergen of Parietaria judaica Pollen

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Antigenic peptides with substituted side chains inhibit immune responses to a number of recall Ags from infectious agents in vitro. Here we show that the same strategy can be applied to peptides derived from a pollen protein, the major allergen of Parietaria judaica (Par j1), a plant responsible for most allergic sensitization in the southern Mediterranean area. Three T cell lines responding to Par j1 protein were used to identify a stimulatory peptide. Two different monosubstituted altered peptide ligands (APL) were identified that bound to the HLA-DR of the responders, did not stimulate the T cell lines on their own, and decreased the response to subsaturating amounts of the unmodified stimulatory peptide. Most important, these APL were able to inhibit the response of these cell lines to intact Par j1 protein. A third monosubstituted peptide bound to the HLA-DR but did not show inhibitory activity. The two APL had a lower affinity than the unsubstituted peptide for the HLA-DR. The last two observations make MHC blockade an unlikely explanation for the observed effect. These results indicate the action of a specific peptide-mediated antagonism that may be useful in controlling the T cell component of an allergic response. The Journal of Immunology, 1999, 162: 1982–1987.

The phenomenon of antagonism is well known for a wide range of receptors (1). Recently it has been applied to the recognition by the TCR of antigenic peptides bound to MHC proteins. Altered peptide ligands have been designed that can inhibit T cell recognition even in the presence of the corresponding unmodified antigenic peptide (2–5). Altered peptides usually incorporate one substitution of a putative T cell contact residue of a known antigenic peptide, and they maintain unmodified MHC binding properties. Antagonism is hypothesized to involve incomplete signaling through the TCR complex either due to conformational or kinetic changes (6–10). Since the discovery of TCR antagonism, several experimental attempts have been made to regulate T cell responses in immune-mediated diseases (11).

However, the phenomenon has been explored in most detail using T cell clones responding to viral or model peptides.

An important application of peptide antagonism could be the control of allergic diseases characterized by specific IgE production after contact with allergens. The ability of IgE to bind mast cells and basophils and the ensuing release of histamine and other mediators are responsible for most, if not all, of the clinical features found in allergic patients (12). The T cell help needed for Ab production is a critical point in developing immune response. The role of T cells in specific IgE production has been extensively assessed and provides some of the strongest evidence for T cell-mediated B cell help (13, 14). Therefore, if T cell help can be down-regulated, it may relieve the resulting allergic response.

There has been a dramatic increase in allergic symptoms over the last few years, and the social cost for their management is heavily affecting the health care system of the developed countries (15). Parietaria (Pellitory of the wall, family Urticaceae) accounts for most of the allergenic sensitization in the southern Mediterranean area (16). The allergen that is the focus of these studies, Par j1, is the predominant allergen protein recovered from the pollen of Parietaria judaica. The importance of Parietaria as source of allergic stimuli is currently under investigation in the United Kingdom, United States, and Australia in regions where the plant also flourishes (17–20). The ability to regulate T cell response could be an extremely important approach for controlling IgE production in P. judaica allergies, therefore opening a new approach to immunotherapy.

Materials and Methods

PBMC separation

Blood was obtained from three patients (BS, DFA, DFL) sensitized to Par j1 as determined by skin prick tests and IgE measurement by radioallergosorbent test (RAST). The three patients were fully informed about the aim of the study and consented to donate blood for research purposes.

Abbreviations used in this paper: wt, wild type; sDR, soluble HLA-DR molecules; APL, altered peptide ligands; RFU, relative fluorescence units.
PBMC were isolated by gradient density separation using Ficoll (Phar- 
icia, Piscataway, NJ) according to standard protocols.

Cell lines

PBMC were cultured in flat-bottom six-well plates (Costar, Cambridge, MA) suspended at $1 \times 10^6$ cells/ml in complete RPMI 1640 (supplemented with 0.2 mM L-glutamine, 100 U penicillin, 100 $\mu$g/ml streptomycin; Life Technologies, Grand Island, NY) plus 10% heat-inactivated human serum AB (HyClone, Logan, UT) and stimulated with purified Par j1 at $5 \mu$g/ml final concentration. Purified Par j1 was a generous gift of Dr. J. Carreia, (Abello, Madrid, Spain). Irradiated autologous PBMC (3000 rad from a $^{60}$Co source) plus Ag were used to stimulate the cells every 7 days for 2 wk. The cells were further expanded using RPMI-2 20 U/ml plus purified allergen for 1 more wk. Before use in the experiments described, the specificity for Par j1 was assessed by a standard thymidine incorporation assay comparing specific proliferation to Par j1 with that to the recall Ag purified protein derivative or a different pollen Ag Lol pI (ryegrass). For the specificity assay the cell lines were divided at $2 \times 10^4$ cells/well in a final volume of 200 $\mu$l of complete medium (RPMI 1640 supplemented as above) in a 96-well flat-bottom microtiter plate and stimulated with Par j1 at $5 \mu$g/ml, Lol pI at $10 \mu$g/ml, or purified protein derivative at $10 \mu$g/ml. After 72 h, each well was pulsed with 1 $\mu$Ci of $[^{3}H]$ for each well. Sixteen hours later, cells were harvested onto glass filters, and radioactivity was measured by liquid scintillation by means of a beta counter. Proliferation values of control cultures have been subtracted, and all cultures were in triplicate. The response of the lines was similar to that described for T cell clones specific for Par j1 (21). Ab-blocking studies were performed using the Ab L243 (IgG2a, anti-HLA-DR) provided as culture supernatants and used at 1:25 dilution on the basis of titration experiments.

Screening of peptides

Peptides were synthesized from various portions of the Par j1, and an immunodominant peptide was identified by its ability to stimulate most of the T cell clones derived from the cell lines (21). This peptide corresponds to residues 47–65 of the Par j1 sequence. Several peptides were designed substituting an alanine or valine at positions that are not likely to be DR anchor residues. The peptides used for the experiments were chosen on the basis of their ability to bind DR while not stimulating a large panel of clones derived from DR3, and DR6. As can be seen, the wt and altered peptides can inhibit the binding of the control peptide to sDR1, sDR3, and sDR4. None of the peptides can inhibit the binding of the control peptide to sDR52a.

Antigenic properties of the altered peptides

The ability or inability of the substituted peptides to stimulate the T cell lines used here was measured. The results are shown in Fig. 2. For all three cell lines, the V6, A11, and A14 substituted peptides were very poor stimulators as compared with the wt peptide. The range of concentrations used was in the same order as that needed to obtain stimulation with wt peptide. This characteristic would make them suitable peptides to act as MHC blockers or TCR antagonists when used in this concentration range. It is not known whether at much higher doses the substituted peptides may start to act as agonists.

Altered peptide inhibition of Ag responses

In light of these results, we tested the ability of these three altered peptides to block the response of the T cell lines to wt peptide and intact Par j1 protein. Inhibition experiments were performed with minor modification to published protocols (5). The three T cell

### Table I. Peptides derived from position 47-65 of the Par j1 sequence

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>wt</td>
<td>IQTAMKTYSDIGGLVSEV</td>
</tr>
<tr>
<td>V6</td>
<td>IQTAMKTYSDIGGLVSEV</td>
</tr>
<tr>
<td>A11</td>
<td>IQTAMKTYSDGGGLVSEV</td>
</tr>
<tr>
<td>A14</td>
<td>IQTAMKTYSDIGGLVSEV</td>
</tr>
</tbody>
</table>

Peptide inhibition experiments were performed as described (5) with some minor modifications. Cell lines having the best proliferation with the wt peptide were chosen for the experiments. Specific cell lines isolated as previously described were cultured for 1 more wk using wt peptide. Cells were divided in 96-microtiter plates as described. Irradiated autologous PBMC or irradiated autologous EBV-transformed cells were incubated with suboptimal doses of wt $(10 \mu$M) and Par j1 $1 \mu$g/ml $(70 \textrm{nM})$ for 3 h at 37°C. Cells were washed twice with PBS to remove unbound peptide or Ag, resuspended in complete media, and monosubstituted peptides (V6, A11, and A14) were added at different concentrations. After 16 h, the cells were washed twice in RPMI 1640, and T cells and complete media were added to obtain a 1:1 ratio of APCs and T cells. After 24 h, $[^{3}H]$thymidine was added, and a standard T cell proliferation assay was conducted as described above.

Results

The recently published sequence of Par j1 (23) was used to identify a peptide able to stimulate T cell lines and clones derived from these lines (21), from three allergic donors. The sequence of the peptide thus identified is shown in Table I and will be referred to as wt. This sequence was used to design a number of altered peptides with single alanine or valine substitutions at positions deemed unlikely to be anchor residues. These peptides were tested on a panel of T cell clones derived from the three donors, and those peptides incapable of stimulating the clones were identified. Three peptides, called V6, A11, and A14 after the single position substituted (Table I), were tested for the necessary criteria to act as antagonists: Class II MHC binding, inability to induce specific proliferation of the T cell, and inhibition of T cell response to the normal Ag.

### Class II MHC binding properties of the peptides

Class II MHC binding of the peptides was studied by competition experiments using empty soluble HLA-DR molecules (sDR) produced in insect cells infected with baculovirus (24, 25). Fig. 1 shows the results of a competition peptide binding assay using sDR1, sDR3, sDR4, and sDR52a. The ability of the wt and altered peptides to inhibit the binding of well-characterized class II-binding (control) peptides to the soluble DR molecules was tested as previously described (26). DR1 and DR3 were tested because they are found in the responding cell lines (line BS is DR1,2 and lines DFA and DFL are DR3). DR4 represents a common HLA allele, and DR52a is the product of a second class II locus associated with DR3 and DR6. As can be seen, the wt and altered peptides can inhibit the binding of the control peptide to sDR1, sDR3, and sDR4. None of the peptides can inhibit the binding of the control peptide to sDR52a.
lines were prepulsed with suboptimal amounts of wt peptide or Par j1 protein and then cultured with the altered peptides. When the cultures are costimulated with the wt peptide and increasing concentrations of two peptides, V6 and A11, the initial response is inhibited in all three cell lines (Fig. 3). Altered peptide A14 had no influence on the proliferation of the cell lines.

Most interestingly, there was also a decrease in proliferation of the cell lines stimulated by the intact Par j1 protein in the presence of V6 or A11 (Fig. 4). The effect was less pronounced for the BS line and the A11 peptide. However, at higher peptide concentrations, the response was inhibited to the same extent as in the other lines. The intact protein induces a higher proliferation rate (five- to

Identity of competitor peptides

FIGURE 1. Peptide binding studies. Each panel shows the results of competition binding experiments with one HLA-DR allele-labeled peptide pair. The HLA-DR allele and the fluorescently labeled binding (control) peptide are identified in the upper right corner of each panel. The identity of the competitor peptide (present at 1 mM) is identified along the x-axis. None, the binding of 1 μM FITC-labeled control peptides with 10 nM purified soluble HLA-DR protein without competition. Control, competition with the unlabeled form of the same peptide used for the binding assay.

FIGURE 2. Stimulation of Par j1-specific T cell lines with peptides. Donors are identified at the top of each panel. The peptides are identified in the inset; wt (triangle), V6 (square), A11 (diamond), and A14 (circle). The y-axis shows the uptake of [3H]thymidine in cpm, and the x-axis shows the concentration of the stimulatory peptide. For comparison, the maximal response for 5 μg/ml (∼0.35 μM) of intact Par j1 was 55,000 cpm for DFA, 53,000 cpm for DFL, and 49,000 for BS. The values reported are the average of triplicates.
sixfold) than does the equivalent concentration of wt peptide (legend Fig. 2). In spite of this increased proliferation, the two altered peptides were able to reduce the response. However, the concentration of APL required to achieve this inhibition was 100-fold higher than that needed for inhibiting the response to the wt peptide.

Affinity of the altered peptides

It is possible that the altered peptides either act as antagonists or directly compete with the wt peptide or Par j1 Ag for MHC class II binding. Even at approximately equimolar concentrations, the APL could act to block the MHC class II if their affinity for the MHC was much greater than that of wt peptide. To test this, the affinity of the three altered peptides for HLA-DR1 was measured directly and compared with wt (Fig. 5). The altered peptides show a decrease in affinity compared with wt.

DR-restriction experiments

To assess the DR restriction of these cell lines, we used the anti-HLA-DR mAb L243. Proliferation experiments were conducted in the presence of the Ab as described in Materials and Methods. As shown in Fig. 6, the addition of L243 to the cultures inhibits T cell responses to both Par j1 and wt. No inhibition was detected when the anti-class I mAb w632 (IgG2a) was added to the cultures (data not shown).

Discussion

We identify a number of peptide antagonists that interfere with allergen peptide stimulation of T cell lines derived from sensitized individuals. More importantly the peptides also inhibited the response of the line to the intact allergen. This is the first report of the use of peptides to inhibit the response to an entire allergen. This inhibition occurred in spite of the high proliferation observed when the cell lines were stimulated with the entire Ag. An increase in proliferation when entire Ag is used as compared with a peptide is not unexpected since processing of entire Ag can be envisaged as being more efficient than that of peptides and may increase other signals sent by the APC (27, 28). It should be pointed out that the lines were subject to a final priming with wt peptide before the inhibition studies. However, since the lines were generated using entire purified Par j1 as stimulator, the effect of this last peptide priming would be minimal unless the response was already heavily skewed toward this particular T cell epitope. Thus, the inhibition of the response to the intact Ag would indicate that the major T cell

FIGURE 3. Peptide inhibition assays using wt peptide as stimulator. The panels left to right are inhibition with the V6, A11, and A14, respectively. Components of the assay are described in the inset. The y-axis shows percentage of maximal proliferation, and the x-axis shows the concentration of inhibitor peptide used. The data shown here are representative of three independent experiments. Maximal responses (100%) were the following for each culture identified by peptide and responder and shown as $10^{-3}$ cpm: for V6, DFL = 40, BS = 37, and DFA = 52; for A11, DFL = 47, BS = 46.5, and DFA = 67; for A14, DFL = 42, BS = 39, and DFA = 57.

FIGURE 4. Peptide inhibition assays using intact Par j1 protein as stimulator. The legend is like that for Fig. 3. Maximal responses (100%) were the following for each culture identified by peptide and responder, and shown as $10^{-3}$ cpm: for V6, DFL = 48, BS = 45.3, and DFA = 70; for A11, DFL = 60, BS = 40.5, and DFA = 74; for A14, DFL = 55, BS = 43, and DFA = 65.
epitope in the protein corresponds to the region from which the peptide was derived. Preliminary data obtained in our lab support this hypothesis. The ability of a modified peptide to inhibit proliferation of a T cell line responding to the entire Ag approaches the natural situation where such a response would be required to the pollen itself to be beneficial. While being a more realistic system, the use of T cell lines that respond to the APC derived from PBMC could introduce unexpected complications into the experimental study of a phenomenon that at its core involves class II MHC, peptide, and TCR. Thus, it should be pointed out that the ability of fixed irradiated autologous EBV lines to act as APC was determined in a number of independent experiments. The proliferative response of the T cell lines to wt peptide was impaired by the two APL (data not shown), excluding possible mechanisms due to class II recycling, peptide impairment of processing, or other effects mediated by the use of PBMC as APC (5, 29, 30), and making it likely that the observed inhibition is due to the APL binding to class II MHC.

Peptides can act in a number of ways to inhibit immune responses (reviewed in Ref. 31). For in vitro cultures, the two most likely mechanisms are peptide blockade of the MHC molecule, or TCR antagonism. It should be pointed out that the substituted peptides were used in a range that maximally represented a twofold excess over wt peptide. This would not be expected to favor MHC blockade. The nature of the inhibition observed here is further clarified by the results with the A14 peptide and by a more detailed analysis of the HLA-DR binding affinities of the altered peptides. The A14 peptide can bind to DR1 and -3, cannot stimulate the T cells, yet does not inhibit the T cell response to either Par j1 or wt peptide. This makes peptide competition for available HLA-DR molecules (MHC blockade) an unlikely explanation for the inhibition shown by the other two peptides. It also rules out other nonspecific effects due only to the presence of extra peptide during the stimulation. Even at equimolar concentration of agonist and antagonist, MHC blockade could occur if the APL affinity is much higher for the class II than that of the wt peptide. The APL show a lower affinity for DR1 as compared with wt peptide. This observation also rules against the occurrence of MHC blockade. To the extent that this observation can be generalized to DR3, this argument would hold for the whole system. Thus, the data from the stimulation with the wt peptide support true peptide antagonism (5) as an explanation for the observed phenomenon.

The nature of the inhibition of the response to the Par j1 protein is less clear. Even at the lowest concentration of altered peptides used, the excess of altered peptide to target was 100-fold. As mentioned above, the fact that the A14 peptide can bind to the DR alleles involved, yet not inhibit the response, does not favor an MHC blockade explanation. A number of possible explanations can be offered for why higher concentrations of altered peptides are needed to see effects equivalent to that observed with the wt peptide experiments. It could be assumed that the processing of intact protein induces the APC to function more efficiently. Factors associated with the nature of the uptake of peptide vs that of intact Ag may play a role. For example, if peptide binding is predominantly limited to surface exchange with preloaded class II, stimulation by peptides may be less efficient than by a protein that is processed by the endosomal pathway. It is also possible that other portions of Par j1 protein may act to boost the response. We have recently described T cells recognizing carbohydrate moieties of Par j1 protein that produce cytokines (32). These T cells could partially offset the effect of the APL on the response to the peptide portion of the processed protein. While the observations with intact Ag are interesting and point to the use of altered peptides in allergic responses, more experiments are needed to further define the nature of this response and its antagonism.

![Graph](http://www.jimmunol.org/)

**FIGURE 5.** Determination of APL affinity. The percentage inhibition of FITC-labeled MP19–31 binding to sDR1 by wt, V6, A11, and A14 peptides is shown at different concentrations of the peptides, respectively.

**FIGURE 6.** Determination of DR restriction. A. Response of the T cell lines to whole Par j1 protein in the presence or absence of anti-DR mAb L243. B. Response of the T cell lines to wt peptide. T cell lines and culture conditions (with or without L243) are the same for A and B and are identified in B.
The nature of the responding T cells is an interesting aspect of this system, and more detailed experimentation will be necessary to define the repertoire. Because antagonism is thought to be a clonal phenomenon, a number of tentative conclusions can be reached. The fact that the lines are antagonized by peptides corresponding to a single region of the Ag would lead to the expectation that the repertoire of responding T cells is limited in these lines. Preliminary analysis of the three lines has indeed shown a limited T cell repertoire in all three lines. However, lines from each individual display a distinct pattern of TCR V gene usage and CDR3 length (De Palma et al., manuscript in preparation). It will be necessary to link these T cells with the B cell responsible for specific IgE secretion. A major B cell epitope has been characterized on Par j1 (33). This may restrict the number of responding B cells, making their identification and the subsequent analysis of their sensitivity to activated T cells easier.

It will also be interesting to investigate the modality of antagonism in these experiments. Biochemical and functional studies will be necessary to determine to what extent altered peptides interfere with specific T cell signaling and lymphokine production. The relation between cytokine production and the inhibition of proliferation studied here will be important in this regard. The identification of the peptide residues that are involved in MHC contact and identification of TCR contact residues will further our understanding of the T cell recognition process. More importantly, studies of additional side chain substitutions at the TCR contact residues should permit the design of better antagonists.

The availability of peptides that are able to down-regulate specific T cell responses could lead to lower IgE production. The use of such peptides could avoid the risks of traditional immunotherapy, in which entire Ag is used. Specific T cells could be regulated without involvement of a further IgE secretion, because antagonist peptides can be designed that do not contain epitopes recognized by IgE. Our observation that these altered peptides bind to a number of common class II MHC alleles implies that they may be of general use. While the use of peptides as tolerizing agents in allergy has been investigated (34, 35), this is the first report of the possible use of peptides to antagonize the response to a whole allergen. The large extent of morbidity due to allergies and the increased mortality due to allergic asthma justify a particular attention toward these diseases, and the data reported here suggest new possibilities for therapies.

Acknowledgments

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tition for antigen presentation in living cells involves exchange of peptide bound by class II MHC molecules. Nature 342:880.