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d healthy controls. *Int. Archs.*

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e clones: induction, antigen
immune clones. *J. Immunol.*

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36K and 65K *Mycobacterium*
J. Immunol. **18**, 849–854.

aracterization of lymphocyte
children, and allergic children
111, 1065–1072.

class II tetramers in tracking
Methods **29**, 282–288.

xtious Epstein-Barr virus by
Sci. USA **69**, 383–387.

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Mapping of Human T-Cell Epitopes of Allergens

Thomas Zeiler and Tuomas Virtanen

Abstract

Allergens are characterized by their ability to be bound by gE. The Swiss-Prot protein database currently lists a partial or complete amino acid sequence of in excess of about 350 allergens. It is not clear how allergens participate in the process of allergic sensitization, the generation of specific T-helper type 2 (Th2) lymphocytes, which play a crucial role in stimulating B lymphocytes to produce allergen-specific IgE.

T-helper (Th) cells play a key role in the regulation of immune responses. The recognition of antigen by T cells is complex and it can trigger qualitatively differential signaling. Therefore, it is conceivable that epitopes or antigenic determinants recognized by Th cells may influence the quality of immune response. The aim of this chapter is to describe the way in which T-cell epitopes can be identified (mapped). This is particularly important because knowledge of the precise T-cell epitopes of allergens can give important information on the pathogenesis of allergy and can help to develop better preparations for the diagnostics and/or immunotherapy of allergy.

Key Words: Allergen; epitope; T-helper cell.

1. Introduction

Allergens are a special group of antigens characterized by their ability to be bound by IgE. Knowledge of the molecular biology of allergens has increased greatly during the past 10–15 yr. The Swiss-Prot protein database lists a partial or complete amino acid sequence of about 350 allergens at the moment (<http://www.expasy.ch/cgi-bin/lists?allergen.txt>; March 1, 2006). Despite the increased information, it is not clear how allergens participate in the process of allergic sensitization, the generation of specific T-helper type 2 (Th2) lymphocytes, which play a crucial role in stimulating B lymphocytes to produce allergen-specific IgE. Although several allergens are enzymes and this property appears

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to be important for their allergenicity (*1*), there is no evidence that any biological property in general would account for the allergenic capacity of proteins (*2,3*).

The specific cells of the immune system that recognize antigens and allergens are B and T lymphocytes. Whereas B cells recognize three-dimensional molecular structures by their receptors (*4,5*), T-helper (Th) cells (CD4⁺) recognize short linear fragments of allergens (approx. 10–20 amino acids) presented on the surface of antigen-presenting cells in association with the major histocompatibility complex (MHC) class II molecules (*6,7*).

Th cells play a key role in the regulation of immune responses (*8*). The recognition of antigen by T cells is not a simple on/off phenomenon and it can trigger qualitatively differential signaling (*9,10*). Therefore, it is conceivable that the specific sites, epitopes, or antigenic determinants recognized by Th cells in allergens may influence the quality of immune response. In other words, the way how allergens are recognized by the cells of the immune system can be involved in favoring Th2 differentiation (*11–13*). In this context, the interplay among the immune system, exogenous allergens, and endogenous proteins can be important (*11*).

In addition to the fact that knowing the T-cell epitopes of allergens can give important information on the pathogenesis of allergy, it can help to develop better preparations for the diagnostics and/or immunotherapy of allergy. For the preparation to be used in immunotherapy, it is important that it contains all the important T-cell epitopes because the beneficial effects of immunotherapy are expected to be mediated via T cells (*14*). This is especially true for modified preparations, such as T-cell epitope-containing peptides or their constructs (*15*).

The conventional way of mapping T-cell epitopes is straightforward but requires basic knowledge of the methods of T-cell culture (*see* previous chapters). In brief, the optimal stimulation concentrations of peptides and allergens should be determined in advance. The mapping should be performed as soon as there are sufficient numbers of allergen-specific T cells, either clones or in lines, because the growth of T cells slows down after repeated stimulations. In some cases, the analysis can be done directly with peripheral blood mononuclear cells (PBMC) (*16,17*) but weak responses may preclude this approach (*18,19*). T-cell epitopes are determined by stimulating the cells with peptides and measuring the responses by the incorporation of ³H-thymidine in a proliferation test.

The mapping can be complemented by measuring the binding of allergen peptides to MHC class II molecules (*19*). The analysis is valuable, for example, by identifying peptides which can bind to several MHC alleles.

A novel alternative for mapping T-cell epitopes is to use peptide/MHC class II tetramers (*20*).

2. Materials

1. Allergens (*see* **Note 1**).
2. Peptides (*see* **Note 2**).
3. The allergen-specific T-cells.

3. Methods

3.1. Measurement of Allergy

The responses of allergic peptides are usually measured in Chapter 2 (also *see* **Note 4**).

4. Notes

1. Natural or recombinant lines and clones. It is advisable to use potent stimulants re-creating specific T-cell lines. These should be tested in advance.
2. Overlapping peptides can be purchased from commercial suppliers but they are expensive. The synthesis with the multiplex method is the use of allergen fragments or by the digestion of allergens into fragments of various lengths. One way to decrease the number of possible epitopes with confidence is to use a computer program (<http://www.imtech.res.in/raghu/imap/>). Whereas the length of peptides varies from 10 to 20 amino acids, the mapping (*17*). We have used peptides of 15–20 amino acids (*18,19*). In this way, it is possible to use five peptides which overlap, the response may be weak, its detection can be improved by mapping of T-cell epitopes in which n is the number of amino acids in the protein and p is the number of peptides. The correct amino acid sequence can be determined by mass spectrometry or by sequencing. If the preparation also allows, it can be used directly for mapping.

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2. Materials

1. Allergens (see Note 1).
2. Peptides (see Note 2).
3. The allergen-specific T-cell lines and clones (see Chapters 3 and 4 and Note 3).

3. Methods

3.1. Measurement of Allergen-Specific Proliferation of T Cells

The responses of allergen-specific T-cell clones and lines on stimulation with peptides are usually measured by a standard proliferation assay as described in Chapter 2 (also see Note 4).

4. Notes

1. Natural or recombinant allergens are needed for creating allergen-specific T-cell lines and clones. It is advantageous that the preparation is pure; contaminants may be potent stimulants resulting in T-cell cultures of undesired specificities. For creating specific T-cell lines and clones, the optimal stimulation doses of allergens should be tested in advance with PBMC. It is often between 10 and 200 µg/mL.
2. Overlapping peptides can be obtained most conveniently from specialized commercial suppliers but they are quite expensive. A more economic way may be the in-house synthesis with the multipin technology (e.g., Mimotopes Pty Ltd). Another possibility is the use of allergen fragments. They can be produced by molecular biology methods or by the digestion of allergenic proteins with enzymes followed by a purification step with high-pressure liquid chromatography (HPLC). This approach yields fragments of various lengths which renders it difficult to localize the epitope accurately. One way to decrease the number of peptides needed can be the prediction of possible epitopes with computer programs like TEPITOPE (21) or through Internet (<http://www.imtech.res.in/raghava/propred/>, <http://www.syfpeithi.de/>) before the actual mapping experiments.

Whereas the length of peptides presented in association with MHC II molecules varies from 10 to 20 amino acids (6,7) the length can influence the results of epitope mapping (17). We have used 16mer peptides overlapping with 14 amino acids (18,19). In this way, it is possible to detect the epitope-specific response with three to five peptides which increases the reliability of the results. With a shorter overlap, the response may be seen with only one or two peptides and if the response is weak, its detection can be difficult. The number of peptides needed for the mapping of T-cell epitopes can be calculated according to the formula $n = (l-o)/(p-o)$ in which n is the number of peptides needed (the nearest integer), l is the number of amino acids in the protein sequence, o is the number of amino acids in the overlap, and p is the number of amino acids in the peptide.

The correct amino acid sequence of peptides to be used in mapping should be verified by mass spectrometry or by other methods. This is usually done by the manufacturer. If the preparation also appears pure by the analytical HPLC, it may be possible to use it directly for mapping. This is especially true for initial screening. However,

because contaminants (e.g., peptide fragments) may affect the results, we have preferred to use peptides purified with HPLC.

Peptides may be poorly soluble in water because of their amino acid composition. In this case, the peptide may be first dissolved in a small volume of dimethyl sulfoxide (DMSO) and then diluted in the culture medium. The final concentration of DMSO on plates should be $\leq 0.1\%$ (v/v).

Before use, the peptides can be sterilized by membrane filtration or by γ -irradiation. Storage is at -70°C .

3. Our experience suggests that the responses of PBMCs against allergens are often very weak. Therefore, we prefer to perform the mapping of T-cell epitopes with T-cell lines and clones.

Epitope mapping with a T-cell line is likely to reveal a more comprehensive repertoire of epitopes than the mapping with T-cell clones only. In the mapping with clones, there is always the possibility that cloning and long-term T-cell culture result in the selection of a limited number of clones detecting few immunodominant epitopes. On the other hand, the responses of clones are usually clear-cut and allow a more detailed analysis of the T-cell response.

It is easier to get well-responding T-cell lines from patients with clinically severe forms of allergy. Therefore, patients' reactivity to the allergen to be studied should be verified by skin prick tests and/or by measuring allergen-specific IgE.

4. A starting point for the optimal stimulation concentration for peptides can be deduced from the molar concentration of the allergen which produced the optimal results in tests with PBMCs (or T-cell lines or clones). For example, if the optimal concentration of an allergen with 160 amino acids is $100\text{ }\mu\text{g/mL}$, the concentration for a 16mer peptide could be $10\text{ }\mu\text{g/mL}$ (the molar concentrations being roughly the same for the peptides and the allergen). Testing with lower and higher concentrations of peptides increases the reliability of the results.

The results of a proliferation test (the counts per minute [cpm] obtained from scintillation counting) of a T-cell clone or line are usually expressed as a stimulation index (SI) according to the formula:

$$\text{SI} = \frac{(\text{mean cpm of wells with T cells, feeder cells, and allergen}) - (\text{mean cpm of wells with T cells only})}{(\text{mean cpm of wells with T cells and feeder cells}) - (\text{mean cpm of wells with T cells only})}$$

The SI with PBMCs is obtained simply by dividing the mean cpm of cells with allergen with the mean cpm of cells without allergen. Although no exact limit can be set for a positive response, $\text{SI} \geq 2$ or 3 with PBMCs or T-cell lines and $\text{SI} \geq 5$ with T-cell clones are often regarded as positive. Especially with PBMCs when the proliferative responses can be very weak, another possibility for determining positive responses is to assign a cutoff value calculated as the mean cpm value of several wells containing unstimulated cells plus three times the standard deviation. This allows the results of individual wells to be determined as positive or negative. Then the difference in the frequency of positive wells between stimulated and nonstimulated cultures (several replicates) is analyzed statistically (17,22).

An alternative for measurement of cytokine

References

1. Reed, C. E. and Kita, H. Allergic respiratory disease.
2. Virtanen, T. and Mäntylä, J. Allergens are lipocalins. In *Lipocalins* (eds), L. Salier, J.-P., eds), *Lipocalins*, abstract.php?chapid=25.
3. Holt, P. G. and Thomas, J. Allergens: unresolved issues.
4. Scheiner O. and Kraft, D. *Allergy* **50**, 384–391.
5. Dudler, T., Chen, W.-Q., and others (1992) High-level expression of allergenically active honey bee venom allergens. *J. Biol. Chem.* **267**, 201–210.
6. Schwartz, R. H. (1985) T-cell products of the major histocompatibility complex.
7. Germain, R. N. (1993) *Immunology* (Paul, W. E., ed.).
8. Abbas, A. K., Murphy, K. M., and T lymphocytes. *Nature* **373**, 79–83.
9. Evavold, B. D. and Allen, J. Proliferation by an altered T cell.
10. Sloan-Lancaster, J. and T cell activation: molecular mechanisms. *Immunol.* **14**, 1–27.
11. Virtanen, T., Zeiler, T., and lipocalins: a consequence of allergen. *Immunol. Today* **20**, 398–401.
12. Kinnunen, T., Buhot, C., and of lipocalin allergen Bos d 5. *Int. Arch. Allergy Immunol.* **117**, 171–176.
13. Virtanen, T. and Mäntylä, J. *Allergen Immunotherapy* (Marcel Dekker, Inc., New York).
14. Till, S. J., Francis, J. N., and immunotherapy. *J. Allergy Clin. Immunol.* **95**, 100–105.
15. Ali, F. R. and Larche, N. Allergy for allergic disease. *Exp. Allergy* **10**, 1–10.
16. Bungy, G. A., Rodda, S. C., and topes of the major fraction of allergen.

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is done by filtration or by γ -irradiation.

MCs against allergens are often mapped of T-cell epitopes with

a more comprehensive repertoire of clones. In the mapping with clones, 3-term T-cell culture result in the few immunodominant epitopes. Usually clear-cut and allow a more

in patients with clinically severe allergy the allergen to be studied should be allergen-specific IgE.

Concentration for peptides can be chosen which produced the optimal results. For example, if the optimal concentration is 100 $\mu\text{g/mL}$, the concentration of allergen being roughly 100 $\mu\text{g/mL}$ with lower and higher concentrations results.

Minute [cpm] obtained from scintillation counter expressed as a stimulation

$$\frac{(\text{mean cpm of wells with T cells only}) - (\text{mean cpm of wells without T cells only})}{(\text{mean cpm of wells with T cells only})}$$

Using the mean cpm of cells with allergen. Although no exact limit can be set for BMCs or T-cell lines and $\text{SI} \geq 5$ is recommended. Especially with PBMCs when other possibility for determining stimulation is the mean cpm value is used three times the standard deviation to be determined as positive or negative wells between stimulated and control wells analyzed statistically (17,22).

An alternative for measuring cellular responses in a proliferation assay is the measurement of cytokines from the cultures after stimulation.

References

1. Reed, C. E. and Kita, H. (2004) The role of protease activation of inflammation in allergic respiratory diseases. *J. Allergy Clin. Immunol.* **114**, 997–1008.
2. Virtanen, T. and Mäntylä, R. A. (2005) Important mammalian respiratory allergens are lipocalins. In *Lipocalins* (Åkerström, B., Borregaard, N., Flower, D., and Salier, J.-P., eds), Landes Bioscience, Georgetown, <http://www.eurekah.com/abstract.php?chapid=2559&bookid=190&catid=15>.
3. Holt, P. G. and Thomas, W. R. (2005) Sensitization to airborne environmental allergens: unresolved issues. *Nat. Immunol.* **6**, 957–960.
4. Scheiner O. and Kraft, D. (1995) Basic and practical aspects of recombinant allergens. *Allergy* **50**, 384–391.
5. Dudler, T., Chen, W.-Q., Wang, S., Schneider, T., Annand, R. R., and Dempsey, R. O. (1992) High-level expression in *Escherichia coli* and rapid purification of enzymatically active honey bee venom phospholipase A₂. *Biochim. Biophys. Acta* **1165**, 201–210.
6. Schwartz, R. H. (1985) T-lymphocyte recognition of antigen in association with gene products of the major histocompatibility complex. *Annu. Rev. Immunol.* **3**, 237–261.
7. Germain, R. N. (1993) Antigen processing and presentation. In *Fundamental Immunology* (Paul, W. E., ed.), 3rd Ed., Raven, New York, pp. 629–676.
8. Abbas, A. K., Murphy, K. M., and Sher, A. (1996) Functional diversity of helper T lymphocytes. *Nature* **383**, 787–793.
9. Evavold, B. D. and Allen, P. M. (1991) Separation of IL-4 production from Th cell proliferation by an altered T cell receptor ligand. *Science* **252**, 1308–1310.
10. Sloan-Lancaster, J. and Allen, P. M. (1996) Altered peptide ligand-induced partial T cell activation: molecular mechanisms and role in T cell biology. *Annu. Rev. Immunol.* **14**, 1–27.
11. Virtanen, T., Zeiler, T., Rautiainen, J., and Mäntylä, R. (1999) Allergy to lipocalins: a consequence of misguided T-cell recognition of self and nonself? *Immunol. Today* **20**, 398–400.
12. Kinnunen, T., Buhot, C., Närvänen, A., et al. (2003) The immunodominant epitope of lipocalin allergen Bos d 2 is suboptimal for human T cells. *Eur. J. Immunol.* **33**, 1717–1726.
13. Virtanen, T. and Mäntylä, R. (2004) Mammalian allergens. In *Allergens and Allergen Immunotherapy* (Lockey, R. F., Bukantz, S. C., and Bousquet, J., eds), Marcel Dekker, Inc., New York, pp. 297–317.
14. Till, S. J., Francis, J. N., Nouri-Aria, K., and Durham, S. R. (2004) Mechanisms of immunotherapy. *J. Allergy Clin. Immunol.* **113**, 1025–1034.
15. Ali, F. R. and Larche, M. (2005) Peptide-based immunotherapy: a novel strategy for allergic disease. *Expert Rev Vaccines* **4**, 881–889.
16. Bungy, G. A., Rodda, S., Roitt, I., and Brostoff, J. (1994) Mapping of T cell epitopes of the major fraction of rye grass using peripheral blood mononuclear cells

- from atopics and non-atopics. 2. Isoallergen clone 5A of *Lolium perenne* group I (Lol p I). *Eur. J. Immunol.* **24**, 2098–2103.
17. Reece, J. C., McGregor, D. L., Geysen, H. M., and Rodda, S. J. (1994) Scanning for T helper epitopes with human PBMC using pools of short synthetic peptides. *J. Immunol. Method.* **172**, 241–254.
 18. Zeiler, T., Mäntyjärvi, R., Rautiainen, J., et al. (1999) T cell epitopes of a lipocalin allergen colocalize with the conserved regions of the molecule. *J. Immunol.* **162**, 1415–1422.
 19. Immonen, A., Farci, S., Taivainen, A., et al. (2005) T cell epitope-containing peptides of the major dog allergen Can f 1 as candidates for allergen immunotherapy. *J. Immunol.* **105**, 3614–3620.
 20. Novak, E. J., Liu, A. W., Gebe, J. A., et al. (2001) Tetramer-guided epitope mapping: rapid identification and characterization of immunodominant CD4+ T cell epitopes from complex antigens. *J. Immunol.* **166**, 6665–6670.
 21. de Lalla, C., Sturniolo, T., Abbruzzese, L., et al. (1999) Cutting edge: identification of novel T cell epitopes in Lol p5a by computational prediction. *J. Immunol.* **163**, 1725–1729.
 22. Taswell, C. (1984) Limiting dilution assays for the determination of immuno-competent cell frequencies. III. Validity tests for the single-hit Poisson model. *J. Immunol. Methods* **72**, 29–40.

6

Determining MHC

Mark Larché

Abstract

T-cell receptors (TcR) recognize antigens presented by major histocompatibility complex (MHC) molecules. T-cells are able to bind within the groove of the MHC molecule recognized by the TcR. The TcR will only express one maternal and one paternal MHC and HLA Class II DR, DP, and DQ. A clone of T cells will bear targets (peptide-MHC complex) on the T-cell surface, a cascade of activation.

When investigating the T-cell response, we provide an example of how to identify which MHC molecule is involved in the T-cell response. Such information is important in the chapter, we provide an example of how to identify which MHC molecule is involved in the T-cell response.

Key Words: MHC; T cell

1. Introduction

1.1. How Antigen-Specificity is Generated

The antigen-specificity of T lymphocytes is generated by the interaction of the T-cell receptor (TcR) with the antigen-MHC complex during development (1). The TcR is a bound antigen receptor.