

Immunodominant T Cell Epitopes Associated with Distinct Immune Responses to the Dermatophyte Antigen Tri r 2: Statistical Approaches to Mapping Studies

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Key Words

Trichophyton · T cell proliferation · Peptides · Hypersensitivity, immediate · Hypersensitivity, delayed type

Introduction

The T lymphocytes that control immune responses to foreign proteins react specifically with linear epitopes, or peptides, consisting of 15–20 amino acids. In keeping with this, peptides derived from antigens will stimulate proliferative responses in vitro in cultures of circulating cells from individuals who have made an immune response. Interpretation of T cell epitope mapping studies using peptides is difficult owing to the low frequency of peptide-specific T cells in the total T cell repertoire, and the wide variation in counts per minute (cpm) observed between replicate wells [1–3]. Antigens derived from the dermatophyte fungus *Trichophyton* elicit immediate (IH) or delayed type (DTH) hypersensitivity skin test reactions in different individuals. Evidence for the role of *Trichophyton* antigens in allergic disease is supported by im-

provement of asthma symptoms after treatment with fluticasone in patients with late-onset asthma associated with chronic dermatophytosis and IH to *Trichophyton* [4]. Thus, *Trichophyton* provides a model system for defining T cell epitopes associated with allergic (IH) and cell-mediated (DTH) responses. Classical allergens do not stimulate strong peripheral blood mononuclear cell (PBMC)-proliferative responses in nonatopic individuals, which has proved problematic in defining differences in immunodominant epitopes between atopic and nonatopic subjects. In contrast, the *Trichophyton rubrum* subtilase homologue Tri r 2 (29 kD) has the unique ability to stimulate strong proliferative responses in PBMC cultures established from individuals with distinct skin tests to this antigen [5, 6]. Furthermore, the association of IH and DTH reactions with distinct serum antibody and T cell cytokine profiles suggests that different T cell subsets mediate these responses [7–9].

The goal of the present study was 3-fold: (1) to examine the intra-assay variability in peptide responses; (2) to address how the peptide response of an individual should be evaluated, and (3) to assess how differences in peptide responses between patient groups can be analyzed statistically.

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Table 1. Variation in counts per minute among replicate wells containing peptides derived from Tri r 2: data from selected individuals

Subject No.	Peptide 5				Peptide 8			
	15 ^a	13 ^a	17 ^a	21 ^a	2 ^b	5 ^b	6 ^b	20 ^a
Well								
1	20,936	5,355	<i>419</i>	<i>961</i>	7,545	<i>216</i>	<i>1,907</i>	<i>667</i>
2	67,120	3,356	1,262	3,776	<i>2,041</i>	4,591	7,737	<i>189</i>
3	51,330	2,111	3,700	14,345	<i>2,302</i>	<i>1,784</i>	<i>723</i>	<i>367</i>
4	65,760	7,759	3,954	<i>1,376</i>	5,275	2,557	<i>793</i>	8,664
5	64,665	5,148	2,572	7,329	<i>778</i>	<i>190</i>	<i>1,553</i>	<i>661</i>
6	46,919	2,830	3,971	4,553	7,606	2,551	10,167	<i>252</i>
7	33,390	9,353	2,382	5,891	7,370	2,265	<i>503</i>	<i>967</i>
8	50,876	6,889	8,164	3,395	<i>3,396</i>	7,064	<i>2,752</i>	2,141
9	40,612	8,708	5,720	4,174	<i>2,758</i>	<i>517</i>	11,360	2,834
10	44,723	2,337	1,080	8,616	3,980	3,994	<i>520</i>	<i>892</i>
11	55,511	4,114	4,091	4,358	6,117	<i>1,956</i>	8,148	<i>1,798</i>
12	50,487	4,147	4,291	4,082	<i>2,078</i>	2,164	11,468	9,584
Mean	49,360	5,428	3,467	5,238	4,271	2,487	4,803	2,414
SD	13,588	2,655	2,144	3,588	2,428	1,975	4,560	3,242
Cutoff value from control wells ^c	7,403	1,891	790	1,500	3,499	2,124	3,707	2,070
Stimulation index	55	35	12	12	21	15	11	18
Standardized index	4.33	3.28	2.06	2.30	3.01	2.26	1.92	2.48

cpm shown in italics represent those less than the cutoff.

^a DTH responders.

^b IH responders.

^c The mean + 3 standard deviations of 60 unstimulated wells.

Materials and Methods

Ten patients with IH and 10 with DTH skin tests to r-Tri r 2 were identified for T cell epitope mapping studies. Skin testing of human subjects using r-Tri r 2 and drawing of blood samples for T cell studies was approved by the University of Virginia Human Investigation Committee. A set of twenty-seven 20-mers (designated P1 through P27) and one 19-mer (P28), each with a 10-amino acid overlap spanning the length of Tri r 2, was designed for mapping T cell antigenic determinants. Peptides were prepared by standard F-moc chemistry using a Symphony automated peptide synthesizer (50 μ M scale; Rainin) and purified to >90% by reverse phase HPLC. Stock solutions of peptides (1 mM) were prepared and peptides were diluted to a working concentration of 7.5 μ M immediately prior to use. PBMC were purified by density gradient centrifugation over Ficoll-Paque and cultured at 3×10^5 cells per well for 5 days at 37°C in a 96-well plate. The evaluation of each individual incorporated 12 replicate wells for cultures stimulated with each peptide (i.e. 28×12 total wells) and 60 unstimulated wells. Tetanus toxoid and PHA were used as positive controls. The medium contained RPMI 1640 with L-glutamine, 10% autologous human serum (non-heat-inactivated), 100 U/ml penicillin and 100 μ g/ml streptomycin. Cells were pulsed with 1 μ Ci/well of [³H]-thymidine during the final 8 h of culture before harvesting and counting. Data (cpm) for each of the 12 pep-

tide-stimulated replicates was log-transformed (base e where the value of e = 2.7). A standardized index value was derived for each peptide using log-transformed data. Experimental groups were compared using the mean standardized index for each peptide. Positive wells were defined as those with cpm > mean + 3 standard deviations of 60 unstimulated wells.

Results and Discussion

To optimize the assay design, cells were plated at high cell numbers (3×10^5 compared with $1-2 \times 10^5$ per well) and 12 replicate wells were incorporated for each of the 28 Tri r 2 peptides. By including a large number (n = 60) of unstimulated wells it was possible to compare differences in background counts between individuals and to determine a cutoff level above which the proliferation in any given well was likely to represent a positive response (table 1). While cpm for unstimulated wells generally showed a normal distribution, it was not uncommon to observe high counts in a minority of these wells from

some of the patients, resulting in high cutoff values (i.e. >3,000 cpm; table 1). Since random T cell proliferation is often observed in cell cultures, a positive peptide response was defined as ≥ 3 positive wells (table 1). Using these criteria, some peptides were more stimulatory (peptide 5) than others (peptide 8). Furthermore, analysis of the primary data showed a high variability in counts among peptide-stimulated replicates (503–11,468 cpm for peptide 8 in patient 6). These results clearly illustrate why analyzing responses with 3 replicates for each peptide could give misleading results. Having determined which peptides elicit a positive response, the magnitude of the response for a specific peptide can be compared between individuals. Proliferation data is typically reported as stimulation indices (mean cpm of stimulated wells divided by mean cpm of unstimulated wells). Reporting data in this way does not account for the skewed nature of the responses. Standardized indices were derived based on log-transformed data (table 1). This method corrects for differences in the distribution of counts and quantitative differences in background counts. Standardized indices and the corresponding stimulation indices are shown in table 1. It is important to note that small differences in standardized

indices represent large differences in absolute counts (compare patients 13 and 15, table 1). The good correlation between stimulation and standardized indices reflects the increased analytical power obtained by incorporating multiple replicates in peptide-stimulated cultures. Indeed, our results demonstrated statistically significant differences in the response to a single peptide using the two-sample t test. By log-transforming the data from peptide-stimulated cultures it is possible to apply statistical methods for comparing between different patient groups. Linear discriminant analysis identified two regions of the molecule which predicted classification of patients into IH and DTH groups. These findings suggest that different regions of the molecule may be relevant to distinct immune responses; more specifically, the lack of T cell responsiveness to an amino-terminal region of Tri r 2 in IH subjects may predispose patients to allergic responses to *Trichophyton*.

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