

Distinct Human T Cell Repertoires Mediate Immediate and Delayed-Type Hypersensitivity to the *Trichophyton* Antigen, Tri r 2¹

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The 29-kDa subtilase homologue, Tri r 2, derived from the dermatophyte fungus *Trichophyton rubrum*, exhibits unique immunologic characteristics in its ability to elicit immediate (IH) and delayed-type (DTH) hypersensitivity skin tests in different individuals. Thus, Tri r 2 provides a model for comparing the T cell repertoire in subjects with distinct immune responses to a single Ag. Recombinant Tri r 2 produced as a GST fusion protein in *Escherichia coli* stimulated strong in vitro lymphoproliferative responses in 10 IH and 10 DTH responders. Patterns of T cell epitope recognition were compared between skin test groups using 28 overlapping peptides (each in 12 replicate wells) derived from Tri r 2 to stimulate T lymphocyte proliferation in vitro. Peptide 5 (P5; aa 41–60) induced the strongest response in DTH subjects and showed the largest difference between DTH and IH responders in proliferation (mean standardized index, 2.22 and 0.82, respectively; $p = 0.0047$) and number of positive wells (81 vs 12). Responses to P5 were associated with diverse HLA haplotypes. These results showed that P5 contains an immunodominant epitope specifically associated with DTH and that this peptide is recognized in a permissive manner. Cross-validated linear discriminant analysis using T cell proliferative responses to two regions of Tri r 2 (aa 51–90 and 231–270) gave a 95% predictive accuracy for classification of subjects into IH or DTH groups. We conclude that different immune responses to *Trichophyton* are mediated by distinct T cell repertoires between individuals with IH and DTH reactions to Tri r 2. *The Journal of Immunology*, 2000, 165: 4379–4387.

Antigens derived from the dermatophyte fungus *Trichophyton* exhibit unique immunologic properties in their ability to elicit immediate (type I) or delayed (type IV) hypersensitivity skin test reactions in different individuals. Immediate hypersensitivity (IH)³ responses are associated with chronic recurrent infections and the presence of serum IgE Abs (1–3). In contrast, acute dermatophytosis, which resolves spontaneously, has been associated with delayed-type hypersensitivity (DTH) (4–6). Thus, it has been proposed that cell-mediated responses to *Trichophyton* play a role in the eradication of infection, while IH responses are nonprotective. A role for fungal Ags in allergic disease is strongly supported by the association between chronic dermatophytosis and allergic respiratory symptoms, and the improvement of late-onset asthma after antifungal therapy in patients with IH to *Trichophyton* (7–10).

Studies in mice and humans have reinforced the concept that distinct CD4⁺ T lymphocyte subsets are involved in different types of immune response to a variety of pathogens, including fungi

(11–15). We have previously reported that short-term T cell lines specific for *Trichophyton* Ags established from DTH and IH subjects produced cytokines consistent with Th1 and Th2/Th0 phenotypes, respectively (16). Thus, the T lymphocyte subsets underlying immediate and delayed responses to a single *Trichophyton* Ag are distinct. Studies using lymphoproliferation assays to map T cell antigenic determinants of Ags associated with DTH have demonstrated differences in the T cell repertoire in humans with different disease states (17–21). However, most studies of T cell responses to allergens have not shown significant differences in T cell repertoires between atopic and nonatopic subjects (22–24).

Purified natural *Trichophyton* Ags (Tri t 1 and Tri t 4) can elicit distinct immune responses in different individuals (25, 26). IH skin tests were associated with high titer anti-Tri t 4 IgG Ab and specific IgE Ab, while DTH reactions were associated with only low titer IgG Ab. We have cloned two *T. rubrum* proteins that have sequence identity with distinct serine proteinases: the 83-kDa prolyl oligopeptidase homologue, Tri r 4, and the 29-kDa subtilase homologue, Tri r 2. In keeping with studies of natural Ags, recombinant Tri r 2 can elicit IH and DTH skin responses (27).

Previous studies using human PBMC cultures to map T cell epitopes have observed a high degree of variation in counts per minute between replicate wells stimulated with antigenic peptide (21, 28). Despite this, many epitope-mapping studies fail to address the intra-assay variability of T cell responses to individual peptides in the assay design or in the analysis. Here, we use a rigorous assay design incorporating 12 replicate cultures for each peptide to demonstrate highly significant differences in T cell repertoires between individuals with IH and DTH reactions to Tri r 2. Furthermore, we show that an amino-terminal immunodominant epitope of Tri r 2 is specifically associated with DTH.

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³ Abbreviations used in this paper: IH, immediate hypersensitivity; DTH, delayed-type hypersensitivity; LDA, linear discriminant analysis; r-Tri r 2, recombinant Tri r 2.

Materials and Methods

Purification of Tri r 2

Recombinant Tri r 2 (r-Tri r 2) was produced in *Escherichia coli* strain BL21 as described previously (27). Briefly, bacteria were transformed with pGEX-4T-3 expression vector containing an 867-bp DNA fragment encoding the carboxyl-terminal 289 aa corresponding to the putative mature form of Tri r 2. Expression of the 29-kDa putative mature form of Tri r 2 as a fusion protein with GST was induced with 0.2 mM isopropyl-1-thio- β -D-galactopyranoside at 37°C. The recombinant protein (GST-Tri r 2) was purified from cell lysates by glutathione affinity chromatography, and protein yields were measured by Bradford assay. The same method was used to produce recombinant GST for use as a negative control in skin test and in vitro lymphocyte cultures.

Immunoassays for IgG and IgE Abs to Tri r 2 fusion protein

Serum IgG and IgE Ab to GST-Tri r 2 were measured using an Ag-binding RIA according to methods described previously (27). Serum samples were diluted 1/2 and 1/10 (IgE Ab assay) or 1/12.5 and 1/50 (IgG Ab assay) and were incubated with ^{125} I-labeled GST-Tri r 2 (~120,000 cpm added). IgE myeloma serum (patient P.S.) diluted 1/200 was used as a carrier in the IgE binding assay. Immune complexes were precipitated with the appropriate antiserum, i.e., sheep anti-human IgE (Binding Site, San Diego, CA) or anti-human IgG (Strategic Biosolutions, Ramona, CA). After incubation for 16 h at 4°C, the precipitates were washed and counted in a gamma counter. Quantitation of anti-Tri r 2 IgG Ab was conducted using a control curve constructed with pooled sera from three patients assigned to contain 2000 U of IgG Ab/ml. Values for IgE Ab measurements were expressed as counts bound per minute above the background.

Classification of IH and DTH responders by skin testing

Sixty patients were skin tested with r-Tri r 2. Patients with IH skin tests to r-Tri r 2 were identified from a population of patients attending the University of Virginia Allergic Diseases Clinic who exhibited positive skin tests to a *Trichophyton* extract (Bayer C, Elkhart, IN; containing *T. tonsurans*, *T. rubrum*, and *T. mentagrophytes* species). Criteria for classifying patients in the IH experimental group were a positive immediate skin test response to *Trichophyton* extract and to purified r-Tri r 2, and the presence of anti-Tri r 2 IgE Ab. Subjects with DTH skin tests responses to r-Tri r 2 were identified from patients attending the clinic who had a DTH skin test response to *Trichophyton* extract or from individuals with a history of dermatophytosis (athlete's foot or onychomycosis). Patients were classified in the DTH group based on a delayed skin test response to both *Trichophyton* extract and r-Tri r 2, and a lack of measurable IgE Ab. Negative subjects were those individuals who had neither a delayed nor an immediate skin test response to *Trichophyton* extract and r-Tri r 2. Recombinant Tri r 2 and GST were filtered through a 0.2- μ m pore size sterile nitrocellulose disposable filter (Micron Separations, Westborough, MA) and diluted in 0.05% human serum albumin in phenol-saline solution. Intradermal skin tests were conducted using 0.02 ml of purified GST-Tri r 2 at 0.1, 1, and 10 μ g/ml. Before intradermal testing, prick testing was conducted using 10 μ g/ml of protein. Skin test sites were examined at 10 min after injection of Ag and at 24 h. All patients were tested with GST as a negative control. Informed consent was obtained before skin testing. Skin testing of human subjects using GST-Tri r 2 and drawing of blood samples for T cell studies and HLA typing (see below) were approved by the University of Virginia human investigation committee.

Preparation of Ag and peptides for T cell studies

Recombinant Tri r 2 and GST were absorbed with polymyxin B agarose (Sigma, St. Louis, MO) before measuring for protein content and filtering as described above. A set of twenty-seven 20-mers (designated P1 through P27) and one 19-mer (P28), each with a 10-aa overlap spanning the entire length of the putative mature form 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, Woburn, MA) and were purified to >90% by reverse phase HPLC. Stock solutions of peptides (1 mM) were solubilized in sterile water or 10% DMSO (P4, P16, and P17). P25, which contained two cysteine residues, was solubilized in the presence of 10 mM DTT. Peptides were diluted to a working concentration of 7.5 μ M immediately before use.

Proliferation assays to whole Ag

Blood (50 ml) was drawn from subjects with IH, DTH, and negative skin tests to r-Tri r 2. PBMC were purified by density gradient centrifugation over Ficoll-Paque (Amersham, Uppsala, Sweden) and cultured (2×10^5

cells/well) with r-Tri r 2 or GST for 5 days at 37°C in quadruplicate in a 96-well plate. Tetanus toxoid and PHA were used as positive controls. Complete medium contained RPMI 1640 (Life Technologies, Gaithersburg, MD) 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 of [^{3} H]thymidine/well during the final 8 h of culture before harvesting and counting. Results were expressed as stimulation indexes for Tri r 2 (the mean of quadruplicate wells containing 1 μ g/ml Tri r 2 divided by the mean of wells containing 1 μ g/ml GST) and tetanus toxoid (the mean of quadruplicate wells containing 10 μ g/ml tetanus toxoid divided by the mean of wells containing no Ag).

Proliferation assays using Tri r 2 peptides

Blood (100 ml) was drawn from 10 patients each with IH and DTH skin tests to r-Tri r 2. Preliminary studies were conducted to determine optimal conditions for PBMC cultures using antigenic peptides and cells derived from a patient with DTH to r-Tri r 2. Cells were plated at different cell numbers ($1-3 \times 10^5$ cells/well), and peptides were tested in each of three concentrations (7.5, 25, and 75 μ M) using quadruplicate cultures for each peptide Ag. Proliferative responses induced by individual peptides were assessed by comparison of mean counts per minute for quadruplicate wells to mean counts per minute for negative control wells (no peptide). Time-course experiments were conducted by harvesting cells after 4, 5, and 6 days of culture. Optimal assay conditions for antigenic peptides were achieved after 5 days of culture using 7.5 μ M peptide to stimulate 3×10^5 cells/well. For epitope mapping studies, each assay incorporated 12 replicate cultures for each of 28 antigenic peptides and 60 wells without peptide as negative controls. Five 96-well plates were used in each assay. Plates 1-4 contained P1-P7, P8-P14, P14-P21, and P22-P28, respectively, with each plate containing 12 control wells. Plate 5 contained an additional 12 control wells and quadruplicate cultures of GST, r-Tri r 2, tetanus toxoid, and PHA.

ELISA of human sera for Abs against synthetic peptides

Individual wells of the ELISA plate (Immulon 4; Dynex, Chantilly, VA) were coated with P5, P8, P16, and P25 (5 μ g/well) in sodium carbonate buffer (15 mM NaHCO₃ and 35 mM Na₂CO₃, pH 9.6) at 4°C overnight. Unoccupied binding sites were blocked with PBS containing 0.05% Tween 20 (PBS-T) and 5% skim milk. For subsequent steps, PBS-T containing 5% skim milk was used as diluent unless otherwise stated. After three washes with PBS-T, 100 μ l of serum was added to each well at a 1/2 dilution (for IgE assay) or a 1/100 dilution (for IgG assay) and incubated for 2 h at 37°C. For the IgG assay, the plate was washed three times with PBS-T before adding 100 μ l of peroxidase-labeled goat anti-human IgG (1/1000; The Binding Site, Birmingham, U.K.) and incubating for 1 h at 37°C. For the IgE assay, after three washes with PBS-T, 100 μ l of biotin-labeled goat anti-human IgE Ab (1/1000; Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added to each well. After incubation for 1 h at room temperature the plate was washed and incubated for an additional hour with 100 μ l/well of streptavidin peroxidase (1/1000). IgG and IgE Ab assays were developed using 100 μ l of 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) in 0.07 M citrate-phosphate buffer (pH 4.2) containing 0.03% H₂O₂, and the OD was read at 405 nm.

HLA class II typing

Genomic DNA was isolated from 2 ml of whole blood using the Puregene DNA isolation kit (PGC Scientific, Gaithersburg, MD). HLA typing was conducted in the Molecular Pathology Laboratory at the University of Virginia. HLA-DRB1 alleles were PCR amplified and sequenced using an ALFexpress automated DNA sequencer (Amersham). Amplification of exon 2 of DRB1 was performed using a mix of group-specific primers. The locus-specific sequencing primer was internal to the group-specific primers. Sequences were evaluated using HLA SequiTyper software (Amersham) and compared with the European Bioinformatics Institute HLA database (European Bioinformatics Institute, Cambridge, U.K.) for allele assignments. HLA-DQB1 alleles were PCR amplified using an array of sequence-specific primers (One Lambda, Canoga Park, CA). PCR-amplified products of exon 2 were visualized by agarose gel electrophoresis. The pattern of amplified bands was evaluated, and alleles were assigned using One Lambda DNA/LMT software.

Molecular modeling of Tri r 2

The SYBYL molecular modeling package (Tripos, St. Louis, MO) was used to determine homology with known structures, for graphic visualization, and for performing all insertions and deletions. The CHARMM-based program CONGEN was used for mutating the proteinase K sequence to

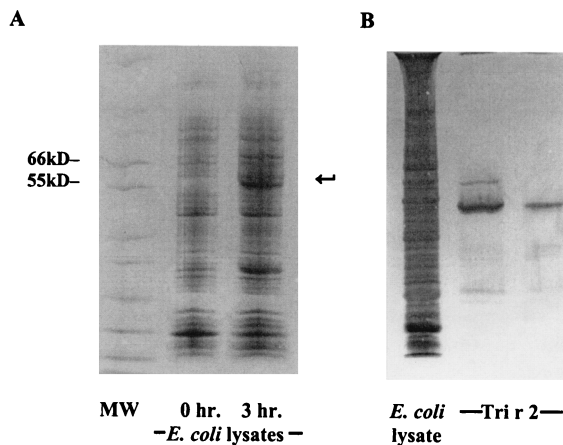


FIGURE 1. SDS-PAGE analysis of recombinant Tri r 2 expressed in *E. coli*. **A**, Analysis of cell lysates 3 h after induction of protein expression showed a 57-kDa band (Coomassie blue staining). **B**, Recombinant Tri r 2 was purified from cell lysates after two passages over a glutathione-Sepharose column (lanes 2 and 3, respectively) and was analyzed on silver-stained SDS-polyacrylamide gel.

that of Tri r 2 and for energy minimizations (29, 30). The established structure of proteinase K (Brookhaven Database no. 2prk.pdb) was used as the starting molecule for modeling of Tri r 2.

Statistical analysis

Data (counts per minute) for each of the 12 peptide-stimulated replicates (28 peptides in total) was log transformed (natural logarithm; base e , where the value of $e = 2.7$) to compare PBMC proliferative responses to Tri r 2 peptides between IH and DTH groups. Mean proliferation values for individual peptides and for five sets of controls on separate assay plates were calculated from log-transformed data. The mean proliferation value for controls was then subtracted from the mean proliferation value for each peptide on the same assay plate to derive corrected mean values for each peptide. These values are referred to as the standardized index for each peptide. Corrected mean values for control wells were derived by subtracting the mean of four sets of controls (48 wells) from the fifth set of controls (12 wells). Mean standardized indexes were used to compare responses between IH and DTH groups. The variance for control wells between different assay plates was not significantly different (Levene's test for homogeneous variances, $p = 0.50$). Responses to peptide 5 were analyzed by two-sample Student's t test. Cross-validated linear discriminant analysis (LDA) was used to predict patient classification. The number of positive wells for each peptide was calculated by scoring the response of each well containing peptide as a positive or a negative event using the mean + 3 SD of 60 control wells as a cutoff.

Results

Definition of patients with IH and DTH to purified Tri r 2

Recombinant Tri r 2 was produced in *E. coli* using the pGEX-4T-3 vector. The resulting GST fusion protein migrated as a 57-kDa band on SDS-PAGE, consistent with a GST moiety fused to the 29-kDa putative mature form of Tri r 2 (Fig. 1A). The presence of

Table I. Serum Abs and PBMC proliferative responses to r-Tri r 2 in individuals with IH and DTH skin tests^a

Patient	Age	Sex ^b	Race ^b	Allergic Disease ^b	IgG ^c (U/ml)	IgE ^c (cpm)	Stimulation Index	
							Tri r 2	Tetanus toxoid
IH subjects								
1	38	M	W	sr	145	2,609	30	430
2	68	M	W	u	950	6,314	8	281
3	60	M	W	pr	4,720	2,293	53	7
4	24	M	W	pr	4,400	2,465	51	426
5	46	M	B	pr	850	451	9	663
6	55	F	W	a	1,900	48,800	36	86
7	75	M	W	s	2,200	3,357	58	283
8	73	F	W	a	1,150	713	46	29
9	61	M	B	a, s	310	2,624	136	126
10	34	F	W	a	600	1,886	38	351
Mean					1,081 ^d	2,745 ^d		
DTH subjects								
11	26	M	W		<20	<400	28	87
12	28	M	W		20	<400	58	750
13	58	M	W		90	<400	23	224
14	29	M	W		35	<400	130	1,057
15	40	F	W		840	<400	157	47
16	38	F	W	a	340	<400	7	220
17	35	M	B		125	<400	40	129
18	25	F	W		500	<400	3	32
19	55	F	W	c	800	<400	<3	12
20	37	F	W	u	40	<400	3	97
21	31	F	W		510	<400	39	79
Mean					139 ^d	<400		
Negative subjects ^e								
22	36	F	W		160	<400	<3	717
23	27	M	W		320	<400	7	135
24	25	F	W		80	471	<3	118
25	25	M	W		60	<400	<3	944

^a Subjects with positive skin tests to r-Tri r 2 were identified by screening 60 individuals with positive skin tests to *Trichophyton* extract with or without a history of dermatophytosis.

^b M, male; F, female; W, white; B, black; sr, seasonal rhinitis; pr, perennial rhinitis; u, urticaria; a, asthma; s, sinusitis; c, cough.

^c Measured by Ag-binding RIA.

^d Geometric mean values.

^e Based on negative skin test reactivity to *Trichophyton* extract and r-Tri r 2.

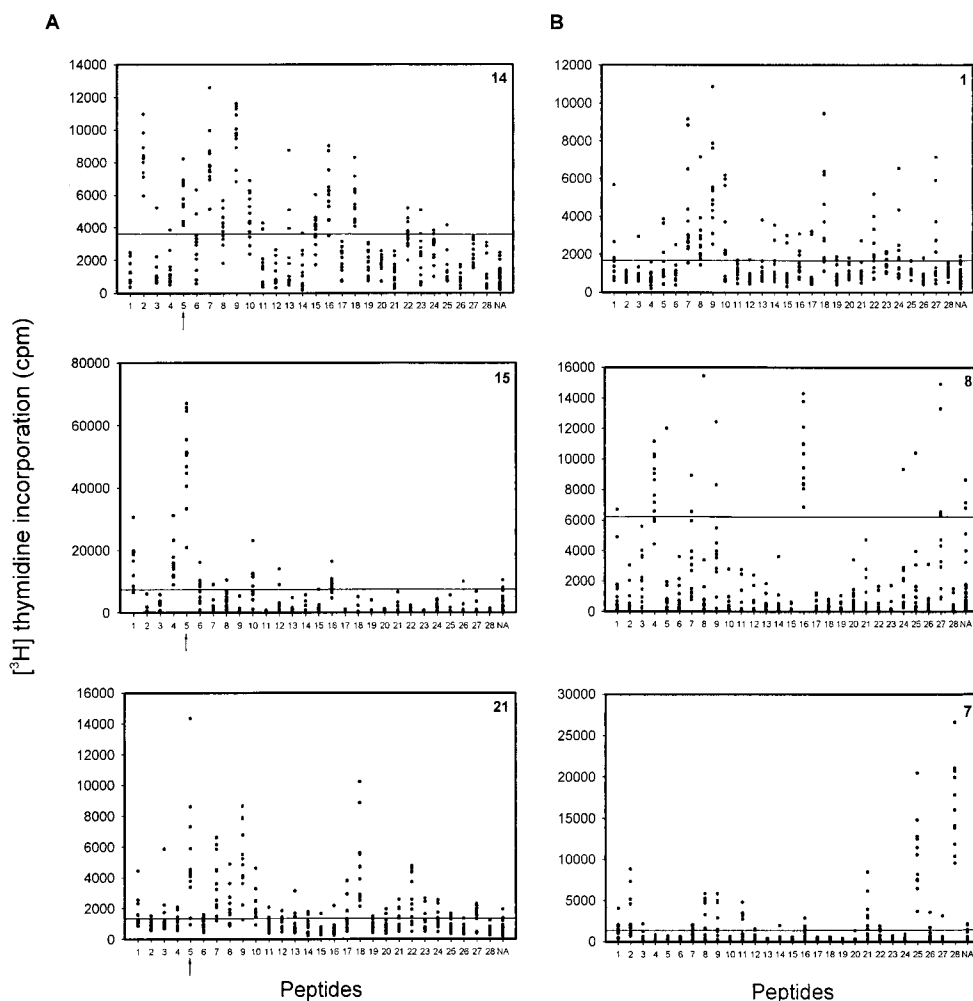


FIGURE 2. Proliferative responses of PBMC to Tri r 2 peptides. A representative sample from each of three DTH (A) and three IH (B) responders is shown. Each point corresponds to incorporated [^{3} H]thymidine (counts per minute) in a single well (12 replicate wells for P1–P28). The final column in each figure represents 60 replicate wells containing no Ag (NA). The horizontal line represents an arbitrary cutoff value (mean + 3 SD of 60 unstimulated wells). The arrows denote P5.

the GST moiety was confirmed by amino-terminal amino acid sequence analysis. Recombinant Tri r 2 was purified by two passages over a glutathione affinity column, and purity was assessed by silver-stained SDS-polyacrylamide gel (Fig. 1B). Recombinant GST was produced by the same method.

Ten patients with IH (mean age, 53 years) and 11 patients with DTH (mean age, 37 years) skin tests to r-Tri r 2 were identified by skin testing 60 individuals with positive skin tests to *Trichophyton* extract (see *Materials and Methods*; Table I). None of the individuals had an immediate or delayed response to GST. Nine patients in the immediate group presented to the University of Virginia Allergic Diseases Clinic with allergic symptoms including urticaria, perennial rhinitis, chronic sinusitis, and asthma. In contrast, only two subjects in the DTH group reported allergic symptoms. Although the majority of patients in both skin test groups gave a history of dermatophytosis, seven patients in the immediate group reported recurrent infections, and three of these patients were receiving antifungal therapy. Immediate sensitivity was associated with significantly higher titers of IgG Ab to Tri r 2 (geometric mean, 1081 U/ml; $n = 10$) compared with the DTH group (geometric mean, 139 U/ml; $n = 11$; $p < 0.05$) and the presence of IgE Ab (Table I).

PBMC proliferative responses to recombinant Tri r 2 and antigenic peptides

Proliferative responses to r-Tri r 2. Recombinant Tri r 2 stimulated strong dose-dependent T lymphocyte proliferative responses

using PBMC from either IH or DTH subjects (stimulation index, 8–136 and 3–157, respectively), while responses to GST were generally low or undetectable (Table I and data not shown). Recombinant Tri r 2 elicited a positive proliferative response in only one of four skin test-negative subjects (Table I).

Proliferative responses to peptides derived from Tri r 2. To optimize the frequency of wells with positive responses for any given peptide, PBMC were plated at 3×10^5 /well, and 12 replicate cultures were performed for each peptide. Epitope mapping studies were conducted in 10 individuals each with IH and DTH responses to r-Tri r 2. All individuals studied responded to one or more peptides, with each showing a unique pattern of recognition. However, the nature of the PBMC proliferative responses to peptides differed; some peptides stimulated positive responses in each of the 12 replicate wells (i.e., counts per minute higher than the mean + 3 SD of 60 control wells) with minimum variability in counts per minute between wells (e.g., P5 and P18, subject 14, Fig. 2). In contrast, the majority of peptides stimulated responses with high variability in both counts per minute and frequency of positive responses among replicate wells (P9 and P27, subject 8, Fig. 2). These responses are referred to as skewed. To examine differences between IH and DTH subjects in the Ag-specific T cell repertoire for Tri r 2, mean proliferative responses to each of the 28 Tri r 2 peptides were compared for both skin test groups. Because of the skewed nature of responses to antigenic peptides, log transformation was necessary to apply statistical techniques for comparison

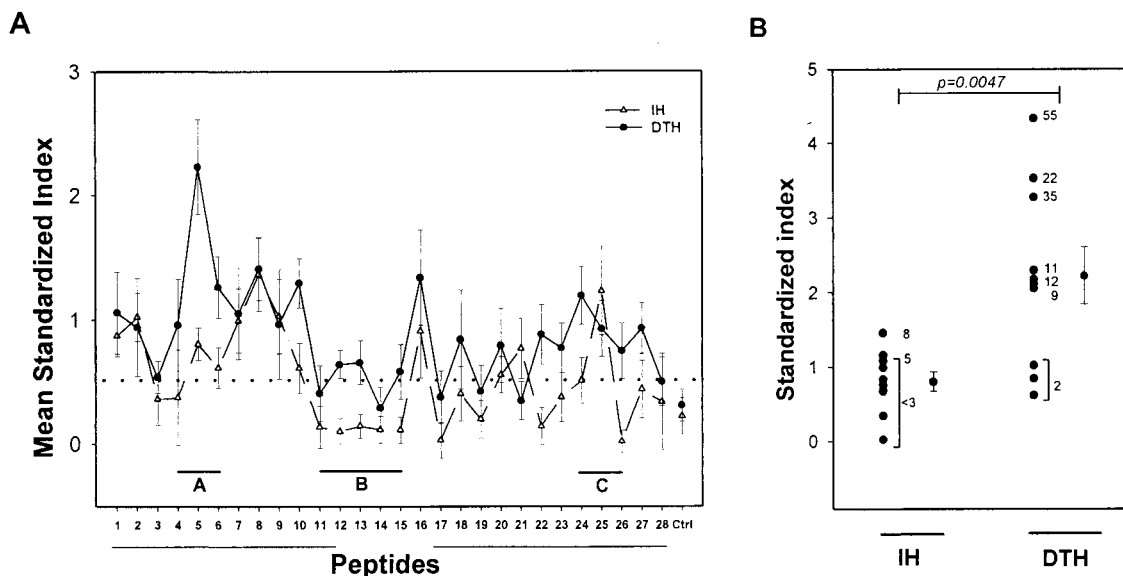


FIGURE 3. Patterns of T cell epitope recognition for Tri r 2 in IH and DTH responders. *A*, Mean standardized indexes for IH and DTH responders (P1–P28). Vertical bars represent the SEM. Horizontal bars represent regions A, B, and C. The dotted line is an arbitrary reference line corresponding to a standardized index value of 0.5. *B*, Standardized indexes for P5 in each skin test-positive individual. Numbers indicate stimulation indexes calculated by conventional methods (the mean counts per minute of 12 wells containing P5 divided by the mean counts per minute of 12 unstimulated wells).

between skin test groups. Data for unstimulated (control) wells showed equal variance between different assay plates ($p = 0.50$), and these data were used to standardize measurements of the responses to peptides for cultures located on the same assay plate (see *Materials and Methods*). Corrected mean values for each peptide were calculated based on log-transformed data for each of 12 replicate wells. We refer to these values as the standardized index. These values were used to derive mean standardized indexes for each peptide. Mean indexes for each skin test group ($n = 10$ for both IH and DTH groups) in cultures stimulated with peptides ranged from 0.02 for P26 (IH group) to 2.23 for P5 (DTH group); the mean indexes for unstimulated cultures were 0.25 (IH) and 0.28 (DTH), representing values approaching zero (Fig. 3A). Comparison between skin test groups identified three distinct regions of the molecule, designated A, B, and C (spanned by P4–P6, P11–P15, and P24–P26, respectively), where responses to peptides differed (Fig. 3A). Differences in regions A and B were characterized by hyporesponsiveness or lack of responsiveness, respectively, for the IH group. In contrast, DTH and IH groups showed a difference in the pattern of recognition in region C. Mean proliferative responses to Tri r 2 peptides were generally higher in DTH than in IH subjects. Peptides 5, 6, 8, 10, 16, and 24 (corresponding to aa 41–60, 51–70, 71–90, 91–110, 151–170, and 231–250, respectively) produced the highest mean proliferation in DTH subjects. In contrast, peptides 2, 8, 16, and 25 (aa 11–30, 71–90, 151–170, and 241–260, respectively) produced the strongest proliferation in the IH group. Although some peptides were stimulatory for both skin test groups (P2, P8, and P16), the response to P5 was specifically associated with DTH. The mean proliferative response to P5 was significantly higher in the DTH group than in the IH group ($p = 0.0047$; Fig. 3B). This peptide not only induced the strongest response in DTH subjects, but also had the largest difference in number of positive wells between the two skin test groups (Figs. 3 and 4). Furthermore, peptides flanking P5 were stimulatory in DTH, but not IH, subjects, suggesting that an amino-terminal region of Tri r 2 (aa 31–70) contains an immunodominant epitope associated with DTH, but not IH, immune responses. Responses to peptides 11–15 (aa 101–160) were generally not measurable in IH

subjects, although peptides 12 and 13 stimulated positive responses in the DTH group. Cross-validated linear discriminant analysis using responses to six peptides (P6, P7, P8, P24, P25, and P26) spanning two distinct regions of the molecule provided a 95% predictive accuracy (19 of 20 patients) for classification of patients in IH and DTH groups. Cells from each subject were typed for HLA-DR (DRB1) and DQ (DQB1) haplotypes (Table II). There was no significant difference in the frequency of HLA-DR and -DQ Ags between IH and DTH subjects. Notably, seven subjects with delayed skin tests who had strong PBMC proliferative responses to P5 (standardized index >2) had diverse HLA class II haplotypes.

Ab binding to Tri r 2 peptides

The epitope-mapping studies were conducted in the presence of autologous serum to minimize in vitro artifacts. To investigate

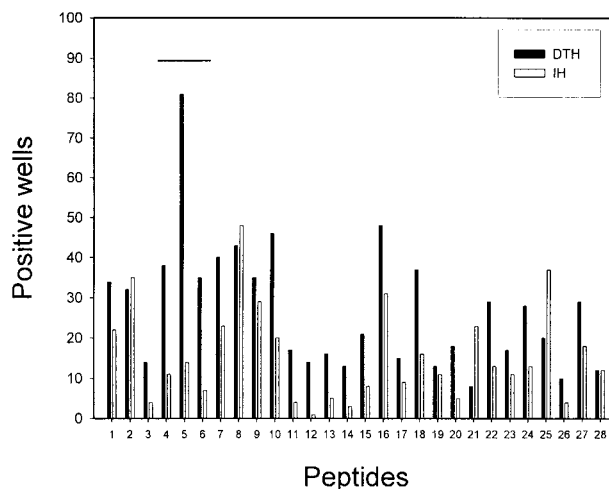


FIGURE 4. Comparison of the frequency of positive wells in different skin test groups. Positive wells were those incorporating counts per minute above the mean + 3 SD of 60 unstimulated wells. The horizontal bar represents the region spanned by P4, P5, and P6.

Table II. Major HLA class II alleles expressed in IH and DTH subjects

Subject ^a	HLA-DR ^b (DRB1)	HLA-DQ ^b (DQB1)
IH to Tri r 2		
1	0404, 0701 (DR4,7)	0302, 0303 (DQ8,9)
2	0101, 0701 (DR1,7)	0202, 0501 (DQ2,5)
3	1101, 1501 (DR11,15)	0302, 0602 (DQ8,6)
4	1301, 1501 (DR13,15)	0602, 0603 (DQ6)
5	1101, 1501 (DR11,15)	0602 (DQ6)
6	0404, 0701 (DR4,7)	0202, 0302 (DQ2,8)
7	1104, 1502 (DR11,15)	0601, 0301 (DQ6,7)
8	0101, 1404 (DR1,1404)	0501, 0503 (DQ5)
9	1102, 1503 (DR11,15)	0301, 0602 (DQ7,6)
10	0701 (DR7)	0202, 0303 (DQ2,9)
DTH to Tri r 2		
11 ^c	0101, 0404 (DR1,4)	0302, 0501 (DQ8,5)
13 ^c	0405, 1301 (DR4,13)	0302, 0603 (DQ8,6)
14 ^c	0701, 1501 (DR7,15)	0202, 0602 (DQ2,6)
15 ^c	0301, 1401 ^d (DR17,14)	0201, 0503 (DQ2,5)
	0310, 1423 ^d (—)	
16 ^c	0804, 1501 (DR8,15)	0402, 0301 (DQ4,7)
17 ^c	0302, 1202 (DR18,12)	0402, 0602 (DQ4,6)
18	0406, 1101 (DR4,11)	0301, 0302 (DQ7,8)
19	0401, 0407 (DR4)	0301 (DQ7)
20	0301, 1501 (DR17,15)	0201, 0602 (DQ2,6)
21 ^c	0404, 0701 (DR4,7)	0202, 0302 (DQ2,8)

^a Numbers correspond to those in Table I.
^b The corresponding HLA serologic specificities where known are shown in parentheses.
^c Subjects with strong PBMC proliferative responses to P5 (standardized index >2).
^d Could not distinguish between these haplotypes.

whether serum Abs influence T cell responses to peptides, IgG and IgE Ab binding to select peptides (P5, P8, P16, and P25) was determined by ELISA. No IgE Ab binding was detected, IgG Ab binding to P8 was measurable in only a single IH subject, and IgG Ab binding to P25 was measurable in one DTH subject (data not shown).

Immunodominant epitopes in the context of the primary and tertiary structure of Tri r 2

Tri r 2 has a high degree of sequence identity with the class D subfamily of subtilases; it contains conserved amino acid motifs flanking aspartic acid, histidine, and serine residues, which form the catalytic triad characteristic of this enzyme family (27). Im-

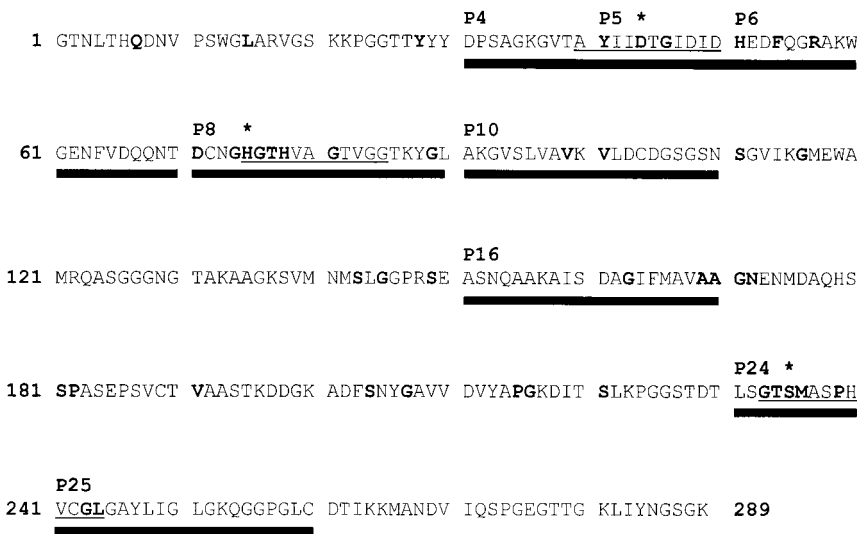
munodominant T cell epitopes of Tri r 2, defined as those peptides stimulating the highest mean proliferative responses in both skin test groups (P4, P5, P6, P8, P10, P16, P24, and P25), spanned each of the conserved amino acid motifs (Fig. 5). The three-dimensional structure of Tri r 2 was predicted to be essentially the same as the structurally conserved regions of the subtilase proteinase K, with most differences residing in external loops (Fig. 6, A and B). Deletion of a single alanine residue was identified in a β strand deep within the active site cleft but outside of the conserved catalytic triad motifs. Immunodominant epitopes of Tri r 2 localized to the active site cleft and to regions distal to this site containing α helices and β strands with connecting loops (Fig. 6C).

Discussion

Epitope-mapping studies of Tri r 2 showed highly significant differences in in vitro T cell proliferation in subjects with IH and DTH to this Ag. Although fungal extracts have been shown to elicit IH and DTH responses in the skin, no single Ag with these properties had previously been identified. Thus, until now it was not possible to compare the T cell repertoire in humans with distinct immune responses to the same Ag.

Most studies that compare patterns of T cell epitope recognition between experimental groups do not optimize study design because they do not account for the high variability in counts per minute among replicate wells for responses to peptides. Furthermore, those studies did not demonstrate statistically significant differences in the T cell repertoire. Recently, two studies have conducted a more stringent analysis of peptide responses to identify T cell antigenic determinants (21, 28). However, those studies assumed that data for control wells were normally distributed and that values above the arbitrary cutoff level had a Poisson distribution. In the present study counts for unstimulated wells varied considerably between individuals and did not show a consistent normal distribution. Based on the skewed distribution of counts per minute for peptide-stimulated replicates, log-transformed data were used to derive standardized indexes for peptide responses in IH and DTH subjects. Control wells were used as a normalizing factor to adjust for differences in background counts and to allow comparison between experimental groups. Statistical methods demonstrated equal variance among control wells of different plates when analyzing data obtained from the whole group ($n = 20$

FIGURE 5. Alignment of immunodominant T cell epitopes with the primary amino acid sequence of Tri r 2. Peptides stimulating the highest mean proliferative response in IH and DTH subjects are labeled according to the position of the first peptide residue in the primary amino acid sequence. Underlined residues represent conserved amino acid motifs flanking catalytic triad residues (*). Conserved residues of the class D subtilase subfamily are in bold.



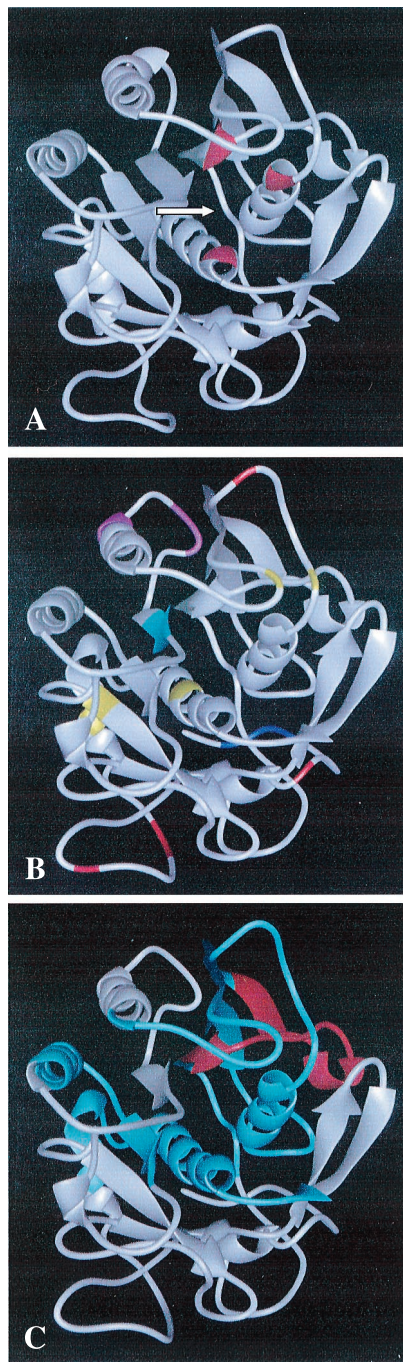


FIGURE 6. Three-dimensional structure of Tri r 2 and molecular localization of immunodominant T cell antigenic determinants. Comparison of the C α backbone structures of proteinase K (A) and Tri r 2 (B) identified deletion of a single residue (turquoise) proximal to the putative active site. Catalytic residues (red) and the active site (arrow) of proteinase K are shown in A. Tri r 2 residues depicted in red represent one or two amino acid insertions or deletions, while those shown in magenta and blue represent insertions of three and four amino acids, respectively. Five cysteine residues of Tri r 2 are shown in yellow (positions 72, 104, 189, 242, and 260). Two disulfide bonds were predicted between residues 72 and 104 and between residues 189 and 260. C, Immunodominant T cell epitopes of Tri r 2; regions corresponding to peptides 4, 6, 8, 10, 16, 24, and 25 (turquoise) and P5 (red) are shown.

patients); furthermore, in the majority of individuals, the intra-assay variance among control wells on different plates was equal, thereby confirming that subtraction of mean values for control

wells was an appropriate method for comparing peptide responses within the same individual and between different subjects. Differences in proliferative responses between skin test groups were identified for three regions of Tri r 2 spanned by P4–P6, P11–P15, and P24–P26 (regions A, B, and C, respectively). The most striking observation was the strong proliferative response to P5 in delayed responders. Furthermore, responses to peptides flanking P5 were lower in the IH group, suggesting that this amino-terminal region of the molecule contained an immunodominant epitope specifically associated with DTH. Interestingly, the three individuals with DTH skin tests who had the lowest responses to P5 also had the lowest T cell responses to whole Ag. Although T cell proliferative responses of IH responders were comparable to those of DTH responders in cultures stimulated with whole Ag, no single peptide was specifically associated with IH. However, the immediate group did show T cell proliferation to multiple peptides. Cross-validated LDA was used to search for the best predictive model of classification into IH and DTH groups. The strategy was to identify the model of two three-peptide-long regions with the best predictive power from all possible combinations. Although P5 alone was by far the best predictor (17 of 20; 85%), responses to this peptide were highly variable. As a consequence, P5 in combination with other peptides that exhibited less variability did not result in the best predictability by LDA. The model with the highest predictive power (P6–P8 and P24–P26) spanned regions A and C of the molecule (Fig. 3A).

The region of Tri r 2 spanned by peptides 11–15 (region B) stimulated only modest responses in delayed responders; however, responses in IH subjects were generally not measurable. In addition, responses to P22 and P26 were markedly lower in IH compared with DTH subjects. Our studies demonstrated a lack of IgE and IgG Ab binding to peptides, which argues against the possibility that inhibition of these responses occurred as a result of a blocking effect of Abs on exogenous loading of peptides onto MHC molecules. The selective hyporesponsiveness to P5 and the lack of response to other peptides in IH compared with DTH subjects are potentially important in light of a previous report of selective anergy to a single T cell epitope derived from *Mycobacterium tuberculosis* in patients with active tuberculosis (18). In that study the authors hypothesized that failure of the immune system to respond to specific antigenic peptides could predispose individuals to develop clinical illness. IH has been associated with chronic dermatophytosis; thus, using *M. tuberculosis* as a paradigm, it is possible that hyporesponsiveness to specific antigenic determinants (e.g., P5) contributes to persistence of infection with *Trichophyton* species. Since chronic dermatophytosis is commonly observed in older patients, selective hyporesponsiveness to specific T cell antigenic determinants may reflect the changes in the T cell repertoire that accompany the aging process. Indeed, in our study individuals with immediate skin tests to Tri r 2 were significantly older than those with delayed responses. It is possible that the complex homeostatic mechanisms that regulate the balance of naive and memory T cells in the total T cell population are undermined with aging; selective loss of T cell clones, including those specific for P5, could predispose individuals to develop IH and dermatophytosis. It has recently been shown that repeated Ag challenge results in a narrowed T cell repertoire relative to the primary repertoire in mice; this results from loss of cells expressing TCRs with lower affinity for Ag (31). Extending this concept to the human system, Ag exposure throughout life may also influence the T cell repertoire. Alternatively, differences in the T cell repertoire might occur early in the development of the immune response; such mechanisms may include an inherent difference in the precursor frequency of Ag-specific T cells among different individuals

as a result of thymic selection of the total T cell population; involvement of different APCs, possibly influenced by the cytokine milieu at the time of Ag exposure; and chemical or physical properties of the Ag. Clearly, antigenic properties play an important role in development of the response. Indeed, it has recently been shown that human T cell cytokine patterns and IgE and IgG4 Ab responses can be differentially regulated by conformational variants of the molecule; furthermore, disulfide bonds and free sulfhydryl groups have been reported to influence recognition by T cells (32, 33). Tri r 2 contains a free sulfhydryl (Cys²⁴²) in the same α -helix as the catalytic serine residue. This helix is situated just below the β strand within which a single alanine residue is deleted. These changes may explain the lack of enzymatic activity of this molecule (27).

HLA typing showed no significant difference in allele frequency between the two skin test groups, suggesting that differences in the T cell repertoire are independent of HLA haplotype. Furthermore, those subjects with the strongest responses to P5 had diverse HLA class II haplotypes, suggesting that P5 is a promiscuous epitope. Studies of mycobacterial and malaria proteins as well as Ags derived from hepatitis B and tetanus toxin have confirmed the presence of promiscuous epitopes that are recognized in association with MHC class II molecules (19, 28, 34–38). In contrast, no epitopes that bind promiscuously to class I MHC molecules have been identified for any pathogen. One possible explanation for the increased response to P5 in subjects with DTH is that P5 is a protective epitope. Identification of peptides that are both protective and promiscuous is relevant to the development of peptide vaccines for chronic dermatophytosis.

Peptides that stimulated strong proliferative responses localized to several different regions of the molecule; these included the active site cleft and regions outside this site containing different secondary structural elements. However, it is not known whether the localization of T cell antigenic determinants within the molecule influences the generation of these determinants *in vivo*. Alignment of T cell epitopes with the conserved amino acid motifs flanking the catalytic triad residues may reflect cross-reactivity with other commonly encountered bacterial and fungal Ags derived from the subtilase family of serine proteinases.

Several groups have reported a phenomenon called T-B reciprocity, which describes a mechanism by which Th cells can preferentially influence the specificity of Abs produced. Recently, Shirai et al. (39) showed that the Th cell reactivity to a region of the hepatitis C virus envelope correlated with the production of Abs to this region. The authors suggested that induction of Abs to this region of the molecule was dependent on Th cells specific for a sequence proximal to the Ab epitope. A lack of Ab binding to immunodominant Tri r 2 peptides suggests that T-B reciprocity does not play a role in the generation of these epitopes, although longer peptides may be required to test this hypothesis.

In summary, we have demonstrated highly significant differences in the T cell repertoires mediating diverse immune responses to the same Ag. Furthermore, we identified an immunodominant T cell epitope in the amino-terminal region of Tri r 2 that was specifically associated with delayed hypersensitivity. These findings demonstrate a selective loss in response to antigenic peptides that may reflect narrowing of the T cell repertoire or failure to maintain adequate numbers of memory T cells. The clinical relevance of different T cell repertoires remains to be determined. It is possible that peptide 5 contains an epitope that specifically promotes the development of a DTH response, making progression to chronic dermatophytosis unlikely. It is not known whether individuals with DTH can subsequently develop immediate responses later in life. If this is the case, then hyporesponsiveness to peptide 5 may reflect

modulation of the T cell repertoire, which, in turn, could play a direct role in predisposing an individual to develop IH. Our findings demonstrate the importance of studying T cell repertoires and provide the first step to understanding how diverse immune responses arise in humans.

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