

Definition of a *Trichophyton* Protein Associated with Delayed Hypersensitivity in Humans

Evidence for Immediate (IgE and IgG4) and Delayed Hypersensitivity to a Single Protein¹

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Dermatophytes of the genus *Trichophyton* cause infections of human skin, nails, and hair. Unlike most Ags, *Trichophyton* can elicit either immediate (IH) or delayed (DH) hypersensitivity skin reactions. Previous studies isolated a 30-kDa Ag (Tri t 1) that caused IH skin tests. The study presented here used skin testing and in vitro T cell proliferation assays to monitor purification of an Ag, designated Protein IV, associated with DH reactions. Protein IV was purified by cation exchange HPLC; amino acid sequence analysis of the N-terminus and nine internal peptides (143 residues) revealed no homologies to Tri t 1 or to any other known proteins. A mAb-based ELISA was developed to measure Protein IV. Protein IV elicited DH skin reactions in subjects with a history of athlete's foot but also caused IH skin reactions. Serologic responses to Protein IV were studied in 59 adults who had been skin tested with *Trichophyton* extract. IH skin reactions were associated with a positive RAST (14/23) as well as with specific IgE (13/23) and IgG4 (14/23) Abs to Protein IV. DH skin tests were not associated with IgE or IgG4 Abs. IgE anti-Protein IV Abs were quantitatively correlated with IgG4 Abs ($r = 0.57$, $p < 0.001$). Specific IgG Abs to Protein IV were highest in IH subjects (gm = 230 U/ml), and lowest in those with DH (gm = 91 U/ml) or negative (gm = 81 U/ml) skin tests; furthermore, the prevalence of IgG Abs increased significantly with age. Protein IV is the first defined protein associated with both DH and IH skin reactions; these reactions are characterized by distinct serologic responses. The results establish that diverse immune responses in humans can be directed against the same protein. *The Journal of Immunology*, 1996, 156: 1695-1701.

Dermatophyte fungi of the genus *Trichophyton* characteristically infect keratinized structures, including the nails, hair shafts, and stratum corneum of the skin. *Trichophyton tonsurans* is the most common cause of ringworm of the scalp (tinea capitis) in North America, predominantly affecting children (1-3). Tinea capitis has increased in prevalence in the United States since the 1970s, creating a significant public health problem (2, 4-6). *T. rubrum* and *T. mentagrophytes* are the most common causes worldwide of athlete's foot and onychomycosis (infection of the nail beds) (1, 7, 8). An estimated 30 to 70% of adults are asymptomatic carriers of these pathogens; the incidence of symptomatic disease increases with age, affecting children only infrequently (9-11).

The nature of the immune response to *Trichophyton* is unusual in that it is not exclusively of one type; both immediate hypersensitivity (IH)³ and delayed hypersensitivity (DH) skin test reactions can be elicited. Delayed reactions to common allergens are very

rare and conversely most Ags associated with DH, e.g., tuberculosis, tetanus, and influenza are not associated with IH. It is apparent that some individuals clear dermatophyte infections effectively and others do not (12, 13). Furthermore, it has been suggested that the severity of the clinical condition is related to the nature of the underlying immune response; in some studies, the presence of highly inflamed lesions has been correlated with DH (14-16). Other reports have suggested that persistent infection is more common in individuals with IH and, conversely, that DH facilitates clearance of the fungus (17-20).

Since the 1930s an association between dermatophytosis and disease in the respiratory tract and skin has been reported among patients with IH skin tests to *Trichophyton* (20-25). We recently reported a series of cases of *Trichophyton* asthma and the improvement of symptoms in patients treated with long-term systemic antifungal therapy (26, 27). The implication is that absorption of fungal Ag can give rise to IgE Ab production, sensitization of the airways, and development of allergic symptoms. The investigation of both the immune response to dermatophytes and the relationship between colonization and allergic diseases such as urticaria, sinusitis, and asthma has been hampered by a lack of purified Ags from these fungi (28). Currently, none of the skin test reagents used for documenting DH or IH reactions to *Trichophyton* have been standardized, and both cellular and serologic responses remain largely undefined (29, 30).

Previous studies from our laboratory reported the purification of a 30-kDa Ag, designated Tri t 1, which is important in IH reactions giving strong wheal and flare skin reactions and serum IgE Abs (31). However, no association between Tri t 1 and DH was found. To investigate the immune response to *Trichophyton*, we purified an 83-kDa *T. tonsurans* protein designated Protein IV, obtained

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³ Abbreviations used in this paper: IH, immediate hypersensitivity; DH, delayed hypersensitivity; RIA, radioimmunoassay; RAST, radioallergen sorbent; gm, geometric mean; CI, confidence interval; SAS, saturated ammonium sulfate; ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid).

N-terminal sequence data, and produced mAbs specific for this Ag. The results demonstrate that this protein elicits DH skin test reactions associated with IgG Abs in some subjects, whereas in other subjects the same protein produces IH skin tests associated with serum IgG, IgE, and IgG4 Abs.

Materials and Methods

Purification of Protein IV

A 60 to 90% saturated ammonium sulfate (SAS) fraction of a *T. tonsurans* mycelial extract was prepared as described previously (31). Culture material was kindly provided by Hollister-Stier Laboratories (Spokane, WA). Total protein content was 13 mg/ml as determined by Bradford assay. Fifty milligrams of fractionated extract was applied to a 5×50 mm Mono S cation exchange column (Pharmacia, Piscataway, NJ). The buffer system used was a 40 mM acetic acid buffer, pH 4.8, with a 0 to 0.5 M NaCl gradient at a flow rate of 0.5 ml/min over 20 min. The eluent was monitored at 280 nm and the peaks analyzed by SDS-PAGE with a 10 to 15% silver-stained gel on a PhastSystem (Pharmacia). Protein IV was eluted in 0.5 M NaCl and the pH of the eluent restored to 7.0 using 0.2 M dibasic sodium phosphate buffer. The fraction containing Protein IV was concentrated by centrifugation to a volume of 300 μ l using a Centricon-10 concentrator (Amicon Inc., Beverly, MA) and eluted from a 1×30 cm Superdex 75 gel filtration column (Pharmacia) in a 50 mM sodium phosphate buffer, pH 7.2. Purity was assessed by SDS-PAGE analysis. The final concentration of HPLC-purified Protein IV was 0.15 mg/ml as determined by Bradford assay.

Trichophyton Ags were also purified by electroelution from an SDS gel with a micro-electroelutator (Amicon) according to the method of LeGendre and Matsudaira (32). Five milligrams of the 60 to 90% ammonium sulfate fractionated *T. tonsurans* extract was applied to eight lanes of an $18 \times 16 \times 0.15$ cm 12% SDS acrylamide gel. Bands ranging from 50 to 100 kDa were excised, electroeluted for 2 h, and concentrated by centrifugation to a volume of 50 μ l. Purity was assessed by SDS-PAGE analysis and yield estimated by the degree of Coomassie staining compared with a bovine gamma globulin standard. Approximately 20 μ g of electroeluted Protein IV was radiolabeled by the chloramine-T technique with 0.5 μ Ci of ^{125} I (Amersham Corp., Arlington Heights, IL), which resulted in a sp. act. of 20 to 30 μ Ci/ μ g of protein.

Amino acid sequence analysis

Amino-terminal amino acid sequence of electroeluted Protein IV was determined by Edman degradation with a gas phase sequencer (Model 470A; Applied Biosystems, Foster City, CA). Six lysylpeptidase-generated fragments of electroeluted Protein IV were also sequenced. Confirmation of the amino-terminal amino acid sequence and an additional sequence of four endoproteinase Asp-N-generated peptides was obtained by blotting Protein IV from a 12% acrylamide gel onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) and sequencing off the membrane. Peptide sequences were compared with the National Biomedical Research Foundation, Swiss-Prot, and GenBank databases using FASTA (33).

Carbohydrate analysis

Carbohydrate analysis was performed as described previously (34). Briefly, HPLC-purified Protein IV was hydrolyzed in trifluoroacetic acid at 100°C for 4 h, dried in vacuo, and reconstituted in 40 μ M 2-deoxy-D-glucose. Monosaccharides were eluted from a 4.6×250 mm CarboPac PA-1 anion exchange column linked to a pulsed amperometric detector (Model PAD-2) with a gold working electrode (Dionex Corp., Sunnyvale, CA). Quantitation of each monosaccharide was obtained by correction relative to glycoproteins of known monosaccharide composition.

Monoclonal antibodies

Five A/J mice were immunized i.p. with 50 μ g of a gel filtration fraction enriched for Protein IV in CFA. Mice were boosted with Ag at weeks 2 and 4. A final intrasplenic injection of 20 μ g HPLC-purified Protein IV was given to the mouse demonstrating the highest IgG Ab titer by radioimmunoassay (RIA) and ELISA (1/51,000). Three days later, this mouse (A/J no. 30) was killed and its spleen cells were fused with SP₂/0 myeloma cells as described previously (35). At 10 days, 200 hybrids were screened by ELISA with a gel filtration fraction of *T. tonsurans* extract enriched for Protein IV. Hybrids producing IgG anti-Protein IV Abs were tested for binding to ^{125} I-labeled Protein IV and cloned by limiting dilution. Six positive clones were expanded and injected into pristine primed mice for ascites production. Clones 4B8-A1, 6G1-A4, 6G2-E11, and 6H7-H1 were

determined to be of the IgG1 isotype by immunodiffusion, and clones 1B8-F10 and 7A9-F8 were of the IgG3 isotype. A/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The use of animals for this study was approved by the Animal Research Committee of the University of Virginia under guidelines for the use and care of animals formulated by the National Council for Medical Research.

mAbs 4B8-A1 and 6G2-E11 were partially purified by 50% ammonium sulfate fractionation of ascites. mAb 4B8-A1 was applied to an Affi-Gel protein A MAPS II column (Bio-Rad) and biotinylated with Enzotint biotinylating reagent (Enzo Diagnostics Inc., New York, NY) according to manufacturer's instructions.

mAb ELISA for Protein IV

Immulon-4 microtiter plates (Dynatech, Alexandria, VA) were coated with mAb 6G2-E11 at 2 μ g/well in 100 μ l of 0.05 M carbonate-bicarbonate buffer, pH 9.6, overnight at 4°C. Plates were washed and blocked with 1% BSA in PBS Tween-20 and then incubated for 1 h with 100 μ l of samples containing unknown quantities of Protein IV. A standard curve was established using doubling dilutions of HPLC-purified Protein IV from 10 μ g/ml (1:15 dilution) to 20 ng/ml. After washing, 100 μ l of a 1/200 dilution of biotinylated mAb 4B8-A1 was added to each well and incubated for 2 h. Assays were developed with 0.01 M 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) in 0.07 M citrate-phosphate buffer, pH 4.2, containing 0.03% H₂O₂ and the OD was read at 405 nm.

Human subjects and skin testing

Sera were obtained from 30 subjects previously skin tested with 0.03 ml Hollister-Stier *Trichophyton* mix containing *T. tonsurans*, *T. rubrum*, and *T. mentagrophytes* species (1:200 w/v). An additional 29 subjects with a history of athlete's foot who responded to an advertisement were skin tested with *Trichophyton* mix and HPLC-purified Protein IV. Sera from 43 children attending the pediatric outpatient clinic were also available.

Protein IV was filtered through a 0.2 μ m sterile nitrocellulose disposable filter (Micron Separations, Inc., Westborough, MA) and diluted in 0.05% human serum albumin in phenol-saline solution. Skin testing was done by prick test with the ammonium sulfate-fractionated *T. tonsurans* extract, estimated to contain 160 μ g/ml protein by Coomassie staining. Intradermal testing was done with 0.03 ml HPLC-purified Protein IV at 0.8 and 8.0 μ g/ml. Test sites were examined at 20 min after injection and at 24 and 48 h. A positive immediate hypersensitivity reaction was defined as a wheal of 6×6 mm or larger on intradermal testing at 20 min. Positive delayed reactions were defined as erythema of ≥ 7 mm diameter at 24 h. Skin testing of subjects was approved by the Human Investigation Committee of the University of Virginia.

Ag binding RIA

Murine and human IgG Abs to Protein IV were measured by an Ag binding RIA using methods previously described (36, 37). Briefly, diluted serum or ascites was incubated with 2 ng of ^{125} I-labeled electroeluted Protein IV for 4 h and then immunoprecipitated with the appropriate antiserum, i.e., goat anti-mouse IgG (Chemicon, El Segundo, CA) or sheep anti-human IgG (The Binding Site, La Jolla, CA). After 16 h at 4°C, the precipitates were washed three times in borate buffered saline and counted on a gamma counter (Micromedic 4/200). Human anti-Protein IV IgG Abs were quantitated with a control curve using serial dilutions of serum from patient A.R., selected because it gave the highest binding to radiolabeled Protein IV. This serum was arbitrarily assigned 1000 U/ml.

Radioallergosorbent test (RAST)

Activated paper disks were coated with 20 μ g of the 60 to 90% ammonium sulfate fraction of *T. tonsurans* extract and IgE Abs were detected by methods previously described (31). A control curve using duplicate doubling dilutions from 1:2 to 1:4096 was constructed with the serum of a patient with IH skin tests (D.M.). The standard serum was arbitrarily assigned 1000 RAST units. Positive results were defined as values >40 U/ml.

IgE and IgG subclass Abs to Protein IV and Tri t 1

Specific IgE Abs to purified proteins were measured by ELISA. Immulon-4 microtiter plates were coated with 2 μ g/well anti-Protein IV mAb 6G2-E11 or anti-Tri t 1 mAb 6B11-C2 in 0.1 M carbonate-bicarbonate buffer, pH 9.6, overnight at 4°C. Plates were washed and blocked by standard techniques and then incubated with 100 μ l of ammonium sulfate-fractionated *T. tonsurans* extract at 20 μ g/well for 2 h. After washing, plates were incubated for 2 h with 100 μ l of serum at 1:2 and 1:20 dilutions, and then 100 μ l of 1:1000 biotinylated goat anti-human IgE (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was added. After incubation for 1 h,

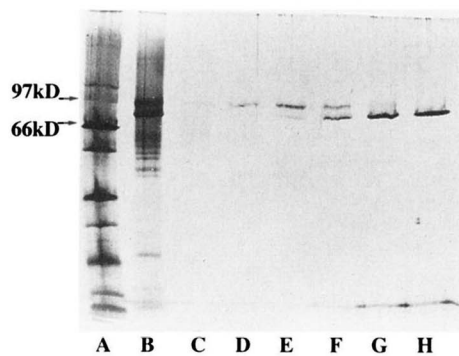


FIGURE 1. SDS-PAGE analysis of purification of Protein IV by cation exchange HPLC. Samples are as follows: A, m.w. markers; B, 60 to 90% SAS fraction of *T. tonsurans* extract; C to F, Mono S fractions eluted with 0.35 M, 0.4 M, 0.46 M, and 0.49 M NaCl; G and H, Protein IV eluted with 0.5 M NaCl. The sample in lane H was concentrated for further analysis.

plates were developed with ABTS as described above. Each assay was quantitated on the basis of a control curve constructed with serum from a subject (J.N.) with IH skin tests. Duplicate doubling dilutions of serum from 1:2 to 1:4096 formed the control curve and this serum was arbitrarily assigned 4000 U/ml. Positive values were defined as those >40 U/ml.

Subclass Abs were measured by a modification of the ELISA used to measure IgE. Abs were detected with subclass-specific biotinylated monoclonal anti-human IgG (Accurate Chemical & Scientific Corp., Westbury, NY) at 1:1000 (IgG1), 1:30,000 (IgG2), 1:4000 (IgG3), or 1:5000 (IgG4) dilution. A control curve was established with serum from a patient (no. 6) with chronic sinusitis and high anti-Protein IV IgG Ab levels. This serum was assigned 8000 U/ml and positive values were defined as those >40 U/ml.

Statistical analysis

Standard regression analysis and Pearson *r* correlation coefficients were used to determine the relationships between anti-Protein IV and anti-Tri t 1 Abs and IgE and IgG4 Abs. Means are presented as geometric means (gm) with 95% confidence intervals (CI). The nonparametric Mann-Whitney *U* test was used to compare mean Ab levels between different age groups.

Results

Purification of Protein IV

Skin testing and in vitro T cell proliferation assays were used to monitor the purification of a *Trichophyton* Ag associated with DH reactions. Preliminary work suggested that delayed reactions were directed against a *Trichophyton* Ag with a molecular size of ~70 kDa. Several prominent high m.w. proteins were clearly visible in the 60 to 90% ammonium sulfate fraction of *T. tonsurans* extract (hereafter referred to as *T. tonsurans* extract) using SDS-PAGE analysis. Consecutive stained bands were assigned numbers I through IX starting with the protein of highest m.w. Proteins ranging in size from 50 to 100 kDa were excised and electroeluted. In vitro cell proliferation studies showed that a 83-kDa protein, designated Protein IV, gave the highest stimulation indices when added to PBMCs from a subject with DH to *Trichophyton*. To obtain Protein IV in its native form, an alternative method of purification was developed because the SDS-PAGE procedure resulted in modified Ag. Native Protein IV was purified by cation exchange HPLC with a Mono S column. *T. tonsurans* extract was applied in a 40 mM acetic acid buffer, pH 4.8. Protein IV was eluted at the end of the salt gradient in 0.5 M NaCl, in keeping with its acidic isoelectric point of ~3.8 (Fig. 1). A secondary size-exclusion step using a Superdex 75 column resulted in purification of Protein IV in its native form in a buffer appropriate for skin

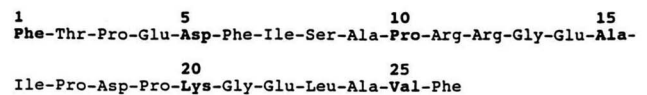


FIGURE 2. Amino-terminal amino acid sequence analysis of Protein IV.

Table I. Carbohydrate composition of *Trichophyton tonsurans* Protein IV^a

Monosaccharide	Mole/Mole of Protein	% Total Carbohydrate
Fucose	0	0
Galactosamine	0	0
Glucosamine	2.5	11.6
Galactose	0.9	3.4
Glucose	3.1	11.7
Mannose	19.5	73.3

^a Quantitative monosaccharide analysis of 10 µg of Protein IV, as determined by amino acid analysis. Results are calculated assuming a m.w. of 83,000 for Protein IV.

Table II. Fluid-phase binding of ¹²⁵I-labeled *Trichophyton* Protein IV by murine mAbs

Clone	¹²⁵ I-Protein IV Bound (cpm) ^a
Protein IV 7A9-F8	3,340
6H7-H1	13,623
6G2-E11	13,212
6G1-A4	11,564
1B8-F10	7,044
4B8-A1	14,630
Tri t 1 6B11-C2	3
2F2-F7	159
Der f 1 6A8-B10	582
Polyclonal mouse antiserum	22,557

^a Binding by Ag-binding RIA with mAb ascites and mouse serum at 1:100 dilution. Values shown are cpm bound minus background.

testing. Purity was confirmed by SDS-PAGE analysis with silver staining.

Amino-terminal sequence analysis revealed no homology of Protein IV to any other known proteins, including Tri t 1 (Fig. 2). Internal amino acid sequence of five lysylpeptidase-generated fragments (59 residues) and four endoproteinase Asp-N-generated peptides (58 residues) was also obtained (data not shown). Quantitative monosaccharide analysis by HPLC showed that Protein IV was a mannose-rich glycoprotein with a monosaccharide composition of 11.6% glucosamine, 3.4% galactose, 11.7% glucose, and 73.3% mannose (Table I); carbohydrate comprised 5% of the total weight of Protein IV. Sialic acid was not detected.

mAbs to Protein IV and development of ELISA

An A/J mouse was selected for anti-Protein IV mAb production. After fusion, hybrids were screened by Ag-binding RIA for anti-Protein IV IgG Abs and six clones were selected for production of ascites. Polyclonal mouse antiserum bound >22% and three clones (6H7-H1, 6G2-E11, and 4B8-A1) bound >13% of the radiolabeled Ag (Table II). Anti-Tri t 1 mAbs (2F2-F7 and 6B11-C2) did not bind to Protein IV. Inhibition ELISA studies suggested that the anti-Protein IV mAbs recognized a group of epitopes on Protein IV with varying degrees of overlap (data not shown). Each mAb was titrated and tested in combination with biotinylated mAb 4B8-A1 by ELISA using Protein IV purified by cation exchange HPLC. Mab 6G2-E11 gave the highest OD reading and was selected as the

Table III. Quantitation of *Trichophyton* Protein IV in commercial skin test extracts, a bronchial challenge preparation, and *Trichophyton* cultures

Sample	Protein ($\mu\text{g/ml}$)	Working Dilution ^a
Skin test extracts ^b		
<i>Trichophyton</i> mix 1:10 w/v	4.3	1:200 w/v
<i>T. mentagrophytes</i> 1:10 w/v	<0.1	1:1000–1:200 w/v
<i>T. mentagrophytes</i> 1:20 w/v	5.1	1:1000–1:200 w/v
<i>T. mentagrophytes</i> 1:500 w/v	1.1	1:500 w/v
<i>T. rubrum</i> 1:20 w/v	14	1:1000–1:200 w/v
Bronchial challenge preparation ^c		
	6.3	10 ⁻⁵ –10 ⁻¹
Culture filtrates ^d		
<i>T. tonsurans</i> ^e	1.1	NA
<i>T. tonsurans</i> , var. <i>sulfureum</i> ^f	10.1	NA
<i>T. rubrum</i> ^e	77.5	NA
<i>T. mentagrophytes</i> ^e	2.5	NA

^a Dilution used for intradermal skin testing or bronchial challenge dose.

^b Extracts obtained from different companies.

^c *T. tonsurans* extract prepared from Hollister-Stier dried mycelial culture.

^d Cultures grown in 25 ml Sabouraud dextrose broth for 4 days (e) or 15 days (f) at 37°C and shaking at 1200 rpm.

^e *Trichophyton* species isolated from skin by the Mycology Laboratory at the University of Virginia.

^f *T. tonsurans* 56186 obtained from American Type Culture Collection (Rockville, MD).

NA, Not applicable.

primary Ab for the Protein IV ELISA and was also used to capture the Ag in assays to detect IgG4 and IgE anti-Protein IV Abs. *Trichophyton tonsurans* extract was highly enriched for Protein IV, containing approximately 450 $\mu\text{g/ml}$ by ELISA. The concentration of Protein IV in the commercially available *Trichophyton* mix (1:10 w/v) was 4.3 $\mu\text{g/ml}$ (Table III); intradermal skin testing at a 1:200 (w/v) resulted in a final concentration of ~0.2 $\mu\text{g/ml}$ Protein IV. The concentration of Protein IV varied considerably between different skin test extracts (range, <0.1 to 14 $\mu\text{g/ml}$). No measurable Protein IV was detected in other fungal skin test extracts, including *Alternaria tenuis*, *Candida albicans*, *Aspergillus* mix, and *Cladosporium herbarum*. Protein IV was detected in culture filtrates of *T. tonsurans*, *T. rubrum*, and *T. mentagrophytes* grown in our laboratory, suggesting that cross-reactivity existed between the three species. The highest concentration of Protein IV was detected in *T. rubrum* cultures (Table III). Fifty house dust samples were collected from mattresses and bedroom floors. Only two samples contained detectable Protein IV by ELISA (data not shown).

Skin test and serologic responses to Protein IV

Twenty-nine volunteers with a history of athlete's foot were skin tested to *Trichophyton* mix and used to evaluate skin test reactivity and serologic responses to Protein IV. Eight subjects had DH skin tests to *Trichophyton* mix and of these, five had DH responses to purified Protein IV that were maximal at 24 to 96 h. Furthermore, a dose response was demonstrated in one subject with DH skin tests; erythema increased from 7 mm diameter at 4 $\mu\text{g/ml}$ to >2 cm at 20 $\mu\text{g/ml}$. All subjects with DH responses or with dual skin tests (IH/DH) had IgG Abs to Protein IV, but none had detectable IgG4 or IgE Abs (Table IV). Nine subjects gave immediate hypersensitivity skin responses to purified Protein IV; these reactions were seen both in subjects with IH skin tests to *Trichophyton* mix (6 of 9) and in subjects with negative skin tests (3 of 10). Among the nine subjects with IH reactions to Protein IV, six had specific IgE Abs and seven had IgG4 Abs to purified Ag. Thus, there were

Table IV. Immediate (IH) and delayed (DH) skin test responses to *Trichophyton* mix and to Protein IV in volunteers

Response to <i>Trichophyton</i> Mix ^a	<i>n</i>	Skin Response to Protein IV		Specific Abs to Protein IV		
		IH	DH	IgG	IgE	IgG4
DH	(8)	0/8	5/8	8/8	0/8	0/8
IH/DH	(2)	0/2	0/2	2/2	0/2	0/2
IH	(9)	6/9	(0/9) ^b	9/9	4/9	7/9
Negative	(10)	3/10	(0/10) ^b	9/10	3/10	4/10

^a Skin tests of *Trichophyton* mix were done with 0.03 ml (1:200, w/v) containing ~0.2 $\mu\text{g/ml}$ Protein IV.

^b Concentration of Protein IV used for skin testing was limited by the IH reaction.

^c Includes four of the six subjects with IH reactions to Protein IV.

^d Includes two of the three subjects with IH reactions to Protein IV.

distinct differences in serology profiles between individuals with IH and those with DH skin tests.

Thirty additional serum samples were obtained from adults previously skin tested to *Trichophyton*; these samples were analyzed with those from the preceding group for anti-Protein IV Abs. Immediate skin test reactions to *Trichophyton* mix and Protein IV were associated with a positive RAST in 14 of 23 subjects, and anti-Protein IV IgE Abs in 13 of 23 subjects (Fig. 3). IgG4 Abs were detectable in 14 of 23 subjects with IH skin test reactions. Among the dual responders, only 1 of 12 had a positive RAST to *Trichophyton*; however, 4 subjects had IgE Abs and two subjects had IgG4 Abs to Protein IV. DH skin tests to *Trichophyton* mix were generally associated with negative RAST and no detectable IgE or IgG4 Abs to Protein IV (Fig. 3 and Table IV). Negative skin test reactions were generally not associated with IgE Abs. However, two subjects with negative skin tests to *Trichophyton* mix had measurable IgG4 Abs to Protein IV (Fig. 3); one such subject exhibited negative skin test reactivity specifically to Protein IV despite detectable levels of anti-Protein IV IgE Abs. There was a strong quantitative correlation between IgE Abs to Protein IV and IgE Abs to Tri t 1 ($r = 0.90$, $p < 0.001$), but there was no correlation between IgG and IgE Abs to Protein IV. However, specific IgG Abs to Protein IV were common in all skin test groups, including subjects with negative skin tests. Mean levels of IgG Abs were significantly higher in subjects with immediate reactions only ($\text{gm} = 230$; CI, 132 to 398; $n = 23$) or in those with a dual reaction ($\text{gm} = 182$; CI, 105 to 316; $n = 12$) compared with those with delayed reactions ($\text{gm} = 91$; CI, 55 to 151; $n = 10$) or negative skin tests ($\text{gm} = 81$; CI, 49 to 135; $n = 14$) ($p < 0.05$).

Sera from 43 children attending the pediatric outpatient clinic were tested for *Trichophyton* Abs to evaluate the effect of age. The prevalence of anti-Protein IV IgG Abs increased from 23% in children under 2 yr to 64% in children aged 4 to 16 yr. Anti-Protein IV IgG Ab levels increased significantly with age ($p \leq 0.03$) (Fig. 4). IgE and IgG4 Abs to Protein IV coexisted in most subjects; 23 of 35 subjects with detectable IgE Abs to Protein IV also had measurable IgG4 Abs. Conversely, anti-Protein IV IgG4 Abs were only detectable in 11 of 67 subjects with no measurable specific IgE Abs. Linear regression analysis showed a significant quantitative correlation between IgE and IgG4 anti-Protein IV Abs ($r = 0.57$, $p < 0.001$) (Fig. 5). Abs to Protein IV of the IgG1, IgG2, and IgG3 subclasses were also assayed by ELISA. There was no significant correlation between anti-Protein IV IgG1, IgG2, and IgG3 Abs and either IgE or IgG4 Abs to Protein IV. In addition, no significant differences in IgG1, IgG2, or IgG3 subclasses were detected between skin test groups. Finally, sera were tested from 11

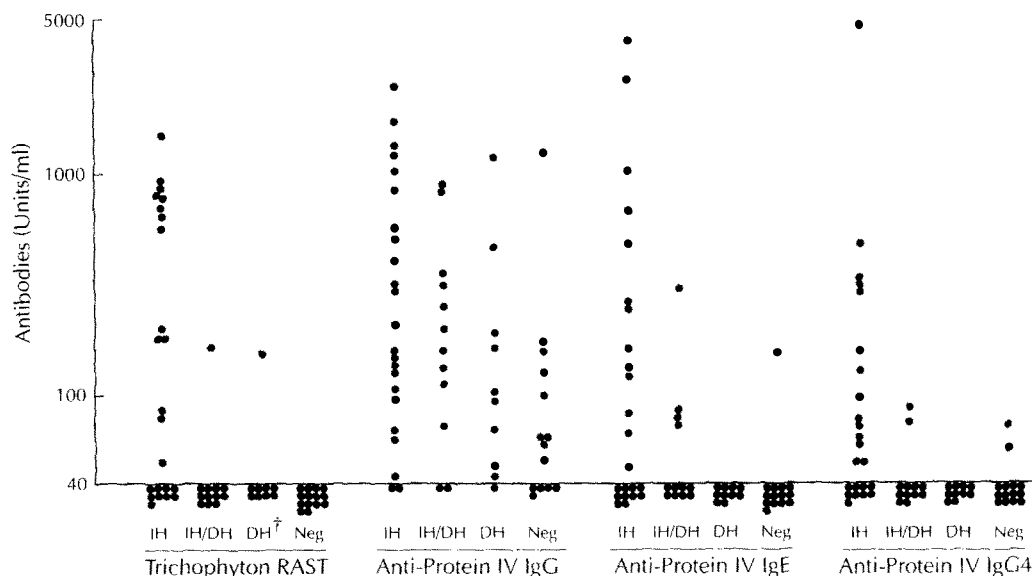


FIGURE 3. Quantitative measurements of *Trichophyton* Abs in 59 subjects with IH, DH, dual (IH/DH), and negative skin test reactions (Neg) to *Trichophyton* mix or to purified Ag. Abs measured were IgE Ab to the 60 to 90% SAS fraction of a *T. tonsurans* extract (*Trichophyton* RAST), IgG Ab to Protein IV measured by Ag-binding RIA, and anti-Protein IV IgE and IgG4 Abs measured by mAb-based ELISA. †One sample was not available for assay by RAST. Values were assigned arbitrary units and units for each type of Ab were not equivalent (see text).

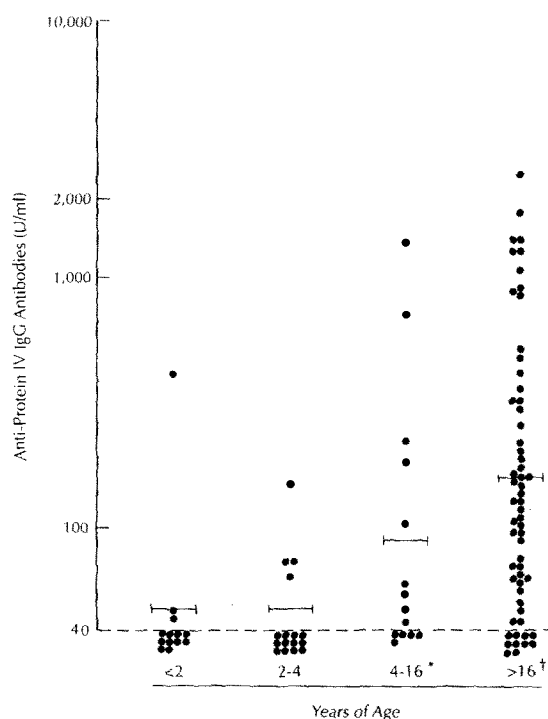


FIGURE 4. Anti-Protein IV IgG Ab levels in children under age 2 yr ($n = 13$), ages 2 to 4 yr ($n = 16$), and ages 4 to 16 yr ($n = 14$), and in adults ($n = 59$). Ab levels were significantly higher above the age of 4 years than in children under age 2 (* $p = 0.014$) or in those between 2 and 4 yr (* $p = 0.02$). Ab levels in adults were significantly higher than in all other age groups († $p < 0.03$). Bars represent geometric means.

asthma patients with a history of *Trichophyton* sensitivity. All subjects had anti-Protein IV IgG Ab levels >350 U/ml. Four of these subjects had high IgG Abs specific to Protein IV (range, 466 to 2000 U/ml) associated with both IgE (range, 52 to 1200 U/ml) and IgG4 (range, 110 to 8,800 U/ml) anti-Protein IV Abs.

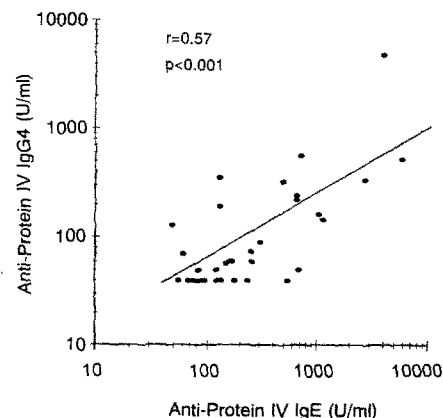


FIGURE 5. Linear regression analysis of the correlation between IgE and IgG4 Abs to Protein IV in sera from 35 subjects (including adults and children) with measurable anti-Protein IV IgE Abs.

Discussion

There is a dichotomy in the immune response to *Trichophyton*; both immediate and delayed hypersensitivity reactions to this fungus are well described. Characterization of Tri t 1 revealed an Ag associated with immediate but not delayed hypersensitivity. Our objective was to purify a dermatophyte Ag which caused DH reactions; however, the protein purified was found to elicit both IH and DH skin responses. Protein IV is an 83-kDa mannose-rich glycoprotein, isoelectric point ~ 3.8 , which is the first defined protein to elicit both IH and DH skin tests in different subjects; purification of an Ag with such capabilities provides a very important tool for studying the immunologic relationship between these two responses. It is well established that differential production of cytokines by Th cells during an immune reaction has important regulatory effects on the nature of the response. Responses in mice that are characterized by substantial IL-2 and IFN- γ but little or no IL-4 or IL-5 production (Th1 responses) result in DH reactions that

culminate in macrophage activation, and IgG2a but no IgE synthesis. The Th2 response is characterized by IL-4 and IL-5 cytokine production and generation of IgG1- and IgE-secreting cells and eosinophilia. In humans, we can hypothesize that those persons manifesting a DH reaction and who are relatively resistant to infection may have an initial response that is principally Th1 in nature, whereas a Th2 type cellular response may predominate in those who have a propensity to develop chronic dermatophytosis. In the past, most preparations used to study T cell responses to *Trichophyton* have been mixtures of proteins. Purification of *Trichophyton* Ags will facilitate in vitro T cell studies that should provide better characterization of the effector cells involved in IH and DH skin test reactions.

The nature of the immunogen may influence the subsequent response; it has been reported that the protein portion of dermatophyte glycopeptides preferentially stimulates cell-mediated immunity, whereas the polysaccharide portion preferentially stimulates humoral immunity (15, 38). Different antigenic epitopes may also play a role in differentiation of the immune response; amino acid sequence analysis and the production of synthetic peptides will aid in the elucidation of such epitopes. It should be stressed that other factors may also influence immune responses; dermatophyte-derived lymphocyte inhibitory factors have recently been shown to suppress T cell-mediated responses (39).

Positive DH skin test reactions were generally elicited at 8 µg/ml Protein IV and no dual reactions occurred to purified Ag; however, the administered dose was limited to 0.8 µg/ml in some subjects with an IH reaction. It is possible that Protein IV would elicit dual reactions at higher concentrations in these subjects. Also, dual skin test reactions are relatively uncommon and skin testing of additional subjects is necessary to confirm that dual reactions are not elicited by a single protein. There are conflicting views on the nature of the early phase that precedes the DH reaction. In contrast to previous studies, we found that four subjects with the dual reaction had anti-Protein IV IgE Abs, implying that the early reaction may be mediated by IgE Abs. Only one of these subjects had a positive RAST for IgE Ab to *Trichophyton*, suggesting that the ELISA method used to detect specific Abs was more sensitive. Monoclonal IgE Abs have been shown to facilitate the initiation of delayed hypersensitivity and it has been proposed that IgE and T cell-mediated hypersensitivity may coexist (40).

The development of mAbs made it possible to assay commercially available skin test extracts for the presence of Protein IV. Wide variations in the concentration of Protein IV between extracts were found, confirming the need for standardization. Furthermore, Protein IV was detectable in the culture filtrates of all species tested, suggesting the existence of cross-reactivity between the species. The results on cross-reactivity are in keeping with previous studies (31, 41). Protein IV was not detected in other fungal skin test extracts by the ELISA method.

We examined the prevalence of Abs in subjects who were skin tested with *Trichophyton* mix and Protein IV. Immediate hypersensitivity reactions were associated with positive RAST and high anti-Protein IV-specific IgE and IgG Abs. Subjects with DH skin tests had lower levels of IgG Abs and no detectable IgE. In addition, low levels of IgG Abs were detected in some subjects with negative skin tests; interestingly, three of these subjects who were skin-test negative to *Trichophyton* mix had immediate reactions to Protein IV, and two of these subjects had measurable anti-Protein IV IgE Abs. The dosage of Protein IV used in the commercial skin test extract was approximately 0.2 µg/ml, suggesting that the dosage discrepancy in specific Ag may explain negative skin test reactivity to *Trichophyton* mix in the presence of an immediate reaction to purified Ag.

Immediate hypersensitivity reactions were associated with anti-Protein IV IgG4 Abs and a quantitative correlation between anti-Protein IV IgE and IgG4 Abs was found to exist. The role of IgG4 Abs in allergic disease is unresolved; current opinion suggests that IgG4 has neither an exclusive role as a protective Ab nor an alternative role to IgE Abs as an anaphylactic Ab (42). Measurements of IgG subclasses to Protein IV revealed a coexistence of IgG1, IgG2, and IgG3 Abs with IgG4 Abs. In addition, OD readings (405 nm) suggested that IgG1 Ab was the most abundant subclass present. Furthermore, only the IgG4 subclass showed distinct distributions between different skin test groups. The IgG4 isotype is the least abundant IgG subclass in humans. IgG4 Abs are monovalent and do not bind complement but can inhibit complement activation of IgG1 Abs in some systems (43). Mean IgG4 levels are increased in patients with atopic dermatitis, asthma, and cystic fibrosis. Furthermore, IgG4 Abs are associated with chronic allergen exposure: the presence of IgG1 Abs in novice beekeepers stung for the first time is well documented, but after subsequent stings, a shift to an IgG4-restricted response occurs (43). The role of IgG4 Abs in dermatophytosis is unclear; the presence of IgG4 Abs to Protein IV and the significant correlation with IgE Abs strongly supports the view that the IH reaction to this protein arises from a Th2 response.

Finally, we examined the levels of anti-Protein IV IgG Abs in different age groups; results showed that levels increased significantly with age. Interestingly, anti-Protein IV IgE Abs were measurable in 3 of 13 children under the age of 2, implying that colonization and immune responses to *Trichophyton* may occur at an early age and that immunologic mechanisms predisposing them to chronic dermatophytosis are established early. As mentioned earlier, *T. tonsurans* is the most prevalent of the *Trichophyton* species in early childhood, whereas *T. rubrum* and *T. mentagrophytes* occur more frequently in adults (1–3, 7, 8). Since we have demonstrated cross-reactivity between *Trichophyton* species, it is plausible that exposure to Protein IV throughout life may occur by infection with *T. tonsurans* in early childhood, with subsequent *T. rubrum* colonization in adult life.

The current results establish that Protein IV mediates two immunologically distinct reactions: DH associated with serum IgG Abs or IH associated with both IgE and IgG4 Abs in addition to IgG Abs. Protein IV is the first defined protein to be associated with both immediate and delayed hypersensitivity reactions in humans.

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