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Trichophytin Extraction: Biological Comparison of Trichophytin Extracted from *Trichophyton mentagrophytes* Grown in a Complex Medium and a Defined Medium

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The extraction of antigens (trichophytin) from *Trichophyton mentagrophytes* var. *asteroides* grown in both a complex medium and a defined medium proved to be reproducible and feasible. These extracts were first evaluated by skin testing in both infected and noninfected guinea pigs. Further evaluation and standardization was accomplished in selected humans by comparing these antigens to a trichophytin known to elicit allergic reactions to the dermatophytes. The trichophytins produced by this extraction procedure gave no false positive (or negative) skin test reactions. These antigens did, however, reliably elicit allergic sensitivity of both the immediate and delayed type. The potency of both complex and defined media antigen was shown to be equal to that of the standard trichophytin.

In 1925 Bloch (1) emphasized the importance of hypersensitivity reactions in dermatophyte infections and concluded that an extractable carbohydrate protein complex (trichophytin) isolated from several dermatophytes was the allergen involved. Subsequently, trichophytins were prepared from both supernatant and mycelial mats of dermatophytes grown in various types of complex media by various crude extraction procedures (2-8). These procedures resulted in complex and nonstandardized trichophytins which elicited false negative and false positive skin tests (5, 8-12). Cruickshank et al. (12-15) demonstrated that their glycopeptide antigen elicited allergic reactions in infected guinea pigs. Skin testing in humans showed that both immediate and delayed allergic reactions could be obtained with their trichophytin, but specificity of the reaction was not completely determined.

We used trichophytin, kindly provided by C. N. D. Cruickshank, for testing infected and noninfected guinea pigs and confirmed that this antigen is nontoxic and does not irritate locally, but elicits allergic reactions. These observations led to a comprehensive study of trichophytin allergy and dermatophyte infection in an all-male volunteer population (16). Initially, the skin test antigen given by Cruickshank and a commercially available antigen were compared. It was observed that 29% of the population did not react to Cruickshank's trichophytin. Some of these men presented no historical, clinical, or mycological evidence of dermatophyte infection. Another 57% exhibited some degree of delayed-type hypersensitivity (DTH) to the trichophytin. Most of those exhibiting delayed reactions presented a past history of infection, but more than 90% were free from infection at the time of the survey. The remaining 14% exhibited immediate-type hypersensitivity with or without coexistent DTH to Cruickshank's trichophytin, and most of these subjects were chronically infected with the dermatophytic fungi. The commercially prepared trichophytin did not give the same reactions in these three skin test reaction groups. Overall, commercially prepared trichophytin gave 25% false positive or false negative reactions.

These and several other observations made in this laboratory led us into several in vitro and in vivo studies in which we attempted to further understand the immunological processes involved in dermatophyte infections. To accomplish these studies required the following: (i) that we produce an antigen that would detect allergic sensitivity to the dermatophytes equally well as the antigen provided by Cruickshank, and (ii) that we develop a simple defined medium for supporting *Trichophyton mentagrophytes* growth to eliminate the lot-to-

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lot variation encountered when using a complex medium.

This study relates a detailed extraction method that closely parallels that of Cruickshank et al. (12–15), and compares on an immunobiological basis antigens produced from complex and defined media.

MATERIALS AND METHODS

Organism. *T. mentagrophytes* var. *asteroides* ATCC 24585 was used in all experiments. The fungus was maintained on Sabouraud agar slants (Difco) at 30 C prior to the preparation of the spores.

Preparation of spores. Microaleuriospores, viable subunits of the fungus, were used as started cultures in all experiments. The spores were harvested from the mycelia according to the method of Reinhardt et al. (unpublished data). Briefly, sterile physiological saline was used to wash the fungal mat from the agar surface. The mat was then shaken at 275 rpm in a 250-ml Erlenmeyer flask, containing no. 3000 glass beads, for 30 min. After gentle passage through a sterile Swinnex syringe filled with no. 3950 Pyrex glass wool, the spores and remaining mycelial fragments were washed three times by centrifugation at 13,000 × *g* in a saline antibiotic wash solution. A fourth washing was accomplished in saline without the antibiotics.

Media. A complex medium and a defined medium were used to grow the fungus. The complex medium (also used by Cruickshank) contained 1% Panmede (Paines and Byrne, Ltd.), 4% dextrose, and 2% vitamin-free casein hydrolysate (Nutritional Biochemicals Corp.) per liter. The pH of this medium was adjusted to 5.5 with 1.0 N NaOH. The constituents of the defined medium (in grams per liter) were as follows. (i) Stock vitamins: dextrose (200.0); thiamine (0.01); riboflavin (0.028); nicotinic (0.094); pantothenic (0.150); pyridoxine (0.0036); biotin (0.0006); folic acid (0.004); cyanocobalamine (0.0012); and inositol (0.0012). (ii) Stock amino acids: L-Leucine (3.0); L-iso leucine (3.0); L-phenylalanine (2.0); L-glutamic acid (6.8); L-tyrosine (1.0); L-arginine (6.8); L-valine (3.0); and L-serine (1.0). (iii) Stock mineral salts: $(NH_4)_2SO_4$ (30.0); $CaCl_2$ $2H_2O$ (1.0); $MgSO_4$ (2.5); $ZnSO_4$ (0.010); $FeCl_3$ (0.10); $CuSO_4$ (0.001); and $MnCl_2$ (0.010). (iv) Stock phosphate buffer: KH_2PO_4 (10.0) and Na_2HPO_4 (40.0). Final concentrations of the above components were one-tenth of that indicated. Stocks (i), (ii), and (iii) were adjusted to pH 6.5 with 1.0 N NaOH and stored at 5 C. To prepare 1 liter of medium, 100 ml of filter-sterilized stocks (i), (ii), and (iii) and 200 ml of stock (iv) were added to 500 ml of sterile water.

Fermentation. (i) New Brunswick fermentor. A New Brunswick bench-top fermentor model B, fitted with an automatic pH controller and an automatic silicone antifoam device, was filled with 10 liters of the complex medium. Prior to inoculation, the instrument was set to maintain a temperature of 30 C, sterile air was bubbled through the medium at a rate of 15 liters/min, and the entire 10 liters was agitated at 200 rpm. Twenty milliliters of a concentrated spore suspension was aseptically introduced into the fermentor. As the amount of growth in the fermentor increased, the rpm was increased to 800 to insure proper agitation. At the end of 7 days, when the pH reached approximately 8.5, the fungus was harvested.

(ii) Fermentation in Fernbach flasks. Fernbach flasks containing 1 liter of either complex (pH 5.5) or defined (pH 6.5) medium were inoculated with 2 ml of a concentrated spore preparation. The flasks were incubated at 30 C in a Psycrotherm incubator shaker (New Brunswick) at 175 rpm to insure proper aeration. At the end of the 7-day growth period, the pH was approximately 8.5.

Antigen extraction. (i) Preparation of the acetone powder. At the end of 6 days of growth, the pH of the culture was adjusted to 6.0 to 6.5 with 1 N HCl. Three times the volume of acetone (Baker analyzed; room temperature) was added in a slow stream with constant stirring. Gentle stirring was continued for 18 h at 28 to 30 C. The acetone was then carefully decanted so as not to allow the mycelial mat to become air-dry. The original volume of acetone was added back to the mat and stirred for 2 h. This procedure was repeated for 2 h. Decantation and acetone extraction were repeated again for 1 h. At the end of the 5-h period, the acetone was removed from the mycelial mat by vacuum, filtering small amounts at a time onto Sharkskin filter paper. The cakes of mycelium were dried in the open at room temperature until no odor of acetone remained. The cakes were then ground with a mortar and pestle to a fine powder in a well-ventilated hood.

(ii) Ethylene glycol extraction. The acetone-dried powder was added in the ratio of 50 g to each liter of prewarmed (40 C) ethylene glycol (Baker analyzed). The viscous material was stirred overnight with a motorized stirrer at room temperature. The suspension was centrifuged at 13,000 × *g* for 15 min at 5 C in a Sorvall RC-2B. The supernatant was saved, and the pellet was again extracted for 18 h in ethylene glycol. At the end of the second extraction both supernatants were pooled and dialyzed against 5 C distilled water for 60 h. The material remaining inside the dialysis membrane was lyophilized.

(iii) Ethanol precipitation. The lyophilized material was dissolved in a minimal amount of distilled water and poured into 3 volumes of cold ethanol. This was allowed to stand at 4 C overnight and centrifuged at 18,000 × *g* for 20 min at 5 C. The supernatant was discarded, and the pellet was dissolved in a minimal amount of distilled water and lyophilized.

(iv) CTAB purification. The ethanol precipitate was dissolved in a minimal amount of 1% sodium borate (pH 8.5). Five percent CTAB (cetyltrimethylammoniumbromide) (Eastman) was added dropwise until the white or tan precipitate no longer formed. This suspension was centrifuged at 2,000 × *g* for 10 min at 5 C. The pellet was saved and the supernatant was processed further. To the supernatant 1 N NaOH was added dropwise until the pH reached 9.5. After standing overnight at 5 C, the pellet was combined with that obtained earlier. The CTAB-antigen complex was washed twice with distilled water and dissolved in a minimal amount of 2 N

acetic acid. Ten times the volume of cold ethanol was then added. After remaining at 5 C overnight, the solution was centrifuged at 13,000 × *g* for 10 min at 5 C. The precipitate was again dissolved in the acetic acid-ethanol solution and recentrifuged. The precipitate was then washed twice with 100% cold ethanol, centrifuged and dissolved in a minimal volume of distilled water, and centrifuged at 13,000 × *g* for 10 min at 5 C. The supernatant was poured through a small column of Dowex AG 50WX8 (hydrogen form) (Bio-Rad) to remove any residual CTAB. The eluate was membrane filtered (0.45 μm; Millipore Corp.) and dialyzed against distilled water at 5 C overnight. The dialyzed material was lyophilized to a powder form and stored at −20 C.

Skin testing. Before skin testing human volunteers, the three antigens (Cruickshank's trichophytin, CM107, and defined [i]) were tested on both infected and noninfected guinea pigs by administering the material intradermally in 100 μg per 0.1 ml of normal saline.

Human volunteers were selected from men in a California correctional facility. Informed consent was obtained before testing. CM107, defined (i), and Cruickshank's trichophytin were used in normal saline without preservatives to avoid any nonspecific reactions from primary irritation or allergic sensitivity to the preservatives. A concentration of 10 μg of Cruickshank's trichophytin per 0.1 ml of saline was confirmed to be optimal for detecting allergic sensitivity in humans. CM107 was first compared to the Cruickshank antigen by administering simultaneously three concentrations (10, 1, and 0.1 μg per 0.1 ml) of one antigen intradermally on the volar aspect of the left forearm and the other antigen on the right forearm. Defined (i) was then compared in the same manner with the now standardized CM107.

The tests were read at 20 min, and a wheal greater than 10 mm plus or minus flare was accepted as a positive, immediate allergic reaction. The skin test sites were also read at 24 and 72 h. The delayed reactions observed at 72 h were erythematous, edematous, indurated, occasionally papulovesicular, and rarely ulcerative. These reactions were measured in millimeters across the transverse aspect of the arm.

RESULTS

Yield of active material. The extraction of mycelium grown in the complex medium gave a yield of 64.4 mg/100 ml. This correlates well with the data of Codner et al. (17), who reported a yield of 70 mg of "active material" per 100 ml. Extraction from the defined medium gave approximately 10% less skin test antigen, 58.0 mg/100 ml of defined medium.

Guinea pig skin tests. No appreciable difference could be detected on guinea pigs concerning the activity of any of the three antigens (Table 1). In the noninfected pigs, no primary irritant or toxic reactions were observed at the sites where any of the three antigens were injected. Guinea pigs exhibiting delayed sensitivity to the antigens typically exhibited erythema, edema, and induration. The mean diameter for Cruickshank's trichophytin in the guinea pigs who had recently recovered from an infection of *T. mentagrophytes* was 13.9 mm compared with a mean diameter of 13.2 mm for CM107 and 13.2 mm for defined (i).

TABLE 1. *Guinea pig skin test data*[a]

Status of animal	Type of antigen	Erythema diameter (mm)		
		24 h	48 h	72 h
Noninfected				
1	CM107	3.0	0	0
2	CM107	2.0	0	0
3	Cruickshank	3.2	0.8	0
4	Cruickshank	2.7	0.7	0
5	Defined (i)	0.0	0	0
6	Defined (i)	3.0	0	0
Infected				
7	CM107	14.0	14.0	14.0
8	CM107	13.0	13.0	13.0
9	Cruickshank	14.6	14.0	14.0
10	Cruickshank	13.2	13.2	13.0
11	Defined (i)	15.0	15.0	15.0
12	Defined (i)	13.0	13.0	13.0

[a] Diameter of skin test reactions in noninfected pigs versus pigs who had recently recovered from an infection with *T. mentagrophytes* ATCC 18748. The antigens were administered in concentrations of 100 μg/0.1 ml of 0.9% saline for injection on the left flank of each pig.

Human skin tests. The antigens were compared by using three concentrations (10, 1, and 0.1 μg per dose) of each material, and at each concentration the reactions were similar for all three of the antigens. The minimal antigen concentration eliciting a reaction and the size and intensity of the reactions were similar; hence, for simplicity only the results of the 10-μg skin test are shown in Table 2.

(i) Nonreactive volunteers. Twenty minutes after injection of the antigens, a slight amount of edema was noted. Reexamination of skin test sites at 24 and 72 h showed no reaction to any of the three antigens.

(ii) Immediate reactors plus or minus DTH. Twelve subjects chronically infected with *T. mentagrophytes* var. *interdigitale*, having in some cases DTH, were tested with the three antigens. There was no significant difference in the mean diameters of the wheal and flare detected with the three antigens. There was no significant difference in the sizes of the delayed reactions seen in these same subjects.

Delayed reactors. In the 15 men of the

TABLE 2. *Human skin test data*

Immunobiologic class	No. of subjects	Antigen (10 μg/0.1 ml)	Diameter of skin test reactions (mm)						
			20 min			24 h		72 h	
			Mean[a]	Wheal[b]	Flare[c]	Mean[d]	Range	Mean	Range
Nonexperienced or virginal (nonreactors)	6	Cruickshank	9.6/0.0	(7–10)	(0–0)	0.0	(0–0)	0.0	(0–0)
		CM107	8.3/0.0	(7–10)	(0–0)	0.0	(0–0)	0.0	(0–0)
		Defined (i)	9.0/0.0	(7–10)	(0–0)	0.0	(0–0)	0.0	(0–0)
Chronically infected (immediate ± DTH)	12	Cruickshank	17.5/42	(10–23)	(17–62)	4.1	(0–12)	2.0	(0–8)
		CM107	18.4/40	(11–25)	(15–70)	3.8	(0–14)	2.0	(0–9)
		Defined (i)	18.5/45	(11–25)	(20–70)	4.2	(0–13)	2.0	(0–11)
Experienced-immune (DTH only)	15	Cruickshank	10.1/4.2	(7–15)	(0–12)	6.0	(0–8)	10.0	(5–17)
		CM107	10.4/3.6	(8–11)	(2–13)	6.4	(0–7)	10.2	(7–15)
		Defined (i)	10.8/5.2	(6–11)	(2–13)	6.7	(0–7)	10.4	(5–13)

[a] Mean diameter of the wheal/flare (mm).
[b] Range of wheal reactions.
[c] Range of flare reactions.
[d] Mean diameter of reaction (mm).

experienced-immune class, a significant DTH reaction was observed at 72 h by using all three antigens. The areas were erythematous, edematous, and indurated. Again, no significant difference in the mean diameter or intensity of the reactions was observed with any of the three antigens.

DISCUSSION

Antigen extraction. As implied previously, a skin test material must be potent, yet possess no local skin irritancy when injected. It is very easy to prepare trichophytin from both cultures of the dermatophytes. It is not advisable to wash the mycelial mat with large quantities of water before extraction because much of the active material is lost in the washes. It is also important to lower the pH of the culture to approximately 6.0, as reported by Cruickshank et al. (12–15), in order to obtain the highest yield of active material. Failure to drop the pH of the culture during early extractions gave lower yields. Lowering the pH may facilitate penetration of the solvent into the mycelia, but the precise action is unknown. It has been shown that the solubility of some glycopeptides is greater above room temperature; thus, this galactose-mannose peptide was extracted at 40 C, which appears to facilitate extraction. Stopping dialysis after 60 h instead of 96 h appears to increase yield of active material and at the same time is adequate to remove primary irritants. Presumably, the molecular weight of the antigen is approximately 30,000 (14) and may thus be lost through prolonged dialysis or inactivated in some manner.

Strict adherence to the ethylene glycol extraction, ethanol precipitation, and CATB purification is required to obtain a good yield of an active material suitable for skin testing. Skin-test-active materials are present in the culture filtrate, but we found it necessary to follow this procedure to completely remove the primary irritants. In our early efforts, the final product often caused nonspecific reactions in guinea pigs that were not infected. In later studies, some final products elicited specific positive reactions in guinea pigs and large reactions in skin-test-positive humans, but, in addition, some false positive reactions in other human skin test nonreactive to Cruickshank purified trichophytin. Again, it should be mentioned that such false positive reactions were indeed nonimmunologic, for such subjects when experimentally infected underwent a primary infection course. On the other hand, one may follow the general procedure and produce a product free of primary irritancy, but unless the important steps outlined above are followed, the yield may be very low. In extraction of both CM107 and defined (i), the exact procedure detailed in Materials and Methods and elaborated on above was followed, and in both cases a good yield of irritant-free antigen was obtained.

The defined medium was patterned after the components known to be present in Panmede. The 10% decrease in the quantity of antigen extracted from the defined medium was expected since this medium was developed with the idea of having only the essential vitamins, amino acids, and minerals necessary to support the growth of the fungus.

Human skin test data. A common problem encountered when using commercial trichophytin or crude extracts is the percentage of false positives due to primary irritants contained in such skin test materials (5, 9–12). None of the noninfected guinea pigs or the six virginal subjects manifested an initial wheal or flare reaction greater than 10 mm. It is most important to note that at the 24- and 72-h readings all visible evidence of the skin test had vanished. These results indicate that, at the concentrations used, the three antigens are free from materials that produce primary irritant reactions in guinea pig and human skin. That the three antigens are similar on an immunological basis is evident when one compares the size of both immediate and delayed reactions in the chronically infected and experienced immune groups (Table 2).

Theoretical implications. The method of antigen extraction reported in this paper is biologically reliable and reproducible. The similarity of skin test reactions (Table 3) proved that the defined medium was not only capable of supporting growth, but also allowed for the production of an immunologically active, irritant-free trichophytin. It appears advantageous to use antigen extracted from a defined medium to facilitate chemical characterization and to insure the purity and reproducibility of trichophytin for other in vitro and in vivo uses.

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