

ISOLATION AND PARTIAL CHARACTERIZATION OF IMMUNOLOGICALLY REACTIVE FRACTIONS FROM CHITINASE DIGESTED CELL WALL OF TRICHOPHYTON MENTAGROPHYTES*

A. Kh. AL-RAMMAHY**, Julia G. LEVY & Barbara KELLY

Department of Microbiology University of British Columbia Vancouver, British Columbia Canada V6T 1W5

Summary

Cell wall preparations of *Trichophyton mentagrophytes* were digested with chitinase following which various fractions were isolated by ultrafiltration and Sephadex gel filtration. All fractions isolated contained both polysaccharide and peptide material. A correlation was seen between those fractions capable of eliciting immediate and delayed skin reactions in sensitized guinea pigs and those capable of stimulating the *in vitro* proliferation of lymphocytes taken from sensitized guinea pigs. These immunologically active fractions also developed precipitin lines with antiserum taken from sensitized animals. A low molecular weight fraction was found to be completely reactive immunologically (UM_{2(a)}), and appeared to have a molecular weight in the range of 2,000–4,000 as assessed by ultrafiltration and gel filtration studies.

Introduction

Different approaches have been followed by many workers in the isolation of the antigenic components of dermatophytes. Most of the methods used were chemical and include; ethylene glycol (3, 4, 8, 12), hot dilute alkali (6), phenol (19). Using these methods, many investigators isolated crude nitrogen containing polysaccharide fractions which were used for detecting skin reactivity in sensitized animals (3, 4) or naturally infected human beings (11, 13, 19, 22).

The immunological roles of the polysaccharide and peptide moieties of this material have been investigated by several workers. According to Barker and co-workers (3,

4), immediate and delayed type hypersensitivity were attributable to the carbohydrate and peptide moieties of the isolated galactomanan-peptide respectively. Nozawa and co-workers (19), showed that proteolytic digestion of phenol water extracted material decreased the delayed hypersensitivity. Glucans isolated by How *et al.*, (12) from *Trichophyton rubrum* and *Microsporum quicqueanum* sensitized guinea pigs and elicited immediate skin reactions. The lipid fraction of the crude trichophyitin of the cell wall of *T. mentagrophytes* was shown by Anderson *et al.*, (1) to cause inflammatory delayed allergic skin reactions in sensitized guinea pigs.

In addition to serological techniques, immediate and delayed type hypersensitivity as measured by skin reactivity has been, until recently, the main immunological test used by other workers to study the antigenic components of the dermatophytes.

Several investigators (2, 11, 23), showed that lymphocyte transformation was a sensitive method for the demonstration of mycotic sensitization and elucidation of the cell mediated immunological responses to dermatophyte antigens.

In this work, we have attempted to fractionate antigenic components from chitinase digests of mycelial cell wall preparations of *T. mentagrophytes* by ultrafiltration and gel filtrations. A study was made on the immunological properties of each fraction thus isolated. A comparison was made between skin test reactions with lymphocyte transformation and immune precipitation as measures of the immune responses of guinea pigs sensitized to whole mycelial cell wall preparations.

Materials and methods

Organism and Growth Conditions

Trichophyton mentagrophytes (TM) var *asteroides* is one of

* This work was supported by grant number 3411 from the Medical Research Council of Canada.

** A. Kh. Al-Rammahy was supported by a scholarship from the Ministry of High Education, Iraq.

the stock culture collections of the Department of Microbiology, University of British Columbia.

The fungus was grown in a 30 litre fermenter in 4% glucose, 1% neopeptone and incubated at 30°C for 90 h with continuous stirring and aeration. The inoculum was prepared by seeding eight two litre flasks, each containing 800 ml of growth medium which were incubated in a shaking waterbath at 30°C for 6 days. All cultures were checked under the microscope to exclude the possibility of contamination.

The culture was treated with merthiolate at a final concentration of 1 : 7000 for 2 h prior to further manipulation. This treatment was shown earlier to effectively kill all fungal cells. The culture was then filtered through two layers of cheese cloth and the fungal mat was washed many times with distilled water until the absorbance at 280 nm of the filtrate was < 0.03.

Preparation of Mycelial Cell Wall

The fungal mat was defatted according to the method of Bartinicki-Garcia & Nickerson (5). Fat free mycelia were freeze dried and the dry weight was 26.25 g. The dried mycelia were then sonicated in citrate phosphate buffer at a setting of 80 (Biosonic) 5 times for 30 seconds each with a 20 second interval between each treatment. Mycelial sonicates were centrifuged at 600 × g in a Sorvall centrifuge. The pellet was exposed to freezing and thawing with liquid nitrogen (7) using glass beads (Operlin 0.1–0.11 mm) in a mortar until 95% breakage of mycelia was demonstrated by microscopic examination. The mycelial cell wall suspension was centrifuged at 10,000 × g and the pellet was washed three times to remove all cytoplasmic debris, and then freeze dried. This dry material was used in the preparation of the antigenic fractions.

Digestion and Fractionation of Cell Wall

The antigenic fractions of the cell wall were prepared by chitinase digestion as illustrated in Fig. 1. The dried cell wall material was digested for 24 h with chitinase (Calbiochem) at a concentration of 0.5 mg/ml of the substrate mixture at 37°C in a shaking waterbath following which the remaining pellet was washed several times with 0.05 M citrate buffer pH 6.0 and the same amount of fresh enzyme was added and incubated for another 6 days with a few drops of toluene to prevent contamination. It is worth mentioning that the 24 h and 7 day digestions did not show obvious differences in terms of the molecular size of the

Cell wall pellet was homogenized in 0.5 M citrate phosphate buffer pH 6.0, incubated with chitinase at 0.5 mg/ml at 37°C in a shaking water bath for 24 hrs or 7 days. Hydrolysates were cleared by centrifugation at 10,000 g.

Hydrolysates were concentrated by ultrafiltration (Amicon)

UM₁₀ passed fraction was concentrated on UM₂ filter

Sephadex G 25 (Fig. 3)

UM₂ (a)
UM₂ (b)
UM₂ (c)
UM₂ (d)
UM₂ (e)

Peaks were concentrated by UM₂ filter

UM passed fractions were freeze dried and desalted on Sephadex G 25

PUM₂ (a) PUM₂ (b) PUM₂ (c)

UM₁₀ concentrated

Sephadex G 150 (Fig. 2)

Peaks were concentrated by UM₁₀ filter

UM₁₀ (a) UM₁₀ (b)

Fig. 1. Preparation of antigenic materials from *T. mentagrophytes* cell walls.

components in the chitinase hydrolysate as shown by ultrafiltration followed by gel filtration.

High molecular weight materials were concentrated by a UM₁₀ Amicon filter (MW > 10,000) and further fractionated on Sephadex G 150 column (100 × 2.5 cm) equilibrated and eluted with 0.85% NaCl in 5 ml fractions. Fig. 2 shows the elution profile. Digested material of molecular weight < 10,000 which passed through UM₁₀ filter was concentrated on a UM₂ filter (MW > 1,000) and fractionated on Sephadex G 25 column (60 × 2.0 cm) in 4 ml fractions (Fig. 3). The fractions thus prepared were concentrated by either ultrafiltration or freeze drying and were stored frozen in 0.85% NaCl.

Quantitative Analysis

Chemical analyses were performed on the digested and sized fractions from the cell wall material to determine their sugar and protein content. Total sugar content was

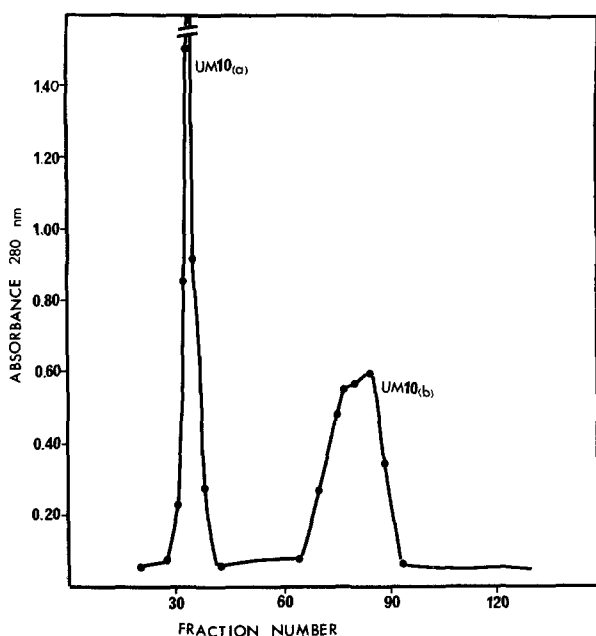


Fig. 2. Elution profile of UM_{10} concentrated material on Sephadex G-150.

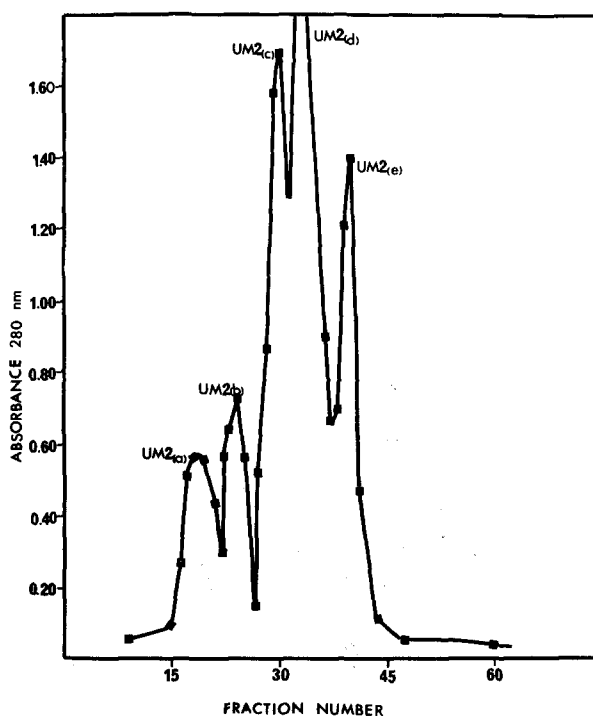


Fig. 3. Elution profile of UM_2 concentrated material on Sephadex G-25.

determined by Anthrone reagent (16) using glucose as a standard. Protein was measured by the method of Lowry *et al.*, (14).

Immunization of Animals

Twelve albino guinea pigs weighing 400–500 g were immunized with the whole dried isolated cell wall material. Each animal received three injections of 0.2 ml of 2.0 mg/ml cell wall antigen in 50% complete Freund's adjuvant (CFA, Difco). Two injections were given intramuscularly and one intraperitoneally. The animals were boosted with the same preparation and amount four weeks after the first injection.

Skin Tests

The isolated antigenic fractions used for skin testing were dissolved in 0.85% NaCl solution. Intradermal injections of 25 μ g of Lowry positive test antigens in 0.2 ml were administered into the skin of sensitized and control animals whose flanks had been shaved and subsequently treated with Nair (Carter-Wallace N.S. Inc., Toronto, Canada). Cutaneous reactions were measured after 30 min for an immediate reaction, after 5 h for an Arthus reaction and after 24 h for the delayed hypersensitivity reaction. Reactions were considered positive when the difference in the diameter of the wheal or induration between sensitized and control animals was > 5 mm.

In vitro Lymphocyte Stimulation

In these studies lymphocyte transformation tests were done *in vitro* to measure the antigen specific lymphocyte stimulation and to correlate the results with those of skin reactivity. The procedures used have been described in detail elsewhere (10). Briefly, 5×10^5 lymph node cells per microtiter well (Linbro) were cultured in 0.25 ml 1640 medium with different concentrations of antigens, and incubated for 24 or 96 h at 37 °C in a humidified incubator supplied with 5% CO_2 . Eighteen hours prior to culture harvest, ^3H -thymidine (New England Nuclear) (2 μ Ci per well in 0.05 ml 1640) was added. Harvesting was done by microharvester, and thymidine incorporation was measured by liquid scintillation counter. All tests were run in triplicate. Comparisons were made between cultures containing antigen, and those containing no antigen, using lymphocytes from immunized and unimmunized animals. Statistical analyses of data were carried out using Student's test.

Table 1. Quantitative analysis of the Anthrone positive and Lowry positive material of the hydrolysate and of the fractions obtained after ultrafiltration and gel filtration.

Fraction No.	Lowry positive mg recovered	Anthrone positive mg recovered	Fraction No.	Lowry positive mg recovered	Anthrone positive mg recovered
UNF ₂₄ h*	218	890.8	UNF ₇ d*	116.2	540.0
UM ₁₀ (a)	112.4	40.0	UM ₁₀ (a)	66.0	39.0
UM ₁₀ (b)	116.25	195.5	UM ₁₀ (b)	29.9	120.0
UM ₂ (a)	28.6	98.65	UM ₂ (a)	10.3	38.7
UM ₂ (b)	3.8	80.20	UM ₂ (b)	2.3	18.9
UM ₂ (c)	4.9	117.5	UM ₂ (c)	6.9	192.9
UM ₂ (d)	21.5	71.5	UM ₂ (d)	4.12	12.8
UM ₂ (e)	15.75	111.78	PUM ₂ (a)	17.3	24.4
PUM ₂ (a)	27.0	105	PUM ₂ (b)	3.9	6.0
PUM ₂ (b)	3.8	0	PUM ₂ (c)	9.8	0
PUM ₂ (c)	2.1	0			

*UNF unfractionated

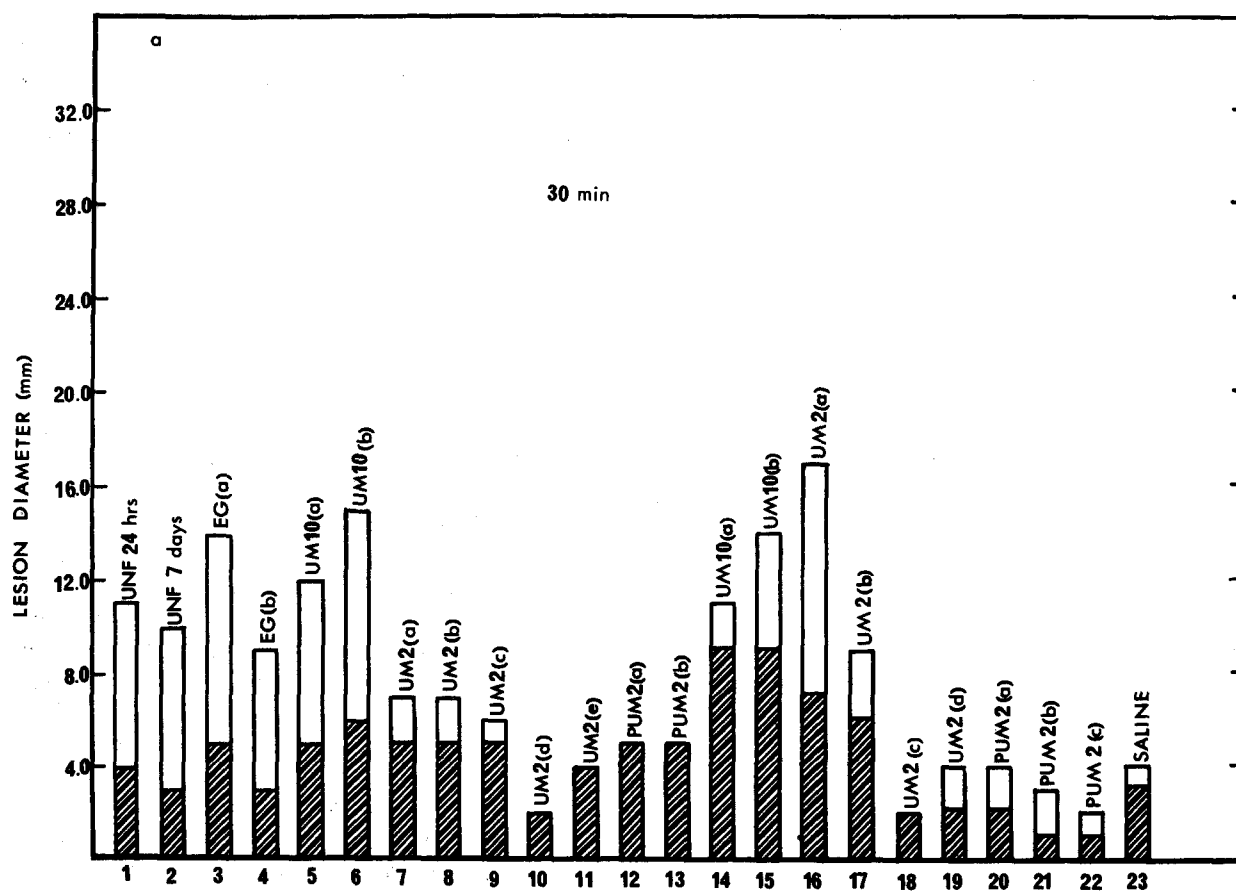


Fig. 4. (a) Cutaneous allergic reactions in immunized guinea pigs (whole column) compared to that of unimmunized animals (shaded) to *T. mentagrophytes* cell wall fractions after 24 hr and 7 days digestion with chitinase 30 minutes after intradermal injection (immediate reaction).

Immunodiffusion

Double diffusion slides using 1% agarose in barbitone buffer were used. All fractions were tested for the presence of precipitable antigens with the antiserum from the immunized guinea pigs. Slides were incubated in a humidifier at 4°C for 48 h, washed with saline for two days with frequent changes, and finally washed with distilled water, dried and stained with Coomassie blue 0.25% in ethanol.

Results

The effect of chitinase on the isolated mycelial wall of TM was monitored by measuring the carbohydrate and protein content of the supernatants after each step of digestion. Table 1 shows the quantitative analysis of the original

hydrolysate and of the different fractions obtained after ultrafiltration and gel filtration. Very little difference was found between the ratio of Anthrone positive to protein positive fractions in the 24 h and 7 day digestions.

The immunological activity of the different fractions as assessed by intradermal skin testing of sensitized guinea pigs is shown in Fig. 4a, b, c, which demonstrate the immediate, Arthus and delayed reactions of the 24 h and 7 day digests and the various fractions derived from them. In the immediate reactions, it was apparent that essentially all fractions of MW > 10,000 gave positive reactions and that the UM₂(a) fraction gave a positive reaction in one instance. Data from the 5 hour readings were not altogether clear since an appreciable level of non-specific erythema was associated with injection of some of the fractions. The delayed reactions, read at 24 h gave clear cut data showing that all fractions down to UM₂(a) gave good delayed

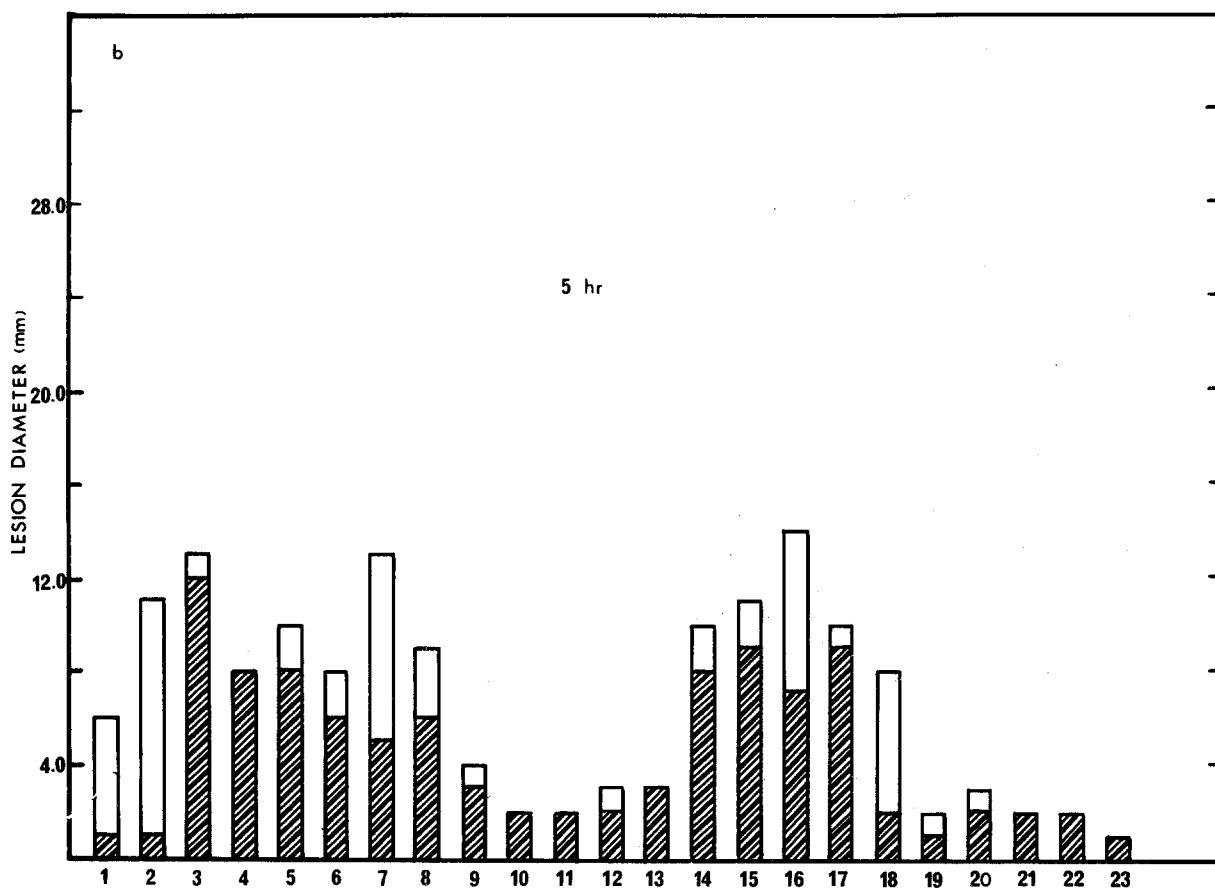


Fig. 4. (b) Cutaneous allergic reactions in immunized guinea pigs (whole column) compared to that of unimmunized animals (shaded) to *T. mentagrophytes* cell wall fractions after 24 hr and 7 days digestion, with chitinase 5 hr after intradermal injection (Arthus reaction).

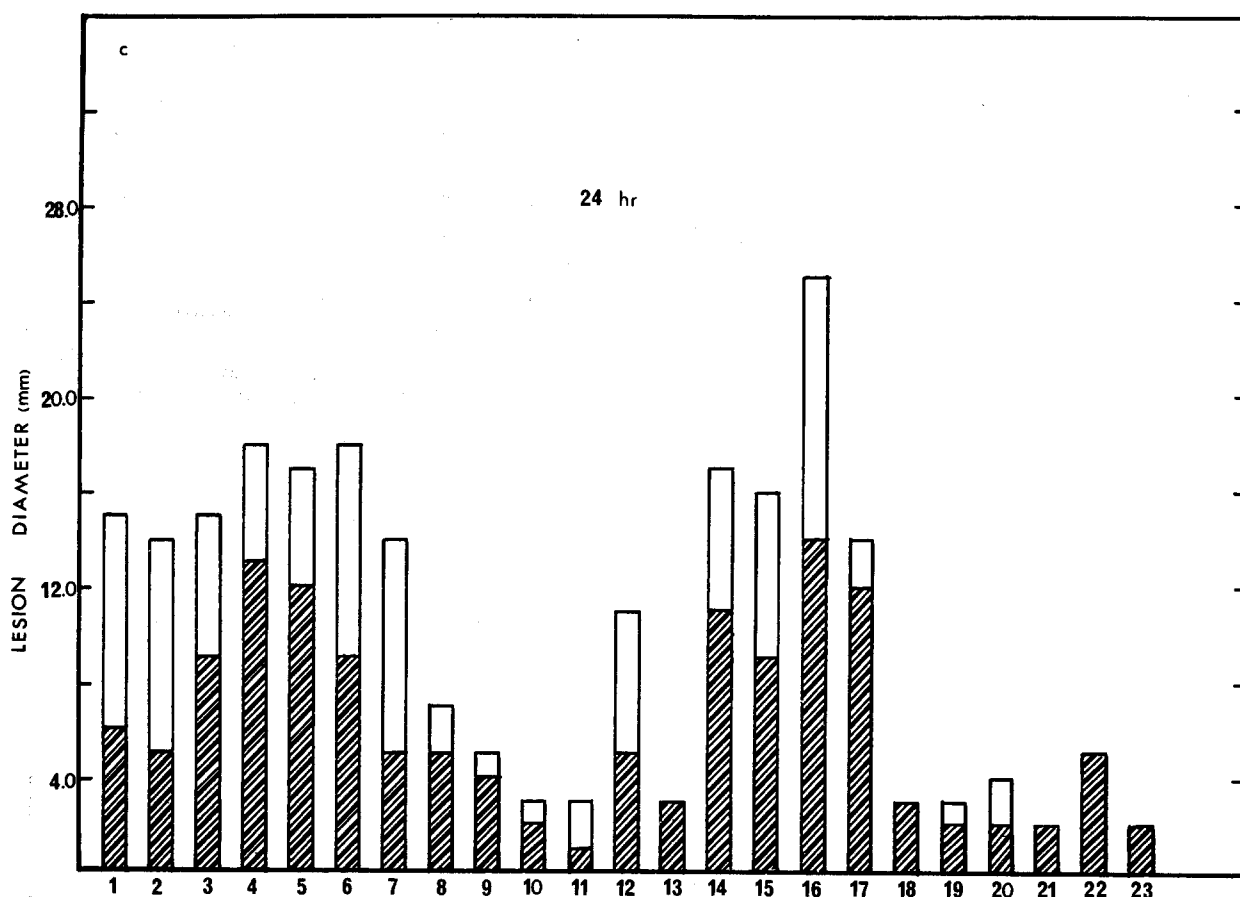


Fig. 4. (c) Cutaneous allergic reactions in immunized guinea pigs (whole column) compared to that of unimmunized animals (shaded) to *T. mentagrophytes* cell wall fractions after 24 hr and 7 days digestion with chitinase 24 hr after intradermal injection (delayed hypersensitivity reaction).

reactions in sensitized animals.

The ability of these fractions to induce lymphocyte transformation when cultured *in vitro* with lymph node cells from sensitized guinea pigs was tested. Table 2 shows the tritiated thymidine incorporation by lymph node cells in the presence of the various fractions. Stimulation indices after 24 h and 96 h incubations were calculated by comparing the ratio of CPM of sensitized cells with and without antigen, and the ratio of CPM of unsensitized cells with and without antigen. Tests were run at both 5.0 and 25.0 $\mu\text{g/ml}$ of antigen. Because the higher dose was optimal for stimulation only these figures are shown. None of the fractions tested caused non-specific proliferation and increased thymidine uptake in unsensitized cells (data not shown), so it was concluded that increased uptakes were indications of specific responsiveness. Stimulation indices of > 2.0 were shown to be significant ($p = < 0.05$), since

SEM for triplicate counts was never greater than 10%.

It can be seen that there is reasonably good correlation between those fractions capable of inducing skin reactivity in sensitized animals and those capable of inducing lymphocyte proliferation, since again, the $\text{UM}_2(\text{a})$ fraction was the one of lowest molecular weight which induced strong positive lymphocyte transformation at both 24 and 96 h.

The ability of these fractions to react with antibody directed to the whole mycelial cell wall of *T. mentagrophytes* was tested by immunodiffusion.

Fig. 5 shows the immunodiffusion analysis with anti-whole mycelial cell wall guinea pig serum and the various fractions. It is of interest to note that the $\text{UM}_2(\text{a})$ fraction, although it is of low molecular weight produces stronger precipitin lines than the equivalent $\text{UM}_{10}(\text{a})$ and $\text{UM}_{10}(\text{b})$ fractions.

Table 2. Tritiated thymidine incorporation by lymph node cells in response to *T. mentagrophytes* cell wall fractions after 24 hr and 7 days digestion with chitinase

Fraction No.	Response at 24 h to 25 µg/ml antigen		Response at 96 h to 25 µg/ml antigen	
	CPM	S.I.*	CPM	S.I.
<i>24 h digest</i>				
UM ₁₀ (a)	39,133	4.99	124,561	9.14
UM ₁₀ (b)	25,200	3.22	108,940	8.00
UM ₂ (a)	17,126	2.18	64,028	4.70
UM ₂ (b)	12,093	NS**	18,558	NS
UM ₂ (c)	7,730	NS	13,793	NS
UM ₂ (d)	9,540	NS	15,231	NS
UM ₂ (e)	10,050	NS	15,401	NS
PUM ₂ (a)	1,910	NS	13,078	NS
PUM ₂ (b)	6,680	NS	14,815	NS
PUM ₂ (c)	6,400	NS	15,305	NS
<i>7 day digest</i>				
UM ₁₀ (a)	29,200	3.73	93,950	6.9
UM ₁₀ (b)	41,330	5.27	11,003	NS
UM ₂ (a)	18,756	2.39	32,310	2.37
UM ₂ (b)	12,093	NS	7,611	NS
UM ₂ (c)	6,466	NS	11,283	NS
UM ₂ (d)	9,363	NS	1,451	NS
PUM ₂ (a)	18,426	2.35	7,701	NS
PUM ₂ (b)	11,566	NS	8,091	NS
PUM ₂ (c)	16,696	2.13	17,996	NS
No Antigen	7,838		13,623	

*Stimulation Index

**Not significant when compared to control counts

Discussion

There has been a considerable amount of work done on the immunological properties of chemically extracted dermatophytic mycelia. By and large, those results obtained with extracted materials have been in agreement when assessed by their ability to cause immediate or delayed skin reactions in sensitized guinea pigs. In the present study cell wall material of *T. mentagrophytes* was freed from cytoplasmic contamination and the lipid content was removed, following which, it was treated with chitinase. It was assumed that enzymatic degradation of cell wall components would not modify the antigenic determinants whereas chemical extraction might do so. In previous studies, the time during which the enzyme was permitted to digest fungal cell walls ranged from 12 h to 7 days (9, 15, 18, 20). Our choice of enzymatic treatment of 24 h and 7 days was based on the assumption that at 24 h digestion might give fractions which would not represent the antigenic components of the cell wall due to the resistance of the cell wall to enzymatic attack.

Essentially all UM₁₀ (high molecular weight) and UM₂(a) fractions from the 24 h and 7 days digestions were active in both skin tests as well as in lymphocyte transformation studies.

Our data support the finding of other investigators (3, 4, 19) who found that peptide or protein rich fractions gave strong delayed skin reactions. It was noticeable that some fractions with high protein contents were slightly more active than fractions containing low amounts of protein. However, this was not a consistent observation, and no conclusions can be drawn from this.

Assuming that lymphocyte transformation is a measure of the T cell activity, our lymphocyte transformation studies are in accordance with the skin test results, for those fractions capable of inducing delayed skin reactions also caused lymphocyte proliferation with sensitized lymphocytes. These results also confirm the observation of Hanifin *et al.*, (11) and Svejgaard *et al.*, (23) who have stated that dermatophytic cell wall extracts can induce proliferation of sensitized lymphocytes.

That these fractions also contained antigenic deter-

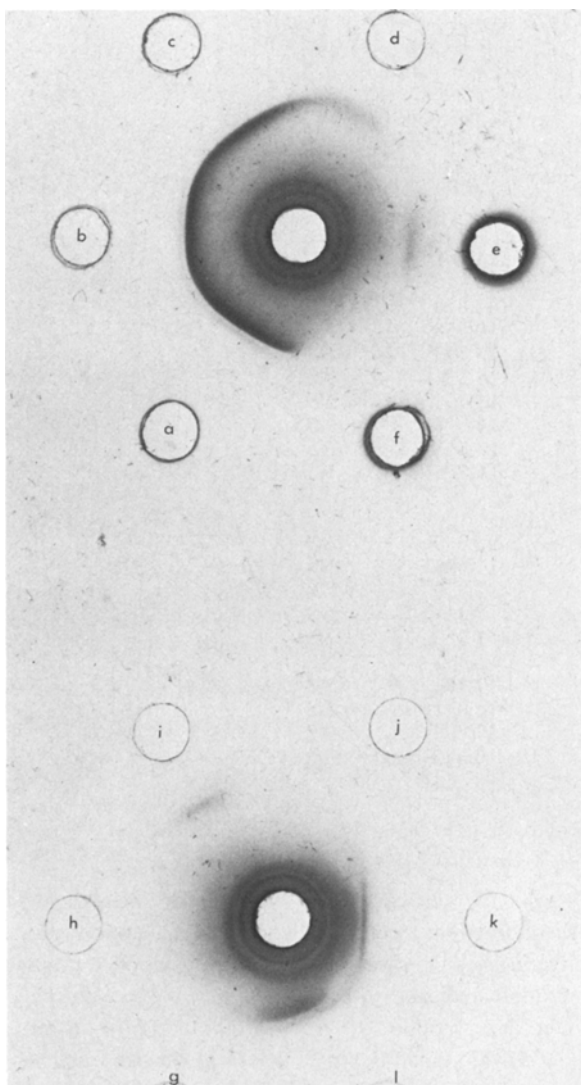


Fig. 5. Immunodiffusion of the fractions after ultrafiltration and gel filtration of the chitinase digest. Antiserum used was that of the immunized guinea pigs. All antigens were tested at two different concentrations (20, 40 μ g).

a & b 24 h digest
c & d 7 day digest
e & f 7 day UM₁₀(a)
g & h 24 h UM₁₀(a)
i & j 24 h UM₁₀(b)
k & l 24 h UM₂(a)

minants capable of reacting with antibody was indicated by their ability to induce immediate and Arthus skin reactions and to form immunoprecipitin lines in the presence

of antibody specific for *T. mentagrophytes* cell wall material. With respect to the size and reactivity of the individual fractions there was no difference between T cell activation and reactivity with antibody, in that the UM₂(a) fraction was the fraction of lowest molecular weight to give positive reactions in all tests.

In summary, digestion of the cell wall of *T. mentagrophytes* with chitinase releases fragments which contain large amounts of Anthrone positive and Lowry positive material. These fragments were shown to exhibit immunological activity by eliciting immediate and delayed type hypersensitivity, by inducing lymphocyte proliferation and by yielding immune precipitates with antisera taken from sensitized guinea pigs. We observed that there was a direct correlation between *in vivo* and *in vitro* test results.

References

1. Anderson, B.A., L. Hellgren & J. Vincent. 1976. Allergic delayed skin reactions from lipid fractions of trichophyton. *Sabouraudia* 14: 237–241.
2. Balogh, E., C. Mezaros & K. Halmy. Die Anwendung des Lymphocytentransformationstestes bei der Untersuchung der mykotischen Sensibilisation. *Mykosen* 14: 207–211.
3. Barker, S.A., C.N.D. Cruickshank & J.H. Holden. 1963. Structure of galactomannan-peptide allergen from *T. mentagrophytes*. *Biochimica Biophysica Acta* 74: 239–246.
4. Barker, S.A., O. Basarab, & C.N.D. Cruickshank, 1967. Galactomannan-peptides of *T. mentagrophytes*. *Carbohydrate Research* 3: 325–332.
5. Bartiniki-Garcia, S., & N.J. Nickerson, 1962. Isolation, composition and structure of cell walls of filamentous and yeast like forms of *Mucor rouxii*. *Biochimica Biophysica Acta* 58: 102–119.
6. Bishop, C.T., F. Blank, & M. Hranisavljevic. 1962. The water-soluble polysaccharides of dermatophytes I. A galactomannan from *Trichophyton granulosum*. *Canadian Journal of Chemistry* 40: 1816–1825.
7. Bleyman, M., & C. Woese. 1969. Ribosomal ribonucleic acid mutation during bacterial spore germination. *Journal of Bacteriology* 97:27–31.
8. Codner, R.C., C.N.D., Cruickshank, M.D. Trotter & S.R. Wood. 1961. The production of trichophytin antigen in submerged culture of *T. mentagrophytes*. *Sabouraudia* 1: 116–122.
9. Domanski, R.E., & R.E. Miller. 1968. Use of a chitinase complex and β -(1–3) glucanase for spheroplast production from *Candida albicans*. *Journal of Bacteriology* 96, 270–271.
10. Gregerson, D.S., B. Kelly & J.G. Levy. (1975). Responses of guinea pig lymphocytes to mitogens, an antigen and mixed leucocyte culture in media with and without mercaptoethanol and foetal calf serum. *Immunology* 29: 237.
11. Hanifin, J.M., L.E. Ray & W.C. Lobitz. 1974. Immunological reactivity in dermatophytosis. *British Journal of Dermatology* 90: 1–8.

12. How, M.J., M.T. Withnall & C.N.D. Cruickshank. Allergic glucans from dermatophytes Part I Isolation, purification and biological properties. *Carbohydrate Research* 25: 341–353.
13. Jones, H.E., H., Reinhardt, M., Rinaldi. 1973. A Clinical mycological and immunological survey for dermatophytosis. *Archives of Dermatology* 112: 40–42.
14. Lowry, O.H., N.J., Rosebrough, A.L., Farr & R.J. Randall, 1951. Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry* 193: 265–276.
15. Moreno, R.E., F. Kanetsuna & L.M. Carbonell. 1969. Isolation of chitin and glucan from the cell wall of the yeast form of *Paracoccidioides brasiliensis*. *Archives of Biochemistry and Biophysics* 130: 212–217.
16. Morris, D. 1948. Quantitative determination of carbohydrates with Dreywood's Anthrone reagent. *Science* 107: 254–255.
17. Noguchi, T., T., Hattori, H. Shimonaka & Y., Ito. 1971. Immunological studies of *T. mentagrophytes*, isolation of immunoglobulins and their immune responses. *Japanese Journal of Experimental Medicine* 41: 401–410.
18. Noguchi, T., T., Banno, Y., Watanabe, Y. Nozawa & Y., Ito. 1975. Carbohydrate composition of the isolated cell walls of dermatophytes *Mycopathologia* 55, 2: 71–76.
19. Nozawa, Y., T., Noguchi, Y., Ito, N., Sudo & S., Watanabe. 1971. Immunological studies on *T. mentagrophytes* *Sabouraudia* 9: 129–138.
20. Nozawa, Y., Y., Kitajima & Y., Ito. 1973. Chemical and ultrastructural studies of isolated cell walls of *Epidermophyton floccosum*. Presence of chitin inferred from x-ray diffraction analysis and electron microscopy. *Biochimica et Biophysica Acta* 307: 92–103.
21. Odds, F.C., L., Kaufman, D., McLaughlin, C., Calloway & S.O. Blumer. 1971. Effect of chitinase complex on the antigenicity and chemistry of yeast-form cell walls and other reactions of *Histoplasma capsulatum* and *Blastomyces dermatitidis*. *Sabouraudia* 12, 138–149.
22. Sorenson, G. W., H., Jones. 1976. Immediate and delayed hypersensitivity in chronic dermatophytosis. *Archives of Dermatology* 112: 40–42.
23. Svejgaard, E., M., Thomsen, N., Morling & A.H., Christiansen. 1976. Lymphocyte transformation in vitro in dermatophytosis. *Acta pathologica et Microbiologica Scandinavica* Sect. C. 84: 511–519.