

CHARACTERIZATION OF IMMUNOLOGICALLY ACTIVE PEPTIDES FROM THE CELL WALL OF *T. MENTAGROPHYTES**

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Abstract

A low molecular weight fraction from chitinase digested cell walls of *T. mentagrophytes* containing both polysaccharide and peptide moieties was found to have immunological reactivity at both the cellular and humoral level. This fraction (UM₂(a)) was further degraded by treatment with either a combination of pronase and carboxypeptidase A or with trypsin. Peptides were separated from the carbohydrate-rich fraction by ultrafiltration. The carbohydrate-rich fraction retained the ability to induce both immediate and delayed skin reactions in sensitized guinea pigs and to stimulate the proliferation of sensitized lymphocytes *in vitro*. The peptide moieties retained reactivity in that they caused delayed reactions and lymphocyte proliferation but were unable to induce immediate or Arthus reactions in sensitized animals. Tryptic peptides from UM₂(a) were purified by ion exchange chromatography. A high proportion of these peptides demonstrated immunological activity at both the cellular and humoral level since they were capable of inducing delayed reactions and/or lymphocyte transformation, as well as being capable of blocking the complement fixation reaction between UM₂(a) and specific antiserum.

Introduction

The peptide moiety of the nitrogen-containing polysaccharides of dermatophytes has been shown by many investigators to be responsible for the delayed-type hypersensitivity reaction (2, 3, 10, 12). Nozawa *et al.*, (12) showed that the peptide contents of the polysaccharide-

peptide complex extracted from the cell wall of *T. mentagrophytes* affected the delayed hypersensitivity reaction. They showed that fractions containing 11.4%–18.7% peptide were much more active than those containing 0.25%–0.81% peptide. These investigators were not certain that the peptide moiety had an effect on the immediate reaction, since the antigenic substances tested contained both polysaccharide and peptide material. Because animals are capable of responding immunologically at both the humoral and cellular level to very small amounts of antigenic material, the data presented by these workers, although indicative of a separate immunological role for the peptide and carbohydrate moieties of dermatophyte cell wall material, are not conclusive.

Analysis of the antigenicity of different protein species isolated from *T. mentagrophytes* have been done by Ito (11), who isolated 22 protein fractions by phenol-water extraction and chromatography. Christiansen and Svejgaard (5) analyzed the antigenic contents of four dermatophyte species by cross-immunoelectrophoresis using submerged cultures homogenized by mechanical methods. They used the whole mycelial extract as the antigen for raising antiserum and for immune electrophoresis. These workers showed 26 antigens from their preparation of *T. mentagrophytes*. Disc electrophoresis was used by Shechter *et al.*, (13) to compare the protein species in culture filtrates and mycelial cell wall extracts of many dermatophytes. They found no definite relationship between the protein fractions from the same organism cultured in two different media and suggested that the culture medium influences the genetic expression of the species.

We have shown in the previous paper that a relatively low molecular weight fraction taken from a cell wall hydrolysate of *T. mentagrophytes* (UM₂(a)), is a peptide containing polysaccharide which can stimulate *in vitro* sensitized lymphocytes and can evoke immediate and delayed skin hypersensitivity as well as form a precipitin

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line with antibody raised in guinea pigs to a preparation of whole mycelial cell wall of *T. mentagrophytes*. In this paper we report a further characterization of that fraction and the immunological roles of the polysaccharide and peptide moieties of this fraction of the cell wall hydrolysate.

Materials and methods

Antigen

UM₂(a) is one of the fractions which was isolated from the cell wall of *Trichophyton mentagrophytes* after chitinase digestion, ultrafiltration and gel filtration. The procedures for digestion and fractionation have been described previously (1). This material is the major low molecular weight fraction which was shown to be immunologically comparable to the higher molecular weight fractions, in that it stimulated high level immediate and delayed skin reactions, formed a precipitin line with specific antiserum, and stimulated the proliferation of sensitized lymphocytes *in vitro*. Therefore, the UM₂(a) fraction was chosen and used for further characterization, in an attempt to understand the immunological roles of the carbohydrate and peptide moieties of this cell wall complex.

Enzyme digestions

Pronase – Carboxypeptidase:

This procedure was carried out in an attempt to remove most of the peptide moiety. The UM₂(a) fraction was digested first with pronase (Calbiochem) at an enzyme concentration of 0.5–1.0% of the weight of the substrate to be digested, and increased during the course of digestion to 2%. Incubation was continued for 6 days at 37 °C at pH 7.8 in the presence of 0.0015 M CaCl₂. The enzyme digest was then put through a UM₁₀ amicon (Amicon Corporation, Lexington, Mass.) filter to remove the enzyme. The filtrate was put through a UM₂ filter and washed three times with distilled water. The UM₂ filtrate was freeze-dried while the retained fraction was subjected for further enzyme treatment with carboxypeptidase A (Worthington biochemical Investigations) in phosphate buffer 0.02 M, pH 8.0 at a concentration of 1% and incubated for 24 h at 37 °C. The enzyme was removed as for pronase. The UM₂ retained fraction was considered to consist mainly of the UM₂(a) carbohydrate moiety. Peptide and carbohydrate contents of all fractions were determined by quantitative ninhydrin and Anthrone tests respectively.

Trypsin: To test the antigenic role of the peptide moiety in UM₂(a), tryptic degradation of the complex was performed. This enzyme (Sigma) was chosen because of the specificity of the enzyme, so that sizable peptides could be recovered from the digest for immunological testing. Previous amino acid analysis of UM₂(a) showed lysine but not arginine to be present at significant levels, so that it was possible that substantial digestion could be achieved. The enzyme content of the incubation mixture was approximately 2% the amount of the substrate. Digestion was carried out in 0.04 M phosphate buffer at pH 7.0 for 24 h at 37 °C. Separation of the enzyme from the peptides was performed as described above by ultrafiltration.

Peptide purification

The tryptic peptides were purified chromatographically according to the method of Canfield (4) for peptide separation using a Dowex 50W X8 resin in a 0.7 × 150.0 cm water-jacketed column equilibrated with starting buffer. Each sample was adjusted to pH 2.8 with formic acid, applied to the column and washed in with three 1.0 ml volumes of starting buffer. Elution was performed using an eight-chambered gradient system with each buffer at a volume of 200 ml. Gradient elution with an increasing pH and salt concentration was used with pyridine-acetate buffers ranging from 0.1 N acetic acid, pH 3.8, up to 2.0 N acetic acid, pH 5.18. Fractions of 4 ml were collected and 0.3 ml from every other tube were taken and analyzed by the quantitative ninhydrin reaction after alkaline hydrolysis according to the method of Hirs *et al.*, (9). Each ninhydrin positive peak was pooled and dried by flash evaporation at 45 °C, washed with distilled water at least three times, freeze-dried and dissolved in normal saline. These fractions were used for skin tests, lymphocyte stimulation, complement fixation tests and amino acid analysis.

Immunization

Guinea pigs to be used for immunological testing were immunized with cell wall material from *T. mentagrophytes* as described previously (1). Serum was collected from immunized guinea pigs, inactivated at 56 °C for 30 min. and stored at –20 °C.

Skin tests and lymphocyte stimulation

These were carried out as described previously (1, 7). All

peptide fractions collected were tested for their immunological reactivity by intradermal skin test and for their ability to stimulate lymphocytes taken from immunized guinea pigs. Unimmunized animals were used as controls.

Complement fixation

Quantitative complement fixation tests were performed by a modification of the method described by Kabat and Mayer (1961). Precise methods have been published elsewhere (6). Lyophilized guinea pig complement (Flow Laboratories) was used at a dilution of 1:100. Veronal buffer at pH 7.5 was used as a diluent. The antigen dilutions used were 0.1, 0.2–3.2 µg/ml and antiserum dilution of 1:100, 1:200–1:1500. A 2% suspension of washed sheep erythrocytes was sensitized by mixing them with an equal volume of 1:100 dilution of hemolysin (Difco) for at least 15 min. The dilution of complement producing 50% hemolysis was obtained by the method of probits as described previously (15).

Hapten Inhibition

Hapten inhibition studies were performed as described by Gerwing and Thompson (6). Briefly, the test involved incubation for 24 h at 4°C of anti whole mycelial cell wall guinea pig serum (at 1:1000) with the various test peptides (at 50 µg per ml). Subsequently, whole antigen (UM₂(a)) at a previously determined optimal concentration (0.25 µg/ml) for complement fixation and guinea pig complement at a dilution of 1:75 was added to each test. Incubation at 4°C was continued for a further 24 h after which time each mixture was assayed for free complement. Appropriate controls of the peptides, antigen and antibody were carried out in each instance.

Results

We have shown in previous work (1) that a low molecular weight fraction (UM₂(a)) from *T. mentagrophytes* cell wall containing both polysaccharide and peptide gave in guinea pigs strong delayed skin reactivity as well as a high degree of stimulation to sensitized lymph node cells after 96 h incubation. It was thought that the elimination of the peptide part would present an opportunity to study the immunological role of the carbohydrate moiety. To this end enzyme digestions were performed on the UM₂(a) fraction. Table 1 shows quantitatively the amount of Anthrone positive and ninhydrin positive material remaining after pronase-carboxypeptidase and trypsin digestions. The results indicate that most of the peptide material was removed by pronase-carboxypeptidase treatment, whereas this was not so with trypsin treatment.

Figure 1 shows the results of those fractions which evoked hypersensitivity reactions in immunized guinea pigs. It can be seen that the material retained in the filter containing mainly carbohydrate elicited both immediate and delayed skin reactions, whereas the peptide fractions elicited only minimal immediate reactions but did give both Arthus and delayed reactions, indicating that both the pronase and trypsin digested peptides retained immunological specificity.

Table 2 shows the *in vitro* response of sensitized lymphocytes to the fractions obtained after enzymatic treatment. The CPM of the sensitized cells in the presence of peptide fragments was compared to cells incubated without peptide. Cells from unimmunized animals were also incubated with and without the test fractions.

All fractions were capable of inducing lymphocyte proliferation of cells from immunized animals at 96 h showing that immunological specificity was retained and

Table 1. Chemical analysis of UM₂(a) fraction after enzyme digestion

Enzyme	(1) Mg CH ₂ O before digestion	(2) Mg Protein before digestion	Mg CH ₂ O after digestion	Mg Protein after digestion and ultrafiltration	Mg Protein passed UM ₂ Amicon filter
Trypsin	62	22	55.5	12.24	9.75
Pronase	62	22	54.0	10.46	—
Pronase + Carboxypeptidase	54	10.46	46.5	2.42	

(1) Anthrone test

(2) quantitative ninhydrin test

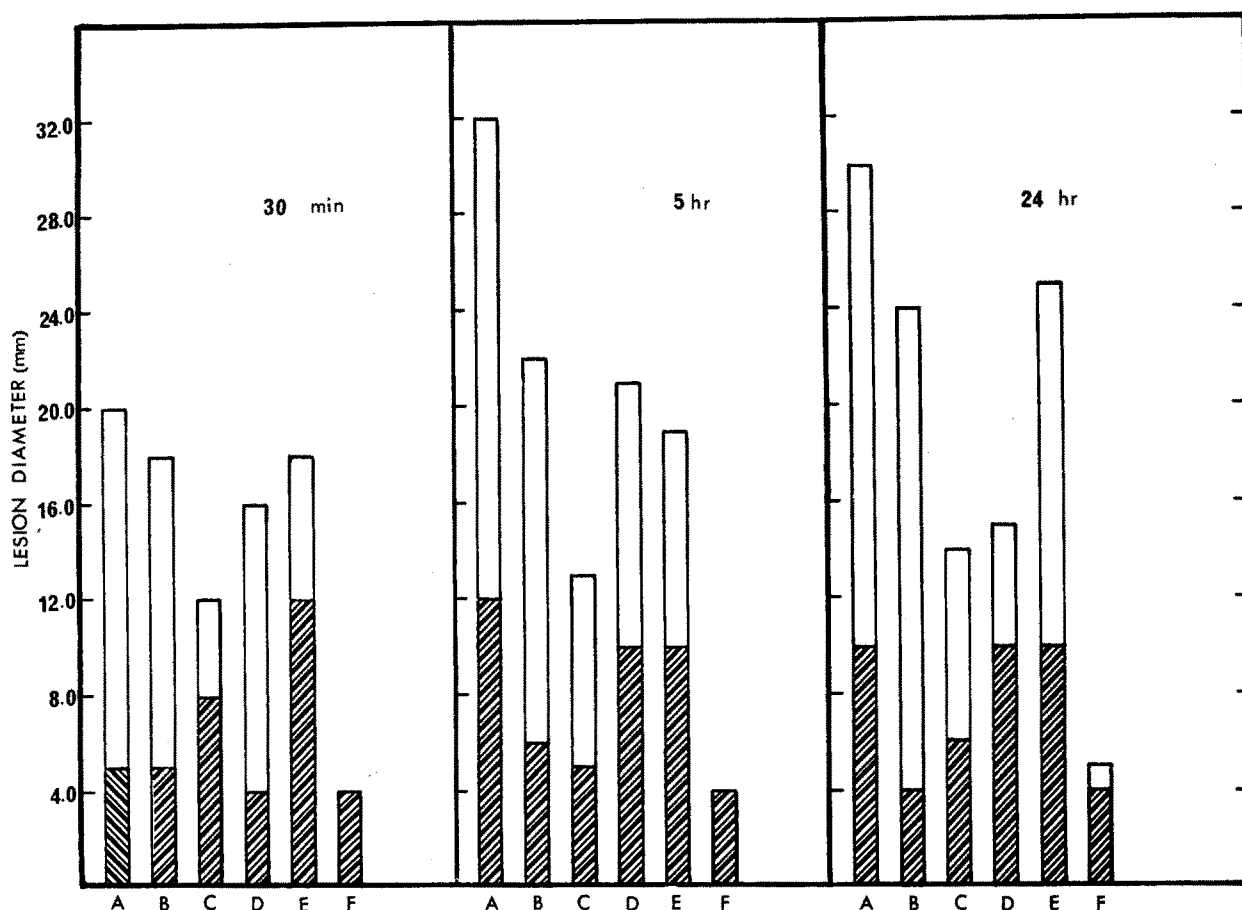


Fig. 1. Cutaneous allergic reactions of the enzyme digests of $UM_2(a)$ fraction in immunized guinea pigs (whole column) compared to that of the unimmunized animals (shaded).

A = $UM_2(a)$ fraction

B = Pronase-carboxypeptidase UM_2 retained fraction (PCUM₂R)

C = Pronase-carboxypeptidase UM_2 passed fraction (PCUM₂P)

D = Trypsin UM_2 retained (TUM₂R)

E = Trypsin UM_2 passed (TUM₂P)

F = Saline

Table 2. Lymph node cell incorporation of tritiated thymidine in response to $UM_2(a)$ sub-fractions after enzyme treatment and fractionation.

Antigen	Lymphocyte response at various antigen levels					
	5 μ g/ml		25 μ g/ml		125 μ g/ml	
<i>24 hr.</i>						
UM ₂ (a)	11,610	NS	19,843	NS	16,943	NS
PCUM ₂ R	15,843	NS	14,636	NS	14,813	NS
PCUM ₂ P	19,096	NS	13,346	NS	13,783	NS
TUM ₂ R	19,050	NS	19,450	NS	19,123	NS
TUM ₂ P	16,143	NS	18,676	NS	12,333	NS
No Antigen	15,193					
<i>96 hr.</i>						
UM ₂ (a)	16,063	2.55	16,280	2.59	17,381	2.73
PCUM ₂ R	20,036	3.19	21,916	3.49	19,260	3.06
PCUM ₂ P	16,343	2.60	16,373	2.60	12,066	1.92
TUM ₂ R	20,466	3.26	22,520	3.59	18,670	2.97
TUM ₂ P	6,766	NS	16,793	2.67	17,890	2.85
No Antigen	6,286					

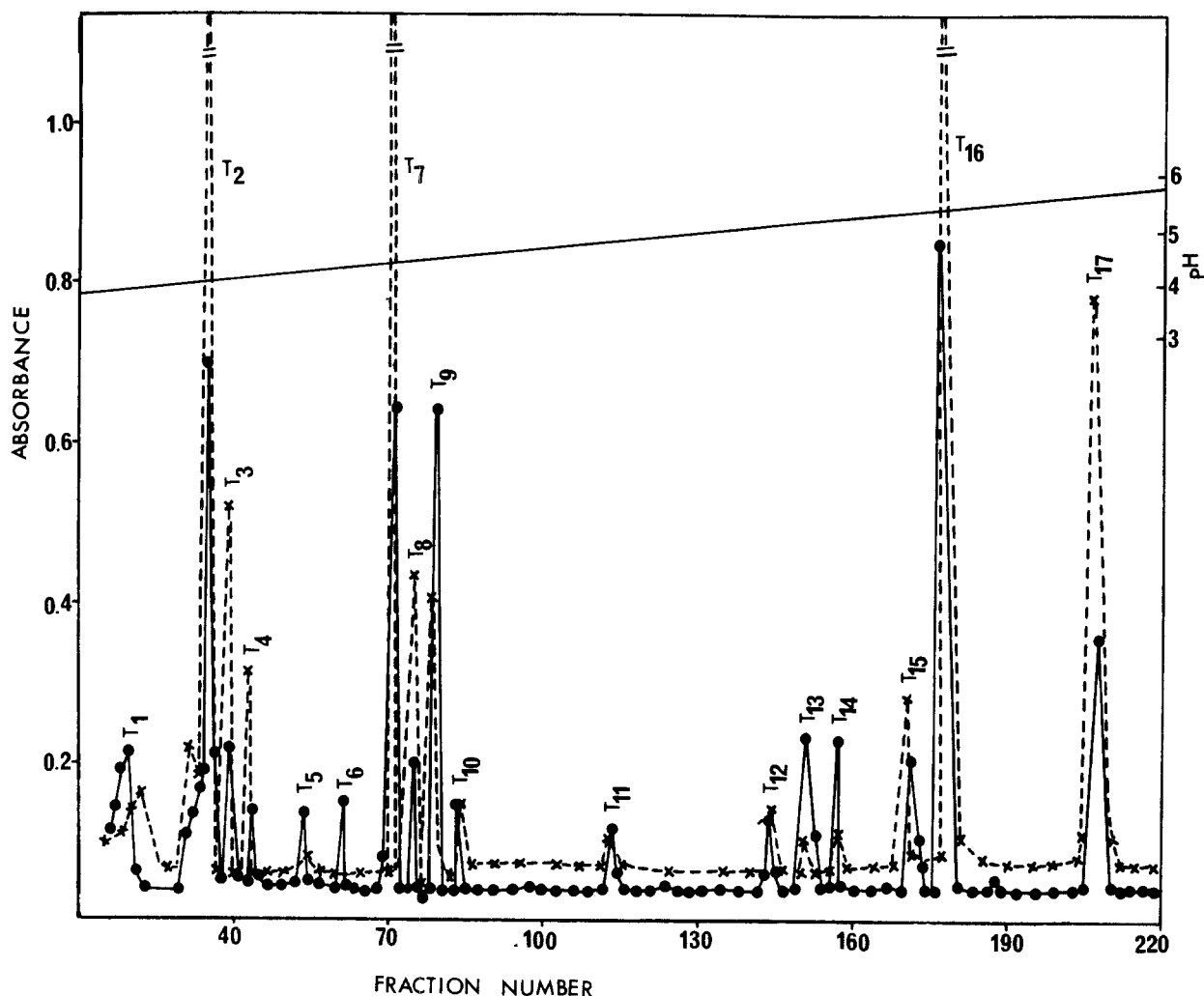


Fig. 2. Elution profile of tryptic peptides on Dowex 50W X8 column. (—●) = absorbance at 570 μ (---x) = absorbance at 440 μ m.

resided in both the carbohydrate-rich and peptide fractions. However, there was virtually no measurable response at 24 h incubation. Cultures of cells from unimmunized animals showed no proliferative response (data not shown).

The elution profile of the purified tryptic peptides after ion exchange chromatography is shown in Figure 2. The skin reactions to the pooled peptide peaks are shown in Figure 3a, b, c. Out of 17 peptides tested, it can be seen that 5 gave fairly good delayed skin reactions while none of them induced significant immediate reactions. Table 3 shows the results of the 96 h lymphocyte response to each peptide. About half of the isolated peptides induced lymphocyte proliferation in culture with immunized cells. Most peptide fractions which showed positive skin reactions were

capable of stimulating proliferation, although there was not an absolute correlation. The results indicated that the *in vitro* assay might be slightly more sensitive than skin testing.

Immunodiffusion and complement fixation studies with anti-whole mycelial cell wall guinea pig serum showed that a number of peptides were apparently large enough to form immune precipitates or to fix complement (data not shown). These peptides were numbers 13, 14, 15 and 16 and were not included in the hapten inhibition test.

The results of the inhibition of complement fixation by tryptic peptides is shown in Table 4. It can be seen that most of the peptides tested (i.e., those which did not fix complement in their own right) were capable of inhibiting the

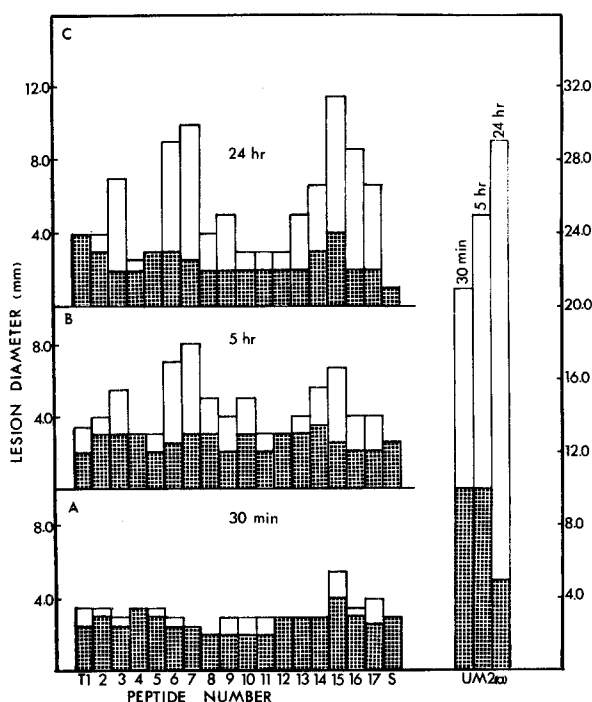


Fig. 3. Cutaneous allergic reactions of the tryptic peptides from UM₂(a) in immunized guinea pigs (whole column) compared to that of unimmunized animals (shaded) at 30 min (A), 5 h (B) and 24 h (C).

Table 3. Sensitized lymph node cell incorporation of tritiated thymidine in response to tryptic peptides from UM₂ (a)

	96 h			
	25 µg/ml		125 µg/ml	
	CPM	S.I.*	CPM	S.I.
UM ₂ (a)	63,076	3.37	52,876	2.69
T ₁	50,563	2.70	18,183	NS
T ₂	49,713	2.65	40,506	2.06
T ₃	49,816	2.67	30,350	NS
T ₄	39,352	2.09	6,933	NS
T ₅	40,970	2.19	14,763	NS
T ₆	32,820	NS	14,873	NS
T ₇	39,763	2.12	53,796	2.73
T ₈	32,466	NS	22,953	NS
T ₉	21,630	NS	21,443	NS
T ₁₀	22,710	NS	11,486	NS
T ₁₁	16,210	NS	2,163	NS
T ₁₂	24,073	NS	18,336	NS
T ₁₃	50,666	2.7	30,980	NS
T ₁₄	31,776	NS	23,090	NS
T ₁₅	29,632	NS	34,060	NS
T ₁₆	44,173	2.36	54,040	2.75
T ₁₇	58,860	3.14	48,618	2.47
No. Ag.	18,740		19,680	

*S.I. - stimulation index

Table 4. Inhibition of complement fixation by tryptic peptides

Peptide No.	% of Inhibition
T ₁	54
T ₂	57
T ₃	85
T ₄	71
T ₅	53
T ₆	71
T ₇	80
T ₈	69
T ₉	100
T ₁₀	79
T ₁₁	68
T ₁₂	75

reaction of UM₂(a) with specific antiserum indicating that these peptides contain at least one intact antigenic determinant recognized by antibodies formed against whole cell wall material.

Discussion

The proteolytic enzyme digestion of the UM₂(a) fraction, followed by ultrafiltration, was shown to be effective in the removal of a substantial amount of peptide from the polysaccharide peptide complex. Whereas either trypsin or pronase on their own removed approximately half the peptide content, the combination of pronase plus carboxypeptidase removed about 90% (Table 1). When the carbohydrate-rich fraction left after proteolytic digestion was tested immunologically it appeared that it was capable of eliciting immediate and Arthus skin reactions in sensitized guinea pigs, both of which are indications of humoral immunity. However, this carbohydrate-rich fraction was also capable of eliciting delayed skin reactions and lymphocyte transformation of sensitized cells to levels comparable with the intact UM₂(a) fraction. These data imply that cellular (T cell) immunity in sensitized animals is directed toward the carbohydrate moiety of the cell wall of *T. mentagrophytes*. These findings are in disagreement with those of Barker and Cruickshank (2, 3), whose work indicated that the humoral response rather than the cell-mediated response was stimulated by the carbohydrate of dermatophyte cell walls. Our findings are also in disagreement with those of Nozawa *et al.*, (12), who found that proteolytic digestion of cell wall material decreased the delayed hypersensitivity reaction by 54%. While our findings are not in agreement with those of other investiga-

tors, it should be emphasized that no conclusions can be drawn on the immunological role of the carbohydrates of this fraction since even after proteolytic digestion, a measurable amount of peptide material remained (approximately 5%) and it is conceivable but unlikely that the cell-mediated reactions demonstrated were attributable exclusively to the peptide moiety remaining.

The peptide materials which were separated from the carbohydrate polymer by filtration through a UM₂ amicon filter appeared to be active immunologically. These fractions were not able to induce immediate skin reactions in sensitized animals, but did cause the development of delayed reactions. This observation is in agreement with the implications of other investigators (2, 3, 12). It was somewhat surprising that the peptide material isolated from the pronase-carboxypeptidase digest was capable of causing skin reactivity and of stimulating lymphocyte transformation, since it was felt that this kind of degradation would probably result in the formation of peptides too small to retain any immunological integrity.

The tryptic peptide fraction was further characterized by ion exchange chromatography. The individual peptides thus isolated were tested for their immunological properties. The data showed clearly that those peptides capable of inducing delayed skin reactions were also those which usually induced lymphocyte transformation, thus indicating a correlation between the *in vivo* and *in vitro* assay for cell-mediated immunity. It should be mentioned that this correlation was not absolute since some peptides reacted positively in only one of the test systems. The reasons for this are not clear. This observation is in general agreement with those of Hanifin *et al.*, (8) who showed the consistency of lymphocyte transformation and delayed skin reactivity. They found that patients with either *T. mentagrophytes* or *T. rubrum* infection who gave delayed skin reactions also had peripheral blood lymphocytes which underwent transformation in the presence of antigen after 5 days *in vitro*.

The finding that these isolated peptides did not elicit immediate skin reactions was not surprising, since most of them were probably too small to be able to affect a classical histamine mediated immediate reaction, since it is assumed that mast cell degranulation will only take place in the presence of aggregated antigen. However, it has been shown previously, (14, 16) that small haptenic peptides containing single antigenic determinants are capable of inducing cell-mediated reactions in sensitized animals.

The possibility that these peptides were functional in humoral immunity was investigated by testing the tryptic

peptides for their ability to inhibit complement fixation by UM₂(a) with specific antiserum and for their ability to form precipitates with the antiserum. It was somewhat surprising to note that virtually all of the isolated peptides were active either in inhibiting the reaction between UM₂(a) and the antiserum, which implies a single antigenic determinant; or by forming a precipitin line with the antiserum which implies a peptide of two or more determinants. Thus, while it may be accurate to state that the peptide fraction does not cause immediate skin reactions in sensitized animals, it is apparent that circulating antibody in sensitized animals does react strongly with the peptides. Thus, it is clear that the peptide portion of the cell wall is active at the level of both humoral and cell-mediated immunity in *T. mentagrophytes* sensitized animals.

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