IMMUNOCHEMICAL STUDIES ON THE ANTIGENIC PROPERTIES OF THE CELL WALL OF TRICHOPHYTON MENTAGROPHYTES

by

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ABSTRACT

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. Amino acid analysis of an immunologically active fraction of low molecular weight indicated that the peptide content comprised a limited array of amino acids.

This fraction, found to be completely reactive immunologically ($UM_2(a)$), appeared to have a molecular weight in the range of 2,000-4,000 as assessed by ultrafiltration and gel filtration studies. This fraction, ($UM_2(a)$) 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 $UM_2(a)$ 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 $UM_2(a)$ and specific antiserum.

TABLE OF CONTENTS

List of Tables

List of Figures.

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Section 1 General Introduction

Dermatophytes

The dermatophytes are species of the genera Trichophyton , Microsporum , and Epidermophyton. The dermatophytic fungi are pathogenic only to the superficial skin, nails and hair in human beings and animals. They do not invade the deeper tissue or internal organs. These infections are known as dermatophytosis (ringworm). They produce relatively non-inflammatory infections in the case of Anthropophillic fungi and inflammatory infections in the zoophilic fungi. However, the disease process is greatly influenced by the host response to the dermatophytic infection.

Acquired Resistance:

Cutaneous infections with dermatophytes may induce resistance to re-infection with the same or another species. This resistance depend on the degree and duration of infection , upon the species and strain of dermatophytes (Zoophylic or Anthropophilic), the host (animal or human), and the site of infection.

In an attempt to understand the lack of pathogenicity of dermatophytes to the deeper tissue, Lorincz et al, (45) showed that dermatophytes were inhibited when implanted in the abdomen of healthy mice, but grew vigorously when transferred to cullure medium indicating the inhibitory properties of the deep tissue environment. Blank et al . (13) demonstrated that dermatophytes grew profusely in all layers of viable full thickness skin explants maintained in short term tissue culture. However, growth was prevented by bathing the explants in fresh

species on the basis of gel diffusion tests with antisera to sonically treated mycelia. However, little definitive information in this area has been forthcoming .

Interest in the purification of the antigenic components of dermatophytes started in 1962 with the work of Barker et al (6) who purified the glycopeptides from the cell wall by extraction with ethylene glycol as shown by Codner et al (20). The extracts were precipitated by cetyl trimethyl ammonium bromide from a borate buffer solution by increasing the pH. The glycopeptides contained the following amino acids; aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, isoleucine, leucine, tyrosine, phenylalanine and lysine. The carbohydrate part of the glycopeptide contained either D-galactose or D-mannose 9.0-20 and 73% respectively. The protein content was 9% . How et al (37) have isolated glucans from four species of dermatophytes, among them was T. mentagrophytes, these glucans were purified from ethylene glycol extracts by mild fractionation at neutral pH, on Bio-Gel P-300 and DEAE Sephadex A-50 . Polysaccharide-peptide complexes from T mentagrophytes were isolated by Nozawa et al. (54) by using the phenol extraction method of Westphal (71). The crude extracts were separated and purified by gel filtration and DEAE cellulose chromatography. In most fractions they found a large amount of serine, threonine, proline, glycine, alanine, and aspartic acid; and small amounts of glutamic acid, valine, isoleucine, lysine and leucine. Each of these polysaccharide-peptide complexes contained mannose, galactose, glucose and glucosamine.

Composition & Chemistry of Dermatophytic Antigens

Over fifty years ago Bloch et al (16) characterized the antigenic components of trichophytine (crude extracts of various dermatophyte preparation) as being a nitrogen containing polysaccharide. Since that time, there have been numerous publications dealing with the isolation and purification of these components and the serology of the antigenic preparations. The early work was directed mainly toward differentiating the different species of dermatophytes according to the chemical and the serological characteristics of their antigenic components.

Sharp (61) found extensive cross reactivity between mycelial extracts from different species by precipitin tests with rabbit antisera. Carbohydrates and protein antigens were isolated by keeney et al (43) from culture filtrates of T. mentagrophytes and T. rubrum. They showed cross reactions in tests for dermal sensitivity. Dermatophytes could be differentiated from other fungi by a fluorescent antibody technique using rabbit antisera to different dermatophyte species (47,48) but could not be used to differentiate between dermatophyte species because of the extensive cross reactivity. Andrieu et al (2) used immunoelectrophoresis for the analysis of 17 species. More than 10 antigens were detected in extracts of each species by using homologous antisera. Many of these antigens cross-reacted with antisera to other species.

Various methods have been used for the isolation and characterization of dermotophyte antigens. Shecter et al (62) separated protein constituents of six species of dermatophytes by disc electrophoresis. However, these fractions were not isolated or characterized. Dyson et al (26) grouped

to be responsible for the accelerated reaction appearing after secondary infection.

Acquired resistance to dermatophytic infection was shown by De Lamater (22) not to be passed on to the offspring of infected pregnant guinea pigs, indicating that the altered host response following infection is probably cell mediated rather than antibody-mediated.

In humans, increased resistance usually folows the severe inflammatory forms of infection by zoophylic species but does not always follow the more chronic infections caused by anthropophillic species (9). The same author pointed out that fungi which do not invade the hair follicles do not seem to give rise to an equivalent immunity when growing in the horny layer of the smooth skin. Resistance in natural infections was also shown by Friedman et al (27) who noticed that from children cured tinea capitis never became reinfected when they returned to a heavily infected environment.

King et al (44) characterized a factor present in normal human serum (serum inhibitory factor). This inhibitory factor was non dialyzable, heat stable et 56 C for 4 hours and is fungistatic.

It would appear that resistance following either experimental or natural infections with dermatophytes is mediated by cell-mediated immunity (since it is not passed to the foetus), as well as soluble factors which may include specific antibody as well as non-specific factors.

human serum. Jessner and Hoffman (40), as well as Per and Braude (57) have also demonstrated the inhibitory effect of serum on the grouth of dermatophytes. They attributed this inhibition to the presence of fungicidal antibodies with in the serum possibly arising from previous subclinical exposure to dermatophytic infection. Bloch (15), showed that cutaneous inoculation of giunea pigs with the zoophylic dermatophytes, Achorion quinkeanum, Trichophyton gypsium or Microsporum Lanosum produced infections which healed spontaneously and resulted in a relative resistance to subsequent infection. He claimed that the immunity was generalized and not confined to the site of the primary infection. It was also noted that prior subcutaneous or intraperitoneal injection of fungus decreased the susceptibility of the skin to cutaneous infection, thus supporting his contention of generalized resistance resulting from prior infection.

Greenbaun (33), on the other hand, claimed that a local immunity was produced in guinea pigs after cutaneous infection with dermatophytes. This resistance to infection was only complete at the site of the spontaneously healed areas. Reinjection at first site demonstrated complete resistance whereas injection of the animal at another site gave rise to typical infectious lesions.

Revalier (58), showed that an autoclaved spore suspension of T. granulosum applied three weeks after the first infection could induce a similar but less pronounced cutaneous reaction when compared to a second infection with live spores. Thus, the altered host response, probably cell-mediated immunity, rather than proliferation of the fungus, appeared

Bishop et al (12) isolated three polysaccharides from many species of dermatophytes. They deproteinized the defatted mycelia by digestion with trypsin, and the polysaccharides were extracted from the insoluble residue by hot alkali. Anderson et al (1) fractionated the crude trichophytin and obtained the following fractions ; polysaccharide-peptide complex, total lipid without free fatty acids, and fatty acids. They showed that, in addition to the polysaccharide-peptide complex, free fatty acids were responsible for positive skin reactions but not the total lipid without the free fatty acids.

From these studies it would appear that many species of dermatophytes contain similar, if not identical, antigens in their cell wall, since cross-reactivity between species is a common observation. Attempts at fractionation of dermatophyte antigens have demonstrated the complexity of these organisms both in their protein antigens and in their glycopepetides which presumably constitute cell wall components.

Hypersensitivity Reactions:

Bloch and his associates (17) recognized the importance of hypersensitivity reactions in dermatophyte infection and concluded that the active principle, extractable from the mycelia of the causative fungi, was a carbohydrate-protein complex. Studies on hypersensitivity in dermatophytosis started in 1902 when Neisser and Plato (51) published their work on the preparation and usefulness of trichophytins. Trichophytin is the antigenic preparation of the fungal mycelia and/or concentrated culture filtrates which is used for cutaneous hypersensitivity testing by intradermal injection. Both immediate and delayed

reactions occur, but the latter is most often associated with infections. Commercial trichophytins are composed mainly of pooled concentrated culture filtrates of 1-15 species. There are more than ten trichophytins available which vary in their manufacturing process (63).

Sensitization resulting from dermatophytic infection is specific for dermatophytes but is not species specific. An infection due to T. gypseum will cause hypersensitivity to trichophytin prepared from this dermatophyte but also to favin prepared from A. schoenleinii and microsporin prepared from M. lanosum (17).

De Lamater (23,24) studied the development of delayed type hypersensitivity during the course of cutaneous infection with M. gypseum in guinea pigs. The trichophytin reaction peaked at 15 days after inoculation and then started to regress. The site of previous infection was relatively anergic as shown by reduced inflammatory reaction to trichophytin. The same author showed that the ability to react to trichophytin increased with age, being greatest in adult and least in newborn guinea pigs.

Ito (38) detected delayed cutaneous reaction in an experimental T. rubrum infection in humans by 14 days after inoculation which disappeared after 35 days at which time an antibody-mediated immediate type urticarial reaction developed. Immediate forms of hypersensitivity have also been demonstrated by others. Cruickshank et al (21) detected immediate reaction to trichophytin in guinea pigs infected with T. mentagrophytes by using Evans blue dye. Hanifin et al (35) compared

the type of cutaneous reaction in patients with T. mentagrophytes and T. rubrum. They found most of the patients with T. mentagrophytes exhibited delayed reaction to trichophytin while most of the patients with T. rubrum lacked delayed reactions but manifested an immediate real reaction to trichophytin.

Patients with chronic dermatophytosis were found to have a relatively specific defect in delayed hypersensitivity to trichophytin and their cell mediated responses to other antigens were somewhat decreased (64).

Cruickshank et al (21) showed that the delayed hypersensitivity reaction to trichophytin could be transferred to non infected guinea pigs with peritoneal exudate cells of sensitized donors, indicating that a classical cell-mediated response was involved. Immediate-type reaction to trichophytin in humans could be transferred by serum indicating a circulating antibody reaction (66). Thus, both cell-mediated and humoral immunity appear to be involved in the immune response to these fungi.

The lack of either standerized or pure homogenous trichophytins for skin testing has severely limited the usefulness of the trichophytin reaction for diagnostic purposes (32).

Immunological Reactivity:

Barker, Cruickshank and their colleagues demonstrated that the immediate reaction was related to the carbohydrate portion of dermatophyte antigens while delayed reaction was related to the peptide moiety of the glycopeptide (6,8,11). It was also shown that yeast or other polysaccharides that had similar structural features in common with the carbohydrate portion gave strong immediate reaction in sensitized

Individuals.

Nozawa et al (55) showed that proteolytic digestion of their phenol extracted, partially purified polysaccharide-peptide complexes from T. mentagrophytes decreased the delayed hypersensitivity reaction but hardly affected the immediate reaction. Precipitin reaction was the same before and after proteolytic enzyme treatment. These authors concluded that this indicated that the antibodies were specific mainly for the carbohydrate moieties.

The nitrogen free polysaccharides isolated by Bishop et al (12) did not elicit cutaneous hypersensitivity reactions in guinea pigs sensitized by cutaneous infection. They showed that each of the three groups of polysaccharides (galactomannan 1 and galactomannan 2 and glucan) reacted with antiserum produced in rabbit from autoclaved mycelia. They monitored the serological reactions by qualitative precipitation in gel, complement fixation and immunoelectrophoresis (29,59)

How et al (37) showed that the ethylene glycol extract of T. rubrum mycelia, the purified glucan, and the mixture of glycopepetides, isolated from the extract, were each capable of sensitizing guinea pigs. All three groups of animals thus sensitized gave good immediate and delayed responses when challenged with the ethylene glycol extract of the mycelium or the glycopeptide components. The glucan, however, elicited significant immediate responses in animals sensitized with the glucan or the ethylene glycol extract, but insignificant delayed responses in all three groups of animals.

Keratinase I & 11 have been shown to have common determinant groups

by gel diffusion analysis using antisera prepared in rabbits to active Keratinase I & 11. (73,74). Grappel et al (30,31) showed that these rabbit antibodies could cause retardation in the growth and alteration in the morphology of dermatophytes. Austwick (3) speculated that these dermatophyte specific antibodies diffusing into the hair bulbs could be responsible for the degenerative changes observed in the intrapapillary hyphae in healing ringworm lesions.

Balogh et al (4) showed a correlation between the degree of positivity of the lymphocyte transformation test, the spread of the mycotic process and the sensitization of the organism. Hanifin et al (35) showed the correlation of positive lymphocyte responses and the presence of delayed but not immediate cutaneous reactions in naturally infected human beings. Svejgaard et al (67) studied the lymphocyte response of patients to different dermatophyte antigens as well as to some bacterial and fungal mitogens. The latter were used to show that the lymphocytes respond normally to these mitogens and patients suffered no functional lympholyte deficiency. They showed that lymphocytes from patients responded more strongly to the dermatophyte antigens than did these from non-immune controls. They observed that in most patients suffering from T. mentagrophytes infection, responses to T. mentagrophytes antigens were significantly stronger than that in other petients. On this basis, these workers felt that the lymphocyte transformation assay could demonstrate specificity to antigens not detected by other assay procedures, which usually showed extensive cross reactivity.

Attempts to characterize antigens of dermatophytes in terms of the reactions they evoke have indicated that the carbohydrate moieties of these antigens may be responsible to a large extent, for humoral immunity, whereas the peptide fractions are thought to be responsible for stimulating cell mediated immunity. Since few investigators have worked with highly purified antigens, such observations cannot be regarded as conclusive.

In this work an attempt was made to isolate the antigenic components of T. mentagrophytes by enzymatic digestion of the mycelial cell wall with chitinase, since chitin has been reported to be one of the major cell wall components of dermatophytes, (53,55,60) and to compare skin testing with lymphocyte transformation and immune precipitation as measures of the immune responses in sensitized guinea pigs. The immunological role of the polysaccharide peptide moieties of the antigenic components of the cell wall of T. mentagrophytes was also investigated.

Section II Isolation and Partial characterization of Immunologically Reactive Fractions from Chitinase Digested cell wall of *Trichophyton mentagrophytes*.

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 (7,8,20,37), hot dilute alkali (12) and phenol (52,54). Using these methods, many investigators isolated crude nitrogen containing polysaccharide fractions which were used for detecting skin reactivity in sensitized animals (7,8) or naturally infected human beings (35,42,64).

The immunological roles of the polysaccharide and peptide moieties of this material have been investigated by several workers. According to Barker and co-workers (7,8) immediate and delayed type hypersensitivity were attributable to the carbohydrate and peptide moieties of the isolated galactomaman-peptide respectively. Nozawa and co-workers (51), showed that proteolytic digestion of phenol water extracted material decreased the delayed hypersensitivity. Glucans isolated by How *et al.*, (37) from *Trichophton rubrum* and *Microsporum quickeanum* sensitized guinea pigs and elicited immediate skin reactions. The lipid fraction of the crude trichophytin 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 (4,35,67), 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 an attempt was made to isolate the antigenic components of T. mentagrophytes by enzymatic digestion of the mycelial cell wall with chitinase, since chitin has been reported to be one of the major cell wall components of dermatophytes, (53,55,60), and to compare skin testing with lymphocyte transformation and immune precipitation as measures of the immune responses in sensitized guinea pigs.

MATERIALS AND METHODS

Organism and Growth Conditions

Trichophyton mentagrophytes Var asteroides is one of 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 has been shown to effectively kill all fungal cells. The culture was then filtered through two layers of cheese cloth and the fungal mat was washed extensively 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 (10). 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 x g in a Sorvall centrifuge. The pellet was

exposed to freezing and thawing with liquid nitrogen using glass beads (Operlin o.1-o.11 mm) in a mortar until 95% breakage of mycelia was demonstrated by microscopic examination. This method has been found to be efficient for breakage by others (14). The mycelial cell wall suspension was centrifuged at 10,000 x 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/(*Streptomyces* Calbiochem) at a concentration of 0.5 mg per cent of the substrate mixture at 37 C in a shaking waterbath following which the remaining pellet was washed several times with 0.05M 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. Microscopic examination of the digests showed that no bacterial contamination had occurred. The 24 h and 7 day digestions did not show obvious differences in terms of the molecular size of the components in the chitinase hydrolysate as shown by ultrafiltration followed by gel filtration.

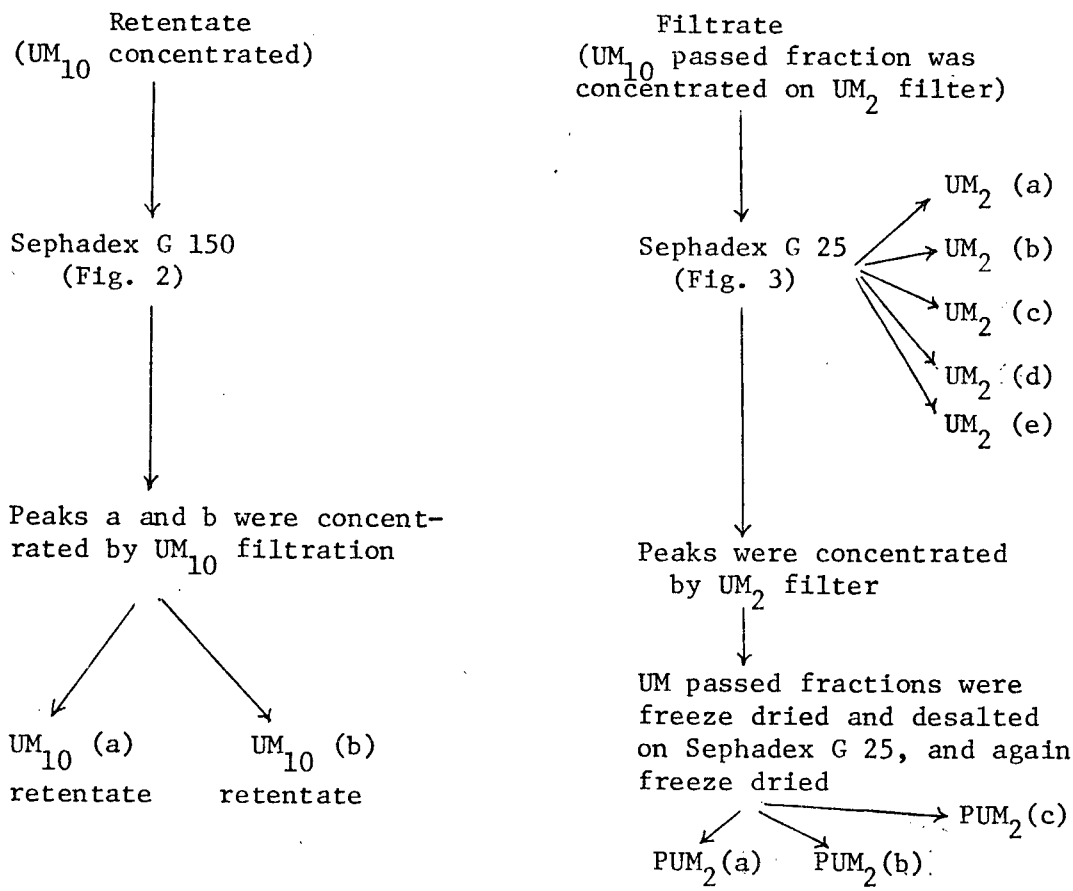
Digests were centrifuged at 30,000 x g for 60 min. prior to fractionation.

High molecular weight materials were concentrated by a UM_{10} Amicon

Figure 1 Preparation of antigenic materials from Trichophyton mentagrophytes cell walls.

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 on Amicon UM_{10} filters



filter (MW > 10,000) and the retentate further fractionated on a Sephadex G 150 column (100 x 2.5 cm) which had been equilibrated and eluted with 0.85% NaCl in 5 ml fractions (Fig. 2). Material in the G-150 peaks (UM_{10} (a) and UM_{10} (b) were again concentrated by UM_{10} filtration and stored at -20°C . Digested material of molecular weight < 10,000 which passed through UM_{10} filters was concentrated on a UM_2 filter (MW > 1,000) and fractionated on Sephadex G 25 column (60 x 2.0 cm) in a 4 ml fractions (Fig. 3). The peaks, UM_2 (a) through (e) were again concentrated by UM_2 filtration, and the rentates were stored at -20°C . The filtrates, which passed through the UM_2 filters (ie., PUM_2 fractions) were concentrated by lyophilization. Chitinase activity was recovered only in the UM_{10} (a) fraction.

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 determined by Anthrone reagent (50) using glucose as a standard. Protein was measured by the method of Lowry et al., (46)

Amino acid analyses were performed on the isolated fractions. Samples were hydrolysed with 6 N HCl in sealed evacuated ampules at 108°C for 20 h . The hydrolysates were repeatedly washed with distilled water and evaporated using a flash evaporator to remove the HCl, and the amino acid composition was determined on a Beckman-Spinco model 120 automatic amino acid analyzer.

Immunization of Animals

Albino guinea pigs weighing 400-500 g were immunized with the whole dried isolated cell wall material. Each animal received three

Figure 2. Elution profile of high molecular weight material (UM_{10} retentate) on Sephadex G-150 .

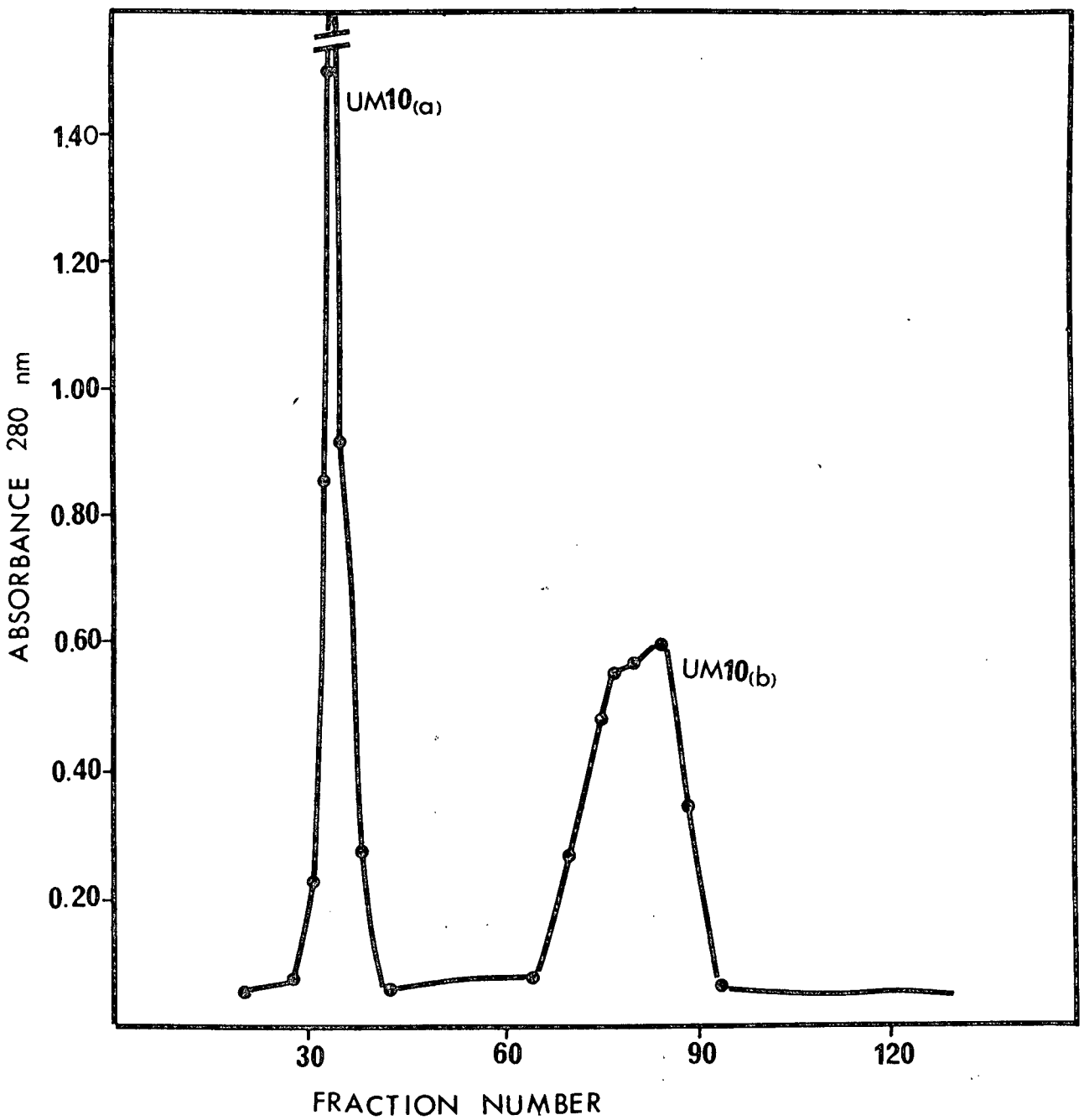
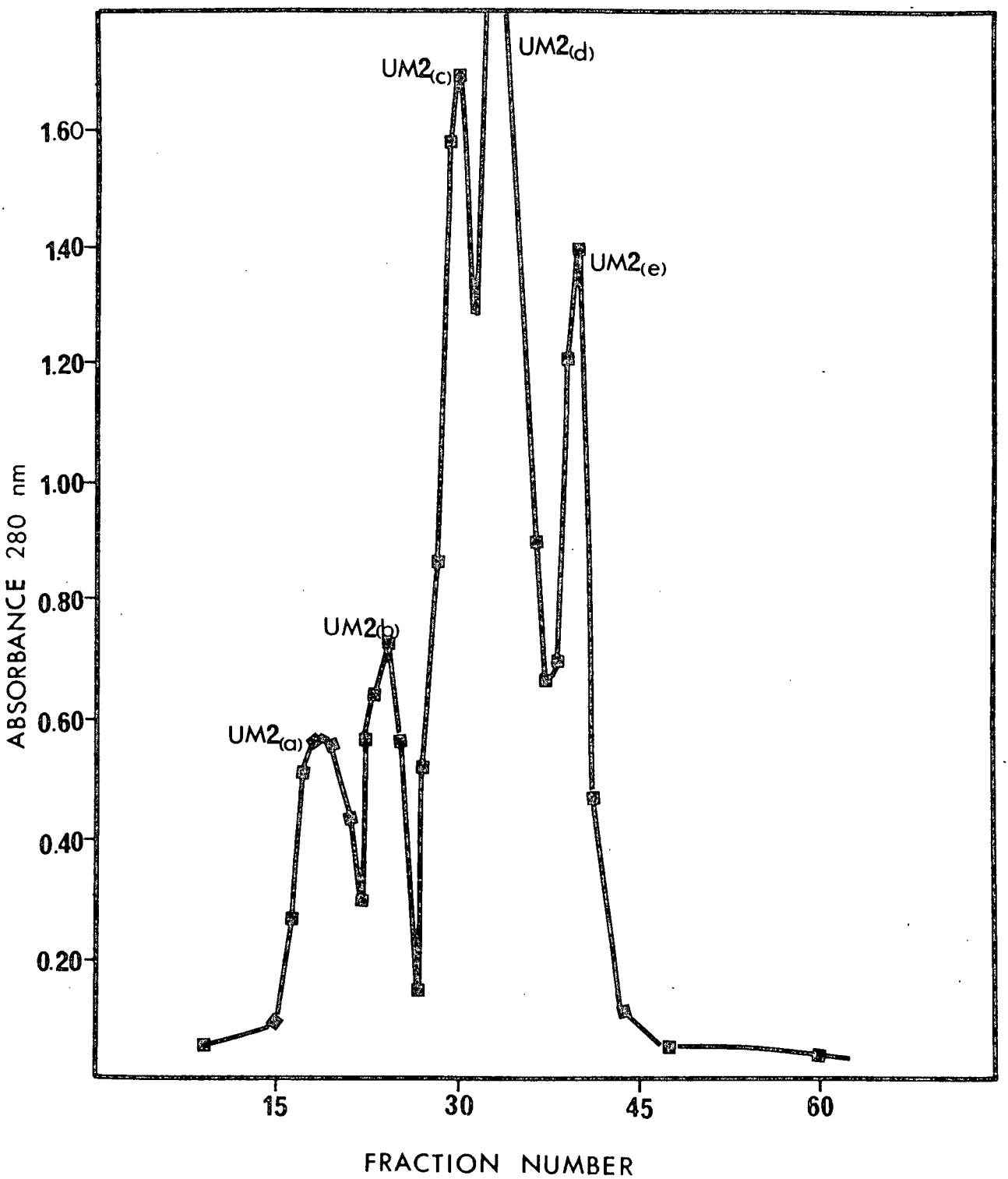

UM10(a)
UM10(b)
1.40
1.20
1.00
0.80
0.60
0.40
0.20
ABSORBANCE 280 nm
30
60
90
120
FRACTION NUMBER

Figure 3. Elution profile of low molecular weight material (UM_2 retentate) on Sephadex G-25 .


UM2(c)
UM2(d)
UM2(e)
UM2(b)
UM2(a)
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
ABSORBANCE 280 nm
15
30
45
60
FRACTION NUMBER

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 intraperitonealy. 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 µg 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). This volume was used in order to minimize inaccuracies due to faulty delivery and leakage at the site of inoculation. Cutaneous reactions were measured after 30 min for an immediate reaction, after 5 h for an Arthus reaction and after 24 h for delayed hypersensitivity reaction. Measurements were made with calipers under strong light. Animals were coded to prevent prejudicial measurement. Reactions were considered positive when the difference in the diameter of the wheal or induration between sensitized and control animals was > 8 mm. Skin measurements after 48 h were not significantly different from those measured at 24 h . For this reason data are routinely presented at 24 h.

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 skin reactivity. Animals used in these studies had been primed with antigen 10 - 20 days prior to sacrifice. The

procedures used have been described in detail elsewhere (34). 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 humidifed incubator supplied with 5% CO_2 . Eighteen hours prior to culture harvest, ^{3}H-thymidine (New England Nuclear) (2 µCi per well in 0.05 ml 1640) was added. Harvesting was done by microharvester, (Otto Hiller, Inc., Madison, Wisc.) 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 t test.

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. Serum was taken from those animals sacrificed and used for lymphocyte transformation studies. Slides were incubated in a humidifer 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 T. mentagrophytes 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 days 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.

It can be seen that both immediate and delayed reactions were evoked from essentially all UM_{10} fractions as well as the $UM_2(2)$ fraction.

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 µg/ml of Lowry protein. Because the higher dose was optimal for stimulation only these figures are shown. None of the fractions tested caused non-specific proliferation in unsensitized animals 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%

Lowry positive material of the hydrolysate and of the fractions obtained after ultrafiltration and gel filtration.

24 Hour Digestion					7 Days Digestion				
Fraction No.	Lowry positive mg recovered	%	Anthrone positive recovered	%	Fraction No.	Lowry positive mg recovered	%	Anthrone positive mg recovered	%
UNF[1]	218	1.4[4]	891	5.6[4]	UNF[1]	116	0.77[4]	540	3.6[4]
EG(a)[2]	26	1.3[4]	37	1.8[4]	-	-	-	-	-
EG(b)[2]	14	0.7[4]	4	0.2[4]	-	-	-	-	-
UM_{10}(a)[3]	112	51.3	40	4.5	UM_{10}(a)	66	56.9	39	7.2
UM_{10}(b)	16	7.3	196	22.0	UM_{10}(b)	30	25.9	120	22.2
UM_2(a)	29	13.3	99	11.1	UM_2(a)	10	8.6	39	7.2
UM_2(b)	4	1.8	80	8.9	UM_2(c)	2	1.7	19	3.5
UM_2(c)	5	2.3	118	13.2	UM_2(c)	7	6	193	35.7
UM_2(d)	22	10.1	72	8.0	UM_2(d)	4	3.4	13	2.4
UM_2(e)	16	7.3	112	12.6	PUM_2(a)	17	14.6	24	4.4
PUM_2(a)	27	12.4	105	11.8	PUM_2(b)	4	3.4	6	1.1
PUM_2(b)	4	1.8	0	0	PUM_2(c)	10	8.6	0	0
PUM_2(c)	2	0.9	0	0					

[1]UNF unfractionated chitinase digest.

[2]EG(a), (b) are the two peaks recovered from Sephadex G-150 after extraction of fresh fungal cell wall with ethylene glycol (date not shown).

[3]See Figure (1) for description of the individual fractions tested.

[4]These %s relate to the amount of mycelial dry weight before chitinase digestion and ethylene glycol extraction. All other %s relate to the amounts recovered from UNF after digestion

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. No major differences in immunological activity between the high molecular weight fractions and UM_2(a) low mol wt) are evident, although slightly lower stimulation indices were observed with this fraction in comparison to the higher molecular weight materials. Fig. 5 shows the dose response titration curve of the UM_2(a) fraction. The maximum lymphocyte response occurred after 96 h incubation with an optimum antigen concentration of 25 µg/ml.

Fig. 6 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 UM_2(a) fraction, although it is of low molecular weight produces stronger precipitin lines than the equivalent UM_{10}(a) and UM_{10}(b) fractions.

The results of amino acid analysis of UM_2(a) and UM_{10}(b) are shown in Table 3. UM_{10}(a) was considered to contain most of the enzyme chitinase and was therefore not analysed. It can be seen that the amino acid ratios of these two fractions are quite distinct from each other. In the UM_2(a) fraction, there is a limited array of amino acids (no Phe or Tyr) indicating that the peptide moiety of this material may be of limited size. This is not surprising since the molecular weight of UM_2(a) may be roughly estimated at somewhere between 2000 and 4000. Moreover, the rather high ratio of proline in this fraction is of interest, since high proline ratios in the cell walls of dermatophytes have been observed by other investigators (60) .

Figure 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). EG. (a), (b) are the two peaks recovered from Sephadex G-150 after extraction with ethylene glycol. Fractions 5-13 are from 1 day citinase digest wherease fractions 14-22 are from the 7 days chitinase digest.

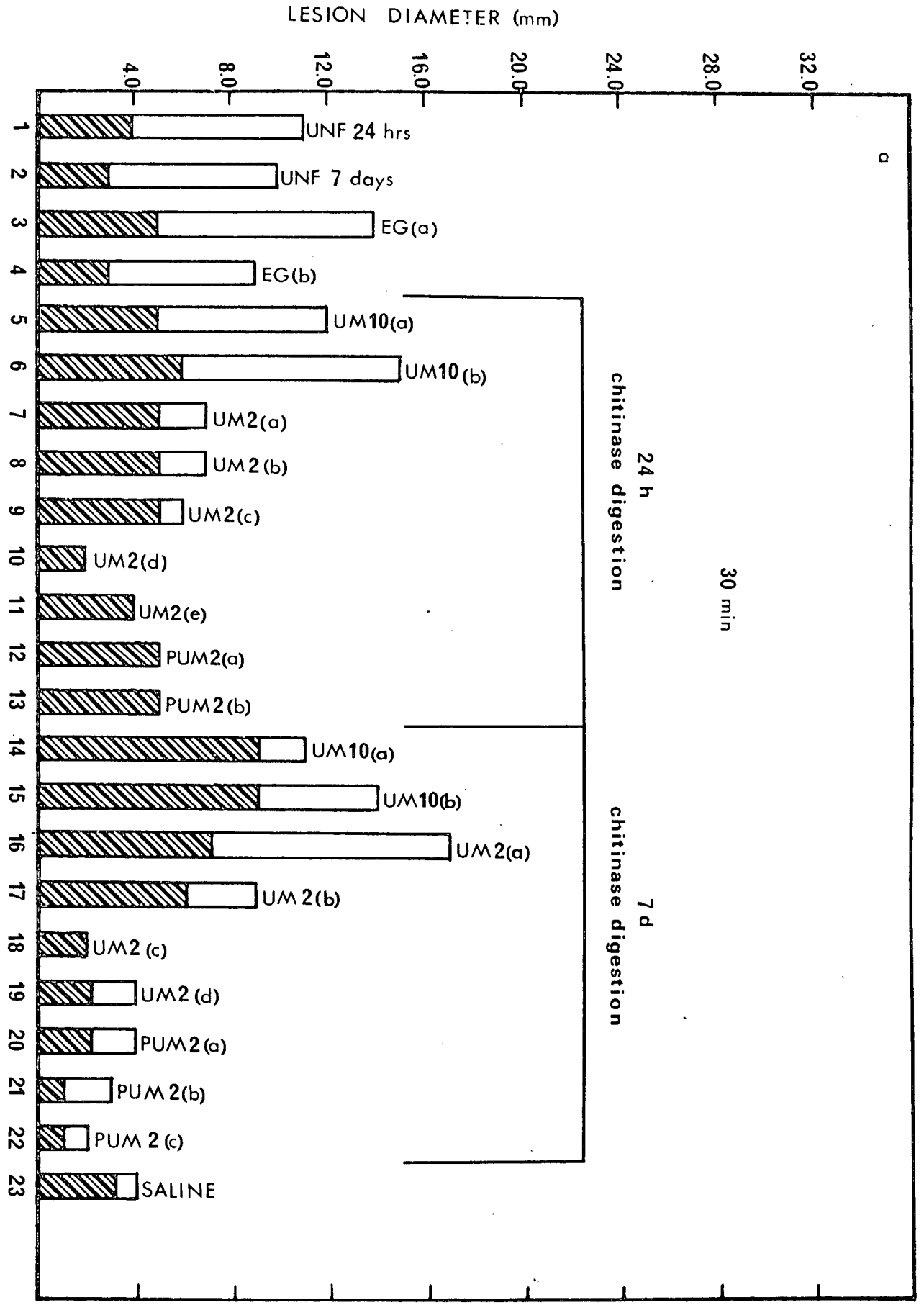
(b)

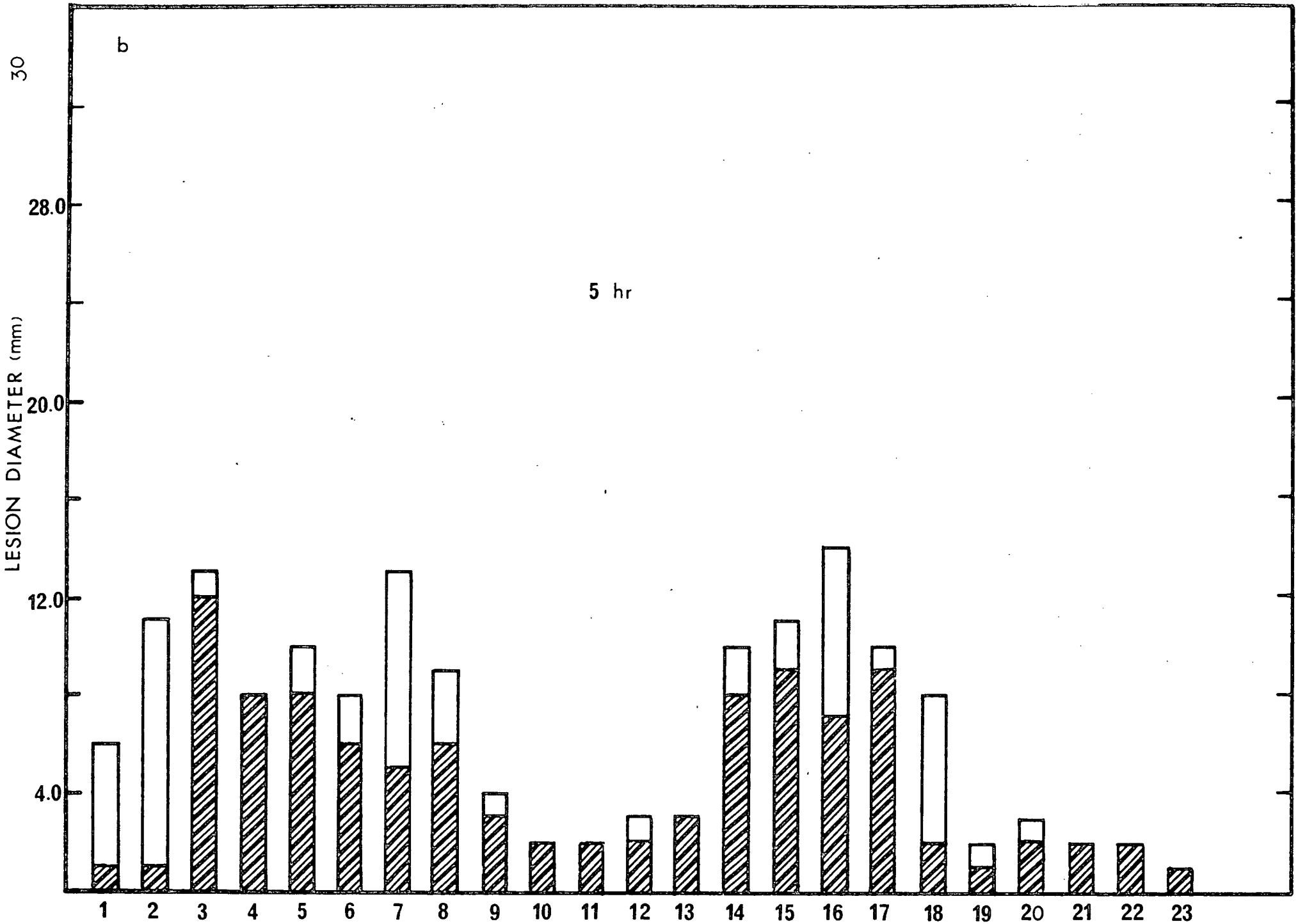
Cutaneous allergic reactions in immunized guinea pigs (whole column) compared to that of unimmunized animals (shaded) to T. mentagrophytes cell wall fractions after 24 hr and 7 days digestion with chitinase 5 hr after intradermal injection (Arthus reaction).

(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 injections (delayed hypersensitivity reaction).

These results represent averages of 12 immunized guinea pigs and 12 unimmunized controls. Standard deviations for individual antigens tested did not exceed 20% when all data were averaged and 10% for unimmunized controls. Each animal received a maximum of 12 injections, 11 test samples and one saline control. Therefore, each antigen was tested 5 times and data represent an average of these measurements.


LESION DIAMETER (mm)
4.0
8.0
12.0
16.0
20.0
24.0
28.0
32.0
1 UNF 24 hrs
2 UNF 7 days
3 EG(a)
4 EG(b)
5 UM10(a)
6 UM10(b)
7 UM2(a)
8 UM2(b)
9 UM2(c)
10 UM2(d)
11 UM2(e)
12 PUM2(a)
13 PUM2(b)
14 UM10(a)
15 UM10(b)
16 UM2(a)
17 UM2(b)
18 UM2(c)
19 UM2(d)
20 PUM2(a)
21 PUM2(b)
22 PUM2(c)
23 SALINE
24 h chitinase digestion
30 min
7 d chitinase digestion
a


b
30
28.0
20.0
12.0
4.0
LESION DIAMETER (mm)
5 hr
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23

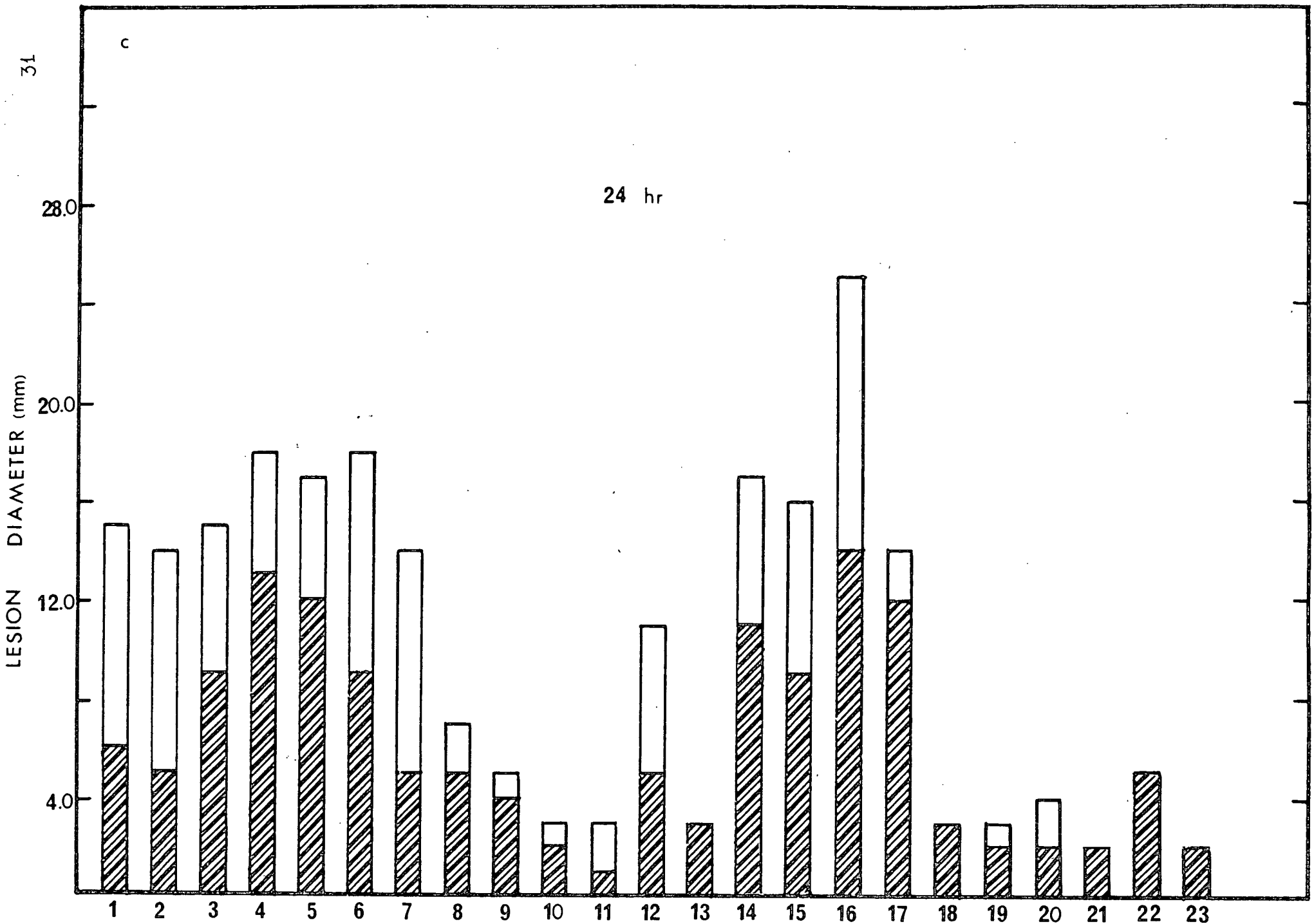

c
24 hr
LESION DIAMETER (mm)
28.0
20.0
12.0
4.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23

Figure 5

UM_2(a) antigen titration by the in vitro lymphocyte transformation. (●——●) (■——■) = tritiated thymidine incorporation by the sensitized cells after 24 hr and 96 hr incubation respectively. (○——○) (□——□) = incorporation by the non-sensitized cells after 24 hr and 96 hr incubation respectively.

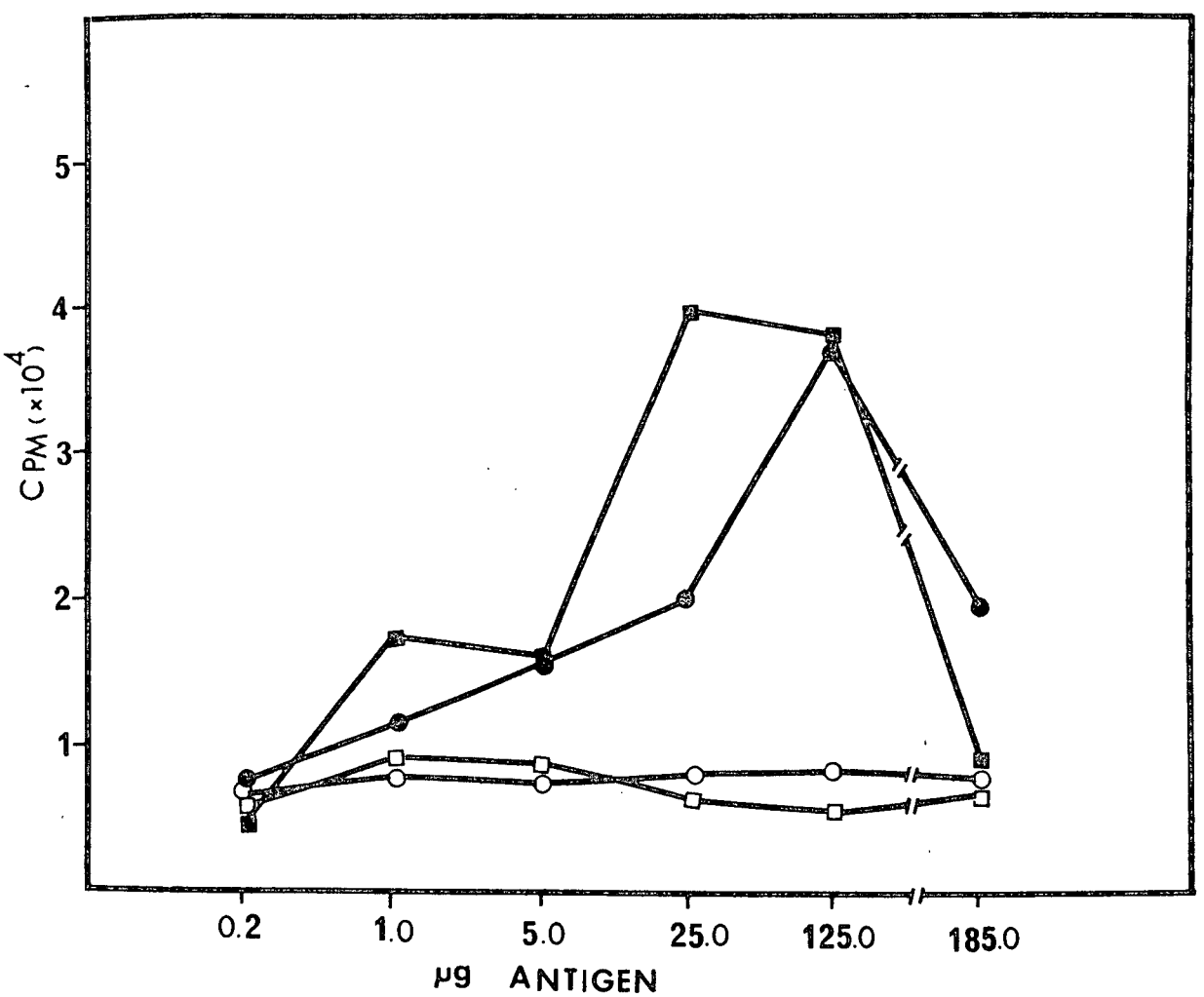

5
4
3
2
1
CPM (×10^4)
0.2
1.0
5.0
25.0
125.0
185.0
μg ANTIGEN

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

Fraction No	Response at 24 h to 25 µg/ml Lowry protein		Response at 96 h to 25 µg/ml Lowry protein	
	CPM	S.I.*	CPM	S.I.
24 h digest				
$UM_{10}(a)$	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
7 days digest				
$UM_{10}(a)$	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	7838		13,623	

* Stimulation Index

** Not significant when compared to control counts

FIGURE 6

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 µg).

a & b 24 h digest

c & d 7 day digest

e & f 7 day UM_{10}(a)

g & h 24 h UM_{10}(a)

i & j 24 h UM_{10}(b)

k & l 24 h UM_{2}(a)

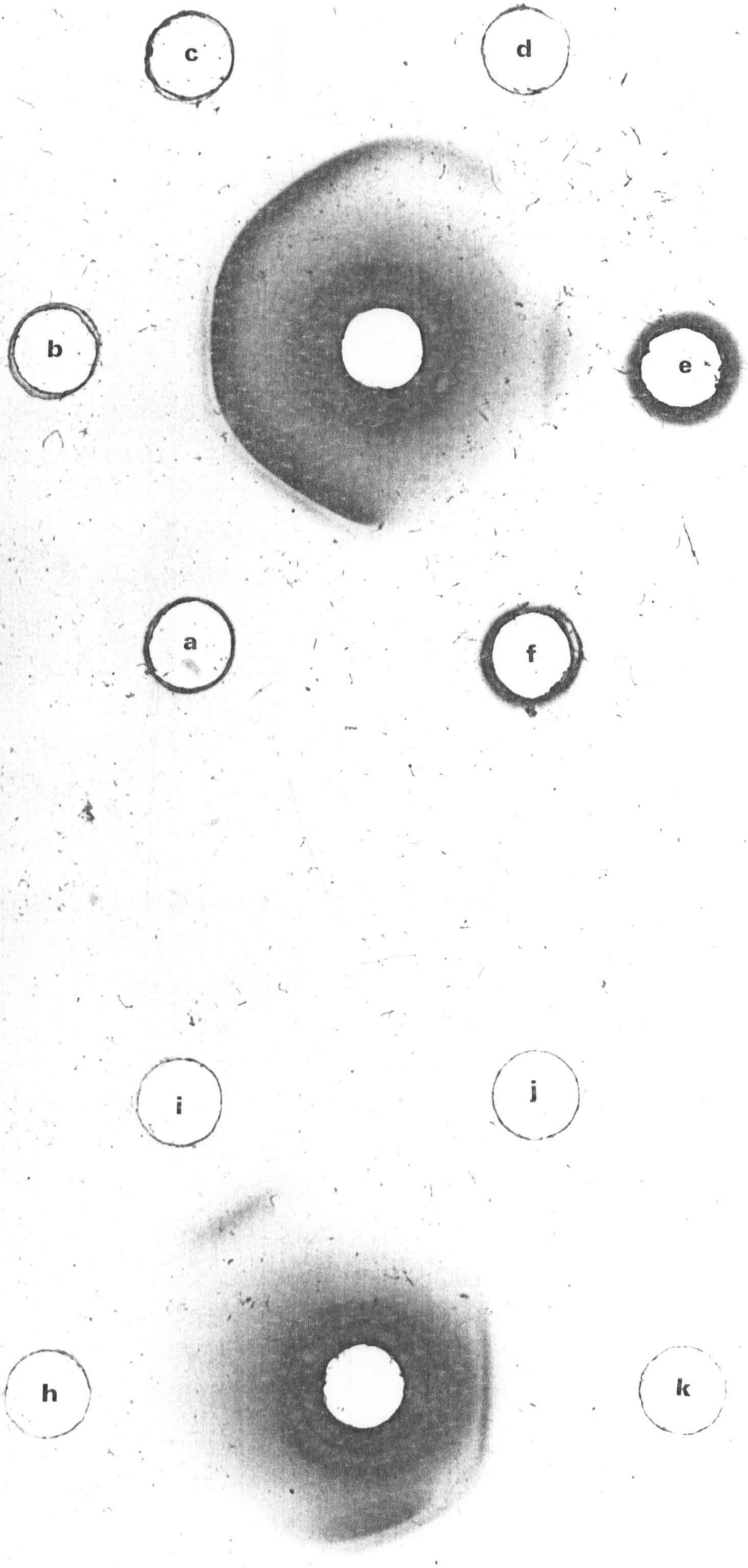

c
d
b
e
a
f
i
j
h
k
g
l

Table 3. Amino Acid compositions of the fractions $UM_{10}(b)$ and $UM_2(a)$.

Amino Acid	$UM_{10}(b)$		$UM_2(a)$	
	µmoles	Molar Ratio*	µmoles	Molar Ratio**
Aspartic acid	.068	2.7	.088	3.9
Threonine	.139	5.4	.063	2.8
Serine	.170	6.7	.061	2.7
Glutamic acid	.084	3.3	.083	3.7
Proline	.026	1.0	.151	6.7
Cysteine	0	-	0	-
Glycine	.115	4.5	.098	4.3
Alanine	.248	9.7	.041	1.8
Valine	.101	3.9	.040	1.8
Methionine	0	-	0	-
Isoleucine	.027	1.0	.023	1.0
Leucine	.169	6.6	.024	1.1
Tyrosine	.063	2.5	0	-
Phenylalanine	.016	.61	0	-
Histidine	.052	2.03	.050	2.2
Lysine	.022	.85	.049	2.2
Arginine	.264	10.30	.018	0.8

* Calculated on the basis of 61 amino acid residues being present in which Pro, Ile, Lys and Phe have a molar ratio of 1.0.

** Calculated on the basis of 25 amino acid residues being present in which Ile, Leu, and Arg have a molar ratio of 1.0

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 agreemnt 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 cytophlasmic 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 wherease 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 (25,49, 53,55,56). Our choice of enzymatic treatment of 24 h and 7 days was based on the assumption that a 24 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, and that prolonged digestion might yield more representative fragments.

Essentially all UM_{10} (high molecular weight) and UM_2(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 (7,8,54) 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 (34), our lymphocyte transformation studies are in accordance with the skin test results, for those fractions capable of inducing delayed skin reactions also causedlymphocyte proliferation with sensitized lymphocytes. These results also confirm the observation of Hanifin et al., (35) and Svejaard et al., (67) who have stated that dermatophytic cell wall extracts can induce proliferation of sensitized lymphocytes.

The amino acid analyses of UM_{10}(b) and UM_2(a) were quite distinct. The UM_{10}(b) fraction contained essentially all the amino acids commonly found in proteins. This fraction is probably composed of a variety of cell wall fragments which may be quite heterogeneous. The UM_2(a) fraction on the other hand had a quite distinctive amino acid composition in that it contained extremely high levels of proline and a limited array of amino acids (phenylalanine and tyrosine were not present). This fraction is of quite low molecular weight, possibly between 2,000 to 4,000 as assessed by its filtration properties. The amino acid composition of this material is suggestive of the possibility of a relatively small peptide being associated with the polysaccharide of the cell wall. It is of interest to note that the analysis of UM_2(a) is in agreement with those reported by others for isolated glycopeptides from the cell wall of T. mentagrophytes.

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.

Section III Characterization of Immunologically Active Peptide from the Cell Wall of *Trichophyton mentagrophytes*

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 (7,8,37,54). Nozawa *et al.*, (54) 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 (39), who isolated 22 protein fractions by phenol-water extraction and chromatography. Christiansen and Svejgaard (67) 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., (62) 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.

I have shown previously that a relatively low molecular weight fraction taken from a cell wall hydrolysate of T. mentagrophytes (UM_2(a), is a peptide containing polysaccharide which can stiumulate in vitro sensitized lymphocytes and can evoke immediate and delayed skin hypersensitivity as well as form a precipitin line with antibody raised in guinea pigs to a preparation of whole mycelial cell wall of T. mentagrophytes. In this section further studies were made on that fraction and the immunological roles of the polysaccharide and peptide moieties of this fraction of the cell wall hydrolysate.

Materials and Methods

Antigen: UM_2(a) is one of the fractions which was isolated from the cell wall of Trichophyton mentagrophytes after chitinase digestion, ultra-filtration and gel filtration. The procedures for digestion and fractionation have been described previously. UM_2(a) material was isolated from 24 hr digests. 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 UM_2(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 UM_2(a) fraction was digested first with pronase (Calbiochem) at an enzyme concentration of 0.5 - 1.0% of the weight of the substrate (calculated as per cent Lowry protein) to be digested, and increased during the course of digestion to 2% . Incubation was continued for 6 days at $37^{o}C$ at pH 7.8 in the presence of 0.0015 M $CaCl_2$. The enzyme digest was then put through a UM_{10} amicon (Amicon Corporation, Lexington, Mass.) filter to remove the enzyme. Subsequent testing of filtrates showed them to be free of enzyme activity. The filtrate was put through a UM_2 filter and washed three times with distilled water.

The UM_2 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 UM_2 retained fraction was considered to consist mainly of the UM_2(a) carbohydrate moiety. Peptide and carbohydrate contents of all fractions were derermined by quantitative ninhydrin and Anthrone tests respectively.

Trypsin: To test the antigenic role of the peptide moiety in UM_2(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 UM_2(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 0.04M 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 (18) for peptide separation using a Dowex 50W X8 resin in a 0.7 x 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. (36). Eluted material accounted for 100 per cent of starting material. 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: Twelve guinea pigs to be used for immunological testing were immunized with cell wall material from T. mentagrophytes as described previously. Serum was collected from immunized guinea pigs at the time of sacrifice inactivated at 56°C for 30 min. and stored at -20°C.

Skin tests and lymphocyte stimulation:

These were carried out as described previously. 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. All tests were run at 10 and 25 µg peptide material. A total of 12 immunized animals were used in this study.

Amino acid analyses

Peptides that were shown to have been antigenic by skin test and lymphocyte transformation and which were shown to be a single peptide by

Polyacrylamide gel electrophoresis:

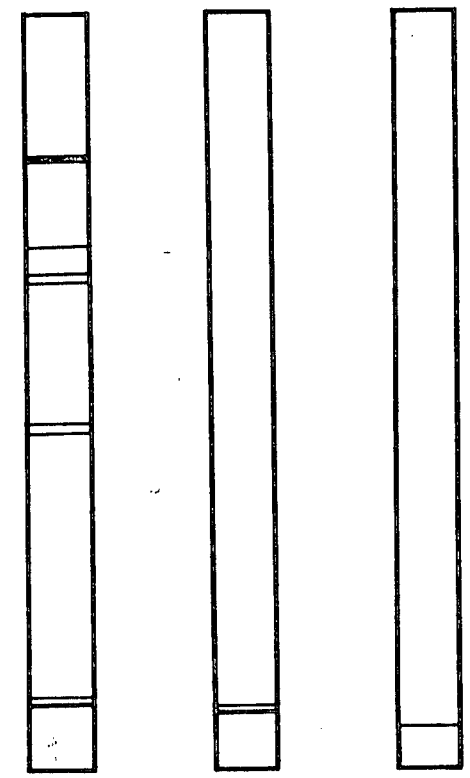
The method of Weber and Osborn (70) was followed. The UM_2(a) fraction was found to contain two subfractions, Fig (7) which differ in their staining intensity. Each subfraction was shown to contain peptide and carbohydrate moieties by coomassie brilliant blue and periodate acid schiff stains respectively.

Thin layer chromatography:

The peptide fractions of pronase and trypsin digests were analyzed by thin layer chromatography. Precoated plates with silica gel G 250 microns were used. The solvent was butanol, acetic acid, water 3:1:1 respectively according to Wünch et al (72)

Figure 7

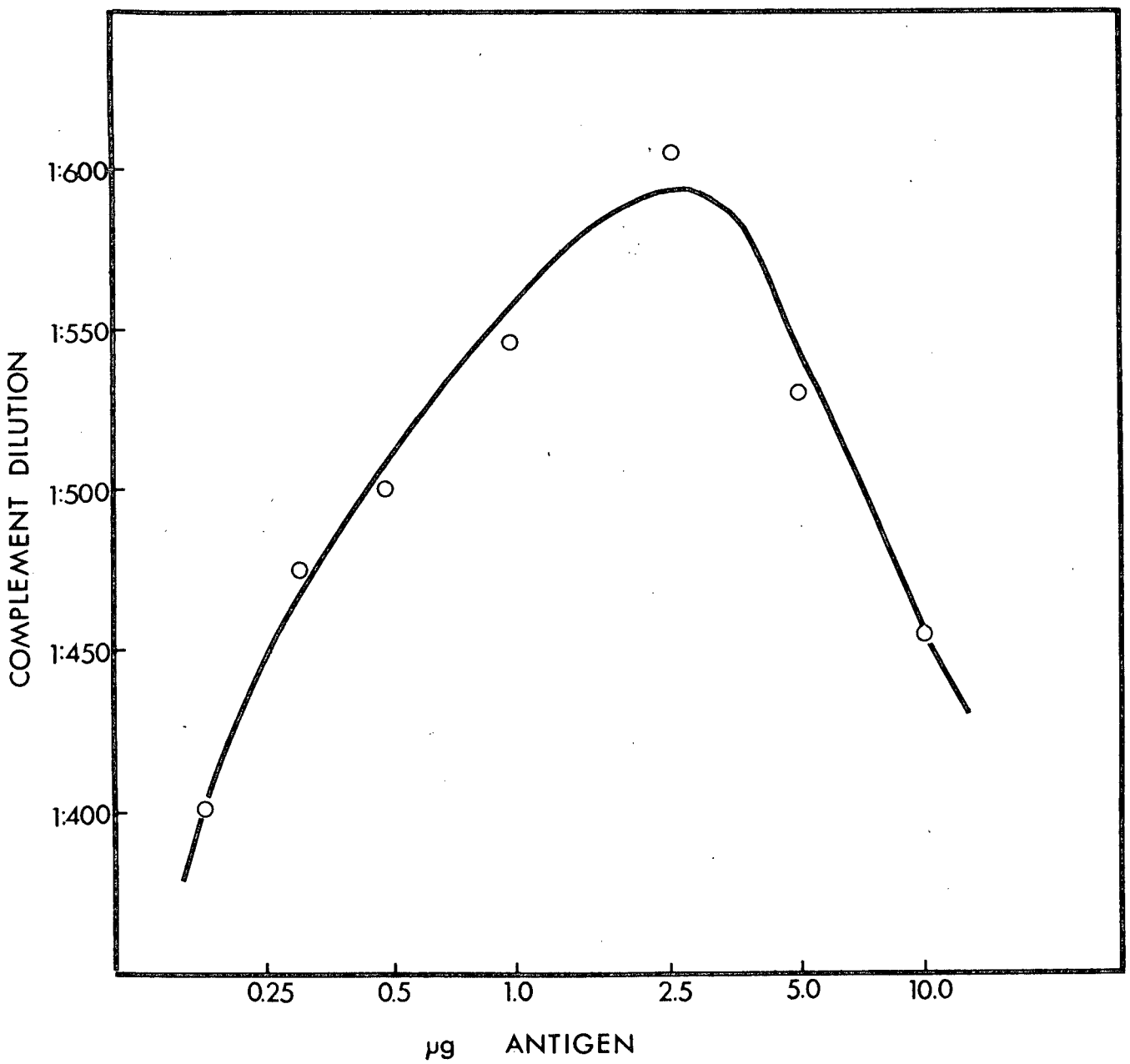
Disc gel electrophoresis pattern of 24 hr (UNF), UM_2(a) fractions. TD is the position of the tracking dye.



24 hr(UNF) UM2(a) TD

Figure 8

Complement fixation titration curve of UM_2(a) fraction.


1:600
1:550
1:500
1:450
1:400
COMPLEMENT DILUTION
0.25
0.5
1.0
2.5
5.0
10.0
µg ANTIGEN

thin-layer chromatography were analyzed for their amino acid composition in the Beckman Spinco automatic amino acid analyzer.

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 (28). 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 dilutions from 1:100 to 1:1600 . 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 (68). Titration of UM_2(a) with guinea pig antiserum is shown in fig. 8 .

Hapten Inhibition:

Hapten inhibition studies were performed as described by Gerwing and Thompson (28). Briefly, the test involved incubation for 24 h at $4^{o}C$ 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 (UM_2(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 $4^{o}C$ 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 section II that a low molecular weight fraction ($UM_2(a)$) from T. mentagrophytes cell wall containing both polysaccharide and peptide gave in guinea pigs strong delayed skin reactivity as well as a 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 $UM_2(a)$ fraction. Table 4 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, wherease this was not so with trypsin treatment.

Figure 9 shows the results of those fractions which evoked hypersensitivity reactions in immunized guinea pigs. It can be seen that the undigested UM_2 retentate material containing mainly carbohydrate induced both immediate and delayed skin reactions, wherease the peptide fractions could not induce immediate but did give both Arthus and delayed reactions, indicating that both the pronase and trypsin digested peptides retained some immunological specificity.

Table 5 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

Table 4 . Chemical analysis of $UM_2(a)$ fraction after enzyme digestion. Figures represent total values for each fraction.

Enzyme	Mg CH_2O before digestion ($UM_2(a)$)	Mg Protein before digestion ($UM_2(a)$)	Mg CH_2O after digestion ($PCUM_2R$)	Mg Protein after digestion and ultrafiltration ($PCUM_2R$)	Mg Protein passed UM_2 Amicon filter ($PCUM_2P$)
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) Anthone test

(2) Quantitative ninhydrin test

(3) no carbohydrate was detectable in this fraction

Figure 9

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 ($PCUM_2R$)

C = Pronase-carboxypeptidase UM_2 passed fraction ($PCUM_2P$)

D = Trypsin UM_2 retained (TUM_2R)

E = Trypsin UM_2 passed (TUM_2P)

F = Saline

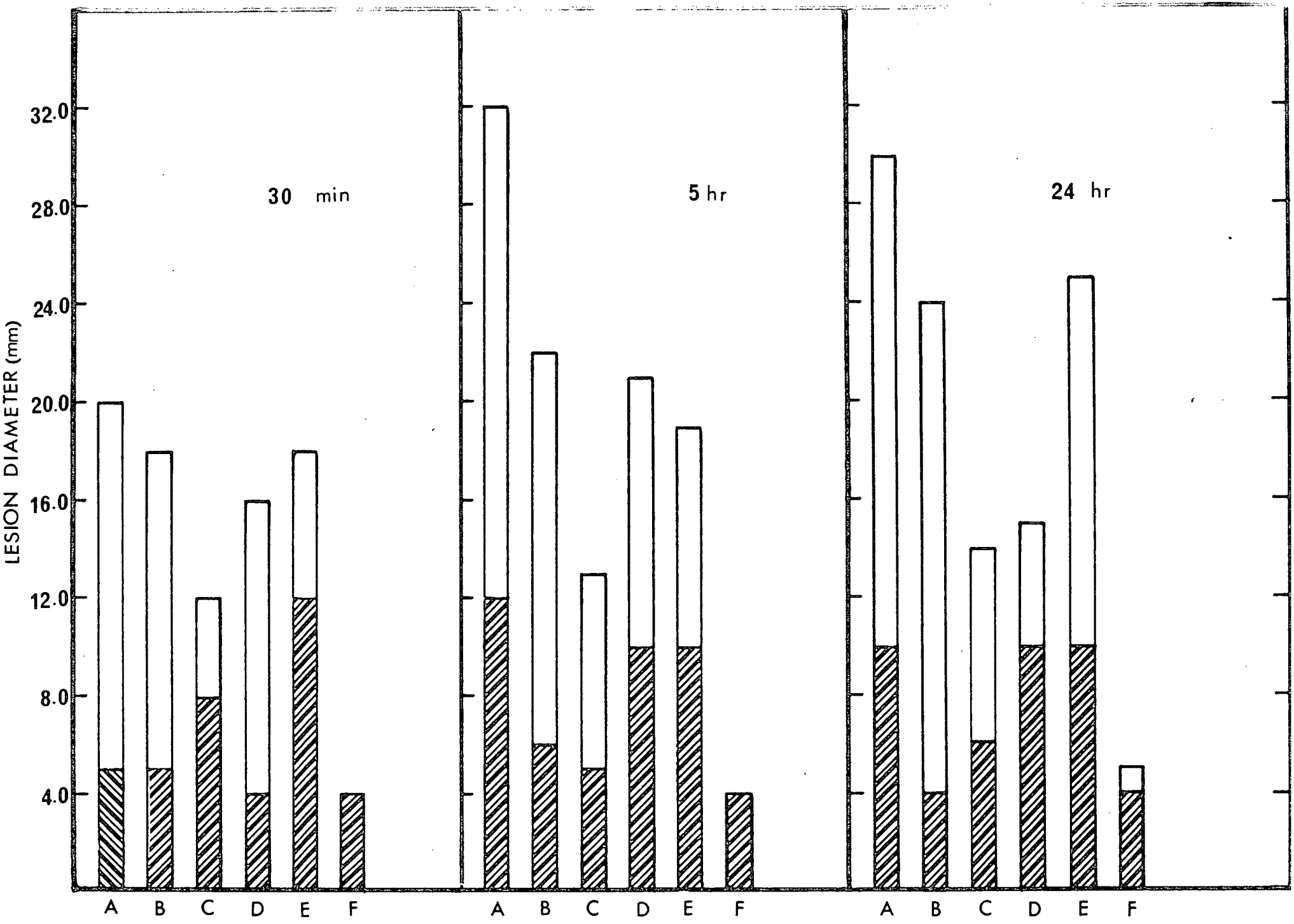

LESION DIAMETER (mm)
32.0
28.0
24.0
20.0
16.0
12.0
8.0
4.0
30 min
5 hr
24 hr
A
B
C
D
E
F
A
B
C
D
E
F
A
B
C
D
E
F

Table 5. Lymph node cell 96 hour incorporation of tritiated thymidine in response to $UM_2(a)$ sub-fractions after enzyme treatment and fractionation.

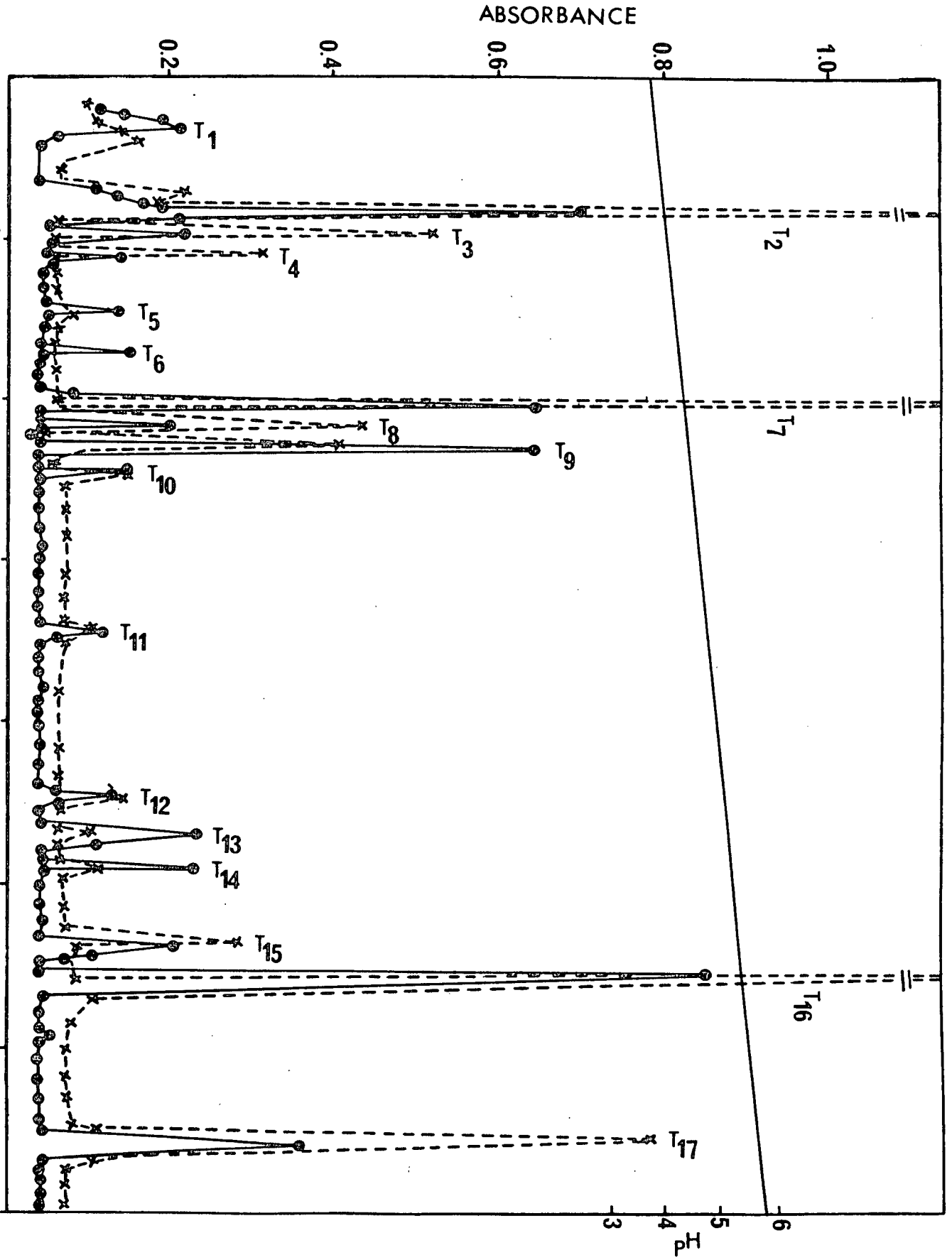
Lymphocyte response at various protein levels

Antigen	5 µg/ml	S.I.*	25 µg/ml	S.I.*	125 µg/1ml	
$UM_2(a)$	16,063	2.55	16,280	2.59	17,183	2.73
$PCUM_2R$	20,036	3.036	21,916	3.49	19,260	3.06
$PCUM_2P$	16,343	2.60	16,373	2.60	12,066	1.92
TUM_2R	20,466	3.26	22,520	3.59	18,670	2.97
TUM_2P	6,766	NS	16,793	2.67	17,890	2.85
No Antigen	6,286					

* Stimulation Index

Figure 10

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


ABSORBANCE
0.2
0.4
0.6
0.8
1.0
40
70
100
130
160
190
220
T1
T2
T3
T4
T5
T6
T7
T8
T9
T10
T11
T12
T13
T14
T15
T16
T17
3
4
5
6
pH

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 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 eultion profile of the purified tryptic peptides after ion exchange chromatography is shown in Figure 10. The skin reactions to the pooled peptide peaks are shown in Figure 12a,b,c . Out of 17 peptides tested, it can be seen that 5 gave fairly good delayed skin reactions while none of them induced reactions at 30 minutes. Table 6 shows the results of the 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.

Except for T_6, T_7, T_{11} and T_{15}, all other peptide peaks showed one spot on thin layer chromatography (Fig 11). The fractions with more than one spot were not analyzed for their amino acid content. The amino acid analyses of some of the peptides shown to have been antigenic by skin test and/or lymphocyte stimulation are presented in Table 7 . Some of the isolated peptides were at too low a yield to permit analyses.

Immunodiffusion and complement fixation studies with anti-whole mycelial cell wall guinea pig serum showed that a number of peptides were

Figure 11 .

Thin layer chromatography of the tryptic peptides.

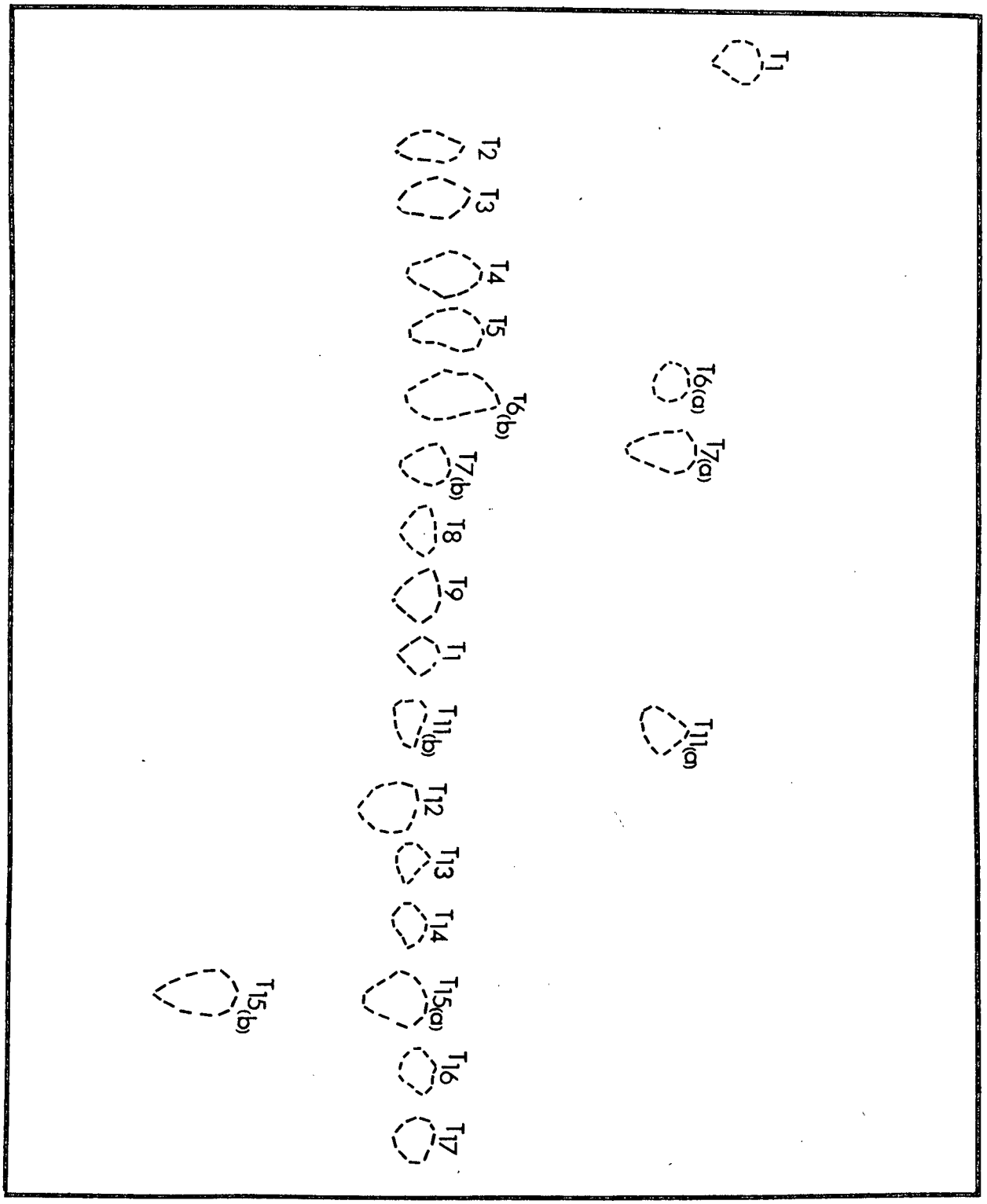
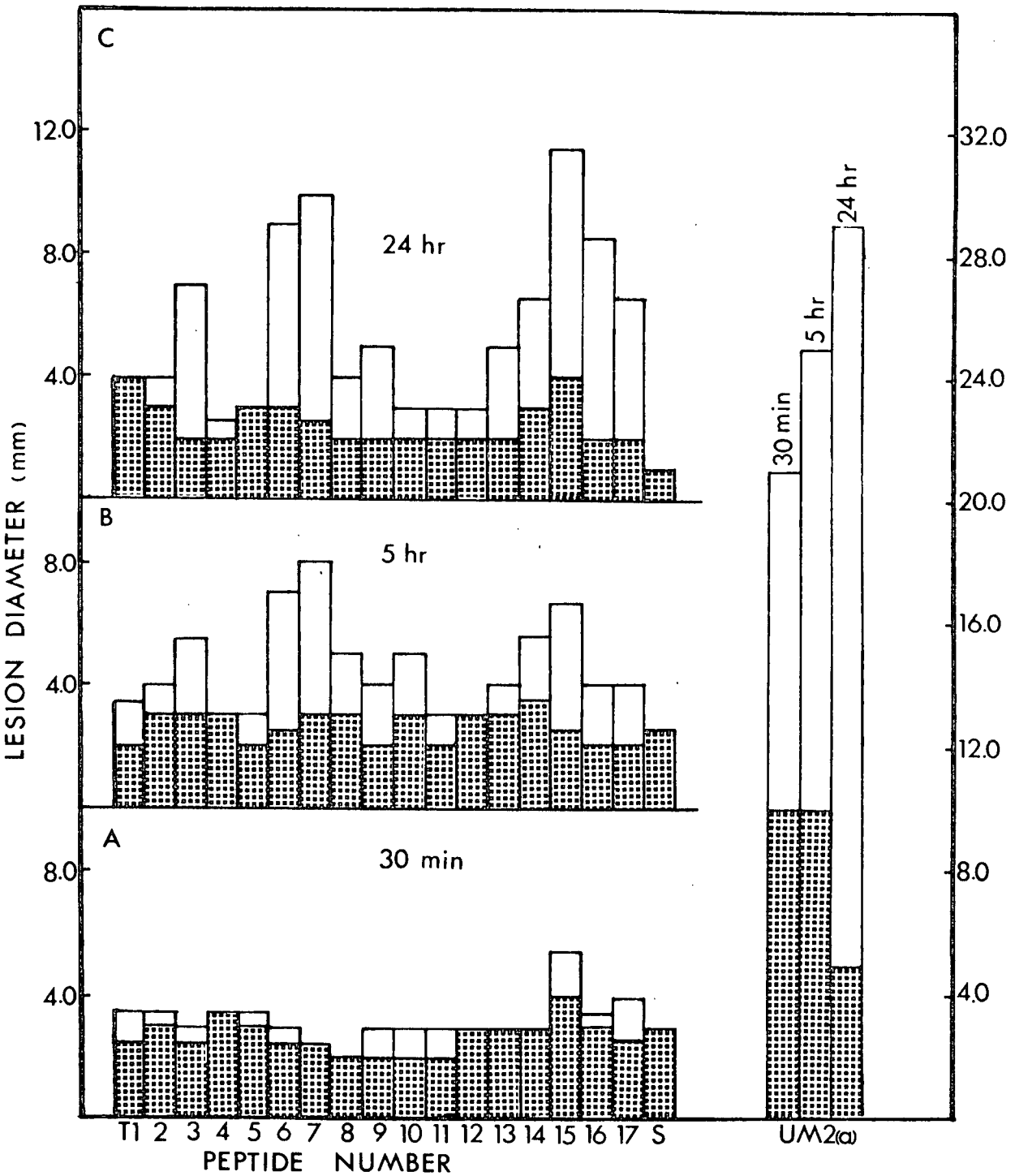

T1
T2
T3
T4
T5
T6(b)
T7(b)
T8
T9
T1
T11(b)
T12
T13
T14
T15(b)
T6(a)
T7(a)
T11(a)
T15(a)
T16
T17

Figure 12 .

Cutaneous allergic reactions of the tryptic peptides from UM_2(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) .


C
12.0
8.0
4.0
24 hr
B
8.0
4.0
5 hr
A
8.0
4.0
30 min
LESION DIAMETER (mm)
T1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 S
PEPTIDE NUMBER
32.0
28.0
24.0
20.0
16.0
12.0
8.0
4.0
30 min
5 hr
24 hr
UM2(a)

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 8 . 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 reaction of UM_2(a) with specific antiserum indicating that these peptides contain at least one intact antigenic determinant recognized by antibodies formed against whole cell wall material.

Table 6. Sensitized lymph node cell 96 hour incorporation of tritiated thymidine in response to tryptic peptides from $UM_2(a)$.

	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	39,253	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	53,769	2.73
T_8	32,466	NS	22,953	NS
T_9	21,630	NS	21,443	NS
T_{10}	22,710	NS	11486	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
N. Ag.	18,740		19,680	

*S.I. - Stimulation index

Table 7. The amino acid compositions of some of the tryptic peptides from $UM_2(a)$

	T_2		T_3		T_{13}		T_{16}		T_{17}		$UM_2(a)$	
	µmoles	molar ratio	µmoles	molar ratio	µmoles	molar ratio	µmoles	molar ratio	µmoles	molar ratio	µmoles	molar ratio
Aspartic Acid	.053	1.04	.071	2.96					.04	1.02	.088	3.9
Threonine			.052	2.16							.063	2.8
Serine	.058	1.15	.069	2.88							.061	2.7
Glutamic acid			.047	1.96	.051	2.21	.020	1.4	.077	1.97	.083	3.7
Proline	143	2.81			1028	1.21					.151	6.7
Glycine							.040	2.7			.098	4.3
Alanine					.065	2.83					.041	1.8
Valine					.040	1.74	.058	4.0			.040	1.8
Isoleucine			.053	2.20			.027	1.8			.023	1.0
Leucine							.030	2.0			.024	1.1
Histidine											.050	2.2
Lysine	.010	0.2			.010	0.43	.011	0.8			.049	2.2
Arginine											.018	0.8

Table 8 . 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

Discussion

The proteolytic enzyme digestion of the UM_2(a) fraction, followed by ultrafiltration, was shown to be effective in the removal of a substantial amount of peptide from the polysaccharide peptide complex. pronase on
Wherease either trypsin or their own removed approximately half the peptide content, the combination of pronase plus carboxypeptidase removed about 90% (Table 4). 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 UM_2(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 (7,8,), whose work indicated that the humoral response rather than the cell-mediated response was stimulated by the carbohydrate of dermatophyte cell walls. Our finding are also in disagreement with those of Nozawa et al ., (54), who found that proteolytic digestion of cell wall material decreased the delayed hypersensitivity reaction by 54% . While these findings are not in agreement with those of other investigators, 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 UM_2 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 (y,8,54). 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.*, (35) 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 induce immediate skin reactions was not surprising, since most of them were obviously too small to be able to effect a classical histamine mediatedimmediate 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, (65,69) 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 UM_2(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 UM_2(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.

The amino acid analyses of some of the isolated peptides are shown in table 7 as well as the analysis of UM_2(a). These data do not constitute

a complete picture of the tryptic peptides associated with this fraction but they do provide information regarding the amino acid composition of the major immunologically active peptides. Peptides T_2 and T_3 were active in the lymphocyte stimulation assay as well as in the complement fixation inhibition test, whereas only T_3 was capable of inducing a positive delayed skin reaction. It is clear that both T_2 and T_3 are unique peptides. Peptides T_{13}, T_{16} and T_{17} caused lymphocyte transformation and delayed skin reactions and were active in the complement fixation test. The analysis of these peptides showed clearly that these also have quite distinct amino acid sequences. From this somewhat limited study of the amino acid compositions of these peptides, it would appear that the peptide portions of the $UM_2(a)$ fraction contain a diverse array of amino acid sequences which constitute antigenic determinants recognized at both the humoral and cellular level of immune responsiveness.

Section 1V General Conslusions

Chitinase Digestion

The digestion of clean cell wall preparation of T. mentagrophytes with chitinase yielded a variety of heterogeneous materials which could be partialy fractionated according to their molecular size by ultrafiltration and gel filtration. All the fractions isolated in this manner contained both peptide and polysaccharide material. When comparisons were made between material which had been digested for either 24 hours or 7 days with chitinase, in terms of the fractions obtained and their immunological activities no differences were observed. This indicates that extensive digestion occurs during the first 24 hours, and prolonged treatment with the enzyme has very little obvious effect.

Immunological activity of various fractions

All the fractions from the 24 hour chitinase digestion obtained after ultrafiltration and sephadex filtration were tested for their ability to evoke immediate, Arthus-type, or delayed skin reactions in guinea pigs sensitized to whole cell wall fragments of T. mentagrophytes. They were also tested for their ability to stimulate lymphocyte proliferation in cells from sensitized guinea pigs. Fractions which had molecular weight of greater than 10,000 (as estimated by their ability to pass through a UM10 filter gave strong skin reactivity and good lymphocyte stimulation. Only one fraction of lower molecular weight (UM_2(a)) showed strong immunological reactivity by both skin testing and in vitro lymphocyte stimulation. This fraction had a molecular weight of between 2 and 10,000 as estimated by its ability to pass through a UM10 filter but not a UM_2(a)filter, and by its elution with the void volume on Sephadex G-25 .

Other UM_2 fractions which were included in Sephadex G-25 columns did not show complete immunological reactivity. As a result of these observations, further studies were carried out on the UM_2(a) fraction since it was the material of minimum molecular size to exhibit complete reactivity.

Studies on UM_2(a)

An attempt was made to determine whether the carbohydrate and peptide moieties of this fraction played different roles in immune responsiveness. UM_2a material was digested with either trypsin or a combination of pronase and carboxypeptidase A. After digestion the material was washed on a UM_2 filter. The retained material contained essentially all the polysaccharide part of the fraction wherease peptides were found in the filtrate. The polysaccharide fraction which had been digested with carboxypeptidase A and pronase retained only about 5% of the original peptide material.

Tests were carried out to determine the immunological reactivity of each fraction. It was found that the polysaccharide rich materials retained their ability to induce both immediate and delayed skin reactions in sensitized guinea pigs as well as to stimulate lymphocyte proliferation. These observations indicate that no separate role can be attributed to the polysaccharide moiety in stimulating either humoral or cell mediated immunity. However, because a small amount of peptide remained in these polysaccharide rich fractions, these observations are not conclusive. The peptide material resulting from the digestions were of relatively low molecular weight since they had passed through a

UM_2 filter. These fractions, however, were found to be capable of stimulating delayed skin reactions and lymphocyte proliferation but not immediate and Arthus reactions. While these observations, superficialy, might be taken to indicate that peptide materials were only involved in cell mediated immunity, this is not necessarily the case. These materials are of low molecular weight. It is thought that mast cell degranulation, which initiates the events leading to an immediate skin reaction, involves the complexing of antigen and antibody on the mast cell surface. It has been shown that haptens and monovalent antigens can block this reaction, since it is thought that multivalency of antigen is required to acheive sufficient perturbation of the mast cell membrane to initiate degranulation. Therefore, the observation that these peptides could not cause immediate skin reactions should not be regarded as proof for their lack of participation in humoral immunity.

Immunological Behaviour of Tryptic Peptides from UM_2(a)

The tryptic digest of UM_2a was chromatographed on an ion exchange column in an attempt to purify individual peptides so that their reactivity could be tested. Isolated fractions were tested for homogeneity by thin layer chromatography. Only those fractions which satisfied this criterion were analysed for their ability to induce skin reactivity in sensitized guinea pigs, to stimulate lymphocyte proliferation, and to block complement fixation between UM_2a and specific antiserum. Essentially no peptide induced immediate or Arthus reactions. However, a very high proportion of them were capable of

inducing delayed reactions, stimulating lymphocyte proliferation or blocking complement fixation. These findings showed that a number of these peptides were reactive at both the humoral and cell mediated level of immune responsiveness, showing clearly that these two elements cannot be separated in terms of the roles of different fractions of T. mentagrophytes cell wall components. The amino acid analyses which were carried out on some of the tryptic peptides isolated showed clearly that the peptide moiety of UM_2a did not constitute a small repeating subunit but rather a complex peptide structure.

Previous reports in this area have indicated that the polysaccharide portions of dermatophyte cell walls are responsible for inducing antibody-mediated (immediate) reactions, whereas the peptide portions are responsible for inducing the cell-mediated reactions. The findings reported here do not support this view, and show that immune responsiveness to dermatophyte antigens can not be classified in this way. Rather, both polysaccharide and peptide moieties of the cell wall appear to stimulate both humoral and cell-mediated immunity.

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