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Isolation of Glycopeptides with Skin Test Activity from Dermatophytes

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By using ethylene glycol extraction of whole submerged cultures, followed by Sephadex G-200 and diethylaminoethyl-Sephadex chromatography, we isolated four distinct glycopeptides from *Trichophyton mentagrophytes, T. rubrum,* and *Microsporum canis.* Chemical analyses revealed that these glycopeptides contained mostly carbohydrate (42.5 to 81.6%) and protein (4.3 to 11.3%), with lesser amounts of phosphorus (0.4 to 6.0%) and hexosamines (0.3 to 0.6%). Based upon total carbohydrate and monosaccharide content, these dermatophyte glycopeptides could be divided into two chemical groups: glucopeptides (DSI_1) and mannopeptides (DSI_2, $DSII_1$, and $DSII_2$). The mannopeptides and glucopeptides of each species of dermatophyte were not significantly different chemically from those derived from the other two dermatophyte species studied. Skin testing of DSI_1-glucopeptides or DSI_2-mannopeptides in immunized guinea pigs indicated that only the DSI_2-mannopeptides elicited a delayed hypersensitivity reaction. Skin testing *T. mentagrophytes* 62-infected guinea pigs with the four purified DS-glycopeptides, as well as earlier fractions from the purification scheme, derived from *T. mentagrophytes, T. rubrum,* and *M. canis,* again indicated that only the DSI_2-mannopeptides of the two *Trichophyton* species elicited a delayed hypersensitivity reaction. The number of infections or duration of infection had no effect on the size of the skin test response. DSI_2-mannopeptides were non-cross-reactive between genera when tested in *Trichophyton*-immunized or -infected guinea pigs and *Microsporum*-immunized guinea pigs.

Factors that influence the course of dermatophyte infection have long been a subject of investigation. DeLamater and Benham (21-24) suggested a role for cellular immunity in recovery from experimental dermatophyte infection in guinea pigs. More recently Lepper (46) described a similar phenomenon in *Trichophyton verrucosum* infection in cattle. Jones and associates (40) reported that the development of delayed hypersensitivity in experimental *T. mentagrophytes* infection of humans was coincident with resolution of the primary lesion. However, the roles of specific and nonspecific host responses in dermatophytoses have not been clearly established (29).

Only four groups of workers have fractionated dermatophyte extracts and associated immunological response with chemically identifiable components (2, 5-9, 12-19, 26, 30-35, 39, 50, 58). Three chemical classes have been examined for immunological reactivity: nitrogen-free polysaccharides, keratinases, and glycopeptides. Bishop and Blank and associates (2, 13-17, 31-35, 58) isolated water-soluble nitrogen-free polysaccharides, which, though of possible taxonomic significance, did not elicit a delayed or immediate hypersensitivity response in sensitized guinea pigs (58). Yu et al. (63, 64) isolated keratinases from *T. mentagrophytes.* Grappel and Blank and associates (19, 26, 30) found that these keratinases elicited delayed hypersensitivity and inhibited the in vitro migration of sensitized guinea pig peritoneal macrophages. However, keratinases have been isolated only from *T. mentagrophytes.* A number of investigators have indicated the presence of immunologically active glycopeptides in dermatophytes (5-9, 12, 18, 38, 39, 50, 51) and other fungi (3, 28, 44, 45, 54-57). Several of these reports (5, 8, 12, 18, 38, 39, 50) described techniques for extracting and purifying dermatophyte glycopeptides. In a few studies, when skin tested in sensitized animals (6, 8, 12), all of the isolated glycopeptides produced a delayed hypersensitivity response in any animal tested regardless of the sensitizing dermatophytes. Some investigators (5) have not tested their glycopeptide fractions for the capac-

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ity to elicit a delayed hypersensitivity response. Only Basarab et al. (12) reported the extraction of glycopeptides from a strain of *T. rubrum* and *Microsporum canis;* all other investigations have dealt only with the glycopeptides of *T. mentagrophytes.*

We believed that the examination of the glycopeptide class of dermatophyte extracts showed the most promise for an investigation of the immune response to dermatophyte infections. It was first necessary to isolate these substances in relatively pure form and from a variety of dermatophyte species, using more than one strain of each species.

MATERIALS AND METHODS

Organisms. Fungi were isolated from patients with dermatophytoses at University Hospitals Clinics, The Ohio State University, Columbus.

Skin or nail scrapings were cultured on Sabouraud dextrose agar (Difco, Detroit, Mich.) and mycobiotic agar (Difco). Fungal isolates were identified as dermatophytes by growth on *Trichophyton* agars (Difco), urease production (53), and their ability to perforate prepubertal hair (1) as well as by morphological criteria (37). Isolates were found to be free of bacterial contamination by culture in brain heart infusion broth (Difco), Mueller-Hinton broth (Difco), and thioglycolate broth (Difco) at 37 and 25°C. After 2 weeks, each broth was subcultured to solid media to confirm the absence of bacterial contaminants.

Three *T. rubrum* strains (numbers 43, 52, and 70) and two *T. mentagrophytes* strains (numbers 38 and 62) isolated by us from patients with dermatophytosis were selected for study. We also studied *T. mentagrophytes* var. *interdigitale* ATCC 24585, *T. rubrum* ATCC 10218, and an *M. canis* isolated from a case of tinea capitis. All cultures were maintained on Sabouraud dextrose agar.

Growth of submerged cultures and extraction. Starter cultures were prepared by inoculating 20 ml of Center mold medium no. 1 (59) in a 125-ml flask modified to contain glucose 4% (wt/vol) instead of sucrose and by the addition of 1% (wt/vol) casein hydrolysates (Calbiochem, La Jolla, Calif.) and 0.357% (wt/vol) *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES) (Calbiochem), pH 7.0. After 2 weeks of incubation at room temperature, the mycelial mat was ground with a sterile mortar and pestle, and about one-fourth was used to inoculate 3 to 4 liters of Center mold medium no. 1 in a 12-liter Pyrex glass carboy, which was incubated at 27 to 30°C for 7 to 14 days. Agitation was provided by a 7.6-cm Teflon stir bar and magnetic stir plate. The submerged culture was extracted by the technique of Barker and Cruickshank and their associates (6–9, 12, 18). The details are provided, since we encountered difficulty in reconstructing their methods. Cultures were adjusted to pH 6.0 with 1 N HCl (52), and 3 volumes of acetone was then slowly added (A-18, Fisher Scientific Co., Fair Lawn, N.J.). After 18 h at room temperature with continuous agitation, the fluid was discarded. The residue was further dried by suspension in 1 liter of acetone for 2 h. This acetone drying step was repeated twice. The acetone-dried rubbery mass was extracted thrice with ethylene glycol, 40 ml/g (E-178, Fisher), at 37°C with constant agitation for 18 h. Particulate matter was removed by centrifugation (16,300 × *g*, 20 min, 4°C), and the three extracts were pooled. Ethylene glycol was removed by dialysis against distilled water at 4°C, using 12,000-molecular-weight cutoff tubing (Arthur H. Thomas Co., Philadelphia, Pa.). The contents of the dialysis bag were lyophilized. The lyophilized residue was dissolved in a small amount of distilled water and centrifuged to remove any insoluble matter. Three volumes of cold ethanol (U.S.P., U.S. Industrial Chemicals Co., New York, N.Y.) was added. After 18 h at 4°C, the polysaccharide-rich precipitate was collected by centrifugation (4,300 × *g*, 15 min, 4°C) and lyophilized to yield a light tan powder designated crude polysaccharide-rich fraction.

This fraction was dissolved in a minimal amount of 1% (wt/vol) sodium borate ($Na_2B_4O_7 \cdot 10H_2O$; Fisher), pH 8.5, and 5% (wt/vol) cetyltrimethylammonium bromide (CTAB; Sigma Chemical Co., St. Louis, Mo.) was added until visible precipitate no longer formed. The precipitate was removed by centrifugation (4,300 × *g*, 15 min, 4°C) and stored at 4°C (52). The pH of the supernatant was slowly raised to 9.5 with 1 N NaOH. After 18 h at 4°C, the second precipitate was collected by centrifugation (4,300 × *g*, 15 min, 4°C) and combined with the residue from the first centrifugation, and the supernatant was discarded. This polysaccharide-borate-CTAB complex was treated to release polysaccharide-containing material by dissolving the complex in a minimal volume of 2 N acetic acid. The crude polysaccharide was precipitated from solution at a final concentration of 90% ethanol (52). After 18 h at 4°C, the precipitate was collected by centrifugation (4,300 × *g*, 15 min, 4°C) and washed first in 1% (vol/vol) acetic acid in ethanol, then ethanol, and finally diethyl ether (Allied Chemical, Morristown, N.J.). After air drying, the washed residue was dissolved in distilled water. Two milliliters of AG-50, hydrogen form (Bio-Rad Laboratories, Richmond, Calif.), was added and mixed by inversion for 10 min. The resin and any insoluble material was removed by centrifugation (400 × *g*, 15 min, room temperature). The supernatant was dialyzed against distilled water at 4°C. The contents of the dialysis bag were lyophilized to yield a powder designated CTAB fraction. CTAB fractions were stored at −20°C over phosphorus pentoxide in vacuo.

Gel chromatography of CTAB fractions. CTAB fractions were chromatographed on Sephadex G-200 gel (Pharmacia, Uppsala, Sweden) columns. Samples were dissolved and eluted from columns (65 to 90 by 2.5 cm) with water containing 0.02% (wt/vol) sodium azide (Fisher). Flow rates varied from 0.1 to 0.5 ml/min. Ten-milliliter fractions were collected and assayed for carbohydrate by the orcinol-sulfuric acid method (61), and protein was assayed by the method of Lowry et al. (47); their absorbance at 260 nm was also measured. Carbohydrate-protein-containing peaks (SI and SII) were pooled, dialyzed against distilled water at 4°C, lyophilized, and stored at −20°C over phosphorus pentoxide in vacuo.

Ion-exchange chromatography of SI and SII fractions. Sephadex G-200 peaks (SI and SII) were individually rechromatographed on columns of dieth-

ylaminoethylene (DEAE)-Sephadex A-50, chloride form (Pharmacia). The gel was hydrated, and the columns (50 by 1.5 cm) were washed with 0.02 M NaCl containing 0.005 M phosphate buffer, pH 7.0, and the antibacterial agent 0.05% (wt/vol) 1,1,1-trichloro-2-methyl-2-propanol (Eastman Kodak, Rochester, N.Y.). Samples were dissolved in 0.02 M NaCl buffer, applied to the column, and eluted, using either a logarithmic or linear NaCl gradient at pH 7.0. A logarithmic gradient was made by filling chambers 1 to 4 of a Buchler Varigrad (Buchler Instruments, Inc., Fort Lee, N.J.) with 80 ml of 0.02 M NaCl buffer and chamber 5 with 75 ml of a 1.5 M NaCl buffer. A linear gradient was formed by adding 150 ml of 0.02 M NaCl buffer to chamber 1 and 135 ml of 2.0 M NaCl buffer to chamber 2 of a Buchler Varigrad.

Ten-milliliter samples were collected and analyzed by the orcinol-sulfuric acid method. Carbohydrate-containing peaks DSI_1, DSI_2, $DSII_1$, and $DSII_2$ were pooled, dialyzed against distilled water, lyophilized, and stored at −20°C over phosphorus pentoxide in vacuo.

Once the elution pattern on DEAE-Sephadex was established for each organism, replicate samples were eluted stepwise with 100 ml of 0.02 M NaCl buffer followed by 100 ml of 1.5 M NaCl buffer on a 20- by 0.9-cm column. The fractions containing the carbohydrate peaks DSI_1, DSI_2, $DSII_1$, or $DSII_2$ (100 ml each) were dialyzed, lyophilized, and stored as described above.

Chemical analyses of DS fractions. (i) Carbohydrates.

(*a*) *Hydrolysis conditions.* For analyses of total carbohydrate, D-glucose, D-galactose, and thin-layer chromatography (TLC), samples were hydrolyzed at 100°C with 2 N H_2SO_4 in either a Teflon-lined screw-cap or heat-sealed ampoule.

For total hexosamine, samples were hydrolyzed with 2 N HCl in Teflon-lined screw-cap tubes at 95 to 100°C.

Experiments indicated that 4 h was adequate for maximum hydrolysis for all carbohydrate assays.

(*b*) *Monosaccharide assays.* For TLC analysis, acid hydrolysates were passed through a mixed bed column (25 by 10 mm) of alternating AG-1, acetate form, and AG-50, hydrogen form (48). Column eluates were lyophilized, and the residue was dissolved in distilled water to a concentration of 4 μg/μl. Five microliters of each sample was spotted and chromatographed according to Hansen (36) with the following modifications. Silica Gel H containing 7.5% magnesium acetate (Analtech, Inc., Newark, Del.) had been treated for 1 h in 0.5 M NaH_2PO_4 and then dried. After sample loading, these plates were developed twice with isopropanol-acetone-0.1 M lactic acid (4:4:2), with air drying between developments. Resolved monosaccharides were detected by using an orcinol-ferric chloride-sulfuric acid spray (42). Standard mixtures of D-glucose, D-galactose, and D-mannose were included in each TLC experiment.

For enzymatic determinations of monosaccharides, hydrolysates were neutralized with 2 N NaOH. The neutral solutions were assayed for glucose with glucose oxidase (4) (Glucostat special, Worthington Biochemicals, Freehold, N.J.) and for galactose with galactose oxidase (4) (Galactostat, Worthington). These assays were modified by the addition of HEPES buffer (Calbiochem) to a final concentration of 50 mM (pH 7.13).

(*c*) *Total carbohydrate assays.* Acid hydrolysates were directly analyzed by the orcinol-sulfuric acid method as described by Svennerholm (61). Preliminary TLC experiments showed that the experimental samples had varying ratios of glucose, galactose, and mannose. For samples of DSI_1, we referred our data to a hydrolyzed standard curve of D-glucose. All other samples were referred to a standard curve composed of equal weights of D-galactose and D-mannose.

(*d*) *Total hexosamines.* Svennerholm's modification (60) of the Elson-Morgan (27) reaction was used. Authentic D-galactosamine, D-glucosamine, and D-mannosamine (Pfanstiehl Laboratories, Inc., Waukegan, Ill.) were hydrolyzed with each group of samples. Hexosamine values of purified DS fractions were calculated by using a glucosamine standard curve.

(ii) Protein. Total protein was estimated on unhydrolyzed samples by the method of Lowry et al. (47), with crystalline bovine albumin (Sigma) as a standard.

(iii) Phosphorus. Phosphorus was estimated by the method of Bartlett (11, 25), with potassium phosphate (Fisher) as a standard.

(iv) Lipid content of DSII fractions. Lipid was estimated by dissolving 1.5 to 2.0 mg of glycopeptide in 2.0 ml of chloroform-methanol (2:1). After solution, 0.20 ml of 0.15 M NaCl was added with mixing. The upper phase was discarded. The lower phase was dried, and the residue was taken as the lipid content.

(v) Boron. Analyses for boron were done by Schwarzkopf Microanalytical Laboratory, Woodside, N.Y.

(vi) Nucleic acids. The ratio of the absorbance at 280 and 260 nm was determined for two to three batches of 11 DS fractions (one DSI_1, six DSI_2, one $DSII_1$, and three $DSII_2$) at a concentration of 1 mg per ml of distilled water. Bovine serum albumin (BSA; Sigma), calf thymus deoxyribonucleic acid (DNA; Worthington), and a purified cell wall glycoprotein of *Candida albicans* B311, kindly supplied by J. Domer, Tulane University, were analyzed in the same manner. *C. albicans* glycoprotein and BSA were tested at 1 mg/ml; DNA was tested at 0.1 mg/ml.

Statistical methods. Comparisons of the chemical composition of each DS-glycopeptide were made by the Statistics Laboratory at the Ohio State University, using a three-way analysis of variance (10). Significant differences between means were determined by applying the Bonferoni procedure (49), selecting a 0.01 level of confidence.

Sensitization of guinea pigs. Dermatophyte-free guinea pigs were sensitized either by artificial immunization or by inoculation with viable spores. For the former, an ethylene glycol extract (3 mg per 350- to 450-g animal) of *T. mentagrophytes* ATCC, *T. rubrum* ATCC, *T. rubrum* 70, or *M. canis* was suspended in 0.15 M NaCl with 1:5,000 merthiolate (Eli Lilly, Indianapolis, Ind.). An equal volume of the suspension was emulsified in Freund complete adjuvant (BBL, Cockeysville, Md.); 0.1 ml was injected into each footpad, and 0.6 ml was injected in the nape of the neck. Control animals were inoculated with saline-Freund complete adjuvant.

The infected animals were inoculated dermally with

spores of *T. mentagrophytes* 62 according to the method of Tagami et al. (62). The lesions and hair were cultured at 7 days and examined microscopically for evidence of fungal infection.

Skin testing with dermatophyte fractions. (i) Immunized animals. Two weeks after immunization, animals were skin tested with 100 μg of the DSI_1 and DSI_2 derived from the sensitizing dermatophytes in 0.1 ml of merthiolated saline (8). They also received 0.1 ml of Dermatophytin (Hollister-Stier, Seattle, Wash.) and merthiolated-saline control. The diameter of induration was measured at 24, 50, and 72 h.

Selected animals from each group were retested, using the DSI_2 fractions derived from the sensitizing dermatophytes, 4 weeks after immunization. The diameter of induration was measured at 3, 6, 24, 48, 72, and 96 h.

(ii) Infected animals. Two weeks after the appearance of lesions, when the lesion had begun to resolve, one group of animals was skin tested with the DSI_1, DSI_2, $DSII_1$, and $DSII_2$ from *T. mentagrophytes* 62, *T. rubrum* ATCC, and *M. canis*. Others were skin tested with the CTAB, SI, SII, DSI_1, DSI_2, $DSII_1$, and $DSII_2$ fractions of *T. mentagrophytes* 62 or *M. canis*. Noninfected controls were tested similarly.

We wanted to determine the effect of reinfection and duration of infection on the size of skin test responses. In these experiments, there were three groups of infected animals. One group was inoculated 10 and another 2 weeks before skin testing. The third group, reinfected animals, was inoculated 10 weeks and again 2 weeks before skin testing. Each animal, as well as uninfected controls, was skin tested with the DSI_2 from *T. mentagrophytes* 62 (homologous), *T. rubrum* 70, and *M. canis* as well as with Dermatophytin.

All dermatophyte fractions were dissolved in 0.15 M sterile saline (nonpyrogenic; Abbott Laboratories, North Chicago, Ill.) to a final concentration of 200 μg/0.1 ml. Sterile nonpyrogenic saline was used as a control. All skin tests were 0.1 ml, and the diameter of induration was measured at 3, 6, 24, 48, and 72 h.

Histology. Indurated 24-h skin tests of the *T. mentagrophyte* 62 DSI_2 fraction injected into a homologously infected animal were biopsied, and the tissue was fixed, embedded, sectioned, and stained with hematoxylin and eosin by conventional procedures.

RESULTS

Gel chromatography. Analysis of the CTAB fractions of *T. mentagrophytes, T. rubrum,* and *M. canis* on Sephadex G-200 resulted in similar elution patterns for all strains. The upper panels of Fig. 1, 2, and 3 illustrate a typical elution pattern of the CTAB fraction from each of these organisms. The major fraction, which contained carbohydrate and protein, was eluted at the void volume and is always designated SI. This peak was isolated from all CTAB fractions. A second carbohydrate-protein peak, SII, was included on Sephadex G-200 columns and was not always detected. A third prominent peak was generally found, but was composed of little or no protein or carbohydrate and was characterized by a relatively strong absorbance at 260 nm. This material was also not consistently present and was not further analyzed.

Ion-exchange chromatography. Using a linear or logarithmic NaCl gradient as eluant, we found two carbohydrate-containing peaks in both SI and SII fractions analyzed on DEAE-Sephadex A-50 (Cl^-) columns. These fractions are designated DSI_1 and DSI_2 if derived from the SI fraction, and $DSII_1$ and $DSII_2$ if derived from the SII fraction.

The DSI_1 fractions were eluted between 0.02 and 0.10 M NaCl, and the DSI_2 fractions were eluted between 0.2 and 0.7 M NaCl. The $DSII_1$ fractions were eluted at 0.02 M NaCl, and the $DSII_2$ fractions were eluted between 0.05 and 0.45 M NaCl.

Table 1 reflects the mass of material (percentage of dry weight) extracted from the acetone residues and present in each step of the purification process. We generally obtained about 40 to 50 g of starting acetone residue per batch. For example, in the case of the DSI_2 fraction of *T. mentagrophytes,* we recovered only 0.032%, which represents an average yield of 16 mg.

Chemical analyses of DS fractions. We wanted to know whether the chemical content of the DS fractions differed between or within species. The results of quantitative chemical analyses of the DS fractions are shown in Table 2 as the mean percentage of dry weight. From these data we see that all DS fractions had a relatively large carbohydrate content (42.5 to 81.6%), with less protein (4.3 to 11.3%) and generally smaller amounts of phosphorus (0.4 to 6.0%) and hexosamines (0.3 to 0.6%).

Qualitative TLC of acid hydrolysates of the DS fractions of each dermatophyte strain indicated the presence of glucose, galactose, and mannose as the predominant monosaccharides. The DSI_1 fractions (Fig. 4A, B) appeared to contain predominantly glucose, and the DSI_2 fractions (Fig. 4C, D) predominantly mannose. In similar analyses, the $DSII_1$ and $DSII_2$ fractions were composed of predominantly mannose and equal amounts of mannose and galactose, respectively. The $DSII_2$ fractions of *T. rubrum* 52, 70, and ATCC also contained trace amounts of a fourth unidentified sugar that migrated ahead of mannose in this system. No attempt was made to identify this sugar.

Quantitative specific enzymatic assays for D-glucose and D-galactose confirmed that the DSI_1 fractions from *T. rubrum* and *M. canis* were predominantly glucose (Tables 2 and 3). The DSI_1 fractions of *T. mentagrophytes* were composed of equal amounts of glucose and mannose. The DSI_2 and the DSII fractions of all strains contained mostly mannose. There was a significant amount of unidentified mass (10.9 to

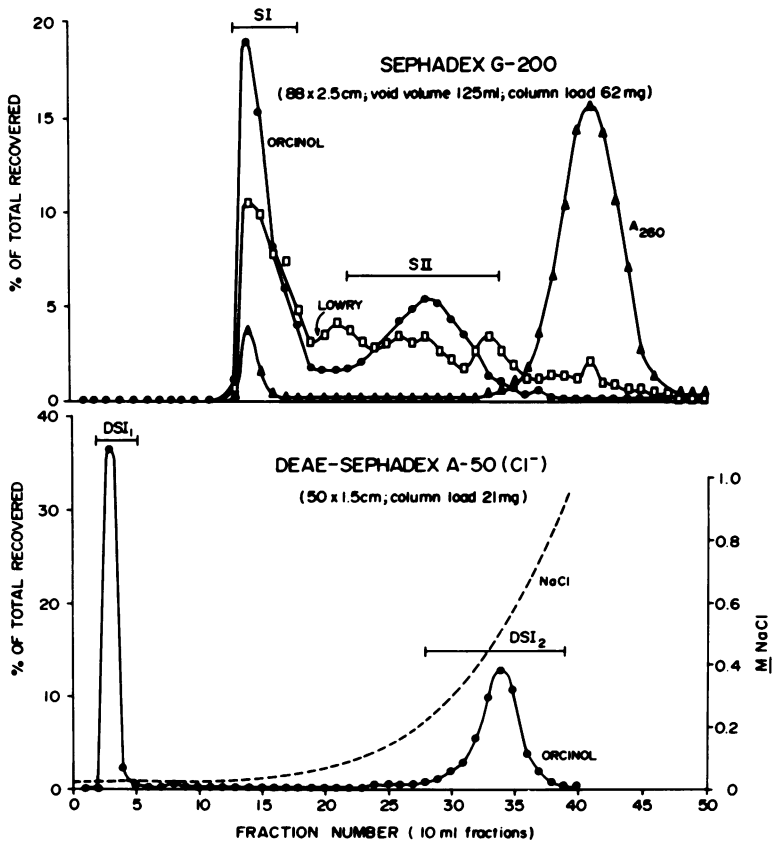

FIG. 1. *Sephadex G-200 and DEAE-Sephadex A-50 (Cl^-) chromatography: T. mentagrophytes 62.*

48.5%) as deduced from total dry weights.

Statistical analyses failed to indicate a significant difference in the total carbohydrate, hexosamine, protein, or phosphorus content between the DS fractions of *T. mentagrophytes, T. rubrum,* or *M. canis.* However, the DSI_1 of *T. mentagrophytes* contained significantly larger amounts of glucose and mannose than the DSI_1 of either *T. rubrum* or *M. canis.* When statistical comparisons were made between the DS fractions of *T. mentagrophytes,* the DSI_1 could be distinguished from the DSI_2, $DSII_1$, and $DSII_2$ fractions. Similarly, the DSI_1 fractions of *T. rubrum* and *M. canis* were distinct from the other DS fractions isolated from each organism. These differences were in the larger total carbohydrate content and predominance of glucose in DSI_1 fractions compared with less total carbohydrate and the predominance of mannose in DSI_2, $DSII_1$, and $DSII_2$ fractions.

The lipid content as percentage of dry weight of the $DSII_1$ fractions from *M. canis, T. mentagrophytes* ATCC, and *T. rubrum* 43 was 2.2, 1.5, and 5.2, respectively. The lipid content of the $DSII_2$ fractions from *T. rubrum* 70 and *T. mentagrophytes* 38 was less than 0.01 and 7.9, respectively. The mean value for these five samples was 3.36 (standard deviation [SD], 3.16).

Three samples of DSI_2 from *T. mentagrophytes* were analyzed for their boron content: one sample from strain ATCC and a sample from two different batches from strain 38. The reported results of the boron content as the percentage of dry weight were 0.32, 0.22, and 0.24, respectively (mean, 0.260; SD, 0.053).

The ratios of the absorbance at 280 and 260 nm were determined for 20 DS fractions as well as for DNA, BSA, and *Candida* glycoprotein. The mean ratio for the DS fractions was 0.80 (SD, 0.10), compared with values of 0.55 for DNA, 0.76 for BSA, and 0.96 for the *Candida* glycoprotein.

Skin testing with DS fractions. (i) Immunized animals. To determine the biological activity of these glycopeptides, we first immunized guinea pigs with the ethylene glycol extracts of *T. mentagrophytes* ATCC, *T. rubrum* ATCC or 70, or *M. canis.* We skin tested each of these animals with the DSI_1 and DSI_2 fractions isolated from the dermatophytes that had been

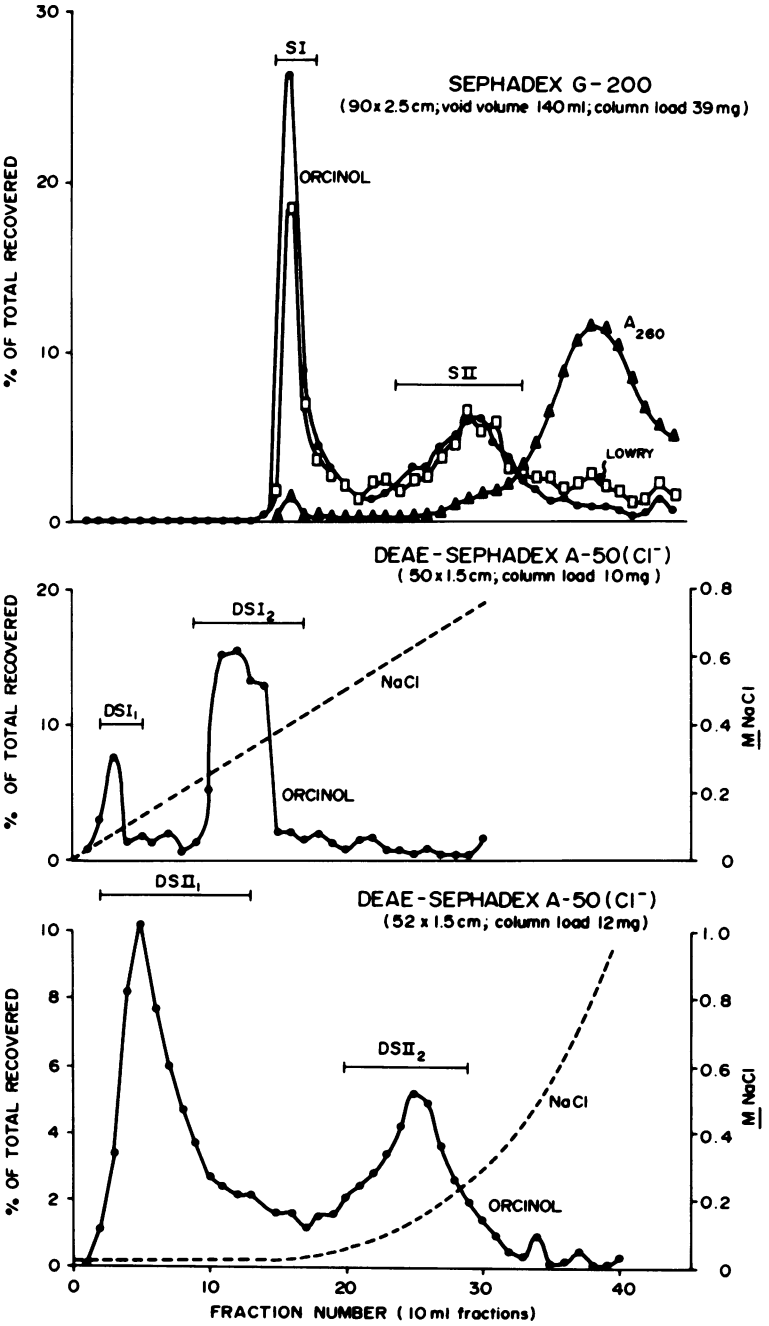

FIG. 2. *Sephadex G-200 and DEAE-Sephadex A-50 (Cl^-) chromatography: T. rubrum ATCC.*

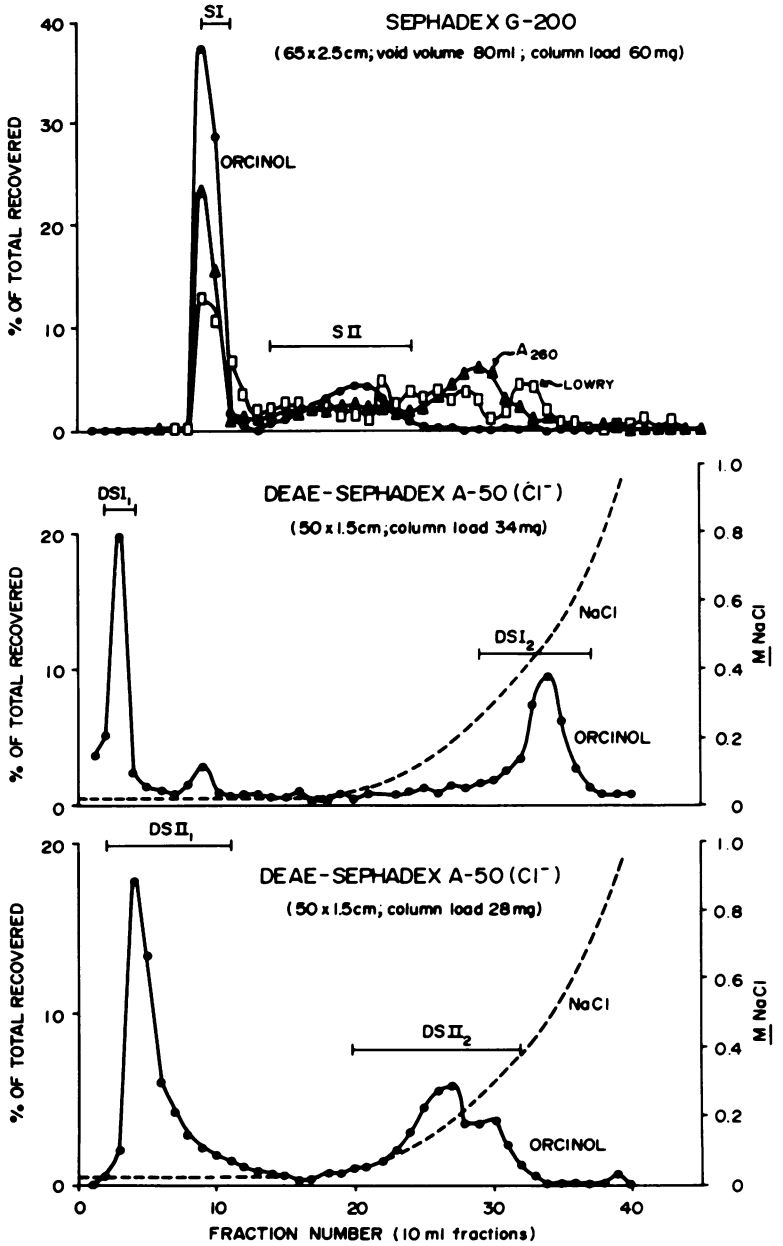

FIG. 3. *Sephadex G-200 and DEAE-Sephadex A-50 (Cl^-) chromatography: M. canis.*

TABLE 1. *Mean fraction yields (percentage of dry weight of starting acetone residues)*[a]

Dermatophyte	Dermatophyte fraction							
	Ethylene glycol extract	CTAB	SI	DSI_1	DSI_2	SII	$DSII_1$	$DSII_2$
T. mentagrophytes[b]	2.32 ± 2.02[c] (11)[d]	0.118 ± 0.090 (13)	0.051 ± 0.003 (7)	0.017 ± 0.016 (3)	0.032 ± 0.026 (4)	0.024 ± 0.021 (8)	0.008 ± 0.002 (3)	0.010 ± 0.007 (3)
T. rubrum[e]	1.06 ± 1.07 (9)	0.114 ± 0.077 (11)	0.055 ± 0.042 (10)	0.028 ± 0.021 (6)	0.019 ± 0.014 (8)	0.018 ± 0.009 (9)	0.007 ± 0.001 (3)	0.005 ± 0.0 (3)
M. canis	ND[f]	0.073 ± 0.024 (4)	0.032 ± 0.016 (4)	0.026 ± 0.009 (3)	0.007 ± 0.002 (3)	0.023 ± 0.006 (4)	0.008 (2)	0.003 (2)

[a] We generally started with 50 g of acetone residue.
[b] Strains 38, 62, and ATCC 24585.
[c] Mean ± standard deviation.
[d] Number of different batches.
[e] Strains 43, 52, 70, and ATCC 10218. Acetone residue weights for strain 70 are estimated.
[f] Not done.

used to immunize them. The results of these skin tests read at 50 h are shown in Table 4.

Though we took an induration of greater than 5 mm in diameter to indicate a positive response to the DS fraction, in this table we report the actual measured values. The value of 5 mm is the mean (plus 2 SD) induration observed after skin testing with these DS fractions in nonimmunized animals.

The only DSI_1 fraction to elicit a response in any animal was isolated from *M. canis,* and then only when tested in *T. mentagrophytes* ATCC-immunized animals. The DSI_2 of *T. mentagrophytes* and *M. canis* only reacted in animals immunized with ethylene glycol extracts from the homologous organism. The DSI_2 fractions of *T. rubrum* ATCC and 70 showed less specificity since they reacted in animals immunized with either the *T. mentagrophytes* ATCC or their homologous *T. rubrum,* eliciting no response in animals immunized with *M. canis.* The Dermatophytin did not elicit a response in any animal.

These skin tests had been read between 24 and 50 h, and we questioned whether the positive DSI_2 responses were the result of a strong Arthus reaction related to the injection of a large amount of carbohydrate-containing material. To test this, one animal from each ethylene glycol group, which had a positive skin test at 2 weeks post-immunization, was retested with the same DSI_2 fraction 2 weeks later. Each skin test was examined at 3, 6, 24, 48, 72, and 120 h. Early responses (3 and 6 h) were minimal (4 to 5 mm) except for the DSI_2 of *M. canis.* This preparation elicited 5- to 18-mm responses in immunized as well as control animals. It appeared that this preparation was toxic to the animals. The late responses again peaked at 48 h, with the diameter of induration of positive reactions ranging from 5 to 20 mm. We found no clear evidence of an Arthus-type response to these preparations. That is, for the majority of the skin tests there was no measurable response at 3 h, a time when a strong Arthus response should have been visible. The only two skin tests that were positive at 3 h were to the injection of saline in control animals and to the *T. rubrum* 70 DSI_2-mannopeptide tests in the *T. rubrum* 70-immunized animals (data not shown). Since this latter response decreased to zero by 6 h, it may have represented an Arthus reaction, but it did not contribute to the induration we observed at 24 or 48 h. The saline response in a control animal was observed in only 1 of 40 animals tested and may represent slow absorption of the test injection.

The DSI_2 from *T. rubrum* 70 produced a relatively strong delayed hypersensitivity response in animals immunized with *T. rubrum* 70

TABLE 2. *Summary of the chemical content of dermatophyte DS-glycopeptides*[a]

Glycopeptide	Carbohydrate				Total hexosamines	Protein	Phosphorus	Unidentified
	Total	Glucose[b]	Galactose[b]	Mannose[b]				
T. mentagrophytes[c]								
DSI_1	81.6 ± 12.2	37.1 ± 30.6	4.2 ± 4.7	46.1 ± 33.1	0.6 ± 0.1	5.8 ± 3.1	1.1 ± 1.0	10.9
DSI_2	50.6 ± 12.6	2.3 ± 0.5	3.6 ± 2.6	42.9 ± 11.5	0.6 ± 0.1	7.8 ± 1.8	5.4 ± 3.6	35.6
$DSII_1$	72.6 ± 4.0	7.1 ± 3.4	16.3 ± 1.7	49.2 ± 1.8	0.4 ± 0.4	6.3 ± 2.5	0.6 ± 0.3	26.1
$DSII_2$	63.3 ± 9.4	3.3 ± 1.8	14.7 ± 4.6	45.3 ± 6.9	0.5 ± 0.1	7.3 ± 1.2	1.2 ± 0.3	27.7
T. rubrum[d]								
DSI_1	78.6 ± 16.3	60.9 ± 34.5	2.3 ± 3.1	12.2 ± 22.3	0.4 ± 0.3	5.6 ± 4.7	0.4 ± 0.4	15.0
DSI_2	53.3 ± 10.5	1.7 ± 0.9	4.6 ± 2.4	46.3 ± 10.3	0.3 ± 0.2	9.1 ± 2.3	4.7 ± 4.2	32.6
$DSII_1$	60.9 ± 15.0	10.3 ± 4.8	13.3 ± 5.1	37.3 ± 7.0	0.5 ± 0.1	8.4 ± 7.6	0.7 ± 0.4	29.5
$DSII_2$	48.0 ± 11.9	2.6 ± 0.4	8.9 ± 2.6	36.4 ± 9.6	0.5 ± 0.1	11.3 ± 4.1	2.3 ± 1.3	37.9
M. canis								
DSI_1	81.2 ± 16.0	74.0 ± 15.6	0.7 ± 1.0	8.5 ± 7.4	0.5 ± 0.1	4.2 ± 3.2	0.6 ± 0.6	13.5
DSI_2	43.4 ± 19.0	3.7 ± 1.2	2.2 ± 2.1	29.2 ± 10.4	0.5 ± 0.4	10.0 ± 4.1	6.0 ± 4.8	40.1
$DSII_1$[e]	77.2	4.3	25.6	47.3	0.5	6.6	0.3	15.4
$DSII_2$[e]	42.5	3.4	11.0	28.1	0.4	7.3	1.3	48.5

[a] Mean percentage of dry weight ± standard deviation; 3 to 11 different batches of DS-glycopeptides.
[b] Percentage of total carbohydrate ± standard deviation.
[c] Strains 38, 62, and ATCC 24585.
[d] Strains 43, 52, 70, and ATCC 10218.
[e] Only one pooled sample.

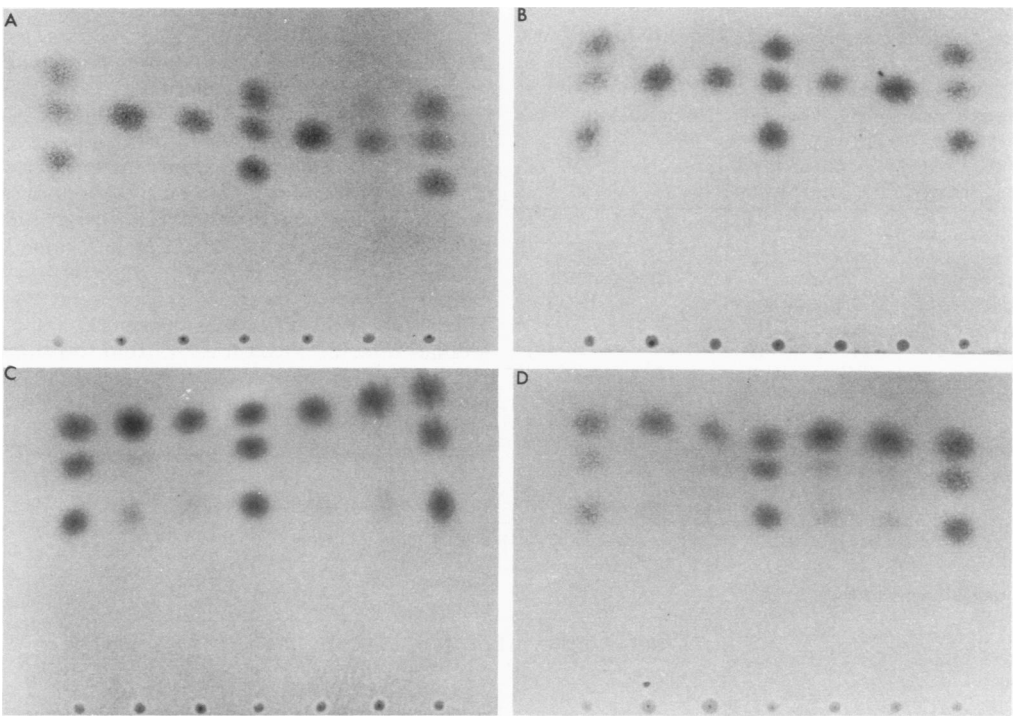

FIG. 4. *TLC of the acid hydrolysates of DSI-glycopeptides. (A) Lanes 1, 4, and 7 contain standard mixture of monosaccharides. From top:* D-*mannose,* D-*glucose,* D-*galactose. Lanes 2, 3, and 5 contain T. mentagrophytes* DSI_1*: strains 38, 62, and ATCC 24585, respectively. Lane 6 contains M. canis* DSI_1*. (B) Lanes 1, 4, and 7 contain a standard mixture of monosaccharides as in (A). Lanes 2, 3, 5, and 6 contain T. rubrum* DSI_1*: strains 43, 52, 70, and ATCC 10218, respectively. (C) Lanes 1, 4, and 7 contain standard mixture of monosaccharides as in (A). Lanes 2, 3, and 5 contain T. mentagrophytes* DSI_2 *from strains as in (A). Lane 6 contains M. canis* DSI_2*. (D) Lanes 1, 4, and 7 contain standard mixture of monosaccharides as in (A). Lanes 2, 3, 5, and 6 contain T. rubrum* DSI_2 *from strains as in (B).*

or ATCC or with *T. mentagrophytes*. This confirmed the nature of our observations reported in Table 4. The DSI_2 from *T. rubrum* ATCC produced a much weaker response in all animals. The DSI_2 from *T. mentagrophytes* ATCC did not elicit a response in either animal tested.

(ii) Infected animals. We recovered only the morphologically identical fungus from skin scrapings and hairs from the developing lesions. Microscopic examination revealed the presence of fungal elements in the scales and hairs (Fig. 5).

In a preliminary titration in an animal infected once, the injection of 200, 100, 50, and 10 μg of homologous DSI_2 elicited 15-, 10-, 7-, and 0-mm-diameter indurations, respectively, at 24 h. Two hundred micrograms was used as the amount of DS-glycopeptides, and earlier fractions, in most skin tests.

The mean indurations using the DS-glycopeptides of three dermatophytes in uninfected control and infected guinea pigs are shown in Table 5. Only the DSI_2 fractions derived from *T. mentagrophytes* 62 and *T. rubrum* ATCC elicited a delayed response at 24 h in animals infected with *T. mentagrophytes* 62. These data support our earlier results with immunized animals which indicated that the DSI_2 fractions were relatively more reactive. Reactions measured at 3 and 6 h do not support the presence of a strong Arthus response, which could have contributed to the 24-h measurements.

To determine whether a more reactive component had been lost during the isolation of the DS fractions, additional groups of animals were tested with intermediate fractions that had been prepared during the isolation sequence. The homologous DSI_2 elicited the strongest delayed hypersensitivity reaction. The induration at 24 h (9 mm) was larger than that produced by an equal mass of any fraction produced earlier in the purification scheme. The homologous SI fraction gave a response (6 mm) that peaked at 48 instead of 24 h. At 3 and 6 h, fractions from *T. mentagrophytes* and *M. canis* gave responses up to 6 mm in diameter in infected animals. Control animals gave similar but smaller values.

We also tested guinea pigs that had been infected twice before skin testing (Table 6). In these experiments, guinea pigs infected with *T. mentagrophytes* 62 were skin tested with the DSI_2-glycopeptides from the homologous organism, *T. rubrum* 70, and *M. canis*. The animals were also skin tested with Dermatophytin. We found no evidence for an Arthus reaction with the DSI_2 from *Trichophyton* spp. There was a suggestion of an Arthus reaction with the DSI_2 from *M. canis*. At 24 h there was evidence for delayed-type hypersensitivity when the glycopeptide from the homologous organism and from *T. rubrum* was used, but very little induration from *M. canis*. This preparation of DSI_2 from *T. rubrum* apparently was irritating as evidenced by the responses at 3 and 6 h. Skin tests with Dermatophytin showed no responses at 24 h. There was little difference in the response of animals injected with the DSI_2 from the homologous *T. mentagrophytes* whether they were infected 2 or 10 weeks or even twice before skin testing.

Histology. The histology of the biopsies from

TABLE 3. *Mean monosaccharide ratios of DS-glycopeptides (glucose/galactose/mannose)*

Source of DS-glycopeptide	Fraction			
	DSI_1	DSI_2	$DSII_1$	$DSII_2$
T. mentagrophytes[a]	1:T[b]:1	1:2:19	1:2:7	1:4:14
T. rubrum[c]	1:T:T	1:3:27	1:1:4	1:3:14
M. canis	1:T:T	1:1:8	1:6:11	1:3:8

[a] Strains 38, 62, and ATCC 24585.
[b] T, Trace.
[c] Strains 43, 52, 70, and ATCC 10218.

TABLE 4. *Mean induration 50 h after skin test of immunized guinea pigs*[a]

Immunizing substance[b]	Mean induration (diam in mm) with given skin test substance[c]									
	Control (saline)	*T. mentagrophytes* ATCC		*T. rubrum* ATCC		*T. rubrum* 70		*M. canis*		Dermatophytin[d]
		DSI_2	DSI_2	DSI_2	DSI_2	DSI_1	DSI_2	DSI_1	DSI_2	
Control (saline)	1	2*	3	ND[e]	3	0	2	2	3*	0
T. mentagrophytes ATCC	0	3*	8	ND	7	3	10	6	5*	0
T. rubrum ATCC	0	1*	1	ND	7	1	5	1	0*	0
T. rubrum 70	0	ND	1	ND	2	3	7	0	ND	0
M. canis	0	ND	2	ND	0	0	0	2	9*	0

[a] Three guinea pigs except as noted (*), where only one guinea pig was tested.
[b] Ethylene glycol extracts.
[c] 100 μg in 0.10 ml of saline.
[d] 0.10 ml of undiluted commercial Dermatophytin.
[e] ND, Not done.

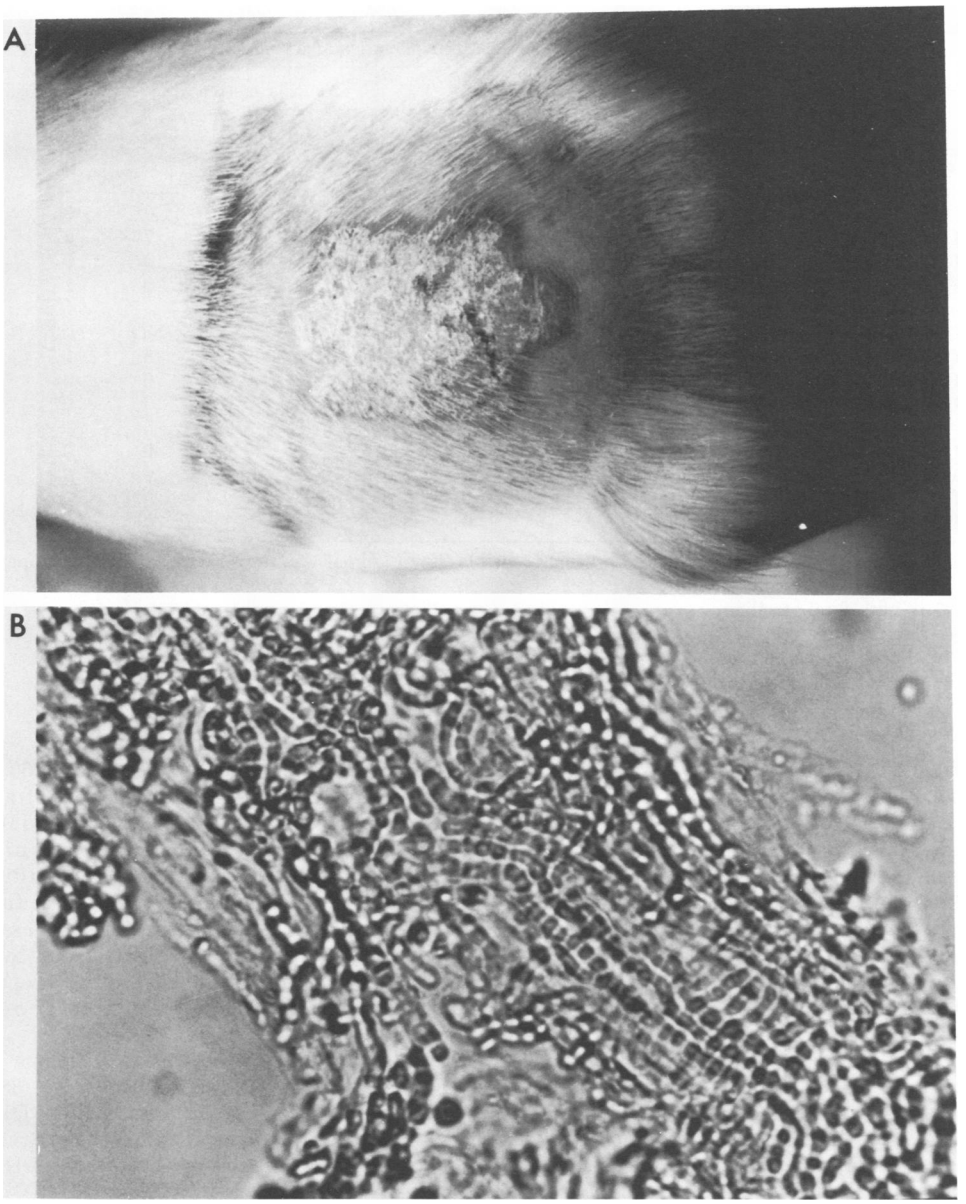

FIG. 5. *(A) Experimental T. mentagrophytes 62 infection of guinea pig shown on day 9 of infection. (B) Skin scraping from lesion shown in (A) (×360).*

indurated skin tests was compatible with a delayed hypersensitivity reaction. Compared with control sections, we saw a relatively moderate cellular infiltrate, which was predominantly mononuclear.

DISCUSSION

We selected an isolation procedure using ethylene glycol extraction because it is a relatively mild treatment that has been shown to yield fractions with immunological activity from fungi (6–8, 18, 20, 26). The use of ethylene glycol is supported by the work of Kaaman et al. (41). Comparing the response of guinea pigs immunized with *T. mentagrophytes* and skin tested with an ethylene glycol or phenol extract from *T. mentagrophytes*, they found that the ethylene glycol extract gave the most consistent results and showed no tendency to produce toxic reactions.

TABLE 5. *Mean induration of skin tests in infected and uninfected guinea pigs*[a]

Skin test substance[b]	Mean induration (diam in mm)									
	Uninfected animals[c]					Animals infected with *T. mentagrophytes* 62[d]				
	3[e]	6	24	48	72	3	6	24	48	72
Control (saline)	0	0	0	0	0	0	0	0	0	0
T. mentagrophytes 62										
DSI_1	2	0	0	0	0	1	0	0	0	0
DSI_2	4	0	0	0	0	3	0	7	6	4
$DSII_1$	0	0	0	0	0	1	0	0	0	0
$DSII_2$	0	0	0	0	0	0	0	0	2	1
T. rubrum ATCC										
DSI_1	ND[f]	ND	ND	ND	ND	ND	ND	ND	ND	ND
DSI_2	1	1	0	0	0	3	1	8	7	4
$DSII_1$	4	4	0	0	0	0	0	0	0	0
$DSII_2$	2	1	0	0	0	0[g]	0[g]	0[g]	0[g]	0[g]
M. canis										
DSI_1	1	1	0	0	0	3	0	0	0	0
DSI_2	1	0	0	0	0	3	0	0	0	0
$DSII_1$	1	1	0	0	0	1	0	0	0	0
$DSII_2$	3	3	0	0	0	0	1	0	0	0

[a] Skin test given 14 days after infection became visible. Each animal was tested with four DS-glycopeptides from three different dermatophytes.
[b] 200 μg in 0.10 ml of saline.
[c] Four animals tested.
[d] Three animals tested unless otherwise noted.
[e] Hours after skin test.
[f] ND, Not done.
[g] One animal.

Sephadex G-200 chromatography of all CTAB fractions yielded similar elution patterns. Each isolate consistently demonstrated a prominent excluded peak, SI, containing carbohydrate and protein. In most samples, SI was followed by another carbohydrate- and protein-containing peak, SII. Barker and associates (6–8, 18, 20, 26), using Sephadex G-100 rather than G-200, found only one homogeneous peak in their CTAB fractions. Using Sephadex G-100, Basarab et al. (12) found two peaks in the eluants from the CTAB fractions of *T. mentagrophytes* and *T. rubrum*. Nozawa et al. (50, 51) used Sephadex G-100 to separate two fractions that they isolated from phenol-water extracts of *T. mentagrophytes*. However, their separation on Sephadex G-100 was apparently poor, and their excluded peak, which was the only one they studied further, was probably contaminated by their included peak. Arnold et al. (5) found only a single carbohydrate peak from trichloroacetic acid extracts of *T. mentagrophytes* when analyzed on Sephadex G-200. Our preliminary experiments indicated that Sephadex G-200 columns were the most efficient means of consistently separating the two carbohydrate-protein peaks, SI and SII, in our CTAB fractions.

In addition, we have demonstrated and readily separated a third peak on Sephadex G-200 which apparently accounts for very little mass but absorbs in the ultraviolet (maximum at 260 nm). Varying amounts of this visibly colored fraction were found in all strains tested. It was our opinion that proper conditions for the separation of this peak were necessary. Small quantities of this material could significantly and artificially contribute to protein determination if assayed by ultraviolet absorption as performed by Nozawa et al. (50, 51). Arnold et al. (5) also reported an apparently similar pigment from *T. mentagrophytes*.

Many different glycopeptide fractions have been isolated from dermatophytes. Ito (38, 39) was able to separate 22 fractions by DEAE-Sephadex chromatography of phenol extracts of *T. mentagrophytes*. Nozawa et al. (50, 51) subdivided their excluded Sephadex G-100 peak into seven fractions on a borate-treated column of DEAE-cellulose. Arnold et al. (5) found five subfractions of their Sephadex G-200 peak. Using a procedure analogous to ours, Barker and associates (6–8, 18, 20, 26) found two or three peaks when analyzing their excluded G-100 fraction. In contrast, we were always able to demonstrate only two peaks on DEAE-Sephadex columns from either the SI (excluded) or SII (included) G-200 fractions derived not only from *T. mentagrophytes* but also from *T. rubrum* and *M. canis*. We, therefore, could reproducibly isolate four post-DEAE-Sephadex fractions from

each strain of each species examined. The chromatographic properties of these fractions indicate that we have eluted two fractions, DSI_2 and $DSII_2$, which were not isolated by Barker and associates (6–8, 18, 20, 26) and apparently not by others (5, 38, 39, 50, 51).

We examined each of the DS fractions chemically. These fractions contained mostly carbohydrate, less protein, and relatively small amounts of phosphorus and hexosamines and are classified as glycopeptides: DSI_1, DSI_2, $DSII_1$, and $DSII_2$. We found no chemical differences between the DSI_1 fractions from one species and the DSI_1 fractions from any other species. Similarly, the DSI_2, $DSII_1$, and $DSII_2$ fractions from any one species were statistically indistinguishable from the same fraction of any other species. We did, however, show that the DSI_1 class of glycopeptides was different from the other three classes of glycopeptides. The carbohydrate component of the DSI_1-glycopeptides is predominantly glucose, and in the other glycopeptides it is predominantly mannose. Therefore we feel that these glycopeptides can be classified as either glucopeptides or mannopeptides.

We attempted to chemically fractionate each DS fraction and were unable to identify a significant amount of material. We considered that the unidentified fractions might contain boron or CTAB. Boron assays could only account for 0.26% of the unidentified mass. If this amount of boron as borate were complexed to CTAB, we calculate that no more than 7% of the dry weight of these fractions could be borate and CTAB.

It is known that skin testing with CTAB elicits a toxic response in humans but not guinea pigs (9). In a human volunteer whom we skin tested with the DSI_2 fractions of *T. mentagrophytes*, *T. rubrum*, and *M. canis*, we observed a reaction that was not attributable to either immediate or delayed hypersensitivity. This supports the possibility that the unidentified portion of our DS-glycopeptide may have contained CTAB. We calculated that the maximum amount of CTAB present in each of our human skin tests was no more than 0.7 μg.

The orcinol method, used for the determination of total carbohydrates, varies in its response to different individual monosaccharides; therefore, we first determined the monosaccharides present and their approximate ratios in each DS-glycopeptide by TLC. To minimize error, we used these data in order to choose the appropriate monosaccharides for a standard in our assay of each DS fraction.

Lipid or nucleic acids might constitute part or all of the unidentified substance present in our preparations. We could attribute no more than 3.61% of the material as lipid mass. Although no

TABLE 6. *Mean induration of skin test with DSI_2 fractions in T. mentagrophytes 62-infected and uninfected guinea pigs*[a]

Skin test substance[b]	Mean induration (diam in mm)									
	Uninfected animals					Infected animals[c]				
	3[d]	6	24	48	72	3	6	24	48	72
T. mentagrophytes 62	6	2	0	0	0	7	4	8	4	2
T. rubrum 70[e]	8	8	0	0	0	ND[f]	ND	ND	ND	ND
M. canis	5	1	0	0	0	4	4	1	0	0
Dermatophytin	1	0	0	0	0	1	0	0	0	0
Saline	3	0	0	0	0	0	0	0	0	0
Skin test substance[b]	Infected animals[g]					Multiply infected animals[h]				
	3	6	24	48	72	3	6	24	48	72
T. mentagrophytes 62	7	1	7	6	5	6	8	10	6	4
T. rubrum 70[e]	ND	ND	ND	ND	ND	4	5	5	4	3
M. canis	3	2	0	0	0	7	4	1	0	0
Dermatophytin	2	0	0	0	0	1	1	0	0	0
Saline	0	0	0	0	0	0	0	0	0	0

[a] Three to four animals tested.
[b] 200 μg in 0.10 ml of saline.
[c] Skin test given 2 weeks after infection became visible.
[d] Hours after skin test.
[e] Two animals tested.
[f] ND, Not done.
[g] Skin test given 10 weeks after infection became visible.
[h] Skin test given 10 weeks after first infection became visible. Infected for the second time 2 weeks before the skin testing.

quantitative assay for nucleic acids was performed, we did compare the absorbance ratio (280/260 nm) of DS fractions with that of materials of known composition. We found that the DS fraction ratios were more comparable to those obtained with protein-containing substances (BSA, *Candida* glycoprotein) than with purified DNA. Regardless of the magnitude of the unidentified fraction, this ratio was relatively constant.

We conclude that the DS fractions may contain trace amounts of boron, CTAB, lipid, and/or nucleic acids. We presume that the bulk of the unidentified fraction is protein and carbohydrate not detected by our techniques.

In experiments with immunized animals, our data suggest that only the DSI_2-mannopeptides elicited a delayed hypersensitivity response (6- to 10-mm mean diameter), whereas the DSI_1-glucopeptides tested at the same dosage did not. This is contrary to the reports of Barker and coworkers (6, 8, 12), who found delayed hypersensitivity responses to all of their glycopeptides with the exception of one from *M. canis* tested in *M. canis*-immunized animals. It is our thought that the glycopeptides isolated by these workers were possibly contaminated with the mannopeptides we identify as DSI_2.

Apparently in only one other investigation (43) were isolated dermatophyte glycopeptides skin tested in infected animals. In our experiments, we skin tested uninfected and infected animals as the lesions regressed, using the four DS-glycopeptides isolated from *T. mentagrophytes* 62, *T. rubrum* ATCC, and *M. canis* (Table 5). Only the DSI_2 fractions of *T. mentagrophytes* 62 and *T. rubrum* elicited a delayed hypersensitivity response in *T. mentagrophytes*-infected animals. Hence the DSI_2-mannopeptide from *T. rubrum* showed cross-reaction in *T. mentagrophytes*-infected animals. Since the DSI_2-mannopeptide from *M. canis* did not react, these findings suggest that there is genus specificity in the delayed reaction to the DSI_2-mannopeptides.

We also tested all DS-glycopeptides and their parent fractions from *T. mentagrophytes* 62 and *M. canis* in infected and uninfected guinea pigs. Barker and associates (6, 8, 12) generally found a loss in skin reactivity in their more purified products. In our work, no subfraction from the earlier steps of the purification scheme elicited a response equal to the purified DSI_2. We did see a delayed hypersensitivity response to the *T. mentagrophytes* SI from which this DSI_2 was derived. Therefore the specific activity of our *T. mentagrophytes* 62 fractions increased with each purification step.

We found that the response of *T. mentagrophytes*-infected guinea pigs to the DSI_2-mannopeptides was essentially the same whether the animals were tested 2 or 10 weeks after infection or even if the animals were infected twice.

Finally, we recognize that the number of animals in each skin test group was small (two to four). However, we found a delayed hypersensitivity reaction to our DSI_2 fractions in each of four different experiments. We believe that within the confines of the experimental design, the use of small groups of animals in four experiments showing reproducibility was more desirable than performing fewer experiments with a larger group size. This in vivo assay of immunological reactivity is qualitative, and in most cases the differences between negative responses (0 mm) and those we called positive delayed hypersensitivity (6 to 10 mm) was clear and reproducible.

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