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Immunochemical Studies on the Human Pathogen *Sporothrix schenckii*: Effects of Chemical and Enzymatic Modification of the Antigenic Compounds upon Immediate and Delayed Reactions

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The rhamnose-containing polysaccharide-peptide compound derived from the cells of the pathogenic fungus *Sporothrix schenckii* has been shown to contain 87.1% carbohydrate and 12.5% peptide and to give rise to both immediate- and delayed-type reactions in sensitized guinea pigs. The capacity to induce immediate-type reaction, passive cutaneous anaphylaxis, was completely lost by degradation of the carbohydrate moiety by periodate, whereas the ability to induce the delayed-type reactions of migration inhibition (in vitro) and the sporotrichin reaction (in vivo) were only slightly affected by periodate treatment. On the other hand, delayed "reactivities" to the compound were considerably reduced by treatment with papain, whereas the immediate-type reaction remained positive. These results lead to a conclusion that the rhamnose-containing polysaccharide of the polysaccharide-peptide antigenic compound plays an important role in the immediate-type reaction, whereas the peptide is largely responsible for the delayed-type reaction.

The chemistry of numerous fungal polysaccharides and glycoproteins has been studied intensively and some comprehensive reviews have appeared (5, 17); however, there is little information concerning the chemistry of human pathogenic fungi (1, 3, 4, 11). *Sporothrix schenckii* is the agent of sporotrichosis which usually causes a subcutaneous infection in man; a definite diagnosis of this mycotic disease has been chiefly made by means of routine mycological procedures. In addition, some immunological diagnoses by skin tests or serological techniques have also been available. The recognition of the importance of the hypersensitivity reaction in sporotrichosis was first due to the work of Widal in 1910 (30). Because the increasing incidence of sporotrichosis is recognized (31), there is urgent need for a more reliable antigen for immunological tests. To date, the crude antigen from concentrated culture filtrates of *S. schenckii* has been commonly used for the serological diagnosis of patients having sporotrichosis (14), and little attention has been given to the relationship between the chemical composition and the immunological activity of antigenic compounds existing in the cells of *S. schenckii* (2, 15, 20).

Our previous paper (19) has reported the isolation and purification of immunologically active polysaccharide-peptide compounds from *S. schenckii* which have been shown to induce both immediate and delayed hypersensitivity reactions (20). In the present study, attention was focused on elucidating the relationship between the chemical composition of the antigen and immediate and delayed hypersensitive reactivities in experimental sporotrichosis. For this purpose, modifications of polysaccharides or peptides moieties of the antigenic compound derived from *S. schenckii* cells were performed; the immunological activities of the modified antigens were examined in sensitized guinea pigs using several criteria: the precipitin test, the migration inhibition test, the sporotrichin test, and passive cutaneous anaphylaxis.

MATERIALS AND METHODS

Organisms. A strain of *S. schenckii* was cultured at 27 C in Sabouraud broth for 4 weeks on a surface culture. The fungal mats were separated from the culture medium by filtration and washed repeatedly with distilled water.

Extraction and purification of antigenic substances. The antigenic substances in this study were extracted according to the method previously described (19) (Fig. 1). Antigenic substances were extracted with 45% phenol (29), and a crude polysaccharide fraction was further purified by chromatography on a Sephadex G-100 column. The main fraction

passing through the column in the void volume was designated CP.

Modification of the CP fraction. For digestion of the peptide moiety in the CP fraction, the CP and papain were dissolved in acetate buffer (pH 5.7) containing 5 mM cysteine-hydrochloride and 1 mM ethylenediaminetetraacetic acid to give 0.5 and 0.01% concentrations, respectively, and incubated at 37 C for 48 h. Gel filtration on a Sephadex G-75 column was then performed to remove small degraded peptides and enzyme (24).

For degradation of the polysaccharide moiety in the CP fraction, the CP was partially degraded with 16 mM sodium periodate (16) at 4 C for 100 h.

Chemical constituents of antigenic compounds. Total sugar content was determined by the anthrone method, and qualitative analysis of monosaccharides was done by gas-liquid chromatography (22). Qualitative and quantitative determinations of amino sugar were carried out according to the previous paper (21). For amino acid analysis, the samples were hydrolyzed at 105 C in sealed ampoules, and the hydrolysates were analyzed using an amino acid autoanalyzer (Hitachi model KLA-3). The amounts of total nitrogen and phosphorus were determined by the Kjeldahl-Nessler and Gomori methods, respectively.

Immunological activities. (i) Preparation of antiserum to CP. Male, albino rabbits (about 3 kg) were inoculated every 7 days for 5 weeks with a mixture of 1.0 ml of Freund complete adjuvant and 1.0 ml of 0.9% antigen (CP) in 0.85% NaCl solution administered intramuscularly in each thigh. They were bled 1 week after the last injection, and anti-CP serum was frozen until use.

(ii) Immunoelectrophoresis. The immunoelectrophoretic technique in agar was essentially as described by Grabar (10).

(iii) Passive cutaneous anaphylaxis. The method was performed principally by Ovary's method (25). Normal, albino guinea pigs, 300 to 400 g, were injected intracutaneously with 0.05 ml of diluted anti-CP serum. At the same time, both the diluent 0.85% NaCl solution (which was used to dissolve the antigen) and nonsensitized rabbit serum were injected as controls. After 5 h the animals were injected intravenously with a mixture of 0.25 mg of antigen and 1% Evans blue dissolved in 1.0 ml of saline. The diameter of the blue staining around the injection site was measured 30 min later.

(iv) Migration inhibition test. Male, albino guinea pigs, 300 to 400 g, were sensitized with 5 mg of dead whole cells emulsified (1:1, vol/vol) in Freund complete adjuvant injected into the thigh muscles each week for 2 weeks. The procedure employed was principally the capillary tube method of George and Vaughan (9) with minor modifications. To obtain peritoneal exudate, the sensitized (5 weeks after sensitization) and nonsensitized guinea pigs were injected intraperitoneally with 20 ml of liquid paraffin. Four days later exudate cells were harvested by washing the peritoneal cavity with chilled Hanks balanced salt solution. The peritoneal exudate cell suspension was sucked into sterile capillaries, and the capillaries were affixed with a high-vacuum grease (Dow Corning Co.) in tissue culture chambers. The chambers were filled with Eagle medium containing 20% normal heat-inactivated calf serum and 0.006% kanamycin with or without antigens (64 μg of antigen per 1.2 ml of nutrient medium). The chambers were incubated at 37 C for 24 h, and the rate of migration of cells from the capillaries was determined by the formula of Carpenter (7). Migration indexes of 80% or less were recorded as positive (6).

(v) Delayed skin test (sporotrichin test). Male, albino guinea pigs, 300 to 400 g, were inoculated intraperitoneally with 10 mg of whole cells suspended in 1 ml of 0.85% NaCl solution. Skin tests were performed 6 weeks after a single sensitizing dose of antigen, using 0.1 mg of antigen in 0.1 ml of saline. Readings were made at 24 h, and the degrees of erythema and induration were recorded in millimeters as the product of the two diameters of the test site reactions measured at right angles to one another. Palpable induration of 7 mm or greater was considered to be a positive reaction according to Edwards (8). Controls consisted of reactions in sensitized guinea pigs injected with the various antigens.

RESULTS

Figure 1 shows the extraction procedure for isolating antigens derived from the cells of *S. schenckii*. The crude polysaccharide fraction was obtained from the water layer; the elution pattern obtained by Sephadex G-100 gel filtration during subsequent purification of the crude polysaccharide fraction is presented in Fig. 2. It was found that the main CP fraction assayed with anthrone coincided with the peptide curve monitored by absorbance at 275 nm. This chromatographic result suggests that the CP fraction is a polysaccharide-rich peptide compound. Actually, the CP contained 87.1% carbohydrate and 12.5% peptide (Table 1). The yield of the CP was about 0.6% of the starting material of *S. schenckii* dried mycelia. Gas-liquid chromatographic analysis of sugar components showed that the CP was composed of rhamnose, mannose, and galactose, and their molar proportion was 2.1:5.6:1.0. The results of chemical analysis suggest that the CP is a rhamnose-containing polysaccharide peptide compound. As for amino acid composition, all fractions contained large quantities of aspartic acid, threonine, serine, glutamic acid, glycine, and alanine (Table 2).

To investigate the effect of alteration in the chemical components of the CP fraction upon their capacity to elicit the hypersensitivity reactions, papain and periodate treatments were performed (Fig. 1). The papain-treated fraction was designated as CP-I and the periodate-treated fraction as CP-II. Analytical data on the modified fractions (CP-I and CP-II) are

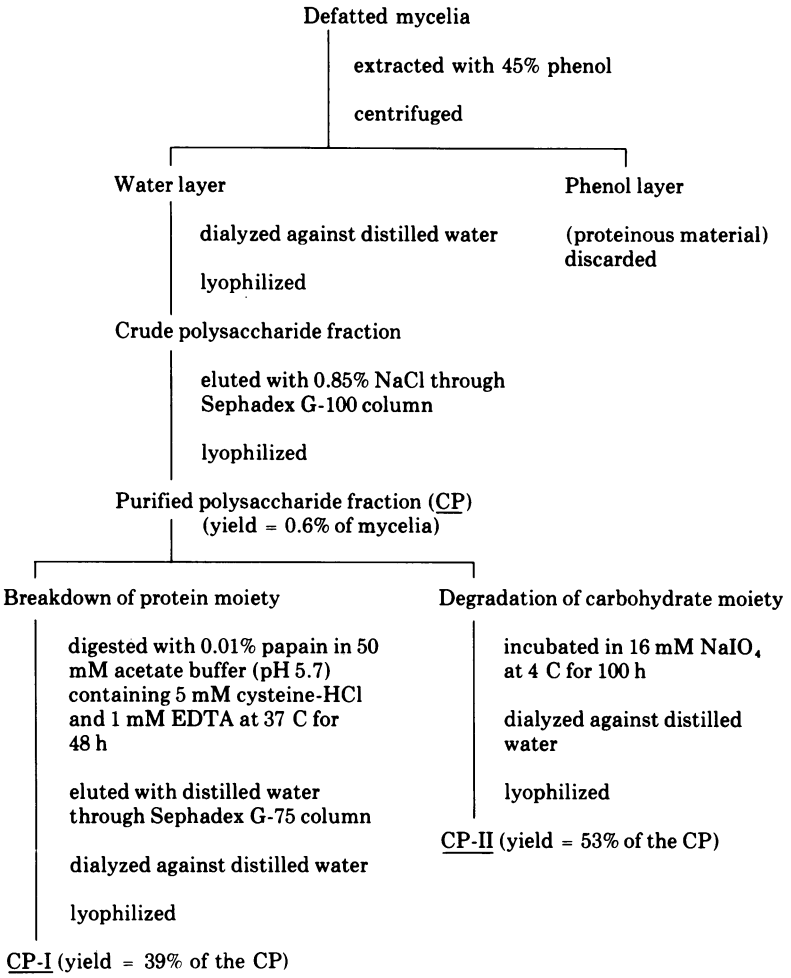

FIG. 1. *Procedure for isolation, purification, and modification of antigenic compounds (CP, CP-I, CP-II) from S. schenckii cells. EDTA, Ethylenediaminetetraacetic acid.*

given in Tables 1 and 2. Little difference of the sugar component was observed between the CP fraction and the enzyme-treated fraction, CP-I. However, the proteolytic digestion caused a 50% reduction in the nitrogen content of the CP fraction. In contrast, treatment with periodate, which disrupted the glycol group of sugar, drastically affected the sugar moiety of the CP. Any carbohydrate component was not detectable by anthrone reaction and gas-liquid chromatography.

For immunological assay the antigens of CP, CP-I, and CP-II were used. Figure 3 shows the results of immunoelectrophoretic analyses using the anti-CP rabbit serum. A few strong precipitin arcs were obtained at the negative electrode with CP and CP-I, whereas CP-II failed to produce a precipitin arc.

Passive cutaneous anaphylactic activities are shown in Table 3. No bluing spot was observed in any of the controls. The dimension of the injected area was greater than 100 mm^2 with CP and CP-I. In contrast, the activity was completely abolished by periodate.

The migration patterns are shown in Fig. 4. The results of the migration inhibition test are shown in Table 4 and are recorded as the migration indexes of 24-h packed cell preparations made from the peritoneal exudates of sensitized guinea pigs. The migration was significantly inhibited by the addition of the CP (average migration index of 10 sensitized guinea pigs = 69.7%) and the periodate-treated CP-II (70.5%). However, papain-treated CP-I showed a weak inhibition of the migration (88.6%). The average migration index in six nonsensitized

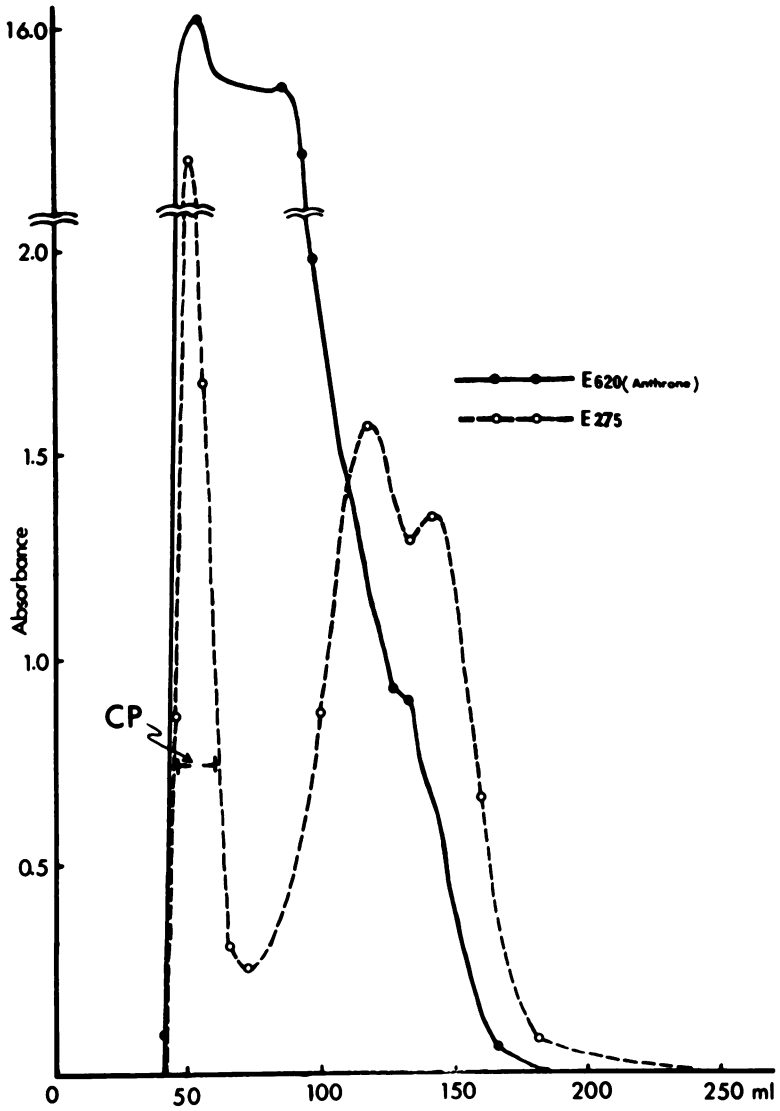

FIG. 2. *Fractionation on Sephadex G-100 column (2.8 by 40 cm) with 300 mg of the crude polysaccharide fraction in 0.85% NaCl solution. The 5-ml fractions were pooled.* ——, *Absorbance at 620 nm;* -----, *absorbance at 275 nm.*

guinea pigs was 93 to 99% to each antigen.

For delayed skin tests, intradermal injection of the antigens was carried out in four guinea pigs sensitized with dead *S. schenckii* cells. Control sites were injected with 0.85% NaCl solution; all antigens used were injected into four nonsensitized guinea pigs as another control. Sites injected with 0.85% NaCl showed reactions of less than 4.6 by 4.3 mm of erythema and no induration in the sensitized guinea pigs. The mean diameter of induration for CP was 12.1 by 11.5 mm, and the delayed skin reactivity of papain-treated CP-I was reduced to less than 20% of the original CP (Table 5). On the other hand, little change was observed in the activity of the periodate-treated CP-II. It was found that there was close correlation between the in vitro and in vivo manifestations of the delayed-type reactions.

DISCUSSION

Many efforts have been made to isolate and identify antigenic substances from fungi and to clarify the relationship between the chemical

nature and immunological activity of fungal antigens. Barker et al. reported that a peptide galactomannan from *Trichophyton mentagrophytes* is responsible for the immediate and delayed hypersensitivity reactions (4). The pure galactomannan of *Trichophyton* has been isolated by Blank and co-workers (11, 26) and was shown to have activities in the complement fixation and precipitin tests; no capacity to elicit the delayed skin test was found. Recently, it has been reported (23) that proteolytic enzyme treatment of antigenic polysaccharide-peptide complexes extracted from mycelia of *T. mentagrophytes* caused a loss of the ability of the extract to elicit a delayed reaction to skin tests in humans with naturally acquired trichophytosis. The immunological properties of protein and polysaccharide antigens of other fungi, *Aspergillus* (3), *Coccidioides*, and *Histoplasma* (1), were also investigated.

TABLE 1. *Chemical composition of whole cells and antigenic compounds extracted from S. schenckii cells*

Composition	Whole cell (%)	Extracted antigenic compound		
		CP	CP-I	CP-II[a]
Total carbohydrate[b]	48.1	87.1	91.6	0
Rhamnose[c]	11.7	21.1	21.6	0
Mannose[c]	14.4	55.9	58.6	0
Galactose[c]	4.7	10.0	11.3	0
Glucose[c]	17.3	Trace	Trace	0
Total nitrogen[d]	6.7	2.0	0.9	4.1
Peptide[e]	35.2	12.5	5.6	25.6
Glucosamine[f]	6.1	Trace	Trace	Trace
Phosphorus[g]	0.6	0.1	Trace	Trace

[a] Contains decomposed substances due to periodation of polysaccharide moiety.
[b] By the anthrone method.
[c] Gas-liquid chromatography.
[d] Kjeldahl-Nessler's method.
[e] Nitrogen content (×6.25).
[f] Elson-Morgan's method.
[g] Gormori's method.

TABLE 2. *Amino acid composition of antigenic compounds extracted from S. schenckii cells*

Amino acid	Antigenic compound (10^{-7} mol)		
	CP	CP-I	CP-II
Aspartic acid	7	6	12
Threonine	20	10	36
Serine	18	8	38
Glutamic acid	4	4	14
Proline	1	Trace	1
Glycine	9	5	21
Alanine	14	6	26
Valine	11	3	18
Isoleucine	3	1	4
Leucine	2	2	5
Tyrosine	1	Trace	1
Phenylalanine	Trace	Trace	Trace
Lysine	3	2	5
Histidine	2	Trace	2
Arginine	1	Trace	Trace

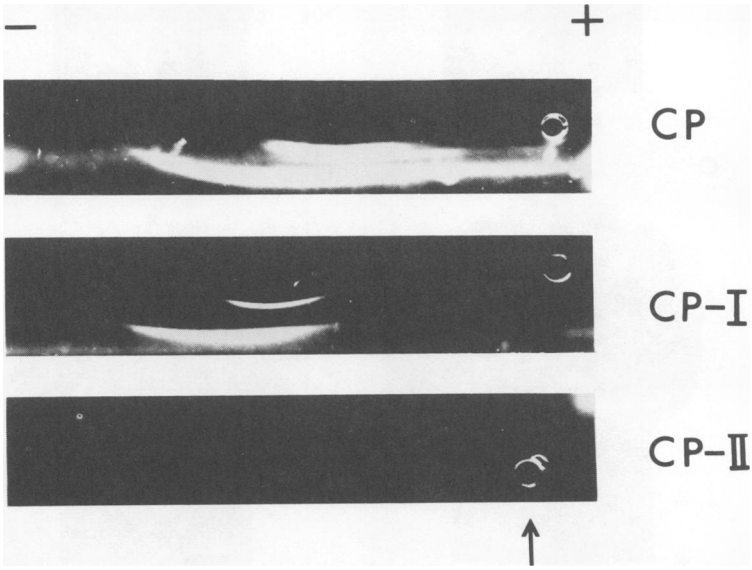

FIG. 3. *Immunoelectrophoregrams of polysaccharide-peptide compounds. The antigen solutions (2 mg/ml) were placed in the wells (arrow). Rabbit anti-CP serum was added to the center trough after electrophoresis of the antigens. Diffusion was at 15 C for 2 days.*

As for *S. schenckii*, the immunologically active substances were isolated from the cells or culture filtrates, and the serological investigations were performed by using these antigens (2, 12, 13, 15, 18–20, 27, 28; S. McMillen and E. R. Laverty, Bacteriol. Proc., p. 115, 1969). Aoki et al. (2), Ishizaki (12), and Lloyd and Bitoon (15) evaluated the use of *Sporothrix* antigen for clinical diagnosis. Aoki et al. found that the polysaccharide extracted from *S. schenckii* cells disrupted with the French press was a rhamnomannan compound (1:1); it was effective for eliciting the sporotrichin reaction and showed cross-reaction with *Aspergillus* antigen. Ishizaki isolated antigens from the culture filtrate of *S. schenckii*, the major fraction of which contained 15% peptide and rhamnose, mannose, and galactose (4:5:1). This fraction proved to be immunologically active by precipitin reaction in agar gel and by the sporotrichin test. Recently, a serologically active peptido-rhamnomannan was isolated from the cells and culture medium of *S. schenckii* by Lloyd and Bitoon (15). They also showed that the sugar composition of the polysaccharide included mannose and rhamnose (5:3) and that 18% peptide was present. Our previous studies have shown that the water layer after 45% phenol extraction from *S. schenckii* cells comprises mixtures of polysac-

TABLE 3. *Passive cutaneous anaphylaxis produced with antigenic compounds extracted from S. schenckii cells*

Fraction	Dimension (mm^2)
CP	157
CP-I	118
CP-II	0
Control	0

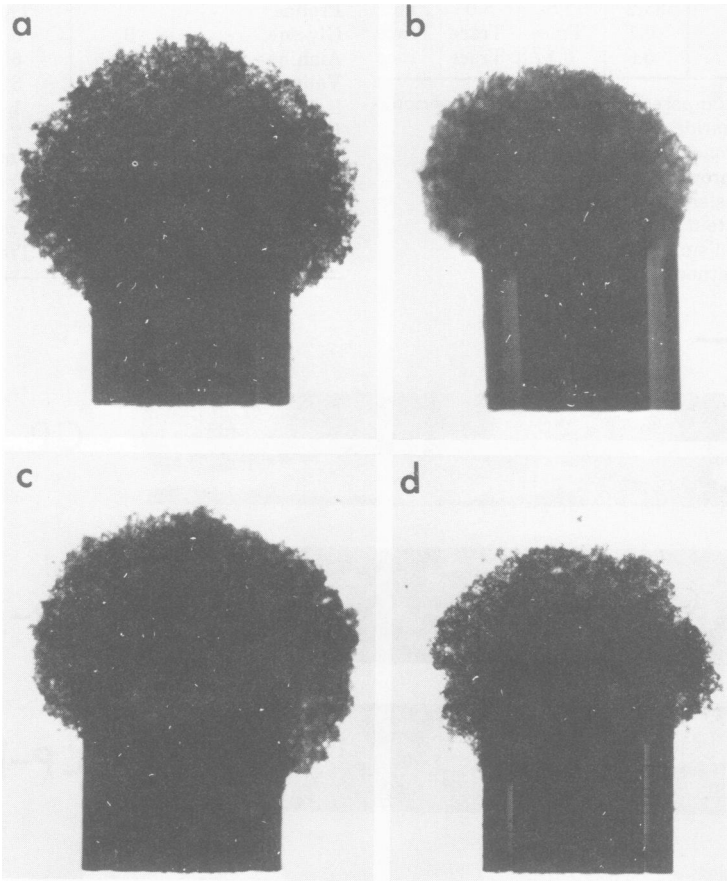

FIG. 4. *Migration patterns of peritoneal exudate cells from guinea pigs sensitized with S. schenckii cells. (a) Control: migration pattern in the absence of antigen; (b) CP; (c) CP-I; (d) CP-II.*

TABLE 4. *Migration inhibition of peritoneal exudate cells induced with antigenic compounds from S. schenckii cells*

Sensitized guinea pig	Migration index (%)		
	CP	CP-I	CP-II
1	79	91	84
2	69	94	55
3	85	94	84
4	67	93	88
5	78	90	79
6	72	75	56
7	51	73	60
8	64	98	62
9	74	97	79
10	58	81	58
Mean ± CI[a]	69.7 ± 7.2	88.6 ± 6.4	70.5 ± 9.4

[a] CI, Confidence interval (significance level, α = 0.05).

TABLE 5. *Comparison of delayed skin reactions induced with antigenic compounds extracted from S. schenckii cells*

Sensitized guinea pig	Induration/erythema (mm)		
	CP	CP-I	CP-II
1	11.5 × 10.9 / 14.6 × 13.1	5.7 × 5.1 / 7.6 × 7.4	9.9 × 9.5 / 13.7 × 13.2
2	10.4 × 10.2 / 13.2 × 11.6	1.5 × 1.5 / 5.4 × 4.9	7.7 × 7.1 / 10.0 × 9.1
3	13.0 × 12.2 / 16.8 × 15.0	0 × 0 / 6.0 × 5.2	10.9 × 10.5 / 15.8 × 15.0
4	13.5 × 12.6 / 19.3 × 17.9	2.3 × 2.3 / 7.3 × 6.5	12.9 × 12.2 / 16.8 × 15.8
Mean	12.1 × 11.5 / 16.0 × 14.4	2.4 × 2.2 / 6.6 × 6.0	10.4 × 9.8 / 14.1 × 13.3
Control[a]	0 × 0 / 2.3 × 2.3	0 × 0 / 2.3 × 2.1	0 × 0 / 3.4 × 2.9

[a] From four nonsensitized animals.

charide and peptide (19) which give rise to both immediate and delayed hypersensitivity reactions in sensitized guinea pigs (20). The present report deals with chemical studies of the purified antigenic polysaccharide-peptide compounds derived from *S. schenckii* cells and with the chemical components responsible for immunological activities. Our main antigenic fraction (CP) consisted of 87.1% of a sugar moiety which contained rhamnose, mannose, and galactose (2.1:5.6:1.0) and 12.5% of a peptide moiety, so the CP is the rhamnose-containing polysaccharide-peptide compound. Therefore, this fraction is an ideal model for studying involvement of polysaccharide and peptide in the immediate- and delayed-type reactions. It has been clearly shown that the CP and the papain-treated CP-I were found to be active by the test of passive cutaneous anaphylaxis. However, the periodate-treated CP-II did not give any bluing spot. On the other hand, treatment with papain (CP-I) significantly abolished the delayed-type reactivities, including both inhibition of peritoneal cell migration and the delayed skin test, whereas treatment of periodate (CP-II) did not abolish the delayed-type reactivities. The results of this study indicated that the polysaccharide moiety could not elicit delayed-type reactions in guinea pigs sensitized with *S. schenckii* cells, but provoked the immediate-type reaction, and also that the peptide moiety elicited only the delayed-type reaction. These experimental results are supported by the precipitin activities in agar gel, which are related to a humoral antibody, since the enzyme-digested fraction (CP-I) showed the same precipitin arcs as those with the undigested fraction (CP). Similar results have been found with the other fungi (1, 3, 4).

In these experiments peptide moiety could not be completely removed by papain digestion so that the delayed-type reaction was not completely abolished. It might be possible that a small proportion of the polysaccharide is concerned with the mediation of the delayed-type reaction. Until the two main components of the molecule can be completely separated, it will not be certain whether only the peptide is concerned in the delayed-type reaction or not. Further studies on purification of the antigen may resolve this question.

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