JOURNAL OF BACTERIOLOGY, June 1967, p. 1741–1748
Vol. 93, [illegible]

Printed in U.S.A.

Nature of the Skin-reactive Principle in Culture Filtrates Prepared from *Paracoccidioides brasiliensis*

ANGELA RESTREPO-MORENO[1] AND JOHN D. SCHNEIDAU, JR.
Department of Microbiology, Tulane University School of Medicine, New Orleans, Louisiana 70112

Received for publication 30 January 1967

Mycelial and yeast-phase culture filtrates prepared from three strains of *Paracoccidioides brasiliensis* exhibited equal reactivity in sensitized guinea pigs. Ethyl alcohol-precipitated fractions obtained from the culture filtrates also showed no difference in reactivity between mycelial and yeast phase when tested in sensitized guinea pigs. Chemical analyses of the ethyl alcohol-precipitated fractions revealed the presence of seven aliphatic amino acids in both the mycelial- and yeast-phase products. Glucose, galactose, arabinose, and glucosamine were also detected, but the relative proportions of these sugars were different for the mycelial phase as compared with the yeast phase. Both the mycelial- and yeast-phase ethyl alcohol precipitated fractions contained 2 to 4% nitrogen, but no protein or nucleic acid could be detected. Removal of nitrogen from the ethyl alcohol-precipitated fractions by chloroform extraction resulted in an almost complete loss of skin reactivity, whereas the material recovered from the chloroform, which contained most of the nitrogen, still exhibited almost as much reactivity as was present prior to extraction. A considerable portion of the reducing substances was removed along with the nitrogen by the chloroform extraction, suggesting a strong chemical link between the carbohydrate and the peptide portions of the active moiety. Since no protein was present in the fractions, it was presumed that the active moiety is a glycopeptide.

The first recorded attempt to demonstrate cutaneous hypersensitivity in patients with South American blastomycosis was that of Fonseca and Area-Leao (13), who injected a mycelial filtrate intradermally into two patients, both of whom reacted to the inoculum. Since then, numerous investigators have employed either mycelial-phase (2, 4, 5, 8) or yeast-phase culture filtrates (1, 16, 19–22) of *Paracoccidioides brasiliensis* to study skin reactivity in infected patients and in the normal population. In most of these investigations, the majority of patients with active disease gave positive reactions to either type of filtrate, but some patients failed to react, and occasional positive reactions were observed in patients with histoplasmosis and sporotrichosis. In a few instances, possibly because of the use of a protein-containing culture medium, patients exhibited positive reactions to the uninoculated broth. In some studies, the filtrates were used directly in patients, without any attempt to test their potency in laboratory animals. In general, the culture filtrates that have been employed have varied greatly from batch to batch with respect to composition and potency. It is difficult to compare the results reported by different investigators, since no quantitated standard was used and since the chemical composition of the antigenic material was not identical in all instances.

Fava-Netto and Raphael (11) reported the use of a "polysaccharide" fraction for skin testing. This "polysaccharide" was actually an extract of autoclaved yeast-phase cells which was originally devised for use in a complement-fixation test. Positive cutaneous reactions were obtained in 87% of proven cases of South American blastomycosis, but many positive reactions also were observed among patients with other diseases and among healthy nurses, physicians, and medical students at a regional hospital. The chemical nature or degree of purity of the extract was not determined, but it is likely that it contained some nitrogen. Recently, considerable interest has been focused on the role of polysaccharides in fungal

[1] Present address: Departamento de Microbiologia y Parasitologia, Facultad de Medicina, Universidad de Antioquia, Medellín, Colombia, S.A.

hypersensitivity, and a number of investigators have prepared polysaccharide fractions from *Histoplasma capsulatum*, *Coccidioides immitis*, and *Blastomyces dermatitidis* (6, 9, 10, 23, 24, 27). In all cases, the fractions employed were strongly reactive in skin tests. Chemical analyses of these polysaccharide fractions were mainly limited to nitrogen determinations and identification of constituent sugars, and in all cases nitrogen was found to be present in the fractions. Attempts to further purify the fractions by removal of the nitrogen resulted in a marked reduction in activity. In pilot experiments in our laboratories, protein fractions prepared from sonically disrupted yeast- and mycelial-phase cells were completely nonreactive in infected animals. The evidence to date indicates that the activity of culture filtrates resides primarily in that portion precipitated by ethyl alcohol. This paper reports the results of an attempt to define more precisely the chemical nature of the substance present in ethyl alcohol-precipitated fractions of culture filtrates that are capable of eliciting positive cutaneous reactions in sensitized animals or humans.

MATERIALS AND METHODS

Fungal strains. Two Brazilian isolates of *P. brasiliensis* were obtained from L. Georg, National Communicable Disease Center, Atlanta, Ga. A third strain was isolated by the senior author from a patient in Medellín, Colombia. Strains were maintained in the mycelial phase on Sabouraud's glucose (BBL) agar at room temperature, and in the yeast phase on Kelley's medium (18) at 37 C.

Preparation of culture filtrates. Each of the three strains of *P. brasiliensis* was grown in the mycelial and yeast phase, in submerged shake cultures in a specially designed dialysate medium. To prepare the dialysate medium, 30 g of dehydrated Trypticase Soy Broth (BBL) was dissolved in 100 ml of sterile distilled water, poured into sterile cellulose casings, and dialyzed at 5 C against 900 ml of sterile distilled water for a period of 18 hr; the material remaining in the casing was then discarded. To the dialysate, 10 g of glucose and 5 g of ammonium sulfate were added. The medium was distributed in 200-ml amounts in heavy-duty 500-ml Ehrlenmeyer flasks and autoclaved at 121 C for 12 min. After cooling, 1.0 ml of a polyvitamin solution containing biotin, choline, folic acid, nicotinamide, pantothenic acid, pyridoxal, thiamine, and riboflavine (BME Vitamin Mixture 100 ×, Microbiological Associates, Bethesda, Md.) was added to each 100 ml of medium.

Yeast-phase cultures of each strain were prepared by inoculating the flasks with 5 ml of a stock suspension of yeast cells, and incubating at 35 C with constant agitation on a Gyrotary shaker (New Brunswick Scientific Co., New Brunswick, N.J.), at 103 strokes per minute. The stock suspension of yeast cells was prepared by transferring the growth from a 7-day-old yeast-phase culture on Kelley's medium to 200 ml of the broth culture medium and incubating for 1 week on the Gyrotary shaker at 35 C. For the preparation of mycelial-phase cultures of each strain, the growth from a 2-week-old Sabouraud's agar slant was suspended in saline, homogenized in a Waring Blendor for 5 min, transferred to a flask with 200 ml of broth medium, and placed on the shaker at 22 C for 1 week. Amounts of 5 ml of this culture were inoculated into each of the flasks containing 200 ml of broth medium and incubated on the shaker at 22 C.

After 4 weeks of incubation, both the yeast- and mycelial-phase cultures were checked for the absence of contamination and uniformity of growth phase; for each strain, those cultures free from contamination were pooled, and Formalin was added to a final concentration of 0.5%. The cultures were then stored for 2 days at 5 C with occasional shaking. The cellular elements were removed first by centrifugation at 1,400 × *g* in the case of the yeast-phase cultures, and by filtration through coarse filter paper for the mycelial-phase cultures. The culture fluids obtained were then passed through 0.45-μ filter pads (Millipore Corp., Bedford, Mass.), and dialyzed against distilled water for 2 days at 5 C with four daily water changes, after which the dialyzed filtrates were reduced to one-tenth the original volume by fan perevaporation. Sodium chloride (0.85%) was then added, the filtration was repeated, and the filtrates were stored in the cold with Merthiolate added to a final concentration of 1:10,000. A control sample of uninoculated broth was processed in an identical manner. Equal volumes of the concentrated filtrate from the mycelial-phase of each strain were pooled, and a pooled yeast-phase culture filtrate was similarly prepared. Prior to fractionation procedures, the pooled filtrates were tested for skin reactivity in infected guinea pigs during the 7th week after inoculation.

Preparation of ethyl alcohol-precipitated fractions of culture filtrates. Pooled mycelial- and yeast-phase filtrates that evoked in infected guinea pigs indurated areas exceeding 10 mm in diameter were extracted by precipitation with 5 volumes of ethyl alcohol according to the techniques described by Pappagianis et al. (24) for the preparation of a polysaccharide fraction from *Coccidioides immitis*. The fractions obtained will hereafter be referred to as "EPF" (ethyl alcohol-precipitated fractions). Each precipitate was dried and stored in vacuo in a desiccator jar. Samples (5 to 10 mg) of each EPF were further extracted by the chloroform-amyl alcohol method of Sevag (31), and the aqueous and chloroform phases were separated by means of a separatory funnel. Both the aqueous phase and the chloroform-amyl alcohol phase were evaporated to dryness, and the residue was stored in vacuo in a desiccator jar. For skin testing, weighed quantities of the dried residues were dissolved in sterile buffered Merthiolate-saline. For all analytical procedures, fractions were dissolved in distilled water.

Analytical methods. Total nitrogen was determined by the Kjeldahl-Nessler method as described by Seibert and Affronti (29), and total phosphorus, by the method of Fiske and Subbarow (12). The ultraviolet-absorption curve at 260 and 280 mμ was determined with a Beckman automatic spectrophotometer. Reducing sugars were estimated by the anthrone method as modified by Seibert and Affronti (30) with glucose or galactose as a standard. Pentoses were determined by the Bial reaction as described by Kabat and Mayer (17), and hexosamines, by the Dische and Borenfreund method (7). *N*-acetyl-amino sugars were estimated by the method of Reissig, Strominger, and Leloir (26). Constituent monosaccharides were identified by one-dimensional paper chromatography. Samples ranging from 1 to 50 mg were hydrolyzed with 2 N HCl for 90 min at 58 C, evaporated to dryness, redissolved in distilled water, and brought to dryness three times over NaOH. The hydrolysates were redissolved in minimal amounts of water and filtered through Millipore pads to remove charred material. Both ascending and descending chromatograms were run on Whatman no. 4 paper at room temperature. Three different solvent systems were used: isopropanol-water (160:40); phenol-water-ammonia (160:40:1); and ethyl acetate-pyridine-water (120:50:40). Quantitative determinations of monosaccharides were carried out after localization of the sugar spots by eluting parallel sugar areas according to the techniques of Putnam (25) and Zamora, Bojalil, and Bastarrachea (33).

For the determination of amino acids, 5-mg samples of the EPF were hydrolyzed in 6 N HCl for 18 hr at 110 C and evaporated to dryness. Residual HCl was removed as described above, and amino acids were identified by one- and two-dimensional chromatograms by use of 10 μliters of the hydrolysate. Two solvent systems were used: butanol-acetic acid-phenol (120:30:50), and phenol-ethyl alcohol-ammonia-water (150:40:1:10); chromatograms were developed with ninhydrin as described by Smith (32).

Sensitization of guinea pigs. Albino male guinea pigs weighing 500 to 600 g were used for all experiments. Each animal was tested on arrival for natural sensitization with undiluted mycelial- and yeast-phase culture filtrates, and only animals with negative reactions were used for subsequent sensitization and skin-testing. Sensitization was accomplished by infecting the animals with suspensions of live yeast-phase cells. For *P. brasiliensis*, each guinea pig was inoculated intratesticularly with a broth suspension of viable yeast-phase cells in two doses of 0.5 ml each, given at 10-day intervals; each dose contained 7.2×10^5 viable units. To investigate cross-reactivity, other animals were infected with *H. capsulatum* by intraperitoneal inoculation of 10^6 viable yeast particles, and with *Sporotrichum schenckii* by intratesticular inoculation of 2×10^7 viable yeast-phase particles in a single dose.

Skin tests on guinea pigs. All animals infected with *P. brasiliensis* were skin-tested during the 7th week after the last inoculation, and animals infected with *H. capsulatum* and *S. schenckii* were tested 4 weeks after inoculation by intradermal injection of 0.1 ml into the skin of the previously shaved abdomen.

For the EPF, weighed quantities were dissolved in buffered, Merthiolate-saline; the quantities given in the test and tables represent the amount of EPF in the 0.1-ml skin-test dose.

Twenty-five guinea pigs infected with *P. brasiliensis* were divided into five groups of five each and skin-tested with the following materials. Group I: mycelial filtrate of *P. brasiliensis* undiluted and in doubling dilutions and up to 1:32. Group II: yeast-phase filtrates of *P. brasiliensis* similarly diluted. Group III: mycelial-phase EPF of *P. brasiliensis* in varying doses up to 500 μg, and undiluted mycelial filtrate. Group IV: yeast-phase EPF of *P. brasiliensis* in varying doses up to 500 μg and undiluted yeast-phase filtrate. Group V: mycelial- and yeast-phase EPF of *P. brasiliensis* before and after extraction with chloroform-amyl alcohol, in amounts of 200 μg (per 0.1 ml.), and the material extracted by the chloroform-amyl alcohol from both the mycelial- and yeast-phase EPF, in amounts of 50 to 200 μg. In testing for cross-reactivity, five guinea pigs infected with *P. brasiliensis*, five infected with *H. capsulatum*, and five infected with *S. schenckii* were all tested with the mycelial- and yeast-phase culture filtrates undiluted and diluted 1:4, and with 100 μg each of the mycelial- and yeast-phase EPF of *P. brasiliensis*. The animals infected with *P. brasiliensis* were additionally tested with histoplasmin (Parke-Davis & Co. Detroit, Mich.) diluted 1:10 and 1:50, and with sporotrichin undiluted and diluted 1:50 [The sporotrichin used was the same material employed by Schneidau et al. (28) in their skin-test survey of Louisiana residents.] *S. schenckii*-infected animals were tested with sporotrichin, and *H. capsulatum*-infected animals, with histoplasmin, in the same dilutions employed in testing the *P. brasiliensis*-infected guinea pigs. Controls consisted of 10 uninfected guinea pigs tested with the highest concentrations of each of the materials employed in the test series. As an additional control, all infected animals were tested with 0.1 ml of the uninoculated broth, dialyzed and concentrated in the same manner as the culture filtrates.

Skin-tests on human volunteers. A total of 65 individuals were skin-tested with 0.1 ml of a 1:100 dilution of the mycelial- and yeast-phase culture filtrates of *P. brasiliensis* and with a 1:100 dilution of histoplasmin. Eighteen positive reactors to histoplasmin and 20 nonreactors were further tested with 10 μg each of the mycelial- and yeast-phase EPF.

RESULTS

Chemical analysis. The precipitates which were readily obtained from the mycelial and yeast filtrates, on the addition of 5 volumes of ethyl alcohol, were soluble in saline or distilled water and were tan in color. Yields ranged from 250 to 400 μg/ml. Attempts to obtain precipitates from uninoculated broth medium were unsuccessful. The supernatant fluid remaining after removal of the precipitate was nonreactive in sensitized animals, and failed to yield more precipitate when additional quantities of ethyl alcohol were added.

Results of chemical analyses of the precipitated materials are presented in Table 1. The fractions were ninhydrin-positive and contained 2 to 4% nitrogen. The ultraviolet-absorption curve failed to show peaks in the regions corre-

TABLE 1. *Analysis of Paracoccidioides brasiliensis ethyl alcohol-precipitated fractions*

Fraction from	Yield	Per cent			
		N	Reducing substances (anthrone)	Pentoses (orcinol)	Phosphorus
	μg/ml				
Mycelial culture filtrate	250	2.70	36.76	8.20	1.60
Yeast culture filtrate	360	3.90	28.90	6.24	0.80

TABLE 2. *Analysis of chromatographically separated components of Paracoccidioides brasiliensis ethyl alcohol-precipitated fractions*

Fraction from	Per cent				
	Glucose	Galactose	Arabinose	Glucosamine	Total
Mycelial culture filtrate	3.52	35.20	4.87	2.12	45.79
Yeast culture filtrate	6.50	10.20	2.43	0.57	19.70

TABLE 3. *Analysis of the ethyl alcohol-precipitated fractions from Paracoccidioides brasiliensis culture filtrates before and after extraction with chloroform-amyl alcohol*

Fraction tested	Nitrogen (%)			Reducing substances (%)		
	Before extraction	After extraction		Before extraction	After extraction	
		Water phase	Chloroform phase		Water phase	Chloroform phase
Mycelial EPF	2.3	0.6	1.1	50	20	21
YEAST EPF	2.6	0.3	2.6	30	16	11

sponding to protein or nucleic acids. Chromatograms of acid hydrolysates of both fractions revealed what appeared to be a polypeptide. The following seven amino acids were detected: leucine, methionine, alanine, glutamic acid, serine, threonine, and glycine. No significant difference in the values obtained for reducing substances was noted when galactose was used as a standard instead of glucose.

Chromatographic analysis of acid hydrolysates of both the mycelial-phase EPF and yeast-phase EPF revealed the presence of glucose, galactose, arabinose, and glucosamine. *N*-acetyl-amino sugars were not detected either by chromatography or by quantitative techniques. Quantitative determination carried out on eluates from paired chromatographic spots revealed differences in the distribution of the component monosaccharides of the mycelial-phase EPF as compared with the yeast-phase EPF (Table 2). Hexosamine figures were very low. Extraction of solutions of the ethyl alcohol-precipitated fractions with chloroform-amyl alcohol according to the method of Sevag reduced the nitrogen percentage from 2.3 to 0.6% in the mycelial-phase EPF and from 2.6 to 0.3% in the yeast-phase EPF. Most of the nitrogen could be accounted for in the chloroform phase. A considerable proportion of the reducing substances was also removed along with the nitrogen by the chloroform extraction (Table 3).

Animal tests. In all animals tested, dermal reactions to the test material began at 6 hr, with diffuse edema and little or no erythema. At 24 hr, the reactions reached their peak, showing a well-defined zone of redness and induration, with tissue necrosis in some cases. At 48 hr, the intensity of the reactions had greatly diminished, although in some instances the induration persisted for longer periods of time. Positive reactions (induration) ranged from 5 to 20 mm in diameter.

The uninfected animals in the control groups did not react to any of the test materials, and none of the infected guinea pigs was sensitive to the uninoculated control broth.

Results of skin tests done on animals infected with *P. brasiliensis* with culture filtrates and with EPF showed that there was no significant difference in reactivity between the mycelial and yeast phases for either the culture filtrates or the EPF. The highest dilution of either culture filtrate capable of evoking a positive reaction was 1:4, and the least amount of either the mycelial- or yeast-phase EPF required to evoke a positive reaction was 100 μg. Solutions of EPF with greatly reduced nitrogen content after treatment with chloroform-amyl alcohol lost practically all of their reactivity, whereas that portion that could be recovered from the chloroform-amyl alcohol mixture retained almost as much reactivity as was originally exhibited by the EPF prior to extraction. (Table 4).

Animals infected with *H. capsulatum* cross-reacted with the undiluted mycelial- and yeast-phase culture filtrates and mycelial phase EPF of *P. brasiliensis*, whereas, in the group of animals infected with *S. schenckii*, cross-reaction was limited to the undiluted mycelial filtrate of *P. brasiliensis*. Animals infected with *P. brasiliensis* did not cross-react with either histoplasmin or sporotrichin in the concentrations tested (Table 5).

Tests on human volunteers. Of the 65 human volunteers tested with the mycelial- and yeast-phase filtrates of *P. brasiliensis* and with histoplasmin, 18 gave reactions of 5 mm or more to histoplasmin and 12 of these reacted to the *P. brasiliensis* filtrates. When the positive histoplasmin reactors were further tested with *P. brasiliensis*, mycelial- and yeast-phase EPF, eight reacted positively to both fractions (Table 6).

TABLE 4. *Comparison of skin reactions observed in guinea pigs[a] infected with Paracoccidioides brasiliensis when tested with ethyl alcohol-precipitated fractions of P. brasiliensis culture filtrates before and after extraction with chloroform-amyl alcohol*

Animal no.	Mycelial-phase EPF				Yeast-phase EPF			
	Before extraction (200 μg)	After extraction			Before extraction (200 μg)	After extraction		
		Water phase (200 μg)	Chloroform phase			Water phase (200 μg)	Chloroform phase	
			200 μg	50 μg			200 μg	50 μg
1	10[b]	0	10	8	8	0	8	5
2	14	2	10	6	12	0	10	8
3	12	0	8	5	12	2	8	6
4	15	3	10	8	10	2	10	6
5	12	0	12	8	10	0	10	5

[a] Animals tested at 7th week postinfection.
[b] Figures indicate diameter of induration in millimeters.

TABLE 5. *Cross-reactions among guinea pigs infected with Paracoccidioides brasiliensis, Histoplasma capsulatum, and Sporotrichum schenckii tested with homologous and heterologous filtrates and with the ethyl alcohol-precipitated fractions prepared from P. brasiliensis culture filtrates*

Test material[a]	Animals infected with														
	H. capsulatum					*S. schenckii*					*P. brasiliensis*				
	1[b]	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Histoplasmin 1:10	10[c]	10	7	5	5	—	—	—	—	—	0	0	0	0	0
Histoplasmin 1:50	5	9	5	0	5	—	—	—	—	—	0	0	0	0	0
Sporotrichin Undiluted	—	—	—	—	—	7	9	9	10	10	0	0	0	0	0
Sporotrichin 1:50	—	—	—	—	—	7	7	8	5	5	0	0	0	0	0
P. brasiliensis MF undiluted	7	7	6	7	5	6	5	5	9	7	10	10	10	12	10
P. brasiliensis MF 1:4	0	0	0	0	0	0	0	0	0	0	7	7	6	10	7
P. brasiliensis YF Undiluted	5	5	7	6	5	0	0	0	0	0	9	9	10	7	10
P. brasiliensis YF 1:4	0	0	0	0	0	0	0	0	0	0	5	5	6	5	6
P. brasiliensis MEPF 100 μg	5	5	5	5	5	0	0	0	0	0	8	8	6	8	8
P. brasiliensis YEPF 100 μg	0	0	0	0	0	0	0	0	0	0	5	7	6	8	5

[a] MF: mycelial filtrate; YF: yeast filtrate; MEPF, YEPF: mycelial and yeast phase EPF, respectively.
[b] Animal number.
[c] Induration at 24 hr in millimeters.

TABLE 6. *Skin reactivity of histoplasmin-positive individuals to Paracoccidioides brasiliensis culture filtrates and ethyl alcohol-precipitated fractions*

No.	Histoplasmin (1:100)	*P. brasiliensis*			
		Mycelial filtrate (1:100)	Yeast filtrate (1:100)	EPF	
				Mycelial (10 μg)	Yeast (10 μg)
1	5	N[a]	N	N	N
2	6	N	N	N	N
3	6	N	N	N	N
4	10	N	N	N	N
5	6	N	N	N	N
6	5	N	N	N	N
7	8	N	6	N	N
8	6	6	5	N	N
9	6	5	N	N	N
10	6	6	N	N	N
11	12	12	10	12	15
12	12	15	5	12	9
13	12	9	5	8	5
14	14	16	5	7	8
15	15	12	9	10	9
16	16	7	5	8	10
17	20	15	10	20	10
18	30	15	5	30	20

[a] Results are expressed as induration at 24 hr in millimeters. N = negative (<5 mm).

Only 1 of 47 histoplasmin-negative individuals tested gave a positive reaction to any of the *P. brasiliensis* test materials.

DISCUSSION

The difficulties experienced in growing *P. brasiliensis* in a liquid, defined medium could explain the paucity of reports dealing with the biological activities of its fractions. The dialyzed culture medium described in this paper has proven to be a valuable tool in investigations which require luxuriant growth of both phases of the fungus. It also has the advantage of being nonantigenic when used in an intradermal test, as shown by animal experiments.

Crude mycelial and yeast filtrates have been used alternatively and sporadically to detect sensitivity to the agent of South American blastomycosis (1, 4, 13, 14, 19, 22) without definite conclusions as to the relative advantages of one preparation over another. In our experiments, no significant difference was noted between the activity of mycelial-phase and yeast-phase preparations for either culture filtrates or the EPF, indicating that either growth form could be employed with equal effectiveness. Both the culture filtrates and the EPF elicited dermal reactions that reached their maximum at 24 hr and faded gradually thereafter. Similar 24-hr reactions have been noted by other investigators who have studied human skin sensitivity to fungal antigens (6, 11, 28). Although both the EPF and the culture filtrates have comparable results, the EPF should be the material of choice for skin testing, since the use of quantitated amounts of a more chemically defined product, in addition to rendering the results more reproducible, would permit a more valid comparison between studies carried out by different investigators.

The lack of reactivity to *P. brasiliensis* test materials observed in histoplasmin-negative individuals, and the fact that histoplasmin-positive persons exhibiting reactions of 10 mm or larger did react to these materials in an area where South American blastomycosis is absent, indicate cross-reactivity between the two fungi, a finding which was anticipated as a result of the tests carried out in guinea pigs with heterologous antigens (Table 5). There appears to be a considerable difference in the degree of hypersensitivity observed in guinea pigs as compared with humans. In guinea pigs, positive reactions were obtained with culture filtrates in dilutions not higher than 1:4 and with the EPF with a skin-test dose not less than 100 μg, whereas, in the histoplasmin-positive humans tested, reactions to the *P. brasiliensis* culture filtrate were observed with dilutions as high as 1:100 and with the EPF in doses as low as 10 μg; similar amounts of skin-test materials have given sizeable reactions in a small number of patients with active South American blastomycosis tested by one of the authors in Medellin, Colombia. Thus, the ratio of the sensitivity of humans as compared with that of guinea pigs was approximately 10:1 or greater in our experiments, which might explain why only minimal or negative reactions were observed for the *P. brasiliensis* fractions tested in histoplasmin-positive guinea pigs. The cross-reactions observed in histoplasmin-positive human subjects emphasize the necessity of testing with other heterologous antigens before attempting to interpret the significance of a positive skin test to paracoccidioidin.

The ethyl alcohol-precipitated fractions extracted from the mycelial and yeast filtrates of *P. brasiliensis* were chemically similar, containing the same seven amino acids and the same three monosaccharides. Although the ratios for the three sugars were quite different for the mycelial-phase EPF, as compared with the yeast-phase EPF, no significant difference in skin reactivity was observed. The finding that both mycelial-phase EPF and yeast-phase EPF contain significant amounts of nitrogen was to be expected in view of the studies of Salvin et al. (27), Markowitz (23), and Zamora et al. (33), who have shown that skin-reactive substances isolated from fungi such as *H. capsulatum*, *B. dermatitidis*, *C. immitis*, *Nocardia asteroides*, and *N. brasiliensis* contain nitrogen incorporated in the carbohydrate fraction in the form of protein or polypeptides. It does not seem likely that the nitrogen in our fractions represents protein, in view of our inability to detect protein by either ultraviolet spectrophotometry or conventional chemical procedures. The fact that only seven aliphatic amino acids were present in the fractions suggests a polypeptide as the source of the nitrogen.

The chemical determinations and skin tests carried out with mycelial- and yeast-phase EPF before and after chloroform extraction indicate that most, if not all, of the skin reactivity resides in the chloroform phase, which also retained most of the nitrogen. This is in accord with the findings of Pappagianis et al. (24) and of Dyson and Evans (9) with polysaccharides from *C. immitis* and *B. dermatitidis*, respectively. It is interesting to note that in our studies the nitrogen-containing chloroform phase carried along a substantial part of the reducing substances, suggesting a strong chemical link between the carbohydrate and the peptide portions of the active moiety. Presumably, some reducing sugars were bound to the nitrogen-containing substances and were extracted with them; thus, it seems likely that the bulk of the nitrogen represents a glycopeptide, and that the glycopeptide is the material primarily responsible for the skin reactivity. It is postulated that the peptide is linked to the polysaccharide in such a fashion as to constitute the "determinant" portion, whereas the polysaccharide would represent just the "core" of the moiety. Studies by Barker et al. (3) with a glycopeptide isolated from *Trichophyton mentagrophytes* lend credibility to this postulate. By chemical means, these authors were able to show that degradation of the carbohydrate part of the glycopeptide caused reduction in the immediate type reactions, whereas a degradation of the peptide caused a loss of delayed-type reactivity. Similar observations have been reported with blood-group substances (15).

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service Research Grant AI-02580 and by Public Health Training Grant 5TIAI-3 from the National Institute of Allergy and Infectious Diseases.

We are indebted to Morris F. Shaffer for many valuable suggestions and to Tom Mitchell for technical assistance.

Miss Restrepo was a Fellow of the Agency for International Development during the time that this investigation was carried out.

LITERATURE CITED

1. ALMEIDA, F. P., AND C. S. LACAZ. 1941. Intradermoreação com paracoccidioidina no diagnóstico do granuloma paracoccidioidico. II. A reação do Montenegro na granuloma paracoccidioidico. Folia Clin. Biol. (Sao Paulo) **13:** 177–182.
2. ALMEIDA, F. P., C. S. LACAZ, AND C. A. CUNHA. 1945. Intradermoreação para diagnóstico da Blastomicose sul-americana (granulomatose-paracoccidioidica). Arquiv. Brasil. Med. **35:** 267–272.
3. BARKER, S. A., C. N. D. CRUICKSHANK, J. H. MORRIS, AND S. R. WOOD. 1962. The isolation of a *Trichophytin* glycopeptide and its structure in relation to the immediate and delayed reactions. Immunology **5:**627–632.
4. CARVALHO, A. 1953. Sôbre õ emprêgo da paracoccidiodina na cidade do Rio de Janiero. Primeiros resultados basados no estudo de 475 individuos. Rev. Brasil. Tuberc. **21:** 73–82.
5. CARVALHO, A. 1958. Sôbre õ uso intradérmico da paracoccidiodina (filtrado e suspenção) como meio auxiliar no diagnóstico na doença de Lutz. Rev. Nac. Tuberc. **2:**131–178.
6. CROSS, F. W., AND A. HOWELL. 1948. Studies on fungus antigens. II. Preliminary report on the isolation of an immunologically active polysaccharide from histoplasmin. Public Health Rept. (U.S.) **63:**179–183.
7. DISCHE, Z., AND E. BORENFREUND. 1950. A spectrophotometric method for the microdetermination of hexosamines. J. Biol. Chem. **184:**517–522.
8. DOUAT, N. E., AND V. M. DIAZ. 1958. Intradermoreações de paracoccidioidina, coccidioidina, e histoplasmina: resultados de testes em 300 individuos. Rev. Brasil. Tuberc. **26:**663–668.
9. DYSON, J. E., AND E. E. EVANS. 1955. Delayed hypersensitivity in experimental fungus infections. The skin reactivity of antigens from the yeast phase of *B. dermatitidis*. J. Invest. Dermatol. **24:**447–454.
10. EDWARDS, P. Q., R. A. KNIGHT, AND S. MARCUS. 1961. Skin sensitivity of human beings to *Histoplasma capsulatum* and *Blastomyces dermatitidis* polysaccharide antigens. Am. Rev. Respirat. Diseases **83:**528–534.
11. FAVA NETTO, C., AND A. RAPHAEL. 1961. A reação intradérmica com polissacáride do *Paracoccidiodes brasiliensis* na blastomicose sul-americana. Rev. Inst. Med. Trop. Sao Paulo **3:**161–165.
12. FISKE, C. N., AND Y. SUBBAROW. 1925. The colorimetric determination of phosphorus. J. Biol. Chem. **66:**375–400.
13. FONSECA, O., AND A. AREA LEAO. 1927. Reaction

cutanée spécifique avec le filtrat de cultures de *C. immitis*. Compt. Rend. Soc. Biol. **97**:1796–1797.

14. FRIEDMAN, L., AND N. F. CONANT. 1953. Immunologic studies on the etiologic agents of South and North American blastomycosis. Mycopathol. Mycol. Appl. **6**:310–316.
15. HOLBORROW, E. J., AND G. LOEWI. 1962. The immune response to blood-group substances. Immunology **5**:278–286.
16. HOUNIE, P., AND R. C. ARTAGAVEYTIA ALLENDE. 1957. Encuestra sobre la sensibilidad al agente de la blastomicosis suramericana. Anales Fac. Med. Montevideo **42**:27–32.
17. KABAT, M. A., AND M. M. MAYER. 1961. Experimental immunochemistry, 2nd ed. Charles C Thomas, Publisher, Springfield, Ill.
18. KELLEY, W. H. 1939. A study of the cell and colony variations of *Blastomyces dermatitidis*. J. Infect. Diseases **64**:293–296.
19. LACAZ, C. S. 1948. Blastomicose Sul-americana. Reações intradérmicas com a paracoccidioidina, coccidioidina e blastomicetina. Rev. Hosp. Clin. **3**:11–18.
20. LACAZ, C. S. 1951. Lesões pulmonarea na blastomicose sul-americana: inquérito preliminar com a piacoccidioidina. Hospital (Rio de Janeiro) **39**:405–422.
21. LACAZ, C. S., M. C. ROCHO PASSOS, C. FAVA NETTO, AND B. MACARRON. 1959. Contribução para o estudo de "Blastomicose-Infecção". Inquérito com a paracoccidiodina. Estudo serológico e clinico radiológico dos paracoccidiodino-positivos. Rev. Inst. Med. Trop. Sao Paulo **1**:245–259.
22. MACKINNON, J. E., R. C. ARTAGAVEYTIA ALLENDE, AND L. ARROYO. 1953. Sobre la especificidad de la intradermoreacción con paracoccidiodina. Anales Frac. Med. Montevideo **38**:363–382.
23. MARKOWITZ, H. 1964. Polysaccharide antigens from *H. capsulatum*. Proc. Soc. Exptl. Biol. Med. **115**:697–700.
24. PAPPAGIANIS, D., E. W. PUTNAM, AND G. S. KOBAYASHI. 1961. Polysaccharide of *Coccidioides immitis*. J. Bacteriol. **82**:714–723.
25. PUTNAM, E. W. 1957. Paper chromatography of sugars, p. 62–72. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 3. Academic Press, Inc., New York.
26. REISSIG, J. L., J. L. STROMINGER, AND L. F. LELOIR. 1955. A modified colorimetric method for the estimation of n-acetyl-amino sugars. J. Biol. Chem. **217**:959–966.
27. SALVIN, S. B., AND R. F. SMITH. 1959. Antigens from the yeast phase of H. capsulatum. III. Isolation, properties and activities of a protein-carbohydrate complex. J. Infect. Diseases **105** 45–53.
28. SCHNEIDAU, J. D., L. M. LAMAR, AND M. A HAIRSTON. 1964. Cutaneous hypersensitivity to sporotrichin in Louisiana. J. Am. Med. Assoc. **188**:371–373.
29. SEIBERT, F. B., AND L. F. AFFRONTI. 1963. Kjedahl-Nessler method for the determination of nitrogen, p. 5–8. *In* Methodology manual for investigation of mycobacterial and fungal antigens. American Thoracic Society, New York.
30. SIEBERT, F. B., AND L. F. AFFRONTI. 1963. Anthrone method for determining polysaccharide, p. 16–20. *In* Methodology manual for investigation of mycobacterial and fungal antigens. American Thoracic Society, New York.
31. SEVAG, M. G. 1934. Eine neue physikalische Entweissungs method zur Darstellung biologische wirksamer Substanzen. Biochem. Z. **273**:418–429.
32. SMITH, I. 1958. Chromatographic techniques. Interscience Publishers, Inc., New York.
33. ZAMORA, A., L. F. BOJALIL, AND F. BASTARRACHEA. 1963. Immunologically active polysaccharides from *Nocardia asteroides* and *Nocardia brasiliensis*. J. Bacteriol. **85**:549–555.