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Cell-mediated immune responses in patients with paracoccidioidomycosis

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SUMMARY

We tested the hypothesis that symptomatic infection with *Paracoccidioides brasiliensis* caused impaired host cellular immune responses. In a cross-sectional study in Colombia, the immune responses of thirty-six patients with paracoccidioidomycosis were compared with those of sixty normal individuals. Patients demonstrated increased skin sensitivity to paracoccidioidin (para) and histoplasmin, and reduced reactivity to candidin and dinitrochlorobenzene as compared to controls. The skin test response to tuberculin (PPD) was similar to that of controls. *In vitro* lymphocyte transformation (LT) and leucocyte migration responses to phytohaemagglutinin and PPD did not differ in patients and controls; these responses to PPD correlated with skin sensitivity in controls, but not in patients. LT and inhibition of leucocyte migration to para were seen in more patients than controls; the latter response correlated with skin sensitivity in controls only. Positive LT to para was associated with absence of antibodies to *P. brasiliensis*. Analysis of symptomatic patients suggests that the prevalence of para skin test positivity was lowest in patients with the longest duration of disease; this implies decrease in specific cell-mediated immunity with prolonged active infection. Analysis of clinically cured patients suggests that the prevalence of para skin sensitivity and LT to para and PPD increased with time elapsed since diagnosis; this implies development or restoration of immunocompetence upon clinical recovery. Results of a preliminary longitudinal study on the immunological responses of six patients with active paracoccidioidomycosis are compatible with the above observations.

INTRODUCTION

Paracoccidioidomycosis, the most prevalent human systemic mycosis in Latin America, is caused by the dimorphic fungus *P. brasiliensis*. The infection can be symptomatic or asymptomatic as demonstrated by skin-test reactivity to antigens prepared from the fungus (Restrepo *et al.*, 1968). Clinical manifestations are those of chronic granulomatous diseases with pulmonary and/or lymph node involvement, lesions in mucocutaneous areas or internal organs (Sampaio, 1972). Patients with paracoccidioidomycosis are defined as persons who have had clinical infection due to *P. brasiliensis* some time in their lives.

In endemic areas, the incident case rate is extremely low among persons who demonstrate skin-sensitivity to paracoccidioidin (Greer & Restrepo, 1976). This implies acquired resistance to *P. brasiliensis* in the majority of persons exposed to the fungus. The important role of the immune system in paracoccidioidomycosis is also suggested by the extensive involvement of the reticular endothelial system in fatal cases (Giraldo *et al.*, 1976). Specific antibodies are present in patients with paracoccidioidomycosis (Correa & Giraldo, 1972), but they do not appear to provide any protective function.

An association of impaired cell-mediated immunity (CMI) with paracoccidioidomycosis has been shown (Mendes & Raphael, 1970; Mendes *et al.*, 1971; Mendes, 1975; Musatti, 1975). Depressed CMI

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may be a consequence of clinical infection or an impaired immune system may predispose an individual to symptomatic infection. An elucidation of the causal relationship of paracoccidioidomycosis and impaired CMI may provide an indication of the diagnostic and/or prognostic values of CMI assays in the disease, and a basis for administering immunotherapy to patients (Rocklin *et al.*, 1970; Bullock, Fields & Brandriss, 1972; Graybill *et al.*, 1973). This study tested the hypothesis that symptomatic infection with *P. brasiliensis* causes depressed host cellular immune responses.

MATERIALS AND METHODS

Study subjects. Patients came from two endemic provinces in Colombia, Valle and Cauca. They were diagnosed on clinical and radiological findings, and one or more of the followings: microscopic observation of clinical specimen(s), cultural isolation of *P. brasiliensis* and immunodiffusion test. Patients were referred to the study by physicians in the University Hospital of Valle and physicians in regional hospital and respiratory centers. Thirty-six patients were studied: eight were diagnosed during our study period of November 1974 to December 1975. Patients' clinical records were reviewed and when possible, their physicians were consulted on their clinical course.

Sixty clinically normal adults who had never been skin-tested with histoplasmin and paracoccidioidin and were serologically negative to the latter were admitted as controls. They were persons participating in routine surveys on systemic mycoses.

In vivo assays for CMI (a) *Skin tests.* These tests consisted of 0·1 ml of each of the followings: standardized mycelial paracoccidioidin (Restrepo & Schneidau, 1967), 40 μg/ml, supplied by A. Restrepo; histoplasmin (1:100, US CDC, Atlanta, Georgia); tuberculin PPD (5TU, Parke–Davis & Co., Detroit, Michigan); and candidin (dermatophytin 'O' 1:100, Hollister-Stier Lab, Spokane, Washington). Antigens were injected in both forearms employing standard techniques. At 48 hr, a PPD reaction of $\geqslant 10$ mm of induration was considered positive; for other antigens, an induration $\geqslant 5$ mm was read positive.

(b) *DNCB Contact sensitization.* This test was performed on thirty-six patients and fifty-seven controls; three controls were unwilling to participate. A dose of 2000 μg of 2,4-dinitrochlorobenzene (DNCB, K & K Lab, Plainview, New York) in 0·1 ml acetone was applied topically within a plastic ring (diameter, 1·5 cm; depth, 1 cm) on the subjects' lower arm. The site was examined for delayed hypersensitivity (DH) response 18–21 days later. Any induration was read as positive response. When none was observed, 100 μg DNCB/0·1 ml acetone was applied on the opposite arm and reaction read at 48 hr. Absence of induration after the second contact was read as negative response.

In vitro CMI assays. These assays were performed on blood samples from thirty-five patients and fifty-nine controls.

(a) *Lymphocyte transformation* (*LT*) *assay.* Peripheral venous blood was drawn into heparin-coated syringes, 100 u of preservative-free sodium heparin (NBC, Cleveland, Ohio) per ml of blood. A volume of blood with 6×10^5 lymphocytes was dispensed into 12×75 mm sterile plastic culture tube (Arthur Thomas Co., Philadelphia, Pennsylvania). Two millilitres of tissue culture medium (TCM) 199 (Difco Lab, Detroit, Michigan) supplemented with 10% foetal calf serum, 0·4 ml of 10% $NaHCO_3$, 10^3 u penicillin and 10^3 u streptomycin per 100 ml medium was added to each tube.

To each set of three tubes, one of the following in TCM was added: phytohaemagglutinin 'P' (PHA, Difco Lab, Detroit, Michigan), 0·1 ml of 1:100 dilution (McMurray, personal communication); tuberculin PPD (Trudeau Institute, Saranac Lake, New York), 10 μg/ml (Miller & Jones, 1973); preservative-free paracoccidioidin (Restrepo & Schneidau, 1967) supplied by A. Restrepo, 40 μg/ml as determined in preliminary studies on patients' blood samples. One set of three tubes contained no stimulant and served as control cultures.

Tubes were incubated at 37°C. After 72–96 hr of incubation, 2·5 μCi of ^{3}H-labelled thymidine (sp. act. 6·7 Ci/mmole, New England Nuclear, Boston, Massachusetts) was added to each tube for a further 18–24 hr of incubation. The cells were harvested (Jung *et al.*, 1970), transferred to glass tubes and washed with 3 ml of methanol. The precipitate was air-dried and dissolved in 0·5 ml of 1 M hyamine hydroxide (New England Nuclear, Boston, Massachusetts) in a 56°C water-bath for 3–4 hr. The cells were washed into counting vials with 15 ml of scintillation fluid (Omnifluor, New England Nuclear, Boston, Massachusetts) and their radioactivity was counted in a scintillation counter (Nuclear Chicago, Chicago, Illinois) for 2 min. Results are expressed as a ratio of ct/min in stimulated cultures to counts in controls cultures.

In PHA- and PPD-stimulated cultures, a LT ratio $\geqslant 10$ was regarded as positive response (McMurray, personal communication). In paracoccidioidin-stimulated cultures, a LT ratio $\geqslant 1{\cdot}4$ was regarded as positive as determined from frequency distributions of LT ratios in patients and controls (Fig. 1).

(b) *Leucocyte migration inhibition assay.* Four millilitres of blood were centrifuged at 100 *g* for 10 min. The buffy coat was removed, washed in 3 ml of TCM prepared as described above, centrifuged at 100 *g* for 10 min and the cells suspended in 1·5 ml of medium. Capillary haematocrit tubes, 7·5 mm long with a 1·2 mm inner diameter (Curtin Scientific Co., Houston, Texas) were filled with the cell suspension, sealed at one end with wax, centrifuged at 100 *g* for 5 min and cut at the cell–fluid interface. The cell-filled portion was embedded in silicone grease in chambers of migration plates (Arthur Thomas Co., Philadelphia, Pennsylvania).

To each set of three chambers, 0·5 ml of TCM with one of the followings was added: PHA, 0·1 ml of 1:100 dilution; PPD, 10 μg/ml; and paracoccidioidin, 40 μg/ml. Plain TCM was added to one set of chambers which served as control cultures. The chambers were covered with glass slips and incubated at 37°C for 24 hr. Area of leucocyte migration was

viewed with a stereoscope mounted with a 0·5 mm squared grid in the ocular. The area was recorded as the number of squares completely traversed by cells. Results are expressed as a migration percentage:

$$\frac{\text{mean migration of cells in control cultures}}{\text{mean migration of cells in test cultures}} \times 100\%.$$

A percentage of < 80 was interpreted as positive migration inhibition (Bice *et al.*, 1974).

Serologic test. The agar gel immunodiffusion test (Crowle, 1961) was done on sera from all patients and controls. Paracoccidioidin, a culture filtrate antigen of *P. brasiliensis* yeasts (Restrepo, 1966), was supplied by A. Restrepo.

Statistical analysis. The two-tailed *t*-test of significance and the Chi-squared test of association at 5% level were used. In the latter test, when the smallest expected frequency number was < 5 and the total number of test ≥ 30 a Yates correction was used.

Longitudinal study. Six freshly diagnosed patients were evaluated over a 9-month period. One patient was followed for 6 months only. These patients received daily oral treatment with sulpha drugs. At 3-month intervals, blood was drawn for immunodiffusion and complement fixation (CF) tests (USDHEW, 1965) and LT studies. On the last visit, skin tests were repeated in all patients and the DNCB test done on those who had not previously given a positive response; reactions were read at 48 hr.

RESULTS

Study subjects

All controls and all but one patient were male. The patients' mean age was 50·8 years and controls' 46·0 years. Twenty-one patients (58·3%) and forty-six controls (76·7%) were agricultural workers. Twenty-five patients (69·4%) and forty-six controls (76·7%) resided in rural areas. Patients and controls were comparable in demographic features.

Upon entry into the study, seventeen patients were symptomatic and nineteen free of clinical symptoms. Precipitating antibodies were demonstrated in sera from fourteen of the symptomatic and seven of the clinically cured patients.

Skin tests

Table 1 shows results of skin tests. Patients showed increased skin sensitivity to paracoccidioidin ($P<0{\cdot}01$) and histoplasmin ($P<0{\cdot}01$), and reduced reactivity to candidin ($P<0{\cdot}05$) when compared to controls. Skin sensitivity to PPD in patients and controls were similar. Analysis of patients and controls who reacted positively to none, one, two or all three of the non-paracoccidioidal antigens: histoplasmin, PPD and candidin by Chi-squared test for proportional trend did not show any difference between these two groups. Complete cutaneous anergy was seen in similar proportion of patients (16·7%) and controls (20·0%).

Skin test reactions to paracoccidioidin and histoplasmin were positively correlated in both patients (coefficient of correlation $r = 0{\cdot}51$) and controls ($r = 0{\cdot}67$). Skin test reactions to paracoccidioidin and candidin were positively correlated in patients ($r = 0{\cdot}38$) but not in controls ($r = -0{\cdot}39$). These indicate that the paracoccidioidin and histoplasmin reactions were not specific; and the candidin reactions were specific in controls only.

DNCB contact sensitization

Ten of thirty-six patients and forty-four of fifty-seven controls reacted positively 18–21 days after primary contact with DNCB. The non-responders were tested again. Eight of twenty-six patients and all thirteen controls showed DH to the second DNCB contact. Overall positive response rates were: 18/36 in patients and 57/57 in controls. Differences between patients' and controls' responses to primary and secondary DNCB contact were significant ($P<0{\cdot}01$).

Lymphocyte transformation

Fig. 1 shows the transformation ratios of lymphocytes in the presence of various stimulants.

Phytohaemagglutinin. Three symptomatic patients demonstrated a LT ratio of less than 10. The mean

TABLE 1. Patients' and controls' skin-test reactivity 48 hr after i.d. administration of four different antigens

Antigen	Positive reactors		Significance
	Patients (total = 36)	Controls (total = 60)	
Paracoccidioidin	16 (44·4%)	10 (16·7%)	$P<0·01$
Histoplasmin	24 (66·7%)	24 (40·0%)	$P<0·01$
Tuberculin	19 (52·8%)	26 (43·3%)	n.s.
Candidin	11 (30·6%)	34 (56·7%)	$P<0·05$

n.s. = difference between patients and controls not significant.

LT ratio in symptomatic patients was 60·4 (±23·6), and in clinically cured patients 69·9 (±9·9). Controls' mean LT was 59·0 (±9·04). There was no significant difference between mean ratios in patient groups and controls.

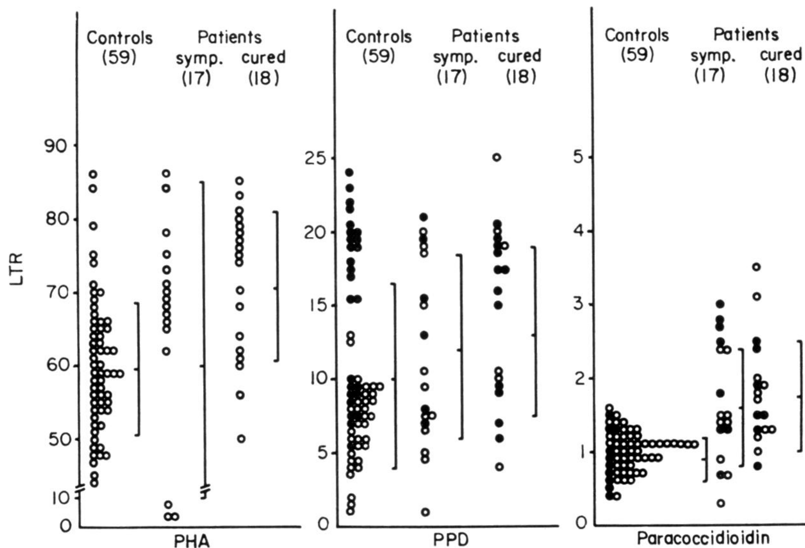

FIG. 1. LT ratio (LTR) is expressed as transformation of stimulated lymphocytes/transformation of unstimulated lymphocytes. Each circle represents the mean of three determinations. In figures showing LT in PPD- and paracoccidioidin-stimulated cultures, black circles represent subjects skin test positive to homologous antigen; open circles, skin test negative subjects. Group means plus or minus standard error are indicated. Note different scales of LTR. Symp. = symptomatic.

Tuberculin. Mean LT ratio in controls was 10·0 (±6·2) and those in symptomatic and cured patients were 11·7 (±6·2) and 12·9 (±5·8) respectively. These ratios did not differ significantly from each other Of the twenty-six controls who were skin-test positive to PPD, sixteen had positive LT response. Of the thirty-three skin-test negative controls, thirty-one showed negative LT response. There was association between *in vitro* and *in vivo* responses to PPD in controls ($P<0·05$). Similar analyses of symptomatic and cured patients did not reveal any correlation between LT and skin test reactivity.

Paracoccidioidin. Controls had mean LT ratio of 0·90 (±0·28). Symptomatic and cured patients had mean LT ratios of 1·58 (±0·82) and 1·68 (±0·75) respectively which were significantly higher than that of controls ($P<0·05$). More patients (twenty in thirty-five) than controls (three in fifty-nine) showed a LT ratio above 1·4 ($P<0·05$). Among both controls and patients, LT and skin test responses did not correlate.

Leucocyte migration inhibition

Fig. 2 shows data on migration of patients' and controls' leucocytes in the presence of various agents.

Phytohaemagglutinin. Leucocytes from controls showed mean migration of 20·8% (±6·8%); leucocytes from symptomatic and clinically cured patients showed mean migration of 18·9% (±6·6%) and 15·2% (±7·3%). All did not differ significantly from each other.

Tuberculin. Mean migration of leucocytes from controls was 77·5% (±9·1%) and from symptomatic and cured patients were 84·8% (±10·0%) and 75·3% (±9·0). These did not differ significantly from each other. Of the twenty-six controls who were skin-test positive to PPD, inhibition of *in vitro* migration was seen in leucocytes from seventeen subjects. Of the thirty-three skin-test negative controls, inhibition of leucocyte migration was seen in eleven. *In vivo* and *in vitro* responses to PPD was positively correlated in controls ($P<0{\cdot}01$), but not in patients.

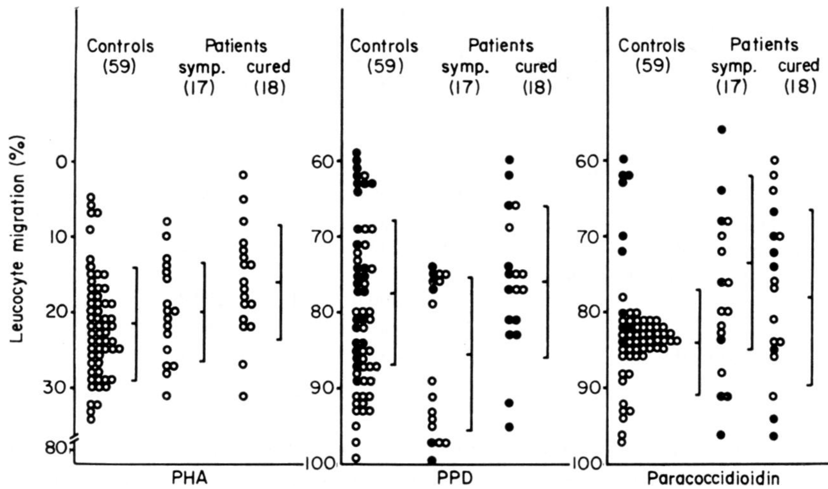

FIG. 2. Leucocyte migration, expressed as mean leucocyte migration in three test cultures with PHA or PPD or paracoccidioidin/mean leucocyte migration in three control cultures see legend for Fig. 1. Symp. = symptomatic.

Paracoccidioidin. Mean migration of controls' leucocytes was 84·0% (±7·5%) and of symptomatic and cured patients' leucocytes 73·6% (±11·4%) and 77·3 (±11·7%) respectively. There was no significant difference in these migration means. Significantly more patients (eighteen in thirty-five) than controls (seven in fifty-nine) showed inhibition of leucocyte migration. Inhibition of leucocyte migration was correlated with cutaneous DH in controls ($P<0{\cdot}01$), but not in patients.

Correlation between cellular and humoral immune responses

With one exception, the *in vivo* and *in vitro* cellular immune responses did not differ in patients with antibodies and patients without antibodies to *P. brasiliensis* (Table 2). The exception was: more serologically negative patients (78·6%) than serologically positive patients (42·9%) showed positive response in paracoccidioidin-induced LT ($P<0{\cdot}05$).

Cellular immune responses in patients with respect to time after diagnosis

Among symptomatic patients, the prevalence of positive skin reactivity to paracoccidioidin appears to be lowest in patients with the longest duration of disease while that to other antigens or DNCB shows no similar trend (Table 3). No distinct pattern in LT or leucocyte migration in the presence of PHA, PPD and paracoccidioidin with regard to time after diagnosis was discerned. Among clinically

TABLE 2. *In vivo* and *in vitro* cellular immune response in patients with and in patients without antibodies to *P. brasiliensis*

Cellular immune response	Patients with antibodies (total = 21) Positive reactors No.	%	Patients without antibodies (total = 14) No.	%	Significance
Skin tests:					
Paracoccidioidin	11	52·4	5	33·3	n.s.
Histoplasmin	16	76·2	8	53·3	n.s.
Tuberculin	10	47·6	9	60·0	n.s.
Candidin	6	28·6	5	33·3	n.s.
DNCB test	10	47·6	8	53·3	n.s.
Lymphocyte transformation:					
PHA	18	90·0	14	100·0	n.s.
PPD	12	57·1	7	50·0	n.s.
Paracoccidioidin	9	42·9	11	78·6	$P<0·05$
Leucocyte migration inhibition:					
PHA	21	100·0	14	100·0	n.s.
PPD	13	61·9	7	50·0	n.s.
Paracoccidioidin	10	47·6	8	57·1	n.s.

cured patients, the prevalence of paracoccidioidin skin test positivity seems to increase with time elapsed since diagnosis and that to other antigens or DNCB remains fairly constant over time. Reactivity of LT to paracoccidioidin and PPD also appears to increase as time after diagnosis increases while that of LT and leucocytes in other tests did not vary with time.

Longitudinal study

Three patterns of skin test reactivity were observed (Table 4). Two patients (group 1) developed DH response to paracoccidioidin after 9 months of treatment. One of them also showed positive DH to histoplasmin. Cutaneous DH to all test antigens in two other patients (group 2) was unchanged. The

TABLE 3. Cellular immune response in symptomatic and clinically cured patients with respect to time after diagnosis

Patients	Time after diagnosis (years)	Cellular immune responses: No. patients positive/no. patients tested — Skin tests: Para.	Skin tests: Histo.	Skin tests: PPD	Skin tests: Candidin	DNCB	Lymphocyte transformation: Para.	Lymphocyte transformation: PPD	Lymphocyte transformation: PHA	Leucocyte migration inhibition: Para.	Leucocyte migration inhibition: PPD	Leucocyte migration inhibition: PHA
Symptomatic	0–1	5/10	4/10	3/10	2/10	4/10	6/10	5/10	8/10	5/10	5/10	10/10
	2–3	3/4	4/4	2/4	1/4	1/4	1/4	2/4	4/4	2/4	2/4	4/4
	4–5	0/1	1/1	1/1	1/1	1/1	0/1	0/1	0/1	0/1	0/1	1/1
	6–7	0/2	2/2	0/2	1/2	1/2	2/2	1/2	2/2	1/2	1/2	2/2
Clinically cured	0–1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
	2–3	1/5	3/5	3/5	1/5	2/5	1/5	2/5	5/5	3/5	3/5	5/5
	4–5	1/5	3/5	4/5	2/5	4/5	3/4	2/4	4/4	1/4	3/4	4/4
	6–7	3/5	3/5	3/5	2/5	4/5	3/5	3/5	5/5	3/5	3/5	5/5
	8	2/3	3/3	2/3	1/3	1/3	3/3	3/3	3/3	2/3	2/3	3/3

remaining two patients (group 3) showed decrease in paracoccidioidin reaction size and concomitant loss of histoplasmin and candidin skin reactivity. In all patients, PPD reaction did not vary.

Sera from group 1 patients showed decrease in CF antibodies (Table 4). Group 2 patients' CF antibody titre was unchanged. Antibody responses from group 3 patients included the above types.

One patient each from groups 1, 2 and 3 did not respond to primary or secondary DNCB contact. These patients all reacted positively when tested 9 months later. Patients' LT ratios fluctuated with time; no trend was discernible.

TABLE 4. Skin reactivity to microbial antigens and antibody responses in patients in longitudinal study

Group	Patient	Time after treatment (months)	Skin tests: Para.	Histo.	PPD	Candidin	Complement fixing antibody titre
1	V.R.	0	0* (−)	0† (−)	0 (−)	0 (−)	1:256
		3					1:128
		6					n.d.
		9	8 (+)	0 (−)	0 (−)	0 (−)	1:64
	J.R.	0	0 (−)	0 (−)	13 (+)	0 (−)	1:256
		3					1:32
		6	12 (+)	11 (+)	15 (+)	0 (−)	1:32
2	H.M.	0	6 (+)	16 (+)	0 (−)	0 (−)	1:64
		3					1:64
		6					1:16
		9	14 (+)	13 (+)	0 (−)	5 (−)	1:64
	J.A.	0	10 (+)	10 (+)	0 (−)	0 (−)	1:16
		3					n.d.
		6					AC
		9	11 (+)	12 (+)	7 (−)	0 (−)	1:16
3	A.T.	0	12 (+)	12 (+)	9 (−)	9 (+)	1:256
		3					1:128
		6					1:64
		9	8 (+)	0 (−)	8 (−)	0 (−)	1:32
	M.H.	0	36 (+)	13 (+)	8 (−)	14 (+)	1:128
		3					1:32
		6					n.d.
		9	8 (+)	0 (−)	6 (−)	0 (−)	1:128

n.d. = Not done, due to insufficient amount of serum; AC = anticomplementary.
* Induration size of skin test reaction in mm.
† Qualitative skin test reaction.

DISCUSSION

The prevalence of paracoccidioidin skin sensitivity in controls was similar to those reported within endemic areas in Colombia (Restrepo *et al.*, 1968; Greer, de Estrada & de Trejos, 1974). Only 44·4% of patients showed positive skin reactivity. This figure is lower than those shown in other studies with patients, which ranged from 53·8–81·0% (Restrepo, 1967; Schneidau, 1972; Greer *et al.*, 1974). Paracoccidioidin skin positivity is related to the severity of clinical infection (Restrepo *et al.*, 1970). As will be discussed below, it might be related to duration of clinical infection.

The high number of patients with positive DH response to histoplasmin reflects either dual infection with *Histoplasma capsulatum* and *P. brasiliensis*, or cross-reactions between histoplasmin and paracoccidioidin (Restrepo, 1967; Greer *et al.*, 1974) or both. In this study, histoplasmin and paracoccidioidin were non-specific.

Patients showed no impairment in PPD skin sensitivity. Mendes & Raphael (1970) and Mendes (1975) applied skin tests of 2TU and observed depressed PPD skin reactivity in their patients. We used a dosage of 5TU. When a reaction size of ⩾15 mm instead of 10 mm was read as positive, there was still no difference between patients and controls. Further trials with either 2TU or 5TU are needed to establish the possibility of non-specific reduction in PPD skin sensitivity in patients with paracoccicioidomycosis.

The positive correlation between candidin and paracoccidioidin reactions in patients only implies that the candidin skin reactivity in some patients may have been induced by paracoccidioidin skin sensitivity. Therefore, patients' reactivity to candidin may have been lower than that observed. Furthermore a selective response mechanism may have been induced by the disease process since skin reactions in controls were not correlated. Clinical paracoccidioidomycosis may have impaired the ability of candida-sensitized lymphocytes to react to homologous antigen while rendering them reactive to paracoccidioidin, a heterologous antigen. Similarly, active infection may have induced the paracoccidioides-sensitized lymphocytes to react to candidin.

Complete cutaneous anergy to non-paracoccidioidal antigens was not unique to patients. The selection of two heterologous mycotic antigens and one bacterial antigen was sufficiently varied to insure the detection of general cutaneous anergy. Our finding differs from that of earlier workers (Mendes & Raphael, 1970; Mendes, 1975). The discrepancy may be due to composition of the patient groups. Only 47·2% of our patients had active infection at time of study, while all patients in earlier studies were symptomatic.

Impairment of induction and sensitization of lymphocytes was shown in patients by the DNCB test. Mendes & Raphael (1970) reported a DNCB response rate of 27·4% in fifty-one patients and Mendes (1975) described a 62% reactivity in sixteen patients. Persons with paracoccidioidomycosis appear to be severely impaired in their ability to be sensitized.

Lymphocytes from only three patients did not show PHA-induced transformation. In contrast, Mendes *et al.* (1971) and Musatti (1975) reported reduction in PHA-induced ^{3}H-labelled thymidine incorporation by purified T lymphocytes from half of their patients. The greater number of positive reactors seen in our study may be due to B-cell transformation since populations of mixed lymphocytes were used.

Tuberculin was employed as a control antigen to assess the relationship between the *in vitro* and *in vivo* CMI assays. Patients and controls demonstrated similar LT and skin test responses to PPD. However, these two assays were correlated in controls only. Dissociation of the two tests in patients reflects malfunction in either cutaneous DH mechanism or lymphocyte transformation. This dichotomy in immunological responses was inapparent when results on either CMI assays were compared with those of controls, or when only one test was performed. Impairment of PPD responses suggests that clinical paracoccidioidomycosis depresses CMI to tuberculosis or non-specific immune mechanisms which may be attachment of antigens onto macrophage, presentation of antigen-macrophage complex to sensitized lymphocytes and the function of an intact vascular system. Our finding indicates the need to do both *in vivo* and *in vitro* assays with the same antigen for the detection of immunological abnormalities in patients.

A filtrate antigen derived from mycelial culture of *P. brasiliensis* was used in inducing LT because of the availability of the antigen. In paracoccidioidomycosis the mycelial phase of the fungus is the infective phase and the yeast phase the morphologic form in the infected host. The paracoccidioidin used may not have the same antigenic components to which lymphocytes were sensitized during disease. Antigens derived from the morphologic phase of the organism as present in the infected host may be more efficient in eliciting immune responses (Deresinski, Levine & Stevens, 1974; Stevens, Levine & Ten Eyck, 1974). Musatti (1975) had employed a cytoplasmic antigen derived from *P. brasiliensis* yeasts in stimulating LT and reported low transformation ratios from patients. Greater induction of LT by paracoccidioidal antigen may be achieved in the future through purification and isolation of different antigenic fractions of the *P. brasiliensis* yeasts. Lack of correlation between skin sensitivity and LT to paracoccidioidin may be due to nature of the antigen or heterogeneity of lymphocytes involved in the responses.

Inhibition of leucocyte migration and cutaneous DH were correlated in controls when PPD was the antig en.Lack of correlation between CMI assays in patients supports our previous remark on the possible impairment of CMI to tuberculosis and/or depression of non-specific immune mechanisms in persons with clinical paracoccidioidomycosis. When paracoccidioidin was the test antigen, inhibition of leucocyte migration and cutaneous DH were also correlated in controls only. This indicates impairment in patients' CMI to paracoccidioidomycosis in either cutaneous DH or release of a leucocyte migration inhibition factor.

In patients, reduction in paracoccidioidin-induced LT but not in cutaneous DH was associated with the presence of antibodies to *P. brasiliensis*. *In vitro* depression may arise from technical nature of the assay, since whole blood was used, and may not necessarily reflect *in vivo* depression. Musatti (1975) reported the existence of a factor in the plasma of patients with paracoccidioidomycosis which inhibited PHA-induced LT. This inhibitory factor was characterized; it may be antibodies. Impairment of LT may be due to both antibodies and inhibitory factor.

Overt disease as the cause of depressed cellular immune responses has been implicated in lepromatous leprosy, Hodgkin's disease and sarcoidosis in which immunological recovery parallels clinical recovery (Chase, 1966; Brown *et al.*, 1967; Turk & Bryceson, 1971). The exact date of onset of disease in most of our patients was unknown or not recorded. Patients might have waited weeks or months before they were diagnosed of having paracoccidioidomycosis. The period between diagnosis and entry into our study in symptomatic patients represents the minimum duration of active infection and in clinically cured patients, a less accurate measure of length of recovery since the date of disappearance of symptoms cannot be assessed correctly. In symptomatic patients the prevalence of paracoccidioidin skin test positivity was lowest in those with the longest duration of illness. In clinically cured patients, the prevalence of paracoccidioidin skin test positivity increased with time elapsed since diagnosis. These observations are compatible with the hypothesis that active infection with *P. brasiliensis* impaired specific CMI and that development or recovery of specific immunocompetence parallels clinical recovery.

Analysis of *in vitro* responses of clinically cured patients suggested that the prevalence of paracoccidioidin-induced LT increased as time elapsed since diagnosis. This reinforces the concept of the development or restoration of specific immunocompetence upon clinical cure. Analysis of *in vitro* responses of symptomatic patients showed no trend with time. Failure to detect any variation may be due to sensitivity of the techniques and nature of the antigen used. In both patient groups, the LT (with the exception of PPD-induced LT in cured patients), leucocyte migration and skin reactivity to non-paracoccidioidal antigens did not vary with time, implying impairment in CMI to non-paracoccidioidal infections or general immune mechanisms may be long-lasting.

Results from the preliminary longitudinal study support the above comments. The various dermal DH responses to paracoccidioidin in patients receiving similar drug therapy emphasize the polarity of possible immunological reactions. Following acute illness, patients may develop specific CMI and achieve clinical cure (group 1), or the cellular immunological state may deteriorate leading to subsequent chronic infection (group 3). In yet other patients (group 2) the immunological reactivity is intermediate between these two poles of manifestation. Fluctuation of humoral immune responses does not seem to reflect *in vivo* cellular immune responses. Skin tests with paracoccidioidin and other fungal antigens may be an accurate and necessary test to supplement serologic tests in assessing patients' immunological recovery. Both DNCB contact sensitization and LT failed to distinguish patients with distinct cutaneous DH reactions.

Further prospective studies on larger number of patients over longer periods of time are required to establish firmly the immunological patterns observed here. Moreover, future studies should be directed to identification of possible genetic, hormonal or nutritional factors that predispose patients with paracoccidioidomycosis to develop different immunological reactions after acute illness. Elucidation of the predisposing factors may eventually aid in the treatment of the disease.

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