Cell-Mediated Immunity to *Pityrosporum Orbiculare* in Tinea Versicolor

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ABSTRACT *Pityrosporum orbiculare*, the presumed etiologic agent of tinea versicolor, was cultured in vitro and antigenic extracts prepared from the cultured organisms. Studies with lymphocytes from human cord blood and peripheral blood of guinea pigs demonstrated that such extracts were not mitogenic. Further studies in guinea pigs indicated that the animals could be sensitized by the injection of *P. orbiculare* extract in Freund's complete adjuvant and that this extract could elicit lymphocyte transformation and delayed skin test responses in sensitized animals. A group of 12 tinea versicolor patients and 15 normal subjects were studied in vitro for cell-mediated immunity to *P. orbiculare* extract. The majority of the subjects tested in both groups demonstrated positive lymphocyte transformation responses to this extract, as well as to standard mitogens and common microbial antigens. However, lymphocytes from tinea versicolor patients produced significantly less leukocyte migration inhibitory factor activity when stimulated by *Candida albicans* and *P. orbiculare* extracts than did lymphocytes from normal subjects. This was also true if only subjects with positive lymphocyte transformation responses to these antigens were considered. Leukocyte migration inhibitory factor responses to streptokinase/streptodornase were not significantly different between the two groups. Therefore, it appears that although both normal subjects and tinea versicolor patients demonstrate prior sensitization to antigens of *P. orbiculare*, the effector function of lymphocytes from most tinea versicolor patients appears to be impaired in that they produce subnormal amounts of the mediator leukocyte migration inhibitory factor when stimulated with antigenic extracts of this organism.

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INTRODUCTION

Lipophilic organisms of the genus *Pityrosporum* are common residents of normal human skin (1). Under artificial culture conditions these organisms are yeast-like in form and lipid dependent, requiring oil or long-chain fatty acids for growth (2). There are two such lipid-dependent species in this genus: *P. ovale* and *P. orbiculare*. This distinction has been based primarily on the finding that the former is morphologically smaller and more ovoid than the latter (3). However, because growth requirements are quite similar (4), because they share common antigens (5), and because the organisms have been converted from one morphologic form to the other by altering the culture medium (6), it has been suggested that both might be variants of the same species.

Pityrosporum orbiculare is considered by most authorities to be the etiologic agent of tinea versicolor (7, 8). This conclusion is based upon several lines of evidence: (*a*) by using the proper media, the organisms can be readily cultivated from scrapings of tinea versicolor lesions; (*b*) the cultured organisms can form germ tubes in culture (6, 9, 10); (*c*) specific antisera to cultured *P. orbiculare* organisms have been found to react with the fungal elements in scrapings of tinea versicolor lesions (11); and (*d*) experimental tinea versicolor infections have been produced in humans through inoculation of cultured *P. orbiculare* (12). However, although this organism appears to produce tinea versicolor, it is commonly found on the skin of normal individuals (3, 4, 9).

Cell-mediated immunity appears to be a major form of defense in other superficial fungal infections such as chronic mucocutaneous candidiasis (13) and in infections caused by dermatophytic fungi (14–16). It has been determined that a large segment of the population has cell-mediated immunity against *Candida* and *Trichophyton* antigens (17), possibly as a result of previous minor episodes of infection that had been

cleared by normal host defenses. Tinea versicolor has an increased incidence in patients on steroid therapy (18) or undergoing immunosuppression for renal transplantation (19), suggesting that host defenses may play a role in preventing the development of this infection as well. However, it is also possible that, in contrast to other cutaneous fungal infections, tinea versicolor may be so superficial in location that the body does not recognize its presence and therefore does not attempt to remove it.

In the present study, antigenic extracts were prepared from *P. orbiculare*, and cell-mediated immunity to these extracts was assessed in normal subjects and in tinea versicolor patients. A more general evaluation of cell-mediated immunity was also carried out in both groups. The objectives of this study were: (*a*) to seek evidence of prior systemic sensitization in both groups of subjects to antigens of this organism, and (*b*) to look for possible defects of cell-mediated immunity that might be associated with this type of infection.

METHODS

Patients. In this study, 12 patients with tinea versicolor were evaluated after obtaining informed written consent. In each case, the original diagnosis was made by the patient's dermatologist and was confirmed in our laboratory after referral in 9 of 12 patients by the typical appearance of the organism in potassium hydroxide preparations of skin scrapings. The other three cases did not have active lesions when seen by us. Most patients were receiving topical therapy at the time of the study. All 12 were in general good health, were ambulatory, and lived at home. Patients were determined to have either extensive or limited tinea versicolor on the basis of skin involvement visually estimated to be $>$ or <50 cm^2. More specific patient data are given in Results.

Results of the various immunologic tests performed on blood samples from this group were compared to results of similar studies performed on a group of 15 normal subjects drawn from the medical students, house staff, and laboratory personnel of this institution. The control group consisted of 3 female and 12 male subjects, ranging in age from 22 to 40 yr. The two groups of subjects were studied concurrently during the period from January to August 1977.

Cord blood. Three human cord blood samples were obtained at the time of delivery using sterile technique.

Animals. Outbred English short-haired guinea pigs were used in certain experiments. Animals were immunized by injection into each footpad of 0.1 ml of an emulsion of *P. orbiculare* extract (protein content 632 μg/ml) in Freund's complete adjuvant. The animals were used 2 mo later and the results compared to studies performed on unimmunized animals of the same age.

An antiserum to *P. orbiculare* was produced by immunizing a rabbit by injection of 0.5 ml of an emulsion of this extract in Freund's complete adjuvant into each hind footpad, followed 2 mo later with an intravenous injection of 1.0 ml of the same extract.

Organisms. Pityrosporum organisms were isolated by culturing skin scrapings from tinea versicolor lesions on mycosel agar (BBL, Div. Becton, Dickinson & Co. Cockeysville, Md.) overlaid with a thin layer of sterile olive oil at 30°C. Subcultures were made from emerging colonies and identified as either *P. ovale* or *P. orbiculare* by microscopic examination. These organisms did not grow on agar or in broth without added lipids. Organisms were grown in bulk in Sabouraud's dextrose broth containing chloramphenicol (20 μg/ml) and cycloheximide (40 μg/ml) and overlaid with 300 ml of sterile olive oil. The cultures were maintained at 30°C with constant stirring for 14 days, at which time the oil portion of the culture was removed and centrifuged to recover the organisms. Morphologic characteristics were again determined microscopically to be certain that no change had taken place during the culture period.

Candida albicans was obtained from a clinical isolate and maintained on Sabouraud's dextrose agar. This organism was cultured in bulk by inoculation of Sabouraud's dextrose broth containing chloramphenicol and cycloheximide. The culture was maintained at 30°C with constant stirring for 3 days at which time the organisms were harvested by centrifugation.

Trichophyton rubrum, Trichophyton mentagrophytes, and *Epidermophyton floccosum* were obtained from clinical isolates and maintained on mycosel agar. The organisms were cultured in bulk by inoculating Sabouraud's dextrose broth containing chloramphenicol and cycloheximide. The cultures were maintained at room temperature without stirring for 4 wk, and the mycelial mats removed by filtration.

Preparation of extracts. Extracts of *P. orbiculare, P. ovale,* and *C. albicans* were prepared in parallel by the same method. After harvesting, the organisms were washed with saline three times to remove broth constituents. The organisms were then extracted with ether, followed by dispersal in phosphate-buffered saline (pH 7.4) and centrifugation. The pellet was ground for 20 min in a mortar and pestle with glass beads and then recombined with the preceding supernate. After stirring at room temperature for 4 h, this suspension was centrifuged at 3,000 *g* for 10 min, and the supernates dialyzed exhaustively against phosphate-buffered saline at 4°C. They were then frozen and thawed once, again centrifuged at 3,000 *g* for 30 min, sterilized by filtration (Millipore Corp., Bedford, Mass.), and stored in aliquots at −20°C. Protein content was determined by the Lowry method. Two extracts (nos. 1 and 2) were prepared from *P. orbiculare* and one each from *P. ovale* and *C. albicans.* Unless otherwise stated "*P. orbiculare* extract" refers to preparation no. 1 (see Table III), inasmuch as this was the *Pityrosporum* extract employed most extensively in these studies.

Because of the possibility that antigenic material could be present in the olive oil, a control preparation was made by extraction of olive oil with ether in the presence of saline. The latter was removed, dialyzed, frozen and thawed, and sterilized by filtration in a manner similar to that used in the preparation of other extracts. In addition, a Sabouraud's broth control preparation was made by diluting Sabouraud's broth in saline. Each of the above was prepared as if the volume of packed organisms used to prepare organism extracts were in reality either all olive oil or all Sabouraud's broth.

Extracts of the dermatophytic fungi were prepared by washing the mycelial mats three times with saline and then grinding them for 20 min with glass beads in a mortar and pestle. The resulting material was mixed with saline, stirred for 4 h at room temperature, frozen and thawed once, centrifuged at 3,000 *g* for 30 min, sterilized by filtration, and stored in aliquots at −20°C.

Other antigens and mitogens. The mitogens used were

phytohemagglutinin (PHA)[1] (The Welcome Research Laboratories, Beckenham, Eng.), concanavalin A (Con A) (Miles Laboratories, Inc., Elkhart, Ind.), and pokeweed mitogen (PWM) (Grand Island Biological Co., Grand Island, N. Y.). Other antigens used were streptokinase/streptodornase (SKSD) (American Cyanamid Co., Lederle Laboratories Div., Pearl River, N. Y.), trichophytin (Dermatophytin, Hollister-Stier Laboratories, Inc., Spokane, Wash.), and a commercial *Candida* extract (Dermatophytin "O," Hollister-Stier Laboratories).

Skin testing. Six tinea versicolor patients underwent skin testing with 0.1 ml of the following preparations: *C. albicans* (Dermatophytin "O" 1:100), SKSD (4 μm/1 μm per ml) and trichophytin (Dermatophytin 1:30). The tests were read at 15 min, 24, and 48 h; 5 mm of induration or greater was considered a positive response. Human subjects were not skin tested with *Pityrosporum* extracts.

Guinea pigs were tested intradermally with 6.4 μg of *P. orbiculare* extract in a 0.1-ml volume, a concentration found to produce little nonspecific inflammation, but still evoke typical delayed hypersensitivity responses in immune animals. Skin tests in guinea pigs were read at 18 h, and 5 mm or more of induration was considered positive.

Lymphocyte transformation. In humans, mononuclear cells obtained from heparinized blood using Ficoll-Hypaque (Ficoll, Pharmacia Fine Chemicals, Piscataway, N. J.; Hypaque, Winghtrop Laboratories, Div. of Sterling Drug, New York) gradients were washed three times in Hanks' balanced salt solution (Grand Island Biological Co.) and suspended at 0.25×10^6/ml in tissue culture medium RPMI 1640 (Grand Island Biological Co.) supplemented with 2 mM glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml), and 15% autologous plasma. In some cases, the autologous plasma was replaced with the same concentration of a different subject's fresh frozen plasma (<4 wk old) that had been absorbed with washed erythrocytes of the lymphocyte donor. Aliquots of 0.4 ml of the mononuclear cell suspension were cultured in triplicate in small test tubes at 37°C and 5% CO_2 with 20 μl of various antigen or mitogen preparations. After 3 days for mitogens or 5 days for antigens, 1.0 μCi of tritiated thymidine (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) was added to each tube and the lymphocytes harvested onto glass fiber strips 18 h later using a cell harvesting apparatus (Chap-100, Adaps, Inc., Dedham, Mass.). Tritiated thymidine uptake was measured in a Beckman scintillation counter (Beckman Instruments, Inc., Palo Alto, Calif.). Data were expressed as counts per minute (cpm) and stimulation ratios (SR) (SR = cpm in stimulated culture/cpm in control culture). A positive response in our system was considered to be a SR of 4.0 or greater and cpm of 3,000 or greater because these values correlated best with skin test results in a series of tests on 12 normal subjects.

Lymphocyte transformation on cord blood lymphocytes was performed in a similar manner except that the blood was drawn into an acid-citrate-dextrose anticoagulant, and 15% fetal calf serum was used in place of autologous plasma. Fetal calf serum was used because it supported lymphocyte transformation better than did cord blood plasma.

Lymphocyte transformation in guinea pigs was assayed by adding 40 μl of heparinized blood obtained by cardiac puncture directly to 0.4 ml of RPMI 1640 supplemented with glutamine, penicillin, and streptomycin. Cultures were harvested at 3 days for mitogens or 5 days for antigens, using Zap-Isoton (Coulter Electronics Inc., Hialeah, Fla.) to remove RBC before harvesting onto glass filter strips as with human lymphocyte cultures.

In preliminary tests, the culture period and concentrations of antigens and mitogens to attain optimal responses were determined. For the most part, dose-response curves for antigens and mitogens were carried out with lymphocytes from normal subjects. However, in the case of the *Pityrosporum* extracts, lymphocytes from tinea versicolor patients were used also.

Leukocyte migration inhibition assay. This procedure is a modification of the indirect leukocyte migration inhibition assay (LMIF) of Clausen (20). Mononuclear cells were suspended at 5×10^6/ml in medium 199 (Grand Island Biological Co.) supplemented with glutamine, penicillin, streptomycin, and 2% autologous plasma. In some cases, a different plasma was substituted for autologous plasma, as above. The cells were cultured for 3 days in 1-ml aliquots with half having 50 μl of an antigen preparation added, and the rest serving as controls. The cultures were centrifuged each day with the supernates being saved and fresh media added after the first and second centrifugations. A control supernate was prepared for each antigen-stimulated supernate by adding the proper amount of antigen at the end of the 3-day culture period. The supernates were dialyzed, lyophilized, and reconstituted at one-fifth their original volume in fresh medium.

Assays of leukocyte migration inhibitory activity in these supernates were performed with human polymorphonuclear leukocytes as indicator cells. These cells were purified using Ficoll-Hypaque centrifugation and hypotonic lysis of contaminating erythrocytes. The resulting cell preparations (consisting of 98–99% polymorphonuclear leukocytes) were suspended at 2×10^8/ml in medium 199 with 25% heat-inactivated horse serum. Agarose plates were prepared in 60-mm plastic Petri dishes (Falcon 1007, Falcon Plastics, Div. BioQuest, Oxnard, Calif.) with 5 ml of medium 199 containing 0.9% agarose (Indubiose A37, Accurate Chemical & Scientific Corp., Hicksville, N. Y.), 10% heat-inactivated horse serum, and added glutamine, penicillin, and streptomycin. 20-μl aliquots of each stimulated and control supernate were incubated with the same volume of polymorphonuclear leukocyte cell suspension for 30 min at 37°C in air, and 5 μl of each resulting cell-supernatant preparation added to each of four 2-mm wells in the agarose plates. Each antigen-stimulated supernate was compared to its own control supernate on the same plate. After incubation for 18 h at 37°C in 5% CO_2, fixation with 3% glutaraldehyde, and removal of the agarose, the plates were read in a blind fashion by two observers. Diameters of the circles of migrating cells were measured directly, and a percent migration inhibition (% *MI*) determined by the following formula: % $MI = 100 -$ diameter-stimulated supernate − well diameter/diameter-unstimulated supernate − well diameter $\times$ 100.

In one experiment, performed at the end of the studies, control supernates for the *P. orbiculare* extract were compared to medium alone in search of excessive leukocyte migration inhibitory activity in the control preparations. Also, in certain cases, negative supernates were again assayed at 1:5 and 1:25 dilutions in an attempt to uncover migration-stimulating activity.

Immunodiffusion tests. The antiserum described above to *P. orbiculare* extract was tested by immunodiffusion for reactivity against a variety of other extracts of microbial origin. This antiserum was tested against the extracts of *P. orbiculare*, *P. ovale*, the three dermatophytes (*T. rubrum*,

[1] *Abbreviations used in this paper*: PHA, phytohemagglutinin; Con A, concanavalin A; PWM, pokeweed mitogen; SKSD, streptokinase/streptodornase; cpm, counts per minute; SR, stimulation ratio; LMIF, leukocyte migration inhibitory factor.

T. mentagrophytes, and *E. floccosum*) and *C. albicans* as described above. In addition, it was also tested against a *Candida* sonicate, purified protein derivative (Connaught Medical Research Laboratory, Willowdale, Ontario, Canada), SKSD, endotoxin (lipopolysaccharide B, Difco Laboratories, Detroit, Mich.), and extracts of *Thermoactinomyces vulgaris*, *Micropolyspora faeni*, and *Aspergillus fumigatus* (kindly supplied by Dr. V. P. Kurup, VA Center, Wood (Milwaukee), Wis.). All tests were carried out in standard agar Ouchterlony plates using 10 μl of both antigen and antiserum in 2-mm wells which were 4 mm apart. A positive reaction was read as the presence of any precipitin lines after 24–48 h at room temperature.

RESULTS

Immunodiffusion tests of cross-reactivity of an antiserum to P. orbiculare with other antigenic extracts. A rabbit serum to *P. orbiculare* produced positive reactions to all the *Pityrosporum* extracts tested, including that of *P. ovale*. This antiserum did react with an extract of *M. faeni* but not with any of the others tested (as listed in Methods).

Cell-mediated immune responses to P. orbiculare extract in guinea pigs. Table I(A) shows that whereas normal guinea pig lymphocytes produced good responses to the standard mitogens PHA, Con A, and PWM, they did not respond to the *P. orbiculare* extract, indicating that this extract did not act as a mitogen in this system. Table I(B) shows that immunized guinea pigs produced positive lymphocyte transformation responses to *P. orbiculare* extract, whereas there was no response with nonimmune animals. As shown in Table I(C), 6.4 μg of *P. orbiculare* extract protein in 0.1-ml vol injected intradermally produced strongly positive delayed skin test responses in immunized animals, but not in those that were not immunized.

Transformation responses of cord blood lymphocytes to Pityrosporum extracts, other antigens, and mitogens. Lymphocytes from three human cord blood specimens were used to further test the possibility that these extracts might be mitogenic. Whereas all three of the cord bloods responded positively by lymphocyte transformation to the standard mitogens PHA and PWM and two of three to Con A, none responded to the *P. orbiculare*, *P. ovale*, or *Candida* extracts, or to SKSD at concentrations employed in the in vitro assay of lymphocyte transformation in adult human subjects.

Transformation responses of lymphocytes from normal subjects and tinea versicolor patients to mitogens and antigens. As shown in Table II, both groups of subjects demonstrated positive lymphocyte transformation responses to standard mitogens, with no significant differences between the groups being observed. Table II also shows that the responses of these subjects to the common microbial antigens *C. albicans*, SKSD, and *T. rubrum* were not significantly different between the two groups. Statistical evaluations were

TABLE I
Immunologic Evaluation in Guinea Pigs of P. Orbiculare Extract

Stimulant	CPM±SEM	SR±SEM
A Lymphocyte transformation responses at 3 days (5 nonimmune animals) to *P. orbiculare* extract and mitogens		
PHA, *1 μg/ml*	62,794±9,910	203.3±41.6
Con A, *20 μg/ml*	31,154±6,393	95.6±17.4
PWM, *1:200*	7,151±762	22.5±3.4
P. orbiculare, *32 μg/ml*	505±86	1.6±0.3
B Lymphocyte transformation responses at 5 days to *P. orbiculare* extract (32 μg/ml)		
Animals		
Nonimmune (n = 10)	502±62	1.6±0.3
Immunized (n = 5)	6,704±3,785	13.0±2.4

C Delayed skin test responses* to *P. orbiculare* extract (6.3 μg in 0.1-cm³ volume)	No. positive/ No. tested	Mean induration ±SE	Range
Nonimmune (n = 10)	0/5	0±0	—
Immunized (n = 5)	5/5	18.6±1.2	15–22

* Skin tests measured as millimeters of induration at 18 h.

TABLE II
Transformation of Lymphocytes from Normal Subjects and Tinea Versicolor Patients to Mitogens and Common Microbial Antigens

Stimulant	Normal subjects	Tinea versicolor patients	Statistical significance*
PHA, *1 μg/ml*			
No. positive/no. tested	13/13	12/12	—
Mean cpm±SE	234,433±18,786	239,218±17,519	NS†
Mean SR±SE	230.9±32.4	305.9±50.8	NS
Con A, *20 μg/ml*			
No. positive/no. tested	13/13	12/12	—
Mean cpm±SE	147,299±23,230	112,598±17,996	NS
Mean SR±SE	134.9±19.0	145.9±40.6	NS
PWM, *1:200*			
No. positive/no. tested	13/13	12/12	—
Mean cpm±SE	57,766±13,547	70,358±21,729	NS
Mean SR±SE	51.8±7.5	66.3±21.5	NS
C. albicans, *5 μg/ml*			
No. positive/no. tested	12/14	11/11	—
Mean cpm±SE	31,453±5,826	27,612±8,914	NS
Mean SR±SE	31.4±6.8	22.4±6.1	NS
SKSD, *100 and 25 U/ml*			
No. positive/no. tested	14/14	10/11	—
Mean cpm±SE	39,104±12,874	27,602±6,029	NS
Mean SR±SE	32.0±7.5	22.5±3.3	NS
T. rubrum, *4 μg/ml*			
No. positive/no. tested	4/14	5/11	—
Mean cpm±SE	5,946±1,330	8,477±1,781	NS
Mean SR±SE	4.6±1.8	7.4±1.7	NS

* Statistical significance by unpaired t test.
† $P > 0.05$.

also carried out with data expressed as $\log_{10}$ of the cpm or SR of these responses and again no significant differences could be found between the two groups.

Transformation responses of lymphocytes from normal subjects and tinea versicolor patients to Pityrosporum extracts. Table III demonstrates that there appears to be reactivity to all three of the *Pityrosporum* extracts in most of the normal subjects, as well as tinea versicolor patients. The responses of tinea versicolor patients were lower than those in normal subjects, but these differences, for the most part, were not significantly different between the two groups. Conversion of the data for extract no. 1 to $\log_{10}$ form did not reveal statistically significant differences between the two groups. In general, subjects with positive responses to *P. orbiculare* extract did not respond in lymphocyte transformation tests either to the Sabouraud's broth control preparation (0 of 9) or to the olive oil control preparation (1 of 14). In the latter case, the one positive subject had a response to this control preparation that was only 8% of his response to *P. orbiculare* extract.

Production of LMIF to antigens and P. orbiculare extract by lymphocytes from normal subjects and tinea versicolor patients. 15 normal subjects and 12 tinea versicolor patients were tested for the ability of their lymphocytes to release the lymphokine, LMIF when incubated with SKSD, or the extracts of *Candida* or *P. orbiculare*. The *P. ovale* extract was not evaluated in this system because there appeared to be definite cross-reactivity between it and the *P. orbiculare* extract, as evidenced by the immunodiffusion tests and by study of individual lymphocyte transformation responses to the two antigens. Positive and negative responses were determined by standard designation of 20% migration inhibition as a positive result. As can be seen in Fig. 1, all normal subjects had positive responses to all of these antigens with the exception of two who had negative *Candida* responses. The latter two subjects were also negative to *Candida* by skin tests and lymphocyte transformation, probably indicating lack of significant exposure to this organism. Only 2 of 12 tinea versicolor patients had positive responses to *P. orbiculare* antigen, whereas the others had negative responses by the above criteria. All normal subjects and 11 of 12 tinea versicolor patients showed positive responses to SKSD antigen. Although, as noted previously, all tinea versicolor patients showed positive responses to *Candida* extract in lymphocyte transformation tests, 7 of 12 patients were negative when tested to *Candida* antigen in the LMIF assay. Table IV shows a comparison of mean LMIF results between the two groups. The mean percent migration inhibition response of the tinea versicolor patients to *P. orbiculare* extract (15.17±2.16) was significantly lower ($P < 0.001$) than that of the normal subjects (31.60 ±2.03). There was a significant difference even if one excludes the single tinea versicolor patient with negative lymphocyte transformation or the two LMIF-negative tinea versicolor patients with possible underlying causes of diminished immunity (i.e., one with advanced age and one taking lithium carbonate [see Table V]). There was also a significant difference in mean LMIF response to *Candida* antigen between the two groups, considering either all subjects or only those

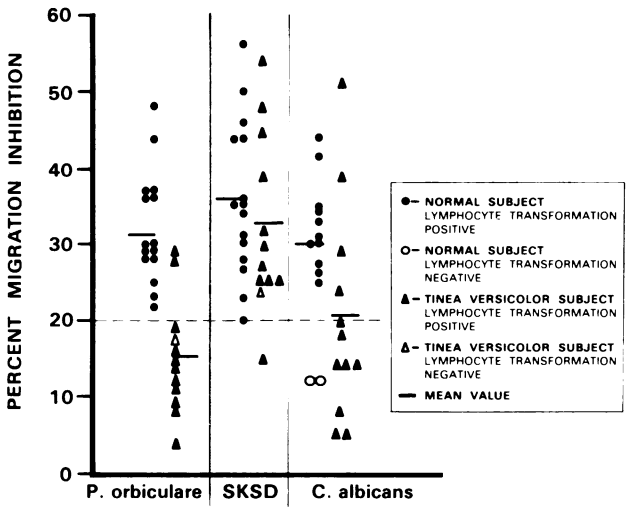

FIGURE 1 Production of LMIF by antigen-stimulated lymphocytes from normal subjects and tinea versicolor patients. Migration inhibition of 20% or more is considered a positive response in this test.

TABLE III
Transformation Responses of Lymphocytes from Normal Subjects and Tinea Versicolor Patients to Pityrosporum Extracts

Stimulant	Normal subjects	Tinea versicolor patients	Statistical significance*
P. orbiculare, no. 1, 32 μg/ml			
No. positive/no. tested	14/14	10/11	—
Mean cpm±SE	22,709±3,888	15,960±4,430	NS‡
Mean SR±SE	21.4±4.2	16.1±5.2	NS
P. orbiculare, no. 2, 44 μg/ml			
No. positive/no. tested	6/6	3/3	—
Mean cpm±SE	14,484±6,182	7,864±1,771	NS
Mean SR±SE	23.5±5.9	5.1±0.3	$P < 0.02$
P. ovale, 27 μg/ml			
No. positive/no. tested	9/11	4/5	—
Mean cpm±SE	15,013±3,767	11,386±4,863	NS
Mean SR±SE	15.8±4.8	7.7±3.3	NS

* Statistical significance by unpaired *t* test.
‡ $P > 0.05$.

with positive lymphocyte transformation. There was no difference in the mean responses of the two groups to SKSD. 6 of the 10 negative *P. orbiculare*-stimulated supernates were assayed at 1:5 and 1:25 dilutions without evidence of any migration-stimulating activity being discovered.

Comparison of control values in lymphocyte transformation and LMIF tests between normal subjects and tinea versicolor patients. There were no significant differences between the two groups in either tritiated thymidine uptake or LMIF production by unstimulated lymphocytes, indicating that high background activity cannot explain reduced responsiveness in the tinea versicolor group. In addition, upon examination of the values for individual patients, an elevated control value did not explain a negative response in any of the cases. This information is most pertinent for the LMIF control values because reduced responsiveness was most evident here and because an increase of activity of the control would reduce the percent migration inhibition demonstrable for a particular antigen.

Effect of substituting normal for patient plasma in lymphocyte transformation and LMIF responses. In an effort to find blocking activity in the plasma of tinea versicolor patients, plasma from normal subjects was substituted for that of the tinea versicolor patients in assays performed in parallel to those containing the patient's plasma. Three tinea versicolor patients had increased responses when other plasma was used, but only one had a marked increase (cpm ↑354%, SR ↑259%). In the LMIF test to *P. orbiculare* extract, 2 of 10 tinea versicolor patients had an increased response (↑20, ↑100%). In one of these patients, the response converted from negative to positive. 3 of 10 patients had increased LMIF responses to *Candida*. In two of these, this result was enough to convert a negative result to positive, but in both cases the percent migration inhibition increased only 4%. In the majority of tinea versicolor patients, it appears that substitution of a normal plasma for the patient's plasma does not produce marked increases in responsiveness.

Results of skin testing in tinea versicolor patients. Six tinea versicolor patients underwent skin testing to *Candida*, SKSD, and trichophytin in order to assess their ability to develop in vivo delayed hypersensitivity reactions. All subjects manifested at least one positive delayed response, and 14 of the 18 tests gave positive delayed responses. Four of the six subjects tested also manifested a wheal and flare reaction to one of the antigens used (one subject had this type of response to *Candida*, two to trichophytin, and one to both *Candida* and trichophytin).

Summary of data from the tinea versicolor patients. Table V shows the clinical data and immunologic responses to *P. orbiculare* extract in the tinea versicolor group. The most common pattern of immunologic responses in these patients was positive lymphocyte transformation, but negative LMIF production to *P. orbiculare* extract. The reduced responsiveness in the LMIF assay did not appear to correlate with age, other medical conditions, or the extent of tinea versicolor involvement.

DISCUSSION

Extracts were prepared from cultured, ether-extracted, and mechanically ground *Pityrosporum* organisms. The initial evaluation in guinea pigs demonstrated

TABLE IV
Summary of Results of LMIF Assays

Antigen	Normal subjects		Tinea versicolor patients		Statistical significance‡
	No. LMIF positive/ no. tested	MI*±SE	No. LMIF positive/ no. tested	MI±SE	
		%		%	
All subjects					
P. orbiculare	15/15	31.6±2.0	2/12	15.2±2.2	$P < 0.001$
SKSD	15/15	35.9±2.6	11/12	32.4±3.4	NS§
C. albicans	13/15	29.8±2.3	5/12	20.1±4.0	$P < 0.05$
Lymphocyte transformation— positive subjects only					
P. orbiculare	14/14	31.3±2.1	2/10	14.6±2.6	$P < 0.001$
SKSD	14/14	36.1±2.8	9/10	31.1±3.2	NS
C. albicans	13/13	32.5±1.6	4/11	18.4±4.0	$P < 0.005$

* Migration inhibition.
‡ Statistical significance of difference between mean values by unpaired *t* test.
§ $P > 0.05$.

TABLE V
Summary of Data from Tinea Versicolor Patients

Patient	Age	Sex	Associated conditions	Degree of tinea versicolor*	Response to *P. orbiculare*	
					LT‡	LMIF
	yr					
1§	31	M	Allergic rhinitis	Extensive	Positive	Negative
2	30	M	None	Limited	Positive	Positive
3§	45	F	None	Limited	Positive	Negative
4§	77	M	Arteriosclerotic cardiovascular disease	Extensive	Positive	Negative
5	23	M	DP‖, warts	Limited	Positive	Negative
6	30	M	DP, manic depressive¶	Limited	Positive	Negative
7	41	M	None	Extensive	Positive	Negative
8	27	M	Warts	Extensive	Positive	Negative
9§	54	M	None	Extensive	Positive	Negative
10	29	M	Recent hepatitis	Extensive	Positive	Positive
11	33	M	None	Limited	Negative	Negative
12	30	M	DP	Limited	Positive	Negative

* Degree of tinea versicolor divided into two categories by visual estimation of combined area of lesions: extensive = >50 cm^2, limited = <50 cm^2.

‡ Lymphocyte transformation.

§ Patients 1, 3, 4, and 9 were related. Patient 4 was the grandfather, and patients 3 and 9 the aunt and uncle, respectively, of patient 1.

‖ Dermatophytosis.

¶ This patient was taking lithium carbonate at the time he was studied.

that such extracts were capable of sensitizing the animals when injected in complete Freund's adjuvant and of eliciting responses indicative of cell-mediated immunity in these immunized animals. Other studies, using guinea pig or cord blood lymphocytes, demonstrated that these extracts were not mitogenic, but functioned as specific antigens. These latter studies also make it appear unlikely that mitogenic quantities of contaminants, such as endotoxin, could be present in these extracts.

In lymphocyte transformation tests on 14 normal subjects and 11 tinea versicolor patients, all were found to react to the first *Pityrosporum* extract employed (*P. orbiculare* extract no. 1), and most responded also to another extract from *P. orbiculare* and one from *P. ovale*. Similar testing of two media control preparations indicated that these subjects were reacting to components of the cultured organisms rather than to media elements. Immunodiffusion tests indicated cross reactions between *P. orbiculare* and *P. ovale*, and between *P. orbiculare* and *M. faeni*, but not with 10 other common antigens tested, including *Candida* and various dermatophytes. It is not possible to determine from the present studies whether normal humans are sensitized to *Pityrosporum* antigens through cross reaction with another antigen or through direct contact with the *Pityrosporum* organisms themselves. The latter seems more likely for the following reasons: (*a*) Both species of *Pityrosporum* are common skin flora in humans (3, 4, 9) and therefore most people are exposed in some manner to their antigens; (*b*) *P. orbiculare* has the capability of invading at least the upper layers of the skin; (*c*) the number of subjects in this study reacting to the *Pityrosporum* extracts is higher than that usually reacting to most other common environmental antigens (17). In a study of antibody responses to *Pityrosporum* antigens, Alexander (5) had difficulty finding any subjects with negative or low titers to either *P. ovale* or *P. orbiculare*. These results were interpreted as evidence that, because most adults had had dandruff at some time in their lives, they had been exposed to *P. ovale*, which has long been associated with this condition. Because antigens of the two organisms cross-reacted in their system, sensitization to *P. ovale* would produce reactivity to *P. orbiculare* also. Inasmuch as most humans seem to have antibody to *Pityrosporum* antigens, we might also expect to find cell-mediated immunity to these antigens. However, because of the cross-reactivity of these two organisms, it is impossible to determine from the present studies which organism (if either) is actually responsible for sensitization. Indeed, as mentioned previously, it is possible that *P. ovale* and *P. orbiculare* may really be variants of the same species, lessening the importance of determining which form produces hypersensitivity in humans.

Tinea versicolor patients, like the normal control subjects, showed vigorous lymphocyte transformation responses to mitogens, indicating adequate numbers and transforming capabilities of lymphocytes. Similarly, in antigen-induced lymphocyte transformation tests to *Candida*, SKSD, and *Trichophyton*, the two groups were comparable, indicating similar exposure of both groups to these common microorganisms. Skin testing in tinea versicolor patients revealed good ability to produce cutaneous delayed hypersensitivity reactions. In lymphocyte transformation studies of reactivity to *Pityrosporum* antigens, most of the subjects in both groups reacted positively to the three preparations, particularly the two *P. orbiculare* preparations. This is evidence that a majority of the subjects tested have contacted these antigens and have lymphocytes capable of recognizing them. However, whereas 11 of 12 tinea versicolor patients had normal LMIF responses to SKSD, only 5 of 12 had normal responses to *Candida*, and 2 of 12 to *P. orbiculare* extract. Therefore, although most of the tinea versicolor patients have lymphocytes that can recognize *Pityrosporum* antigens, their populations of specific effector lymphocytes for these antigens are either absent or malfunctioning.

We have undertaken preliminary studies to determine why lymphocytes from tinea versicolor patients fail to generate significant amounts of LMIF activity in response to *Pityrosporum* antigens. One possibility is that the background activity of resting lymphocytes might be too high to permit an incremental response, as reported for monocyte chemotactic factor production by lymphocytes of patients with Wiskott-Aldrich syndrome (21). However, this does not appear to be the case in tinea versicolor, either for background-tritiated thymidine uptake in lymphocyte culture or for the amount of LMIF released by unstimulated lymphocytes. Another possibility is that the patients might have a serum blocking factor, as reported in chronic dermatophytic infections by Walters et al. (14). In most cases, we were unable to increase responses by lymphocytes from tinea versicolor patients by substitution of normal plasma for the patient's plasma in these assays. However, it is still possible that there was a factor in the plasma that damaged these lymphocytes for a longer period of time than that required for the assay. This plasma factor would have to be rather selective, however, because most of the tinea versicolor patients responded normally to SKSD. Finally, it is possible that lymphocytes from tinea versicolor patients produce a migration-enhancing factor that balances the migration-inhibiting effect in their stimulated supernates. We were unable to uncover such a factor by assaying dilutions of negative *Pityrosporum*-stimulated supernates, but it is still possible that both factors could be present in the same concentrations in these supernates.

Our data provide evidence that active infections are not necessary to produce the immunologic defects found in this group of tinea versicolor patients: (*a*) several patients appeared to have defective responses against *Candida* without being infected by this organism; (*b*) the degree of tinea versicolor infection did not correlate with the lymphocyte responses to *Pityrosporum* antigen. Therefore, it is possible that there may be a certain population of humans who either lack the ability to respond to this type of antigen or who are predisposed toward the loss of this ability. A genetic predisposition has previously been demonstrated for the superficial fungal infection tinea imbricata, where susceptibility appears to be inherited as an autosomal recessive trait (22). We would expect that individuals with an inherited predisposition to tinea versicolor would be the first to be affected in a temperate climate. Because tinea versicolor is much more common in tropical climates (23), it would be interesting to determine whether the infection in patients from warmer climates could overcome intact host defenses because of improved growing conditions, or whether these patients also have abnormal cell-mediated immune responses to antigens of the infecting organism(s).

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