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Skin Testing of Guinea Pigs and Footpad Testing of Mice With a New Antigen for Detecting Delayed Hypersensitivity to *Cryptococcus neoformans*

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This study was undertaken to evaluate the potential of a cryptococcal culture filtrate antigen, cryptococcin C184, for detecting delayed hypersensitivity in *Cryptococcus neoformans*-injected animals. The antigen was tested on guinea pigs which had received saline or *C. neoformans* and on animals sensitized to *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Candida albicans*, or *Sporothrix schenckii*. A delayed-type hypersensitivity response was elicited by cryptococcin C184 in *C. neoformans*-injected guinea pigs, whereas no indurations or erythemas were seen at 48 h after skin testing of saline controls or heterologously sensitized guinea pigs. Besides being specific for *Cryptococcus*, the antigen showed a high degree of sensitivity and was reproducible. Footpad tests were conducted with the antigen on mice which had previously received either 10^5 viable *C. neoformans* cells or saline. Delayed hypersensitivity was indicated in the *C. neoformans*-injected mice by the increase in thickness of antigen-injected footpads when compared with the saline-injected footpads. In control mice, antigen- and saline-injected footpads were comparable in thickness 24 h after injection. Mice sensitized to *B. dermatitidis* were footpad tested with C184, and no cross-reactivity was demonstrated.

Most workers agree that the host's major defense against *Cryptococcus neoformans* is cell-mediated immunity (1, 2, 4). Studies on delayed hypersensitivity have been limited to some extent because of the lack of a sensitive and specific antigen for the detection of the delayed response. Salvin and Smith in 1961 (12) reported a *C. neoformans* cell-extract antigen which was capable of detecting skin hypersensitivity in guinea pigs. Their antigen was subsequently used in preliminary testing in man by Muchmore et al. (7). The cell-extract antigen showed no cross-reactivity when skin tested on guinea pigs injected with *Candida albicans* or *Histoplasma capsulatum* (12). Preparation of the cell extract antigen was achieved by forcing viable *C. neoformans* cells through a small orifice of a pressure cell under a pressure of 60,000 lb/in^2. This method was cumbersome, and it was difficult to achieve comparable amounts of cell breakage upon repeating the procedure.

Atkinson and Bennett (3) prepared a urea extract of cryptococcal cells which has been used as a skin-test antigen. This antigen cross-reacted in *H. capsulatum*- and *Blastomyces dermatitidis*-injected guinea pigs. In addition to cross-reactivity, the antigen stimulated a rise in anticryptococcal antibody titers.

This study was undertaken to evaluate the potential usefulness of a cryptococcal culture filtrate antigen, prepared in our laboratory, for detecting delayed hypersensitivity to *C. neoformans* in guinea pigs and mice. Specific goals were to: (i) examine the reproducibility of the method used to prepare the antigen; (ii) determine whether skin testing with the antigen would induce anticryptococcal agglutinins or enhance intradermal reactivity; (iii) assess the extent of cross-reactivity, if any, in animals sensitized to other mycotic agents; and (iv) establish a mouse model for studying cellular immunity in cryptococcosis.

MATERIAL AND METHODS

Animals. Hartley strain guinea pigs of both sexes weighing 700 to 900 g were used. Mice used for sensitization and footpad testing were six-week-old CBA/J inbred strain. All animals were born and raised in the animal facilities of the Department of Microbiology, University of Oklahoma, Norman, Okla.

Organisms. *C. neoformans* isolate 184, previously described (8), was used both for sensitization of animals and preparation of the skin-test antigen. In

specificity studies, groups of five guinea pigs were sensitized with: *Candida albicans*, CDC strain MI-023; *Histoplasma capsulatum*, Scritchfield strain (5); *Blastomyces dermatitidis*, strain 242, isolated from a human case of blastomycosis; or *Sporothrix schenckii*, a recent isolate from a case of sporotrichosis in Mooreland, Okla. *C. neoformans* and *Candida albicans* were maintained on Sabouraud glucose agar at 35 C. The yeast phases of *B. dermatitidis* and *S. schenckii* were grown on brain heart infusion agar containing 10% human blood, and *H. capsulatum* yeast was grown on hemoglobin agar (Difco Laboratories, Inc., Detroit, Mich.) at 35 C.

Sensitization of animals. Guinea pigs were sensitized with viable yeast cells in complete Freund adjuvant (H37Ra) (Difco Laboratories, Inc.). For preparation of inocula, organisms were grown for 72 h at 35 C, harvested, and washed with sterile physiological saline solution (PSS). Total numbers of cells were determined by hemacytometer counts. Appropriate dilutions were made to give stock concentrations of 2×10^7 cells/ml for all sensitizing agents except for *C. albicans*, which was prepared to contain 5×10^8 cells/ml. A stable emulsion was made by mixing 1 volume of stock cell suspension with 1 volume of complete Freund adjuvant. Each guinea pig was sensitized by injecting 0.2 ml of the emulsion into each of the front footpads and 0.6 ml subcutaneously into the neck region. For controls, guinea pigs were given saline-adjuvant emulsion in the same manner. Skin tests were applied 12 days after initial injection of organisms.

Mice were injected intraperitoneally with 3×10^5 viable *C. neoformans* cells contained in 0.5 ml of saline. Control mice were given the same volume of saline. To sensitize mice to *B. dermatitidis*, 2.5×10^7 viable *B. dermatitidis* yeast cells in complete Freund adjuvant were injected subcutaneously. Footpad testing was done on day 14 or 21 after injection of cells or saline.

Antigens. The intradermal test material under investigation, cryptococcin C184, was prepared according to the procedure that Murphy and Cozad used for preparation of their HA antigen (8). Briefly, this included growing *C. neoformans* cells at 30 C for 72 h in neopeptone dialysate broth and then killing the cells by adding Formalin to a final concentration of 2%. The cell free filtrate was collected and dialyzed against four changes of PSS. Three different batches of antigen were prepared and designated C184-1, C184-2, and C184-3.

The sensitivity of each group of guinea pigs to homologous antigens was determined by using: histoplasmin H-42 (14, 16), diluted 1:25; 50 μg of merthiolate-killed *B. dermatitidis* yeast cells (15); *S. schenckii* yeast cells, prepared according to Nielsen (10); and *Monilia albicans* antigen (Hollister and Stier Laboratories, Dallas, Tex.), diluted 1:10. Additionally, for controls, animals were skin tested with sterile PSS and neopeptone dialysate broth which had been previously dialyzed against four changes of PSS.

Intradermal testing of animals. The abdomens of the guinea pigs were shaved the day before application of skin tests. Three test sites were placed on each side of the animal. A portion (0.1 ml) of antigen was injected intradermally into each site. Erythemas and indurations were measured at 24 and 48 h after injection. A skin test was considered to be positive when the induration was 5 mm or greater at 48 h.

The method used in footpad testing of mice was primarily that of Youmans and Youmans (17). The 53 *C. neoformans*-injected mice and 40 control animals were footpad tested with 0.03 ml of cryptococcin C184 in the right rear pad and the same volume of PSS in the left rear pad. Twenty mice previously injected with *B. dermatitidis* received cryptococcin C184 in the right rear pad and *B. dermatitidis* antigen in the left pad. Another 10 mice which had been given *B. dermatitidis* were footpad tested with *B. dermatitidis* antigen in right pad and saline in left pad as a control.

Histological studies. Histological sections were made from guinea pig specimens collected 48 h after injection of the skin test antigens. Tissues were fixed in buffered Formalin, sectioned, and stained with hematoxylin and eosin.

Agglutination test. Serial twofold dilutions of guinea pig sera were made with PSS so that each tube contained 0.5 ml of diluted serum. To each serum dilution was added 0.5 ml of a suspension of washed, Formalin-killed *C. neoformans* cells. Formalin-killed cells prepared by the procedure of Neill et al. (9) were diluted to give 60% transmission by using tubes (0.5 by 4 inch [about 1.3 by 10.2 cm]) in a Spectronic 20 (Bausch & Lomb) spectrophotometer set at 500 nm. The tubes were mixed and incubated for 2 h at 37 C then overnight at 4 C before reading for agglutination. Known positive and known negative serum samples were run with each battery of tests.

Statistical analyses. Means, standard error, and paired and unpaired *t* tests programed on the Hewlett-Packard calculator model 9810A were used in analyses of data.

RESULTS

Skin testing of guinea pigs. Three different cryptococcin C184 preparations and dialyzed medium were compared as to skin reactivity in guinea pigs which had been injected with *C. neoformans* in complete Freund adjuvant 12 days before skin testing (Table 1). Whereas two of the preparations, C184-1 and C184-2, elicited positive reactions in 95% of the guinea pigs tested, the third batch, C184-3, produced positive results in only 85% of the animals. The erythemas and indurations were greater at 24 h than at 48 h after testing; however, the 48-h indurations were considered to be the best index to use in determining positive reactors, because it was less likely that a Jones-Mote reaction would influence the induration size at 48 h. The dialyzed medium induced an erythematous response in 3 of the 20 sensitized guinea pigs at 24 h, but this had faded by 48 h after skin testing. One of the 10 control animals reacted to the dialyzed medium with a 4-mm erythema at 24 h; however, the reaction was not detectable at 48 h. None of the three antigen preparations

TABLE 1. *Comparison of skin test reactivity of three cryptococcal culture filtrate preparations in guinea pigs*[a]

Skin test antigen	No. of guinea pigs	Skin test reactivity (mm)			
		24 h		48 h	
		Mean erythema	Mean induration	Mean erythema	Mean induration
C184-1	20	14.5 ± 2.1[b] (100)[c]	12.7 ± 2.3 (100)	9.2 ± 1.9 (100)	9.4 ± 2.9 (95)
C184-2	20	13.2 ± 1.9 (100)	10.6 ± 1.9 (100)	8.5 ± 2.4 (100)	7.7 ± 2.6 (95)
C184-3	20	13.5 ± 3.5 (100)	11.0 ± 3.5 (90)	8.9 ± 3.6 (95)	7.4 ± 3.9 (85)
Dialyzed medium	20	1.3 ± 3.6 (15)	0 (0)	0 (0)	0 (0)

[a] Animals were injected 12 days earlier with 2×10^7 viable *C. neoformans* cells in Freund complete adjuvant.
[b] Standard deviation.
[c] Percentage of animals with reactions of 5 mm or greater.

elicited any kind of a reaction in saline-treated animals.

Histological sections were made from skin test sites of two *C. neoformans*-infected and two control (uninfected) animals. There was a marked mononuclear infiltration in the tissue of all positive skin test sites (Fig. 1a). In contrast, no infiltration was noted in dialyzed medium sites on sensitized guinea pigs (Fig. 1b). Tissues from all sites of control animals appeared to be uniform, with no noticeable infiltration of granulocytes or mononuclear cells (Fig. 1c).

Ten sensitized guinea pigs were skin tested a second time 14 days after the first skin test. The paired *t* test was used to compare the 48-h indurations induced by a first application of C184-1 with 48-h indurations induced by the same antigen on a second application. The same comparison was made by using C184-2. There was no significant difference in reaction sizes of

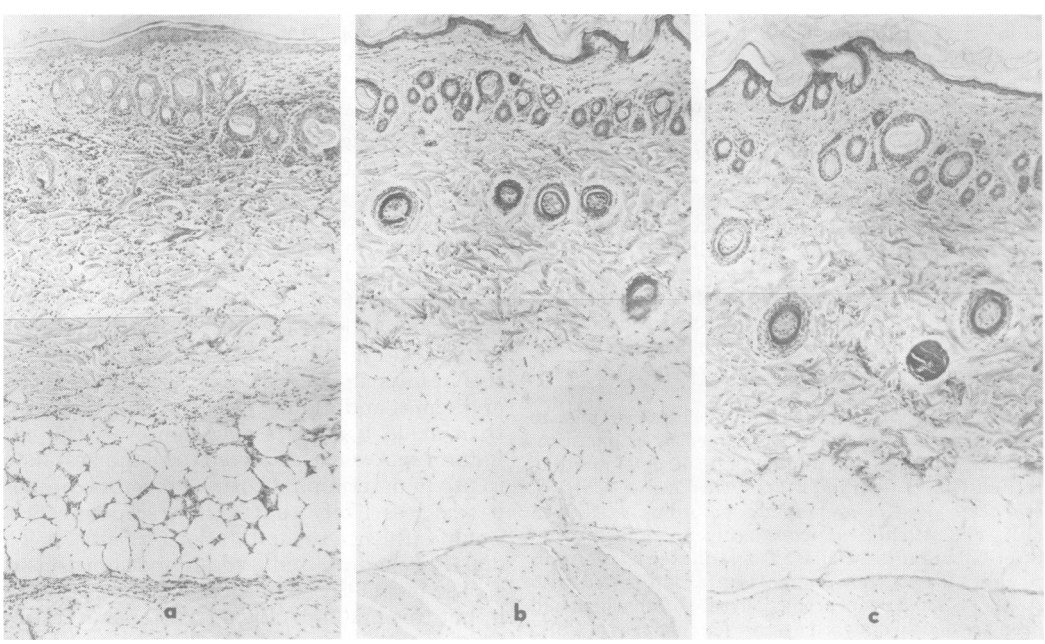

FIG. 1. *Histological sections of skin-test sites obtained 48 h after injection of (a) C184 cryptococcin into C. neoformans-injected guinea pigs; (b) dialyzed medium into C. neoformans-injected guinea pigs; and (c) C184 cryptococcin into saline-treated animals (controls).*

first and second skin tests with either antigen preparation. One group of 10 control animals was skin tested as many as four different times with C184 preparations at multiple sites, and all animals remained nonreactive.

To determine whether skin testing with C184 might induce humoral antibody titers, agglutination tests were run on sera collected from eight guinea pigs before initial injection of *C. neoformans* cells, 11 days after injection of cells, and 7 days after the first skin test was applied. Serum samples were collected from control animals at the same time periods. Agglutinins were not detected in any of these sera. A positive control serum was run at the same time as the test samples and had an agglutination titer of 1:512. A second skin test was administered 14 days after the first. Ten days after the second skin test, agglutinin titers were again negative for three sensitized and three control animals.

Studies were next done to determine whether lyophilization would have any effect(s) upon the skin test reactivity of the C184 antigen. Lyophilized preparations of the C183-3 were reconstituted to the original volume and then compared with the reactivity of the same antigen before lyophilization. In the 10 *C. neoformans*-injected guinea pigs, 90% were positive to both the original and lyophilized antigens. Furthermore, there was no significant difference in the mean indurations produced, i.e., 7.3 mm as compared with 7.4 mm. When the lyophilized antigen was concentrated twofold, 100% of the guinea pigs reacted, showing a mean induration of 10.9 mm.

Cryptococcin C184-1 was used in skin testing guinea pigs sensitized to *H. capsulatum*, *B. dermatitidis*, *S. schenckii*, or *Candida albicans*. There were no detectable reactions to the cryptococcin in any of the heterologously injected animals; however, the homologous antigen induced both induration and erythema (Table 2).

Footpad testing of mice. Mice which had been injected 21 days previously with viable *C. neoformans* cells were footpad tested with cryptococcin C184-2 in the right rear footpad and saline in the left. Control animals injected with saline only were footpad tested in the same manner. The mean differences of the cryptococcin pads and the saline pads are shown in Fig. 2. The vertical bars represent the standard error. The cryptococcin and saline pads were approximately the same size immediately after injection of 0.03 ml of material. Both pads began to reduce in size over the next 12-h period. The size of saline pads decreased more rapidly than the cryptococcin pads in *C. neoformans*-injected animals. This is reflected by the greater mean differences in *C. neoformans*-injected animals over control mice at 6 h. By 12 h after injection, there was essentially no difference in footpads of *C. neoformans*-injected animals and saline-treated mice; however, the cryptococcin pads in sensitized animals began to swell between 12 and 18 h and showed the greatest mean increase (0.45 mm) over saline pads at 36

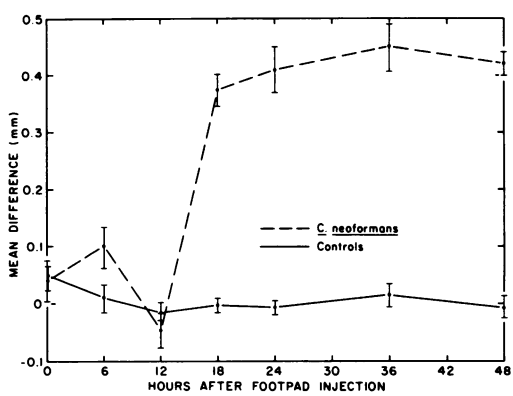

FIG. 2. *Mean difference (thickness of cryptococcin-injected footpad minus thickness of saline-injected footpad) of 53 CBA/J mice injected 21 days previously with 3 × 10⁵ viable C. neoformans cells compared with mean difference of 40 saline-injected mice as controls. Vertical bars indicate standard error of the mean.*

TABLE 2. *Skin test reactivity of cryptococcal culture filtrate antigen in heterologously injected guinea pigs*

Skin-test antigen reactions (mm)	Guinea Pigs injected with:					
	C. neoformans[a]	*H. capsulatum*[b]	*B. dermatitidis*[b]	*S. schenckii*[b]	*Candida albicans*[b]	Saline[b]
Cryptococcin C184-1						
Erythema	10.1 ± 1.7	0	0	0	0	0
Induration	10.4 ± 4.2	0	0	0	0	0
Homologous antigen						
Erythema		12.2 ± 1.8	2.0 ± 1.7	6.8 ± 1.6	6.8 ± 1.6	0
Induration		17.2 ± 2.8	6.3 ± 1.2	12.2 ± 3.3	7.4 ± 2.0	0

[a] Mean diameter ± one standard deviation of readings from 22 guinea pigs.
[b] Mean diameter ± one standard deviation of readings from five guinea pigs.

h after injection of antigen. At 48 h, the cryptococcin footpads still had a statistically significant increase in size over the saline pad.

Since Spencer and Cozad (15) had defined a model system for detection of delayed hypersensitivity for *B. dermatitidis* in mice, we decided to determine the extent of cross-reactivity, if any, to cryptococcin in *B. dermatitidis*-sensitized mice. Thirty mice were injected subcutaneously with 2.5×10^7 *B. dermatitidis* cells in Freund complete adjuvant 14 days before footpad testing. Twenty mice were footpad tested with *B. dermatitidis* antigen in one rear footpad and cryptococcin in the other. Ten mice were footpad tested with blastomyces antigen in one pad and saline in the other. There was a maximal mean increase of 0.3 mm in blastomyces antigen footpads over saline footpads. In animals footpad tested with blastomyces antigen and cryptococcin, the blastomyces pads showed a mean increase of 0.41 mm over the cryptococcin-injected pad.

Twenty-four-hour measurements of 93 saline-injected mouse footpads were compared with the corresponding blastomyces antigen footpad or cryptococcin footpad measurements in *B. dermatitidis*-sensitized animals by using an unpaired *t* test. Footpads injected with blastomyces antigen were significantly larger (99.5% confidence level) than saline-injected footpads 24 h after injection, but cryptococcin-injected pads were significantly smaller (99.5% confidence level) than saline pads (Table 3).

DISCUSSION

The cryptococcal culture filtrate antigen evaluated in this study appears to have some potential as a useful skin-testing tool. It was relatively simple to make, and the three different batches compared showed only slight variations in the degree of reactivity. The variability could possibly be attributed to quantitative differences in the three preparations. For example, C184-3 was reactive in 90% of the sensitive guinea pigs in one group, whereas 100% of those animals gave positive skin test reactions to a twofold concentrate of that antigen. Chemical studies are now in progress to further elucidate whether or not concentration was the cause of the variation in reactivity.

Being able to lyophilize and reconstitute the antigen without any detectable change in skin reactivity will permit quantitation of the amount of material used in skin testing and provides an efficient means of storing the antigen.

From the histological studies, it was evident that the C184 antigen was detecting a tuberculin-type hypersensitivity specific for *C. neoformans*. The tissue findings in the positive test sites were comparable to those described by Martins and Raffel (6) for a delayed tuberculin-type sensitivity in guinea pigs sensitized with bacillus Calmette-Guerin vaccine and skin tested with tuberculoprotein.

Cryptococcin C184 appeared to have a high degree of sensitivity. This was reflected by the fact that 95% of the *C. neoformans*-injected animals gave positive skin tests with C184-1 and C184-2 preparations, and 85% of those animals reacted to C184-3. In a second group of *C. neoformans*-injected guinea pigs, C184-3 gave positive results in 90% of the animals, but, when concentrated twofold, 100% of the animals had positive skin test reactions.

There was no evidence that the antigen would induce anticryptococcal agglutinin titers when used for skin testing in guinea pigs previously sensitized by means of a subcutaneous injection of *C. neoformans* in complete Freund adjuvant. In fact, the sensitizing injection did not stimulate detectable agglutinin responses. This could have been due to the possibility that excess circulating cryptococcal polysaccharide had neutralized, in vivo, any antibody that had been produced. It is possible that skin testing with C184 cryptococcin could not initiate a humoral response but could enhance a response which had already been stimulated; therefore, further study must be done to eliminate these possibilities.

Repeated skin testing with C184 did not stimulate a cellular immune response in previously negative animals. Furthermore, cryptococcal-sensitized animals responded with equivalent mean indurations to two successive applications of C184 antigen, which indicated that the antigen did not enhance an already established cellular response.

The specificity of this cryptococcal culture filtrate antigen was definitely superior to that reported for the urea extract cryptococcin (3) and was equal to, if not better than, the specificity data published on the cell extract antigen of Salvin and Smith (12).

TABLE 3. *Unpaired t-test data comparing blastomyces- or cryptococcin-injected footpads with saline-injected footpads*

Comparison		*t* test	Probability
20 blastomyces-sensitized mice footpad tested with:	93 control mice footpad tested with:		
Blastomyces	Saline	5.9	<0.005
Cryptococcin	Saline	−3.2	<0.005

In many ways, the mouse is a better experimental animal for studying cryptococcosis than is the guinea pig (11); therefore, the antigen was assayed for its ability to detect a delayed hypersensitive response in mice previously injected with *C. neoformans*. Results of the mouse studies showed that delayed hypersensitivity could be induced in CBA/J mice by injection of viable *C. neoformans* cells and that delayed hypersensitivity could be detected with cryptococcin C184. The greatest mean increase in footpad thickness in *C. neoformans*-sensitized mice was at 36 h after injection of cryptococcin. Spencer and Cozad (15), using a particulate antigen, reported the greatest increase in footpad size at 48 h. Most workers (13, 17) have been reporting footpad measurements at 24 h after injection of test material and have been considering an increase of 0.3 mm or greater indicative of hypersensitivity. Since there was a sufficient increase in footpad thickness (0.41 mm) at 24 h after injection of the cryptococcin into sensitized mice to consider the test positive, the 24-h readings were used in cross-reactivity studies.

The experimentation on cross-reactivity of cryptococcin with other mycotic agents in mice has been limited; however, the data obtained indicated there was no cross-reactivity in *B. dermatitidis*-sensitized mice. As an ancillary issue, it was observed that the blastomyces-sensitized animals were able to clear C184 cryptococcin from footpads more readily than 93 control mice could clear saline from footpads (Table 3). Because of the experimental approach used, further investigation must be done to firmly establish this observation.

Since it has been established that delayed hypersensitivity to *C. neoformans* could be stimulated and detected in the mouse system, the murine model could be used as an effective tool for studying the role of delayed hypersensitivity in cryptococcosis.

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