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Comparison of Skin Reactivity of Spherule and Mycelial Coccidioidins Produced by Different Strains of *Coccidioides immitis* (37899)

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(Introduced by H. Baer)

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The usefulness of coccidioidin as a measure of prior infection with *Coccidioides immitis* has been well established (1). Most currently used coccidioidins and all licensed coccidioidins are produced from autolyzed mycelial phase cultures. However, the spherule forms are predominant in infected tissues. The significance of whether coccidioidin is derived from spherule or mycelial phases of growth, or whether strain differences play a role in the properties of coccidioidin has not yet been clearly defined. It is the purpose of this paper to compare spherule and mycelial coccidioidins produced by several strains from the standpoint of reactivity and specificity in guinea pigs sensitized with each of the strains.

Materials and Methods. Strains of *Coccidioides immitis* included Woodville, a soil isolate associated with human infections in California (2); Silveira, isolated from a patient in the San Joaquin Valley with nonfatal disseminated coccidioidomycosis (3); and Strain 46, isolated from a fatal human case of coccidioidal meningitis (3).

Mycelial coccidioidin was produced at 34°C by inoculating 1–2 × 10^6 viable cells in a 1-liter Erlenmeyer flask which contained 200 ml of asparagine broth (1). Four-week-old mycelia killed with 0.5% (v/v) formalin and dried *in vaccuo* were used for sensitization. Filtrates harvested at 16 weeks postinoculation were used in these studies. Volumes lost during incubation because of evaporation or assimilation into the mycelial mat were restored with water after final sterilization through a 0.22-μm membrane filter. Spherule coccidioidin (spherulin) was produced by the method of Levine *et al.* (4) from mature spherules which had been grown in a chemically defined medium. It was necessary to repeat passage in the medium for some strains before mycelia were reduced to less than 2% of the population. Spherules sedimented by centrifugation (2000*g*) were allowed to lyse at 34°C in five times their packed volume of distilled water for 40 days (5). In order to remove unlysed spherules and cellular debris from the spherulin, the mixture was centrifuged at 20,000*g* for 20 min and the supernatants were passed through a 0.22-μm Millipore filter. Spherules used for sensitization were killed with 0.5% (v/v) formalin.

Groups of eight guinea pigs were sensitized as previously described (6) with organisms, coccidioidin, or both, incorporated in complete (CFA) or incomplete (IFA) Freund's adjuvants as follows: 1. Mycelia or spherulins in IFA; 2. Mycelia or spherules in CFA; 3. Mycelia or spherules with respective coccidioidin in IFA; 4. Mycelial or spherules with respective coccidioidin in CFA. Each emulsion was made using equal volumes of oil and aqueous (saline) phase. The oil phase consisted of Arlacel A:Drakeol 6 VR (35 parts:65 parts). When CFA was used, each milliliter of final emulsion contained 10 mg of heat-

killed BCG organisms. Final emulsions where applicable, also contained in each ml: 3.5 mg spherules, 5 mg mycelia, 1 mg spherulin, or 5–8 mg mycelial coccidioidin. Each guinea pig was sensitized by the subcutaneous injection in the nuchal area of 0.2 ml of respective emulsion. Animals were skin tested 6–10 weeks after sensitization. Reactions were read at 24 hr and recorded in millimeter diameter of erythema which was coincident with induration.

Results. Dose-response curves for each of the coccidioidin preparations in homologously sensitized groups of four animals are shown in Fig. 1. The dose-response curves permitted the selection of a dilution of each reagent which would give a reaction of 13–15 mm in homologously sensitized animals. An exact measure was not possible because of the somewhat flat nature of the curves. The total weight of substance and other properties in each of the selected doses have been calculated and presented in Table I. As can be seen, the micrograms/dose varied with strain. In the next experiments, comparisons of six reagents (mycelial and spherule coccidioidins of three strains) were made using the selected single dose of each, as shown in Table I. The groups of animals in these studies were those sensitized with organisms and coccidioidin in IFA (OR) and in CFA (ORB) since these groups were consistently highly reactive (Fig. 2). There were eight animals in each group of sensitized guinea pigs. Four in each group had been skin tested 2 weeks previously. Animals which had a prior skin test were often less reactive than those which had not had a prior skin test. This was especially true of the Strain 46 and Silveira groups sensitized with mycelial mat plus coccidioidin in CFA (ORB), and for the Woodville group sensitized with spherules plus spherulin in CFA (ORB). In such instances as the other groups sensitized with the Silveira spherule preparations, a single prior skin test did not appear to affect the result (Fig. 2). In the groups of animals sensitized with spherulin preparations, all selected skin-test doses gave

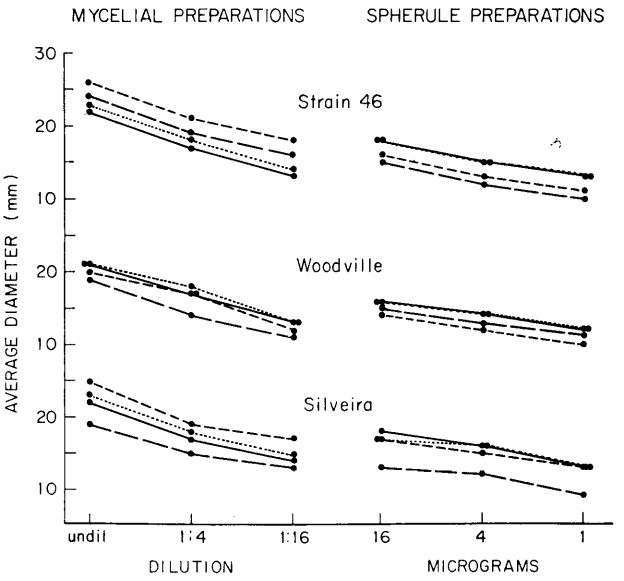

FIG. 1. Dose-response curves in guinea pigs sensitized with mycelial or spherule preparations in Freund's adjuvants and tested with homologous strain coccidioidin or spherulin. Micrograms of total weight of mycelial coccidioidin per undiluted test dose (0.1 ml) varied with the strain as follows: Strain 46 (1680 μg), Woodville (1152 μg), Silveira (1040 μg). Sensitizing preparations: _____ = Organisms; _ _ _ = Organisms + BCG; ..... = Organisms + reagent; _ _ _ = Organisms + BCG + reagent.

TABLE I. Characteristics of Spherule and Mycelial Coccidioidins at Equipotent Skin-Test Doses.[a]

	Mycelial coccidioidin			Spherule coccidioidin		Ratio
Strain	Dilution used in skin test	Total weight (μg/dose)	Total NDCP (μg/dose)	Total weight (μg/dose)	Total CP (μg/dose)	Weight coccidioidin/dose / Weight spherulin/dose
46	1:24	70	6.9 (1.3)	4	3.2 (1.5)	17.5
Woodville	1:8	144	18.4 (2.7)	8	3.6 (2.0)	18.0
Silveira	1:20	52	3.7 (1.1)	3	2.5 (1.2)	17.3

[a] Doses were selected to yield a reaction diameter of 13–15 mm in animals sensitized with homologous organisms and coccidioidin or spherulin in BCG containing water in oil emulsions. Legend in Fig. 1 contains information on the weight of substance in undiluted mycelial coccidioidin from which information in third column was derived. NDCP = nondialyzable carbohydrate and protein; CP = carbohydrate and protein; numbers in parentheses indicate the protein value.

equivalent reactions. Thus, antigenic differences that may have existed between the three strains, whether used as spherulins or mycelial coccidioidins, were not expressed by skin testing in guinea pigs. Such uniform reactivity of the reagents was not as clear in guinea pigs sensitized with Woodville and Silveira mycelial preparations in IFA. In these two groups there was a suggestion that the spherulins of Strain 46, Woodville, and Silveira were not as reactive as the corresponding mycelial coccidioidins. More uniform reactivity was observed when the sensitization vehicle was CFA instead of IFA.

Discussion. Sensitivity was readily induced to coccidioidin prepared from mycelial-phase or spherule-phase organisms. This could be accomplished by the injection of coccidioidin or organisms alone, or in com-

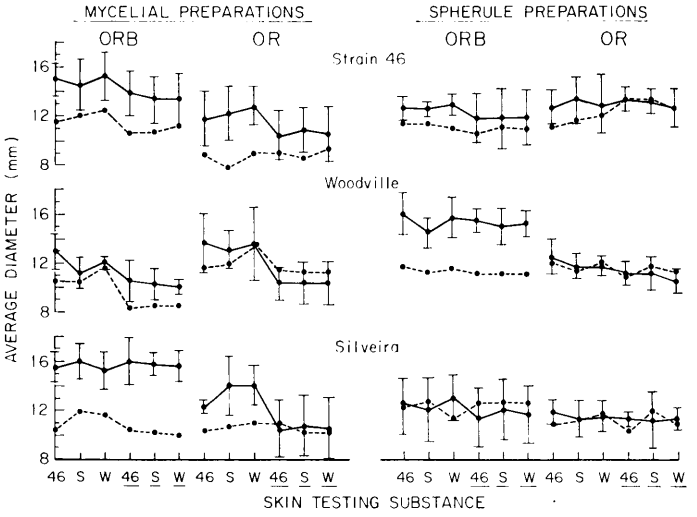

FIG. 2. Skin test activity of coccidioidin and spherulin from single strains of *Coccidioides immitis* in guinea pigs sensitized with mycelial or spherule preparations. Sensitizing preparation: O = mycelial mat organisms or spherule organisms, respectively; R = reagent (coccidioidin or spherulin); B = *Mycobacterium bovis* (BCG). Skin-testing substances were compared adjusted to biological equivalence. S = Silveira strain; W = Woodville strain. The data for spherulin are above the underlined symbols. Solid lines denote animals which have not had a prior skin test. Error bars denote ±1 standard deviation.

bination with each other in complete (CFA) or incomplete (IFA) Freund's adjuvant. Levine has reported that on a weight basis spherulin is more active than coccidioidin derived from mycelia of *C. immitis* (4). These observations have been confirmed, and on the basis of total soluble solids contained in each equipotent dose, we have determined that for strains Silveira, Woodville, and 46, the spherulin was 17–18 times more reactive (Table I). It has been demonstrated (8, 9) that the dialyzable constituents contain skin-reactive substances, so the comparison to the total soluble substances is a valid means of comparison. Experiments are in progress to compare the reactivity of the nondialyzable constituents on a total weight basis to that of spherulin.

The work presented here supports the contention of Smith (1) that significant strain differences of *C. immitis* did not exist from the standpoint of skin reactivity. In the study presented here with three strains isolated in California, differences could not be detected regardless of whether the coccidioidins were prepared from the mycelial or spherule phases of growth. Thus, once mycelial or spherule skin-test reagents were standardized to equipotency in animals sensitized with homologous preparations, they were virtually equivalent in reactivity in guinea pigs sensitized either with mycelial or spherule preparations (Fig. 2). Spherulin may have been slightly less reactive than coccidioidin in guinea pigs sensitized with mycelia and coccidioidin in IFA (Fig. 2, group OR), but in all other groups of animals, reactivity of spherulins and coccidioidins was comparable. Differences in precipitating antigens have been reported between strains and different forms of the same strain (10), but these were not reflected in skin test reactions. Since an observed skin reaction is the summation of a number of interacting specific antigen–lymphocyte systems, it is not possible to discriminate such differences. Another reason may be that nonprecipitating dialyzable antigens may account for much of the skin reactivity. Furthermore, antigens capable of precipitating do not necessarily elicit skin reactions. The shared complement of antigens may vary quantitatively as well as qualitatively, but always seem to react to a common total in heterologously sensitized guinea pigs even though the doses were selected for equipotency in the homologous systems. It would be interesting to isolate purified antigens and make similar comparisons.

Studies in man, in which comparisons were made between spherulin and conventional coccidioidin, indicate that spherulin is capable of detecting reactions that coccidioidin cannot detect (7). One reason for this may be that spherulin, being devoid of locally irritating or toxic concentrated media components or other dialyzable substances that are found in coccidioidins, can be used at higher active concentrations without toxicity contributing to the reaction. However, Levine *et al.* (7) have indicated that qualitative differences in skin reactivity may be detected in man with spherule and mycelial coccidioidins.

Skin tests administered in guinea pigs 2 weeks prior to second skin tests caused a decrease in reactivity in some groups of animals (Fig. 2). A number of factors may be responsible for this observation. Presumably the number of circulating sensitive lymphocytes has reacted and decreased, perhaps sequestered in lymph nodes (11) and not yet generated to the levels seen in guinea pigs skin tested for the first time. This phenomenon is being quantitated for coccidioidin at the present time in order to determine the stability of the sensitive state in animals skin tested at varying intervals.

Summary. Coccidioidin produced from mycelial and spherule phases of growth of three strains of *Coccidioides immitis* were compared for skin reactivity. Spherule coccidioidin was about 17 times more active on a weight basis than mycelial coccidioidin. When each of the coccidioidins was adjusted to equipotency in guinea pigs sensitized with the homologous strain and form of organisms, all of the skin-test preparations were virtually equivalent in reactivity in heterologously as well as homologously sensitized animals. Skin testing sometimes rendered animals less reactive to a second skin test.

1. Smith, C. E., Whiting, E. G., Baker, E. E., Rosenberger, H. G., Beard, R. R., and Saito, M. T., Amer. Rev. Tuberc. **57**, 330 (1948).

2. Winn, W. A., Levine, H. B., Broderick, J. E., and Crane, R. W., N. Engl. J. Med. **268**, 867 (1963).

3. Friedman, L., Smith, C. E., Roessler, W. G., and Berman, R. J., Amer. J. Hyg. **64**, 198 (1956).

4. Levine, H. B., Cobb, J. M., and Scalarone, G. M., Sabouraudia **7**, 20 (1969).

5. Scalarone, G. M., Levine, H. R., Chaparas, S. D., and Cobb, J. M., Sabouraudia, In press. (1973).

6. Chaparas, S. D., Fuller, V. J., and Seligmann, E. B., Jr., Proc. Soc. Exp. Biol. Med. **140**, 556 (1972).

7. Levine, H. B., Gonzalez-Ochoa, A., and Ten Eyck, D., Amer. Rev. Respir. Dis. **107**, 379 (1973).

8. Anderson, K. L., Wheat, R. W., and Conant, M. R., Appl. Microbiol. **22**, 294 (1971).

9. Ibrahim, A. B., and Pappagianis, D., Infec. Immun. **7**, 786 (1973).

10. Rowe, J. R., Newcomer, V. D., and Wright, E. T., J. Invest. Dermatol. **41**, 225 (1963).

11. Schlossman, S. F., Levin, H. A., Rocklin, R. E., and David, J. R., J. Exp. Med. **134**, 741 (1971).

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