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Delayed-Type Hypersensitivity Responses to a Cell Wall Fraction of the Mycelial Phase of *Coccidioides immitis*

EDWARD R. WARD, JR.,[1] REBECCA A. COX,[2*] JOHN A. SCHMITT, JR.,[1] MILTON HUPPERT,[3] AND SUNG H. SUN[3]

Brooks Aerospace Medical School, San Antonio, Texas 78223

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A skin test-active fraction was isolated from the mycelial-phase cell walls of *Coccidioides immitis*. This alkali-soluble, water-soluble antigen (C-ASWS) elicited positive reactions in 22 of 24 (92%) of the *Coccidioides*-sensitized guinea pigs whereas only 14 (54%) of the same guinea pigs reacted to commercial coccidioidin (BioCox). None of the 21 *Histoplasma*-sensitized guinea pigs cross-reacted with the C-ASWS antigen. Footpad tests in mice actively infected with *Coccidioides* further established the efficacy of the C-ASWS antigen in eliciting a delayed-type hypersensitivity response. One-microgram doses of C-ASWS produced reactions comparable to 100-μg doses of nondialyzable coccidioidin (Smith's lot 64 D4). The C-ASWS fractions isolated from three different *C. immitis* strains showed similar reactivity in terms of the number of positive reactions produced in *Coccidioides*-sensitized guinea pigs. However, the induration responses (diameter in millimeters) elicited by the C-ASWS fraction of one strain were significantly less than those elicited by the C-ASWS fractions of the other two *C. immitis* strains.

The need for an improved antigen reagent for testing in vivo and in vitro parameters of the immune response in coccidioidomycosis has generated an abundance of research efforts directed toward this end. Most recently, workers have investigated the efficacy of preparations from culture filtrates of the spherule/endospore phase of *Coccidioides immitis*. Their results have demonstrated that spherulin indeed is superior to the classical mycelial-phase coccidioidin (2-5). Spherulin, however, has the same inherent problem as coccidioidin in that it does not lend itself to dry-weight measurement and must be standardized on an empirical basis.

In 1973, Cox and Larsh (1) extracted an alkali-soluble, water-soluble cell wall fraction from the yeast phase of *Blastomyces dermatitidis* which compared favorably with two blastomycin preparations in the guinea pig model. Using a modification of this technique, we have obtained a skin test-active fraction (C-ASWS) from the mycelial-phase of *C. immitis*. (C-ASWS is used to designate the alkali-soluble, water-soluble fraction extracted from *C. immitis* mycelial cell walls and to distinguish it from the alkali-soluble, water-soluble fraction that Cox and Larsh [1] extracted from yeast-phase cells of *Blastomyces dermatitidis*.)

This study compared the C-ASWS antigen to commercial coccidioidin in both *Coccidioides*-sensitized and *Histoplasma*-sensitized guinea pigs. The C-ASWS also is compared with coccidioidin (Smith's lot 64 D4) in mice infected with *C. immitis*.

MATERIALS AND METHODS

Cultures. Three isolates of *C. immitis* were used. *C. immitis* strain 46 was made available by H. B. Levine, School of Public Health, Berkeley, Calif. *C. immitis* ATCC 7366 and *C. immitis* K9-71X, an isolate from a dog's lung taken at necropsy, were obtained from the Epidemiology Division, U.S. Air Force School of Aerospace Medicine, Brooks Air Force Base, Tex. The human isolate of *Histoplasma capsulatum* (Scritchfield) was obtained from the Missouri State Chest Hospital, Mount Vernon, Mo. Stock cultures of these organisms were maintained on Sabouraud dextrose agar (Difco, Detroit, Mich.) at 25 C.

Cell wall fractionation. Mycelial growth for fractionation was obtained by inoculating 10^8 arthrospores (estimated by hemocytometer counts) suspended in sterile physiological saline into a 2.8-liter low-form flask containing 1,000 ml of 1% yeast extract (Difco)-2% glucose broth. After 96 h of incubation in a gyratory incubator (New Brunswick Scientific, New Brunswick, N.J.) at 30 C, 13.5 ml of 37% formaldelhyde was added to each culture. The flasks then were placed at 4 C for 24 h. The formalin-killed mycelium was harvested by filtration through a

[1] Present address: Department of Botany, The Ohio State University, Columbus, Ohio 43210.
[2] Present address: Research Immunology, San Antonio State Chest Hospital, San Antonio, Tex. 78223.
[3] Present address: Mycology Research Laboratory, Veterans Administration Hospital, Long Beach, Calif. 90801.

Whatman filter paper (no. 40) and washed three successive times by suspending in distilled water and centrifuging at 10,000 × *g* for 5 min. The mycelium was then resuspended in distilled water to approximately 10 times its packed volume, mixed with an equal volume of glass beads (0.45 to 0.55 mm in diameter), and shaken in a Braun model MSK homogenizer for 120 s at 2,000 rpm. Heat effects during cell breakage were minimized with a carbon dioxide cooling device. The homogenized suspension was centrifuged for 10 min at 27,000 × *g*, 4 C, in a Sorvall model RC2-B refrigerated centrifuge. The supernate was discarded and the cell wall pellet was resuspended in distilled water and washed three times by centrifugation at 27,000 × *g* for 10 min each at 4 C.

To further purify the cell walls of cytoplasmic material, the cell wall pellets were resuspended in 0.01 M potassium phosphate buffer (pH 7.2), containing 100 μg of trypsin (Difco) per ml, to approximately 10 times their packed volume. The suspension was incubated for 3 h in a water bath shaker at 30 C. The trypsin-treated cell walls were washed in distilled water by centrifugation at 27,000 × *g* for 10 min at 4 C until the filtered supernate had an absorbance of 0.05 or less at a wavelength of 280 mm on a Gilford model 540 spectrophotometer. The brown, non-cell wall material was removed from the upper surface of the cell wall pellet after each wash. To solubilize the cell wall antigen, the trypsin-treated cell walls were hydrolyzed in 1 N NaOH (1.0 ml/mg) for 3 h at 25 C. The cell wall suspension was centrifuged at 27,000 × *g* for 10 min at 4 C, and the alkali-soluble supernate was passed through a membrane filter (0.45-μm pore size, Millipore Corp., Bedford, Mass.). The filtrate was dialyzed against several changes of distilled water at 4 C until the pH of the dialysate was 7.0. The alkali-soluble, water-insoluble precipitate, which formed during dialysis, was removed by centrifugation. The supernate (C-ASWS) was again filtered through a 0.45-μm pore size (Millipore) membrane, lyophilized, and stored at −20 C.

Sensitization of guinea pigs. A total of 113 male Hartley inbred guinea pigs, each weighing 400 to 600 g, were used in this study. Fifty-four were immunized with *C. immitis* strain 46; 15 were immunized with *C. immitis* ATCC 7366; 15 were immunized with *C. immitis* K-971X; and 21 were immunized with yeast-phase cells of *H. capsulatum* (Scritchfield). Eight guinea pigs were not immunized and were used as controls. Formalin-killed *C. immitis* mycelial cells and yeast-phase cells of *H. capsulatum* were lyophilized, ground with mortar and pestle, and emulsified in complete Freund adjuvant containing *Mycobacterium tuberculosis* H37RA (Difco) to a final concentration of 12.0 mg/ml. Each animal was injected with 1.0 ml of the emulsion: 0.2 ml in each of the front footpads and 0.6 ml subcutaneously into the posterior neck region. Skin tests were performed 4 weeks after injection.

Skin tests. The C-ASWS preparations were resuspended in physiological saline to a concentration of 100 μg/0.1 ml. For comparative studies, the *Coccidioides-* and *Histoplasma*-sensitized guinea pigs were skin tested with 0.1 ml of commercial coccidioidin 1:100 (BioCox, Bio Products Research Laboratories, Inc. Tempe, Ariz.), the C-ASWS fractions prepared from three different strains of *C. immitis* (C-ASWS-46; C-ASWS-ATCC; C-ASWS-K9-71X), and with Histoplasmin 1:100 (Parke, Davis and Co., Detroit, Mich.). The skin test sites were routinely alternated for each antigen. Indurations of 5 mm or greater in diameter were considered positive, irrespective of erythema.

Dose response. Fifteen guinea pigs were sensitized with *C. immitis* strain 46. Three weeks later, each guinea pig was skin tested with 10-, 25-, 50-, and 100-μg doses of C-ASWS prepared from *C. immitis* strain 46. Skin test sites were alternated for each antigen concentration.

Footpad tests. Randomized mice were infected intranasally with 10^3 viable arthrospores of *C. immitis* followed by treatment with amphotericin B. Survivors of the infection (test group) and noninfected mice (controls) were injected intradermally in the right and left hind footpads with 0.05 ml of physiological saline or with C-ASWS-46 or coccidioidin (Smith's lot 64 D4). The doses for the two antigens are expressed in terms of nondialyzable material (dry weight). Footpad thickness was measured at 48 h, using calipers.

RESULTS

The delayed-type hypersensitivity responses of two groups of guinea pigs that had been sensitized to *C. immitis* strain 46 (24 guinea pigs) and *H. capsulatum* (21 guinea pigs) are summarized in Table 1. Each group was tested with the C-ASWS antigen (strain 46, 100 μg), commercial coccidioidin (BioCox, 1:100), and Histoplasmin (Parke-Davis, 1:100). Of the *Coccidioides*-sensitized group, the C-ASWS antigen elicited 91.6% (22/24) positive reactions at both 24 and 48 h. The average induration of positive reactors was 12.8 and 12.3 mm, respectively. Commercial coccidioidin elicited 58.3% (14/24) positive reactions at 24 h and 29.2% (7/24) at 48 h. The sensitivity of the C-ASWS antigen was accompanied by a high degree of specificity as evidenced by the lack of cross-reactions in any of the 21 guinea pigs sensitized with *H. capsulatum*. Although coccidioidin exhibited a low degree of sensitivity, only one positive reaction (8 mm) was produced in the heterologously sensitized group of guniea pigs, and this reaction subsided to less than 5 mm at 48 h. Of the *Histoplasma*-sensitized guinea pigs, 19 (90.4%) reacted at 24 h and 20 (95.2%) reacted at 48 h to histoplasmin.

Table 2 presents the results obtained from skin testing three groups of guinea pigs (15 per group). Each group was sensitized with one of three *C. immitis* strains (45, ATCC 7366, or K9-71X) and then skin tested with the C-ASWS preparations obtained from each of the three

TABLE 1. *Skin test reactivity of C-ASWS antigen, commercial coccidioidin, and histoplasmin in sensitized guinea pigs*

Reactivity	Skin test antigen[a]					
	C-ASWS (100 μg)		Coccidioidin (1:100)		Histoplasmin (1:100)	
	24 h	48 h	24 h	48 h	24 h	48 h
Coccidioides-sensitized						
No. of positive reactors/total	22/24	22/24	14/24	7/24	0/24	0/24
Positive reactors (%)	91.6	91.6	58.3	29.2	0	0
Avg induration[b] (mm)	12.8	12.3	8.6	9.0	0	0
Histoplasma-sensitized						
No. positive	0/21	0/21	1/21	0/21	19/21	20/21
Positive reactors (%)	0	0	4.8	0	90.4	95.2
Avg induration (mm)	0	0	8.0	0	7.6	7.2

[a] Each guinea pig was inoculated with 0.1 ml of coccidioidin (1:100), histoplasmin (1:100), and C-ASWS antigen (100 μg).

[b] Expressed as the mean induration of positive reactors only.

TABLE 2. *Comparison of skin test reactivity of C-ASWS antigens prepared from three stains of C. immitis*

Reactivity	C-ASWS antigens prepared from strain:					
	46		ATCC 7366		K9-71X	
	24 h	48 h	24 h	48 h	24 h	48 h
Strain 46						
No. of positive reactors/total	15/15	15/15	14/15	15/15	14/15	14/15
Positive reactors (%)	100	100	93.3	100	93.3	93.3
Avg induration (mm)	14.0	14.5	14.0	14.8	9.8	10.2
Strain ATCC 7366						
No. of positive reactors/total	15/15	15/15	15/15	15/15	15/15	15/15
Positive reactors (%)	100	100	100	100	100	100
Avg induration (mm)	16.4	16.4	18.2	18.2	10.8	10.5
Strain K9-71K						
No. of positive reactors/total	15/15	15/15	15/15	15/15	15/15	15/15
Positive reactors (%)	100	100	100	100	100	100
Avg induration (mm)	15.8	16.1	15.3	15.8	12.7	12.8

strains. This experiment was designed to test C-ASWS antigen in both the homologous and heterologous systems. Positive reactions were obtained in all ATCC 7366-sensitized and K9-71X-sensitized animals by antigens prepared from each of the three strains. One guinea pig immunized with strain 46 did not respond to the C-ASWS, ATCC 7366, or K9-71X antigen. Maximal skin test responses (millimeters of induration) were elicited by the C-ASWS antigens from strain 46 and ATCC 7366 as compared with that of strain K9-71X.

Table 3 contains a statistical analysis of the induration response data presented in Table 2, with t values and significance (P) of the results of the three C-ASWS antigens compared with each other within each group of sensitized guinea pigs. These values indicate that C-ASWS antigen prepared from strain K9-71X was significantly less reactive (in terms of the induration elicited) than antigens prepared from the other two strains except in the homologous system at the 48-h reading. There were no significant differences between the reactivities of antigens from strains 46 and ATCC 7366 in the strain 46-sensitized group or the strain K9-71X-sensitized group. ATCC 7366 antigen was, however, significantly more reactive than antigen 46 in the homologous system.

Figure 1 depicts the mean indurations produced by skin testing 15 *C. immitis* strain 46-sensitized guinea pigs with C-ASWS 46 at con-

TABLE 3. *Statistical analysis of induration responses presented in Table 2*

Sensitizing strain	Antigens compared	*t* values[a]		*P* values	
		24 h	48 h	24 h	48 h
46	46 vs. K9-71X	5.40	3.19	<0.001	<0.001
	46 vs. ATCC 7366	0.80	−0.49	NS[b]	NS
	K9-71X vs. ATCC 7366	−6.78	−8.49	<0.001	<0.001
ATCC 7366	46 vs. K9-71X	3 94	3.89	<0.001	<0.001
	46 vs. ATCC 7366	−2.35	−4.26	<0.02	<0.001
	K9-71X vs. ATCC 7366	−8.96	−9.84	<0.001	<0.001
K9-71X	46 vs. K9-71X	2.16	2.00	<0.02	NS
	46 vs. ATCC 7366	−1.96	−1.22	NS	NS
	K9-71X vs. ATCC 7366	−6.74	−9.94	<0.001	<0.001

[a] *t* values were obtained by the means test for unpaired data.
[b] NS, Not significant.

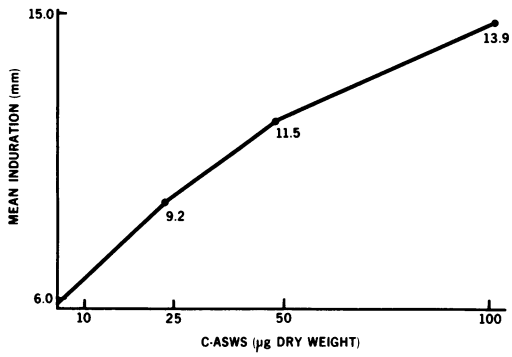

FIG. 1. *Mean indurations obtained from skin tests of 15 Coccidioides immitis-sensitized guinea pigs with 10-, 25-, 50-, and 100-μg doses of C-ASWS.*

centrations of 10, 25, 50, and 100 μg/0.1 ml. Mean indurations of 6.0, 9.2, 11.5, and 13.9 mm were obtained for C-ASWS skin test doses at these concentrations, respectively. These results establish that the intensity of delayed-type hypersensitivity responses elicited is directly proportional to the dose concentration of C-ASWS used for skin testing. Positive reactions were elicited in 73% (11/15) of the guinea pigs when skin tested with a 10 μg/0.1-ml dose. Fourteen of the 15 guinea pigs reacted to doses of 25, 50, and 100 μg/0.1 ml; one guinea pig failed to react to any of the concentrations used.

A comparison of C-ASWS 46 antigen and coccidioidin (Smith's lot 64 D4) in the murine model of coccidioidomycosis is presented in Tables 4 and 5. Both antigens elicited significant reactions at a 0.1-mg dose. C-ASWS, however, produced significant reactions at 0.01- and 0.001-mg doses. These data indicate that C-ASWS antigen is at least tenfold stronger on a dry-weight basis than Smith's lot 64 D4 coccidioidin. It is interesting to note that these results closely parallel those obtained by Levine et al. (4) when comparing spherulin with coccidioidin-64 in the murine model. There were no significant differences in the footpad thickness of noninfected (control) mice at 24 or 48 h after injection of saline versus coccidioidin, saline versus C-ASWS, or coccidioidin versus C-ASWS.

TABLE 4. *C-ASWS antigen (strain 46) compared with coccidioidin (Smith's lot 64 D4) in the murine model of coccidioidomycosis*

Antigen	Dose (mg)	No. of readings	Footpad thickness (mm) at 48 h[a]
Saline	0	10	3.15 ± 0.24
Coccidioidin	0.1	14	3.61 ± 0.21
	0.01	13	3.15 ± 0.32
C-ASWS	0.1	8	3.88 ± 0.23
	0.01	10	3.75 ± 0.26
	0.001	9	3.67 ± 0.25

[a] Expressed as average ± standard deviation.

DISCUSSION

The present study demonstrates that an alkali-soluble, nondialyzable fraction (C-ASWS antigen) extracted from the cell walls of *C. immitis* elicits a delayed hypersensitivity response in *Coccidioides*-sensitized guinea pigs and mice actively infected with coccidioidomycosis.

A comparison of the relative reactivities of the cell wall antigen with that of commercial coccidioidin in sensitized guinea pigs strongly favors the C-ASWS antigen. The detection rates of positive reactors experienced when skin testing with C-ASWS antigen were considerably greater than those obtained with coccidioidin.

TABLE 5. *Statistical analysis of footpad responses of infected mice to saline, coccidioidin, and C-ASWS*

Groups compared		*t* values	Probability
Saline vs.	Coccidioidin (0.1 mg)	4.91	<0.005
	Coccidioidin (0.01 mg)	0.03	NS[a]
	C-ASWS (0.1 mg)	6.44	<0.005
	C-ASWS (0.01 mg)	5.31	<0.005
	C-ASWS (0.001 mg)	4.58	<0.005
Coccidioidin (0.1 mg) vs.	C-ASWS (0.1 mg)	2.75	<0.01
	C-ASWS (0.01 mg)	1.47	NS ($P < 0.10$)
	C-ASWS (0.001 mg)	0.61	NS
Coccidioidin (0.01 mg) vs.	C-ASWS (0.1 mg)	5.59	<0.005
	C-ASWS (0.01 mg)	4.82	<0.005
	C-ASWS (0.001 mg)	4.07	<0.005

[a] NS, Not significant.

In comparing three different C-ASWS antigen preparations in both their homologous and heterologous systems, some variation in antigen reactivity was noted. All three *C. immitis* strains (46, ATCC 7366, and K9-71X) appeared to be equally effective as immunogens, but the C-ASWS antigen prepared from strain K9-71X generally elicited lower averages of induration irrespective of the immunizing strain. Presumably, this is attributable to either strain variations in the amounts of active alkali-soluble cell wall components or perhaps to fluctuations in the reproducibility of the procedure for isolating the antigen. Presently, studies are being conducted to resolve this question.

C-ASWS antigen appears to offer some advantages as a research tool in cellular immunity as it relates to coccidioidomycosis. First, it shows a high degree of reactivity in eliciting delayed hypersensitivity responses in *Coccidioides*-sensitized guinea pigs and mice. Secondly, it can be administered in high concentrations without eliciting either nonspecific inflammatory responses or cross-reactions with *Histoplasma*-sensitized guinea pigs. Thirdly, dry-weight measurements of dosage will allow a more precise standardization from lot to lot than methods presently used to standardize conventional skin test preparations.

We are presently evaluating the efficacy of this antigen in the in vitro lymphocyte transformation and indirect macrophage inhibition assays, using lymphocytes obtained from patients with proven coccidioidomycosis. Additionally, studies are in progress to further characterize the nature of C-ASWS, both antigenically and biochemically.

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