Cross-Reactivity Between Antigens of *Coccidioides immitis*, *Histoplasma capsulatum*, and *Blastomyces dermatitidis* in Lymphocyte Transformation Assays

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The cross-reactivity of four *Coccidioides* antigens, three *Histoplasma* antigens, and two *Blastomyces* antigens were determined in lymphocyte transformation assays of 11 coccidioidin-reactive, histoplasmin-nonreactive subjects (group I), 13 coccidioidin-nonreactive, histoplasmin-reactive persons (group II), and 13 subjects who were skin test negative to both antigens (group III). Mycelial and yeast (or spherule)-phase antigens of the three fungi were included. Significant cross-reactivity was obtained with both coccidioidins, spherulin, and the alkali-soluble, water-soluble cell wall antigen of *C. immitis*, to the extent that the responses of histoplasmin-antigen persons were not statistically different (P > 0.05) from those of coccidioidin-active persons. In contrast, optimal dilutions of *Histoplasma* mycelial and yeast-phase lysates effectively distinguished (P < 0.01) responses of histoplasmin- and coccidioidin-reactive persons. The alkali-soluble cell wall antigen of *H. capsulatum* showed extensive cross-reactivity at most concentrations and was markedly stimulatory to lymphocytes of skin test-negative persons. Blastomycin elicited significant cross-reactions in histoplasmin-sensitive subjects and to a lesser extent in coccidioidin-sensitive subjects. The alkali-soluble cell wall antigen cross-reacted in cultures of histoplasmin-reactive persons but not in those of coccidioidin-reactive persons. All antigens effectively distinguished (P < 0.001) homologous responses of skin test-positive persons (groups I and II) from those of skin test-negative persons (group III). The extensive cross-reactivity in lymphocyte transformation assays in the absence of cross-reactivity in skin tests suggests that these two immune responses may be mediated by different T lymphocyte populations, may be elicited by different antigenic components, or both.

Antigens presently available for immunological studies of coccidioidomycosis, histoplasmosis, and blastomycosis are heterogeneous mixtures containing common antigenic components (10, 13, 18, 21, 22, 30, 31). Accordingly, extensive cross-reactivity occurs between antigens of *Coccidioides immitis*, *Histoplasma capsulatum*, and *Blastomyces dermatitidis* (3–5, 7, 29). Cross-reactivity is most extensive between *Histoplasma* and *Blastomyces*, to a lesser extent between *Histoplasma* and *Coccidioides*, and to an even lesser extent between *Blastomyces* and *Coccidioides*. It is not uncommon that the reaction to the heterologous antigen is stronger than that obtained with the homologous antigen. This is especially true in serological assays (5).

The lymphocyte transformation (LT) assay is commonly used to measure T (thymus derived) lymphocyte function in fungal diseases (1, 6, 11, 16, 20, 26, 27). Briefly, this assay consists of culturing lymphocytes in vitro with antigen for 5 to 7 days during which sensitized T cells recognize and respond to specific antigens by undergoing an increased mitotic division, as measured by radioisotope uptake. Previous reports have suggested that LT assays may prove useful in the diagnosis of fungal diseases (15, 23). The potential value of LT as a diagnostic tool will depend, in part, upon the specificity of responses obtained with fungal antigens. The present study was, therefore, undertaken to assess the extent of cross-reactivity between antigens of *C. immitis*, *H. capsulatum*, and *B. dermatitidis* in LT assays of healthy subjects with known skin test reactivity.

MATERIALS AND METHODS

Study groups. Thirty-seven healthy subjects were divided into three groups on the basis of their skin test reactivity to coccidioidin (1:100, Cutter Labs, Berkeley, Calif.) and histoplasmin (1:100, Parke-Davis, Dallas, Tex.). Group I contained 11 subjects who reacted
inoculation of skin histoplasmin. Group II contained 13 subjects who were negative to coccidioidin but positive to histoplasmin. Group III contained 13 persons who were nonreactive to both coccidioidin and histoplasmin. The mean skin test responses of the three study groups are summarized in Table 1.

**Antigens.** Antigens from the saprophytic and parasitic (tissue) phases of *C. immitis*, *H. capsulatum*, and *B. dermatitidis* differ qualitatively and quantitatively (10, 18, 19). For this reason, LT assays were performed with antigens derived from mycelial and yeast (or spherule) phases of the three fungi. Antigens from *Coccidioides* included: coccidioidin TS, a tolune-induced lysate of mycelial-phase cells of *C. immitis* Silveira (28), obtained from Demosthenes Pappagianis, University of California, Davis; coccidioidin XV-77, a mycelial-phase lysate concentrated by ultrafiltration (19), obtained from Milton Huppert, Veterans Administration Hospital, San Antonio, Tex.; spherulin, an aqueous extract of the spherule/endo-sporic phase of strain Silveira obtained from Berkeley Biologicals, Berkeley, Calif. (lot 4A); and C-ASWS, an alkali-soluble, water-soluble cell wall extract of mycelia (32). *Histoplasma* antigens included: histoplasmin H-42, a mycelial-phase autolysate obtained from the Center for Disease Control, Atlanta, Ga.; histoplasmin YP, a yeast-phase lysate obtained from Marilyn Sutcliffe, Veterans Administration Hospital, Nashville, Tenn.; and H-ASWS, the alkali-soluble cell wall antigen obtained from yeast cells. *Blastomyces* antigens included: blastomycin KCB-26, an autolysate of mycelia obtained from Coy Smith, University of Kentucky, Lexington; and B-ASWS, an alkali-soluble, water-soluble extract of yeast-phase cells (9).

Streptokinase-streptodornase (SK-SD, American Cyanamid Co., Lederle Laboratories Div., Pearl River, N.Y.) and *Candida* antigen, obtained from M. Sutcliffe, were included in LT assays to compare the responses of the three study groups to antigens unrelated to *Coccidioides*, *Histoplasma*, and *Blastomyces*. Mitogens phytohemagglutinin (PHA, Difco, Detroit, Mich.) and concanavalin A (ConA, Sigma Chemical Co., St. Louis, Mo.) were included for the same reason.

Before LT assays, antigens were dialyzed at 4°C against a 40-fold volume of sterile, distilled water to remove preservatives. The nondialyzable antigens were lyophilized and reconstituted back to their original volume in tissue culture medium. Since the available activity of the antigens was limited, no attempts were made to determine the amount (as dry weight) of nondialyzable material present. Rather, we chose to refer to the reconstituted antigens as undiluted stocks from which the indicated dilutions were made. The exceptions to this were the ASWS preparations which were quantitated on a dry-weight basis. The protein content of each antigen, determined by the method of Lowry et al. (25), was as follows: coccidioidin TS, 0.64 mg/ml; coccidioidin XV-77, 32.8 mg/ml; spherulin, 0.72 mg/ml; C-ASWS, 0.32 mg/ml; histoplasmin H-42, 1.12 mg/ml; histoplasmin YP, 0.20 mg/ml; H-ASWS, 0.73 mg/ml; blastomycin KCB-26, 0.83 mg/ml; and B-ASWS, 0.71 mg/ml.

**LT assays.** Sixty milliliters of heparinized blood (20 U/ml) was collected in sterile Vacutainer tubes. The blood was carefully layered over Ficoll-Hypaque and centrifuged at 450 × g for 40 min as described by Boyum (2). The mononuclear cells (interface layers) were pooled and washed twice in Hanks balanced salt solution and once in tissue culture medium 199 (TC199, Grand Island Biological Co., Grand Island, N.Y.). Lymphocytes were counted in a Neubauer hemacytometer and suspended to a concentration of 2 × 10⁶ per ml of TC199 supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (2 mM), and 30% plasma pooled from healthy subjects. One-tenth milliliter of the cell suspensions was dispensed into a series of microtiter wells (Microtest II, Falcon Products, Cockskeville, Md.) containing 0.1 ml of the indicated antigen dilution or, for controls, 0.1 ml of TC199. Antigens and controls were assayed in quadruplicate. The cultures were incubated at 37°C under a 5% CO₂-humidified atmosphere for 5 days. PHA and ConA were added to appropriate wells on day 2 to obtain optimal mitogen responses (72-h cultures). The cultures were pulsed with 0.5 μCi of [³H]thymidine (2 Ci/mmol, Amersham/Searle, Amer- sham, England) and harvested 5 h later on glass fiber disks using a MASH II harvester (Microbiological Associates, Bethesda, Md.). The disks were transferred to scintillation vials containing 3 ml of a toluene-based scintillation fluid and counted for radioactivity. LT responses were calculated as total counts per minute.

**Statistical analysis.** Because of the heterogeneity of the variances, the data were analyzed by the Mann-Whitney rank-sums test (17).

**RESULTS**

LT responses to mitogens, SK-SD, and *Candida.* LT responses of the three study groups were comparable to mitogens PHA and ConA and to antigens SK-SD and *Candida* (Fig. 1) with the exception of PHA responses (P < 0.05) in group II (histoplasmin reactive) when compared with group III (skin test negative) subjects.

**LT responses to Coccidioides antigens.** Transformation responses to the four *Coccidioides* antigens are depicted in Fig. 2 to 5. Six to seven dilutions of each antigen were routinely included in each assay to assess the optimal

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<th>Table 1. Skin test responses of groups I, II, and III to Coccidioidin and Histoplasmin</th>
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*Induration (millimeters) at 24 and 48 h after intradermal inoculation of coccidioidin 1:100 and histoplasmin 1:100.

*Range of induration responses.
concentration in terms of sensitivity (responses of coccidioidin-reactive subjects) and specificity (responses of histoplasmin-reactive and skin test-nonreactive subjects). Coccidioidin TS (Fig. 2) and coccidioidin XV-77 (Fig. 3) were similar in their biological activities, i.e., both showed extensive cross-reactivity in histoplasmin-sensitive persons with the exception of coccidioidin TS at a dilution of 1:500. LT responses to spherulin (Fig. 4) indicated this antigen to be more sensitive, in terms of the magnitude of responses in coccidioidin-reactive persons, but without specificity in histoplasmin-reactive persons. A slight stimulatory effect was observed to spherulin 1:10 as evidenced by the response (1,800 cpm) of skin test-negative persons. LT responses to C-ASWS (Fig. 5) were similar to those obtained with spherulin except that a 1-μg level of the cell wall antigen showed some degree (P < 0.05) of specificity in assays of histoplasmin-sensitive subjects. The nonspecific stimulatory effect of the ASWS antigen is consistent with previous studies (8, 12).

LT responses to Histoplasma antigens. The results obtained with three Histoplasma antigens are shown in Fig. 6 to 8. Cross-reactivity with histoplasmin H-42 (Fig. 6) and YP (Fig. 7) occurred in coccidioidin-reactive subjects but was significantly lower than the cross-reactivity observed with Coccidioides antigens. Histoplasmin YP appeared to be somewhat less cross-reactive in coccidioidin-sensitive persons since the responses of groups I (coccidioidin reactive) and III (skin test negative) were comparable (P > 0.05) at dilutions of 1:10, 1:50, and 1:500. Transformation responses to H-ASWS are
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FIG. 5. LT responses of coccidioidin skin test-positive subjects (△), histoplasmin skin test-positive subjects (○), and skin test-negative persons (●) to C-ASWS.

FIG. 6. LT responses of coccidioidin skin test-positive subjects (△), histoplasmin skin test-positive subjects (○), and skin test-negative persons (●) to histoplasmin H-42.

FIG. 7. LT responses of coccidioidin skin test-positive subjects (△), histoplasmin skin test-positive subjects (○), and skin test-negative persons (●) to histoplasmin YP.

FIG. 8. LT responses of coccidioidin skin test-positive subjects (△), histoplasmin skin test-positive subjects (○), and skin test-negative persons (●) to H-ASWS.

FIG. 9. LT responses of coccidioidin skin test-positive subjects (△), histoplasmin skin test-positive subjects (○), and skin test-negative persons (●) to blastomyces KCB-26.

Shown in Fig. 8. With the exception of the 1-μg dose, responses of histoplasmin-reactive subjects were not significantly different from those of coccidioidin-reactive persons. The nonspecific stimulatory effect of H-ASWS exceeded that of all other antigens studied.

LT responses to Blastomyces antigens. Blastomycin KCB-26 (Fig. 9) elicited significant reactions in histoplasmin-sensitive and in coccidioidin-sensitive persons. Essentially no response was obtained in lymphocytes of skin test-negative subjects. Unfortunately, we cannot rule out the possibility of dual sensitivity since an antigen is not currently available for assessing skin test reactivity to Blastomyces. However, consistently the responses of histoplasmin-reactive persons were greater, in terms of total counts per minute, to H. capsulatum antigen than to Blastomyces antigens. The same was true for coccidioidin-reactive persons. It is also pertinent to point out that, without exception, responses of the 13 histoplasmin-reactive subjects were greater to blastomycin KCB-26 than to any of the four Coccidioides antigens, a finding that suggests cross-reactivity in LT assays is
more pronounced between *Histoplasma* and *Blastomyces* than between *Histoplasma* and *Coccidioides*. In contrast to blastomycin KCB-26, B-ASWS (Fig. 10) did not elicit cross-reactions in coccidioidin-reactive subjects. Significant cross-reactivity was, however, observed in histoplasmin-sensitive persons.

**DISCUSSION**

Previous studies have documented extensive cross-reactivity between antigens of *C. immitis*, *H. capsulatum*, and *B. dermatitidis* in skin tests and in serological assays (3-5, 7, 29). The results of the present study extend their cross-reactivity to include LT assays. Studies with four *Coccidioides*, three *Histoplasma*, and two *Blastomyces* antigens showed that (i) reactions of healthy, coccidioidin-sensitive persons were significant but not statistically different from the responses of healthy, histoplasmin-sensitive persons with the exception of coccidioidin TS (1:500) and C-ASWS (1 μg); (ii) histoplasmin-reactive subjects responded strongly to histoplasmin H-42 and YP, and their responses were statistically greater than the cross-reactions obtained in coccidioidin-sensitive persons; and (iii) blastomycin KCB-26 elicited significant cross-reactions in histoplasmin-reactive subjects and to a lesser extent in coccidioidin-reactive subjects, whereas B-ASWS showed cross-reactivity with histoplasmin-reactive subjects only. All antigens effectively distinguished responses between skin test-reactive (groups I and II) and nonreactive (group III) subjects. These results are similar to the patterns of cross-reactivity obtained with antigens of *Coccidioides*, *Histoplasma*, and *Blastomyces* in skin tests and in serological assays.

Quantitative and qualitative differences have been reported in studies of antigens from the mycelia and yeast (or spherule) phases of these three dimorphic fungi (10, 18, 19). Recently, Huppert et al. (19) demonstrated 26 protein-containing bands in coccidioidin (XV) using two-dimensional immunoelectrophoresis with anti-coccidioidin. With this reference system, Huppert et al. (18) showed that spherulin contained 10 immune precipitates in common with coccidioidin, C-ASWS contained 8, histoplasmin H-42 and H-ASWS contained 9 and 6, respectively, while blastomycin KCB-26 and B-ASWS contained, respectively, 12 and 5 protein-containing bands that precipitated with anti-coccidioidin. Five immunoprecipitates were common to all antigens, though differences were observed in the relative amounts in each antigen. The presence of common antigenic components among these fungi no doubt contributes to the cross-reactivity observed in immunological assays. Until physiochemical, immunochemical procedures, or both are employed to fractionate antigens of *Coccidioides*, *Histoplasma*, and *Blastomyces*, cross-reactivity will continue to diminish the efficacy of these antigens.

Both yeast-phase and mycelial-phase extracts of the three dimorphic fungi were included in LT assays. Statistically, we were unable to demonstrate any significant differences between the two phases in regard to their sensitivity and specificity. It seems reasonable to assume that antigens from the mycelial phase and those from the yeast (or spherule) phase both contain the component(s) responsible for LT responses in homologously and heterologously sensitized persons. Whether or not the component(s) that elicits LT responses in homologously sensitized persons is identical to or dissociable from that which elicits cross-reactions is not yet known and awaits fractionation studies.

In previous studies directed towards isolating a more chemically defined antigen from *Blastomyces*, we reported on the efficacy of the ASWS cell wall extract of yeast-phase cells in skin tests, in assays for macrophage inhibitory factor, and in LT assays in the guinea pig model (9, 14). Throughout those studies B-ASWS proved to be more sensitive and specific than blastomycin antigens. In subsequent experiments, the ASWS cell wall fraction of mycelial-phase cells of *C. immitis* was examined for biological activity (8, 11, 12, 32). Comparative studies of C-ASWS with several different coccidioidins and with spherulin showed C-ASWS to be highly sensitive in eliciting delayed-type hypersensitivity responses in sensitized guinea pigs and in infected mice (32). LT and indirect macrophage inhibitory factor assays were limited to healthy, coccidioidin skin test-positive persons,
patients with coccidioidomycosis, and healthy, skin test-negative subjects (8, 11, 12). Responses to C-ASWS were comparable to or better than those obtained with coccidioidin TS and spherulin with the limitation that C-ASWS had a nonspecific stimulatory effect upon LT in healthy, skin test-negative persons. Previous studies with the ASWS cell wall fraction of *H. capsulatum* have not been reported. In the present study, H-ASWS was highly cross-reactive in LT assays of coccidioidin-sensitive persons and was almost mitogenic in lymphocyte cultures of skin test-negative persons, thereby lessening the potential usefulness of this antigen. Cross-reactivity and nonspecific stimulation of lymphocytes also occurred with B-ASWS and C-ASWS, but to a lesser extent. Despite apparent shortcomings of the ASWS cell wall antigens, they do offer the decided advantage of being standardized on a dry weight or chemical basis (9, 32). Mycelial and yeast lysates are rarely reproducible from batch to batch, the exception being spherulin, which can be standardized on a dry-weight basis (24).

In addition to establishing the extent of cross-reactivity of antigens from *Coccidioides*, Histoplasma, and Blastomyces, the results of the present study suggest that cross-reactivity can occur in LT assays in the absence of cross-reactivity in skin tests. We purposely selected donors who were skin test reactive to coccidioidin but negative to histoplasmin, donors who were reactive to histoplasmin but not to coccidioidin, and donors who were nonreactive to both antigens. The extensive cross-reactivity observed in LT assays in the absence of skin test reactivity to 1:100 dilutions of the heterologous antigens suggests that the T-lymphocyte population that mediates LT responses differs from that which mediates skin test reactivity. Alternatively, it can be argued that the antigen component(s) which elicits LT responses differs from that which elicits skin test responses. It is pertinent to point out that the coccidioidin and histoplasmin antigens used in skin testing differed from those antigens used in LT assays. However, 7 of 13 healthy, histoplasmin skin test-positive subjects were skin tested with and were negative to first strength spherulin (bioequivalent to coccidioidin 1:100), yet these same 7 persons showed significant cross-reactivity to spherulin in LT assays. The question of cross-reactive components versus distinct T-cell subpopulations cannot be resolved until monomolecular antigens are isolated from these dimorphic fungi.

The results of this study diminish the likelihood that LT assays will prove useful in distinguishing clinical infection between these three systemic fungi. Rather, the extensive cross-reactivity between antigens of *Coccidioides*, Histoplasma, and Blastomyces coupled with the depressed LT responses of most fungal patients to antigens of the infecting fungus (1, 6, 11, 16, 20, 26, 27) severely limits any diagnostic application of this assay.

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LITERATURE CITED


