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Use of an Alkali-Soluble Water-Soluble Extract of *Blastomyces dermatitidis* Yeast-Phase Cell Walls and Isoelectrically Focused Components in Peripheral Lymphocyte Transformations

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An alkali-soluble water-soluble extract of *Blastomyces dermatitidis* yeast-phase cell walls was tested for its ability to elicit a response in lymphocytes isolated from the peripheral blood of *Blastomyces*-infected guinea pigs. Sequential preparations of the antigen were reproducible and specific in the in vitro lymphocyte transformation assay. Cross-reactivity of the antigen was not evident in lymphocyte transformation assays on lymphocytes obtained from *Histoplasma*-infected guinea pigs or from animals sensitized with complete Freund adjuvant. Fractionation of the antigen was accomplished on an isoelectric-focusing column, using a sucrose density gradient support. Components were assayed for activity in skin testing and lymphocyte transformation. Comparison of column fractions to the whole antigen showed greater response to the whole antigen in in vivo and in vitro assays.

Parameters used in evaluating cell-mediated immunity (CMI) include in vivo skin testing and in vitro assays for migration inhibition factor and mitotic responses by lymphocytes causing lymphoblast formation. These cellular reactions are important in host defense to some mycotic diseases (4, 8).

The role of CMI in host defense against blastomycosis is unknown. No antigen has been found that is sensitive and specific in monitoring the disease. Culture filtrate antigens have been isolated, but are highly cross-reactive, difficult to reproduce, and demonstrate little or no sensitivity (2, 3, 10).

Our laboratory has concentrated on antigenic extracts of the organism itself rather than the metabolic byproducts found in other blastomycins. An alkali-soluble water-soluble extract of *Blastomyces dermatitidis* yeast-phase cell walls (referred to as B-ASWS) isolated by Cox and Larsh was shown to be more specific than cytoplasmic fractions of the yeast cells in its ability to elicit in vivo responses (2). Recently, we reported the sensitivity and specificity of B-ASWS in in vivo and in vitro tests for CMI (6).

Our purpose in this study was to establish the reactivity of the antigen in the peripheral lymphocyte transformation assay and to evaluate the reproducibility of the biological activity of sequential preparations of the antigen. Further fractionation of the antigen by isoelectric focusing was undertaken with the hope of obtaining a more sensitive component. Fractions were assayed for biological activity in in vitro and in vivo assays.

MATERIALS AND METHODS

Antigen preparation. *B. dermatitidis* strain SCB-2 (ATCC 26199) was maintained in the yeast phase at 37°C on brain heart infusion agar (Difco) slants and transferred weekly. Log-phase growth cultures (72 h old) were used to inoculate brain heart infusion broth cultures. Broth cultures were incubated at 37°C on a gyratory shaker (120 rpm) for 72 h. Cells were killed by addition of formaldehyde (final concentration, 0.2%) and were placed at 4°C for 24 h. Cells were harvested by repeated washings with distilled water and centrifugation at 10,000 × *g* for 10 min at 4°C. Isolation and fractionation of the cell walls were accomplished according to the method of Cox and Larsh (2). Washed cells were mechanically disrupted for 120 s, using a Braun model MSK homogenizer fitted with a carbon dioxide cooling device to minimize heat accumulation. The cell wall fraction was collected by centrifugation and washed 10 times in distilled water. Crude cell walls were treated with trypsin (100 μg/mg) and hydrolyzed with 1 N NaOH for 3 h at 25°C. The preparation was centrifuged, and the alkali-soluble supernatant was filtered (0.45-μm membrane filter [Millipore Corp.]) and dialyzed against repeated changes of distilled water at 4°C. After the removal of the water-insoluble glucan by centrifugation, the nondialyzable B-ASWS was filtered, using a PM10 ultrafiltration membrane. The PM10 residue was

eluted, lyophilized, and stored at −20°C. For in vitro assays, B-ASWS was suspended in TC 199 medium (Grand Island Biological Corp.) to a concentration of 1 mg/ml. Stock solutions were stored at 4°C under a CO_2 atmosphere.

Other antigens used in the study were histoplasmin (H-42), phytohemagglutinin (PHA-M; Difco), and purified protein derivative (PPD) obtained from the National Institutes of Health. The H-42 was dialyzed for 72 h against three changes of sterile distilled water at 4°C, lyophilized, and reconstituted to original concentration in TC 199 before use.

Results of preliminary studies determined optical concentrations of antigens used in the study (e.g., those concentrations which elicited blastogenic responses in cells from sensitized guinea pigs and showed minimal toxicity for cells of normal, nonsensitized animals as demonstrated by radioisotope incorporation).

Animals. Three groups of Hartley inbred guinea pigs (400 to 600 g) were sensitized via footpad inoculation. Twenty-two animals received 5×10^7 viable yeast-phase cells of *B. dermatitidis* (strain SCB-2) in Freund complete adjuvant; 16 were sensitized with 10^8 viable yeast-phase cells of *Histoplasma capsulatum* (Scritchfield isolate) in Freund complete adjuvant; and 10 control animals received saline in Freund complete adjuvant. Each guinea pig received 0.2 ml in the front footpads and 0.6 ml, subcutaneously, in the nuchal area.

LT assay. Lymphocyte transformation (LT) assays were initiated 21 days after infection, and animals were monitored weekly through week 8. Ethylenediamine tetraacetic acid-anticoagulated blood (1 mg/ml) was obtained from guinea pigs by cardiac puncture and was diluted with an equal volume of Hanks balanced salt solution (GIBCO). Four milliliters of the diluted blood was layered on three milliliters of Ficoll-Paque gradient (Pharmacia) and centrifuged at 4°C for 45 min at 450 × *g* (1). The interfacial lymphocyte layer was harvested, washed three times (250 × g, 10 min) in TC 199 medium, counted, and adjusted to a concentration of 2×10^6 lymphocytes per ml in TC 199 supplemented with 10% heat-inactivated fetal calf serum, L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). One-tenth milliliter of the cell suspension was introduced into microtiter plates (Falcon), for a final cell concentration of 2×10^5 cells. Triplicate cultures were incubated at 37°C in a 5% CO_2 humidified atmosphere for 5 days (13). The antigen-treated cells received 5, 10, and 25 μg of B-ASWS 1:200 and 1:500 H-42, 10 μg of PPD, and 1:100 of the mitogen PHA-M. PHA-M was added 48 h after the beginning culture. Sixteen hours before harvest, 0.06 μCi of [^{3}H]thymidine (specific activity, 6.7 Ci/mmol; New England Nuclear) was added to each culture. The cultures were harvested, using a Multiple Automated Sample Harvester (MASH II; Microbiological Associates). Ten washes of distilled water followed by an ethanol rinse were used per well. Precipitates were collected on a glass-fiber filter and placed in liquid scintillation vials containing 10 ml of toluene-Spectrafluor (Amersham/Searle) cocktail. Samples were counted in a Beckman LS100C scintillation counter. The transformation responses are expressed as the ratio of counts per minute of antigen-stimulated cultures to those of nonstimulated cultures—blastogenic indexes (BI).

Skin testing. Skin tests were performed only in *Blastomyces*-infected guinea pigs. In vivo testing was initiated 2 weeks after sensitization. The visceral area of each animal was shaved, and 0.1 ml of B-ASWS (100 μg), PPD (10 μg), and isoelectrically focused ASWS components (100 μg) were injected intradermally. Indurations of 5 mm or greater at 24 h were considered positive.

Statistical analysis. Data were submitted to a one-way analysis of variance. The level of significance of the LT responses was determined, using the Student's *t* test for unpaired data. Reproducibility of sequential preparations of the antigen was obtained using a paired *t* analysis.

Isoelectric focusing. Using an LKB Produktor 440 column, a 1% solution of ampholytes (aliphatic polyamino-polycarboxylic acids of low molecular weight) was superimposed on a linear sucrose gradient (0 to 45%) support according to standard techniques (7). Twenty milligrams of B-ASWS reconstituted in sterile physiological saline was introduced in the heavy-gradient suspension. Focusing was carried out for 24 h with a final voltage of 600 V. The anode solution was removed, and the column was eluted, using an ISCO model 328 fraction collector. The column effluent was collected in 2-ml amounts and monitored at 280 nm during collection, and pH values were determined for each 2-ml sample. Ultraviolet light absorption curves were superimposed on plots of the linear pH gradient to determine isoelectric points. Sample components were recovered from ampholyte-sucrose solutions by dialysis. Fractions were lyophilized and stored at −20°C until used.

Chemical analysis. Protein was determined by the method of Lowry et al. (11), using bovine serum albumin as a standard. Carbohydrate determinations were done on samples hydrolyzed with 1 N HCl at 110°C for 7 h. The hydrolysates were neutralized with NaOH and filtered. The procedure of Park and Johnson (14) was used to determine total reducing groups, using alpha-D-glucose as a standard.

RESULTS

Lymphocyte responsiveness. The BI of the three animal groups to PHA-M and PPD are given in Fig. 1. Responses to the nonspecific mitogen PHA-M were obtained in all three groups. A greater response to PPD at 10 μg was obtained in the control group with a mean BI of 11.82. The *Histoplasma* and *Blastomyces* groups yielded a BI of 8.16 and 6.16, respectively. Counts per minute of control (nonstimulated) cultures were comparable. No stimulatory effects were found in peripheral lymphocytes from nonsensitized guinea pigs tested with PPD.

The responses of the three groups to the B-ASWS antigen are in Fig. 2. Each point is an average of responses compiled from two experimental groups (four animals each) sampled alternately for 8 weeks. A significantly greater

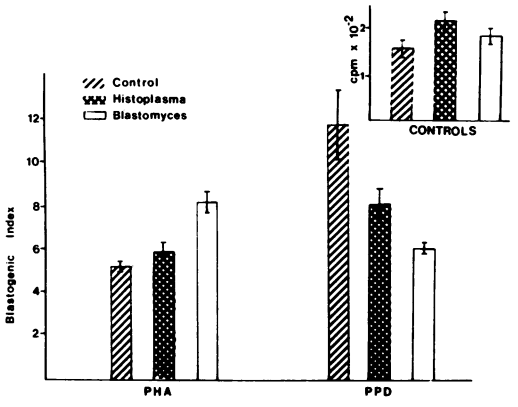

FIG. 1. *Average BI (vertical axis) of lymphocytes from Blastomyces-infected animals, Histoplasma-infected animals, and control animals to a nonspecific mitogen PHA and PPD. Control or unstimulated cultures are expressed as counts per minute. Standard error of the mean is shown as vertical bars on the histogram.*

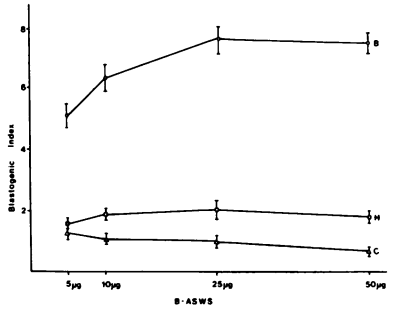

FIG. 2. *Lymphocyte responses (expressed as BI) of Blastomyces-infected animals (B), Histoplasma-infected animals (H), and control animals (C) to various concentrations of B-ASWS. Vertical bars depict average indexes ± standard error.*

response was obtained in the *Blastomyces* group ($P \leq 0.005$) than in either the nonsensitized group or the *Histoplasma* group. The homologously infected animals yielded BI values of 7.47, 7.56, 6.28, and 5.14 at 50, 25, 10, and 5 μg, respectively. Some toxicity was apparent at the 50-μg level. No significant difference was observed at the 5-μg level of B-ASWS when *Histoplasma*-infected animals were compared to the nonsensitized group ($P > 0.1$). However, marginal responsiveness was noticed in the *Histoplasma*-infected animals at 10 μg of B-ASWS (BI = 1.85) and in the presence of 25 μg of B-ASWS (BI = 2.02). BI values of greater than 2 were considered significant.

The *Histoplasma* group was tested with H-42 at two dilutions with a BI of 8.2 at the 1:200 dilution. This maximal response was obtained with lymphocytes isolated 2 weeks postinfection. Control animals exhibited no significant response to H-42.

Sequential antigen preparation. Sequential isolations of the cell wall antigen of *B. dermatitidis* were assayed for reproducibility in the peripheral LT assay. Antigen prepared at different times (A and B) were tested at three concentrations: 5, 10, and 25 μg (Fig. 3). Average BI values obtained from lymphocytes isolated from 20 *Blastomyces*-inoculated guinea pigs were 7.56, 6.28, and 5.14 (preparation A) and 7.56, 6.53, and 5.17 (preparation B) at 25, 10, and 5 μg of B-ASWS. No significant differences were observed ($P > 0.05$).

Isoelectric focusing. In a preliminary screening column, using ampholytes ranging from pH 3.5 to 10, three bands absorbing at 280 nm were found in the acid pH range. For better resolution, ampholytes in a range of pH 3.5 to 5 were chosen for the antigen preparation. Two major peaks at 280-nm absorbance with isoelectric points (pI) of 4.01 and 4.69, respectively, were found. An initial peak 1 was located at the interface of the anode solution but had no activity in either the LT system or skin testing.

The BI values obtained in the *Blastomyces* group to peak 2 (pI 4.01) and peak 3 (pI 4.69) and the whole ASWS antigen are shown in Fig. 4. An increasing response was obtained to peak 3 with a maximal response at the 50-μg level. Peak 2 maximal response occurred at the 25-μg concentration. The whole ASWS was found to be more responsive than either peak.

Skin test. Using an in vivo parameter, skin testing, the antigen elicited responses in *Blastomyces*-infected guinea pigs. Eighty-three percent of the animals reacted to B-ASWS (100

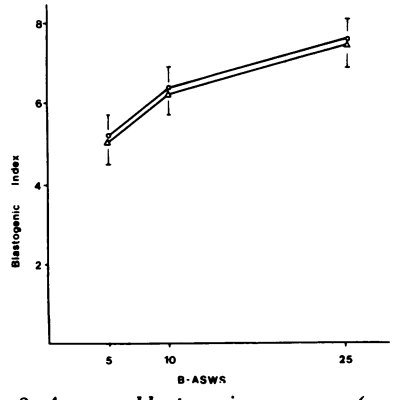

FIG. 3. *Average blastogenic responses (expressed as BI of lymphocytes from Blastomyces-infected guinea pigs to two sequential preparations of B-ASWS (A and B). Each point represents an average of 20 samplings. Vertical bars depict mean ± standard error.*

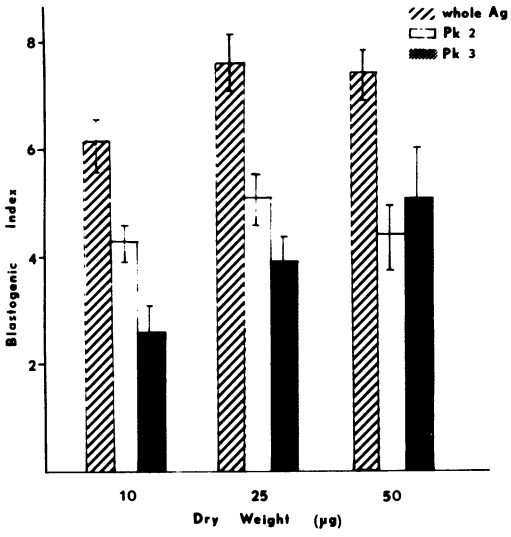

FIG. 4. *Comparison of BI values of lymphocytes isolated from Blastomyces-infected guinea pigs to B-ASWS (whole antigen) and isoelectrically focused fractions (peak 2 and 3). All antigens run at 100 µg (dry weight) were toxic to the cells. Vertical bars show standard error of the mean.*

µg), with an average induration of 12.8; 50% reacted to peak 3 and 33% to peak 2 with indurations of 9.8 and 11.5, respectively. No increase in sensitivity was shown with the fractions.

Chemistry. B-ASWS had a protein-carbohydrate ratio of 1.56:1. The ratio of protein to polysaccharide for the isoelectrically focused fractions was essentially the same at 1.12:1 for peak 2 and 1.15:1 for peak 3.

DISCUSSION

CMI plays a major role in the host defense to mycotic infections (4, 5, 8, 12). The cellular competency of blastomycotic patients is unknown. No antigen is available that is capable of eliciting cellular responses. In contrast to broth culture filtrate blastomycins, ASWS antigen from *B. dermatitidis* yeast cells has yielded a sensitive and specific antigen that can monitor cellular responses in experimental animal models of blastomycosis. The application of B-ASWS in assays of cellular immunity becomes an important tool in evaluating the disease.

The B-ASWS antigen did induce significant blastogenic responses in homologously infected animals. Average indexes of 5.14 at 5 µg to 7.56 at 25 µg were observed in the homologously infected animal (Fig. 2). The greatest index found in the *Histoplasma* group was 2.02 in response to 25 µg of B-ASWS. This was not considered a significant BI. These results correlate well with previous data when the antigen was used to stimulate lymphocytes obtained from lymph nodes (6). Even though indexes obtained in the lymph node lymphocyte assay were lower, they were still significantly greater than the indexes of the heterologous groups. The differences could be attributable to the lymphocyte populations and sources of cells.

Since the antigen can be standardized on a dry weight basis, it was necessary to determine if the biological activity was comparable in two different preparations. Preparation A and B compared favorably at all three concentrations in their ability to elicit lymphocyte responses as measured in an LT assay. The protein-carbohydrate ratios of these two preparations have been analyzed and have elicited responses that are comparable. In previous work (unpublished data) two preparations were compared in the MIF assay and were found to elicit similar responses.

The antigen has been chemically fractionated by other investigators, using disc gel electrophoresis (3) and preparative polyacrylamide gel electrophoresis (10). Isoelectric focusing was chosen to increase the resolution of components and for ease in recovery of the samples. Banding patterns of the antigen focused in the acid pH range. Equivalent dry weights of the peaks demonstrated lesser reactivity than the same dry weight of the whole antigen. Perhaps two or more individual components of the antigen are necessary for maximal biological activity.

In summary, further fractionation of the antigen gave three components, one nonstimulatory and two stimulatory but less so than the whole antigen. The antigen was shown to be specific and sensitive in the peripheral LT assay, and it is capable of detecting various levels of immune responsiveness in animal models of blastomycosis. The peripheral blood assay in microtiter was found to be superior in that the degree of response was reflected by the higher indexes obtained using peripheral blood as a lymphocyte source (indexes of 2 to 3 in lymph node lymphocytes and indexes of 5 to 8 in peripheral blood lymphocytes), and greater differences in BI values were noticed when homologously infected animals were tested as compared with heterologously infected animals or nonsensitized animals. Use of the antigen in patient care may provide information about the lymphocyte subpopulations and the functional activity of the T cell. B cell activity, as measured by routine serological methods, may only be a measurement of the degree of infection and not a correlation to immunity. The study of host response to blastomycosis will be advanced once an antigen is available.

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