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In Vivo and In Vitro Cell-Mediated Immune Responses to a Cell Wall Antigen of *Blastomyces dermatitidis*

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An alkali-soluble, water-soluble cell wall fraction of *Blastomyces dermatitidis*, designated B-ASWS, was evaluated as an antigen for detecting in vivo (skin tests) and in vitro migration inhibition factor (MIF) production and lymphocyte transformation (LT) responses in *Blastomyces*-infected guinea pigs. The biological activity of B-ASWS was compared with that of blastomycin KCB-26. The superiority of B-ASWS, in terms of its sensitivity and specificity, was evident in in vivo and in vitro assays. Skin test responses were obtained in 21 of the 24 *Blastomyces*-infected guinea pigs, whereas only one of the 14 *Histoplasma*-infected guinea pigs were significantly greater than those obtained using cell populations from *Histoplasma*-infected or noninfected guinea pigs. The con- MIF and LT in peritoneal exudate cells and lymph node cells of homologously infected animals. In each biological system, the responses of the *Blastomyces*-infected guinea pigs were significantly greater than those obtained using cell populations from *Histoplasma*-infected or non-infected guinea pigs. The contrasting efficacy of B-ASWS, as compared with blastomycin KCB-26, suggests that the cell wall antigen will be a useful tool for detecting cell-mediated immune responses in blastomycosis.

At present, there is not an antigen available for diagnostic, epidemiological, or immunological studies of blastomycosis. Blastomycin, a broth culture filtrate of mycelia-phase cells of *Blastomyces dermatitidis*, is known to be a complex heterogeneous mixture containing multiple antigenic components (4, 13). The inefficacy associated with this antigen is attributed to its lack of sensitivity and specificity (1-3, 15). Similar problems are encountered with culture filtrates of *Histoplasma capsulatum* and *Coccidioides immitis*, designated histoplasmin and coccidioidin, but to a much lesser extent (7, 12, 21, 22).

Numerous efforts have been made to obtain a more reliable antigen for blastomycosis (6, 10, 11, 15). Most of these were directed towards isolating the skin test active component(s) from blastomycin preparations. Although this approach may yield biologically active components, sophisticated and time-consuming techniques that may or may not prove to be practical for a large-scale production of a skin test antigen are required. Studies in our laboratory have, therefore, centered on isolating an antigen directly from *B. dermatitidis* cells rather than from culture filtrates of this organism. In a previous paper, we reported on a skin test active antigen that was obtained from yeast-phase cells by alkaline hydrolysis (3). This alkali-soluble, water-soluble cell wall antigen, designated B-ASWS, exhibited sensitivity and specificity in eliciting skin test responses in sensitized guinea pigs. The present report extends these studies to in vitro assays of lymphocytes and peritoneal-exudate (PE) cells of experimentally infected guinea pigs in an effort to further establish the efficacy of B-ASWS.

MATERIALS AND METHODS

Antigens. *B. dermatitidis* strain SCB-2 (ATCC 26199) was maintained in the yeast phase at 37°C on brain heart infusion (Difco) agar slants and transferred weekly. Three-day-old yeast cultures (log-phase growth) were inoculated in brain heart infusion broth and incubated at 37°C on a gyratory shaker (120 rpm) for 72 h. The cells were killed by the addition of merthiolate (final concentration, 1:10,000) and refrigerated at 4°C for 24 h. Yeast-phase cells were harvested by centrifugation at 10,000 × *g* for 10 min at 4°C and washed repeatedly in distilled water. Isolation and fractionation of the cell walls was accomplished as described previously (3). Cells were mechanically disrupted in a Braun model MSK homogenizer for 120 s. The cells walls were collected by centrifugation and washed 7 to 10 times in distilled water. Crude cell walls were treated with trypsin (100 μg/mg) and then hydrolyzed with 1 N NaOH for 3 h at 25°C. The suspension 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. The water-insoluble glucan was removed by centrifugation, and the non-dialyzable B-ASWS was collected on a PM10 ultrafiltration membrane (Amicon). The PM10 residue was eluted, lyophilized, and stored at −20°C. For in vitro studies, B-ASWS was suspended to a concentration of 1 mg/ml of TC199 medium (GIBCO) and stored at 4°C. The protein content of this antigen, using the method of Lowry et al. (14), was 0.70 mg of protein per mg of B-ASWS.

Blastomycin KCB-26 was kindly supplied by Coy Smith, University of Kentucky, Lexington. Prior to the in vitro studies, this antigen was dialyzed for 72 h against daily changes of sterile distilled water at 4°C to remove preservatives. The non-dialyzable antigen was lyophilized, reconstituted to its original volume in TC199 medium and filter sterilized. The dry weight of blastomycin KCB-26 was determined to be 1.89 mg/ml (of non-dialyzable material) and contained 0.64 mg of protein per ml. Histoplasmin (1:100) was obtained from Parke, Davis & Co. This antigen was dialyzed for 72 h against daily changes of sterile distilled water at 4°C and then lyophilized. The lyophilized material was reconstituted in TC199 medium to one-tenth of its original volume for the in vitro studies. Purified protein derivative (PPD) was obtained from the National Institutes of Health. Phytohemagglutin M was purchased from Difco.

In a series of preliminary studies, the optimal concentration of each antigen (e.g., that concentration which elicited migration inhibition factor [MIF] production and blastogenic responses in cells from sensitized guinea pigs and exhibited minimal toxicity for cells of normal, nonsensitized animals) was determined.

Animals. Male and female inbred guinea pigs (Hartley) weighing 600 to 800 g were infected via footpad inoculation with viable yeast-phase cells or saline suspended in an equal volume of Freund complete adjuvant containing *Mycobacterium* H37Ra (Difco). Twenty-four guinea pigs received 5×10^7 yeast cells of *B. dermatitidis* strain SCB-2; 14 guinea pigs received 10^8 yeast cells of *H. capsulatum* (Scritchfield isolate); and 20 animals (noninfected controls) were inoculated with a saline-adjuvant mixture only. Each guinea pig was injected subcutaneously with a total of 1 ml, 0.2 ml in each front footpad and 0.6 ml in the nuchal area.

Skin test. Skin tests were performed 2 weeks after sensitization. A 0.1-ml portion of B-ASWS (100 μg), KCB-26 (1:25), histoplasmin (1:100), and PPD (10 μg) was injected intradermally into the shaved visceral surface of each animal. All three groups of guinea pigs were skin tested with each antigen. Indurations of 5 mm or greater at 24 h were considered positive.

Our purpose in skin testing the three guinea pig groups prior to performing the in vitro assays was to eliminate from the test groups any animal that did not exhibit a response of 10 mm or greater to the homologous skin test antigen. Because it was reported that skin testing may suppress in vitro lymphocyte transformation (LT) responses (23), the LT and MIF assays were not initiated until 7 days after the skin tests had been read. These assays were continued up to 8 weeks after the animals had been sensitized and/or infected.

MIF assay. The direct MIF was performed using the agarose-droplet method of Harrington and Statsny (9). Seventy-two hours after mineral oil had been injected intraperitoneally, the guinea pigs were anesthetized with ether and exsanguinated by cardiac puncture. PE cells were collected in Hanks balanced salt solution (GIBCO) that was freshly supplemented with 10 U of heparin per ml. When present, erythrocytes were lysed by suspending the cells in 0.89% NH_4Cl (pH 7.2) for 10 min at 25°C (20). Afterwards, the pellet was washed twice by centrifugation ($250 \times g$, 10 min) in TC199 medium. The cells were suspended in a twofold volume of a mixture containing TC199 medium, 15% guinea pig serum and 0.4% agarose. A 1-μl amount of the cell suspension was dispensed into each of eight wells on a microtiter tissue culture chamber (Lab-Tek). Medium, with or without antigen, was added to duplicate wells on each of three chambers so that each antigen dilution was assayed, using six agarose-cell droplets. To minimize subjectivity, the chambers were coded so that the person reading the MIF assays would not have knowledge of how the antigens had been distributed. The chambers were incubated for 18 to 24 h in a humidified 37°C, 5% CO_2 environment. The distance of macrophage migration was measured by using an inverted phase-contrast microscope fitted with an ocular micrometer. The ratio of the distance of macrophage migration in the presence of antigen to that of the controls was determined, and the results were expressed as the percentage of migration inhibition (MI). Values of 20% or greater were considered significant.

LT assay. Axillary lymph nodes were removed and washed in cold TC 199 medium supplemented with L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). The nodes were minced in TC199 medium, and the resulting lymphocyte suspension was passed through 150-mesh stainless-steel gauze. The lymphocytes were washed twice by centrifugation in TC199 medium, counted, and diluted to a concentration of 2×10^6 per ml of TC199 medium containing 10% heat-inactivated fetal calf serum. One-milliliter amounts were dispensed into a series of culture tubes to which was added 0.1 ml of antigen or, for controls, 0.1 ml of TC199 medium. Triplicate cultures were used to assay antigens; controls were performed in quadruplicate. The cultures were incubated at 37°C in a 5% CO_2-humidified atmosphere. At 48 h, 1.0 μCi of [^{3}H]thymidine (specific activity, 6.7 Ci/mmol) was added to each culture. After an 18-h pulse, the cultures were harvested by successive centrifugations in 0.89% NaCl, 5% trichloroacetic acid, and methanol. The precipitates were solubilized in 1 ml of NCS (Amersham/Searle), quantitatively transferred to scintillation vials containing 9 ml of a toluene-based scintillation fluid, and counted in a Beckman LS100C scintillation counter. The results are expressed as blastogenic index (BI), e.g., the ratio of the counts per minute of antigen-stimulated cultures to that of the controls. BI values greater than 1.00 were considered significant.

Statistical analyses. Statistical analyses of the

results were obtained by testing the LT and MIF responses in a one-way analysis of the variance. The level of significance ($P < 0.05$) was determined by using Student's t test for unpaired data.

RESULTS

Skin test. The skin test responses of the *Blastomyces*-infected, *Histoplasma*-infected and noninfected (control) animals are presented in Table 1. Of the 24 *Blastomyces*-infected guinea pigs, 21 (87%) were skin test positive to B-ASWS (100 μg). Only 7 (30%) of these same guinea pigs responded to blastomycin KCB-26 (1:25 or 75.6 μg). All of the *Histoplasma*-infected guinea pigs were skin test positive to histoplasmin (1:100); 1 (7%) of these cross-reacted to B-ASWS; 12 (85%) cross-reacted to blastomycin KCB-26. It is noted that the numbers of homologous and heterologous reactions obtained with the B-ASWS preparation used in the present study and with preparations used in the previous study (3) were not significantly different ($X^2 = 0.94$, $P > 0.05$). Nor were the numbers of heterologous reactions obtained from KCB-26 used in this study and from the blastomycin KCB-25 used in the previous study statistically different, e.g., 85% for KCB-26 and 74% for KCB-25. On the other hand, the two blastomycins differed significantly ($X^2 = 8.87$, $P < 0.01$) in their sensitivity; i.e., only 30% of the reactions elicited by KCB-26 were homologous, whereas 65% of the reactions elicited by KCB-25 were homologous. These results further establish the lack of reproducibility of blastomycins, even when prepared identically and used at similar concentrations (1:25 dilution). Increasing doses of blastomycin increased sensitivity, but this was concomitant with decreased specificity.

Since each guinea pig was inoculated with an adjuvant containing *Mycobacterium* H37Ra, all three guinea pig groups were expected to respond to PPD (10 μg). However, only one of the *Blastomyces*-infected guinea pigs was skin test positive to this antigen. These guinea pigs did develop significant in vitro responses to PPD, but they were less than those obtained for the other two test groups.

All guinea pigs were assayed within 8 weeks of sensitization, and 10 to 11 animals were selected for study. In most instances, PE cells and lymph nodes were assayed from the same animal.

MIF assay. The MIF responses of the three guinea pig groups to PPD (10 μg) are given in Fig. 1. The MI (expressed as the percentage of inhibition) of the *Blastomyces*-infected guinea pigs was significantly lower ($P < 0.01$) than those of the noninfected and *Histoplasma*-infected guinea pigs. Nevertheless, an inhibition index of 30% was obtained. That the concentration of PPD used was not toxic was established in direct assays of PE cells obtained from healthy, nonsensitized guinea pigs.

The MIF responses of these same guinea pigs to 10, 50 and 100 μg of B-ASWS is indicated in Fig. 2. Of the 12 *Blastomyces*-infected guinea pigs tested, 6 had a positive MI index to the 10-μg dose; 9 responded to the 50-μg dose; and 10 responded to the 100-μg dose. The specificity of these responses is evident in that PE cells from the control and *Histoplasma* groups failed to produce MIF to B-ASWS. The differences between the responses of the *Blastomyces*-in-

TABLE 1. *Skin test responses of guinea pigs to B-ASWS, blastomycin KCB-26, Histoplasmin, and PPD*

Skin test antigen	Skin test responses					
	Blastomyces infected[a]		*Histoplasma* infected		Noninfected	
	No. of positive reactors/guinea pigs tested[b]	Avg induration (mm)[c]	No. of positive reactors/guinea pigs tested	Avg induration (mm)	No. of positive reactors/guinea pigs tested	Avg induration (mm)
B-ASWS	21/24	14.5	1/14	13.5	0/20	0
Blastomycin KCB-26	7/24	10.9	12/14	12.4	0/20	0
Histoplasmin	0/24	0	14/14	15.8	0/20	0
PPD	1/24	12.5	10/14	12.9	17/20	14.9

[a] Guinea pig groups.
[b] Each positive reactor had an induration of 5 mm or greater at 24 h.
[c] Average induration (millimeters of diameter) of positive reactors only.

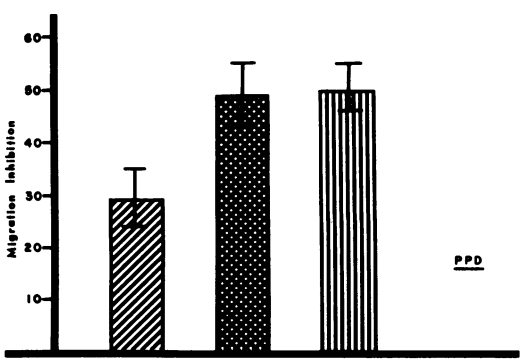

FIG. 1. *MIF response (expressed as the percentage of MI, vertical axis) of PE cells from 12 Blastomyces-infected (////), 10 Histoplasma-infected (IIIII), and 10 noninfected (×××) guinea pigs to PPD (10 μg). Vertical bars depict mean ± standard error.*

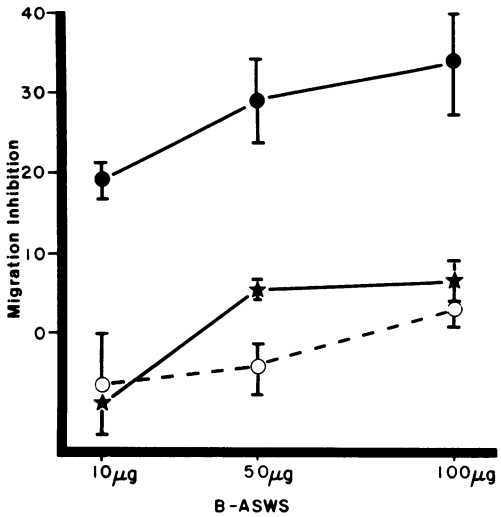

FIG. 2. *MIF responses (expressed as the percentage of MI, vertical axis) of PE cells from Blastomyces-infected (●), Histoplasma-infected (★), and noninfected (○) guinea pigs to 10-, 50-, and 100-μg concentrations of B-ASWS. Vertical bars depict mean ± standard error.*

fected guinea pigs and the control animals and those between the *Blastomyces*-infected and *Histoplasma*-infected groups were highly significant ($P < 0.001$). The mean MI of the 10 *Histoplasma* guinea pigs to histoplasmin was 15.6 ± 2.6, which, when compared with responses obtained in control groups, was significant at the 0.01 level. Although the MI of 15.6 was less than that designated as a positive response (20%), the homologous reaction of *Histoplasma* guinea pigs to histoplasmin was significantly greater than the heterologous reaction to B-ASWS at the 50- or 100-μg level ($P < 0.005$). Of interest, the *Histoplasma*-infected guinea pig that had a positive skin test to B-ASWS did not have a positive MIF response to this antigen, even though an MI of 31% was obtained with histoplasmin.

The results obtained with blastomycin KCB-26 are presented in Fig. 3. A significant difference ($P < 0.001$) between the *Blastomyces*-infected and noninfected guinea pigs when tested with the 1:100 dilution was obtained, but not to 5- or 10-fold-lower concentrations of this antigen. It is noteworthy to mention that the 1:100 concentration of blastomycin (18.9 μg [dry weight]) elicited an MI index comparable to those obtained with the 10-μg dose of B-ASWS. Further comparisons of the two antigens (on a dry-weight basis) could not be made because higher concentrations of blastomycin proved toxic.

LT assay. Lymph node cells obtained from each guinea pig were assayed for their LT responses to B-ASWS, blastomycin KCB-26, PPD, and the mitogen phytohemagglutin M. These results are shown in Fig. 4 through 6. There was no difference in the mitogenic responses of the three groups to phytohemagglutin M (2.5 μg of protein). Again, the *blastomyces*-infected guinea pigs had a significantly lower ($P < 0.01$) response to PPD (BI = 1.97) than did the control group (BI = 7.58) or the *Histoplasma*-infected guinea pigs (BI = 4.89). PPD was not found to have a stimulatory effect on lymphocytes of healthy, nonsensitized guinea pigs.

The LT responses obtained with B-ASWS are depicted in Fig. 5. The blastogenic responses of the *Blastomyces*-infected guinea pigs were significantly greater ($P < 0.001$) than those of the noninfected and heterologously infected guinea pig groups to concentrations of 50 and 100 μg. This latter guinea pig group had a mean BI of 1.27 ± 0.08 for histoplasmin, which, when compared with the control, was significant at the 0.001 level. The almost linear response obtained for the *Blastomyces*-infected guinea pigs that received the three concentrations of B-ASWS reflects the dose dependency of these reactions. Of the 13 homologously infected guinea pigs, 7 had a positive LT response to the 10-μg dose, 10 responded to 50 μg, and 12 responded to 100 μg of B-ASWS. There was no detectable difference in the blastogenic re-

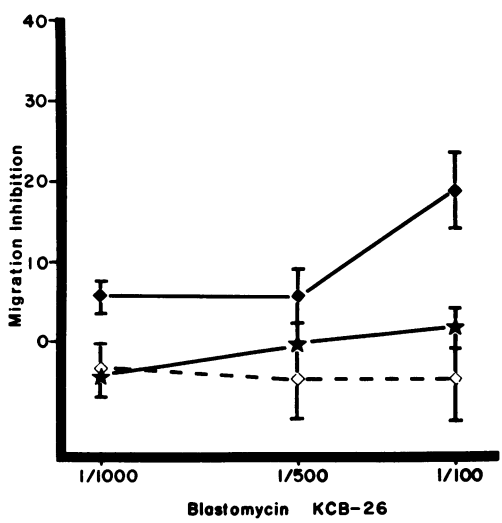

FIG. 3. *MIF responses (expressed as the percentage of MI, vertical axis) of PE cells from Blastomyces-infected (◆), Histoplasma-infected (★), and noninfected (◇) guinea pigs to 1:100, 1:500 and 1:1,000 dilutions of blastomycin KCB-26. Vertical bars depict mean ± standard error.*

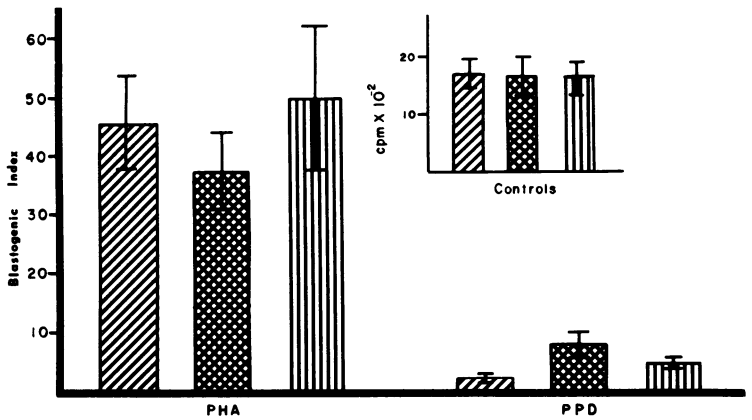

FIG. 4. *LT responses (expressed as BI, vertical axis) of 13 Blastomyces-infected (///), 11 Histoplasma-infected (IIIII), and 11 noninfected (×××) guinea pigs to PPD (10 μg) and PHA (1:1,000 dilution). Vertical bars depict mean ± standard error.*

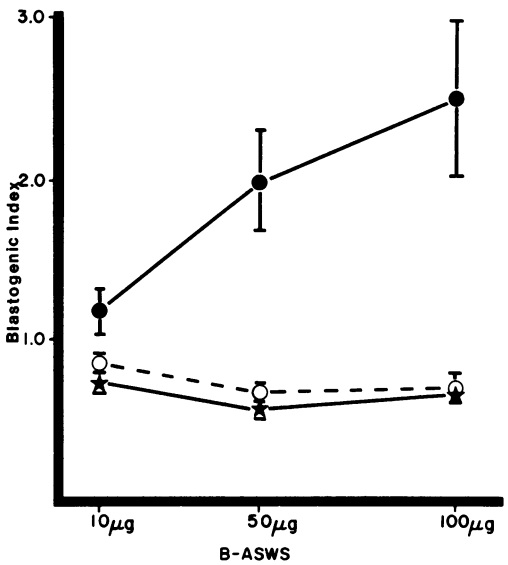

FIG. 5. *LT responses (expressed as BI, vertical axis) of Blastomyces-infected (●), Histoplasma-infected (★), and noninfected (○) guinea pigs to 10-, 50-, and 100-μg concentrations of B-ASWS. Vertical bars depict mean ± standard error.*

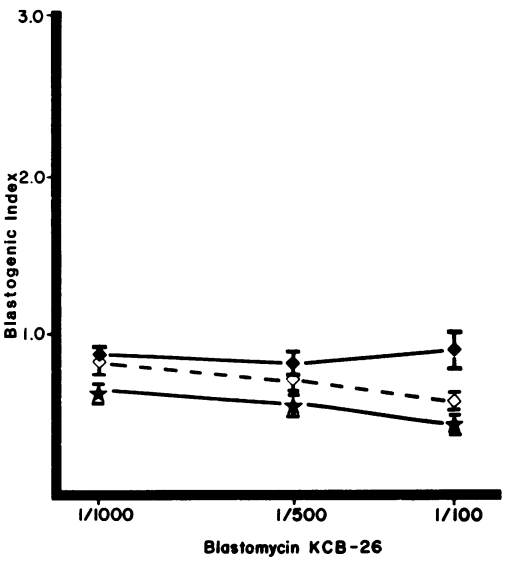

FIG. 6. *LT responses (expressed as BI, vertical axis) of Blastomyces-infected (◆), Histoplasma-infected (★), and noninfected (◇) guinea pigs to 1:100, 1:500, and 1:1,000 dilutions of blastomycin KCB-26. Vertical bars depict mean ± standard error.*

sponses of guinea pigs assayed early after infection (3 weeks) and those assayed at 8 weeks.

In contrast to the responses obtained with B-ASWS, blastomycin KCB-26 failed to elicit significant levels of blastogenesis (Fig. 6), regardless of the concentration used. Only 4 of the 13 *Blastomyces*-infected guinea pigs responded to the 1:100 dilution, and only 2 responded to the 1:500 and 1:1,000 dilutions. Higher concentrations exhibited marked toxicity for lymphoctes. It should be mentioned that B-ASWS and blastomycin KCB-26 were somewhat toxic for lymphocytes of heterologously infected or sensitized guinea pigs, yet these same concentrations were optimal for stimulating blastogenesis in lymphocytes of *Blastomyces*-infected guinea pigs. A similar finding was reported to occur with old tuberculin (17).

DISCUSSION

Our present knowledge of virtually every aspect of blastomycosis remains meager. This is largely attributed to the lack of a suitable antigen, e.g., one that exhibits a reasonable degree

of sensitivity and specificity. Because cellular immunity is thought to play a key role in the host's resistance to this disease, there exists a need for an antigen that is capable of detecting cell-mediated responses. In our experience, and those of others, blastomycin was proven to be ineffective. In the present study and in a previous report, blastomycin elicited a greater number of skin test responses in *Histoplasma*-infected guinea pigs than in homologously infected animals. The highest concentration (18.9 μg) of blastomycin elicited MIF production but not LT responses. Possibly higher concentrations of this antigen might prove to be more effective, but the presence of toxic components precluded their use. Herein lies an inherent difficulty in studies ultilizing broth culture filtrates.

The in vivo and in vitro results obtained with B-ASWS are in contrast to those obtained with blastomycin. Cell populations (PE and lymph nodes) from *Blastomyces*-infected animals released MIF and underwent a significant blastogenic response to all three concentrations of B-ASWS tested, although the responses obtained with the 10-μg dose were borderline. The extent to which the skin tests represent a delayed-type hypersensitivity response, as opposed to an Arthus-type reaction, was a major concern in our studies. The high doses of B-ASWS required to elicit the skin test responses and the early (3 to 6 h) indurations observed support the presence of an Arthus-type response. On the other hand, the persistence of the indurations (maximal at 24 h) and the predominantly mononuclear response observed at 24 and 48 h (3) are consistent with a tuberculin delayed-type hypersensitivity response. The results obtained in the in vitro assays tend to further strengthen our supposition that responses to B-ASWS are, at least in part, T-cell mediated (19, 25). The extent of blastogenesis obtained in lymphocytes from *Blastomyces*-infected guinea pigs was low, yielding indexes of only 1.2, 2.0, and 2.5 for concentrations of 10, 50, and 100 μg, respectively. This is, in part, attributed to the high background counts (1,640) of control (non-stimulated) cultures which, in turn, may have resulted from our use of fetal calf serum rather than homologous serum (8, 17). Nevertheless, the LT responses of these guinea pigs were significantly higher ($P < 0.001$) than those of the *Histoplasma*-infected or control groups.

Our studies of the B-ASWS cell wall antigen were not limited to *B. dermatitidis*. Recent studies have demonstrated that the alkali-soluble cell wall fraction of mycelial-phase cells of *C. immitis* exhibits a similar degree of specificity and sensitivity (24). In contrast to the B-ASWS cell wall fractions of *B. dermatitidis* and *C. immitis*, that obtained from yeast-phase cells of *H. capsulatum* was found to lack specificity, as evidenced by the number of cross-reactions obtained in *Blastomyces*-infected guinea pigs (unpublished data).

It is becoming increasingly evident that alkaline hydrolysis of fungal cell walls solubilizes components that are capable of inducing as well as detecting delayed-type hypersensitivity responses. A report by Domer (5) provides evidence that an ethylenediamine-soluble glycoprotein of *H. capsulatum* cell walls elicits in vivo and in vitro cell-mediated responses in experimentally infected guinea pigs. The specificity of this alkali-soluble cell wall antigen has not been reported. Similarly, studies by Reiss et al. (18) established that a peptidoglucomannan of *Candida albicans*, released during alkaline hydrolysis, was effective in eliciting skin test responses and in vitro MIF responses. The extent to which these alkali-soluble cell wall antigens of these different fungi might be chemically similar remains to be determined. It is clear, however, that continued studies of these antigens will provide new information as to the antigenic and biochemical makeup of fungal cell walls.

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