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Stimulation and Suppression of Cell-Mediated Immunity by Endosporulation Antigens of *Coccidioides immitis*

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The culture filtrate of *Coccidioides immitis* induced to replicate in its parasitic phase in vitro, termed endosporulation antigens (EA), was assayed for ability to stimulate human lymphocyte blastogenesis in vitro. Stimulation of lymphocytes from skin test-positive healthy subjects by EA was comparable to stimulation by spherulin at optimal dilutions, but EA are 500 times more potent when compared on the basis of weight. Both preparations slightly stimulated lymphocytes from skin test-negative subjects. Heating or dialysis of EA enhanced the effect on skin test-positive subjects, but concentration depressed it. Concentrated EA also depressed nonspecific stimulation caused by phytohemagglutinin. Dialysis of concentrated EA reduced the ability to depress responses. EA from an avirulent strain of *C. immitis* were as stimulatory as EA from a virulent strain, but concentrating the former did not produce as much depression as concentrating the latter did. A survey of subjects with an optimal dose of EA in lymphocyte transformation showed that EA could separate skin test-positive from -negative subjects as well as spherulin could. The survey also showed that a Δcpm (the difference of incorporated counts of tritiated thymidine per minute in the presence or absence of the reagent) of 10,000 is useful for this separation. These results also indicate the presence of suppressive substances in EA which are only partially dialyzable and which were significantly more prominent in a preparation from the virulent strain.

Coccidioidin, a culture filtrate of *Coccidioides immitis* in its saprophytic mycelial phase, and spherulin, a lysate of spherules (parasitic phase, spherule-endospore cycle) in distilled water, are the standard reagents for the in vivo detection of human cell-mediated immunity to this fungus (14, 15) and are the only coccidioidal reagents commercially available. Coccidioidin was also shown to be useful in the in vitro assay of human cell-mediated immunity (16), although spherulin was subsequently shown to be a more potent reagent for this purpose (4, 5). However, spherulin requires several steps in its preparation which use live organisms, and, as it is a lysate, it is relatively difficult to produce large volumes of this reagent (9). We sought a parasitic-phase antigen preparation that could be prepared simply, safely, inexpensively, and in large quantities for in vitro studies. We therefore tested a culture filtrate of the organism that had been induced to replicate in its parasitic phase in vitro (2). The studies reported here describe an in vitro assay of human cell-mediated immunity in which a preparation derived from such cultures was used. We term this preparation endosporulation antigens (EA).

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MATERIALS AND METHODS

Antigens and mitogens. Spherulin without preservative was prepared as previously described (6, 9) and used in lymphocyte cultures at a final concentration of 23 μg/ml. Phytohemagglutinin-P (PHA) (Difco Laboratories, Detroit, Mich.) was used at a final concentration of 34 μg/ml in the cultures.

EA were prepared from different strains of *C. immitis* as follows. *C. immitis* cultures passaged in the endospore-spherule phase (9) were used. Endospores were harvested from 7-day-old cultures, and 0.8×10^6 to 2.0×10^6 endospores per ml were inoculated into basal spherule medium (BSM) (10). During incubation for 72 to 96 h at 35°C, the endospores matured into spherules (1.0×10^6 to 1.6×10^6/ml) which ruptured and released their endospore progeny. The endospores were removed by centrifugation at $800 \times g$ for 20 min, and the supernatant was further purified by centrifugation at $1,560 \times g$ for 20 min and filtration through 0.45-μm and then 0.22-μm filters. Lot 1 of EA was prepared

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from *C. immitis* Silveira (1) and held at 4°C for 3 years. A second lot of EA from this strain was prepared, stored at 4°C, and utilized within weeks. EA from *C. immitis* 46 (1) were prepared in the same way.

Batches of EA were heated at 56°C for 30 min, dialyzed at 4°C against 0.9% saline for 48 h, or concentrated 10-fold by dialysis against a 1:1 mixture of Aquacide I and II (Calbiochem, La Jolla, Calif.), or subjected to a combination of these procedures. BSM was treated similarly for the controls. Weights of nondialyzable solids were determined by placing samples of the concentrate in the chamber of a temperature-compensated refractometer (Series TSB, American Optical Corp., Buffalo, N.Y.) and reading with an incandescent light source. Unless otherwise specified below, lot 1 of EA from the Silveira strain was used as the stimulus.

Lymphocyte cultures. As previously detailed (5, 11), mononuclear cells, isolated by centrifugation in Ficoll-Hypaque, were washed with phosphate-buffered saline, counted, and suspended in tissue culture medium 199 containing 50 μg of gentamicin per ml and 20% autologous serum. Each mitogen or reagent (or medium 199 for controls) was added in a 0.05-ml portion to 1 ml of medium containing 6×10^5 cells, and the mixture was divided into three 0.25-ml cultures in tubes. Tritiated thymidine was added to cultures containing PHA and to controls on day 3 and to antigen cultures and controls on day 6. The cells were then harvested on filters on day 7, and the counts per minute (cpm) of [^{3}H]thymidine incorporated in the cells was quantitated in a scintillation counter. The mean cpm of triplicate samples was determined, and the culture response was expressed as Δcpm, i.e., the mean cpm of stimulated cultures minus the mean cpm of unstimulated cultures. A subtraction that resulted in zero or a negative number was considered a zero, for simplification. The experimental plan, within the limitations of availability of reagents and subjects, was that individuals would act as their own controls. The plan would allow individual comparisons between different reagents.

In vivo studies. Determination of the virulence of *C. immitis* strains was performed in mice by intranasal challenge with dilutions of arthroconidia, as previously described (1).

Subjects. Healthy adult subjects were skin tested with a 0.1-ml intradermal dose of a 1:100 dilution of coccidioidin (Cutter Laboratories, Berkeley, Calif.) and Usual Test Strength spherulin (Berkeley Biologicals, Berkeley, Calif.). Induration of ≥5 mm was considered a positive response.

Statistics. Student's *t* test was used for comparisons between group means and means of paired samples. Significant *P* values are presented in the text, tables, or both.

RESULTS

Comparison of EA with spherulin, dose-response studies, and effect of treatments on EA. In an initial study, 10 subjects were studied (Table 1). Spherulin was a potent stimulant of lymphocytes from skin test-positive subjects. Responses were similar to those caused by PHA, a mitogen that stimulates the majority of T lymphocytes (13). EA stimulated the lymphocytes of skin test-positive subjects as well as spherulin did. EA and EA diluted 1:10 gave similar responses, and it was only with a 1:100 dilution that a diminution in the response caused by EA was noted.

TABLE 1. Lymphocyte transformation results with healthy subjects

Reagent	No. of subjects	Skin test result	Mean Δcpm ± SE
PHA	3	+	33,578 ± 1,970[a]
Spherulin	3	+	23,987 ± 4,730[b]
EA (undiluted)	3	+	17,835 ± 451[c]
Spherulin	7	−	2,325 ± 597[b]
EA (undiluted)	7	−	837 ± 316[c]
EA (1:2 dilution)	7	−	1,975 ± 568

[a] PHA results in skin test-negative subjects not significantly different from results in skin test-positive subjects (PHA results in seven skin test-negative subjects, Δcpm ± SE = 28,809 ± 7,122).

[b] Skin test-positive versus skin test-negative results, $P < 0.001$.

[c] Skin test-positive versus skin test-negative results, $P < 0.001$.

A small amount of mitogenic activity or stimulation due to cross-reactivity in the host to related antigens results from the exposure of lymphocytes to spherulin, as seen in the skin test-negative subjects. The "nonspecific" stimulation of lymphocytes from skin test-negative subjects seen with EA was comparable to or slightly less than the stimulation seen with spherulin. The nonspecific stimulation caused by EA was greater at a 1:2 dilution than with undiluted EA, and this increase was noted with six subjects. With further dilutions to 1:4, 1:10, and 1:100, the stimulation was decreased. The PHA stimulation of lymphocytes from skin test-negative subjects served as a positive control in these experiments.

The effect of various treatments of EA was examined in a related study to determine whether treatment would enhance or depress stimulation. In all three skin test-positive subjects, either dialyzing or heating the undiluted EA enhanced the stimulation, dialysis by 36% (mean) and heating by 66% ($0.1 > P > 0.05$ for both procedures, compared with untreated EA). Concentrating the EA 10-fold, however, markedly reduced the stimulation of the lymphocytes of skin test-positive subjects (>90% reduction in all three, $P < 0.01$). Neither dialysis nor concentration of EA affected the low Δcpm of lymphocytes from skin test-negative subjects.

Because heating the EA is an easier procedure to perform than dialysis, heat-treated EA were selected for another study on dose response. Heated undiluted EA were superior to heated

EA diluted 1:10 or 1:100 in stimulating lymphocytes from three skin test-positive subjects. However, the heated EA were still a potent stimulant even at a 1:100 dilution (mean Δcpm, 19,410). Studies with lymphocytes from seven skin test-negative subjects, in which heated EA were compared with spherulin, showed results indistinguishable from those in Table 1. Both heating and dialyzing the EA neither enhanced stimulation of lymphocytes by skin test-positive subjects nor affected the results with skin test-negative subjects compared with either procedure alone. Studies with four subjects (two skin test positive, two negative) proved that the components of the medium used to make EA were not the source of the stimulation (mean Δcpm with BSM, 337). Heat-treated EA became the standard preparation for subsequent studies.

Comparison of EA from different isolates and lots. The Silveira strain of *C. immitis* is virulent; i.e., the lethality it produces in mice after intranasal challenge is one of the highest of any strain studied (1). The 50% lethal dose for this strain in 1976, when the lot of EA used in the studies described thus far was prepared, was 49 arthroconidia. This assay was repeated in 1980 at a time when a second lot of EA was prepared from this strain after conversion to its endospore-spherule phase. The strain had been passaged in vitro in its saprophytic (mycelial-arthroconidial) phase in the 4-year interval, but it had not lost virulence; the repeat 50% lethal dose was 38 arthroconidia. *C. immitis* 46, by contrast, is relatively avirulent; the 50% lethal dose determined for this strain was 478 arthroconidia.

In studies with six subjects, heat-treated EA from strain Silveira lot 1, used in the preceding studies, strain 46, and a second lot prepared from the Silveira strain in 1980 were compared. No differences were seen with these three reagents in this experiment, and the results were the same as those noted above. Concentrating the EA from strain Silveira lot 2 ablated (i.e., Δcpm, zero or negative) the response in all subjects, as was seen earlier with lot 1.

Although these similar results might have been anticipated, a 10-fold concentrate of EA from strain 46 gave different results (Table 2). That strain 46 EA are as stimulatory as EA from strain Silveira lot 1, as mentioned earlier, is also seen when the data from this experiment are compared with the data presented previously. However, the depression of response with concentrated EA from strain 46 was not as profound (mean, 56% decrease compared with response to heated EA in skin test-positive subjects) as noted earlier with concentrated EA from either lot of strain Silveira (depression, >90 to 100% versus untreated EA). The depression due to the concentrated Silveira strain EA was significantly ($P < 0.01$) greater than that due to the strain 46 preparation.

The medium used to prepare the EA was concentrated 10-fold and added to undiluted EA. When lymphocytes from two subjects were incubated with this mixture, the concentrated medium had no effect on the stimulation response to EA (Table 2). These results indicate that the depression seen with concentrated EA was not due to toxicity caused by concentration of the components in BSM.

Effect of EA from different strains on mitogen responses. The preceding studies suggested that some inhibitory substance or substances present in EA were being increased by 10-fold concentration and depressing the stimulation caused by other substances in the mixture. This was studied further by testing the effect of the concentrates on response to the nonspecific mitogen PHA.

In experiment 1 (Table 3), it was demonstrated that concentrated EA contain substances which almost totally ablate responses to another stimulus such as PHA ($P < 0.01$). The depression of response in the two skin test-negative subjects suggests that this action is nonspecific. Dialysis of the concentrated EA considerably lessened this suppressive activity; i.e., it reduced the suppression of PHA responsiveness from >90 to 65% in the four subjects (Table 3, column 6).

The depression of a nonspecific mitogen response by concentrated EA was confirmed in experiment 2 (Table 3). We also demonstrated

TABLE 2. Lymphocyte transformation results with EA from *C. immitis* 46

Subject	Skin test result	Δcpm		
		Heated EA[a]	Concentrated EA[a]	Heated EA + concentrated BSM
1	−	2,659	228	ND[b]
2	+	13,089	1,310	ND
3	+	28,354	9,015	30,225
4	+	33,094	18,440	37,286
5	+	35,333	19,499	ND

[a] Concentrated EA versus heated EA for skin test-positive subjects, $P < 0.01$.
[b] ND, Not done.

TABLE 3. Effect of EA from different isolates and lots on mitogen stimulation

Expt no.	Subject	Skin test result	Δcpm					
			PHA	PHA + concentrated EA (S1)[a]	PHA + dialyzed and concentrated EA (S1)[a]	PHA + concentrated EA (S2)[a]	PHA + concentrated EA (46)[b]	PHA + concentrated BSM
1	1	+	22,103	92	8,038			
	2	+	17,873	65	5,398			
	3	+	23,065	282	11,712			
	4	−	12,202	51	1,315			
	5	−	11,211	77	ND[c]			
2	1	+	73,800	ND		0	ND	ND
	2	+	58,932	ND		1,150	ND	ND
	3	+	47,911	0		ND	17,521	ND
	4	+	42,865	0		ND	12,012	33,473
	5	+	24,845	ND		ND	ND	27,178
	6	+	47,507	ND		ND	13,969	26,085
	7	−	31,877	1,305		1,499	37,378	33,778
	8	−	61,910	1,261		1,719	48,210	47,542
	9	−	36,432	ND		ND	32,205	38,257

[a] Isolate from *C. immitis* Silveira, lot 1 (S1) or lot 2 (S2).
[b] Isolate from *C. immitis* 46.
[c] ND, Not done.

that concentrated EA prepared from lot 2 of strain Silveira acted similarly to the concentrated EA from lot 1 in depressing response ($P < 0.01$ for both). However, the concentrated EA from strain 46 (Table 3, column 7) was not as suppressive (37% decrease of response in six subjects). This decrease was significantly less than that caused by concentrated EA from Silveira lot 1 or 2 ($P < 0.02$ for either comparison). This is consistent with the lower degree of depression of the response to the stimulatory components in EA from strain 46 caused by concentrating EA from strain 46, as seen earlier (Table 2). Finally, the depression of PHA responses by concentrated EA is not caused by the concentration of suppressive or toxic factors in BSM (the medium used to prepare EA). When cells from six subjects were studied, three had slightly lower PHA responses in the presence of concentrated BSM, and three had slightly higher responses (Table 3, column 9).

Weight analyses. That the depression of response caused by concentrated EA is not due to a change in tonicity of the medium of lymphocyte cultures is indicated by the similar weights of nondialyzable solids in EA and in the medium used to prepare them. These weights were determined to be 1.5, 2.2, 1.4, and 1.8 μg/ml for BSM alone, for EA from strain Silveira lots 1 and 2, and for strain 46, respectively. The small differences, reflecting the balance between the gain of extracellular products from fungal growth and the loss of medium components used for growth, could not explain the depression of response which was seen only with the concentrated EA. A correlation between the depression caused by EA from different strains and the weights of those EA was also lacking.

Survey for sensitivity and specificity of in vitro responses. A project to study the effect of a coccidioidal vaccine in humans (P. L. Williams, S. P. Sorgen, D. L. Sable, D. Pappagianis, H. B. Levine, S. K. Brodine, and D. A. Stevens, Proc. 25th Annu. Meet. Coccidioidomycosis Study Group, abstr., no. 12, 1980) provided the opportunity to compare in vitro transformation responses to spherulin and to heated EA in skin test-positive and -negative donors before vaccination. In this phase, no attempt was made to have subjects act as their own controls. As shown in Fig. 1, if a Δcpm of 10,000 is used as the cutoff, 94% of skin test-positive subjects exceeded that response when EA were used as the stimulant. A single skin test-positive subject gave a Δcpm response of <1,000. In contrast, only 75% of skin test-positive subjects exceeded a Δcpm of 10,000 when spherulin was used as the stimulant. Two subjects gave a Δcpm response of 1,000 to 10,000 with spherulin.

A Δcpm of 10,000 appears to be a useful cutoff to separate skin test-positive from skin test-negative subjects, as is shown by studies with skin test-negative subjects (Fig. 2). Of skin test-negative subjects, 95% produced Δcpm responses of <10,000 with EA. One subject had a Δcpm of >10,000. In contrast, 100% of skin test-negative subjects tested with spherulin had a Δcpm of <10,000. Both EA and spherulin show nonspecific stimulatory activity, as is seen in the finding that 47 and 55%, respectively, of skin

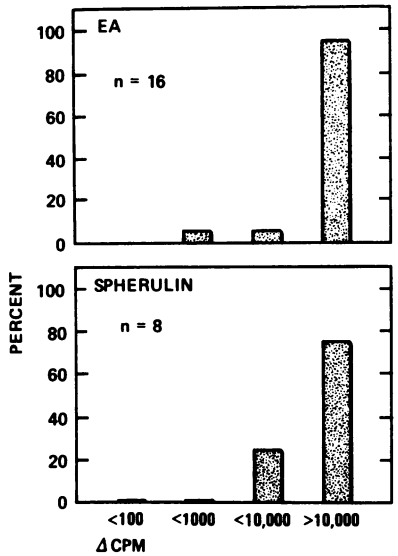

FIG. 1. Comparison of in vitro lymphocyte transformation responses to EA and to spherulin in skin test-positive healthy subjects at several Δcpm levels. Δcpm = mean cpm of incorporated tritiated thymidine in triplicate stimulated cultures minus cpm in unstimulated cultures; n = number of subjects.

test-negative subjects had a Δcpm of >1,000 with these reagents.

DISCUSSION

We have shown in these studies that extracellular antigens produced by *C. immitis* during spherule growth and endospore release are extremely potent in eliciting in vitro responses in lymphocytes from subjects with prior coccidioidal infection. EA were as potent as spherulin was in these studies when both were used at apparently optimal dilutions. By combining data from previous studies (3, 6) in which spherulin prepared in similar fashion was used, the optimal concentration of spherulin appears to be from 20 to 50 μg of nondialyzable solids per ml in the culture mixture. By comparison, in this study, EA were optimal and equivalent to spherulin at concentrations from 0.064 to 0.085 μg of nondialyzable solids per ml of culture mixture. Thus, EA are approximately 500 times as potent as spherulin in stimulation in vitro when compared by weight. Spherulin is more potent than coccidioidin when compared by weight (4, 5).

Both EA and spherulin were shown to be effective in separating skin test-positive and skin test-negative subjects on the basis of in vitro stimulation results. In this survey, spherulin appeared to be slightly less sensitive but slightly more specific.

A small amount of nonspecific stimulation of lymphocytes from skin test-negative subjects was noted with both EA and spherulin. This is in agreement with previous studies of spherulin (3, 4, 6) and has also been previously noted with coccidioidin (4, 6). This result might be caused by a nonspecific mitogen present in the preparations or to cross-reaction by cells sensitized to other fungal antigens. Subjects who, by skin test, show evidence of prior infection with *Histoplasma capsulatum* but not *C. immitis* are one group previously demonstrated to have lymphocyte stimulation in vitro by coccidioidal antigens (3, 7).

Concentration of the EA produced suppression of responses or toxicity to responding cells. Preparations of EA made from a virulent strain of *C. immitis* were markedly more suppressive than was a preparation made from a strain attenuated in virulence when the strains were grown under identical conditions. No such differences were noted concerning the ability of these preparations in undiluted form to stimulate lymphocytes from immune subjects.

Analysis of these studies supports the interpretation that the stimulation seen with unmodified EA reflects a balance of both stimulation and depression and that there are both stimulatory and depressing substances in EA. In initial experiments, a 1:10 dilution stimulated responses in cells from skin test-positive subjects that were indistinguishable from responses to undi-

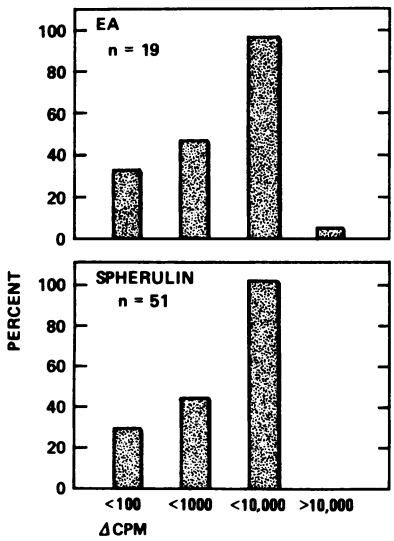

FIG. 2. Comparison of in vitro lymphocyte transformation responses to EA and to spherulin in skin test-negative healthy subjects at several Δcpm levels. Δcpm = mean cpm of incorporated tritiated thymidine in triplicate stimulated cultures minus cpm in unstimulated cultures; n = number of subjects.

luted EA; in all dilution experiments, log dilutions of EA gave relatively flat dose-response curves. In one experiment, a higher stimulation of lymphocytes from skin test-negative subjects was seen with a 1:2 dilution than was seen with undiluted material. Also, concentrated EA depressed the response to a nonspecific mitogen. The studies with dialyzed preparations are of special interest in this regard. Dialysis of the stimulatory undiluted EA enhanced their stimulation, and dialysis of the inhibitory concentrated EA decreased the inhibition. Both of these findings strongly suggest that the suppressive substance or substances are at least partially dialyzable, a property that could be useful in further separation studies. Because heating also enhanced stimulation by EA, at least one inhibitory substance is likely to be heat labile. These studies also suggest that both the stimulatory material and the suppressive substances in EA are stable for at least 3 years in storage at 4°C.

Other coccidioidal preparations that have been reported to be useful in detecting lymphocyte blastogenesis in vitro are lysates of saprophytic-phase organisms, either toluene induced (12) or concentrated by ultrafiltration (8), and an alkali- and water-soluble extract of mycelial walls (3). The latter preparation was as stimulatory to cells from skin test-positive subjects as spherulin was, and, like spherulin, it showed some nonspecific stimulation of lymphocytes of subjects with negative coccidioidal and histoplasmal skin tests (3). The toluene lysate was more stimulatory than spherulin was in one study (4) but less stimulatory in another (3), and nonspecific stimulation was demonstrated in one study (4) but not confirmed in another (3). The toluene lysate when concentrated by ultrafiltration was less stimulatory than spherulin was, but nonspecific stimulation was not seen (3).

In our study, EA were shown to be a potential source of potent coccidioidal antigens that can be produced simply and in large quantities. Thus, they may be a useful tool for further studies of antigen characterization and separation. Such studies could address the problem of in vitro cross-reactivity of histoplasmin-positive, spherulin-negative (or coccidioidin-negative) subjects with current coccidiodial preparations. In addition, EA could be a source of skin test antigens.

These studies also suggested the presence of one or more factors produced by *C. immitis* that depress lymphocyte response, are toxic for immune cells, or both. Further efforts to separate the active components are needed, and separation is now being attempted with chromatographic methods.

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LITERATURE CITED

1. **Chaparas, S. D., H. B. Levine, G. M. Scalarone, and D. Pappagianis.** 1975. Cellular responses, protective immunity, and virulence in experimental coccidioidomycosis. Pan Am. Health Organ. Sci. Publ. **304**:80–87.
2. **Converse, J. L.** 1955. Growth of spherules of Coccidioides immitis in a chemically defined liquid medium. Proc. Soc. Exp. Biol. Med. **90**:709–711.
3. **Cox, R. A.** 1979. Cross-reactivity between antigens of *Coccidioides immitis*, *Histoplasma capsulatum*, and *Blastomyces dermatitidis* in lymphocyte transformation assays. Infect. Immun. **25**:932–938.
4. **Cox, R. A., E. Brummer, and G. Lecara.** 1977. In vitro lymphocyte responses of coccidioidin skin test-positive and -negative persons to coccidioidin, spherulin, and a *Coccidioides* cell wall antigen. Infect. Immun. **15**:751–755.
5. **Deresinski, S. C., R. J. Applegate, H. B. Levine, and D. A. Stevens.** 1977. Cellular immunity to Coccidioides immitis: in vitro lymphocyte response to spherules, arthrospores and endospores. Cell. Immunol. **32**:110–119.
6. **Deresinski, S. C., H. B. Levine, and D. A. Stevens.** 1974. Soluble antigens of mycelia and spherules in the in vitro detection of immunity to *Coccidioides immitis*. Infect. Immun. **10**:700–704.
7. **Graybill, J. R., and R. H. Alford.** 1976. Variability of sequential studies of lymphocyte blastogenesis in normal adults. Clin. Exp. Immunol. **25**:28–35.
8. **Huppert, M., N. S. Spratt, K. R. Vukovich, S. H. Sun, and E. H. Rice.** 1978. Antigenic analysis of coccidioidin and spherulin determined by two-dimensional immunoelectrophoresis. Infect. Immun. **20**:541–551.
9. **Levine, H. B., J. M. Cobb, and G. M. Scalarone.** 1969. Spherule coccidioidin in delayed dermal sensitivity reactions of experimental animals. Sabouraudia **7**:20–32.
10. **Levine, H. B., J. Cobb, and C. E. Smith.** 1960. Immunity to coccidioidomycosis induced in mice. Trans. N.Y. Acad. Sci. **22**:436–449.
11. **Nikoskelainen, J., D. V. Ablashi, R. A. Isenberg, E. U. Neel, R. G. Miller, and D. A. Stevens.** 1978. Cellular immunity in infectious mononucleosis. II. Specific reactivity to Epstein-Barr virus antigens and correlation with clinical and hematologic parameters. J. Immunol. **121**:1239–1244.
12. **Pappagianis, D., C. E. Smith, G. S. Kobayashi, and M. T. Saito.** 1961. Studies of antigens from young mycelia of Coccidioides immitis. J. Infect. Dis. **108**:35–44.
13. **Robbins, J. H.** 1964. Tissue culture studies of the human lymphocyte. Science **146**:1648–1654.
14. **Smith, C. E., E. G. Whiting, E. E. Baker, H. G. Rosenberger, R. R. Beard, and M. T. Saito.** 1948. The use of coccidioidin. Am. Rev. Tuberc. **57**:330–360.
15. **Stevens, D. A., H. B. Levine, S. C. Deresinski, D. R. Ten Eyck, and A. Restrepo.** 1977. Epidemiological and clinical skin testing studies with spherulin, p. 107–114. *In* L. Ajello (ed.), Coccidioidomycosis: current clinical and diagnostic status. Symposia Specialists, Miami.
16. **Zweiman, B., D. Pappagianis, H. Maibach, and E. A. Hildreth.** 1969. Coccidioidin delayed hypersensitivity: skin test and in vitro lymphocyte reactivities. J. Immunol. **102**:1284–1289.