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Experimental Induction of Anergy to Coccidioidin by Antigens of *Coccidioides immitis*

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Failure to react to coccidioidin (anergy) often occurs in patients with disseminated coccidioidomycosis. One possible reason may be desensitization by excessive amounts of antigen. This was studied experimentally by injection of soluble and hyphal antigens of *Coccidioides immitis* into coccidioidin- and tuberculin-sensitive guinea pigs. Guinea pigs sensitized by injection of killed hyphal cells of *C. immitis* in complete Freund adjuvant were subsequently injected daily either with soluble coccidioidal antigen administered intraperitoneally or with hyphal antigen administered either subcutaneously or intraperitoneally. Gradual loss of cutaneous reactivity to coccidioidin occurred, but the reactivity to tuberculin remained unimpaired. The rapidity of desensitization was roughly proportional to the dose of antigen with desensitization occurring as early as 6 days after beginning injections. This anergic state was temporary, and reactivity returned several days after discontinuing injection of antigen. Injection of coccidioidal antigen led to production of coccidioidal complement-fixing antibody, but there was no consistent relationship between the antibody titer and state of cutaneous reactivity to coccidioidin. Peritoneal exudate or pulmonary alveolar cells from desensitized animals migrated freely in the presence of coccidioidin but were inhibited in the presence of tuberculin. Heat treatment did not impair the capacity of the soluble or hyphal antigen to induce anergy, thus suggesting that the antigen active in complement fixation was perhaps not involved in desensitization. Polysaccharide obtained by ethanol precipitation of dialyzed coccidioidin failed to induce anergy. Dialysis of the soluble coccidioidal antigen caused the loss of the desensitizing activity. Thus, specific desensitization could be induced by administration of large doses of coccidioidal antigen but dialyzable components appear important in this desensitization.

Delayed hypersensitivity (DH) is an allergic response often detectable in coccidioidomycosis. It is elicited in human beings or experimental animals by the intradermal (i.d.) injection of coccidioidin. Patients with disseminated coccidioidomycosis often show a loss of cutaneous reactivity to coccidioidin, and this anergy represents an unfavorable prognosis (26). Patients with disseminated coccidioidomycosis may also exhibit high complement fixation (CF) titers (25, 26). Since the CF titer is often low in cases of primary coccidioidomycosis, appearance of high CF titer is usually regarded as a sign of dissemination and hence poor prognosis (25). Recovery from disseminated disease often results in the gradual fall of CF titer and the reestablishment of coccidioidin sensitivity (26).

According to Smith et al. (26), the cause of the poor sensitivity during disseminated coccidioidomycosis could be either desensitization by excessive amounts of circulating antigen or inability of the body to react to any cutaneous stimulation. The present study was undertaken to explore the role of excess coccidioidal antigen in the induction of anergy to coccidioidin. Guinea pigs doubly sensitized to coccidioidin and tuberculin were used to study the specificity of the induced anergy. Capillary macrophage migration inhibition tests (2) were carried out to correlate the state of reactivity to coccidioidin in the intact guinea pig with the in vitro system.

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MATERIALS AND METHODS

Antigens. Soluble-coccidioidal antigen lot F1-68 (coccidioidin) was prepared by growing *Coccidioides immitis* strain Silveira in glucose yeast extract (GYE) medium (2% glucose, 1% yeast extract; Difco) at 34 C for 3 days. The mycelium was then removed by filtration of the culture through Whatman no. 1 filter paper. The filtrate was sterilized by passing through a Seitz filter, preserved with aqueous Merthiolate (final concentration 1:10,000), and stored at 4 C (19). The undialyzed and dialyzed coccidioidin lot F1-68 (dialyzed against several changes of distilled water) contained 13 mg and 10 mg (dry weight), respectively, of solids per milliliter.

Hyphal antigen lot 3D-34 was prepared by growing *C. immitis* strain Silveira in GYE for 3 days. The mycelial mat was collected on Whatman no. 1 paper in a Buchner funnel and washed three times with distilled water. It was killed by suspension in 0.5% Formalin in saline (0.185% formaldehyde, vol/vol) to give a final concentration of 30 mg (dry weight) of solids per ml.

Tuberculin was obtained from the United States Department of Agriculture, Agriculture Research Service, Hyattsville, Md. It was a filtrate from three strains of *Mycobacterium tuberculosis* var. *hominis*, which contained 150,000 ± 3,000 tuberculin units per 10 ml (1 tuberculin unit = 0.00002 mg trichloracetic acid-precipitable protein). Tuberculin protein derivative (PPD) was purchased from Parke Davis and Co., Detroit, Mich.

Antisera. CF-positive and precipitin-positive human sera were obtained from patients infected with *C. immitis*.

Sensitization of guinea pigs. Adult Hartley strain guinea pigs (Simonsen Laboratories, Gilroy, California), weighing 600 to 700 g, were sensitized to coccidioidin and tuberculin by injecting into each hind foot pad 3.0 mg of hyphal antigen 3D-34 in complete Freund adjuvant containing killed *M. tuberculosis* $H_{37}Ra$ (Difco Laboratories, Detroit, Michigan). Three guinea pigs were used for each dose of antigen and for each control group. Sensitization was achieved in 10 days.

Skin tests. DH was elicited by i.d. injection of 0.1 ml of coccidioidin or tuberculin into shaved areas of the backs of guinea pigs with a tuberculin syringe fitted with a 26-gauge needle. The skin test dose represented 130 μg (total dry weight) of coccidioidin or tuberculin. Control tests were carried out on the opposite side by using 0.1 ml Merthiolated saline or GYE. The skin tests were read at 6 to 8 h for immediate reactivity and at 24 and 48 h for the DH response. The cutaneous reactivity was quantitated by measuring the diameter of erythema and induration, and by the thickness of a skin fold at the test site minus the normal skin fold thickness (approximately 4.0 mm).

In vitro macrophage migration inhibition tests. The capillary macrophage migration inhibition technique (2) was applied to peritoneal exudate or pulmonary alveolar cells. Pulmonary alveolar cells were harvested by the pulmonary lavage technique of Myrvik et al. (17). Medium 199 with Earle salt base (Grand Island Biological Co.) containing normal guinea pig serum (15%), 40 U of penicillin, 50 μg of streptomycin, and 50 μg of neomycin per ml was used (6). Coccidioidin or tuberculin (130 μg per ml) was added to each chamber to study the specific inhibition of macrophage migration. In some experiments PPD (50 μg per ml) was used.

Induction of anergy to coccidioidin with excess coccidioidal antigens. After recording the initial cutaneous reactivities of the sensitized guinea pigs to coccidioidin and tuberculin, one group of animals was injected daily intraperitoneally (i.p.) with either 130 mg or 65 mg, respectively, of soluble antigen Lot F1-68 until the animals showed no cutaneous reactivity to coccidioidin. Sensitized control animals received 130 mg or 65 mg of GYE daily i.p. as long as the test animals received F1-68. The second group of animals was injected daily i.p. or subcutaneously (s.c.) with 7.5 mg, 15 mg, or 130 mg, respectively, of hyphal antigen lot 3D-34 until the animals showed no cutaneous reactivity to coccidioidin. Sensitized control animals in this case received no injected material. Attempts to prolong anergy already established by injection of killed hyphae were carried out by a daily i.d. injection of 130 μg of coccidioidin F1-68. Serum samples obtained from each group of animals prior to sensitization and on days 9, 18, 24, and 30 were tested for CF titer. The body weights of the animals were recorded every third day.

Serological and other methods. Complement fixation tests were carried out by a modification of the Kolmer quantitative method (25). Precipitin tests were performed by the method of Smith et al. (25) in 7- by 70-mm test tubes. Immunodiffusion tests were carried out by the method of Huppert and Bailey (10). Protein was measured by the method of Lowry et al. (15) with crystalline bovine serum albumin as standard. Carbohydrate was estimated by Fairbairn's (3) modification of the anthrone method using mannose as standard (20). Polysaccharide was precipitated from dialyzed coccidioidin by the addition of 5 volumes of 95% ethanol as described by Pappagianis et al. (20). After drying, the precipitate was dissolved in 1:10,000 Merthiolated saline to give a final concentration of 1.3 mg per ml. This contained 90 μg of "protein" per ml by Lowry's method (15) and 9.6 mg of carbohydrate per ml as mannose by the anthrone method (3).

RESULTS

Induction of anergy with soluble antigen of C. immitis. Figure 1 illustrates the results of desensitization with untreated soluble coccidioidal antigen lot F1-68. Injections of 130 mg of undialyzed F1-68 daily i.p. resulted in the induction of anergy to coccidioidin on day 9, at which time the animals still possessed their initial reactivities to tuberculin. Injection of one-half this dose, i.e., 65 mg of F1-68 per day,

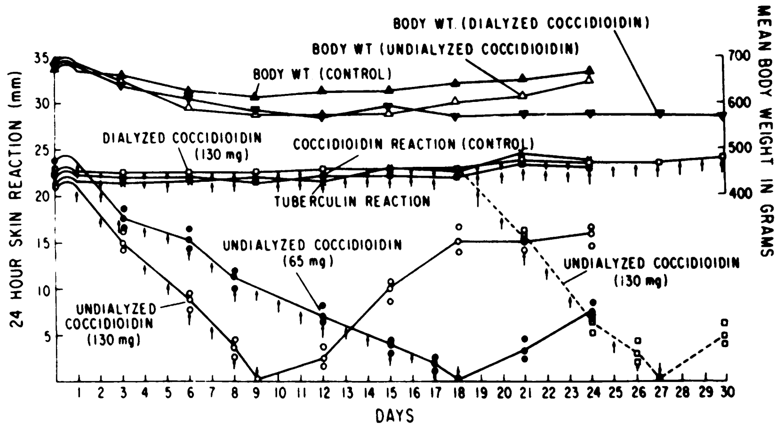

FIG. 1. *Induction of anergy by repeated i.p. injection of coccidioidin. Arrow indicates day of injection. Recordings were only made for animals that received 130 mg of undialyzed coccidioidin daily. Lines represent mean readings of skin tests and body weight in three guinea pigs.*

took twice as long, i.e. 18 days, for the induction of anergy. Anergy so induced was transient in that reactivity to coccidioidin returned within about 3 days after discontinuation of the daily injection of F1-68.

Capillary macrophage migration inhibition tests with pulmonary alveolar cells from normal, sensitized, and anergic animals showed that the cells from the latter animals migrated as freely as the cells from normal animals in the presence of coccidioidin, whereas the cells from sensitized animals were inhibited in the usual manner (Fig. 2). However, in the presence of tuberculin or PPD, cells from the anergic animals were inhibited as well as the cells from the sensitized animals (Fig. 3). Cells from the normal animals were not affected by these concentrations of tuberculin or PPD.

The soluble antigen F1-68 appeared to be somewhat toxic to the animals, in that the animals that received F1-68 lost more weight (approximately 15% on the average) than the control ones that received only GYE (approximately 7% on the average). Both groups of animals slowly recovered the lost weight upon discontinuation of daily injection of F1-68 or GYE (Fig. 1).

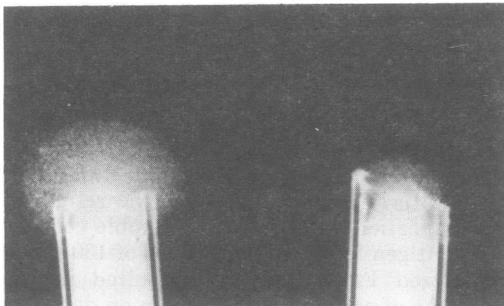

FIG. 2. *Pulmonary alveolar cells in the presence of coccidioidin. Left: Cells from an anergic guinea pig. Right: Cells from a sensitized guinea pig.*

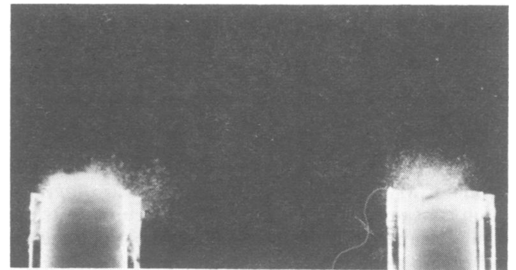

FIG. 3. *Pulmonary alveolar cells in the presence of OT. Left: Cells from an anergic guinea pig. Right: Cells from a sensitized guinea pig.*

Complement fixation titers at the time of anergy varied from animal to animal. Some animals showed high titers, such as 1:32 to 1:512, whereas others showed a titer of 1:16 or less. Some sensitized control animals receiving GYE exhibited high CF titers, such as 1:32 to 1:256, at the time when they reacted strongly to coccidioidin. Such CF titers developed also in sensitized animals that did not receive GYE or F1-68. The CF titers of the anergic animals did not change significantly at the time they re-established sensitivity to coccidioidin. Similarly, the CF titers of the sensitized control animals remained unchanged after discontinuation of daily injection of GYE (Table 1). Immunodiffusion tests with guinea pig serum were positive and appeared to represent the line of reaction associated with CF type antibody. However, tube precipitin tests were negative.

TABLE 1. *Serum CF titers of coccidioidin-sensitive and desensitized guinea pigs before and after i.p. injections of coccidioidin*[a]

Guinea pigs treatment	Guinea pig no.	CF titer				
		Before antigen treatment	Days after beginning treatment			
			9	18	24	30
Undialyzed coccidioidin[b] (65 mg) daily for 18 days	1	1:4	1:256	1:256	1:128	NT[c]
	2	1:4	1:512	1:512	1:128	NT
	3	1:8	1:16	1:16	1:32	NT
Undialyzed coccidioidin (130 mg) daily for 9 days	4	1:2	1:8	1:8	1:4	NT
	5	1:4	1:32	1:64	1:64	NT
	6	1:2	1:128	1:128	1:128	NT
Dialyzed coccidioidin (130 mg) daily for 30 days	7	1:2	1:128	1:128	1:64	1:32
	8	1:2	1:32	1:64	1:64	1:64
	9	1:4	1:16	1:32	1:16	1:8
Undialyzed GYE[d] (130 mg) daily for 24 days	10	1:4	1:32	1:64	1:64	NT
	11	1:2	1:8	1:8	1:16	NT
	12	1:8	1:128	1:256	1:128	NT

[a] Following initial skin test.
[b] Coccidioidin lot F1-68.
[c] NT = not tested.
[d] Glucose yeast extract broth.

Effect of dialysis and heat. From the results shown in Fig. 1 it can be seen that daily i.p. injection of 130 mg of dialyzed F1-68 for 30 days did not depress skin reactivity to coccidioidin, whereas daily administration of 130 mg of undialyzed F1-68 induced anergy in 9 days. Guinea pigs that had received 130 mg of dialyzed F1-68 daily for 18 days were still reactive in the coccidioidin test. Subsequent treatment with daily injection of 130 mg of undialyzed F1-68 for 9 days resulted in anergy. The anergy so induced was temporary and was lost in about 3 days after discontinuation of injection of the undialyzed F1-68.

Since the dialyzed F1-68 failed to induce anergy while the undialyzed material was active, it was felt that the low-molecular-weight substances which left the dialysis tubing might be important in the induction of anergy. In one experiment the dialysate of F1-68 was collected, concentrated in a flash evaporator at 34 C, and 1:10,000 Merthiolate in saline was added to give a final concentration of 13 mg (dry weight) of dialyzable solids per ml. One hundred thirty milligrams of this material was then injected i.p. daily into coccidioidin- and tuberculin-sensitive guinea pigs. This material induced anergy on day 9. Anergy to coccidioidin was also induced by reconstituting the dialyzed F1-68 and the dialysate of F1-68 in equal volumes and injecting 130 mg of this mixture daily for 9 days into coccidioidin- and tuberculin-sensitive guinea pigs. It should be pointed out, however, that thorough dialysis is necessary to remove the components active in induction of anergy. The dialyzed F1-68 and its dialysate were both as effective as undialyzed F1-68 in eliciting DH reaction in coccidioidin-sensitized guinea pigs.

Anergy induced by the dialysate of F1-68 and the reconstituted F1-68 was temporary, fading within about 3 days after the discontinuation of daily injection of these reagents.

Since the coccidioidal CF antigen appears to be heat labile, it was of interest to study the effect of heat on the induction of anergy. Heating of undialyzed F1-68 at 60 C for 1 h or autoclaving for 25 min at 121 C (15 lb of pressure) did not destroy its capacity to induce anergy. Other observations, such as in vitro DH tests and body weights made on these animals remained similar to the ones reported earlier for untreated F1-68.

Action of alcohol-precipitated polysaccharide. Polysaccharide obtained by the addition of five volumes of 95% ethanol to dialyzed F1-68 was active as a skin test reagent for detecting DH when used at a dose of 130 μg per 0.1 ml. However, it did not desensitize coccidioidin-sensitive guinea pigs. These efforts to desensitize with polysaccharide are summarized in Fig. 4.

Induction of anergy by i.p. or s.c. injection of hyphal antigen of C. immitis. As indicated in Fig. 5, daily i.p. injection of 7.5 mg of hyphal antigen lot 3D-34 into guinea pigs sensitized to coccidioidin and tuberculin resulted in induction of anergy to coccidioidin on day 18. The reactivity to tuberculin, however, remained un-

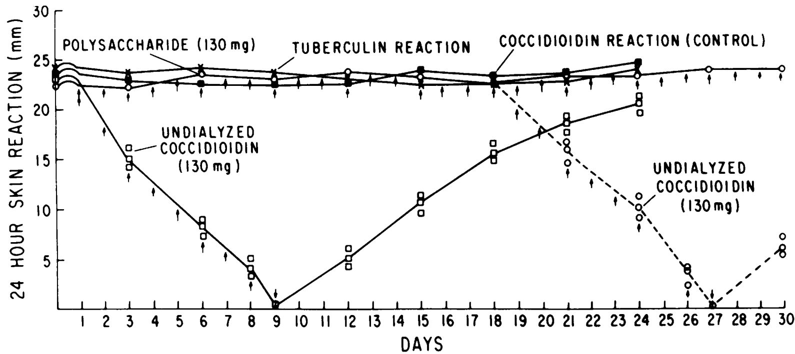

FIG. 4. *Induction of anergy by repeated i.p. injection of polysaccharide of C. immitis. Ar'ow indicates day of infection; lines represent mean readings of skin tests in three guinea pigs.*

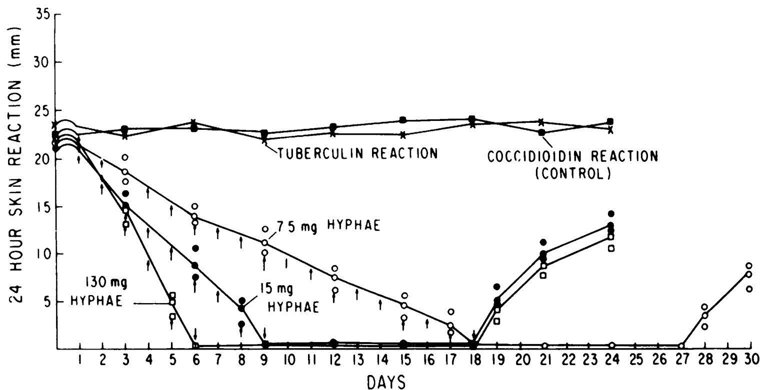

FIG. 5. *Induction of anergy by repeated i.p. injection of killed hypahe of C. immitis. Arrow indicates day of injection; lines represent mean readings of skin tests in three guinea pigs.*

changed. Increasing the dose of 3D-34 from 7.5 to 15 mg per day decreased the time of induction of anergy by one half, i.e., 9 days instead of 18. Increasing the dose further to 130 mg per day further shortened the time to only 6 days for the induction of anergy. Anergy induced by all three doses (7.5, 15, and 130 mg) of 3D-34 was found to be temporary, even though it was more lasting than that induced by soluble antigen (Fig. 1, 4). Thus, after discontinuation of daily injections of the 7.5- or 15-mg doses of 3D-34 DH allergy to coccidioidin returned in 9 days. Anergy induced by 130 mg of 3D-34 lasted for 12 days, after which sensitivity to coccidioidin slowly returned. Thus, whole cells provided more prolonged desensitization when compared with comparable weights of soluble antigen. Anergy induced by i.p. or s.c. injection of 130 mg of hyphal antigen daily for 6 days could be sustained for at least 6 weeks by daily i.d. injection of 130 μg of F1-68.

Capillary macrophage migration inhibition tests performed with pulmonary alveolar cells from animals rendered anergic by hyphal antigen showed migration of these cells in the presence of coccidioidin and inhibition in the presence of tuberculin. Similar cells from sensitized control animals were inhibited by both coccidioidin and tuberculin.

Animals that received hyphal antigen lost more weight (approximately 30% on the average) than those that received soluble antigen. These animals also took longer (greater than one week) to regain their lost weight after the discontinuation of antigen administration.

Complement fixation titers varied among the anergic animals, some exhibiting high CF titers, i.e., 1:32 to 1:256, others showing lower titers. Some of the sensitized control animals exhibited high CF titers, i.e., 1:32 to 1:256, at a time when they showed strong sensitivity to coccidioidin (Table 2). Immunodiffusion tests were positive, whereas tube precipitin tests were negative.

Anergy to coccidioidin was also induced by the s.c. injection of all three doses (7.5, 15, and 130 mg) of hyphal antigen 3D-34. The time of induction of anergy was the same (namely 18, 9,

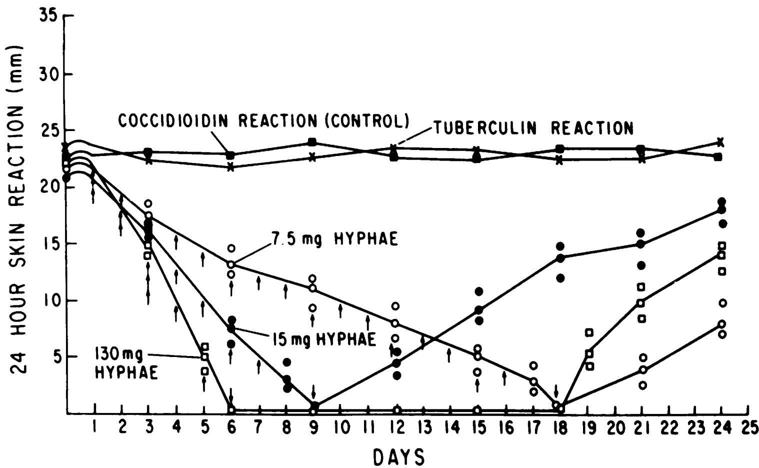

FIG. 6. *Induction of anergy by repeated s.c. injection of killed hyphae of C. immitis. Arrow indicates day of injection; lines represent mean readings of skin tests in three guinea pigs.*

TABLE 2. *Serum CF titers of coccidioidin-sensitized and desensitized guinea pigs before and after i.p. injections of killed hyphae*[a]

Guinea pigs treatment	Guinea pig no.	CF titer				
		Before antigen treatment	Days after beginning treatment			
			9	18	24	30
7.5 mg daily for 18 days	13	1:4	1:64	1:64	1:128	1:64
	14	1:4	1:32	1:32	1:64	1:64
	15	1:2	1:8	1:16	1:8	1:8
15 mg daily for 9 days	16	1:2	1:16	1:16	1:8	NT[b]
	17	1:4	1:256	1:256	1:128	NT
	18	1:2	1:64	1:64	1:32	NT
130 mg daily for 6 days	19	1:4	1:128	1:128	1:128	NT
	20	1:8	1:256	1:128	1:128	NT
	21	1:2	1:32	1:32	1:16	NT
Sensitized controls (not injected)	22	1:4	1:32	1:64	1:32	NT
	23	1:4	1:256	1:256	1:128	NT
	24	1:4	1:16	1:32	1:32	NT

[a] Following initial skin test.
[b] NT = not tested.

and 6 days, respectively) as with i.p. injection. However, the duration of anergy when the two smaller doses were employed, i.e., 7.5 mg and 15 mg, was shorter, i.e., about 3 days, when compared with that achieved by injecting the same dose i.p., i.e., 9 days. The duration of anergy upon daily s.c. injection of 130 mg of 3D-34 was 12 days, the same as that noted after i.p. injection of the same dose (Fig. 6).

The body weight of animals injected s.c. declined less than that of animals that received antigens i.p. and returned to normal more rapidly after discontinuation of antigen injection.

Other observations, such as in vitro DH tests and serological tests made on these animals, were similar to the ones reported for the animals tested i.p.

Heating of hyphal antigen lot 3D-34 at 60 C for 1 h or by autoclaving at 121 C (15 lb of pressure) for 25 min did not impair the capacity to induce anergy.

DISCUSSION

The injection of coccidioidal antigen in large amounts led to desensitization only to coccidioidin in guinea pigs doubly sensitive to coccidioidin and tuberculin. This reflects the specificity of anergy. These observations are in agreement with those of Uhr and Pappenheimer (30), who found that guinea pigs sensitized to diphtheria toxoid and ovalbumin selectively lost their reactivity to the corresponding antigen used for desensitization. Similar specific desensitization was demonstrated by Oliveira-Lima

(18) in humans previously sensitized to both tuberculin and streptococcal antigens. Thus, repeated intramuscular injection of large doses of tuberculin (together with killed tubercle bacilli) led to loss of reactivity to tuberculin but not to streptococcal antigens. Similar observations indicating the apparent specificity of desensitization have been made by Kligman (12) and Feingold and Benjamini (4).

The specificity of anergy induced by coccidioidal antigens supports the suggestion put forth by Smith et al. (26) that the anergy induced in disseminated coccidioidomycosis is due to desensitization by excessive amounts of specific antigen. This does not exclude the alternative suggested by the same authors that anergy may in some patients be caused by the inability to respond to any cutaneous antigenic stimulus with DH.

This specific nature of anergy was further correlated in vitro by the capillary macrophage migration inhibition test which has been shown by earlier workers to represent an in vitro correlate of CH (1, 2, 29). It was found that the pulmonary alveolar or peritoneal exudate cells of anergic animals were not inhibited in the presence of coccidioidin but were inhibited in the presence of tuberculin or PPD. Similar cells from sensitized animals were inhibited in the presence of both coccidioidin and tuberculin. These observations are in agreement with those of other workers (14, 29).

The anergy induced to coccidioidin was transient. This observation conforms to the findings of several other investigators who used various antigens. Thus, Rothschild et al. (22) found that continued injection of large amounts of tuberculin was necessary to maintain a state of anergy to tuberculin in guinea pigs. A temporary state of unresponsiveness was also induced by other workers (4, 7, 12, 17, 24, 30).

The temporary nature of anergy to coccidioidin was paralleled by the in vitro results of macrophage migration inhibition tests. Thus, when injections of antigen were discontinued and sensitivity to coccidioidin returned, peritoneal exudate or pulmonary alveolar cells from such animals also became susceptible to in vitro inhibition by coccidioidin. However, maintenance of the anergy by daily i.d. injections of 130 μg of coccidioidin (F1-68) also maintained unreactivity of peritoneal cells to coccidioidin in the capillary migration test.

This return of reactivity in guinea pigs (and their cells) after discontinuation of exposure to antigen may be comparable to the return of coccidioidin sensitivity in humans recovering from disseminated coccidioidomycosis (26). In such patients there may be reduction of fungal antigens and hence re-establishment of coccidioidin sensitivity.

The demonstration that dialysis of coccidioidin removed components important in desensitization suggested that small, perhaps nonimmunogenic molecular species were of importance in connection with the phenomenon of DH. Restoration of dialyzed to undialyzable components also restored the capacity to desensitize. Similar observations have been made by Feingold and Benjamini (4) and Feingold et al. (5) who were successful in hyposensitizing flea bite-sensitive guinea pigs against DH by the injection of large doses of hapten derived from collected oral secretion of the flea. Their attempts to hyposensitize flea bite-sensitive guinea pigs by injecting nondialyzable portions of extracts of whole fleas, however, failed, indicating the role of low-molecular-weight substances in hyposensitization. It is of some interest that our nondialyzable material, although not capable of desensitizing in the doses tested, nevertheless was active in eliciting DH when injected i.d. Whether this is a quantitative or qualitative difference between nondialyzable and dialyzable components (the latter may far exceed the molar concentration of the former) is not certain from these studies. However, Stewart and Kimura (27) noted that the nondialyzable portion of their coccidioidin was not capable of eliciting the DH response in sensitized subjects.

Heating or autoclaving of undialyzed coccidioidin had no effect on the induction of anergy. Heat treatment also did not affect the capacity of the soluble antigen to elicit DH reactions in sensitized guinea pigs (findings similar to those of Smith et al. [26]). Thus, it appears that the heat labile antigen important in complement fixation is not involved in desensitization.

The polysaccharide obtained from dialyzed coccidioidin did not induce anergy in coccidioidin- and tuberculin-sensitive guinea pigs despite its capacity to elicit DH. This may be similar to the observation that nondialyzable portions of coccidioidin were also inactive for induction of anergy. Pappagianis et al. (20) estimated the mean molecular weight of the ethanol-precipitated polysaccharide prepared from coccidioidin as approximately 31,700. This may provide an estimate of the size of the fraction that is inactive in desensitization. However, it does not clarify the apparent paradox that the polysaccharide as well as dialyzed coccidioidin can elicit DH but do not induce anergy at the concentration tested.

The induction of anergy by whole, killed

hyphal cells appeared comparable to the effect of soluble coccidioidin. This was evident both with local deposition by s.c. injection and with the antigen given by the i.p. route which is presumably more widely distributed. The latter may be analogous to the case report on cutaneous anergy without systemic disease (16).

The time of induction of anergy by both routes of injection was the same (i.e., 6, 9, and 12 days, respectively) depending on the quantity of hyphal cells used. However, the intraperitoneal administration provided longer sustained desensitization with the smaller doses. The reason may be that the locally deposited antigen may be either quickly eliminated from the system or is more effectively blocked from entering the circulation, e.g., by an inflammatory cell response, whereas the intraperitoneally introduced antigens may remain free long enough to interact with cells important in DH. However, when a large dose such as 130 mg of hyphal antigen per day was used, the route of injection made no difference as to the duration of anergy. Perhaps the local inflammatory response was insufficient to nullify the immunologic effect of large doses of hyphal antigen.

Heating or autoclaving the hyphal antigen did not alter its capacity to induce anergy by either route of injection. As in the case of soluble antigen, this indicated that the antigen active in complement fixation was, perhaps, not involved in desensitization.

The loss of weight of the guinea pigs that received either soluble or hyphal antigens of *C. immitis* was greater than the loss in weight of the sensitized control animals. This suggested a toxic effect of coccidioidal components. There are previous indications of a toxic nature of coccidioidal antigens (8, 19). In a separate experiment not included in these data 2 of 10 animals that received hyphal antigen i.p. died while the experiments were in progress, possibly as a result of a toxic effect.

The CF titers varied from animal to animal in our guinea pig model. Some anergic animals exhibited high CF titers, whereas others did not. Similarly, some sensitized control animals showed high CF titers at the time they reacted strongly to coccidioidin. Moreover, some anergic animals continued to show high CF titers even after regaining reactivity to coccidioidin. These observations suggest, at least indirectly, that the CF antibody appears to play no role in either the induction or maintenance of anergy to coccidioidin in the uninfected sensitized host. It is possible, however, that CF antibody and excess antigen may act synergistically to induce anergy, the CF antibody acting perhaps by immunological enhancement (11).

The induction of anergy to coccidioidin by administration of large amounts of antigen may result from either the elimination (by death) or the paralysis (due to antigen overloading) of the specific skin reactive cells, e.g., small lymphocytes (9) present in the circulation. Stulbarg and Schlossman (28) observed that increasing the concentration of their antigen α-DNP-$(Lys)_{11\text{-}15}$ to a certain concentration resulted in a rise in stimulation index (the ratio of antigen induced thymidine-2-^{14}C incorporation to thymidine-2-^{14}C incorporation in antigen-free cultures). However, the stimulation index fell upon further increase of the concentration of that antigen. A lack of skin reactive cells has also been implicated in the poor DH reactivity in sarcoidosis (29). Schlossman et al. (23) showed that the specifically sensitive lymphocytes may be sequestered in lymph nodes and thus be unavailable for reaction at the cutaneous site of testing for DH. Thus, in the presence of excess coccidioidal antigen, the skin reactive cells could either remain paralyzed or would be constantly eliminated from the circulation, and hence would not be able to take part in DH reactions. Since the elimination or paralysis of the cells is specific, other skin reactive cells specific for tuberculin would be free to participate in DH reaction to tuberculin.

It should be emphasized that the present studies were carried out in guinea pigs sensitized by killed mycelial cells and the model may not fit the picture of anergy observed in the infected host in which the endospore-spherule form of *C. immitis* prevails. Additional studies on anergy in coccidioidomycosis should be carried out on infected hosts utilizing both conventional mycelial coccidioidin and antigens derived from spherules which Levine et al. (13) have clearly shown differ from the mycelial coccidioidin.

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