

Control of Tissue Reactions in Monkeys Vaccinated with Viable *Coccidioides immitis* by Prevaccination with Killed *Coccidioides immitis*

J. L. CONVERSE, G. A. DEAUVILLE, E. M. SNYDER, J. G. RAY, AND M. E. SEAQUIST

U.S. Army Biological Laboratories, Fort Detrick, Frederick, Maryland

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ABSTRACT

CONVERSE, J. L. (U.S. Army Biological Laboratories, Fort Detrick, Frederick, Md.), G. A. DEAUVILLE, E. M. SNYDER, J. G. RAY, AND M. E. SEAQUIST. Control of tissue reactions in monkeys vaccinated with viable *Coccidioides immitis* by prevaccination with killed *Coccidioides immitis*. *J. Bacteriol.* **90**:783-788. 1965.—Control of undesirable tissue reactions resulting from the subcutaneous injection of 150 viable arthrospores of *Coccidioides immitis* (strain D-76) was obtained by four injections of formalin-killed arthrospores 14, 12, 8, and 4 weeks (total dose, 36 mg) before injection of the viable arthrospores. Only 6 and 12% of these vaccinated animals exhibited ulceration and lymphadenopathy, respectively, as compared with 100 and 83% of the animals receiving only the viable vaccine. Agar-gel immunodiffusion precipitin titers of approximately 1:64 were evident 3 months after vaccination in animals receiving both vaccines, as compared with 1:128 in those injected with the viable vaccine alone. The above data indicated that somatic reactions to injection of a viable vaccine could be eliminated by preinjection of a killed vaccine. However, 6 months after vaccination, respiratory challenge (7,500 strain Cash arthrospores) indicated that this treatment also impaired the protective effect of the viable vaccine. All animals receiving both vaccines developed mild pulmonary coccidioidomycosis, whereas only 50% of the animals receiving only the viable vaccine were infected. In addition, the group receiving both vaccines demonstrated a more rapid and higher postchallenge precipitin titer. All vaccinated animals (those receiving the killed, the viable, or a combination of the two vaccines) survived for 4 months after challenge, as compared with 88% mortality (50% within 14 days) in the nonvaccinated controls.

As a result of evidence that second respiratory infections with *Coccidioides immitis* rarely, if ever, occur in nature (Smith, 1957), and that dissemination beyond the regional lymph nodes is extremely rare in naturally or laboratory-acquired primary cutaneous coccidioidomycosis (Wilson, Smith, and Plunkett, 1953; Guy and Jacobs, 1926; Trimble and Doucette, 1956; Wright, Newcomer, and Nelson, 1959; Johnson et al., 1964; Meis, 1961; Winn, 1961, 1964; Harrell and Honeycutt, 1963; Goodman and Schabarum, 1963), studies on a viable vaccine against coccidioidomycosis were initiated. Previous studies of such a vaccine by Pappagianis et al. (1959) and Converse et al. (1962a) in mice, and by Pappagianis et al. (1960) in cynomolgus monkeys, had indicated the feasibility of this method of protection against the disease.

It was reported (Converse, Castleberry, and

Snyder, 1961) that: (i) protection against pulmonary challenge with *C. immitis* could be obtained in rhesus monkeys by subcutaneous injection of 10 viable arthrospores; (ii) that this protection was not strain-specific; and (iii) that no dissemination beyond the regional lymph nodes occurred with a vaccine dose of this size. It was noted, however, that in some instances (particularly in the higher vaccine doses) untoward tissue reactions (ulcerated vaccination site and axillary lymphadenopathy) resulted from the injection of the viable vaccine.

We indicated (Converse et al., 1962b) that various strains of *C. immitis*, used as viable vaccines, exhibited substantial differences in tissue reactions to inoculation, and (Castleberry, Converse, and Soto, 1964) that these tissue reactions could be controlled in dogs by oral amphotericin B therapy at the time of vaccination.

The object of the present study was to control these tissue reactions to the viable vaccine by pre-injection of a killed vaccine.

MATERIALS AND METHODS

Strains of C. immitis. Strains Cash and D-76 were grown in the liquid medium of Roessler et al. (1946) for 14 days at 34 C, on a reciprocating shaker with a 4.5-inch (11.4-cm) stroke, and operating at approximately 100 excursions per minute.

Killed vaccine. The arthrospores of strain Cash were suspended in 0.5% aqueous formaldehyde and held at room temperature for 48 hr. After they were checked for viability, the spores were washed to remove the formaldehyde and resuspended (12 mg/ml) in incomplete Freund's adjuvant (9 parts Bayol F to 1 part Arlacel A).

Viable vaccine. The arthrospores of strain D-76 were diluted in 0.85% saline to a concentration of 150 viable spores per 0.5 ml. This particular strain (D-76), shown previously to be exceedingly active in producing tissue reaction upon subcutaneous injection, was used as the viable vaccine in this study, to insure ulceration and lymphadenopathy upon injection.

Vaccination. The killed vaccine was injected subcutaneously at intervals of 0, 2, 6, and 10 weeks (total dose, 36 mg) at various sites; 0.5 ml of the viable vaccine (150 spores) injected subcutaneously in the right forearm 30 days after the last injection of killed vaccine.

Respiratory exposure. *C. immitis* Cash, used as the challenge organism, was administered 6 months after injection of the viable vaccine. The arthrospores were resuspended in 8% aqueous glucose and aerosolized in a 115,000-liter test tank. The animals' heads were exposed to the aerosol for approximately 4 min. The theoretical inhaled dose (7,500 arthrospores) was estimated from the average breathing rate and lung volume of the monkeys, the length of exposure, and the cloud concentration. The cloud concentration was determined by viable plate counts of the contents of the in-line filters after withdrawal of measured amounts of the aerosol.

Serology. Serological response of the animals to the vaccines and to the respiratory challenge was followed by the agar-gel immuno-diffusion precipitin test of Ray and Kadull (1964) and by the complement-fixation test.

Skin hypersensitivity. Coccidioidin skin tests were administered intrapalpebrally. The coccidioidin was prepared by growing arthrospores for 7 days at 34 C with shaking in the liquid medium of Goldschmidt and Taylor (1958). The cultures were then stored for 3 weeks at 5 C and Seitz filtered. The undiluted filtrate was used for the tests.

Histopathology. Tissues for histological study were fixed in 10% formaldehyde, impregnated with paraffin, sectioned, and stained with the Giemsa, Gomori silver methenamine, and Ziel-Nielson acid-fast stains.

Culture of histological material. Mycological cultures were made on glucose-peptone-yeast extract-agar slants of the right axillary lymph nodes, the lungs, and any suspicious lesions, for recovery of the fungus.

Four groups of monkeys (seven to nine in each group) were injected with the formaldehyde-killed vaccine, the viable vaccine, the formaldehyde-killed vaccine followed by the viable vaccine, or were left unvaccinated as controls. All animals were observed, during the 6-month interval between the inoculation of the viable vaccine and the respiratory challenge, for development of ulceration of the vaccination site or axillary lymphadenopathy. Their serological response to the various vaccination regimens was also recorded. Similar observations were made for an additional 4-month period after respiratory challenge, to determine the efficiency of the vaccines, and then all animals were killed and complete necropsies were performed. Two additional groups of monkeys (total of 13), receiving either the viable vaccine or the formaldehyde-killed vaccine followed by the viable vaccine, were unchallenged by the respiratory route and were maintained for histological comparison.

RESULTS

Prechallenge vaccination period. During the period of vaccination (Table 1), only 6 and 12% of the animals receiving both the killed and viable vaccines exhibited ulceration and lymphadenopathy, respectively, as compared with 100 and 83% of those receiving only the viable vaccine, indicating substantial control of tissue reaction to the viable vaccine by preinjection of killed vaccine.

This control was also indicated by the serological response of these animals at 3 months postvaccination. As shown in Fig. 1, highest mean titers ($\pm 1:128$) developed in animals receiving the viable vaccine, the lowest ($\pm 1:16$) in those vaccinated with the killed vaccine, and intermediate levels ($\pm 1:64$) in the group receiving both

TABLE 1. Control of tissue reactions to the injection of a viable *Coccidioides immitis* vaccine

Immunization	Tissue reaction to viable vaccine	
	Ulcerated vaccination site	Axillary lymphadenopathy
Formalin-killed vaccine* plus viable vaccine†	1/17‡ (6%)	2/17 (12%)
Viable vaccine alone	12/12 (100%)	10/12 (83%)

* Total dose: 36 mg (0, 2, 6, and 10 weeks).

† Strain D-76, 150 viable spores.

‡ Number reacting/number tested.

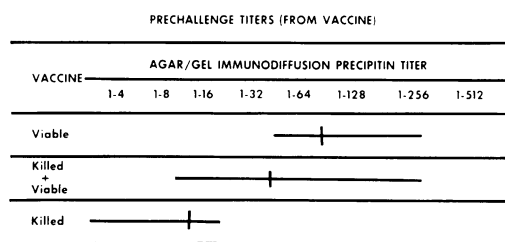


FIG. 1. Serological response of monkeys to various vaccination regimens. Horizontal lines indicate range of titers in each group; the vertical lines, the mean titer.

the killed and the viable vaccine. All vaccinated animals exhibited positive skin reactions to the injection of coccidioidin at this time.

Postchallenge period. The serological response of vaccinated monkeys to the respiratory challenge is shown in Table 2. At the time of challenge, the group receiving only the viable vaccine had maintained essentially its maximal vaccination titer (1:64), in contrast to the lowered titers (1:2 to 1:16) of the other two groups. Two facts are evident from the data in Table 2, which can later be correlated with the histological findings:

the higher the prechallenge titer (shown in the 0 column), the slower the development of the disease, as reflected by the acceleration of the post-challenge titer; and the lower the prechallenge titer, the greater the maximal titers developed after challenge. Note that the group receiving only the viable vaccine maintained its prechallenge titer until 60 days after challenge, in contrast to the other two groups, which exhibited an immediate rise in titer.

The clinical record of the experimental and control animals after challenge is shown in Table 3. All animals receiving the viable vaccine (either with or without the killed vaccine) remained in good flesh and healthy in appearance and actions, throughout the 4-month observation period. Some apathy and poor appetite were noted among the group receiving only the killed vaccine, but otherwise they were healthy in appearance and appeared to recover from the effects of the challenge dose. As seen in the mortality column, all of the 24 vaccinated animals survived the respiratory challenge.

This was in striking contrast to the evidence of extremely severe illness in the nonvaccinated control animals. This group suffered an 88% mortal-

TABLE 2. Serological response* of vaccinated monkeys to the respiratory challenge with *Coccidioides immitis*

Vaccine	Days after respiratory challenge					
	0	15	45	60	90	120
Viable.....	1:64	1:64	±1:64	±1:256	±1:512	1:256
Killed plus viable.....	1:16	±1:256	±1:512	1:1,024	1:2,048	1:2,048
Killed.....	1:2	±1:256	±1:1,024	±1:4,096	1:2,048	±1:2,048

* Values reported are the mean titer for each group (the mean and the median titers were essentially the same in each instance); ± indicates an incomplete reaction.

TABLE 3. Clinical record of vaccinated and nonvaccinated monkeys after respiratory challenge with *Coccidioides immitis*

Group	Vaccine	Clinical observation	Avg maximal titer		Mortality (dead/total)	Lung pathology*
			Prechallenge	Postchallenge		
1	Viable	Healthy	±1:128	±1:1,024	0/9	+†
2	Killed plus viable	Healthy	±1:64	±1:4,096	0/8	+
3	Killed	Some loss of appetite, but healthy in appearance	±1:16	±1:4,096	0/7	++
4	None (control)	Loss of weight, extreme debilitation, coughing, rapid respiration	Negative	1:16,384‡	7/8	++++

* Relative extent of lung involvement (+, minimal; ++, moderate; +++++, severe).

† This group contained five animals that were not infected.

‡ Value for the one surviving control.

TABLE 4. Mortality* and serological response of nonvaccinated controls of respiratory challenge with *Coccidioides immitis*

Animal no.	Immunodiffusion titer on day										
	11	14	15	20	39	42	49	53	60	90	120
7C-23	Died										
T-61	Died										
7C-21		Negative, died									
T-66		Hemo,† died									
T-65			1:8	Hemo, died							
T-68			1:16		1:512, died						
T-27			Negative			±1:2,048	1:1,024	1:1,024, died			
6C-17			1:4			1:1,024	1:4,096		1:2,048	1:16,380	±1:2,048

* Day of death as indicated.

† Hemolyzed.

ity during the 4-month observation period; 50% of them died by the 14th day postchallenge. As seen in Table 4, other deaths occurred on the 20th, 39th, and 53rd days. The mortality and serological record of the nonvaccinated controls indicated a direct relationship between length of survival and height of titer; that of the one surviving animal reached a maximum of 1:16,380 by the 90th day postchallenge. Note also that three of the four animals tested on the 15th day had developed titers of 1:4 to 1:16.

In general, the histological findings on autopsy (Table 3) correlated with the serological response and with clinical signs and symptoms. The seriousness of the disease increased stepwise in groups 1 through 4, as indicated by clinical symptoms, serological response, and extent of pathological involvement of the lungs. Although the mean maximal serological titer was the same ($\pm 1:4,096$) for groups 2 and 3, the titers increased more rapidly in group 3 (see Table 2). The extent of lung involvement in *infected* animals in groups 1 and 2 was equal (+); however, approximately half the animals in group 1 were not infected (all animals in groups 2, 3, and 4 developed pulmonary coccidioidomycosis).

Histological sections from representative animals in each of the four groups are shown in Fig. 2. Although lung sections in the first three groups appear similar, considerably more evidence of caseous necrosis may be noted in the center of the lesions in the lungs of animals in group 3 (receiving only the killed vaccine). Note, also, the almost complete destruction of lung tissue in the non-vaccinated control animals in group 4.

Although histological evidence indicated that 79% of the vaccinated, challenged animals (19 of 24) contracted pulmonary coccidioidomycosis, only 26% of these (5 of 19) exhibited positive lung cultures 4 months after exposure. As indicated by negative culture of the right axillary lymph nodes, 73% of the animals (22 of 30) receiving the viable vaccine were probably free from the vaccine strain at 10 months after vaccination.

Histological examination of the 13 vaccinated, unchallenged control monkeys revealed one animal with a generalized infection. Lesions containing the fungus were found in the lungs, liver, bronchial and axillary lymph nodes, and in the skin at the site of vaccination. Two other animals in this group showed minimal dissemination granulomata, in the pancreatic lymph node of one, and in the inguinal node of the other. No other lesions were found in either of these animals.

DISCUSSION

In ranking the effectiveness of the three vaccines used in this study, it appears, from the data presented here and particularly from former studies (Converse et al., 1961), that the viable vaccine was the most effective, the combination of the killed and the viable next, and the killed vaccine least effective. This is borne out by the increasing indications of disease in the three groups of animals, in the order named above (clinical symptoms, serological response to pulmonary challenge including both height and acceleration of titer, and the pathological changes in the lungs).

The three disseminations of infection beyond the regional lymph nodes (axillary) noted in the

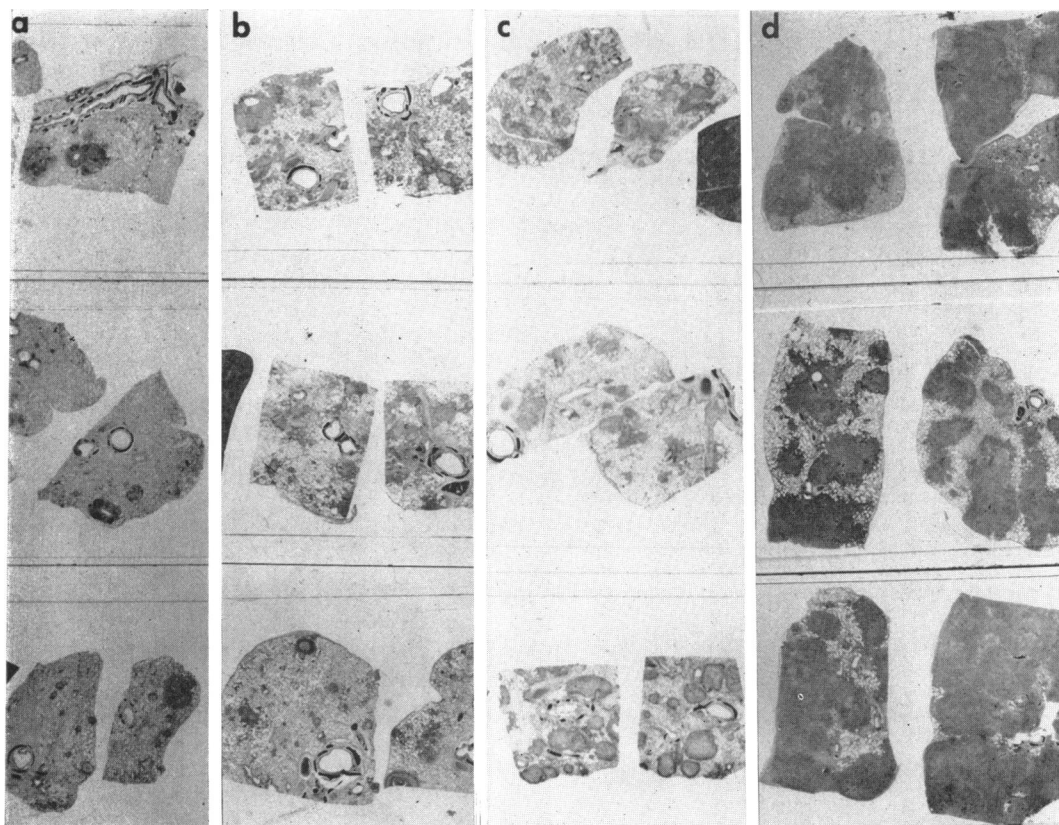


FIG. 2. Representative lung sections of vaccinated and nonvaccinated monkeys. (a) Viable vaccine. (b) Killed plus viable vaccine. (c) Killed vaccine. (d) Nonvaccinated controls. Note the almost complete lung destruction in the nonvaccinated controls (d) and the central necrosis of the lesions in animals receiving the killed vaccine (c). Many of the lesions appearing in the vaccinated animals (a, b, and c) were not coccidioidal, but were caused by lung mite (*Pneumonyssus simicola*) infections.

vaccinated, unchallenged control animals must be attributed to the viable vaccine, since animal-to-animal transmission of coccidioidomycosis is not known to occur. It should be noted, however, that a vaccine dose 15 times greater than necessary (Converse et al., 1961) and a particularly aggressive strain (D-76) of the fungus were used as the viable vaccine in this study. The high dose and the highly virulent strain were used in this instance so that any control of tissue reaction to its injection by the preinoculation of a killed vaccine would be absolutely clear-cut. It is thought, however, that the one generalized infection in the vaccinated, unchallenged group was a result of individual susceptibility of the monkey involved, since the fungus was still in evidence at the site of vaccination 10 months after injection. This has never occurred before in any of our studies; all other animals exhibited complete healing of the vaccination site.

Production of a high degree of antibodies by injection of killed *C. immitis* has not been previously reported. It is thought that the relatively high titers and the unequivocally positive skin-test reactions in the animals receiving only the killed vaccine were, in the present study, due to the use of incomplete Freund's adjuvant.

It is evident from the data presented that the viable vaccine can be controlled, by preinjection of a killed vaccine, to such an extent that the undesirable tissue reactions do not occur. However, it also appears that, to a certain extent, its protective effect was also impaired. Although the infectious dose of *C. immitis* received in nature is unknown, it is logical to believe that it would be more on the order of 2 or 3 to possibly 50 arthrospores, rather than 7,500 arthrospores as used in this study. It is hoped that a repetition of this experiment, with the use of a more realistic exposure dose (50 to 100 arthrospores) and a lower

viable vaccine dose (10 arthrospores) of a milder vaccine strain, will prove as successful as former studies with the viable vaccine.

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