JOURNAL OF BACTERIOLOGY
Vol. 87, No. 1, pp. 81-85 January, 1964

EXPERIMENTAL PRIMARY CUTANEOUS COCCIDIOIDOMYCOSIS IN THE MONKEY[1]

JOHN L. CONVERSE, STEVEN P. PAKES,[2] ERNEST M. SNYDER, AND MERIDA W. CASTLEBERRY[3]

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

Received for publication 12 August 1963

ABSTRACT

CONVERSE, JOHN L. (U.S. Army Biological Laboratories, Fort Detrick, Frederick, Md.), STEVEN P. PAKES, ERNEST M. SNYDER, AND MERIDA W. CASTLEBERRY. Experimental primary cutaneous coccidioidomycosis in the monkey. J. Bacteriol. 87:81-85. 1964.—Primary cutaneous coccidioidomycosis was studied in the monkey (*Macaca mulatta*) to find a suitable strain of *Coccidioides immitis* for use as a viable vaccine. Intradermal inoculation (medial surface of the right forearm) produced more severe vaccination reactions (draining vaccination site and axillary lymph node hypertrophy) than did subcutaneous injection. A subcutaneous vaccine dose of 10 arthrospores resulted in less reaction than a 100-spore dose. Moreover, dissemination beyond the regional lymph nodes did not occur after injection of ten spores of even the most virulent strains of *C. immitis*. Two of the five strains tested (Silveira, M-11, D-76, Cash, and a colonial isolate from Cash designated CW1) exhibited very mild vaccination reactions and appeared to have been cleared from the tissues upon necropsy at 10 months postvaccination. These two strains (Cash and CW1) appear promising for further immunological studies with a viable vaccine.

Studies on experimental primary cutaneous coccidioidomycosis were initiated with the objective of developing a safe viable vaccine against the pulmonary form of the disease. Epidemiological evidence of the lack of second infections in thousands of patients with a diagnosis of primary pulmonary coccidioidomycosis (Smith, Pappagianis, and Saito, 1957), and the lack of evidence of systemic dissemination in approximately 18 proven cases of primary cutaneous infection (Wilson, Smith, and Plunkett, 1953; Guy and Jacob, 1926; Coccidioidomycosis Coop Study, 1961) have indicated the feasibility of such a vaccine.

We reported (Converse, Castleberry, and Snyder, 1961) that the subcutaneous inoculation of *Coccidioides immitis* in the forearm of rhesus monkeys protected them from a later respiratory challenge with the same fungus. It was also shown that as few as ten arthrospores gave this protection without dissemination beyond the axillary lymph nodes, and that the protection was not strain-specific.

In the work reported here, primary cutaneous coccidioidomycosis in monkeys was studied with five *C. immitis* strains of graded virulence in man and in various experimental animals. The objective of the present study was threefold: to find a strain that could be safely used as a viable vaccine (i.e., causing no dissemination beyond the regional lymph nodes), to find one that would exhibit less severe side effects (i.e., causing a mild vaccination reaction, and less involvement of the axillary lymph nodes), and to compare the effects of subcutaneous and intradermal inoculation of the fungus.

MATERIALS AND METHODS

Organisms. The following *C. immitis* strains were used: Silveira, isolated from a recovered primary pulmonary human infection; Cash, from a nonfatal, extrapulmonary disseminated, human infection; M-11, a rodent isolate from Arizona; D-76, isolated from a disseminated dog infection by R. E. Reed of the University of Arizona; and a colonial isolate of strain Cash (CW1), with altered virulence (low) and altered colonial morphology. All strains were harvested as arthrospores from 14-day submerged growth (34 C with shaking) in the liquid synthetic medium of Roessler et al. (1946).

[1] Animals maintained in compliance with the "Principles of Laboratory Animal Care" as promulgated by the National Society for Medical Research, 1961, Bio-Medical Purview 1:14.

[2] Present address: School of Veterinary Medicine, Ohio State University, Columbus.

[3] Present address: Research and Nutrition Laboratory, Fitzsimmons U.S. Army General Hospital, Denver, Colo.

Experimental animals. Ten groups of rhesus monkeys (*Macaca mulatta*), of both sexes, each weighing approximately 3 kg, were inoculated, either subcutaneously or intradermally, in the medial surface of the right forearm, with saline suspensions (0.5 ml) of either 10 or 100 viable arthrospores of each of the five strains. They were housed two per cage.

Pathogenesis. The monkeys were observed for a period of 10 months for clinical signs of disease and for gross evidence of tissue reaction to the injections. At 10 months, all animals were sacrificed for complete necropsies. Serological titers were obtained from terminal bleeding, and cultures were made, routinely, from the inoculation site, the right axillary lymph node, and the lung. Any suspected lesions in other organs or tissues were also cultured.

Histopathology. Histopathological studies were made on sections of the inoculation site, the right axillary lymph node, and all the body organs, with the Giemsa as well as specific fungus stains. Lung sections were also checked with fluorescent-antibody techniques. Tissues for microscopic study were fixed in 10% formalin, impregnated with paraffin, and sectioned before staining.

RESULTS

Subcutaneous versus intradermal inoculation. Intradermal vaccination with viable *C. immitis* produced twice as many open and draining lesions at the site of inoculation as did subcutaneous vaccination (Table 1). Moreover, in the intradermal vaccines, small, multiple, skin lesions (similar in appearance to those formed in sporotrichosis infections) developed progressively, from the vaccination site to the axillary lymph node.

Approximately an equal number of monkeys exhibited axillary lymphadenopathy in both groups, but this condition was first noted by the fourth week in the intradermal vaccines as compared with the ninth week in the subcutaneous vaccines, indicating a much greater rate of progression of the fungus from the vaccination site to the regional lymph nodes via the intradermal route. For these reasons, subcutaneous vaccination was chosen as the route of inoculation for further studies.

Vaccine dose level. Subcutaneous dose levels of 10 or 100 viable fungi produced equal numbers of open lesions at the site of inoculation (Table 2); however, the higher dose resulted in almost five times as many visibly enlarged lymph nodes as were noted in the 10-spore group. Moreover, the rate of progression of the fungus from vaccination site to regional lymph node was greater in the high-dose than in the low-dose group (6 weeks versus 9 weeks, respectively).

Comparison of strains. Subcutaneous inoculation of *C. immitis* strains Silveira, D-76, M-11, Cash, or CW1 resulted in significant differences in tissue response and serological response (Table 3). Open lesions at the vaccination site and visibly enlarged axillary lymph nodes were noted in approximately one-half of the monkeys receiving strains Silveira and D-76 as compared with one-fourth of those vaccinated with strain M-11. These conditions were not evident in the groups inoculated with either strain Cash, or the

TABLE 1. *Tissue response of monkeys to subcutaneous and intradermal inoculation of Coccidioides immitis arthrospores*

Time	Subcutaneous		Intradermal	
	Draining lesions	Enlarged lymph nodes	Draining lesions	Enlarged lymph nodes
weeks				
4	0/8	0/8	3/8	2/8
6	2/7	0/7	4/8	3/8
7	0/7	0/7	4/8	4/8
9	0/7	2/7	5/8	4/8
21	1/7	2/7	0/8	3/8
Total incidence of response per total inoculated	3/7 (43%)	4/7 (57%)	7/8 (88%)	4/8 (50%)

TABLE 2. *Tissue response of monkeys to subcutaneous inoculation of 10 or 100 Coccidioides immitis arthrospores*

Time	10 Spores		100 Spores	
	Draining lesions	Enlarged lymph nodes	Draining lesions	Enlarged lymph nodes
weeks				
4	0/20	0/20	1/16	0/16
6	2/20	0/20	2/15	4/15
7	1/20	0/20	1/15	4/15
9	2/20	1/20	1/15	6/15
21	1/20	2/20	1/15	4/15
Total incidence of response per total inoculated	4/20 (20%)	2/20 (10%)	3/15 (20%)	7/15 (47%)

TABLE 3. *Tissue response of monkeys to subcutaneous inoculation of arthrospores of various strains of Coccidioides immitis*

Strain	Response	Time (weeks)					Total incidence of response / Total inoculated
		4	6	7	9	21	
Silveira	Draining lesion	0/8	2/7	0/7	0/7	1/7	3/7 (44%)
	Enlarged lymph node	0/8	0/7	0/7	2/7	2/7	4/7 (57%)
D-76	Draining lesion	0/6	1/6	1/6	1/6	0/6	1/6 (17%)
	Enlarged lymph node	0/6	2/6	2/6	3/6	2/6	3/6 (50%)
M-11	Draining lesion	1/8	1/8	1/8	2/8	0/8	2/8 (25%)
	Enlarged lymph node	0/8	2/8	2/8	2/8	1/8	2/8 (25%)
Cash	Draining lesion	0/6	0/6	0/6	0/6	0/6	0/6 (0%)
	Enlarged lymph node	0/6	0/6	0/6	0/6	0/6	0/6 (0%)
CW1	Draining lesion	0/7	0/7	0/7	0/7	0/7	0/7 (0%)
	Enlarged lymph node	0/7	0/7	0/7	0/7	0/7	0/7 (0%)

TABLE 4. *Serological response of monkeys to subcutaneous inoculation of arthrospores of various strains of Coccidioides immitis*[a]

Immunization dose (spores)	Strain				
	Silveira	D-76	M-11	Cash	CW1
10	+1:8	+1:128	±1:32	Negative	+1:2
	+1:8	Negative	Negative	Negative	Negative
	±1:64	Negative	Negative	Negative	Negative
	±1:64	Negative	±1:32		Negative
100	±1:128	+1:64[b]	±1:128	Negative	Negative
	±1:128	+1:256	Negative	Negative	Negative
	+1:512[c]	+1:512	Negative	Negative	+1:8
	NT[d]		±1:128		

[a] Each entry represents individual monkey.
[b] This monkey was immunized with formalin-killed arthrospores prior to injection with viable D-76.
[c] Disseminated infection.
[d] Not tested (early death: disseminated infection).

colonial isolate of strain Cash designated strain CW1.

As shown in Table 4, at the termination of the experiment (10 months postvaccination), precipitin titers were much higher in animals vaccinated with strains Silveira or D-76 (maximal, 1:512) than in the M-11 vaccinees (maximal, 1:128). The titers in those receiving strains Cash or CW1 were either negative or at a very low level (1:2 to 1:8). As expected, the higher dose levels produced higher titers. The absence of a titer in some of these animals does not necessarily indicate absence of protection, since neither the precipitin nor the complement-fixation level is a measure of immunity in coccidioidomycosis. (A positive delayed skin hypersensitivity reaction to the injection of coccidioidin was noted in all animals at 16 weeks postvaccination.)

Histopathological studies. Upon necropsy of the 100-spore group at 10 months, the highest incidence of residual fungi was noted in the tissues of animals inoculated with the Silveira strain and, in decreasing order, in the M-11, D-76, and Cash strains. Three of the four animals receiving strain Silveira exhibited systemic dissemination beyond the axillary lymph node; one of the M-11 vaccine group showed minimal dissemination. It is important to note that those injected with strains D-76, Cash, and CW1 remained free from dissemination even at this high dose.

A similar picture was exhibited by animals receiving the ten-spore dose, with two important

TABLE 5. *Histological changes resulting from inoculation of 100 Coccidioides immitis arthrospores*

Strain	Vaccination site	Right axillary lymph node	Lung	Other
Silveira	+	+	−	Liver
	+	−	−	
	+	+	+	Liver, spleen, kidney, adrenal glands
	+	+	+	Liver, inguinal lymph nodes, thoracic abscess
M-11	−	NE*	−	
	+	+	−	Left axillary lymph node
	+	+	−	
	−	+	−	
D-76†	−	−	−	
	−	−	−	
	−	+	−	
Cash	−	−	−	
	−	+	−	
	−	−	−	
CW1	−	−	−	
	−	−	−	
	−	−	−	

* Not examined.

† Node of first animal in this group showed no histological changes, except the presence of giant cells. Nodes of the second and third animals both contained caseous granulomata; however, no spherules were in evidence in the second one. Node cultures of both the second and third were positive for *C. immitis*.

TABLE 6. *Histological changes resulting from inoculation of ten Coccidioides immitis arthrospores**

Strain	Vaccination site	Right axillary lymph node
Silveira	−	+
	+	NE†
	+	−
	+	+
M-11	−	−
	+	+
	−	−
	−	−
D-76	−	−
	−	−
	−	−
	−	+
Cash	−	−
	−	−
	−	−
CW1	−	−
	−	−
	−	−
	−	−

* No changes were observed except at vaccination site and in right axillary lymph node.

† Not examined.

exceptions. No systemic dissemination occurred with any of the strains at this dose level, and no residual fungi were found at the skin site or in the regional lymph nodes of any of the animals inoculated with strains Cash or CW1. There was approximately 98% corroboration of the presence of residual fungi in the tissues by positive cultures.

An observation of particular importance was noted on examination of the three monkeys receiving subcutaneous inoculations of 100 viable strain D-76 arthrospores. The first monkey in this group (see Tables 4 and 5), prior to subcutaneous inoculation with viable D-76 arthrospores, had received several inoculations of a killed vaccine consisting of *C. immitis* arthrospores treated with 0.5% formalin. Clinical as well as histopathological observations of this animal indicated that the killed vaccine limited the development of the fungi in the viable vaccine. The two animals *not* receiving the killed vaccine exhibited noticeable swelling at the vaccination site; caseous, axillary lymphadenitis; positive axillary lymph node cultures; and high precipitin titers (1:256 and 1:512). In contrast, the animal receiving prior vaccination with the

killed product, in addition to lack of gross or microscopic evidence of tissue reaction, exhibited negative cultures to the fungus and a comparatively low precipitin titer (1:64).

DISCUSSION

As reported previously with strain Silveira (Converse et al., 1961), none of the strains used in this study exhibited any dissemination beyond the regional lymph nodes when used at the 10-spore dose level; nor did strains D-76, Cash, or CW1 at the 100-spore dose level.

Progression of the fungus from the vaccination site to the axillary lymph nodes proceeds at a much slower rate when inoculated subcutaneously than when the intradermal route is used. This would be a distinct advantage if *C. immitis* were used as a live vaccine, since more time would be allowed for buildup of immunity before any possible escape of the fungus past the regional lymph nodes.

It is also evident that the various strains of *C. immitis* elicit different degrees of tissue reaction after subcutaneous inoculation. Strains Cash and CW1 appear particularly mild in this respect. Used at the ten-spore dose (shown previously to give protection equal to that of much higher doses), the undesirable effects from vaccination reaction would be held to a minimum, and it appears that the fungus would be cleared from the body. Moreover, strain CW1, in addition to exhibiting extremely low virulence for mice, appears to die out when injected into either mice or monkeys. If either of these two strains remains viable in the animal host long enough to stimulate immunity before being cleared from the tissues, a successful and safe viable vaccine against human coccidioidomycosis might be assured. This point is presently being investigated.

Another approach to reducing vaccination reaction and increasing the safety of a viable vaccine, the preinjection of a formalin-killed vaccine before use of a viable vaccine, appears promising, since this regimen seems to slow down, and possibly even inhibit, the multiplication of the viable vaccine. Admittedly, the data in support of this are few, but this also is being more extensively studied.

ACKNOWLEDGMENT

The authors wish to express appreciation to John G. Ray for establishing the precipitin titers on animals in these studies.

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