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AN EXPERIMENTAL VIABLE VACCINE AGAINST PULMONARY COCCIDIOIDOMYCOSIS IN MONKEYS

AUGUST 1963

UNITED STATES ARMY
BIOLOGICAL LABORATORIES
FORT DETRICK

U.S. ARMY BIOLOGICAL LABORATORIES
Fort Detrick, Frederick, Maryland

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AN EXPERIMENTAL VIABLE VACCINE AGAINST PULMONARY COCCIDIOIDOMYCOSIS IN MONKEYS

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Project 1C022301A068

August 1963

The work reported here was performed under Project 4B11-02-066, "Bacterial and Fungal Agent Research," Task -02, "Bacterial and Fungal Laboratory Research." The expenditure order was 2079. This material was originally submitted as manuscript 5190.

FOREWORD

The authors wish to thank Mr. John G. Ray and Mr. Louis F. Maire, III for performing the serological tests and Mr. Robert K. Castle and Mr. Eugene F. Bishop for making the photographs.

ABSTRACT

Monkeys (Macaca mulatta) vaccinated by subcutaneous injection in the forearm with from 10 to 10^8 viable Coccidioides immitis arthrospores were protected against respiratory challenge with approximately 7000 viable arthrospores administered six months after vaccination. Protection was evident from: the healthy appearance throughout four months post-respiratory challenge; negative chest X-rays at 15, 30, 60, and 120 days; and only very minor histopathological pulmonary changes on autopsy at 120 days, with negative lung cultures in 80 per cent of the animals. This was in striking contrast to the outward clinical appearance of severe disease (loss of weight, accelerated respiration, severe coughing, general debilitation); positive X-rays; massive pulmonary destruction, positive lung cultures and death of five of nine of the monkeys that were unvaccinated or had received non-viable arthrospore vaccines. The appearance of spherules (very few in number, accompanied by very minor pathological changes) in the lungs of some of the "dissemination controls" (subcutaneous viable vaccination without respiratory challenge) indicated possible dissemination from the primary cutaneous infection, although oral transmission from the cutaneous lesions could not be ruled out.

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I. INTRODUCTION

The apparent protection afforded by primary pulmonary coccidioidomycosis against secondary, exogenous reinfection, and the absence of reported cases of systemic dissemination of Coccidioides immitis from foci of primary cutaneous infections, suggested the subcutaneous inoculation of this organism as a viable vaccine, to establish immunity against the disease.

Smith[1] and his coworkers, in several very definitive epidemiological studies involving follow-ups of thousands of human pulmonary Coccidioides infections, found no evidence of second infections with the fungus.

Documented primary, cutaneous infections with C. immitis are few, and are limited almost entirely to personnel working with the organism. Among these are the report of Wilson et al[2] of a mortician contracting a lesion on the finger from embalming the body of a fatal case of coccidioidal granuloma; Guy and Jacob's[3] description of the infection in a patient inoculated by the prick of a cactus thorn; and several unpublished laboratory accidents (self-inoculation). Winn[4] and Meis[5] have treated an additional 12 cases of primary cutaneous coccidioidomycosis. In the majority of these patients, infection was mild and limited to focal lesions at or near the site of inoculation. In no instance was dissemination of the organism noted beyond the regional lymph nodes of the affected appendage.

There have been numerous experimental studies of primary cutaneous coccidioidomycosis in animals: the dog and the rabbit,[6-8] the guinea pig,[9-11] and mice.[12] In these studies, systemic dissemination was extremely rare. Moreover, the few visceral lesions noted were small focal areas, unaccompanied by any clinical evidence of illness.

Several investigators (Pappagianis et al[12] with subcutaneous vaccination, using a highly virulent strain of C. immitis; Converse et al[13] with intraperitoneal vaccination using a strain of low virulence) have studied the resistance of mice, previously inoculated with the viable organism, to a second infection via the intraperitoneal route with a virulent strain. Substantial resistance to the challenge dose was indicated in both of these studies, by histopathological evidence as well as mortality rates. Pappagianis et al[14] reported a similar resistance to second infection in preliminary studies with cynomolgus monkeys (Macaca iris).

This report concerns an extensive study of the immunogenic response of rhesus monkeys (Macaca mulatta) to subcutaneous vaccination with viable C. immitis arthrospores, and the pathogenesis of primary cutaneous infections in the monkey.*

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* Animals were maintained in compliance with the "Principles of laboratory animal care" as promulgated by the National Society for Medical Research.

II. MATERIALS AND METHODS

A. ORGANISM[15]

The organisms used in this study were Coccidioides immitis, strain Silveira, isolated from a recovered primary pulmonary human infection; strain Cash, from an extrapulmonary disseminated nonfatal infection; and strain M-11, a rodent isolate from Arizona, all used as viable vaccines. Strain Cash, killed with 0.5 per cent formalin, was used as the nonviable vaccine, and strain Silveira as the respiratory challenge organism. Strains Cash and M-11 arthrospores were harvested from a 14-day submerged growth (34°C with shaking) in the liquid synthetic medium of Roessler et al.[16] Strain Silveira was harvested by vacuum as dry arthrospores from a 5- to 8-week growth on modified (0.1 per cent yeast extract) Sabouraud's agar, after desiccation of the medium.

B. IMMUNIZATION

Monkeys of both sexes, weighing approximately three kilograms, were immunized by subcutaneous injections of viable or nonviable arthrospores (0.5 ml saline suspension) in the medial surface of the right forearm. They were housed two monkeys per cage.

C. RESPIRATORY EXPOSURE

Respiratory challenge of the animals was obtained by inhalation of a dry cloud of arthrospores aerosolized in a 4800-liter exposure chamber (head exposure only) by means of compressed air. The exposure dose was calculated from the average volume of monkey lungs, the respiration rate, the exposure time, and the cloud density.

D. PATHOGENESIS

The course of the infections (primary cutaneous as well as pulmonary) was followed by: clinical observation, coccidioidin skin-hypersensitivity tests, determination of precipitin (Ppn) and complement-fixation (CF) titers, frontal chest X-rays, and gross and histopathological studies and mycological culture of autopsy material at death, or upon sacrifice at four months post-respiratory challenge.

Tissues for microscopic study were fixed in 10 per cent formalin, impregnated with paraffin, sectioned, and stained with Giemsa or the Gomori silver methenamine stains. The fluorescent antibody technique was used as a further check on the presence of the organism in the tissues.

III. RESULTS

Monkeys, in groups of four animals each, were given either a single injection of 10, 100, or 1000 viable strain Silveira arthrospores, or three injections (30 days apart) of formalin-killed arthrospores (total dose, 10^9 spores). Six months after vaccination these four groups of animals, together with a group of nonvaccinated control monkeys, were challenged by the respiratory route with a calculated inhaled dose of approximately 7000 viable strain Silveira arthrospores. An additional group of monkeys receiving the viable vaccine (10 to 10^8 spores in ten-fold increments) but not challenged by the respiratory route, was maintained as "dissemination controls."

Vaccination with viable arthrospores resulted in subcutaneous lesions one to three centimeters in diameter. Draining lesions (Figure 1) at the vaccination site and enlargement of the right axillary lymph nodes (Figure 2) were noted in approximately 50 per cent of the animals. The open lesions were healed and the majority of the axillary lymph nodes had returned to normal size six months following vaccination (Figure 3). At that time, all monkeys that received the viable vaccine exhibited skin hypersensitivity to coccidioidin (Table I), while the reactions of those vaccinated with the killed product remained doubtful (some erythema, but no induration). With one exception, the precipitin and complement-fixation titers of all vaccinated animals had either become negative or were at a low level (1:4 to 1:64). The animal in question was shown, on autopsy, to have four active subcutaneous head lesions, which probably were responsible for the high titers (CF: 1:256, Ppn: 1:512).

Following respiratory exposure, the nonvaccinated control monkeys, and those vaccinated with the formalin-killed product, exhibited extreme debilitation (Table II), suffering from loss of appetite, emaciation, and pronounced accelerated respiration, accompanied by coughing. Widespread, wispy infiltration throughout all lobes of the lungs and visible consolidation in some areas were noted in X-rays of these animals 15 days after respiratory exposure (Figure 4). The majority of the unvaccinated controls showed a marked rise of both CF and Ppn titers during the four-month holding period. Approximately half of the unvaccinated control group and of the formalin-killed vaccine group died from pulmonary coccidioidomycosis within four months after respiratory exposure.

In contrast, monkeys vaccinated with the viable preparations exhibited no visible clinical symptoms of the disease following respiratory challenge. The X-rays were negative throughout the four-month holding period, and the majority of serological titers remained at a low level (1:4 to 1:64). No deaths occurred in this group.

Figure 1. Vaccination Site on Right Forearm of a Monkey Receiving 10^5 Viable C. Immitis, Strain Silveira, Arthrospores (34 Days Post-immunization).

Figure 2. Right Axillary Lymph Node on the Monkey Shown in Figure 1 (30 Days Post-immunization).

Figure 3. Healed Vaccination Site and Decreased Swelling of the Axillary Lymph Node at 6 Months Post-immunization. The monkey shown in Figures 1 to 3 was an extreme case. Tissue reaction to vaccination was much less in those receiving the lower doses (10 to 1000 spores).

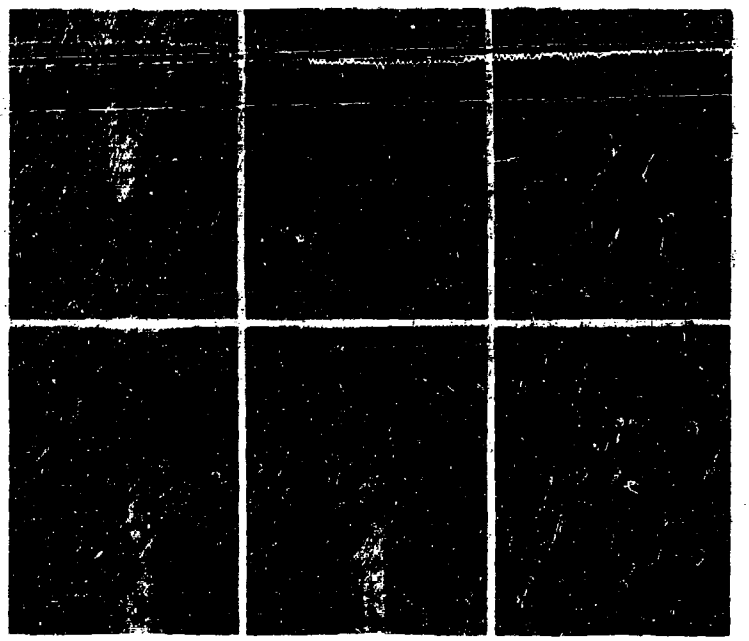

Figure 4. Top row: Three Nonimmunized Control Monkeys (15 days after Respiratory Exposure to Approximately 7000 Arthrospores) Showing Pneumonic Infiltration Throughout the Whole Lung. Note consolidated area in animal at top, left. Bottom Row: From left, animals receiving 10, 100, and 1000 viable-spore vaccinations (15 days after respiratory exposure to approximately 7000 arthrospores). Note lack of evidence of infection.

TABLE I. SEROLOGICAL RESPONSE OF MONKEYS TO CUTANEOUS AND PULMONARY COCCIDIOIDOMYCOSIS

Immunization	PRE-CHALLENGE Skin Test	CF[a] Titer	Precipitin[a] Titer	POST-RESPIRATORY CHALLENGE Maximum CF Titer	Maximum Precipitin Titer
	-	-	-	128	64
	-	-	-	256	256
Unvaccinated	-	-	-	256	256
(controls)	-	-	-	256	512
	-	-	-	256	1024
	+	-	8	64	16
Viable Vaccine	+	-	8	8	16
Dose, spores	+	-	4	16	16
10	+	32	64	64	64
	+	-	4	8	16
	+	16	32	32	32
	+	-	4	8	8
100	+	-	2	8	8
	+	256	512[b]	512	1024
	+	16	32	32	32
	+	-	8	128	128
1000	+	-	4	-	4
	±[c]	-	-	16	32
Nonviable	±	-	-	64	16
Vaccine	±	-	-	64	64
	±	-	-	8	16

a. Six months post-immunization.
b. This animal shown to have four subcutaneous head lesions on autopsy, probably explaining the high titers.
c. Questionable results (erythema but no induration).

TABLE II. CLINICAL RESPONSE OF MONKEYS TO PULMONARY COCCIDIOIDOMYCOSIS

IMMUNIZATION	CLINICAL SYMPTOMS	X-RAY	MORTALITY, dead/total
Unvaccinated (controls)	Loss of appetite, energy. Emaciation. General debilitation. Accelerated respiration. Coughing.	Widespread wispy infiltrations throughout all lobes of the lungs by the 15th day post-respiratory challenge. Later consolidated areas.	2/5
Viable Vaccine	None	Negative	0/12
Nonviable Vaccine	Same as unvaccinated controls.	Less involvement than controls. Delayed development (30 versus 15 days).	3/4

Upon autopsy (Table III), the lungs of the unvaccinated control group and formalin-killed vaccine group were bosselated in appearance, and were covered with surface lesions. Large palpable consolidated areas present throughout all lobes of the lungs were caseous and necrotic upon section (Figure 5). There was extensive adhesion of the lungs to the pleura and diaphragm. Histopathological examination revealed granulomas with spherules in the lungs of every animal in these two groups, and extrapulmonary dissemination in more than half of them. All lung cultures were positive for C. immitis.

In five animals of the viable vaccine groups, histopathological lung sections revealed several self-contained focal lesions containing spherules. This minimal involvement, because of the character of the lesions and the time intervals involved, was attributed to the vaccine injection rather than the respiratory challenge. This was further indicated by the increase in the number of animals showing this condition with increases in the viable vaccine dose. Moreover, the only positive lung cultures in this group occurred in animals receiving the highest vaccine dose.

TABLE III. HISTOLOGICAL RESPONSE OF MONKEYS TO PULMONARY COCCIDIOIDOMYCOSIS

IMMUNIZATION	GROSS PATHOLOGY	HISTOPATHOLOGY		LUNG
	Lung	Lung	Other	CULTURE
Unvaccinated (controls)	Bosselated appearance.	+	-	NC[a]
	Scattered surface lesions and large palpable nodules throughout all lobes.	+	Kidney, hilar LN	+
		+	-	+
	Large consolidated areas (caseous necrosis).	+	Kidney	+
	Extensive adhesions.	+		+
Viable Vaccine Dose, spores		-	-	-
		-	-	-
10	Few small circumscribed focal lesions, 2 to 3 mm in diameter (generally ascribed to mite infestations).	-	-	-
		-	-	-
		+	-	-
100		-	-	-
		-	-	-
		+	-	-
	Minor adhesions in 2 of the 12 monkeys in these groups.	+	4 subcutaneous head lesions	+
		-	-	-
1000		+	-	+
		+	-	+
Nonviable Vaccine	Same as controls.	+	Liver	+
		+	Spleen, hilar LN	NC
		+	hilar LN	+
		+	hilar LN	NC

a. Not cultured.

Eighty per cent of the "dissemination controls" (vaccinated but not challenged by the respiratory route) exhibited this same minimal systemic dissemination of the organism from the cutaneous infection to the lung (Figure 6) but no physical signs of illness. In three of those, receiving vaccine doses in the 10^5 to 10^8 range, dissemination was noted in other organs (spleen, kidney, or liver).

In these same studies, other monkeys were vaccinated with viable arthrospores of strains Cash and M-11. These animals exhibited resistance to pulmonary challenge similar to that shown by the monkeys receiving viable strain Silveira vaccine, indicating that the protective antigen of the viable vaccine was not strain-specific.

Figure 5. Left: Histological Sections of Three Nonimmunized Animals Following Respiratory Exposure to Approximately 7000 Arthrospores. Note the consolidated necrotic lesions in the lung sections. Right: Lung sections of three animals vaccinated with 100 or 1000 viable arthrospores. These animals received the same respiratory exposure as those shown on the left. The large cavitated lesion shown in the center section was shown (microscopically) to be a lung mite lesion.

Figure 6. C. immitis Spherules in Tissues of Dissemination Control. Top, left to right, skin at vaccination site and right axillary lymph node. Bottom, left to right, the lung and the spleen.

IV. DISCUSSION

It was evident from the data presented that the subcutaneous injection of viable C. immitis engendered an immunity in monkeys that enabled them to resist subsequent pulmonary infections with the organism. This immunity was not strain-specific, at least with the three strains tested, including two highly virulent strains and one of very low virulence (M-11) for mice.

A dose of ten viable organisms gave equal protection to that of much higher doses, and accomplished this with less severe primary lesions and without dissemination beyond the axillary lymph node.

The fact that the injection of 10 viable organisms resulted in solid immunity, whereas the injection of approximately 10^9 nonviable organisms failed to induce an immunity, indicates that protective antibodies are formed only in response to multiplication of the organism in the tissues (possibly in combination with some specific tissue element). Although the injection of a nonviable vaccine appeared to delay the progress of the disease somewhat, the end result of respiratory challenge was the same as that noted in the nonvaccinated controls.

As expected, there was a direct relationship between skin hypersensitivity and resistance to pulmonary infection. The interesting point, however, was that injection of nonviable vaccine limited the formation of complement-fixing and polysaccharide-precipitating antibodies following respiratory exposure, to the same extent as the viable vaccine (Table I), but it did not limit the spread of infection in these animals. This indicates that (a) the protective antibodies are distinct and separate from those operative in the CF and Ppn reactions, and (b) that formalin treatment of the arthrospore inactivates the antigen responsible for formation of protective antibodies, but has no effect on those stimulating the CF and Ppn antibodies. Thus, multiplication of the organism in the tissues is necessary for the formation of protective antibodies, but not necessary for the organisms' action on CF and Ppn response.

A correlation can be seen (Table III) of an increase in the number of animals showing positive histopathological findings (spherule-containing granulomas) in the lung, with an increase in the number of viable organisms in the vaccine dose. Table III also shows that the only positive lung cultures were found among the animals receiving the higher viable-spore vaccine dose. Since all of the animals received approximately equal respiratory challenge doses, it is our opinion that these lesions resulted from the immunizing, rather than the challenge, dose. Thus the histopathological changes noted in the lungs of the vaccinated, challenged monkeys and in the vaccinated, unchallenged dissemination controls are strongly indicative that systemic dissemination, although subclinical, can result from primary cutaneous infection with C. immitis.

Either the number of organisms escaping from the axillary lymph node was so small, or the time element was such, that sufficient immunity was developed before the organism could exert its full virulence in the lung, consequently resulting in self-contained, subclinical infection. This may be the reason that systemic dissemination from primary cutaneous coccidioidomycosis has never been recognized in human infections.

Transmission of the organism, via the mouth, from the draining vaccination lesion to the lungs cannot be ruled out, but this is extremely unlikely in light of results of experimental intravenous infections in monkeys,[17] in which the character of the lung lesions resulting from haematogenous spread of the organism was quite different from that of lesions resulting from impingement in the lung of an organism from an aerosol. The lung lesions noted in the present study were very similar to those reported by Blundell[17] as resulting from haematogenous spread of the organism. However, there was no doubt that the head lesions of one monkey (Table III) and lesions of the ramos of the mandible and the eyelid of one of the dissemination controls resulted from direct transmission from the arm lesion at the site of vaccination.

Further studies are in progress, to evaluate the safety of a 10-spore viable vaccine, to establish statistically the nondissemination from a subcutaneous dose of this magnitude, and to examine the tissue response to injection of various strains of the organism. Preliminary evidence[18] has indicated strain differences, dose level differences, and inoculation site differences in the monkeys' response to experimental primary cutaneous infections with *C. immitis*.

LITERATURE CITED

1. Smith, C.E. "The public health significance of coccidioidomycosis," Proc. Symposium on Coccidioidomycosis, U.S. Public Health Service, 1957.

2. Wilson, J.W.; Smith, C.E.; and Plunkett, O.A. "Primary cutaneous coccidioidomycosis: The criteria for diagnosis and report of a case," Calif. Med. 79:233-239, 1953.

3. Guy, W.H., and Jacobs, F.M. "Granuloma coccidioides," Arch. Dermat. & Syph. 14:596, 1926.

4. Winn, W.A. Discussion from the floor, Trans. 6th Ann. VA-AF Coccidioidomycosis Study Group, 1961. p. 29.

5. Meis, P.R. Discussion from the floor, Trans. 6th Ann. VA-AF Coccidioidomycosis Study Group, 1961. p. 29.

6. Rixford, E. "Case for diagnosis presented before the San Francisco Medico-Chirurgical Society, March 5, 1894," Occident. Med. Times 8:326, 1894.

7. Rixford, E. "A case of protozoic dermatitis," Occident. Med. Times 8:704-707, 1894.

8. Ophuls, W. "Further observations on a pathogenic mould formerly described as a protozoon (Coccidioides immitis, Coccidioides pyogenes)," J. Exp. Med. 6:443-486, 1905.

9. Davis, D.J. "Coccidioidal granuloma with certain serologic and experimental observations," Arch. Dermat. & Syph. 9:577-588, 1924.

10. Chipman, E.D., and Templeton, H.J. "Coccidioidal granuloma," Arch. Dermat. & Syph. 21:259-278, 1930.

11. Guy, W.H., and Jacobs, F.M. "Granuloma coccidioides," Arch. Dermat. & Syph. 16:308-311, 1927.

12. Pappagianis, D.; Smith, C.E.; Berman, R.J.; and Kobayashi, G.S. "Experimental subcutaneous coccidioidal infection in the mouse," J. Invest. Dermatol. 32:589-598, 1959.

13. Converse, J.L.; Castleberry, M.W.; Besemer, A.R.; and Snyder, E.M. "Immunization of mice against coccidioidomycosis," J. Bacteriol. 84:46-52, 1962.

14. Pappagianis, D.; Miller, R.L.; Smith, C.E.; and Kobayashi, G.S. "Response of monkeys to respiratory challenge following subcutaneous inoculation with Coccidioides immitis," Am. Rev. Respirat. Diseases 82:244-250, 1960.

15. Friedman, L.; Smith, C.E.; Roessler, W.G.; and Berman, R.J. "The virulence and infectivity of twenty-seven strains of Coccidioides immitis," Am. J. Hyg. 64:198-210, 1956.

16. Roessler, W.G.; Herbst, E.J.; McCullough, W.G.; Mills, R.C. and Brewer, C.R. "Studies with Coccidioides immitis: I. Submerged growth in liquid mediums," J. Infect. Dis. 79:12-22, 1946.

17. Blundell, G.P.; Castleberry, M.W.; Lowe, E.P.; and Converse, J.L. "The pathology of Coccidioides immitis in the Macaca mulatta," Am. J. Pathol. 39:613-630, 1961.

18. Converse, J.L.; Pakes, S.P.; Snyder, E.M.; and Castleberry, M.W. "Experimental primary cutaneous coccidioidomycosis in the monkey," Trans. 7th Ann. VA-AF Coccidioidomycosis Study Group. 1962.