EXPERIMENTAL VIABLE VACCINE AGAINST PULMONARY COCCIDIOIDOMYCOSIS IN MONKEYS[1]

JOHN L. CONVERSE, MERIDA W. CASTLEBERRY,[2] AND ERNEST M. SNYDER

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

Received for publication 21 June 1963

ABSTRACT

CONVERSE, JOHN L. (U.S. Army Biological Laboratories, Fort Detrick, Frederick, Md.), MERIDA W. CASTLEBERRY, AND ERNEST M. SNYDER. Experimental viable vaccine against pulmonary coccidioidomycosis in monkeys. J. Bacteriol. **86**:1041–1051. 1963.—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 6 months after vaccination. Protection was evident from: the healthy appearance throughout 4 months after 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% of the animals. This was in striking contrast to the outward clinical appearance of control monkeys that were unvaccinated or had received nonviable arthrospore vaccines. These monkeys showed 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 animals. 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.

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 (1957), 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 Wilson, Smith, and Plunkett's (1953) report of a mortician's contracting a lesion on the finger from embalming the body of a fatal case of coccidioidal granuloma; Guy and Jacobs' (1926) description of the infection in a patient inoculated by the prick of a cactus thorn; and several unpublished laboratory accidents (self-inoculation). Winn (1961) and Meis (1961) 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 (Rixford, 1894*a*, *b*; Ophuls, 1905); the guinea pig (Davis, 1924; Chipman and Templeton, 1930; Guy and Jacobs, 1927); and mice (Pappagianis et al., 1959). 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. (1959) with subcutaneous vaccination with a highly virulent strain of *C. immitis*, and Converse et al. (1962*a*) with intraperitoneal vaccination with a

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

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 by mortality rates. Pappagianis et al. (1960) reported a similar resistance to second infection in preliminary studies with cynomolgus monkeys (*Macaca irus*).

This report describes 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.

MATERIALS AND METHODS

Organism (*Friedman et al., 1956*). *C. 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) were used as viable vaccines. Strain Cash, killed with 0.5% formalin, was used as the nonviable vaccine, and strain Silveira was used as the respiratory challenge organism. Strains Cash and M-11 arthrospores were harvested from 14-day submerged growth (34 C with shaking) in the liquid synthetic medium of Roessler et al. (1946). Strain Silveira was harvested by vacuum as dry arthrospores from 5- to 8-week growth on modified (0.1% yeast extract) Sabouraud agar, after desiccation of the medium.

Immunization. Monkeys (*Macaca mulatta*) of both sexes, weighing approximately 3 kg, were immunized by subcutaneous injections of viable or nonviable arthrospores (0.5 ml of a saline suspension) in the medial surface of the right forearm. They were housed with two monkeys per cage.

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.

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 4 months after respiratory challenge.

Tissues for microscopic study were fixed in 10% 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 fungus in the tissues.

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). At 6 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 tenfold increments) but not challenged by the respiratory route, was maintained as "dissemination controls."

Vaccination with viable arthrospores resulted in subcutaneous lesions 1 to 3 cm in diameter. Draining lesions (Fig. 1) at the vaccination site and enlargement of the right axillary lymph nodes (Fig. 2) were noted in approximately 50% of the animals. The open lesions were healed, and the majority of the axillary lymph nodes had returned to normal size 6 months after vaccination (Fig. 3). At this time, all monkeys which received the viable vaccine exhibited skin hypersensitivity to coccidioidin (Table 1); the reactions of those vaccinated with the killed product remained doubtful (some erythema, but no induration). With one exception, the Ppn and CF titers of all vaccinated animals either had 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).

After respiratory exposure, the nonvaccinated

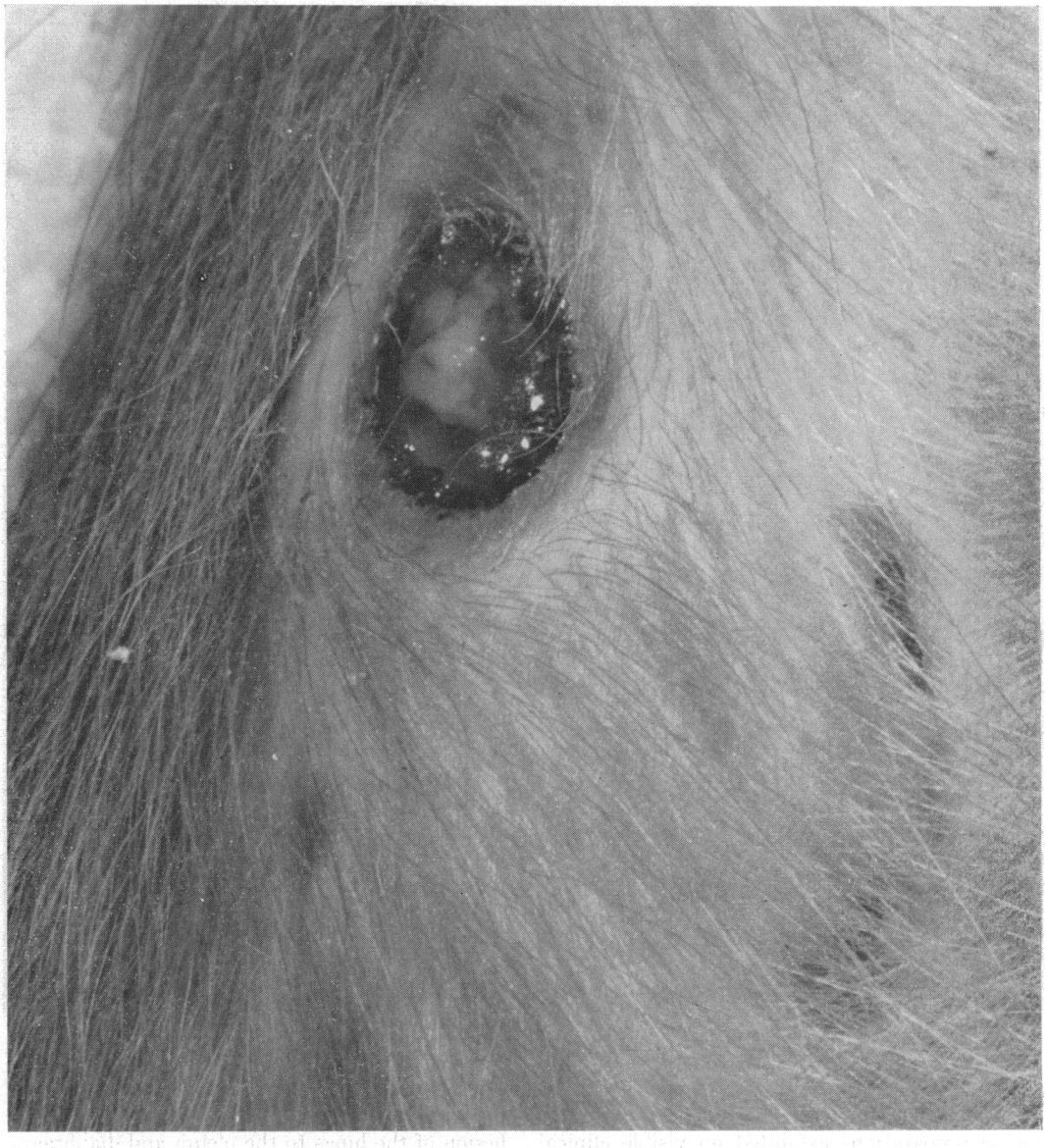

FIG. 1. *Vaccination site on right forearm of a monkey receiving 10^8 viable Coccidioides immitis strain Silveira arthrospores (14 days postimmunization).*

control monkeys and those vaccinated with the formalin-killed product exhibited extreme debilitation (Table 2), 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 (Fig. 4). The majority of the unvaccinated controls showed a marked rise in both CF and Ppn titers during the 4-month holding period. Approximately half of the unvaccinated control group and of the formalin-killed vaccine group died from pulmonary coccidioidomycosis within 4 months after respiratory exposure.

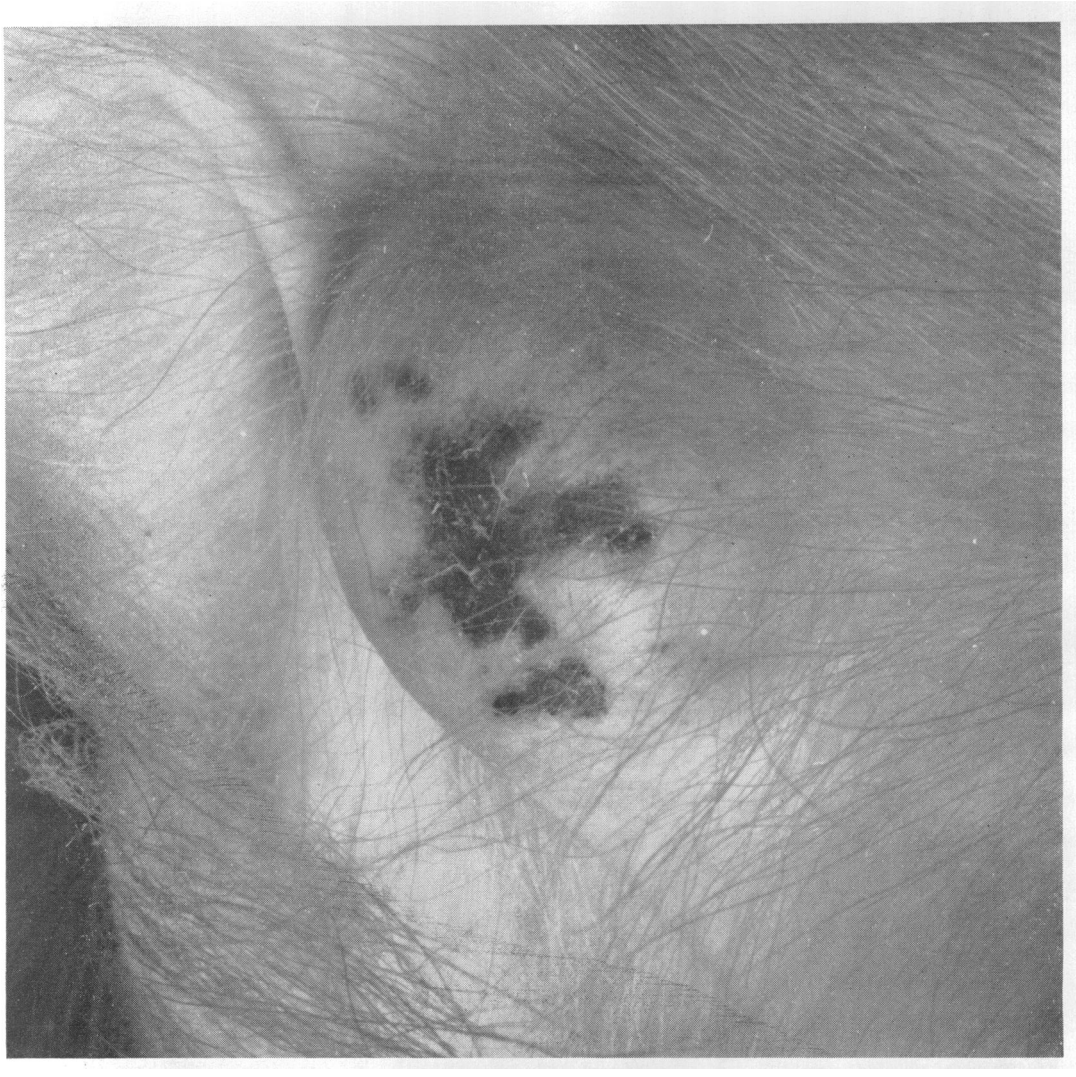

FIG. 2. *Right axillary lymph node on the monkey shown in Fig. 1 (30 days postimmunization).*

In contrast, monkeys vaccinated with the viable preparations exhibited no visible clinical symptoms of the disease after respiratory challenge. The X rays were negative throughout the 4-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.

Upon autopsy (Table 3), 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 (Fig. 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

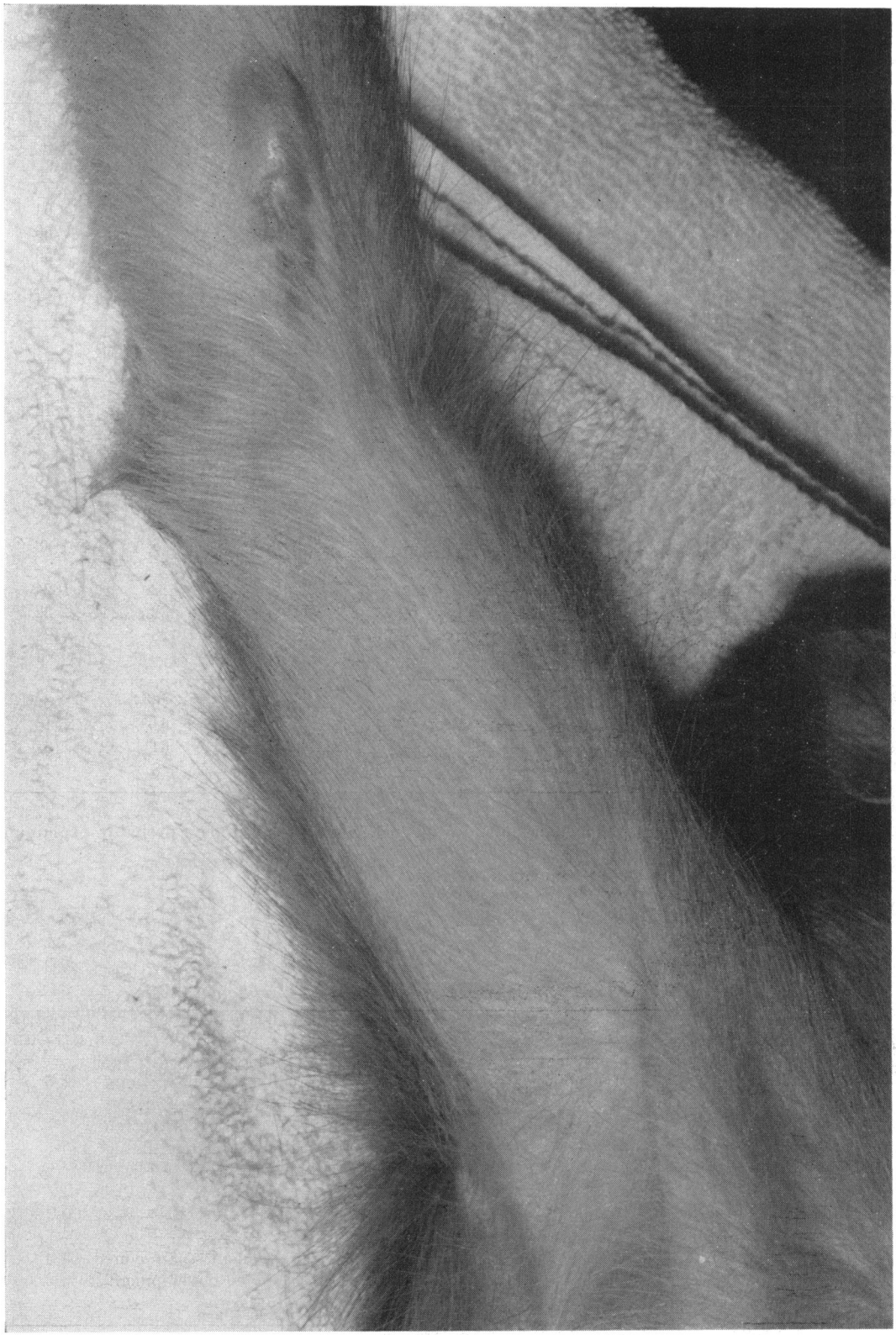

FIG. 3. *Healed vaccination site and decreased swelling of the axillary lymph node at 6 months postimmunization. The monkey shown in Fig. 1 to 3 was an extreme case. Tissue reaction to vaccination was much less in those receiving the lower doses (10 to 1000 spores).*

TABLE 1. *Serological response of monkeys to cutaneous and pulmonary coccidioidomycosis*

Immunization	Prechallenge			After respiratory challenge	
	Skin test	CF* titer	Precipitin* titer	Maximal CF titer	Maximal precipitin titer
Unvaccinated (controls)	−	−	−	128	64
	−	−	−	256	256
	−	−	−	256	256
	−	−	−	256	512
	−	−	−	256	1024
Viable vaccine dose (spores)					
10	+	−	8	64	16
	+	−	8	8	16
	+	−	4	16	16
	+	32	64	64	64
100	+	−	4	8	16
	+	16	32	32	32
	+	−	4	8	8
	+	−	2	8	8
1000	+	256	512†	512	1024
	+	16	32	32	32
	+	−	8	128	128
	+	−	4	−	4
Nonviable vaccine	±‡	−	−	16	32
	±	−	−	64	16
	±	−	−	64	64
	±	−	−	8	16

* Postimmunization, 6 months.

† This animal was shown to have four subcutaneous head lesions on autopsy, probably explaining the high titers.

‡ Questionable results (erythema but no induration).

TABLE 2. *Clinical response of monkeys to pulmonary coccidioidomycosis*

Immunization	Clinical symptoms	X ray	Mortality (dead/total)
Unvaccinated (controls)	Loss of appetite and energy, emaciation, general debilitation, accelerated respiration, coughing.	Widespread wispy infiltrations throughout all lobes of the lungs by the 15th day after 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 vs. 15 days).	3/4

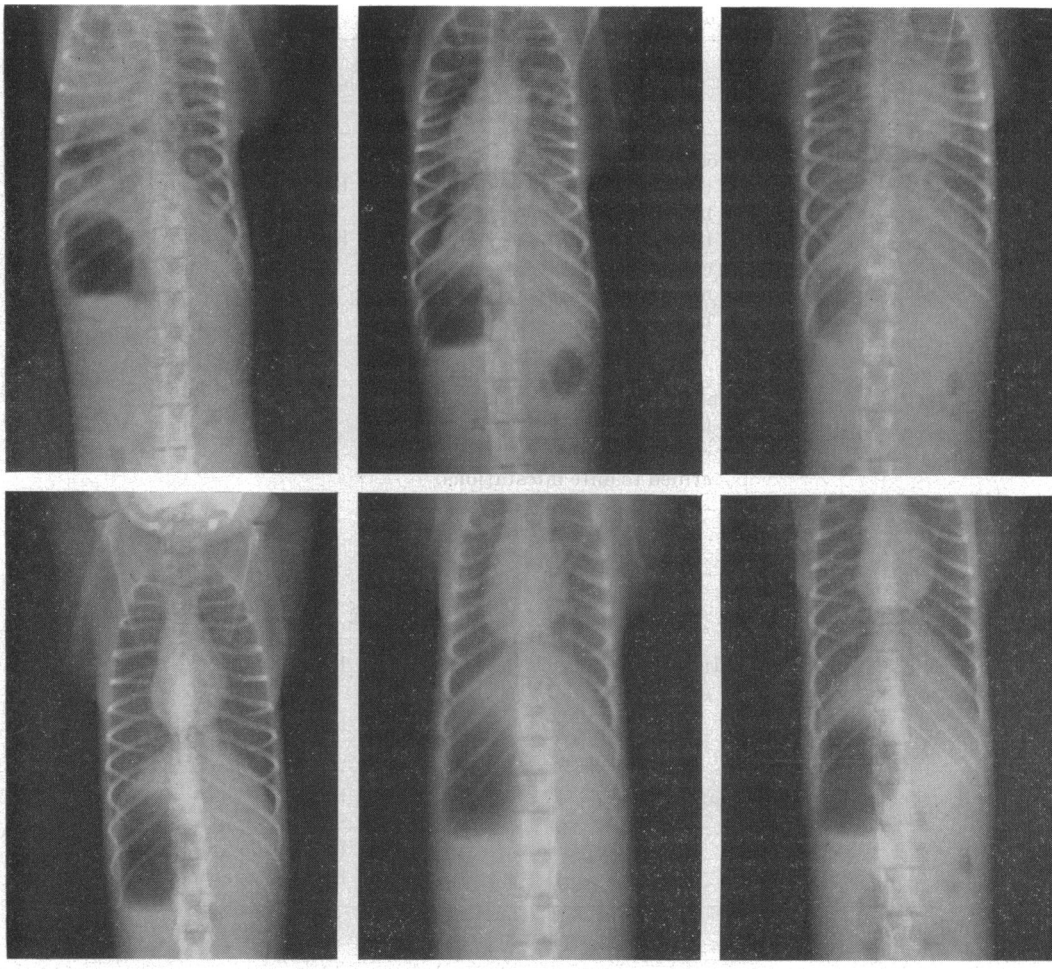

FIG. 4. *Top row: three nonimmunized control monkeys (15 days after respiratory exposure to approximately 7000 arthrospores) showing pneumonic infiltration throughout the whole lung. Note consolidated area in animal at top left. Bottom row: from left, animals receiving 10, 100, and 1000 viable spore vaccinations (15 days after respiratory exposure to approximately 7000 arthrospores). Note lack of evidence of infection.*

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.

Of the "dissemination controls" (vaccinated but not challenged by the respiratory route), 80% exhibited this same minimal systemic dissemination of the organism from the cutaneous infection to the lung (Fig. 6) but with 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.

TABLE 3. *Histological response of monkeys to pulmonary coccidioidomycosis*

Immunization	Gross pathology, lung	Histopathology		Lung culture
		Lung	Other	
Unvaccinated (controls)	Bosselated appearance, scattered surface lesions and large palpable nodules throughout all lobes, large consolidated areas (caseous necrosis), extensive adhesions	+	—	NC*
		+	Kidney, hilar lymph node	+
		+	—	+
		+	Kidney	+
		+		+
Viable vaccine dose (spores)				
10	Few small circumscribed focal lesions, 2 to 3 mm in diameter (generally ascribed to mite infestations)	−	—	−
		−	—	−
		−	−	−
		−	—	−
100		+	−	−
		−	−	−
		−	—	−
		+	—	−
1000	Minor adhesions in 2 of the 12 monkeys in these groups	+	Four sc head lesions	+
		−	—	−
		+	—	+
		+	−	+
Nonviable vaccine	Same as controls	+	Liver	+
		+	Spleen, hilar lymph node	NC
		+	Hilar lymph node	+
		+	Hilar lymph node	NC

* Not cultured.

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 fungus. 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 protection equal 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 fungus 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 after respiratory exposure to the same extent as did the *viable* vaccine (Table 1), but it did not limit the spread of infection in these animals. This indicates that (i) the protective antibodies are distinct and separate from those operative in the CF and Ppn reactions, and (ii) that formalin treatment

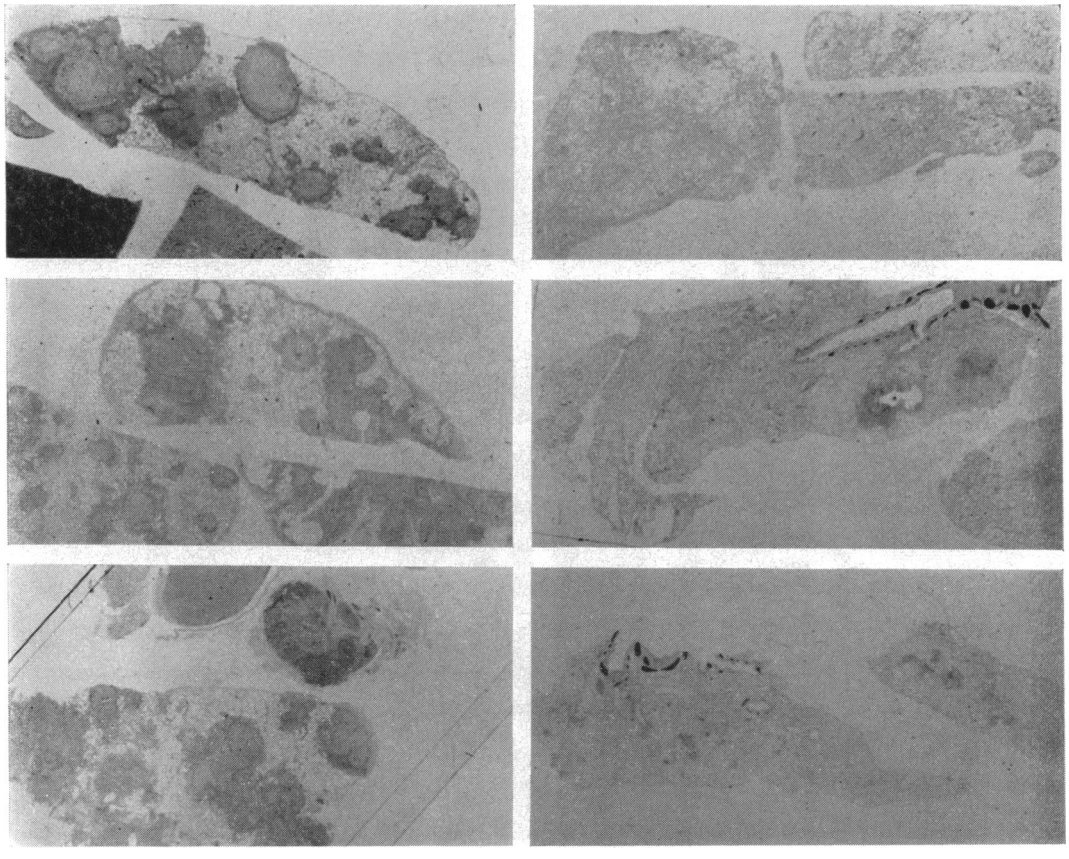

FIG. 5. *Left: histological sections of three nonimmunized animals after respiratory exposure to approximately 7000 arthrospores. Note the consolidated necrotic lesions in the lung sections. Right: lung sections of three animals vaccinated with 100 or 1000 viable arthrospores. These animals received 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.*

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 3) 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 3 also indicates 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.

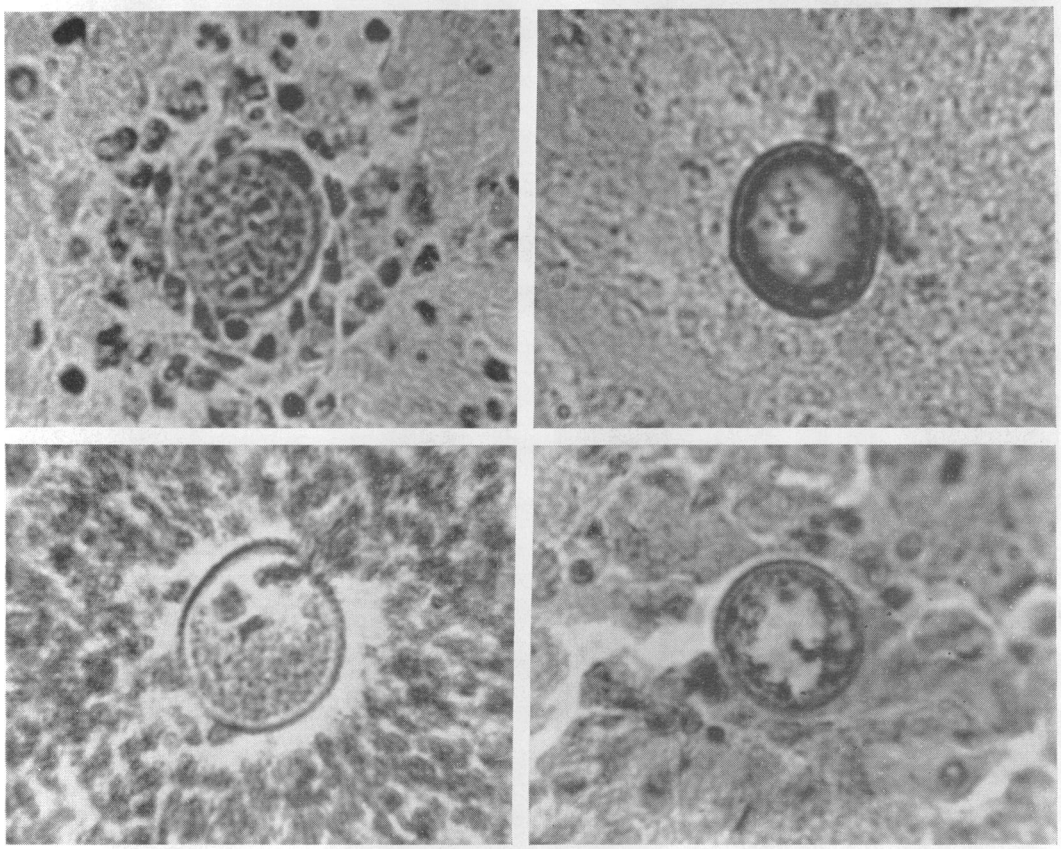

FIG. 6. *Coccidioides immitis spherules in tissues of dissemination control. Top (left to right): skin a vaccination site and right axillary lymph node. Bottom (left to right): the lung and the spleen.*

Transmission of the fungus to the lungs, via the mouth, from the draining vaccination lesion cannot be ruled out, but this is extremely unlikely in light of results of experimental intravenous infections in monkeys (Blundell et al., 1961), in which the character of the lung lesions resulting from hematogenous spread of the fungus was quite different from that of lesions resulting from impingement, in the lung, of a fungus spore from an aerosol. The lung lesions noted in the present study were very similar to those reported by Blundell as resulting from hematogenous spread of the fungus. However, there was no doubt that the head lesions of one monkey (noted in Table 3), 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 ten-spore viable vaccine, to establish statistically the nondissemination from the subcutaneous dose of this magnitude, and to examine the tissue response to injection of various strains of the organism. Preliminary evidence (Converse et al., 1962*b*) has indicated strain differences, dose level differences, and inoculation site differences in the monkeys' response to experimental primary cutaneous infections with *C. immitis*.

ACKNOWLEDGMENTS

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

A portion of the information contained in this manuscript was presented at the International Conference on Mycobacterial and Fungal Antigens, sponsored by the George Washington University Medical School, the American Thoracic

Society, and Chas. Pfizer & Co., Inc. Some of the information presented will be published in the Proceedings of the Conference.

Literature Cited

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

Chipman, E. D., and H. J. Templeton. 1930. Coccidioidal granuloma. A.M.A. Arch. Dermatol. Syphilol. **21**:259–278.

Converse, J. L., M. W. Castleberry, A. R. Besemer, and E. M. Snyder. 1962*a*. Immunization of mice against coccidioidomycosis. J. Bacteriol. **84**:46–52.

Converse, J. L., S. P. Pakes, E. M. Snyder, and M. W. Castleberry. 1962*b*. Experimental primary cutaneous coccidioidomycosis in the monkey. Trans. 7th Ann. Veterans Administration–Armed Forces Coccidioidomycosis Study Group, Los Angeles, Calif.

Davis, D. J. 1924. Coccidioidal granuloma with certain serologic and experimental observations. A.M.A. Arch. Dermatol. Syphilol. **9**:577–588.

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

Guy, W. H., and F. M. Jacobs. 1926. Granuloma coccidiodes. A.M.A. Arch. Dermatol. Syphilol. **14**:596.

Guy, W. H., and F. M. Jacobs. 1927. Granuloma coccidioides. A.M.A. Arch. Dermatol. Syphilol. **16**:308–311.

Meis, P. R. 1961. Discussion from the floor. Trans. 6th Ann. Meeting Veterans Administration–Armed Forces Coccidioidomycosis Coop Study, p. 29.

Ophuls, W. 1905. Further observations on a pathogenic mould formerly described as a protozoon (*Coccidioides immitis, Coccidioides pyogenes*). J. Exptl. Med. **6**:443–486.

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

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

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

Rixford, E. 1894*b*. A case of protozoic dermatitis. Occident Med. Times **8**:704–707.

Roessler, W. G., E. J. Herbst, W. G. McCullough, R. C. Mills, and C. R. Brewer. 1946. Studies with *Coccidioides immitis*. I. Submerged growth in liquid mediums. J. Infect. Diseases **79**:12–22.

Smith, C. E. 1957. The public health significance of coccidioidomycosis. Proc. Symp. Coccidioidomycosis, U.S. Public Health Service.

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

Winn, W. A. 1961. Discussion from the floor. Trans. 6th Ann. Meeting Veterans Administration–Armed Forces Coccidioidomycosis Coop Study, p. 29.