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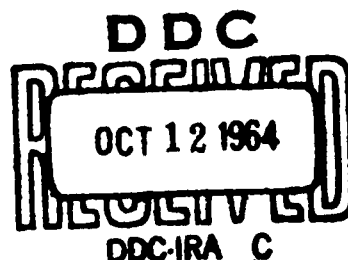
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TECHNICAL MANUSCRIPT 147

IMMUNIZATION AGAINST COCCIDIOIDOMYCOSIS BY KILLED CELL AND CELL FRACTION VACCINES

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ABSTRACT

Aerosol and subcutaneous vaccination with killed arthrospores and subcutaneous vaccination using a boivin-type fraction were compared for their efficacy in protecting rhesus monkeys against lethal aerosol challenge with *C. immitis*. Complete protection against lethal challenge was produced in the six monkeys subcutaneously vaccinated with killed arthrospores. Four of the six monkeys in each of the other immunized groups also survived. Only two of the six challenged controls survived. All animals were infected as a result of challenge. No dissemination was observed in animals immunized with the boivin-type fraction. The skin test reaction may be useful in detecting a response to immunization, but thus far the agar gel precipitin and CF reactions have not proved useful in detecting resistance to infection.

I. INTRODUCTION

Immunization against experimental coccidioidomycosis began with the use of a killed, whole-cell antigen. Negroni and Bonfiglioli¹ investigated the immune response in guinea pigs using dead material. Vogel and co-workers² at Duke University found that intramuscular immunization with a killed spherule-endospore suspension increased resistance of guinea pigs to pulmonary infection. The disease not only occurred less frequently but was less severe in the immunized than in the non-immunized control animals, Friedman^{3,4} later reported that killed arthrospore vaccines administered subcutaneously protected mice against lethal disease but not against infection with Coccidioides immitis. In the latter experiments the challenge dose was administered intraperitoneally.

After Pappagianis and co-workers⁵ observed that respiratory exposure appeared to be a more severe test of immunized animals than challenge by parenteral inoculation, Levine⁶ adopted intranasal instillation as the method of challenge. Killed arthrospores, mycelial and spherule antigens introduced intramuscularly into mice all protected against lethal intranasal challenge. Mice that received the spherule-endospore vaccine appeared to have infections that were milder than mice immunized with other test vaccines.

Extending his studies to larger animals, Levine⁷ tested the efficacy of killed spherule vaccine in cynomolgus monkeys challenged with 200 air-borne organisms. A total dose of 9 milligrams (dry weight) of vaccine was used for immunizations that were given alternately subcutaneously and intramuscularly at 1, 11, 28, and 54 days. The animals developed a limited delayed hypersensitivity to coccidioidin after immunization. This response was seen as a mild induration and erythema at 24 hours. Survival ratios after respiratory challenge were high in the vaccinated group and relatively low in the non-vaccinated control group. The establishment of infection was not prevented in either group and the benefit derived from immunization appeared to be chiefly the prevention of fatal disease.

The killed whole-cell vaccines thus afforded partial but not complete protection against coccidioidomycosis in both large and small animals. Their action was to reduce mortality rather than to prevent infection entirely. Moreover, the intact-cell antigens had objectional side effects.⁷ They were slowly absorbed; often nodules and sometimes necrosis developed at the site of inoculation.

Hampson,⁸ working with Conant, had also obtained similar results with killed intact-cell vaccines. Therefore, cell components of C. immitis were investigated in the hope that through isolation and purification of the active principle, protective capacity of vaccines could be increased and undesirable side effects reduced. Several immunologically active fractions obtained in chemical extraction studies have afforded small animals protection comparable to that of killed whole-cell vaccines.

The purpose of this investigation was to test a killed, intact-cell antigen and a highly active, cell-fraction antigen for the protection they afforded when given subcutaneously. The immunizing capacity of the killed, intact-cell antigen given subcutaneously was also compared with that of the same antigen given as an aerosol. The monkey was selected as the test animal. This report describes the results of immunization with the whole-cell and fractional vaccines against lethal challenge.

II. MATERIALS AND METHODS

Twenty-nine monkeys, Macaca mulatta, of both sexes, weighing five to seven pounds, were used for this investigation.* These animals were divided into four groups of six animals each and one group of five animals. The groups contained animals that were (a) aerosol-immunized with dead C. immitis arthrospores and subsequently challenged, (b) aerosol-immunized with dead arthrospores and not challenged, (c) subcutaneously immunized with dead C. immitis arthrospores and challenged, (d) subcutaneously immunized with a bovin-type C. immitis cell fraction and challenged, and (e) non-immunized and subsequently challenged.

Arthrospores were produced by growing C. immitis strain Silviera on Sabouraud's medium in six-inch Petri dishes at 34°C and 95 per cent relative humidity for at least two weeks. After good growth had been obtained, the temperature was increased to 40° to 45°C and the relative humidity was reduced. This low relative humidity fluctuated, but was usually below 20 per cent. The agar was allowed to dry completely. The arthrospores were then harvested by vacuum and collected in a paper extraction thimble. After harvesting, the arthrospore product was placed in a desiccator with calcium chloride to remove any residual moisture. To obtain a near-uniparticulate product, the harvested arthrospores were sifted through a No. 40 (openings 420 microns) U. S. Standard Sieve Series wire mesh screen. The resultant product was essentially uniparticulate, the particles having a mass median diameter of approximately five microns. This dry product was sealed in glass jars and stored at 4°C. For immunization, arthrospores were placed in 0.5 per cent formaldehyde for several days at room temperature, thoroughly washed, dried, and examined for viability. Any clumps that resulted from these treatments were broken, and the final killed product was again essentially uniparticulate with a mass median diameter of approximately five microns.

* In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

Dried killed arthrospores were used in the immunization as an aerosol in a small plastic box of 60-liter capacity (Figure 1). The box was fitted with four ports, two on each side, that allowed the heads of the monkeys to be inserted from a prone position. These openings were covered by rubber dam material. The animals were immobilized by small straight-jackets. A bacterial air filter equalized air pressure and helped contain the aerosol as much as possible. The spores were aerosolized into the box with compressed air from a small hopper with a rotating brush. The brush dispersed the spores into the air space of the hopper, and then the compressed air forced these air-borne spores into the aerosol box. The aerosol dose of dead spores that each monkey inhaled was calculated from their average lung capacity, their breathing rate, and the concentration of spores as an aerosol in the box. The latter was calculated from the efficiency of the disseminating device (50 per cent efficiency), the size of the box, and the estimated number of spores actually disseminated. The spores were aerosolized for a 20-second period every three minutes. The animals were exposed for a total of ten minutes for each immunization. The calculated inhaled aerosol immunization dose per monkey was eight milligrams. The immunizing dose was larger than that used by Levine⁷ because rhesus monkeys were used. Each monkey received three separate immunizations spaced at 0, 2, and 7 weeks for a 24-milligram calculated inhaled dose.

For subcutaneous immunization, the killed arthrospore product was weighed and suspended in saline. Each animal received an immunizing dose of eight milligrams inoculated into the lateral or medial surface of the thigh. Three immunizing doses at different times were given for a total of 24 milligrams.

The boivin-type C. immitis antigen was prepared from killed arthrospores of the same batch that was used for other immunizations. The spores were (a) treated with acetone to remove the soluble toxic or inhibitory fats, (b) treated with pyridine to remove the inert polysaccharides and proteins, (c) vibrated with glass beads in a Waring blender, and (d) centrifuged at 30,000 rpm for 30 minutes. The resultant sediment, designated boivin fraction III⁸ was combined with pertussis vaccine. Three separate subcutaneous immunizing doses of eight milligrams each were given with this material.

To prevent any inadvertent immunizations of the control animals by an aerosol of dead arthrospores in the environment, they were housed in another building until immunization of other animals had been completed.

The animals were challenged 25 days after the last immunization. The method used for aerosol challenge has been previously described.¹⁰

All animals, including the non-immunized controls, were skin-tested with undiluted coccidioidin before immunization. The coccidioidin used was produced in this laboratory according to the method of Smith.¹¹ Five strains of C. immitis were used, including Silveira, Cash, and M-11 strains. The

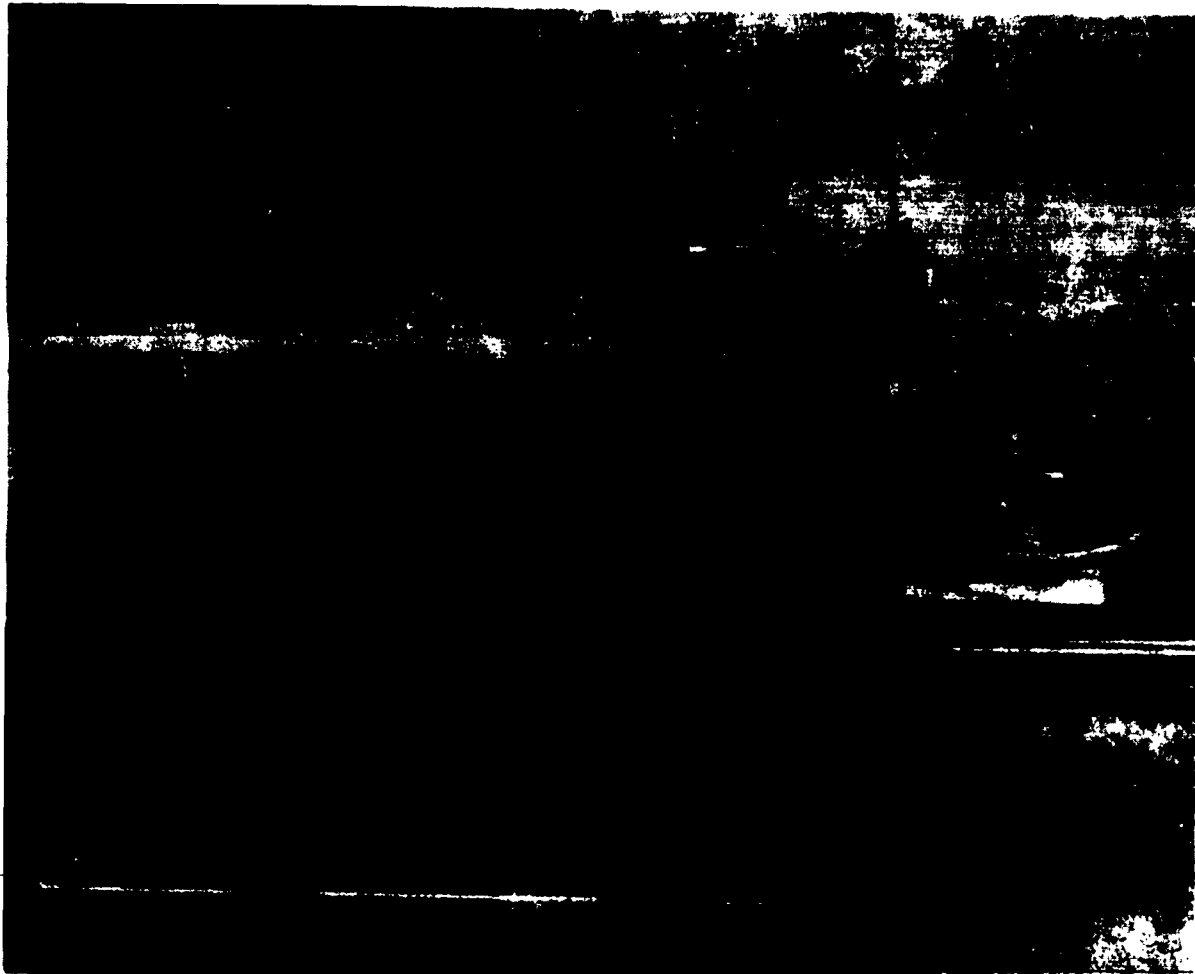


Figure 1. Aerosol Immunization Chamber with Disseminating Apparatus.
(FD Neg C-6771)

a indicates exposure port; b, bacterial air filter;
c, air pump; d, hopper; and e, aerosol delivery tube.

coccidioidin was compared with a standard lot supplied by Dr. Smith and appeared slightly stronger than the standard Smith lot. Skin tests were given 13 days after the first immunization, 12 days after the second immunization, 7 days after the third, and 69 days after challenge. Skin tests were given in the upper eyelid, using 0.1 milliliter of coccidioidin. Readings were made 24 and 48 hours after injection. Both erythema and induration were considered in all readings. The monkeys were also skin-tested with old tuberculin* before immunization and then every month thereafter until challenge.

Blood samples were collected from all animals after immunization and after challenge to determine whether agar gel precipitins or complement-fixing (CF) antibodies developed. The animals were bled after immunization was completed (Table I). Animals surviving challenge were also bled 67 days and 81 days after exposure. The techniques used in the agar gel precipitin and CF tube tests have been described by Ray and Kadull** and others.^{10,12} Sera were not pretreated for the agar gel precipitin test. For the CF determinations, sera were pretreated with complement in an effort to eliminate the anti-complementary activity usually found in such sera.¹²

The mean inhaled challenge dose was 260 viable particles (range 100 to 680). After challenge, animals were housed in individual cages in a gas-tight, air-conditioned cabinet system. All animals were autopsied at death or after sacrifice. Animals were sacrificed by injection of Nembutal*** at the conclusion of the experiment. Pathologic examination was made of all tissues collected at autopsy. The experiment was terminated 81 days after challenge.

The time sequences for skin tests, immunizations, challenge, and sacrifice are listed in Table I.

* Tuberculin (Intradermic) for diagnosis of tuberculosis in animals. Fort Dodge Laboratories, Inc., Fort Dodge, Iowa.

** Ray, J. G., Jr., and Kadull, P. J. "The agar gel precipitation inhibition technique in coccidioidomycosis antibody determination." To be published.

*** Nembutal (Sodium) Veterinary, Abbott Laboratories, North Chicago, Ill.

TABLE I. TIME SEQUENCE OF MONKEY IMMUNIZATION EXPERIMENT

Day	Procedure
-7	Skin test
0	First immunization
13	Skin test
15	Second immunization
27	Skin test
48	Third immunization
55	Skin test
71	Blood sampling
73	Challenge
139	Blood sampling
142	Skin test
153	Blood sampling
153	Sacrifice

III. RESULTS

Pre-experiment skin-test reactions with coccidioidin and tuberculin were negative. All tuberculin skin-test results remained negative for the duration of the experiment. Skin-test reactions with coccidioidin after the first immunization were also all negative. At the third coccidioidin skin testing, 27 days after immunization begun, slight induration of less than 5 by 5 mm was noted in three animals that were injected subcutaneously with dead *C. immitis* arthrospores. This reaction was seen at 24 hours; no reactions were seen 48 hours after testing. In all other animals, skin-test reactions were negative. The fourth skin test, 55 days after the first immunization, produced approximately similar results in

the group subcutaneously immunized with whole cells. After challenge, all surviving animals produced positive indurations upon skin testing. The indurations were larger than 5 by 5 mm and were easily seen at 24 and 48 hours, except in one animal in which the induration faded after 24 hours. The aerosol-immunized, non-challenged animals continued to show negative response to coccidioidin throughout the experiment.

Neither agar gel precipitins nor complement-fixing antibodies were detected in any of the animals after immunization and before challenge. After challenge, agar gel precipitins and CF antibodies closely paralleled each other throughout the experimental period in all test animals. Titers in both tests were high at the first postchallenge bleeding and tended to remain high during the test period. The agar gel precipitin titers for the first test period ranged from 1:64 to 1:512 with a median at 1:512. The range at the second test period was 1:128 to 1:1024, but the median remained at 1:512. Complement-fixation titers generally were higher than those for the agar gel precipitin test. Titers in the first postchallenge test samples ranged from 1:512 to 1:2048 with the median at 1:2048. In the second test period, the range was 1:512 to 1:8192, but the median remained at 1:2048. The aerosol-immunized, non-challenged control animals continued to have negative titers for the duration of the experiment. No significant differences were observed among the immunized groups in antibody occurrence or in CF titers and disseminated disease. No serologic comparisons were attempted between the immunized groups and the non-immunized control group because only two of the control groups survived to the blood sampling dates. However, it was noted that the highest CF titer occurred in a non-immunized control animal (E22). Both surviving animals of this group had disseminated disease.

In the non-immunized group, four of the six animals died following challenge. Deaths occurred on Day 11, Day 35, and two on Day 54 after challenge. Two animals of the aerosol-immunized group and two animals immunized with the boivin-type antigen died following challenge. No death occurred during the course of the experiments in the group subcutaneously vaccinated with killed arthrospores.

Pathologic examination of tissue from autopsy revealed pulmonary coccidioidomycosis in all challenged animals, immunized and non-immunized. The methods of vaccination did not seem to influence the severity of the lung lesions when they were examined microscopically. There were no marked differences among the immunized and non-immunized groups in the severity of the lung involvement. The infection involved both lungs of all animals. Discrete or coalescing nodular lesions were observed in most or all lobes of both lungs. Hilar lymph nodes were enlarged in animals in all challenge groups. Dissemination was observed in surviving animals in the non-immunized group and in some animals in the group immunized with whole-cell vaccine. No dissemination was observed in animals immunized with the boivin-type fraction.

The aerosol-vaccinated unchallenged control animals exhibited no macroscopic nor microscopic lesions compatible with coccidioidomycosis.

Table II summarizes the results obtained in each experimental and control group with reference to time of death and presence of extra-pulmonary, granulomatous lesions found in the remaining sacrificed animals.

TABLE II. RESPONSE OF IMMUNIZED AND NON-IMMUNIZED (CONTROL) MONKEYS TO RESPIRATORY CHALLENGE WITH LETHAL DOSE OF AEROSOLIZED C. IMMITIS

Antigen and Method of Administration	Number of Animals	Arthrospore Dose Range	Morbidity ^{a/} and Mortality Response		
			Per Cent Infected	Per Cent Deaths	Per Cent Disseminated ^{b/}
Aerosol immunization with dead <u>C. immitis</u> arthrospores	6	110-232	100	33	25
Subcutaneous vaccination with dead <u>C. immitis</u> arthrospores	6	120-210	100	0	50
Subcutaneous vaccination with boivin-type fraction	6	294-320	100	33	0
Non-immunized	6	100-680	100	66	100
Aerosol-vaccinated with dead <u>C. immitis</u> arthrospores non-challenged controls	5	-	0	0	0

a. Surviving animals sacrificed after 80 days.

b. Surviving animals only considered.

IV. DISCUSSION

In human coccidioidomycosis, the coccidioidin skin test and the precipitin and complement-fixation reactions are proved diagnostic aids.¹³ Thus far, however, only the skin test has been found useful in experimental immunological studies with C. immitis. Positive skin reactions have been elicited in guinea pigs immunized with killed cell vaccines,^{2,8} and there is some evidence for development of similar skin hypersensitivity in monkeys.

Levine and associates⁷ reported very mild and modified skin reactions to coccidioidin in cynomolgus monkeys after immunization with a killed spherule vaccine and before challenge with live organisms. The reaction consisted of a small area of erythema, ranging from 2 by 2 mm to 8 by 5 mm, which persisted for 24 hours. Induration was only sporadically present. In the present investigation, skin reactions to coccidioidin were observed in animals after immunization only in the group injected subcutaneously with killed arthrospore suspension. The response was similar to that reported by Levine; a slight erythema in four of six animals and faint indurations in three animals were visible at 24 but not at 48 hours.

Although the coccidioidin skin test has proved useful in experimental immunological studies with C. immitis, the agar gel precipitin and tube complement-fixation reactions appear to have little value. No agar gel precipitins or complement-fixing antibodies were detected in blood of animals during or following immunization, not even in the group that developed marginal skin test reactions. Also, no quantitative relationship was found after challenge of immunized animals between antibody occurrence and development of progressive disease.

Immunizations using both the whole-cell and fractionated cell antigen and using both the subcutaneous and aerosol methods of administration afforded protection against lethal disease but did not protect against infection with C. immitis. The best protection against lethal infection was provided by the subcutaneously administered killed whole-cell antigen. Some deaths occurred among animals in the other two groups, but the number was less than in the non-immunized controls. The C. immitis cell-fraction vaccine provided the best protection against spread of infection from the lungs and development of systemic disease. The present study also provided some evidence, although slight, for a relationship between skin hypersensitivity and resistance to lethal challenge. No deaths occurred among animals that developed a positive skin test after immunization, but some deaths occurred among those failing to exhibit skin hypersensitivity.

Calculated inhaled doses of test animals ranged from 100 to 680 viable particles. The doses were calculated from weights of the respective animals and concentration of organisms in the air of the test chamber at the time of animal exposure. Some difference in dosage was expected, since all animals could not be exposed at the same time and there was a gradual decay of

aerosolized organisms in the test chamber. The differences in dosage were not considered significant in this test. Previous experimental respiratory infection studies indicated that the LD₅₀ for monkeys occurred between 100 and 600 organisms.¹⁴ Dosage levels in the thousands of organisms are required for LD values approaching 100 per cent.

An attempt was made to use an equal-sized immunizing dose in all three test groups. The entire 24-milligram immunizing doses of killed whole cells and cell-fraction antigens were delivered by subcutaneous injection. The delivery of a 24-milligram immunizing dose of dry, dead arthrospores by aerosol for each animal was assumed. Palm *et al*¹⁵ reported 90 per cent retention by monkeys of inhaled particles in the range of approximately five microns in diameter. It was expected that some spores would be lost by the natural cleansing action of the respiratory tract in ridding itself of foreign particles. The amount of loss by this action was not known, and no effort was made to compensate for it.

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