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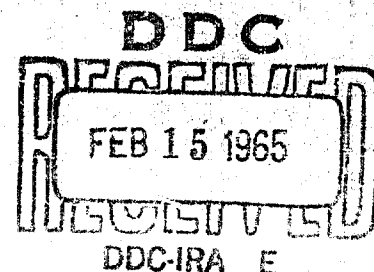
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TECHNICAL MANUSCRIPT 182

COCCIDIOIDIN SENSITIVITY
IN CONTROL, IMMUNIZED,
AND INFECTED GUINEA PIGS

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COCCIDIOIDIN SENSITIVITY IN CONTROL, IMMUNIZED,
AND INFECTED GUINEA PIGS

James T. Sinski

Special Operations Division
DIRECTOR OF DEVELOPMENT

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ABSTRACT

Delayed hypersensitivity to undiluted coccidioidin developed in guinea pigs immunized with dead C. immitis arthrospores and guinea pigs infected with an aerosol of C. immitis arthrospores at weeks 1 and 2. Delayed hypersensitivity in control animals sensitized by repeated intradermal testing developed at weeks 3 and 4. The delayed hypersensitivity responses were characterized grossly by indurations larger than 25 mm² and could be seen at 6 and 24 hours after testing. Retesting reduced the size of the 24-hour indurations compared with virginal reactions. The retest delayed reactions in infected animals had indurations at 24 and 48 hours that were larger than those in the other groups.

In those animals that were skin-test-positive but not challenged, no tube precipitins, agar gel precipitins, complement fixing antibodies, anaphylaxis, or immediate hypersensitivity was detected. Because of the lack of precipitins and the inability to produce anaphylaxis, the early phase of the hypersensitivity seen at 6 hours was not considered an Arthus reaction.

I. INTRODUCTION

Skin-test sensitivity to broth culture filtrates from Coccidioides immitis growth was produced in guinea pigs with the filtrate itself and with killed, dried, mycelium of the fungus. Those observations were made in 1927¹ before the use of coccidioidin as we know it today. For skin-test purposes the fungus was inoculated into peptone broth prepared with placenta meat extract.³ Both immediate and delayed reactions were produced in these sensitized animals.

Coccidioidin as used today is made with an asparagine synthetic medium devised to avoid antigenic and nonspecific characteristics.³ Use of coccidioidin made with asparagine synthetic medium resulted in positive skin tests in animals after injection with killed arthrospores or killed spherules. Some investigators reported that guinea pigs intramuscularly injected with killed spherules⁴ and killed arthrospores⁵ developed delayed hypersensitivity. These investigators, however did not mention observations to determine whether any other type of immunologic response was elicited by these injections of killed cellular elements. Levine et al.⁶ as well as Sinski et al.⁷ have reported very mild and modified skin reactions to coccidioidin in cynomolgus and rhesus monkeys after immunization with a killed spherule and an arthrospore vaccine respectively. In the latter study, reactions read after 24 hours consisted of a small area of erythema and induration that appeared sporadically.

The purpose of the present investigation was to observe more precisely in guinea pigs* the type of sensitivity to coccidioidin developed in (i) control animals, (ii) animals after injection with whole, killed arthrospores of Coccidioides immitis, and (iii) aerosol-infected animals. Animals were observed for immediate, Arthus, and delayed reactions.

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

II. MATERIALS AND METHODS

Male Hartley strain guinea pigs, born and raised at the Fort Detrick Animal Farm, were used. At the beginning of the investigation each animal weighed about 300 grams and was tattooed on both ears with an identifying number. The animals were divided into three groups.

The control group, consisting of ten nonimmunized animals, were skin tested weekly in the same spot on the abdomen with full strength coccidioidin plus merthiolate 1:10,000 v/v. The control group was divided into two subgroups of 5 animals each. Each subgroup was similarly skin tested several months later to verify the results obtained with the first subgroup.

The second group consisted of 25 animals, one subgroup of 20 and one of 5. The larger subgroup was immunized with three injections; each injection contained 8 mg of whole, killed arthrospores (dry weight) suspended in 0.5 ml of saline. Injections were given subcutaneously in the shoulder at 0, 1, and 5 weeks. Skin testing, which was repeated in the same spot on the abdomen began immediately after the first immunization. The subgroup of 5 animals was treated similarly to the larger group, but the investigations were separated by several months. The second subgroup was used to verify the results obtained with the first subgroup.

The third group consisted of only 5 animals. They were neither skin tested nor immunized before being infected with an aerosol of dry C. immitis arthrospores. The arthrospores of C. immitis strain Silveira were grown on Sabouraud's medium and harvested as previously described.⁸ For immunization, the harvested arthrospores were killed with formalin vapors. The spores were examined for viability by plate counts.

The coccidioidin was produced in this laboratory according to the method of Smith.³ Five strains of C. immitis were used including Silveira, Cash, M-11 strains, and two isolates of a Duke University strain. The coccidioidin was compared with a standard lot supplied by Dr. Smith, using infected guinea pigs and monkeys. The lot of coccidioidin used in this investigation produced indurations in infected animals that were within 1 mm of those produced in the same animals with Smith's standard coccidioidin. In monkeys the coccidioidin used in the present investigation produced an induration as shown by closure of the eye lid to a degree similar to that resulting from the Smith standard lot injected intradermally into the other eye lid of the animal. All standardizations used 0.1 ml of each product. To assure uniformity throughout the experiment, the coccidioidin used in this investigation was produced in one batch of 500 ml. Although this batch was divided into smaller units for storage, all skin tests during the investigation were given with the same batch.

Skin tests were given weekly in the same spot on the abdomen of each animal. The spot just below the skin test was marked with dye, and this dye spot marking was repeated but persisted from week to week. Skin tests were read with particular reference to the development of (i) immediate allergy, (ii) Arthus reaction, and (iii) delayed hypersensitivity. Readings were therefore made at 15 minutes, 6, 24, and 48 hours after skin testing. Reactions of 25 mm² or larger were considered positive.

In addition to repeated weekly skin tests in the same area on immunized and infected animals, comparisons were made with the skin reactions in a virginal site after these animals had developed definite sensitivity. The first virginal site chosen was the thigh; the second virginal skin tests were made on the back.

As further aids to determine the nature of the skin-test reaction in the immunized animals, they were (i) scratch-tested with coccidioidin, (ii) sera were tested for the presence of tube precipitins, agar gel precipitins, and complement fixing antibodies⁹ and (iii) at the conclusion of the experiment several noninfected animals were given one to two ml of coccidioidin intracardially to observe possible development of anaphylaxis. At the termination of the investigation, all the challenged animals were autopsied and examined for coccidoidal lesions.

III. RESULTS

A. REACTIONS IN CONTROL ANIMALS

In control animals that were repeatedly skin-tested in the same spot with undiluted coccidioidin plus merthiolate no immediate allergic reaction, i.e., wheal and flare, was seen in any of the animals at any time during the experiment (Figure 1). An edema did develop at 15 minutes in most of these animals after the first skin test. This reaction was first observed at the first week after the experiment began (as the second skin testing). The reaction continued to be evoked upon subsequent retesting for the duration of the experiment. At weeks 12 and 13, when skin tests were made in virginal areas as well as the retest areas, these edematous reactions developed at both sites to a size greater than 25 mm² and were generally about equal in size.

Positive 6-hour reactions were recorded at week 4 and following week 7. This reaction was characterized by an induration rather than an edema. There were no individual indurations seen at 6 hours that were positive before week 4. No necrosis nor hemorrhage was seen in any of these animals at the 6-hour readings.

The average indurations at 24 hours were negative in control animals except for those at week 8. Then, the average induration at 24 hours was 25 mm². Although average indurations of 25 mm² did not occur until week 8, individual positive indurations (not shown in Fig. 1) were observed at week 3. During weeks 12 and 13, when virginal skin tests were made as well as retests, the average virginal 24-hour reaction at week 13 was larger than 25 mm². All other average readings at 24 hours were below that considered positive. The average indurations read at 48 hours in these control animals skin-tested with coccidioidin were negative. This lack of response was noted in both the retest and virginal areas. Only on very few occasions was an individual retest positive.

B. REACTIONS IN IMMUNIZED ANIMALS

The same general pattern of development of skin-test response was seen in animals subcutaneously injected with dead *C. immitis* arthrospores (Figure 2). No evidence of immediate allergy was seen at 15 minutes in these immunized animals. After week 1 test period, an edema was seen at the 15-minute readings, and this reaction continued to be an average size of 100 mm² at each testing. The average 15-minute responses to virginal and retests at weeks 10 and 11 were similar. Slight, but negative, average 6-hour indurations were observed at week 1. At week 2, positive, average 6-hour indurations of 630 mm² were observed. Average 6-hour indurations

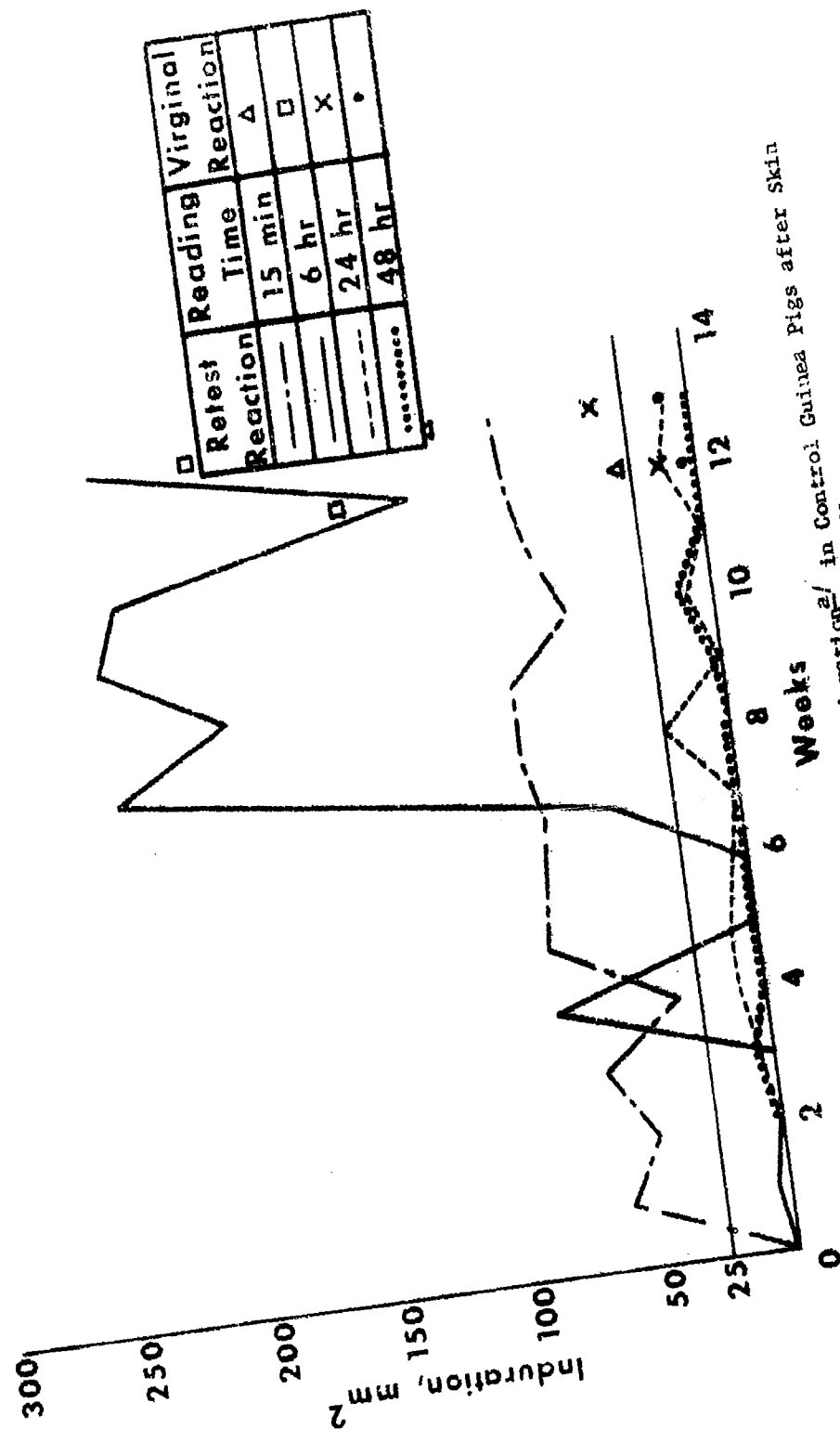


Figure 1. Average Size of Induration^{2/} in Control Guinea pigs after Skin Testing with Undiluted Coccidioidin.

a. measurement of edema for 15 minute reactions

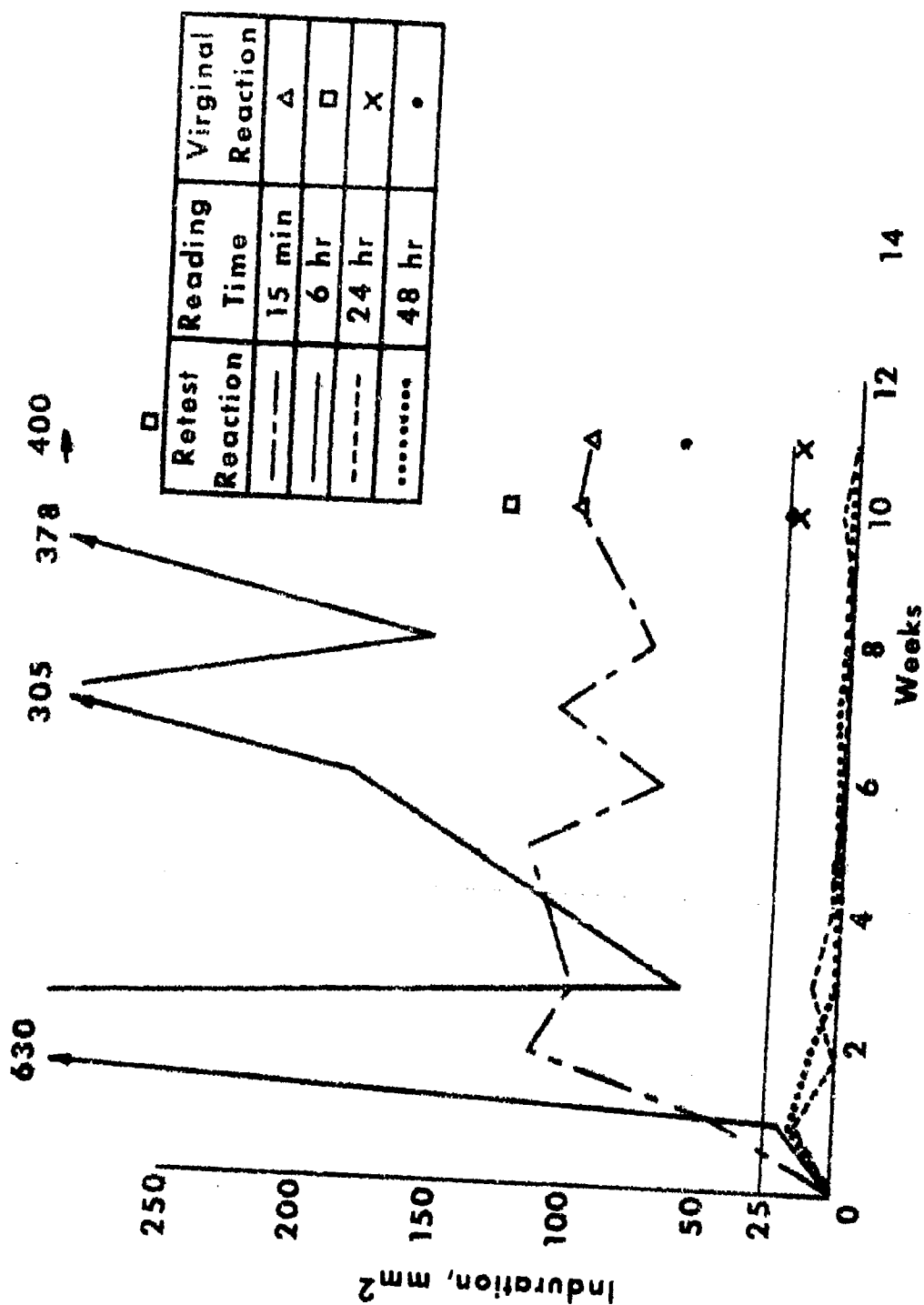


Figure 2. Average Size of Induration^a in Immunized Guinea Pigs after Skin Testing with Undiluted Coccidioidin.

a. measurement of edema for 15 minute reactions

were all positive after week 2. At weeks 10 and 11, when retest and virginal responses were compared, the average virginal responses seen at 6 hours were not as large as the retest responses. The average induration at week 10 in the retest area was 378 mm²; the average virginal induration was 130 mm². The average induration at week 11 in the retest area was 400 mm² while the average virginal induration was 265 mm². The average induration at 24 hours in these sensitized animals was negative, although there were individual positive reactions at 24 hours after the first skin test. At weeks 10 and 11 the average indurations observed at 24 hours in virginal sites were positive; the average retest indurations, however, were almost negative at these time periods.

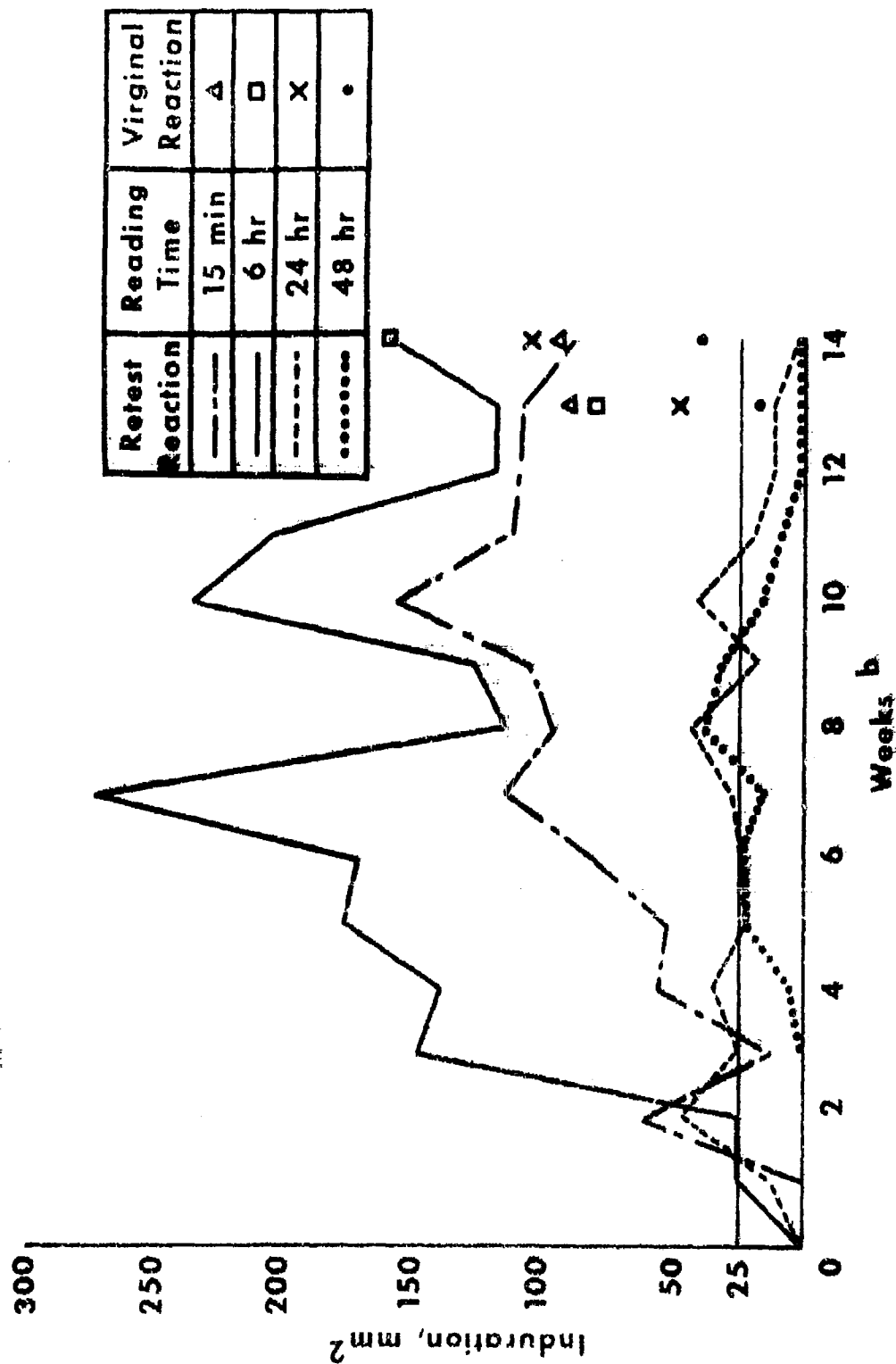
Average indurations in those sensitized animals seen at 48 hours were also negative. Individual 48-hour reactions were positive at week 1 in this sensitized group, but these soon became negative. Only at weeks 10 and 11, when skin tests were given in virginal areas, were the individual reactions (not shown in Fig. 2) positive. At week 10 the average induration was 25 mm² and at week 11 the average size of induration was 21 mm².

C. REACTIONS IN INFECTED ANIMALS

Animals infected with an aerosol of *C. immitis* developed an edema reaction at 15 minutes (Figure 3). The magnitude of this reaction did not change at weeks 13 and 14, when virginal and retest areas were used. No wheal and flare reaction developed in these animals at any time. In these infected animals the average 6-hour indurations were positive by the first week after exposure. These average-size indurations fluctuated about a level of 150 mm².

By week 1 test period, or 10 days after infection, two animals had 24-hour positive coccidioidin reactions. Following week 2 the average induration seen at 24 hours in this infected group was generally positive. After week 10 the average indurations were negative in retest areas. At weeks 13 and 14, the 24-hour virginal test reactions were positive.

The time of development of average positive indurations during the span of the experiment at which 48-hour reactions were seen was longer than the time of development of the average 24-hour positive indurations. The average induration at week 6 was large enough to be considered positive. Before that, only one animal had an induration at 48 hours large enough to be considered positive. After week 9 in this infected series the average induration size recorded at 48 hours began to drop. It was only at week 14 in the virginal skin test sites that the average induration was positive (37 mm²).



Skin-test results were similar in the additional control and immunized groups, and thus the observations made on the original animals were verified.

All scratch tests for allergic response, using coccidioidin as the antigen, were negative.

In the control and immunized groups of animals all serologic determinations, tube precipitin, agar gel precipitin, and complement fixation reactions were negative on representative serum samples from animals exhibiting positive skin-test response. Also, in animals of the control and immunized groups that gave positive delayed skin test reactions, the intracardial introduction of one or two ml of full strength coccidioidin failed to produce anaphylaxis.

Upon autopsy all animals that had been exposed to aerosols were found to be infected with C. immitis.

IV. DISCUSSION

Delayed hypersensitivity to coccidioidin was observed in individual guinea pigs that were immunized or infected as early as weeks 1 and 2 after these experimental conditions were applied. It was only at weeks 3 and 4 that the delayed hypersensitivity response was observed in individual control animals that had been skin-tested weekly with full strength coccidioidin. As described by Boughton and Spector¹⁰ for the tuberculin reaction in guinea pigs, the delayed hypersensitive response is biphasic with peak activities at 3 hours and subsiding at 5 hours and another peak beginning at 8 hours and subsiding at 24 to 48 hours. Although no variation in the gross sizes of the indurations between 6 and 24 hours were observed in the present investigation, the definite early response was seen. A second interpretation of the results obtained in the present experiment is based on the observations of Salvin.¹¹ The early portion of the hypersensitive reaction observed in the present investigation at 6 hours after skin testing could be an Arthus-type response.

The development of delayed hypersensitivity to full strength or potent coccidioidin in the present series of control guinea pigs is at variance with the observations made by Smith et al.³ In their investigations 1 ml. of undiluted coccidioidin was given intracutaneously in one series and 5.0 ml intraperitoneally in another series at weekly intervals four times. Other guinea pigs were given 0.1 ml undiluted coccidioidin daily for three weeks. The route was not mentioned. A month after injections ceased the animals still did not react to 0.1 ml of undiluted coccidioidin. The reason for the variance between Smith's results and the present results is not known. Differences in amounts, routes, time schedules, or the particular batch of coccidioidin may account for the difference. Although Hirsch and D'Andrea¹ did develop delayed hypersensitivity to repeated skin tests, the composition of the medium they used differed from that established by Smith et al.³ and that used in this investigation.

The development of delayed hypersensitivity to undiluted coccidioidin does seem consistent with the work of Howell¹² in which normal guinea pigs were sensitized by undiluted histoplasmin, blastomycin, and an autoclaved filtrate of Candida albicans.

In animals that react poorly to coccidioidin and therefore must be skin-tested repeatedly with undiluted skin-test antigens, the possibility of developing delayed hypersensitivity from the coccidioidin injections must be considered. The use of diluted coccidioidin in man or other animals is not known to sensitize by repeated injections,^{3,12} and these might be used in such poorly reacting animals. But if dilute antigens are used the possibility of getting false negative reactions must also be considered.

Because the animals did develop delayed hypersensitivity and then were skin-tested in the same site the retest phenomenon is in effect. Sites at which positive skin tests have been elicited exhibit an accelerated reaction on reinjection of skin test material. Arnason and Wahsman¹⁵ in their recent investigations of the retest reaction in delayed sensitivity have shown that the retest reaction reaches its acme at 6 to 8 hours. Similarly, in the present investigations the 6-hour reactions were prominent and the 24- to 48-hour reactions were much reduced and average size indurations were negative. When skin tests were made in virginal areas the inhibition of the 24-hour reaction did not occur and positive reactions were observed.

The 6-hour reaction was not considered an Arthus reaction for several reasons. Although an edema, an early characteristic of Arthus reaction, did develop after 15 minutes, no necrosis nor hemorrhage was seen in any of the skin tests for the 6-hour reading. Precipitins were not detected in the sera of those animals from which blood was drawn. It must be admitted, however, that the method used for detection of precipitins was not as sensitive as that used by Salvin.¹¹ Also, representative animals did not go into anaphylactic shock when full strength coccidioidin was introduced into the circulatory system. These tests were negative, and therefore they indicate that the 6-hour reaction may be a manifestation of the biphasic nature of the delayed response, although an attempt to show positively that this reaction was a delayed one was not attempted in guinea pigs. The transfer of the sensitivity developed in the present investigation by mononuclear cells or serum would aid in the characterization of this reaction.

Although edema was produced in most animals upon repeated skin testing with full strength coccidioidin, the significance of this reaction was not investigated.

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