IMMUNITY TESTS IN COCCIDIOIDAL GRANULOMA*

JEAN V. COOKE, M.D.

SAN FRANCISCO

Human infection by the organism now known as *Coccidioides immitis* was first reported in 1892 by Posadas and Wernicke in Buenos Aires. These observers regarded the parasites as protozoa, but later (1896) Rixford and Gilchrist gave the organism the binominal designation of *Coccidioides immitis*, and Ophüls succeeded in obtaining pure cultures in 1900. Other cases have been recognized from time to time, and recently MacNeal and Taylor¹ have collected twenty-four cases from the literature. In one of these reported by Brown, however (Case 18), the diagnosis was not confirmed culturally or by microscopic examination. All the reported cases except the first have occurred in the United States, and in a large proportion of them the infection can be traced to the San Joaquin Valley in California. The disease affects chiefly adult males, only one woman and one child being included in the cases observed. Only two patients are known to have recovered.

The infection is characterized by the formation of successive nodular or suppurative foci which may affect any part of the body, but more often are localized in the bones and joints, lymph-nodes, lungs and skin. The clinical symptoms depend on the anatomical distribution of the lesions. Histologically, the process resembles tuberculosis and belongs to the group of the infectious granulomata, the disease usually being termed, therefore, coccidioidal granuloma. In sections and in pus from the lesions the organism occurs as refractile, doubly-contoured, spherical bodies varying from 3 to 50 microns in diameter. These differ from the parasites found in blastomycosis in that budding forms are never seen in the animal body. In the tissues *Coccidioides immitis* multiplies by endosporulation and mycelium is never found, but when transplanted on artificial culture mediums the spherical bodies form mycelium almost immediately. Growth appears usually in less than a week and is more rapid at incubator temperature. Preparations from cultures show septate branching hyphae on which after a period of time chlamydospores develop. Dogs, rabbits and guinea-pigs can easily be infected with pus from one of the lesions or with emulsions from cultures. For more details concerning the clinical, pathological

* Submitted for publication Sept. 29, 1914.

* From the Pathological Laboratory of the University of California Hospital.

1. MacNeal and Taylor: Jour. Med. Research, 1914, xxx, 261.

and microbiological features of the infection reference may be made to the papers of Ophüls,² Wolbach,³ Hektoen,⁴ Brown⁵ and MacNeal and Taylor.¹

The purpose of this paper is to add two other cases to the list of those already observed, and to report the results of a series of experiments designed to demonstrate specific immune substances in the blood of one of these patients.

CASE REPORT

History.—A Portuguese man (M. M.), aged 25, was admitted to the surgical wards of the University of California Hospital, Dec. 1, 1913, with multiple abscesses. He was born in the Azores Islands, but had been in this country for many years. During the three months preceding his present illness he had been employed as a milker on a ranch near Newman, Cal. (San Joaquin Valley). His family history and past history were both negative. He remembered no previous illnesses and had not been associated, so far as he knew, with anyone affected with ulcerations on the skin or with abscesses.

The history of his illness follows: About nine months previous to admission he noticed a painful lump on his right wrist. This was opened by a physician and exuded pus for a considerable length of time. Later his right knee began to swell and after three months suppurated. It was aspirated by another physician and placed in a plaster cast, but as the rapid suppuration continued he was removed to a hospital and the joint was resected. The wound did not heal after this operation but continued to discharge pus. During this time other suppurative foci appeared over the right shoulder, left arm, left ankle below the external malleolus, and two foci beneath the scalp.

Physical Examination.—On admission (Dec. 1, 1913) the right knee showed numerous sinuses and the resected joint was mobile and painful; the right foot showed a sinus at the first distal metatarsal joint, running up the inner side of the foot to an ulcer over the Achilles tendon; the right wrist was riddled with sinuses discharging pus, the carpus seemed disintegrated and the lower end of the radius was swollen; left foot showed a discharging sinus below the outer malleolus, the left ulna was thickened and just below its middle showed a painful soft fluctuating mass about 6 cm. in diameter; two sinuses in the scalp ran down to the skull, one over the frontal bone just to the right of the median line and the other about 7 cm. posterior to the first; the left knee joint was distended and painful. The ulcerated areas at the sinus openings showed flat irregular granulations at the edges and were bathed in a purulent secretion.

The patient appeared considerably emaciated and was quite weak. Physical examination was otherwise negative except for a moderate enlargement of the cervical, axillary and inguinal lymph-nodes and a slightly impaired resonance at the left pulmonary apex.

Course.—The left knee gradually became more swollen and was aspirated several times. December 24 it was opened and 1,000 c.c. of pus removed. During the next two months the patient became weaker and more emaciated, all the lesions continued to discharge abundant purulent material, and new suppurative foci appeared on the left hip, right axilla and left shoulder.

March 8 the patient left the hospital against advice. He died a few days later and no necropsy was permitted.

2. Ophüls: Jour. Am. Med. Assn., 1905, xlv, 1291.

3. Wolbach: Jour. Med. Research, 1904, xiii, 53.

4. Hektoen: Jour. Am. Med. Assn., 1907, xlix, 1071.

5. Brown: Jour. Am. Med. Assn., 1907, xlviii, 743.

During his stay in the hospital his temperature ranged between 37 and 38 C., and the leukocytes varied from 12,000 to 20,000; hemoglobin was 46 to 60 per cent. (Dare); urine was negative for sugar and albumin. Blood-cultures taken on three occasions were negative. Wassermann was negative. The spherical doubly-contoured organisms were demonstrable in fair numbers in every specimen of pus examined. One specimen of sputum was reported to contain coccidioides, but this could not be verified as no more sputum could be obtained.

Medication.—Two doses of neosalvarsan, 0.6 gm., were given intravenously and also large doses of potassium iodid, without any apparent effect.

The Parasite.—Numerous examinations of pus from the various lesions showed pus cells and rather numerous refractile doubly-contoured spheres which varied considerably in size; some were no larger than a red blood corpuscle, while the largest one measured was 40 microns in diameter. All gradations between these sizes were seen. The spheres were for the most part composed of a coarsely granular protoplasm in which little structure could be distinguished. Some cells, however, appeared filled with small spheres each of which had a sharply defined individual capsule, and occasionally the smaller bodies could be seen escaping from one of these sporangium-like forms. No budding forms were ever observed.

Cultures at incubator temperature showed a definite tenacious growth at the end of twenty-four hours, which after a few days spread over the whole surface. Old cultures, especially those kept at room temperature, developed abundant aerial hyphae and appeared fluffy. In block preparations on glucose agar, the spore-like bodies from the pus at the end of twenty-four hours showed thread-like outgrowths of mycelium which later became branched and segmented. In older cultures the extremities of the segments became bulbous or clubbed and chlamydospores developed on some of the hyphae.

Male guinea-pigs were inoculated both with a few drops of pus from one of the lesions and with a small amount of an emulsion of a two-months-old agar culture. After a few weeks there occurred in both cases the typical marked enlargement of the testes and the animals were killed. At autopsy suppurative foci were found in the testes, lungs and liver, from which the spherical doubly-contoured bodies were readily demonstrable and pure cultures were recovered.

The patient was an Italian man aged 29, and the history obtained was very meager. Nine months previous to his admission, while at Bakersfield (San Joaquin Valley), he noticed a swelling of the cervical glands. He had been treated for some time in a tuberculosis sanatorium.

Physical examination showed an emaciated man who breathed with difficulty on account of large nodular masses in the neck. On the forehead above the left eye was a large sinus about 4 cm. in depth from which thick yellowish pus was discharging.

At the base of this sinus was necrotic bone. On the left shoulder were two discharging sinuses which extended beneath the clavicle and a similar open sinus was present in each supraclavicular fossa. In the neck extending downward from the angles of the jaw and in the posterior cervical triangles could be felt large, somewhat softened nodular masses. Both lips showed several healed scars. Aside from the special lesions noted, physical examination was negative. On the fourth day after admission the patient died from asphyxiation. Examination of pus from the discharging lesions showed numerous refractile spherical, doubly-contoured bodies, varying somewhat in size, but as a rule about three times as large as a pus cell. Many of these spheres were apparently filled with numerous small spore-like bodies. No budding forms could be found. Cultures gave a mold-like growth.

An additional case of coccidioidal granuloma was observed in July, 1914, at the Santa Clara County Hospital, San Jose, Cal., by Dr. Elizabeth G. Lewis, to whom I am indebted for the following clinical and post mortem notes:

At necropsy the numerous walnut-sized masses in the neck noted in the clinical examination were abscesses filled with yellowish pus, and there was also a large postpharyngeal abscess containing similar material. The apex of each lung was firmly bound by adhesions and formed part of the wall of an abscess which was discharging through a sinus above the clavicle. Section of the lung itself, however, showed no gross lesion and nothing noteworthy was noted in the examination of the rest of the viscera.

Tissue saved for microscopic study was unfortunately thrown away by mistake. The only material available for examination was an agar culture from the pus. This corresponded in all respects to the cultures of *Coccidioides immitis* from the case above described.

ANTIBODIES

This patient was under observation for some time and attempts were made to ascertain what demonstrable antibodies were formed in response to the infection. The antigens used for this purpose were made from the strain of *Coccidioides immitis* obtained from the patient and consisted of the two forms of the organism—(1) the mycelial growth from artificial culture mediums, and (2) the spore-like bodies from one of the purulent lesions. The mode of preparation of these antigens was as follows:

1. *Antigen from mycelial growth.*—A considerable number of agar cultures of *Coccidioides immitis* about four weeks old were used. The cotton plugs were treated with a few drops of formaldehyd solution and sealed. After twenty-four hours the tenacious growth was removed with a platinum spatula and dried over sulphuric acid. It was then powdered by grinding for several hours in an agate mortar.

2. *Antigen from the organism in the body.*—One thousand cubic centimeters of rather thick pus from an abscess (knee) was treated with 162 c.c. of anti-formin and allowed to stand with occasional shaking for five hours. It was then centrifuged and the sediment washed five times with 0.85 per cent. NaCl solution, after which the reaction was neutral to litmus. Microscopic examination showed that the sediment was composed almost entirely of the spherical doubly-contoured bodies. Cultures remained sterile. This sediment was dried over sulphuric acid and the residue ground for several hours in an agate mortar. A second specimen of 500 c.c. of pus was similarly treated with anti-formin, centrifuged and washed. A rather thick emulsion of the spherical bodies was made in 0.85 per cent. NaCl solution. This latter was used in the agglutination tests and was also used as antigen in some of the complement fixation tests.

Controls.—A similar powder was made in exactly the same manner from cultures of a strain of blastomyces from one of the Chicago cases. This organism showed many budding forms in culture in addition to mycelial growth.

Normal serums used in all the controls were from ambulatory patients not suffering from any infectious disease.

COMPLEMENT FIXATION TESTS

The antigens used for these tests consisted of 0.5 per cent. emulsions of the powdered growth of coccidioides and blastomyces in 0.85 per cent. NaCl solution and in addition an emulsion of the spore-like forms of coccidioides obtained from the pus. A rabbit-antisheep

hemolytic system was used in all tests and the complement and hemolysin were always titrated before use. In the tests the inactivated patient's serum with antigen and $1\frac{1}{2}$ units of complement were incubated for one hour and then two units of hemolysin (1:2,000) and 1 c.c. of a 5 per cent. suspension of sheep corpuscles added. The tubes were reincubated for two hours and placed in the icebox. The final readings were made the following day. Two normal serums were used as controls. On titration it was found that the emulsions of the dried mycelium of coccidioides inhibited hemolysis in amounts larger than 0.07 c.c., and that of blastomyces in amounts larger than 0.05 c.c., while the coccidioides "spores" did not inhibit in dilutions of 0.3 c.c. Varying amounts of serum up to 0.5 c.c. were tested and the results were entirely negative. Another similar series was carried out, using inactivated serum collected the same day the tests were done. This also gave negative results.

CUTANEOUS REACTIONS

Since certain antigens like tuberculin, typhoidin and others cause a specific skin reaction, it was thought of interest to try similar reactions with coccidioides in the present case. Boughton and Stober⁷ have reported an apparently specific skin test in a case of blastomycosis, although it is stated that the patient was recovering from the infection at the time the test was done. Davis,⁸ however, was unable to obtain a positive skin reaction in guinea-pigs experimentally infected with a similar organism. In applying the tests both the intradermal method and scarification with a von Pirquet chisel were used. The following antigens were employed in each test: (1) A rather thick suspension of the powdered mycelial growth of coccidioides suspended in 0.85 per cent. NaCl solution; (2) a 5 per cent. glycerin emulsion of the same dried powder concentrated to one-tenth its original volume; (3) a concentrated glycerin bouillon filtrate from a four-weeks-old *coccidioides* culture prepared in a manner analogous to that used for making "old tuberculin"; (4) a rather thick suspension in 0.85 per cent. NaCl solution of the powdered spherical bodies from the pus; (5) and (6) suspensions of dried blastomyces growth prepared in a similar manner to (1) and (2). Controls of 0.85 per cent. NaCl solution and 50 per cent. glycerin were also used. A small drop of each of these was placed on a small area of scarification on the skin of the arm made with a von Pirquet chisel and allowed to dry. If the material had not dried at the end of five minutes, the excess was gently removed with absorbent cotton. No indication of any reaction appeared around any of

7. Boughton and Stober: THE ARCHIVES INT. MED., 1914, xiii, 599.

8. Davis: Jour. Infect. Dis., 1911, viii, 190.

the areas in the five succeeding days. The tests were repeated one month later and on two control cases with the same result.

In the intradermal tests, a very small amount of each of the antigens was injected in the superficial layers of the skin of the arm. In all except the controls and (3) the concentrated broth filtrate, there was formed an indurated red area of reaction from 5 to 10 mm. in diameter which at the end of forty-eight hours became pustular. The pus was sterile and the lesions had practically disappeared at the end of a week. This same reaction occurred in six control patients on whom the same tests were done and undoubtedly resulted from irritant substances present in the organisms themselves. The same tests were repeated after the various antigens had been diluted until no reaction was caused in control patients and the diluted antigens caused no reaction in the case of coccidioid granuloma.

AGGLUTINATION TESTS

The suspensions of coccidioides used in these tests were obtained as above described by treating a specimen of pus with antiformin, and consisted of a considerable number of the spherical doubly-contoured bodies in salt solution. The hanging drop method was used and two series of preparations made; one was incubated at 37 C. and the other left at room temperature. The dilutions made of the serum were 1:5, 1:10, 1:20, 1:40 and 1:80, and in each experiment the mixtures were made of (1) immune serum plus suspension, (2) normal serum plus suspension, and (3) salt solution plus suspension. Both the incubated series and that left at room temperature were examined at intervals of one hour for the first six hours, and again at the end of twenty-four hours. The results were entirely negative.

PRECIPITIN TESTS

For these tests 0.3 gm. of the powdered mycelial growth of coccidioides was thoroughly ground with 30 c.c. of 0.85 per cent. NaCl solution to which 0.5 per cent. phenol had been added. This suspension was allowed to stand for several days, being shaken at intervals, and then centrifuged. The faintly opalescent supernatant fluid was employed in the tests. Similar extracts were made from the powdered coccidioides "spores" and from dried blastomyces cultures.

The first series of tests was carried out by stratifying the antigen above the serum in such a manner as to give a sharp line of contact. The tubes were incubated and readings made at the end of one hour and two hours. The various antigens were used in their full strength and also diluted 1:2, 1:5, 1:10 and 1:20, with 0.85 per cent. NaCl solution. As controls a solution of 0.85 per cent. NaCl solution with

0.5 per cent. phenol and normal serums were used. In all, serums from twelve control patients were tested. Table 1 gives the results of these experiments.

Another series of precipitin tests was carried out in the usual way by mixing various amounts of serum with the same antigens used in the previous experiment. The technic used was as follows: Varying amounts of clear serum from the patient with coccidioidal granuloma were added to a constant amount of the antigen extracts in small test-tubes and the mixture diluted to a uniform volume by the addition of 0.85 per cent. NaCl solution. The tubes were then incubated for two

TABLE 1.—RESULTS OF PRECIPITIN TESTS

Extract, 0.5 c.c.	Serum from Case of Coccidioides, 0.5 c.c.	Normal Serum * 0.5 c.c.
Coccidioides culture	Heavy ring	No precipitate
Coccidioides 1:2½	Distinct ring	No precipitate
Coccidioides 1:5	Faint ring	No precipitate
Coccidioides 1:10	No precipitate	No precipitate
Coccidioides 1:20	No precipitate	No precipitate
Coccidioides "spores"	Distinct ring	No precipitate
Coccidioides "spores" 1: 2½ ..	Faint ring	No precipitate
Coccidioides "spores" 1: 5	No precipitate	No precipitate
Coccidioides "spores" 1: 10	No precipitate	No precipitate
Coccidioides "spores" 1: 20	No precipitate	No precipitate
Blastomyces	No precipitate	No precipitate
Blastomyces 1: 2½	No precipitate	No precipitate
Blastomyces 1: 5	No precipitate	No precipitate
Blastomyces 1: 10	No precipitate	No precipitate
Blastomyces 1: 20	No precipitate	No precipitate
NaCl solution 0.85 per cent. + phenol 0.5 per cent.	No precipitate	No precipitate

* Eleven other normal serums were tested with the same result.

hours at 37 C. and placed in the ice-chest. The readings were made at the end of twenty-four hours. The usual controls were introduced as may be noted from Table 2.

SUMMARY

In a case of coccidioidal granuloma studied no specific complement-fixing bodies or agglutinins could be found in the blood-serum using cultures of *Coccidioides immitis* and emulsions of the same organism from human lesions as antigens. No specific skin reaction could be demonstrated. Precipitins, however, could be demonstrated in the serum even when diluted 1:160, when an extract of dried cultures of the organism was used as precipitinogen. These precipitins were apparently specific, since they could not be demonstrated when normal

serum was tested with the same antigen or when the specific immune serum was tested with an antigen similarly prepared from the closely related organism, blastomyces.

The presence of specific precipitins in this infection must be verified by the examination of other cases. It is suggested that this reaction might be applied as a means of diagnosis in cases of deep-seated infec-

TABLE 2.—ADDITIONAL PRECIPITIN TESTS

	Extract Coccidioides Culture	Extract Coccidioides "Spores"	Extract Blastomyces Culture	NaCl Solution 0.85 Pct.
Immune serum 1: 10	+	+	0	0
Immune serum 1: 20	+	+	0	0
Immune serum 1: 40	+	+	0	0
Immune serum 1: 80	+	0	0	0
Immune serum 1: 160	+	0	0	0
Immune serum 1: 320	0	0	0	0
Normal serum 1: 10*	0	0	0	0
Normal serum 1: 20	0	0	0	0
Normal serum 1: 40	0	0	0	0
Normal serum 1: 80	0	0	0	0
Normal serum 1: 160	0	0	0	0
Normal serum 1: 320	0	0	0	0
NaCl solution 0.85 per cent. and phenol 0.5 per cent.	0	0	0	0

+ = precipitate; 0 = no precipitate.

* A series of nine other normal serums was tested with negative results.

tion where there are no discharging lesions from which the spherical doubly-contoured bodies can be demonstrated. It might also serve as a means of differentiating coccidioidal granuloma from blastomycosis in obscure cases.

Experiments are now being carried out to determine whether specific immune substances are formed in infected animals.

36 Farnsworth Lane.