The Use of Immunofluorescent Techniques In Diagnosis of Clinical Histoplasmosis*

Sarah H. W. Sell, M.D., F.C.C.P., Amos Christie, M.D. and Nancie S. Schweikert, M.D.**

INTRODUCTION

Fr from the early misconception that human histoplasmosis was a disseminated, uniformly fatal fungus disease,\(^1,2\) a benign or primary form was described in 1945\(^3\) and its relationship to pulmonary calcification in non-reactors to tuberculin was firmly established. During recent years, a therapeutic agent, amphotericin B, has been developed which is especially effective for the more serious, progressive and disseminated varieties of the disease. Herein lies the challenge to the skill of the physician since early and accurate diagnosis must, as it is in other infectious diseases, be at a premium in the improvement of morbidity and mortality.

The inadequacy of diagnostic methods to verify the clinical impression of active histoplasmosis has been a source of frustration to the clinician. The fastidious nature of this fungus with its peculiar requirements for cultural growth makes this means an irritatingly delayed period in the diagnosis. Two or three weeks might be necessary, particularly in relatively unsophisticated microbiologic hands. Morphologic studies of biopsy material are not entirely satisfactory since the identification of the ring or yeast cell forms of the fungus, particularly in blood or bone marrow, as well as liver, spleen and lymph node, are not easy to identify even for the experienced pathologist. Skin testing has its limitation since most individuals living in endemic areas have positive skin tests. Conversion, however, has the same significance as tuberculin testing with recent conversion, particularly in young children, indicating active disease. Unfortunately, in the disseminated varieties of histoplasmosis, the skin test, as in overwhelming infection with tuberculosis, is likely to be negative. Immunologic methods, such as complement fixation, have been developed by us and by others,\(^4\) but because the response is an acute phase reaction, it is frequently possible to miss the rise, or in other cases the need to wait for paired sera necessitates delay in the accumulation of weight of evidence to support the diagnosis.

All of these diagnostic dilemmas then underline the need for other techniques to be readily available in order to establish the early and accurate diagnosis of human histoplasmosis. The toxicity and the economic considerations in the use of amphotericin B make it necessary to be certain before undertaking a therapeutic regimen in a hospital which might require six to 12 weeks. In the primary or relatively benign forms of the disease, speed in diagnosis is not so important, but the differential diagnosis with its involvement of malignant conditions and other granulomatous disease, or with virus and atypical primary pneumonias, make the need for accurate and early diagnosis equally demanding. It is to this end that immunofluorescent techniques\(^5,6\) were explored for usefulness in diagnosis of human histoplasmosis.

The present report presents adapted immunofluorescent techniques and examples from groups of cases in which we have found them to be useful in clarifying the diagnosis of human histoplasmosis.

METHODS

Antiserum for Histoplasma capsulatum was prepared in rabbits by the intravenous injection of six-day old yeast phase cells.\(^\ast\) These were washed three times and packed by centrifugation. A 1:100 suspension of the sedimented cells was prepared in saline which contained 0.5 per cent formalin. Amounts of 1, 2, and 5 ml of the suspension were given on each of three successive days in a week\(^7\) for three weeks. The fourth week, the rabbits were challenged with 1 ml of a 1:100 suspension in saline of living yeast-phase organisms. Ten days later, the blood was collected, serum separated and pooled. The complement fixation titer was found to be >1:256 with yeast-phase antigen and 1:128 with histoplasmin.

Antihistoplasma globulin was prepared and tagged with fluorescein isothiocyanate by the method described by Cherry et al.\(^8\) Specificity was insured by adsorption with yeast-phase cells of Blastomyces dermatitidis, Candida albicans and Coccidioides immitis until there was no cross-staining with these strains.\(^6\) For identification of cultures, aqueous suspensions of the growth were spread thinly onto non-fluorescent glass slides and quickly air-dried. After direct staining by the fluorescein-tagged globulin,\(^9\) the slides

---

\(^*\) From the Department of Pediatrics, Vanderbilt University School of Medicine, Nashville, Tennessee.

\(^\ast\) Supported by Public Health Service Research Grant No. CC 00106 from National Communicable Disease Center, Atlanta, and the Davidson County Tuberculosis Association, Nashville.

---

\(^\ast\) Strain A-28 obtained from Dr. William Kaplan at National Communicable Disease Center, Atlanta.
were examined with a Leitz ultraviolet microscope which was fitted with a mercury lamp (HBO-200) light source and OC-1 filter (Fig 1).

To gain experience with immunofluorescent techniques in tissues, we followed Procknow's procedures for producing experimental histoplasmosis in mice. The fresh and fixed sections were used for the preliminary studies. The problem of the non-specific fluorescence, especially in phagocytes, was diminished by adsorption of the reagent with equal volumes of mouse liver powder. In addition, counter-staining with lissamine-rhodamine in bovine albumin was helpful. By trial and error, we found that a final dilution of 1:8 was effective. The technique consisted of adding together equal parts of the immunofluorescent reagent and lissamine-rhodamine (1:4) which were then applied to the slide for staining. Careful rinsing was essential to remove the excess stain while retaining the integrity of the tissue attached to the slide. Routine paraffin sections of human biopsies, cut at 3μ thickness, were stained satisfactorily after removal of the paraffin. Fresh biopsy material was minced and spread thinly on the slides to dry in air. In agreement with others, we found that the staining reagent which was adsorbed with mouse-liver powder had a short fluorescent life, e.g. two to three months even when stored in the freezer. Examples of the fluorescent antibody staining of *Histoplasma capsulatum* in human tissues are shown in Figure 2.

The following cases illustrate the usefulness of the immunofluorescent techniques in establishing the diagnosis of histoplasmosis in patients whose differential diagnosis presented serious problems for the clinician.

**Case Reports**

**Case 1:**

A four and one-half-month-old infant was considered to be normal until, at two months of age, he developed pneumonia characterized by coughing, rapid respiration and fever. Treatment with penicillin for four weeks was not helpful. He refused feedings, lost weight and failed to progress in motor development. Two days before admission to Vanderbilt Pediatric Service, he became excessively irritable and vomited.

Physical examination revealed a seriously ill, poorly nourished and developed infant with elevated temperature, excessive irritability, pallor and marked hepatosplenomegaly.

Helpful diagnostic procedures included a chest roentgenogram which revealed bilateral pulmonary peribronchial infiltrates and hemogram which showed hemoglobin of 8.7 gm per cent with lymphocytosis and elevated sedimentation rate. Repeated bone marrow aspirations failed to reveal *H. capsulatum*. Repeated skin tests with histoplasmin gave negative results. Complement fixation titer was reported to be 1:5. Surgical biopsies of the liver and peritoneal fluid were obtained. Smears and sections were stained immediately with the immunofluorescent reagent. *H. capsulatum* was identified. The cultures which grew in ten days from both liver and peritoneal fluid were identified, in the early yeast phase, by the immunofluorescent techniques and were later verified by the routine cultural methods. The cultures of the bone marrow aspirations were all negative after four weeks' incubation.

**Course:** This child was treated with triple-sulfa, but since he failed to improve, a course of amphotericin B was given. His recovery was striking. The hepatosplenomegaly cleared in six months. His growth and development have proceeded normally during the three-year period since that time.

**Comment:** The clinical impression of histoplasmosis, on admission of this child, was difficult to verify since the skin tests and bone marrow aspirations were negative. The recognition of *H. capsulatum* in the material from biopsy of the liver and peritoneal fluid gave the positive answer more quickly than the routine sections or cultures. The identification of the cultures in the yeast phase gave an answer.
earlier than the routine methods which depended upon the development of typical tuberculous chlamydospores.

Case 2: A 20-month-old child had been seen in our Well Baby Clinic since birth. At age 13 months, the routine skin test for tuberculin was negative, but histoplasmin was strongly positive. The child was asymptomatic, growing and developing normally until age 19 months when she had mild respiratory symptoms with cough and elevated temperature. Later, wheezing was noted. Over a two-week period, dyspnea developed as the wheezing increased. Chest roentgenograms showed bilateral nodular parenchymal lesions with fluid in the interlobar fissures. The differential diagnoses included metastatic disease (e.g. Wilm's tumor), lymphoma, or granulomatous disease. On admission to the wards, her physical examination revealed her to be a well-developed, thin, tachypneic child in mild respiratory distress. The pertinent physical findings included posterior cervical lymphadenopathy with slight erythematous rash over the anterior chest. Generalized ronchi were heard over both lung fields with wheezes, but no rales. The liver and spleen were not enlarged. Extensive diagnostic procedures failed to confirm any of the likely diagnoses. Bone marrow aspirations were repeatedly negative by immunofluorescent methods and cultures. It happened that six serum samples from this child, collected for another study, were available for complement fixation titers against \textit{H. capsulatum}. The results indicated that since age 18 months she had high titers, e.g. 1:512 with yeast phase, and 1:128 with mycelial phase antigens. The diagnosis of histoplasmosis was made, but this child was carefully observed since the x-ray findings in the chest films gave such strong suggestion of malignant disease. She was sent home on triple-sulfa therapy. At age 23 months, a soft, nontender supraclavicular node appeared. Biopsy of the caseous node revealed \textit{H. capsulatum} by immunofluorescent techniques, but the cultures were negative. By the age of two and one-half years, her chest was clearing with calcifications becoming evident. She was growing normally again.

Comment: This child, at the time of admission to the hospital, did not look as sick as her chest films indicated. The differential diagnosis between malignant disease and histoplasmosis was difficult. The findings of the repeatedly high titers for the complement fixation tests and the identification of the fungus in the lymph node biopsy were confirmatory evidence of histoplasmosis in this young child whose skin test was known to be positive since age 13 months. Her subsequent course has confirmed the diagnosis.

Case 3: A four-year-old boy was considered to be a normal child until one week before the referral to our ward when he developed cough and dyspnea. A chest film taken by the local physician revealed a mediastinal mass. During the week, the dyspnea had increased to the point of respiratory distress each time he tried to eat. There was no temperature elevation or hemoptysis.

The pertinent physical findings included a well-nourished, well-developed boy with suprasternal retraction and respiratory stridor. Roentgenograms of the chest revealed a mass interposed between the trachea and esophagus. This was thought to represent a duplication cyst of the trachea or a malignant mass. The skin test for histoplasmin was strongly positive while tuberculin was negative.

Therapy began with an exploratory thoracotomy. A large, abscessed node was found and drained. Biopsies were taken from the node and the surrounding lung. Following drainage, there was dramatic improvement of the respiratory distress symptoms.

The fresh biopsy from the lung revealed \textit{H. capsulatum} by the immunofluorescent method, but no organism could be identified in the caseous node. Later, cultures and methenamine silver stains of the sections of lung confirmed the finding of \textit{H. capsulatum}, but none was found in the node.

The complement fixation test on this child was negative with \textit{H. capsulatum} antigens. All other cultures were negative. He was treated with triple-sulfa. The clearing was rapid. He has been observed for three years with an uneventful course.

Comment: Before surgery, the question of malignancy or duplication cyst of the trachea made diagnosis urgent. The positive skin test for histoplasmin was suggestive, but not diagnostic in this endemic area in a four-year-old child. The early identification of \textit{H. capsulatum} in the lung biopsy was important in settling the diagnosis in this child.

**DISCUSSION**

The three patients reported here offer examples of the clinical dilemmas which can be posed by human histoplasmosis. Each case represented a group of patients with comparable problems. The immunofluorescent techniques open up new areas of diagnostic aid. Positive findings can be helpful, indeed. The widespread use of fluorescent-antibody methods by Public Health laboratories had made widely available the equipment and skill in use of the procedures.

Recently, others have reported on the usefulness of these techniques in tissues from animal infections and in retrospective study of routine tissue blocks.

**SUMMARY**

The need for improved diagnosis in human histoplasmosis stimulated the adaptation of immunofluorescent methods to the identification of \textit{H. capsulatum} in cultures and biopsies from patients. Three illustrative cases are presented in which early diagnosis was improved by application of this technique.

**ACKNOWLEDGMENT:** During the late 1930's, Chuck Smith and I were intimately involved in the rapidly expanding Public Health movement in California. It was natural when I came to Vanderbilt and observed the prevalence of pulmonary calcifications in nontuberculin reactors that I would write my friend whose contributions to the "cocci" problem were well known to me. In his answer dated December 30, 1943, Chuck reminded me, "P.S. Old histoplasmosis holds a soft spot, probably because I know so little about it, but DeMonbreun did do his epochal transmission studies at Vanderbilt and the status of histoplasmosis now is like that of coccidiodal granuloma before Gifford and Dickson showed mild infections occurred." The rest of the story is history. My affection and admiration for my old friend were unbounded, but I was never able adequately to acknowledge his contributions to my own work. I think he would be pleased to know that we are still trying to clarify concepts and develop new methods of study—things which he always did so well.

A. C.
REFERENCES


7 Kaplan, W.: Personal communication.


Reprint requests: Dr. Christie, Vanderbilt University Hospital, Nashville 37203.