

A MODIFICATION OF THE INDIRECT FLUORESCENCE TEST (IFT) FOR PARACOCCIDIOIDOMYCOSIS

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A modification of the indirect fluorescence test (IFT) for serological diagnosis of Paracoccidioidomycosis is described in which formolized budding forms (yeast cells) of the fungus Paracoccidioides brasiliensis are used as antigen. A further modification introduced is a less elaborate technical procedure of the test without lowering the sensitivity of the reaction. The test may be considered as adequately accurate and easy to perform in any laboratory with immunofluorescence facilities.

INTRODUCTION

Paracoccidioidomycosis is a serious chronic and progressive disease caused by the fungus *Paracoccidioides brasiliensis* and mainly attacks the lungs, mucosa of the mouth and nose with frequent spread to the lymph nodes, adrenal glands and other viscera.

Though the infection is relatively easy to diagnose by histological scrutiny of biopsied tissues, and in stained preparations from squashed tissues and of sputum, certain immunological tests are available to aid the diagnosis (complement fixation, electroimmunophoresis, immunofluorescence, precipitin and intradermal test). The comparative sensitivity of the available serological reactions for paracoccidioidomycosis has been reviewed in detail by Fava Netto¹.

Franco *et al.*² describe an indirect fluorescence test using as antigen broken-up cellular walls of yeast forms of *P. brasiliensis*, which remain in the sediment after preparation of the polysaccharide antigen for the complement fixation test. According to Franco *et al.*² the results obtained with the immunofluorescence test

compare favourably with those obtained by the complement fixation test.

Patients and suspects for the disease are frequently seen in our clinic and those diagnosed for the infection are treated in our hospital. To extend the scope of our immunofluorescence unit to such cases, a modification of the indirect fluorescence test for paracoccidioidomycosis was introduced. The modification was aimed for a simpler way in preparing the antigen, and a less elaborate technical procedure of the test, without affecting the sensitivity of the reaction.

MATERIALS AND METHODS

Reagents required:

- a) phosphate buffered saline (PBS) pH 7.2
- b) 2 per cent. formalin in phosphate-buffered saline pH 7.2
- c) 10 per cent. glycerine in phosphate-buffered saline pH 8.0
- d) the antigen
- e) anti-human immunoglobulin-fluorescein conjugate
- f) Evans blue 1 per cent. aqueous solution

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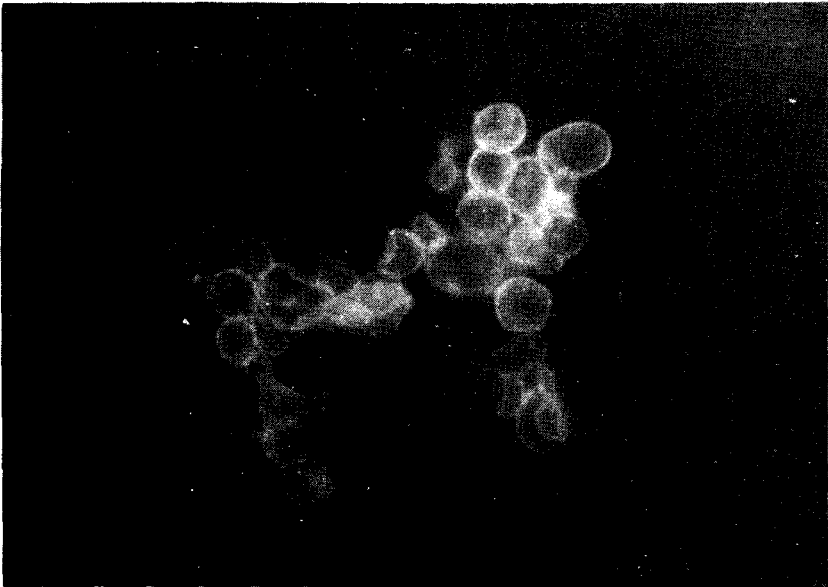
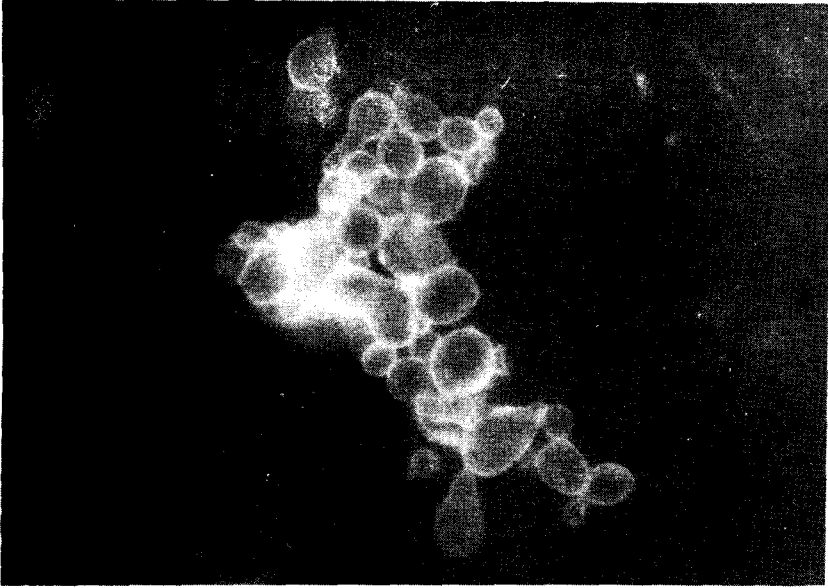


Fig. 1 and 2. — Photomicrograph showing the appearance of yeast forms of *P. brasiliensis* after exposure to sera from patients with active form of Paracoccidioidomycosis. Note the well defined fluorescence around the cell wall and the overall fluorescence in some of the smaller cells.

1 – The antigen: this is prepared from 30 days old cultures of *P. brasiliensis* grown on nutrient agar at 37°C. The cultures are overlaid with 2 per cent formalin in PBS, to cover the fungus colonies, and the tubes are left on the bench for 24 hours to kill the budding forms (yeast cells). After 24 hours the mould is scraped carefully from the agar surface, the suspension is transferred into a suitable glass container into which some glass beads have been added, and the emulsion is agitated by hand or in a mechanical shaker for 10 minutes to separate the yeast cells. The emulsion is filtered twice through six layers of surgical gauze and the filtrate is washed five times with PBS by spinning in a centrifuge at 3,000 rpm, for three minutes between each wash. After the last spin the supernatant fluid is discarded and the sediment from each of the tubes is pooled. A drop of the concentrate is examined under a coverslip with the microscope for density of the yeast cells and the presence of particles of impurity. The antigen concentrate is then diluted with PBS to give between 30 – 50 yeast cells per microscope field (x 45 objective and x 10 eyepiece) and is stored in a refrigerator at 4°C. During the storage the yeast cells tend to form small clumps; these can be broken-up by adding a few glass beads into the bottle and agitating for two minutes, after which the cells are sufficiently dispersed and ready for distribution on the test slide.

2 – The immunoglobulin-fluorescein conjugate: anti-human IgG was used obtained from Wellcome Diagnostic Reagents, England. Prior use the lyophilised conjugate was diluted with PBC to 1:40; as counterstain 10 µmg of Evans blue was added to each one ml of the diluted conjugate.

3 – The sera: in the test trial 42 sera were used obtained from 26 patients with various stages of the infection. Some of the sera were taken before the treatment, in the course of the treatment and on completion of the therapy. An equal number of sera from normal persons were used as controls. For the test the sera were diluted to a concentration of 1:10; 1:20; 1:40; 1:80; and 1:160. Sera which were reactive in the last dilution were diluted further to the end titre.

4 – The test: a drop of antigen is placed in the centre of each of 10 ARALDITE rings marked on a microscope slide and the slides are dried on a hot plate at 45°C for minutes. This treatment fixes the yeast cells sufficiently to the glass surface without the danger of them

being removed during washing in PBS. On each of five antigen fields a drop of the diluted serum is placed, the first field holding the lowest and the last the highest serum dilution, and the slides are incubated in a humid chamber at room temperature for 45 minutes. After incubation the sera are removed from the slides with a gentle jet of PBS and are washed in PBS for five minutes with a gentle agitation on a rotating table. After washing each slide is dipped into distilled water to remove the PBS and the slides are dried in an upright position with the help of a hair dryer. After drying each of the antigen fields is covered with a drop of immunoglobulin-conjugate and the slides are incubated for further 45 minutes (humid chamber, room temperature). At the end of the second incubation period the immunoglobulin is removed with a jet of PBS and the slides are washed for five minutes in PBS (with slight agitation). After the wash each slide is shaken vigorously to remove excess PBS and the moisture underneath the slides is wiped away with hygienic tissue. The test fields are sandwiched between 10 per cent glycerine in PBS (pH 8.0) and a coverslip ready for examination. The preparations were examined by incident light illumination with the VICKERS PHOTOPLAN microscope using 200 watt high pressure mercury vapour lamp and BG 12 as exciter filter with OG 1 as a barrier filter.

RESULTS

With reactive sera there was a well pronounced degree of greenish fluorescence mainly around the cell wall. Some of the smaller cells showed a distinct overall fluorescence whilst in the larger cells the fluorescence was confined to the cell wall only, with a reddish-grey staining inside the cell (Fig. 1 and 2). With the non-reactive (control sera) the yeast cells appeared reddish-grey with a faint whitish outline around the cell wall (Fig. 3). There was no difficulty in differentiating the reactive samples from the non-reactive.

DISCUSSION

In the 22 patients with clinically active form of the infection, acute or chronic, the test was positive in titres ranging from 1:10 to 1:360 which compared favourably with the stages of the disease in these patients. In the remaining four cases, where the therapy was discontinued because of clinical inactivity of the disease, the



Fig. 3 — Appearance of the yeast cells after exposure to sera from normal persons. The cells are only dimly outlined with a faint whitish line around the cell wall.

test was negative. On one of these patients the test was repeated some months later and was found to be positive in a titre of 1:10. Judging from the clinical condition of this patient the positive test suggested a relapse of the infection though this was not possible to confirm by a mycological scrutiny.

It is well known that treatment for Paracoccidioidomycosis may extend over a long period and that relapses after apparently successful therapy quite often. A serological test therefore is an useful aid in the assessment of activity of the infection and on the effects of therapy. The modified version of the reaction

described here appears to be adequately sensitive, and relatively simple to perform, in any laboratory with the facility for immunofluorescence.

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RESUMO

Descreve-se uma modificação da reação de imunofluorescência indireta para o diagnóstico sorológico da paracoccidioidomicose na qual células formolizadas da fase leveduriforme do Paracoccidioides brasiliensis foram empregadas como antígeno. Outra modificação foi a de simplificar a técnica da reação sem prejuízo da sua sensibilidade. O teste pode ser considerado como suficientemente sensível e de fácil execução em qualquer laboratório equipado para reações de imunofluorescência.

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