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Isolation of *Candida* Protoplasts from a Case of *Candida* Endocarditis

RICHARD ROSNER
St. Joseph's Hospital, Paterson, New Jersey

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ABSTRACT

ROSNER, RICHARD (St. Joseph's Hospital, Paterson, N.J.). Isolation of *Candida* protoplasts from a case of *Candida* endocarditis. J. Bacteriol. **91**:1320–1326. 1966.—A case of endocarditis caused by *Candida tropicalis* is described. Even though the patient was receiving adequate therapy, and all routine blood cultures were negative for growth, the patient continued to give clinical evidence of active, progressive endocarditis. The isolation of osmotically fragile bodies from blood cultures placed in an osmotically controlled medium is described in detail. The role of these bodies, called protoplasts, in the active disease process of this patient is discussed in relation to the criteria for the implication of protoplasts in the disease process. Several explanations as to what caused the in vivo formation of protoplasts of *C. tropicalis* in this patient are discussed.

An ever increasing amount of literature has appeared concerning L forms, L variants, spheroplasts, and protoplasts since Kleineberger (6) first described large bodies. In recent years, the above-mentioned names have been used so interchangeably that the presumed boundaries which might exist between these forms have become so artificial and slight as to be confusing. Several investigators, too numerous to review in this report, distinguish L forms as stable forms which occur naturally, whereas L variants are unstable forms which occur naturally and which tend to revert back to the parent organism. Tulasne et al. (15) suggested that the stable L forms be referred to as "type A" L forms and the unstable forms be referred to as "type B" forms. A similar classification was made earlier by Deines and Weinberger (2*a*), who described two types of L forms for the genus *Proteus*. One type, which remained stable as the L form, was classified as type 3A; the other L form, which tended to revert back to the bacilli, they designated as type 3B. Many authors have since used this designation.

Hurwitz et al. (4) and Michael and Braun (13) described bodies which they termed as spheroplasts. These bodies, unlike true L forms, are produced artificially and do not occur in nature. Spheroplasts may also contain cell wall fragments or even a modified cell wall. Still another form, designated a protoplast by such authors as Weibell (16), McQuillen (10), and Brenner (2), can be induced artificially and may also occur naturally. This form differs from a spheroplast in that it completely lacks a cell wall.

The relationship between protplasts, spheroplasts, L forms, and L variants was reviewed by McQuillen (11) and Kleineberger-Nobel (7).

Most of the current literature seems to favor the term protoplast to describe those atypical forms either created artificially or isolated in nature. For this reason, the term protoplast will be applied to the atypical forms isolated from the patient described in this report.

Protoplasts have been isolated or produced artificially from a wide variety of bacteria. Mattman et al. (12) were able to demonstrate protoplasts from the mycobacteria. Kinsky (5), as well as Bachmann and Bonner (1), isolated protoplasts from hypha-forming fungi, and Eddy and Williamson (3) and Nêcas (14) demonstrated protoplasts in yeasts.

The direct relationship between protoplasts and disease is not clearly understood. Mattman et al. (12) demonstrated and grew protoplasts of *Mycobacterium tuberculosis* from the spinal fluid of a patient with tuberculous meningitis. They were unable to demonstrate or grow typical rod forms of the organism from the same spinal fluid. Recently, at the annual convention of The American Federation of Clinical Research, P. Charache of Johns Hopkins School of Medicine presented a paper dealing with 10 patients from whom protoplasts were isolated during a disease process. Dr. Charache demonstrated the direct relation-

ship between protoplast isolation and the disease process in each of the 10 patients.

The case presented in this report deals with the isolation of protoplasts of *Candida* from multiple blood cultures of a patient known to have a candidal endocarditis, and the subsequent demonstration of these forms in material obtained at postmortem.

CLINICAL CASE HISTORY

In 1958, a 34-year-old Negro male was admitted to a general hospital. Heart catheterization and closed mitral commissurotomy were performed. The patient did well until 1963, at which time he was admitted to this hospital with congestive heart failure. From 1963 to late 1964, the patient was admitted to this hospital on three more occasions with the same complaint. His next-to-last admission was in September 1964. At this time, the patient developed a fever of 103 F. Sputum cultures revealed large numbers of β-hemolytic *Streptococcus pyogenes*, and the patient was placed on penicillin therapy for 10 days. Six blood cultures drawn during this admission failed to reveal any growth. The patient recovered and was discharged. One month later, in late November 1964, the patient was again admitted with congestive heart failure and, for the first time, a pulmonary infarct. The patient stated that 2 weeks prior to admission he had dental work with no antibiotic coverage. On admission, the patient was febrile and presented a temperature of 104 F. During his first 12 hr of hospitalization, six blood cultures were taken, and then the patient was started on penicillin therapy. Five days later, all six blood cultures were reported positive for *Candida tropicalis*. Penicillin therapy was discontinued. In light of the bacteriological findings it was decided that the patient had a candidal endocarditis complicating his previously damaged mitral valve. The patient was started on amphotericin B therapy, with the antifungal agent being administered intravenously in 20- to 30-mg doses with slow increments to 50-mg doses daily. The patient was kept on this therapy for 11 weeks. Despite the fact that all blood cultures taken after the 15th day of hospitalization were reported as negative for growth, the patient continued to run a low-grade fever, and general improvement of the endocarditis was extremely slow. It was believed that the fever was due to the effects of the drug.

Therapy was discontinued after 11 weeks to better assess the patient's clinical picture. Daily blood cultures continued to be negative for growth during the post-therapy period. Despite the negative blood cultures, the patient continued to give evidence of active endocarditis. For this reason, on post-therapy day 12, it was decided to culture the patient's blood for osmotically fragile bodies. Three special blood culture flasks were inoculated on post-therapy day 12, and one flask per day on days 13 through 18. After 48 hr, the bacteriology laboratory reported the presence of "atypical forms" in the special "L" flasks. By post-therapy day 18, the patient was again presenting a fever of 103 F, and was placed on amphotericin B therapy as before. The patient's condition remained about the same except for a slowly rising blood-urea nitrogen (BUN). On 1 April 1965, therapy was discontinued due to a very high BUN. On 15 April 1965, just after completing dinner, the patient suddenly expired. An autopsy was performed on the morning of 16 April. Cause of death was listed as a large embolus found in the left anterior ascending coronary artery. Several large vegetative lesions were noted on the mitral valve.

MATERIALS AND METHODS

Blood cultures were collected by two technologists, one of whom flamed the mouth of the blood culture flask while the other collected the blood by venipuncture. The venipuncture site was first degermed by iodine and 70% alcohol.

Blood culture flasks were prepared by placing 50 ml of thiglycolate medium without indicator or dextrose in a 125-ml Erlenmeyer flask. A cotton plug was used as a closure. The flask was autoclaved, cooled, and then incubated for 24 hr to ensure sterility. After autoclaving, a piece of aluminum foil was wrapped over the cotton plug and top of the flask to prevent evaporation. All flasks were stored at room temperature.

The osmotically controlled, or "L" thioglycolate flasks were prepared in the same manner except for the medium used. "L" thioglycolate medium was prepared by dissolving 2.4 g of thioglycolate medium without dextrose or indicator in 90 ml of distilled water. This solution was gently heated to dissolve the medium. When cool, 20 g of sucrose crystals and 10 ml of horse serum were added. The medium was sterilized by filtration and was dispensed into flasks.

After a blood culture flask had been inoculated, it was incubated for 48 hr. After this incubation period, a sterile cotton swab was used to inoculate a blood-agar plate subculture and to prepare a smear for Gram stain. The blood culture flask was then reincubated. The subculture plate was incubated under a 10% carbon dioxide atmosphere for 48 hr. This subculture procedure was repeated after the blood culture flask had incubated for 5, 8, and 14 days.

RESULTS

Blood cultures obtained during the first 15 days were all found to contain *C. tropicalis*. The organism was identified by the criteria described by Conant et al. in 1954 (*Manual of Clinical Mycology*, 2nd ed., W. B. Saunders & Co., Philadelphia).

All blood cultures collected after this 15-day period were negative for growth. Post-therapy regular thioglycolate blood cultures were also negative for growth; however, eight of the nine "L" thioglycolate blood cultures were found to contain small, gram-variable, slightly pleomorphic, coccoidlike bodies which would not grow on blood-agar under a variety of conditions and were definitely not bacteria. These bodies could not be demonstrated in the regular thioglycolate blood culture flasks, in uninoculated "L" thioglycolate flasks, or in "L" thioglycolate flasks

inoculated with the author's blood. Daily Gram stains of the "L" thioglycolate blood cultures revealed a slow morphological change from these small, coccoid forms to rodlike forms, to branching, filamentous forms, and eventually to large oval forms which finally developed into true, single-budding yeast forms (Fig. 1–9). This transformation took 103 days in the osmotically controlled medium.

Tissue sections were prepared from the mitral

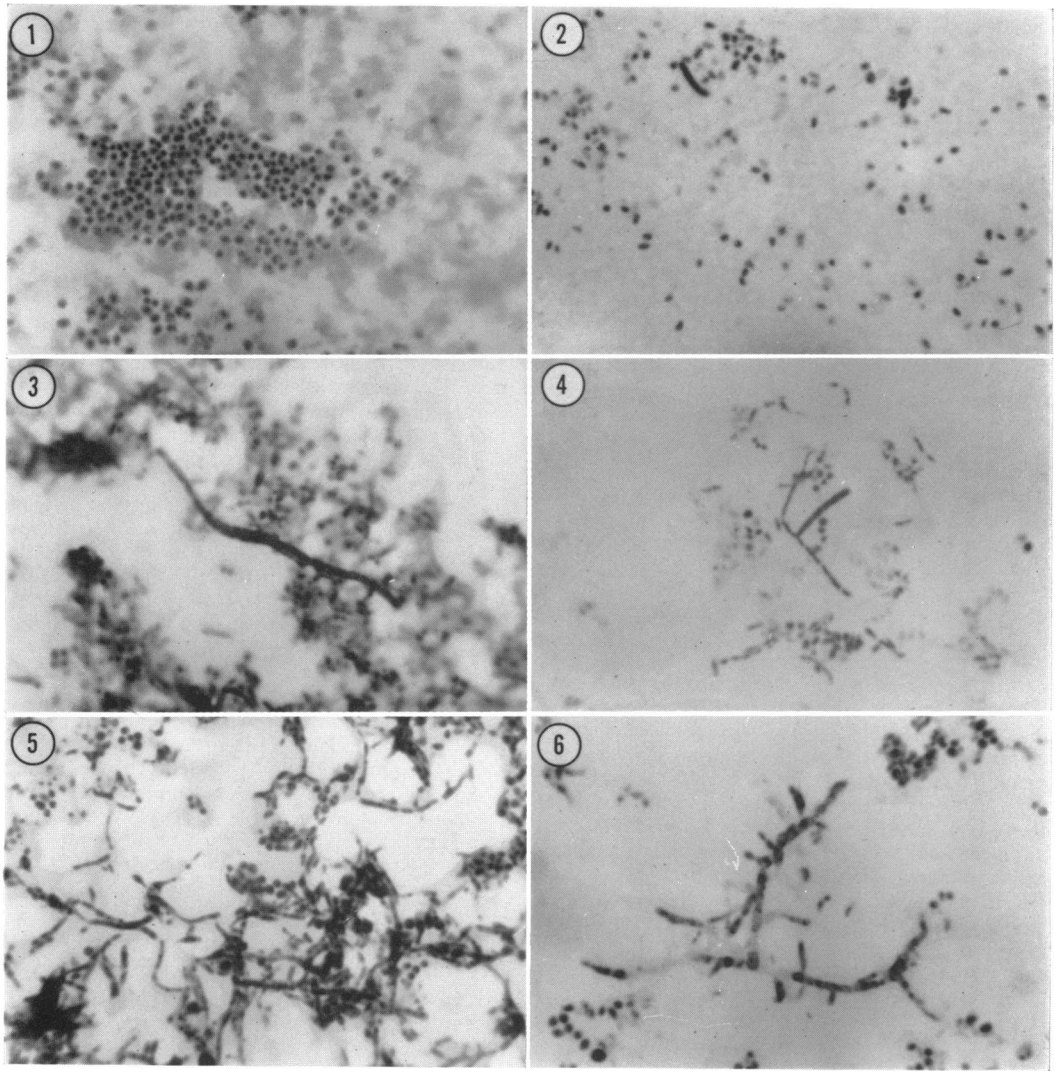


FIG. 1. *Gram stain prepared from a 48-hr-old "L" blood culture flask. × 2,000. The organisms appear as small, gram-variable, coccoidlike cells.*

FIG. 2. *Gram stain of a 13-day-old "L" blood culture flask. × 2,000. A deeply stained rodlike form can be seen in the upper center of the field.*

FIG. 3. *Gram stain prepared from a 21-day-old "L" blood culture flask. × 2,000. Abranching hyphal form can be seen in the center of the field along with the small gram-variable, coccoid forms.*

FIG. 4. *Same culture as Fig. 3. A hyphal form without branching is visible.*

FIG. 5. *Gram stain prepared from a 29-day-old "L" blood culture flask. × 2,000. Several hyphal forms are present along with the original coccoidlike forms.*

FIG. 6. *Gram stain prepared from a 37-day-old "L" blood culture flask. × 2,000. A multi-branching hyphal form with what appears to be a single budding yeast cell in the middle of the hyphae.*

valve lesions and from the embolus. These sections were stained with periodic acid-Schiff stain and examined for the presence of yeast cells which demonstrated a cell wall and cells in which no cell wall could be noted. In both the mitral valve lesions and the embolus, large numbers of *Candida* cells could be seen, along with several pleomorphic structures which did not demonstrate any cell wall material (Figures 10–19).

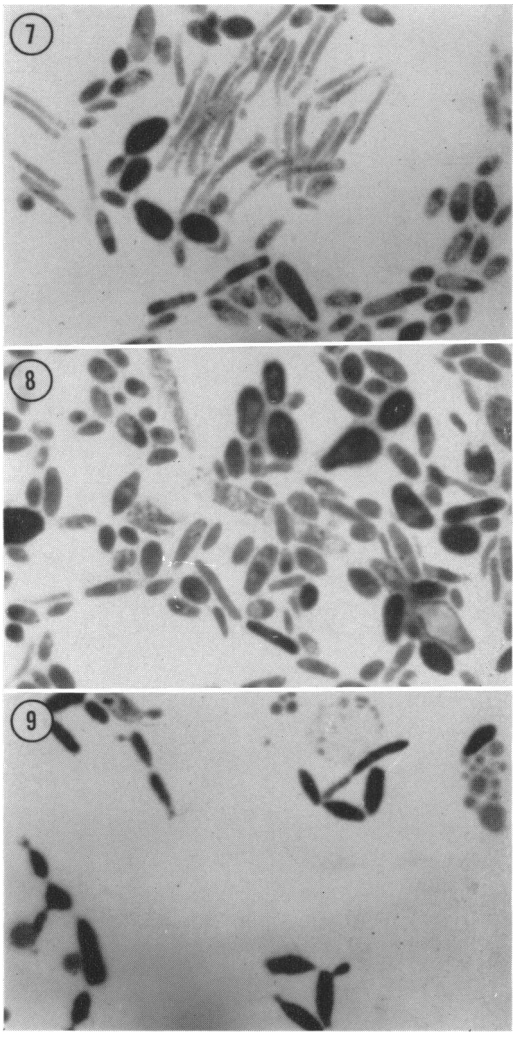


FIG. 7. *Gram stain prepared from a 69-day-old "L" blood culture flask.* × *2,000. The hyphal forms have broken into poorly stained granular rod forms and large oval forms.*

FIG. 8. *Gram stain prepared from a 91-day-old "L" blood culture flask.* × *2,000. The granular rod forms have almost completely disintegrated. The oval forms have diminished in size and now stain more deeply than before.*

FIG. 9. *Gram stain prepared from 103-day-old "L" blood culture flask.* × *2,000. Typical single-budding yeast cells are present along with the small coccoid forms which were present originally.*

DISCUSSION

The decision to use an osmotically controlled medium in conjunction with regular media was based on the apparent discrepancy between the bacteriological findings and the clinical picture presented by the patient. Dr. Charache, in her paper dealing with the role of protoplasts in disease, mentioned four criteria which can be used to implicate protoplasts in a given disease process. One of these criteria deals with a clinical course which is not consistent with the bacteriological findings once the patient is placed on adequate therapy. The patient in this report did not present a clinical course consistent with the bacteriological findings; therefore, it was decided to attempt to isolate osmotically fragile bodies from the blood of the patient. Once these forms were found in the special media, Dr. Charache's criterion of consistent clinical and bacteriological findings was fulfilled. The three other criteria discussed by Dr. Charache were: (i) visualization of the atypical forms directly from the clinical material; (ii) failure of these atypical forms to grow under conditions which would readily support the growth of the parent organism; and (iii) ability of the atypical forms to survive in an osmotically controlled medium, and their ability to revert back to the parent organism first isolated from the disease process.

Visualization of atypical forms was accomplished after the patient had received 5 days of penicillin therapy and 11 weeks of amphotericin B therapy. Unfortunately, these forms were not looked for prior to this time. Once the attempt to isolate these forms was started, however, they were observed in eight of nine consecutive blood cultures. These forms were consistent with the general description of protoplasts in that they required an osmotically controlled medium for survival; they were small, gram-variable, pleomorphic forms; they would not grow on medium which was not osmotically controlled; and they lysed when placed in ordinary media. Tissue sections obtained at autopsy and stained with periodic acid-Schiff stain revealed large numbers of *Candida* cells with easily demonstrable cell walls, along with many atypical, pleomorphic forms which lacked any cell wall material.

After prolonged incubation in an osmotically controlled medium, the atypical forms underwent morphological changes, and finally reverted back to the original yeast form which was identified as *C. tropicalis*. Subculturing of the atypical

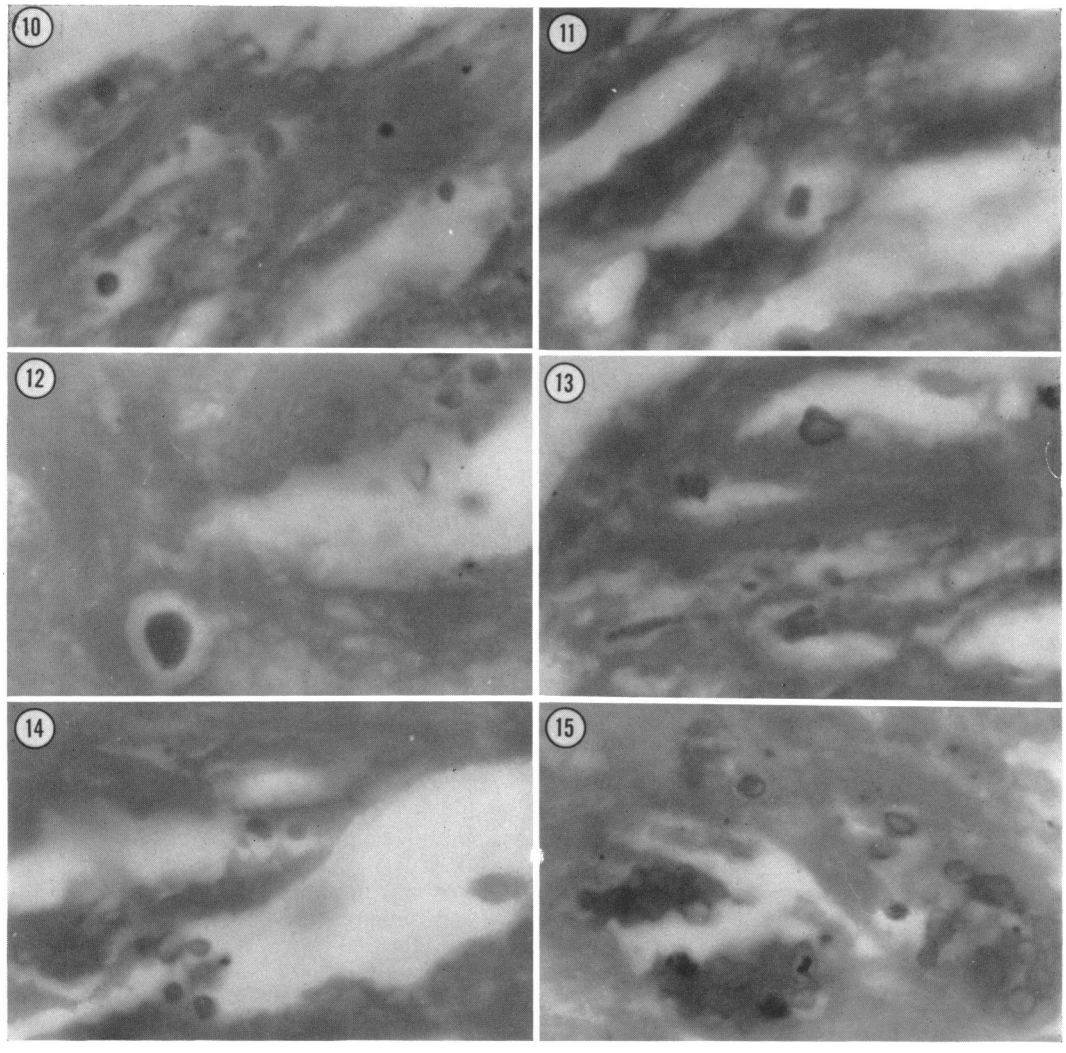


FIG. 10. *Tissue sections of lesion on mitral valve stained with PAS. × 2,000. A cell in the lower left section of the photograph demonstrates a cell wall; however, the cell in the middle right section of the photograph does not indicate any cell wall.*

FIG. 11. *Tissue section of lesion on mitral valve stained with PAS. × 2,000. There is no indication of any cell wall in the cell in the center of the photograph.*

FIG. 12. *Same section as Fig. 11. Cell walls are clearly visible in the cells in the extreme upper right of the photograph, while the cell wall is definitely not present in the cell in the lower center of the photograph.*

FIG. 13. *Same section as Fig. 11. Cell walls are definitely present in the cells in the upper section of the photograph. A pleomorphic, rodlike form which does not demonstrate any cell wall is visible in the lower center of the field.*

FIG. 14. *Same section as Fig. 11. Cells demonstrate cell walls. In the upper section of the photograph are two cells which do not demonstrate any cell walls.*

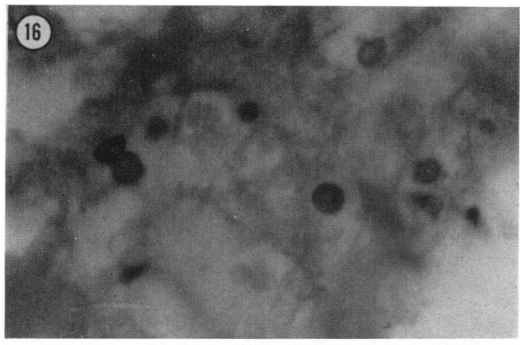
FIG. 15. *PAS stain of lesion from mitral valve. × 2,000. Note the large clusters of Candida cells which clearly demonstrate cell walls.*

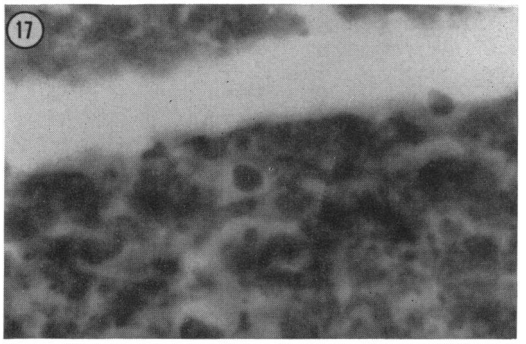
forms from the osmotically controlled medium to any of several regular media was unsuccessful until typical yeast cells appeared in the osmotically controlled medium.

With the final reversion of the atypical forms back to the original organism, all four criteria discussed by Dr. Charache were fulfilled.

The most important aspect of this case deals

with the possible reasons for the in vivo formation of protoplasts. Unfortunately, any explanations can be arrived at only by assumptions, because so little is known about protoplast formation. In this report, attempts to isolate protoplasts were begun only after the patient had been placed on two different antibiotics.





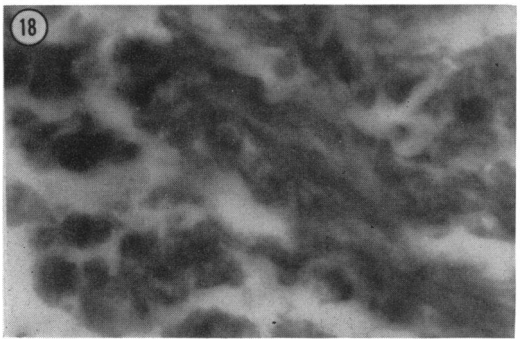


FIG. 16. *Same section as Fig. 15. Note the presence of many large cells which demonstrate cell walls. In the extreme right of the field is a small cell which lacks any cell wall.*

FIG. 17. *PAS stain of the embolus.* × *2,000. Two cells, one in the center portion of the photograph and one in the upper right section, both of which lack a stained cell wall.*

FIG. 18. *Same section as Fig. 17. A small cell in the center of the field appears to be surrounded by a clear area. There is no cell wall visible on this cell.*

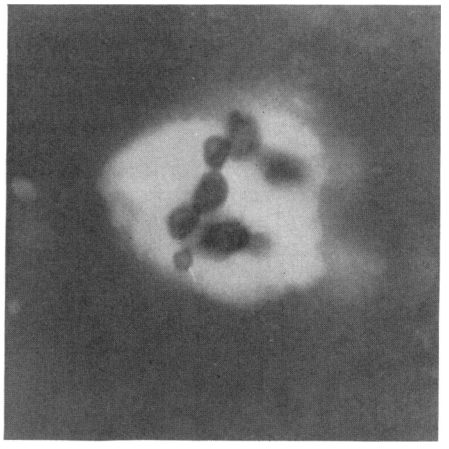


FIG. 19. *Same section as Fig. 17. A cluster of typical Candida cells are present in another "clear" area.*

There are three possible reasons for the formation of protoplasts in this patient. The first reason assumes the natural occurrence of protoplasts. Several investigators are of the opinion that L forms, or protoplasts, can and do occur in nature. Many investigators, such as Smith et al. (Proc. Soc. Exptl. Biol. Med. **96**:550, 1957) and McKay and Truscott (Ann. N.Y. Acad. Sci. **79**:465, 1960) feel that the pleuropneumonia-like (PPLO) group of organisms are nothing more than naturally occurring L forms, or protoplasts, of various bacteria. Several attempts to isolate protoplasts from cultures of *C. tropicalis* in osmotically controlled media have proved unsuccessful in this laboratory.

Another possible explanation for the in vivo formation of protoplasts in this patient involves the action of amphotericin B on *C. tropicalis*. This drug, like other polyene drugs, has been shown to act on the cell membrane of an organism in such a manner as to cause leakage of vital cytoplasmic material out of the cell. Amphotericin B is not known to have any direct effect on the cell wall; however, like other drugs, it may temporarily interfere with cell wall synthesis. The effect of amphotericin B on the protoplasts of fungi such as *Neurospora crassa* in vitro has been studied by Kinsky (5). Kinsky demonstrated that the protoplast of *N. crassa* would shrink and become crenated in the presence of amphotericin B; however, many of the protoplasts would not lyse. Relatively high doses of this drug were required to cause lysis of the protoplasts.

The third possible explanation for the formation of protoplasts involves the use of penicillin. This patient received 10 days of penicillin therapy during his next-to-last admission, and 5 days of

penicillin during his last admission. On his last admission, blood cultures taken during the time the patient was receiving penicillin were positive for *C. tropicalis*. Penicillin is well known for its ability to cause protoplast formation in gram-positive organisms. There is no reference in the current literature concerning the ability of penicillin to cause in vivo protoplast formation in *Candida*. Unfortunately, no attempt was made to isolate protoplasts from the patient just after penicillin therapy but prior to amphotericin B therapy.

A sequence of events which could account for the formation of, and allow the persistence of, protoplasts in this patient can be logically hypothesized, but cannot be proven experimentally. It appears most likely that the in vivo formation of the protoplasts was caused by penicillin, as this drug is well known for its ability to cause protoplast formation. The ability of the protoplasts to survive 11 weeks of amphotericin B therapy can be explained by the results obtained by Kinsky (5), in which it was shown that very high concentrations of amphotericin B were needed to cause lysis of fungal protoplasts. It is perfectly safe to assume that some of the protoplasts present in the mitral valve lesion did not come in contact with a sufficiently high dose of amphotericin B to cause them to lyse, and therefore they were able to survive. These protoplasts, when liberated from the lesion into the blood stream, were lysed rapidly owing to the concentration of amphotericin B in the blood. Once therapy was discontinued, however, the concentration of amphotericin B in the blood dropped to the point where the protoplasts were able to survive, and thus were isolated in blood culture.

The isolation of atypical forms which would not survive in any media except an osmotically controlled media, and which failed to demonstrate any cell wall, fulfills the definition of a protoplast. The isolation of protoplasts from multiple blood cultures of a patient with active endocarditis and the failure to isolate any typical forms of the parent organism at the same time implicates these protoplasts in an active disease process. This implication is further supported by the observation of these protoplasts in both a vegetative lesion on the mitral valve and an embolus obtained at autopsy. These visual observations, along with the eventual reversion of the protoplasts back to the parent organism, fulfill the four criteria set down by Dr. Charache in her paper dealing with the implication of protoplasts in the disease process.

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