DISSOCIATION OF CANDIDA ALBICANS BY LITHIUM CHLORIDE AND IMMUNE SERUM¹

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Received for publication October 20, 1939

INTRODUCTION

Recognition of the phenomenon of bacterial dissociation has clarified much of the confusion caused by morphologic, pathogenic and antigenic changes in old cultures kept on artificial media. It was felt that similar changes occurred in yeast-like fungi and might account for many of the irregularities noted in descriptions based on studies of old strains. Martin and Jones (1940), for example, observed that about one-fourth of the strains of *Candida*² obtained from the Centraalbureau voor Schimmelcultures differed from the original descriptions in certain features. While it was thought that these cultures were members of the six species described by them, they could be classified only provisionally by the methods adapted to the study of freshly isolated strains. The following investigations were made, therefore, to determine whether or not dissociation could be produced in freshly isolated strains of *Candida albicans* and to observe any changes in morphology, biologic reactions or pathogenicity.

¹ Aided by a grant from the John and Mary R. Markle Foundation.

² In accord with a decision reached at an informal meeting of medical mycologists attending the Third International Congress for Microbiology, September 2-9, 1939 in New York City, we have substituted the generic term *Candida* for *Monilia*. Although Berkhout's original description of the genus *Candida* will require emending, it was the consensus of opinion of those present that the existing confusion would be greatly clarified if workers in this field would use only one generic term while awaiting official action by the Rules Committee of the International Botanical Congress.

HISTORICAL

Lithium chloride as a dissociative agent was first used in the study of the dissociation of ascosporeogenous yeasts, *Saccharomyces*, by Fabian and McCullough (1934) and Wickerham and Fabian (1936). Negroni (1935) studied cultural variations of *Candida albicans* in the presence of phenol, formaldehyde, and by cultivation at different pH levels and different temperatures. Mackinnon (1936) noted changes in morphology and pathogenicity of 3 strains of *Candida albicans* by cultivating the fungus in a medium rich in carbohydrate. Cavallero (1936) changed the gross morphologic characteristics of *Candida albicans* by using starvation media but reversion occurred when the variants were subcultured again on rich media. Kurotchkin's (1936) results were irregular but he was able to obtain rough colonies by culturing the fungi in media containing high concentrations of peptone or carbohydrate. Dissociation of species of *Candida* has been studied also by Besta (1938), Cavallero (1938) and Redaelli, Ciferri and Cavallero (1939).

Although some of these earlier investigators demonstrated dissociation among these forms, they did not make a complete study of changes in pathogenicity and used a limited number of strains, some of which already had shown macroscopic evidences of dissociation.

CULTURES STUDIED

Eighteen cultures of *Candida albicans* 14 strains from the vagina and 4 from sputum, were isolated from patients at Duke Hospital. These strains were identified by the methods outlined by Martin, Jones, Yao and Lee (1937) and Jones and Martin (1938) and the purity and colony characteristics of each strain were checked by repeated platings on Sabouraud's and blood agar. Each strain developed smooth creamy colonies showing only round or slightly oval budding cells on Sabouraud's and blood agar, no surface growth on Sabouraud's broth incubated at 37°C. for 48 hours, acid and gas in glucose and maltose, acid in sucrose with no change in lactose, and numerous chlamydospores on corn-meal agar. Rabbits injected intravenously with 1.0 ml.

of a 1 per cent suspension developed renal abscesses and died in 2-5 days. Subcutaneous injections of rabbits with 0.1 ml. of a 4 per cent suspension resulted in subcutaneous abscesses in 24 to 48 hours and hard nodules in 4 days.

METHODS OF DISSOCIATION

Each strain was cultured at 37°C. in Sabouraud's broth containing 0.25 per cent lithium chloride and 5 strains were cultured in Sabouraud's broth containing 3 per cent immune rabbit serum. These cultures were transferred to fresh media of the same composition in 0.1 ml. amounts every 48 hours and Sabouraud's and blood agar plates streaked at each transfer. This process was continued until rough colonies appeared on the streaked plates. The rough colonies were studied by the same methods used for the identification of freshly isolated strains (plate I).

Dissociation was induced in 14 of the eighteen strains. Although 4 strains, 1 from sputum and 3 from the vagina, were transferred in lithium chloride broth through 18 transfers for 36 days, no rough colonies appeared.

COMPARISON OF THE SMOOTH AND ROUGH FORMS

Growth on Sabouraud's agar plates. Individual colonies of the smooth forms were grayish-white, creamy, rounded up and had regular borders (plate II, fig. 1). The rough forms were white, flat, dry and somewhat larger than the smooth forms (plate II, fig. 2). Both types of colonies appeared on the plates streaked from the lithium chloride or immune serum broth, but each type bred true on subculture after isolation. The rough colonies lost some of the dry appearance if the plates were kept for several days, but when transplanted to fresh media, the characteristic dryness reappeared. The colonies of the rough forms on Sabouraud's agar plates resembled those of *C. krusei*, but were much larger.

Growth on blood agar. Although only two types of colonies were noted on Sabouraud's agar (i.e., smooth and rough), an intermediate type of colony could be distinguished on blood agar. This colony appeared prior to the appearance of the rough

colonies and was white and creamy with stellate borders. When transferred to Sabouraud's agar, it gave a creamy growth, indistinguishable from the smooth forms. This stellate growth was considered intermediate between the smooth and rough forms (plate II, fig. 4). The rough colonies appeared as dull-gray thin spreading growths with irregular borders. When these were transferred, the entire colony came off the surface of the agar.

Growth in Sabouraud's broth. Growth of the smooth forms in Sabouraud's broth resulted in a homogenous sediment in the bottom of the tube, the supernatant fluid remaining relatively clear. There was no growth on the surface of the broth. The rough forms produced little growth in the bottom of the tube but a pronounced surface "veil." This top growth appeared within 24 hours and extended 5-6 mm. above the surface of the broth. Although this surface growth developed faster, it was similar to that produced by *Candida krusei* and was considered to be the first indication of dissociation (plate II, fig. 3).

MICROSCOPIC MORPHOLOGY

Sabouraud's agar. Examination of the early growth of the smooth forms on Sabouraud's agar showed round or slightly oval budding cells (plate III, fig. 3). The interior of the cell, when examined in unstained mounts, was composed of finely granular protoplasm. Microscopic examination of the cells from early colonies of the rough forms on Sabouraud's agar, however, showed elongated and bizarre shapes, often approaching the appearance of mycelial filaments (plate III, fig. 4). The interior of the cells in unstained mounts appeared to be filled with numerous oil droplets.

Corn-meal agar. Corn-meal agar slide cultures of the smooth strains showed a well-developed mycelium with numerous chlamydospores, frequently in clusters, at the ends of the branches (plate III, fig. 1). The character of the mycelial development of the rough forms was somewhat different from that of the smooth. The mycelial growth was brush-like with clusters of elongate blastospores on the mycelium and no chlamydospores.

There were also large numbers of thread-like septate filaments characteristic of true mycelium (plate III, fig. 2).

FERMENTATIONS

The method proposed by Martin, Jones, Yao and Lee (1937) was used in this experiment. The carbohydrate broth solutions were inoculated from a sugar-free medium and sealed with sterile vaseline. Final readings were made after 10 days incubation at 37°C. Both the smooth and rough forms produced acid and gas in glucose and maltose, acid only in sucrose and failed

TABLE 1

ANTIGEN NUMBER	ANTI-S SERUM		ANTI-R SERUM	
	S Antigen	R Antigen	S Antigen	R Antigen
1	1:640	1:640	1:320	1:640
2	1:640	1:640	1:320	1:640
3	1:640	1:320	1:640	1:320
4	1:320	1:320	1:320	1:640
5	1:320	1:320	1:320	1:640
6	1:320	1:320	1:320	1:320
7	1:640	1:640	1:640	1:640
8	1:640	1:320	1:320	1:640
9	1:640	1:640	1:320	1:320
10	1:640	1:640	1:320	1:640
11	1:640	1:640	1:640	1:640
12	1:640	1:640	1:320	1:640
13	1:640	1:640	1:320	1:640
14	1:640	1:640	1:640	1:640

to ferment lactose. Although the fermentation reactions were the same for both the smooth and rough forms, it was noted that the rough form fermented more slowly.

VIRULENCE

Benham (1932), Stovall and Bubolz (1932) and many others have shown *Candida albicans* to be pathogenic for rabbits when injected intravenously. In our experiments, intravenous injection of 1.0 ml. of a 1 per cent suspension of all the smooth strains killed rabbits within 2 to 5 days. The rough forms, how-

ever, were not pathogenic for rabbits in doses 10 times as large (5 ml. of a 2 per cent suspension).

Subcutaneous injection of 0.1 ml. of a 4 per cent suspension of the smooth form produced localized abscesses with erythema

TABLE 2
Summary of characteristics of smooth and rough forms

	SMOOTH	INTERMEDIATE	ROUGH
Sabouraud's agar	Smooth, creamy. Rounded up colonies	Smooth, creamy	Flat, dry, spreading. Colonies larger than smooth
Blood agar	Small gray creamy colonies	White, stellate creamy colonies	Dry, flat, irregular borders
Sabouraud's broth	Homogenous sediment	Homogenous sediment	Liquid clear. Extensive surface growth
Microscopic morphology	Round or oval budding cells	Oval or irregular cells	Large, elongated and irregular cells
Mycelial formation	Branching, tree-like. Numerous chlamydospores	Same as smooth	Brush-like, no chlamydospores
Carbohydrate fermentation	Glucose AG; Maltose AG; Sucrose A; Lactose O	Same as smooth	Same as smooth. Somewhat slower
Pathogenicity	1 ml. of a 1 per cent suspension killed rabbit, 2-5 days	Required 2-ml. of a 1 per cent suspension to kill rabbit, 2-5 days	Not pathogenic
Agglutination	Marked cross agglutination of rough and smooth forms. Intermediate not tested		

and the formation of hard nodules in 4 days. With the exception of 2 cases, in which there was a temporary area of erythema subsiding in 24 hours, equal amounts of the rough forms produced no reaction.

AGGLUTINATIONS

A serological study of the smooth and rough forms was made in an effort to determine their antigenic relationship. Immune rabbit sera were prepared against 3 smooth and 3 rough strains by intravenous injection of increasing doses of heat-killed vaccine three times weekly until a satisfactory titer was established. Antigen of smooth and rough forms was prepared by washing the growth from a 24-hour Sabouraud's slant with saline. A 1:1000 dilution by volume of packed cells was used. The results of the agglutinations are shown in table 1.

The agglutination reactions did not separate sharply the smooth from the rough forms. Reciprocal agglutinin-absorption tests with one smooth form and its corresponding rough variant showed complete absorption in both cases.

NONSPECIFIC AGGLUTINATION

Much importance has been attached to nonspecific agglutination among the dissociants of bacteria and fungi. No effort was made to test this phenomenon beyond its appearance in cold and heated saline. There appeared to be some tendency toward spontaneous clumping of the rough variant in cold saline. This was not seen in the smooth form. Heating to 100°C. did not increase the effect.

A summary of the characteristics presented by the smooth and rough forms is shown in table 2.

DISCUSSION

Because previous methods of study did not allow immediate identification of freshly isolated cultures of yeast-like fungi, it was necessary to subculture them for extended periods on various media. It was conceivable that cultures maintained on different media for varying periods of time might become partially dissociated and be described as new species because of slight morphologic variations. Such a condition was found to exist when Martin and Jones (1940) studied many old cultures which could not be identified readily with the six species most commonly isolated from man: *Candida albicans*, *C. tropicalis*, *C. pseudo-*

tropicalis, *C. krusei*, *C. parakrusei*, and *C. stellatoidea*. Before these cultures could be identified by the methods of Martin, Jones, Yao and Lee (1937) it was necessary to streak 48-hour Sabouraud's broth cultures on blood agar plates to separate the smooth and rough variants. By picking the smooth colonies, cultures were obtained which agreed with the descriptions of the most commonly encountered species and could be identified.

Laboratory proof that extreme variations did occur in the genus *Candida* and made specific identification difficult was demonstrated by the dissociation of freshly isolated strains of *C. albicans* in the presence of lithium chloride or immune rabbit serum. The change in gross cultural characteristics, microscopic morphology, and loss of pathogenicity separated sharply the rough and normal smooth variants of *C. albicans*. The smooth variants, however, gave the constant diagnostic characters for *C. albicans* as described by Martin and Jones (1937). In view of these findings, therefore, old cultures must be studied for possible dissociative changes and where both smooth and rough colonies can be separated, the former should be used for classifying the fungus.

SUMMARY

Eighteen freshly isolated cultures of *Candida albicans*, 14 strains from the vagina and 4 from sputum, were cultured in lithium chloride or immune rabbit serum to induce dissociative changes.

Fourteen of these strains produced variants which seemed permanent and differed from the parent form in gross and microscopic morphology and pathogenicity.

It was concluded that these variants were true rough phases of *Candida albicans* and were analogous to dissociants that occur among the bacteria.

APPENDIX

Sabouraud's glucose agar. Bacto-glucose 40 grams, Fairchild's peptone 10 grams, agar 25 grams, distilled water 1000 ml. Melt in autoclave, filter through gauze, tube and autoclave at 15

pounds pressure for 15 minutes. No pH adjustment necessary.

Sabouraud's glucose broth. Same as Sabouraud's glucose agar, with the exception that no agar is added. No pH adjustment necessary.

Beef extract agar. Difco beef extract 3 grams, sodium chloride 5 grams, Difco peptone 10 grams, agar 25 grams, distilled water 1000 ml. Melt in autoclave, filter through gauze and titrate to pH 7.6. Tube and autoclave. Final pH 7.4.

Beef extract blood agar. Same formula as beef extract agar, with the addition of approximately 10 per cent sterile citrated sheep blood.

Carbohydrate broth. Same basic formula as beef extract agar except that no agar is added. After dissolving, heat to boiling and titrate to pH 7.2. Add 100 ml. of indicator (below), tube and autoclave. Final pH 7.0.

Indicator solution. Brom-thymol-blue 0.04 gram, distilled water 100 ml. Titrate to the point that 1 drop of 1 N NaOH or 1 N HCl will cause a complete change in color.

Corn-meal agar. Corn meal 62.5 grams, water 1500 ml. Heat to 60°C. for one hour. Add 19 grams of agar and melt in autoclave, make up to 1500 ml. with water, filter through gauze, tube and sterilize. No pH adjustment necessary.

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PLATE 1. DIAGRAMMATIC REPRESENTATION OF THE METHODS USED TO
DISSOCIATE FRESHLY ISOLATED STRAINS OF *CANDIDA ALBICANS*

(Walter A. Mickle and Claudius P. Jones: Dissociation of *Candida Albicans*)

PLATE 2

FIG. 1. Moist smooth colony of *Candida albicans* on Sabouraud's glucose agar incubated at 37°C. for 48 hours.

FIG. 2. Dry rough spreading colony of dissociated strain of *Candida albicans* on Sabouraud's glucose agar incubated at 37°C. for 48 hours.

FIG. 3. Development of the smooth and rough variants of *Candida albicans* on Sabouraud's glucose broth incubated at 37°C. for 3 days.

FIG. 4. Smooth and intermediate colonies of *Candida albicans* cultured on blood agar plate and incubated at 37°C. for 4 days.

(Walter A. Mickle and Claudius P. Jones: Dissociation of *Candida Albicans*)

PLATE 3

FIG. 1. Development of mycelium with numerous chlamydospores typical of the freshly isolated smooth strain of *Candida albicans* on cornmeal agar slide cultures.

FIG. 2. Development of septate mycelium with clusters of elongate blastospores typical of the rough variant of *Candida albicans* on cornmeal agar slide cultures.

FIG. 3. Round and oval budding cells of the normal smooth culture on Sabouraud's glucose agar.

FIG. 4. Elongate cells of the rough variant on Sabouraud's glucose agar.

(Walter A. Mickle and Claudius P. Jones: Dissociation of *Candida Albicans*)