FACTORS IMPORTANT FOR THE GROWTH OF HISTOPLASMA CAPSULATUM IN THE YEAST CELL PHASE ON PEPTONE MEDIA

I. BLOOD AND BLOOD DERIVATIVES¹

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Received for publication March 29, 1951

Factors of importance in the cultivation of *Histoplasma capsulatum* Darling in its yeast cell phase have been the subject of investigation from the time it was first shown, by Demonbreun (1934), that this fungus is the causative agent of the systemic mycotic infection, histoplasmosis. In a classical presentation of the morphology, life cycle, and pathogenicity of *H. capsulatum*, Demonbreun showed that *in vitro* cultures of this organism always grow in mycelial form at room temperature, but can be made to grow in a yeast cell phase (the *in vivo*, tissue form) at 37 C under suitable conditions. He reported that although the mycelial form flourishes on many of the commonly used nutrient media, "good growth of the yeastlike form of the fungus . . . [is] . . . obtained only on blood agar or other media rich in proteins." Additional emphasis on the desirability of using blood-containing media for yeast cell growth is to be found in the publications of Conant (1941) and Campbell (1947). Heavy growth of well-encapsulated yeast cells in both liquid and solid tryptose medium fortified with 5 per cent plasma was reported by McVickar (1947). In the first of a series of interesting articles, Salvin (1947) described excellent yeast cell development in a semisolid medium, devoid of blood, blood derivatives, or other proteins. In his 1949 publication, Salvin gave the formula of a medium, completely synthetic save for the presence of agar; the presence of biotin, and either a sulfide or sulfhydryl group in this medium, was regarded by Salvin as essential for the multiplication of *H. capsulatum* in its yeast cell phase. A recent brief note (Salvin, 1950) discusses growth in liquid media containing no protein.

In 1946 an investigation was begun in these laboratories on the factors important in bringing about growth of this fungus in the yeast cell phase. Attention was first directed to attempting to define the nature of the role of the enhancing action of blood and blood derivatives. The elucidation of this aspect of the problem, briefly presented earlier (McVickar, 1948), is described in detail in this paper.

EXPERIMENTAL METHODS AND RESULTS

The basal medium: Preliminary tests with a small number of different types of peptones indicated that certain of these peptones could support some growth of *Histoplasma* in the yeast cell phase if a very heavy inoculum was used in

¹ Aided in part by a research grant from the National Tuberculosis Association.

streaking the plate. In order to find the most suitable peptone the test was therefore extended to include eighteen different peptone media. Each peptone was used in 1 per cent concentration, together with 1 per cent glucose and 2 per cent agar. Quadruplicate plates of each medium were streaked in as standardized a manner as possible with equal amounts of a very heavy suspension of yeast cells, sealed with "Parafilm," and incubated at 37 C. Readings of the plates for amount and distribution of yeast cell growth were carried out 1, 2, 4, 6, and 14 days after inoculation. Many of the peptones supported no growth whatever; on most of the others, multiplication of the inoculated cells occurred only at the very densest portion of the streaking. Difco proteose-peptone was found to support the heaviest growth, and accordingly was chosen as the basal medium for all subsequent experiments.

Inoculum: *H. capsulatum*, V.U. strain #603, isolated in yeast cell phase from a case of fatal histoplasmosis, was used in all the studies presented here. Material for inoculation was obtained from stock cultures which were maintained at 37 C on basal medium in sealed petri dishes, and transferred every five days. For use in the experiments to be described later, the basal medium was inoculated with an evenly dispersed suspension of yeast cells that had been brought to a standard, photometrically-determined density, then suitably diluted so that after inoculation of the basal medium the number of yeast cells per ml was approximately 3,000.

Technique for testing the ability of a substance to effect yeast cell growth: This technique is based on the observation that in the standard inoculated basal medium pour plate, in which each cell of the inoculum is well-isolated, little or no growth of any kind (either mycelial or yeast cell) takes place when the plates are incubated at 37 C. If blood, or plasma, or other substances of similar activity, are present, numerous yeast cell colonies develop. In the earliest experiments, the substance to be tested for its ability to support yeast cell growth was added directly to the inoculated basal medium before the agar had solidified. This technique, however, has certain disadvantages: (1) it requires an inordinate amount of test material; (2) detailed observations, particularly with the microscope, cannot be made if the substance to be tested is deeply pigmented or particulate; and (3) in testing the activity of a large number of substances at the same time, which is frequently necessary for purposes of comparison, it is difficult to be certain that all test media contain identical amounts of inoculum which has been exposed for the same length of time to the temperature of melted agar. Accordingly, a suitable technique, which would lack these objectionable features, was devised.

The substance to be tested is added in desired concentration to plain, autoclaved, cooled 2 per cent agar, then the medium is pipetted in 20 ml amounts into flat-bottomed petri plates and allowed to solidify. Using sterile precautions, 1 by 2 cm blocks are cut from the test medium and placed upon the surface of a plate of inoculated basal medium. (These blocks adhere to the basal medium so firmly that no change in position occurs during subsequent handling.) After the block has been placed in position upon the basal medium, the plate is sealed

with parafilm, incubated at 37 C, and observed at the end of 1, 2, 4, 6, and 14 days. If the test block contains a substance which does not bring about yeast cell growth, the plate remains clear. If yeast cell growth is stimulated, numerous colonies develop underneath, and in the immediate vicinity of, the test block; a rough estimation of the degree of activity of the test substance can be made by measuring the area over which yeast cell growth occurs. A diagrammatic representation of the "block method" technique is shown in figure 1; figure 2 is a photograph of a typical positive test plate.

It is apparent that when this simple technique is used (1) only a small amount of substance is needed for testing; (2) observations can readily be made on the development of yeast cell colonies in the basal medium, no matter how opaque the test substance; and (3) all pour plates of basal medium in the same test contain identical concentrations of organisms which have been exposed to the temperature of melted agar for approximately the same length of time, since

Figure 1. Diagrammatic representation of the "block method" for testing the ability of a substance to stimulate the growth of *H. capsulatum* in its yeast cell phase. A and B represent a plate culture before and after five days' incubation at 37 C, respectively. a and b are the agar blocks containing substances to be tested on the basal medium, in which each small dot represents an individual isolated yeast cell.

the inoculated agar for all plates comes from the one inoculated flask of basal medium. The technique possesses further advantages from an experimental standpoint, for the test block can be placed upon, or removed from, the inoculated basal medium at any time desired, and each plate serves as its own control. Considering the purposes for which it is employed, this block method is to be preferred over the more familiar cylinder, or paper disc technique, to which it is comparable.

Tests on the activity of different components of blood: The original plan of investigation on the ability of blood to stimulate the growth of *H. capsulatum* in the yeast cell phase was predicated on the assumption that blood contains some essential vitaminlike substance, or substances. In order to discover which component of blood is active in this respect, a simple fractionation of whole blood was conducted. The blood used throughout these experiments was recently

drawn, Wassermann positive, citrated blood, discarded by the blood bank of Vanderbilt University Hospital. (A small-scale test showed that Wassermann positive blood is neither more nor less effective than Wassermann negative blood, in enhancing yeast cell growth.) Since preliminary tests by the block method demonstrated that the plasma derived from this blood was about as effective as the whole blood itself, further fractionation was confined to the

Figure 2. Results of a positive test by the "block method." (The agar block has been removed in order to show better the yeast cell colonies beneath it.) Note the almost complete absence of colonies other than in the vicinity of the test block.

plasma. Plasma was first centrifuged, to remove completely the cellular elements, then passed through a Sela #02 porcelain filter for sterilization. Usually the filtration was attended by coagulation of the fibrine; this necessitated a second passage through a filter. Plasma lost none of its ability to stimulate yeast cell growth as a result of the procedures of clarification.

Further fractionation was begun by dialyzing 200 ml of clarified plasma in a Visking cellophane sac against repeated changes of distilled water at 4 C for 72 hours, or until no further change in volume occurred following a fresh change

of distilled water. The collected dialysates, representing the plasma filtrate, were concentrated *in vacuo* at temperatures not allowed to exceed 37 C. The undialyzable portion remaining in the sac was centrifuged; following decantation of the supernatant, the sedimented euglobulin was washed repeatedly with cold, distilled water, and brought into solution in physiological saline. The supernatant was then half-saturated with ammonium sulfate to bring down the pseudoglobulin fraction, which was collected by centrifugation, washed repeatedly with half-saturated ammonium sulfate solution, dialyzed free of ammonium sulfate, and taken up in normal saline. The final fraction, albumin, was precipitated from the supernatant (after removal of the pseudoglobulin) by full saturation with ammonium sulfate, dialyzed free of ammonium sulfate, and dissolved in normal saline. All four fractions were reconstituted in concentrations equivalent to those of the original plasma.

These components, together with a sample of the clarified whole plasma from which they had been obtained, were tested simultaneously by the block method for their ability to stimulate growth of *H. capsulatum* in the yeast cell phase. The filtrate, euglobulin, and pseudoglobulin were all devoid of activity. In the albumin fraction alone lay the ability to make the yeast cell inoculum multiply. Further purification of the albumin, by three more ammonium sulfate precipitations, resulted in no impairment of its activity. The effectiveness of plasma in the cultivation of the yeast cell phase of *H. capsulatum* was thus demonstrated to be a reflection, primarily at least, of its content of albumin.

On *a priori* grounds it seemed unlikely that so large a molecule as albumin could be functioning as a growth substance essential for multiplication of the yeast cells. Consideration was given to the possibility that the activity might be a function not of the albumin itself but rather of some other substance which remained firmly bound to the albumin throughout the fractionation procedures. Such a substance perhaps could be utilized by the well-isolated individual yeast cells of the inoculum if they were in intimate contact with it, as would be the case in an inoculated pour plate into which plasma (albumin) had been directly incorporated. However, the conditions of the block test itself rendered this concept difficult to accept, since it would be necessary to postulate migration of albumin, bearing the hypothetical growth substance, from the test block into the underlying basal medium. That such a passage did not in fact occur was demonstrated by unchanged activity of the albumin test block in spite of the interposition of a cellophane membrane (Visking cellophane, and also Dupont #300 cellophane) between the block and the inoculated medium.

It thus became apparent that, contrary to original conjecture, the effectiveness of plasma was not due to the presence of an essential growth substance. Since the test block of albumin did bring about the development of yeast cell colonies in the underlying agar, even though no substance could pass from the block into the agar below, it could be concluded only that passage took place in the opposite direction—that some substance, or substances, detrimental to the growth of *H. capsulatum* in the yeast cell phase, passed from the seeded nutrient agar into the test block, to be adsorbed by the albumin. As a possible further check

on the validity of this concept, several activated charcoals were tested by the block method. All specimens were found to be effective, to a greater or lesser extent. Norit decolorizing carbon² (purified by extraction with hot acid and repeated washings with distilled water) displayed the greatest activity, with an optimal concentration of 1 per cent. Although the activity of charcoal (also manifested through cellophane membrane) was unequivocal, the yeast cell colonies that grew in the inoculated basal medium were fewer in number, and took longer to develop, than with plasma or albumin.

Mention must be made of the vagaries in the behavior of *H. capsulatum*, with regard to its dependence on plasma for growth in the yeast cell phase. Data have been accumulated which indicate that such factors as age of the inoculum, length of time which elapses before autoclaved media are inoculated, and so forth, are of importance. These factors will be considered in subsequent papers. Such inconsistent behavior, whatever its causes, has necessitated setting up each experiment in quadruplicate and repeating each experiment several times at widely separated intervals of time.

DISCUSSION

It has been shown that the effectiveness of plasma, and therefore presumably of blood and certain other protein-containing materials, in making possible the growth of *H. capsulatum* in its yeast cell phase on peptone media, is due to the adsorption from the medium of some substance (or substances) toxic to the yeast cells. The presence in peptones of substances inhibitory for various other so-called "fastidious" pathogenic microorganisms has been reported by many others, notably Drennan and Teague (1917) and Meyer and Batchelder (1926) for *Pasteurella pestis*, Wright (1929, 1933) and Dubos (1930) for the *Pneumococcus*, O'Meara and Macsween (1937) for a variety of pathogenic bacteria, Pollock (1947) for *Hemophilus pertussis*, and Schuhardt *et al.*, (1949) for *Brucella*. Several of these authors also showed that at least one of the functions of serum or plasma in stimulating the growth of pathogenic bacteria, was that of the adsorption of inhibitory substances present in the nutrient medium. Pollock (1947) was able to show that it was the albumin fraction which was solely responsible for this adsorption. The detoxifying activity of albumin has been taken advantage of by Davis and Dubos (1947) in developing a medium for growing *Mycobacterium tuberculosis*.

The facts reported in this paper are the result of repeated observation and experimentation on a single strain of *H. capsulatum*. It should not be inferred that precisely similar results are to be obtained with all strains of this organism. Nevertheless, the basic principle of the detoxifying action of plasma has been found to be valid for ten different strains of *Histoplasma* tested in our laboratory, although these ten strains have shown a widely varying sensitivity to the toxic content of peptone.

² Eastman-Kodak Company, Rochester, New York.

ACKNOWLEDGMENT

It is a pleasure to acknowledge the skillful technical assistance of Marya Grimes in this study.

SUMMARY

The effectiveness of blood and similar protein-containing materials, in promoting the growth of *Histoplasma capsulatum* in its yeast cell phase on peptone media at 37 C, is shown to be due primarily to the ability to adsorb from the medium substances toxic for this organism. The activity of plasma in this respect is due to its content of albumin.

A simple, effective, and convenient technique for testing such activity is described.

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