TISSUE CULTURES OF MOUSE PERITONEAL EXUDATES INOCULATED WITH *BLASTOMYCES DERMATITIDIS*[1]

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Observations on mouse monocytes, maintained in cell culture and inoculated with *Histoplasma capsulatum*, have been previously reported (Howard, 1959). *H. capsulatum* differs from other fungi pathogenic for man in that it is primarily a parasite of cells of the reticulo-endothelial system. Thus the fungus was ideally suited to preliminary studies on the usefulness of tissue culture techniques for the investigation of problems of the host-parasite relationship in the human mycoses. It was thought to be of interest to extend the observations to include *Blastomyces dermatitidis*, a fungus which is not characteristically a parasite of phagocytic cells, yet has certain biologic features in common with *H. capsulatum*. Both *B. dermatitidis* and *H. capsulatum* possess two morphologic forms: a yeast form in the tissues of an infected host and a mycelial form on culture media incubated at room temperature. On suitable culture media incubated at 37 C, the organisms may be converted to their yeastlike tissue phases.

The present study reports observations on the behavior of *B. dermatitidis* in tissue cultures of mouse peritoneal exudates and compares these observations with those obtained under similar experimental conditions with *H. capsulatum*.

MATERIALS AND METHODS

Most of the materials and methods used in these experiments were described in detail in a previous paper (Howard, 1959) and are, therefore, considered only briefly in this paper.

Tissue culture methods. Suspensions of mononuclear cells were obtained by washing the peritoneal cavity of mice with chilled Hanks balanced salt solution (BSS) containing 5 units heparin per ml, 5 days after intraperitoneal injection of 1.0 ml of a 10 mg per cent solution of glycogen. Cells were washed 2 times in balanced salt solution and pipetted into 16 by 125 mm screw cap tubes containing 5 by 43 mm cover slips which had been coated and fixed to the side of the tubes with a few drops of Formvar (0.5 per cent polyvinyl formol in ethylene dichloride). After the cells had settled onto the plastic-coated cover slips, the balanced salt solution was replaced with 1.5 ml of a nutrient growth medium which consisted of 40 per cent filtered normal human serum and 60 per cent balanced salt solution.[3]

Organism. The strain of *B. dermatitidis* used in these studies was a recent isolate from a patient at the Wadsworth Veterans Administration Hospital, Sawtelle, California. Stock cultures of the fungus in the yeast phase were maintained on slants of a defined medium consisting of, per liter[4]: glucose, 10 g; NH_4Cl, 1.9 g; Na_2HPO_4, 0.568 g; KH_2PO_4, 0.136 g; $MgSO_4 \cdot 7H_2O$, 0.254 g; agar, 20 g. The cultures were stored in the refrigerator and transferred every 2 months.

Mycelial phase cultures of the fungus were grown on Sabouraud's glucose agar, stored in the refrigerator and transferred approximately twice a year.

Inoculation of cell cultures. Suspensions of yeast cells of *B. dermatitidis* were standardized and inoculated into cell cultures in the manner previously described for *H. capsulatum* (Howard, 1959).

Inoculation with the mycelial phase of the

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[3] The balanced salt solution used in preparing the nutrient growth medium was the supernatant balanced salt solution from the first washing of the exudate cells ("conditioned BSS" of Barski, Messore, and Lepine, 1955).

[4] The medium was developed by Dr. D. L. McVickar (*personal communication*) and is similar in some respects to an ammonium sulfate glucose medium employed by Levine and Ordal (1946).

fungus was made in the following manner. A saline suspension of the spores and mycelial fragments was prepared from a month-old culture grown on Sabouraud's glucose agar at room temperature. Samples (0.1 ml) of this suspension were inoculated into culture chambers after the cells had settled onto the cover slips.

Examination of cultures. Cover slips were removed at periodic intervals after preparation of the cell cultures. The cover slips were immersed in warm saline for 20 min, fixed in methanol for 5 min, and stained by the May-Greenwald-Giemsa technique (Hanks, 1955). In some experiments cover slips were stained by the periodic acid-Schiff method (Baker, 1957).

RESULTS

Inoculation of cell cultures with yeast cells of B. dermatitidis. Inoculation of peritoneal exudate

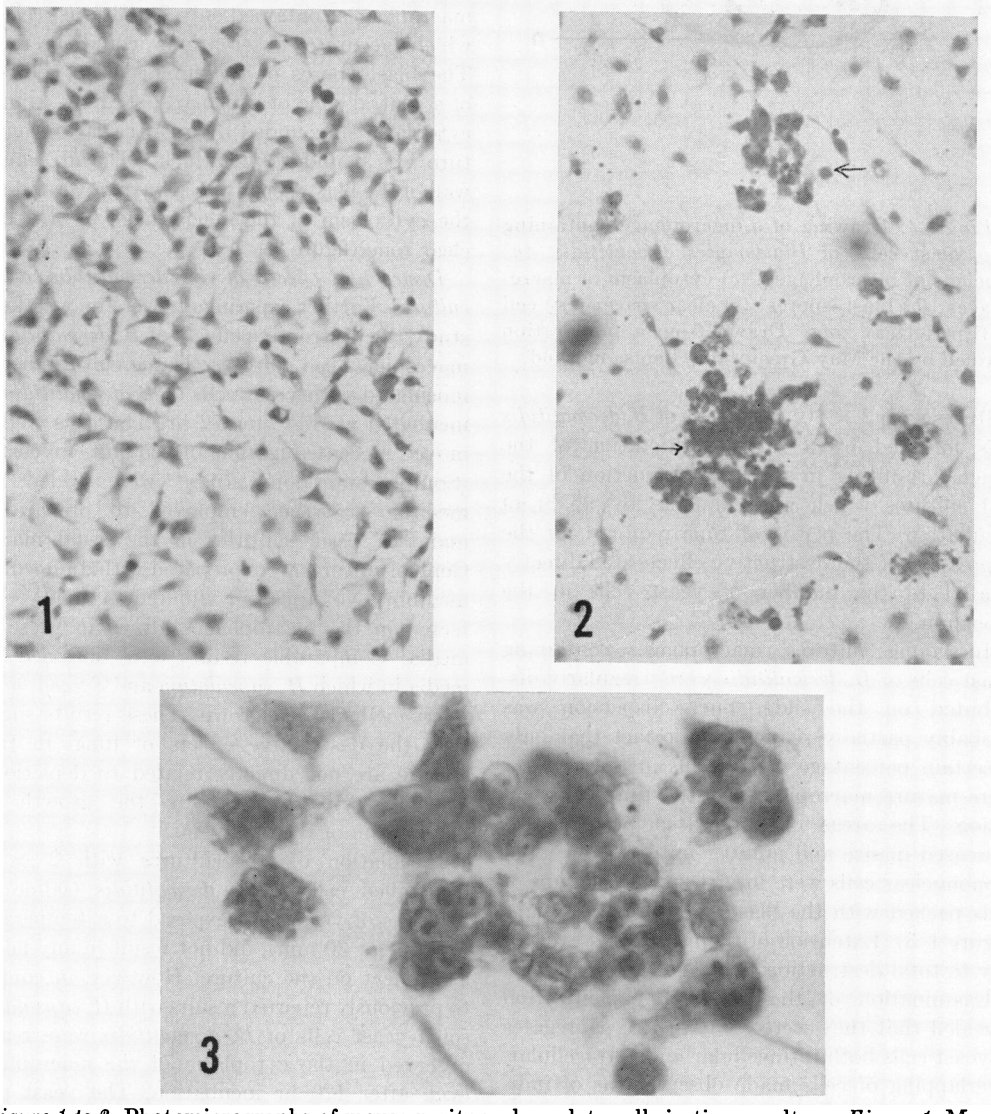

Figure 1 to 3. Photomicrographs of mouse peritoneal exudate cells in tissue culture. *Figure 1.* Macrophages from an uninfected culture 48 hr after preparation (100×). *Figure 2.* Macrophages from a similar culture 48 hr after infection with yeast cells of *Blastomyces dermatitidis* (100×). Note scattered areas of heavy parasitization. *Figure 3.* One area of figure 2 at higher magnification (450×). Note cell walls and retraction of cytoplasm of blastospores. Preparations stained by periodic acid-Schiff method.

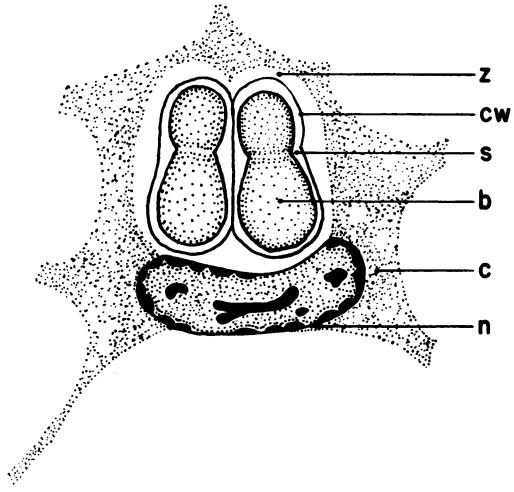

Figure 4. Drawing of a macrophage containing two yeast cells of *Blastomyces dermatitidis*. (*n*) Nucleus of macrophages; (*c*) cytoplasm of macrophages; (*b*) blastospores; (*s*) clear space; (*cw*) cell wall; (*z*) clear zone. Drawn from a preparation stained by the May-Greenwald-Giemsa method.

cultures with 2×10^5 yeast cells of *B. dermatitidis* was followed by a rapid proliferation of the fungus, resulting in marked destruction of the cell cultures which was complete by 96 hr of incubation. The period of time required for the expression of the destructive effects was directly related to the number of yeast cells in the inoculum.

In young cultures, macrophages containing yeast cells of *B. dermatitidis* were irregularly distributed on the slide. Such dispersion was probably partially related to the fact that only a certain percentage of the cells in the cultures were mature macrophages at the time of inoculation. The areas of parasitized macrophages increased in size and number until the sheets of mononuclear cells were interrupted by clumps of cells packed with the blastospores of the fungus (figure 1–3). Extension of the process led eventually to total destruction of the cultures.

Examination of the areas of parasitization revealed that they were composed of aggregates of yeast cells both intracellular and extracellular. Overlapping of cells made observations of individual blastospores within the aggregates very difficult (figure 3). Only rarely were macrophages found to be infected with less than five blastospores regardless of the size of the inoculum. The impression was gained that macrophages ingested clumps of yeast cells either present in the inoculum or resulting from extracellular proliferation. Evidence of bursting of the macrophages was seen. Such evidence indicated that ingested aggregates continued to proliferate within the macrophage.

Observations of the morphology of *B. dermatitidis* within the macrophages were difficult because of the aggregate-forming tendencies of the fungus (figure 3). However, occasional macrophages contained only 2 to 3 yeast cells, on which accurate observations could be made. The blastospores of the fungus were composed of a central zone of irregularly staining, granular cytoplasm surrounded by a clear space, which in turn was bounded by a thick cell wall. The cell wall of the blastospore was always separated from the cytoplasm of the macrophage by a delicate clear zone (figure 4).

Destructive effects of B. dermatitidis on cell cultures. Further experiments were undertaken to study the destructive effects of *B. dermatitidis* on macrophage cell cultures. Tissue culture medium inoculated with yeast cells of *B. dermatitidis* was incubated at 37 C for 72 hr. The cells were removed by centrifugation and 25 units mycostatin (Squibb and Sons, Inc.)[5] were added. The medium was then employed to prepare and maintain tissue cultures in the usual manner. Control cultures were prepared with uninoculated medium. No apparent difference could be detected in the macrophages grown in these two media. Comparable results were obtained with media in which *H. capsulatum* and *Saccharomyces cerevisiae* had been grown. These results suggest that the destructive effects of fungi in tissue culture are not directly related to depletion of essential cell nutrients by the growth and metabolism of the organisms.

Inoculation of cell cultures with 2×10^5 heat-killed cells of *B. dermatitidis* (saline suspensions of yeast cells exposed to a temperature of 60 C for 30 min), did not result in any noticeable effect on the culture. However, in contrast to previously reported results with *H. capsulatum*, some yeast cells of *B. dermatitidis* were readily observed in the cytoplasm of the macrophages even after 120 hr incubation. The yeast cells, although recognizable as *B. dermatitidis*, were

[5] This amount of mycostatin has been shown to have no obvious effect on monocytes in tissue culture (Howard, 1960).

markedly distorted in that the cytoplasm was shrunken and failed to stain as intensely as that of viable cells. The cell walls were often flattened on one or more sides giving the appearance of having been folded inward or indented. That such cells did not represent viable yeast cells which had survived the heat treatment was indicated by the failure of the supernatant medium to yield any growth of *B. dermatitidis* on subculture to Sabouraud's glucose agar incubated at room temperature.

These observations suggest that the destructive effects of *B. dermatitidis* on cultures of mouse peritoneal exudates were related directly to the rapid proliferation of the fungus intracellularly and extracellularly.

Inoculation of cell cultures with hyphal fragments of B. dermatitidis. Inoculation of macrophage cultures with the mycelial phase of *B. dermatitidis* resulted in conversion of the inoculum to the yeast phase. The sequence of morphologic changes, shown in figure 5*a–d*, was nearly identical to that observed with *H. capsulatum* (Howard, 1959). Hyphal fragments were composed of a blue granular cytoplasm with prominant red staining granules scattered randomly throughout (figure 5*a*). After 24 hr incubation, the red-staining granules were concentrated in definite packets separated from one another by clear spaces (figure 5*b*, 6). The granules often coalesced to form a large central granule. Hyphae at this stage gave a marked banded appearance (figure 6). At times the walls of the clear spaces between the packets bulged outward (figure 5*c*). The packets gave the appearance of a chain of oidia (figure 6). After 72 to 120 hr incubation, the chain of oidia fragmented into individual elements (figure 5*d* and 6). It is believed that these individual elements represent blastospores of *B. dermatitidis*.

Occasionally the ends of the hyphal fragments became swollen. Such terminal swellings, how-

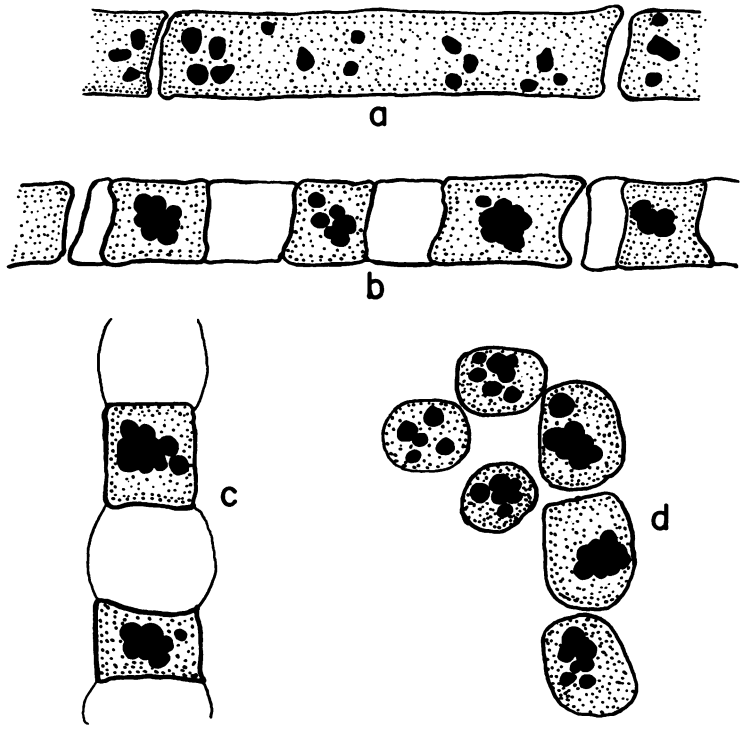

Figure 5a–d. Drawing of the various stages of development of blastospores from hyphal fragments of *Blastomyces dermatitidis*. (*a*) Fragment of mycelium with granular cytoplasm and prominent deeply stained granules. (*b*) Condensation of cytoplasm and granules into packets which give the appearance of a chain of oidia. (*c*) Walls of hyphal fragments between oidia bulging outward. (*d*) Chain of oidia shortly after fragmentation into individual elements. All drawings made from preparations stained by the May-Greenwald-Giemsa method.

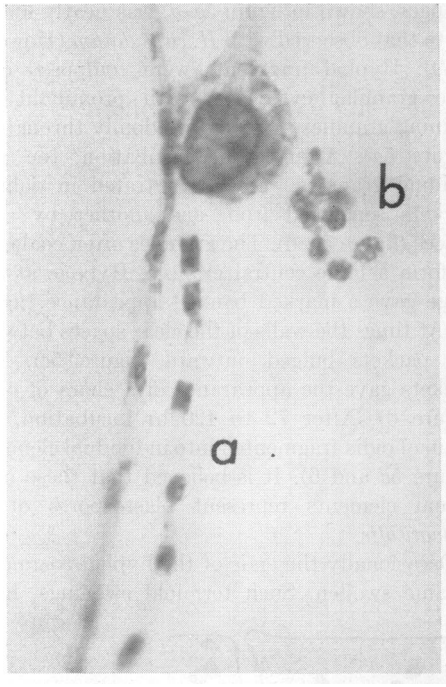

Figure 6. Photomicrograph showing hyphal fragment composed of individual packets or oidia (*a*) and a chain of oidia shortly after fragmentation into constituent elements (*b*) (450×). Preparation stained by the May-Greenwald-Giemsa method.

ever, did not seem to represent blastospores for they often formed germ tubes and continued to grow as hyphal filaments. The walls of the swellings became thickened, and the cells thus formed resembled chlamydospores. Similar structures have been observed in cultures in vitro (other than tissue cultures) incubated at 37 C (Howard, *unpublished observations*).

DISCUSSION

The marked destruction of mouse macrophages by yeast cells of *B. dermatitidis* is in contrast to the observation that this fungus has little or no effect on fibroblasts, in culture, from rat and chicken tissues (Duque, 1946-1947). The difference in effect is probably related to differences in the strain of fungus studied, size of inoculum, and to the fact the fibroblasts proliferate in cell culture, whereas macrophages do not increase appreciably in numbers in tissue culture.

The marked tendency of the yeast cells of *B. dermatitidis* to remain in aggregates during proliferation hindered effective observations on intracellular parasitism. The tendency of *B. dermatitidis* to form aggregates is characteristic of the fungus when grown on most, if not all, in vitro culture media (Conant et al., 1954). It was usually impossible or at least very difficult to discern exactly how many yeast cells were present within the macrophages. It was equally difficult to be sure if a clump of yeast cells was completely obscuring the macrophage in which they resided or whether the clump simply represented an aggregate of proliferating extracellular forms. Thus it was not considered possible to present the observations in the form of numerical data based on percentage of infected macrophages or relative numbers of intracellular parasites. These observations have led to the general conclusion that *B. dermatitidis* is not so well suited to studies involving tissue cultures of peritoneal exudate cells as is *H. capsulatum* (Howard, 1959).

The morphology of the yeast cells of *B. dermatitidis* observed in tissue culture was not different from that of published descriptions (Conant et al., 1954). The outer clear space which always surrounds the organism when in the intracellular location has been observed by other investigators interested in studying fungi as they appear within phagocytic cells (Wang and Schwarz, 1959; Young, 1958). The differentiation of the blastospores into several distinct zones with individual staining properties suggests that cytochemical studies of the yeast cells of *B. dermatitidis* might be productive of useful information concerning their structure and composition.

In spite of a large amount of study devoted to the dimorphism of *B. dermatitidis*, only a single reference to the sequence of morphologic events which attend the conversion of the fungus could be found. In a study designed to show the basic similarity between *B. dermatitidis* and *Blastomyces brasiliensis*, Conant and Howell (1942) reported their observation on the conversion of *B. dermatitidis* from the filamentous to the yeast-like type of growth. These observations were summarized by the authors as follows:

"Small fragments of hyphae were seen to concentrate their protoplasmic content in one or two cells from which round or pyriform structures were formed. These bodies continued to grow by budding until they developed a mass of large, thick-walled, round cells. After breaking away

from the mass, individual cells enlarged, continued reproducing by single buds and attained the character of the tissue form of *Blastomyces dermatitidis*."

The present work confirms and extends the observations of Conant and Howell (1942). The close biologic relation of *B. dermatitidis* to *H. capsulatum* is further emphasized by the complete similarity of the morphologic events accompanying conversion of the mycelial to the yeast forms.

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SUMMARY

Peritoneal exudates of mice maintained in tissue culture have been observed after inoculation with *Blastomyces dermatitidis*, and the observations compared with those obtained under similar experimental conditions with *Histoplasma capsulatum*. Inoculation with 2×10^5 yeast cells of *B. dermatitidis* produced an overwhelming destruction of cell cultures. The period of time required for the expression of the destructive effects was directly related to the number of yeast cells in the inoculum.

The marked tendency of yeast cells of *B. dermatitidis* to remain in aggregates during proliferation hindered effective observations on intracellular parasitism. It was not considered possible to present the observations in the form of numerical data based on percentage of infected macrophages or relative numbers of intracellular parasites. It was concluded that *B. dermatitidis* is not so well suited to studies involving tissue cultures of peritoneal exudate cells as is *H. capsulatum*.

Inoculation of macrophage cultures with the mycelial phase of *B. dermatitidis* resulted in the conversion of the inoculum to the yeast phase. The sequence of morphologic changes involved in conversion was studied in detail and found to be very similar to that reported for *H. capsulatum*.

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