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EARLY HOST-YEAST CELL RELATIONSHIP IN EXPERIMENTAL MURINE CRYPTOCOCCOSIS

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EARLY HOST-YEAST CELL RELATIONSHIP IN EXPERIMENTAL MURINE CRYPTOCOCCOSIS

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CHAPTER I

INTRODUCTION

Cryptococcosis (torulosis, European blastomycosis, Busse-Buschke disease) is a disease of varied manifestations which was first recorded in 1894 by Busse. The relationship between the host and the parasite remains poorly understood. This disease, caused by the yeast-like fungus Cryptococcus neoformans, is the most common systemic mycosis in Europe (Mathais, 1960) and, although not the most common mycosis in the United States, it is associated with about 0.2% of all autopsies at most large hospitals (Sandfelder, 1971). The history of the disease and studies of the yeast producing the disease have been well reviewed by Skinner (1950), Lodder and Kreger van Rij (1952) and Price (1971).

The Cryptococcal Cell

Although evidence for a perfect state of C. neoformans has been reported several times, most recently by Shadomy (1971), no conclusive evidence has been published to justify removing this species from the Deuteromycetes. The genus

Cryptococcus is described by Lodder and Kreger van Rij (1952) as consisting of spherical or oval cells which are encapsulated, form buds, form pseudomycelium without ascospores, produce extracellular starch, and are nonfermenters. C. neoformans is characterized by its inability to assimilate nitrogen, ability to grow at 37C and virulence for mice. With the exception of a single report of disease produced by C. albidus, only the species C. neoformans is known to cause disease in man or other animals. This yeast varies in size from 4-7 μm and, exceptionally, 2.5-20 μm in diameter.

Unlike Blastomyces dermatitidis and Paracoccidioides brasiliensis, C. neoformans is a uninucleate yeast. It shares this feature only with Histoplasma capsulatum among the common pathogenic fungi. Ultrastructural studies have demonstrated that it is a typical eukaryotic cell with finely dispersed chromatin enclosed by a nuclear membrane. The cytoplasm has been described as containing mitochondria (which sometimes occur as ring-shaped structures), endoplasmic reticulum, vacuoles and mesosomes (Al Doory, 1971). The cell is enclosed by a cytoplasmic membrane, surrounding which is a thick cell wall enclosed by a capsule with a fine fibrillar structure. No significant structural differences have been described between cells found in lesions in diseased animals and cells grown on artificial media. The cells observed in lesions are usually larger and have thicker capsules than the cells cultured on artificial media.

Portal of Entry

Most investigators believe that the portal of entry of C. neoformans in man is the respiratory tract. This theory has been supported by the finding of organisms in soil in the size range (<10 μm) believed requisite for inhalation and retention in the human respiratory tract (Ishaq, Bulmer and Felton, 1968). The worldwide occurrence of the organisms in soil suggests that man is continuously exposed to them. Some investigators have isolated C. neoformans from the skin and oral cavity of healthy humans (Benham, 1935). Sandfelder (1971) believes that human infection occurs generally by exposure to sites with heavy concentrations of the yeast, as in avian habitats frequented by pigeons and other birds. This suggestion is supported by Walter and Atchison (1966) who found that sera of pigeon breeders reacted more strongly with cryptococcal antigen than did the sera from nonpigeon breeders.

Although the evidence is mounting that this disease is airborne, little proof to associate source of infection with disease has been published. Symmers (1967) reported bird excreta containing cryptococci in the neighborhood of a hospital where patients with debilitating disease developed cryptococcosis. Hickie and Walker (1964) reported the development of a case of cryptococcosis in a debilitated patient who was attended by the same nursing personnel as a cryptococcal patient who had many organisms in his sputum

and skin lesions. If this patient were the source of infection, it would be the first documented case of man-to-man transmission of the disease and would raise considerable question over the size of the infectious particle and the portal of entry of the organism. Although cryptococci have been handled in laboratories for almost 100 years, no laboratory worker has ever been reported to have developed the disease.

The greatest support for a respiratory portal of entry for this organism has been the finding that mice can be infected by this route (Ritter and Larsh, 1963), and that cryptococci have been reported rarely as normal flora in man (Sandfelder, 1971). Also, noteworthy is the fact that primary cutaneous cryptococcosis is almost unknown, although soil contaminated wounds would be expected to produce such lesions.

The Lesion of Cryptococcosis

Cryptococcosis was first reported as a disease which resulted in osteolytic lesions (Busse, 1894). Its more important organ involvement, cryptococcal meningitis was not recognized until 1907 (Turk, 1907). In most of the early reports, the disease was characterized by the presence of large gelatinous lesions which, in many cases, were described grossly as being similiar to myxomas (Curtis, 1896; Frothingham, 1902). Since the gelatinous lesions were partially solubilized during preparation of tissues for

microscopic examination, it was believed by Stoddard and Cutler (1916) that the yeast produced enzymes that digested the tissue around them (histolysis). Based on this observation, they named the organism Torula histolytica. The histolysis theory was rejected in later monographs by Freeman (1931), and Littman and Zimmerman (1956). It was Freeman who first described the large cryptococci-filled structures as gelatinous lesions. Gelatinous lesions of this type can be produced in experimental animals and they may become so massive as to account for 20% of the animal's total body weight (Price, 1971). Classically, there is no evidence of acute inflammatory cells in these lesions and a minimal number of macrophages are seen. It is this type of lesion that has impressed workers with the unresponsiveness of the host to the etiologic agent (Freeman, 1930). The gelatinous-type lesion is especially common in disease of the central nervous system and may account for considerable displacement of brain tissue as the lesion increases in mass.

The second type of lesion commonly seen in cryptococcosis was described as granulomatous by Freeman (1931). This lesion is characterized by the presence of small numbers of cryptococci and large numbers of chronic inflammatory cells. The granulomatous-type lesion is recognized by the absence of an observable yeast-cell capsule, which makes a disease with this type lesion difficult to diagnose. This type of response has led to the belief that

cryptococcosis is a chronic disease similar to tuberculosis and histoplasmosis. If the usual host response to the organism is this type of inflammatory process, and if as many individuals are infected as has been suggested by Littman and Walter (1968), the granulomas must resolve more readily than those seen in tuberculosis and histoplasmosis since no evidence of residual granulomas has been seen, with the possible exception of that reported by Baker and Haugen (1955). The lack of necrosis in these lesions is associated with infrequent calcification, another characteristic of cryptococcal granulomas.

With the possible exception of cases that had an associated acute inflammatory response, neither gelatinous nor granulomatous cryptococcosis has apparently been cured without the aid of newer antifungal drugs. This suggests that a granulomatous response does not result in resolution of the disease after it has become clinically obvious.

The first acceptable evidence that cryptococcal capsular polysaccharide was a prominent feature of lesions was provided by Levine, Hirano and Zimmerman (1964), who demonstrated ultrastructurally cryptococcal polysaccharide in experimental murine cryptococcosis of the central nervous system. The material was found remote from the yeast cells and unlike the situation with tuberculosis and histoplasmosis, phagocytosis of the yeast cells was an infrequently observed phenomenon.

Predisposing Factors in Cryptococcosis

Coexisting disease is said to be more frequent in cryptococcal meningitis than in pulmonary cryptococcosis (Campbell, 1966; Spickard *et al*, 1963). There appears to be a significant relationship between cryptococcosis and leukemic and lymphomatous disorders, with a concomitancy as high as 80% (Hutter and Collins, 1962). Iatrogenic damage to reticuloendothelial tissue is believed to increase susceptibility to this disease (Baker, 1971). As with most mycotic diseases, corticosteroids and the cytotoxic drugs used in chemotherapy of malignant disease are believed to greatly increase susceptibility to cryptococcosis.

Virulence Factors

With one possible exception (Bulmer and Sans, 1968), no virulence factors have been identified in *C. neoformans*. One toxin has been identified by Stypulkowski *et al* (1971) and it has been shown to have powerful antimitotic activity. These authors reported longer survival times in patients with malignant disease when associated cryptococcal disease was present. Bulmer and Sans (1968) demonstrated that cryptococcal capsule was antiphagocytic in *in vitro* studies with human leukocytes. At the present time, however, there is no general understanding as to how the organism produces disease other than by its presence and the host's response to the organisms, mechanisms first proposed by Stoddard and Cutler (1916). Cortisone has been considered by some workers

to increase the susceptibility of mice to fungus disease (Sandfelder, 1971). Colchicine, another antiinflammatory agent similiar to cortisone, is believed to interfere with phagocytosis (Berlin and Ukena, 1972).

Animal Models

The mouse has been used by most investigators (Baker, 1971) to study experimental cryptococcosis. It is considered by some (Stoddard and Cutler, 1916) to be a good model for the human disease, although Carbonell (1971) states that the abscess formation seen in mice has not been observed in man. Mice develop both gelatinous (Price, 1971) and granulomatous (Stoddard and Cutler, 1916) types of lesions, as does the human.

Experimental animals dying of cryptococcosis exhibit primarily central nervous system disease usually involving extensive cerebral cortical lesions with resulting displacement and herniation of the central nervous tissue. Stoddard and Cutler (1916) described experimental granulomatous meningitis with resulting hydrocephalus. Rats (Curtis, 1896), rabbits, and dogs (Stoddard and Cutler, 1916) have been used to study this disease.

Portals of Entry in Experimental Disease

The mouse may be infected by various routes. Because the respiratory tract is believed to be the natural portal of entry for cryptococcosis, various investigators have

infected animals by nasal instillation (Basu Mallik et al, 1966), by aerosol (Price, 1971) and exposure to soil containing cryptococci (Ritter and Larsh, 1963). These methods of infection have the disadvantage that early lesions are very difficult to locate microscopically in tissue. Subcutaneous injection of organisms was used by Bergman (1961) to study the early events in experimental murine cryptococcosis. Other routes of inoculation which have been used to study host response are intraperitoneal, intravenous and intracerebral injection of organisms. Intramuscular injection was used by Berry (1969) to study macrophage turnover in experimental histoplasmosis because of the (a) ease of locating early lesions in this tissue, (b) well established lymphatic drainage of limb muscles in mice (Barone, Bertrand et Desenclos, 1950) and (c) ease of locating the lymph nodes that drain this area.

Objectives of this Investigation

Because the ultrastructure of lesions caused by fungi is still an unexplored area (Carbonell, 1971), it was decided to characterize the yeast-host relationship at both light and electron microscopic levels, with emphasis on the early inflammatory reactions in the lesions. An ultrastructural investigation has been made in only one case of human pulmonary cryptococcosis (Caulet et al, 1969), and only once in experimentally induced disease (Levine, Hirano and Zimmerman, 1964). In the present study, an experimental

lesion was followed after inoculation of cryptococci into skeletal muscle. Observations on the early changes in the yeast cells and host cells and their subsequent interactions as the lesion developed were explored. The mouse was used in this investigation. It is assumed that it mimics the human in its response to the organisms, although it is probable that mice are more susceptible to the disease than man.

In order to evaluate the role of the cell wall, capsule, other diffusable products, and the presence of structures the size of yeast cells in the host, experiments were designed to evaluate the response of mice to (a) heat-killed cryptococci, (b) saline, (c) latex spheres 6-14 m in diameter, and (d) diffusable products produced by cryptococci isolated from the host inside a diffusion chamber implanted into the mice. The effect of cortisone acetate and colchicine pretreatment of animals on the development of early lesions was also explored.

CHAPTER II

MATERIALS AND METHODS

Organism

Two strains of Cryptococcus neoformans were used in these studies. One strain, C1A, has been used extensively in this laboratory and produces large, gelatinous cryptococomas (Price, 1971) in mice. The other strain, PH, is a recent isolate from a gelatinous osteolytic lesion in man. Both isolates were maintained by twice weekly subculturing on Brain Heart Infusion (BHI) agar (Difco).

Experimental Animals

White Swiss mice were obtained locally from Dr. Allen J. Stanley, Department of Physiology and Biophysics, University of Oklahoma Health Sciences Center and were used in these studies. Mice of both sexes were equally represented. At the begining of each experiment all mice weighed an average of 35 gm. All animals were maintained in the University of Oklahoma Health Sciences Center Laboratory Animal Facility.

Diffusion Chamber

In order to evaluate any changes caused by products elaborated *in vivo* by *C. neoformans* cells, these organisms were placed in diffusion chambers which were then implanted into the peritoneal cavity of mice.

Preparation of Cells used in the Implant

Cryptococci, grown on BHI agar for 48 hours were suspended in 0.85% NaCl (saline) to a final concentration of 10^7 cells/ml as determined by an American Optical Hemacytometer and used in experiments.

Construction of the Diffusion Chambers

The diffusion chambers (Milipore Filter Corp. Bedford, Mass) consisted of a plastic ring 1.0cm in diameter onto which two membranes (pore size, 0.22 μm) were cemented with Milipore Cement to form a chamber. All chamber components (two membranes and ring) and the chamber assembly tool were sterilized by treatment with 70% ethanol and allowed to dry under sterile conditions. The dry components were then assembled, cemented together, filled with 0.1 ml of the yeast suspension, and sealed. A total of 20 chambers were constructed, 10 filled with PH strain cells and 10 filled with C1A strain cells. Ten of these (five of each strain) were implanted into animals on the day of construction of the chambers. The remaining 10 chambers were individually placed into BHI broth-filled culture tubes and incubated

for 3 days at 25C. All chambers were constructed at the same time to insure that minimal differences would exist among any of the individual chambers.

Contamination Testing

To determine if diffusion chambers were contaminated with unwanted organisms or if the construction would prevent escape of C. neoformans the culture tubes containing the diffusion chambers were inspected after incubation at 25C for 3 days. The broth was examined for bacterial or fungal growth by observing it for turbidity and staining samples with Gram's stain.

Diffusion Chamber Implantation

Ten mice were anaesthetized with nembutal (sodium pentobarbital, Abbot laboratories), placed in dorsal recumbency and shaved from xiphoid to pubis. The exposed skin was cleaned with 70% ethanol and an incision was made through the skin. The skin was separated from the muscle of the abdominal wall, the muscle was incised and the loaded chamber placed in the peritoneal cavity. The incision was then closed and the animal was allowed to recover under the heat of a 60 watt light to prevent hypothermia. Following recovery the mice were caged and moved to the laboratory animal facility.

The mice were observed twice weekly. Any change in behavior or physical state was recorded. Animals dying

during a 90-day period were examined postmortem and portions of the cerebrum, lungs, heart, liver, spleen, and kidneys and contents of diffusion chambers were prepared and inoculated onto Sabouraud Dextrose agar (Difco) and BHI agar and incubated at 25C to detect C. neoformans. The remaining portions of tissues were fixed in 10% neutral buffered formalin for future histologic studies. Animals surviving 90 days post-implantation were sacrificed and examined in a similiar manner. The implanted diffusion chambers were removed, separated and one half of each diffusion chamber (one membrane) was fixed in glutaraldehyde, post-fixed in osmium and prepared for electron microscopy.

Fixation of Tissues and Yeasts

One of the problems in ultrastructural studies of fungi is the difficulty of penetrating the cell wall with fixatives (Carbonell, 1971). Because of the good preservation of mammalian tissues with glutaraldehyde followed by post-fixation with Palade's osmium fixative (Palade, 1952), glutaraldehyde was selected as the primary fixative for electron microscopic studies of C. neoformans grown on agar media. For this purpose 24-hour cultures on BHI agar plates were cut into 3 mm cubes with a razor blade and placed directly into the primary fixative. Because of the difficulty of paraffin sectioning glutaraldehyde-fixed tissues and the better preservation of fungi with more rapidly penetrating fixatives (Carbonell, 1971), 4% formaldehyde in phosphate

buffer was prepared from paraformaldehyde (Sabatini, 1953). This later fixative was prepared freshly before animals were to be sacrificed and was used as a perfusate and primary fixative, followed by post-fixation with osmium.

Development of Cryptococcal Lesions

To study the development of primary cryptococcal lesions, mice were inoculated with C. neoformans, strain PH. Because of the difficulty of accurately delivering a small number of yeast cells to any area of the lung, the triceps muscle was selected as the inoculation site since it is a well circumscribed organ with a known lymphatic drainage (Barone et al, 1950). This experimental model is similiar to the one used by Berry (1969) for studying the host response to Histoplasma capsulatum. This is an established model for studying fungus and chronic inflammatory disease alike.

Preparation of Cells

Forty-eight-hour cultures of BHI-grown PH strain cells were suspended in saline and adjusted to 10^8 cells/ml. A series of ten-fold serial dilutions (1:10, 1:100, 1:1000, 1:10,000, 1:100,000, 1:1,000,000 and 1:10,000,000) was then made and these dilutions were cultured on BHI agar to establish a viable cell count/total cells present. The 1:1000 dilution was used for inoculation of animals.

Inoculation of Animals

White Swiss mice were anaesthetized with 0.5 mg sodium nembutal administered intraperitoneally. The right forelimb was then shaved and swabbed with 70% ethanol. Suspensions containing 10^3 cells or latex spheres in 0.05 ml saline were injected into the triceps muscles while the process was observed under a dissecting microscope. The animals were allowed to recover under the heat of a 60 watt light bulb.

Animals were separated into seven groups and injected with 0.05 ml of each of the following: (1) saline, (2) heat-killed (60C for 1 hr) PH strain cells suspended in saline, (3) living PH strain cells suspended in saline (4) latex (styrene divinylbenzene copolymer lates, Dow Chemical Co., Midland, Mich.) spheres 6-14 μm in diameter, suspended in saline, (5) living PH strain cells inoculated into animals pretreated with 0.005 mg colchicine injected intramuscularly 4 hours prior to inoculation, (6) latex spheres injected into colchicine-treated animals and (7) living PH strain cells inoculated into animals pretreated with 5.0 mg cortisone acetate injected subcutaneously 24 hours prior to inoculation.

Evaluation Schedule

Throughout the study, a total of 84 animals in all experimental groups (12 in each) were sacrificed at 1,2,4,8, 16,32 and 70 hours post-injection. The saline control animals were dropped from the study after 16 hours post-

injection because of the lack of substantial response and the early reversal of changes produced in these animals.

Tissue Preparation

Animal sacrifice. Mice were anaesthetized using 0.5 mg sodium nembutal administered intraperitoneally. They were then placed in dorsal recumbency and an incision was made from xiphoid to mentalis. The skin was reflected and the thoracic cage was opened using two lateral incisions. The right atrium was then cut and the animals were perfused through the left ventricle with 20 ml of cold (4C) Sabatini's buffered formaldehyde (Pease, 1964). Following perfusion, the skin was removed from the right forelimb and the limb and deep axillary nodes were removed at the level of the acromion. The limb was then immersed in 50 ml of fixative and stored at 4C overnight. The next day, the triceps was removed by sectioning the upper muscle mass along the humerus. The muscle was then sectioned transversly into 3 mm slices. Sections containing lesions, as observed with a dissecting microscope, were selected for light and/or electron microscopic study. The brain, lungs, liver, and spleen from one animal of each group were also taken and fixed in 10% neutral buffered formalin.

Light microscopic study. The 3 mm slices were dehydrated in alcohol, cleared in xylene, infiltrated with paraffin and cut at 5 µm using a Baush and Lomb rotary microtome. Sections were stained with alcian blue,

mucicarmine, Gomori's methenamine-silver (Emmons, 1970) or hematoxylin and eosin (Lillie, 1954).

Electron microscopic study. The 3 mm triceps slices of animals injected with living cells were cut into 3 mm cubes, washed in three changes of Sabatini's buffer, and post-fixed for three hours in Palade's osmium. Following post-fixation, the tissues were washed three times in osmium buffer, dehydrated in alcohol, cleared in propylene oxide, and infiltrated in either 6:4 Epon 812 or Araldite 502 epoxy resin. The infiltrated tissues were placed in gelatin capsules and polymerized for 24 hours at 60C. Sections for electron microscopic examination were cut at 100 nm on a Porter Blum MT-1 Ultramicrotome, mounted on copper grids and examined with an RCA EMU-3G electron microscope at an accelerating voltage of 50 KV.

Screening Resin Blocks for Lesions

Because of the small number of cells inoculated into the mice, the majority of lesions were less than 1 mm in diameter. Approximately 50 epoxy resin blocks were prepared from each animal and sometimes only one of them contained tissue with lesions. Because of the amount of time required to prepare thick sections for light microscopy this technique was abandoned and examination of razor blade trimmings from the block was substituted. Because of their large size, the yeast cells in the trimmings could be visualized at magnifications of 200X when placed under a light microscope.

This technique allowed for rapid evaluation of the blocks and for selective trimming of the block faces to include small lesions. Blocks with observable lesions were selected for thin sectioning and ultrastructural study.

Thick Sections from Epoxy Resins

To provide for a better comparison between paraffin-imbedded light microscopic sections and epoxy resin-imbedded electron microscopic sections, some specimens were cut at 1.5 μm on a Porter Blum MT-1 Ultramicrotome using glass knives. Sections were placed on glass microscope slides and stained with a 1.0% crystal violet solution (Pease, 1964), dehydrated, cleared, mounted in permount and examined by light microscopy.

Thin Sections from Epoxy Resins

Epon and Araldite epoxy blocks were cut at 100 nm (gold-silver interference color) on a Porter Blum MT-1 Ultramicrotome using glass or diamond knives. Sections were collected on clean #300 mesh copper grids and stained with uranyl acetate and lead citrate.

Identification of Cells in Lesions

Microscopic morphology of cultured C. neoformans cells has been described previously by Edwards et al (1967) and Al Doory (1971). Any cell with a prominent cell wall, lipid inclusions, mitochondria with thick cristae, and an extracellular capsule was considered to be characteristic of

this yeast.

Of the cells in the inflammatory exudate, neutrophils were considered to be those cells with a multilobed nucleus and dense cytoplasm with small lysosomal granules. Eosinophils were identifiable by their distinctively structured lysosomes. Macrophages included all cells with a kidney-shaped to round nucleus and evidence of phagocytic activity (phagocytized material within the cytoplasm). These criteria conform to those established by other workers in the field (Papadimitriou and Spector, 1971).

CHAPTER III

RESULTS

Morphology of C. neoformans

PH strain Cryptococcus neoformans cells cultured on BHI agar and examined by light microscopy appeared as small, budding yeast cells 4-7 µm in diameter with prominent refractile bodies within the cytoplasm. When cells were suspended in India ink and examined, small capsules measuring up to 3 µm in thickness were visible on a few of the cells. Strain C1A had a similar morphology. The thickness of the capsule decreased with incubation time.

Ultrastructural examination of the yeasts proved to be extremely difficult due to the problem of obtaining good infiltration of the imbedding resin into the cells. A solution to the problem was sought by using various fixatives (glutaraldehyde, formaldehyde, acrolein and osmium) and by extending the dehydration, clearing and infiltration times and temperatures. None of these modifications, including infiltration with resin for 24 hours at 60C improved the results significantly. In addition to the problem with infiltration of resin, the hardness of the cell wall made sectioning of these cells difficult, even when diamond

knives were used. Even the best sections showed considerable distortion of the yeast cells.

When sections were studied ultrastructurally, the yeast cells were observed to have buds and contain a granular cytoplasm with prominent mitochondria, the most conspicuous cytoplasmic organelles (Appendix, Fig.1). The mitochondria were typical for yeast and had cristae which were very thick in comparison to those seen in mammalian cells. Structures identifiable as nuclei were rarely seen. The yeast cell wall appeared as a thick border surrounding the cell and was fibrillar in nature. The capsule was not prominent in these cells but portions of it could be seen as a granular to fibrillar layer surrounding the cell wall.

Animals Containing Diffusion Chambers

Of the 10 animals receiving diffusion chamber implants with C1A and PH cells, only three (1 C1A and 2 PH strain) survived to the 90th day following surgery. Three animals demonstrated erosion and necrosis of the skin overlying the implant within the first week and died with bacterial peritonitis secondary to open abdominal wounds. C. neoformans cells could not be recovered from any of the internal organs of these animals. Of the remaining 4 animals which did not survive to the 90th day, one died on day 14, one on day 20, one on day 28 and one on day 89. Organisms could not be isolated from the organs of any of the animals, although microscopic examination of the diffusion chambers indicated

that yeast cells were present. Cultural examination of the diffusion chambers revealed the presence of viable cells. No evidence of disease was found in the animals that were sacrificed on the 90th day. Microscopic examination of these chambers revealed yeast cells on the inner surface of the diffusion chamber membranes. Ultrastructural examination of these membranes revealed yeasts within a granular matrix lying alongside the membrane. The yeasts were similar to those seen in culture.

Lesion Development in Untreated Animals

One Hour Post-Injection

Gross examination of animals. One hour after injection of the mice with living or dead yeasts or latex spheres there was no difference in the gross appearance of any of the animals. No lesions were readily observable except for those of the site of injection.

Microscopic appearance of the lesions in animals which received living cells of C. neoformans. Microscopic examination of tissue sections from these animals revealed small yeast cells (<5 μm in diameter) in the interstitial spaces between muscle fibers, sometimes within the perimysium (Appendix, Fig. 2). Inflammatory cells were seen frequently at this time although separation of the perimysium indicated the presence of edema, probably secondary to inoculation trauma. Vacuolization of the individual muscle

fibers was observed, probably also as a result of trauma.

Microscopic appearance of the lesions in animals which received heat-killed cells of C. neoformans. At this time period there were no detectable differences between lesions produced by live cells and those produced by heat-killed cells.

Microscopic appearance of the lesions in animals which received latex spheres. When sections of tissues from animals removed one hour after injection with latex were characterized, accumulations of the spheres were noted in the perimysium, with minimal signs of edema or degeneration of the muscle parenchyma (Appendix, Fig. 3).

Ultrastructure of the lesions at one hour. One hour following inoculation of live C. neoformans, the cells were difficult to locate ultrastructurally. They were most frequently found adjacent to muscle fibers that were degenerating and, in their size and lack of conspicuous capsule, did not differ greatly from those seen in culture. However, a thin layer of capsule was beginning to develop on the cells (Appendix, Fig. 4). Especially noticeable in these cells was the amorphous, granular cytoplasm with complex, membrane-bound bodies 2-3 μm in diameter. Frequently, distorted mitochondria with thick cristae were seen in the yeasts. As with the light microscope studies, no inflammatory cells were seen at this time. Occasional yeast were noted along muscle fibers that were intact and

well preserved.

Lesions produced by heat-killed cells did not differ significantly from those seen in animals inoculated with live yeast cells.

Summary of observations in animals at one hour following injection. Mice injected with living or dead C. neoformans cells, latex spheres of approximately the same size as the yeast, or sterile saline responded with a small amount of edema as represented by separation of the muscle fibers surrounding the site of injection. The injected particles tended to accumulate in the muscle perimysium. Degeneration of the skeletal muscle was evidenced by homogenization of the fibers. Inflammatory cells were absent or present in very small numbers at this time period.

Two Hours Post-Injection

Gross examination of the animals. Gross examination of the animals two hours following injection of yeast cells or latex spheres revealed no significant changes different from those seen at the first hour.

Microscopic appearance of the lesions in animals which received living cells of C. neoformans. No changes were observed that suggested that these lesions were different from those seen in the animals one hour following injection.

Microscopic appearance of the lesions in animlas which received heat-killed cells of C. neoformans. No significant differences were seen between these tissues and those of the one hour group.

Summary of findings in animals at two hours following injection. Examination of tissue from the triceps muscle of mice injected with live C. neoformans, heat-killed C. neoformans, latex spheres or sterile saline revealed edema, characterized by separation of the muscle fibers. The yeast from tissues were identical morphologically to those seen on BHI culture.

Four Hours Post-Injection

Gross examination of the animals. No detectable lesions were seen on external examination of the animals four hours following injection. In grossly sectioned, fixed muscle, light colored areas, in the center of the muscle, were sometimes seen. These lesions had indistinct borders, were generally spherical in shape and measured approximately 1 mm in diameter.

Microscopic appearance of the lesions in animals which received living cells of C. neoformans. Microscopic examination of tissues from these animals revealed an accumulation of yeasts with early inflammatory changes which consisted of edema and infiltration with acute inflammatory cells. Phagocytosis of the majority of cells was a conspicuous feature in most animals. Only a few of

these animals failed to respond with infiltration of the lesion by phagocytic cells and in still others even though inflammatory cells were present, the yeast cells were not phagocytized. Vacuolization of the muscle fibers was associated with these lesions. The live yeasts had begun to develop a capsule which could be seen in sections which were stained with alcian blue or mucicarmine (Appendix, Fig. 5,6). This was the last time period that live cells with a small capsule were seen. In addition to an increase in capsule size, the yeast cells themselves were noticeably larger (an increase in size from 4-7 μm to 8-10 μm).

Microscopic appearance of the lesions in animals which received heat-killed cells of C. neoformans. The inflammatory response of these animals was idenical to that seen in animals inoculated with living cells. However, the heat-killed yeasts failed to develop capsules or enlarge in size.

Microscopic appearance of the lesions in animals which received latex spheres. Examination of tissues from these animals revealed an accumulation of polymorphonuclear inflammatory cells similiar to that seen in the animals inoculated with living cells. Phagocytosis of the spheres was also noticed.

Ultrastructure of the lesions at four hours. Tissues from animals inoculated with living yeasts were prepared for ultrastructural examination. At the fourth hour following

inoculation, phagocytosis was more prominent than at any other time during the observation periods. Numerous well preserved inflammatory cells were seen, of which neutrophils were the most common. Eosinophils were frequently noted closly associated with mononuclear cells which contained numerous mitochondria (Appendix, Fig. 7).

Neutrophils containing as many as four phagocytized yeasts were observed (Appendix, Fig. 8), many of which were collapsed forms. No actual fusion of lysosomes with phagosomes could be detected in any of these cells. It was common to see yeasts in varying stages of degeneration within a single phagocyte; the most distorted yeasts within the phagocyte were the most degenerative-appearing cells ultrastructurally. These changes in the yeasts consisted of loss of mitochondria, coarse granulation of cytoplasm and the appearance of amorphous, electron dense material. At this time period, muscle was seen frequently adjacent to the lesion. It was intact, with minimal or no degenerative changes. Ultrastructurally, there seemed to be two types of collapsed or crescent-shaped yeasts. One type demonstrated no detectable change in the cell wall; the other demonstrated breakage of the outer parts of the wall at the points of collapse (Appendix, Fig. 9). As had been noted at the light microscopic level, the early development of the large, extracellular capsule (frequently fibrillar in nature) was noted at the fourth hour postinoculation. Extensive

degenerative changes were occasionally seen in the phagocyte following engulfment of the yeast (Appendix, Fig. 10). In these cases the yeasts usually did not appear to have undergone the degenerative changes noted above. Lipid vacuoles continued to be a conspicuous feature within the yeasts at this stage of development.

Summary of findings in animals at four hours following inoculation. The fourth hour marked the begining of phagocytosis of the yeasts by the polymorphonuclear phagocytes that had appeared at the site of inoculation immediately prior to this time period. The cells were capable of phagocytizing four or more yeasts or latex spheres. Frequently the yeasts appeared to have collapsed, an action which was sometimes associated with breakage of the outer parts of the yeast's cell wall and was usually associated with degeneration of yeast cell contents (loss of mitochondria and cell organelles).

Eight Hours Post-Injection

Gross examination of the animals. Gross examination of these animals prior to sacrifice revealed nothing unusual. They exhibited no physical or behavioral changes. Lesions were obvious in grossly sectioned triceps muscle. The lesions were pale, spherical structures, 1.0-1.5 mm in diameter, within the center of the muscle mass. They were more prominent in animals which had been inoculated with live yeast cells.

Microscopic appearance of the lesions in animals which received living cells of C. neoformans. Microscopic examination of tissue from animals inoculated with live yeast cells revealed early abscess formation. Phagocytosis was less prominent than it was at the fourth hour following inoculation. The yeast cell capsules were larger than they had been previously (Appendix, Fig. 11,12). At this time period numerous collapsed, crescent-shaped yeasts were seen in phagocytic cells within the lesions. This form of yeast cell was seen frequently in older lesions. Prominent spicules were frequently seen radiating from the yeast cell wall. This stage of lesion development was characterized also by an accumulation of neutrophils around individual yeasts. It was at this time period that difficulty was encountered in getting the mounting resin infiltrated into the yeast cells. This resistance to resin infiltration correlated with the increase in the size of the yeast cell wall.

Microscopic appearance of the lesions in animals which received heat-killed yeast cells. The lesions produced in response to injection of heat-killed cells contained more inflammatory cells than did the lesions seen at the fourth hour.

Microscopic appearance of the lesions in animals which received latex spheres. Phagocytosis of latex spheres was diminished from that seen in the lesions four hours following injection. A wide variability in the number of inflammatory

cells was noted.

Ultrastructure of the lesions at eight hours. At eight hours post-inoculation, phagocytosis of live yeast cells was not as conspicuous as had been seen previously. Neutrophils appeared to surround individual yeasts (Appendix, Fig. 13). Yeasts were encapsulated at this stage and the cell wall was thickened considerably. No degranulation of lysosomes within the phagocytic cells was seen. Also of interest was the observation that the phagocytes which contained phagocytized yeasts did not seem to accumulate around the remaining extracellular yeasts. Thus, the characteristic features of the lesions eight hours post-inoculation were the lack of recent phagocytosis, the encircling of the yeasts by neutrophils, the continued development of capsule and thickening of the cell wall.

Summary of findings in animals at eight hours following injection. The lesions at eight hours following injection were more cellular than earlier lesions due to an increased infiltration of acute inflammatory cells. The lesions of animals that received heat-killed cells were essentially unchanged from those observed at four hours following injection. The animals that received living C. neoformans had lesions that were detectably larger than those in animals receiving heat-killed cells. Microscopically, the yeasts were now larger in size, due to an increase in both the size of the cell and its capsule. In addition

phagocytosis of the yeasts was not as prominent when compared to the 4 hour specimens. As the yeasts enlarged they became resistant to the penetration of embedding resin and difficulty was encountered in sectioning for ultrastructural study. The lesions produced in latex-injected animals were essentially unchanged from those seen at four hours.

Sixteen Hours Post-Injection

Gross examination of the animals. In some of the animals (those inoculated with living C. neoformans), grossly recognizable enlargement of the muscle surrounding the site of injection was seen at this time period. Gross examination of the fixed and sectioned muscle from animals injected with live yeasts revealed lesions similar to those described at four hours except that the lesions were larger (2-3 mm in diameter) and had a better defined border.

Microscopic appearance of the lesions in animals which received living cells of C. neoformans. Microscopic examination of tissue from these animals revealed a large accumulation of inflammatory cells. An area of many large, heavily encapsulated, budding yeasts was frequently restricted to a part of these lesions, most commonly the periphery. The capsules were readily recognizable with the mucicarmine and alcian blue stained preparations (Appendix, Fig. 14). The peripheral part of the lesion closely resembled the gelatinous type lesions described by Freeman

(1931). A small percentage of cells were collapsed and crescent-shaped. Phagocytosis of the yeasts was not seen. Observation of tissues stained by the methenamine silver technique revealed yeast cells that were not seen with the other staining methods. The cells that were seen only in methenamine silver preparations were scattered throughout the collection of inflammatory cells and appeared non-encapsulated. Because of the close approximation of neutrophils in these lesions, phagocytosis of yeasts could not be discerned. The methenamine silver-stained yeasts were small (2-4 μm in diameter) and frequently distorted.

Microscopic appearance of the lesions in animals which received heat-killed yeast cells. Examination of tissue from animals injected with heat-killed cells showed early abscess formation. Yeasts were observed only in tissues stained by the methenamine silver method. The abscesses were not significantly smaller than those observed in the animals which had been injected with living cells.

Microscopic appearance of the lesions in animals which received latex spheres. Examination of the muscle tissue from animals that received latex spheres again showed considerable variability and revealed, in some animals, lesions consisting of latex spheres and inflammatory cells, while in other animals there was accumulation of latex spheres with no inflammatory cells.

Ultrastructure of the lesions at sixteen hours. By the sixteenth hour following injection of living yeasts,

exudate surrounded the yeast cells. Cells within the exudate resembled those seen at four and eight hours except that many of them demonstrated "U" shaped invaginations of the cell wall (Appendix, Fig. 15). Also prominent was the lack of capsular material, large electron-lucent vacuoles and the mitochondria with the thick cristae which are usually characteristic of the yeast. Many of the neutrophils associated with the lesions were degenerating and contained coarsely granulated cytoplasm. Although fibroblasts were infrequent, fine collagen fibrils were becoming a conspicuous part of the peripheral (gelatinous) part of the lesions (Appendix, Fig. 16). Toward the margin of the lesion, where the yeasts were numerous and large in size, macrophages were seen which contained many vacuoles containing fibrillar material which was structurally similar to cryptococcal capsule. Sections of large yeasts in these lesions were difficult to make because of the problem of infiltrating them with resin.

Summary of the animals at sixteen hours following injection. The sixteenth hour after injection was characterized by the consistent appearance of macrophages in the lesions. Continued growth of the yeasts occurred in the lesions produced by the inoculation of live C. neoformans. This proliferation occurred most commonly at the periphery of the lesions and was characterized by accumulation of numerous large, heavily encapsulated cells. Many small yeasts were

also seen in the lesions of these animals. These small forms were found within the central parts of the lesions and were surrounded by neutrophils. The small yeasts became infiltrated with resin readily and had thin cell walls and little capsular material. The cell walls were frequently collapsed to form small "U" shaped structures. The animals injected with heat-killed yeasts or latex spheres demonstrated lesions similar to those seen at the eighth hour following injection.

Thirty-two Hours Post-Injection

Gross examination of the animals. Grossly, there were no differences between these animals and those examined at sixteen hours following injection. Observation of gross sections of the fixed muscle revealed 2-3 mm lesions in the animals inoculated with living yeast cells. At this time period the lesions appeared more circumscribed than did those at earlier time periods.

Microscopic appearance of the lesions in animals which received living cells of C. neoformans. Microscopic examination of the tissue from the group of animals which received living yeasts revealed large areas consisting almost entirely of yeasts. Some of the cells were surrounded by several phagocytes. These large gelatinous, yeast-containing, areas had displaced muscle fibers and the large collections of acute inflammatory cells noted previously. In a few areas, encapsulated yeasts appeared within the dense

collections of acute inflammatory cells. Macrophages were now prominent in the peripheral part of the lesions and, in some areas, they demonstrated faintly-staining mucicarmine-positive material within their cytoplasm (Appendix, Fig. 17). The macrophages appeared to have several vacuoles of the faintly-staining material within their cytoplasm which increased the size of the cytoplasm and gave it a "foamy" appearance. Most of the yeasts showed evidence of active budding and were surrounded with capsules that, in sections stained by the alcian blue and mucicarmine techniques, were very dense. Only in the methenamine silver-stained sections were numerous nonbudding, nonencapsulated, small yeast cells seen in the center of the lesions surrounded by degenerating neutrophils.

Microscopic appearance of the lesions in animals which received heat-killed yeast cells. Heat-killed cells of C. neoformans produced lesions similiar to those seen with live cells except that there was no gelatinous marginal proliferation and the yeast cells were frequently broken and distorted and visible only in tissue stained with methenamine silver.

Microscopic appearance of the lesions in animals which received latex spheres. At 32 hours animals which received latex spheres demonstrated lesions indistinguishable from those seen in animals examined at sixteen hours postinjection.

Ultrastructure of the lesions at thirty-two hours. At this time period a thick cortex of cryptococcal cells surrounded areas of exudate. Although many different techniques were attempted the cortical part of the lesions were resistant to infiltration of the imbedding resin. Consequently, only the central part of the lesions, and the cortical part of the lesions that contained very few yeast cells, could be examined. The large yeasts in such areas never became infiltrated with resin and thus sectioning for ultrastructural examination was difficult. Outside the cell wall of these large yeasts was a capsule which consisted of two layers, the inner layer was dense and granular and the outer was less dense and fibrillar. Surrounding these cells were many macrophages with complex cytoplasmic membranes and phagosomes containing material consisting of fibrils similiar in appearance to the outer, less dense capsule of the yeasts. Frequently, bodies within the phagosomes could be seen which appeared to be cell wall remnants of yeasts (Appendix, Fig. 18). Macrophages with the phagosomes containing fibrillar material appeared identical to the macrophages seen at the light microscopic level which contained intracytoplasmic mucicarmine-positive material within their cytoplasm.

Summary of reactions in animals at thirty-two hours following injection. Heat-killed yeasts and latex spheres were associated with lesions identical to those seen at

sixteen hours. Living C. neoformans produced lesions by the thirty-second hour that were characterized by areas of large heavily encapsulated (8-15 μm) yeasts. These areas had many macrophages which contained a substance with the staining characteristics of cryptococcal capsular material and gave the macrophages a "foamy" appearance. Other areas of the lesion contained closely packed neutrophils, macrophages, and small yeast cells which were demonstratable light microscopically, only by methenamine silver staining. The large yeasts were resistant to infiltration with imbedding resin and could not be sectioned for ultrastructural study. Examination of the "foamy" macrophages at the electron microscopic level revealed phagocytic cells with many phagosomes containing fibrillar material similiar in appearance to the less dense part of the yeast capsule. Also seen were residual bodies similiar in appearance to collapsed yeast cell walls.

Seventy Hours Post-Injection

Gross examination of the animals. At seventy hours following injection only the animals which had received living yeast cells demonstrated obvious external manifestations of the lesions within the muscle. Gross sectioning of the fixed muscle of these animals revealed the presence of 2-3 mm lesions. No external lesions were detectable in the animals which had received heat-killed cells or latex spheres and sectioning of the muscle revealed changes no different

from those seen at thirty-two hours.

Microscopic appearance of the lesions in animals which received living cells of C. neoformans. Microscopic examination of tissue from animals which had received living yeasts revealed enlargment of the gelatinous component of the lesions and retraction of the collections of neutrophils and yeast cell debris. At this time period, macrophages and collagen gave an organized appearance to the central part of the lesion. Even deep within the central part of the lesions the cytoplasm of the macrophages demonstrated a "foamy", vesicular appearance. Frequently, small, red-staining bodies were seen within these cells in tissues stained by the mucicarmine technique. Surrounding, and within the gelatinous, peripheral part of the lesion, were many similiar macrophages. For the first time in the development of the lesions, yeasts could be seen in the lymph nodes. These cells resembled those seen in the original lesions and they were surrounded by macrophages in the subcapsular sinus. In one of the animals, yeasts were also seen in the perivascular connective tissue of the lung. Foamy macrophages were also associated with these lesions.

Microscopic appearance of the lesions in animals which received heat-killed cells or latex spheres. Tissues of animals in this group remained unchanged from those examined at earlier time periods.

Ultrastructure of the lesions at seventy hours. At the seventieth hour following inoculation of living C. neoformans cells, ultrastructural examination revealed no significant differences from results observed at the thirty-second hour.

Summary of the animals at seventy hours following injection. The animals injected with either heat-killed or latex spheres demonstrated lesions identical with those observed at thirty-two hours. The animals inoculated with living yeasts demonstrated lesions similiar to those seen at thirty-two hours except that the cortical part of the lesions had enlarged due to an increase in the number of heavily encapsulated yeasts and the accumulation of "foamy" macrophages.

Lesion Development in Treated Animals

Cortisone Acetate-Treated Animals

Changes in the yeast cell. Pretreatment of mice with cortisone acetate had no detectable affect on the yeast cells in the lesions.

Development of the lesion. Four hours post-injection of yeasts, there was an infiltration of neutrophils into the lesions. Associated with the infiltration of neutrophils was phagocytic activity that correlated well with that seen in the untreated animals at the fourth hour. No differences in response were noted at any other time periods. There

seemed to be little or no detectable difference between this group and the untreated animals inoculated with live C. neoformans cells when observed grossly.

Colchicine-Treated Animals

Animals injected with latex spheres. No gross changes were seen in these animals one hour after injection. In gross sections of fixed muscle the latex spheres appeared as white to yellow-colored particles in the center of the muscle mass. Microscopic examination of the lesions one to four hours post-injection revealed numerous neutrophiles, edema, vacuolar degeneration of the skeletal muscle fibers and non-phagocytized latex spheres. From four hours to seventy hours post-injection a gradual disappearance of the inflammatory cells was observed.

Animals inoculated with living yeast cells. Gross examination of animals at one to four hours post-inoculation revealed no detectable lesions. In gross sections of the fixed tissues no changes were observed at one and two hours post-inoculation, other than occasional bleeding which probably resulted from trauma induced by inoculation. By the fourth hour post-inoculation some of the animals demonstrated translucent, white lesions 0.5 to 1mm in diameter. Microscopic examination of these lesions revealed more edema than the earlier non-treated or cortisone treated animals had shown. This transudate was more acidophilic than that seen previously. Phagocytosis seemed to be a less prominent feature

at the fourth hour post-inoculation but many neutrophils with phagocytized cells were seen. Vacuolization of the muscle fibers and degeneration of the skeletal muscle nuclei were also slightly more conspicuous. Gross examination of the animals eight through seventy hours post-inoculation revealed increasing masses in the triceps muscle which reached a maximum at seventy hours post-inoculation. These lesions were white, translucent and well demarcated from the surrounding tissue by the end of the observation period.

CHAPTER IV

DISCUSSION

Cryptococcosis occupies a position between the mycotic diseases caused by virulent fungi and those caused by the more opportunistic fungi. This intermediate position is due to the lesser virulence of C. neoformans and the weak proliferative response to the yeast on the part of the host (Sandfelder, 1971). Littman and Walter (1968) believe, however, that cryptococcosis is a common subclinical entity. Since C. neoformans exists in the environment in a size range small enough (<10 μm) to penetrate deep into the lung, it has been postulated that man frequently becomes infected with this yeast (Littman and Walter, 1968). Presumably, this type of infection is followed with an acute inflammatory response by the host which may terminate the infection or, in rare instances, fail to terminate the initial stages of disease and lead to the appearance of clinically recognized disease. The present study involved the characterization of this presumably initial lesion and the factors which may alter it in an experimental animal model reported to be similiar to the situation in human disease (Stoddard and Cutler, 1916).

Although several authors have reported on the ultra-

structural morphology of the yeast C. neoformans (Al Doory, 1971; Edwards et al, 1967 and Carbonell, 1971), the PH strain used in this study has never been described ultrastructurally. The difficulty of sectioning pathogenic fungi for electron microscopy has been described by Carbonell (1971). This present study revealed that the use of even the most vigorous penetrating fixatives left most of the yeast cell contents unfixed. This difficulty made it impossible to infiltrate some of the yeasts with the imbedding resins required for electron microscopy. Examination of the PH strain revealed that it was typical of the strains described previously ultrastructurally using cultures maintained on artifical media (Al Doory, 1971; Edwards et al, 1967).

The Milipore diffusion chamber implant experiments were designed to study the effects of diffusable products from C. neoformans on the host, e.g. toxic symptoms, disease, or death. None of the animals which received implants developed any of these signs that could be detected by observation of the living animals or by gross and microscopic studies of their organs. These results are compatible with those of Price (1971) who demonstrated that large gelatinous lesions, termed cryptococcomas, weighing as much as 20% of the total body weight of the mouse, produced no evidence of disease other than that produced by large space-occupying lesions. Diffusion chamber experiments under the conditions described demonstrate that this yeast can be in an environment protected

from phagocytosis and survive any lethal serum factors. Survival was based upon both cultural and morphologic evidence. Thus, phagocytosis was not required to protect the yeasts from any fungicidal properties of the host's serum. In addition to the survival of the yeasts, no toxic changes were detected in the mice during this time period. These results lend further support to the belief by certain investigators that such toxic products are not elaborated (Freeman, 1930).

The traumatic introduction of 10^3 C. neoformans cells into the skeletal muscle of mice initiated a well-timed, acute, inflammatory response with early signs of edema beginning the first hour after injection. Yeasts 4-7 μm in diameter and without detectable capsule were seen in dialated perimysial septa at this time and very closly resembled cells seen in culture. During this time the yeasts in tissue had a conspicuously thin cell wall and prominent lipid vacuoles, characteristic of cultured cells.

Four hours after injection of live cryptococci into untreated mice phagocytosis became a prominent feature of the lesion. The fact that cryptococci can be phagocytized by human neutrophils was established in 1968 by Bulmer and Sans. Light microscopic examination of the muscle tissue containing the cryptococcal lesions at this time period revealed that many of the yeasts were within phagocytic cells. Because most of the yeasts were alive when they were injected, it is

tempting to believe that most of the cells that were phagocytized were also alive. Evidence for the viability of the phagocytized cells was basically on morphologic evidence. Yeasts with mitochondria similiar in structure to those seen in cultured cells and in nonphagocytized cells were assumed to be alive. Mitochondrial morphology is one of the more sensitive indicators of cell death and autolysis (Hayat, 1970). Frequently, a single phagocytic cell was seen to have ingested more than one yeast cell and there was evidence of sequential degenerative changes in these phagocytized yeast cells. These changes consisted of loss of mitochondria, increased density of the cytoplasm and bending of the cell wall, especially where it was in contact with an adjoining phagocytized yeast cell. These sequential degenerative changes can be an indication that the neutrophil of the mouse has the capability of destroying cryptococci.

Although the incorporation of lysosomes into phagosomes containing the yeast cells was never observed, some of the neutrophils contained no detectable lysosomal granules in their cytoplasm, indicating that the lysosomes may have been incorporated into the phagosome at an earlier time. Not all of the phagocytes containing phagocytized yeasts appeared to kill the yeast cells as demonstrated by degenerative changes. In some cases, the neutrophil appeared degenerative and the cryptococci appeared viable. The lesions seen at the fourth hour following injection were puzzling

because of indications that some neutrophils had the ability to kill the phagocytized yeasts while others did not. From light and electron microscopic observation of these lesions it was assumed that most of the yeasts were phagocytized by the fourth hour.

The eighth hour post-infection was characterized by a change in the yeasts from small, thinly encapsulated cells to larger, more heavily encapsulated cells. After this change took place, phagocytosis by neutrophils was no longer obvious; the neutrophils were noted in rosette formation around the yeasts when sections of muscle taken from infected mice at the eighth hour after injection were examined microscopically. This arrangement of neutrophils is believed not to have an adverse effect on the yeasts, as degeneration of yeasts was not noted. None of the yeast cells contained within the rosettes appeared to be degenerating, which suggests that the host was incapable of killing the yeast cells.

The sixteenth hour after inoculation of live yeast cells was characterized by the development of a thick cortical portion in the nonencapsulated abscess. The yeasts in the cortical part of the abscess were believed to have been derived from the cells that were not killed by the rosette formations seen in the eighth hour. The most obvious difference between the cortex and the central part of the lesion was the small number of yeasts in the latter and the abundance of cells in the former. Not only were yeast cells

more abundant in the cortical region of the lesion but they were larger and had thick capsules. The yeasts were now characteristic of those seen in tissues of infected animals. The differences between yeasts grown on artificial media and those observed in infected tissues have been noted since the time of Sanfelice (1895). These differences consist of an increase of both yeast cell and capsule size in the organisms observed in lesions in animals. Although various media have been designed to grow cryptococci similiar in morphology to those found in infected tissues, success has not yet been achieved.

The large yeasts with thick cell walls and large capsules were difficult to section for electron microscopic examination. These large yeasts were surrounded with macrophages; accumulations of neutrophils were seen in the central part of the lesions surrounding yeasts which had thin capsules. These yeasts were detectable only on light microscopic sections stained by the methenamine silver technique. These small yeasts were infiltrated with resin, easily sectioned and found to have thin cell walls and thin capsules. Many of these small cells were located inside phagosomes of neutrophils, forming the majority of the cell population of the central part of the lesion. As seen at the four hour period the "U" shaped invaginations of the yeast cell walls were noted which probably represent the collapse of yeast cell walls due to phagocytic activity. These

invaginated yeast contain cytoplasmic organelles with good morphology.

The thirty-second and seventieth hours following injection were characterized by a continued increase in the size of the cortical portion of the lesions. It was similiar to that described at the sixteenth hour except there were more yeast cells and a greater amount of extracellular capsule was present. Thin sections were obtained from this part of the lesion and examined with the electron microscope. Due to the thickness of the cryptococcal cell wall and its impermeability, no sections could be made of the yeast cells themselves, but sections of the capsules surrounding these yeast cells were obtained. Ultrastructural examination revealed that the capsule consisted of two distinct zones. The inner zone was a very dense fibrillar structure while the outer zone was less dense and consisted of fibrils radiating peripherally from the inner zone. Occasionally the capsule appeared granular instead of fibrillar. This difference seemed to depend on the type of fixation used although it was not completly predictable. Surrounding this capsule were macrophages with many vacuolar structures containing fine, reticulated material. The material possessing this reticulated pattern was comparable in appearance to that seen in the outer, less dense capsule of the yeast cells. Light microscopic examination of this material stained with the mucicarmine technique revealed staining of the vacuoles in these cells. Although the process

forming these structures was never observed, it is believed that these vacuoles contained capsular material from the yeast cells. The structures resembled capsule material ultrastructurally and stained with the mucicarmine technique, indicating mucopolysaccharide content.

The development of the cortical part of the cryptococcal lesion represented a failure by the host to contain the infection. Once these large, heavily encapsulated cells developed in the lesion, the host phagocytes (macrophages and neutrophils) lost the ability to ingest the yeast cells and could only phagocytize the capsular material surrounding the yeasts. During the observation period, the yeasts ability to produce capsule seemed to exceed the ability of the macrophages' ability to ingest it. This resulted in the inability of the macrophages to approach the cryptococcal cell.

Although four day lesions were well circumscribed, it became obvious early in the post-inoculation period that yeast cells were being carried via the lymphatics where they appeared without signs of inflammation in the subcapsular sinus of regional lymph nodes. Yeast cells not only were found in the lymph nodes, they appeared in the lungs of the animals. This could be explained by the failure of the lymph nodes to contain the infection thus allowing yeast cells to enter the circulatory system with returning lymph. Because of the large size (>10 μm) of some of these cells, it would have been difficult for them to traverse the capillary bed of the

lung and they would be filtered out in the lung after leaving the right heart.

In comparing the exudation of inflammatory cells in lesions produced by viable cryptococci with the exudation seen when dead cryptococci and latex spheres were injected, there was no appreciable difference between these lesions during the first sixteen to thirty-two hours except for the production of capsule by the living cells and for the increase in size and number of these cells. The injection of all cryptococci (living or dead) and latex spheres produced more inflammation than was seen when sterile saline was injected.

Findings from these investigations confirm what many authors have observed (Carbonell, 1971). Specifically, cells of C. neoformans produce no tissue changes other than that caused by compression of the tissue secondary to the growing cells or damage produced by the host in responding to the yeast cells. The absence of tissue damage by this yeast makes this organism difficult to find in some lesions and helps explain the classic absence of calcification of old cryptococcal lesions. It should be reemphasized that no product elaborated by these yeast cells has been proven to be toxic to man or animals. The difficulty in explaining human disease is not so simple however. There still remains no satisfactory answer as to why more people are not seen with cryptococcosis. No cases have been found in the literature which could be described as early subclinical cases.

If this form of cryptococcosis is as common as some authors believe (Littman and Walter, 1968), it seems that it would have been reported from autopsies in people who died from other causes.

C. neoformans is unique among the pathogenic fungi in that it is encapsulated. This capsule has been described in lesions by Levine et al (1964) and Caulet et al (1969). Cryptococcal capsule is unique when compared with capsules of other infectious agents. It seems to become a prominent part of the lesion with no active, digestive degeneration of it by neutrophils or macrophages. This was noted by Levine et al (1964) who observed the prominent role of the capsule in disease and believed it to be an inherent characteristic of the brain lesions. In this study the capsule was also seen to become a prominent part of the lesion. There was no apparent damage inflicted to the phagocytes by the phagocytized material and the presence of the fibrillar pattern of the capsule within the phagosomes indicated that the phagocytes could not process or digest this material.

In animals injected with living cells and sacrificed eight hours post-injection obvious retraction of the capsule had occurred. This phenomena was noticed by Stoddard and Cutler (1916) and led them to suggest that it represented digestion of the tissue around the yeasts. Ultrastructural studies of lesions also revealed some retraction of this capsular material. However, because of the irregularity of

this capsular retraction at electron microscopic levels, and the failure of the capsule to retract in some cells, it is suggested that the space represents fixation retraction. There was no evidence to support a digestive process in the tissue of the lesion.

It has been popular to incriminate corticosteroids for decreasing the resistance of the host to fungal infection. Supposedly this drug restricts the incorporation of the phagosome and lysosome (Merkow et al, 1971). Such a mechanism would function if the cell normally killed all yeasts and the killing was dependent on the incorporation of lysosomes into the phagosomes. However, in these and previous experiments (Price, 1971), cryptococcosis in mice was found to be an infection that never resolves. Because no experimental animals can apparently eliminate the infecting cells, previous workers have used the rate of dissemination as an index of increased susceptibility in mice treated with corticosteroids. In the present study, the response of animals pretreated with cortisone acetate was no different from that of untreated mice. Phagocytes of animals treated with colchicine phagocytized yeasts at a rate comparable to that seen in untreated animals. Neither of the drugs appeared to have any effect on the development of lesions, the population of cells seen in the lesions or the behavior of the phagocytic or yeast cells in the lesions.

CHAPTER V

SUMMARY

The objective of this study was to characterize the reactions between Cryptococcus neoformans and the inflammatory cells of mice in vivo, and to observe the host's reaction to these yeast cells which were isolated from the host by a membrane that could not be penetrated by inflammatory cells of the host.

For this purpose, 10^6 yeast cells were placed in Milipore diffusion chambers which were implanted into the peritoneal cavity of mice. The animals were followed for a period of 90 days. No infection of tissues outside the chamber resulted and no behavioral or physical changes in the mice were noted during this time period. After the animals were sacrificed, all chambers were found to contain viable C. neoformans.

The role of inflammatory cells in early experimental lesions was studied by inoculating mice with 10^3 C. neoformans cells in the triceps muscle. The lesions were followed grossly, microscopically and ultrastructurally for a period of seventy hours to study changes that evolved in the yeast and inflammatory cells of the host. Edema was noted in the

first two hours post-inoculation. Four hours post-inoculation neutrophils entered the lesion and began phagocytizing the yeast. During the fourth hour the yeast cells began to develop capsules and lose the lipid vacuoles that were prominent in cultured cells. The development of capsule continued and, by the eighth hour, the neutrophils were incapable of phagocytizing the yeast cells. The neutrophils were now seen to surround the yeast cells. As the lesions aged, the yeast cells began to enlarge and produce large amounts of capsule. This enlarging focus was seen to compress the accumulation of neutrophils and associated small, nonencapsulated yeast cells. By the thirty-second hour post-inoculation macrophages began to appear and phagocytize the capsular material.

Comparison of these lesions with lesions produced by the injection of heat-killed yeast cells and latex spheres of a similiar size demonstrated that the major difference in the lesions produced was the inability of the dead cells or latex spheres to reproduce and grow in size.

Pretreatment of animals with colchicine or cortisone acetate did not appear to have any detectable affect on the development of lesions produced by living C. neoformans.

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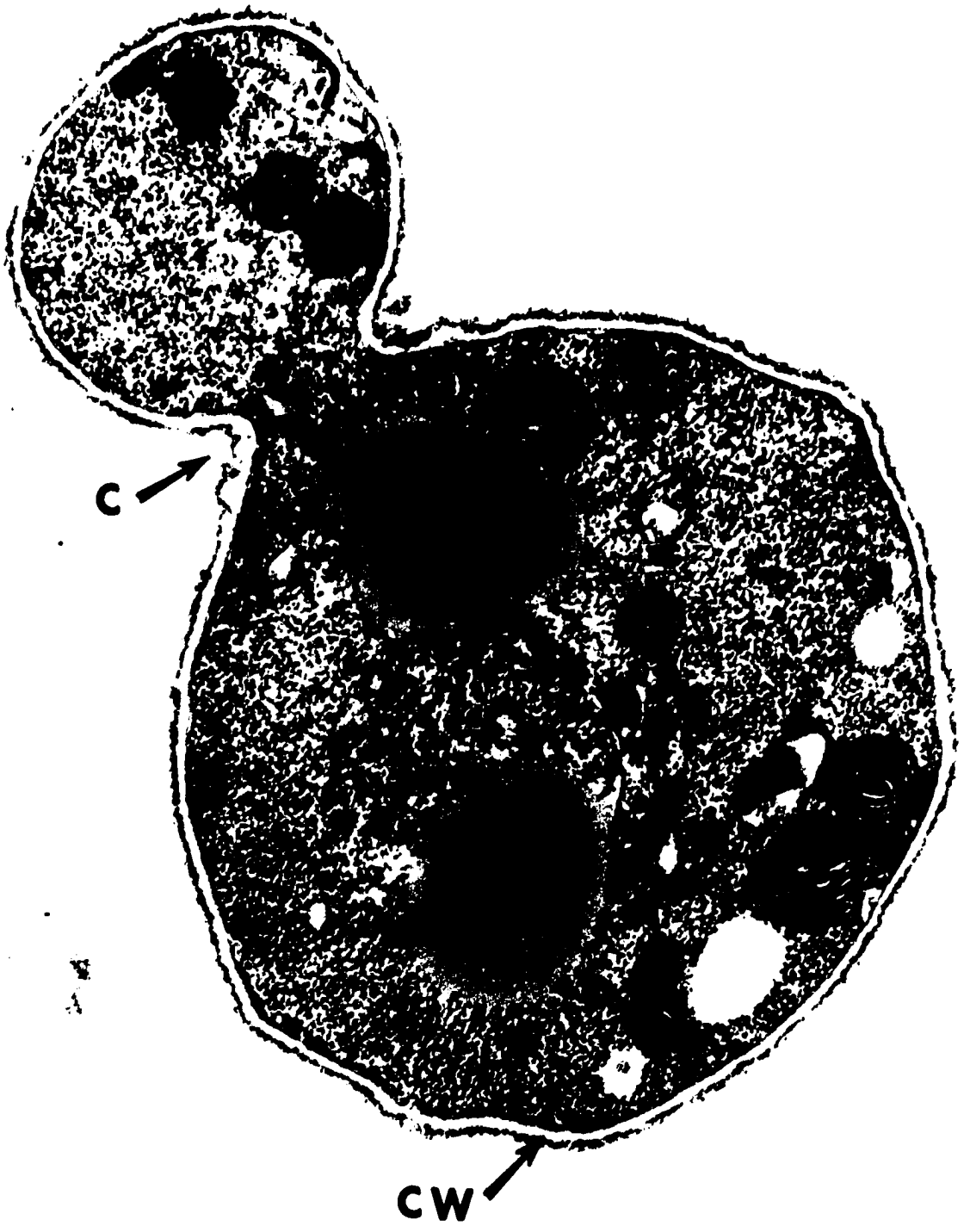
APPENDIX

Figure 1. BHI-grown Cryptococcus neoformans cell. 38,000X. An electron micrograph of a budding yeast cell with a small amount of capsule (C) around the thin cell wall (CW) is seen. The cytoplasm is filled with lipid vacuoles (L) and mitochondria (M). No nuclear structures are visible.



(1)

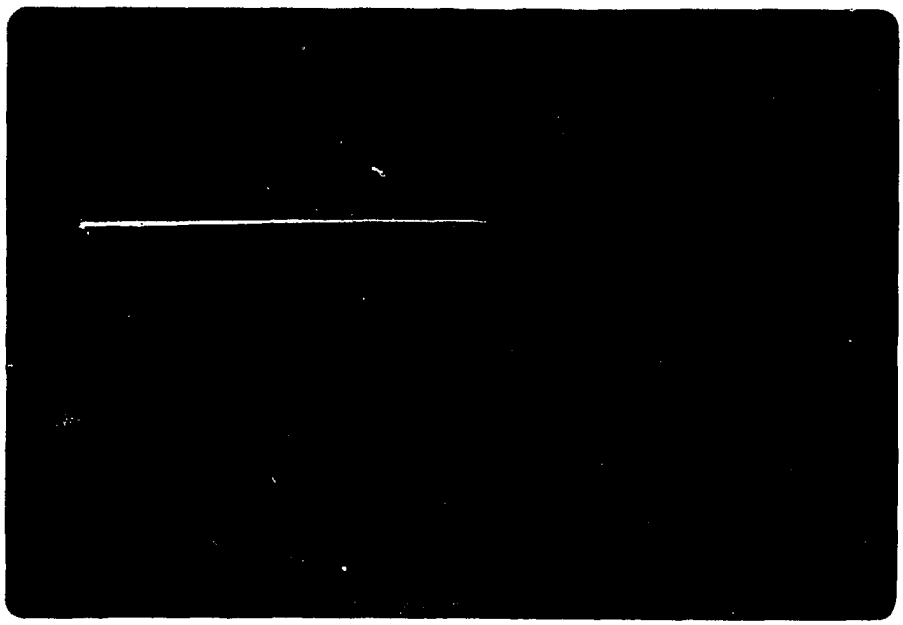


Figure 2. Cryptococci in muscle perimysium one hour after injection. hematoxylin and eosin stain. 800X.

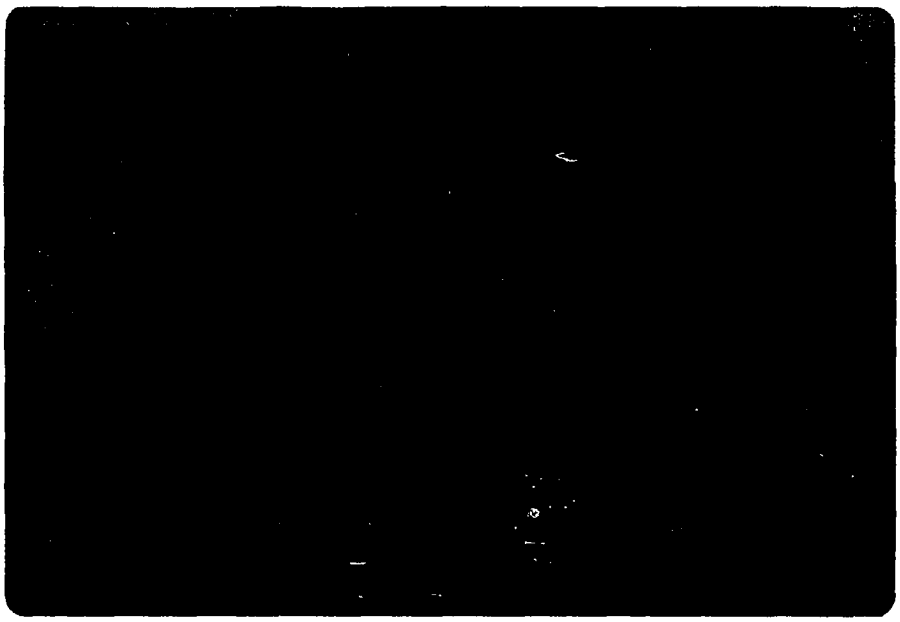


Figure 3. Latex spheres in muscle one hour after injection. hematoxylin and eosin stain. 1,000X.

Figure 4. Cryptococcus neoformans cell one hour after inoculation. 22,000X. A small, thin walled yeast cell with a thin layer of capsule (C) and a complex, membrane-bound structure (arrow) is depicted. Yeast cells with this type of inclusion were seen infrequently when grown on artificial media.


C

Figure 5. Cryptococci in muscle four hours after inoculation. alcian blue stain. 1,000X.

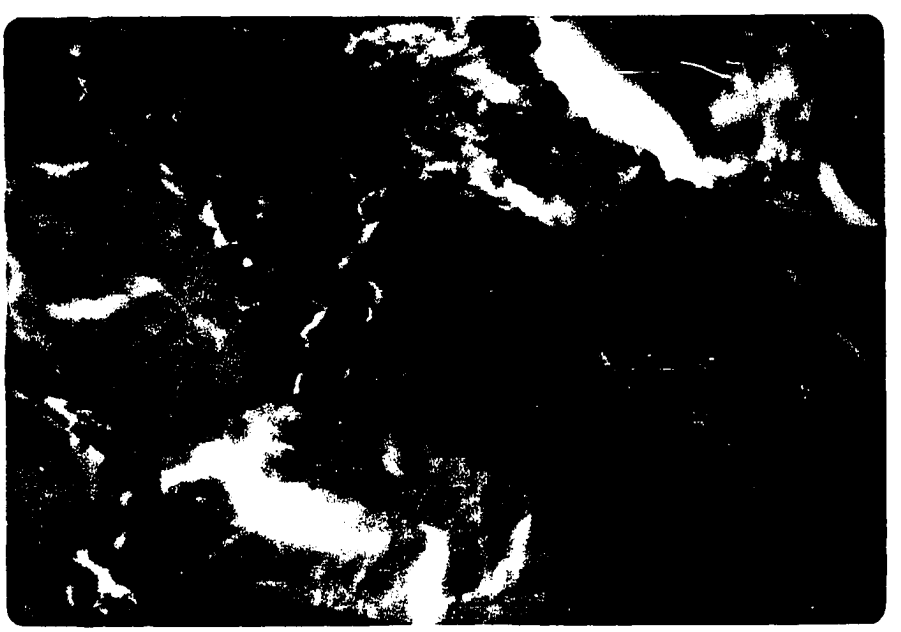


Figure 6. Cryptococci in muscle four hours after inoculation. mucicarmine stain. 1,000X.

Figure 7. Eosinophil contact four hours after inoculation. 18,000X. Eosinophil contact with outer cells in inflammatory exudate was commonly observed. The eosinophil is recognizable by its characteristic granules (G)

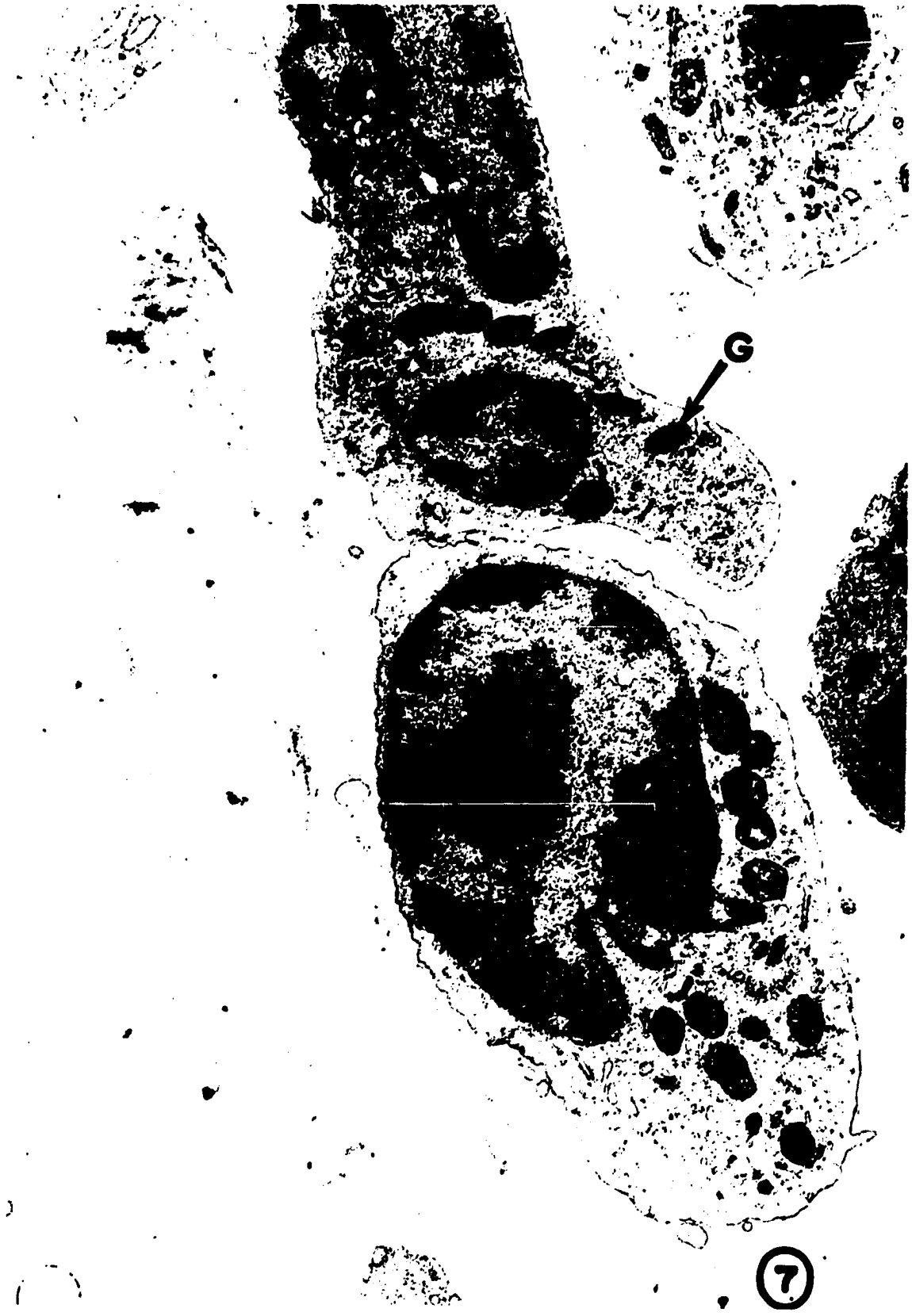
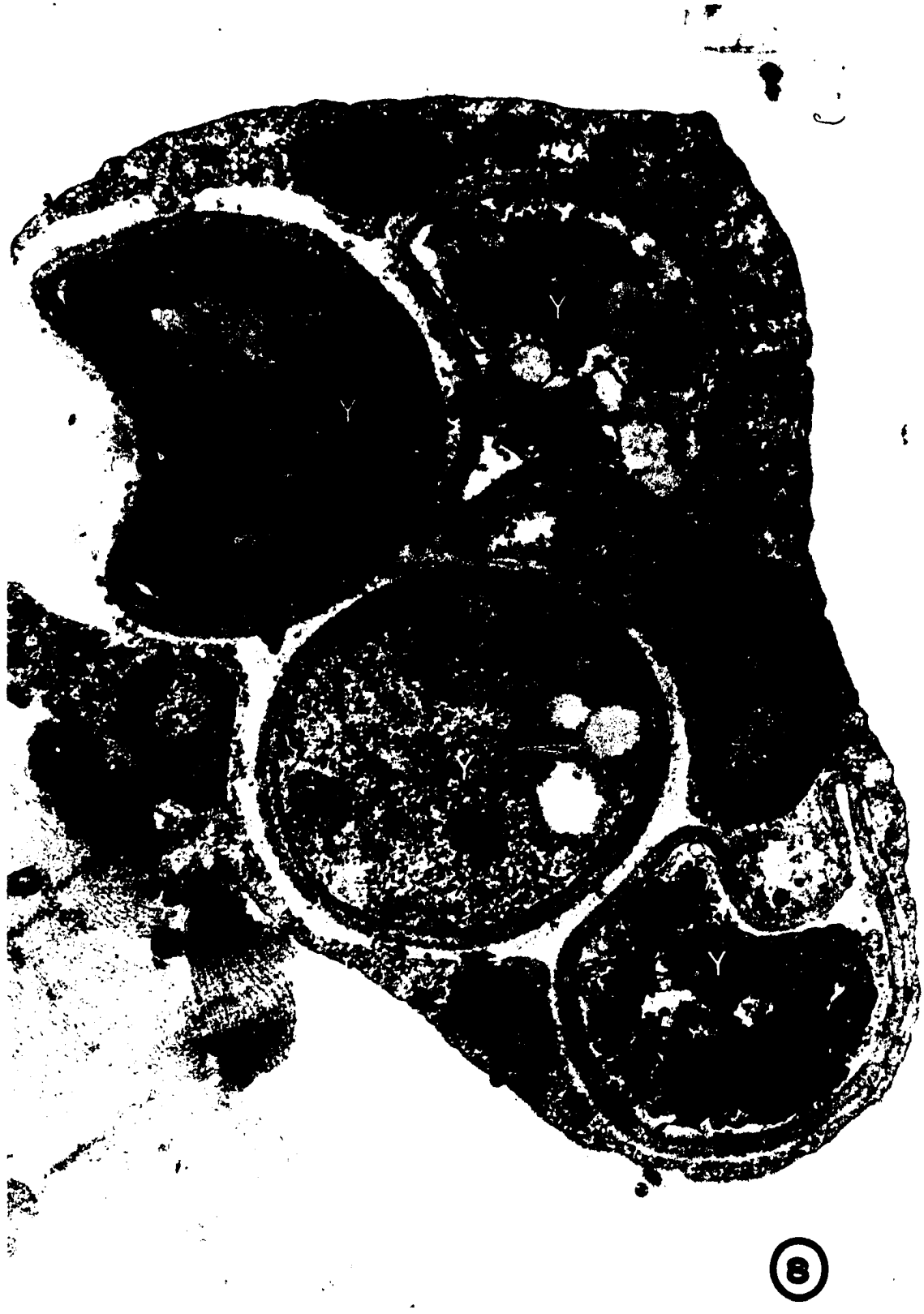

G
7

Figure 8. Neutrophil with four phagocytized yeast cells. 32,000X. The neutrophil is recognized by its multiple nuclear components (N). Within the phagocyte are four yeast cells (Y) in different stages of degeneration. The cell walls have become deformed in the more degenerate appearing cells. The cell wall may bend without breaking.



(8)

Figure 9. Crescent formation with accompanying breakage of the cell wall. 48,000X. Two yeast cells are seen within a phagosome of a neutrophil. The lower cell has collapsed and the cell wall (CW) has broken. Because the phagosome membrane still outlines the original spherical shape of the yeast cell, this breakage probably occurred after fixation of the phagocyte. The upper yeast cell has obvious mitochondria (M).

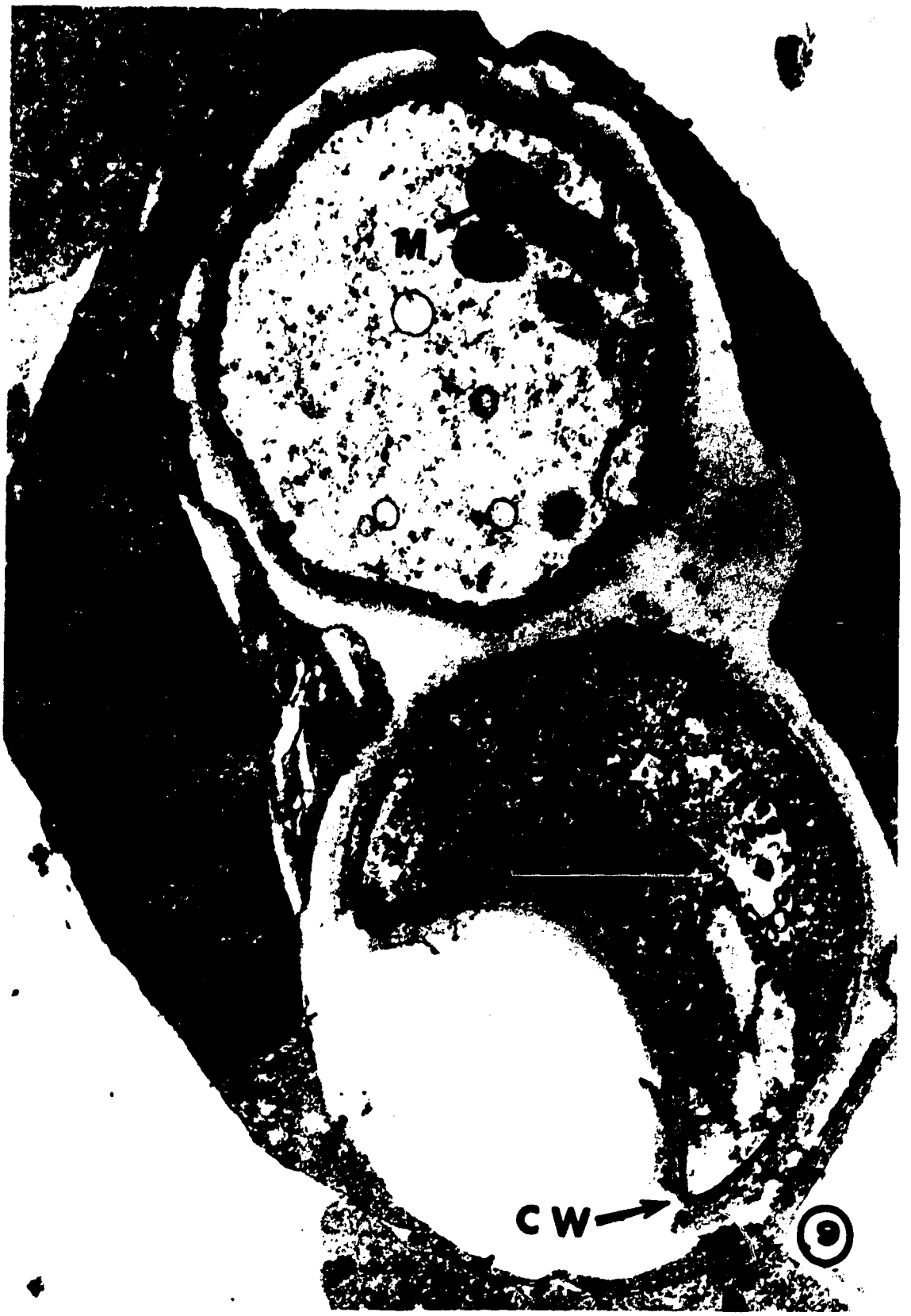

M
CW

Figure 10. Phagocytosis of yeast cells by neutrophils four hours after inoculation. 10,000X. Two well preserved yeast cells are seen. Mitochondria are obvious in both cells. One phagocyte seems to be undergoing degeneration as evidenced by the vesicular appearance of its cytoplasm (C). A small amount of capsule (arrow) surrounds the yeast cell. Both yeast cells have a thin cell wall (CW).


CW
C
10

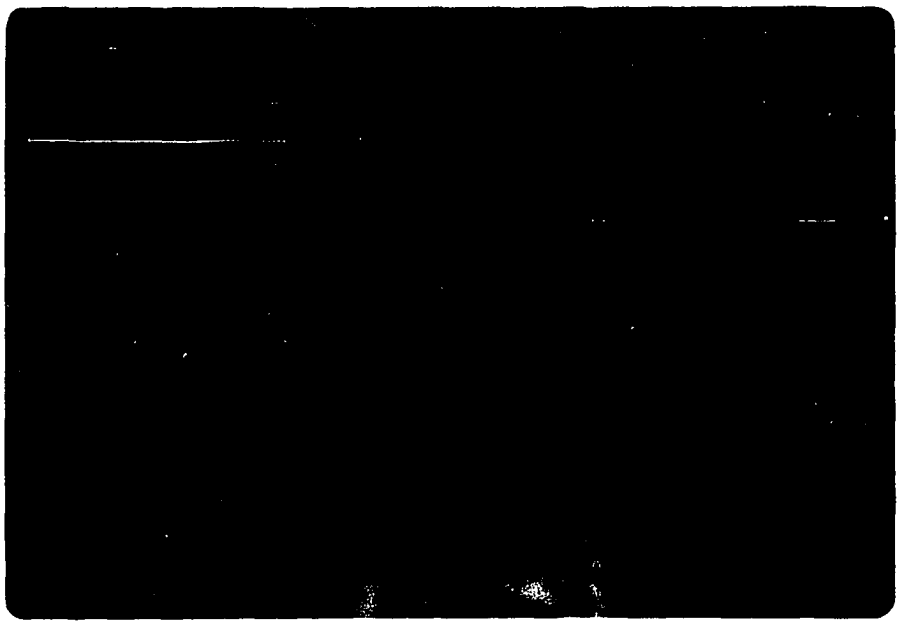
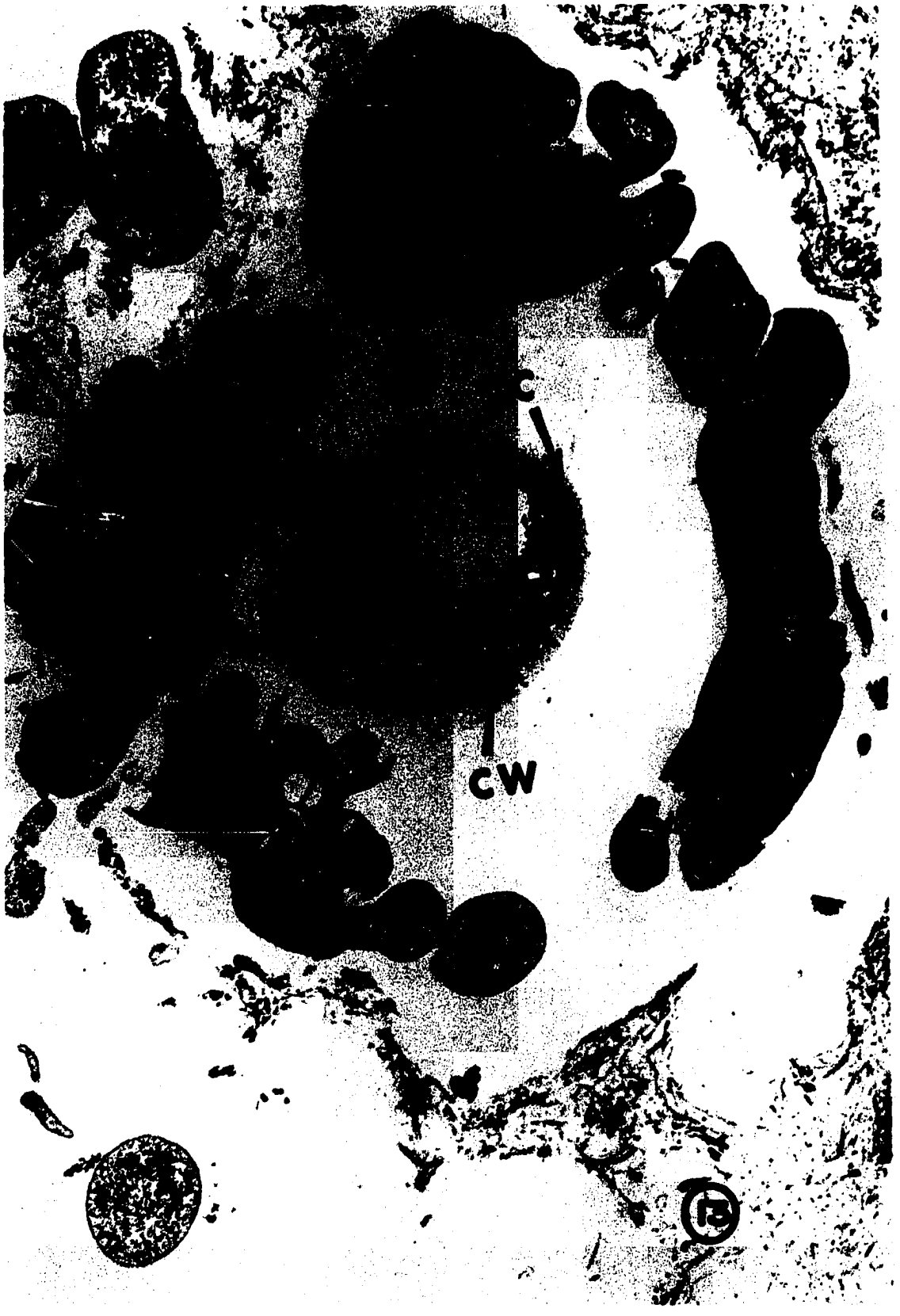


Figure 11. Cryptococci in muscle lesion eight hours after inoculation. mucicarmine stain. 1,000X.

Figure 12. Cryptococci in muscle lesion eight hours after inoculation. alcian blue stain. 800X.

Figure 13. Rosette of neutrophils surrounding cryptococci eight hours after inoculation. 8,500X. Four neutrophils are seen surrounding one yeast cell. The yeast cell wall (CW) is surrounded by a small amount of capsule (C). This rosette formation was a characteristic finding in eight-hour specimens and indicates an inability of the neutrophils to phagocytize these cells.


CW

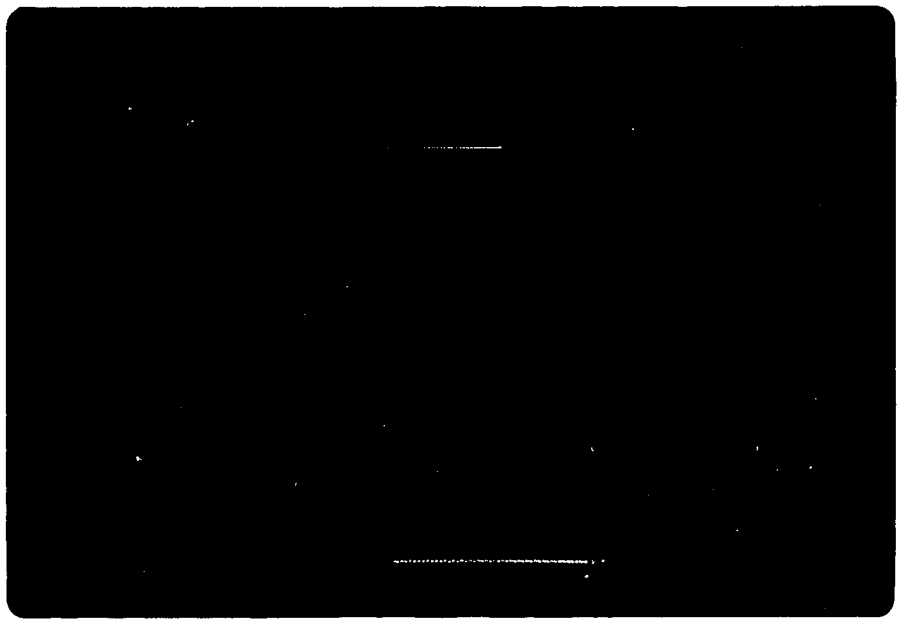


Figure 14. Peripheral part of abscess at 16 hours after inoculation. alcian blue stain. 1,000X.

Figure 15. "U" shaped invagination of cryptococcal cell wall at sixteen hours after injection. 29,000X. The yeast cell is surrounded by neutrophils that may be identified by their lysosomal granules (arrow). The yeast cell wall (CW) has begun to collapse, yet the mitochondria (M) are well preserved.


CW

Figure 16. Foamy macrophages with capsule and collagen fibrils. 30,000X. This is a section through the capsule of a large, heavily encapsulated yeast cell. The capsule consists of dense and less dense areas (arrows). The fibrils of the less dense capsule resemble those seen in the phagosomes (C) of the surrounding macrophages. Collagen (CC) is seen also in this lesion.

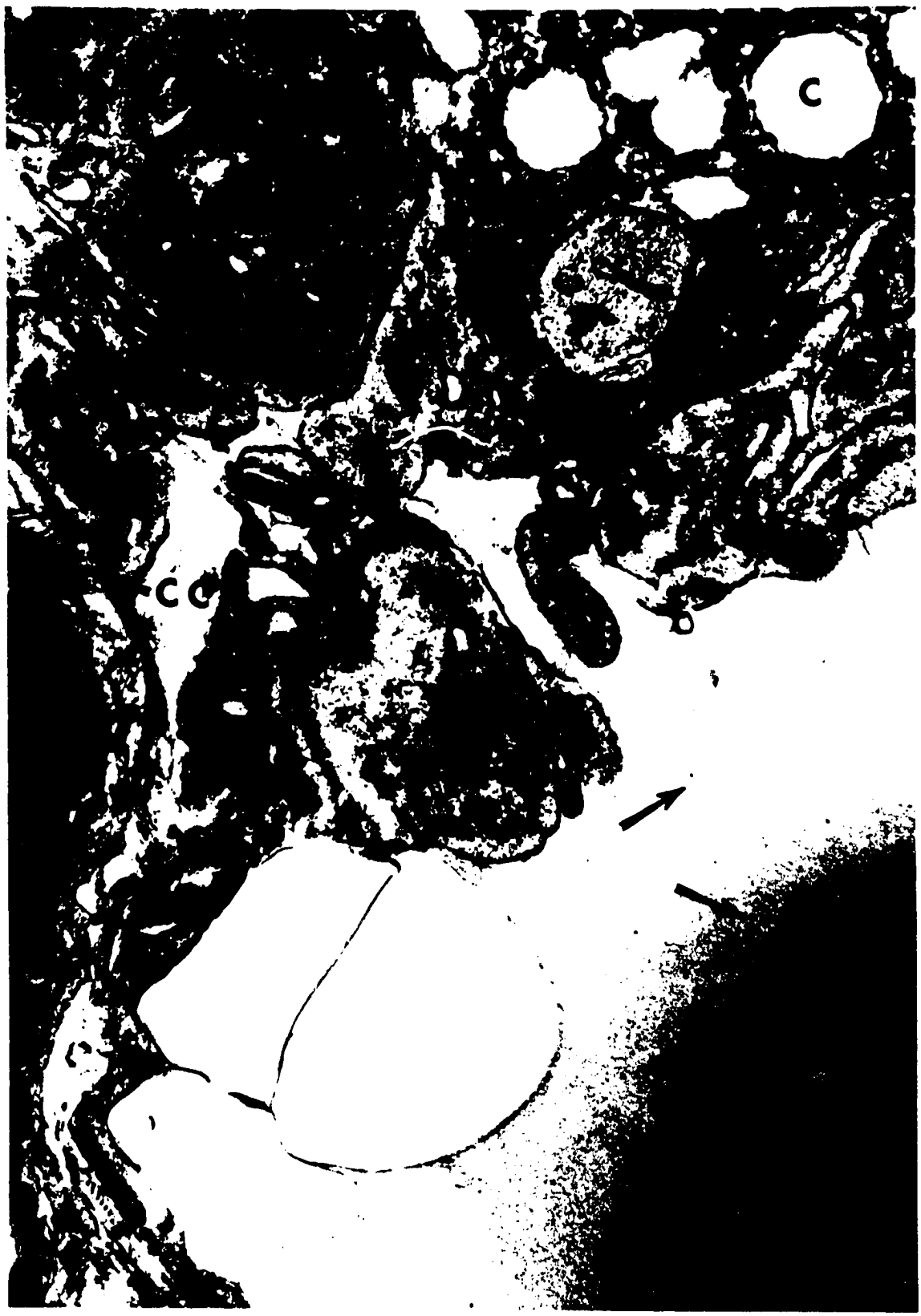

C
C
C

Figure 17. Capsular material within the cytoplasm of macrophages. mucicarmine stain. 1,000X.

Figure 18. Macrophages and large yeast cell at thirty-two hours after injection. 12,000X. This micrograph closly resembles that in figure 16 except that a residual body formed from a yeast cell wall is seen in one of the macrophages (arrow). This folding of the cell wall is similiar to the initial folding seen in degenerating yeast cells four hours after inoculation.