Virulence of *Cryptococcus neoformans*

Regulation of Capsule Synthesis by Carbon Dioxide

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Abstract

Cryptococcus neoformans **is variably encapsulated in vitro, whereas in tissues it develops a large capsule. We observed that cells of a strain with thin capsules, when growing in a standard fungal culture medium, became heavily encapsulated when incubated in serum-free cell culture medium (Dulbecco's modified Eagle's medium [DME]). Capsule size was quantitated physically by measuring cell volume, and chemically by determining the content of a capsular monosaccharide, glucuronate. The CO_2/HCO_3^- couple stimulated capsule development, resulting in visible enlargement by 3 h after exposure to high CO_2/HCO_3^-. The amount of capsule per cell was directly proportional to the total millimolar CO_2/HCO_3^- concentration between 24 and 2.4 mM at pH 7.35, but at constant P_{CO_2} (40 torr) and varying $[HCO_3^-]$, the cells were heavily encapsulated down to pH 6.8. Concentration of CO_2/HCO_3^- in the physiologic range increased elaboration of polysaccharide into the medium and slowed the cell generation time from 2 to 6 h. Four other first-passage clinical isolates were all heavily encapsulated in DME with CO_2/HCO_3^-, but variably encapsulated in DME without CO_2/HCO_3^-. Exposure of yeast to increased CO_2/HCO_3^- caused a marked reduction in complement-mediated phagocytosis by mouse macrophages. A stable clone was isolated which contained capsular polysaccharide, but lacked the CO_2-inducible phenotype. This clone was avirulent for steroid-treated rabbits. Thus, the prevailing CO_2 concentration in mammalian tissues may be one stimulus for capsular polysaccharide synthesis. This could serve as an adaptive mechanism favoring parasite survival in the host.**

Introduction

Synthesis of a polysaccharide capsule is a critical determinant of virulence for *Cryptococcus neoformans.* Isolates from human cerebrospinal fluid (CSF)[1] have large capsules which are highlighted in India ink preparations (1). Acapsular mutants have reduced virulence or are avirulent for experimental animals (2–4). Whether the amount or thickness of the capsule determines virulence is controversial (5). In general, the organism is thickly encapsulated when observed in mammalian tissues (1). However, upon culture in vitro in various fungal media, capsule thickness is variable and is strain-dependent (1, 6).

While performing phagocytosis measurements on a strain of *C. neoformans,* designated H99 (7), originally isolated from a human with meningitis, we observed that yeasts cultured in mammalian cell culture medium were highly resistant to engulfment under conditions where yeast cells cultured in a standard fungal medium were readily phagocytized by mouse macrophages. This difference correlated with enlargement of the capsules of yeasts growing in cell culture medium. The observation led to an investigation of the effect of medium components on capsule development. We found that the capsule was a dynamic structure whose size is highly dependent on medium composition: notably (but not exclusively) on the medium CO_2/HCO_3^- concentration. The results of this analysis are presented using methods that quantitate the amount of cellular and medium capsular polysaccharide. The findings show that capsular polysaccharide biosynthesis is markedly stimulated upon exposure to CO_2/HCO_3^-.

Methods

C. neoformans strains. Most experiments were done with the H99 strain serotype A, isolated from human CSF and maintained on Sabouraud's agar slants. Virulence of this isolate has been demonstrated in rabbits (7). H99 cells from slants were grown in suspension in yeast nitrogen broth (YNB) (Difco Laboratories, Inc., Detroit, MI) with 55.5 mM glucose in glass tubes incubated at 37°C in a slanted position and were passaged every day. Log-phase cells from these tubes were used to seed various media for experiments. Other strains (MC, F396, and KEH) were fresh isolates from human CSF. P3109 was isolated from human lung. The isolates were cultured in the same way as for H99.

Media. Dulbecco's modified Eagle's medium (DME) (Gibco Laboratories, Grand Island, NY) with 25 mM glucose, penicillin G 100 U/ml, and streptomycin 100 μg/ml was prepared with 22 mM $NaHCO_3$ and 25 mM sodium 3-(*N*-morpholino)propanesulfonic acid (NaMOPS), pH 7.3 (DME[+$NaHCO_3$]), or without bicarbonate but with 47 mM NaMOPS, pH 7.3 (DME[−$NaHCO_3$]). To DME with varying concentrations of $NaHCO_3$, organic buffers (BIS-TRIS, PIPES, MOPS, and Hepes) at a given pH were added to make up a total millimolar concentration (HCO_3^- + buffer) equal to 47. Bis(2-hydroxyethyl)iminotris(hydroxymethyl) methane (BIS-TRIS) was used at pH 6.1, piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) at pH 6.8, MOPS at pH 7.3, and Hepes at pH 7.4 and above. Control experiments with variable concentrations of each of the organic buffers showed that these compounds alone did not affect capsule enlargement of the yeast cells. Agar plates were prepared by mixing twice concentrated DME media with twice concentrated (3.6% wt/vol) Bacto agar (Difco Laboratories, Inc.) at 45°C before pouring. DME without phenol red was prepared from individually sterilized groups of medium components obtained

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1. *Abbreviations used in this paper:* BIS-TRIS, bis(2-hydroxyethyl)iminotris(hydroxymethyl) methane; CBA, Columbia blood agar; CSF, cerebrospinal fluid; DME, Dulbecco's modified Eagle's medium; MOPS, 3-(*N*-morpholino) propane sulfonic acid; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid); YNB, yeast nirogen broth.

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commercially (amino acids and vitamins, Gibco Laboratories) or prepared in the laboratory (salts, glucose, and pyruvate).

Carbon dioxide concentration was controlled by using CO_2-sensing incubators at 37°C at constant relative humidity (model 3326, Forma Scientific, Marietta, OH). The incubators were calibrated by using an infrared spectrophotometer (IL200 CO_2 monitor, Instrumentation Laboratory, Inc., Lexington, MA) to sample incubator air each day. Once set at a given CO_2 concentration, the incubators maintained a constant environment and did not require further readjustments. The spectrophotometer was standardized with a commercial 5% CO_2, 95% N_2 gas mixture.

Media in 25-cm^2 or 75-cm^2 Falcon flasks were equilibrated in an incubator having an appropriate P_{CO_2} and seeded with log-phase *C. neoformans* cells at $\sim 10^4$ cells per ml. The culture flasks were incubated without agitation until the cells were harvested.

Cell volume measurements. Two methods were used. First, direct measurements of the diameter of the cell body plus capsule thickness were made microscopically at × 600 on India ink (Marion Scientific, Kansas City, MO) preparations by using a calibrated micrometer eyepiece. The volume was calculated from the measured diameter by assuming a spherical geometry. Increase in cell volume could occur because of capsule expansion or cell body enlargement or both. Cell body diameters were constant under the different growth conditions studied (~ 5 μm) with the exception that the cell bodies of cryptococci grown in YNB were significantly smaller. This was related to the low pH of YNB (5.4). Cell body diameters increased to ~ 5 μm when yeasts were cultured in YNB at pH 7.3. Changes in cell volume resulting from growth in DME media resulted from changes in capsule size.

A second method that employed measurements of packed cell volume and numbers of particles was used. This technique is analogous to erythrocyte volume measurements (8). Cryptococci washed three times by centrifugation (7,000 *g*) with phosphate-buffered saline (PBS) were suspended in 100 μl of PBS containing 20% formalin. This treatment killed the yeast without altering the capsule thickness based on microscopic measurements of cells suspended in India ink. Two 25-μl capillary pipettes were loaded with cell suspension and the ends were sealed in a flame. The pipettes were centrifuged at 1,500 *g* for 15 min and the percentage of packed cell volume was measured. Enough cells were used to give packed volumes of >10%. 25 μl of the concentrated cell suspension was added to 3.0–10.0 ml of PBS and the absorbance was measured at 520 nm. Absorbance was converted to cell number by using a standard curve. The curve (Fig. 1) was generated by measuring absorbance for a series with known cell densities, determined with an electronic particle counter of stationary-phase H99 cells. This preparation is virtually free of cell clumps and budding cells. Determining cell number in the experimental samples by light scattering instead of direct particle counting was used because electronic cell counts underestimated the true cell number by scoring groups of two or more cells as one particle. The curves relating absorbance to particle number on stationary cells with thick and thin capsules were essentially identical. This indicated that the capsule did not increase the absorbance and hence the same standard curve was used for cryptococci cultured in media with varying CO_2/HCO_3^- concentrations. The mean cell volume was calculated by the following method: MCV = (PCV/CC)10^{12}, where MCV = mean cell volume in micrometers cubed; PCV = packed cell volume (milliliters per 100 ml); CC = cell count (number of cells per milliliter); and 10^{12} = cubic micrometers per milliliter.

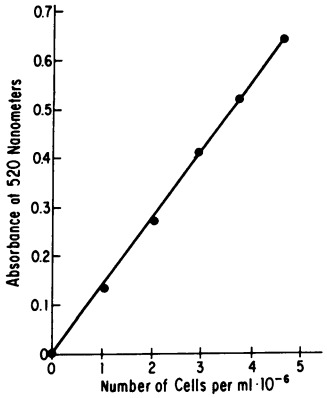

Figure 1. Standard curve relating light scattering to cell number. *C. neoformans* H99 cells from stationary-phase culture were washed in PBS by centrifugation and resuspended at 5 × 10^6 cells/ml. Dilutions were made to give 4, 3, 2, and 1 × 10^6 cells/ml. A known dilution of each suspension was counted electronically in triplicate and absorbance at 520 nm was measured.

Electronic cell counts. Yeast particles were counted electronically using a Coulter ZB_1 counter (Coulter Electronics Inc., Hialeah, FL) equipped with a 100 μm aperture tube. Appropriate settings were determined by generating a volume distribution curve using log-phase H99 cells growing in YNB (9). For counting, 500-μl samples were diluted into 10 ml of Isoton (Curtin-Matheson Scientific, Marietta, GA) containing 0.5% NaClO to kill the yeasts.

Electronic cell counts were used to measure growth rates and to quantitate cell carbohydrate and glucuronic acid content. These experiments were done before light scattering was adopted as a measure of cell number. Because electronic cell counts only slightly underestimated cell number, these experiments were not repeated using light scattering to measure cell number.

Total cell carbohydrate. A modification of the phenol sulfuric acid method was used (10). Yeast cells washed three times by centrifugation (7,000 *g*) with PBS were resuspended in a small amount of PBS. 25 μl of 80% wt/vol phenol was added to 500-μl cell suspension with shaking. Then 2.5 ml of concentrated H_2SO_4 was added, and the glass tubes were shaken again and immersed in boiling water for 15 min. After cooling, absorbance at 480 nm was determined against a blank cuvette in the reference beam containing all additions except for phenol. Nanomoles of carbohydrate as mannose was determined from a standard curve and was expressed per million cells based on electronic cell counts of the sample.

Cell glucuronic acid content. The method of Blumenkrantz and Asboe-Hansen was used (11). 3 ml of concentrated H_2SO_4 with 12.5 mM tetraborate was added to 500 μl of washed yeast cells with mixing on ice. After boiling for 15 min the tubes were cooled and 50 μl of 0.15% *m*-phenylphenol (K & K Biochemicals, Plainview, NY) in 0.5% NaOH solution was added. The tubes were shaken and the absorbance at 520 nm was determined against a blank containing all additions except for *m*-phenylphenol solution. The absorbances were measured within 15 min because the pink color that develops fades with time. Nanomoles of glucuronic acid in the samples was determined from a standard curve and values were calculated per million cells based on electronic cell counts.

In some experiments glucuronate in the culture medium was measured. A sample of cell suspension growing in DME without phenol red was counted to determine cell density and centrifuged to remove yeast cells. The supernatant medium was dialyzed against PBS to remove medium glucose using Spectrapor 3787-H47 dialysis tubing (A. H. Thomas Co., Philadelphia, PA). Glucuronate per milliliter in the dialysate was determined and related to the number of cells per milliliter of the original suspension. The sample volume change during dialysis was negligible.

Phagocytosis of cryptococci by macrophages. H99 cells were grown overnight in medium with 50 μCi per ml of $Na_2{}^{35}SO_4$, specific activity = 43 Ci/mg of S, (ICN, K & K Laboratories, Plainview, NY). Labeling the cells with $^{35}SO_4$ had no effect on CO_2/HCO_3^--induced encapsulation. Yeast cells were washed with PBS three times by centrifugation and resuspended at 2 × 10^5 cells/ml in DME(+$NaHCO_3$) for addition to macrophages. Peritoneal exudate cells were from outbred Swiss mice (Harlan Sprague Dawley, Inc., Walkersville, MD). These mice had received 10^7 colony forming units *Mycobacterium bovis*, strain BCG, (Trudeau Institute #1029, Saranac Lake, NY) i.p. 2–5 wk previously, and 10% proteose peptone (Difco Laboratories, Inc.) i.p. 3 d previously. The cells were added to 16-mm diam culture wells (Costar 3524, Cambridge, MA) in DME(+$NaHCO_3$) at 3 × 10^6 cells per well (12). After 1 h nonadherent cells were removed by rinsing,

and 0.5 ml of DME(+$NaHCO_3$) with given concentrations of normal mouse serum was added to each well. Then 0.5 ml of yeast cell suspension was added and the plates were incubated at 37°C in humidified 5% CO_2, 95% air for 90 min. Extracellular yeasts were removed from macrophages by rinsing three times with PBS, and then macrophages were lysed by adding 1.0 ml of 0.5% deoxycholate. Released cryptococci were resuspended and 500 μl was mixed with 10 ml of Biofluor (New England Nuclear, Boston, MA) and counted in a liquid scintillation spectrometer (Beckman LS-250, Palo Alto, CA). The percentage of phagocytosis was calculated from triplicates of the counts per minute recovered from macrophages divided by the counts per minute added to macrophages × 100. Differences in counting efficiency between samples were negligible as determined by the external standard ratio method. Time course experiments showed maximum uptake by macrophages by 90 min. Normal mouse serum used as opsonin was from outbred Swiss mice. Blood was collected on ice by cardiac puncture, clotted, and separated, and the serum was stored frozen at −85°C. Washed exudate cells were >80% mononuclear phagocytes based on Giemsa stains and phagocytosis of opsonized zymosan.

Cryptococcal meningitis. An animal model of chronic meningitis in corticosteroid-treated rabbits has been described (7, 13). *C. neoformans* was grown on Columbia blood-agar base (CBA) containing 100 μg/ml of chloramphenicol at 35°C for 4 d. Cells were taken up on a sterile swab and suspended in PBS at $1–3 \times 10^7$ cells per 0.3 ml. Yeast suspension (0.3 ml) was injected intracisternally into sedated (100 mg of Ketaject, Bristol Laboratories, Syracuse, NY, and 20 mg of Rompun, Cutler Laboratories, Shawnee, KA) New Zealand White male rabbits (Franklin Farms, Wake Forest, NC). Rabbits were treated with 2.5 mg/kg·d of cortisone acetate i.m. daily, beginning 1 d prior to yeast inoculation. Colony counts of the inoculum were done on CBA. At intervals thereafter CSF was obtained by cisternal puncture for examination on India ink preparations and quantitative colony counts were made on CBA. Total leukocyte counts were made by using a hemocytometer on all CSF samples. All fluids were streaked on DME(+$NaHCO_3$) agar plates and incubated at 37°C in humidified 95% air, 5% CO_2.

Results

Induction of capsule formation. In experiments to measure phagocytosis of *C. neoformans* strain H99 by mouse macrophages, we noted that yeast cells grown in DME(+$NaHCO_3$) were resistant to engulfment when fresh 10% normal mouse serum was used as opsonin. India ink preparations disclosed a striking enlargement in the capsules of cells grown in DME(+$NaHCO_3$) as compared to YNB (Fig. 2). In a series of experiments H99 cells grown in YNB were transferred to DME that lacked various components, and after a 20-h incubation (log-phase cells), capsule size was evaluated on India ink preparations. When $NaHCO_3$ was deleted from DME and the flasks were incubated at ambient CO_2 concentration (0.3 torr), stimulation of capsule production was lost. Other factors tested that had no effect on capsule size were (*a*) the glucose concentration of DME between 5 and 50 mM, (*b*) deletion of the DME vitamins except for thiamine which is required for growth of *C. neoformans* (6), (*c*) addition of DME amino acids to YNB, (*d*) adjusting the sodium and phosphate

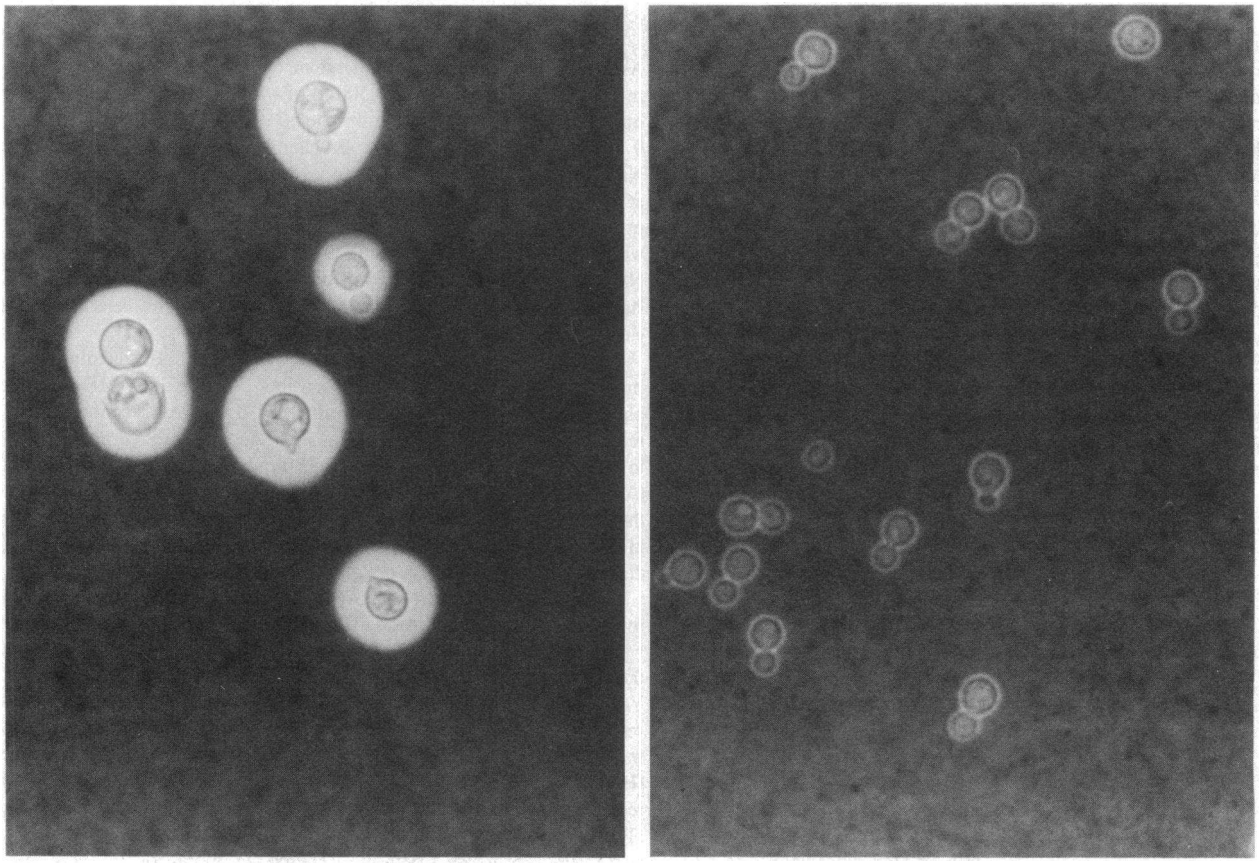

Figure 2. Tissue culture medium stimulates capsule formation by *C. neoformans.* H99 cells were cultured overnight in DME(+$NaHCO_3$), *left,* or YNB, *right,* and India ink preparations were made (× 400). YNB contained no $NaHCO_3$ under ambient P_{CO_2}, pH 5.4. DME(+$NaHCO_3$) contained 22 mM $NaHCO_3$ and 25 mM MOPS, pH 7.3, under 40 torr P_{CO_2}.

concentrations of YNB to 154 and 0.9 mM, respectively, and (*e*) buffering the pH of YNB at 7.3. Bicarbonate (22 mM) and CO_2 (40 torr) added to YNB stimulated capsule formation if (*a*) the pH was buffered at 7.3, and (*b*) an amino acid such as asparagine was added as the source of nitrogen in place of $(NH_4)_2SO_4$. At present it is not clear why $(NH_4)_2SO_4$ suppresses capsule enlargement.

Enlargement of the cell capsule upon exposure to CO_2/HCO_3^- was quantitated by cell volume measurements and by chemical determinations of both total cell carbohydrate and cell glucuronic acid content. In these experiments H99 cells were grown overnight in DME with or without $NaHCO_3$ under 40 torr or 0.3 torr P_{CO_2}, respectively. Then the cells were harvested, washed with PBS, and used for the measurements shown in Table I. By each method CO_2/HCO_3^- led to an increase which was commensurate with the enlargement of the capsule observed directly on India ink preparations. Cell volume measured by centrifugation proved to be a simple quantitative measure of this phenomenon whereas direct microscopic measurements were tedious. Glucuronate comprises ~14% of the capsular polysaccharide of the serotypes studied thus far (14); its concentration in other cell compartments would be expected to be negligible relative to the capsule. This supposition is supported by measurements on an acapsular mutant of *C. neoformans* strain 602 (3), whose glucuronate content was consistently below the level of detection of the assay used (<10 nmol per sample). The percent cell glucuronate of the total cell carbohydrate is the same for yeasts growing with or without high carbon dioxide (7%, Table I). This is consistent with a quantitative increase without necessarily any alteration of capsular monosaccharide stochiometry. The increase induced by CO_2/HCO_3^- is less when the chemical methods are compared to cell volume measurements. This may mean that the enlarged capsule has less densely packed carbohydrate. Indeed, we commonly observed India ink carbon particles deposited within the outer substance of the capsule after growth at high CO_2 tension.

Time course of capsule enlargement. H99 cells grown in YNB were transferred to DME(−$NaHCO_3$) and DME(+$NaCO_3$). At frequent intervals the yeasts were examined in India ink and samples of the medium were assayed for macromolecular glucuronate (Fig. 3). After a lag period of 3 h yeast cells growing in DME(+$NaHCO_3$) began to develop thickened capsules. Thereafter the proportion of cells with capsular thickening steadily increased. This morphologic change was mirrored by the elaboration of capsular carbohydrate into the carbon dioxide-rich medium. Because cells in the two media grew at different rates, the medium glucuronate values were calculated per million cells based on cell counts made at 2-h intervals. The CO_2/HCO_3^- induction phenomenon appears to be associated with a marked increase in polysaccharide synthesis resulting in enlargement of the capsule and release of capsular polysaccharide into the medium. This change is evident by ~4–6 h after exposure of the cells to physiologic CO_2 concentration.

CO_2/HCO_3^- concentration requirements and effects of compounds affecting carbon dioxide metabolism. H99 cells growing in YNB were transferred to DME with varying $NaHCO_3$ and CO_2 concentrations at constant pH (7.3). After a 20-h incubation cell volumes were measured by the centrifugation method. In all experiments India ink examinations were also performed and in each case increases in cell volume were accompanied by capsular thickening as observed directly under the microscope. During the 20-h incubation the cells remained in log phase as determined by cell counts at time zero and at the time of cell harvest. In addition, medium pH under all conditions was measured at time 0 and at 20 h. In no case was there a change >0.2 pH unit during the incubation. The CO_2 concentration was directly related to capsule thickness between 0.3 and 40 torr (Fig. 4 *A*). As shown in Fig. 4 *A* the total millimolar CO_2 concentration (bicarbonate plus CO_2 [millimolar concentrations given in parentheses]) markedly affected capsule size between 2.4 and 24 mM. At higher

*Table I. Effect of Carbon Dioxide on Capsular Polysaccharide of C. neoformans, Strain H99**

	Medium $CO_2/NaHCO_3$ concentrations	
Type of measurement	0.3 torr/0 mM	40 torr/22 mM
Cell volume by India ink exclusion (μm^3)	125±21	1,167±208
Cell volume by centrifugation method (μm^3)	212±22	710±45
Total cell carbohydrate (*nmol mannose/10^6 cells*)	96±8.6	311±41
Total cell glucuronate (*nmol/10^6 cells*)	5.1±0.6	19.6±2.2

* Yeast cells were cultured for 20 h in DME(+$NaHCO_3$) or DME(−$NaHCO_3$). At the time of harvest the cells were in log phase. Each value is based on a minimum of four separate experiments and are means±SEM.

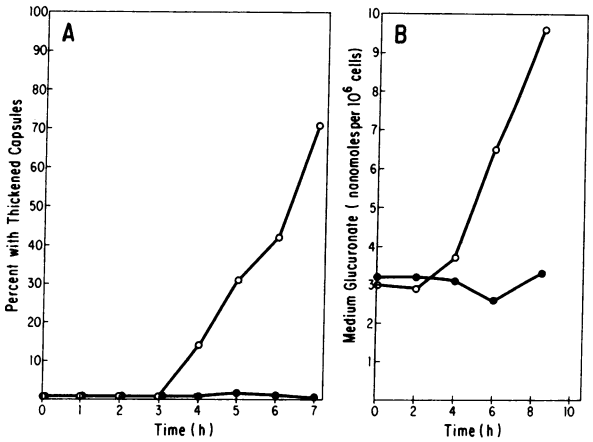

Figure 3. Time course of capsule stimulation by CO_2/HCO_3^-. (*A*) Log-phase H99 cells growing in YNB were washed and resuspended in DME(+$NaHCO_3$) (○) or DME(−$NaHCO_3$) (●) at 2 × 10^5 cells/ml. At 1-h intervals India ink preparations were made and the cells were scored for appearance of thickened capsules. At least 200 cells were examined at each time point. (*B*) Conditions were the same as in *A* except that the cells were resuspended at ~5 × 10^6/ml in spinner flasks. At each time point medium was removed for cell count, centrifugation was performed to remove cells, and dialysis against PBS was done to remove glucose. Dialysates were assayed for glucuronic acid, expressed as nanomoles per 10^6 cells. DME(+$NaHCO_3$) contained 22 mM $NaHCO_3$ and 25 mM MOPS, pH 7.3, under 40 torr P_{CO_2}. DME(−$NaHCO_3$) contained 47 mM MOPS, pH 7.3 under ambient P_{CO_2}.

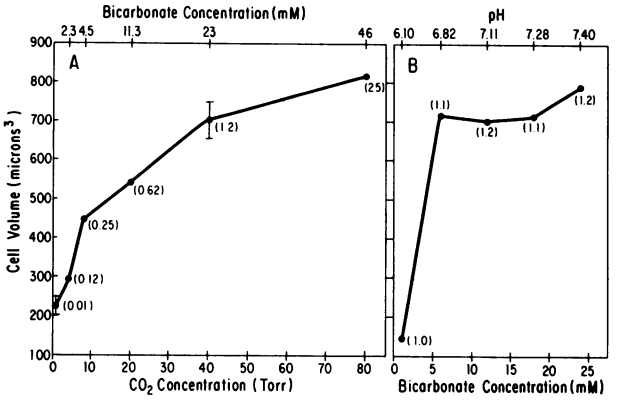

Figure 4. Effect of carbon dioxide, bicarbonate, and pH on capsule formation. H99 cells were incubated overnight in DME with CO_2 and $NaHCO_3$ concentrations as shown, and washed in PBS, and their average cell volumes were measured by the centrifugation method. (*A*) Each point represents a separate experiment with the incubator P_{CO_2} set and calibrated at a given concentration. Organic buffer used was MOPS, pH 7.35, at 48.0, 45.7, 43.5, 36.7, 25.0, and 2.0 mM respective to the increasing $NaHCO_3$ concentrations shown. In each experiment, cells in DME(+$NaHCO_3$) and DME(−$NaHCO_3$) were included in their respective incubators (points with standard error bars). (*B*) Incubator P_{CO_2} was held constant at 40 torr. Buffer systems used were: 1 mM $NaHCO_3$ + 46 mM BIS-TRIS, pH 6.10; 6 mM $NaHCO_3$ + 41 mM PIPES, pH 6.82; 12 mM $NaHCO_3$ + 35 mM MOPS, pH 7.11; 16 mM $NaHCO_3$ + 31 mM MOPS, pH 7.28; 24 mM $NaHCO_3$ + 23 mM Hepes, pH 7.40. In all experiments, medium pH was measured at the beginning and at the time of harvesting the cells. Overnight growth did not lead to a pH change >0.2 U under any of the conditions tested. The numbers in parentheses are the millimolar concentrations of dissolved CO_2 calculated from the Henderson–Hasselbach equation. The total millimolar CO_2 concentrations referred to in the text are the sums of bicarbonate concentrations plus dissolved CO_2 concentrations in millimolar.

concentrations (49 mM, Fig. 4 *A;* 100 mM, not shown) there was little further increase in cell volume. In contrast, when the P_{CO_2} was held constant at 40 torr and the bicarbonate concentration was varied, the cells had thickened capsules between 6.0 (pH 6.8) and 24 (pH 7.4) mM bicarbonate (Fig. 4 *B*). Of the three variables—medium pH, bicarbonate, and dissolved CO_2, only changes in the dissolved CO_2 concentration influenced capsule size, with one exception. For comparisons the millimolar concentrations of dissolved CO_2 calculated from the Henderson–Hasselbach equation are given in parentheses at each data point. The exception is at low pH (6.1) with 40 torr P_{CO_2} and 1.0 mM $NaHCO_3$ where the effect is lost despite 1.0 mM dissolved CO_2 (Fig. 4 *B*). At low medium pH, it may be that some supervening factor blocked the accelerated capsule production at a step unrelated to the process affected by CO_2. The low pH did not inhibit cell growth and in fact cell counts showed more rapid proliferation as pH dropped from 7.3 to 6.1. In the physiologic pH range, however, it appeared that dissolved CO_2 or H_2CO_3 rather than HCO_3^- or the extracellular pH induced encapsulation. The effect of alkaline pH could not be studied during log phase in that cell growth was inhibited above pH 7.5. The same findings as shown in Fig. 4, *A* and *B* were obtained if cell glucuronate was quantitated as an independent method of capsule size measurement (results not shown).

Factors affecting carbon dioxide metabolism were tested. The addition of biotin 50 ng/ml, avidin 1 U/ml, carbonic anhydrase 50 μg/ml, or acetazolamide 100 μg/ml, did not affect capsule size in cells grown in either DME(−$NaHCO_3$) or DME(+$NaHCO_3$). Given the effect of carbonic anhydrase on the kinetics of CO_2 hydration, we predicted that encapsulation might be accelerated. However, this may require enzyme penetration into the cells, which may not have occurred under the conditions we used. Conversely, we postulated that acetazolamide might inhibit the rate of capsule expansion. The observed negative result does not rule out a role for carbonic anhydrase in that this enzyme in yeast differs from mammalian carbonic anhydrase and may not be inhibited by acetazolamide (15). Furthermore, carbonic anhydrase and acetazolamide would be expected to affect the rate of enlargement and this change might not have been detected in our experiments. Inhibition of either transcription with 10 μM actinomycin D, or oxidative phosphorylation with 5 μM antimycin A, completely blocked encapsulation in DME(+$NaHCO_3$). Under these conditions the cells remained viable as determined by plate counts on Sabouraud's agar, but replication was completely inhibited.

Relationship between growth rate and capsule formation. When *C. neoformans* cells were transferred to medium containing CO_2/HCO_3^-, their rate of replication decreased. For example, in the experiments shown in Fig. 4 *A*, although the number of cells per milliliter at time zero in the different media was equal, at the time of harvest the cell concentration was inversely related to the total CO_2 concentration. Growth curves were measured for H99 cells in DME(+$NaHCO_3$) or DME(−$NaHCO_3$) (Fig. 5 *A*). The generation time in DME(−$NaHCO_3$) was 2.1 h compared to ~6 h in DME(+$NaHCO_3$). This finding raised the possibility that relatively slowly replicating cells might develop large capsules irrespective of carbon dioxide concentration. India ink exam-

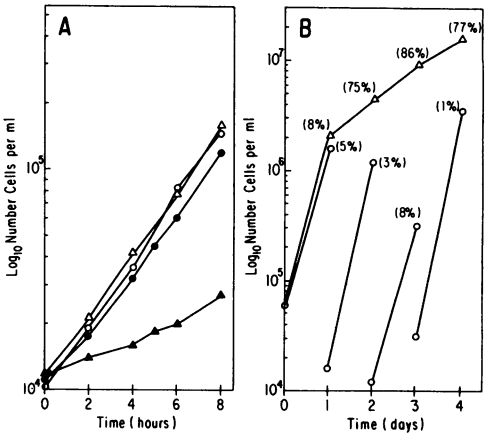

Figure 5. Correlation between capsule induction and slowed cell replication. (*A*) Growth curves are shown for H99 wild-type cells (closed symbols) and the H99 C3D clone (open symbols) in DME(+$NaHCO_3$) (▲, △) and DME(−$NaHCO_3$) (●, ○). (*B*) At the times shown H99 cells growing in DME(−$NaHCO_3$) were diluted to the densities shown with fresh medium (○), or centrifuged and resuspended at the same density in fresh medium (△). The percentages in parentheses are the proportion of cells with thickened capsules as determined on India ink preparations. DME(+$NaHCO_3$) contained 22 mM $NaHCO_3$ and 25 mM MOPS, pH 7.3 under 40 torr P_{CO_2}. DME(−$NaHCO_3$) contained 47 mM MOPS, pH 7.3 under ambient P_{CO_2}.

inations were done on cells during the log phase versus stationary phase in DME(−$NaHCO_3$) (Fig. 5 *B*). Cells maintained in the log phase by daily dilution into fresh medium did not develop thickened capsules through a 4-d culture period. However, if the cells were resuspended at high density daily in fresh medium without dilution, a high percentage of the yeasts developed thick capsules. This was associated with a much reduced growth rate, as shown in Fig. 5 B. The same result was obtained when the experiment was repeated in stoppered flasks containing separate cups filled with 20% NaOH to trap CO_2. At present it is not clear what factor(s) or lack of nutrients induce capsule enlargement under these culture conditions. However, the experiments show that CO_2/HCO_3^- is not necessarily required. Under all conditions thus far tested there has been a direct correlation between slower replication rate and capsule enlargement.

Isolation of a C. neoformans clone lacking carbon dioxide responsiveness. H99 cells were passed in DME(+$NaHCO_3$) daily over a 2-wk period. India ink examinations showed the emergence of cells with narrow capsules. A sample of this culture was plated onto DME(+$NaHCO_3$) agar and incubated under CO_2 at 40 torr for 3 d. The plates showed two colony types: opaque colonies with glistening surfaces and translucent colonies which were mucoid. On India ink preparations the cells from these colony types had thin and thick capsules, respectively. A thin capsule clone, chosen at random and designated C3D, was studied further. Characteristics related to capsular carbohydrate are given in Table II. C3D was phenoloxidase positive and released cryptococcal antigen into the medium. Therefore it was not an acapsular mutant of *C. neoformans.* Measurements of cell volume, total carbohydrate, and glucuronate content were comparable to the values obtained for H99 wild-type growing in DME(−$NaHCO_3$) with the exception that the glucuronate content was only ~2% of total carbohydrate. This may indicate a qualitative difference in the capsule of the C3D clone compared to the parent strain. Unlike the parent H99 strain, the C3D clone did not respond to CO_2/HCO_3^- by increasing its capsule size. Growth curves for C3D are shown in Fig. 5*A:* C3D cells grew rapidly at equal rates in both DME(+$NaHCO_3$) and DME(−$NaHCO_3$). This may explain why C3D-like cells emerge after continuous culture in DME(+$NaHCO_3$). If C3D is a spontaneous mutant lacking the CO_2-inducible character, this genotype would be selected because of its more rapid growth rate in DME(+$NaHCO_3$). The C3D clone has remained stable during more than 150 transfers in DME(+$NaHCO_3$) and upon transfer to DME(−$NaHCO_3$) or Sabouraud's agar. Stationary-phase C3D cells do not develop thickened capsules. The C3D clone resembles the hypocapsular mutants isolated by Jacobson et al. (16). These clones, produced by mutagenization, synthesize capsular polysaccharide but retain thin capsules under conditions where wild type cells develop thickened capsules.

Capsule induction in other C. neoformans strains. Four recent clinical isolates from CSF (MC, F396, KEH) or lung tissue (P3109) were cultured overnight in YNB, DME(−$NaHCO_3$), and DME(+$NaHCO_3$). Cell volume measurements in comparison to H99 strain and H99 (C3D) are shown in Fig. 6. In all cases growth in YNB led to cells with relatively small capsules while the DME(+$NaHCO_3$) grown cells were thickly encapsulated. For MC, P3109 and KEH strains capsule thickening occurred in DME(−$NaHCO_3$) but not to the extent produced by culture in DME(+$NaHCO_3$). F396 cells were thickly encapsulated in DME with or without CO_2/HCO_3^-. The different *C. neoformans* strains demonstrated a variability in the encapsulation process in DME(−$NaHCO_3$). This variability ranged from slight thickening (e.g., H99 and P3109) to partial thickening (e.g., MC and KEH) to thickly encapsulated cells (F396). Again, it is clear that encapsulation can be induced by factors other than high carbon dioxide tension and that this depends on the strain and the phase of the growth curve (in the case of the H99 strain).

*Table II. Capsular Carbohydrate Measurements Comparing C. neoformans Strains Growing in DME(+$NaHCO_3$)**

Assay	*C. neoformans* strain		
	H99 parent strain	H99/C3D clone	Acapsular mutant (strain 602)
Phenoloxidase test‡	+	+	+
Cryptococcal polysaccharide titer of culture medium§	1:64,000	1:8,192	Negative
Cell volume by centrifugation method *(μm^3)*‖	681	128	ND¶
Total cell glucuronate *(nmol/10^6 cells)*	24.3	2.8	None detected
Total cell carbohydrate *(nmol mannose/10^6 cells)*	423	144	ND

* Organisms were cultured in DME(+$NaHCO_3$) overnight. At the time of harvest cells were in log phase. Measurements were made on the three strains cultured under identical conditions during the same time period in one experiment.

‡ Organisms were streaked on caffeic acid agar plates and incubated in the dark at 25°C for 4 d.

§ Yeast cells were removed by centrifugation and serial dilutions of the supernatant mediums were made. The titer was the greatest dilution giving agglutination of cryptococcal polysaccharide (serotypes A, B, and C) coated latex beads.

‖ Cell volume of H99/C3D done by India ink exclusion was 110 μm^3. For H99 parent strain cultured under the same conditions see Table I.

¶ ND, not done. Because these cells were massively clumped when grown in liquid medium, it was impossible to perform these assays accurately.

Phagocytosis of H99 cells grown in DME(+$NaHCO_3$) or DME(−$NaHCO_3$). To test the potential significance of the carbon dioxide-induced encapsulation process, phagocytosis experiments were performed that used activated mouse macrophages. For H99 cells cultured in YNB, phagocytosis in serum-free medium was nil (Fig. 7). Addition of 10% fresh normal mouse serum led to phagocytosis of 90–100% of the yeasts under conditions of the assay. Phase-contrast microscopy showed that cryptococci were internalized, and not simply attached to the phagocytes. Opsonization by normal mouse serum was completely blocked by the treatments shown in Fig. 7 *A*. These data are compatible with activation of the alternative complement pathway and engulfment via one of the macrophage C3b receptors (17–19).

H99 cells were cultured overnight in DME(+$NaHCO_3$) or DME(−$NaHCO_3$). Although cells grown in DME(−$NaHCO_3$) were readily phagocytized, CO_2/HCO_3^- exposed cells were engulfed with low efficiency (Fig. 7 *B*). The decreased phagocytosis

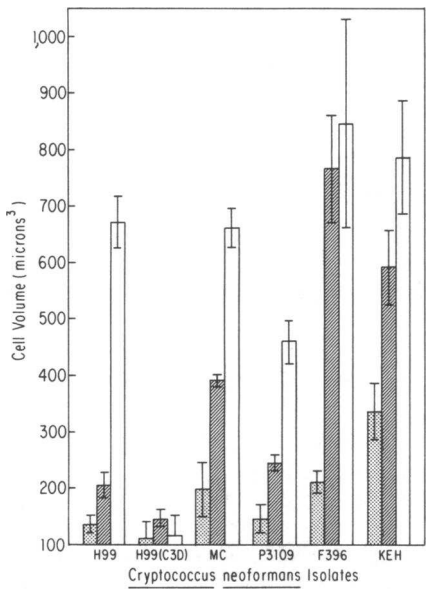

Figure 6. Capsule size of five strains of *Cryptococcus neoformans* growing in media with and without CO_2/HCO_3^-. Log-phase cells growing in YNB were transferred to YNB (■), DME(−$NaHCO_3$), (■), or DME(+$NaHCO_3$) (□), and cultured overnight. Cells were harvested and washed in PBS, and cell volumes were measured by using the centrifugation method. YNB contained no $NaHCO_3$ under ambient P_{CO_2}, pH 5.4. DME(+$NaHCO_3$) contained 22 mM $NaHCO_3$ and 25 mM MOPS, pH 7.3, under 40 torr P_{CO_2}. DME(−$NaHCO_3$) contained 47 mM MOPS, pH 7.3, under ambient P_{CO_2}. Bar heights are means of at least three experiments±SEM. For source of strains see Methods.

for DME(+$NaHCO_3$) grown cells could not be overcome by increasing the serum concentration (Fig. 7 *C*). At high serum concentrations (25% and 50%), the capsule of DME(+$NaHCO_3$)-

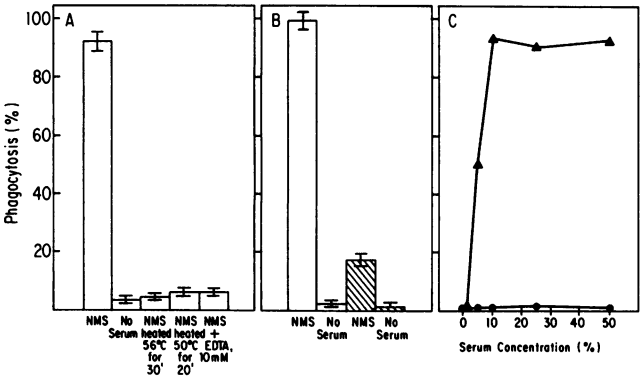

Figure 7. Phagocytosis of *C. neoformans* H99 by mouse peritoneal macrophages. (*A*) Effect of normal mouse serum (NMS) and treatments to NMS on uptake of H99 cells cultured overnight in YNB. NMS concentration was 10%. (*B*) Open bars are for H99 cells cultured overnight in DME(−$NaHCO_3$); hatched bars are for H99 cells cultured overnight in DME(+$NaHCO_3$). NMS concentration was 10%. (*C*) Uptake at varying NMS concentrations for H99 cells cultured overnight in DME(−$NaHCO_3$) (▲) or DME(+$NaHCO_3$) (●). Values are means of triplicate cultures. YNB contained no $NaHCO_3$ under ambient P_{CO_2}, pH 5.4. DME(+$NaHCO_3$) contained 22 mM $NaHCO_3$ and 25 mM MOPS, pH 7.3, under 40 torr P_{CO_2}. DME(−$NaHCO_3$) contained 47 mM MOPS, pH 7.3, under ambient P_{CO_2}.

cultured cells became visible under the light microscope as in a Quellung reaction. This did not occur if the serum was heated at 56°C for 30 min. A Quellung reaction was observed by Diamond et al. (17), who used cryptococci obtained directly from the CSF of humans with meningitis.

It will be important to determine whether the differences observed for mouse peritoneal macrophages occur with macrophages (and perhaps neutrophils) from sites more relevant to cryptococcal infections, namely lung and CSF. This question is being pursued by using rabbits where macrophages from lung and CSF are more readily available. In the case of the central nervous system, it is clear that opsonins are present in the CSF of rabbits with cryptococcal meningitis. This opsonic activity can be measured using macrophages isolated from the CSF and cultured in vitro with cells of the H99/C3D clone (Perfect, J. R., unpublished data). Experiments employing CSF and alveolar macrophages for phagocytosis of H99 cells cultured with and without CO_2/HCO_3^- remain to be performed.

Animal virulence experiments. The H99 strain causes cryptococcal meningitis when injected intracisternally into rabbits immunosuppressed with cortisone (7). Isolation of the H99-derived C3D clone provided the opportunity to test its virulence and stability in vivo. The combined results of three experiments are shown in Fig. 8. 24–48 h after intracisternal inoculation of yeast, CSF from nearly all rabbits receiving wild-type organisms were positive by India ink for thickly encapsulated yeasts. Based on the findings presented in Fig. 4, the concentrations of CO_2 and bicarbonate in CSF would be expected to provide ample stimulus for encapsulation of H99 cells. These concentrations, measured on human CSF by a number of investigators, are ~47 torr and 24 mM, respectively (20). The levels in rabbit CSF would not be expected to differ significantly from other mammals, including humans. Rabbits receiving H99 cells developed chronic meningitis with 10^3–10^4 cryptococci per milliliter of CSF throughout the 3-wk period. 5 of 12 rabbits given H99 cells died during this time. All were heavily infected and exhibited focal neurologic signs. This is the typical course of infection in steroid-treated animals (7,

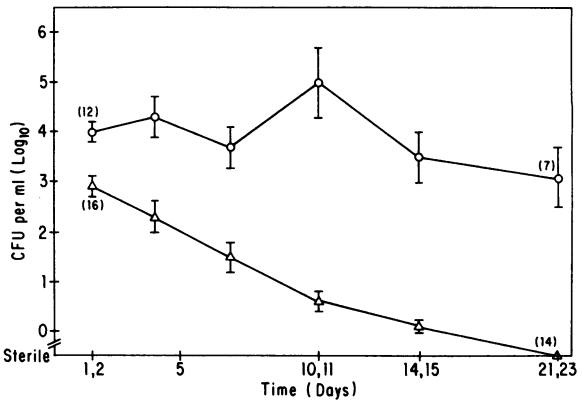

Figure 8. Chronic meningitis in steroid-treated rabbits injected intracisternally with *C. neoformans* H99 or the H99/C3D clone. Data points represent mean colony-forming units (CFU) per milliliter of CSF±SEM at times shown for three separate experiments. Rabbits received ~10^7 CFU H99 parent strain (○) or H99/C3D clone (△) at time zero. Numbers in parentheses are the number of living animals at the times CSF was sampled. One rabbit in each group died before day 1. These deaths may have been related to trauma from intracisternal injection, or to anesthesia.

13). For 16 rabbits given H99/C3D cells CSF colony counts were lower by one $\log_{10}$ at 1–2 d. Later in the infection the counts progressively decreased such that all but one animal had sterile CSF by days 14–15. Each CSF sample was examined with India ink and in no instance were large capsule cryptococci seen from C3D-injected rabbits. In addition, all CSF samples were plated on DME(+$NaHCO_3$) and DME(−$NaHCO_3$) agar media. In all cases C3D isolates retained their narrow capsule phenotype on both media. The parent H99 isolates grew as narrow capsule cells on DME(−$NaHCO_3$) and as thickened capsule cells on DME(+$NaHCO_3$) agars. Two C3D rabbits died, one on day 21 with sterile CSF and the other on day 15. The latter animal developed gram-negative bacillary meningitis and had $\sim 10^2$ cryptococci per milliliter of CSF at death despite having had sterile CSF on days 7 and 11. The 14 surviving C3D rabbits were observed for 30–60 d. None relapsed. The clearance of C3D cells from the subarachnoid space was not associated with augmented CSF leukocytosis compared to animals infected with H99 wild-type cells. Total leukocyte counts were similar for both groups between days 1 and 7. Subsequently, leukocyte counts fell in C3D animals mirroring the fall in yeast cell counts whereas in the H99 wild-type group CSF leukocytosis persisted at a relatively constant level. It is clear that the C3D clone, which lacks the ability to develop a thickened capsule upon CO_2 exposure, is completely avirulent under the conditions of this animal model for cryptococcal meningitis. It remains to be determined whether the loss of virulence is related to the loss of CO_2 responsiveness or to an as yet undefined property of C3D cells.

Discussion

Microbiologists are familiar with the observation that cryptococci in CSF or tissue usually have large capsules, which may decrease in size when the yeasts are cultured in vitro. For example, Littman (1) noted that the degree of encapsulation of *C. neoformans* decreased upon transfer from in vivo sites to laboratory culture medium. In quiescent 7–30-d cultures capsule thickening occurred in the presence of thiamine (an essential vitamin for growth of *C. neoformans*), and optimal concentrations of glutamate (1). Farhi et al. (21) found that pH 5.0 inhibited, whereas neutral pH promoted, capsule production in a medium containing salts, glucose, glutamate and thiamine. Glucose concentration and medium osmolarity were reported by Dykstra et al. (22) to influence capsule thickness. However, the levels required to induce the effect were not commensurate with those present in extracellular fluid. Iralu (23) noted that strains of *C. neoformans* could be made to produce large capsules by cultivation for 2–7 d at 36°C on Sabouraud's dextrose agar or brain heart infusion agar under 10% CO_2 or in a candle jar. More recently, Anna (24) described that incubation of cryptococci in serum led to capsule thickening. Taken together the preceding studies suggested that capsule production is stimulated by conditions present in extracellular fluid (i.e., neutral pH, serum, high CO_2 tension). However, the relationship between CO_2, bicarbonate, and pH was not studied. Moreover, the degree of encapsulation was difficult to quantitate, and the results relied on qualitative observations or tedious microscopic measurements on cells suspended in India ink.

For the wild-type H99 strain, we found that encapsulation was induced by exposure to CO_2/HCO_3^- in medium of composition similar to extracellular fluid. This process was quantitated chemically by measuring the glucuronic acid content of the cells and physically by measurement of cell volume. By using these methods we characterized the encapsulation process as follows: (*a*) capsule expansion can be detected within 4 h after exposure to CO_2/HCO_3^-; (*b*) of the three variables—carbon dioxide, bicarbonate, and pH—capsule expansion varies with the carbon dioxide concentration in the physiologic pH range; at pH 6.1 capsule expansion is blocked even in the presence of tissue CO_2 tension; (*c*) capsule thickening is accompanied by a marked increase in the rate of elaboration of capsular polysaccharide into the medium; (*d*) yeast cells undergoing capsule expansion reproduce at a much slower rate; and (*e*) capsule expansion occurs in the absence of physiologic concentrations of CO_2/HCO_3^- when the yeast cells are replicating slowly at high cell density. Under these latter conditions, a number of other factors may come into play, such as limiting O_2 concentration or depletion of medium nutrients.

The inverse relation between multiplication rate and capsule expansion raises the question as to whether the effect of CO_2/HCO_3^- is primary or secondary. At this stage of our knowledge, which is cause and which is effect remain unknown. Carbon dioxide or other signal(s) may stimulate polysaccharide synthesis; as a result of diverted carbon flux the cells might then reproduce more slowly. Alternatively, these stimuli may directly slow cell multiplication by some means; decreased glucose catabolism might then provide more glucose to be channeled into capsular polysaccharide production. Resolution of these questions will require an understanding of the biochemical regulation of capsule synthesis.

Bulmer's group proposed that cryptococcal encapsulation is a dynamic process, one that is responsive to the environmental niches occupied by this species (21). Our findings confirm and expand this notion. Upon entry into the lung, narrowly encapsulated cells from the environment are exposed to a CO_2 concentration that would result in maximal elevation of capsular polysaccharide synthesis. Whether the newly synthesized carbohydrate is structurally different is under study. At what level does this regulation occur? We favor modulation of a previously assembled enzyme system inasmuch as uninduced cells synthesize capsule but at a much lower rate. The enzymes responsible for capsule production have not been studied nor is the site of synthesis known. Regulation of glycogen synthesis in baker's yeast bears consideration as a model of control. The key regulatory enzyme, a glucosyl transferase, is activated by glucose-6-phosphate and shows marked inhibition by monovalent anions such as chloride (25, 26). It is possible that the CO_2 effect that we describe could be related to chloride. Dissolved CO_2 readily enters the cell, hydrates (yeast carbonic anhydrase), and dissociates to bicarbonate based on the prevailing intracellular pH. Released protons are buffered. This may produce chloride efflux if the cells are impermeable to cations which otherwise could enter to balance newly generated bicarbonate anions. At equilibrium the fallen steady-state chloride concentration would allow the hypothetical mannosyl transferase to become active. In this regard, it is intriguing that Farhi et al. (21) and later Dykstra et al. (22) showed that high extracellular NaCl concentration markedly suppressed encapsulation of all strains studied. Increased intracellular glucose-6-phosphate concentration, by activating the transferase, could induce encapsulation in the absence of high CO_2 tension. This would occur when cell

replication slows upon entry into stationary phase with decreased glucose flux down the glycolytic pathway.

Alternatively, the CO_2 effect could be related to lowering of cytosolic pH if a rate-limiting step in capsule synthesis is highly pH sensitive. However, significant stimulation of capsule formation occurs at <5 mM total CO_2 concentration (Fig. 4 *A*). Considering the high degree of cytosolic buffering capacity, it seems likely that the pH change to this concentration of CO_2 is negligible once a steady state is reached. Nevertheless an effect on intracellular pH must be considered as a means through which CO_2 induces capsule synthesis.

The degree of encapsulation of different strains of *C. neoformans* has never been convincingly shown to affect virulence (5, 22), but the ability to regulate encapsulation has not been studied in this regard. Selection of a stable phenotype lacking the regulatory property enabled us to approach this question. The results suggest that the CO_2-induced regulatory property may be a virulence factor. Log-phase H99 cells exposed to high CO_2 become highly resistant to complement-mediated phagocytosis whereas air-grown cells, or H99/C3D cells grown in high CO_2, are efficiently phagocytized. A large inoculum of C3D cells given intracisternally to steroid-treated rabbits is cleared from the CSF by ~14 d after failing to produce the chronic meningitis that results from the same number of wild-type cells. No revertants were found when CSF was repeatedly examined by India ink preparations or plating the cryptococci on DME agar medium with CO_2/HCO_3^- to detect heavily encapsulated colonies. In the case of some *C. neoformans* stains (e.g., F396), factors other than CO_2 may regulate the rate of capsule synthesis. It is clear that the expression of virulence is multifaceted and the CO_2 regulatory property is but one of these factors.

The encapsulation process may be a regulated function which is adaptive for the success of this parasite. We speculate that, in the saprophytic environment, encapsulation is suppressed. This favors rapid growth and, perhaps, mating of a and alpha types. Once small infectious particles are inhaled, encapsulation is stimulated by the high CO_2 concentration found in lung and other tissues. In addition to CO_2, other stimuli may contribute to accelerate capsular polysaccharide synthesis as well. Under these conditions the organism replicates more slowly but gains a critical advantage: it becomes more resistant to phagocytosis. This would favor survival in the lungs and allow dissemination to the meninges and other tissues.

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