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Dissociation of a Hydrophobic Surface from Phagocytosis of Encapsulated and Non-Encapsulated *Cryptococcus neoformans*

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Cryptococcus neoformans is surrounded by a capsular polysaccharide that inhibits phagocytosis of the yeast by macrophages. This capsular polysaccharide also confers several physicochemical properties to the cell surface, including a negative surface charge and a hydrophilic surface. The present study was designed to determine whether a hydrophobic surface was necessary or sufficient for phagocytosis of *C. neoformans* cells. The hydrophobic nature of the cell surface was measured by hydrophobic interaction chromatography on octyl-Sepharose. Liability to phagocytosis was determined by use of mouse peritoneal macrophages. The surface properties of *C. neoformans* cells were modified by (i) preincubation of cryptococcal cells with nonimmune serum or immune anticapsular serum, (ii) chemical modification of the carboxyl and *O*-acetyl groups in the capsular polysaccharide, and (iii) use of various serotypes of *C. neoformans* with different degrees of *O*-acetyl and xylosyl substitution. The results showed that it was possible to experimentally vary the surface hydrophobic-hydrophilic characteristics of the cell surface; however, the antiphagocytic character of the capsule remained unchanged. The results further suggest that a hydrophobic surface was neither necessary nor sufficient for phagocytosis of *C. neoformans* cells by macrophages.

The molecular basis for cell adhesion has received considerable attention. In general, two basic mechanisms are proposed for cell adhesion. Cell adhesion may be based upon interaction between a ligand on a cell and a specific receptor for that ligand on a second cell (20). Alternatively, cell attachment may be dependent upon nonspecific interactions, such as hydrophobic interaction (14, 18), charge, or other physicochemical effects.

Phagocytosis is a specific example of cell adhesion, and similar adhesion mechanisms have been proposed to explain attachment and ingestion of particles by phagocytic cells. Several authors have argued that phagocytosis is largely nonspecific and can be explained on thermodynamic grounds which predict that phagocytes will engulf particles with overall surface properties that are more hydrophobic than those on the surface of the phagocyte (16, 18). Equally compelling are arguments that phagocytosis is more complex and involves the specific and coordinated interaction between particle-bound ligands such as immunoglobulin G or opsonically active complement fragments and their corresponding receptors on the plasma membranes (3).

Many microorganisms are surrounded by antiphagocytic capsules (4). The mechanisms proposed to explain the antiphagocytic effects of capsular polysaccharides largely parallel those for cell adhesion. Thus, several reports have suggested that the antiphagocytic surfaces of cells of *Salmonella typhimurium* (12) and numerous other encapsulated bacteria (19) are due to the apparent hydrophilic character of the microbial capsule as shown by hydrophobic interaction chromatography (HIC) (12) or contact angle (19). In contrast, Kozel and Gotschlich (9), McGaw and Kozel (13), and Wilkinson et al. (21, 22) have proposed that the antiphagocytic action of the capsule is due to a masking by the capsule of potential opsonins that are bound to sites beneath the capsular surface in such a way that these opsonic ligands are unable to interact with their receptors on the phagocyte. This masking mechanism does not require that antiphagocytic surface determinants necessarily be hydrophilic, but does require that the macrophage lack recognition capabilities for the capsular surface (9).

In ongoing studies in my laboratory, I have examined the antiphagocytic nature of the *Cryptococcus neoformans* capsule. The present study was designed to determine whether a hydrophobic surface was necessary or sufficient for phagocytosis of this yeast. Our studies took

advantage of several features of *C. neoformans* and its capsular polysaccharide. First, both normal and immune rabbit immunoglobulin G will bind to *C. neoformans*, yet only immune anticapsular immunoglobulin G is opsonic for encapsulated strains (8, 11). Second, the cryptococcal polysaccharide contains side chains that include glucuronic acid and *O*-acetyl groups. Each of these determinants confers distinct physicochemical properties to the capsular surface. By appropriate chemical modification of these groups, it is possible to modify the physicochemical features of *C. neoformans*. Finally, there is a natural variation between cryptococcal serotypes in the amount of *O*-acetyl and xylosyl substitution. *O*-Acetylation varies from 10.3% in serotype D to 3% in serotype C, whereas, xylose substitution varies from a xylose/mannose residue ratio of 1:3 in serotype D to 4:3 in serotype C (1, 2). In the course of these studies, I found that it was possible to experimentally vary the surface hydrophobic-hydrophilic character of *C. neoformans*; however, the antiphagocytic character of the capsule remained unchanged.

MATERIALS AND METHODS

***C. neoformans* strains.** *C. neoformans* isolates of serotypes C (ATCC 24066; American Type Culture Collection, Rockville, Md.) and D (ATCC 24067) were the encapsulated strains used throughout this study. *C. neoformans* 602 is a non-encapsulated strain whose characteristics have been described in detail elsewhere (5, 6). All cells were grown in a yeast extract dialysate medium (6) and were killed with Formalin before use (5). The procedure for purification of cryptococcal polysaccharide has been described previously (6).

Carbohydrate modification procedures. Whole encapsulated cryptococci were carboxyl reduced by the method of Taylor and Conrad (17). This procedure converted glucuronic acid side groups to glucose, with a concomitant loss of charge on the polysaccharide (9). The pH was carefully maintained below 7.5 throughout the reduction procedure. Two treatments were done to ensure complete reduction. Physical and chemical characteristics of carboxyl-reduced soluble polysaccharide and carboxyl-reduced whole cells have been described previously (9).

Removal of *O*-acetyl groups was accomplished by adjusting aqueous solutions of polysaccharide or aqueous suspensions of whole cells to 0.1 N NaOH with 1.0 N NaOH. The alkaline pH was maintained for 60 min at room temperature, followed immediately by neutralization with 1 N HCl and extensive washing with (whole cells) or dialysis against (soluble polysaccharide) distilled water. Two treatments were done to ensure complete hydrolysis of *O*-acetyl groups. Physical and chemical characteristics of de-*O*-acetylated soluble polysaccharide and de-*O*-acetylated whole cells have been described previously (9). Cells were both de-*O*-acetylated and carboxyl reduced by first de-*O*-acetylating the cells as described above, followed by carboxyl reduction.

Opsonization and phagocytosis of *C. neoformans* cells. *C. neoformans* isolates to be opsonized with normal serum were incubated for 2 h at 37°C with 1 ml of serum per 10^7 cells. Cells opsonized with capsular antiserum were incubated for 2 h at 37°C with four times the amount of antiserum necessary to produce 50% phagocytosis of the encapsulated cells. Dilutions of antiserum were made with phosphate-buffered saline (PBS, pH 7.2). After incubation with normal serum or capsular antiserum or both, the cells were washed twice with PBS and resuspended in 0.15 M NaCl (saline) at 10^8 cells per ml.

Cells to be used in phagocytosis experiments were further diluted to 10^6/ml in Hanks balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.), pH 7.2, containing antibiotics (100 U of penicillin and 100 μg of streptomycin per ml [GIBCO]).

Unstimulated peritoneal macrophages were obtained from 8- to 12-week-old Swiss mice (Microbiological Associates, Walkersville, Md.). The procedure for collection and culture of macrophages has been described previously (10). Monolayers were prepared in four-chamber tissue culture chamber-slides (model 4804; Lab-Tek Products, Div. Miles Laboratories, Inc., Westmont, Ill.) and incubated for 24 h at 37°C in 2.6% CO_2 before use. Each monolayer contained approximately 2.5×10^5 macrophages. For phagocytosis assays, the culture medium was decanted and each monolayer was washed two times with warm (37°C) Hanks balanced salt solution. One milliliter of Hanks balanced salt solution containing 10^6 appropriately prepared yeast cells was added to each chamber.

Phagocytosis of *C. neoformans* cells was determined after incubation of cells with macrophages for 1 h at 37°C. After incubation, the slides were washed, fixed, and stained as previously described (10). Slides were examined microscopically, and 200 macrophages per monolayer were observed for ingested cryptococci. Results are presented as mean values from at least four monolayers and are reported as the percentages of macrophages with ingested cells (percent phagocytosis).

Serum and antiserum. Calf serum (GIBCO) was used as the source of normal serum. Human serum was obtained from normal volunteers after obtaining informed consent. Capsular antiserum was prepared in rabbits with a methylated bovine serum albumin conjugate of cryptococcal polysaccharide as antigen. The procedures for preparation of methylated bovine serum albumin (7) and immunization of rabbits (9) have been described previously.

HIC. HIC was carried out with octyl-Sepharose (Pharmacia Fine Chemicals AB, Uppsala, Sweden) in a variation of the HIC procedure described by Stjerstrom et al. (16). Octyl-Sepharose was prepared according to the directions of the manufacturer. *C. neoformans* cells were mixed with 1 ml of the gel for 30 min at room temperature in 20 ml of the attachment buffer, 1 M $(NH_4)_2SO_4$ in 0.01 M phosphate buffer (pH 6.8). The cells and gel were then poured onto a sieve with a 38-μM opening size, and the buffer containing unbound cells was collected. Cells adherent to the gel were eluted by washing with 20 ml of buffers with stepwise decreasing concentrations of $(NH_4)_2SO_4$ and increasing concentrations of Triton X-100. Elution buffers consisted of 0.01 M sodium phosphate (pH 6.8) and 0.5 M $(NH_4)_2SO_4$, 0.5 M $(NH_4)_2SO_4$–0.05% Triton X-100, 0.25 M $(NH_4)_2SO_4$–0.075% Triton X-100, 0.10 M $(NH_4)_2SO_4$–0.09% Triton X-100, or 0.10% Triton X-

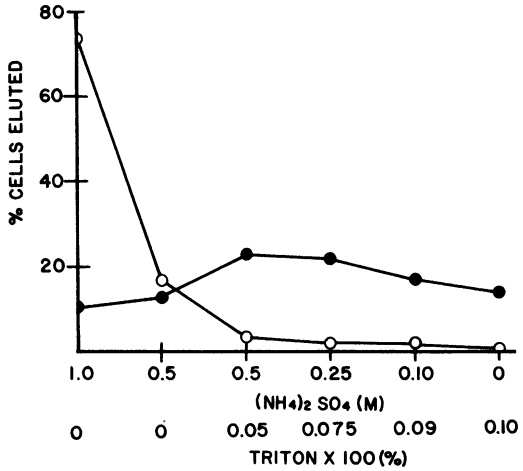

FIG. 1. Elution of cells of *C. neoformans* serotype D after HIC on octyl-Sepharose. Yeast cells were unopsonized (○) or preincubated with normal bovine serum (●).

100. The buffer was collected after each elution step, and the number of eluted cells was determined with a particle counter (Electrozone/Celloscope; Particle Data, Inc., Elmhurst, Ill.). Data are reported as the percentage of total cells eluted by each buffer. HIC of treated cells is reported as the mean from three replicate experiments.

Macrophages to be examined for HIC were collected from mice as described above and incubated overnight at 37°C. Adherent cells were removed by gentle scraping with a rubber policeman and washed two times with saline, and cell densities were determined by microscopic examination in a hemacytometer.

RESULTS

An initial experiment was done to assess the pattern of *C. neoformans* binding to the hydrophobic octyl-Sepharose gel. Preliminary experiments had shown that unopsonized cells of serotype D were hydrophilic, whereas cells of serotype D preincubated with normal bovine serum were relatively hydrophobic. Unopsonized or normal serum-opsonized cells of serotype D were mixed with octyl-Sepharose and eluted with a stepwise gradient of decreasing ammonium sulfate and increasing Triton X-100. The results (Fig. 1) showed that unopsonized *C. neoformans* cells did not bind to the gel and were found free in the initial attachment buffer. In contrast, cells of serotype D that were preincubated with normal bovine serum bound to the gel and were eluted when the $(NH_4)_2SO_4$ concentration was reduced to 0.5 to 0.1 M and the Triton X-100 concentration reached 0.05 to 0.09%. All subsequent experiments were done with a similar elution system. For the purposes of data reduction, data are reported as the percentages of cells adherent to the octyl-Sepharose after the initial incubation of cells and gel in 1.0 M $(NH_4)_2SO_4$. Thus, a high percentage of adherent cells reflects a hydrophobic surface, whereas a low percentage of adherent cells indicates that the cell surface has little tendency for hydrophobic interaction. HIC of macrophages cultured for 24 h showed that 40% of the macrophages were adherent to the gel after the initial incubation in 1.0 M $(NH_4)_2SO_4$. Most of the adherent cells were eluted with 0.5 M $(NH_4)_2SO_4$. Thus, macrophages showed a hydrophobic interaction that was intermediate between unopsonized and normal serum-opsonized cells of serotype D.

Non-encapsulated cryptococci are effectively opsonized by immunoglobulin G found in normal nonimmune serum (11). Consequently, we examined the effect of normal bovine serum on phagocytosis and HIC of the non-encapsulated strain 602. *C. neoformans* cells were preincubated with heat-inactivated normal bovine serum, washed with saline, and incubated with octyl-Sepharose or monolayers of macrophages. The results (Table 1) showed that neither unopsonized nor normal serum-opsonized cells of strain 602 showed an appreciable binding to the octyl-Sepharose, suggesting the presence of a hydrophilic surface on both cells. In contrast, preincubation of non-encapsulated cells with normal serum effected a marked increase in phagocytosis of the yeast. Thus, opsonization produced a significant increase in phagocytosis without a concomitant alteration in surface hydrophobicity.

We next examined the effects of anticapsular serum on phagocytosis and surface hydrophobicity of encapsulated cells of serotype D. Encapsulated cryptococci were preincubated with anticapsular serum in the presence or absence of heat-inactivated normal human serum, washed with saline, and incubated with octyl-Sepharose or monolayers of macrophages. The results (Table 2) showed that (i) normal human serum increased the hydrophobicity of the cell surface, (ii) anticapsular serum had no effect on HIC of the yeast, and (iii) anticapsular serum modulated the ability of normal serum to increase cell

TABLE 1. Effect on normal serum on HIC and phagocytosis of non-encapsulated *C. neoformans* 602

Cell treatment	Mean ± SEM % cells adherent to octyl-Sepharose	Mean ± SEM % phagocytosis
Untreated strain 602	12 ± 2.4	8.5 ± 1.8
Strain 602 opsonized with nonimmune bovine serum	15 ± 1.9	85 ± 2.4

TABLE 2. Effects of nonimmune human serum and anticapsular rabbit serum on HIC and phagocytosis of cells of *C. neoformans* serotype D

Cells opsonized with:		Mean ± SEM % cells adherent to octyl-Sepharose	Mean ± SEM % phagocytosis
Nonimmune serum	Anticapsular serum		
Absent	Absent	13 ± 2.4	0.1 ± 0.5
Present	Absent	31 ± 5.4	0.7 ± 0.5
Absent	Present	12 ± 2.9	78 ± 2.5
Present	Present	15 ± 3.1	81 ± 2.2

surface hydrophobicity. Phagocytosis occurred only in the presence of capsular antiserum. Nonimmune serum neither enhanced nor modulated phagocytosis. These results showed no correlation between a hydrophobic surface and phagocytosis of the yeast since unopsonized cells were relatively hydrophilic and were not engulfed, cells opsonized with normal serum were relatively hydrophobic yet remained resistant to phagocytosis, and cells opsonized with anticapsular serum were relatively hydrophilic but were engulfed at a high rate.

The cryptococcal capsule contains several constituent groups that might contribute to the hydrophilic or hydrophobic character of the cell surface. Furthermore, these groups are easily altered or eliminated by chemical modification. *O*-Acetyl side chains on the polysaccharide may contribute to hydrophobic interaction, and the carboxyl groups on glucuronic acid side chains contribute to a hydrophilic surface (9). In addition, the extent of *O*-acetylation varies with serotype. Serotype D is the most heavily *O*-acetylated, and serotype C contains a lower density of *O*-acetyl groups. As a consequence, we examined the effects of de-*O*-acetylation, carboxyl reduction, and preincubation with heat-inactivated normal bovine serum on the HIC and phagocytosis of encapsulated cells of serotypes C and D. The results (Table 3) showed that cells of serotype C are more hydrophilic than cells of serotype D, and this hydrophilic character of serotype C is largely unchanged by chemical modification. In contrast, the hydrophobic character of serotype D is increased markedly by reduction of the carboxyl groups. Preincubation of modified and unmodified yeast cells with heat-inactivated normal bovine serum increased the hydrophobic interaction with octyl-Sepharose. This effect was greatest with cells of serotype D and least apparent with unmodified and de-*O*-acetylated cells of serotype C. Despite the fact that the hydrophobic character of the cell surface could be experimentally controlled by selection of serotype, chemical modification, or preincubation with nonimmune serum, all encapsulated cells of serotypes C and D remained completely resistant to phagocytosis.

DISCUSSION

These studies were carried out to determine whether a hydrophobic surface, as shown by HIC, was necessary or sufficient for phagocytosis of encapsulated *C. neoformans* cells. The results suggest that a hydrophobic surface, as shown by HIC, was not necessary for effective phagocytosis. Opsonization of encapsulated cells with small amounts of anticapsular serum had little effect on the hydrophilic character of the encapsulated cells, yet these opsonized cells were readily ingested by macrophages. Similarly, normal serum-opsonized cells of strain 602 had little tendency toward hydrophobic interaction, yet high-level phagocytosis occurred with

TABLE 3. Effects of chemical modification and nonimmune bovine serum on HIC and phagocytosis of cells of *C. neoformans* serotypes C and D[a]

Cell treatment	Not serum treated		Serum treated	
	% Cells adherent to octyl-Sepharose	% Phagocytosis	% Cells adherent to octyl-Sepharose	% Phagocytosis
Untreated serotype C	7.0 ± 1.3	0	12 ± 0.8	0.1 ± 0.5
De-*O*-acetylated serotype C	7.7 ± 1.7	0.1 ± 0.5	11 ± 1.4	0.5 ± 0.6
Carboxyl-reduced serotype C	9.3 ± 0.8	0	19 ± 1.7	0
De-*O*-acetylated + carboxyl-reduced serotype C	11 ± 1.6	0	39 ± 0.8	0
Untreated serotype D	18 ± 3.0	0	81 ± 3.4	0.4 ± 0.7
De-*O*-acetylated serotype D	15 ± 1.4	0.1 ± 0.5	60 ± 3.1	0.5 ± 0.8
Carboxyl-reduced serotype D	46 ± 4.8	0	80 ± 3.2	0
De-*O*-acetylated + carboxyl-reduced serotype D	24 ± 2.0	0	67 ± 2.6	0

[a] Results are expressed as mean ± standard error of the mean.

the opsonized non-encapsulated cells. The possibility must be considered that some other assay of surface hydrophobicity might show that these phagocytosis-sensitive cells exhibit a significant but weak tendency toward hydrophobic interaction that is not detected by HIC. However, two points should be made in this regard. First, HIC has been of considerable value as a measure of surface hydrophobicity in studies of other microbial surfaces (12, 16). Second, these phagocytosis-sensitive cells exhibited substantially less binding to octyl-Sepharose than did the macrophages themselves. Thus, our results provide an exception to arguments that phagocytes will engulf only particles that are more hydrophobic than themselves.

It should be emphasized that this lack of association between hydrophobicity and phagocytosis is based on an overall estimate of hydrophobicity as shown by HIC. This approach is fundamentally similar to those used in other studies of the relationship between surface properties and phagocytosis (12, 15, 16, 18, 19). None of these studies provides information on the role of hydrophobic interaction in the microenvironment of the Fc receptor and the Fc fragment, and it is possible that hydrophobic interaction may play a role in this receptor-ligand binding.

It is apparent from the results shown in this study that a hydrophobic surface is not sufficient for phagocytosis of encapsulated cryptococci. Carboxyl reduction (Table 3) or preincubation with nonimmune human or bovine serum (Tables 2 and 3) increased the tendency of encapsulated cells toward hydrophobic interaction, yet the cells remained resistant to phagocytosis. It could be argued that the hydrophobic properties of these modified cells were inadequate for phagocytosis; however, the tendency for hydrophobic interaction by these phagocytosis-resistant cells greatly exceeded the hydrophobic properties of cells ingested by macrophages, such as cells of strain 602 opsonized with normal serum (Table 1) or encapsulated cryptococci opsonized with anticapsular serum (Table 2). These results suggest that the phagocytosis-inhibiting properties of the cryptococcal capsule cannot be explained solely on the basis of a hydrophilic surface which might be produced by the capsule.

A strong circumstantial argument has been made for hydrophilicity as a mechanism for inhibition of phagocytosis by microbial capsules (12, 18, 19). The crucial question is whether hydrophilicity and resistance to phagocytosis are causally related or whether these are epiphenomena in which the antiphagocytic capsule is coincidentally hydrophilic in nature. In the case of *C. neoformans*, the hydrophobic and hydrophilic properties of the opsonized or unopsonized capsule could be dissociated from the antiphagocytic properties of the polysaccharide. There is a similar report that *Escherichia coli* O118:K$^-$ has a hydrophobic uncharged surface, yet this strain is poorly phagocytosed (15). Additional studies of a broader spectrum of microorganisms using either structural derivatives of encapsulated microorganisms or mutant strains with variations in capsular structure are needed to further assess the causal or circumstantial association between resistance to phagocytosis by capsular polysaccharides and a hydrophilic surface as shown by assays for overall surface hydrophobicity, such as contact angle or HIC.

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