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# Concomitant but Not Causal Association Between Surface Charge and Inhibition of Phagocytosis by Cryptococcal Polysaccharide

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The mechanism by which capsular polysaccharides inhibit phagocytosis is not clearly understood. We investigated the association between a negative surface charge and inhibition of phagocytosis by the capsular polysaccharide of *Cryptococcus neoformans*. A two-polymer aqueous-phase system containing phosphate ions was used to assess surface charge. Opsonins such as normal bovine serum and normal human immunoglobulin G reduced the surface charge on non-encapsulated cryptococci and simultaneously enhanced phagocytosis. These same opsonins had no effect on phagocytosis or surface charge of encapsulated cryptococci. $F(ab')_2$ fragments of normal human immunoglobulin G neither enhanced phagocytosis nor altered the surface charge of non-encapsulated cryptococci. Addition of purified cryptococcal polysaccharide to non-encapsulated cells inhibited phagocytosis of the yeast and induced a strong negative charge at the yeast surface. Chemical modification to reduce the surface charge of either purified cryptococcal polysaccharide or intact encapsulated cryptococci produced a small loss of phagocytosis-inhibiting activity; however, all treated polysaccharide preparations retained a significant ability to inhibit phagocytosis of the yeast. These results indicated that the association between surface charge and inhibition of phagocytosis was largely circumstantial, and presence of a negative surface charge could not account for the powerful antiphagocytic action of cryptococcal polysaccharide.

We have recently published data indirectly suggesting that the capsule of *Cryptococcus neoformans* masks the presence of opsonic proteins that have bound to the cell wall (8, 11). Implicit in this inhibition mechanism is the assumption that the capsule presents a new surface to the macrophage for which the macrophage has no receptors. The physicochemical characteristics of the capsular surface that are responsible for this recognition failure are not known.

A strongly negative surface due to uronic acid in the capsular polysaccharide is a dominant biophysical characteristic of the surface of encapsulated cryptococci. Data from several reports suggest that the surface charge of either the phagocytic cell (12) or the particle (3, 10, 16) may play a role in phagocytosis. Most mammalian cells carry a net negative charge; consequently, presence of a negative charge induced by the cryptococcal capsule could give rise to a net repulsive force between cryptococci and phagocytic cells. Indeed, Luderitz et al. (9) note that most capsular antigens are negatively charged, and they suggest that resistance to phagocytosis is due to this surface charge.

The present study was undertaken (i) to assess the role of encapsulation and opsonization on surface charge and phagocytosis of *C. neoformans*, (ii) to utilize appropriate chemical modification reactions to reduce the uronic acid residue, and (iii) to assess the effect of this reduction on phagocytosis and surface charge of encapsulated cryptococci. Our results showed that encapsulation was strongly associated with inhibition of phagocytosis and a negative surface charge. However, the association between inhibition of phagocytosis and surface charge was largely circumstantial, and surface charge can account for only a small part of the profound inhibition of phagocytosis produced by cryptococcal polysaccharide.

## MATERIALS AND METHODS

**Yeast strains and soluble polysaccharide.** *C. neoformans* strain 613 is a moderately encapsulated isolate. *C. neoformans* strains 602 is a non-encapsulated strain that has surface receptors for the soluble polysaccharide; thus, addition of purified polysaccharide to strain 602 allows experimental variation in the extent of encapsulation on the yeast (4). The characteristics of these strains have been described elsewhere

in detail (5). Yeasts used in phagocytosis assays were Formalin killed (6) and used as a suspension in Hanks balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.) containing antibiotics (100 U of penicillin and 100 μg of streptomycin per ml; GIBCO) and buffered with sodium bicarbonate to pH 7.2. The procedure for purification of cryptococcal polysaccharide has been described previously (5). Polysaccharide was prepared for use as a saline solution.

**Phagocytosis assays.** 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 (6).

Monolayers were prepared in four-chamber tissue culture chamber/slides (model 4804 Lab-Tek Products, Westmont, Ill.) and incubated for 24 to 48 h at 37°C in 2.6% $CO_2$ before use. Each monolayer contained approximately $2.5 \times 10^5$ macrophages.

For phagocytosis assays, the culture medium was decanted, and each monolayer was washed two times with warm (37°C) Hanks balanced salt solution. A test yeast suspension was warmed for 2 min at 37°C in a water bath, and 1 ml was immediately added to each chamber. The yeast suspensions consisted of: (i) $10^6$ yeast cells; (ii) a source of opsonin; (iii) when required by an experimental protocol, 0.25 ml of cryptococcal polysaccharide in saline; and (iv) enough Hanks balanced salt solution to give a final volume of 1 ml. In some experiments, yeast cells were preincubated with a source of opsonin for 30 min at 37°C, sedimented, resuspended to $10^6$ yeast cells per ml, and added (1 ml) to macrophage monolayers.

Phagocytosis was determined after incubation of monolayers with yeast cells for 1 h at 37°C. After incubation, the slides were washed, fixed, and stained as previously described (6). Slides were examined microscopically, and 200 macrophages per monolayer were observed for ingested yeasts. The large size of cryptococcal cells permitted a clear differentiation between attached and ingested yeasts (6). Results are presented as mean values from at least four monolayers and report the percentage of macrophages with ingested yeasts (percent phagocytosis). Statistical analysis of phagocytosis data was done by analysis of variance.

**Serum and serum components.** The sources of opsonins for *C. neoformans* were (i) normal bovine serum (GIBCO; lot A790120), (ii) heat-inactivated normal bovine serum, and (iii) immunoglobulin G (IgG) isolated from normal human serum by diethylaminoethyl cellulose chromatography (7, 21). $F(ab')_2$ fragments of normal human IgG were prepared by the techniques of Spiegelberg and Weigle (15).

**Phase systems.** Two-polymer aqueous-phase systems were prepared from aqueous stock solutions of 40% (wt/wt) polyethylene glycol 6000 (J. T. Baker Chemical Co., Phillipsburg, N.J.) and 20% (wt/wt) dextran T-500 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) by the techniques described by Walter (19). Isotonic phase systems described by Walter (19) with the following compositions were used in our study: (i) 5% (wt/wt) dextran and 4% (wt/wt) polyethylene glycol 6000 containing 0.11 M sodium phosphate buffer, pH 6.8; (ii) the same polymer concentrations, but 0.09 M sodium phosphate buffer and 0.03 M NaCl; (iii) the same polymer concentrations, but 0.06 M sodium phosphate buffer and 0.075 M NaCl; (iv) the same polymer concentrations, but 0.03 M sodium phosphate buffer and 0.12 M NaCl; and (v) the same polymer concentrations, but 0.01 M sodium phosphate buffer and 0.15 M NaCl. Samples (200 ml) of the above phase systems were mixed at 20°C, and the phases were allowed to separate. The top (polyethylene glycol-rich) and bottom (dextran-rich) phases were collected, and 2 ml of top phase and 2 ml of the bottom phase were pipetted into individual tubes.

Yeast cells were prepared for partition experiments by incubating them with appropriate opsonins or other reagents for 30 min at 37°C. The cells were sedimented by centrifugation, washed one time with distilled water, and resuspended in distilled water at a concentration of $10^8$ cells per ml unless otherwise noted. Fifty microliters of yeast suspension was added to each phase tube, and the tubes were mixed by inversion 20 times. The phases were allowed to separate for 20 min at 20°C, and a 200-μl sample was withdrawn from the upper phase and diluted in 20 ml of saline. A Coulter Counter was used to determine cell concentrations. Data are reported as the percentage of cells added that partitioned into the top phase. All results presented are averages from at least three replicates.

**Potential of top phase relative to bottom phase.** Bulk phase potential differences were measured by a modification of the techniques described by Reitherman et al. (14). Electrodes were Pasteur pipettes filled with 1% agar containing 3 M KCl. A Tektronics type 502 dual-beam oscilloscope was used to measure the potential differences. Data are reported as the mean of at least five determinations.

**Immunofluorescence.** Indirect immunofluorescence was used to assay binding of cryptococcal polysaccharide to strain 602. Cells of strain 602 were incubated with cryptococcal polysaccharide followed by rabbit cryptococcal antiserum and stained with fluorescein-conjugated antiserum to rabbit immunoglobulins (lot 7500; Cappel Laboratories, Downingtown, Pa.) by previously described procedures (4). All slides were read in a "blind" fashion.

**Reduction of cryptococcal polysaccharide.** Purified cryptococcal polysaccharide was applied to a column of Dowex 50 ($H^+$) cation exchanger, and the effluent was lyophilized to yield the free acid. This form of cryptococcal polysaccharide was esterified with ethylene oxide and reduced with $NaBH_4$ by the procedure of Sutherland (17) to convert uronic acid to hexose residues.

Whole cells of encapsulated cryptococci were carboxyl reduced by carbodiimide treatment (18) followed by reduction with sodium borohydride. Aqueous suspensions of whole cells were made 2 mM with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, and the pH was maintained at 4.75 on a Radiometer pH stat with 0.1 N HCl until $H^+$ uptake ceased. The treatment with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide was repeated, and the cells were washed with distilled water and reduced with $NaBH_4$ as described by Aspinall (1).

## RESULTS

**Assay of surface charge in two-polymer aqueous-phase systems.** Reitherman et al. (14) reported that phosphate partitions unequally between the phases of a dextran-polyethylene glycol phase system. Thus, in the presence of phosphate and appropriate polymer concentrations, partitioning of cells between the phases may be based primarily on surface charge (19). A preliminary experiment was done to determine the effects of various phosphate concentrations on partitioning of strains 613 and 602. Strain 613 was presumed to be negatively charged due to the uronic acid in the capsular polysaccharide. The potential difference between the phases was also determined to allow a comparison between the partitioning of yeast cells in a given phase system with the electrostatic potential in the phase system. The results (Fig. 1) showed that high phosphate concentrations induced a distinct potential in the system. Encapsulated strain 613 partitioned completely into the upper polyethylene glycol-rich phase at high phosphate concentrations, whereas non-encapsulated cells showed less affinity for the upper phase. Neither cell type showed any appreciable affinity for the upper phase in the absence of a potential difference between the two phases. Thus, partitioning in the phase system containing 0.11 M phosphate was considered to be a consequence of interaction between the electrostatic potential in the phase system and presence of a negative charge on the yeast surface. This "charged" phase system was used to assay cell surface charge in all subsequent experiments.

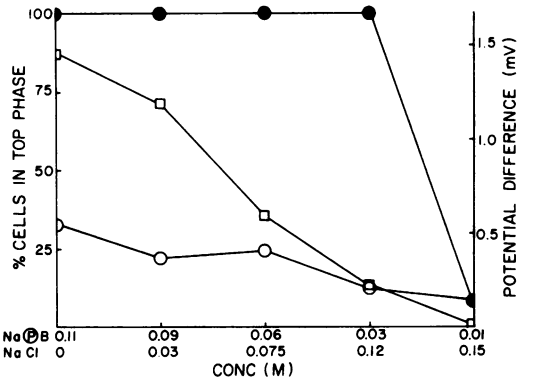


FIG. 1. *Effect of phosphate ions on the potential difference (□) between dextran-rich and polyethylene glycol-rich phases and the partitioning of encapsulated (●) and non-encapsulated (○) cryptococci in each phase system.*

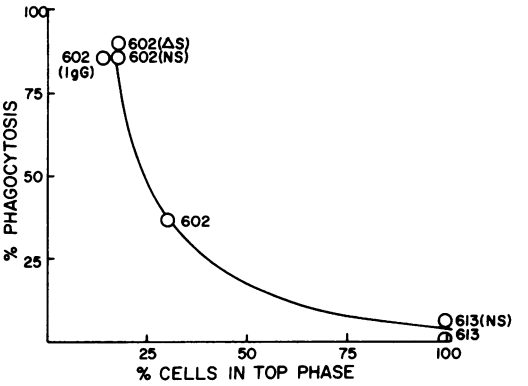


FIG. 2. *Partitioning in a charged phase system and phagocytosis of encapsulated (613) and non-encapsulated (602) cryptococci in the presence of no opsonins, normal bovine serum (NS), heat-inactivated bovine serum (ΔS), and normal human IgG.*

**Association between surface charge and phagocytosis of *C. neoformans*.** Normal nonimmune serum and IgG isolated from nonimmune serum are opsonic for non-encapsulated *C. neoformans* (7). An experiment was done to determine how opsonization might influence surface charge and to compare this effect with susceptibility of the opsonized cells to phagocytosis. Cells of strain 613 or strain 602 were incubated for 30 min at 37°C with normal bovine serum (10 ml per $10^8$ cells), heat-inactivated bovine serum (10 ml per $10^8$ cells), normal human IgG (50 mg per $10^8$ cells), or saline. The cells were washed in distilled water and assayed to determine surface charge and susceptibility to phagocytosis. The results (Fig. 2) showed good correlation between increased phagocytosis and reduced surface charge as shown by partitioning in a charged phase system.

Previous studies in our laboratory demonstrated that $F(ab')_2$ fragments of normal human IgG are unable to enhance phagocytosis of non-encapsulated cryptococci (7). Accordingly, cells of strain 602 were incubated for 30 min at 37°C with normal human IgG (50 mg per $10^8$ cells) or $F(ab')_2$ fragments of normal human IgG (50 mg per $10^8$ cells) in the absence of any additional serum components. The cells were washed with distilled water and assayed to determine surface charge and susceptibility to phagocytosis. The results (Table 1) showed that not only did $F(ab')_2$ fragments fail to opsonize the yeast, but they also failed to alter the surface charge of the yeast.

The previous experiments suggested a strong association between reduced surface charge and increased phagocytosis of *C. neoformans*. Since cryptococcal polysaccharide is highly charged due to the uronic acid residue, we attempted to

assess the ability of purified cryptococcal polysaccharide to inhibit phagocytosis and any concomitant effect the polysaccharide might have on partitioning of the yeast in the charged phase system. Cells of strain 602 were incubated for 30 min at 37°C with normal bovine serum (1 ml per $10^7$ cells) and various concentrations of cryptococcal polysaccharide. The cells were then washed, added to the charged phase system to assess changes in surface charge, and added to monolayers of macrophages to determine susceptibility to phagocytosis. The results (Fig. 3) showed that inhibition of phagocytosis at increasing concentrations of cryptococcal polysaccharide coincided with an increased surface charge on the yeast.

**Reduction of cryptococcal polysaccharide.** The previous experiments demonstrated a strong association between surface charge and inhibition of phagocytosis, yet the experiments did not prove that inhibition of phagocytosis was in fact due to a negative surface charge. As a consequence, cells of the encapsulated strain 613 that had been treated to reduce the carboxyl groups were examined for their susceptibility to phagocytosis. If inhibition of phagocytosis is causally related to surface charge, the reduced cells should show little or no resistance to phagocytosis. Cells of strain 613 were complexed with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, and the uronic acid was reduced to glucose with sodium borohydride. Partitioning of the treated and untreated cells in a charged phase system showed a total loss of surface charge on the reduced cryptococci (Table 2). Reduction in the capsular surface charge produced a significant ($P > 0.05$) increase in phagocytosis, but phagocytosis of reduced encapsulated cryptococci never reached the levels observed with non-encapsulated cryptococci.

The previous experiment suggested that factors other than surface charge were responsible for inhibition of phagocytosis by cryptococcal

TABLE 1. *Effect of F(ab')$_2$ fragments on phagocytosis and partitioning of strain 602*

| Cells[a] | Phagocytosis[b] | Partition coefficient[c] |
|---|---|---|
| Strain 602 | 37 ± 14 | 31 ± 1 |
| IgG-opsonized strain 602 | 87 ± 5 | 25 ± 3 |
| F(ab')$_2$-opsonized strain 602 | 35 ± 16 | 31 ± 2 |

[a] Cells of strain 602 were opsonized with IgG from normal serum or F(ab')$_2$ fragments of normal IgG.
[b] Mean percent phagocytosis ± standard deviation.
[c] Percentage of cells in the top phase ± standard deviation. Partitioning was determined in a phase system containing 0.11 M phosphate.

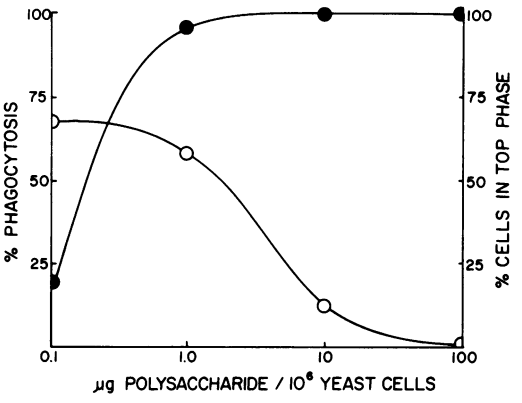


FIG. 3. *Inhibition of phagocytosis of non-encapsulated C. neoformans* (○) *and induction of surface charge* (●) *by cryptococcal polysaccharide.*

TABLE 2. *Effect of carbodiimide activation and sodium borohydride reduction on phagocytosis and partitioning in a charged phase system of encapsulated C. neoformans*

| Treatment | Phagocytosis[a] | Partition coefficient[b] |
|---|---|---|
| Untreated | 3 ± 1 | 95 ± 2 |
| EDC activated, borohydride reduced | 32 ± 5 | 4 ± 1 |

[a] Mean percent phagocytosis ± standard deviation.
[b] Percentage of cells in the top phase ± standard deviation. Partitioning was determined in a phase system containing 0.11 M phosphate.

polysaccharide. This interpretation was reinforced when purified cryptococcal polysaccharide that had been treated to reduce the carboxyl groups was examined for the ability to inhibit phagocytosis of non-encapsulated strain 602. Cells of strain 602 were incubated for 30 min at 37°C with normal bovine serum and various concentrations of either untreated or reduced cryptococcal polysaccharide. The cells were then added to the charged phase system to assess changes in surface charge and added to monolayers of macrophages to determine susceptibility to phagocytosis. The reduced polysaccharide was uncharged since it did not alter the partitioning of strain 602 in the charged phase system (Fig. 4). It could be argued that this failure of reduced polysaccharide to alter the surface charge of strain 602 was due to an inability of reduced polysaccharide to bind to the yeast; however, examination of reduced-polysaccharide-treated strain 602 by indirect immunofluorescence showed that the polysaccharide did, in fact, bind to cells of strain 602. The data further showed that the reduced polysaccharide had a slightly diminished ability to inhibit phagocytosis of strain 602 at a concentration of 1 μg of

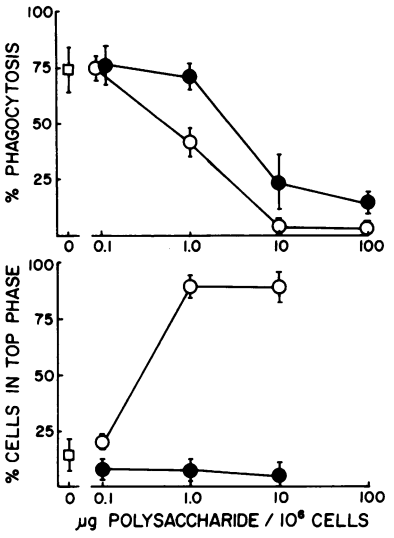


FIG. 4. *Effect of unmodified* (○) *and reduced* (●) *cryptococcal polysaccharide on inhibition of phagocytosis and partitioning in a charged phase system of non-encapsulated C. neoformans. Data shown as mean ± standard deviation.*

polysaccharide per $10^6$ yeast cells; however, the reduced polysaccharide did not differ significantly from untreated polysaccharide at concentrations of 10 and 100 μg of polysaccharide per $10^6$ yeast cells. Thus, surface charge may contribute to inhibition of phagocytosis at very low densities of inhibiting polysaccharide; however, as the level of inhibitory polysaccharide increases, surface charge is no longer necessary for maximal inhibition of phagocytosis.

## DISCUSSION

Charge repulsion between surfaces of like charge is one of the few mechanisms by which a capsule could introduce a repulsive force between a microorganism and a phagocytic cell (2, 13). Most mammalian cells carry a net negative charge; therefore, the presence of charged groups such as the uronic acid or phosphate groups found in many microbial capsules could give rise to a net repulsive force. Several investigators have studied with mixed results the association between surface charge and inhibition of phagocytosis by capsular polysaccharides. Magnusson et al. (10) found that a negative charge characterizes the surface of rough *Salmonella typhimurium* strains that are susceptible to phagocytosis, whereas the absence of charge characterizes the surface of smooth strains that are resistant to phagocytosis. Stendahl et al. (16) later noted that the presence of a negative charge external to the lipopolysaccharide, for example, the charged K antigen of *Escherichia coli*, seems to be related to increased resistance to phagocytosis.

In the present study, we found an inverse correlation between surface charge and phagocytosis of *C. neoformans*. Successful opsonization was associated with a reduction in cell surface charge. $F(ab')_2$ fragments that bound to the yeast but failed to opsonize similarly failed to reduce the surface charge. Addition of purified cryptococcal polysaccharide to non-encapsulated cryptococci effected a decrease in phagocytosis and a concomitant increase in cell surface charge.

The bulk of evidence suggests that encapsulation is associated with marked changes in the physicochemical environment at the cell surface, such as alterations in surface charge or hydrophobicity. Given the large size of the capsule relative to the microbial cell itself, this is not surprising. The crucial unanswered question is which, if any, of these physicochemical alterations are causally related to inhibition of phagocytosis. Two interpretations for the association between surface charge and inhibition of phagocytosis were possible. First, inhibition of phagocytosis by cryptococcal polysaccharide could be directly related to induction by the polysaccharide of a negative charge barrier around the yeast that prevents effective contact between the opsonized yeast and macrophages. Alternatively, there might be no causal relationship between the presence of a negative surface charge and inhibition of phagocytosis. The charged phase system could be nothing more than a very sensitive technique for assay of polysaccharide on the surface of the yeast without any mechanistic implications for inhibition of phagocytosis.

Chemical modification of the surface charge demonstrated that the association between surface charge and inhibition of phagocytosis was largely circumstantial. Reduction of surface charge did produce a partial loss of the phagocytosis-inhibiting activity; however, this loss in biological function was not as profound as might be inferred from measurements of cell surface charge. If inhibition of phagocytosis were directly related to surface charge, reduction of uronic acid to glucose should have abrogated the antiphagocytic effect. This was not the case since the reduced polysaccharide retained a significant ability to inhibit phagocytosis.

The small contribution made by charge to inhibition of phagocytosis could be due to one of at least two mechanisms. First, charge could introduce a significant repulsive force between the yeast cell and the macrophage that acts

synergistically with the remaining factors that account for the bulk of the antiphagocytic effect. Alternatively, charge could be a necessary factor in maintaining the three-dimensional structure of the capsule. We (7, 8) and others (20) have previously suggested that one action of the capsule is masking the presence of opsonic proteins that have bound to the cell wall. Loss of charge could produce a reduction in capsule size. Thus, a partial "unmasking" of opsonins bound to the cell wall might occur with reduced encapsulated cells (Table 2), and more reduced than untreated polysaccharide might be required to occlude opsonins bound to non-encapsulated cryptococci (Fig. 4). Current studies in our laboratory are designed to distinguish between these two possible modes of action.

## ACKNOWLEDGMENTS

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