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Factors Influencing the Phagocytosis of *Clostridium difficile* by Human Polymorphonuclear Leukocytes

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Phagocytosis of *Clostridium difficile* by human polymorphonuclear leukocytes (PMNs) and the possible role of the clostridial toxins in this process were investigated. Phagocytosis of *C. difficile* was independent of aerobiosis and clearly depended on opsonization. Either complement or antibodies to *C. difficile* could serve as opsonins. Toxigenic strains of *C. difficile* were more resistant to phagocytosis than were nontoxigenic strains. Pretreatment of PMNs with as much as 10,000 units of toxins from culture filtrates of *C. difficile* for 2 h had no effect on either the phagocytic activity of PMNs or their viability as determined by trypan blue exclusion. In contrast, treatment of human embryonic intestinal cells with the same amount of toxin under identical conditions resulted in cell death.

Clostridium difficile causes diarrhea and pseudomembranous colitis in humans (3, 4, 7). The development of these diseases is often associated with antibiotic therapy, for example, with clindamycin (8), ampicillin (28), and cephalosporins (30). Occasionally, colitis caused by *C. difficile* can occur in the absence of such antibiotics (12).

C. difficile produces two antigenically and biologically distinct heat-labile toxins that appear to be involved in the pathogenesis of the disease. Toxin A, also referred to as the enterotoxin, causes accumulation of hemorrhagic fluid in ligated rabbit ileal loops and death in laboratory animals (2, 21, 31). Toxin B, a cytotoxin, alters the morphology of a variety of cultured cells (2, 31). The cytotoxin appears to act synergistically with the enterotoxin to cause increased morbidity and mortality as compared with that caused by the enterotoxin alone (21).

Both toxins appear to be large protein complexes with molecular weights of approximately 500,000 as determined by gel filtration (4, 25, 31). The observations that the enterotoxin can agglutinate rabbit erythrocytes at 4°C and that a monoclonal antibody can precipitate the enterotoxin have led some investigators to propose that the enterotoxin is composed of subunits (19, 20). Rolfe and Finegold (25) suggested that the cytotoxin, also, is a multimeric protein since dissociation of the toxin resulted in the appearance of a single protein band of 50,000 daltons.

The mode of intoxication by either of the toxins is unclear. Both toxins contain cytotoxic activity, albeit to different degrees (31). The initial cytopathic event caused by both toxins is an alteration of the morphology of the treated cell, an event that can occur as rapidly as 30 min after treatment of cells with either the enterotoxin or the cytotoxin (27). After the appearance of the altered morphology, cells treated with either toxin will exhibit a loss of intracellular potassium and an inhibition of protein synthesis (27). These observations, coupled with those demonstrating a loss of cell surface fibronectin (1) and a decentralization of the cell nucleus (29) after treatment of cells with toxin, suggest that the initial step in the intoxication process involves disruption of the integrity of the cell membrane.

The effects of the *C. difficile* toxins on the cells of the immune system have not been investigated. Polymorphonuclear leukocytes (PMNs), which are the first line of defense against extracellular bacterial pathogens, have been demonstrated to be located in the pseudomembrane as well as in the intestinal muscosal layer underlying the pseudomembrane (10, 23, 32). Since the PMN can exert its phagocytic activity anaerobically as well as aerobically (18, 22), the question arises as to how *C. difficile* can persist to cause disease in the presence of PMN infiltrates. In this communication, we describe the interaction of PMNs with *C. difficile*. Furthermore, evidence is presented to demonstrate the lack of an effect of the *C. difficile* toxins on the phagocytic activity and viability of PMNs.

MATERIALS AND METHODS

Strains, media, and culture conditions. *C. difficile* VPI 10463, which was obtained from T. D. Wilkins (Virginia Polytechnic Institute and State University, Blacksburg, Va.), was the major strain used in this study. Four other isolates (strains 707, 717, 4217, and 4326) of *C. difficile* were clinical isolates that were provided by S. Allen (Indiana University School of Medicine, Indianapolis, Ind.). All strains were maintained in chopped meat broth and grown under anaerobic conditions in thioglycolate broth at 37°C. Strains were classified as either toxigenic (Tox^+) or nontoxigenic (Tox^-) by the demonstration of toxins in the culture medium.

Preparation and assays of toxins. Toxins used in the study were obtained from cultures of *C. difficile* VPI 10463, a strain known to be toxigenic (31). Brain heart infusion broth in which *C. difficile* had been propagated for 96 h was centrifuged at 3,000 × *g* for 20 min. The resulting cell-free medium was filtered through a 0.45-μm-pore-size membrane filter (31). The presence of the enterotoxin was determined by fluid accumulation in rabbit ileal loop assays (31). The cytotoxin was detected by using human embryonic intestinal (HEI) cells or Chinese hamster ovary cells that had been grown in a microtiter cell culture plate. Tenfold serial dilutions of the culture filtrate were incubated for 24 h at 37°C with 2×10^5 cells. Cells were then washed, stained with crystal violet, and examined microscopically. One unit of cytotoxin activity is defined as the highest dilution of the

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culture filtrate causing morphological changes in 50% of the cells.

Radiolabeling of *C. difficile* for phagocytosis assays. For the preparation of radiolabeled bacteria, a 0.3-ml sample of *C. difficile* that had been grown in thioglycolate broth was inoculated into 30 ml of brain heart infusion broth containing 5 μCi of [^{3}H]uridine per ml. After growth overnight, the bacteria were harvested by centrifugation at 3,000 × *g* for 20 min. Cells were washed twice with phosphate-buffered saline (PBS) and then suspended in PBS-G (PBS containing 6.88 mM glucose) to yield a suspension of approximately 10^9 bacteria per ml as determined spectrophotometrically.

Preparation of anti-*C. difficile* serum. New Zealand rabbits were injected intramuscularly with 0.5 mg of heat-killed *C. difficile* (strain VPI 10463) in complete Freund adjuvant. Subsequent injections were given in incomplete Freund adjuvant on a weekly basis for 4 weeks. Serum was collected and was determined to contain antibodies to *C. difficile* by enzyme-linked immunosorbent assay with *C. difficile* as the test antigen.

Opsonization. Normal human serum or anti-*C. difficile* serum was used to opsonize suspensions of *C. difficile*. A 1-ml sample of bacteria containing 10^9 bacteria was mixed in a polypropylene tube with 0.2 ml of the opsonin for 30 min at 37°C with shaking on a rotary shaker (10 rpm). The cells were centrifuged at 2,000 × *g* for 15 min and then suspended in 1 ml of Earle balanced salt solution containing 6.88 mM glucose (EBSS-G).

Preparation of PMNs. Fresh human whole peripheral blood was obtained from healthy adult donors who gave their informed consent. Heparinized venous blood (100 ml) was mixed with 20 ml of 6% dextran in 0.9% NaCl and allowed to sediment for 60 to 90 min at room temperature. The supernatant fluid containing the leukocytes was collected, and the PMNs were separated from other leukocytes by centrifugation through a 10-ml cushion of Histopaque (Sigma Chemical Co., St. Louis, Mo.). The resulting pellet was suspended in cold distilled water to lyse any contaminating erythrocytes. After the addition of NaCl to a final concentration of 0.9%, the cells were pelleted by centrifugation and washed with PBS-G. After centrifugation, the PMNs were suspended in EBSS-G at a final concentration of 10^8 cells per ml. Cell viability was >95% as determined by trypan blue exclusion.

Phagocytosis assay. The phagocytosis assay was based on that described by Verhoef et al. (34). A 0.1-ml sample of a suspension of opsonized or nonopsonized bacteria (approximately 10,000 cpm) was added to a polypropylene tube followed by the addition of 0.1 ml of a suspension of PMNs. A control tube consisting of bacteria and buffer was also prepared. The tubes were then placed on a rotary shaker and mixed at 10 rpm for 30 min at 37°C. The reaction was terminated by the addition of 3 ml of PBS-G. Radiolabeled bacteria that had associated with PMNs were recovered by differential centrifugation at 160 × *g* for 5 min. The phagocytic cell pellet was washed three times with PBS-G, and the final PMN pellet was used to determine the amount of radioactivity by liquid scintillation counting. All values were corrected for the amount of unassociated bacteria that might have been pelleted by centrifugation by subtracting the amount of radioactivity that was present in the pellet of the control tube containing bacteria and buffer. Typically, this value was 60 cpm, which represented about 0.6% of the input radioactivity. The extent of phagocytosis of *C. difficile* at any sampling time was calculated by the following formula: percentage of phagocytosis = (counts per minute in PMN cell pellet/total input counts per minute) × 100. All assays were done in triplicate and were repeated on separate days with phagocytes from different human donors.

TABLE 1. Factors influencing phagocytosis of *C. difficile*[a]

Environment	Opsonin	% Phagocytosis[b]
Aerobic	None	4.3 ± 1.4
	Antiserum	45.1 ± 2.5
	HI antiserum	42.5 ± 1.3
	Normal serum	16.4 ± 1.8
	HI normal serum	3.5 ± 0.6
Anaerobic	None	4.2 ± 1.6
	Antiserum	52.5 ± 3.6

[a] *C. difficile* was incubated with buffer, 10% anti-*C. difficile* serum, or 10% normal human serum for 30 min at 37°C. In some cases, sera were heat inactivated (HI) at 56°C for 30 min before use in opsonization. PMNs and bacteria were allowed to equilibrate under either aerobic or anaerobic conditions before the initiation of the phagocytosis assays. Ratio of bacteria to PMNs in the assays was 10:1.

[b] The values represent the mean ± the standard deviation.

Toxin treatment of PMNs. Various dilutions of the toxin preparation were made in PBS-G. PMNs (10^8 cells) were pelleted by centrifugation and suspended in 1 ml of the diluted toxin preparation. As controls, PMNs were resuspended in PBS-G alone. After incubation at 37°C for the indicated time intervals, the PMNs were recovered by centrifugation at 200 × *g* for 5 min. Cells were washed with PBS-G twice and then suspended in EBSS-G at a final concentration of 10^8 cells per ml before phagocytic activity was measured.

Anaerobic conditions and preparation of materials for anaerobic experiments. All anaerobic procedures were conducted in an anaerobic chamber (model 1024; Forma Scientific) that was filled and purged with an anaerobic gas mixture (10% CO_2, 85% nitrogen, 5% hydrogen). The chamber contained a catalyst (palladium-coated alumina) to remove residual oxygen. All materials used in the anaerobic chamber were prereduced by incubation in the chamber for 24 h before use. Suspensions of phagocytic cells and bacteria were allowed to equilibrate in the anaerobic chamber before the phagocytosis assays were performed.

RESULTS

Phagocytosis of *C. difficile*. Phagocytosis of *C. difficile* (strain VPI 10463) by human PMNs depended on opsonization of the bacteria (Table 1). Only 4.3% of the nonopsonized bacteria were associated with PMNs. In contrast, *C. difficile* that had been treated with either anti-*C. difficile* serum or normal human serum was quite susceptible to phagocytosis. The vast majority of opsonizing activity present in the anti-*C. difficile* serum was due to antibody since heat treatment of this serum removed very little, if any, of the opsonizing activity. On the other hand, the opsonizing activity of the normal human serum was heat labile and was presumably complement. Similar results were obtained when normal rabbit serum was used instead of normal human serum (data not shown).

Since *C. difficile* is an anaerobic microorganism that inhabits an anaerobic environment, the effect of anaerobiosis on phagocytosis of *C. difficile* was investigated. The results of phagocytosis assays conducted under anaerobic conditions were similar to those obtained when the assays were conducted under aerobic conditions (Table 1). The slight

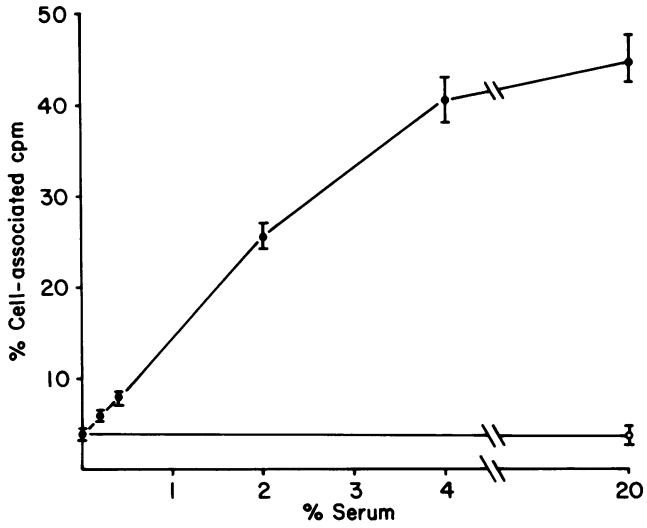

FIG. 1. Effect of various concentrations of anti-*C. difficile* serum on phagocytosis of *C. difficile*. Radiolabeled *C. difficile* cells were incubated with the indicated concentrations of heat-treated anti-*C. difficile* serum (●) or heat-treated normal human serum (○) at 37°C for 30 min before use in the phagocytosis assay. Assays were conducted with a ratio of bacteria to PMNs of 10:1. Bars show standard deviation.

difference in the extent of phagocytosis under anaerobic conditions and under aerobic conditions was determined by the Student *t* test to be insignificant at the 5% significance level. Since anaerobiosis had no effect on the extent of phagocytosis, all subsequent assays for phagocytosis were conducted aerobically.

The degree of phagocytosis of *C. difficile* also depended on the concentration of the opsonin used to treat the bacteria. An increase in the concentration of anti-*C. difficile* serum from 0.2 to 4% resulted in a 10-fold increase in the degree of phagocytosis (Fig. 1). The increase in the extent of phagocytosis was linear up to a serum concentration of 4%, beyond which value no significant increase in phagocytosis was observed. At a concentration of 20%, normal rabbit serum that was heat treated had no effect on the phagocytic activity of PMNs. A similar relationship between the extent of phagocytosis and the concentration of complement was also observed when normal human serum served as the opsonin (data not shown).

Finally, phagocytosis of *C. difficile* that had been opsonized with normal human serum depended on the ratio of bacteria to PMNs (Table 2). A change in the ratio of bacteria per PMN from 5:1 to 10:1 resulted in an increase in the percentage of input bacteria that associated with PMNs. At a ratio of 20:1, PMNs ingested a smaller percentage of the input bacteria as compared with the percentage ingested

TABLE 2. Effect of various bacteria/PMN ratios on phagocytosis[a]

Ratio of bacteria to PMNs	Cell-associated ^{3}H radioactivity (cpm)	% Phagocytosis
5:1	303	10.4 ± 2.5
10:1	928	16.6 ± 1.8
20:1	1,344	10.1 ± 1.6

[a] Radiolabeled *C. difficile* that had been opsonized with 10% normal human serum was incubated with PMNs at the indicated ratios. The results are the average of two separate experiments ± the standard deviation.

TABLE 3. Phagocytosis of toxigenic and nontoxigenic strains of *C. difficile*[a]

Isolate	Phenotype	Opsonized	% Phagocytosis[b]
10463	Tox$^+$	−	4.3 ± 1.4
		+	16.4 ± 1.8
717	Tox$^+$	−	6.3 ± 0.5
		+	14.4 ± 0.2
707	Tox$^-$	−	4.6 ± 1.7
		+	23.1 ± 1.4
4217	Tox$^-$	−	8.0 ± 0.9
		+	30.2 ± 6.5
4326	Tox$^-$	−	4.4 ± 1.6
		+	26.0 ± 1.2

[a] Various strains of *C. difficile* that had been classified as either positive or negative for toxin production was treated with normal human serum (opsonized, +) at a final concentration of 10% or with buffer (opsonized, −) and then used in phagocytosis assays.

[b] The values represent the mean ± the standard deviation.

when a 10:1 ratio was used. However, at a 20:1 ratio, no additional bacteria were able to associate with PMNs. Since phagocytosis assays employing a ratio of bacteria to PMN of 10:1 yielded the greatest percentage of phagocytosis, this ratio was used in all further experiments.

Phagocytosis of nontoxigenic strains of *C. difficile*. Since the nonopsonized, toxigenic strain (VPI 10463) of *C. difficile* was not ingested to any great extent by PMNs (Table 1), we examined the possibility that the antiphagocytic property of *C. difficile* could be due to its ability to produce the cytotoxin and the enterotoxin. To this end, several isolates of *C. difficile* that had been classified as either Tox$^+$ or Tox$^-$ were used in the phagocytosis assays (Table 3). All nonopsonized isolates of *C. difficile* were poorly ingested by PMNs; values for phagocytosis ranged from 4.3 to 8.0%. No significant differences could be detected in the degrees of phagocytosis of nonopsonized Tox$^+$ and Tox$^-$ strains. However, when the isolates were opsonized with normal human serum before the phagocytosis assay, a noticeable difference in the degree of phagocytosis between toxigenic and nontoxigenic strains was observed. All the nontoxigenic strains were more susceptible to phagocytosis than were the toxigenic strains. The sensitivity to phagocytosis of the Tox$^-$ strains was 50 to 100% greater than that of Tox$^+$ strains.

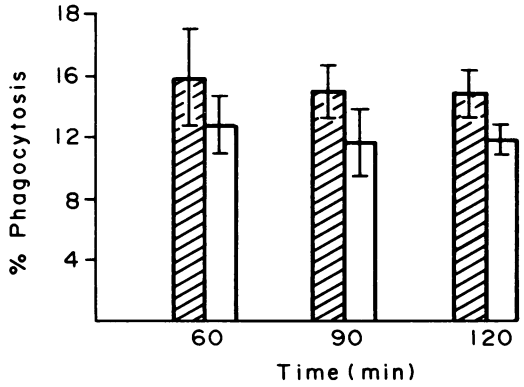

FIG. 2. Effect on phagocytosis of pretreatment of PMNs with *C. difficile* toxins. PMNs were incubated with 500 units of *C. difficile* toxins at 37°C for the indicated times before being assayed for phagocytic activity. Control PMNs were incubated in buffer. *C. difficile* that had been opsonized with 10% normal human serum was used in the phagocytosis assays. Symbols: ▨, treated; □, control. Bars show standard deviation.

TABLE 4. Effect of *C. difficile* toxins on viability of PMNs[a]

Toxin units	% Viability
0	98
100	95
1,000	97
10,000	96

[a] PMNs were incubated at 37°C for 18 h with the indicated amounts of *C. difficile* toxin. Viability was determined by the percentage of cells that excluded trypan blue.

Effect of toxins of *C. difficile* on phagocytic activity of PMNs. Since Tox^+ strains of *C. difficile* were less susceptible to phagocytosis than Tox^- strains were, we examined the possibility that the clostridial toxins could be damaging the PMNs, thereby reducing their ability to ingest bacteria. Therefore, PMNs were pretreated with diluted culture filtrates containing 500 units of cytotoxin for various periods of time. As a control, another set of PMNs were incubated in buffer. After washing, the phagocytic activity of the PMNs was determined by using a toxigenic strain of *C. difficile* that had been opsonized with normal human serum. The results (Fig. 2) demonstrate that pretreatment of PMNs with clostridial toxins for as long as 2 h did not have any inhibitory effect on phagocytosis. Rather, the toxins appeared to have a slight stimulatory effect on the phagocytic activity of PMNs. However, this stimulatory effect was not observed when *C. difficile* opsonized with anti-*C. difficile* serum was used in the phagocytosis assays (data not shown). To ascertain that the toxin preparation was active at the time of treatment, we also used samples of the toxins to treat HEI cells. In all cases, the toxins were cytotoxic to HEI cells. Increasing either the length of time of treatment of PMNs with toxins to 4 h or the concentration of toxin to 50,000 units did not have any adverse effect on the phagocytic activity of the PMNs (data not shown).

Effect of toxins on PMN viability. Since the phagocytic activity of PMNs declines greatly 6 h after their isolation from venous blood, the effects of long-term exposure of PMNs to the clostridial toxins could not be investigated in this manner. Instead, the effect of the toxins on the viability of PMNs was determined by trypan blue exclusion. For comparative purposes, HEI cells were treated with the same dilutions of the toxins under identical conditions. Cells were stained and examined microscopically 1, 2, 3, and 18 h after incubation with the toxins.

PMNs that had been treated with these concentrations of toxin for as long as 18 h were still viable (Table 4). After 18 h in the presence of 10,000 units of toxin, 96% of the PMNs excluded trypan blue. In comparison, 98% of PMNs incubated in buffer were viable after 18 h. Furthermore, no difference in the morphology of PMNs incubated with toxins or with buffer was evident at any of the sampling times.

Figure 3 illustrates the time course of the effects of the same concentrations of the toxin on HEI cells. Approximately 100% of the cells that had been treated with 1,000 or 10,000 units of toxin exhibited morphological changes within 2 h of treatment. After 3 h of treatment, all the HEI cells treated with 100 units of toxin were morphologically altered. HEI cells that had been incubated with either 10 units or 1 unit of toxin demonstrated significant cytotoxicity after 18 h of incubation.

In other experiments not shown here, the cytotoxin activity of the culture filtrate was neutralized by antiserum to culture filtrates of *C. difficile* (a kind gift of T. D. Wilkins). In addition, incubation of the cytotoxin with PMNs did not inactivate the cytotoxin since the cytotoxin that had been recovered from the mixture was still active against HEI cells.

DISCUSSION

A wide variety of bacterial toxins are inhibitory or cytotoxic to PMNs. Staphylococcal alpha-toxin can impair the ability of PMNs to ingest and kill opsonized *Staphylococcus aureus* (16). The pneumolysin produced by *Streptococcus pneumoniae* inhibits chemotaxis of phagocytes and can, at high concentrations, lyse PMNs (17). The alpha-hemolysin of *Escherichia coli* prematurely activates PMNs, thereby preventing phagocytosis of bacteria (6). The adenylate cyclase of *Bordetella pertussis* has been shown to be inhibitory to PMNs (9) and to the cytotoxic activity of natural killer cells (E. L. Hewlett, D. L. Smith, G. A. Myers, R. D. Pearson, and H. D. Kay, Clin. Res. **31**:365A, 1983). Since colitis as a result of infection by *C. difficile* is characterized by large numbers of PMNs infiltrating the mucosal layers of the intestine, we investigated the possibility that the cytotoxic properties of the *C. difficile* toxins inhibit, in some manner, the interaction of PMNs with *C. difficile*. Therefore, the parameters of phagocytosis of *C. difficile* by human PMNs and the effect of the toxins of this bacteria on the activity of PMNs were investigated.

All the isolates of *C. difficile* that were tested proved to be very resistant to phagocytosis. To achieve optimal phagocytosis of *C. difficile*, we had to treat the bacteria with opsonins. Either complement or antibodies to *C. difficile* were effective opsonins, and the degree of phagocytosis depended, in part, on the concentration of the opsonin. However, in contrast to other bacteria such as *Bacteroides* spp. (5, 33), the opsonizing properties of complement and of antibodies were not even additive, much less synergistic. Furthermore, phagocytosis of *C. difficile* was independent of aerobiosis. This result was expected since phagocytosis and killing of other anaerobic bacteria occur equally well under both anaerobic and aerobic conditions (18, 22).

The fact that *C. difficile* requires opsonization for phagocytosis to occur to any great extent indicates the presence of an antiphagocytic determinant on the bacteria. The identity of the antiphagocytic determinant is unknown, but it is neither the enterotoxin nor the cytotoxin since nontoxigenic strains of *C. difficile* are resistant to phagocytosis. Preliminary data indicate that treatment of *C. difficile* with pronase

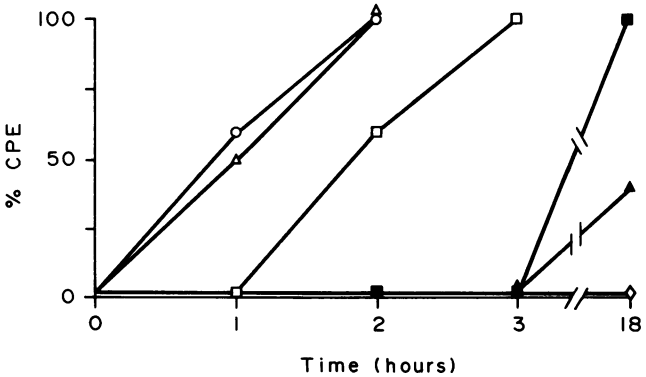

FIG. 3. Time course of treatment of HEI cells with clostridial toxins. Human intestinal cells were incubated with 10,000 units (○), 1,000 units (△), 100 units (□), 10 units (■), 1 unit (▲), or 0 units (◇) of toxin at 37°C for the indicated times. The cells were examined for cytopathic effect (CPE) by staining with crystal violet.

or with 10 mM EDTA, a procedure that removes cell surface carbohydrates (24), had no effect on the extent of phagocytosis. It is conceivable that a polysaccharide capsule may be the antiphagocytic determinant. *Bacteroides fragilis*, among other bacteria, contains a polysaccharide capsule that is antiphagocytic (18). Although it is unclear whether *C. difficile*has a capsule, other members of the genus *Clostridium*, for example, *C. perfringens*, possess a capsule.

Although all nonopsonized isolates of *C. difficile* associated poorly with PMNs, PMNs ingested opsonized *C. difficile* efficiently. Of interest was the observation that the nontoxigenic isolates of *C. difficile* were ingested more efficiently than were the toxigenic isolates of *C. difficile*. Two reasons could exist for the increased sensitivity of the Tox^- strains to phagocytosis. The first is that the toxins of the Tox^+ strains of *C. difficile* were inhibiting the phagocytic activity of PMNs, or possibly killing PMNs. Surprisingly, pretreatment of PMNs with high titers of the toxins of *C. difficile* had no effect on phagocytosis. Remarkably, even after treatment of PMNs with the toxins for as long as 18 h, no evidence of any response to the toxins could be obtained. In fact, after treatment of PMNs with 10,000 units of the toxins, no loss of viability could be demonstrated by the trypan blue exclusion method. This lack of response by PMNs is in contrast to that by the HEI cell line in which approximately 100% of the cells demonstrated a cytopathic response within 2 h of treatment with 10,000 units of the toxin. Moreover, 50% of the HEI cells treated with 1/10,000th of the amount of toxin used to treat PMNs showed changes in morphology after 18 h of treatment. Thus, the greater resistance of opsonized Tox^+ strains of *C. difficile* to phagocytosis as compared with opsonized Tox^- strains was not due to the elaboration and subsequent action of the enterotoxin and cytotoxins. An alternative explanation is that, for some reason, nontoxigenic strains of *C. difficile* are more efficient at fixing complement than are the toxigenic strains. Increased fixation of complement would result in greater degrees of phagocytosis. The fixation of complement by the various isolates of *C. difficile* is currently under investigation.

The results demonstrating that *C. difficile* toxins have no effect on phagocytosis are in contrast to those of Gemmell (15), who reported that purified cytotoxin inhibited the phagocytic activity of PMNs. There are no obvious reasons to explain these discrepancies. One possibility is that different strains of *C. difficile* were used in these two studies. Conceivably, the ability to inhibit PMN function could be strain specific.

The *C. difficile* toxins have been shown to be cytotoxic to a variety of eucaryotic cells, such as Chinese hamster ovary cells (11), human lung fibroblast cells (13, 14), and HEI cells (this study). To our knowledge, no cell line has been reported to be resistant to the effects of the cytotoxin. The lack of effect of the cytotoxin on human PMNs was repeatable with PMNs from the same donor. Moreover, similar results were obtained with PMNs from various human donors.

The reason(s) for this lack of response of PMNs to the cytotoxin is not known, but it is not due to inactivation of the toxin by the release of enzymes from the PMNs since active cytotoxin was recovered after incubation with PMNs for 2 h. Although several biochemical events, such as decreased synthesis of DNA, RNA, and proteins (13, 26) and increased levels of cyclic GMP (35), have been reported in cells treated with the cytotoxin, it is unclear which of these, if any, is the primary target of the cytotoxin. Florin and Thelestan (14) demonstrated that, after the interaction of the cytotoxin with the cell surfaces, the cytotoxin appears to be internalized by endocytosis. Thus, it is possible that PMNs lack a receptor on the plasma membrane for the cytotoxin. Alternatively, it is possible that the cytotoxin binds to the membrane of PMNs and is internalized, but is inactivated once inside the phagocyte. Such inactivation should prevent any development of cytopathology. Experiments to test these possibilities are in progress.

In summary, *C. difficile* appears to have an antiphagocytic determinant, and the toxins of *C. difficile* do not have any effect on the phagocytic ability of PMNs. The persistence of *C. difficile* in the inflammatory exudate that is present in antibiotic-associated colitis does not appear to be a result of the action of the cytotoxin on PMNs, but rather may be attributable, in part, to the antiphagocytic properties of *C. difficile*.

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