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EFFECTS OF CLINDAMYCIN ON ADHERENCE OF CLOSTRIDIUM DIFFICILE TO HUMAN EMBRYONIC INTESTINAL CELLS

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An in vitro assay system, consisting of monolayers of human embryonic intestinal cells (HEI) and Clostridium difficile, was used to observe cell surface and cytoplasmic interactions. Microorganism test conditions include toxin B positive (#938) and toxin B negative (#789) strains, each harvested from standard sheep blood agar (SBA), casamino acids agar (CAS), and CAS containing subinhibitory concentrations of clindamycin (CAS-CL). Effects of toxin B were seen when organisms were harvested from SBA. Adherence and internalization were observed with organisms harvested from CAS and CAS-CL. Evidence of adherence and/or movement into HEI cells was observed at 15, 30, 60, and 90 min time periods using the in vitro internalization assay, conventional scanning electron microscopy, transmission electron microscopy, and field emission scanning electron microscopy of freeze fractured preparations. Quantitative organism determinations, and the observed activity of microorganisms harvested from CAS and CAS-CL, revealed their capability to adhere to and/or enter HEI cells. The toxin B producer, 938, harvested from CAS-CL, entered the cytoplasm and remained intact within it.

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PREFACE

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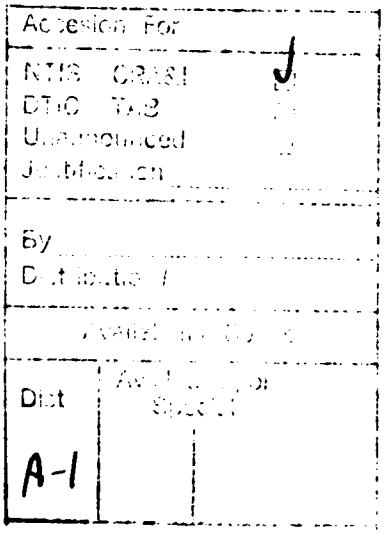

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1. INTRODUCTION

Clostridium difficile, the etiologic agent of pseudomembranous colitis, unpredictably colonizes the bowels of certain hospitalized patients following the administration of antibiotics or during the course of a hospital stay.[1] Enterotoxins produced by this microorganism are capable of damaging the bowel mucosa[2] but, heretofore, it has not been determined whether, (as with microorganisms such as Escherichia coli) adhesive factors help this microorganism colonize and proliferate prior to toxin production.[3,4,5,6,7]

Previous studies using the hamster model clarified some assumptions about proper conditions for colonization. In the hamster, diet, colonization, and competition for colonization sites, were shown to affect the outcome of an experimentally induced infection. More specifically, administration of a high percentage of protein in the diet, or total food deprivation for several days, hampered the animal's ability to resist establishment of C. difficile cecitis in the presence or absence of antibiotics.[8] In addition, when antibiotics were administered during the course of a high percentage protein diet, experimental lifespans were shortened compared to the administration of antibiotics or increased protein alone. Evidence suggests a synergistic action of diet with antibiotics.[8,9]

Indirectly, a colonization mechanism has been elucidated in the following experiments. The presence of a nonpathogenic strain of C. difficile in the bowel prior to challenge with a pathogenic strain, protected the bowel from colonization.[10,11,12,13] It was shown that this protection was not immunologic but resulted from exclusion of the pathogenic strain from the mucosa.[14] Although increased survival time of cefoxitin-treated hamsters was observed when prior treatment with a noncytotoxigenic strain was employed, no protection was seen when cytotoxigenic and noncytotoxigenic strains were administered simultaneously. These observations show that attachment sites were blocked by nonpathogenic variants.[8] Colonization appears to be site dependent and may be influenced by the selective production of cell wall components resulting from microbial consumption of available nutrients.[15,16]

This in vitro study notes the effects of a high percentage of protein in the growth media, and exposure to clindamycin, on the behavior of C. difficile on HEI cells. The role of selected nutrients and clindamycin in the predisposition of C. difficile to attach to cell surfaces, enter, the survive within HEI cells is shown. A combination of selected nutrients and the presence of clindamycin, in addition to the microenvironment, may be critical to the development of pseudomembranous colitis. As no

valid explanation for relapse has yet been postulated, one explanation which must now be considered is survival within non-phagocytic cells in response to selected microbial nutrients and clindamycin.

2. MATERIALS AND METHODS

2.1 Organisms used.

Clostridium difficile strains, isolated from patient population at the Medical College of Virginia, Virginia Commonwealth University, include *C. difficile* strain 938 from a symptomatic adult, *C. difficile* 789, isolated from an asymptomatic child and N6, isolated from an asymptomatic infant. Assays for the production of toxin B showed that 938 and N6 caused rounding of HEI cells in 24 hours and this rounding was specifically inhibited by *C. difficile* antitoxin. Strain 789 did not cause rounding of HEI cells after 48 hours.

Typical colonies chosen for identification were irregular, convex, gray, had a matt surface and rough edge, and fluoresced under a 366 nm lamp. Gram-stains of the microorganisms from blood agar revealed gram-positive rods with terminal/subterminal spores. Gas-liquid chromatography of chopped meat broth culture produced short chain fatty acids, acetic, propionic, iso-butyric, butyric, iso-valeric, valeric, isocaproic, lactic, succinic, and small amounts of caproic. The microorganisms were lipase and lecithinase negative, did not digest meat, and had a musty odor. Toxin B, from the cytotoxigenic strains, could be neutralized with *C. difficile* antitoxin prepared against a partially purified culture filtrate containing toxin B and organism cell wall components. This antitoxin was prepared in the goat and obtained from Dr. Tracey Wilkins, Virginia Polytechnic Institute and State University. Toxin B producers were defined as those producing rounding of HEI cells in 4-48 hours. All *C. difficile* isolates had minimum inhibitory concentrations of 1.0 µg/mL when tested against clindamycin by agar dilution susceptibility.[22]

2.2 Organism preparation for adherence assay.

Microorganisms were grown on CAS consisting of 1% casamino acids (Difco, Detroit, Michigan), 2% agar (Difco) and 0.5% sheep blood (Flow Labs, McLean, Virginia). To expose the organisms to subinhibitory concentrations of clindamycin, 0.05 µg/mL clindamycin was added to the CAS media. Organisms were harvested at 48 hr in Eagle's minimal essential media (MEM, Flow) containing 1% fetal calf serum (GIBCO, Grand Island, New York), pH 5.5, washed once and resuspended in MEM 1% fetal calf serum (FCS). Antitoxin at a 1:10 dilution in 0.05 mL MEM was used in an attempt to block adherence. This mixture was incubated at 37°C in an anaerobe jar with the atmosphere generated by an

anaerobe gas pack (Becton Dickinson Microbiology Systems, BDMS, Cockeysville, Maryland) for 15 minutes. The preparations were centrifuged at 1500 rpm for 5 min and resuspended in MEM with 1% FCS, pH 5.5.

2.3 Tissue cells used.

Human embryonic intestinal cells #CCL6 were obtained from the American Type Culture Collection. They were maintained in HAM's F12 medium (GIBCO), containing 10% FCS (GIBCO) and penicillin/streptomycin, 50 units and 50 µg/mL respectively (Difco).

2.4 Assay for cytotoxigenicity.

Procedures for the determination of cytotoxigenicity were followed as previously described.[7] Briefly, the cytotoxin assay tested supernatant dilutions from each microorganism for rounding of HEI cells from 4-48 hours after inoculation. If rounding was observed, an this rounding was blocked using antitoxin to toxin B, then the organisms were considered cytotoxigenic.

2.5 Adherence assay.

The adherence assay was performed using C. difficile grown on CAS as previously described. Earlier studies measured adherence of seven different isolates under five different conditions, in duplicate.[7] Briefly, microorganisms were inoculated onto cell monolayers, incubated anaerobically on a rotating platform for 15 min at 37°C, washed, stained with Giemsa stain, and read. The percentage of cells (number of cells out of 100 with adherent microorganisms) with microorganisms adhering was calculated. Areas with low background adherence were chosen for these counts.

To determine whether adherence depended on microbe viability, both viable and nonviable microorganisms were used initially. Viable C. difficile were taken from CAS plates incubated anaerobically for 48 hrs. Nonviable microorganisms were taken from blood agar plates stored aerobically for three days. Subcultures from these plates failed to produce growth when plated on SBA and incubated anaerobically at 37°C for 48 hrs. Gram stains confirmed intact organisms.

2.6 Internalization assay.

Adherence assay procedures were followed with several exceptions: assays were carried out in 24 wells plates (BDMS); confluent monolayers were inoculated with approximately 5×10^5 organisms; five wells per test condition were harvested; and incubation was in gas pak pouches (BDMS) generating an anaerobic environment. At 30 min, 60 min, and 90 min, monolayers were

washed to remove loose microorganisms and a 1% solution of Triton X100 was placed on the monolayer and incubated at room temperature for 5 minutes. After 5 min, the microorganisms were harvested, dilutions were made, and microorganisms were subcultured to SBA and incubated anaerobically for 48 hours at which time colony counts were established. To the same 24-well tissue culture plate, in different test wells, clindamycin at 10 ug/mL was added to kill any microorganisms attached to the monolayer surface. After incubation in an anaerobe pouch for 90 minutes, these wells were washed to remove dead organisms and a 1% solution of Triton X100 was added. After 5 minutes, the microorganisms were harvested, diluted, and plated on SBA for subsequent colony counts. To determine the percentage of internalized microorganisms, a ratio of internalized organisms to total cell associated organisms was obtained ([internalized/total cell associated] x 100). Microorganisms 789 and 938, harvested from CAS and CAS-CL, were used.

2.7 Scanning electron microscopy.

The interaction of C. difficile with HEI cells was examined using scanning electron microscopy (SEM). Organisms 938 and 789 were grown on SBA and CAS. Both strains exhibited adherence in the adherence assay system when harvested from CAS, and both were tested in the presence and absence of C. difficile antitoxin. HEI cells were maintained in HAM's F12 medium with 10% FCS. Approximately 3 x 10^3 cells in 1 mL of media were placed in each compartment of a 24 well tissue culture plate (BDMS) with glass coverslips in each well, covered and incubated overnight at 37°C in an atmosphere enriched to 7% CO_2. Preceding the addition of microorganisms, the monolayer was rinsed and the media changed to HAM's F12 with 1% FCS, no antibiotics. Each microorganism, 938 and 789, was suspended in phosphate buffered saline (PBS, Flow Labs) containing 300 µg/mL cysteine (Sigma, St. Louis, Missouri), washed once and resuspended in PBS with cysteine. Approximately 1 x 10^9 microorganisms were placed in each well and allowed to incubate in an anaerobic environment for 30, 60, and 90 minutes, generated by a gas pak in an anaerobic jar. At the end of each incubation period, gluteraldehyde fixative (2% in 0.1 M sodium cacodylate buffer) was added to the cells following removal of 50% of the HAM's F12 media. Following incubation at 37°C for 15 min, this mixture was replaced with 2% gluteraldehyde in 0.1 M cacodylate buffer. Following incubation at 37°C for 15 min, the wells were rinsed with cacodylate buffer at 25°C and stored at 4°C. The round glass coverslips in each well, covered with a monolayer of cells and microorganisms, were dehydrated in alcohol and freon, dried by critical point drying, mounted onto copper supports using silver paste, and coated with gold palladium for examination using scanning microscopy.

Antitoxin was added to the reaction mixture by placing 10 uL into 1 mL of microorganism suspension and incubating anaerobically 5 to 10 minutes prior to their inoculation onto HEI cells. Controls were incubated 90 minutes prior to fixation. HEI cells, HEI cells plus antitoxin, and microorganisms plus antitoxin were used as controls.

2.8 Field emission scanning electron microscopy, cryopreservation, and freeze fracture.

The adherence assay was performed as previously described.[7] Preparations were rinsed in buffer and fixed in 3% gluteraldehyde at room temperature after a 60 minute incubation at 37°C. Cryopreservation (938, CAS) in liquid nitrogen was performed, and etching was used to achieve clarity of the image. Freeze fracture (938, CAS-CL) was performed by freezing the coverslip containing the cell/microorganism layer in liquid nitrogen and manually fracturing the coverslip inside a cold chamber.

2.9 Preparation for transmission electron microscopy.

The adherence assay samples were fixed as for scanning electron microscopy (see above). Following fixation in gluteraldehyde, the coverslips were washed three times in cold cacodylate buffer. This buffer, containing 1% osmium tetroxide, was added and incubated 1 hour at 4°C. The preparations were then washed three times in 50% ethanol, followed by a 10 minute wash in 70% ethanol, followed by three 10 minute washes in 100% ethanol, and three 10 minute washes in propylene oxide (Fisher, USA). A mixture of embedding plastics, 1/4 Polybed A, 1/4 Polybed B, (Polysciences, Inc., Warrington, Pennsylvania) and 1/2 propylene oxide with no catalyst was placed on the coverslips and left overnight or until the propylene oxide evaporated.

2.9.1 Embedding on a monolayer.

In preparation for embedding, tops were broken away from embedding capsules (Polysciences, Inc.) which were than filled with the Polybed mixture containing polymerization catalyst. The capsules were incubated at 60°C for approximately two hours until moderately viscous. A prepared coverslip was then placed face down onto the face of the capsule and together they were turned upside down quickly onto a spot of Polybed on a plastic petri dish lid (BDMS). Following a 48 hour incubation period at 60°C, the plastic petri lids were dipped quickly into liquid nitrogen and four way pressure applied manually to each side of the vial until the vial seemed ready to snap off. Two to three liquid nitrogen treatments were sometimes necessary to accomplish this. The finished preparation contained a fixed monolayer on a flat surface.

2.9.2 Ruthenium red staining.

Organisms N6, 789, and 938 monolayer preparations were stained with ruthenium red (RR) during the fixation process. Coverslips were washed three times in MEM containing 1% FCS plus 0.05% RR. One volume of 3% gluteraldehyde in buffer with 0.05% RR was added to one volume MEM plus 1% FCS and placed in the wells. Following incubation at room temperature for 10 min, the fluid was replaced with 2% gluteraldehyde containing 0.05% RR. This mixture was incubated at 4°C for 10 min, washed three times in cacodylate buffer plus 0.05% RR, then incubated at 4°C for ten min in 1% osmium tetroxide with 0.05% RR. After three washes in cacodylate buffer with 0.05% RR, dehydration was carried out having RR in the 50% ethanol solution only. Coverslips were embedded as described.

2.9.3 Cutting on a monolayer.

Blocks were trimmed and aligned to allow immediate cutting of thin sections from the flat surface of each block. RR staining served as an aid to visualize the breadth of the monolayer while cutting the block. Sections were cut from underside to top side of the monolayer resulting in approximately 60 sections per preparation which were collected on copper grids, stained with filtered uranyl acetate solution (saturated in 50% ethanol) for 1 min, and then by Reynolds lead acetate for 5 min. All sections from each monolayer were viewed and pictures were taken of representative views from all depths of the monolayer. At least one complete block was cut and viewed for each growth condition of each of the three microorganisms.

3. RESULTS

3.1 Cytotoxigenicity assay.

The in vitro assay for the presence of toxin B showed rounding of 100% HEI cells at 24 hours by strain 938. Strain 789, however, caused no rounding of HEI cells after 48 hours.

3.2 Adherence assay.

Adherence assays previously performed on seven different isolates in duplicate, gave an average of cells with microorganisms attached of 60%. The preparative adherence assay performed prior to this set of experiment showed 62% and 67% attachment for 938 and 789 respectively. In preliminary experiments comparing attachment of viable microorganisms to attachment of nonviable microorganisms, only viable microorganisms attached, indicating that an active process was at work in the attachment phenomena.

3.3 Internalization assay.

As seen in Table 1 and Figure 1, the ratio of internal to total cell associated organisms was highest with 938 harvested from CAS-CL at 60 minutes. Substantiation of this data from the in vitro adherence assay was achieved by reproducibility of the in vitro results (data not shown), and electron micrograph results.

3.4 Scanning electron microscopy - Growth on SBA.

Cytotoxigenic effects on HEI cells were observed when 938 was harvested from (SBA) standard growth media. HEI cells became round, lost their cellular structure, and appeared generally unhealthy. In Figure 2, cells have detached from the coverslip, cytoplasmic streaming is apparent, and bleb formation is evident on the cell surface. This cytopathic effect was successfully blocked by C. difficile antitoxin as seen in Figure 3. The noncytotoxigenic strain, 789, when grown on SBA, did not damage the monolayer. Both strains were tested in the presence and absence of antitoxin, which had no adverse effects on the cells. Incubation times for all conditions was 90 minutes.

3.5 Scanning electron microscopy - Growth on CAS.

Microorganisms harvested from CAS showed a different type of interaction at the cell surface. Intimate points of association were observed with 789 at 30 minutes in the presence and absence of antitoxin. As seen in Figure 4, areas of association suggest membrane/cell wall association rather than fibrillar adherence. The toxin B producer, 938, harvested from CAS, damaged the cells by 30 minutes, clung to cellular debri and intimately associated with portions of the HEI membrane, as seen in Figure 5.

3.6 Transmission electron microscopy.

The toxin B negative strain, 789, harvested from CAS, showed membrane association with HEI microvilli, seen in Figure 6. This association was not hampered when microorganisms were preincubated with antitoxin. Progressive interaction, Figure 7, resulted in attachment to microvilli, and microorganism cell wall dissolution. With 789 harvested from CAS-CL, cell wall morphology thickened and interaction with microvilli was not observed as depicted in Figure 8.

Figures 9 and 10 represent 938 harvested from CAS. The microorganism entered the cell and once in the cytoplasm, disintegrated within a closed vacuole.

Three strains of C. difficile, 789, 938, and N6, when harvested from CAS-CL, exhibited thickening of the cell wall but did not adhere to microvilli. N6, a toxin B producer, remained intact once it entered the cytoplasm of the HEI cell as shown in Figure 11. Incubation times for transmission EM preparations was 15 minutes.

3.7. Field emission scanning electron microscopy

In Figure 12, the cryopreserved preparation of 938 harvested from CAS, confirmed the intimate interaction seen with conventional scanning EM. In Figure 13, 938 harvested from CAS-CL, confirmed by freeze fracture the intact nature of microorganisms with HEI cells. (Microorganisms shown with arrows).

4. DISCUSSION

Adults develop colitis following the administration of antibiotics, whereas infants harbor C. difficile in the bowel but rarely show signs of disease. In addition, historical data has shown that both intravenous and intramuscular administration of antibiotics are associated with the development of pseudo-membranous colitis, but a direct correlation of disease symptoms to dose does not exit.[17] Indirectly related to antibiotic administrations, predisposing environmental conditions in the bowel have been implicated in the development of pseudomembranous colitis. These include disruptions in the protective layer of the bowel, selected antibiotic administration, changes in health or nutritional status of the host, food deprivation, and a designated diet or change in diet.[13] Changes in the organism, instead of in the environmental conditions in the bowel, have not yet been considered. C. difficile strains differ in their ability to produce cytotoxin, in the presence of cell wall components such as adhesins, in their response to the immediate environment, in the use of selected nutrients, and in their proximity to the bowel mucosa. Organism response to conditions cannot be discounted as contributive to the development of pseudomembranes.

Initial results, presented here, suggest a direct effect of available nutrients (amino acids) and clindamycin on the microorganisms. Entrance into, and survivability with HEI cells occurred with toxin B producer, 938, when harvested from media containing amino acids and clindamycin. Ratios of internalized microorganisms to total cell associated microorganisms were determined. C. difficile 938, harvested from CAS-CL were internalized and survived in high numbers at 60 minutes. When grown on CAS containing no clindamycin, levels of internalization

and survival were not as great. Electron microscopy observations showed an interface confined to an area on the microorganism surface corresponding to a complementary area on the tissue cell surface.

Adhesins have been implicated as an important initiating factor in colonization by enteroxigenic Escherichia coli.[18] In the case of C. difficile, such an adhesin may play a role in disease production, by serving as the initial anchor to the intestinal epithelium. Response of the microorganism to an environmental signal and/or regulatory control, and its role in disease production remain to be determined.[19,20,21]

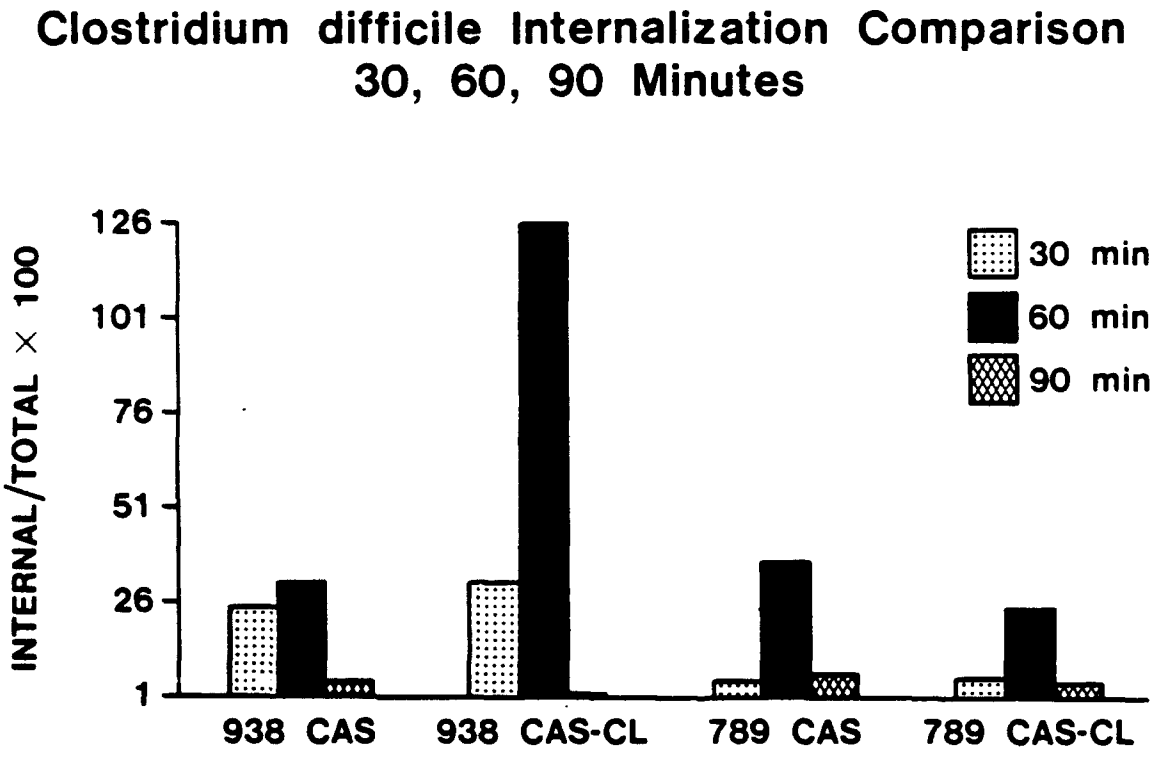

Figure 1. Internalization of Clostridium difficile by human embryonic intestinal cells. Ratio of internalized microorganisms vs. total cell associated microorganisms at 30, 60, and 90 minutes. The greatest ratio is seen with C. difficile 938 harvested from CAS-CL.

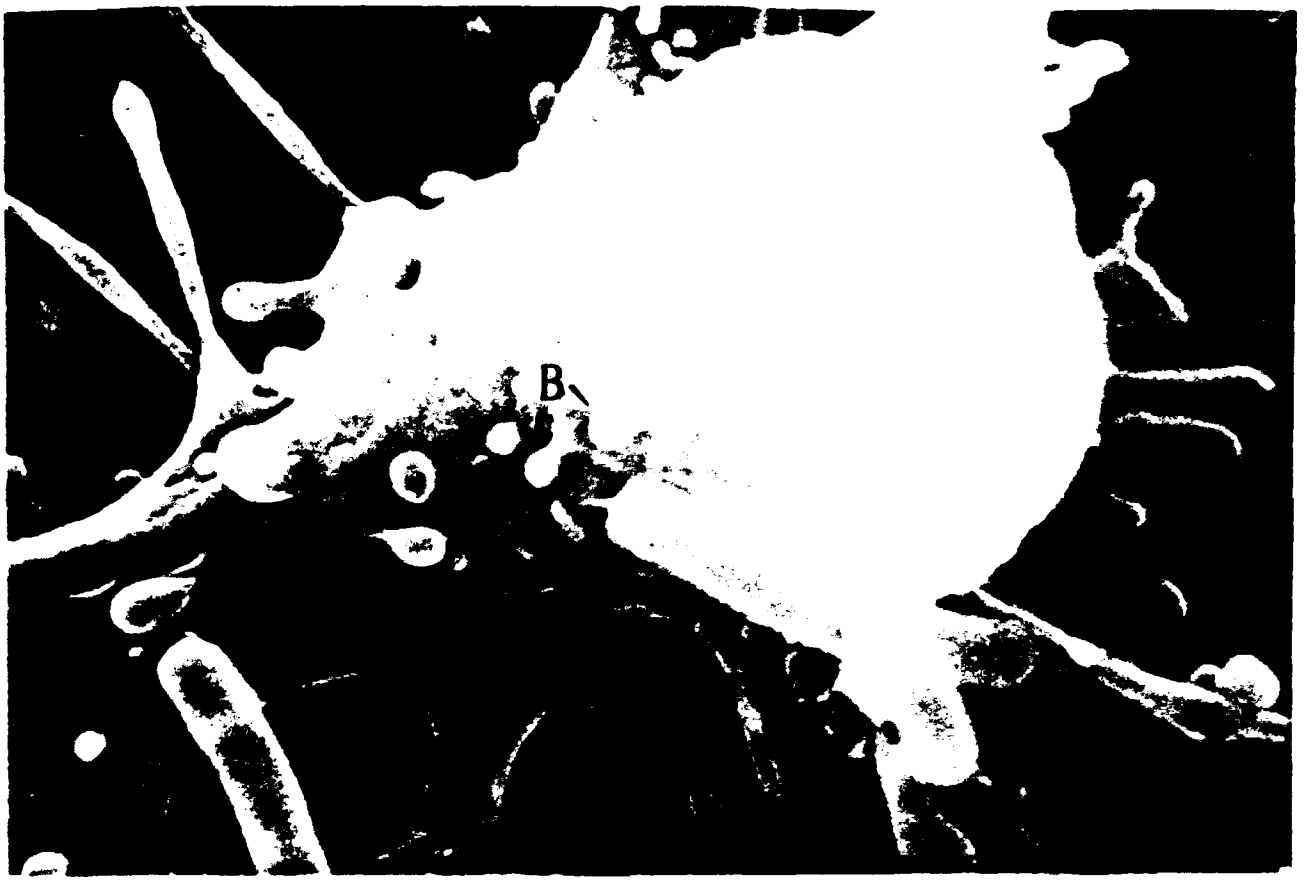

Figure 2. Toxic effects of Clostridium difficile, harvested from sheep blood agar, on human embryonic intestinal cells after 90 minutes. Arrow A points out loss of cell shape represented by cytoplasmic streaming. Arrow B denotes protrusive membrane blebs. Bar = 1 micron.

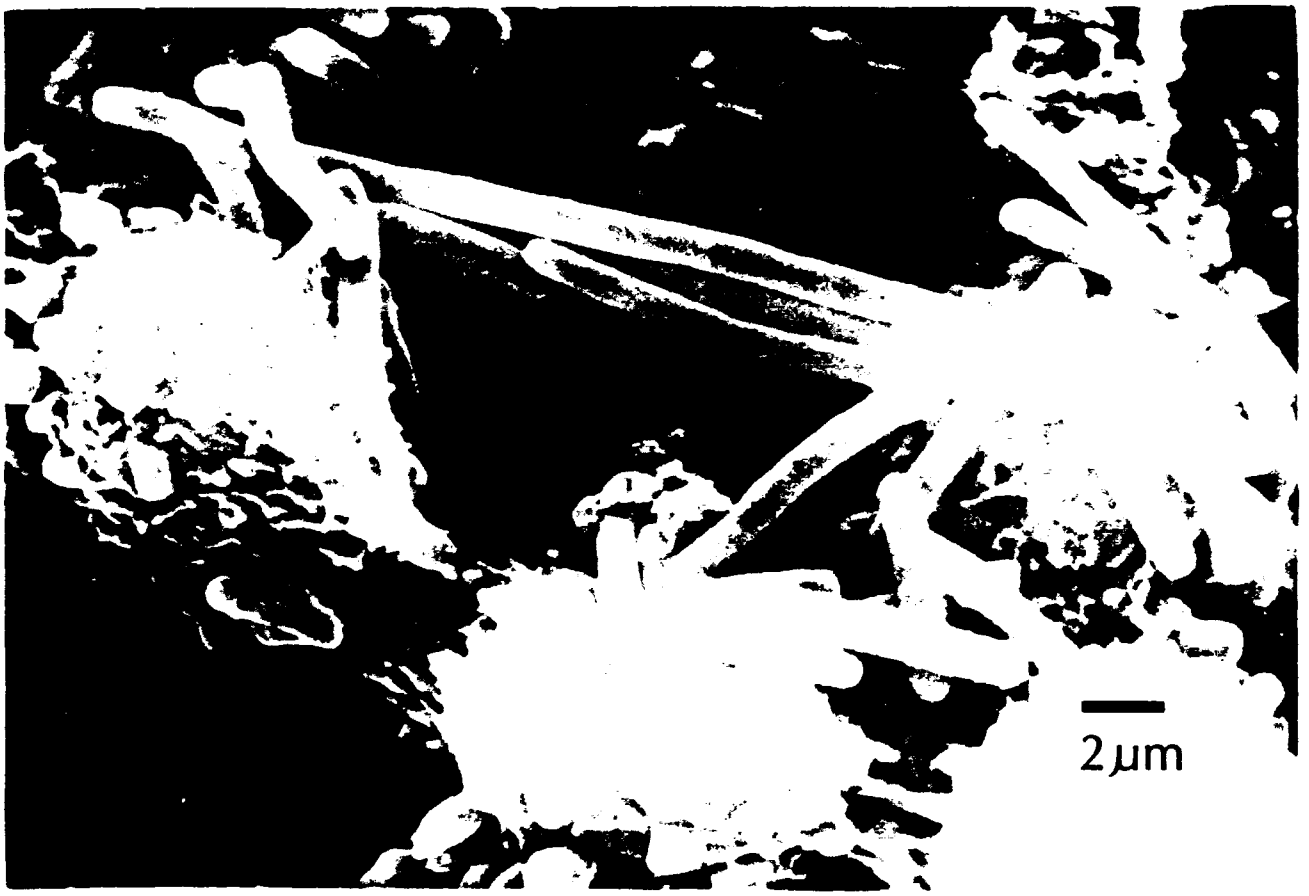

Figure 3. Toxic effects on human embryonic intestinal cells by Clostridium difficile 938 were blocked by Clostridium difficile antitoxin. The organism was grown on sheep blood agar and preincubated with antitoxin prior to a 90 minute incubation with the cells. Bar = 2 microns.

Figure 4. Adherence of Clostridium difficile 789, harvested from casamino acids agar, to human embryonic intestinal cells after 30 minutes. Outer surface association is indicated by arrow. Bar = 0.25 microns.

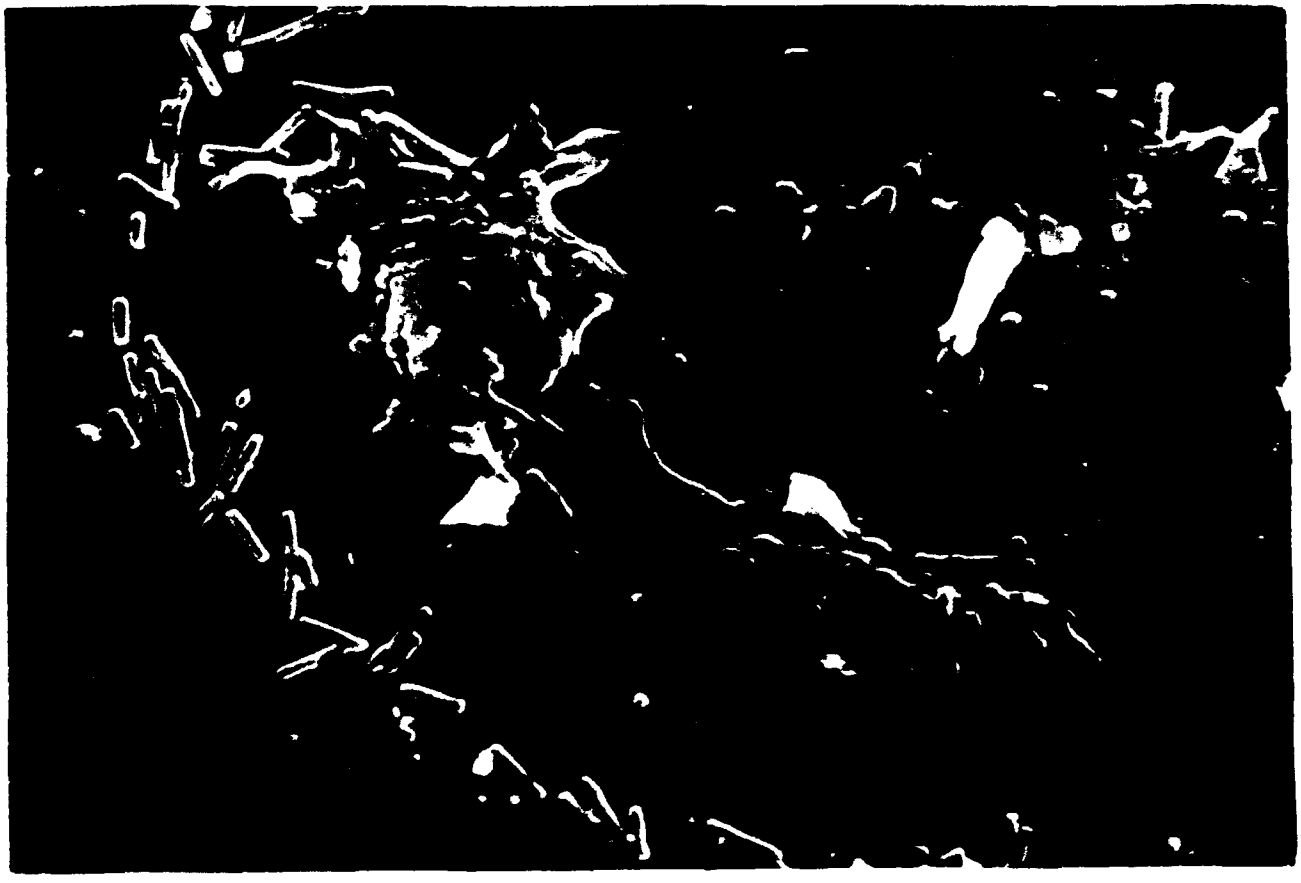

Figure 5. Interaction of Clostridium difficile 938, harvested from casamino acids agar, with human embryonic intestinal cells after 30 minutes. Arrows indicate area of membrane association. Bar = 4 microns.

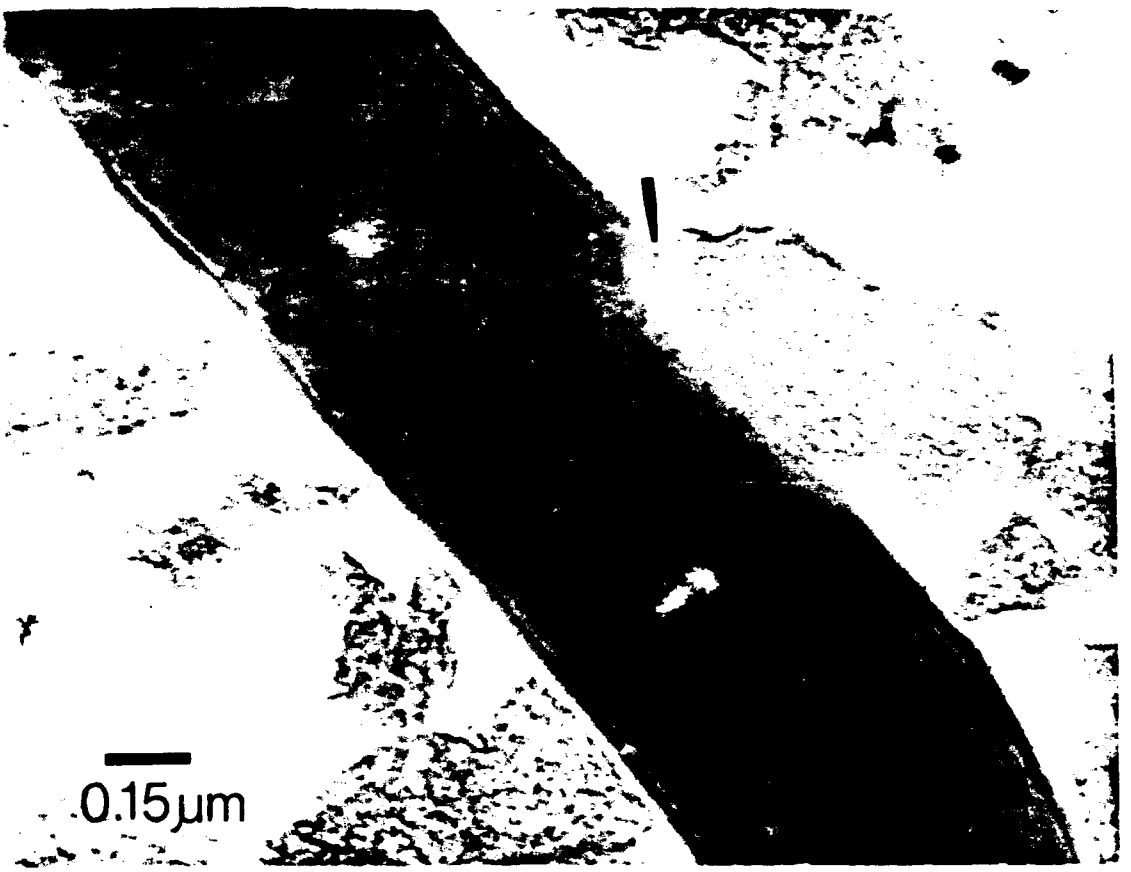

Figure 6. Transmission Electron Micrograph of Clostridium difficile 789, harvested from casamino acids agar, within human embryonic intestinal cell microvilli, after 15 minutes. Arrow indicates apparent fusion of bacterial cell wall and mammalian outer membrane. Bar = 0.15 micron.

Figure 7. TEM of Clostridium difficile 789, harvested from casamino acids agar. Association with human embryonic intestinal cell microvilli and disassociation of microbial outer cell wall integrity is apparent at 15 minutes. Bar = 0.2 micron.

Figure 8. TEM of Clostridium difficile 789, harvested from casamino acids agar containing 0.05 µg/mL clindamycin. The cell wall has a thickened appearance and, although adjacent to human embryonic intestinal cell microvilli, is not intimately associated with it. An electron dense substance can be seen at the periphery of the microbe. Bar = 0.1 micron.

Figure 9. TEM of Clostridium difficile 938, harvested from casamino acids agar, entering a human embryonic intestinal cell at 15 minutes. Bar = 0.2 micron.

Figure 10. TEM of Clostridium difficile 938, harvested from casamino acids agar, following entrance into a human embryonic intestinal cell. Disintegration of the microorganism is apparent after 15 minutes. Bar = 0.2 micron.

Figure 11. TEM of Clostridium difficile N6, harvested from casamino acids agar containing 0.05 µg/mL clindamycin, entering a human embryonic intestinal cell and remaining intact. Bar = 0.2 micron.

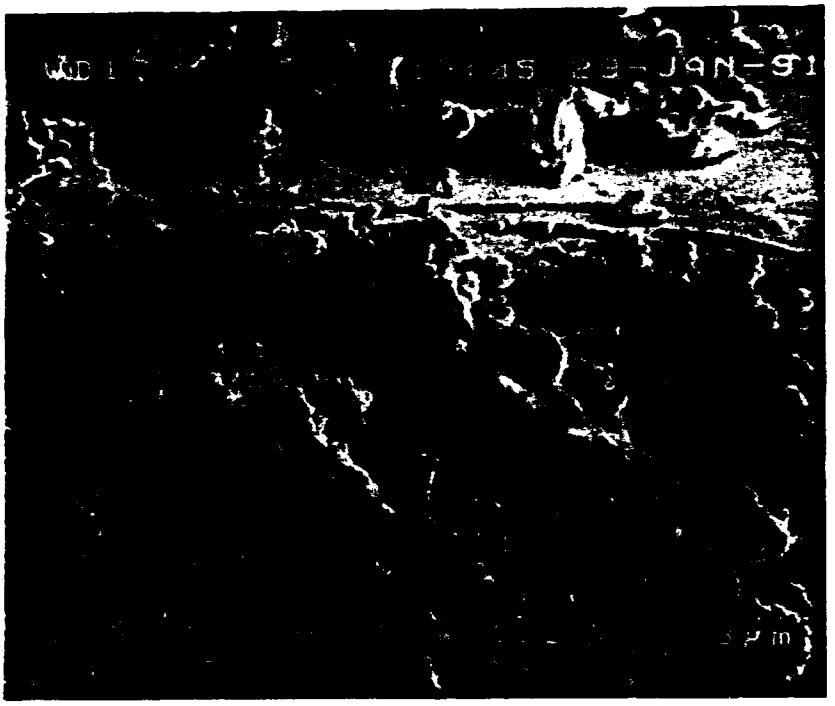

Figure 12. Field emission scanning electron microscopy of Clostridium difficile 938, harvested from casamino acids agar, confirming the intimate association seen by conventional scanning electron microscopy. Interaction was stopped by fixation at 60 minutes and the sample was cryopreserved prior to observation. Bar = 2.73 microns.

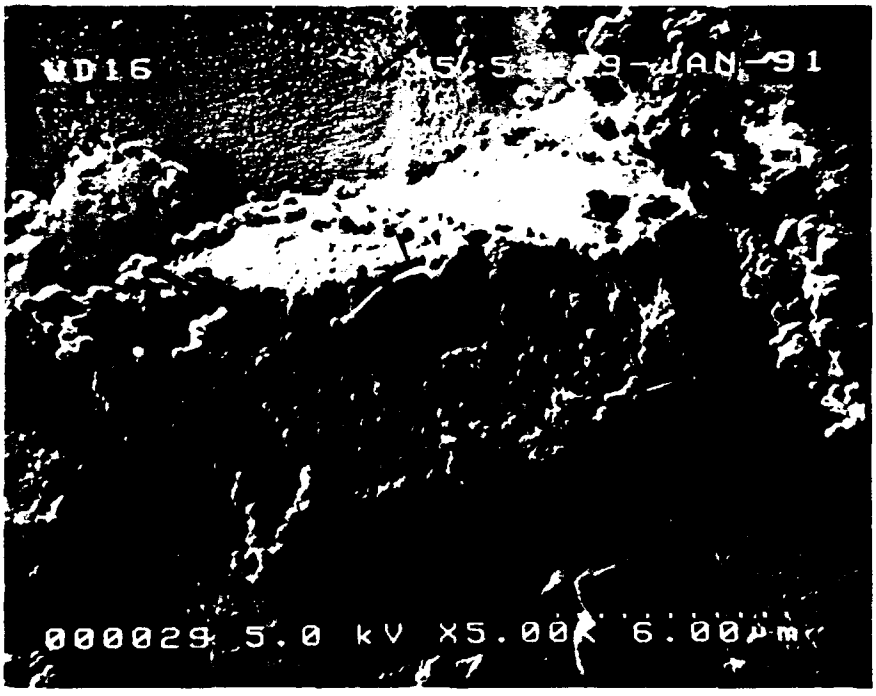

Figure 13. Field emission scanning electron micrograph of a freeze fractured preparation of Clostridium difficile, harvested from casamino acids agar containing 0.05 µg/mL clindamycin, intact and within human embryonic intestinal cells after 60 minutes. Organisms, as shown by the arrows, are numerous and appear unharmed. Bar = 6.0 microns.

Table 1. Numbers of total cell associated *Clostridium difficile* and internalized *Clostridium difficile*. Percentages were obtained by dividing the number of internalized organisms by the number of total cell associated organisms and multiplying by 100.

Organism/ conditions	Total cell-associated organisms CFU/mL	Internalized organisms CFU/mL	Percentage internal/ total x 100
938 CAS			
30 min	5.20×10^4	1.30×10^4	25.0
60 min	5.80×10^4	1.74×10^4	30.0
90 min	1.55×10^5	4.50×10^3	2.9
938 CAS-CL			
30 min	4.00×10^4	1.20×10^4	30.0
60 min	3.50×10^4	4.40×10^4	125.7
90 min	1.00×10^6	4.75×10^3	0.5
738 CAS			
30 min	1.40×10^4	4.80×10^2	3.4
60 min	5.00×10^3	1.75×10^3	35.0
90 min	2.85×10^3	1.30×10^2	4.5
789 CAS-CL			
30 min	2.00×10^4	8.00×10^2	4.0
60 min	7.20×10^3	1.78×10^3	24.7
90 min	1.75×10^4	4.50×10^2	2.6

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