Multiple effects on *Clostridium perfringens* binding, uptake and trafficking to lysosomes by inhibitors of macrophage phagocytosis receptors

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*Clostridium perfringens* is a Gram-positive, anaerobic bacterium that is the most common cause of gas gangrene (clostridial myonecrosis) in humans. *C. perfringens* produces a variety of extracellular toxins that are thought to be the major virulence factors of the organism. However, *C. perfringens* has recently been shown to have the ability to survive in a murine macrophage-like cell line, J774-33, even under aerobic conditions. In J774-33 cells, *C. perfringens* can escape the phagosome and gain access to the cytoplasm. Since the receptor that is used for phagocytosis can determine the fate of an intracellular bacterium, we used a variety of inhibitors of specific receptors to identify those used by J774-33 cells to phagocytose *C. perfringens*. It was found that the scavenger receptor and mannose receptor(s) were involved in the phagocytosis of *C. perfringens*. In the presence of complement, the complement receptor (CR3) was also involved in the binding and/or uptake of *C. perfringens*. Since the receptor inhibition studies indicated that the scavenger receptor played a major role in phagocytosis, *C. perfringens* binding studies were performed with a Chinese hamster ovary (CHO) cell line expressing the mouse SR-A receptor. The cell line expressing the SR-A receptor showed a significant increase in *C. perfringens* binding in comparison to the non-transfected CHO cells. In the absence of opsonizing antibodies, the Fc receptor was not used to phagocytose *C. perfringens*. Forcing the macrophages to use a specific receptor by using combinations of different receptor inhibitors led to only a slight increase in co-localization of intracellular *C. perfringens* with the late endosome-lysosome marker LAMP-1. Carbohydrate analysis of *C. perfringens* strain 13 extracellular polysaccharide confirmed the presence of mannose and negatively charged residues of glucuronic acid, which may provide the moieties that promote binding to the mannose and scavenger receptors, respectively.

**INTRODUCTION**

*Clostridium perfringens* is a ubiquitous, anaerobic spore-forming bacterium, found at high levels in the large intestine, on the skin, and in soil and freshwater sediments (Rood & Cole, 1991). In humans, *C. perfringens* causes anaerobic cellulitis, gas gangrene, enteritis necroticans (Pigbel) and food poisoning (Rood & Cole, 1991). Gas gangrene, or clostridial myonecrosis, is an infection that originates in ischaemic tissues in which the blood supply has been cut off due to trauma or circulatory blockages. Once the infection begins, it rapidly spreads to healthy tissues and, if left untreated, the disease is always fatal due to the release of toxins into the bloodstream resulting in severe shock and cardiac stress (Asmuth *et al.*, 1995; Stevens *et al.*, 1988).

As a species, *C. perfringens* makes 13 different toxins, which are thought to be the major virulence factors of the diseases it causes (Rood, 1998). However, we recently presented evidence that *C. perfringens* can persist inside macrophages, even under aerobic conditions, perhaps by escaping the phagosome and gaining access to the cytoplasm (O’Brien & Melville, 2000). *C. perfringens* was not killed by the cells of a clone, J774-33, of the macrophage-like murine cell line J774A.1 under aerobic or anaerobic conditions, while the non-pathogenic bacterium *Bacillus subtilis* was killed by J774-33 cells under both conditions. *C. perfringens* is also capable of surviving in the presence of mouse peritoneal and bone marrow derived macrophages (D. K. O’Brien & S. B. Melville, unpublished results). Electron microscopic evidence indicated that *C. perfringens* can escape the phagosome of J774-33 cells and both activated and unactivated mouse peritoneal macrophages (O’Brien & Melville, 2000). Therefore, the ability to escape the phagosome and persist in the cytoplasm of macrophages may be important in the earliest stages of a gangrene infection, when bacterial numbers are low and phagocytic cells are present.

One factor that is important in intracellular trafficking...
and/or survival inside macrophages is the receptor used to phagocytose the bacterium. For example, phagocytosis using the Fc and mannose receptors leads to a strong oxidative burst but phagocytosis using the complement receptor alone avoids the oxidative burst (Aderem & Underhill, 1999). The receptor(s) used by macrophages to phagocytose *C. perfringens* has never been identified. Therefore, we used inhibitors of receptor binding to identify the specific receptors used by J774-33 cells to phagocytose *C. perfringens*. We have found that the scavenger, mannose and complement receptor (CR3) were all involved in the phagocytosis process. We have also determined that *C. perfringens* strain 13, the strain used in these studies, produces a polysaccharide capsule that is likely recognized by phagocytosis receptors on the surface of macrophages.

**METHODS**

**Bacterial strains and mammalian cell lines.** The bacterial strains used in this study were *C. perfringens* strain 13 (obtained from D. Duncan, Hershey Foods Technical Center, Hershey, PA, USA) and *B. subtilis* JH642 (obtained from J. Hoch, University of California at San Diego, San Diego, CA, USA). *C. perfringens* was grown in a Coy anaerobic chamber (Coy Laboratory Products) in PGY medium (30 g proteose peptone, 20 g glucose, 10 g yeast extract and 1 g sodium thioglycollate l⁻¹) (Melville et al., 1994). *B. subtilis* was grown in Luria broth (LB) (10 g tryptone, 5 g NaCl and 5 g yeast extract l⁻¹).

J774-33 cells, a highly phagocytic clone of J774A.1 cells, were used in these assays (O’Brien & Melville, 2000). J774-33 cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) with 4.5 g l⁻¹ of both glucose and L-glutamine supplemented with 10 % FBS and 1 % sodium pyruvate, in an atmosphere of 5 % CO₂ at 37 °C. Chinese hamster ovary cells (CHO) and CHO cells transfected with the murine class A-I scavenger receptor (SR-A receptor) were obtained from M. Krieger (Krieger & Herz, 1994). CHO cells were grown in Ham’s F-12 growth medium containing 1 % penicillin/streptomycin, 3 % newborn calf lipoprotein deficient serum, 250 μM mevalonate, 40 μM mevinolin and 3 μg AcLDL ml⁻¹.

**Phagocytosis receptor inhibition assays.** *C. perfringens* bacteria were grown to exponential phase and then washed three times in PBS and added to J774-33 cells grown in 24-well tissue culture plates containing 12 mm round glass coverslips at an m.o.i. of 6–16:1. CHO cells were harvested with PBS containing 10 mM EDTA and 1 % trypsin and seeded into 24-well tissue culture plates containing coverslips and allowed to grow to confluency before infecting with *C. perfringens* at an m.o.i. of 10:1. Inhibitors were added 30 min prior to the addition of the bacteria. The inhibitors were added at a final concentration of 1 mg mannann ml⁻¹, 7 μg fucoidin ml⁻¹, 2 % (w/v) methyl α-D-mannopyranoside, 1:200 dilution of rat anti-mouse antibody against the complement receptor CR3 (CD11b) and a 1:200 dilution of rat anti-mouse antibody against the Fc receptor (CD16/CD32) (Pharmingen). Complement was removed from the serum by heat-treating FBS (56 °C for 30 min). Complement activity was restored by adding a 1:100 dilution of pooled normal guinea pig complement (Cedarlane) back to the heat-inactivated serum. For anaerobic experiments, J774-33 cells were placed in an anaerobic chamber 2 h prior to the start of the experiments to allow the media to become anaerobic. For all experiments, the bacteria were added and at various time points the coverslips were washed twice for 5 min in PBS and then fixed in 2:5 % paraformaldehyde for 20 min at room temperature. The J774-33 cells were then permeabilized by dipping the coverslips in cold (−20 °C) methanol for 10 s. To visualize the macrophage cytoplasm, coverslips were stained for 45 min at 37 °C with CellTracker Green CMFDA (Molecular Probes). To visualize the bacteria and J774-33 nuclei, coverslips were stained for 10 min in the dark at room temperature with the nucleic acid stain propidium iodide (Molecular Probes). Coverslips were mounted on slides with ProLong Anti-Fade mounting medium (Molecular Probes). Macrophages were examined using a Zeiss LSM 510 laser confocal microscope. To produce three-dimensional images using the confocal microscope, a mean of 20 cross-sectional images were captured over a vertical distance of 8–15 μm. The bacteria were identified as cell-associated if they were either attached to the surface of the macrophage or inside the macrophage.

**Phagocytosis of polystyrene microspheres.** To determine if the receptor inhibitors affected the ability of J774-33 cells to phagocytose inert particles, we used 2 μm fluorescent carboxylate-modified microspheres (FluoSpheres; Molecular Probes) as a control for phagocytosis receptor inhibition assays. These microspheres are made from polystyrene and contain a red fluorescent dye. To reduce the nonspecific binding of the beads, bovine serum albumin was added at a concentration of 50 μg ml⁻¹ in PBS containing the beads and shaken overnight at room temperature to coat the beads. The beads were then washed in PBS and added to J774-33 cells at a final concentration of 0:0025 % bead volume ml⁻¹. Phagocytosis receptor inhibition assays were then carried out for 60 min and processed as described above for bacteria.

To show that the decrease in phagocytosis in the presence of various inhibitors was due to specific inhibitor effects, we used 2 μm fluorescent microspheres coated with purified mouse IgG as a control. The covalent coupling of purified mouse IgG (Chemicon) to the fluorescent microspheres was performed as described by Molecular Probes. Inhibition assays were done as described above using mouse-IgG-coated microspheres, in the presence or absence of rat anti-mouse antibody against the Fc receptor (CD16/CD32) at a 1:200 dilution.

**Co-localization of *C. perfringens* and the late endosome-lysosome marker LAMP-1.** To determine if the presence of various inhibitors affected the co-localization of intracellular *C. perfringens* with the late endosome-lysosome marker glycoprotein LAMP-1, co-localization assays were performed as previously described (O’Brien & Melville, 2000). *C. perfringens* cells were opsonized by the addition of a 1:10 dilution of rabbit anti-*C. perfringens* serum (O’Brien & Melville, 2000) to bacteria in PBS. It was shown recently that rabbit IgG binds tightly to murine Fc receptors (Ober et al., 2001). For opsonization, the bacteria-antibody solution was placed on a rotator at room temperature for 30 min, washed three times with PBS, incubated for 30 min in PBS, then washed once more.

**Electron microscopy.** J774-33 cells were grown in DMEM until nearly confluent in 50 ml tissue-culture-treated flasks and the flasks were then incubated at 4 °C. *C. perfringens* strain 13 cells, grown to mid-exponential phase and washed three times in PBS, were then added at an m.o.i. of ~10:1 and incubated at 4 °C for 30 min to allow attachment to occur. The infected macrophages were placed in an incubator (5 % CO₂ at 37 °C), and at times 0, 10 and 20 min after being transferred to the 37 °C incubator, the infected macrophages were fixed with 2:5 % glutaraldehyde. Samples were processed for electron microscopy as previously described (O’Brien & Melville, 2000). Thin sections were viewed and photographed on a JEOL 2000EX electron microscope at 60 kV.

**Isolation of capsular polysaccharide.** The isolation of capsular polysaccharide from strain 13 was done according to Lee &
Cherniak (1974). To summarize, 4 l bacteria were grown overnight, harvested, and dried using increasing concentrations of ethanol and lastly acetone. Cells were boiled for 5 min and then stirred in an ice-water bath for 3 h. After centrifugation, the supernatants were removed and carbohydrates were precipitated overnight by adding two volumes of 95% ethanol. The precipitate was then harvested by centrifugation and washed in increasing concentrations of ethanol and lastly acetone. The pellet was resuspended in 0.01 M Tris/0.1 M NaCl buffer (pH 7.5) and was extracted with chloroform/butanol (9:1, v/v) until no protein was present at the solvent interface. Samples were then loaded onto a Q-Sepharose ion-exchange column to separate nucleic acids from the sample. The polysaccharide was then eluted using a linear gradient of 0–1 M NaCl. Fractions from the Q-Sepharose column were tested for the presence of hexoses using an anthrone assay (Dische, 1962). Fractions containing hexoses were lyophilized and used for glycosyl composition analysis.

Glycosyl composition analysis. To determine the sugar composition of the capsular material isolated from C. perfringens, glycosyl composition analysis was performed according to York et al. (1986). Briefly, the sample was hydrolysed with 1 M methanolic-HCl for 16 h at 80°C. The released methylglycosides were dried down and N-acetylated using methanol and acetic anhydride (1:1, v/v) for 15 min at 45°C. The acetylated sample was trimethyl-silylated (TMS) with Tris-Sil and resolved on a 30 m DB-1 column (0.25 mm × 0.25 mm, i.d.; J&W Scientific) in a Hewlett Packard 5985 GC-MS system using myo-inositol as an internal standard. The following temperature conditions were used: an initial temperature of 160°C, then raised to 200°C at 2 min⁻¹, and increased to 260°C at 10 min⁻¹. To aid in the identification of the sugars, a standard of derivatized methylglycosides was run alongside the samples.

Media and reagents. Mannan, fucoidin, mevalonate, mevinolin, methyl α-D-mannopyranoside and 2-mercaptoethanol were purchased from Sigma. Rat anti-mouse antibody against the complement receptor CR3 (CD11b) and rat anti-mouse antibody against the Fc receptor (CD16/CD32) were purchased from Pharmingen. DMEM with 4-5 g 1⁻¹ of both glucose and L-glutamine supplemented with 10% FBS and 1% sodium pyruvate, newborn calf serum and Ham’s F-12 growth medium were all from Biowhittaker. AcLDL was obtained from Biomedical Technologies. Anti-C. perfringens rabbit polyclonal antibodies were obtained from Applied Diagnostics International. Anti-LAMP-1 rat monoclonal antibody 1D4B was developed by J. T. August and was obtained from the Developmental Hybridoma Bank, developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA. Texas-red-X-conjugated goat anti-rat IgG and FITC-conjugated goat anti-rabbit IgG were both obtained from Molecubes.

RESULTS

C. perfringens phagocytosis is mediated by a variety of receptors on J774-33 cells

Since macrophages are unable to kill C. perfringens and the receptor used for phagocytosis can influence the fate of intracellular bacteria, we used specific inhibitors to block receptors known to function during the phagocytosis of bacteria by macrophages. The number of cell-associated bacteria (intracellular and surface-bound) seen with J774-33 macrophages under aerobic conditions with different receptor inhibitors was determined using laser confocal microscopy. To do this, the cytoplasm of the J774-33 cells was stained with CellTracker Green and the bacterial (and J774-33) nucleic acids were stained with propidium iodide. A representative image is shown in Fig. 1(a).

The addition of fucoidan, an inhibitor of the scavenger receptor on macrophages (Bermudez et al., 1997), led to the highest level of inhibition of bacterial cell association, 80% (Fig. 2a), indicating that the scavenger receptor played a major role in the process. The addition of methyl α-D-mannopyranoside resulted in a 50% reduction in the number of cell-associated bacteria (Fig. 2b), while mannan only inhibited attachment and uptake by 20% (Fig. 2c). The use of heat-treated serum (HTS), i.e. inactivated complement, led to about a 50% reduction in the number of

[Image 348x331 to 519x712]
cell-associated bacteria over the course of 60 min (Fig. 2d). The addition of guinea pig complement to the assay using HTS restored most of the binding and internalization that was lost with HTS (Fig. 2e). The addition of a blocking antibody to the complement receptor (CR3) resulted in comparable amounts of inhibition when compared to the use of HTS (compare Fig. 2f to Fig. 2d). In contrast, blocking the Fc receptor (FcR) with an FcR-specific antibody led to only a slight decrease in the number of cell-associated bacteria (Fig. 2g), indicating that the FcR did not play a role in \textit{C. perfringens} uptake in the absence of opsonizing antibodies.

The \textit{C. perfringens} receptor inhibition assays were also performed under anaerobic conditions (Fig. 3), because the anaerobic \textit{C. perfringens} has been shown to survive better and is more cytotoxic to macrophages in the absence of oxygen (O’Brien & Melville, 2000). The results were similar to those seen under aerobic conditions, with two exceptions: (1) the addition of methyl \(\alpha\)-D-mannopyranoside led to a significantly greater level of inhibition of \textit{C. perfringens} attachment and internalization than was seen under aerobic conditions (compare Figs 3b and 2b), and (2) the addition of guinea pig complement to the HTS did not fully restore the binding of \textit{C. perfringens} during the first 50 min of the assay (Fig. 3e).

**Fig. 2.** Number of cell-associated bacteria detected after infecting \textit{J774-33} cells with \textit{C. perfringens} under aerobic conditions in the presence of specific inhibitors of phagocytosis receptors. Cell-associated bacteria were defined as those that were either attached to the surface or were intracellular, as determined by laser confocal microscopy (see Methods). Open circles represent conditions in the absence of inhibitors. The results when the receptor was blocked with the reagent listed above each graph are shown as closed circles. For comparison, the same curve for standard conditions is shown for each of the graphs. At each time point, the number of attached and intracellular bacteria was determined for 100 macrophages. Each inhibitor treatment was tested in at least three independent experiments; representative results are shown. HTS, heat-treated serum; MMP, methyl \(\alpha\)-D-mannopyranoside.

**Binding of \textit{C. perfringens} to SR-A transfected CHO cells**

Additional evidence that the scavenger receptor SR-A was involved in the binding and uptake of \textit{C. perfringens} was obtained using mouse SR-A transfected CHO cells. The percentage of cell-associated bacteria per coverslip in the presence and absence of fucoidin was determined (Fig. 4). We found a mean of 25 cell-associated bacteria per coverslip when SR-A transfected CHO cells were infected with \textit{C. perfringens}. The mean number of cell-associated bacteria decreased to 7 when fucoidin was added to transfected CHO cells (Fig. 4). This indicated that the scavenger receptor was involved in the binding of \textit{C. perfringens} and that fucoidin could decrease the number of cell-associated bacteria. Wild-type CHO cells infected with \textit{C. perfringens} in the presence and absence of fucoidin gave a mean of 4 and 3\textsuperscript{5} cell-associated bacteria per coverslip, respectively. The lack of inhibition seen in the presence of fucoidin is consistent with the fact that there are no SR-A receptors on CHO cells.
Efficient internalization of \textit{C. perfringens} requires the simultaneous activity of the mannose, CR3 and scavenger receptors

The data in Figs 2 and 3 represent the numbers of cell-associated bacteria present during the course of the assay, including intracellular bacteria and bacteria bound to the surface of the macrophage. To differentiate between binding to a receptor and subsequent internalization by the macrophage, the percentage of intracellular to extracellular (i.e. on the macrophage surface) bacteria was determined for 100 macrophages. Each inhibitor treatment was tested in at least three independent experiments; representative results are shown. HTS, heat-treated serum; MMP, methyl \(\alpha\)-D-mannopyranoside.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{Number of cell-associated bacteria detected after infecting J774-33 cells with \textit{C. perfringens} under anaerobic conditions in the presence of specific inhibitors of phagocytosis receptors. Open circles represent conditions in the absence of inhibitors. The results when the receptor was blocked with the reagent listed above each graph are shown as closed circles. For comparison, the same curve for standard conditions is shown for each of the graphs. At each time point, the number of attached and intracellular bacteria was determined for 100 macrophages. Each inhibitor treatment was tested in at least three independent experiments; representative results are shown. HTS, heat-treated serum; MMP, methyl \(\alpha\)-D-mannopyranoside.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4}
\caption{Number of cell-associated bacteria detected after infecting CHO and SR-A transfected CHO cells under aerobic conditions for 60 min in the presence and absence of fucoidin. The data are represented as the number of cell-associated bacteria per coverslip. Results shown are the mean (±SD) of at least three independent experiments. Statistically significant from SR-A transfected CHO cells in the absence of fucoidin, using the Student's unpaired \(t\)-test (two-tailed); *, \(P<0.05\); **, \(P<0.01\).}
\end{figure}

Red fluorescent polystyrene microspheres were used as phagocytosis controls to determine if the addition of receptor inhibitors resulted in a generalized inhibition of phagocytosis by the J774-33 macrophages. The cytoplasm
of J774-33 cells was stained with CellTracker Green and confocal microscopy was used to determine the percentage of cell-associated microspheres that were intracellular (a representative image is shown in Fig. 1b). In the absence of any treatment, 18% of the cell-associated microspheres were intracellular (Fig. 6), which was about fivefold lower than the ratio of internal versus cell-bound for untreated C. perfringens (Fig. 5). In contrast to the results seen with C. perfringens, the ratio of internal versus bound microspheres was unchanged by the addition of any of the receptor inhibitors tested (Fig. 6), indicating that addition of the inhibitors did not result in a generalized blockage of phagocytosis by the J774-33 cells. Even though there are two mechanisms of phagocytosis (Caron & Hall, 1998), both utilize filamentous actin polymerization; this feature of phagocytosis was not affected by the addition of receptor inhibitors.

In order to demonstrate that our inhibitor treatments could block phagocytosis and were specific for the receptors we were testing, microspheres were coated with mouse IgG before addition to the macrophages to stimulate their uptake via the FcR (see Methods). The addition of a blocking antibody specific to the FcR resulted in a fourfold decrease in internalization of the IgG-coated beads (Fig. 6), indicating that blocking the FcR receptor led to an inhibition of phagocytosis via that specific receptor.

Co-localization with the late endosome-lysosome marker LAMP-1 in the presence of inhibitors of receptors

We looked to see if the rate of co-localization with LAMP-1, an indicator of phagosome/lysosome fusion, would change in the presence of receptor inhibitors and/or combinations of inhibitors. A mixture of inhibitors was used to force the bacteria to be taken up by a single receptor. For these assays, J774-33 cells were infected with C. perfringens and the amount of co-localization with LAMP-1 was measured using laser confocal microscopy (Fig. 7). The non-pathogenic bacterium B. subtilis was used as a positive control, and was found to co-localize with the LAMP-1 marker 63% of the time, while only 9% of the C. perfringens (with untreated J774-33 cells) co-localized with LAMP-1 (Fig. 7). Of the single inhibitors added, only the addition of fucoidin (i.e. functional CR3 and mannose receptors) gave a statistically significant increased level of LAMP-1 co-localization in comparison to untreated J774-33 cells (Fig. 7). Combinations of two inhibitors were added to permit the uptake of C. perfringens to occur by only a single receptor; however, no significant difference was seen in the amount of co-localization with LAMP-1 as compared to the untreated J774-33 cells (Fig. 7). Although the addition of fucoidin gave increased levels of co-localization, the combination of fucoidin and either
methyl α-D-mannopyranoside or HTS did not show a statistically significant increase in co-localization with LAMP-1. Possibly, the complement and mannose receptors need to act together to achieve the most effective internalization of bacteria. When *C. perfringens* was opsonized with antibodies, a statistically significant increase in the number of *C. perfringens* co-localized with LAMP-1 was seen in comparison to the untreated J774-33 cells. Interestingly, while fucoidin treatment or opsonization of *C. perfringens* gave a statistically significant increase in co-localization efficiencies, the levels were only one-third of that seen with the *B. subtilis* control (Fig. 7).

**Electron microscopy of macrophage-bound *C. perfringens* shows macrophages bind to an extracellular matrix on the surface of the bacteria**

We have presented evidence that *C. perfringens* can bind to at least three different receptors on macrophages. Therefore, we wanted to visualize the interactions of *C. perfringens* with these receptors on the surface of J774-33 cells. To increase the number of surface-bound *C. perfringens*, attachment to the macrophages was allowed to occur at a temperature (4 °C) in which phagocytosis was inhibited. After binding of the bacteria to the macrophage had occurred, the cells were shifted to 37 °C and fixed at later time points. The contact points between the macrophages and the bacteria were visualized by transmission electron microscopy. Representative images at 0, 10 and 20 min after being transferred to 37 °C are shown in Fig. 8. At 0 min, all of the bacteria were seen as extracellular, indicating that the low temperature was effective at blocking uptake of the bacteria, but not binding to the surface. By 20 min, nearly all of the cell-associated bacteria examined were intracellular, indicating that phagocytosis proceeded once the macrophages were transferred to the warmer temperature. Interestingly, very few of the intracellular bacteria had escaped the phagosome after 20 min. This is in contrast to our previously reported results (O’Brien & Melville, 2000) and this may be due to the exposure of the macrophages and the bacteria to a low temperature of 4 °C. The macrophages bound the bacteria at a fibrous layer external to the bacterial cell wall (arrows in insets of Fig. 8).

**Composition analysis of the *C. perfringens* strain 13 extracellular polysaccharide indicates the presence of residues that may interact with phagocytic receptors**

The polysaccharide capsule of *C. perfringens* was purified and subjected to carbohydrate composition analysis (see Methods). After analysis, it was found that the polysaccharide contained six sugar residues: glucose (38.6%), galactose (23.1%), mannose (19.5%), glucuronic acid (9.5%), N-acetylgalactosamine (8.7%), and trace amounts of N-acetylgalactosamine (0.6%). All of these sugar residues have been found in the bacterial capsule of another strain of *C. perfringens* (Kalelkar et al., 1997), indicating that the extracellular fibrous layer on strain 13 is probably a capsular polysaccharide.

**DISCUSSION**

The results of these studies indicate that the scavenger, mannose and CR3 receptors (in the presence of complement) were used for binding and phagocytosis of *C. perfringens* by J774-33 cells (Figs 2 and 3). J774-33 cells were used in this study since they readily bind and phagocytose *C. perfringens* and other Gram-positive bacteria (O’Brien & Melville, 2000). J774 cells are known to express the scavenger receptor (Ding et al., 1998) and the Fc receptor (Martin & Weis, 1993) and some clones express the mannose receptor (Fiani et al., 1998). They also express the complement receptor CR3 (Hall et al., 1991), but not CR1 or CR2 (Martin & Weis, 1993).

At least one of these receptors has the capacity to bind two different ligand types. For example, besides binding C3bi, CR3 has a lectin-binding site that recognizes α-methylpyranosides, among other carbohydrates (Ehlers, 2000). Therefore, in the experiments where methyl α-D-mannopyranoside was added, there may have been inhibition of binding to both the mannose receptor and CR3. The FcR was not used for phagocytosis of *C. perfringens* and fixed at later time points. The contact points between the macrophages and the bacteria were visualized by transmission electron microscopy. Representative images at 0, 10 and 20 min after being transferred to 37 °C are shown in Fig. 8. At 0 min, all of the bacteria were seen as extracellular, indicating that the low temperature was effective at blocking uptake of the bacteria, but not binding to the surface. By 20 min, nearly all of the cell-associated bacteria examined were intracellular, indicating that phagocytosis proceeded once the macrophages were transferred to the warmer temperature. Interestingly, very few of the intracellular bacteria had escaped the phagosome after 20 min. This is in contrast to our previously reported results (O’Brien & Melville, 2000) and this may be due to the exposure of the macrophages and the bacteria to a low temperature of 4 °C. The macrophages bound the bacteria at a fibrous layer external to the bacterial cell wall (arrows in insets of Fig. 8).

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by J774-33 cells in the absence of opsonizing antibodies. \textit{C. perfringens} infections are often of sudden onset after exposure to the bacterium; times as short as 6 h after wounding have been reported for the onset of gas gangrene (Stevens, 1997). Therefore, it is questionable whether the humoral immune response plays a significant role in most myonecrotic infections, since it takes several days for high levels of antibodies to be produced.

The data in Figs 2 and 3 depict the effects receptor inhibitors had on the total number of cell-associated bacteria, including attached and intracellular bacteria. In order to determine if the inhibitors affected uptake in a manner independent of binding to the receptor, we ascertained the percentage of cell-associated bacteria that were intracellular 60 min post-infection (Fig. 5). All of the inhibitors tested, except for the anti-FcR antibody, resulted in a decreased level of uptake of cell-associated bacteria. However, even when the total number of cell-associated bacteria was only slightly affected, as with the addition of mannan (Fig. 2), the internalization of the bacteria in the presence of mannan was still affected (Fig. 5). This suggests that while the scavenger, mannose and complement receptors are all involved in the binding and internalization of \textit{C. perfringens}, the most effective internalization of the bacteria occurred when all of the receptors were involved in the phagocytosis process. The total amount of inhibition seen with the addition of inhibitors in separate experiments adds up to greater than 100\% (Figs 2 and 3), indicating that there was some lack of specificity in their effects on the specific receptors we have identified as taking part in binding \textit{C. perfringens}.

Of the three receptors used for phagocytosis of \textit{C. perfringens} the scavenger receptor was the most important

The most dramatic decrease in the number of cell-associated bacteria was seen when J774-33 cells were infected in the presence of the scavenger receptor inhibitor fucoidin. To examine the role that the SR-A scavenger receptor plays in the phagocytosis of \textit{C. perfringens}, we used CHO cells transfected with the murine SR-A receptor. We found that binding of \textit{C. perfringens} to CHO cells was enhanced greater than eightfold in the presence of the SR-A receptor (Fig. 4). When fucoidin was added the number of cell-associated bacteria decreased greater than threefold. This indicates that fucoidin can act as a specific inhibitor of \textit{C. perfringens}.
binding. This supports our J774-33 inhibitor studies that showed the scavenger receptor is important in the binding and uptake of C. perfringens.

Phagocytosis control experiments

Polystyrene microspheres were used as phagocytosis controls in these assays. The fact that the uptake of the microspheres was not affected by the addition of inhibitors while C. perfringens was (Figs 5 and 6) showed that phagocytosis in general was not affected by the addition of inhibitors. As a second control, we showed that uptake of IgG-coated microspheres could be blocked by the addition of anti-FcR antibodies, indicating that this receptor is efficiently inhibited by the addition of a blocking antibody. The FcR receptor was used because it was not used for the internalization of C. perfringens (Fig. 5), while all the other receptors tested were involved in the phagocytosis process. Therefore, the results in Fig. 6 showing that the anti-FcR antibody could block uptake of IgG-coated microspheres reinforce the results seen in Figs 2 and 3 showing that the FcR was not used for phagocytosis in the absence of opsonizing antibodies.

LAMP-1 co-localization studies

The addition of fucoidan and opsonization of C. perfringens showed statistically significant increases in the levels of co-localization with the late endosome-lysosome marker LAMP-1 in comparison to the untreated J774-33 cells. However, the biological significance of this increase is questionable, since the non-pathogenic bacterium B. subtilis was found to co-localize with LAMP-1 at a rate three times higher than that observed with fucoidin treatment or with the opsonized C. perfringens (Fig. 7). Since we have demonstrated in a previous report that J774-33 cells can kill B. subtilis, but not C. perfringens (O’Brien & Melville, 2000), and intracellular B. subtilis co-localized with LAMP-1 at a high (63%) level, the lower level of co-localization seen with the fucoidin treatment and opsonized C. perfringens probably does not reflect a significant increase in killing efficiency.

The capsule of strain 13 may be the target of phagocytosis receptors on J774-33 cells

What do these receptor experiments tell us about the surface properties of C. perfringens? We have obtained high-magnification electron microscopy images of C. perfringens attached to J774-33 cells that show the contact points on the macrophage surface appear to bind to an extracellular matrix material located immediately outside the Gram-positive cell wall of C. perfringens (Fig. 8, left panels). This is similar in appearance to the distinct patchy areas of bacteria and macrophage contact we described in an earlier report (O’Brien & Melville, 2000). The extracellular matrix on the surface of strain 13 was also seen when the bacteria were internalized by macrophages (Fig. 8, middle and right panels). For unknown reasons, the fibrous material can vary in length and appearance (Fig. 8).

Many strains of C. perfringens are known to produce an extracellular capsule (Cherniak & Frederick, 1977; Kalelkar et al., 1997), but strain 13, the strain used in these experiments, did not show evidence of a capsule when examined with the India ink negative stain (S. Melville, unpublished results). However, the extracellular fibrous material seen in high-magnification electron micrographs of bacteria attached to the outer membrane of J774-33 cells (Fig. 8, left panels) indicated that this strain might possess a tightly bound capsule on its surface. To determine if a capsule was present, extracellular polysaccharide from strain 13 was purified and characterized.

The purified polysaccharide from strain 13 cells contained glucose, galactose, mannose, glucuronic acid, N-acetylgalactosamine and N-acetylglucosamine. This is the first description of a capsule being associated with C. perfringens strain 13. Also, the residues found in the polysaccharide of strain 13 are the same residues found in the capsule polysaccharide of C. perfringens strain ATCC 12919 (Kalelkar et al., 1997), indicating that the material from strain 13 is most likely a polysaccharide capsule. The presence of mannose and glucuronic acid would be consistent with residues that would bind the mannose and scavenger receptors, respectively, which we have identified as receptors involved in the binding and phagocytosis of C. perfringens. For example, the mannose in the capsule is likely the residue that the mannose receptor recognized on the surface of the bacteria. Glucuronic acid would impart an overall net negative charge to the capsule polysaccharide oligomers, which are characteristic of one group of substrates bound by the scavenger receptor (Gough & Gordon, 2000). Further evidence that strain 13 can synthesize a capsule was provided by the recent publication of the complete genome sequence of strain 13, in which many genes that could be involved in capsular biosynthesis were identified (http://w3.grt.kyushu-u.ac.jp/CPE/).

Interestingly, the capsule of strain 13 does not appear to inhibit phagocytosis by macrophages, unlike capsules in other Gram-positive bacteria such as Streptococcus pneumoniae and Bacillus anthracis. In fact, J774-33, mouse peritoneal, and bone marrow derived primary macrophages phagocytose the encapsulated form of strain 13 very efficiently (Figs 2 and 3; and O’Brien & Melville, 2000). Therefore, the capsule of C. perfringens strain 13 may not play an anti-phagocytic role in the host. Since we have reported that C. perfringens can persist in the presence of macrophages despite being phagocytosed (O’Brien & Melville, 2000), it may actually be advantageous for C. perfringens to be readily phagocytosed by a macrophage. Because C. perfringens is not killed by the process, the intracellular environment may provide more nutrients and a lower oxygen concentration than is found outside of the macrophage (James et al., 1998), which would be advantageous to an anaerobic bacterium.
ACKNOWLEDGEMENTS

We thank Katherine Traughton (UT Memphis) for electron microscopy assistance, the Complex Carbohydrate Research Center (CCRC) (University of Georgia, Athens, Georgia) for the analysis of C. perfringens polysaccharide, M. Krieger for the generous gift of SR-A transfected and untransfected CHO cells, and David Popham for critical reading of the manuscript. CCRC is supported in part by the Department of Energy-funded (DE-FG09-95ER-20097) Center for Plant and Microbial Complex Carbohydrates.

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