Role of Carbohydrate-mediated Adherence in Cytopathogenic Mechanisms of *Acanthamoeba*

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*Acanthamoeba* keratitis is a vision-threatening corneal infection. It is caused by parasites of the genus *Acanthamoeba* (1, 2). The mechanism by which *Acanthamoeba* produce keratitis has not been fully elucidated. Contact lens wear is thought to be the leading risk factor. However, the disease also occurs, if less frequently, in non-contact lens wearers. It is believed that minor corneal surface injury caused by contact lens wear or other noxious agents and exposure to contaminated solutions, including lens care products and tap water, are two major factors in the pathogenesis of the keratitis. The adhesion of the parasite to the host cells is thought to be a critical first step in the pathogenesis of infection (3–5). Studies aimed at the characterization of the molecular mechanisms by which amoebae adhere to corneal epithelium have shown that: (i) the adhesion of *Acanthamoeba* to corneal buttons in organ culture and to monolayer cultures of cornal epithelium can be inhibited by methyl-α-D-mannopyranoside (α-Man) but not by several other monosaccharides (6, 7), and (ii) a mannose-binding protein is present on the surface membranes of *Acanthamoeba* (8). *In vitro*, *Acanthamoeba* parasites have been shown to produce a cytopathic effect (CPE)1 on a variety of cell types (9–11) including rabbit and human corneal stromal and epithelial cells (5, 12, 13). The parasite’s ability to cause cytolysis and necrosis of host tissues is clearly a key component of *Acanthamoeba* infection but our understanding of how this is achieved is still rudimentary. In the present study, we show that the *Acanthamoeba* lectin binds α-Man and α-1–3-β-mannobiose with highest affinity and that the lectin-mediated adhesion of the amoeba to host cells is a prerequisite for the amoeba-induced cytolysis of target cells.

EXPERIMENTAL PROCEDURES

Characterization of the Carbohydrate-binding Specificity of *Acanthamoeba* Lectin

An *Acanthamoeba* strain derived from an infected human cornea (MEEI 0184; *Acanthamoeba castellani* based on morphological characteristics) was used throughout this study. The parasites were axenically cultured in a proteose peptone/yeast extract/glucose medium (14). To characterize the sugar binding properties of the amoeba lectin a solid-phase assay was used (8). Briefly, wells of microtiter plates were coated with bovine serum albumin-α-D-mannopyranosylphenylisothiocyanate (Man-BSA) (Sigma), 15–25 mol of α-D-mannopyranosyl/mole of albumin, 0.03 μg/ml (50 μl/well in 0.1 M sodium carbonate buffer, pH 9.6, 4°C, overnight); nonspecific binding was blocked with 1% BSA in phosphate-buffered saline (1 h, room temperature), a 50-μl aliquot of 3S-labeled *Acanthamoeba* (5 × 10⁶ cells/ml in phosphate-buffered saline; 1–2 cpm parasite) >95% trophozoites was added to each well, and the radioactivity in aliquots of solubilized material was determined in a scintillation counter.

To characterize the specificity of the amoeba lectin, a range of saccharides were tested for their ability to inhibit the adhesion of amoebe to Man-BSA (see Table I). The 25 saccharides tested were purchased from Sigma, V-Labs Inc. (Covington, LA), and Pfannstiel Laboratories Inc. (Waukegan, IL), and included α1–3-β-mannobiose, α1–6-β-mannobiose, α1–3–α1–6-β-mannotriose, and α1–3–α1–6-β-mannopentose (Table I). Serial dilutions of sugars were tested to calculate the concentration required for 50% inhibition of the binding.

To determine whether any other lectin, besides the mannose-binding protein is present on the amoeba surface, the solid-phase assays were performed using microtiter wells coated with a number of neoglycoproteins including N-acetyl-α-glucosamine-BSA (GlCNAc-BSA), N-acetyl-galactosamine-BSA, fusoc-BSA, and galactose-BSA. These neoglycoproteins contained 15–30 mol of sugar/mol of albumin.

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1 The abbreviations used are: CPE, cytopathic effect; α-Man, methyl-α-D-mannopyranoside; Man-BSA, α-1–3-D-mannopyranosylphenylisothiocyanate/bovine serum albumin; GlCNAC-BSA, N-acetyl-α-glucosamine-bovine serum albumin; ACM, *Acanthamoeba*-conditioned medium; PMSF, phenylmethylsulfonyl fluoride.

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Characterization of the Role of the Mannose-binding Protein in the Cytopathogenic Mechanisms of Acanthamoeba

To characterize the role of carbohydrate-mediated host-parasite interactions in the cytopathogenic mechanisms of Acanthamoeba, in vitro cytopathic assays were performed using cell cultures of corneal epithelium as target cells.

Immortalization of Rabbit Corneal Epithelial Cells—Since primary cultures of corneal epithelium have limited life span and can only be passaged 2 to 3 times without significant alteration in cell morphology, corneal epithelial cells with extended life span were produced. For this, the primary corneal epithelial cell cultures prepared as described earlier (15, 16) were infected with SV40 large T antigen cDNA (17). Retroviruses were used to transduce both the 3-phosphotransferase gene conferring G418 antibiotic resistance and the SV40 large T antigen cDNA into the primary cultures. For infection, irradiated producer cells (2 × 10⁶ cells/100-mm dish) were added to confluent primary cultures of corneal epithelium. Two to three days after infection, the transformed cells were selected by adding G418 (500 μg/ml) into the culture medium. To promote the growth of the selected cells, initially they were co-cultured with irradiated fibroblast cells. After 10–12 days, when the cultures reached 30–40% confluence, G418 as well as fibroblasts were withdrawn. At this stage, cells were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12 (1:1) containing fetal bovine serum (15%), dimethyl sulfoxide (0.5%), gentamicin (40 μg/ml), epidermal growth factor (10 ng/ml), insulin (5 μg/ml), and cholera toxin (0.1 μg/ml). When these cells reached over 50% confluence, they were passaged with a split ratio of 1:2. Upon phase-contrast microscopy, no fibroblastic cells could be detected subsequent to this first passage. The entire cell population was epithelioid. The cultures reacted positively with mAb AE5 which reacts with keratin K3, a marker for corneal epithelial cells. This further established the epithelial origin of the immortalized cells.

Cytopathic Assay—To evaluate the Acanthamoeba-induced CPE on corneal epithelial cell cultures, the parasites (>95% trophozoites) were rinsed three times in a serum-free medium supplemented with 0.4% BSA (18) and aliquots of the parasite suspension (0.2 × 10⁵ to 1 × 10⁶ parasites/ml; 300 μl/well for 24-well plates; 1 ml/well for 6-well plates) were added to wells of confluent cultures of epithelium which had been rinsed and preincubated in the serum-free medium for 2 h. The plates were then incubated at 37 °C in a CO₂ incubator and were periodically examined under a phase-contrast microscope for the presence of cell-free plaques in the monolayer for up to 28 h. Control wells contained the target cells in the medium without the parasites. To evaluate the effect of saccharides on Acanthamoeba-induced CPE, the assays were performed in the presence of sugars (0.03–100 mM). To estimate the relative inhibitory potency of each sugar, the CPE assays were terminated when approximately 50–70% cell loss occurred in the test wells. Percent specific release was calculated using a formula: O.D. of epithelial cells incubated with amoeba × 100/(Eq. 1) O.D. of control cells

To determine whether the mechanisms modulating the CPE produced by intact amoeba and the amoeba-conditioned medium (ACM) are distinct, the CPE assays were also performed using the ACM in the presence and absence of saccharides. To produce the ACM, exponentially growing parasites (>95% trophozoites) in the protease peptone/yeast medium were rinsed and then incubated in the serum-free medium supplemented with 0.4% BSA (2 × 10⁵ to 1 × 10⁶ cells/ml), 37 °C, 24 h in the presence and absence of saccharides (α-Man and GlcNAc, 50 mM). At the end of the incubation period, the parasites were removed by centrifugation and the supernatants were analyzed for their ability to produce CPE. In some cases saccharides were added to the ACM after it was produced.

Measurement of Acanthamoeba-induced Target Cell Lysis

Cell lysis was quantified by measuring ⁵¹Cr release from prelabeled cells. For radiolabeling, confluent monolayer cultures of corneal epithelium in 24-well plates were incubated in cell culture medium containing Na²⁴⁵CrO₄ (460 μCi/ml, 12 μCi/ml, NEN Life Science Products) for 18–20 h. At the end of the labeling period, the cells were extensively rinsed and were then incubated with Acanthamoeba (1 × 10⁵ parasites/ml) in the presence or absence of saccharides (50 mM α-Man or GlcNAc). The cultures were periodically examined under a phase-contrast microscope for CPE. When approximately 50–70% cell loss occurred in the monolayers, aliquots of cell-conditioned media from each well were analyzed for specific ⁵¹Cr release. To determine specific ⁵¹Cr release values, counts/min released in control wells incubated in media alone without the parasites were deducted from the counts/min released in the test wells. Percent specific release was calculated using a formula: E/T × 100, where E is counts/min released in test wells minus the counts/min released in control wells, and T is the total counts/min (counts/min cells + counts/min supernatant in control wells). Counts/min of cells was determined by incubation of the control wells with 0.5% Triton X-100. Conditioned media from each well was also analyzed for lactate dehydrogenase release using an lactate dehydrogenase assay kit purchased from Sigma. In each case, there were four wells/group. The same procedure was used to determine whether ACM possesses cytoytic activity.

Analysis of Proteinases by Zymography

To determine whether the mannose-mediated host-parasite interactions influence the levels of secretory proteinases, conditioned media obtained by incubating the amoeba with primary cultures of corneal epithelium in the presence and absence of saccharides (α-Man and GlcNAc, 50 mM) were also analyzed by zymography.

RESULTS

The Amoeba Lectin Has the Highest Affinity for α-Man and Man(α1–3)Man Units

In solid-phase assays, binding of amoebae to Man-BSA was dose-dependent (Fig. 1). Acanthamoeba did not bind to a number of other glycoproteins including galactose-BSA, fucose-BSA, N-acetyl-D-galactosamine-BSA, and GlcNAc-BSA (Fig. 1). To characterize the carbohydrate binding properties of the amoeba lectin, sugar inhibition studies were performed. Concentrations of sugars required for 50% inhibition were calculated from inhibition curves and are listed in Table I. Among various monosaccharides tested, α-Man was the most potent inhibitor of amoeba binding to Man-BSA. Compared with α-Man the inhibitory potency of methyl-β-mannopyranoside and β-mannose was 8- and 10-fold less, respectively. Epimers of β-mannose, i.e. altrose (C-3) and talose (C-4), were not inhibitory but glucose (C-2) was a weak inhibitor. Compared with D-mannose, the inhibitory potency of D-glucose was 3.5-fold lower. D-Lyxose, which is equivalent to D-mannose with the C-5 substituent missing, was not inhibitory. The reduction of the mannose to D-mannitol by sodium borohydride completely destroyed its inhibitory activity. γ-D-mannosamine, nor N-acetyl-D-mannosamine were inhibitory. D-Glucosamine was also not inhibitory. However, GlcNAc and α-D-fucose were weak inhibitors. Their inhibitory potencies were 26- and 14-fold lower, respectively, compared with that of α-Man. Both mannobiose, the mannnotriose, and the mannopentose were also potent inhibitors; the inhibitory potency of D-mannose nor N-acetyl-D-mannosamine were inhibitory.
Mannose-mediated Host-Parasite Interactions Play a Role in Acanthamoeba-induced Cytopathogenic Mechanisms

Acanthamoeba trophozoites have been shown to produce a CPE on a variety of cultured mammalian cells (5, 9–13). To determine whether the carbohydrate-mediated adhesion of Acanthamoeba to corneal epithelium is a prerequisite for the parasite-induced target cell damage, CPE assays were performed in the presence and absence of various saccharides. The pathogenic ocular isolate of Acanthamoeba used in the present study produced extensive CPE on both immortalized (Fig. 2) as well as primary (Fig. 3) rabbit corneal epithelial cells in culture. Within 8–10 h incubation with the parasites, small cell-free plaques were seen in the monolayers (Fig. 2, 8 h). With the continued incubation with the amoebae, the size of the cell-free areas increased (Fig. 2, 16 h), and eventually the monolayer surrounding the large plaques lifted entirely from the culture dish resulting in almost complete loss of the cell layer (Fig. 2, 24 h). The extent of the monolayer destruction depended on the concentration of amoebae, the nature of cell cultures (primary versus immortalized), the length of the incubation period, and the composition of the media used for the assay. When immortalized cultures were used, an amoeba concentration of 2 × 10⁵ cells/ml was required to completely destroy the monolayer within 20 h. All CPE assays were performed in a serum-free medium supplemented with 0.4% BSA. If BSA was omitted from the media, the destruction of the monolayer occurred approximately twice as fast (data not shown).

Upon phase-contrast microscopy, it was evident that when amoebae were incubated with monolayers, they adhered to the cells within minutes. All saccharides which inhibited amoeba binding to Man-BSA also inhibited the amoeba binding to corneal epithelial cells (not shown). In vitro CPE assays, for the most part, revealed a direct correlation between the ability of the sugar to inhibit the Acanthamoeba binding to Man-BSA and to inhibit the amoeba-induced CPE (Table I). α-Man, both mannobioses, the mannotriose, and the mannopentose which were potent inhibitors of amoeba binding to Man-BSA were also potent inhibitors of amoeba-induced CPE; saccharides such as mannitol, mannosamine, N-acetylated mannosamine, methyl-α- and methyl-β-galactopyranoside which did not inhibit amoeba binding to Man-BSA also did not inhibit amoeba-induced CPE. One striking exception was GlcNAc which was a weak inhibitor of the amoeba adhesion to Man-BSA but a potent inhibitor of CPE (see below).

Primary cultures were relatively less susceptible to amoeba-induced CPE in that 1 × 10⁶ parasites/ml were required to completely destroy the monolayer within 24 h. This parasite concentration is five times higher than that required to produce the same degree of CPE when immortalized cultures were used as target cells. Only a limited number of saccharides were tested for their ability to inhibit the amoeba-induced CPE on primary cultures. At a sugar concentration of 5 mM or less, α-Man, both mannobioses, the mannotriose, the mannopentose, and GlcNAc inhibited CPE. Methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, and α-L-fucose were not inhibitory up to 100 mM concentration (Fig. 3).

α-Man and GlcNAc Modulate Acanthamoeba-induced Cytopathic Effect by Distinct Mechanisms

As described above, GlcNAc, a weak inhibitor of amoeba adhesion to Man-BSA, was a potent inhibitor of amoeba-induced CPE. The inhibitory potency of GlcNAc in the amoeba adhesion assay was 26 times less than that of α-Man (Fig. 4, top panel; Table I). In contrast, in the CPE assays, the inhibitory potency of both α-Man and GlcNAc was nearly identical (Fig. 4, bottom panel; Table I). Amoebae did not adhere to the cell

α1–3-Man-mannobiose was approximately three times greater than that of α1–6-Man-mannobiose and α-Man alone. α1–3,α1–6-Man-mannotriose and α1–3,α1–6,α1–3,α1–6-Man-mannopentose were slightly better inhibitors than α1–3-o-mannobiose (Table I).

![Fig. 1. Amoebae bind to Man-BSA but not to other neoglycoproteins. 35S-Labeled amoebae were allowed to bind to microtiter wells coated with increasing amounts of various neoglycoproteins. □, manno-BSA (Man-BSA); ○, N-acetyl-D-glucosamine-BSA; ●, fucose-BSA; ■, galactose-BSA; ▲, N-acetyl-D-galactosamine-BSA. Inset: 35S-labeled amoebae in varying concentrations (a, 0.5 × 10⁶; b, 1 × 10⁶; c, 2.5 × 10⁶; d, 5 × 10⁶ parasites/ml) were allowed to bind to microtiter wells coated with Man-BSA (0.625 μg/ml). Mean values are reported (n = 6 in each group).

Table I

| Saccharide                  | Inhibition of binding to Man-BSA | Inhibition of CPEa |
|----------------------------|----------------------------------|-------------------|
|                            | concentration in mM              | at ≥50% inhibition |
| d-Mannose                  | 20                               | 5                 |
| Methyl-α-D-mannopyranoside | 1.96                             | 0.31              |
| Methyl-β-D-mannopyranoside | 16                               | NDa               |
| Man(α1–3)Man               | 0.74                             | 0.31              |
| Man(α1–6)Man               | 2.4                              | NDa               |
| Man(α1–3)Man(α1–6)Man      | 0.62                             | 0.31              |
| Man(α1–3)Man(α1–6)Man(α1–3)Man(α1–6)Man | 0.44 | NDa               |
| Methyl-α-D-galactopyranoside | >50                             | ≤50               |
| N-Acetyl-D-glucosamine (GlcNAc) | 50      | 0.31              |
| α-L-Fucose                 | 28                               | >100              |

a Immortalized rabbit corneal epithelial cell cultures were used as target cells. In a limited number of CPE assays with primary cultures, methyl-α-mannopyranoside, both mannobioses, the mannotriose, the mannopentose, and N-acetyl-d-glucosamine were inhibitory at a sugar concentration of 5 mM or less; α-galactose, β-galactose, and α-L-fucose were not inhibitory up to 100 mM concentration.

b ND, not determined.

Note that GlcNAc is a weak inhibitor of amoeba binding to Man-BSA but a potent inhibitor of CPE.
Fig. 2. Acanthamoeba-induced cytopathic effect. Acanthamoebae (2 × 10^6 parasites/ml) were added to confluent cultures of immortalized corneal epithelium in 24-well plates, and the cultures were incubated in a CO₂ incubator for varying periods. At the end of the incubation period, the plates were stained with Giemsa and photographed. Top panel shows the photographs of the wells and lower panel shows the light micrographs of representative areas. Approximate cell density in each well, as measured by scanning the plates in a computer-assisted Bio-Image scanner, is shown in the top right panel. A value of 1.0 was assigned to the cell density of the plates incubated in media alone (Cont.). The values for cultures incubated with amoebae are expressed in change in the density with respect to control plates. Note that during the early phase (8 h) small cell-free plaques appeared in the monolayer; with continued incubation the size of cell-free areas increased (16 h), and eventually monolayer surrounding the large plaques lifted entirely from the culture dish resulting in almost complete loss of the cell layer.

Fig. 3. α-Man inhibits Acanthamoeba-induced cytopathic effect. Confluent primary corneal epithelial cultures in 6-well plates were incubated with Acanthamoebae (1 × 10^6 parasites/ml) in the presence or absence of various saccharides (0.1 mM) for 20 h. The plates were then stained with Giemsa and scanned to estimate approximate cell density. A value of 1.0 was assigned to the cell density of the plates incubated in media alone (Cont.). The values for cultures incubated with amoebae in the presence and absence of saccharides are expressed as change in the density with respect to control plates. Data are expressed as mean ± S.E. (n = 4 in each group). Photographs of the plates are shown in the bottom panel. Note that methyl-α-mannopyranoside (α-Man) markedly inhibited the amoeba-induced CPE, whereas α-L-fucose (α-Fuc) and β-galactose (β-Gal) had little effect. α-Man also inhibited CPE at a lower (5 mM) concentration (not shown, results similar to those shown above for 100 mM concentration).

Monolayer when α-Man was present. In addition, α-Man almost completely inhibited amoeba-induced CPE. Cell-free plaques were not detected in the presence of α-Man (Figs. 3 and 5, top panel, group M). α-Man, however, did not inhibit the lifting of the intact monolayer from the cell substratum upon “prolonged” (24–28 h) incubation of the cultures with amoebae (Fig. 5, middle and bottom panels, groups M). In the absence of α-Man, nearly complete loss of cell layer occurred around 20 h; in the presence of α-Man (50 mM), after an approximate 24-h incubation with the amoebae, the cell layer began to lift from the periphery (Fig. 5, middle panel, group M) and continued to roll inwards until the entire layer was lifted from the dish (Fig. 5, bottom panel, group M). This occurred more than 4 h after the complete loss of the cell layer had occurred in the cultures incubated without the sugar. When GlcNAc (50 mM) was present in the media, amoebae adhered tightly to the monolayer. Despite this, the cell layer remained attached to the culture dish during the entire assay period of 28 h (Fig. 5, middle and bottom panels, groups G). GlcNAc markedly delayed, but did not completely inhibit plaque formation. Upon careful examination of the cultures incubated with amoebae in the presence of GlcNAc for 28 h, small plaques could be seen in the monolayer (Fig. 5, bottom panel, group G). Since CPE assays were performed in the serum-free media, incubations were not continued beyond 28 h.

α-Man but Not GlcNAc Is a Potent Inhibitor of Ameoba-induced Cytolysis—Cell lysis as measured by ⁵¹Cr release revealed that Acanthamoeba possesses cytolytic activity (Fig. 6, E + A). The extent of cytosis, however, did not correlate with the extent of CPE; when approximately 50% cell loss had occurred in the CPE assays, percent specific ⁵¹Cr release was about 12.5%. Greater than 70% of amoeba-induced cytosis was inhibited by α-Man (50 mM) (Fig. 6, E + A + M). In contrast, GlcNAc was only a weak inhibitor of amoeba-induced cytosis with inhibition values ranging between 10 and 20% (Fig. 6, E + A + G). Similar observations were made using LDH release assays (data not shown).

GlcNAc but Not α-Man Inhibits ACM-induced CPE—Early studies have shown that cell-free ACM also has the ability to produce CPE. The CPE-inducing ability of ACM is likely to be independent of host-parasite interactions, and related to the cytotoxic secretory factors of Acanthamoeba. To determine the role of secretory factors of the parasites in the amoeba cytopathogenic mechanisms, ⁵¹Cr release and cytotoxic assays were also performed using cell-free ACM. ⁵¹Cr release assays revealed that ACM lacks cytolytic activity (Fig. 6, E + ACM). Also, ACM failed to produce cell-free plaques in the monolayer.
**Cytopathogenic Mechanisms of Acanthamoeba**

![Figure 4](http://www.jbc.org/)

**Top,** α-Man but not GlcNAC is a potent inhibitor of *Acanthamoeba* adhesion to Man-BSA. Microtiter wells coated with Man-BSA were incubated with 35S-labeled amoebae (50 μl, 5 × 10^5 parasites/ml, 2 counts/min/parasite) in the presence and absence of varying concentrations of α-Man and GlcNAC for 2 h. Amoeba binding to each well was determined as described under “Experimental Procedures.” A value of 1.0 was assigned to the binding value of control wells incubated with amoebae in media alone. The values for wells incubated with amoebae in the presence of sugars are expressed as change in binding values with respect to controls. Note that α-Man inhibited the adhesion of amoebae to Man-BSA coated wells in a dose-dependent manner. Compared with α-Man, GlcNAC was approximately a 25 times weaker inhibitor of *Acanthamoeba* adhesion to Man-BSA (concentration required for 50% inhibition: α-Man, 1.96 mM; GlcNAC, 50 mM, see Table I).

**Bottom,** both α-Man and GlcNAC are equally potent inhibitors of *Acanthamoeba*-induced CPE. Confluent cultures of corneal epithelium in 24-well plates were incubated with *Acanthamoeba* in the presence and absence of varying concentrations of α-Man and GlcNAC for 16 h. At the end of the incubation period, the plates were stained with Giemsa and scanned to estimate approximate cell density. A value of 1.0 was assigned to the cell density of the plates incubated in media alone (Cont.). The values for cultures incubated with amoebae are expressed as change in the density with respect to control plates. Note that both α-Man and GlcNAC inhibited the amoeba-induced CPE in a dose-dependent manner; the inhibition was: 60% at 0.31 mM concentration for α-Man, 1.96 mM; GlcNAC, 50 mM, see Table I).

**DISCUSSION**

To further determine the role of proteinases in the cytopathogenic mechanisms of *Acanthamoeba*, the effect of proteinase inhibitors on amoeba- and ACM-induced CPE on corneal epithelial cells was analyzed. Serine proteinase inhibitors, PMSF (0.5 mM) as well as aprtinin, (5 units/ml), almost completely inhibited both amoeba- and ACM-induced CPE (not shown, results identical to those of CPE assays performed in the presence of GlcNAC, Fig. 5). The metalloproteinase inhibitor 1,10-phenanthroline was toxic to corneal epithelial cells, and therefore it was not possible to evaluate its CPE inhibitory potential.

**Proteinase Inhibitors Inhibit both Amoeba- and ACM-induced CPE**

The goals of the present study were to define more precisely the carbohydrate binding properties of the *Acanthamoeba* mannos-specific lectin and to determine whether carbohydrate-mediated adhesion of amoebae to host cells is a necessary prerequisite for the parasite-induced CPE. The results of the...
saccharide inhibition assays revealed that among monosaccharides, the amoebic lectin has the highest affinity for α-Man. The inhibitory potency of D-mannose was 10-fold less than that of α-Man. Our results, that the epimers of D-mannose, altrose (C-3), and talose (C-4) were not inhibitory and glucose (C-2) was only a weak inhibitor, suggest that the configuration of α-Man is necessary for optimal binding interactions. The fact that α-lyxose was not inhibitory supports the notion that the CH₂OH substituent is also essential for binding. The results of inhibitory potencies of both mannobioses, the mannotriose, and the mannopentose suggest that Man(1–3)Man disaccharide is the most complimentary to the carbohydrate-binding site of the amoebic lectin being three times more potent than the corresponding α-1–6-disaccharide. Elongation of the Man(1–3) chains by additional mannone residues increased the affinity only slightly.

In vivo, pathology of Acanthamoeba infection is characterized by the infiltration and necrosis of the infected tissue (1, 2) and, as described earlier, the parasite has also been shown to cause CPE on a variety of cell types in vitro (5, 9–13). Although it has been presumed that the adhesion of amoeba to host cells is a critical first step in the pathogenesis of infection, several studies have reported that cell-free ACM contain destructive proteases (23–25) and phospholipases (26). Thus, one could argue that if the amoebae elaborate digestive enzymes, target cell loss may well be independent of host-cell contact. The present study revealed that the trophozoite-induced target cell loss resulted from at least four sequential steps: (i) adhesion of amoeba to target cells; (ii) cytolysis of target cells; (iii) development of cell-free plaques in the monolayer which increase in size with time; and (iv) detachment of the monolayer surrounding the plaques from the culture dish.

What is the likely mechanism by which amoeba produce cell-free plaques in the monolayer? It appears that the first...
Confluent cultures of corneal epithelium were incubated with: media ACM was prepared in the media containing the sugar. Note that filtrate and retentate obtained by filtration of ACM and ACM the media alone. GlcNAc did not inhibit the CPE if it was added to the ACM prepared in only if it was present in the media used for the preparation of ACM. did not inhibit the ACM-induced CPE and GlcNAc inhibited the CPE not result in the loss of CPE inhibitory activity of the ACM. ACM produced in the presence of the sugar by Centricon filtration did Centricon tubes. Note that: (i) almost all of the CPE-inducing ability of parasites/ml, and ACM prepared in the serum-free media containing 50 mM GlcNAc (lane ACM+G) compared with that prepared in the media alone (lane ACM). In contrast, the zymography pattern of the ACM prepared in the presence of α-Man was indistinguishable from the ACM prepared in media alone (not shown). C, components P1, P2, and P4 were susceptible to 1,10-phenanthroline, an inhibitor of metalloproteinases.

The actual mechanisms of Acanthamoeba-induced cytolysis and CPE are not known. It has been reported that pathogenic strains of Acanthamoeba produce significantly higher quantities of phospholipases (26), fibrinolytic enzymes (23), and cysteine proteinases (25) compared with nonpathogenic strains. Moreover, a correlation between pathogenicity and proteinase activity has been reported for a number of protozoans (27) including Entamoeba histolytica (28, 29), Giardia lamblia (30), Leishmania amazomen (31), and Trypanosoma cruzi (32). In the present study, we found, not only that Acanthamoebae secrete proteinases but also that carbohydrate-mediated adhesion of amoebae to host cells has a profound effect on the nature of the proteinases secreted by the parasite and/or host cells. We found that a specific metalloproteinase (P3) was secreted into the culture media only upon mannose-mediated direct contact of the parasite to target cells. Although it remains to be determined whether P3 is produced by the host cells or the parasite and whether it is the expression, the activity, or merely the secretion of P3 which is elevated upon the adhesion of amoeba to the host cells, it is clear that contact-mediated cross-talk between the parasite and the host cells is a key component of Acanthamoeba infection.

Studies by Alizadeh and co-workers (33) have shown that the Acanthamoeba-induced cell death is due, at least in part, to apoptosis. A recent study has shown that target cells killed by E. histolytica also undergo DNA fragmentation characteristic of apoptotic death (34). Killing of the host cells by E. histolytica
in vitro occurs only upon direct contact which is mediated by a Gal/GalNAc-specific amoebic surface lectin (35). The contact-dependent cytolyis of E. histolytica has been attributed at least in part to amoebic pore-forming proteins and phospholipase A activity, both of which are thought to influence cytopathogenicity by disrupting target cell membranes thereby rendering the cell permeable to attack by other amoebic enzymes or toxins (36, 37). The mechanism by which Acanthamoeba induce apoptotic death of the target cells has not been elucidated. Our studies suggest that the mannose-modified adhesion of amoebae to host cells followed by secretion of proteinases, especially P3, are likely to be key events.

An unexpected finding of the present study was the observation that GlcNAc which is neither an inhibitor of the adhesion of amoeba to target cells nor an inhibitor of amoeba-induced cytolsys is a potent inhibitor of amoeba-induced CPE. It appears that GlcNAc inhibits CPE indirectly by influencing the expression and/or secretion of the molecules involved in cytopathogenic mechanisms of Acanthamoeba because: (i) the GlcNAc only inhibited the CPE when it was added to the culture medium used to produce ACM and not when it was added to the ACM after it was produced in the media alone; and (ii) removal of GlcNAc from the ACM by Centricon filtration did not result in the loss of CPE inhibitory activity of the ACM.

It appears that GlcNAc inhibits CPE indirectly by influencing the expression and/or secretion or the molecules involved in the pathogenic mechanisms of Acanthamoeba. This is supported by the observation that glucose inhibition assays, regardless of the mechanism, the use of GlcNAc in conjunction with α-Man-based saccharides may have therapeutic potential specially because the saccharides are nontoxic and can be delivered topically to the eye either in the form of eye drops or from contact lenses designed to deliver small continuous doses of drugs into the eye.

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