Acanthamoeba Binds to Extracellular Matrix Proteins in Vitro

Vicki R. Gordon,* Elikplimi K. Asem,† Michael H. Vodkin,* and Gerald L. McLaughlin*

Purpose. To identify host-tissue amoeba interactions that may be important in the pathogenesis of Acanthamoeba keratitis, the ability of the opportunistic pathogen Acanthamoeba polyphaga to bind various components of the extracellular matrix (collagen type IV, laminin, or fibronectin) was examined in vitro.

Methods. A. polyphaga, isolated from a case of human amoebic keratitis, was used in the studies. In the experiments, 96-well plates were coated with 0-, 5-, 10-, 20-, or 50-μg/ml solutions of the basal lamina proteins laminin or collagen type IV, the extracellular matrix protein fibronectin, or casein (control). Amoeba were metabolically labeled with 35[S]-methionine, and 1×10⁴ labeled amoeba in phosphate buffered saline (PBS) were seeded per well and allowed to bind for 20 min. After washing with PBS, bound amoeba were solubilized with 1% sodium dodecyl sulphate (SDS) and scintillation counting was used to determine the number of bound amoeba.

Results. Counts from casein and protein-free controls were not significantly different from each other (P>0.05). There was a significant increase in the binding of 35[S]-labeled A. polyphaga to collagen IV, laminin, and fibronectin over controls (P<0.0001) and the binding was concentration-dependent. The rank order of binding was collagen>>laminin>fibronectin. Alpha-methyl-mannopyranoside, but not fucose, inhibited binding of labeled A. polyphaga to collagen IV, laminin, and fibronectin in a concentration-dependent manner.

Conclusion. In summary, the binding assays show that Acanthamoeba bind preferentially to collagen, laminin, and fibronectin, in that order, and that the adherence process is inhibited by mannoside. Invest Ophthalmol Vis Sci. 1993;34:658-662.

Some species of Acanthamoeba are opportunistic pathogens that cause amoebic keratitis, a vision-threatening and painful eye infection that has been correlated with contact lens wear.¹ Acanthamoeba keratitis is difficult to treat effectively, and an understanding of the pathogenesis of the disease will aid in the development of more effective treatments. Adherence is often the first step in the virulence process for microbial pathogens, and several reports have described the binding characteristics of Acanthamoeba to corneas,²,³ primary cultures of corneal cells,⁴,⁵ and corneal fibroblasts.²,⁶ The addition of carbohydrates such as α-methyl-D-mannopyranoside has been reported to inhibit the binding process to these complex substrates.²,⁴,⁷ However, the host tissue constituents that are important to the establishment of this infection are not known. We have previously reported that the amoeba tend to bind and invade at the junctures between the corneal epithelial cells in cornea cup models.² However, no reports have appeared describing the binding of Acanthamoeba to purified molecules of either the extracellu-
Acanthamoeba Binding to Proteins

Acanthamoeba polyphaga (CDC:0187:1) isolated from a case of human keratitis was grown axenically at 25°C in peptone-yeast extract-glucose (PYG) medium.8 Inoculation size and culture times were adjusted to allow harvest at the late log phase of growth, ie, the slowing growth phase before cyst formation begins, but after many of the amoeba have detached from the flask. We have extensive experience with growth characteristics of Acanthamoeba and confirm with each experiment that at least 95% trophozoites are used. Suspended late log phase trophozoites were harvested, washed, counted using a hemocytometer, and suspended in buffer for labeling and binding studies.

Metabolic Labeling
A. polyphaga (5×10^6 in 100 μl of buffer [0.85% NaCl, 10 mM Hepes, pH 6.8]) was incubated for 45 min with 100 μCi of [35S]methionine (specific activity 40 TBq/mmol; Amersham Corp., Arlington Heights, IL). The mixture was gently agitated with finger taps every 15 min. The metabolically labeled amoeba were resuspended and centrifuged in a micro centrifuge (Fisher Scientific model 235C) by flick spin (briefly depressing the start button in the off position two times quickly) two times with 1 ml of PBS (0.8% NaCl, 8.2 mM Na2HPO4, 2.7 mM KCl, 1.5 mM KH2PO4, pH 7.4) and suspended in PBS at a density of 1×10^6 cells/ml.

Coating of 96-Well Plates
The plates used in the described experiments were irradiated 96-well tissue culture clusters from Costar (Cambridge, MA), but the relative degree of binding was unaltered using untreated tissue culture plates (data not shown). Culture plate wells were coated with varying concentrations of collagen, laminin, fibronectin, or casein. Collagen type IV and laminin were isolated from mouse tumor cells (Engelbreth-Holm-Swarm) and were supplied by Collaborative Biomedical Products (Bedford, MA). The fibronectin used for most experiments was derived from the rat and was supplied by Calbiochem (San Diego, CA), but human fibronectin from Collaborative Biomedical Products was also used with similar results (VRG, data not shown). Casein was purchased from Sigma Chemical Co. (St. Louis, MO). The plate wells were coated for 1 hr at room temperature or overnight at 4°C with 100 μl of 0-, 5-, 10-, 20-, or 50-μg/ml solutions of either collagen type IV, laminin, fibronectin, or a casein control. Four wells were used for each protein concentration and for the control. All solutions were made using PBS except collagen type IV, which was dissolved in 0.05N HCl. The wells were decanted and rinsed two times with 200 μl PBS, by inverting the unlined microtiter plates rapidly and tapping the fluid onto absorbent napkins.

Amoeba Binding to Proteins
To determine the binding characteristics of A. polyphaga in the coated and uncoated wells, a 100-μl aliquot containing 1X10^4 washed, labeled amoeba was added to each well. Aliquots of labeled amoeba were also removed from the metabolically labeled amoeba stock for scintillation counting. The integrity of the seeded amoeba was determined by direct observation; at least 95% intact trophozoites were used in all experiments. By observation, the amoeba had settled to the bottom of the wells within 5 min, but the attachment process was allowed to proceed for a total of 20 min before decanting the supernatant and sequentially rinsing the wells two times with 200 μl PBS as described above. The amoeba remaining in each well were solubilized in 100 μl of 1% sodium dodecyl sulfate (SDS) for 10 min. After several mixes with a pettte, half of the solubilized material was removed from each well and scintillation counting was used to determine the degree of amoeba binding. The data were analyzed by analysis of variance (ANOVA) using the Graphpad Instat statistical package. Student’s t-test was used where applicable.

Protein Binding in the Presence of α-Methylmannopyranoside
Plate wells were coated with 10-μg/ml solutions of collagen type IV, laminin, fibronectin, or casein, as described above. Varying concentrations of α-methylmannopyranoside (20, 100, or 200 mM) or 10 mM fucose (Sigma) were prepared in 0.01 M NaHPO4, 0.12 M NaCl, 1 mM MgCl2, 0.01 M NaHPO4, pH 6.8. The addition of 50 μl of sugar solution to each well was immediately followed by the addition of 1X10^4 labeled amoeba in 50 μl to make final sugar concentrations of 10, 50, or 100 mM. Amoeba were allowed to bind for 20 min, washed, and scintillation counts of solubilized cells were determined as described above. The data were analyzed by ANOVA. No humans were used.

RESULTS
Amoeba binding was assessed using microtiter plate wells coated with proteins. In preliminary experi-
ments, representative microtiter plate wells were examined using an inverted microscope. Amoeba in un-washed wells coated with collagen, laminin, or fibronectin were typically more flattened than in uncoated wells or wells coated with casein. Cells were also inspected microscopically to estimate the uniformity and efficiency of the incubation and washing procedures and the relative degree of cell binding; these qualitative assessments agreed well with quantitative data. For quantitative comparisons, low cell densities (1X10^4 cells/well) were used to minimize amoeba-amoeba interactions in the time frame of the experiment. For comparative binding experiments, quadruplicate counts were averaged.

Results are summarized for a typical experiment (Table 1). The counts bound to casein-coated wells were not significantly different from uncoated or buffer-coated control wells or the scintillation counter blank. However, collagen, laminin, and, to some extent, fibronectin served as efficient substrates for amoeba binding. In wells coated with 5 vs 10 µg/ml solutions of protein, there was a significant (P < 0.0005) increase in bound counts (bound amoeba) for collagen, laminin, and fibronectin. Maximum counts were observed in wells coated with 20 µg/ml of these proteins, although the degree of binding was not significantly different from the binding to wells coated with 10 µg/ml of these proteins. A small but consistent drop in binding was observed for wells coated with 50 µg/ml of these proteins, and this drop was statistically significant at the 95% confidence level for collagen and laminin, relative to binding to the corresponding 10 and 20 µg/ml-coated wells. The reason for this drop is not known, but 10 µg/ml protein concentrations were selected for routine binding experiments. For collagen at 10 µg/ml, 58% of the amoeba counts were bound, while laminin, fibronectin, casein, and uncoated wells had 48%, 12%, 0, and 0 bound, respectively. The average numbers of amoeba bound for each condition are summarized in Table 1.

Our results demonstrate for the first time that Amoeba binds to extracellular matrix proteins known to be a part of the eye and conjunctiva. The data also show that the binding is inhibited by

### Table 1. Amoeba Binding to Varying Concentrations of Extracellular Matrix Proteins or Casein

<table>
<thead>
<tr>
<th>Concentration µg/ml</th>
<th>Protein µg/ml</th>
<th>0</th>
<th>5 µg/ml</th>
<th>10 µg/ml</th>
<th>20 µg/ml</th>
<th>50 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caein</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Collagen</td>
<td>0.368 ± 0.06</td>
<td>58.0 ± 2.2</td>
<td>59 ± 7.6</td>
<td>44.2 ± 3.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibronectin</td>
<td>0.78 ± 0.15</td>
<td>11.8 ± 0.8</td>
<td>12.6 ± 0.92</td>
<td>8.8 ± 2.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laminin</td>
<td>0.358 ± 0.5</td>
<td>47.6 ± 9.2</td>
<td>47.8 ± 4.2</td>
<td>43.2 ± 8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Metabolically labeled amoeba (1 x 10^9 per well) were allowed to adhere 20 min to protein-coated wells. Scintillation counts of washed wells were used to determine the number of [35S]-labeled amoeba bound.

* Numbers represent the mean number of amoeba (x10^2) bound in quadruplicate observations, ± standard deviation. Maximal binding (50 x 10^3 amoeba) was observed at 20 µg/ml collagen. Background counts ranged between 36 and 57 cpm. An average background count of 42 cpm was subtracted from each well.

### Table 2. Effects of α-Methyl-Mannopyranoside (AMM) and Fucose on Amoeba Binding. (Numbers = Percent of Sugar-Free Control)

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Collagen</th>
<th>Laminin</th>
<th>Fibronectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>10 mmol/l fucose</td>
<td>105% ± 13%*</td>
<td>90% ± 5%*</td>
<td>95% ± 11%*</td>
</tr>
<tr>
<td>10 mmol/l AMM</td>
<td>69% ± 3%</td>
<td>52% ± 2%</td>
<td>16% ± 4%</td>
</tr>
<tr>
<td>50 mmol/l AMM</td>
<td>19% ± 5%</td>
<td>21% ± 5%</td>
<td>9% ± 5%†</td>
</tr>
<tr>
<td>100 mmol/l AMM</td>
<td>11% ± 4%</td>
<td>16% ± 2%</td>
<td>0.6% ± 2%†</td>
</tr>
</tbody>
</table>

35[S]-met-labeled amoeba (1 x 10^4) were allowed to bind for 20 min to protein-coated 96-well plate wells. The 100% values for collagen, laminin, and fibronectin were 464, 440, and 199 cpm, respectively. Numbers represent the percentage of bound counts relative to sugar-free controls for each protein. Background binding (43 cpm) was subtracted from all samples. *There was no significant difference between the binding of amoeba in the presence or absence of fucose.

† Values are not significantly different from zero binding (P > 0.05).

The binding of amoeba to the extracellular matrix proteins was greatly suppressed by the presence of α-methyl-mannopyranoside (AMM) (Table 2). The decrease in amoeba binding in the presence of AMM was concentration-dependent. AMM (10mM) decreased amoeba binding to fibronectin, laminin, and collagen IV by 83, 48, and 32%, respectively. A 50-mM AMM concentration decreased binding to collagen and laminin by 80 and 79%, respectively, while binding to fibronectin was completely inhibited. As in the first experiment, no amoeba were bound to uncoated wells (data not shown). The binding of amoeba to fibronectin in the presence of 50 and 100 mM AMM was not significantly different (P>0.05) from the binding seen in uncoated wells. Inhibition of binding by mannosyl sugars has been previously reported for the binding of Acanthamoeba to both corneal tissue and yeast. There was no significant difference (P>0.05) in the binding of amoeba to collagen, laminin, or fibronectin in the presence or absence of 10 mM fucose. Table 2 summarizes an experiment that evaluates the degree of amoeba binding to the tested proteins in the presence of sugars, expressed as a percentage of sugar-free controls.

### DISCUSSION

Our results demonstrate for the first time that Acanthamoeba binds to extracellular matrix and basal lamina proteins known to be a part of the eye and conjunctiva. The data also show that the binding is inhibited by
Acanthamoeba Binding to Proteins

\(\alpha\)-methyl-mannopyranoside. The relative degree of binding suggests that collagen, laminin, and, to a lesser degree, fibronectin are excellent receptors for amoeba. Several researchers have begun to describe the distribution of these molecules in the normal and wounded cornea.\(^{10-13}\) Knowledge of the distribution of these proteins in infected and uninfected corneas will better reveal their possible role in the pathogenesis of Acanthamoeba keratitis. Recent studies indicate that parasitic microorganisms, bacteria, and yeasts use attachment to adhesive proteins, such as fibronectin, laminin, and other extracellular matrix proteins, to enhance their infectivity.\(^{13,14}\) Significantly, the binding of several different protozoan parasites to fibronectin promotes the adherence of the pathogens to host cells.\(^{15-17}\) Elucidation of the nature of interactions between amoeba and matrix proteins may suggest interventions to the process and to amoebic keratitis.

The mechanism of interaction between A. polyphaga and the extracellular matrix glycoproteins is not known. A number of reports postulate that, like host cells, bacteria, yeast, and protozoa have specific binding sites for extracellular matrix glycoproteins.\(^{18-21}\) The lack of binding of Acanthamoeba to casein or plastic and the inhibition of amoeba binding to fibronectin, laminin, and collagen by \(\alpha\)-methyl-mannopyranoside may indicate a common adhesive mechanism between Acanthamoeba and the three active substances. The literature also suggests that a mannose-sensitive binding process is important to the binding, feeding, and pathogenesis of Acanthamoeba.\(^{4,7,20}\) which is consistent with the current data. The glycosylation pattern of fibronectin and collagen IV are not similar, and the glycosylation pattern for laminin is not known.\(^{22}\) The carbohydrates on fibronectin are mostly the result of N-linked glycosylation, though O-linked side chains are also present. Internal mannose, N-acetylgalactosamine, and galactose residues are on the N-linked chains. Some of the side chains bind to concanavalin A,\(^{23}\) a lectin that binds terminal and internal mannose residues, as well as glucose and N-acetylgalactosamine.\(^{24}\) Collagen IV has short, hydroxylsine O-linked glycosylation with terminal glucose or galactose residues.\(^{22}\) The mannose inhibitable binding of Acanthamoeba to the extracellular matrix proteins may be due to a lectin-like carbohydrate-binding molecule on the surface of the amoeba. The rank order of A. polyphaga adhesion to the extracellular matrix glycoproteins in the present communication (collagen>laminin>fibronectin) could be due to variations in the number(s) of binding sites for a particular receptor protein per amoeba or variation in the affinities between the postulated adhesive and receptor(s). Casein, which was used as a protein control, is not glycosylated and may leave questions as to whether the binding was specific for the protein or the carbohydrate moieties. Additional studies may help to distinguish between these and other possible explanations. It should also be possible to determine the relative abundance and distribution of these and other putative receptor molecules in areas associated with amoebic keratitis.

**Key Words**

adhesion, Acanthamoeba, collagen IV, fibronectin, laminin.

**References**


