A mannose binding protein is involved in the adherence of *Acanthamoeba* species to inert surfaces

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Abstract

Some carbohydrates are known to decrease the attachment of *Acanthamoeba* sp. to biological surfaces. By a method based on the reduction of a tetrazolium salt (XTT) by the mitochondrial dehydrogenases of the parasites, D-mannose and α-D-mannopyranoside have been shown to reduce *Acanthamoeba* attachment to inert surfaces, indicating that the mannose binding protein of *Acanthamoeba* trophozoites is involved in adherence to inert surfaces. The reduction in attachment is dose dependant and is not linked with a potential toxicity of the carbohydrates. All the species of *Acanthamoeba* tested were concerned by this mannose binding protein, but the adhesion of *A. culbertsoni* was also reduced by the presence of glucose.

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Keywords: *Acanthamoeba*; Adherence; Inert surface; Mannose

1. Introduction

Some species of the genus *Acanthamoeba* cause keratitis, an ulcerative infection of the cornea, mainly associated with contact lens wear [1]. The contact lens acts as a mechanical vector transferring amoeba, that may have become attached to the lens through inadequate cleaning, onto the ocular surface. The predisposition of *Acanthamoeba* for lens attachment can be affected by the type of material used in the lens, the type of lens wear, and the presence of bacterial biofilm on the lens surface [2]. Once in the ocular environment, parasite binding to the corneal epithelium is believed to be a critical first step in the pathogenesis of keratitis [3]. The adherence of *Acanthamoeba* to the physical components and/or biofilms components is also believed to take place in the process occurring in water biofilms [4]. Moreover, both trophozoites and cysts can serve as reservoirs for bacteria with human pathogenic potential [5].

Previous works have dealt with the attachment of *Acanthamoeba* to different surfaces, in particular contact lenses, in order to reduce the risk of keratitis associated with these materials [6,7]. Some of these studies have used metabolically labelled trophozoites (using 35S methionine or 35S cysteine) to quantify the adherent *Acanthamoeba* by measuring the radioactivity in a scintillation counter [7]. Other authors have counted the adsorbed amoebae on the studied surface by direct microscopic observation [6]. The aim of this work is to apply a simple and reproducible semi-quantitative method to evaluate the adherence of *Acanthamoeba* to inert sur-
faces, then use this method to determine Acanthamoeba adherence in the presence of carbohydrates, as carbohydrates have been shown to inhibit the attachment of these amoeba to biological surfaces [7,8].

2. Materials and methods

2.1. Acanthamoeba strains

Acanthamoeba castellanii ATCC 30234, A. culbertsoni ATCC 30171, A. polyphaga ATCC 30461 and A. castellanii ANPV-1-92, A. hatchetti ABAT-A3 and A. polyphaga V8A (generous gift from Pr Scaglia, Department of Microbiology, Pavia, Italy) were grown at room temperature for three days in 250 ml tissue culture flasks (Nunc, Denmark) containing 25 ml of antibiotic free peptone-yeast extract-glucose (PYG) broth [9].

2.2. Adherence assays

The amoebae were harvested by tapping the tissue flasks containing a monolayer of cells, pelleted with centrifugation, and resuspended in phosphate buffered saline (PBS) following two washes in that same buffer. The adherence test was then performed in 96 well-tissue plates untreated for cell culture (Evergreen Scientific, Los Angeles, CA, USA). Tetrazolium salt 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) was used to assess the adherence of the amoeba to the wells of the plates. The principle of the test was based upon the reduction of XTT tetrazolium to tetrazolium formazan by mitochondrially active Acanthamoeba trophozoites in the presence of an electron-coupling agent called menadione. This method is usually employed in our laboratory for the study of adherence of Candida strains [10]. The trophozoites of Acanthamoeba were counted with a hemocytometer and added to the wells of a 96-well plate as inocula of $2 \times 10^4$ in 200 µl PBS. This number of amoebae was selected to obtain confluence after a 1 h incubation period. The trophozoites were allowed to adhere to polystyrene for 1 h at 27°C. Half of the wells were then gently washed twice with PBS in order to remove non-adherent trophozoites. Thereafter, 600 µg/ml XTT (Sigma) and 0.26 mM menadione (Sigma, St Louis, MO, USA) were added to each well (washed and unwashed), and the plate incubated for 3 h at 37°C without shaking. Plates were then gently agitated and XTT formazan was measured at an absorbance of 492 nm (microplate reader LP 400, Sanofi Diagnostics Pasteur). The metabolic activity was measured in both washed and unwashed wells. Absorbance units obtained in washed wells corresponded to the mitochondrial dehydrogenase activity of adherent trophozoites whereas those obtained in unwashed wells corresponded to the dehydrogenase activity of the overall population. The adherence capacity of the Acanthamoeba strains was calculated by taking the average of the absorbance units collected in the washed wells and dividing it by the average of the absorbance units collected in unwashed wells, then multiplied by 100. Background formazan values were obtained with wells containing PBS only or PBS plus XTT and menadione. These values did not exceed 0.006 absorbance units which were not significant. All experiments were performed at least twice in triplicate. The percentage of adherent trophozoites was directly verified by microscope for each strain in one of the wells from each test. Prior to these experiments, the correspondence between some concentrations of Acanthamoeba trophozoites (from $0.62 \times 10^3$ to $4 \times 10^4$ per well) and the absorbance values of tetrazolium formazan after reduction by mitochondrial parasitic dehydrogenases was determined.

2.3. Effect of carbohydrates on Acanthamoeba attachment

For inhibition of adherence assays, the Acanthamoeba strains were grown in PYG medium as described above. Before the adherence test, the trophozoites were pre-incubated for 30 min in PBS supplemented with 100 mM of each saccharide [7]. The various saccharides used were D-mannose, D-glucose, D-galactose, D-lactose and L-rhamnose (all chosen to be major carbohydrates in biofilms), and α-D-mannopyranoside. The adherence assays were then performed in PBS supplemented with 100 mM of each compound throughout the experiments. To determine if the inhibitory effect of some carbohydrates on adherence was dose dependent, concentrations of 10 and 1 mM of these carbohydrates were tested.

2.4. Evaluation of the toxicity of carbohydrates on Acanthamoeba

The toxicity of each carbohydrate on Acanthamoeba trophozoites has been evaluated by examination of the wells with an inverted microscope after 1, 6, 24 and 48 h of incubation. Any detectable changes in morphology or motility was noted. Moreover, evaluation of cell death was determined with toluidine blue staining.

2.5. Statistical analysis

For statistical analysis, the Mann–Whitney test was used to determine whether saccharides had a significant effect on the adherence of Acanthamoeba strains to the tested surface.
3. Results

3.1. Adherence assays

Table 1 shows the correspondence between the concentrations of *Acanthamoeba* trophozoites and the absorbance values (A\textsubscript{492 nm}) of the tetrazolium formazan obtained after reduction of XTT by the parasitic mitochondrial dehydrogenases.

### Table 1
Concentrations of *Acanthamoeba* trophozoites and absorbance values (A\textsubscript{492 nm}) obtained after reduction of XTT by mitochondrial parasitic dehydrogenases of the tested strains

<table>
<thead>
<tr>
<th><em>Acanthamoeba</em> trophozoites concentration (per well)</th>
<th>A. castellanii ANPV-1-92</th>
<th>A. polyphaga V8A</th>
<th>A. hatchetti ABA-A3</th>
<th>ATCC 30234</th>
<th>A. polyphaga ATCC 30461</th>
<th>A. culbertsoni ATCC 30171</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 × 10^4</td>
<td>0.649 ± 0.012</td>
<td>0.856 ± 0.023</td>
<td>0.658 ± 0.013</td>
<td>0.682 ± 0.024</td>
<td>0.679 ± 0.017</td>
<td>0.764 ± 0.028</td>
</tr>
<tr>
<td>2 × 10^4</td>
<td>0.532 ± 0.011</td>
<td>0.619 ± 0.015</td>
<td>0.557 ± 0.009</td>
<td>0.616 ± 0.020</td>
<td>0.548 ± 0.011</td>
<td>0.566 ± 0.022</td>
</tr>
<tr>
<td>1 × 10^4</td>
<td>0.302 ± 0.007</td>
<td>0.524 ± 0.011</td>
<td>0.327 ± 0.005</td>
<td>0.555 ± 0.019</td>
<td>0.318 ± 0.009</td>
<td>0.387 ± 0.017</td>
</tr>
<tr>
<td>5 × 10^3</td>
<td>0.209 ± 0.005</td>
<td>0.312 ± 0.005</td>
<td>0.221 ± 0.003</td>
<td>0.399 ± 0.014</td>
<td>0.218 ± 0.004</td>
<td>0.212 ± 0.008</td>
</tr>
<tr>
<td>2.5 × 10^3</td>
<td>0.139 ± 0.004</td>
<td>0.211 ± 0.003</td>
<td>0.200 ± 0.003</td>
<td>0.272 ± 0.008</td>
<td>0.150 ± 0.004</td>
<td>0.145 ± 0.007</td>
</tr>
<tr>
<td>1.25 × 10^3</td>
<td>0.099 ± 0.002</td>
<td>0.138 ± 0.003</td>
<td>0.172 ± 0.004</td>
<td>0.156 ± 0.003</td>
<td>0.105 ± 0.003</td>
<td>0.101 ± 0.003</td>
</tr>
<tr>
<td>0.62 × 10^3</td>
<td>0.075 ± 0.002</td>
<td>0.099 ± 0.001</td>
<td>0.056 ± 0.001</td>
<td>0.048 ± 0.001</td>
<td>0.080 ± 0.001</td>
<td>0.079 ± 0.002</td>
</tr>
</tbody>
</table>

Values are means ± standard deviations.

3.2. Effects of carbohydrates on *Acanthamoeba* attachment

Considering the six tested saccharides, D-mannose and α-D-mannopyranoside partially inhibited the adhesion of *Acanthamoeba* trophozoites to inert surfaces (Table 2). The decrease of the attachment of *A. polyphaga* ATCC 30461 was weak when compared with other strains. In addition, the incubation of the trophozoites

### Table 2
Effect of saccharides on the adherence capacity (in %) of *Acanthamoeba* strains

<table>
<thead>
<tr>
<th>Saccharide</th>
<th>A. castellanii ANPV-1-92</th>
<th>A. polyphaga V8A</th>
<th>A. hatchetti ABA-A3</th>
<th>ATCC 30234</th>
<th>A. polyphaga ATCC 30461</th>
<th>A. culbertsoni ATCC 30171</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>55 ± 3</td>
<td>57 ± 2 NS</td>
<td>54 ± 2 NS</td>
<td>34 ± 0.7</td>
<td>51 ± 1 NS</td>
<td>50 ± 2 NS</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>53 ± 4</td>
<td>55 ± 1 NS</td>
<td>54 ± 1 NS</td>
<td>40 ± 1</td>
<td>59 ± 2 NS</td>
<td>54 ± 2 NS</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>43 ± 1</td>
<td>42 ± 2 NS</td>
<td>40 ± 2 NS</td>
<td>26 ± 2</td>
<td>38 ± 4 NS</td>
<td>38 ± 1 NS</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>51 ± 3</td>
<td>48 ± 2 NS</td>
<td>49 ± 1 NS</td>
<td>37 ± 1</td>
<td>53 ± 1 NS</td>
<td>47 ± 2 NS</td>
</tr>
<tr>
<td>D-Lactose</td>
<td>64 ± 3</td>
<td>70 ± 4 NS</td>
<td>69 ± 3 NS</td>
<td>49 ± 3</td>
<td>69 ± 2 NS</td>
<td>70 ± 3 NS</td>
</tr>
<tr>
<td>L-Rhamnose</td>
<td>47 ± 4</td>
<td>44 ± 6 NS</td>
<td>48 ± 3 NS</td>
<td>11 ± 0.7</td>
<td>53 ± 3 NS</td>
<td>54 ± 4 NS</td>
</tr>
<tr>
<td>α-D-Mannopyranoside</td>
<td>55 ± 3</td>
<td>34 ± 0.7 NS</td>
<td>53 ± 1 NS</td>
<td>52 ± 2 NS</td>
<td>15 ± 1 NS</td>
<td>53 ± 2 NS</td>
</tr>
</tbody>
</table>

Control: adherence of the strain without carbohydrate.
Adherence capacity significantly decreased (\(^\wedge\)): Mann–Whitney test, \(p \leq 0.001\).
NS: non-significant.
Values are means ± standard deviations.

### Table 3
Effect dose-dependant of D-mannose and α-D-mannopyranoside on the adherence capacity (in %) of *Acanthamoeba* strains

<table>
<thead>
<tr>
<th>Saccharide</th>
<th>Control 100 mM</th>
<th>Control 10 mM</th>
<th>Control 1 mM</th>
<th>α-D-Mannopyranoside 100 mM</th>
<th>α-D-Mannopyranoside 10 mM</th>
<th>α-D-Mannopyranoside 1 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. castellanii ANPV-1-92</td>
<td>55 ± 3</td>
<td>34 ± 0.7</td>
<td>53 ± 1 NS</td>
<td>52 ± 2 NS</td>
<td>15 ± 1 NS</td>
<td>53 ± 2 NS</td>
</tr>
<tr>
<td>A. polyphaga V8A</td>
<td>53 ± 4</td>
<td>40 ± 1</td>
<td>52 ± 2 NS</td>
<td>53 ± 1 NS</td>
<td>28 ± 1 NS</td>
<td>54 ± 3 NS</td>
</tr>
<tr>
<td>A. hatchetti ABA-A3</td>
<td>43 ± 1</td>
<td>26 ± 2</td>
<td>41 ± 1 NS</td>
<td>42 ± 2 NS</td>
<td>15 ± 0.6 NS</td>
<td>39 ± 3 NS</td>
</tr>
<tr>
<td>A. castellanii ATCC 30234</td>
<td>51 ± 3</td>
<td>37 ± 1</td>
<td>49 ± 2 NS</td>
<td>51 ± 1 NS</td>
<td>25 ± 0.9 NS</td>
<td>48 ± 2 NS</td>
</tr>
<tr>
<td>A. polyphaga ATCC 30461</td>
<td>64 ± 3</td>
<td>49 ± 3</td>
<td>62 ± 3 NS</td>
<td>63 ± 3 NS</td>
<td>40 ± 2 NS</td>
<td>68 ± 3 NS</td>
</tr>
<tr>
<td>A. culbertsoni ATCC 30171</td>
<td>47 ± 4</td>
<td>11 ± 0.7</td>
<td>48 ± 3 NS</td>
<td>48 ± 1 NS</td>
<td>10 ± 0.5 NS</td>
<td>47 ± 4 NS</td>
</tr>
</tbody>
</table>

Control: adherence of the strain without carbohydrate.
Adherence capacity significantly decreased (\(^\wedge\)): Mann–Whitney test, \(p \leq 0.001\).
NS: non-significant.
Values are means ± standard deviations.
of all strains with α-D-mannopyranoside showed a more significant decrease of the adhesion. Interestingly, the adhesion of *A. culbertsoni* is also partially inhibited by D-glucose at the concentration of 100 mM (Table 2).

The quantification of adherence using the XTT tetrazolium method was verified by a microscopic determination of the percentage of adherent trophozoites in one well of each test for all strains and these two approaches perfectly correlated (data not shown).

As far as D-mannose and α-D-mannopyranoside are concerned, concentrations of 10 and 1 mM were then tested and no significant effect was noted on *Acanthamoeba* attachment for these low concentrations (Table 3).

### 3.3. Evaluation of the toxicity of carbohydrates on *Acanthamoeba*

The trophozoites of each *Acanthamoeba* strain were incubated with the concentrations of 100, 10 and 1 mM for D-mannose and α-D-mannopyranoside. Regular microscopic examinations showed no detectable changes in morphology or motility. Toluidine blue staining did not reveal the presence of dead cells.

### 4. Discussion

*Acanthamoeba* sp. are known to adhere to various substrates such as epithelial cells [11], extracellular matrix proteins [12], cellulose fibres [13], or hydrogel lenses [6]. Yang et al. [7] have shown that *A. castellanii* binds to mannose-containing glycoproteins of corneal epithelium and that a mannose binding protein of 136 kDa present on the surface membrane of *Acanthamoeba* is involved in the first step leading to the pathogenesis of *Acanthamoeba* keratitis. Cao et al. [8] have then performed inhibition assays using various carbohydrates. Their results revealed that *A. castellanii* adherence to host cells is mediated by an amoeba lectin with a high affinity for α-mannose and Mannose (α1–3) mannose units. But *Acanthamoeba* trophozoites could possess other surface membrane molecules involved in the adhesion to epithelial cells [14].

We report in this study that the use of XTT-menadione is a simple, reliable and reproducible semi-quantitative method to determine the adherence capacity of *Acanthamoeba* strains to inert surfaces. The results obtained by this method show that all the tested *Acanthamoeba* strains depend on a mannose binding receptor for their adherence to inert surfaces like plastic. Therefore, the surface molecule of the amoebae which binds to mannose or to mannose containing residues is implicated in the adhesion to biological components as well as in the adhesion of *Acanthamoeba* trophozoites to inert surfaces. Moreover, within a species such as *A. polyphaga*, the influence of carbohydrates on the attachment could be different. The inhibition of the binding of *Acanthamoeba* was specific: for *A. castellanii*, *A. polyphaga* and *A. hatchetti*, only D-mannose and α-D-mannopyranoside reduced the attachment. In the case of *A. culbertsoni*, glucose could also be involved in the process of binding to inert surfaces. The inhibition of the attachment of this last species to biological surfaces has not yet been investigated in the literature. To add to complete the opinion of Kenett et al. [14], we can say that *Acanthamoeba* trophozoites may possess different surface membrane molecules involved in adhesion to epithelial cells, but also in adhesion to other surfaces.

We also demonstrated that the inhibition of the attachment of *Acanthamoeba* to the tested surface is dose dependent, with an effect of the carbohydrates at 100 mM and no action at 1 and 10 mM. Moreover, the modification of the attachment due to the presence of certain carbohydrates is not explained by the possible toxicity of these sugars.

Further studies are ongoing in our laboratory to assess the effect of saccharide composition on the attachment of *Acanthamoeba* in water systems biofilms.

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### References


