**Alginate Inhibition of the Uptake of *Pseudomonas aeruginosa* by Macrophages**

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*Pseudomonas aeruginosa* alginate was purified and characterized in terms of uronic acid, carbohydrate and protein content, as well as by infra-red spectroscopy and gel electrophoresis. Added exogenous bacterial alginate inhibited the uptake and degradation of both viable and non-viable radiolabelled non-mucoid *P. aeruginosa* by resident mouse peritoneal macrophages. Alginic acid (from seaweed) inhibited the same parameters to almost the same degree. Bacterial alginate also inhibited the uptake of fluorescent-labelled zymosan and latex particles. Starch, at equivalent viscosity to the alginate, inhibited the uptake and degradation of radiolabelled non-viable *P. aeruginosa* to a greater extent, but Dextran T500 had no effect. This suggests that the viscous nature of alginate exerts a non-specific inhibitory effect on the uptake and subsequent degradation of phagocytosible particles.

**INTRODUCTION**

Bronchopulmonary disease is the leading cause of morbidity and mortality among patients with cystic fibrosis (CF). The respiratory tract of most patients ultimately becomes permanently colonized by *Pseudomonas aeruginosa*, which once acquired is never eradicated, although it may disappear temporarily from the sputum following treatment. Colonization by *P. aeruginosa* is associated with pulmonary damage (Hoiby, 1974). Initial infection is usually with a classic non-mucoid strain but subsequently mucoid strains emerge and predominate, with concomitant pulmonary deterioration and a poor prognosis for the patient. Such mucoid strains elaborate an exopolysaccharide (alginate) of L-guluronyl and D-mannuronyl moieties, in differing proportions according to strains, and with various degrees of acetylation (Pugashetti *et al.*, 1982).

The continued presence of the bacterium implies evasion of the host defence mechanisms. The mucoid nature of the bacteria in the CF lung may partly be responsible for this evasion. *In vitro* studies have indicated that alginate reduces *P. aeruginosa* uptake by rabbit neutrophils (Schwarzmann & Boring, 1971), guinea-pig alveolar macrophages (Ruhén *et al.*, 1980) and murine peritoneal macrophages (Oliver & Weir, 1983; Maresz-Babczyszyn *et al.*, 1984), as well as reducing human neutrophil function (Laharrague *et al.*, 1984; Meshulam *et al.*, 1984). However, few unambiguous or quantitative data exist, particularly with reference to intracellular degradation, and there is no clear evidence as to mechanisms by which such interference might be achieved. The present paper provides detailed quantitative evidence of an effect of alginate on phagocytosis, and indicates that evasion of host defences may be partly a function of the physical, rather than biochemical, properties of the alginate.

**METHODS**

*Bacteria.* *Pseudomonas aeruginosa* strains were a gift from Dr T. Pitt (Central Public Health Laboratory, 61 Colindale Avenue, London, UK). Strain 217m is a mucoid, alginate-producing strain and 217nm is an isogenic

**Abbreviations:** CF, cystic fibrosis; HISS, heat-inactivated swine serum.
non-mucoid revertant of this strain. Working cultures were grown at 37 °C on Pseudomonas Isolation Agar (PIA; Difco), stored at 4 °C and subcultured weekly. Bacteria were grown in Nutrient Broth (Difco) at 37 °C overnight prior to radiolabelling. Alginate was readily removed from mucoid cultures in the necessary washing procedures prior to phagocytosis experiments. Consequently all experiments involving phagocytosis of bacterial particles were performed with washed non-mucoid cells with the addition of suitable quantities of isogenic alginate, when appropriate.

**Extraction and purification of alginate.** A batch modification of the method of Sherbrooke-Cox et al. (1984) was used. Mucoid bacterial growth (48 h) from six Petri dishes of PIA was suspended in 500 ml double-distilled water. This suspension was centrifuged at 20,000 g for 1 h and the pellet, containing mainly bacterial cells, discarded. The supernatant was stirred with 500 g Dowex 1 × 2 resin (BDH) for 1 h, followed, stepwise, by 500 ml each of double-distilled water, 0-5, 0-8 and 1-0 M-sodium chloride solutions, separated in turn from the resin using a Buchner funnel (Whatman filter no. 1). Each fraction was dialysed against double-distilled water, assayed for uronic acid by the method of Knutson & Jeanes (1968), using o-mannurono-6,3-lactone (Sigma) as standard, and the uronic-acid-containing fractions (0-8 M and 1-0 M) were combined and lyophilized.

**Characterization of alginate.** Uronic acid content was determined as previously (Knutson & Jeanes, 1968). Total carbohydrate was assayed by the method of Dubois (1956) and protein by the Lowry method. Infra-red spectra were measured with an SP3-100 double-beam infra-red spectrophotometer (Pye Unicam), using samples in potassium bromide discs. Viscosity of preparations was determined in an Ostwald viscometer and expressed as change in viscosity with concentration (up to 200 µg ml⁻¹). Average molecular size and size distribution were assessed by polyacrylamide gel electrophoresis (PAGE), according to the method of Cowman et al. (1984). Alginate (10 × experimental concentration) was sterilized by autoclaving at 115 °C for 10 min prior to use in uptake experiments. Alginic acid (Sigma) from *Macrocytis pyrifera*, dextran (Pharmacia) and starch (BDH) were sterilized in the same way.

**Macrophage collection and culture.** Macrophages were obtained by peritoneal lavage of normal Swiss mice (T0 strain) with 10 ml cold sodium-bicarbonate-buffered medium 199 (Flow) containing 50 U penicillin ml⁻¹, 50 µg streptomycin ml⁻¹ and 10 U heparin ml⁻¹. Pooled peritoneal fluid was centrifuged at 400 g for 5 min, the sedimented cells were resuspended in medium 199 as before to a concentration of 10⁶ ml⁻¹, and 1 ml samples were placed in capped plastic test tubes. These tubes were incubated at 37 °C in a humidified atmosphere of 5% (v/v) CO₂ in air for 1-2 h. Non-adherent cells were then removed by washing four times with warm phosphate-buffered saline (8-0 g NaCl, 1-15 g Na₂HPO₄, 12-0H₂O, 0-2 g KCl, 0-2 g KH₂PO₄ 1⁻¹, pH 7-3; PBS). After washing, the cells were cultured in 1 ml medium 199 (with antibiotics as before) containing 10% (v/v) heat-inactivated swine serum (HISS). Macrophages appeared as a sheet of spread cells within a few hours, and experimental work began after overnight incubation at 37 °C. Macrophages for experiments to determine the effect of alginate on the viability of the macrophage cultures were radiolabelled by the addition of 1 µCi (37 kBq) L-[¹⁴C]leucine ml⁻¹ (Amersham) during overnight incubation. Excess label was removed by four washes with warm PBS containing 10 mM-leucine.

**Preparation of particles for phagocytosis.** ¹⁴C-labelled bacteria were prepared by incubation of bacteria in 9 ml Davis-Mingoli (DM) salts buffer (pH 7-2) plus 1 ml Nutrient Broth with 20 µCi L-[¹⁴C]leucine for 18 h at 37 °C. Labelled bacteria were washed with DM containing 5 µg leucine ml⁻¹, to free the intracellular pools of radioactive leucine. Bacteria were killed by 24 h exposure to 1% (v/v) formalin at 4 °C followed by four further washes with DM plus leucine; they were non-viable when plated out on PIA. Labelled killed bacteria were counted in a Helber chamber and adjusted to a final concentration of 10⁷ particles ml⁻¹ in medium 199 with 10% HISS. Latex beads of 0-81 µm mean diameter were labelled by the incorporation of the fluorescent compound perylene (Aldrich) according to the method of Kwan et al. (1976). The perylene-labelled latex particles were washed five times with PBS and resuspended in medium 199 with 10% HISS to give a final concentration of 2 × 10⁶ particles ml⁻¹. Zymosan (Sigma; from yeast cell walls) particles were labelled by covalent linking with the fluorochrome fluorescein, and washed and counted as before (2 × 10⁷ particles ml⁻¹).

**Phagocytosis.** Medium was sucked off the macrophage cultures and 1 ml prewarmed bacterial or particle suspension, in medium 199 with serum, with or without sterilized alginate, was added to each tube. Experimental tubes were set up, in triplicate, at 20:1 ratios of bacterial or particle to macrophage, and incubated at 37 °C in a 5% CO₂-in-air atmosphere. At the end of the incubation period the medium was removed from each tube, the macrophages were washed twice with warm PBS, and after 15 min in saline containing 0-1% (v/v) Triton X-100 the lysed cell fraction was removed from the tubes by vortexing. Medium, washes and cell fractions were precipitated with trichloroacetic acid (TCA; 5% (v/v), final concn) after the addition of 0-1 ml 10% (w/v) BSA as carrier to each fraction, and then centrifuged at 3000 g for 10 min. Samples of the acid-soluble fraction were taken and the rest discarded. The TCA precipitates were dissolved in 90% (v/v) formic acid and again samples taken. Radioactively-labelled samples were dispensed into vials containing Scintillant 299 (Packard) and radioactivity was measured in a Packard Tricarb 460c scintillation counter, using a channels ratio method for quench correction. The fluorescence of fluorescein is pH-dependent, being maximal in neutral or alkaline conditions, and for this reason all fluorescent samples were neutralized to pH 7 with 4 M-Tris/HCl buffer (pH 9) before detection in
an LS-3 fluorescence spectrometer (Perkin-Elmer). The fluorescence emission of both perylene and fluorescein was measured at 540 nm; excitation of fluorophores was at 435 nm and 495 nm respectively. Data are expressed as the means ± sd of triplicate determinations and are representative of several separate experiments. A two-tailed t-test was used for statistical analysis. P values >0.05 were not considered significant.

Uptake was calculated as:

\[
\text{TCA-soluble } ^{14} \text{C or fluorescence in cells, washes and medium} + \text{TCA-insoluble } ^{14} \text{C or fluorescence in cells}\}
\]

\[
\times 100
\]

Total \(^{14} \text{C or total fluorescence}\)

Degradation was calculated as:

\[
\frac{\text{TCA-soluble } ^{14} \text{C or fluorescence in cells, washes and medium}}{\text{Total } ^{14} \text{C or total fluorescence}} \times 100
\]

Macrophage-associated material was assessed as TCA-precipitable material in the lysed macrophage fraction, as a percentage of the total in the system.

RESULTS

Characterization of bacterial alginate

Virtually all carbohydrate could be accounted for as uronic acid and only a trace of protein could be detected. Infra-red spectroscopy gave typical alginate spectra (Evans & Linker, 1973; Sherbrock-Cox et al., 1984), with a distinct absorbance band at 1650 cm\(^{-1}\), characteristic of the carboxyl group. Other peaks of absorbance occurred at 3500, 1440, 1280 and 1060 cm\(^{-1}\). The viscosity of four alginate preparations at 37 °C was 0.0219 ± 0.0015 cSt/(μg ml\(^{-1}\)). Alginate preparations compared by PAGE using a 5% (w/v) gel (Cowman et al., 1984) showed a single broad band of large-molecular-mass material, indicating similar average molecular size. Alginate had no marked effect on the viability of macrophages in culture. Macrophage lysis at the highest concentration of alginate used in phagocytosis experiments (1 mg ml\(^{-1}\)) increased over the time course but reached only 9% at 24 h.

Uptake and degradation of \textit{P. aeruginosa} in the presence of added bacterial alginate

Parallel experiments with washed mucoid and with non-mucoid cultures gave equivalent results; for this reason data are given only for experiments where alginate was added to washed non-mucoid cultures.

Fig. 1 is representative of five experiments using non-viable (formalin-treated) non-mucoid bacteria with different preparations of alginate. Uptake and degradation for the control continued at a steady rate up to 24 h at 36% and 33% of the bacterial load respectively. Alginate inhibited the uptake and degradation of radiolabelled \textit{P. aeruginosa} in a dose-related manner (Fig. 1, Table 1). Alginate at a concentration of 1 mg ml\(^{-1}\) had a particularly marked effect; at 4 h the uptake was 60% of the control and the inhibitory effect continued to increase slightly until 24 h (42% of control). Alginate could also reduce intracellular accumulation (Fig. 1c). D-Mannurono-6,3-lactone, at an equivalent uronic acid concentration to 1 mg purified alginate ml\(^{-1}\), had no inhibitory effect on the uptake (93-5% of control at 9 h time point) or degradation (99-2% of control at 9 h time point) parameters. In all experiments autolysis of non-viable bacteria increased slightly over the time course but never exceeded 5%, even at the 24 h time point. For this reason data were not corrected for this parameter.

Addition of bacterial alginate at 1 mg ml\(^{-1}\) showed a similar inhibitory effect on the uptake and degradation of viable non-mucoid \textit{P. aeruginosa} (Fig. 2) to that observed with non-viable cells. The autolysis parameter was more significant with viable bacteria due to increased bacterial metabolic turnover.
Fig. 1. (a) Uptake (●), (b) degradation (○), and (c) macrophage-associated TCA-precipitable pool (▲), of non-viable, non-mucoid radiolabelled *P. aeruginosa* by murine peritoneal macrophages in the presence of exogenous isogenic alginate (*, P < 0.05; **, P < 0.01).

Fig. 2. Uptake (●), degradation (○), macrophage-associated TCA-precipitable pool (▲), and bacterial autolysis (▲), of viable non-mucoid radiolabelled *P. aeruginosa* by murine peritoneal macrophages with (a) and without (b) the addition of exogenous isogenic alginate at 1 mg ml⁻¹. (*, P < 0.05; **, P < 0.01).

Table 1. Uptake/degradation of *P. aeruginosa* (non-mucoid, non-viable) by macrophages in the presence of exogenous isogenic alginate

The results are means ± SD of three replicates.

<table>
<thead>
<tr>
<th>Alginate (mg ml⁻¹)</th>
<th>Uptake (%)</th>
<th>Degradation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>24 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>0</td>
<td>1.55 ± 0.3</td>
<td>32.4 ± 0.9</td>
</tr>
<tr>
<td>0.04</td>
<td>1.04 ± 0.65</td>
<td>28.3 ± 2.4</td>
</tr>
<tr>
<td>0.2</td>
<td>1.67 ± 0.21</td>
<td>28.0 ± 0.74</td>
</tr>
<tr>
<td>0.4</td>
<td>1.98 ± 0.15</td>
<td>22.3 ± 0.19</td>
</tr>
<tr>
<td>0.8</td>
<td>1.35 ± 0.28</td>
<td>18.2 ± 3.12</td>
</tr>
<tr>
<td>1.0</td>
<td>1.20 ± 0.42</td>
<td>14.15 ± 3.93</td>
</tr>
</tbody>
</table>
Effect of bacterial alginate on uptake of other particles

As little or no degradation occurred with the zymosan or latex particles, uptake and the macrophage-associated TCA-precipitable pool were very similar. Consequently, only data relating to the TCA-precipitable pool are presented; this is a good approximation of uptake. Macrophage association of zymosan particles was initially greater than the equivalent measurement with radiolabelled non-mucoid *P. aeruginosa*, being maximal by 4 h (22%) and maintained at this level until 24 h (Fig. 3a). Latex association (Fig. 3b) paralleled zymosan over the first 4 h, but continued at that level for the time course of the experiment (24 h), so that a much larger fraction of the latex suspension was finally taken up by the macrophages (79% at 24 h). Alginate inhibited the uptake of both types of fluorescent-labelled particles; this effect was marked over the whole time course for the 1 mg ml⁻¹ concentration, but for the 0.04 mg ml⁻¹ and 0.2 mg ml⁻¹ concentrations it was only really noticeable at the 24 h time points. The inhibitory effect of alginate at 1 mg ml⁻¹ was maximal at 1 h (42% of the control) for zymosan and at 4 h (44% of the control) for latex. Subsequently the effect was diminished and relatively steady at around 70% of the control for both types of particle. In all experiments with zymosan and latex, a control incubation with alginate inhibited the uptake of radiolabelled *P. aeruginosa* to the expected extent.

Inhibition of uptake by other polymers

Starch also inhibited the uptake and degradation of radiolabelled non-mucoid *P. aeruginosa* in a dose-related fashion (Table 2). Starch at 3% (w/v) is of equivalent viscosity to purified alginate at 1 mg ml⁻¹, and starch at this concentration was more effective than the bacterial alginate control in inhibiting phagocytosis. The addition of seaweed alginic acid at 0.04, 0.20 and 1.0 mg ml⁻¹ inhibited uptake to 92, 64 and 34% of the control respectively at 24 h. The inhibitory effect was also significant at 9 h and on degradation and macrophage-associated TCA-precipitable material, the other parameters of phagocytosis. Dextran T500 at 10 mg ml⁻¹ had no significant effect on uptake (103% of the control at 24 h) or degradation (100% of the control at 24 h).
Table 2. Uptake/degradation of *P. aeruginosa* (non-mucoid, non-viable) by macrophages in the presence of exogenous starch polymer

The results are means ± SD of three experiments.

<table>
<thead>
<tr>
<th>Starch (%, w/v)</th>
<th>0 h</th>
<th>9 h</th>
<th>24 h</th>
<th>0 h</th>
<th>9 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.81 ± 0.13</td>
<td>16.5 ± 0.10</td>
<td>36.8 ± 0.97</td>
<td>1.27 ± 0.1</td>
<td>13.3 ± 0.17</td>
<td>32.2 ± 1.5</td>
</tr>
<tr>
<td>1.5</td>
<td>1.48 ± 0.2</td>
<td>6.33 ± 0.29</td>
<td>13.38 ± 1.35</td>
<td>1.04 ± 0.14</td>
<td>5.5 ± 0.16</td>
<td>11.3 ± 1.14</td>
</tr>
<tr>
<td>3.0</td>
<td>1.25 ± 0.1</td>
<td>3.78 ± 0.23</td>
<td>5.91 ± 0.26</td>
<td>0.94 ± 0.08</td>
<td>3.48 ± 0.19</td>
<td>5.38 ± 0.23</td>
</tr>
</tbody>
</table>

* Values obtained with 1 mg alginate ml⁻¹.

**DISCUSSION**

Phagocytosis, as a basic eukaryotic cell function, contributes to host defence. The process is mainly accomplished by professional phagocytes, namely the macrophage and the neutrophil, and can be considered as ingestion, killing and degradation, normally leading to the inactivation and removal of ingested material. The respiratory tract in CF apparently offers an environment conducive to the selection and maintenance of mucoid *P. aeruginosa* (Doggett, 1969). Mucoid strains are rarely eliminated; hence production of alginate may confer a selective advantage by protecting the bacteria from the defence mechanisms of the host.

Our results indicate that purified exogenous alginate inhibits the uptake and subsequent degradation of both non-viable and viable radiolabelled non-mucoid *P. aeruginosa* by resident mouse peritoneal macrophages. The uptake parameter in our experiments is a measure of the large-molecular-mass material associated with the macrophage, as well as the small-molecular-mass material released from the bacteria within the macrophage. Degradation is purely a measure of the latter, and hence is a measure of the material digested by the macrophage. Little quantitative information with reference to intracellular degradation is available in the literature. Many widely used methods, including particle counting (Oliver & Weir, 1983, 1985; Grasso et al., 1984) are unsatisfactory (Kavet & Brain, 1980) since they do not allow assessment of the proportion of endocytosed material that has disintegrated. Uptake and degradation of bacteria paralleled each other very closely in our experiments; this implies that alginate has a primary effect on the association and ingestion of the bacteria rather than on killing or digestion. The dose-related inhibitory effect on the macrophage-associated large-molecular-mass pool (Fig. 1c) reinforces this supposition. Meshulam et al. (1984) similarly concluded that the reduced neutrophil bactericidal activity for mucoid *P. aeruginosa* was a function of reduced ingestion since the rate of intracellular killing was similar for mucoid and non-mucoid strains. Oliver & Weir (1983) concluded that alginate inhibits binding, as assessed by particle counting, of non-mucoid *P. aeruginosa* and *Staphylococcus albus* to mouse macrophages, another early event in the process of phagocytosis.

Other workers have found a similar inhibitory effect at relatively high concentrations in a variety of cell types and states of activation (Schwarzmann & Boring, 1971; Ruhen, 1980; Oliver & Weir, 1983, 1985). The variation in the effective concentrations of preparations is probably a function of different isolation and purification techniques leading to different physical states of the alginate polymer.

Bacterial alginate also inhibited the uptake of zymosan and latex particles. Each type of particle had quite different uptake kinetics to the non-mucoid bacteria, yet inhibition was marked, particularly at the 1 mg ml⁻¹ level. Grasso et al. (1984) similarly found a dose-related inhibition of the phagocytosis of *Saccharomyces cerevisiae*. It would seem that if a particle is phagocytosible, then bacterial alginate, certainly at relatively high concentrations, can inhibit the process. In addition, commercial seaweed alginic acid, similar in constitution to bacterial alginate, inhibited uptake and degradation to almost exactly the same degree.

If bacterial alginate exerts its effect on uptake or events preceding uptake, then which...
Properties of the alginate polymer are relevant to the inhibition? The D-mannuronic acid component usually predominates in bacterial alginate (Pugashetti et al., 1982), and when the mannuronic acid monomer was tested at the same uronic acid concentration as the alginate, phagocytosis was unaffected, implying that the antiphagocytic properties of the polymer are due to its molecular size or configuration, rather than its chemical constituents.

Starch at equivalent viscosity was actually more effective than alginate at inhibiting the uptake and degradation of *P. aeruginosa* (Table 2). Starch polymer, unlike the polyanionic alginate, is not highly charged, so these experiments indicate that charge is probably not of importance in the inhibition of phagocytosis. Dextran T500, a non-viscous polymer of sugars, had no significant effect on phagocytosis, suggesting the importance of viscosity in the inhibitory process.

In conclusion, alginate apparently exerts a non-specific effect on the uptake and subsequent degradation of *P. aeruginosa* by macrophages. Inhibition of phagocytosis requires relatively large amounts of alginate, and this implies a gross effect on macrophage behaviour rather than a subtle effect on biochemistry. However, alginate may have other roles to play in the evasion of host defences which also account for the increased survival of the bacteria within the CF lung. In recent work we have shown scavenging of free radicals, similar to those developed in the respiratory burst of phagocytes, by alginate. Alginate apparently has many varied effects apart from inhibition of phagocytosis at high concentration, among which its radical-scavenging properties would seem to be important.

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**REFERENCES**


