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The effect of *Pseudomonas* alginate on rat alveolar macrophage phagocytosis and bacterial opsonization

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SUMMARY

Alginate obtained from a mucoid strain of *Pseudomonas aeruginosa* was shown to inhibit the phagocytosis of an isogenic non-mucoid revertant by rat alveolar macrophages. Phagocytosis of *Staphylococcus albus*, binding of sensitized sheep erythrocytes to Fc receptors and uptake of latex particles were also inhibited. These results suggest that the alginate acts as a barrier, surrounding the macrophage preventing the attachment of bacteria or other particles to the plasma membrane. This conclusion was supported by showing that alginic acid, a polysaccharide from seaweed structurally similar to alginate also inhibited the phagocytosis of non-mucoid *Ps. aeruginosa*. The alginate also inhibited opsonisation of the non-mucoid revertant by a non-agglutinating hyperimmune serum. It is proposed that alginate confers a selective advantage on mucoid producing forms of *Ps. aeruginosa* by impairing the host immune response by its action on alveolar macrophages and opsonization of bacteria.

Keywords alveolar macrophages cystic fibrosis *Pseudomonas* alginate phagocytosis opsonization

INTRODUCTION

Strains of *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis (CF) have been found in culture to produce alginate, a D-mannuronic and D-guluronic acid polymer linked by *O*-acetyl groups (Doggett *et al.*, 1966). The presence of these mucoid strains in the lungs of CF patients is associated with a poor prognosis (Høiby, Andersen & Bendixen, 1975) and contributes to the persistence of the infection (Doring & Høiby, 1983). This suggests that the alginate produced by the bacteria may be a contributory factor in the pathogenesis of pseudomonas infection in CF.

Failure to eradicate mucoid *Ps. aeruginosa* strains is a major problem in the treatment of CF and is not thought to be due to a general immune defect (di Sant'Agnese & Davis, 1976). However, it is possible that immune function in the lung may be impaired and exacerbated as a result of alginate production by *Ps. aeruginosa*.

Previous work in this laboratory has shown that the alginate obtained from a mucoid producing strain of *Ps. aeruginosa* inhibited the binding of a non-mucoid revertant to mouse peritoneal and pulmonary macrophages (Oliver & Weir, 1983). Schwartzmann & Boring (1971) have reported that alginate can inhibit the phagocytosis of non-mucoid strains by rabbit neutrophils and preliminary studies have been reported on guinea-pig alveolar macrophages (Ruhen, Holt & Papadimitriou, 1980).

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The purpose of this study was to extend these observations and to investigate these effects in more detail as the pathogenic properties of alginate are not understood.

We used alginate separated from a mucoid strain of *Ps. aeruginosa* to determine its effect on the opsonization of a non-mucoid revertant strain and the phagocytosis of opsonized bacteria and other particles by rat alveolar macrophages.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (250 g) were obtained from the Centre for Laboratory Animals, University of Edinburgh.

Bacteria. Ps. aeruginosa strains were obtained from Dr J. R. W. Govan, Bacteriology Department, University of Edinburgh. Strain 492a is a mucoid, alginate producing strain isolated from the sputum of a CF patient and 492a Rev 1 is an isogenic non-mucoid revertant of this strain. The bacteria were grown for 24 h at 37°C on nutrient agar (Columbia agar base, Oxoid) and harvested by scraping from the plate with a glass slide. Bacterial concentrations were calibrated with a spectrophotometer (SP30; Pye Unicam) at 540 nm. A reading of 0·3 was equivalent to 5×10^8 bacteria/ml.

Staphylococcus albus. A strain of *S. albus* was obtained from the departmental teaching collection. The organisms were grown in horse digest broth, harvested in log phase and killed by 24 h exposure to 0·5% formalin at 4°C. Concentrations were calibrated spectrophotometrically as above ($0{\cdot}42 = 2 \times 10^7$ bacteria/ml).

Extraction of alginate. The alginate was extracted from *Ps. aeruginosa* 492a, as previously described (Oliver & Weir, 1983).

Preparation of sensitized sheep erythrocytes (EAG). EAG were prepared as described elsewhere (Glass, Stewart & Weir, 1982). The anti-sheep erythrocyte antibody used was a murine monoclonal (IgG2b–MAS 013c, clone Sp2; Sera-Lab. Ltd., Crawley Down, Sussex, UK).

Preparation of hyperimmune serum. Heat killed *Ps. aeruginosa* 492a Rev 1 were washed in saline adjusted to a final concentration of 5×10^9 bacteria/ml and mixed with an equal volume of Alhydrogel (2% solution) (Miles Laboratories). The mixture was incubated at 37°C for 1 h, washed and resuspended in saline to give a final concentration of 5×10^9 bacteria/ml. Five rats, each received four weekly i.p. injections of 1 ml of bacteria. They were killed and exsanguinated 1 week after the last injection. The serum was collected, pooled and heat inactivated at 56°C for 30 min and stored at −20°C.

Opsonization of bacteria. Hyperimmune serum was diluted 1:80 with Hanks' balanced salt solution (HBSS) containing 0·1% gelatin (GHBSS) and 9 ml was added to 1 ml of a suspension of 5×10^9 bacteria/ml. The mixture was incubated for 20 min in a shaking water bath at 37°C, washed and finally resuspended in HBSS to give a final concentration of 5×10^8 bacteria/ml.

Inhibition of opsonization. Serum was diluted 1:40 with GHBSS and mixed with an equal volume of alginate at different concentrations. Opsonization was then carried out as described above.

Macrophages. Animals were killed with sodium pentobarbitone (50 mg/animal) and exsanguinated. The trachea and lungs were removed and lavaged using a tracheal cannula with Dulbecco's phosphate-buffered saline (DPBS), containing heparin (10 units/ml), pre-warmed to 37°C. Fifty millilitres of lavage fluid was collected and centrifuged at 200*g* for 10 min. The sedimented cells were resuspended in Eagles MEM without serum (buffered with sodium bicarbonate and supplemented with glutamine, final concentration 2 mM) to give a final viable cell count of 2×10^5 macrophages/ml.

Preparation of monolayers. Monolayers were prepared as previously described (Oliver & Weir, 1983). Non-adherent cells were removed by washing with warm Eagles MEM.

Phagocytosis. Macrophage monolayers were overlaid with 1 ml of bacterial suspension in HBSS and incubated for 30 min at 37°C. Unphagocytosed organisms were removed by vigorous washing with HBSS. Coverslips were air dried, fixed in methanol and stained with May–Grunwald–Giemsa. Triplicate coverslips were used and 200 macrophages counted on each coverslip. The results were expressed as the percentage of macrophages that had phagocytosed five or more bacteria.

Fc receptor assay. Two millilitres of EAG ($2{\cdot}5 \times 10^7$ red cells/ml) were added to each monolayer, centrifuged for 4 min at 30*g* and incubated for 30 min at 22°C (room temperature). Non-adherent erythrocytes were removed by gentle washing with HBSS and the coverslips dipped into 1% formal-HBSS to prevent red cell lysis. Triplicate coverslips were used and on each coverslip 200 macrophages were counted. Results were expressed as the percentage of macrophages binding two or more erythrocytes.

Phagocytosis of latex. The macrophage monolayers were overlaid with 1 ml of HBSS and 20 μl of latex suspension (DIFCO Laboratories, Surrey, UK) (adjusted to read 192 at 540 nm on a spectrophotometer) and incubated for 1 h at 37°C. Non-phagocytosed particles were removed by repeated washing with HBSS. Triplicate coverslips were used and on each coverslip 200 macrophages counted. The results were expressed as the percentage of cells that had taken up five or more latex particles.

Inhibition studies. Cell monolayers were incubated with 1 ml of alginate at physiological concentrations (Ohman & Chakrabarty, 1982), diluted in HBSS for 30 min at 37°C. The cells were washed and assays performed as described above. In other assays the alginate was added with the indicator cells to the macrophage monolayers.

Calculations. Percentage inhibition was calculated as the difference between control (C) and test (E) divided by the control × 100:

$$\frac{C-E}{C} \times 100.$$

Statistics. Statistical analysis was performed by the Student's *t*-test.

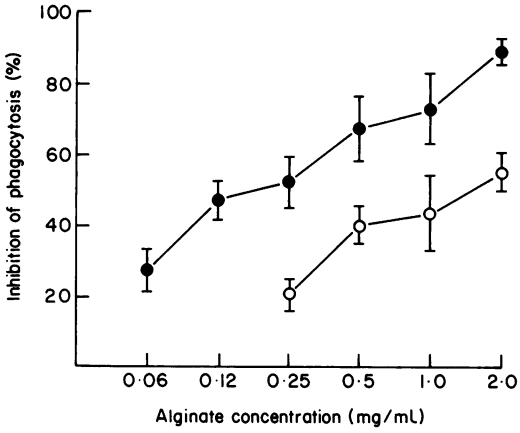

Fig. 1. Inhibition of phagocytosis of non-mucoid *Ps. aeruginosa* by alginate. O——O = pre-treatment of macrophages with alginate; ●——● = addition of alginate with bacteria. Each point represents the mean of four experiments ± 1 s.e.

Table 1. Effect of alginate on the phagocytosis of *S. albus*

	Inhibition of phagocytosis (%) ± 1 s.e.	
Concentration (mg/ml)	Pre-treatment of macrophages with alginate	Addition of alginate with bacteria
0·25	40·5 ± 5	45·6 ± 4·1
0·5	52·1 ± 2·5	54·5 ± 2
1·0	61·3 ± 8·3	72 ± 3·2
2·0	80·5 ± 4·4	87 ± 5·1

Table 2. Effect of alginic acid on phagocytosis of *Ps. aeruginosa* 492a Rev 1

Concentration (mg/ml)	Inhibition of phagocytosis (%) ± 1 s.e.	
	Pre-treatment of macrophages with alginic acid	Addition of alginic acid with bacteria
0·3	29 ± 6	34·8 ± 11
0·6	37 ± 10	44·8 ± 7
1·25	41 ± 11	56·7 ± 6
2·5	63 ± 7	77·3 ± 4
5	78·7 ± 5·2	92·7 ± 0·3
10	86·6 ± 3·3	95·5 ± 4·5
20	100 ± 0	100 ± 0

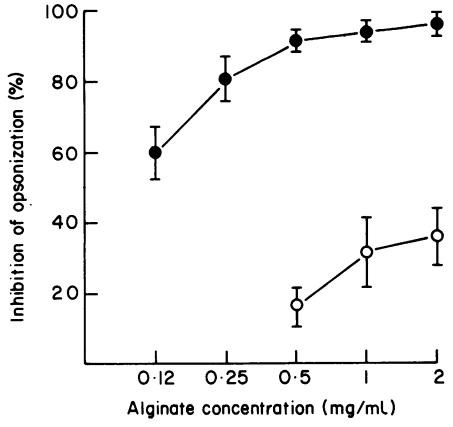

Fig. 2. Inhibition of bacterial opsonisation by alginate. ●——● = non-mucoid *Ps. aeruginosa*; ○——○ = *S. albus*. Each point represents the mean of five experiments (*Ps. aeruginosa*) or three experiments (*S. albus*) ± 1 s.e.

RESULTS

The effect of alginate on phagocytosis

For phagocytosis to occur *Ps. aeruginosa* required to be opsonized with hyperimmune serum, whereas *S. albus* could be opsonised with heat-inactivated normal serum presumably because of the presence of anti-staphylococcal antibody. It is not possible to use mucoid *Ps. aeruginosa* in this assay as the washing procedure after opsonisation causes removal of the alginate, so that the bacteria are no longer mucoid.

Pseudomonas alginate inhibited the phagocytosis ($P < 0{\cdot}05$) of non-mucoid *Ps. aeruginosa* (Fig. 1) and *S. albus* (Table 1) when pre-incubated with the macrophage monolayer for 30 min. This inhibitory effect was reduced by 50% if the macrophages were vigorously washed five times after alginate treatment. The phagocytic capacity of the macrophages returned to normal when these macrophages were re-incubated in Eagles MEM alone for 30 min. Inhibition reached a maximum of 90% when the alginate was added to the macrophage monolayer together with the opsonized bacteria (Fig. 1).

Alginic acid, isolated from *Macroystis pysifera* kelp (Sigma) inhibited the phagocytosis of the non-mucoid revertant in a dose-dependent fashion (Table 2) indicating that the effect of alginate was not due to bacterial derived toxic impurities in the preparation.

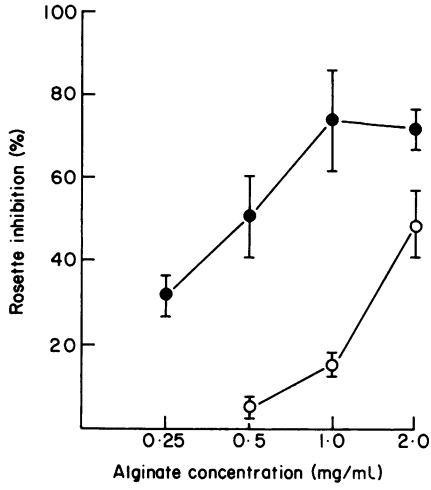

Fig. 3. Inhibition of rosette formation by sensitized sheep erythrocytes by alginate. O——O = pre-treatment of macrophages with alginate; ●——● = addition of alginate with sheep erythrocytes. Each point represents the mean of four experiments ± 1 s.e.

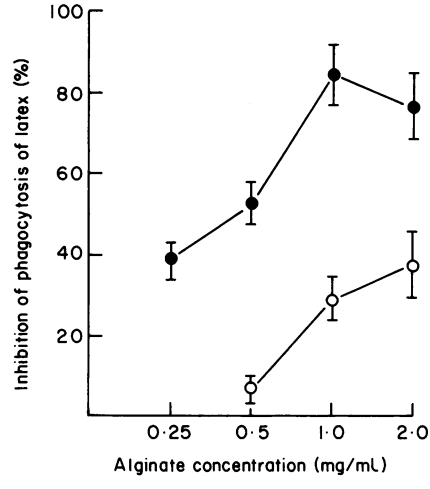

Fig. 4. Inhibition of phagocytosis of latex particles by alginate. O——O = pre-treatment of macrophages with alginate; ●——● = addition of alginate with latex. Each point represents the mean of three experiments ± 1 s.e.

Effect of alginate on opsonization

Opsonization of the non-mucoid revertant by hyperimmune serum was inhibited ($P < 0{\cdot}05$) at 0·12 mg/ml and 0·25 mg/ml and almost totally inhibited ($P < 0{\cdot}001$) at concentrations over 0·5 mg/ml (Fig. 2). The inhibitory effect was not due to inadequate pelleting of the bacteria in the alginate and serum mixture during the washing procedure as colony counts showed that there was no difference in the control and experimental bacterial concentrations of the pellets. Inhibition of opsonisation of *S. albus* was less than that observed with *Ps. aeruginosa*. This may be due to the fact that unopsonized *S. albus* are phagocytosed by alveolar macrophages.

Effect of alginate on Fc receptors

The effect of alginate on Fc receptors was studied with an antibody dilution that gave 30–40% binding of sensitized sheep erythrocytes to the alveolar macrophages.

Alginate reduced ($P < 0{\cdot}05$) the binding of sensitized sheep erythrocytes to the macrophages (Fig. 3). This inhibition was greater when the alginate was incubated together with the sheep erythrocytes than when the macrophages were pre-treated for 30 min.

The effect of alginate on phagocytosis of latex particles

Alginate inhibited phagocytosis of latex particles in a dose-dependent fashion. When the macrophages were pre-incubated for 30 min, phagocytosis was inhibited ($P < 0{\cdot}05$) at 1 mg/ml and 2 mg/ml (Fig. 4). Inhibition was increased at all concentrations used when the alginate was added with the latex to the macrophages ($P < 0{\cdot}05$) at 0·25 mg/ml and 0·5 mg/ml and $P < 0{\cdot}01$ at 1 mg/ml and 2 mg/ml (Fig. 4).

DISCUSSION

Chronic lung infection is a major problem in patients with CF, although systemic infection is rare. The fact that these patients are immunologically competent and have high levels of circulating antibody against the infective bacteria (di Sant'Agnese & Davis, 1976; Høiby & Wilk, 1975) suggests the possibility of local impairment of immunity in the lung. Studies on alveolar macrophages from CF patients have been contradictory. Thomassen *et al.* (1980) showed that there was no intrinsic cellular defect of alveolar macrophages, while other reports showed them to have a decreased capacity to phagocytose candida (Cole, 1979; Sordelli *et al.*, 1982) or an increased phagocytic capacity (Cassino *et al.*, 1980). It has been claimed that the pathophysiology of lung disease in cystic fibrosis is the result of a genetically inherited metabolic defect in the monocyte/macrophage population (Wilson & Fudenberg, 1982).

The respiratory tract in CF offers an environment conducive to the production of mucoid *Ps. aeruginosa* (Macone *et al.*, 1981). As mucoid strains are rarely eliminated production of alginate may confer a selective advantage by protecting the bacteria from the host's immune response. It has been reported that mucoid *Ps. aeruginosa* survive more effectively in the pulmonary environment than their non-mucoid revertants (Govan, Fyfe & Baker, 1983) and that non-mucoid forms are more susceptible to phagocytosis than mucoid forms (Gosciniak *et al.*, 1980). Alginate has been shown to inhibit phagocytosis of bacteria by rabbit neutrophils (Schwartzmann & Boring, 1971) and similar results have been obtained with guinea-pig alveolar macrophages (Ruhen *et al.*, 1980).

An important function of alveolar macrophages is the elimination of foreign particles from the lung by phagocytosis (Green & Kass, 1964). Our studies show that phagocytosis is inhibited when the macrophages are pre-incubated with alginate. Inhibition was increased when alginate was added with the bacteria and decreased when the cells were vigorously washed to remove excess alginate. This suggests that the alginate was coating the macrophage and acting as a barrier to prevent attachment and phagocytosis. It is unlikely that the effect is due to impurities in the preparation. Alginic acid, a chemically similar compound showed a similar inhibitory effect. Further evidence for the barrier effect of the alginate comes from our studies on the binding of sensitized sheep erythrocytes to Fc receptors and phagocytosis of latex particles. Both binding and phagocytosis were inhibited by the alginate supporting the view that the alginate binds non-specifically to the macrophage and prevents particle attachment. Greater inhibition was observed when the alginate was added along with the indicator cells. The test particles may be trapped by the mucoid material and prevented from coming into contact with the macrophage as a result of its viscous nature.

Opsonization of bacteria facilitates phagocytosis and any impairment of this immune defence mechanism would be advantageous to the bacteria. The alginate from mucoid *Ps. aeruginosa* was shown to inhibit opsonization by specific antibody of the non-mucoid revertant. In fact at high concentrations of alginate opsonization was almost totally blocked. This result conflicts with the work of Baltimore & Mitchell (1980), who found that opsonization of two non-mucoid strains, which required antibody for opsonization was not inhibited by the addition of mucoid material. However, they used a different technique for alginate separation and measurement of opsonization, which was not distinguished from phagocytosis.

Pseudomonas alginate has recently been shown to be immunogenic (Pier, Matthews & Eardley, 1983; Bryan, Kureishi & Robin, 1983). However, the evidence suggests that mucoid bacteria are not effectively opsonized and that such antibody may even be harmful if it forms immune complexes in CF sera (Berdischewsky *et al.*, 1980; Moss & Hsu, 1982).

If the *in vitro* inhibitory effect of alginate on phagocytosis and opsonization, reported in this study, occurs *in vivo*, it may partly explain the predominance of mucoid *Ps. aeruginosa* in CF. These effects are likely to exacerbate chronic infection with *Ps. aeruginosa* by protecting the bacteria from the host's immune defences.

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