Effects of recombinant GM-CSF and IgA opsonisation on neutrophil phagocytosis of latex beads coated with P6 outer membrane protein from *Haemophilus influenzae*

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Abstract

Background—IgA is the major antibody class in mucosal secretions, yet its biological functions remain poorly understood and its role as an opsonin for neutrophils has been the subject of controversy. It has been reported that treatment of neutrophils with granulocyte-macrophage colony stimulating factor (GM-CSF) induces the cells to phagocytose particles opsonised with IgA. A study was performed to investigate the effects of GM-CSF and IgA opsonisation on the ability of human neutrophils to recognise and phagocytose latex beads coated with the P6 outer membrane protein of *Haemophilus influenzae*.

Methods—Human neutrophils with and without preincubation with 100 pmol/l GM-CSF, were incubated with non-opsonised P6-coated latex beads or beads opsonised with IgA purified from the blood of a bronchiectatic patient with high titres of IgA anti-P6. Phagocytosis was measured by counting internalised beads during microscopic examination.

Results—The phagocytosis of IgA opsonised beads by untreated neutrophils (mean (SE) 2.1 (0.43) beads/cell) was significantly greater than that of non-opsonised beads (mean (SE) 1.3 (0.30) beads/cell). Treatment of neutrophils with GM-CSF resulted in increased phagocytosis of non-opsonised beads (mean (SE) 2.1 (0.39) beads/cell) but opsonisation with IgA increased this further (mean (SE) 3.4 (0.53) beads/cell).

Conclusions—Human neutrophils recognise and phagocytose non-opsonised particles coated with bacterial antigen. Antibodies of the IgA isotype opsonise for neutrophil phagocytosis of particles coated with bacterial antigen but this behaviour is enhanced, in an additive fashion, by treatment of the cells with GM-CSF. The results suggest that IgA and GM-CSF are important cofactors for neutrophil recognition and elimination of bacterial pathogens.

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Non-typeable *Haemophilus influenzae* is an important cause of respiratory tract infection

in several lung diseases including chronic bronchitis, bronchiectasis, and cystic fibrosis.¹⁻⁵ The sputum and serum of patients with chronic obstructive pulmonary diseases contain IgG and IgA antibodies to outer membrane proteins of the patients' own isolates of *H influenzae*.⁶ Nevertheless, the functional capabilities of these antibodies and their role in protection from infection are not clear. The relatively high concentrations of immunoglobulin A (IgA) in the lung,⁷ and the association of IgA deficiency with recurrent infections, suggest that this immunoglobulin class might have an important role in the antimicrobial defences of the lungs and other mucosal sites.

The biological effects of IgA have, however, remained obscure. Some reports have suggested that human IgA is unable to act as an opsonin for human neutrophils,⁸⁻¹¹ while others have presented evidence that it can.¹²⁻¹⁵ Weisbart *et al*¹⁶ showed that neutrophils treated with granulocyte-macrophage colony stimulating factor (GM-CSF) or granulocyte colony stimulating factor phagocytosed significantly greater numbers of casein-coated latex beads that had been opsonised with IgA antibodies to casein. This effect was associated with the increased expression, on the neutrophils, of high affinity receptors for IgA. These results would suggest that the opsonic effect of IgA may depend upon the IgA receptor status of the neutrophils. The release of colony stimulating factors during infections could therefore significantly increase the ability of neutrophils at inflammatory sites to recognise and dispose of bacteria opsonised with IgA.

The purpose of the present study was to investigate the effects of GM-CSF on the ability of neutrophils to phagocytose an IgA opsonised surface antigen of *H influenzae*. The P6 outer membrane protein was chosen for study because it is present in all strains of *H influenzae*.¹⁷ It is antigenically conserved among strains¹⁷ and it is known to be the target of functional human antibodies.¹⁸

Methods

PURIFICATION OF P6 OUTER MEMBRANE PROTEIN

H influenzae P6 outer membrane protein was isolated from the respiratory tract secretions (expectorated sputum) of patients with

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bronchiectasis by a modification of a method described previously.¹⁸ *H influenzae* was cultured from the secretions on brain/heart infusion agar plates supplemented with 10 µg/ml hemin and 10% (v/v) nicotinamide adenine dinucleotide (NAD). The organisms were verified, by biotyping and serotyping, to be non-typeable *H influenzae*. The bacteria were grown overnight in brain/heart infusion broth containing hemin and NAD maintained at 36°C in 5% CO₂/95% air. The bacteria were harvested by centrifugation at 11 000g for 30 minutes at 4°C and resuspended in 15 mmol/l NaCl, buffered to pH 7.2 with 0.01 mol/l phosphate buffer and centrifuged again at 11 000g before resuspension in buffer B comprising 0.1 mol/l Tris/HCl, pH 8.0 containing 0.5 mol/l NaCl, 1% (w/v) sodium dodecyl sulphate (SDS), and 0.1% (w/v) β-mercaptoethanol. The suspension was sonicated with a Lucas Dawes Ultrasonics Soniprobe and then incubated at 37°C for 30 minutes before centrifugation at 21 000g for 30 minutes. The supernatant was discarded and the pellet resuspended in 30 ml of buffer B. This procedure was repeated twice and the resuspended material was incubated at 37°C for one hour with 10 µg/ml ribonuclease A (Sigma, Poole, Dorset, UK). The preparation was centrifuged at 21 000g and the pellet resuspended in buffer B and incubated again with ribonuclease A for one hour at 37°C. Following centrifugation (21 000g, 30 minutes), the pellet was resuspended in 0.1 mol/l sodium tetraborate buffer, pH 9.5, and incubated at 65°C for 30 minutes before further centrifugation at 100 000g for one hour at 30°C. The supernatant was collected and concentrated approximately ten fold by pressure filtration with an Amicon PM10 membrane to remove residual SDS. This final procedure was repeated four times before assessment of purity by SDS polyacrylamide gel electrophoresis.

ISOLATION OF IgA ANTIBODIES

Venous blood was collected from a patient with radiologically proven bronchiectasis who regularly produced sputum infected with non-typeable *H influenzae*. The blood of this subject was known to contain high titres of IgA antibodies to outer membrane proteins of *H influenzae*, determined by enzyme linked immunosorbent assay and Western blotting.¹⁹ The venous blood was allowed to clot and the serum dialysed exhaustively with 0.01 mol/l phosphate buffer, pH 8.0, containing 0.01 mol/l NaCl (PBS). The dialysed serum was applied to a 9 × 2 cm column packed with DEAE-Sephacel (Pharmacia, Uppsala, Sweden) which had been equilibrated with PBS. The serum was pumped onto the column at 0.3 ml/min followed by elution at the same rate with PBS. The column eluate was monitored by spectroscopy at 280 nm and, after non-bound protein had been recovered, the column was eluted with a linear salt gradient of 0.01–0.1 mol/l NaCl in 0.01 mol/l phosphate buffer, pH 8.0, with continuous collection of 0.5 ml fractions. The column

was finally eluted with 0.01 mol/l phosphate buffer, pH 8.0, containing 0.5 mol/l NaCl. All material collected was investigated for the presence of IgA and IgG using commercially available radial immunodiffusion plates (The Binding Site, University of Birmingham Research Institute, UK). The IgG was detected only in the unbound protein fractions and IgA was collected in the final elution with 0.5 mol/l NaCl. The IgA was purified further by immunoadsorption. Sheep antihuman IgA (The Binding Site, Birmingham) was coupled to cyanogen bromide activated sepharose (Pharmacia) using the manufacturer's recommended protocol to give 30 mg antibody on 2 ml of gel, which was equilibrated in a column with 0.05 mol/l Tris/HCl buffer, pH 8.8, containing 0.1 mol/l NaCl. The IgA-containing material obtained from the ion exchange column was dialysed with 0.05 mol/l Tris/HCl, pH 8.8, and 0.1 mol/l NaCl, pumped onto the column, and the eluate monitored at 280 nm until the absorbance returned to the baseline level. The column was then eluted with 0.2 mol/l glycine/HCl, pH 2.5, with 0.1 mol/l NaCl, and the material collected onto solid Tris. When the absorbance at 280 nm had again returned to baseline the collected material was dialysed with 1.5 mmol/l NaCl and concentrated ten fold on a Speedvac before being stored in aliquots at –40°C. Assay of this material with radial immunodiffusion plates (The Binding Site, Birmingham) showed it to contain 0.01 mg/ml IgA.

ISOLATION OF BLOOD NEUTROPHILS

Venous blood from five healthy volunteers was collected into lithium heparin anticoagulant. Neutrophils were isolated by centrifugation on Percoll density gradients.²⁰ Each blood sample was diluted with an equal volume of 0.15 mol/l NaCl solution and layered carefully onto 2 ml of 1.075 g/ml Percoll (Pharmacia AB, Uppsala, Sweden) in 0.15 mol/l NaCl which had been layered over 3 ml of 1.096 g/ml Percoll. The tubes were centrifuged at 400g for 25 minutes and the neutrophils harvested from the interface between the two Percoll layers. The cells were washed twice in Tris buffered RPMI 1640 medium (Flow Laboratories, Rickmansworth, UK), counted, and resuspended in medium. The cells were >96% neutrophils and viability, assessed by exclusion of trypan blue, was >98%. All reagents were assayed for endotoxin with the KabiVitrum Coatest (Flow Laboratories) and contained less than 20 ng/l.

PHAGOCYTOSIS ASSAY

Polystyrene beads of average diameter 30 µm (Sigma, Poole, Dorset, UK) were washed and suspended in a 1 mg/ml solution of P6 in 0.01 mol/l carbonate/bicarbonate buffer, pH 9.6, overnight at 4°C. The polystyrene beads coated with P6 were washed with 0.015 mol/l NaCl and the purified IgA was added and mixed gently for one hour at room temperature. The beads were washed with 0.15 mol/l NaCl and resuspended in hepes buffered

Figure 1 Frequency distributions of polystyrene beads phagocytosed by human neutrophils. (A) Beads coated with P6 phagocytosed by control cells not treated with GM-CSF; (B) P6-coated beads phagocytosed by cells treated with GM-CSF; (C) P6-coated beads opsonised with IgA phagocytosed by cells not treated with GM-CSF; (D) P6-coated beads opsonised with IgA phagocytosed by cells treated with GM-CSF.

RPMI 1640 cell culture medium (Flow Laboratories).

The neutrophil preparations from the five donors were suspended in hepes buffered RPMI 1640. One half of each neutrophil preparation was incubated with 100 pmol/l GM-CSF (5×10^7 CFU/mg obtained from Genzyme Biochemicals, Maidstone, UK) for 30 minutes at 37°C; the remainder were incubated with medium only. The GM-CSF treated and untreated neutrophils (25 μ l at 5×10^6 /ml) were added to 10^8 P6-coated beads (25 μ l), with and without opsonisation with IgA, in the wells of Multitest slides

Figure 2 Mean (SE) numbers of polystyrene beads phagocytosed by neutrophils from five healthy subjects. Controls were unopsonised beads and cells not treated with GM-CSF (-IgA, -GM-CSF). Results are also shown for unopsonised beads with cells treated with GM-CSF (-IgA, + GM-CSF; $p < 0.01$ v controls), IgA opsonised beads with cells not treated with GM-CSF (+ IgA, -GM-CSF; $p < 0.05$ v controls), and IgA opsonised beads with cells treated with GM-CSF (+ IgA, + GM-CSF; $p < 0.02$ v all other treatments).

(Flow Laboratories). All treatments were incubated, in quadruplicate at 37°C for 20 minutes, after which the slides were washed gently with 0.15 mol/l NaCl solution and stained with Diff-Quik (Travenol). Each preparation was examined at $\times 400$ magnification under oil immersion and the number of beads/cell counted in at least 200 cells/well, care being taken to exclude beads merely adherent on the surface of the cells. Differences in the number of beads/cell between the four treatments of cells from the five subjects were tested with the Student's t test for paired data.

Results

Figure 1 shows the frequency distribution of the numbers of P6-coated beads/cell in neutrophils from one subject. The distribution of beads in all treatments was not normally distributed but opsonisation of beads with IgA antibodies or the treatment of the cells with GM-CSF resulted in a shift in the distribution as more beads were phagocytosed by the neutrophils. The shift in distribution was particularly striking when neutrophils treated with GM-CSF were incubated with beads that had been opsonised with IgA.

The results show that a significant number of neutrophils in all treatments contained no beads, although beads were often observed to be adherent to the surface of the cells. It is possible, therefore, that these adherent beads were destined to be internalised had the period of phagocytosis been prolonged.

Figure 2 shows the average number of beads phagocytosed by neutrophils obtained from the five donor subjects. The phagocytosis of IgA opsonised beads by untreated neutrophils (mean (SE) 2.1(0.43) beads/cell) was significantly greater than that of non-opsonised beads (mean (SE) 1.3(0.30); $p < 0.01$). Treatment of neutrophils with GM-CSF also resulted in increased phagocytosis of non-opsonised beads (mean (SE)

2.1(0.39); $p < 0.05$) but opsonisation with IgA increased this further (mean (SE) 3.4(0.53); $p < 0.02$).

Discussion

H influenzae is implicated as a major pathogen in several lung diseases associated with airway infections.¹⁻⁵ The bronchial secretions of patients with bronchiectasis, especially purulent sputum, contain high concentrations of total IgA²¹ and the serum and sputum titres of specific IgA antibodies to *H influenzae* are also increased.¹⁹ The P6 outer membrane protein (Mr 16.6 kDa) is a major antigenic determinant of *H influenzae*,^{18,22} the molecular structure being highly conserved in all strains of the bacterium.¹⁷ The P6 protein and IgA isolated from patients with *H influenzae* infections are therefore convenient and appropriate reagents for studies of the factors affecting neutrophil recognition and phagocytosis of pathogenic organisms.

Antibodies of the IgA isotype are considered to be an important component of the host immune defence system, but their role as opsonins for neutrophil recognition of antigens has been controversial.⁸⁻¹⁵ In the present study phagocytosis of IgA opsonised beads by untreated neutrophils was significantly greater than that observed for controls where untreated neutrophils were incubated with non-opsonised P6-coated beads. These results therefore support previous reports¹²⁻¹⁵ that blood neutrophils can recognise and respond to IgA antibodies opsonising an antigen and imply an important interaction between IgA antibody production and neutrophil recruitment in response to bacterial infections. This behaviour is presumably mediated by binding to the IgA Fc receptors normally expressed on blood neutrophils.^{12 15 16 23}

Our results with P6 outer membrane protein from *H influenzae* and P6 reactive IgA confirm those of Weisbart *et al*¹⁶ in showing that phagocytosis by neutrophils of antigen coated particles is particularly efficient if the cells are treated with GM-CSF and the antigen is opsonised with IgA antibodies. Weisbart *et al* also showed that the effect of GM-CSF on phagocytosis was accompanied by a change in the characteristics of surface receptors for IgA Fc from low to high affinity. Although they recorded increased phagocytosis by neutrophils in response to IgA opsonisation or treatment of the cells with colony stimulating factors, these effects were not statistically significant. They therefore suggested that the recognition by neutrophils of opsonising IgA was only significant after an increase in receptor affinity resulting from exposure of the cells to colony stimulating factors, indicating synergy. In contrast to the report of Weisbart *et al*, however, we observed significant increases in phagocytosis of P6-coated beads opsonised with IgA or after incubation of the cells with GM-CSF at the same concentration as that used in their study.¹⁶ Furthermore, our results suggest that the

combined effects of these agents were additive and no evidence for synergy was observed.

It therefore appears that colony stimulating factors have the potential to stimulate the phagocytosis by neutrophils of both opsonised and non-opsonised bacteria. The mechanism responsible for this "upregulation" of phagocytosis of non-opsonised P6 remains unknown. Non-opsonised phagocytosis of bacteria may be mediated through the recognition by neutrophils of carbohydrate or protein components of bacteria.^{24,25} Since the P6 outer membrane protein is not glycosylated, peptide sequences on this protein are candidates for recognition by the neutrophils. Integrins have been identified as receptors for some bacterial ligands. The P6 sequence does not contain an Arg-Gly-Asp sequence,²⁶ but this does not preclude recognition of this protein by receptors of the integrin family²⁷ or other adhesion molecules on neutrophils. Neutrophil adherence is increased by GM-CSF²⁸ and the upregulation of non-opsonised phagocytosis by GM-CSF would be consistent with an increased expression or affinity of adhesion molecules which recognise the P6 protein. Further studies will be required to identify the neutrophil receptor and the target peptide on P6.

Various cells in the lung, including fibroblasts, epithelial cells, and activated macrophages and lymphocytes, have the potential to express GM-CSF.²⁹⁻³¹ Further studies should elucidate the mechanisms which determine the production of GM-CSF by lung cells and the role of this cytokine in regulating the antimicrobial effects of IgA and neutrophils. The stimulating effects of GM-CSF on phagocytosis of bacteria by neutrophils and its ability to prolong neutrophil survival³² suggest a potential therapeutic role for this cytokine in augmenting the immune response within the lung. The effective concentrations of GM-CSF within the lung are not known at present, however, and this would need to be established before such therapy could be considered. In addition, the effects of GM-CSF on other cells will be wide ranging and some may have harmful consequences. The benefits of supplementation therapy to enhance phagocytosis would therefore need to be balanced against any potential detrimental effects in the lung.

These observations contribute to our understanding of the human immune response to non-typeable *H influenzae*. Previous studies have shown that the sputum and serum of patients with chronic lung diseases contain IgA antibodies to *H influenzae*,^{6,19,33} but the functional capabilities of these antibodies have not been established. The present study has shown that IgA antibodies which recognise a specific surface antigen on *H influenzae* are capable of stimulating opsonophagocytosis by neutrophils. The observation that these antibodies recognise the P6 outer membrane protein may be particularly relevant in view of the interest in P6 as a vaccine antigen.⁵

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