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Alveolar Macrophages Bind and Phagocytose Allergen-Containing Pollen Starch Granules Via C-Type Lectin and Integrin Receptors: Implications for Airway Inflammatory Disease[1]

Andrew J. Currie,*† Geoffrey A. Stewart,* and Andrew S. McWilliam[2]*†

Recent studies suggest that IgE-independent mechanisms of airway inflammation contribute significantly to the pathophysiology of allergic airway inflammatory diseases such as asthma. Such mechanisms may involve direct interactions between inhaled allergens and cells of the respiratory tract such as macrophages, dendritic cells, and epithelial cells. In this study, we investigated receptor-mediated interactions occurring between alveolar macrophages and allergen-containing pollen starch granules (PSG). We report here that PSG are released from a range of grass species and are rapidly bound and phagocytosed by alveolar macrophages. Human monocyte-derived dendritic cells also bound PSG but no internalization was observed. Phagocytosis of PSG was dependent on Mg^{2+} and Ca^{2+} and was inhibited by neo-glycoproteins such as galactose-BSA and *N*-acetylgalactose-BSA. Partial inhibition of phagocytosis was also seen with the Arg-Gly-Asp-Ser (RGDS) motif and with an anti-CD18 mAb (OX42). The combination of both neo-glycoprotein and anti-CD18 achieved the greatest degree of inhibition (>90%). Together, these data suggest a role for both C-type lectins and β_2-integrins in the binding and internalization of PSG. The consequences of this interaction included a rapid up-regulation of inducible NO synthase mRNA and subsequent release of NO by alveolar macrophages. Thus, receptor-mediated recognition of inhaled allergenic particles by alveolar macrophages may represent a potential mechanism for modulating the inflammatory response associated with allergic airway diseases such as asthma. ***The Journal of Immunology,*** **2000, 164: 3878–3886.**

The respiratory tract (RT)[3] contains a number of cell types including epithelial cells and APC such as dendritic cells (DC) and alveolar macrophages (AM) (1, 2), all of which are capable of interacting with inhaled allergens. There is now clear evidence implicating AM in the inflammation associated with allergic airway diseases such as asthma. Thus, AM from asthmatic patients are more "activated" than those from normal subjects and express elevated levels of ICAM-1 and LFA-1 (3). Stimulated AM from asthmatics produce more GM-CSF, TNF-α, IL-8, and leukotrienes than AM from nonasthmatics (4, 5), and it has also been shown that AM influence the production of IL-5 by $CD4^+$ T cells (6, 7). In addition, AM from allergic asthmatics exhibit an increased expression of the costimulatory molecule CD80 and are more efficient at Ag presentation than AM from normal subjects (8). Thus, by virtue of their anatomical location and activatable phenotype, AM have the potential to play a significant role in initiating and regulating airway inflammation following exposure to allergenic material.

The mechanism(s) by which inhaled allergenic material gains access to the RT in a respirable form is not fully understood. As whole pollen grains are too large to penetrate into the lower RT (9), an understanding of the nature of respirable allergenic material is essential. Recent studies have described the presence in pollen of small starch granules (<5 μm) which possess similar allergenic activity as whole pollen and which are capable of initiating an asthmatic episode (10–13). Besides pollen starch granules (PSG), allergenic activity has also been associated with house dust mite fecal pellets (14, 15), mould spores (16), and fragments of animal dander (17). These allergenic particulates (AP) are all of a size which would allow ready inhalation into the deep lung and hence have the potential to initiate inflammatory responses. To date, there is no information relating to the biological sequelae of inhalation of these AP.

In this study, we have chosen to investigate interactions between AM and PSG. Previous work has demonstrated that PSG are released from pollen from plants such as rye grass (*Lolium perenne*) and birch following exposure to atmospheric moisture and has implicated PSG as the causative agent of thunderstorm-associated asthma epidemics (13, 18, 19,). PSG range in size from ~0.6 to 2.5 μm, making them easily respirable and indeed inhalation of PSG by asthmatics has been shown to elicit significant bronchoconstriction (10). Although not well characterized, it is known that PSG are composed primarily of starch and complex carbohydrates and PSG from rye grass pollen are known to contain the major allergen Lol p 5, to which most rye grass-allergic individuals are sensitized

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[3] Abbreviations used in this paper: RT, respiratory tract; AM, alveolar macrophage; PM, peritoneal macrophage; DC, dendritic cell; PSG, pollen starch granule; MFI, mean fluorescence intensity; nMFI, normalized MFI; MOI, multiplicity of infection; AP, allergenic particle; MSFM, macrophage serum-free medium; iNOS, inducible NO synthase; Gal, galactose; Man, mannose; GalNAc, *N*-acetylgalactose; Gluc, glucose; Fuc, fucose; MFR, Man-Fuc receptor; M-ASGP-BP, macrophage asialoglycoprotein-binding protein; L-NNA, N^G-nitro-L-arginine.

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(20). We have recently demonstrated that rye grass PSG contain significant quantities of (1→3)-β-D-glucan (21). PSG from birch pollen have been identified as a major source of the allergen Bet v 1, suggesting that PSG constitute a major source of both tree and grass pollen allergens in the environment (13).

Interactions between AP and AM may be mediated by a variety of mechanisms. However, due to the large number of surface receptors expressed by AM, we hypothesized that interactions would likely involve receptor-mediated events. Of particular importance to this study are the carbohydrate-binding receptors such as the C-type lectins or the β-glucan receptor. These receptors have specificities for terminal sugar groups rarely found in mammalian systems but which are common constituents of pollen, bacteria, and fungi (22), suggesting the possibility that they play a role in innate recognition of AP such as PSG. The interaction of AP with receptors on AM has the potential to stimulate production of various mediators including NO. Increased production of NO has been implicated in the pathogenesis of asthma (23, 24) and may function by selectively inhibiting the proliferation of Th1 cells (25). Despite evidence that allergen challenge of the airways rapidly leads to an increase in exhaled NO in asthmatic individuals (26, 27), there is no information regarding NO release by AM or other airway cells following direct interactions with AP.

Therefore, the aims of this investigation were to characterize the nature of the interactions between AM and PSG and to investigate the sequelae of this interaction in terms of NO production.

Materials and Methods

Animals

Inbred PVG rats (6–10 wk) were used throughout. Animals were housed on low-dust bedding to minimize background airway inflammation as detailed previously (28) and fed ad libitum on autoclaved food. Animals were serologically free of Sendai virus and other known pathogens. All animal work was conducted with relevant ethics approval from the TVW Telethon Institute for Child Health Research Ethics Committee adhering to the guidelines of the National Health and Medical Research Council of Australia.

Reagents

Pollen (nondefatted) from rye grass (*L. perenne*), bermuda grass (*Cynodon dactylon*), canary grass (*Phalaris arundinacea*), corn (*Zea mays*), johnson grass (*Sorghum halepense*), orchard grass (*Dactylis glomerata*), timothy grass (*Phleum pratense*), and Kentucky bluegrass (*Poa pratensis*) were purchased from Greer Laboratories (Lenoir, NC). All other reagents, unless specified, were purchased from Sigma-Aldrich (NSW, Australia). Carboxylated fluorescent latex beads (Fluoresbrite Carboxy YG, 3 μm diameter) were obtained from Polysciences (Warrington, PA). Anti-rat CD18 (OX42) was prepared from cell culture supernatant by $NaSO_4$ precipitation and extensively dialyzed against PBS before final purification on a protein A column.

Cell preparations

AM were harvested by bronchoalveolar lavage. Rat tracheas were exposed and catheterized, and lungs were lavaged with five aliquots (10 ml) of PBS (37°C) containing 10% (v/v) heat-inactivated FCS (Trace; Biosearch, Perth, Australia) and 0.2% (w/v) lignocaine. Lavage fluid was centrifuged at 500 × *g* for 6 min and cells were pooled by resuspending in 5 ml of 0.14 M NH_4Cl for 5 min to lye RBC. The remaining cells (>98% AM, by ED1 immunohistochemistry and >90% viable, by trypan blue exclusion) were then washed once in RPMI 1640 containing 5% (v/v) heat-inactivated FCS (R5) and once in PBS before being resuspended in R5 and the density adjusted to 2×10^5 cells/ml. All culture media contained antibiotic and antimycotic (100 μg/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B).

Peritoneal macrophage (PM), mast cell, and neutrophil isolation was performed. The peritoneal cavity of each rat was lavaged with ~50 ml of ice-cold PBS. Recovered cells were centrifuged at 500 × *g* for 5 min and resuspended in 1 ml of PBS before being layered onto a 30%:50%:80% discontinuous Percoll gradient and centrifuged at 600 × *g* for 20 min at 20°C. PM (>90% viable, >85% pure after staining cytospins with Diff Quik; Lab Aids, NSW, Australia) recovered from the 30%:50% interface and peritoneal mast cells (>90% pure after staining cytospins with Diff Quik) recovered from the pellet were washed in PBS and resuspended in R5 at a density of 2×10^5 cells/ml. Separate animals were injected i.p. with 2 ml of a 7.5% (w/v) solution of sodium caseinate (Upstate Biotechnology, Lake Placid, NY) in PBS to induce a sterile neutrophil infiltration into the peritoneum. After 4 h, peritoneal lavage was performed, and the recovered cells were layered onto a 30%:50%:80% discontinuous Percoll gradient and centrifuged as above. Neutrophils (>90% pure after staining cytospins with Diff Quik) were recovered from the 50%:80% interface and washed once in PBS and resuspended in R5 as above.

The cell lines P815 (mastocytoma, ATCC TIB-64), EL4 (T cell lymphoma, ATCC TIB-41), and 3T3 (fibroblast, ATCC CRL-6474), kindly provided by Dr. Delia Nelson (Department of Medicine, University of Western Australia), were grown and maintained in R10 for two passages and washed once in PBS before being resuspended in R5.

Human monocyte-derived DC, kindly prepared by Dr. Debbie Cooper (Department of Cell Biology, Institute for Child Health Research, Perth, Western Australia), were derived from T cell- and B-cell-depleted peripheral blood monocytes obtained from healthy donors by culture in the presence of GM-CSF and IL-4 according to the method of Sallusto and Lanzavecchia (29). DCs were used after 7 days in culture and had a typical DC morphology, were CD14 negative, MHC class II^{high} and CD1a positive as ascertained by flow cytometry.

PSG extraction

PSG (≤3 μm) were isolated from whole grass pollen following exposure of intact pollen grains to osmotic stress. Briefly, 500 mg of pollen was added to 50 ml of pyrogen-free water (Baxter Healthcare, Perth, Australia) with antibiotic and antimycotic and 0.05% (v/v) Tween 20. The resulting suspension was vortexed for ~3 min and rotated for 2 h at 4°C in a 50-ml Falcon tube (Becton Dickinson Labware, Mountain View, CA). Whole pollen and pollen fragments were removed by centrifugation at 50 × *g* for 3 min, and the remaining filtrate was passed through a 20-μm nylon filter (Nytal GG; Swiss Screens, Perth, Australia). This filtrate (<5% pollen) was then centrifuged at 2500 × *g* for 10 min and the pellet was resuspended in 20 ml of sterile water. Ten-milliliter volumes were filtered through 25-mm polycarbonate filters with a pore size of 3 μm (Nucleopore; Australian Biosearch). The final filtrate was centrifuged as before and the resulting pellet was resuspended in 1 ml of sterile water containing antibiotic and antimycotic and stored at 4°C. To determine the number and purity of PSG, a 10-μl aliquot of the suspension was diluted 1/100 with Gram's iodine (KI/I_2), and granules were counted on an improved neubauer chamber. The above extraction procedure yielded ≈2×10^8 PSG with a final purity of ≥99%.

To fluorescently label isolated PSG, granules were pelleted and resuspended in 2 ml of 0.1 M $NaHCO_3$ (pH 9.0) containing 1 mg/ml FITC. After incubation for 1 h at room temperature, labeled PSG (FITC-PSG) were washed twice in 20 ml of PBS before being resuspended in 1 ml of PBS. FITC-PSG were used immediately.

Phagocytosis assay

Cells were placed into 2-ml Teflon well inserts (Savillex, Minnetonka, MN; 2×10^5 cells/1.5 ml medium/well) and either used immediately or, in the case of AM and PM, cultured for 24 h unless otherwise stated. To investigate receptor temperature dependence and to prevent phagocytosis, cells were cooled to 4°C for 40 min before addition of FITC-PSG. Parallel cultures were maintained at 37°C in the absence or presence of various inhibitors that were added 40 min before the addition of FITC-PSG. FITC-PSG were adjusted to an multiplicity of infection (MOI) of either 20 or 40 in a final volume of 200 μl of culture medium before addition to each well. After a 4-h incubation at either 37°C or 4°C, cells were centrifuged at 500 × *g* for 5 min in 3-ml flow cytometry tubes and resuspended in 750 μl of fixative solution (2% (v/v) formaldehyde, 12 mM NaN_3, and 55 μM propidium iodide in PBS). Cells remained in fixative overnight at 4°C before analysis by flow cytometry using a Coulter Epics XL flow cytometer (Coulter, Palo Alto, CA). A minimum of 2500 cells (gated based on propidium iodide uptake) were counted from each tube. To quantify the level of FITC-PSG binding/phagocytosis, the specific background autofluorescence of fixed control cells (cells that had not been exposed to FITC-PSG) was used as a threshold level. Cells with a fluorescence intensity higher than this threshold level (positive cells) were considered to be binding/phagocytosing PSG. The percentage of cells binding/phagocytosing was calculated as the number of positive cells with respect to the total number of cells counted. The level of binding/phagocytosis by positive cells was quantified as a mean fluorescent intensity (MFI). To allow for variations in

fluorescent intensity of labeled PSG from different pollen species and between experiments, the MFI of positive cells was then normalized (nMFI) against the MFI of labeled PSG alone. The net phagocytosis of PSG for each point was calculated as the difference in nMFI between cells incubated with FITC-PSG at 37°C and the nMFI of cells incubated with FITC-PSG at 4°C. The percentage of inhibition of phagocytosis in the presence of various inhibitors was then calculated as follows:

$$\% \text{ inhibition} = 100\times[1-(\text{net phagocytic nMFI in presence of inhibitor/net phagocytic nMFI in absence of inhibitor})].$$

Fluorescent microscopy, confocal microscopy, and fluorescence quenching

Cells were visualized by fluorescent microscopy using a Zeiss Axiovert 135 microscope (Oberkochen, Germany) after first being cytocentrifuged and mounted under coverslip in vectorshield mounting medium (Molecular Probes, Eugene, OR). In a series of preliminary experiments designed to determine the degree of PSG internalization by AM, the cell surface fluorescence resulting from adherent FITC-PSG was quenched with trypan blue before flow cytometry. The degree of PSG internalization was also confirmed by confocal microscopy using a Bio-Rad MRC 1000/1024UV laser scanning confocal microscope running Cosmos software (Richmond, CA). The 488-nm line of the argon laser was used in combination with a polychroic beam splitter. The microscope used was a Nikon Diaphot 300 inverted microscope (Melville, NY) equipped with a ×60 water immersion objective.

NO determination

AM were washed in PBS and resuspended at 5×10^5 cells/ml in macrophage serum-free medium (MSFM) (Life Technologies, Grand Island, NY) and cultured in 200-μl volumes in a 96-well plate (Falcon 3072; Becton Dickinson Labware) for 48 h. Nonadherent cells were removed by washing once with PBS and remaining AM were preincubated in MSFM with or without 50 μg/ml polymyxin B sulfate. Isolated rye grass PSG (nonfluorescently labeled) were then added to each well at a ratio of 40 PSG per macrophage. Aliquots of culture medium were taken from each well at 0, 3, 6,12, 24, and 48 h after addition of PSG and assayed in triplicate using Griess reagent with $NaNO_2$ as a standard. In other wells, PSG were added for only 3 h and then cells were washed twice with PBS before addition of MSFM with or without polymyxin B sulfate. Supernatants from these wells were collected and assayed after a 48-h incubation. LPS was added to separate wells at 100 ng/ml with 1% (v/v) normal rat serum for 48 h as a control for both polymyxin B activity and AM activation.

Detection of inducible NO synthase (iNOS) expression by semiquantitative reverse transcription-PCR

RNA was extracted from AM after 0-, 3-, 6-, 12-, 24-, and 48-h exposure to PSG using RNazol B. cDNA was transcribed using 250 ng of oligo(dT)15 (Biotech International, Australia) and 2.5 U of avian myeloblastosis virus-reverse transcriptase (Promega, Madison, WI) in the presence of 12.5 U of ribonuclease inhibitor (Biotech International) in a final volume of 46 μl. The primer sequences for rat iNOS were as published (30) and as follows: forward primer, 5′-CCC TTC CGA AGT TTC TGG CAG CAG C-3′ and reverse primer, 5′-GGC TGT CAG AGC CTC GTG GCT TTG G-3′. These primers were specific for rat iNOS, yielding a 222-bp product from iNOS mRNA. The primer sequences for rat β-actin were as follows: forward primer, 5′-ATG CCA TCC TGC GTC TGG ACC TGG C-3′ and reverse primer, 5′-AGC ATT TGC GGT GCA CGA TGG AGG G-3′. For the PCR reactions, the reaction mixture contained 1 μl of cDNA, 50 ng of forward and reverse primers sequences of either iNOS or β-actin, 1× PCR buffer (Biotech International), 0.2 mM each of dNTPs (Biotech International), 2 mM $MgCl_2$, and 0.5 U of Platinum *Taq* DNA polymerase (Life Technologies) in a total volume of 12.5 μl overlaid with mineral oil. The PCR was run on a programmable thermocycler (Perkin-Elmer, Norwalk, CT) as follows: an initial denaturation step of 94°C for 5 min followed by 35 cycles (25 cycles for β-actin) of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 2 min. This cycle number resulted in a PCR product that was in the linear range of PCR amplification. PCR products were electrophoresed on an ethidium bromide-stained 1.5% (w/v) agarose gel (Progen, Australia). Gel photographs were scanned with a UMAX Vista S-6 scanner using Photoshop software (Adobe System) on a Macintosh computer. Densitometry was performed using Scan Analysis software (Biosoft, Ferguson, MO). Data were expressed as a ratio of the density of iNOS product relative to the density of the β-actin product for each sample.

Table I. *PSG released from various grass pollen species are bound and phagocytosed by AM*

Pollen Species	Relative Release of PSG from Whole Pollen[a]	Relative Level of PSG Binding/Phagocytosis[b]
Rye grass	1.0 ± 0.03	1.0 ± 0.01
Bermuda grass	0.8 ± 0.03	0.9 ± 0.01
Timothy grass	5.5 ± 0.44	0.9 ± 0.02
Bluegrass	0.8 ± 0.09	0.7 ± 0.01
Orchard grass	4.1 ± 0.67	1.1 ± 0.03
Johnson grass	4.1 ± 0.01	1.1 ± 0.03
Corn	1.9 ± 0.03	1.3 ± 0.1
Canary grass	0.8 ± 0.08	1.1 ± 0.03

[a] The relative PSG release was calculated by comparing the total number of PSG released from a fixed quantity of each pollen species (50 mg in 10 ml water) to that released by rye grass. Data are presented as means ± SEM ($n = 3$).

[b] The relative level of PSG binding and phagocytosis was calculated by comparing the nMFI of AM resulting from a 4-h incubation with FITC-PSG (MOI, 20) from each pollen species to that resulting from rye grass FITC-PSG. Data are presented as means ± SEM ($n = 3$).

Statistical analysis

Statistical differences were calculated on original data by the unpaired Student's *t* test (two tailed) for comparing the effect of each inhibitor shown in Table II against an individual internal control. A threshold value of $p < 0.05$ was considered to be significant.

Results

AM bind and phagocytose PSG

To test whether AM were able to phagocytose PSG from a range of grass species, including rye grass, bermuda grass, canary grass, corn, johnson grass, orchard grass, timothy grass, and Kentucky bluegrass (all representing the genera *Poaceae*), AM, cultured for 24 h, were incubated with FITC-PSG (MOI, 20) from each pollen species for 4 h at 37°C. Data in Table I shows that PSG were released from all grass pollen species in similar numbers and that AM were equally capable of interacting with PSG from all grass species. As PSG from all pollen types tested appeared similar in their interaction with AM, and given the relative importance of rye grass as a source of allergen, further investigations focused on PSG isolated from rye grass. Analysis of PSG phagocytosis by flow cytometry showed that PSG were uniformly labeled with FITC (Fig. 1, *A* and *C*). AM precultured for 24 h interacted strongly with FITC-PSG and >70% of cells showed binding and/or phagocytosis after 4 h of incubation (Fig. 1*B*) with each AM binding between 10 and 40 PSG (Fig. 1, *B*, *E*, and *F*). Binding of FITC-PSG resulted in a marked increase in the MFI (Fig. 1*D*, *gate II*, solid line) which was easily distinguishable from background cellular autofluorescence (Fig. 1*D*, *gate I*, dashed line). Confocal microscopy and trypan blue quenching of surface fluorescence (data not shown) confirmed that most PSG associated with AM after 4 h were internalized and not adherent to the cell surface.

PSG bind to several cell types but are only phagocytosed by AM and PM

Both AM and PM cultured for 24 h were highly phagocytic for PSG, although AM exhibited approximately twice the level of phagocytosis. In contrast, freshly isolated (ex vivo) AM and PM showed little or no phagocytosis, suggesting that the receptors for PSG phagocytosis are either not expressed or are inactive on freshly isolated macrophages (Fig. 2*A*). PSG binding at 4°C by either AM or PM did not significantly increase after 24 h in culture. Binding at 4°C could not be further inhibited by any of the potential inhibitors listed in Table II. The relative binding and phagocytosis of PSG by human monocyte-derived DC, rat mast

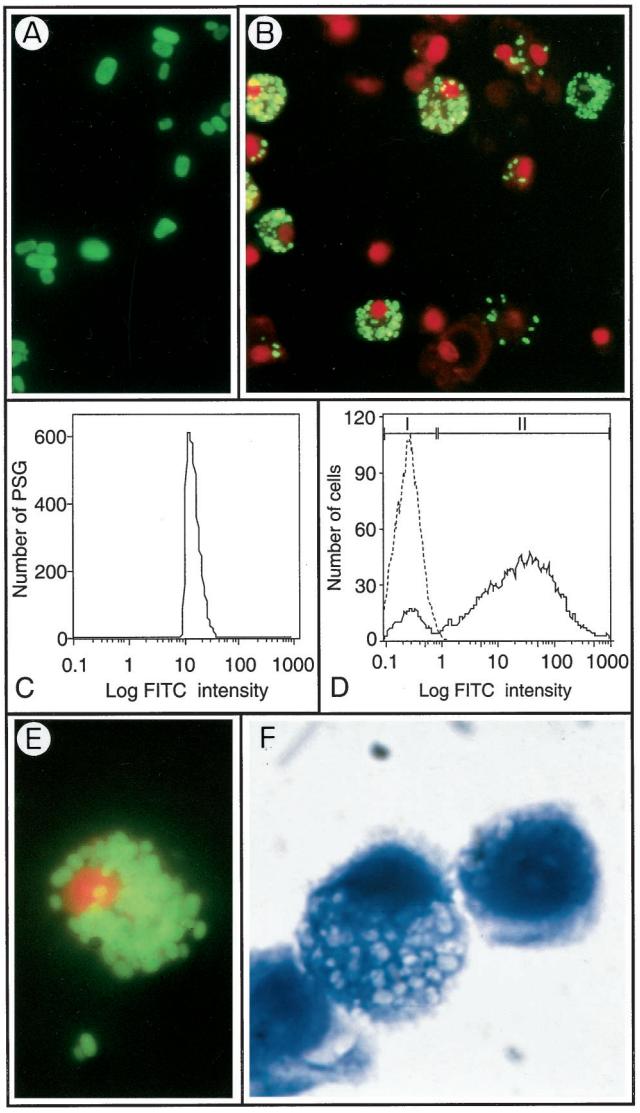

FIGURE 1. Rye grass PSG bind to and are phagocytosed by AM. PSG were isolated from whole pollen and labeled with FITC before addition to AM for 4 h at 37°C. *A*, Isolated FITC-PSG viewed at ×1000 magnification using a fluorescent microscope. *B*, Rye grass FITC-PSG (green) interacted strongly with AM (red nuclei) as seen at ×350 magnification using a fluorescent microscope. *C*, Rye grass FITC-PSG were uniformly labeled as shown by the tight distribution of fluorescence when measured by flow cytometry. *D*, Binding and uptake of FITC-PSG by AM resulted in a strong increase in the MFI of positive cells (*gate II*, solid line), which was easily distinguishable from the level of AM autofluorescence of negative cells (*gate I*, dashed line). *E*, Rye grass FITC-PSG bound and phagocytosed by AM viewed at ×1000 magnification under fluorescent light and under normal light after staining with Diff-Quik (*F*).

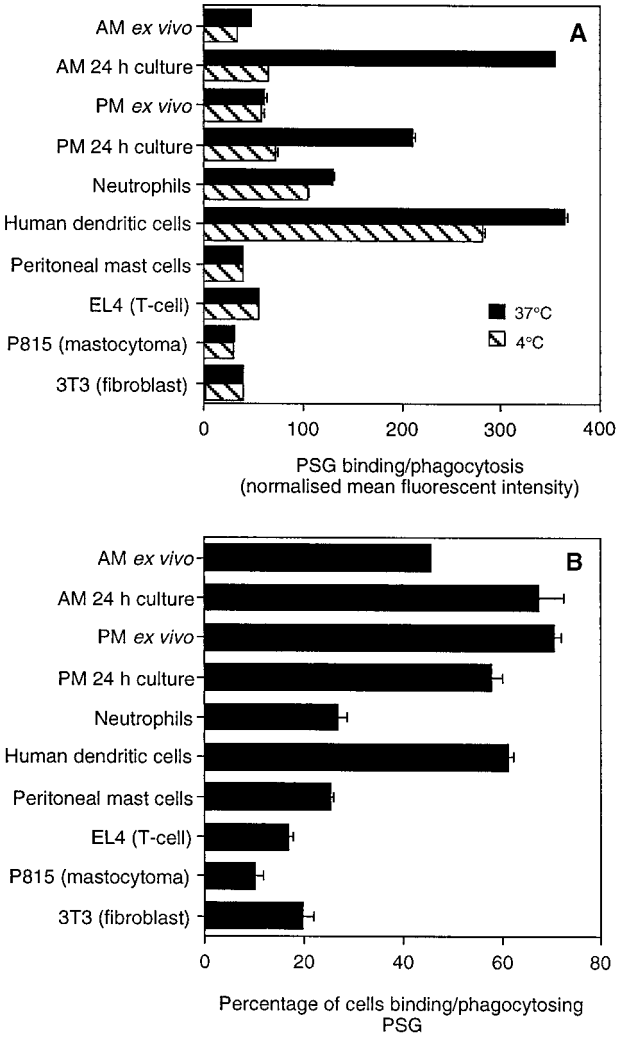

FIGURE 2. Rye grass PSG are avidly phagocytosed by AM and PM and are strongly bound by human DC. Both freshly isolated and cultured AM and PM, human DC, neutrophils, peritoneal mast cells, and the cell lines EL4, P815, and 3T3 were incubated with FITC-PSG (MOI, 40) for 4 h at 37°C or at 4°C before fixation and analysis by flow cytometry. The level of binding and phagocytosis was quantified as a nMFI (*A*) and as the percentage of AM interacting with PSG (*B*; for cells incubated at 37°C only). Data are presented as means ± SEM ($n = 3$) and are representative of four experiments for AM and PM and two experiments for other cell types.

cells, rat neutrophils, and cell lines EL4 (T cell lymphoma), P815 (mastocytoma), and 3T3 (fibroblast) were investigated (Fig. 2, *A* and *B*). Unlike all other cell types tested, DC strongly bound rye grass PSG at 4°C with a similar level of interaction at 37°C, suggesting temperature-independent binding and thus the possible involvement of different receptors to those on AM. Many individual DC showed binding of >20 PSG with >60% of all DC showing some degree of binding. Rat neutrophils bound more PSG than either AM or PM at 4°C with some cells binding >5 PSG. However, <30% of neutrophils were responsible for this binding and no internalization was observed. Rat peritoneal mast cells and all three cell lines tested were poor binders of PSG (<5 PSG/cell) at both 37°C and 4°C. This low level of binding was present on only a small percentage of binding cells (<30% of mast cells and <20% of cell lines).

AM phagocytose PSG in a time-, temperature-, and dose-dependent manner

At 37°C, phagocytosis of PSG by AM commenced within 15 min and continued for 4 h (Fig. 3*A*). AM, maintained at 4°C, again showed temperature-dependent binding with no subsequent increase in the nMFI or percentage of positive cells after 15 min (Fig. 3*B*). The ability of AM to internalize PSG after a 4-h incubation was inhibited completely by cytochalasin B, which also reduced the nMFI and percentage of positive cells to a level equivalent to that found for AM maintained at 4°C (nMFI = 100, ≈50% positive cells, Fig. 3*B*), suggesting that PSG binding is also cytoskeleton dependent (Fig. 3, *A* and *B*). Since the majority of PSG associated with AM after 4 h were internalized, all subsequent

Table II. *Effect of different neo-glycoproteins, sugars, and matrix components on the phagocytosis of PSG by AM*[a]

Inhibitor Used	Inhibitor Concentration	% Inhibition of PSG Phagocytosis
Man-BSA	50 μg/ml	65.4 ± 13.1*
Fuc-BSA	50 μg/ml	55.2 ± 3.2*
GalNAc-BSA	50 μg/ml	60.9 ± 7.7*
Gal-BSA	50 μg/ml	67.9 ± 4.9*
Gluc-BSA	50 μg/ml	55.2 ± 3.9*
BSA alone	50 μg/ml	0.0 ± 12.6
Sialic acid	10 mM	−15.9 ± 9.4
N-acetylglucosamine	10 mM	7.6 ± 8.5
Laminarin	10 mg/ml	−32.4 ± 7.6*
Mannan	10 mg/ml	7.0 ± 7.9
Fucoidan	10 mg/ml	21.6 ± 15.2
Laminan	50 μg/ml	1.4 ± 8.2
Fibronectin	50 μg/ml	23.1 ± 2.8
RGD-ser	50 μg/ml	36.2 ± 12.6*

[a] AM were incubated with inhibitor for 40 min prior to the addition of FITC-labeled rye grass PSG at an MOI of 40 for 4 h. Inhibition of phagocytosis was calculated using a control in which no inhibitor was present. Results show the mean percentage of inhibition of phagocytosis ± SEM ($n = 3$) and are representative data from three separate dose-response experiments.

*, $p < 0.05$, when comparing the level of phagocytosis in the presence of inhibitor to phagocytosis in the absence of inhibitor; i.e., an individual control was used for each inhibitor.

experiments were quantified at 4 h. To determine the maximum uptake of PSG, AM cultured for 24 h were incubated with increasing doses of FITC-PSG (Fig. 3, *C* and *D*). Phagocytosis at 37°C was dose dependent and appeared to reach saturation at a nMFI of 300 and an MOI of 80. The maximum percentage of AM binding and phagocytosis (~75%) was achieved at an MOI of 40–80, suggesting that the receptors involved in PSG phagocytosis were widely expressed on AM cultured for 24 h (Fig. 3*D*). Increasing ratio of PSG to AM resulted in an increase in the percentage of positive cells (Fig. 3*D*) but not in the nMFI of individual AM (Fig. 3*C*).

AM up-regulate phagocytosis of PSG in culture independently of general phagocytic function

Since AM cultured for 24 h strongly phagocytosed PSG (Fig. 2*A*), the effect of culture time on PSG phagocytosis was investigated. AM were found to specifically up-regulate their ability to phagocytose PSG within 12 h of culture, after which time the level plateaued (Fig. 4). This increase in phagocytic capacity for PSG was not a result of an overall increase in phagocytic function since the phagocytosis of fluorescent latex beads was not similarly up-regulated (Fig. 4). The binding of both PSG and latex beads by AM (incubated in the presence of particles at 4°C) was not affected by time in culture and was equivalent in terms of nMFI (Fig. 4). The increase in PSG phagocytosis observed in culture was not affected by the culture medium used nor by the presence of FCS up to 20% (v/v). Similarly, culturing in the presence or absence of glucose had no effect on phagocytosis (data not shown).

Characterization of PSG phagocytic receptors, divalent cation requirement, and inhibition profile

Incubation with EDTA prevented any internalization of PSG at concentrations of EDTA ≥2 mM (Fig. 5). Incubating with EGTA resulted in a partial inhibition of PSG phagocytosis with a maximum of 80% inhibition at 10 mM EGTA (Fig. 5). Since phagocytosis of PSG required divalent cations, we then investigated the

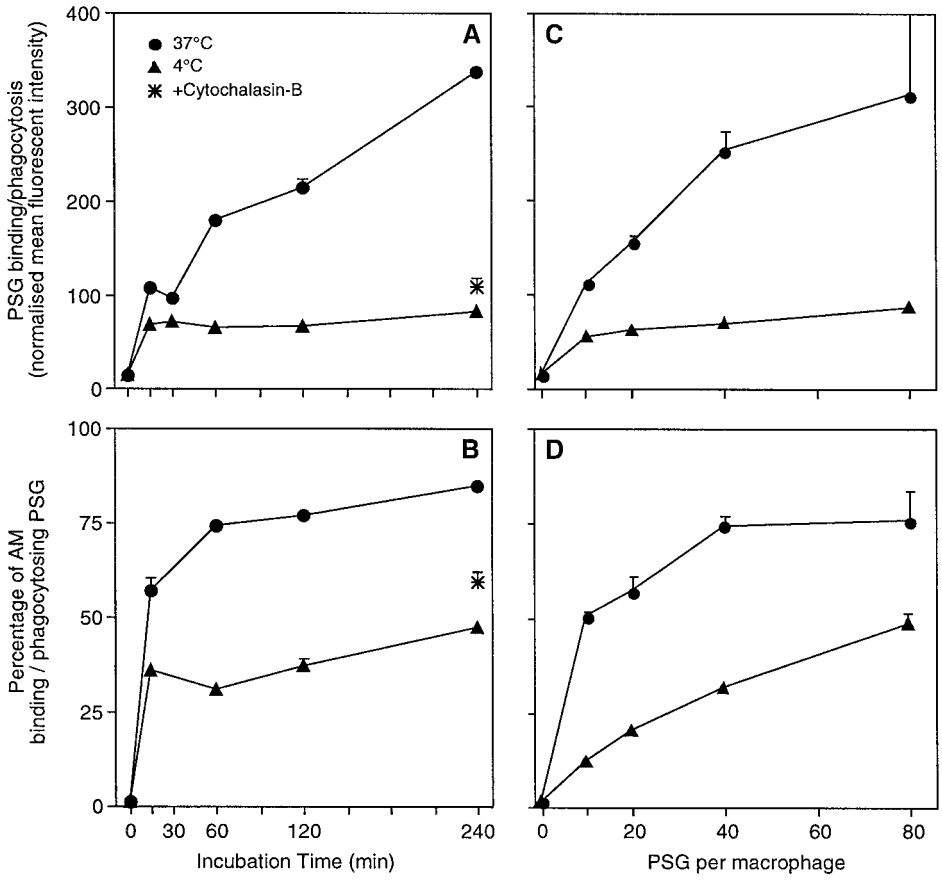

FIGURE 3. Time- and dose-dependent binding/phagocytosis of rye grass PSG by AM. AM, cultured for 24 h, were incubated with rye grass FITC-PSG (MOI, 40) for varying periods of time at either 37°C or 4°C before fixation and analysis by flow cytometry. Cytochalasin B (20 μg/ml) was added to AM maintained at 37°C, 40 min before addition of FITC-PSG. The level of binding and phagocytosis was calculated as a nMFI (*A*) and as the percentage of AM interacting with FITC-PSG (*B*). To determine maximum PSG phagocytosis, AM were incubated with 0, 10, 20, 40, or 80 FITC-PSG per macrophage for 4 h at either 4 or 37°C, and the level of PSG binding and phagocytosis was calculated as a nMFI (*C*) and as the percentage of AM binding/phagocytosing (*D*). Data are presented as means ± SEM ($n = 3$) and are representative of three experiments.

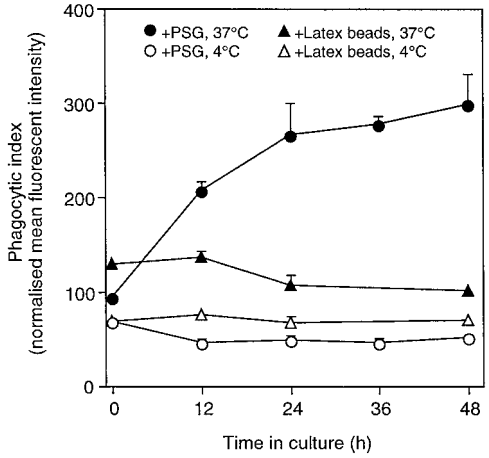

FIGURE 4. Phagocytosis of rye grass PSG by freshly isolated AM is inefficient but is specifically up-regulated in culture independently of the general phagocytic capacity of the cells. AM were harvested and either used immediately or cultured for 12–48 h and then used in a phagocytosis assay. AM from each time point were incubated with FITC-PSG at 4 or 37°C for 4 h before fixation. Alternatively, AM were incubated with fluorescent latex beads at 4 or 37°C for 4 h before fixation as a measure of phagocytic capacity. The level of binding and phagocytosis of both PSG and latex beads was calculated as a nMFI. Data are presented as means ± SEM ($n = 3$) and are representative of two experiments.

potential role of both lectin and integrin receptors in PSG phagocytosis by incubating AM with increasing concentrations of potential inhibitors (Table II). A range of neo-glycoproteins was able to significantly inhibit PSG phagocytosis (Table II); inhibition was achieved at 50 μg/ml with galactose-BSA (Gal-BSA, 68% inhibition), mannose-BSA (Man-BSA, 65% inhibition), and *N*-acetylgalactose-BSA (GalNAc-BSA, 61% inhibition). Glucose-BSA

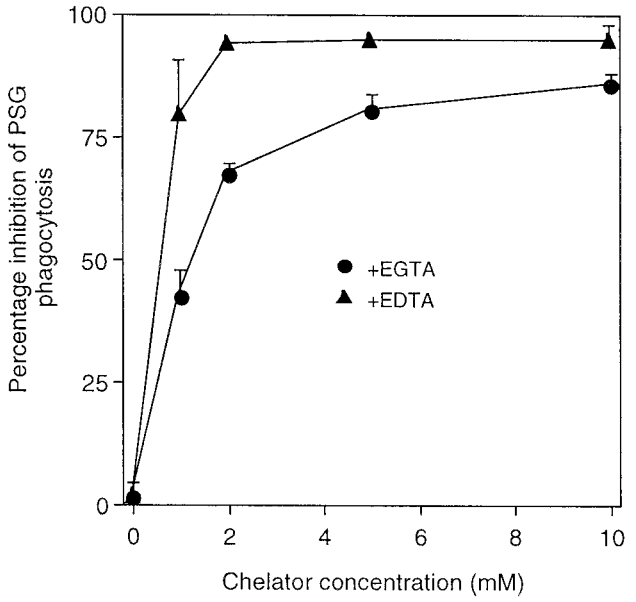

FIGURE 5. AM require divalent cations to phagocytose rye grass PSG. AM were incubated for 40 min in the presence of increasing concentrations of either EDTA or EGTA before the addition of FITC-PSG (MOI, 40) and then incubated for another 4 h before fixation and analysis by flow cytometry. The inhibition of net PSG phagocytosis was then calculated. Data are presented as means ± SEM ($n = 3$) and are representative of three experiments.

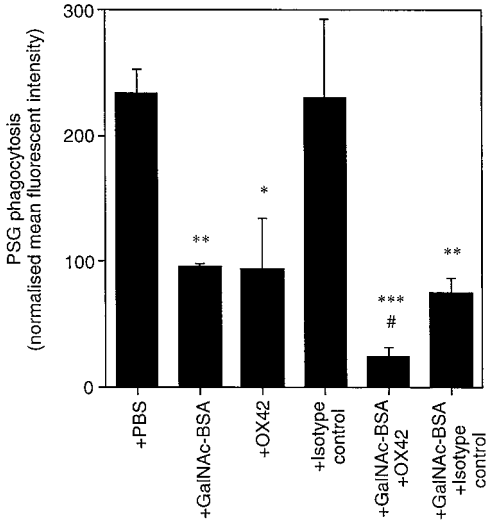

FIGURE 6. Phagocytosis of PSG is inhibited by both lectin and integrin inhibitors in an additive manner. AM cultured for 48 h were incubated with either GalNAc-BSA or OX42 (protein A purified) or both for 40 min before addition of FITC-PSG (MOI, 40) and then incubated for 4 h before fixation and analysis by flow cytometry. A commercially available rat IgG2a was used as an isotype control for OX42 and was tested both alone and in combination with GalNAc-BSA. The net phagocytosis for each treatment was calculated by subtracting the background level of binding at 4°C. Data are presented as means ± SEM ($n = 3$). ***, $p < 0.0005$, **, $p < 0.005$, *, $p < 0.05$, comparing all to PBS treatment. #, $p < 0.05$, comparing AM + GalNAc-BSA + OX42 to + GalNAc-BSA + isotype control.

(Gluc-BSA) and fucose-BSA (Fuc-BSA) led to a 55% and 52% inhibition, respectively. No inhibition occurred with BSA alone.

The inhibition of PSG phagocytosis by Man-BSA and Fuc-BSA suggested the possible involvement of the macrophage Man-Fuc receptor (MFR). However, mannan failed to inhibit phagocytosis (Table II). In addition, culturing AM in the presence of rat IFN-γ (100 U/ml), a potent MFR down regulator, had no effect on PSG phagocytosis. This was despite an observed down regulation of MFR mRNA by reverse transcription-PCR in these cells (no Ab is available for rat MFR) (data not shown).

Sialic acid, an inhibitor of sialic acid receptor binding, and *N*-acetylated-glucosamine, an inhibitor of β-glucan binding to CR3 (CD11b/CD18), showed no significant inhibition of PSG phagocytosis at concentrations as high as 10 mM (Table II). Similarly, there was no inhibition of PSG phagocytosis with laminan. Incubation with either fibronectin or the peptide Arg-Gly-Asp-Ser (RGDS; inhibitors of β_2-integrins) resulted in partial inhibition of PSG phagocytosis (Table II). Further evidence in support of the involvement of β_2-integrins was provided by the significant inhibition obtained using a blocking mAb directed against CD18 (OX42) (Fig. 6). When OX42 was used in combination with GalNAc-BSA, PSG phagocytosis was further inhibited (>90% inhibition), suggesting that both lectins and integrins act in an additive manner in recognition of PSG (Fig. 6). These data suggest that both lectin-like receptors and β_2-integrins may modulate PSG phagocytosis.

AM binding/phagocytosis results in an up-regulation in iNOS mRNA and the production of NO

Incubation of AM with PSG resulted in an up-regulation of the expression of iNOS mRNA within 6 h of exposure to PSG, with expression peaking after a 12-h exposure (Fig. 7*A*). The mRNA for

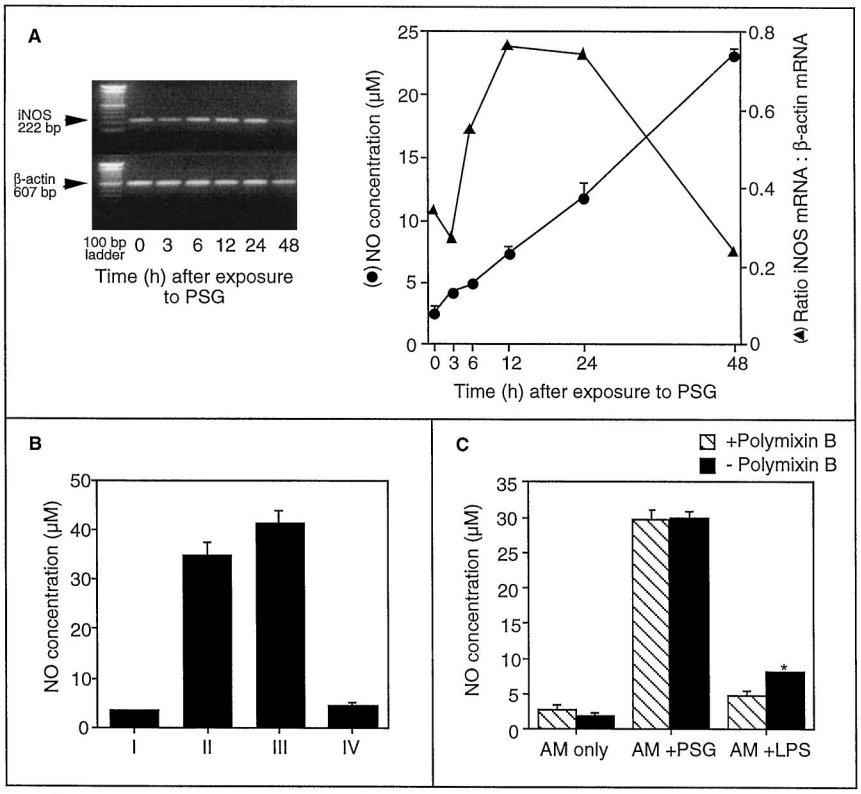

FIGURE 7. AM up-regulate iNOS mRNA and produce NO following exposure to rye grass PSG. *A*, Adherent AM were exposed to rye grass PSG in the presence of polymyxin B sulfate for 0, 3, 6, 12, 24, and 48 h, after which time mRNA was extracted for reverse transcription-PCR for iNOS, and supernatants were assayed for NO in the form of NO_2^- by Greiss assay. Data are presented for NO concentration as means ± SEM ($n = 5$). Data presented for reverse transcription-PCR are representative of two experiments. *B*, AM were incubated with rye grass PSG in the absence (*column II*) or presence of the NOS inhibitor L-NNA, at concentrations sufficient to inhibit constitutive NOS and neuronal NOS (25 nM, *column III*) or iNOS (10 μM, *column IV*). A control of AM incubated with 10 μM L-NNA alone was included (*column I*). Data are presented as means ± SEM ($n = 3$). *C*, AM were incubated alone or with rye grass PSG or LPS (100 ng/ml) for 48 h in either the presence or absence of polymyxin B sulfate and supernatants assayed for NO by Greiss assay. Data are presented as means ± SEM ($n = 3$), *, $p < 0.005$, comparing NO resulting from LPS stimulation in the presence or absence of polymyxin B sulfate.

iNOS remained constant until 24 h, and then returned to background levels by 48 h (Fig. 7*A*). Subsequently, NO was first detected in the culture supernatant at 6 h and levels increased to 22 μm by 48 h. Alternatively, AM that were exposed to PSG for only 3 h, before the granules were removed and media replaced, also produced a significant amount of NO after 48 h. However, the final concentration of NO produced was approximately half that above (12.12 μm; data not shown). Further evidence to support the involvement of iNOS, and not other forms of NOS, as the main source of increased NO was provided by the inhibition of NO production by N^G-nitro-L-arginine (L-NNA) (Fig. 7*B*). Incubation of AM in the presence of 25 nM of L-NNA, which is sufficient to inhibit both neuronal NOS and constitutive NOS activity, failed to inhibit PSG-induced NO production, whereas incubation with 10 μM L-NNA, which is sufficient to inhibit iNOS activity, completely blocked PSG-induced NO production (Fig. 7*B*). The overall stimulation of AM by PSG was not due to exogenous LPS associated with the particles since AM exposed to PSG in the presence of 50 μg/ml polymyxin B sulfate showed similar NO production. This concentration of polymyxin B sulfate was sufficient to significantly inhibit NO produced after exposure to 100 ng of LPS (Fig. 7*C*).

Discussion

The respiratory tract is continuously exposed to AP present in the inhaled air, and these may include PSG, house dust mite fecal pellets, and fungal spores. However, little is known about the nature of the interactions occurring between AP and cells of the RT and the possible consequences of such interactions. In this study, we have used PSG to characterize receptor-mediated interactions occurring between AM and AP. We have demonstrated for the first time that PSG, released from several species of grass pollen, were able to bind to and be phagocytosed by AM. PSG were also strongly bound by human monocyte-derived DC, suggesting the potential for antigenic sampling of allergens within the PSG by DC of the airway lining. Using a flow cytometry-based phagocytic assay, we further demonstrated that PSG interact with AM via two different receptor types. Thus, phagocytosis was totally inhibited by EDTA, partially inhibited by a range of neo-glycoproteins; partially inhibited by an anti-CD18 Ab (OX42), and totally prevented in the presence of both neo-glycoproteins and OX42. Taken together, these results suggest a role for both C-type lectins and integrins in PSG phagocytosis by AM.

Macrophages are known to express a wide range of C-type lectins on their surface (31), and in our system, PSG phagocytosis was inhibited by Gal-BSA and GalNAc-BSA as well as by Man-BSA and, to a lesser extent, Fuc-BSA and Gluc-BSA. Man/Fuc-binding lectins, such as the MFR have been well documented on mature macrophages (32, 33); however, the MFR does not appear to play a role in PSG phagocytosis since phagocytosis was not inhibited by mannan, a known inhibitor of MFR. Furthermore, neither IFN-γ nor dexamethasone altered the level of phagocytosis despite the fact that such mediators are known to regulate MFR mRNA and protein expression (33–35).

The strong inhibition of PSG phagocytosis observed in the presence of Gal-BSA and GalNAc-BSA suggests that a Gal-binding lectin may be involved in this system. Rat macrophages are known to express a type II, C-type lectin specific for Gal and *N*-acetylgalactosamine residues known as the macrophage asialoglycoprotein-binding protein (M-ASGP-BP) (36, 37). This lectin is not expressed on resident AM and PM (36, 38) but has been found on thioglycolate-elicited PM where it is thought to function both in tumor recognition and in receptor-mediated endocytosis of Gal-terminated glycoproteins (39, 40). The reported lack of expression of M-ASGP-BP on resident macrophages is consistent with the deficiency of PSG phagocytosis reported in this study when using freshly isolated AM and PM; however, we are unable to conclude that M-ASGP-BP is involved in this system since other lectin-like

receptors with similar specificity have been described. For example, a type II Gal-binding C-type lectin, designated DC immunoreceptor, was recently identified and shown to be expressed on both activated and nonactivated DC and macrophages (41). In addition, Haltiwanger and Hill (42) have isolated an as yet uncharacterized lectin-like receptor from rat AM which bound to Fuc-BSA and was eluted by Gal. This lectin, with a mass of 46 kDa, was similar in size to M-ASGP-BP (42 kDa) but, unlike M-ASGP-BP, was not immunologically cross-reactive with rat hepatic lectin (42, 43).

Regardless of the specificity of the lectin-like receptor involved in the phagocytosis of PSG, it is clear that carbohydrate recognition systems present on macrophages are able to interact with AP. Considering the carbohydrate content of many purified allergens from both pollens and house dust mites and of the AP that carry them into the RT, such interactions are worthy of further investigation.

Since PSG phagocytosis required both Mg^{2+} and Ca^{2+} and was significantly inhibited by OX42 and RGD peptide, we surmised that a β_2-integrin contributed to PSG phagocytosis. In support of this, binding was temperature dependent and inhibited by cytochalasin B, which may also be indicative of β_2-integrin activity (44, 45). Freshly isolated murine AM have been shown to be deficient in their expression of CR3; however, the receptor is strongly expressed after 24–48 h in culture (46). We found that rat AM behave in the same manner with regard to expression of CD18 (OX42 staining). Furthermore, the level of PSG phagocytosis strongly correlated with the expression of CD18 on both AM and PM.

The levels of both CR3 and LFA-1 are elevated on AM and eosinophils from asthmatic individuals, implicating β_2-integrins in the pathogenesis of asthma (47–51). In addition, ligation of CR3 on the surface of cells such as eosinophils has been shown to result in a rapid cellular degranulation (52). Thus, direct interactions between β_2-integrins and AP, such as PSG, have the potential to exacerbate asthmatic airway inflammation. The involvement of CR3 in the binding and phagocytosis of several diverse ligands is widely documented (53). If indeed CR3 is involved in this system, it would provide a possible explanation for the significant augmentation of PSG phagocytosis observed after incubation with laminarin (Table II). Recent studies have shown that binding of small soluble β-glucans, such as laminarin, to the lectin domain of CR3 generates a primed state of the receptor for up to 18–24 h (54). This "primed" state may mediate the cytotoxicity of neutrophils, macrophages, and NK cells toward iC3b-opsonized tumor cells (55, 56). Thus, it is possible that exposure of AM to laminarin resulted in a priming of CR3 with a resultant increase in PSG phagocytosis. The potential priming of PSG phagocytosis by this mechanism is particularly relevant because we have recently shown that PSG contain significant amounts of (1→3)-β-D-glucan (21). Therefore, the potential exists for PSG to augment their own phagocytosis via interactions with the CR3 lectin domain.

A hallmark of asthmatic airway inflammation, following allergen challenge, is the production of several proinflammatory cytokines, chemokines, and mediators, such as NO, by cells of the RT. However, despite a knowledge of the major effectors involved in airway inflammation, the mechanisms leading to their production are poorly understood. For example, it well documented that exhaled NO is increased in asthmatic individuals (57, 58) and is implicated in the pathophysiology of the disease (25); however, the cellular source of exhaled NO remains to be identified (59, 60). In our study, the interaction of PSG with AM resulted in a significant up-regulation of iNOS mRNA and, consequently, to a time-dependent release of NO. Data are starting to accumulate suggesting that human AM are able to produce significant NO when given the appropriate stimulus (61); however, more work is needed to define the contribution of interactions between allergens and AM in the production of exhaled NO. Based on the rapid phagocytosis of PSG by AM (Fig. 3) and on the subsequent production of NO, results from this study suggest that receptor-mediated interactions between inhaled AP and AM may represent a potent mechanism for the induction/production of exhaled NO.

Production of NO following PSG phagocytosis could be the result of signaling events following recognition by CR3 as has been previously described for other ligands (62) or may be due to receptor recognition by C-type lectins. Since (1→3)-β-D-glucan has been previously shown to elicit NO production by macrophages (63), the (1→3)-β-D-glucan associated with PSG may also be responsible for the production of NO observed in this study. Although there is little available information regarding the production of NO following stimulation of C-type lectins, we found that both AM and PM produce NO in a carbohydrate-specific manner following exposure to the neo-glycoproteins, Gal-BSA and GalNAc-BSA (data not shown). Production of NO by murine macrophages following stimulation with glycosylated BSA has been previously reported (64). However, the role of C-type lectins in NO signaling remains to be fully characterized.

In conclusion, the inhalation of AP such as PSG, house dust mite fecal pellets, and mould spores into the lower airways may result in the initiation and/or perpetuation of allergic airway inflammation. However, the mechanism by which these AP interact with cells of the RT and subsequent production of proinflammatory mediators is poorly understood. This study is the first to demonstrate specific receptor-mediated events occurring between pollen-derived AP and cells of the RT. The strong interaction between PSG and AM or DC shown in this study highlights the importance of resident airway cells in the early recognition of allergenic material. This is particularly relevant as such interactions occur in an IgE-independent manner, suggesting that innate mechanisms may contribute to recognition of allergens within the RT. Such mechanisms, acting via specific receptors, have the potential to influence allergen-induced airway inflammation and thus the phenotype of diseases such as asthma and rhinitis. Furthermore, these interactions result in the release of large amounts of NO, supporting the notion that AM may be an important source of exhaled NO present in asthmatic individuals following allergen challenge. Increasing our understanding of the nature of interactions occurring between AP and cells of the RT may pave the way for new therapies based on inhibition of such interactions or of the subsequent mediator release.

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