Tissue-Specific Fc γ and Complement Receptor Expression by Alveolar Macrophages Determines Relative Importance of IgG and Complement in Promoting Phagocytosis of *Pseudomonas aeruginosa*

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ABSTRACT. Because the expression of IgG Fc receptors and complement receptors on macrophages may vary in a tissue-specific manner, we used monoclonal antibodies and flow cytometry to define the expression and function of opsonin receptors on fresh normal and cystic fibrosis (CF) bronchoalveolar lavage (BAL) macrophages. Using flow cytometry to separately analyze individual types of cells, we then determined the relative contributions of IgG and complement to phagocytosis of *Pseudomonas aeruginosa* by fresh BAL cells, avoiding alterations in receptor expression due to *in vitro* purification or culturing techniques. Neither normal nor CF BAL macrophages express appreciable amounts of the complement receptors CR1, CR2, or CR3. These results were confirmed by immunohistochemical staining of fixed lung sections. BAL macrophages express a high-affinity IgG receptor, Fc γ RI, that is not found on neutrophils (PMN). In contrast, chemoattractant-stimulated blood PMN express large amounts of CR1 and CR3 but do not express Fc γ RI. These results correlate with phagocytosis assays, which show that phagocytosis by macrophages is enhanced by relatively low concentrations of IgG but that the addition of complement does not further increase their phagocytosis. In contrast, low concentrations of IgG alone do not promote phagocytosis by PMN, whereas addition of complement markedly enhances phagocytosis by PMN. These results may explain the previously reported sensitivity of macrophages rather than PMN to the "blocking" effects of anti-*Pseudomonas* antibodies from CF patients, and emphasize the pathologic significance of interference with IgG and complement mediated opsonization in the lung in CF. (*Pediatr Res* 35: 68-77, 1994)

Abbreviations

AM, alveolar macrophage
BAL, bronchoalveolar lavage
CD, cluster of differentiation
CF, cystic fibrosis
CR, complement receptor
Fc γ R, receptor for the Fc portion of IgG
FEV₁, forced expiratory volume in 1 s
FMLP, N-formyl-methionylleucylphenylalanine

HBSS, Hanks' balanced salt solution
LY, lucifer yellow
LPS, lipopolysaccharide
PMN, polymorphonuclear leukocyte
PMSF, phenylmethylsulfonyl fluoride
TBS, 0.05 M Tris-buffered saline, pH 7.6
FACS, fluorescence-activated cell sorter

Optimal host defense requires the interactions of opsonins bound to the surface of the targets of phagocytosis with specific receptors on the surface of the phagocyte. The primary opsonins involved in promoting phagocytosis of bacteria are IgG and fragments of the third component of complement. These proteins can potentially interact with three different Fc γ receptors (Fc γ RI, II, and III) and four different C3 fragment receptors (CR1, -2, -3, -4). The specificity and affinity of each of these receptors is unique, and their functional activities differ and may change under different physiologic conditions. Thus, to understand the importance of any individual opsonin, it is critical to know which receptors are expressed on the phagocytic cells the target is likely to encounter *in vivo*. It has recently become clear that macrophages express Fc γ and complement receptors in a tissue-specific manner (1-5) and that macrophages derived *in vitro* from peripheral blood monocytes may not express the same receptors as tissue macrophages at sites of infection or inflammation (6). To understand the interaction of the host with an infectious agent, it is therefore necessary to study those opsonins and receptors actually involved in phagocytosis at the site of infection.

In CF, chronic pulmonary infection with *Pseudomonas aeruginosa* remains a major clinical problem (7). The relationship between the primary CF defect and this infection remains elusive, as do the reasons why the host's defenses fail to eradicate these bacteria. Our previous work has shown that proteases in the lung remove an important complement receptor, CR1, from phagocytes and an important opsonin, iC3b, from the bacteria (8, 9). This suggests that these mechanisms may severely impair phagocytosis and killing of the bacteria *in vivo*. Other investigators have shown that IgG antibodies from CF patients have decreased opsonic activity and may actually inhibit or "block" the opsonic activity of other antibodies (10-15). Interestingly, this blocking activity is most apparent with macrophages, and has been reported with neutrophils in only one study (13). To better understand the relative importance of IgG antibody *versus* comple-

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Receptor Expression on Normal Alveolar Macrophages

Fig. 1. Receptor expression on normal alveolar macrophages. Separate aliquots of fresh BAL cells were stained with antibody to the indicated receptor or with the appropriate control antibody as described in Materials and Methods. From each aliquot, 10 000 cells were analyzed in the FACS, which was gated for cells with volume and 90° scatter greater than activated neutrophils. For each receptor, the histogram with the antibody to the receptor is shown by the solid line and the control is shown by the dashed line. Results are from a single representative BAL sample.

Table 1. Receptor expression on normal alveolar macrophages and blood neutrophils

Receptor	CD designation of antigen	Clone	Fresh BAL macrophages % cells positive (mean $\pm$ SEM, $n = 10$)	Chemoattractant stimulated normal blood neutrophils % cells positive (mean $\pm$ SEM, $n = 4$)
CR1	35	3D9	6.8 $\pm$ 1.5	88.4 $\pm$ 3.8
CR2	21	HB5	5.4 $\pm$ 2.1	30.0 $\pm$ 6.6
CR3	11b	M1/70	6.7 $\pm$ 1.3	88.8 $\pm$ 4.3
CR4	11c	S-HCL-3	34.7 $\pm$ 5.7	63.4 $\pm$ 11.5
Fc γ RI	64	32.2	38.5 $\pm$ 7.3	1.3 $\pm$ 1.0
Fc γ RII	32	IV.3	75.0 $\pm$ 5.1	97.1 $\pm$ 0.2
Fc γ RIII	16	NKP 15	53.3 $\pm$ 7.6	91.2 $\pm$ 1.2
HLA-DR		L243	95.8 $\pm$ 0.2	0.8 $\pm$ 0.7

* Receptor expression on fresh normal BAL macrophages ($n = 10$) and FMLP-stimulated purified blood neutrophils ($n = 4$). For each sample, the number of cells with fluorescence greater than the appropriate control for each antibody was determined as described in Materials and Methods.

ment *in vivo* and to test the hypothesis that macrophages are more sensitive to the effects of "blocking" antibodies because they are more dependent on the interaction of IgG with high-affinity Fc γ R than neutrophils, we determined which Fc γ and complement receptors are expressed on alveolar macrophages. We also determined the roles of IgG and complement in promoting phagocytosis by these macrophages as compared with neutrophils. To be sure that our results reflected the true physiologic situation *in vivo*, and to avoid alterations in receptor expression due to *in vitro* handling (16, 17), we used fresh BAL cells that were not purified or cultured *in vitro*. Rather, we used the ability of the flow cytometer to selectively analyze specific types of cells. The results show that fresh human alveolar macrophages express a high-affinity Fc γ R (type I), but only minimal amounts of the functional complement receptors CR1, CR2, or CR3. These results correlate with the results of flow cytometry phagocytosis assays on fresh BAL cells, which show that complement deposition on the bacteria fails to enhance phagocytosis by the macrophages, which is mediated primarily by IgG. These observations may provide an explanation for the sensitivity of macrophages to the effects of "blocking antibodies" in CF.

MATERIALS AND METHODS

Human subjects. Nine stable cystic fibrosis patients with mild to moderate lung disease (mean $\pm$ SEM FEV₁ = 82 $\pm$ 10% of

predicted) were recruited from the Cystic Fibrosis Center of Rainbow Babies and Childrens Hospital. Their mean ($\pm$ SEM) age was 19.2 $\pm$ 1 y. No patient was febrile or taking corticosteroids at the time of study. Ten healthy normal volunteers were recruited from hospital staff and students. Their mean age was 27.7 $\pm$ 2.5 y. All subjects gave informed consent and all protocols were approved by the Institutional Review Board of University Hospitals of Cleveland. Heparinized peripheral blood samples of 12–24 mL were obtained from each CF patient just before bronchoscopy was performed. Equivalent blood samples were drawn from normal donors at the same time BAL was performed on normal volunteers.

Bronchoalveolar lavage. Subsegmental lavage was performed using a flexible fiberoptic bronchoscope on awake patients, although some CF patients were lightly sedated with small doses of Demerol and Versed. Local anesthesia was achieved with topical Lidocaine. Multiple aliquots of 30–50 mL of saline, not exceeding 200 mL total, were instilled through the bronchoscope. Sixty to 80% of the instilled fluid was recovered in all cases. Lavage fluid was immediately filtered through surgical gauze to remove mucus plugs and debris. All subsequent steps were performed at 0–4°C. Lavage cells were harvested by centrifugation at 200 $\times$ g for 10 min and washed three times in HBSS without Ca²⁺ or Mg²⁺, supplemented with 0.1% bovine serum

Receptor Expression on Normal Blood Neutrophils

Fig. 2. Receptor expression on purified blood neutrophils stimulated with 10^{-8} M FMLP for 60 min at 37°C . Procedure and presentation are the same as in Fig. 1 except that the cytometer gates were set to include the neutrophils.

Table 2. Immunohistologic detection of complement receptors and HLA-DR on normal alveolar macrophages*

Antigen	% Cells positive (mean $\pm$ SEM; $n = 5$)
CR1	10.2 ± 2.9
CR2	4.7 ± 1.8
CR3	11.8 ± 1.3
CR4	34 ± 4.4
HLA-DR	85 ± 3.7
Control (anticasein)	5.5 ± 0.93

* Receptor expression on morphologically recognizable macrophages in tissue sections of fresh frozen lung specimens. Slides were coded so the observer did not know the antibody with which they were stained. For each antibody, at least 30 cells were graded in each of five lung specimens.

albumin and 10^{-4} M PMSF, then held on ice in this solution. Cell counts were performed using a hemocytometer and differential counts were performed on Wright's stained cytocentrifuge preparations. Viability of lavage cells was assessed by trypan blue exclusion and/or by flow cytometry as described previously (8). Incubation with monoclonal antibodies for determination of receptor expression or with opsonized bacteria for phagocytosis assays was accomplished within 4 h of performing the BAL.

Isolation of peripheral blood neutrophils. PMN were prepared from heparinized peripheral blood samples as previously described, using Percoll density gradients followed by hypotonic lysis of contaminating erythrocytes (8). Purity and viability of these preparations exceeded 95%. PMN were resuspended in HBSS without Ca^{2+} or Mg^{2+} , which was supplemented with 0.1% gelatin (HBSS gel). Cells were stimulated by incubation with the synthetic chemoattractant FMLP at 10^{-8} M for 1 h at 37°C with intermittent vortexing.

Flow cytometry and immunofluorescent assessment of receptor expression. Separate aliquots of 3×10^5 to 10^6 cells each in HBSS gel were incubated with monoclonal antibodies to Fc γ RI, CR, and HLA-DR, or with control antibodies, on a rotator at 4°C for 30 min. The primary antibodies used and the sources from which they were obtained were anti-Fc γ RI (produced by clone 32.2) from Medarex Inc., West Lebanon, NH; anti-Fc γ RII (produced by clone IV.3) from Dr. Michael Fanger, Dartmouth University, Hanover, NH; anti-Fc γ RIII (Anti-leu 11a) from Becton Dickinson, Mountain View, CA; anti-CR1 (produced by clone 3D9)

(8, 18); anti-CR2 (produced by clone HB-5) from Becton-Dickinson; anti-CR3 (Mac 1 antigen on CD11b, produced by clone M1/70) (8, 17); anti-Leu M5 (CD11c) from Becton Dickinson; and anti-HLA-DR from Becton Dickinson. The antibodies to Fc γ RIII and HLA-DR were purchased as FITC conjugates. When these antibodies were used, FITC-conjugated nonimmune antibodies of the same isotypes (IgG1 and IgG2a), which were also purchased from Becton Dickinson, were used as controls. For nonconjugated primary antibodies, an irrelevant primary antibody, mouse IgG1 anticasein, was used as a control. This antibody was the kind gift of Dr. Charlotte Kaetzel, Case Western Reserve University. No differences were observed when control nonconjugated antibodies of other isotypes were substituted for the anticasein monoclonal. After reaction with primary antibody, cells were washed three times with HBSS containing 0.1% bovine serum albumin, 10^{-4} M PMSF, and 0.01% NaN_3 and then stained with FITC-conjugated F(ab')_2 fragments of goat anti-mouse immunoglobulin as previously described (8, 18). Cells were then washed three additional times and held on ice in PBS containing 0.01% NaN_3 until examination in the flow cytometer, generally within 1 h.

Flow cytometry was performed with a Becton Dickinson FACS analyzer equipped for measurement of green and red fluorescence, light scatter at 90° , and true cell volume by the electrolyte displacement method. All data were recorded and processed by a dedicated Consort 30 computer. Analysis gates were set using true cell volume and 90° scattered light characteristics of resting and FMLP-stimulated peripheral blood neutrophils from each individual. BAL cells falling within these gates were considered neutrophils, cells with greater scatter and volume were considered macrophages. Each set of gates was then used to selectively analyze 5 000 to 10 000 cells of each type from the BAL samples of the CF patients. Neutrophils and macrophages were analyzed concurrently in all CF patients. Only macrophages could be analyzed in BAL samples of the normal donors because there were not sufficient neutrophils in these samples (8). Background fluorescence was determined on samples reacted with the anticasein control antibody and the same FITC conjugate, or with the isotype matched directly conjugated control antibodies for anti-Fc γ RIII and anti-HLA-DR. The percentage of positive cells in the sample stained with any given primary antibody was then determined by computerized subtraction of the background histogram, or by determining the number of cells with fluorescence greater than (to the right of) a discriminator set to include (to its

Fig. 3. Phase contrast photomicrographs of sections stained by immunoperoxidase reveal intense staining of alveolar macrophages (arrows) with anti-HLA-DR (A), but not with anti-CR1 (C), anti-CR2 (D), or anti-CR3 (E). Weak staining was seen in 30% of alveolar macrophages with anti-CR4 (F). Positive controls included a section of lymph node stained with anti-CR1 (B). Follicular lymphocytes (upper right) but not paracortical lymphocytes (lower left) reveal intense staining with anti-CR1 (B) and CR2 (not shown). Original magnification $\times 150$.

left) 98% of the cells stained with the control antibody. In all cases, results determined with these two different methods agreed within 5%. *t* tests were used to evaluate the statistical significance of differences in receptor expression between CF and normal cells.

Immunohistochemical staining of fixed lung tissue. Cryostat sections (4 μ m) of normal lungs obtained at autopsy within 12 h postmortem were air dried, fixed by immersion in acetone for 10 min, and equilibrated and washed three times in TBS. Sections of spleen, lymph node, and kidney were also employed as specificity controls. Fifty microliters of each monoclonal antibody were then applied to the sections. Primary murine antibodies were diluted at 1:5 for immunofluorescence and 1:20 for immunoperoxidase from the commercial stock solutions, with the exception of anti-CR1 (3D9), from ascites, and anti-CR3 (Mac-1) hybridoma culture supernatant. These latter antibodies were used at 1:50 and undiluted, respectively, for immunofluorescence and at 1:200 and 1:4, respectively, for immunoperoxidase. All dilutions were in TBS; incubations with primary antisera were performed at 25°C for 30 min for immunofluorescence and at 4°C for 22 h for immunoperoxidase. Slides for immunofluorescence were subsequently stained, after three washes with TBS, with a 1:10 dilution of an Fc-specific fluoresceinated goat IgG fraction of anti-mouse IgG, applied for 30 min at 25°C. For immunoperoxidase, slides were stained with the Vectastain Elite Mouse Kit (Vector Laboratories, Burlingame, CA), using biotin-avidin-peroxidase methods exactly as recommended by the manufacturer. All slides were mounted in 50% glycerol in PBS, pH 7.5. Coded specimens from five lungs were examined microscopically by a single experienced observer who was unaware of the antibodies used. Both immunoperoxidase and immunofluores-

cent stained specimens were examined in parallel, and excellent agreement was obtained with the two different procedures.

Bacteria, opsonization, and phagocytosis assays. Nonmucoid Fisher-Devlin immunotype (FT) 1 *Pseudomonas aeruginosa* and a mucoid strain were clinical isolates from CF patients obtained from the CF Core Center at Case Western Reserve University. Bacteria from overnight broth cultures were heat killed at 60°C for 30 min, washed twice with PBS containing Ca^{2+} , Mg^{2+} , and 0.2% glucose by centrifugation at $12\,000 \times g$ for 10 min, and labeled with the fluorescent reagent LY (Sigma) by incubation for 90 min at room temperature as described by Sveum *et al.* (19). Bacteria were then washed three times and aliquots were stored frozen in suspension at -80°C as previously described (9). Bacteria were thawed just before use, washed in PBS-glucose, then incubated on a rotator at $10^7/\text{mL}$ for 45 min at 37°C with the indicated concentration of a previously described (20) murine IgG3 monoclonal antibody specific for the O-side chain of FT 1 LPS. Alternate sources of antibody included a polyvalent human intravenous immunoglobulin prepared from plasma of normal donors with high titers of anti-*Pseudomonas* antibodies (*Pseudomonas* Immune Globulin, Cutter, Inc., Berkeley, CA) and polyvalent human hyperimmune globulin prepared from the plasma of volunteers immunized with a *Pseudomonas* polysaccharide conjugate vaccine (Nosocuman, Dr. Alan Cross, Walter Reed Army Institute of Research, Washington, DC). These preparations had equivalent titers of antibody to *Pseudomonas* LPS as determined in an enzyme-linked immunosorbent assay (20). In some experiments a murine IgG2b monoclonal against *Pseudomonas* mucoid exopolysaccharide, kindly supplied by Dr. Gerald Pier, Channing Laboratories, Boston, MA (21), was employed with a mucoid strain of bacteria. In indicated

Fig. 4. Phagocytosis of fluorescently tagged *P. aeruginosa* by fresh normal alveolar macrophages and FMLP-stimulated purified blood neutrophils. Washed, LY-tagged FT 1 bacteria were preopsonized as described in the figure when washed again and incubated with BAL cells (open bars) or neutrophils (cross-hatched bars) as described in Materials and Methods. The antibody used was a monoclonal murine IgG3 against the LPS O-side chain high molecular weight polysaccharide. Note that 15 µg/mL corresponds to 10^{-7} M. Mixtures were analyzed in the FACS with gates set for macrophages or stimulated neutrophils respectively. From each sample, 5000 cells were analyzed and the arithmetic mean fluorescence was determined by the Consort 30 computer. The mean fluorescence for BAL cells and neutrophils run in the absence of bacteria was subtracted as background from other samples with the same type of phagocyte. Results shown are the mean $\pm$ SEM of six experiments.

experiments, bacteria were concurrently treated with antibody and with pooled normal human serum as a source of complement (5% final serum concentration). This serum pool contained no detectable antibody to *P. aeruginosa* as determined with a 1:100 dilution in an enzyme-linked immunosorbent assay using whole mucoid bacteria as an absorbent. Sera from chronically infected CF patients are generally positive in this assay at dilutions $\geq 1:10\,000$. Opsonization with serum was also performed on a rotator for 45 min at 37°C. Bacteria were then washed twice and resuspended in HBSS containing Ca^{2+} , Mg^{2+} , and 0.1% gelatin. Although we did not quantify the amounts of C3b, iC3b and C3d on the bacteria used in these studies, our previous results (9) suggest that all three of these fragments are fixed onto *P. aeruginosa* under these conditions. Phagocytosis assays were performed as described previously (9). Briefly, 2×10^7 opsonized, LY-tagged bacteria were incubated at 37°C with 10^6 BAL cells or isolated peripheral blood PMN in a total volume of 0.24 mL of HBSS containing Ca^{2+} , Mg^{2+} , and 0.1% gelatin. Parallel mixtures were incubated at 0°C to allow estimation of the proportion of bacteria attached to the surface of the phagocytes but not ingested. After 30 min of gentle agitation, 2.0 mL of cold PBS was added and phagocytic cells were separated from most of the free bacteria by three differential centrifugations at $180 \times g$ for 5 min. The cell mixtures were analyzed by fluorescence flow cytometry using volume and 90° light scatter gating as described above, which also served to eliminate any remaining free bacteria from analysis. From each sample, the fluorescence of 5 000 cells within each set of volume and light scatter gates was recorded by the cytometer, and the arithmetic mean fluorescence was determined automatically by the computer, allowing linear comparison of fluorescence data.

RESULTS

Opsonin receptors on fresh alveolar macrophages from normal subjects. A representative set of fluorescence histograms for separately immunostained aliquots of fresh bronchoalveolar lavage macrophages from a normal donor is shown in Figure 1. The result for each antireceptor antibody is shown by the solid line, and the result for the corresponding control is shown by the dashed line in each block. The fluorescence value on the x axis is directly proportional to the number of antireceptor monoclonal antibody molecules bound to each cell, and hence also to the number of receptor molecules expressed on each cell (22). The values on the y axis show the number of cells with each fluorescence value. It is readily apparent that the histograms for anti-CR1 and anti-CR2 do not differ appreciably from the corresponding controls, although there is some positive fluorescence for anti-CR3 and a larger difference in the positive fluorescence for anti-CR4 as compared with its control. In contrast, there is relatively less overlap between the histogram for anti-Fc γ R1 with its control, even less for anti-Fc γ R2 and III, and essentially no overlap with anti-HLA-DR. As summarized in Table 1, the results from this subject taken together with results from 9 other normal subjects show that fewer than 10% of alveolar macrophages exhibited specific binding of anti-CR1, anti-CR2, or anti-CR3 antibodies. This suggests that expression of these receptors (CR1, CR2, and CR3) is negligible. In contrast, about 30–40% of cells express CR4 and Fc γ R1, with mean fluorescence values of less than 10, whereas the percentages of cells expressing Fc γ R2 and III are higher. It should be noted that these figures may underestimate the actual percentage of positive cells, particularly for the Fc γ R because for Fc γ R1, for example, the macrophages stain as a single population, but there is some overlap with the

Fig. 5. Effects of different sources of antibody on phagocytosis of fluorescent tagged *P. aeruginosa* by fresh normal alveolar macrophages (open bars) and purified blood neutrophils (cross-hatched bars). Procedures are the same as in Figure 3 except that the indicated sources of antibody were substituted for the monoclonal antibody used in that study. The upper two panels show the effects of human polyclonal antiserum; the lower panel is for a monoclonal murine IgG2b antimucoid exopolysaccharide. With this antibody only, a mucoid strain of *P. aeruginosa* was used. Results shown are for a single representative experiment.

histogram for the control antibody, suggesting that some cells expressing relatively few receptors may have fluorescent values that overlap with cells that have the highest nonspecific binding of the control antibody. Similar observations have been reported by Kindt *et al.* (5). There were no subpopulations of macrophages as defined by light scatter and/or volume characteristics that had distinctive differences in CR or Fc γ R expression in either the CF or normal BAL specimens. The expression of HLA-DR is essentially universal. The latter observation confirms that the cells being analyzed are macrophages because neutrophils and epithelial cells do not express class II histocompatibility antigens (23). Comparable fluorescence histograms for chemoattractant-stimulated normal blood neutrophils are shown in Figure 2. A summary of the results with chemoattractant stimulated neutrophils for this subject and 3 other normal donors is also provided in Table 1 as a comparison. These results are consistent with our results for CR expression on stimulated blood neutrophils published previously (8). The chemoattractant exposure mimics that to which PMN would be exposed as they are recruited from the circulation to the site of infection (8, 18). Differences from the results with the macrophages are readily apparent: most notably,

the neutrophils are markedly positive for CR1 and CR3, only slightly positive for Fc γ R1, and very markedly positive for Fc γ R2 and Fc γ R3. In further contrast to the macrophages, the neutrophils are uniformly negative for HLA-DR.

Immunohistochemistry of CR expression on fixed lung tissue. To be sure that our findings on BAL cells were representative of alveolar macrophages *in situ*, and to confirm the relative lack of expression of CR1, 2 or 3 *in vivo*, we performed immunohistochemical analysis of fixed lung tissue using the same antibodies. The results of sections from 5 lungs, coded so that the observer did not know which antibody was used, are summarized in Table 2, and a representative set of micrographs is shown in Figure 3. Most morphologically recognized intralveolar macrophages (arrows) stained intensely for HLA-DR. Alveolar macrophages were uniformly negative for CR2 and only weakly and infrequently positive for CR1 and CR3, in good agreement with our FACS data. The percentage of cells positive for CR4 was also in good agreement with the FACS data. In contrast, using lymphoid tissue for positive controls showed strong staining of follicular cells in lymph nodes and spleen for CR1 and CR2, and of medullary macrophages in lymph nodes for CR1, CR3, and CR4. The positive staining for CR1 and CR2 in these control tissues suggests that their absence from the alveolar macrophages is not due to postmortem proteolysis.

Role of IgG and C3 fragments in promoting phagocytosis of *P. aeruginosa* by fresh alveolar macrophages from normal subjects. Having determined the pattern of opsonin receptor expression on the BAL macrophages, we next evaluated the relative efficacy of IgG and complement in promoting phagocytosis of *P. aeruginosa* by these cells as compared with chemoattractant-stimulated blood PMN. We first used a mouse monoclonal IgG3 antibody to the high molecular weight polysaccharide O-side chain of the LPS of Fisher-Devlin immunotype 1 *P. aeruginosa* with the homologous bacteria as the targets. These assays were performed with bacteria rendered fluorescent with lucifer yellow, which does not lose its fluorescence in intracellular acidified compartments. Thus, greater relative fluorescence indicates a greater number of bacteria associated with the phagocyte. Separate experiments performed at 0°C to allow attachment but not internalization of opsonized bacteria showed that in every case, internalized bacteria accounted for >90% of the total cell-associated bacteria. Free bacteria were excluded from the analysis by the volume and light scatter gates which were set for each type of phagocyte. For comparison, chemoattractant-stimulated blood neutrophils were included in parallel in all experiments. Control experiments showed that incubation of normal and CF cells under the conditions of phagocytosis assay did not alter their patterns of receptor expression.

There was little phagocytosis of nonopsonized bacteria by either alveolar macrophages (open bars) or neutrophils (PMN, cross-hatched bars), as shown at the left of Figure 4. With the addition of the lowest concentration of specific antibody, 15 μ g/mL (10^{-7} M), phagocytosis by the alveolar macrophages increased from 0.57 ± 0.04 to 2.5 ± 0.35 ($p < 0.001$ by *t* test), although there was no increment in phagocytosis by the neutrophils. With increasing inputs of antibody, phagocytosis by both types of cells increased, but the macrophages showed little additional enhancement of phagocytosis when the antibody concentration was increased from 75 μ g/mL to 150 μ g/mL, consistent with saturation of high affinity Fc γ R. In contrast, the PMN did not appear to exhibit a saturation phenomenon at these concentrations, and the highest input of antibody gave the greatest enhancement of phagocytosis.

To determine the effects of complement, we incubated the bacteria, with or without antibody, with 5% pooled normal human serum. These results are shown in the right half of Figure 4. With either macrophages or PMN, serum alone did not cause much enhancement of phagocytosis above the minimal level seen with nonopsonized bacteria, confirming that this serum pool did not contain functional antibody to these bacteria. When

Receptor Expression on Cystic Fibrosis Alveolar Macrophages

Fig. 6. Receptor expression on fresh BAL macrophages from a CF patient. Procedures and presentation are the same as in Figure 1.

Table 3. Receptor expression on CF BAL macrophages*

Receptor	% Cells positive (mean $\pm$ SEM, $n = 7$)
CR1	10.5 $\pm$ 0.6
CR2	0 $\pm$ 1.3
CR3	4.9 $\pm$ 2.6
CR4	20.9 $\pm$ 7.0
Fc γ RI	20.0 $\pm$ 7.0
Fc γ RII	32.2 $\pm$ 7.3
Fc γ RIII	30.0 $\pm$ 7.0
HLA-DR	80.3 $\pm$ 2.9

* Receptor expression on fresh BAL macrophages from CF patients ($n = 7$). Procedures and presentations are as in Table 1.

serum was used together with 15 μ g/mL antibody, phagocytosis by the macrophages doubled, reaching 4.4 relative fluorescence units. In contrast, the addition of serum to this small amount of antibody had a much more dramatic effect with the neutrophils, increasing phagocytosis over 20-fold, from less than 1 to over 23 units. In the presence of serum, higher inputs of antibody caused even greater enhancement of phagocytosis by the neutrophils, but there was little additional effect with the macrophages. To be sure that the effect of serum was, in fact, due to complement fixation, we performed control experiments in which opsonization was performed in the presence of 10 mM EDTA. As seen on the extreme right, this abolished the enhancement of neutrophil phagocytic activity caused by serum, and the results were not different from those obtained with the same input of antibody alone (150 μ g). Similar results were also obtained when the serum was heat-inactivated. The fact that EDTA and heat inactivation abolished the effects of serum in the opsonization step, before the opsonized bacteria were washed and added to the phagocytes, provides strong evidence that these effects are due to complement.

To rule out the possibility that the observed results were peculiar to the isotype of mouse antibody used or the specific epitope it recognizes on the bacteria, we performed similar experiments with two sources of polyclonal human immunoglobulin. We also performed similar experiments with a mucoid strain of *P. aeruginosa* and a mouse monoclonal antibody specific for mucoid exopolysaccharide. As shown in Figure 5, the results for both alveolar macrophages and PMN are qualitatively quite similar to those reported above regardless of whether the bacteria were opsonized with Nosocuman, a human polyclonal

hyperimmune globulin prepared from plasma of normal volunteers immunized with pseudomonas polysaccharides conjugated to a protein carrier (*top panel*), or with the mouse IgG2b monoclonal to mucoid exopolysaccharide, even though with the latter antibody a mucoid strain of bacteria was used (*bottom panel*). The results with the immune globulin prepared from plasma of donors with naturally occurring high titers of anti-*Pseudomonas* antibodies (*middle panel*) differ slightly, in that there is less overall enhancement of phagocytosis in the presence of serum as well. Even with this preparation, however, increasing the antibody concentration increased phagocytosis by the macrophages more than with the PMN, and the addition of serum did not increase phagocytosis by the macrophages more than antibody alone. The quantitative differences between the results with these antibodies and those in Figure 4 may relate to the subclasses and/or affinities of the specific antibodies in these preparations. More importantly, the qualitative similarity of the results with these other sources of antibody and with a different strain of bacteria to the results obtained with the mouse monoclonal antibody to the pseudomonas LPS O-side chains used above suggests that our observations are primarily related to the pattern of opsonin receptor expression on the phagocytes, rather than to peculiarities of any given source of antibodies or bacterial strain.

Receptor expression and phagocytosis by CF alveolar macrophages. Because one of our major goals is to better understand the actual opsonin-receptor interactions in the milieu of the chronically infected lung in cystic fibrosis, we next studied bronchoalveolar lavage samples from patients with that disease. Although as the chronic *Pseudomonas* infection progresses, neutrophils become the predominant type of cell recoverable on bronchoalveolar lavage, we were able to obtain BAL specimens with a mean ($\pm$ SEM) content of 36.6 $\pm$ 4.5% macrophages and 60 $\pm$ 4.9% neutrophils by studying patients with stable mild disease. The ability of the flow cytometer to selectively analyze each of these types of cells was then used to determine how they could perform *in vivo*, avoiding *in vitro* separation or culturing techniques that might alter their patterns of receptor expression or phagocytic function.

CF BAL macrophages fell within the same volume and 90° light scatter ranges as those from normal subjects. The fluorescence histograms for receptor expression on CF BAL macrophages are quite similar to those obtained with normal macrophages, as shown in the representative histograms in Figure 6. The results for seven patients are summarized in Table 3. There

Fig. 7. Comparison of phagocytosis of fluorescent-tagged FT 1 *P. aeruginosa* by fresh BAL macrophages (open bars), fresh BAL neutrophils (hatched bars) and FMLP-stimulated blood neutrophils (cross-hatched bars) from a single CF patient. Procedures are as in Figure 4, except that each BAL sample was analyzed twice, once with gating on neutrophils and once with gating on macrophages. Results are for a single representative experiment.

were no statistically significant differences in expression of the complement receptors or Fc γ RI, but the percentage of CF cells expressing Fc γ RII, Fc γ RIII, and HLA-DR were lower than the percentage of normal BAL macrophages expressing these proteins ($p < 0.001$, < 0.05 , and < 0.001 respectively). The decreased expression of Fc γ R, especially Fc γ RII, on the macrophages of some of the CF patients may reflect internalization during phagocytosis *in vivo*, and/or the effects of proteases (8, 25). We have not yet directly evaluated the extent to which either or both of these mechanisms (or others as well) contribute to the observed decrease in Fc γ R expression. Receptor expression on BAL neutrophils was analyzed concurrently with that on the macrophages in each CF BAL specimen. The results are essentially identical to those we obtained previously and have already presented in detail in ref. 8. Basically, the CF BAL neutrophils are quite similar to chemoattractant-stimulated blood neutrophils (Fig. 2), except for greatly reduced CR1 and Fc γ RIII expression. In no case did CF blood or BAL neutrophils stain positively for Fc γ RI.

The effects of antibody and complement in enhancing phagocytosis by CF BAL macrophages are shown in the open bars in Figure 7, and are also similar to those obtained with normal macrophages. Once again, phagocytosis appeared to be optimized at the intermediate input of antibody, with additional antibody causing only a slight additional increment. Serum alone had no effect, and the addition of serum to the antibody caused essentially no enhancement above the level seen with each input of antibody alone. Results with eight other CF patients' alveolar macrophages were also similar (not shown). Results with chemoattractant-stimulated CF blood neutrophils, shown in the cross-hatched bars, were similar to those for chemoattractant-stimulated blood neutrophils from normal donors shown in Figure 4. The results with CF BAL neutrophils, shown in the middle bar in each group, differ from those with the blood neutrophils from the normal donors (shown in Fig. 4) or from those obtained with blood neutrophils from the same CF patient. The most striking

difference is that the CF BAL neutrophils, in contrast to the blood neutrophils, show little enhancement of phagocytosis upon the addition of serum. This is consistent with the markedly decreased CR1 expression on CF BAL neutrophils we observed in these and previous studies (8) and emphasizes the pathophysiologic significance of those earlier observations.

DISCUSSION

The problem of chronic lung infection with *P. aeruginosa* in CF remains enigmatic. Because CF patients do not have an increased frequency of infection outside of the respiratory tract, and no systemic immunologic defect has been identified, we have focused on studying the local host defense mechanisms as they function *in situ*, in the milieu of the chronically infected lung. The major cells involved in defense of the lung against bacterial infection are neutrophils and alveolar macrophages. The phagocytic activity of these cells is primarily determined by the receptors they express for opsonins on the surface of their targets, and by the functional capabilities of these receptors. Both the expression and function of these receptors may be altered dramatically by a wide variety of extracellular signals. For example, complement receptor expression by neutrophils is rapidly and markedly increased by chemoattractants (18). Macrophages in tissues also express a very different array of complement and immunoglobulin receptors than do the blood monocytes from which they are derived (3, 4). Monocytes, when cultivated *in vitro*, may express different receptors depending on which supplements are added to the culture media (6, 16). Moreover, 5-fold changes in Fc γ receptor expression and differences in phagocytic activity can be induced during isolation of monocytes (17). Although the factors that determine receptor expression *in vivo* have not been completely defined, it is clear that receptor expression varies in a tissue-specific manner (3, 4). In the present work, we have taken advantage of the ability of the flow cytometer to analyze selectively individual types of cells in complex

mixtures, and have used immunofluorescent staining to determine Fc γ and complement receptor expression, and fluorescent tagged bacteria to determine the phagocytic activity of these cells, all within a few hours of their removal from the lung. This approach obviates the need for adherence, other purification procedures, or artificial culture media that may alter receptor expression and/or function *in vitro* (16, 17). Thus we believe that our results are indicative of the true state of these cells as they function *in vivo* at the site of infection.

With normal alveolar macrophages, we found minimal or no expression of complement receptors CR1, CR2 or CR3. This differs markedly from blood monocytes either at rest or after stimulation or culture *in vitro* (2), but is similar to reports from other laboratories for alveolar macrophages (3, 4). Studies using the same monoclonal antibodies to stain fresh-frozen lung tissue specimens also failed to show any appreciable staining, confirming that the apparent lack of expression of these receptors on cells obtained by bronchoalveolar lavage was, in fact, representative of their status *in vivo*. We did find moderate staining with the Leu-M5 antibody, which recognizes the CD11c-chain of p150/95, the "third" member of the Mac-1/LFA-1 family of leukocyte surface adherence proteins (24), which has been identified tentatively as CR4 (4). This is also consistent with the results of Myones *et al.* (4), who found that p150/95 was the most abundant member of this family on tissue macrophages. This is in marked contrast to neutrophils, which, after exposure to chemoattractants as would occur during their migration into tissues *in vivo*, express relatively large amounts of CR1 and CR3. We also found differences in the expression of Fc γ R on alveolar macrophages *versus* neutrophils, since the macrophages expressed the high affinity IgG receptor, Fc γ RI, in addition to Fc γ RII and III; whereas the neutrophils express only Fc γ RII and III (5, 8, 18, 25). Although it has been reported that blood neutrophils from some subjects with acute streptococcal infection express Fc γ RI (26), this receptor was not found on blood or BAL neutrophils from the stable CF patients or normal subjects studied for this report.

The results of the assays of phagocytosis of selectively opsonized *P. aeruginosa* are consistent with the results of the immunofluorescence assays for receptor expression. There was little phagocytosis of unopsonized bacteria by either macrophages or neutrophils, but even low concentrations (15 μ g/mL) of IgG increased the uptake of bacteria by the alveolar macrophages. Additional enhancement of phagocytosis occurred when the concentration of IgG was increased to 75 μ g/mL, but no further enhancement occurred with the highest concentration, 150 μ g/mL. These results are consistent with saturation of high affinity Fc γ R. Normal blood neutrophils, which do not express Fc γ RI (26), did not show saturation. With alveolar macrophages, the addition of complement caused no further enhancement of phagocytosis above the level due to antibody alone. In contrast, serum treatment of antibody coated bacteria increased phagocytosis by the neutrophils very dramatically, to levels much higher than achieved by the macrophages under any condition. The observations that the effects of serum with the neutrophils were abolished by heat inactivation or 10 mM EDTA strongly suggest that they were, in fact, due to complement fixation onto the bacteria. The fact that complement deposition on the bacteria so dramatically increased phagocytosis by the chemoattractant-stimulated neutrophils while having essentially no effect with the alveolar macrophages is consistent with the expression of CR1 and CR3 on the former but not on the latter. The lack of complement-mediated effects with the alveolar macrophages, which do express p150/95 (Leu M5), suggests that this molecule does not mediate complement dependent phagocytosis under these conditions. Thus, although this molecule is capable of promoting complement dependent adherence under some conditions *in vitro* (4), its true functional role *in vivo* and its identity as "CR4" remains to be defined.

The results with cystic fibrosis alveolar macrophages are quite

similar to normal alveolar macrophages in both the receptor expression and phagocytosis assays. The pattern of HLA-DR expression on the CF cells demonstrates the power of the flow cytometer to selectively analyze the macrophages in these samples, which contained up to 70% neutrophils, since the latter do not express these class II histocompatibility antigens (23). Consistent with previous results on receptor expression (8, 9), fresh BAL neutrophils from the CF patients did not show as much complement-mediated enhancement of phagocytosis as did blood neutrophils, emphasizing the functional importance of the destruction of CR1 mediated by elastase *in vivo* (8, 9).

These results allow assessment of the relative importance of IgG and C3-derived opsonins *in vivo*, and explain some previously reported immunologic abnormalities in CF. Electron microscopy studies by Thomassen *et al.* (27) had shown that CF BAL macrophages were quiescent and did not appear actively involved in phagocytosis. Subsequently, it has been shown that both CF sera and lung fluids contain poorly functional antibodies against *P. aeruginosa* that can actually inhibit or "block" phagocytosis (10–15). Curiously, this "blocking activity" is most notable in assays employing macrophages rather than neutrophils (11–14). Our results, which suggest that macrophages are more dependent on IgG and Fc γ R for phagocytosis than are neutrophils, suggest an explanation for those observations. Because Fc γ RI shows more selectivity for human IgG of subclasses 1 and 3 than do the lower affinity Fc γ RII and III (28), interference with binding of these subclasses to the bacteria is likely to result in decreased phagocytosis by macrophages. This may occur in the lung in CF because of an excess of antibodies of other subclasses (13, 14), and/or because of binding to the bacteria of antibodies with degraded or otherwise altered Fc γ domains (15). Further studies using monoclonal antireceptor antibodies and/or complexes of antigen with antibodies of defined isotypes will be necessary to define more completely the role of each Fc γ R and IgG subclass in promoting *Pseudomonas* phagocytosis.

When considered in the context of the chronic pulmonary infection in CF, in which neutrophils predominate, our observations that the maximal phagocytic activity of BAL macrophages and CF BAL neutrophils is so much lower than blood neutrophils are especially meaningful. Because the phagocytic activity of neutrophils is so dependent on complement, these results emphasize the importance of the complement receptor-opsonin mismatch caused by elastase in the CF lung (8, 9). Thus, these analyses should set the stage for investigations of the potential efficacy of immunologic strategies aimed at improving the subclass distribution and function of the IgG in the lung in CF to take advantage of macrophage Fc γ receptors, and of the use of protease inhibitors to restore the proper functioning of the complement receptors on neutrophils and their specific opsonic ligands.

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