Priming of Blood Neutrophils in Children with Cystic Fibrosis: Correlation between Functional and Phenotypic Expression of Opsonin Receptors before and after Platelet-Activating Factor Priming

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Blood phagocyte opsonin receptor CR1 (CD35) and CR3 (CD11b) functions were examined in cystic fibrosis (CF) patients with endobronchial Staphylococcus aureus or Pseudomonas aeruginosa chronic infection, CF patients without infection, heterozygous, non-CF patients with chronic pulmonary infection, and healthy controls. Circulating and platelet-activating factor (PAF)—primed phagocyte luminescence responses to complement-opsonized zymosan were increased in both groups of infected CF and non-CF children relative to uninfected CF children and healthy control children and adults. The ratio between circulating and PAF-primed phagocyte responses was significantly elevated in all children with CF, and in these, the ratio could serve as an indicator of response to antibiotic treatment. The ratios of circulating and PAF-primed phenotypic expression for CR1, CR3, and FcγRII (CD16), but not FcγRII (CD32), correlated with the functional ratios. Phagocyte opsonin receptor response capacity might be used for evaluation of inflammation and infection in CF patients.

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Informed consent was obtained from patients or their parents if children were less than 12 years old. The study followed French and US Department of Health and Human Services human experimentation guidelines.

R.C.A. is the inventor of and has a royalty interest in the patent that covers the CORE/MORE luminescence system but is not otherwise associated with EOE, Inc. (Little Rock, AR).


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colony stimulating factor, have shown that opsonin receptor-independent and –dependent neutrophil oxidative function in whole blood correlate with patient clinical status and state of marrow stimulation. The ratio of circulating-to-C5a- or PAF-primed opsonin receptor expression-dependent activity provides an objective gauge of the in vivo state of immune activation or inflammation and follow-up of therapeutic effects [20, 21].

In the present study, we evaluated whether functional and phenotypic analysis of opsonin receptor mobilization capacity could be used to monitor the inflammatory and the infectious state in children with CF. We compared blood phagocyte opsonin receptor functional capacity and antigenic expression as measured by flow cytometry on blood specimens of young CF children with and without evidence of pulmonary infection with Staphylococcus aureus or P. aeruginosa (CF homozygotes) and compared the results with those from their parents (CF heterozygotes), with non-CF children with pulmonary infection, and with healthy control children and adults.

Materials and Methods

Study population. Seventy patients with CF were recruited for the study. CF was diagnosed according to standard criteria, including a sweat chloride test [22]. Genotyping of CF patients was done as previously described [11]. In the CF population tested, 73% had the allelic frequency of the ΔF508 mutation, including 50% ΔF508 homozygote, 23% ΔF508 heterozygote with an unknown mutation, and 27% with mutations other than ΔF508 at both alleles. Patient clinical status was evaluated by the Shwachman scoring system [23] and by chest radiographic Brasfield score [24]. The severity of lung disease was evaluated according to chest radiographs and computed tomography (CT) and classified as follows: no or minimal lesions (n = 23), predominantly obstructive lesions (n = 28), and segmental or diffuse disease without bronchiectasis (n = 19). Whenever possible, in patients >6 years old, the pulmonary function was also determined by the forced expiratory volume in 1 s (FEV1), expressed as a percentage of the mean normal value corrected for age and height [25]. The effect of gentamicin was sequentially followed in 3 patients.

The lung infection was determined on the basis of bacteriologic analysis of sputum that was collected as described in [26]. In brief, dental cotton-wool swabs are placed immediately before expectoration between the cheek and the gum and under the tongue at the level of the excretory salivary glands. Once the plugs are in place, control of a physiotherapist. The subsequent expectorant or sputum (of young nonexpectorating children) is collected at the bottom of the throat with a sterile stick without aspiration in order to avoid aspiration lung disease. Unlike oropharyngeal specimens, which do not reliably predict the presence of bacterial pathogens in the lower airways [27], such “protected” sputum provides bacteriologic data similar to that obtained in bronchial secretions collected by transbronchial aspiration [28]. Sputum cultures were made at 1-month intervals. On the basis of sputum bacterial qualitative and quantitative analysis, bronchial infection was defined as bacterial counts of ≥10^6 cfu/L in ≥3 cultures at 1-month intervals. The results enabled CF patients to be stratified into 3 groups as follows.

Group 1, designated uninfected, comprised 25 CF children (12 boys and 13 girls) with a mean age (±SEM) of 3.0 ± 1 years with no history or evidence of P. aeruginosa infection. Two of these children had an S. aureus infection >6 months before the test, but at the time of testing, sputum cultures were negative and all of the children were stable and clinically well.

Group 2 included 14 CF children (7 boys and 7 girls) with a mean age of 9.0 ± 1.2 years. All were chronically infected with S. aureus >6 months, including 5 with mixed S. aureus and Haemophilus influenzae infections.

Group 3 included 31 CF children (15 boys and 16 girls) with a mean age of 11.0 ± 0.8 years. These children were colonized with P. aeruginosa; 23 had mixed infection with S. aureus [11], H. influenzae [6], or mycobacteria [6].

Two groups of children were used as non-CF controls. The first comprised 10 non-CF children (4 boys, 6 girls; mean age 6.5 ± 2.0 years) with chronic recurrent pulmonary infections with S. aureus [5], H. influenzae [4], and Aspergillus species (1) and whose blood was investigated in our laboratory for diagnosis, but not confirmed, of chronic granulomatous disease. The second group was 11 healthy children (7 boys, 4 girls; mean age 9.4 ± 3.0 years) who had blood drawn for a prospective epidemiologic study at the Necker Enfants Malades Hospital; 0.1 mL was sent to our laboratory. The CF status of these 2 groups of control children was not known.

The CF heterozygote group consisted of 20 parents of CF children (14 mothers, 6 fathers). All were without chronic infection or inflammatory disease at the time of testing. Results were compared with those of 35 healthy adults recruited from volunteers of the Necker Hospital blood donation center. Like the control children, the CF carrier status of the adults was not known.

Measurement of phagocyte oxidase and MPO activities. K,EDTA-anticoagulated whole blood was tested within 1 h of collection. Activity-specific chemilumininogenic substrates yield luminescence by different deoxygenation mechanisms and are dependent on different products of phagocyte oxidative metabolism [29]. Basal and PMA-stimulated phagocyte NADPH oxidase, that is NADPH (O2 oxidoreductase) activities, were measured as the luminescence product of dimethylbiacridinium (DBA\(^+\); lucigenin) reductive deoxygenation. Basal and PMA-stimulated MPO (Cl\(_2\), H\(_2\)O\(_2\)) oxoreductase activities were measured as the luminescence product of luminol deoxygenation. The assay reagents (CORE/MORE System; EOE, Little Rock, AR) included (1) blood-diluting medium (BDM), a 5 mM 2-(\(-\)-morpholine)ethanesulfonate (MES) buffered salt solution deficient in divalent cations; (2) lucigenin (dimethylbiacridinium; DBA\(^+\)\) balanced salt solution (DBSS), a 5 mM 3\((\text{-morpholinopropane sulfonic acid buffered salt solution containing 139 mM}\ E/L Na\(^+\), 5.0 mM\ E/L K\(^+\), 1.3 mM Ca\(^++\), 0.9 mM Mg\(^++\), 142 mM\ E/L Cl\(^-\), 0.8 mM H\(_2\)PO\(_4\), plus 0.2 mM 10,11\-dimethyl-9,9\-biacridinium dinitrate, 5.5 mM D-glucose, and 0.05% human albumin; (3) luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) balanced salt solution (LBSS), a 5 mM MES buffered salt solution containing 139 mM\ E/L Na\(^+\), 5.0 mM\ E/L K\(^+\), 1.3 mM Ca\(^++\), 0.9 mM Mg\(^++\), 142 mM\ E/L Cl\(^-\), 0.8 mM H\(_2\)PO\(_4\), 0.15 mM luminol, and 5.5 mM D-glucose; and (4) prefabricated low-dose and high-dose PMA-coated tubes containing 10 pmol and 5 nmol PMA/
Cystic Fibrosis Blood Phagocyte Function

Whole blood (0.1 mL) was diluted in BDM (9.9 mL) and loaded into the luminometer injector (Autolumat LB953; EG & G Bertold, Wildbad, Germany). Basal and stimulated oxidative and MPO deoxygination activities were measured following the injection of 0.1 mL of the BDM-diluted blood (1 μL of whole blood equivalent) into uncoated and PMA-coated tubes containing 0.5 mL of DBSS or LBSS, respectively. Both concentrations of PMA trigger oxidative activation. At low concentration (10 pmol/tube; 17 nM final concentration), PMA stimulates specific degranulation only; at high concentrations (5 nmol/tube; 8.3 μM final concentration), PMA stimulates specific and azurophilic degranulation with MPO release.

Luminescence activities were measured in triplicate over 20 min. Under the conditions of testing and especially at this 10⁻³ blood dilution, both the quenching effect of red blood cells on luminescence photometric measurement and the interference of plasma factors can be ruled out, and luminescence is proportional to the number of phagocytes [30, 31]. Luminescence activity was thus normalized with respect to total phagocyte count per microliter (neutrophils, basophils, eosinophils, and monocytes per microliter of blood, with specific activity expressed as counts/20 min/phagocyte) [19].

Phagocyte opsonin receptor functional expression: ratio of circulating-to-PAF-primed opsonin receptor-dependent activities. Circulating opsonin receptor–dependent luminescence activity was measured by exposing blood phagocytes to a non–rate-limiting quantity of opsonin in the presence of a non–rate-limiting concentration of luminol as the luminogenic substrate. PAF-primed activity was measured under the same test conditions, except that an optimum quantity of PAF was included to ensure maximal opsonin receptor expression. In addition to the previously described (CORE/MORE) reagents, we used the following agents for analysis: zymosan (Z), human complement-opsonized zymosan (hC-OpZ), and human IgG-opsonized zymosan (hIg-GOpZ), all at 2 x 10⁹ U/mL yeast cell wall, and PAF tubes precoated with 50 pmol of 1-O-hexadecyl-2-O-acetyl-sn-glycero-3-phosphorylcholine. Whole blood was diluted 1/100 as described above, and 0.1 mL of diluted blood (i.e., a 1-μL equivalent of whole blood) was injected into uncoated and PAF-coated tubes containing 0.5 mL of LBSS and either 0.1 mL of unopsonized zymosan or human complement–or IgG-opsonized zymosan. Functional circulating activity is defined as the initial (i.e., first 10 min) blood luminescence response stimulated by zymosan in the absence of exogenous primer.

The initial circulating and PAF-primed responses are opsonin receptor–dependent functional activities measured as the luminescence product of phagocyte oxygenation reactions. As such, activity is proportional to the quantity and metabolic capacity of the phagocytes tested. Expressing activity as the ratio of circulating-to-primed opsonin receptor–dependent luminescence normalizes for differences in phagocyte metabolic capacity and provides a robust gauge of in vivo immune activation than can be achieved using the circulating activity per phagocyte alone [21].

Flow cytometry cell surface analysis of CR1 (CD35), CR3 (CD11b), FcγRII (CD32), and FcγRIII (CD16) phenotypic (antigenic) expression. Whole blood (100 μL) was mixed with 20 μL of fluorescein isothiocyanate–conjugated anti-CD35 (mouse IgG1, clone E11; CLB, Amsterdam), anti-CD11b (mouse IgG1a, clone BEAR1; Immunotech, Marseille, France), anti-CD16 (mouse IgG1, clone 3G8; Immunotech), or anti-CD32 (mouse IgG2A, clone 2E1; Immunotech) and incubated at room temperature for 15 min. Flow cytometry lysing solution (2 mL FACS; Becton Dickinson, San Jose, CA) was added to each mixture, and after incubation for 10 min at room temperature, the samples were centrifuged (1200 g, 10 min). The supernatants were discarded, and pellets were washed twice in PBS before being fixed in 400 μL of formaldehyde solution (1% in PBS) and held until flow cytometric analysis. For evaluation of primed opsonin receptor expression, the whole blood was preincubated with PAF (final concentration, 10 μM) before treatment with the antigen-specific monoclonal antibody preparations and formaldehyde fixation. All samples were analyzed for fluorescence by flow cytometer (FACScan; Becton Dickinson Immunocytometry Systems, Mountain View, CA). Neutrophils were distinguished from lymphocytes and monocytes in the whole blood samples by their characteristic forward-sideways scatter. Measurements were made after gating on neutrophils or monocytes as indicated. IgG1 was used as a negative control.

Statistical analysis. Statistical analysis was performed using a software package (Statistica; Statsoft, Tulsa, OK). Comparisons were made by analysis of variance (ANOVA) or Student’s t test, paired or unpaired, and by χ² or Fisher’s exact test. For multiple comparisons, P values were multiplied by the number of comparisons made. Simple regression analysis and Pearson r correlation coefficient were used to determine the relationships between variables. Data are expressed as mean ± SEM.

Discriminate analysis was performed to determine the relationship of activity to membership in the 4 groups: control adults, CF parents (CF heterozygotes), healthy control children, and uninfected CF children (CF homozygotes). Orthogonal (independent) discriminate functions were evaluated from an optimal linear combination of variables and were equal to the number of groups minus one. Multiple discriminate analysis assumes that the groups under study have a multivariate normal distribution with equal covariance matrices.

Results

Patient clinical status. The group of CF children infected with P. aeruginosa clearly differed from the groups of uninfected CF children and S. aureus–infected CF children on the basis of both Shwachman and Brasfield scores. The 2 latter groups significantly differed only by Brasfield score (table 1). Likewise, and as expected, P. aeruginosa–infected CF children had more severe lung disease than those infected with S. aureus (table 1) with more lesions seen on chest radiography and CT (100% vs. 38%, P < .001), a higher incidence of bronchiectasis (38% vs. 0% P < .001), and a significantly lower FEV₁ (43.5% ± 4% vs. 73% ± 7%, P = .02).

Leukocyte count and phagocyte opsonin receptor–independent oxidative activities. Relative to healthy control children, both the uninfected and infected non-CF children and the CF children showed mild leukocytosis (P < .01), but no significant dif-
ference was observed between CF children without detectable infection and those chronically infected with *S. aureus* or *P. aeruginosa* (table 2). Differential phagocyte counts did not show any significant disparity between the various groups of children in the ratios between monocytes and neutrophils or eosinophils and neutrophils (data not shown), thus enabling expression of luminescence activities per phagocyte.

Basal and PMA-stimulated and PMA-stimulated NADPH oxidase activities, measured as lucigenin luminescence, did not significantly differ among the groups (table 2). Likewise, myeloperoxidase activities, measured as luminol luminescence, did not significantly differ except in the group who showed higher PMA-stimulated oxidative activities than all other groups. However, the differences observed were statistically significant only with the high-dose PMA and relative to healthy control children but not to the infected non-CF or CF children.

Because the uninfected CF children were younger than the control children, we tested whether phagocyte oxidative activity functions might be affected by age. No significant differences were found between healthy control children (ages 2–10 years) versus adults either in NADPH-dependent basal activities (basal or triggered by PMA) or in MPO-dependent oxidant generation. Moreover, in each group of CF children, no significant correlation (r < .15) could be found by regression analysis between age and these luminescent parameters.

**Functional opsonin receptor expression measured as circulating and PAF-primed activities.** Opsonin receptor–dependent luminal luminescence activities stimulated by complement-opsonized zymosan in the absence (circulating activity) or in the presence of PAF (primed activity) were highest for the non-CF–infected children followed by *P. aeruginosa*–colonized CF children. Of interest, both circulating and PAF-primed opsonin receptor–dependent activities were significantly increased in *S. aureus*–infected CF children compared with uninfected CF children. As shown in table 3, the ratios of circulating-to-PAF–

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**Table 1.** Clinical characteristics and lung radiographic and computed tomography (CT) findings in cystic fibrosis (CF) patients.

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>Uninfected CF patients</th>
<th>CF patients infected by</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 25)</td>
<td><em>S. aureus</em> (n = 14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. aeruginosa</em> (n = 31)</td>
</tr>
<tr>
<td>Age, years (range)</td>
<td>3 ± 1 (1–7)</td>
<td>9 ± 1.2 (3–20)</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>12/13</td>
<td>7/7</td>
</tr>
<tr>
<td>CF Shwachman score (normal range, 86–100)</td>
<td>91 ± 1.6 (20)</td>
<td>82 ± 3.6 (12)</td>
</tr>
<tr>
<td>CF Brasfield score (normal value, 0)</td>
<td>1.4 ± 0.2 (12)</td>
<td>5.7 ± 1.3 (12)</td>
</tr>
<tr>
<td>FEV 1% (in patients ≥6 years old)</td>
<td>74 ± 5 (5)</td>
<td>77 ± 3.3 (7)</td>
</tr>
<tr>
<td>Lung radiographic and CT lesions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No or mild lung lesions</td>
<td>17</td>
<td>6</td>
</tr>
<tr>
<td>Obstructive lung disease</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Bronchiectatic lung disease</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

NOTE. Data are mean ± SE; no. in parentheses is no. of subjects unless otherwise indicated.

*a* P < .01 vs. children not infected with *Staphylococcus aureus*.

*b* P < .05 vs. children not infected with *S. aureus*.

*c* P < .01 vs. children infected with *S. aureus*.

*d* P < .001 vs. children infected with *S. aureus*.

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**Table 2.** Phagocyte basal and PMA-stimulated oxidative activities measured by chemiluminescence (CL) in whole blood of control adults, parents of children with cystic fibrosis (CF), healthy control children, non-CF children with pulmonary infection, uninfected CF children, and CF children infected with *Staphylococcus aureus* or *Pseudomonas aeruginosa*.

<table>
<thead>
<tr>
<th>Leukocytes/µL</th>
<th>Adults</th>
<th>Non-CF children</th>
<th>CF patients infected with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls (n = 35)</td>
<td>CF parents (n = 20)</td>
<td>Healthy (n = 10)</td>
</tr>
<tr>
<td>Basal CL</td>
<td>6835 ± 313</td>
<td>7784 ± 423</td>
<td>7839 ± 525</td>
</tr>
<tr>
<td>Luminol-dependent</td>
<td>42 ± 5</td>
<td>33 ± 3</td>
<td>38 ± 4</td>
</tr>
<tr>
<td>Lucigenin-dependent</td>
<td>42 ± 4</td>
<td>39 ± 5</td>
<td>37 ± 3</td>
</tr>
<tr>
<td>PMA-induced CL</td>
<td>0.017 nmol PMA/µL</td>
<td>144 ± 16</td>
<td>226 ± 92</td>
</tr>
<tr>
<td>8.3 nmol PMA/µL</td>
<td>2052 ± 267</td>
<td>1791 ± 188</td>
<td>1212 ± 162</td>
</tr>
<tr>
<td>Lucigenin-dependent</td>
<td>0.017 nmol PMA/µL</td>
<td>779 ± 40</td>
<td>899 ± 58</td>
</tr>
</tbody>
</table>

NOTE. Data are mean ± SE; CL is expressed in counts/20 min/phagocyte. Statistical significance calculated by analyses of variance.

*a* P < .01 vs. controls.

*b* P < .01 vs. healthy non-CF children.
primed activities did not significantly differ between control adults and healthy control children. These findings are in accord with those in other studies [18, 20].

In vivo exposure to inflammatory mediators decreases blood phagocyte opsonin receptor response capacity and narrows the difference between circulating and PAF-primed activities, increasing the value of the ratio measured in vitro. The ratio was significantly increased in all groups of CF patients and in infected non-CF children relative to healthy control children. Furthermore, the ratio was significantly increased for P. aeruginosa–infected CF children relative to uninfected and S. aureus–infected CF children. Of interest, children colonized with P. aeruginosa >1 year (n = 21) had significantly higher circulating (5973 ± 732) and PAF-primed (12.046 ± 898) opsonin receptor activities than did CF children infected <1 year (n = 10): 3936 ± 662 (P < .05) and 8164 ± 877 (P < .01), respectively. However, the circulating/PAF-primed ratios did not differ between the 2 groups (0.48 ± 0.03 vs. 0.46 ± 0.05, respectively). Likewise, this ratio did not significantly differ between CF children infected with P. aeruginosa alone (n = 8) and those with associated pathogens (n = 23): 0.49 ± 0.05 vs. 0.46 ± 0.02, including S. aureus (n = 11), H. influenzae (n = 6), or mycobacteria (n = 6).

Stratification according to severity of lung disease (defined in table 1) was informative only in the P. aeruginosa group, because there were too few cases in other groups. By this measure, the circulating/PAF-primed ratio was apparently higher in patients with segmental or diffuse bronchiectasis than in those with obstructive lung disease (0.50 ± 0.05 vs. 0.45 ± 0.03), but the difference was not significant.

With regard to the classical inflammation markers, no significant correlation was found between the circulating/PAF-primed ratio and blood sedimentation rate (r = .01, P = .91) or plasma levels of fibrin (r = -.016, P = .95) and C reactive protein (r = .07, P = .78).

Table 3. Phagocyte opsonin receptor–dependent activity measured by chemiluminescence (CL) in whole blood of control adults, parents of children with cystic fibrosis (CF), healthy control children, non-CF children with pulmonary infection, uninfected CF children, and CF children infected with Staphylococcus aureus or Pseudomonas aeruginosa.

<table>
<thead>
<tr>
<th>Adults Controls (n = 35)</th>
<th>CF parents (n = 20)</th>
<th>Healthy (n = 11)</th>
<th>Infected (n = 10)</th>
<th>Uninfected CF children (n = 25)</th>
<th>CF children infected with S. aureus (n = 14)</th>
<th>P. aeruginosa (n = 31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opsonin receptor–dependent activity</td>
<td>Circulating activity</td>
<td>3258 ± 357</td>
<td>3747 ± 506</td>
<td>2351 ± 174</td>
<td>7099 ± 522^a</td>
<td>3107 ± 257^b</td>
</tr>
<tr>
<td></td>
<td>PAF-primed activity</td>
<td>9212 ± 628</td>
<td>9626 ± 716</td>
<td>8904 ± 404</td>
<td>12317 ± 956^a</td>
<td>7852 ± 628</td>
</tr>
<tr>
<td></td>
<td>Circulating/PAF-primed ratio</td>
<td>0.34 ± 0.02</td>
<td>0.37 ± 0.03</td>
<td>0.29 ± 0.02</td>
<td>0.58 ± 0.05^a</td>
<td>0.41 ± 0.02^a</td>
</tr>
</tbody>
</table>

NOTE. Opsonin receptor–dependent activity is measured within whole blood after stimulation by human complement–opsonin zymosan in absence (circulating activity) or presence of PAF (PAF-primed activity). Data are mean ± SE. CL is expressed in counts/20 min/phagocyte. Statistical significance is calculated by analysis of variance.

^a P < .01 vs. healthy non-CF children.
^b P = .07 vs. uninfected CF children.
^c P < .05 vs. uninfected CF children.
^d P < .01 vs. uninfected CF children.
^e P = .004 vs. uninfected CF children.

adult controls and healthy control children but was not statistically significant by ANOVA.

Again, we tested for a possible relationship between age and opsonin receptor–dependent activity. Correlation coefficients between age and opsonin receptor circulating activity (r = .01) or PAF-primed activity (r = .10) were not significant by regression analysis between opsonin receptor–dependent activity and age. In addition, when all variables were adjusted for age, between-group differences remained statistically significant by ANOVA, especially those between uninfected CF children and healthy control children.

The effect of antibiotic therapy (gentamicin) was studied over time in 3 children with CF who were newly infected with P. aeruginosa (table 4). In 2 children who responded well to this therapy as reflected by decreased leukocyte count, negative sputum cultures, and improved FEV1 scores, the circulating/PAF-primed ratio returned to levels comparable to those in healthy control children. In contrast, in the third child, in whom no significant change in leukocyte count and no improvement of pulmonary infection was found, this ratio remained unchanged.

Multiple discriminant analysis. Multiple discriminant analysis offers an analytic approach for distinguishing group membership based on the combination of measurements taken and determines the relative contribution of each variable tested to group membership [19]. Although infected CF children are clearly differentiated from controls by conventional statistical techniques, the differences between uninfected CF children and controls are less obvious and can be studied in composite form by discriminant analysis. For the present study, group membership (i.e., control adults, control children, CF heterozygotes [parents], and uninfected CF homozygotes [children]) was the single dependent variable—the categorical or nominal variable. The independent variables, the metrics or measured values, included basal activity, PMA-stimulated activity, circulating activity, ratio of circulating to PAF-primed activity, and leukocyte count.
Table 4. Effect of antibiotics on opsonin receptor-dependent phagocyte circulating and PAF-primed activities in CF children chronically infected with *Pseudomonas aeruginosa*.

<table>
<thead>
<tr>
<th>Time after treatment initiation</th>
<th>Before treatment</th>
<th>4 days</th>
<th>7 days</th>
<th>11 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1, beneficial effect of antibiotics on pulmonary infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leukocytes/μL</td>
<td>26,800</td>
<td>12,100</td>
<td>16,000</td>
<td>10,900</td>
</tr>
<tr>
<td>Opsonin receptor-dependent luminol CL</td>
<td>7895</td>
<td>6142</td>
<td>2814</td>
<td>1312</td>
</tr>
<tr>
<td>Circulating activity</td>
<td>15,480</td>
<td>12,118</td>
<td>9611</td>
<td>6406</td>
</tr>
<tr>
<td>PAF-primed activity</td>
<td>0.51</td>
<td>0.51</td>
<td>0.29</td>
<td>0.21</td>
</tr>
<tr>
<td>Circulating/PAF-primed ratio</td>
<td>0.51</td>
<td>0.51</td>
<td>0.29</td>
<td>0.21</td>
</tr>
<tr>
<td>Case 2, beneficial effect of antibiotics on pulmonary infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leukocytes/μL</td>
<td>9700</td>
<td>ND</td>
<td>5250</td>
<td>ND</td>
</tr>
<tr>
<td>Opsonin receptor-dependent luminol CL</td>
<td>4952</td>
<td>ND</td>
<td>2961</td>
<td>ND</td>
</tr>
<tr>
<td>Circulating activity</td>
<td>8437</td>
<td>ND</td>
<td>10,845</td>
<td>ND</td>
</tr>
<tr>
<td>PAF-primed activity</td>
<td>0.58</td>
<td>ND</td>
<td>0.27</td>
<td>ND</td>
</tr>
<tr>
<td>Circulating/PAF-primed ratio</td>
<td>0.58</td>
<td>ND</td>
<td>0.27</td>
<td>ND</td>
</tr>
<tr>
<td>Case 3, no effect of antibiotics on pulmonary infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leukocytes/μL</td>
<td>13,500</td>
<td>13,100</td>
<td>13,500</td>
<td>ND</td>
</tr>
<tr>
<td>Opsonin receptor-dependent luminol CL</td>
<td>5371</td>
<td>7339</td>
<td>4898</td>
<td>ND</td>
</tr>
<tr>
<td>Circulating activity</td>
<td>10,351</td>
<td>13,217</td>
<td>9419</td>
<td>ND</td>
</tr>
<tr>
<td>PAF-primed activity</td>
<td>0.52</td>
<td>0.56</td>
<td>0.52</td>
<td>ND</td>
</tr>
<tr>
<td>Circulating/PAF-primed ratio</td>
<td>0.52</td>
<td>0.56</td>
<td>0.52</td>
<td>ND</td>
</tr>
</tbody>
</table>

NOTE. Opsonin receptor-dependent activity was measured in whole blood after stimulation by human complement-opsonized zymosan in absence (circulating activity) or presence of PAF (PAF-primed activity). Chemiluminescence (CL) is expressed in counts/20 min/phagocyte. ND, not done.

Two of the three computed discriminant functions or roots were statistically significant for assigning group membership. The root 1 and root 2 values (abscissa) plotted against the frequency (ordinate) are shown in figure 1A and 1B, respectively. The first root, which represents 88% of the explained variance, was weighed most heavily by the leukocyte count and the ratio of circulating-to-PAF-primed activity. Figure 1A plots the homozygous CF children and control children discriminant root 1 scores versus frequency. The second root, which represents 20% of the explained variance, was weighed most heavily for circulating and PMA activities and discriminated healthy CF heterozygotes from healthy control adults (figure 1B). The results illustrate the utility of composite luminescence analysis to gauge relatively small differences in levels of immune activation.

**Relationship of phagocyte parameters to CF genotype.** The wide heterogeneity in the clinical expression of CF and differences in the clinical evolution of each patient prompted us to investigate the potential influence of CF genotype on blood phagocyte function in age-matched uninfected CF children. The
ΔF508/ΔF508 CF genotype has been associated with the most severe forms of the CF pancreatic insufficiency relative to ΔF508/other genotype. As shown in table 4, the ΔF508/ΔF508 CF children had a small but significant increase in leukocytes relative to the non-ΔF508 CF children (P = .03). However, for the age-matched uninfected CF children, no genotype-specific differences were observed relative to the Shwachman index or any of the phagocyte luminescence activities, including the circulating-to-PAF-primed activities ratio.

**Phenotypic versus functional opsonin receptor (CR1, CR3, and FcγR) expression.** In order to verify whether the increased functional activity observed in the absence of infection could be related to an increased expression of these receptors, we measured phenotypic (physical) expression of phagocyte opsonin receptors in uninfected CF patients in parallel with phagocyte circulating and PAF-primed oxidative activities.

Stimulation of phagocyte respiratory burst metabolism by untreated zymosan can occur via CR3 (the integrin CD11b/CD18) ligation of mannan sites on zymosan or possibly by a CR3-independent mannan-specific phagocyte receptor mechanism. Activation of phagocyte metabolism by complement-opsonized zymosan, which contains bound C3bi and C3b as opsonins, can proceed via both CR3 and CR1 (CD35) ligation mechanisms. Respiratory burst activation by IgG-opsonized zymosan, which contains bound IgG, can proceed via FcγRII- and FcγRIII-dependent mechanisms.

Phenotypic expressions of CR1, CR3, FcγRII, and FcγRIII on whole blood phagocytes were measured using receptor-specific fluorescent monoclonal antibodies and by quantifying neutrophil staining by flow cytometry. As depicted in figure 2, phenotypic expression of CR1 (CD35) in unstimulated circulating blood phagocytes was similar in uninfected CF neutrophils (n = 8) and in controls (n = 8) (23.4 ± 2.8 vs. 19.5 ± 2.1 mean fluorescence intensity units [MFIU]). Likewise, both CR3 (CD11b) (20.6 ± 3.1 vs. 19.4 ± 2.1 MFIU) and FcγRII (CD32) (19.8 ± 4.5 vs. 18.8 ± 0.8 MFIU) were similar in CF children and in controls; in contrast, FcγRIII (CD16) was significantly lower in CF children than in controls (147.9 ± 19.5 vs. 219.3 ± 31.3 MFIU, P < .01).

As shown in figure 2, the circulating activities of phagocytes from CF children were significantly greater than in controls in response to unopsonized (CR3) (P < .05) and complement-opsonized zymosan, which contains bound C3bi and C3b as opsonins, can proceed via both CR3 and CR1 (CD35) ligation mechanisms. Respiratory burst activation by IgG-opsonized zymosan, which contains bound IgG, can proceed via FcγRII- and FcγRIII-dependent mechanisms.

**Figure 2.** Functional and phenotypic opsonin receptor analysis in CF children and controls. Upper panels: Blood circulating phagocyte opsonin receptor–dependent oxidative activities measured as luminol luminescence after activation with untreated zymosan (Z) (via CR3), complement-opsonized zymosan (hC-OpZ) (via CR3 + CR1), or IgG-opsonized zymosan (hI-OpZ) (via FcγRII plus FcγRIII and possibly CR3). Luminescence is expressed as counts/20 min/phagocyte. Lower panels: Flow cytometry phenotypic analysis after immunostaining whole blood phagocytes with fluorescence isothiocyanate–labeled specific antibodies directed against CR3 (CD11b), CR1 (CD35), FcγRII (CD16), and FcγRIII (CD32) and measured as cell fluorescence gating for neutrophils. Data are mean ± SEM fluorescence intensity units from 8 independent experiments, each including 1 control and 1 child with CF. Differences between controls and CF children were analyzed by Student’s t test (* P < .05).
sonized zymosan (CR1) \( P < .05 \). The IgG-opsonized zymosan-activated respiratory burst metabolism of CF children was also significantly increased compared with controls \( P < .05 \).

We repeated the phenotypic measurements of opsonin receptor expression in the absence and presence of PAF in order to generate the phenotypic ratio of circulating-to-PAF–primed opsonin receptor expression. The results of these flow cytometry measurements of antigenic circulating and PAF-primed expression are presented in figure 3. Exposing whole blood to PAF triggered an increase in the membrane expression of CD11b, CD35, and CD16, but not CD32, in the blood neutrophils of healthy control and CF children. When measurements were gated on monocytes from controls, no significant PAF-induced up-regulation for monocytes was observed (data not shown).

As illustrated in figure 3, the neutrophils of uninfected CF children showed decreased mobilization of opsonin receptors in response to PAF exposure. Stated differently, the phenotypic ratios of circulating-to-PAF–primed opsonin receptor expression of blood neutrophils in CF children were increased relative to those of healthy control children. As shown in figure 4, the phenotypic ratios determined by flow cytometry correlate with the functional ratios determined by luminescence analysis, thus demonstrating that blood phagocytes of uninfected CF children show diminished opsonin receptor response capacity.

**Discussion**

Airway inflammation is an important component of CF lung disease, and its evaluation in the inflammatory and infectious status in CF patients is crucial for long-term prognosis and for adjustment of therapy. Although sputum and throat cultures are not reliable or sensitive indicators of infection, especially in very young children [27, 32], for lack of a better approach, they are routinely used to evaluate lung infection in CF. Armstrong et al. [33] examined bronchoalveolar lavage (BAL) fluid in newly diagnosed infants with CF and showed that development or persistence of infection is accompanied by increased inflammatory markers that are decreased in the absence of or with clearance of infection [33]. Therefore, a noninvasive rapid technique for evaluating inflammation and infection in young

![Figure 3](https://academic.oup.com/jid/article-abstract/179/1/151/877655)
Figure 4. Correlation between functional activity and phenotypic expression of circulating and PAF-primed opsonin receptors. Ratios between circulating (C) and PAF-primed (or maximal, M) for functional activity (abscissa) are plotted against values for phenotypic C/M ratio (ordinate) for each opsonin receptor tested: Z with CD11b (A), hC-OpZ with CD35 (B), and hI-OpZ with CD16 (C). Significant correlation determined by Pearson $r$ coefficients and probabilities. Healthy control children and CF children shown by open and solid symbols, respectively.

children with CF would have value as a diagnostic and therapy management tool.

Our data, obtained using luminescence measurement of blood phagocyte oxygenation activities, indicate that clinically well and apparently uninfected CF children show a decreased ability to mobilize opsonin receptors in response to exogenous PAF. This is shown by a higher ratio between circulating and PAF-primed phagocyte opsonin responses to human complement–opsonized zymosan and indicative of in vivo immune activation (i.e., inflammation). Of note, uninfected CF children were differentiated from healthy control children on the basis of blood phagocyte analysis alone. Our data show that the NADPH-oxidase activity of whole blood phagocytes is not different in CF children in the absence or presence of infection, confirming the findings of our previous study on isolated neutrophils from children with CF [11]. The classical inflammatory markers, including blood sedimentation rates and plasma levels of fibrin or C-reactive protein, were not related to whole blood oxidative activities.

**Influence of infection and clinical status.** In terms of clinical status, the group of children infected with *S. aureus* was intermediate between uninfected children and those infected with *P. aeruginosa*, who had the highest ratio. The study of the influence of infection on blood phagocyte luminescent activities shows that both *S. aureus* and *P. aeruginosa* infection increase MPO-dependent luminescent activities in response to high doses of PMA. Likewise, circulating and PAF-primed opsonin receptor–dependent activities were increased in both groups of infected children relative to uninfected CF children. Similar observations were made in the group of non-CF children with chronic pulmonary infection. Such increases are consistent with increased marrow stimulation and neutrophil production [21]. Our results also indicate that infection with pathogens, especially with *P. aeruginosa*, worsens the situation by further increasing the ratio of circulating-to-PAF–primed activities, which was already increased in uninfected CF children. As illustrated by the effect of gentamicin treatment on newly infected CF children, successful eradication of *P. aeruginosa* resulted in marked improvement of the luminescent parameters, except in a child resistant to gentamicin. In 2 children, the circulating-to-PAF–primed opsonin receptor–dependent activity was normal (and even lower than that in well CF patients). The profound effect of antibiotics on oxidative metabolism may be complex and should be investigated with more patients.

Among the other therapeutic agents that may influence this ratio, antiinflammatory drugs, such as ibuprofen, have been proposed for CF patients [34]. However, none of our patients received such treatment. In contrast, all CF patients in our clinical center receive vitamin E supplementation because of chronic vitamin E deficiency [35]. Although one would expect a modulatory effect of vitamin E treatment on oxidative phagocyte activities, we did not observe any influence of vitamin E levels on blood phagocyte oxidant production.

**Influence of CF genotype.** The small and statistically insignificant increase in the activity of CF heterozygotes relative to controls may be related to CF genetic expression and is still not elucidated. Some have questioned whether CF heterozy-
gotes could have a genetic advantage (recently reviewed in [9]) or are particularly susceptible to pulmonary dysfunctions. In a study involving 315 parents of CF children, a possible association between CF heterozygosity and increased airway reactivity was found [36]. Information regarding the role of CF transmembrane conductance regulator gene expression in CF neutrophil function is incomplete [37]. The fact that we did not find a correlation between the inflammatory index and the type of CF transmembrane conductance gene mutation is consistent with previous reports that found no clear relationships between genotype and phenotype, especially with regard to airway inflammation [38].

Phenotypic and functional expression of opsonin receptors. The capacity of blood neutrophils to mobilize opsonin receptors from intracellular pools, that is, the transition from circulating to PAF-primed opsonin receptor expression was assessed by functional and phenotypic measurements in whole blood to avoid artificial increases of opsonin receptor expression during the process of isolation and purification of phagocytes from blood [39].

Taken together, our data demonstrate that the phenotypic CR3, CR1, and FcγR membrane expressions do not significantly correlate with the unopsonized, complement- and IgG-opsonized zymosan-dependent oxidative responses to unprimed circulating blood phagocytes. Functional activity measurements are dependent on both the opsonin receptor–dependent activation and the specific metabolic capacity of the phagocytes activated. The specific metabolic capacity per phagocyte is changed by immune activation status [19–21]. The ratio of circulating to PAF-primed activities, which gauges the in vivo state of immune activation, is a measurement of the phagocyte’s capacity to mobilize its opsonin receptors in response to exogenous priming agents such as PAF. Since phagocyte metabolic capacity is the same for both circulating and primed activities, the ratio is relatively unaffected by the specific metabolic capacity per phagocyte. An increased ratio indicates a diminished opsonin receptor response capacity, that is a loss in opsonin receptor reserve, and is consistent with in vivo immune activation. Of interest, the functional opsonin receptor–dependent approach to analysis correlates with phenotypic measurements of opsonin receptor reserve. A previous study of basal and FMLP-primed CR1 and CR3 expression failed to show any difference between the circulating neutrophils of CF patients and those of controls, whereas neutrophils isolated from BAL fluid have maximally up-regulated expression of CR3 with a concomitant decrease in CR1 due to in situ proteolysis [40]. The previous study was on adult CF patients chronically infected with P. aeruginosa, and neutrophils were purified from blood before testing; however, functional and phenotypic ratios between circulating and primed opsonin receptor expression were not measured. In a recent study, measurement of CD11b on circulating neutrophils by FACS analysis in chronically infected CF patients did not show any up-regulation compared with uninfected control or acutely infected non-CF bronchiectasis patients [41].

PAF primes maximum opsonin receptor expression [42–44]. The quantity of PAF required for maximum receptor expression is far less than that required to completely activate respiratory burst metabolism [45]. Treatment of blood phagocytes with PAF increased the physical expression of CR1, CR3, and FcγRII. The only receptor not up-regulated by PAF was FcγRII. There is a parallel between the degree of CR1, CR3, and FcγRIII mobilization in response to chemotactic and immunomodulator agents [42, 43].

The functional activities primed by exposure to C5a, LTB4, or FMLP are of the same relative magnitude as those obtained with PAF [21, 45]. In vitro and in vivo studies demonstrate that granulocyte macrophage colony-stimulating factor treatment increases neutrophil oxidative burst in response to cross-linking FcγRII (CD32) without changing the phenotypic expression of FcγRII per neutrophil [46]. Our data also show increased blood phagocyte responsiveness to IgG-opsonized zymosan without change in the phenotypic expression of FcγRII. The nature of IgG opsonin-dependent phagocyte activation is complex. FcγRII (CD32) and FcγRIII (CD16) act synergistically in neutrophil activation [47]. FcγRIII (CD16) is anchored to the phagocyte membrane via a glycosyl-phosphatidylinositol linkage that is cleaved upon activation and released as a soluble form in plasma [48, 49]. In the present study, we observed decreased membrane expression of CD16 in CF children, which might reflect shedding in response to in vivo inflammatory activation. Although no change in PAF-induced FcγRII (CD32) expression was observed, PAF treatment increased the FcγR-mediated oxidative activity of blood phagocytes. Taken together, these observations suggest that the neutrophil priming observed in CF children is not the exclusive consequence of opsonin receptor up-regulation and that the functional differences, which are greater than the phenotypic differences, reflect both opsonin receptor–dependent and post-opsonin receptor activity increases.

From a clinical perspective, our findings indicate that inflammatory activation of circulating phagocytes occurs early in the clinical course of CF when CF is not detectable by classical laboratory tests. Of great interest for our present study was a recent report of CF and non-CF patients with bronchiectasis that showed that CF neutrophils have a decreased L-selectin shedding capacity in response to IL-8 and FMLP, whereas no difference between groups was observed in unprimed neutrophils under basal conditions [41]. Taken together, these results add to the accumulating evidence that neutrophils from CF patients display a different response pattern than those from control subjects [41]. Evaluation of the inflammatory state by opsonin-dependent receptor chemiluminescence could therefore be of great value in CF patient treatment. Infection with various pathogens and the subsequent chronic infection with P. aeruginosa increase the intensity of activation and are as-
associated with increased respiratory burst activity and oxidant production. Such findings corroborate recent reports suggesting that early management of the inflammatory state might delay disease progression and pulmonary impairment in CF patients [34].

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References

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