A Simple Assay to Measure Phagocytosis of Live Bacteria

Heike Bicker,1 Conny Höflich,1 Kerstin Wolk,2 Katrin Vogt,1 Hans-Dieter Volk,1 and Robert Sabat2*

1 Institute of Medical Immunology and 2 Interdisciplinary Group of Molecular Immunopathology, Dermatology/Medical Immunology, University Hospital Charité, Berlin, Germany; * address correspondence to this author at: Interdisciplinary Group of Molecular Immunopathology, Dermatology/Medical Immunology, University Hospital Charité, Campus Charité Mitte, Charitéplatz 1, D-10117 Berlin, Germany. Fax +49 30 450 518964; e-mail robert.sabat@charite.de.

BACKGROUND: The phagocytosis of pathogens is essential for fighting infections. No assay is available, however, to measure both engulfment and degradation of bacteria under conditions similar to those in vivo. We sought to develop a flow cytometric assay to measure the engulfment and degradation of live bacteria by human blood monocytes and granulocytes.

METHODS: We generated enhanced green fluorescent protein (EGFP)-expressing Escherichia coli by transforming E. coli with the plasmid vector pEGFP. We used these bacteria in a flow cytometric assay to measure both engulfment and degradation of living bacteria by monocytes and granulocytes in human whole blood from fresh, heparinized venous blood samples. To determine whether the test detected differences between healthy individuals and patients with secondary immunodeficiencies, we compared the phagocytosis of monocytes and granulocytes measured in blood samples from immunosuppressed kidney transplantation patients and from patients with postoperative sepsis in immunopathysis with phagocytosis measured in samples from age-matched healthy individuals.

RESULTS: In samples from healthy individuals, we found that in both monocytes and granulocytes bacterial degradation was negatively correlated with the age of the sample donor. Furthermore, we detected decreased bacterial engulfment in granulocytes from septic patients and decreased bacterial degradation in monocytes from immunosuppressed kidney transplantation patients.

CONCLUSIONS: This flow cytometric assay measures the engulfment and degradation of live bacteria by human blood monocytes and granulocytes. By means of this assay we detected significant differences between healthy controls and patients with secondary immunodeficiencies that may contribute to the increased incidence of infection complications seen in these patients.

Phagocytosis, the engulfment and degradation of particles such as bacteria, is an essential function of the immune system. The impairment of phagocytosis leads to severe infections, as can be seen in patients with primary and secondary immunodeficiencies (1–6). Phagocytosis assays currently used in medical diagnostics analyze only the engulfment of dead bacteria, the surfaces of which are loaded with fluorescent stains (7, 8). Other phagocytosis tests can be applied to measure only bacterial degradation, but these methods are based on counting viable bacteria after phagocyte lysis and thus are costly in terms of time and effort (9). Therefore, there is a need for a simple and reliable assay to measure both bacterial engulfment and bacterial degradation under conditions similar to those in vivo.

Enhanced green fluorescent protein (EGFP) is a mutant form of the wild-type green fluorescent protein from the jelly fish Aequorea victoria that has been optimized for brighter fluorescence and higher expression in cells (10). The EGFP excitation maximum is 488 nm, and its emission maximum 507 nm (10). We hypothesized that engulfment of living bacteria with intracellular expressed EGFP would cause an increase in phagocyte green fluorescence representing the engulfment of these cells. Over time this fluorescence intensity will decline owing to bacterial destruction and release of EGFP to an environment characterized by low pH and activated proteases. The percentage reduction of the green fluorescence will reflect the degradation ability of the phagocytes. Based on this idea we attempted to create a simple and reliable flow cytometric assay to measure both engulfment and degradation of living bacteria by monocytes and granulocytes in human whole blood.

To generate EGFP-expressing bacteria we transformed Escherichia coli with the plasmid vector pEGFP. In this vector, the lac promoter expresses the EGFP fusion protein, and the vector contains an ampicillin resistance gene for propagation and selection of transformed bacteria. (For detailed information on the generation of EGFP-expressing E. coli and control–E. coli, see the Data Supplement that accompanies the online version of this Brief Communication at http://wwwclinchem.org/content/vol54/issue5.)

We established the test for use on whole blood samples to avoid time-consuming cell separation and also to perform the test under near in vivo conditions. Based on results of preliminary studies (see Supplemental Fig. 1a in the online Data Supplement), we used heparinized venous blood, diluting 100 μL of a freshly
drawn sample with 400 μL of assay medium (for data about the influence of whole blood dilution on test values, see Supplemental Fig. 1b in the online Data Supplement). The assay medium was composed of RPMI 1640 medium supplemented with heat-inactivated fetal calf serum, L-glutamine, heparin, and penicillin/streptomycin. We used penicillin/streptomycin in the assay medium to inhibit further bacterial growth during the assay (see Supplemental Fig. 1c in the online Data Supplement). Next, we added 10 μL of freshly recultivated EGFP– or control–E. coli suspension, corresponding to a bacterial concentration of 6 × 10^6 E. coli/mL in the assay (for data about the influence of bacterial number on test values, see Supplemental Fig. 1d in the online Data Supplement). Samples were then carefully resuspended and incubated at 37 °C and spun at 600 rpm for 15 min (t15 min) or for an additional 3 hours and 45 min at 37 °C without rotation (t4h ). Based on kinetic study results, which showed that the maximum green fluorescence intensity of monocytes and granulocytes incubated with EGFP–E. coli was highest after 15 min and thereafter diminished continuously, we assigned 15 min (t15 min) to be the measuring time-point for bacterial engulfment and 4 hours (t4h ) to be the measuring time-point for bacterial degradation (see Supplemental Fig. 2 in the online Data Supplement).

After 15 min and 4 hours, respectively, engulfment and degradation reactions were stopped by cooling the samples for 10 min on ice, followed by washing with ice cold PBS (1×, without calcium and magnesium). The remaining cell pellet was then incubated with the antibody mix anti-CD14 Phycoerythrin 5 (PC5)/anti-CD33 PCs for 20 min at 4 °C in darkness, followed by erythrocyte lysis and leukocyte fixation via incubation with 1 mL fluorescence-activated cell-sorting (FACS) lysing solution for 15 min. After removal of the lysing solution by centrifugation for 5 min at 200g and decantation of the supernatant, another washing step with PBS supplemented with heat-inactivated fetal calf serum and sodium azide was performed. Cells were then analyzed using a FACS Calibur (Becton Dickinson), measuring at least 50 000 phagocytes by setting a live cell gate according to the characteristic scatter properties of monocytes and granulocytes. A detailed protocol of the assay and further information about human blood sample origin, instrumental setup of the flow cytometer, and statistical analysis can be found in the online Data Supplement.

The commercially available Phagotest® reagent set (Orpegen Pharma), which analyzes the uptake of FITC-labeled heat-inactivated E. coli, was frequently used in parallel with the EGFP assay described herein. To allow better comparison between both tests, the Phagotest reagent set was used in a manner partially modified from the manufacturer’s instructions (as described in the online Data Supplement). In samples from both patients and healthy individuals, FITC intensity of monocytes and granulocytes in the Phagotest reagent set increased beyond that of our assay after 15 min (data not shown).

Data analysis was performed as follows. First, phagocytes were separated from lymphocytes using respective forward- and side-scatter properties. Then, monocytes were distinguished from granulocytes by means of CD14 and CD33 expression and respective side-scatter properties (Fig. 1A). Next, the mean green fluorescence intensities (mfi) of monocytes and granulocytes incubated with EGFP– and control–E. coli were analyzed (Fig. 1, B and C). Bacterial engulfment and degradation were then calculated using the following formulas:

\[
\text{bacterial engulfment (mfi)} = \frac{\text{mfi}_{\text{EGFP–}{E.\, coli}} (t4h) - \text{mfi}_{\text{control–}{E.\, coli}} (t15\,\text{min})}{\text{mfi}_{\text{control–}{E.\, coli}} (t15\,\text{min})} \times 100, \quad \text{for specific mfi (t4h)}
\]

\[
\text{bacterial degradation (%) } = \frac{\text{mfi}_{\text{control–}{E.\, coli}} (t4h) - \text{mfi}_{\text{EGFP–}{E.\, coli}} (t4h)}{\text{mfi}_{\text{control–}{E.\, coli}} (t4h)} \times 100, \quad \text{for specific mfi (t15\,\text{min})}
\]

It should be noted that the changes seen in phagocytic green fluorescence intensities are most probably caused by bacterial engulfment and degradation, respectively. However, these data can also be interpreted as initial bacterial association and subsequent dissociation from the surface of the phagocytes.

Next we investigated intraassay and interassay variances, the influence of blood storage before test initiation on test values, and the stability of the samples before flow cytometric measurement. CVs were significantly lower than 20% for all evaluation parameters, indicating that our assay was reproducible. For example, the mean (SE)/CV for engulfment of monocytes was 56.2 (6.9)/12.3 for sample 1, and 76.7 (4.2)/5.5 for sample 2; engulfment by granulocytes was 55.4 (2.9)/5.3 for sample 1, and 64.3 (1.8)/2.8 for sample 2; degradation by monocytes was 52.6 (7.4)/14.1 for sample 1, and 29.2 (2.0)/6.8 for sample 2; and degradation by granulocytes was 66.0 (2.5)/3.9 for sample 1, and 80.6 (0.7)/0.9 for sample 2 (n = 5/sample). Interassay values can be found in Supplemental Fig. 3 in the online Data Supplement. Furthermore, we found that the storage condition of blood samples before test initiation should be constant, and flow cytometric measurement should be performed immediately after completion of the test procedure, because phagocytosis values decrease with time (see Supplemental Fig. 3 in the online Data Supplement).
Fig. 1. Gating of monocytes and granulocytes and analysis of phagocytosis.
Whole blood samples were incubated with EGFP− or control−E. coli for 15 min and 4 hours each. Cellular reactions were then stopped, and cells were stained with anti-CD14 and anti-CD33 monoclonal antibodies labeled with PC5. Following another washing step and erythrocyte lysis, samples were measured via flow cytometry. (A), After gating according to scatter properties, monocytes and granulocytes were separated according to side scatter properties and staining intensities for CD14 and CD33. (B), Green fluorescence intensities of monocytes and granulocytes incubated with EGFP− or control−E. coli for 15 min (lines, control−E. coli; gray area, EGFP−E. coli). (C), Green fluorescence intensities of monocytes and granulocytes incubated with EGFP− or control−E. coli for 4 hours (lines, control−E. coli; gray area, EGFP−E. coli). (D) In samples from 12 healthy individuals (4 females, 8 males, mean/minimum/maximum age 49.6/27/61 years) bacterial degradation of EGFP-expressing E. coli by peripheral blood monocytes and granulocytes was analyzed and results were correlated with age (r, Spearman rank correlation coefficient).
Subsequently, we analyzed bacterial engulfment and degradation by human blood monocytes and granulocytes from 12 healthy individuals. In contrast to granulocytes, the monocytic engulfment decreased with age ($r = -0.614$, $P < 0.05$, Spearman rank correlation; see Supplemental Fig. 4 in the online Data Supplement). Interestingly, in both monocytes and granulocytes bacterial degradation was negatively correlated with age (monocytes: $r = -0.661$, $P < 0.05$, granulocytes: $r = -0.697$, $P < 0.05$, both Spearman rank correlation; Fig. 1D).

We then investigated whether the test detected differences between healthy individuals and patients with secondary immunodeficiencies. We measured the phagocytosis of monocytes and granulocytes from immunosuppressed kidney transplantation patients and patients with postoperative sepsis in immunoparalysis [a state in which the immune system is not able to successfully fight off infections (11–13)] and compared these results to those for samples from respective age-matched healthy individuals. As shown in Table 1, granulocytes from septic patients in immunoparalysis demonstrated significantly decreased bacterial engulfment. Furthermore, monocytes from kidney transplantation patients showed significantly decreased bacterial degradation. Interestingly, no significant differences were found between healthy individuals and the analyzed patients as detected with the Phagotest reagent set (data not shown).

We have shown that this flow cytometric assay can measure the engulfment and degradation of live bacteria by human blood monocytes and granulocytes. By means of this assay we detected significant differences between healthy controls and patients with secondary immunodeficiencies. These differences may contribute to the increased incidence of infection complications seen in these patients (14, 15).

It would be interesting to test whether our assay would reveal subtle engulfment values, but severely diminished values for the degradation capacity, in both monocytes and granulocytes in patients with chronic granulomatous disease caused by deficiency in NADPH oxidase activity (16). Another interesting application would be the analysis of patients undergoing therapy with anti–tumor necrosis factor (17).

**Grant/Funding Support:** We would like to thank the Deutsche Forschungsgemeinschaft for financial support of our work.

**Financial Disclosures:** None declared.

**Acknowledgment:** We would like to thank Elizabeth Wallace for accurately proofreading the manuscript.

**References**

4. Homef MW, Wick MJ, Rhen M, Normark S. Bac-

---

**Table 1. Bacterial engulfment and degradation by peripheral blood monocytes and granulocytes from kidney transplantation patients and septic patients in immunoparalysis compared to respective age-matched healthy controls.a**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patients (n = 7)</th>
<th>Healthy controls (n = 7)</th>
<th>P</th>
<th>Patients (n = 7)</th>
<th>Healthy controls (n = 7)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Engulfment, mfi</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monocytes</td>
<td>55.2 (6.06)</td>
<td>53.4 (9.52)</td>
<td>NS</td>
<td>27.9 (5.72)</td>
<td>43.5 (4.73)</td>
<td>NS</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>27.7 (3.21)</td>
<td>32.3 (3.09)</td>
<td>NS</td>
<td>17.5 (3.91)</td>
<td>36.1 (4.25)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Degradation, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monocytes</td>
<td>39.9 (5.96)</td>
<td>68.4 (11.40)</td>
<td>&lt;0.05</td>
<td>34.4 (6.81)</td>
<td>53.8 (6.40)</td>
<td>NS</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>57.1 (7.13)</td>
<td>77.2 (3.62)</td>
<td>NS</td>
<td>53.5 (4.92)</td>
<td>68.8 (6.02)</td>
<td>NS</td>
</tr>
</tbody>
</table>

*a Engulfment and degradation of EGFP-E. coli by monocytes and granulocytes analyzed with the phagocytosis assay as described in the text. Data are given as mean (SE) (P indicates significance level between patient and respective healthy control group; NS, not significant, Mann Whitney U-test)*


DOI: 10.1373/clinchem.2007.101337