by Rebbeck et al. (7) for US Caucasians. In that specific study on 94 healthy volunteers, 3.2% appeared to be homozygous for this mutation. We did not find any CYP3A4-V homozygotes among the 199 individuals studied. The allele and genotype frequencies were in Hardy-Weinberg equilibrium ($P = 0.432$); the absence of homozygotes in our study population of 199 individuals is consistent with a Hardy-Weinberg distribution. In Swedish Caucasians, 3 CYP3A4-V alleles were found recently among 39 individuals studied, giving an allelic frequency of 3.8% (15), whereas Sata et al. (9) reported an allelic frequency of 4.2% in 59 white subjects. These data are more in agreement with our results.

CYP3A4 is the most abundant form of the cytochrome P450 enzyme family present in human liver and is involved in the metabolism of many drugs (3, 4, 16). The recently described A(−290)G genetic polymorphism in the 5′ regulatory region potentially alters the transcription efficiency and thus the overall enzymatic activity of CYP3A4. Initially, the effect of this mutation on transcription was believed to be a decrease, based on the clinical presentation of prostate cancer (7) and drug-induced leukemia (12). Later experiments, in which protein expression and enzymatic activity in liver samples were compared, suggested that the CYP3A4-V mutation had no effect on transcription (14). This was supported by experiments on the 6β-hydroxylation of testosterone in three microsomal liver samples from individuals heterozygous for the CYP3A4-V allele (15), although this conclusion was later questioned by others (17). Experiments in which the promoter region of CYP3A4 was fused to the luciferase reporter gene, followed by expression of these constructs in HepG2 and MCF7 cells, indicated that the CYP3A4-V polymorphism increases CYP3A4 transcription compared with the CYP3A4 wild-type allele (18). Further studies are needed to show that the CYP3A4-V polymorphism will lead to increased CYP3A4 enzymatic activity not only in cell culture systems but also in individuals.

In conclusion, we have described and validated a fast and simple PCR-RFLP analysis that can be applied for specific screening for the CYP3A4-V allele. This assay could greatly facilitate studies on the effect of this polymorphism in endogenous processes, environmental susceptibility to cancer, and individual ability to metabolize drugs.

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References


Phagocytes and Oxidative Burst: Reference Values for Flow Cytometric Assays Independent of Age, Andreas Lun,1* Markus Schmitt,2 and Harald Renz3 (1 Institute of Laboratory Medicine and Pathobiocchemistry and 2 Clinic of Pediatric Pneumology and Immunology, University Hospital, Charité, Campus Virchow-Klinikum of the Humboldt-University, Augustenburger Platz 1, 13353 Berlin, Germany; 3 Department of Clinical Chemistry and Molecular Diagnostics, Clinic of the Philippus University Marburg, Baldingerstrasse, 35033 Marburg, Germany; 2) author for correspondence: fax 49-30-45069900, e-mail andreas.lun@charite.de)

The main function of neutrophils is to provide a front line of defense against invasive bacteria. Disturbances in the functioning of neutrophils lead to repeated and life-threatening infections caused by bacteria and fungi (1, 2). Pathological neutrophil functions are detected as perma-
ent inborn metabolic defects of NADPH oxidase with oxidative burst [chronic granulomatous disease (CGD)](2, 3), glutathione peroxidase (4), and adhesion molecules (2, 5). Moreover, transient disturbances of phagocytosis may be detected in systemic infections (5–9), acute pancreatitis (10), tuberculosis (11), and Wegner granulomatosis (12), as well as under special conditions such as in newborns (6, 13, 14), very old persons (15), or in persons undergoing therapies with cytokines, prednisolone, or anesthetics (3–12, 14, 16).

The following clinical and laboratory findings indicate that assessment of granulocyte function is needed: increased susceptibility to bacterial infections, therapy-resistant infections, recurrent infections with nonpathogenic microorganisms, lymphadenitis, abscesses of liver or lung, osteomyelitis, recurrent stomatitis, or gingivitis. Granulocytopenia and defects of B cells or complement compartment must be excluded (17). Phagocytosis, adhesion molecules CD18 and CD11b for leukocyte adhesion defect I or CD15s for leukocyte adhesion defect II, and production of oxygen radicals upon stimulation for CGD can be tested by flow cytometric determinations. Disturbances such as Chédiak-Higashi syndrome, hyper IgE syndrome, or glycogenesis type Ib need other techniques.

One of the most common inherited granulocyte defects is CGD. The nitroblue tetrazolium dye reduction assay, the gold standard for diagnosis of CGD in the past (18–20), has been replaced to a large extent by flow cytometry-based procedures (18, 21–23). Commercially available flow cytometric assays have been used routinely in clinical laboratories. However, age-dependent reference values are not known or have been reported based on very small collectives (15, 24). Therefore, the aim of this study was to establish age-dependent reference values for phagocytosis and oxidative burst activities, both assessed by flow cytometry. In addition, the diagnostic efficiency of oxidative burst activities to detect CGD was examined in patients and heterozygous carriers of the disease.

The capacities of phagocytosis and oxidative burst were tested in 288 individuals. In all cases, acquired or inherited immune deficiency syndrome had been excluded by history and/or extensive analysis of cellular and humoral immune functions. The age distribution was 1 month to 54 years.

To test the diagnostic efficiency, a group of seven patients with CGD (six males and one female) and six heterozygous carriers of CGD was evaluated. In all seven CGD patients investigated, the disease manifested during the first 2 years of life. The main clinical problems had been recurrent cervical purulent lymphadenitis with surgical interventions (n = 5) and liver granulomas (n = 4). Intrathoracic granulomas with abscesses (n = 2) and biliary lymphadenopathy (n = 2) were also common. Two patients suffered from pulmonary tuberculosis, one from a long-lasting Bacille Calmette-Guérin inflammation after vaccination. In each case, bladder granulomas attributable to an *Escherichia coli* infection and diffuse small granulomas in the upper gastrointestinal tract could be observed. One boy presented with wound-healing disturbances after herniorrhaphy.

Oxidative burst and phagocytosis were measured quantitatively by fluorometric analysis using commercial methods (PHAGOBURST® and PHAGOTEST®; Orpegen Pharma) in heparinized whole blood (25). The analyses were performed within 4 h of sampling. Blood samples were transported and stored at room temperature.

In the oxidative burst activity experiments, upon stimulation with unlabeled opsonized bacteria (*E. coli*) as a particulate stimulus or the protein kinase C ligand phorbol 12-myristate 13-acetate (PMA) as a strong stimulus, granulocytes produced reactive oxygen metabolites. Radical formation was measured at 37 °C by conversion of dihydroorhodamine 123 to rhodamine 123 as a fluorogenic substrate (26, 27). A sample without stimulus served as negative background control. The reaction was stopped by addition of lysing solution, which removed erythrocytes and partially fixed leukocytes. After a washing step, DNA staining was performed to exclude aggregation artifacts from bacteria or cells. Cells were analyzed by flow cytometry (FACScan; Becton Dickinson), using Cell Quest software (Becton Dickinson) for data acquisition and analysis, and the results were expressed as mean fluorescence intensity (MFI) plotted as histograms. Cali-brite™ beads (Becton Dickinson) were used to adjust instrument settings and set fluorescence compensation. Forward and sideways scatter were used to select granulocytes. The percentage of cells that did not produce oxygen radicals was between 1% and 15%. In all test series, blood samples taken from healthy adult donors were analyzed as an internal control.

In the phagocytosis experiments, bacteria were opsonized with antibodies and complement of pooled serum samples and subsequently bound by leukocytes. Because phagocytosis is temperature-dependent, control experiments were performed on ice. After 10 min of incubation at 37 °C, phagocytosis was interrupted by placing the samples on ice and adding ice-cold quenching solution; uningested bacteria were excluded from analysis. After several washing steps, red blood cells were lysed, followed by incubation with DNA staining solution to exclude aggregation artifacts from bacteria or cells. Data were acquired as in the oxidative burst test.

To test the analytic precision for phagocytosis and oxidative burst activities, 20 samples were analyzed in duplicate, and the CV was calculated using the following formula: CV (%) = (SD/mean value) × 100, where

\[
SD = \sqrt{\frac{\sum_{i=1}^{n} (\text{pair differences})^2}{\text{number of pairs} \times 2}}
\]

The CVs for the burst test were 14% and 11% for stimulation by *E. coli* and PMA, respectively, with MFI values of 498 arbitrary units (AU) and 610 AU. For phagocytosis, the CV was 15%, with a MFI of 610 AU. In addition, 20 stored measuring protocols were gated and evaluated twice using Cell Quest, and the CVs were calculated from duplicates, using the formula described above, to estimate the impact of gating of granulocytes on
the imprecision of the methods. The CV for gating was 15%.

Statistical analyses were carried out using SPSS for Windows.

Phagocytosis reference limits were calculated for all age groups together; the 2.5th and 97.5th percentiles for the MFI were 496 and 2738 AU, respectively (Fig. 1A, left panel). The distributions of values between the different age groups did not differ significantly; the mean values were between 1000 and 1250 AU.

In the oxidative burst experiments, without stimulation neutrophils did not produce relevant amounts of oxygen radicals as indicated by a MFI of 33 AU (97.5th percentile). For stimulation of granulocytes by opsonized E. coli, a reference interval of 138–964 AU (2.5th to 97.5th percentiles) was calculated (Fig. 1A, middle panel). There were no significant differences between age groups. A slightly different pattern was found after stimulation with PMA (Fig. 1A, right panel). Overall, the values were higher than those for stimulation with E. coli.

To test the diagnostic efficiency of the test system for oxidative burst, a group of seven patients with known CGD and six heterozygous carriers of CGD was tested. As depicted in Fig. 1A, in all CGD patients the oxygen burst activity, expressed as fluorescence intensity after stimulation with E. coli or PMA, was below the 1st percentile of the reference collective. Heterozygous individuals presented with values between the 1st and 50th percentiles of the reference interval. However, carriers can be easily identified by their unique pattern of fluorescence activity. Carriers possessed two distinct subsets of neutrophils; one subset revealed normal function, whereas the other revealed the defect in oxygen radical formation. Fig. 1B illustrates this phenomenon in a family with affected and unaffected siblings. Whereas the patient revealed the expected deficiency in oxygen radical formation, the father showed normal values for stimulation with both E. coli and PMA. The mother and sister as carriers revealed the typical “split population”.

In this study, we aimed to establish age-dependent reference values for fluorometric assays of granulocyte functions as tested by flow cytometry. Although there was high interindividual variation in all age groups, significant differences between age groups were not detected. Therefore, reference values can be provided independent of age. The diagnostic efficiency was then tested in patients and carriers of CGD, and the data clearly indicated that the reference values allowed for correct identification of this disease in all patients. Reference values have also been presented by other authors, but they differ from our data (15, 23, 24, 28). There are several reasons that preanalytical and analytical issues may account for such differences. Important aspects include the equipment and reagents used for lysing of red blood cells (29). The above-mentioned factors have an effect on the test result. However, in our study all of these aspects have been taken into consideration and allowed us to define reference values that are at least useful to identify patients with CGD, the most common hereditary leukocyte defect. In daily routine, it is important to include internal controls, which may consist of a sample from a healthy control donor.

For testing of granulocyte functions, flow cytometric tests have advantages compared with conventional assay systems: they allow exact quantification of functions, and results are obtainable within a short period of time, usually within the same day.
References


C-Reactive protein (CRP) is a marker of acute phase response to inflammation that increases rapidly within hours of disease onset. CRP measurements have been used for many years in the management of a variety of clinical situations, such as bacterial infections, ischemic necrosis of tissue, and active inflammatory conditions (1).

Recent studies of CRP concentrations within the conventional reference interval have suggested new clinical applications. CRP is a prognostic risk factor for coronary heart disease in both patients with angina (stable or not) and in apparently healthy subjects (2), and distinguishes rapidly destructive vs slowly progressive osteoarthritis (3).

In neonates, CRP >1–2 mg/L could be associated with serious disease, usually bacterial infection (4). In most of these studies, the authors have proposed a cutoff point for CRP of 2–3 mg/L. Recent reports show median values in healthy controls of 0.58–1.72 mg/L, and 95 percent confidence ranges of ~0.1–6.0 mg/L (5,6). Nevertheless, these results can be influenced by the sensitivity of the method used for CRP quantification.

Among the many commercially available assays for CRP, the most common use fluid-phase or particle-enhanced turbidimetric or nephelometric procedures (7).

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