Rapid Detection of Intracellular p47phox and p67phox by Flow Cytometry; Useful Screening Tests for Chronic Granulomatous Disease

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Running short title: Flow cytometry for p47phox and p67phox

Abstract

  Chronic granulomatous disease (CGD) is caused by defects of NADPH oxidase. The diagnosis of CGD can be made by analysis of NADPH oxidase activity, however, identification of the CGD subgroups is required before performing mutation analysis. The membrane-bound subunits, gp91phox and p22phox, can be quickly analyzed by flow cytometry, unlike the cytosolic components, p47phox and p67phox. We evaluated the feasibility of flow cytometric detection of p47phox and p67phox with specific monoclonal antibodies in two patients with p47phox deficiency and 7 patients with p67phox deficiency. Consistent with previous observations, p47phox and p67phox were expressed in phagocytes and B cells, but not in T or natural killer cells, from normal controls. In contrast, patients with p47phox and p67phox deficiency showed markedly reduced levels of p47phox and p67phox, respectively. These techniques will be useful to rapidly assess the expression of the cytosolic components, p47phox and p67phox, and represents important secondary screening tests for CGD.

Introduction

Chronic granulomatous disease (CGD) is a primary immunodeficiency disease of phagocytes caused by defects of nicotinamide dinucleotide phosphate (NADPH) oxidase [1, 2]. NADPH oxidase is an enzyme responsible for the production of reactive oxygen species that are needed to kill pathogenic bacteria and fungi. Patients with CGD have increased susceptibility to infections, as well as hyperinflammation and granuloma formation at sites of infection [3]. The NADPH oxidase is a multicomponent system including a membrane-bound flavocytochrome b558, comprised of gp91phox and p22phox, cytosolic components, comprised of p40phox, p47phox, and p67phox, and a small GTP-binding protein comprised of Rac1 or Rac2 [1, 2]. About 70% of CGD cases are caused by mutations in the *CYBB* gene that encodes gp91phox and is located at Xp21.1 [4]. The remaining 30% of cases include four other subgroups of the disease, all of which are inherited in an autosomal recessive manner [1, 2]. Mutations in the *NCF1* gene cause p47phox deficiency, the second most common subtype of CGD, which accounts for 25% of CGD patients. The other cases occur due to mutations in the *NCF2*, *CYBA*, or *NCF4* genes that encode p67phox, p22phox, or p40phox, respectively. To date, p40phox deficiency has only been reported in one individual [5].

A diagnosis of CGD can be achieved by measurement of neutrophil superoxide production via NADPH oxidase. The most recognized test is the nitroblue tetrazolium test, which is based on visual inspection of phagocytes. Because of its high reliability and sensitivity, the dihydrorhodamine (DHR) assay using flow cytometry has largely replaced the nitroblue tetrazolium test in many laboratories [6]. Despite the need for specific

technical skills, the DHR assay is able to distinguish between patients with absent to greatly diminished production of superoxide, which is commonly observed in males with X-linked CGD and patients with residual superoxide production, which is frequently observed in autosomal recessive and variant X-linked CGD [1, 2]. However, further identification of CGD subgroups is necessary before genetic analysis, which is often labor-intensive and time-consuming. The membrane-bound subunits, gp91phox and p22phox, can be analyzed by flow cytometry using monoclonal antibodies (mAbs), such as 7D5 that identifies the extracellular domain of flavocytochrome b558 [7, 8]. In contrast, the cytosolic components, p47phox and p67phox, are usually investigated by immunoblot analysis that requires a large sample of blood and takes longer to perform. We therefore assessed the feasibility of flow cytometric analysis of intracellular p47phox and p67phox expression and demonstrate its usefulness as a secondary screening test for CGD.

Materials and methods

Patients

We studied 9 affected patients with autosomal recessive CGD: 2 with p47phox deficiency and 7 with p67phox deficiency. All patients but p67-4 were born to non-consanguineous Japanese parents. We also evaluated 3 patients with X-linked CGD (gp91phox deficiency) and 10 healthy adult volunteers. Case presentations of patient p47-1 and p47-2 and the sibling cases of p67-1.1 and p67-1.2 have been reported elsewhere [9, 10]. Patients p67-2.1 and p67-2.2 were also sibling cases. Approval for the study was obtained from the Human Research Committee of Kanazawa University Graduate School of Medical Science, and informed consent was obtained in accordance with the Declaration of Helsinki.

Flow cytometry

For the analysis of p47phox and p67phox expression, peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque gradient centrifugation from patients and controls immediately after blood collection or after overnight shipment at ambient temperature. Granulocytes were recovered from the pellet of the gradient after lysis of any erythrocytes. PBMCs were stained for cell-surface antigens before cell membrane permeabilization using the following mAbs: fluorescein isothiocyanate-conjugated anti-CD14 and anti-CD56; phycoerythrin-Cy5-conjugated anti-CD20 and anti-CD3 (all from Becton Dickinson, San Diego, CA). Granulocytes were stained with fluorescein isothiocyanate-conjugated anti-CD16b mAb (Beckman Coulter, Fullerton,

CA). After washing, cells were fixed and permeabilized with Cytofix/Cytoperm Plus kit

(Becton Dickinson) and were incubated with anti-p47phox (clone 1, Becton Dickinson),

anti-p67phox mAb (clone D-6, Santa Cruz Biotechnology Inc, Santa Cruz, CA) or

purified mouse IgG1 (Becton Dickinson) at 4°C for 20 min. Cells were then reacted with

phycoerythrin-conjugated anti-mouse IgG1 (Southern Biotech, Birmingham, AL) at 4°C

for 20 min. Stained cells were analyzed with a FACSCalibur flow cytometer using

CellQuest software (BD Bioscience, Tokyo, Japan) [11]. Because the anti-p47phox and

anti-p67phox mAbs belong to the mouse IgG1 subclass, all antibodies for surface staining

were chosen from mouse IgG2 or IgM to avoid cross-reaction. For analysis of gp91phox

and p22phox, whole blood was stained with 7D5 mAb (MBL, Nagoya, Japan).

Analysis of the production of reactive oxygen species

Peripheral blood was loaded with 1 μM of DHR 123 at 37˚C for 5 min, and

stimulated with 100 ng/mL of phorbol myristate acetate at 37˚C for 30 min. After lysis of

erythrocytes, production of reactive oxygen species of granulocytes was quantified by

measuring intracellular rhodamine using flow cytometry, as previously described [9, 12].

Mutation analysis of NCF1 and NCF2

DNA was extracted from blood samples using standard methods. The *NCF1* and

NCF2 genes were amplified from specific primers as previously described [9].

Sequencing was performed on purified polymerase chain reaction products using the ABI

Prism BigDye Terminator Cycle sequencing kit on an ABI 310 or 3100 automated

sequencer (Applied Biosystems, Foster, CA) [13].

Results

Mutation analysis and ROS production

Table 1 presents the clinical and sequencing data of the patients. Patients p67-2.1 and p67-2.2 were compound heterozygotes bearing Gln260X and Arg395Trp mutations in *NCF2*. The former is a novel nonsense mutation. Two distinct *NCF2* mutations, Tyr394Asp and Asp408fs, were demonstrated in patient p67-3. The effect of the novel missense mutation Tyr394Asp was evaluated using the web-based analysis tool, Mutation@A Glance (http://rapid.rcai.riken.jp/mutation/) [14], and was found to be deleterious on the basis of the SIFT program [15]. Patient P67-5 was a compound heterozygote bearing Trp22X and Arg102X mutations. The former is a novel nonsense mutation.

Upon stimulation with phorbol myristate acetate, DHR-loaded neutrophils from normal controls showed significant increases in fluorescence with tight peaks. Patients with gp91phox deficiency exhibited virtually no neutrophil DHR activity. In contrast, patients with p47phox or p67phox deficiency exhibited variable activity ranging from almost none to a moderate increase in the fluorescence after stimulation with characteristic wider peaks compared with those found in gp91phox deficiency.

Intracellular expression of p47phox and p67phox

Consistent with previous observations [2], p47phox and p67phox were expressed intracellularly in phagocytes and B cells, but not in T or natural killer (NK) cells, from normal controls (Fig. 1). This pattern of lineage-specific expression was the same as that

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observed for gp91phox expression. However, we observed a marked difference in p47phox or p67phox expression among neutrophils, monocytes and B cells, which was in contrast to the expression of gp91phox. The p47phox levels in neutrophils were relatively low compared with monocytes and B cells, whereas the p67phox levels in B cells were considerably lower compared with neutrophils and monocytes. Because neutrophils exhibited decreased viability and tended to aggregate after overnight shipment and cell isolation, we decided to use PBMCs for intracellular staining of p47phox and p67phox in clinical samples in order to avoid non-specific staining that might lead to misdiagnosis.

The patients with p47phox deficiency exhibited markedly reduced levels of p47phox expression in both monocytes and B cells (Fig. 2). Conversely, the patients' monocytes and B cells expressed p67phox at levels that were comparable to normal controls. On the other hand, the patients with p67phox deficiency exhibited considerably reduced levels of p67phox expression in monocytes and B cells (Fig. 2). Although the expression of p47phox in the monocytes appeared to be marginally decreased, p47phox expression in the patients' B cells was comparable to normal controls. Expression of the membrane-bound component, gp91phox, determined by 7D5 mAb, was normal in all patients with p47phox and p67phox deficiency.

Expression of p47phox or p67phox in carrier parents

To evaluate the ability of our flow cytometric analysis technique to detect carriers, we examined p47phox expression in monocytes from the parents of p47-1 and p67phox expression in monocytes from the mother of p67-2.1 and p67-2.2 and the parents of p67-3, all of whom were heterozygous carriers (Fig. 3). The pattern of DHR and gp91phox

expression from the mother of gp91-1 showed a bimodal distribution consistent with her

obligate X-linked CGD carrier status. In contrast, and consistent with previous reports [2],

the NADPH oxidase activities of neutrophils from carriers of the p47phox and p67phox

deficiency were normal. The expression of p47phox or p67phox in their monocytes

appeared to be decreased slightly, compared with normal controls (Fig. 3).

Discussion

In the present study, we have developed practical flow cytometric assays for p47phox and p67phox. Although analysis of the expression of the individual subunits by flow cytometry has been described previously [16], our approach differs from it on two counts. First, we used commercially available mAbs that are specific for p47phox and p67phox. This potential advantage may allow the assay to be widely used in many laboratories. Second, we analyzed the intracellular expression of p47phox and p67phox in monocytes and B cells, as well as in neutrophils. B cells contain all of the components of the phagocyte NADPH oxidase and generate superoxide in response to a variety of stimuli; albeit at a lower level than neutrophils [17]. Our lineage-specific analysis may provide more accurate and reliable data. Previous studies using Western blot analysis have demonstrated that p47phox and p67phox expression in B cells were reduced by about 6 and 70%, respectively, compared with neutrophils [17]. The reasons neutrophils from normal individuals exhibited lower levels of p47phox compared with monocytes and B cells in our assay are presently unclear. Further studies using other anti-p47phox antibodies that recognize different epitopes will be necessary to resolve this issue.

The NADPH subunits including p47phox, p67phox and p40phox exist as a tight complex in the cytoplasm in the resting state [18]. However, loss of one of the p47phox or p67phox proteins did not generally affect the expression of the other, unlike the membrane-bound components, gp91phox and p22phox. Because gp91phox and p22phox act to stabilize each other, and the absence of either protein generally leads to down-regulation of the other [19, 20]. In fact, the 7D5 mAb that binds exclusively to an

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extracellular domain of gp91phox has been proven not to stain the cell surface on neutrophils deficient in p22phox, thus indicating its screening capability for both gp91phox and p22phox deficiency [8]. Therefore, the combined flow cytometric analysis of the surface staining by 7D5 mAb and our assay for intracellular p47phox and p67phox would be a valuable tool for detecting subgroups of CGD.

Mutations in the gp91phox gene cause X-linked CGD and account for approximately 70% of all cases [4]. The remaining 30% are due to mutations in the other subunit and are inherited in an autosomal recessive manner [1]. In Japan, p47phox-deficient CGD has been documented in relatively few patients, unlike Western countries [21]. Because of the infrequency of such diseases, our study was limited to include a small number of patients, in particular, those with p47phox deficiency. However, the vast majority of patients with p47phox deficiency carry the homozygous 2-bp deletion in the *NCF1* gene, which was also detected in our patients. This mutation results from recombination events between the wild-type gene and the pseudogene [22, 23]. Patients with this mutation have been reported to exhibit undetectable levels of p47phox protein expression [23], which was consistent with our results. On the other hand, although a variety of mutations in the *NCF2* gene have been demonstrated in patients with p67phox deficiency, the p67phox protein appears to be undetectable by Western blot analysis in most cases [23]. Interestingly, the p67phox protein was absent in patients p67-1.1, p67-1.2, p67-2.1, p67-2.2, p67-4 and p67-5, whereas residual p67phox expression was detected in patient p67-3 who exhibited the significant remaining NADPH oxidase activity. It should be noted that there are mutations in the *NCF1* or *NCF2* genes that leave the protein expression intact but destroy the enzymatic activity [23]. In such cases, albeit

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rare, our flow cytometric analysis of protein expression as well as Western blot analysis cannot contribute to identification of the CGD subgroups, and mutation analysis is required. Further investigations will be required to assess the sensitivity and limitations of the assay and its utility in determining the phenotype-genotype correlation.

Most female carriers of X-linked CGD can be identified by flow cytometry. A mosaic pattern of oxidase-positive and -negative neutrophils from the basis of the DHR assay or analysis of gp91phox expression is observed due to random X-chromosome inactivation. In contrast, the detection of carriers of autosomal recessive CGD is usually performed by genetic analysis. It is well known that the NADPH oxidase activity is normal in obligate carriers [2]. The expression of p47phox and p67phox proteins, assessed by Western blot analysis, has also been reported to likely be normal in carriers of p47phox and p67phox deficiency, respectively [2]. Our flow cytometric analysis demonstrated slightly decreased expression of p47phox and p67phox in monocytes from obligate carriers of p47phox and p67phox deficiency, respectively, compared with normal controls. However, these subtle differences of protein expression do not allow the detection of carriers in the clinical settings. Therefore, genetic testing remains appropriate for identification of carriers of autosomal recessive CGD.

In summary, our study demonstrated rapid and sensitive detection of intracellular p47phox and p67phox by flow cytometry, which is useful as a secondary screening test for CGD. This technique would be also valuable in the evaluation of chimerism in patients treated with stem cell transplantation or gene therapy.

Acknowledgments

Conflict of Interest

The authors declare that they have no conflict of interest.

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Table 1 Clinical and sequencing data

Subjects	Age[a]	Clinical features	Gene	Nucleotide mutation	Effect
p47-1	31 y	Skin infections, otitis, colitis	*NCF1*	73_74delGT[d]	Tyr26fs
p47-2	13y	BCGitis Williams syndrome	*NCF1*	73_74delGT 7q11.23 deletion	Tyr26fs
p67-1.1[b]	8 y	Perianal abscess, lymphadenitis	*NCF2*	1223delA[d]	Asp408fs
p67-1.2[b]	2 y	Perianal abscess	*NCF2*	1223delA[d]	Asp408fs
p67-2.1[c]	1 mo	Aspergillus pneumonia	*NCF2*	778C>T 1183C>T	Gln260X Arg395Trp
p67-2.2[c]	3 y	Sepsis	*NCF2*	778C>T 1183C>T	Gln260X Arg395Trp
p67-3	6 mo	Aspergillus pneumonia	*NCF2*	1180T>G 1223delA	Tyr394Asp Asp408fs
p67-4	10 m	BCGitis	*NCF2*	304C>T[d]	Arg102X
p67-5	5 y	BCGitis lymphadenitis	*NCF2*	66G>A 304C>T	Trp22X Arg102X

[a] Data at the time of sample collection.
[b,c] Sibling cases.
[d] Homozygous mutation.

Figure Legends

Figure 1   Expression of p47phox, p67phox and gp91phox in blood leukocyte populations from normal individuals.

Shown are the results of intracellular expression of p47phox (top) and p67phox (middle), and surface expression of gp91phox analyzed by 7D5 mAb (bottom) in CD16b^{+} neutrophils, CD14^{+} monocytes, CD3^{+} T, CD20^{+} B, and CD56^{+} NK cells from a healthy control. Thin lines indicate control Ab; thick lines represent mAbs specific for the NADPH subunits.

Figure 2   Analysis of DHR and expression of p47phox, p67phox and gp91phox in CGD patients.

In the DHR assay, granulocytes were analyzed using DHR 123 as a fluorescent probe before (thin lines) and after (thick lines) stimulation with phorbol myristate acetate. Expression of gp91phox in granulocytes, as assessed using 7D5, and expression of p47phox and p67phox in CD14^{+} monocytes and CD20^{+} B cells are shown.

Figure 3   DHR assay and expression of p47phox or p67phox in carrier parents.

Rhodamine expression in unstimulated and phorbol myristate acetate-stimulated granulocytes obtained from obligate carriers. Expression of gp91phox analyzed using 7D5 in granulocytes and the expression of p47phox or p67phox in CD14^{+} monocytes are shown.
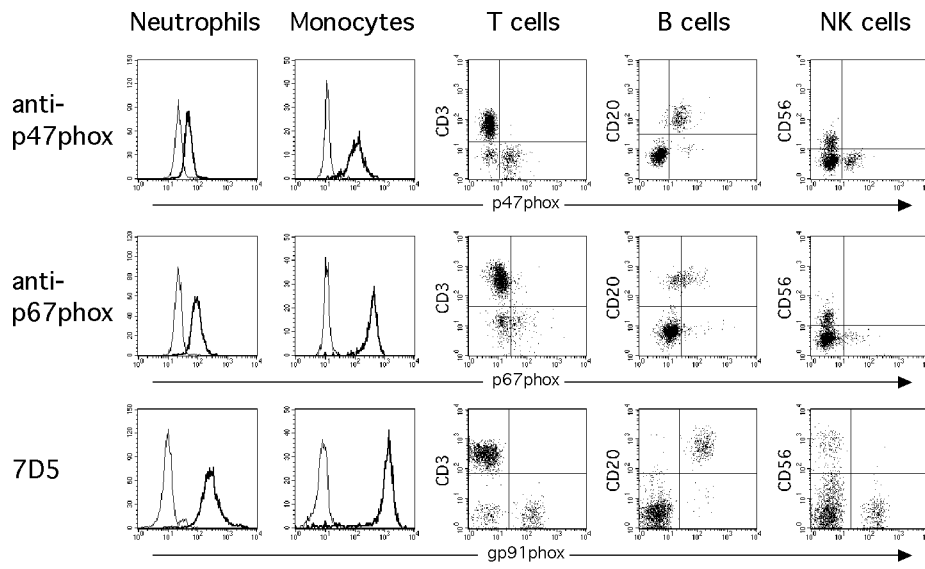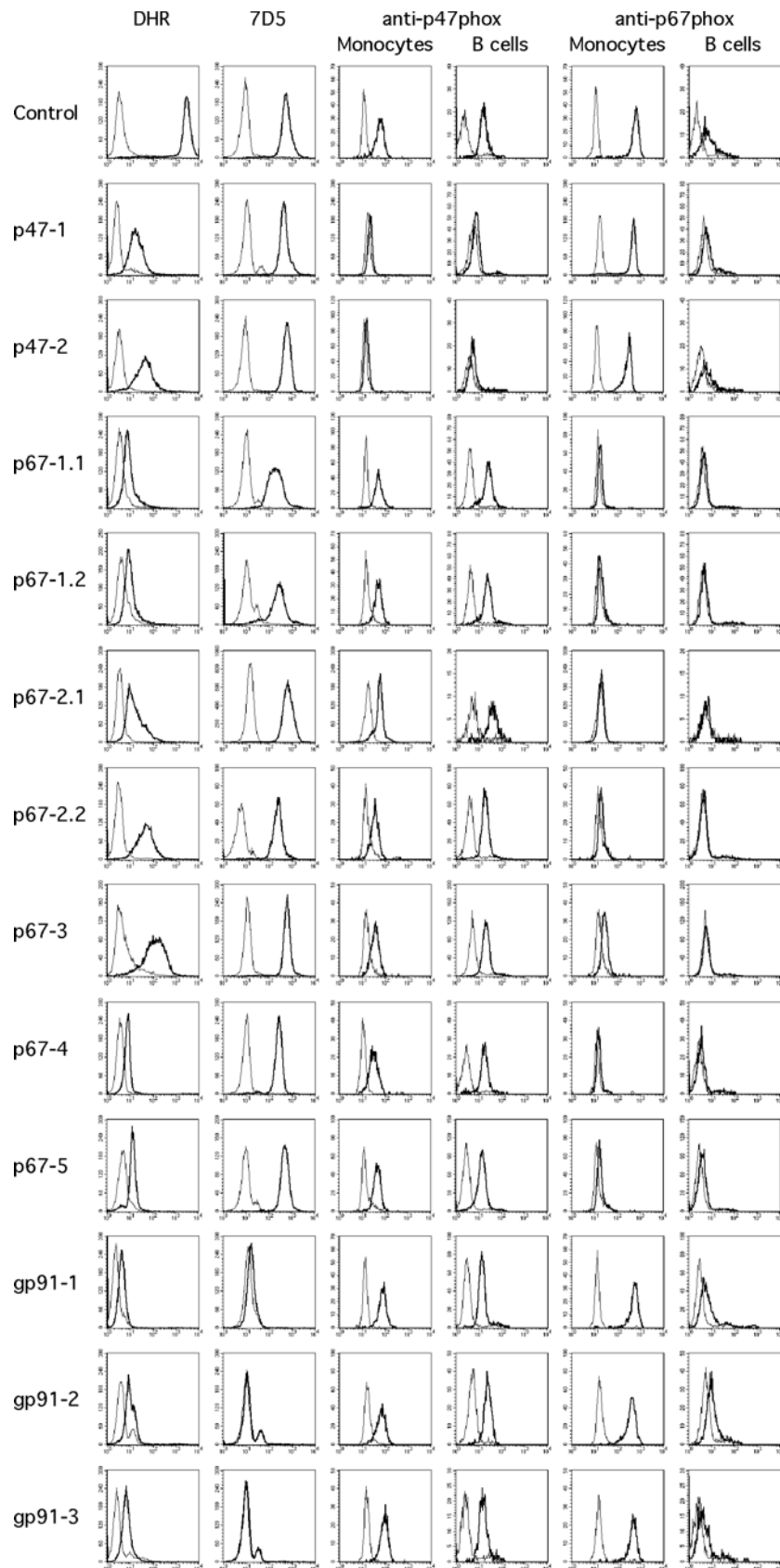
Figure 1

Figure 2

Figure 3