Comparison of two methods for the diagnosis of chronic granulomatous disease - neutrophil oxidative burst measured by the nitroblue tetrazolium slide test versus the dihydrorhodamine 123 flow cytometric assay

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Abstract
Objective: The neutrophil respiratory burst is crucial for the ability of the host to kill ingested microorganisms. The detection of this activity is an essential part of the laboratory investigation of patients with suspected chronic granulomatous disease (CGD). In this study the traditional qualitative nitroblue tetrazolium (NBT) slide test was compared with a quantitative whole blood dihydrorhodamine 123 (DHR 123) assay using flow cytometry.

Methods: A total of 20 samples submitted to Labplus at Auckland Hospital were screened for CGD by both the NBT and the DHR 123 assays.

Results: While the NBT method was able to demonstrate reduced NADPH oxidase in CGD patients, it is highly subjective and cannot identify carrier states of X-linked-type CGD. In contrast, the quantitative whole blood dihydrorhodamine 123 (DHR 123) flow cytometric method evaluated in this study was more rapid, allowing the proportion of affected cells to be determined, and was able to identify the carrier state of X-linked CGD.

Conclusions: The DHR 123 assay proved to be a more sensitive and more convenient method for the measurement of neutrophil oxidative burst activity and showed a number of advantages over the qualitative NBT slide test for the diagnosis of CGD.

Key words: chronic granulomatous disease, nitroblue tetrazolium slide test, dihydrorhodamine 123, flow cytometry, neutrophil respiratory burst

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Introduction
Polymorphonuclear neutrophils (PMNs) destroy microorganisms by producing reactive oxygen species (ROS) during the respiratory burst, a normal host defence mechanism that controls infections. The process is controlled by the multicomponent enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, with the production of reactive oxygen species with the formation of superoxide anion ($O_2^-$) (1). Intracellularly the $O_2^-$ is converted by superoxide dismutase (SOD) to oxygen and hydrogen peroxide ($H_2O_2$), with the latter converted by myeloperoxidase (MPO) to hypochlorous acid and chloramines inside the phagolysosome. These toxic products of the oxidative burst are a part of the powerful $O_2^-$-dependent antimicrobial system of PMNs (1, 2).

The clinical implication for impaired function of cellular NADPH oxidase, can be seen in patients with chronic granulomatous disease (CGD) (1).

In this genetic disorder, neutrophils and monocytes recognise and ingest, but are not able to kill certain microorganisms, because phagocytes fail to generate $O_2^-$. This rare condition is associated with recurrent bacterial and fungal infections and as a result patients develop serious, often life-threatening, and unusually persistent infections (1). CGD patients most commonly present with upper respiratory tract infections, pneumonia and abscesses of the skin, tissue and other organs, arthritis, osteomyelitis, cellulitis, and impetigo. Granulomas form due to the accumulation of white blood cells in the infected areas, even in cases when antibiotics have eliminated the infection-causing organism.

Patients with CGD are especially susceptible to infections caused by catalase positive organisms such as S. aureus, E. coli and Aspergillus species as catalase produced by these organisms destroys any endogenously generated $H_2O_2$. In contrast, the neutrophils of CGD patients are usually able to destroy catalase negative organisms such as pneumococci, streptococci and others because of the $H_2O_2$ formed against the organism within the phagolysosome. CGD can be inherited as either an X-linked or autosomal recessive disorder (Figure 1) with the genetic lesion caused by deletion, frame-shift, nonsense or missense mutations in the gene or gene promoters (3).

Most clinical cases of CGD present in childhood and early diagnosis is crucial as it may require patients to be placed on antibiotic prophylaxis in an attempt to protect against infections before they occur. Since NADPH is a cofactor for the production of superoxide dismutase, other conditions which affect the formation of NADPH can also lead to impaired neutrophil function. This can sometimes also be seen in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiencies (4). The detection of the defect in the neutrophil respiratory burst activity is an important part of the investigation of patients with CGD (5). The NBT slide test has been the traditional laboratory test for the diagnosis of CGD and has been offered for many years at Labplus. In the NBT test, the blue dye formazan is produced by the reduction of the NBT dye, a reaction dependent upon neutrophil NADPH oxidase (2). The NBT test is dependent upon the microscopic evaluation of stained neutrophils for the presence of intracellular formazan. The NBT method was replaced recently by the dihydrorhodamine 123 (DHR) assay, a flow cytometry method that uses an anticoagulated whole blood specimen. DHR 123 is a fluorogenic substrate for the respiratory burst and it is oxidised to rhodamine by cellular hydrogen peroxide. Intracellular rhodamine formation is fluorescent and is detected and measured using a flow cytometer. In this study, the NBT slide test and the whole blood DHR assay were compared.
Materials and methods
All patient and control samples for the NBT assay were collected by laboratory staff by finger-prick. Blood was collected into plain capillary tubes at the patient’s bedside. Samples used for the DHR assay had been previously collected by venesection into EDTA anticoagulant.

Nitroblue tetrazolium test (NBT)
The NBT test involves the microscopic assessment of the ability of patient neutrophils to reduce the yellow soluble redox dye NBT to form blue-black formazan, an insoluble material that precipitates intracellularly. The degree of production of formazan provides a qualitative means of identifying the presence of neutrophil superoxide anion $\text{O}_2^-$ following stimulation.

Complement was used as opsonin for the NBT test and was provided using fresh pooled human serum. A NBT solution of 0.28% in normal saline and a zymosan (yeast) solution of 1% in normal saline was used. The glass adherence reagent consisted of 0.5mL of fresh human serum, 0.6mL NBT solution and 0.3mL normal saline. The zymosan reagent was prepared using 0.5mL of fresh human serum, 0.6mL of NBT solution and 0.3mL zymosan solution.

Two clean glass slides were labelled “glass” and “zymosan” for both the “normal” and “patient” samples and two circles drawn on each slide and whole blood from both the patient and a normal control were applied to the slides. Slides were placed horizontally in a petri dish with moistened filter paper and incubated at 37˚C for 25 minutes. Any blood clots were gently washed off the slides with normal saline, care being taken to avoid scraping of the cell layer that could disturb the adherent neutrophil monolayer. To the areas of the slides labelled “glass”, glass adherence reagent was added. To the “zymosan” area of the slides a few drops of zymosan reagent was added. Slides were again placed in a humidity incubator at 37˚C for 20 minutes and rinsed with normal saline and allowed to dry.

Slides were fixed in methanol for 2 minutes and stained with 0.1% neutral red for 10 minutes. Excess stain was rinsed off with water and slides left to dry.

Adequacy of the counter stain of the adherent neutrophils was checked under the microscope prior to being lacquered. Slides were examined microscopically and the number of adherent PMN cells showing NBT reduction among 100 consecutive PMNs was recorded as a percentage.

Dihydrorhodamine flow cytometry assay
Neutrophils are first stimulated using phorbol 12-myristate 13-acetate (PMA). This initiates intracellular $\text{O}_2^-$ production and dihydorhodamine 123 (DHR) is oxidised by the neutrophil reactive oxygen species (ROS) to rhodamine 123 (RHO) releasing a fluorescent green signal which can be measured by flow cytometry. Since RHO also binds to cellular membranes, the fluorescent signal is exclusively contained within the cells (1). Normal bloods produce a strong fluorescence whereas patients with an abnormality in $\text{O}_2^-$ production produce a weak level or no fluorescence at all. Carriers of the X-linked CGD gene show the presence of two peaks that represent the presence of a single defective CGD gene and a functionally normal gene. In these cases the results show a strong positive peak and a separate weaker/negative peak. DHR is reportedly the most effective flow cytometric probe for assessing the oxidative burst in human granulocytes and is specifically responsive to H$_2$O$_2$ accumulation (1).

In the method stock DHR 123 (Sigma) was dissolved in phosphate buffered saline (PBS) to give a working solution of 30µg/mL. CD45 antibody (Becton Dickinson) was conjugated with peridinin-chlorophyll-protein complex (CD45 PerCP). Phorbol 12-myristate 13-acetate (PMA) (Sigma) was dissolved in dimethyl sulphoxide (DMSO) (Sigma) to form a 1.6mM solution. Stock FACS lyse solution (Becton Dickinson) was diluted 1 in 10 in deionised water to give a working solution.
Residual EDTA anticoagulated whole blood was used and all specimens were tested within 24 hours from the time of collection. Samples requiring testing the next day were stored at 2-8°C overnight. Phorbol 12-myristate 13-acetate (PMA) was used to activate neutrophils in the assay. Three fluorescence activated cell sorting (FACS) tubes were labelled as “blank”, “resting” and “stimulated” for patient and control samples. 10µL of CD45 antibody conjugated with peridinin-chlorophyll-protein complex (CD45-PerCP) was added to each tube. 50µL of mixed patient and control bloods were added to each of the three patient and control FACS tubes. 12.5µL of PBS was added to the “blank” and “resting” tubes and 12.5µL of working PMA solution added to FACS tubes labelled “stimulated” and incubated at 37°C for 15 min. 12.5µL of PBS was then added to tubes labelled “blank” and 12.5µL of working DHR solution to the “resting” and “stimulated” sample tubes. After 5 minutes at 37°C, 1.0 mL of FACS lyse solution was added to all tubes. Tubes were left in the dark at room temperature for 15 min and the cells washed twice with 2ml of PBS. Samples were immediately analysed using the Becton Dickinson FACSCANTO II (San Jose, CA, USA) flow cytometer.

Results

NBT test:
For the adherence slides labelled with “glass”, the NBT reduction was seen as a blue to purple/black formazan staining surrounding the polymorphs (Figure 2A). For the “zymosan” stimulated slides, NBT reduction was seen as a blue to purple/black precipitate in the polymorphs that had ingested the yeast and reduced the NBT (Figure 2B).

The NBT test took an average of 3.5 hours to perform and sixteen tests showed results within the normal range values (>30% glass adherent cells and >90% zymosan stimulated cells). Four test results fell outside of the normal range values for the dye reduction in the “glass” adherent and “zymosan” stimulated neutrophil groups.

These four samples showed either no reduction of the NBT dye or the percentage of cells that reduced the dye was lower than the normal range, producing glass adherent neutrophils with formazan deposits in fewer than 30% (Figure 3A) and the lack of formazan production (Figure 3B).

Figures 2A & 2B. Nitroblue tetrazolium blue formazan staining of neutrophils (A) and the blue to purple black precipitate in the zymosan stimulated neutrophils (B) in a normal patient.

Figures 3A & 3B. Reduced or no formazan staining of neutrophils (A) and no blue/black deposit staining in zymosan stimulated neutrophils(B) in patients with CGD.
DHR assay
Data was collected from the reagent “blanks”, “resting” and “stimulated” FACS tubes with the flow cytometer recording 10,000 neutrophil events. At analysis the scattergram of CD45 expression vs. side scatter was first displayed and the neutrophil population was identified by its typical location and selected by gating (Fig. 4). Gating of CD45 positive cells in this region selects for neutrophils and excludes debris and other cells present in the samples. A histogram of rhodamine fluorescence was obtained for the cells in the gated region of the flow scatterplot (Figures 5-7) for each of the samples.

At analysis the neutrophil populations of both patient and control samples was readily identifiable on the CD45 scattergram (Figure 4). The “blank” samples showed the lowest rhodamine fluorescence (Figure 5A, 6A, and 7A), with “resting” neutrophils showing a slight increase in fluorescence uptake (Figure 5B, 6B, 7B). PMA stimulated neutrophils produced a large increase in rhodamine fluorescence in samples from patients with normal NADPH oxidase activity (Figure 5C). The same level of fluorescence was not seen in samples from patients with X-linked CGD (Figure 6C). Two separate fluorescence patterns were observed in two samples (Figure 7C) from patients known to be X-linked CGD carriers.

![Figure 4. A flow cytometry dot plot of CD45 vs. side scatter of a whole blood specimen. White cells are differentiated by their characteristic CD45 expression - intensity staining on the x axis, and granularity - side scatter on the y axis. In this "resting" sample, the neutrophil population has been gated.](image)

![Figure 5. Flow cytometry histograms showing DHR rhodamine fluorescence for normal neutrophils. (A) “blank”, (B) “resting” neutrophils, and (C) “stimulated” neutrophils.](image)

![Figure 6. DHR pattern in X-linked CGD. (A) “blank”, (B) “resting” and (C) “stimulated” neutrophils.](image)
Figure 7. DHR pattern for CGD carrier state. (A) "blank", (B) "resting" and (C) "stimulated" neutrophils.

Table 1. Results for NBT slide stain and DHR 123 flow cytometric assays.

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Discussion

CGD is a rare inherited disorder of phagocytic cells that can be attributed to a variety of genetic mechanisms. The main characteristic is a defect in the enzymes that produce superoxide and other free radicals, resulting in a failure of phagocytes to destroy engulfed microorganisms. Patients with CGD demonstrate vulnerability to infections caused by catalase-positive organisms that are able to break down neutrophil and monocyte-derived hydrogen peroxide. The lack of the formation of the superoxide free radical in CGD reduces the ability of the host’s immune system to fight off infections. Most cases of CGD are inherited X-linked disorders, although examples of autosomal recessive CGD are also seen. The genetic lesions that cause CGD are variable and may include deletion, frame-shift, nonsense or missense mutations. Female carriers of the X-linked type of CGD exhibit unequal representation of both normal and mutated phagocytes as a result of lyonisation and may be either asymptomatic or have a mild form of CGD.

The activity of neutrophils in the NBT test depends upon the ability of the cells to both adhere to glass and to produce oxidative radicals. The reduction of NBT relies upon cellular NADPH oxidase and therefore the test enables an evaluation of the critical early stages of the respiratory burst (2). In the NBT “glass” slides, the adherence of the neutrophils to the glass surface in the presence of opsonin causes some neutrophils to take up and reduce the NBT. These slides allowed an evaluation of the proportion of neutrophils able to reduce NBT and produce blue formazan staining, in unstimulated cells. To assess the effect of stimulation on the oxidative burst capability of neutrophils, zymosan was used to activate neutrophils in the “stimulated” slides. Zymosan is derived from the cell wall of yeast, and in the presence of complement uptake by phagocytic cells is enhanced. Neutrophil phagocytosis of the zymosan causes activation of NADPH oxidase demonstrated by the reduction of NBT to intracellular formazan.

The results of the NBT test showed sixteen patients with normal results; however, the presence of a carrier state could not be excluded by this method on these samples. Four patients showed results outside of the normal range for the neutrophil reduction of NBT dye, by demonstrating less than 30% of the glass adherent cells with formazan staining and/or less than 90% zymosan stimulated cells showing formazan deposits. For these samples results were consistent with patterns suggestive of either a CGD carrier or disease state in the patients. Clinical care for laboratory test results is important and repeat testing to confirm initial findings is standard laboratory practise in such cases.

In contrast to the time consuming process of sample collection at the patient’s bedside and the time taken to perform the NBT slide test, the DHR 123 assay was a quicker method to perform. The assessment of the respiratory burst by this method relies upon change in the fluorescence of resting neutrophils following stimulation. In stimulated cells increased fluorescence is indicative of the production of H₂O₂ in peripheral blood neutrophils (6). The DHR assay utilises PMA as a strong non-physiological stimulant to activate membrane-associated NADPH oxidase by enhancing protein kinase C, which in turn stimulates superoxide and hydrogen peroxide production (1,6). PMA causes a sustained respiratory burst with high levels of fluorescence seen in samples from normal individuals (Fig. 5D). The DHR assay utilises PMA as a strong non-physiological stimulant to activate membrane-associated NADPH oxidase by enhancing protein kinase C, which in turn stimulates superoxide and hydrogen peroxide production (1,6). PMA causes a sustained respiratory burst with high levels of fluorescence seen in samples from normal individuals (Fig. 5C). The DHR assay utilises PMA as a strong non-physiological stimulant to activate membrane-associated NADPH oxidase by enhancing protein kinase C, which in turn stimulates superoxide and hydrogen peroxide production (1,6). PMA causes a sustained respiratory burst with high levels of fluorescence seen in samples from normal individuals (Fig. 5D). The DHR assay utilises PMA as a strong non-physiological stimulant to activate membrane-associated NADPH oxidase by enhancing protein kinase C, which in turn stimulates superoxide and hydrogen peroxide production (1,6). PMA causes a sustained respiratory burst with high levels of fluorescence seen in samples from normal individuals (Fig. 5D).

CD45 is a protein antigen expressed on all haemopoietic white cells and “gating” the CD45 antigen positive cells in each sample was performed as part of the DHR assay in this study. The CD45 expression together with side scatter properties (complexity of cells) allows for the better selection of peripheral blood neutrophils.

In the DHR 123 assay FACS lyse solution was used to stop the reactions and to lyse red cells while partially fixing the white cells prior to fluorescence measurement. The purpose of the inclusion of a “blank” was to check each sample for background fluorescence, while the “resting” tube was used to set the negative marker. The “stimulated” samples from the sixteen patients which produced strong fluorescence (Figure 5C) following processing would be interpreted as “Normal activity of neutrophils”. Samples from the two patients that showed a lack of response to stimulation with PMA and no change in fluorescence intensity (Figure 6C), would be interpreted as “Results consistent with CGD”. Two other samples showed some response with PMA and the flow histogram showed two fluorescence peaks indicating two populations of neutrophils, one with normal oxidative burst activity and the other either lacking or showing a reduced level of activity (Figure 7C). The interpretative comment provided for these patients would be “Results consistent with carrier status for X-linked CGD”. All samples testing positive in the DHR assay were repeat tested as per the laboratory protocol for the assay.

In contrast to the manual NBT slide method, the DHR assay was faster to perform but it required a different skill set and a flow cytometer. The number of cells evaluated for fluorescence in the flow cytometer was set at 10,000 which greatly exceeded the 100 cells examined in the manual NBT test. The use of EDTA anticoagulated blood samples for the DHR assay has a number of advantages over samples required for the NBT test. The DHR assay eliminates the need to collect bedside patient samples making laboratory testing for CGD at Labplus available not only for hospitalised patients but also for those who are not physically onsite (e.g. outpatient clinics and community collection centres). Due to the specialised nature of this test, most medical laboratories in New Zealand refer samples to specialist testing laboratories. Sending blood samples rather than patients for testing is more convenient for the patient and more cost effective for hospitals. Samples as small as 0.5 ml of blood can be used in the DHR assay making the method ideal for studies of the respiratory burst in children, the most common age group investigated for CGD. Other advantages of the DHR assay over the NBT test include its ability to more clearly identify CGD patients and to establish the carrier state of the X-linked forms of the condition.

Limitations on the successful testing for CGD via the DHR method relate to the relatively short lifespan of PMNs. Samples for testing must arrive in the laboratory on the same day of collection which could have implications for testing from outside of the Auckland area. For these the parallel testing of a “transport control” from a normal patient may help to validate patient results. In addition to this, patients with inflammatory states or current infections are negative indicators for the use of the DHR assay for CGD screening. These conditions can make the DHR results difficult to interpret requiring repeat testing following the cessation of inflammation or infection.

Conclusions

In this study, the qualitative NBT slide test and the quantitative DHR 123 flow cytometric assays were compared for their diagnostic performance for the diagnosis of CGD. Both methods measured the ability of neutrophils to mount a “neutrophil respiratory burst and results of the NBT test and those from the DHR fluorescence test showed good correlation. The results from both methods lead to similar clinical interpretations, the only exception being that the NBT was not able to identify or exclude carrier states of X-linked CGD.
The qualitative NBT slide test is time consuming and labour intensive, requiring technical skills that include the visual inspection of a limited number of peripheral blood neutrophils. Samples used for the NBT test require bedside blood collection, restricting testing to inpatients bringing inconvenience to patients and increased costs. The DHR method is a quantitative measurement of the respiratory burst and is an assay that is quick to perform requiring only small volumes of EDTA anticoagulated blood. The DHR assay provides distinctive histogram patterns that make the interpretation of results less subjective and is able to differentiate between X-linked CGD and X-linked CGD carrier patients. At LabPlus, the DHR assay has become the method of choice for screening and/or diagnosis of patients for CGD.

This study was only able to include a small number of CGD and carrier state patient samples because of the scarcity of cases and the low number of requests for CGD screening tests. The original study was also not able to test the performance of the DHR assay against samples from the less common autosomal recessive form of CGD. Since the original study, three cases of autosomal recessive CGD have been tested using the DHR assay with the results obtained consistent with previously reported NBT results on these patients.

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