Hiroshima Journal of Medical Sciences
Vol.34, No.1, 53~60 , March , 1985
HIJM 34-8

Flow Cytometric Assessment of Neutrophil Oxidative Metabolism in Chronic Granulomatous Disease on Small Quantities of Whole Blood : Heterogeneity in Female Patients

Kazuyuki TAGA[1], Hidetoshi SEKI[1], Toshio MIYAWAKI[1], Tamotsu SATO[1], Noboru TANIGUCHI[1], Kyoichi SHOMIYA[2], Takao HIRAO[3] and Tomofusa USUI[4]

1) Department of Pediatrics, Kanazawa University School of Medicine, Kanazawa 920, Japan
2) Department of Pediatrics, Hamamatsu Medical Center, Hamamatsu 430, Japan
3) Division of Infection and Immunity, Shizuoka Children's Hospital, Shizuoka 420, Japan
4) Department of Pediatrics, Hiroshima University School of Medicine, Hiroshima 734, Japan

(Received December 3, 1984)

Key words: Neutrophil, Oxidative metabolism, Chronic granulomatous disease, Flow Cytometry

ABSTRACT

A rapid and sensitive flow cytometric assay is presented for the quantitative estimation of the oxidative metabolic activity of individual polymorphonuclear leukocytes (PMN) on less than 100 μl of whole blood. This procedure is a simplified version using whole blood of the method of Bass et al (J. Immunol. 130:1910, 1983) that estimated the metabolic burst activity of phorbol myristate acetate (PMA)-stimulated individual PMN as the intracellular generation of a fluorescence product by a flow cytometric assay. With this method, almost all the PMN from normal subjects responded to PMA as a single cell population generating bright intracellular fluorescence. PMN from a boy with chronic granulomatous disease (CGD), could not respond to PMA with any increase of their fluorescence intensity. His mother had two distinct PMN populations one functionally normal and the other defective, indicating a random lyonization in the carrier mother and the X-linked recessive mode of inheritance. In two female patients with CGD from unrelated families, their PMN responded to PMA, as a whole, with a minimal increase in the fluorescence intensity, but the metabolic defects in their PMN were not so complete as seen in a classical X-linked CGD boy. But, PMN from two female sibling patients from the other family responded to PMA as a single uniform cell population with a weak but definite fluorescence intensity. However, the genetic background of these female patients with CGD remains unclear, since PMN dysfunction could not be identified in their mothers with this method.

Phagocytosis by polymorphonuclear leukocytes (PMN) elicits a burst of oxidative metabolism that is intimately associated with the bactericidal activity of the cells[1]. PMN from patients with chronic granulomatous disease (CGD), which can not kill most of bacteria and fungi, do not undergo this "respiratory burst"[14,16,23]. The measurement of the respiratory burst activity of PMN by the methods, such as the quantitative nitroblue tetrazolium (NBT) test, the superoxide measurement by cytochrome C reduction assay, and the luminol-dependent chemiluminescence assay, is generally considered a requisite for the definite diagnosis of CGD[2,3,10,15,20,22]. It has been reported that the phenotypic expression of PMN in heterozygotes for the classical X-linked recessive form of CGD will range, by chance, from normal to defective levels[24]. Thus, the precise evaluation of the oxidative metabolic activity in individual PMN by

single cell analysis is mandatory not only for the diagnosis of CGD but for the detection of various carrier states for CGD. The histochemical PMA-stimulated NBT slide test[25] can afford the informations on individual cells, but the manual microscopic counting is time-consuming and might suffer from many sources of bias. Recent advance in flow cytometry offers a rapid and reliable technique for evaluating the nature in large numbers of individual cells.

In this work, the oxidative burst activity of individual PMN on stimulation with PMA was analyzed by flow cytometry on 100 μl or less of whole blood using a simplified version of the method of Bass et al[4]. The complete lack of oxidative burst activity in a boy with CGD and the presence of two PMN populations, one functionally normal and the other defective, in his mother indicate an X-linked recessive inheritance in this pedigree. PMN from two female patients with CGD from unrelated families responded to PMA with a minimal oxidative burst activity, but their PMN defect was not so complete as seen in a classical X-linked CGD boy. PMN from the other two female sibling patients in the other family responded to PMA as a single uniform cell population with a partial, but definite, oxidative burst activity of about one-fourth of the controls. However, PMN dysfunction could not be demonstrated in any of the mothers of these female patients.

MATERIALS AND METHODS

Patients and specimens. After informed consent was obtained from the parents, heparinized venous blood was drawn from patients with CGD and their mothers. Four female patients with CGD (Case 1, 14 years; Case 2a, 13 years; Case 2b, 11 years; and Case 3, 6 years) from three unrelated families and a 7-year-old CGD boy in another family were evaluated. Cases 2a and 2b were siblings. All of them had been suffering from frequent suppurative infections since their early infancy. The clinical severity in Cases 2a and 2b was seemingly a little milder compared to that in other patients with CGD. The diagnosis of CGD was confirmed by the histochemical and quantitative NBT test, the luminol-dependent chemiluminescence assay, the measurement of superoxide generation, and the bacterial killing test. The levels of glucose 6-phosphate dehydrogenase, myeloperoxidase, phagocytosis, and chemotaxis of PMN were all within normal ranges. They have no other members suggestive of CGD or of increased infectious tendency in their relatives. All the mothers of these patients were in good health. Detailed clinical studies of Case 1, Cases 2a and 2b have been published elsewhere[18,28]. Healthy male volunteers, aged from 20 to 30 years, were included as normal controls in this work.

Reagents. PMA (phorbol myristate acetate) were purchased from Sigma Chemical Co. (St. Louis, MO) and DCFH-DA (2',7'-dichlorofluorescin diacetate) was a product of Eastman-Kodak Co. (Rochester, NY). EDTA and gelatin were purchased from Wako Pure Chemical industries, Ltd. (Osaka, Japan). Other reagents were of analytical grade. Falcon plastic tube (No. 2001; Becton, Dickinson, and Co., Oxnard, CA) and PBSg (Ca- and Mg-free Dulbecco's phosphate-buffered saline, pH 7.4, containing 5 mM glucose and 0.1% gelatin) were used throughout study. Immediately before use, stock solutions of PMA (2 mg/ml) and DCFH-DA (5 mM) in ethanol were diluted with PBSg to give an indicated concentration.

DCFH oxidation by PMN and flow cytometry. As this assay was based on the principle that the intracellular nonfluorescent DCFH (2',7'-dichlorofluorescin) is oxidized into fluorescent DCF (2',7'-dichlorofluorescin) largely by H_2O_2 generated in PMA-stimulated PMN factors affecting DCFH oxidation by PMN in whole blood were evaluated by the preliminary experiment on respect to concentrations of DCFH-DA and PMA, and to time-course of generation of intracellular DCF fluorescence. Then, the following "standard" procedure was employed. An aliquot (0.1 ml or less) of heparinized whole blood diluted in 1.9 ml PBSg containing 5 μM DCFH-DA was incubated for 15 min at 37°C in a shaking water bath. After 15 min of preincubation, EDTA was added to give a final concentration of 5 mM. The incubation of the mixture was continued in the presence or absence of 100 ng/ml PMA for a further 25 min. After that, erythrocytes were lyzed with cold 0.87% NH_4Cl and the sedimented cells were resuspended in 2 ml of cold phosphate buffered saline containing 0.1% sodium azide. The granulocyte cluster on the cytogram of CRT display of Ortho Spec-

trum III (Ortho Diagnostic Systems, Inc., Westwood, MA) was gated by four corner tiggering and the intensity of intracellular DCF fluorescence in individual PMN and the corresponding cell counts were estimated. For convenience, the results were expressed as percentage of fluorescence positive cells within total evaluated cells.

RESULTS

Time-course of generation of intracellular fluorescence (Fig. 1) PMN from healthy subjects were stimulated in the presence of 100 ng/ml PMA and 5 μM DCFH-DA, to give rise to a maximal generation of intracellular fluorescence. A plateau effect was observed beyond these concentrations of the reagents. In this experimental condition, the percentage of fluorescece positive cells attained to a plateau after 10 min of stimulation in normal subjects and a mother of an X-linked CGD boy. However, in two female sibling patients with CGD, Case 2a and 2b, the percentage of positive cells increased with incubation time and appeared to reach a peak after 30 min or later. The intracellular fluorescence of normal PMN increased with time and reached a maximum intensity around 20-25 min of stimulation and declined thereafter. Without PMA stimulation, there was no appreciable generation of intracellular fluorescence in PMN even from healthy subjects.

Table 1. Intracellular DCFH Oxidation by Neutrophils from Patients with CGD and Their Mothers in a "Standard" Assay

Donors	% of posotive cells			
	without PMA (Resting)		with PMA	
Controls (n=10)	0.68±0.38(SD)		94.8±2.2(SD)	
Range	(0.2~1.4)		(90.8~98.5)	
Male CGD (7yrs)	0.1		0.1	
Mother		0.1		38.4
Female CGD (n=3)*				
Case 1 (14 yrs)	1.1	2.7		
Mother		1.7		96.1
Case 2a (13 yrs)	1.5		81.2**	
Case 2b (11 yrs)	1.9		78.3**	
Mother		0.7		93.2
Case 3 (6 yrs)	1.9		1.9	
Mother		2.3		97.8

*Processed after several hours of transportation. Case 2a and 2b were female sibling patients.
**Mean fluorescence intensity of positive cells was approximately one-fourth of controls (see Fig. 1 and Fig. 3).

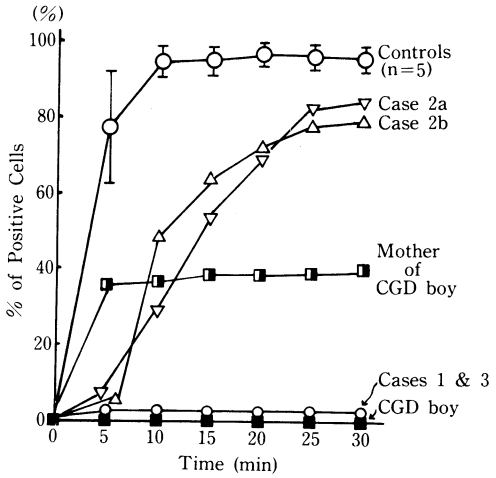

Fig. 1. Time-course of the increasing percentage of fluorescence-positive PMN in normal controls and patients with CGD on stimulation with PMA. After a 15 min-preincubation of specimens in 5 μM DCFH-DA, PMA (100 ng/ml) was added at time zero. Cases 1 and 3 were unrelated female patients with another pedigree. The percentage of fluorescence-positive cells were evaluated by flow cytometry using an Ortho Spectrum III. Although the percentage of positive cells increased with time in Cases 2a and 2b, the intensity of fluorescence of their PMN was much weaker than that of controls.

Flow cytometry of stimulated PMN from patients with CGD (Table 1) Almost all the PMN from healthy subjects, when stimulated with PMA, generated strong intracellular DCF fluorescence and responded as a single cell population (Fig. 2A). But his mother had two distinct PMN populations; one was composed of exclusively defective cells and the other was comparable in the fluorescence intensity with normal controls (Fig. 2C). In two unrelated female patients, Case 1 and 3, from different families, the analytical pattern of stimulated PMN was similar to, but not identical with, that of a male patient with CGD. Their PMN responded to PMA, as a whole, with a minimal increase in fluorescence intensity, but some neutrophils, though

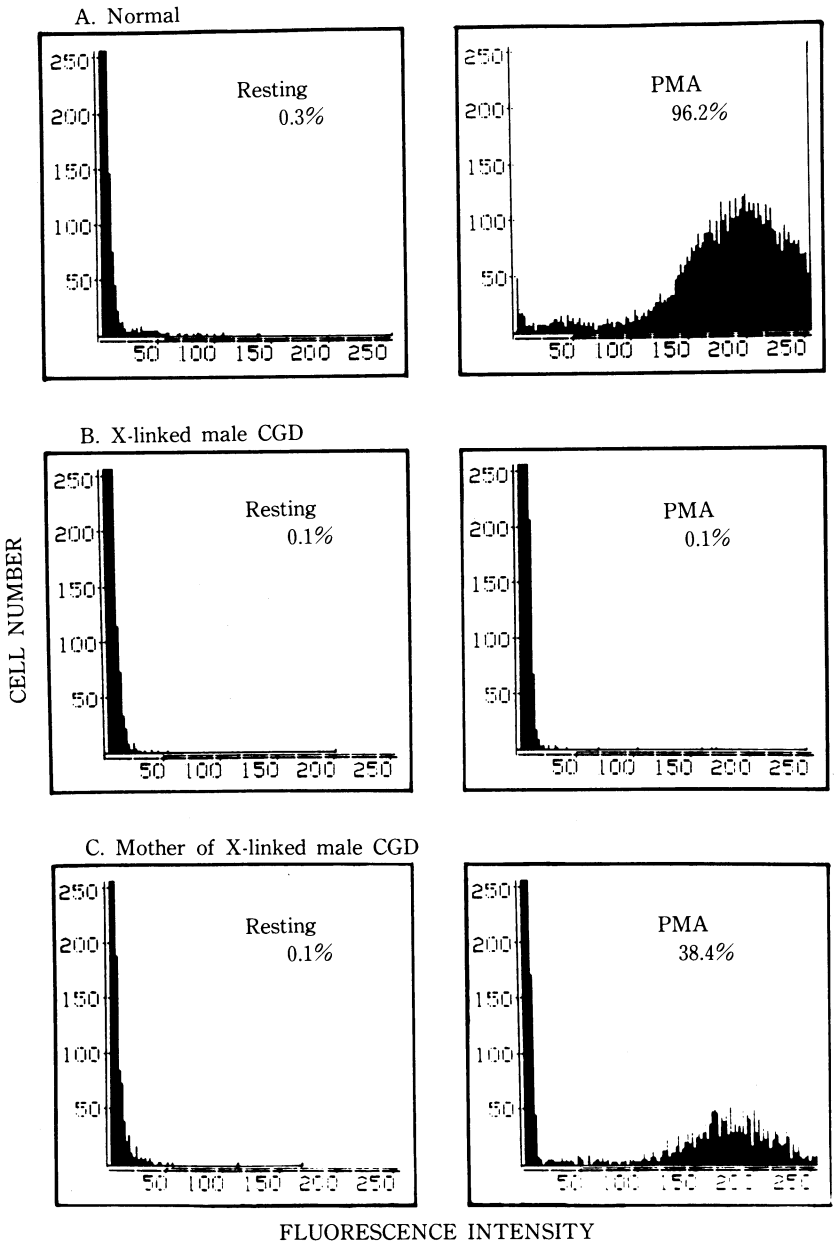

Fig. 2. Fluorescence distribution on flow cytogram of resting (without PMA) and PMA-stimulated PMN from a normal donor (A), a boy with the classical X-linked form of CGD (B), and his asymptomatic heterozygous mother (C) in a "Standard" assay. The histogram represented numbers of cells on the ordinate as a function of fluorescent intensity on the abscissa.

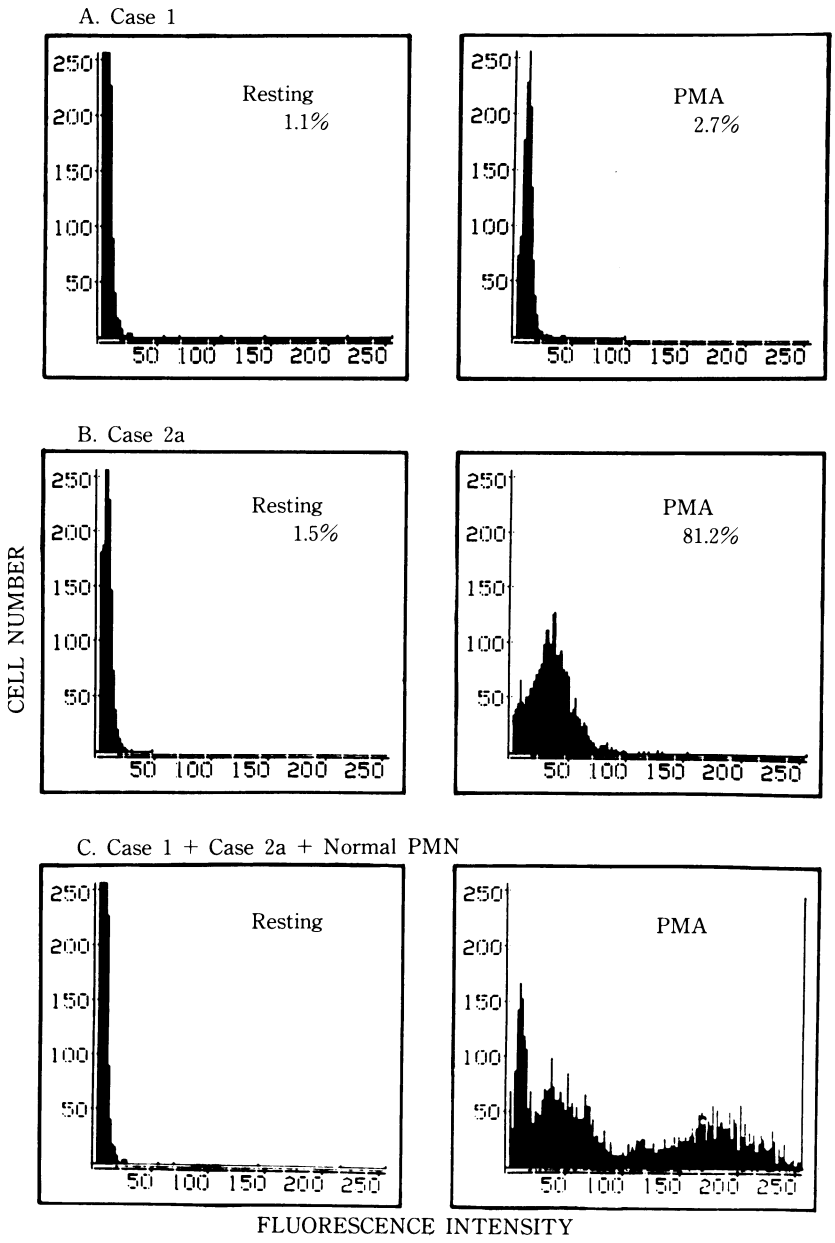

Fig. 3. Fluorescence distribution of resting and PMA-stimulated PMN from Case 1, a female patient with CGD (A), Case 2a, one of the female sibling patients (B), and in a mixed blood specimen of Case 1, Case 2a and a normal donor (C). The experimental design was the same as in Fig. 2, but the blood specimens were processed after several hours of transport.

only a few percent of the total, were able to generate definite intracellular fluorescence on stimulation. Thus, their PMN defects were not so complete as seen in the classical X-linked form of CGD (Fig. 3A). PMN from the mothers of these two female patients, however, responded to PMA normally as a single cell population. In the other two female sibling patients, Cases 2a and 2b, almost all PMN were partially activated with PMA and identified as a single uniform cell population with a mean fluorescence intensity of approximately one-fourth of controls (Fig. 3B). PMN response in their mother appeared to be within low normal ranges.

Effect of cell mixing on DCFH oxidation The presence of two or more PMN populations different in their oxidative metabolic activity in a specimen, such as heterozygous mother for the X-linked form of CGD, raises the question that H_2O_2 released from normal cells, might diffuse into defective cells and generate a fluorescent product of DCF within defective cells. However, that is not a case with PMN from the mother with an X-linked classical CGD boy (Fig. 2C). In addition, when blood specimens from Case 1, Case 2a, and a healthy control were combined in a mixture and stimulated with PMN, three cell populations comparable in fluorescence intensity to each of donor cells were identified by flow cytometry (Fig. 3C).

DISCUSSION

The oxidative metabolic activity of PMN is usually evaluated by using isolated PMN population. The separation of PMN is time-consuming and difficult to carry out on microscale. In addition, the isolation procedure itself tends to cause some artificial activation of PMN. Several authors reported the whole blood method for evaluating PMN oxidative metabolic activity and for screening of CGD[11,21,29]. Recent advance in flow cytometry offers several advantages over either standard spectrophotometric or histochemichal methods. Flow cytometry allows the rapid and precise estimation of the function levels in large numbers of cells on single cell basis as well as the average function of total cell population. Bass et al[4] reported that a combined use of DCFH-DA and PMA might permit a quantitative evaluation of the oxidative metabolic activity of individual PMN by flow cytometry. The procedure used in this work is a simplified whole blood version of the method and is based on the principle that the trapped intracellular polar DCGH is oxidized to fluorescent DCF mainly by H_2O_2 generated in PMN on stimulation with PMA. Thus, in the mixed cell population of functionally different cells, the possibility arises that H_2O_2 released from intact PMN, might diffuse into defective cells and generate the intracellular fluorescent product. In this work, however, PMN were stimulated with PMA in the presence of serum and erythrocytes which are known to be rich in catalase. The coexistence of normal PMN might not exert any appreciable effects on the fluorescence intensity of defective cells, as shown in Fig. 3C. In this experiment, almost all PMN from healthy subjects responded to PMA as a single cell population with strong fluorescence of intracellular DCF. Both magnitude and time course of the intensity of intracellular fluorescence varied from day to day to some degree in the same control subject, probably due to his daily physical conditions, but this in no way detracts from the usefulness of this assay in screening for patients with CGD.

The mode of genetic transmission in the majority of affected male patients with CGD is believed to be an X-linked recessive inheritance[30]. The complete lack of metabolic burst activity in a boy with the classical X-linked form of CGD and the presence of two distinct PMN populations, one normal and the other defective, in his mother indicated a random lyonization effect on the X chromosome in the obligate heterozygous mother for the X-linked recessive disease. But the situation in most female patients with CGD is much complicated because PMN dysfunction usually can be detected in neither parent. In some female patients, an X-linked mode of transmission was suggested, but in others, an autosomal recessive inheritance was proposed by family studies[8,13,19]. All the mothers of the female patients reported here showed no abnormality in their PMN function with this method and the pedigree studies of these female patients by conventional assay techniques could not demonstrate any PMN dysfunction in their fathers and relatives examined (data not shown). Thus, we could not identify the genetic background of these female patients with

CGD.

Female CGD patients in this study, however, showed two distinct patterns of PMN activation by flow cytometric analyses. PMN from two unrelated female patients, Cases 1 and 3, responded to PMA with a minimal increase of intracellular fluorescence. Flow cytometric pattern of their PMN activation seemed to be similar to, but not completely identical with, that of the classical X-linked male CGD. If these female patients with CGD would be heterozygous for the X-linked CGD with an extreme lyonization, their mothers also would be heterozygous with an extreme lyonization pattern in the opposite way. From the results of the present work, however, we could not settle whether it is the case or not. In the other two female sibling patients, almost all the PMN responded to PMA as a single uniform population with a weak but definite intracellular fluorescence. Such partial activation and time-couse of superoxide generation were demonstrated in a male patient with a variant form of X-linked CGD reported by Lew et al[17]. Additional mild variants of X-linked CGD have been reported and some workers claimed that at least two X-linked forms of CGD might exist with different biochemical abnormalities[5,6,17,27].

Recent studies suggest that three enzymes, cytochrome b, flavoproteins, and quinones, cooperate intimately in the superoxide/H_2O_2 generation system and dysfunction in this electron transport system has been implicated as functional defects in CGD patients[7,9,26]. It was suggested that the expression of the superoxide/H_2O_2 generating system is controlled by at least three genetic loci, one or more of which are located on the X chromosome. However, the relationship between the expression of these enzymes and the mode of transmission remains unsettled[12]. In the present work, the genetic heterogeneity in female patients with CGD was demonstrated on the basis of the flow cytometric patterns of stimulated PMN, but a more refined technique must be required for the definite identification of various heterozygous states for CGD.

ACKNOWLEDGEMENTS

We are indebted to Japan Ortho Diagnostic Systems Inc. (Tokyo, Japan) for the use of an Ortho Spectrum III.

This work was supported in part by a grant (No. 58440046) from the Ministry of Education of Japan.

REFERENCES

1. **Babior, B.M.** 1978. Oxygen-dependent microbial killing by phagocytes. N. Engl. J. Med. **298**: 659-725.
2. **Babior, B.M., Kipnes, R.S. and Curnutte, J.T.** 1973. Biological defence mechanisms: The production by leukocytes of superoxide, a potential bactericidal agent. J. Clin. Invest. **52**: 741-744.
3. **Baehner, R.L. and Nathan, D.F.** 1968. Quantitative nitroblue tetrazolium test in chronic granulomatous disease. N. Engl. J. Med. **278**: 971-976.
4. **Bass, D.A., Parse, J.W., DeChatelet, L.R., Szejda, P., Seed, M.C. and Thomas, M.** 1983. Flowcytometric studies of oxidative product formation by neutrophils. J. Immunol. **130**: 1910-1917.
5. **Borregaard, N. Cross, A.R., Herlin, T., Jones, O.T.G., Segal., A.W. and Valerius, N.H.** 1983. A variant form of X-linked chronic granulomatous disease with normal nitroblue slide test and cytochrome b. Eur. J. Clin. Invest. **13**: 243-248.
6. **Borregaard, N., Johansen, K.S. and Esmann, V.** 1979. Quantitative of superoxide production in human polymorphonuclear leukocytes from normals and 3 types of chronic granulomatous disease. Biochem. Biophys. Res. Commun. **90**: 214-219.
7. **Borregaard, N. and Tauber, A.I.** 1984. Subcellular localization of the human neutrophil NADPH oxidase; b-cytochrome and associated flavoprotain. J. Biol. Chem. **259**: 47-52.
8. **Buescher, E.S.** 1983. Inheritance of chronic granulomatous disease, p. 660-661. *In* J.I. Gallin (ed), Recent advances in chronic granulomatous disease. Ann. Intern. Med. **99**: 657-674.
9. **Crawford, A.R. and Schneider, D.L.** 1982. Identification of ubiquinone-50 in human neutrophils and its role in microbicidal events. J. Biol. Chem. **257**: 6662-6667.
10. **Curnette, J.T., Whitten, D.M. and Babior, B.M.** 1974. Defective superoxide production by granulocytes from patients with chronic granulomatous disease. N. Engl. J. Med. **290**: 593-597.
11. **DeChatelet, L.R. and Shirley, S.** 1981. Evaluation of chronic granulomatous disease by a chemiluminescence assay of microliter quantities of whole blood. Clin. Chem **27**: 1739-1741.
12. **Hamers, M.N., De Boer, M., Meerhof, L.J., Weening, R.S. and Roos, D.** 1984. Complementation in monocyte hybrids revealing genetic heterogeneity in chronic granulomatous disease. Nature. **307**: 553-555.
13. **Hitzig, W.H. and Seger, R.A.** 1983. Chronic

granulomatous disease, a heterogenous syndrome. Human. Genet. **64**: 207-215.

14. **Holmes, B., Page, A.R. and Good, R.A.** 1967. Studies of the metabolic activity of leukocytes from patients with a genetic abnormality of phagocytic function. J. Clin. Invest. **46**: 1422-1432.
15. **Homan-Muller, J.W.T., Weening, R.S. and Holmes, B.** 1975. Production of hydrogen peroxide by phagocytizing human granulocytes. J. Lab. Clin. Med. **85**: 198-207.
16. **Kaplan, E.L., Laxdal, T. and Quie, P.G.** 1968. Studies of polymorphonuclear leukocytes from patients with chronic granulomatous disease of childhood; Bactericidal capacity for streptococci. Pediatrics. **41**: 591-599.
17. **Lew, P.D.L., Southwitk, F.S. Stossel, T.P., Whitin, J.C., Simons, E. and Cohen, H.J.** 1981. A variant of chronic granulomatous disease; Deficient oxidative metabolism due to a low-affinity NADPH oxidase. N. Engl. J. Med. **305**: 1329-1333.
18. **Matsuura, R., Kagosaki, Y., Tanaka, H., Sakao, T., Kobayashi, Y. and Usui, T.** .1980. A female case of chronic granulomatous disease (CGD) associated with chronic idiopathic thrombocytopenia. Hiroshima. J. Med. Sci. **29** 83-86.
19. **Mills, E.L. and Quie, P.G.** 1983. Inheritance of chronic granulomatous disease, p. 25-53. *In* J.I. Gallin and A.S. Fauci (ed.), Advance in Host Defence Mechanisms: Vol. 3. Chronic granulomatous disease. Raven Press, New York.
20. **Mills, E.L., Rholl, K.S. and Quie, P.G.** 1980. Luminol-amplified chemiluminescence: A sensitive method for detecting the carrior state in chronic granulomatous disease. J. Clin. Microbiol. **12**: 52-56.
21. **Nakamura, M., Nakamura, M.A., Okamura, J. and Kobayashi, Y.** 1978. A rapid and quantitative assay of phagocytosis-connected oxygen consumption by leukocytes in whole blood. J. Lab. Clin. Med. **92**: 568-573.
22. **Newburger, P.G., Cohen, H.J., Rothchild, S.B., Hobbins, J.C., Malawista, S.E., and Mahoney, M.J.** 1979. Prenatal diagnosis of chronic granulomatous disease. N. Engl. J. Med. **300**: 178-181.
23. **Quie, P.G., White, J.G., Holmes, B. and Good, R.A.** 1967. In vitro bactericidal capacity of human polymorphonuclear leukocytes. Diminished activity in chronic granulomatous disease of childhood. J. Clin. Invest. **46**: 668-679.
24. **Repine, J.E., Clawson, C.C., White, J.G. and Holmes, B.** 1975. Spectrum of function of neutrophils from carriors of sex-linked chronic granulomatous disease. J. Pediatr. **87**: 901-907.
25. **Repine, J.E., Rasmussen, B. and White, J.G.** 1979. An improved nitroblue tetrazolium test using phorbol myristate acetate-coated coverslips. Am. J. Clin. Pathol. **71**: 582-585.
26. **Segel, W.S., Cross, A.R., Garcia, r.c., Borregaard, N., Valerius, N.H., Soothill, J.F. and Jones, T.G.** 1983. Absence of cytochrome b-245 in chronic granulomatous disease. N. Engl. J. Med. **308**: 245-251.
27. **Seger, R., Tiefernauer, L., Bohler, M., Matsunaga, T., Belohradsky, B., Schaad, U., Wahn, V. and Hitzig, W.** 1982. Three families with partial cytochrome b deficiency: A new cause of recurrent bacterial infections. Eur. J. Pediatr. **139**: 311 (Abstract).
28. **Somiya, K., Hirata, Y., Nakano, M., Okamoto, K., Sato, S. Morita, T.** 1981. Two female siblings with chronic granulomatous disease. Acta. Pediat. Jpn. (in Japanese) **85**: 398-407.
29. **Takeshi, K., Matsumoto, T., Shibata, R. and Minakami, S.** 1979. Simple and rapid method for the diagnosis of chronic granulomatous disease, measuring hydrogen peroxide and superoxide anions released from leukocytes in whole blood. Clin. Chem. Acta. **92**: 329-335.
30. **Windhoret, D.B., Page, A.R., Holmes, B., Quie, P.G. and Good, R.A.** 1968. The pattern of genetic transmission of the leukocyte defect in fatal granulomatous disease of childhood. J. Clin. Invest. **47**: 1026-1036.