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CASE REPORTS

Chronic Granulomatous Disease in Two Chinese Families

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SUMMARY **Two Chinese families with X-linked chronic granulomatous disease (CGD) are reported. The first case was an 11-month-old male baby and the second a 2-month-old male baby. Both patients presented with persistent infections caused by *Staphylococcus* and *Candida* since birth. Neutrophil functions were studied in patients and a number of family members. Chemotaxis and phagocytosis were normal in every subject. Slide and spectrophotometric nitroblue tetrazolium (NBT) tests of both patients were abnormal and remained unchanged in spite of treatment with ascorbic acid, levamisole, sulfomethoxazole, trimethoprim and isoniazide. Mothers were proved to be carriers as evidenced by the presence of both normal and CGD phagocytes in the slide NBT test. During the 2-month follow-up period, the percentage of normal phagocytes from the mother of case 1 varied from 12% to 73%, which correlated with the fluctuation of spectrophotometric NBT value. The slide NBT test of the mother of case 2 was nearly normal in face of the presence of CGD phagocytes. Both carrier mothers were healthy and asymptomatic.**

Chronic granulomatous disease (CGD) is a disorder of phagocyte function inherited in either X-linked or autosomal recessive fashion.[1,2] The incidence is estimated to be 1 in one million in Caucasians,[3] but the figure is not known for Orientals. Although several cases of CGD have been found in Japan,[4] there is still no report in Chinese. Clinically, the disease is characterized by repeated severe infections caused by catalase-positive aerobic bacteria and fungi, widespread abscesses and granulomatous reactions, and often, if not intensively treated with antibiotics, leads to death in early childhood. Functionally, the disease is characterized by a total absence of respiratory burst leading to prolonged survival of phagocytosed bacteria.[5]

CGD may be suspected when a patient presents with a history of persistent and severe infections in the face of normal serum immunoglobulins, complement and cell-mediated immunity.[6] Definite diagnosis of CGD depends upon the demonstration of abnormal respiratory burst of the phagocytes despite intact other phagocyte functions.[2]

We here report 2 cases of CGD manifested with persistent staphylococcal and candidial infections since birth. All neutrophil functions were normal except defective nitroblue tetrazolium test and bacterial killing. Moreover, a sex-linked inheritance pattern was demonstrated.

REPORT OF CASES

Patient 1

LHJ, an 11-month-old male infant, was first admitted to a local hospital at the age of 3 weeks because of fever, suppurative lymphadenitis, and impetiginous skin lesions. At 5 months of age he was hospitalized again for fever, anemia, bloody mucoid stool, perianal and skin abscesses. At 9 months of age suppurative lymphadenitis and skin abscesses recurred, and anal fistula was also found. Each time the patient was treated with strong antibiotics for a long period. In January, 1988 when he was 11-months old, he was admitted to our hospital with the same complaints. Physical examination revealed an active and moderately developed infant. Several

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pigmented scars were found on the forechest and cervical area. The bilateral inguinal lymph nodes were inflammed with pus coating. Multiple anal fistulae with draining pus were noted. No hepatomegaly and no skin rashes was found. Laboratory examinations included WBC 30,600/mm^3 (51% neutrophils), ESR 43 mm/hr and CRP 3.16 mg/dl. Immunological studies showed increased serum immunoglobulins (IgG 1,440 mg/dl, IgA 282 mg/dl, IgM 351 mg/dl and IgE 933 IU/ml), elevated serum complement (C3 264 mg/dl and C4 72.7 mg/dl) and normal cell-mediated immunity in terms of T-cell subsets and mitogen responses. Chemotaxis and phagocytosis of PMN were normal but NBT test and bacterial killing were impaired (see later). Erythrocyte G-6-PD activity was 10.7 U/gHb (normal range 4.6-13.5 U/gHb) and myeloperoxidase of PMN was normal. Pus cultures yielded *Staphylococcus epidermidis, Escherichia coli, Klebsiella pneumonia,* and *Proteus mirabilis.* Skin biopsy showed granulation tissue and fibrosis.

His father, mother, and grandmother, aunts and uncles of maternal side had no history of repeated pyogenic infection of skin or other organs. The serum immunoglobulins, complement, erythrocyte G-6-PD and myeloperoxidase of PMN of those relatives were normal.

Patient 2

SML, a 2-month-old male baby, was referred to our hospital due to uncontrolled perianal abscess and candida peritonitis. He was born smoothly with a birth body weight of 2,740 gm. At 1 week of age he was first admitted to a local hospital because of vomitting and poor activity. He was treated with antibiotics but fever developed on the 7th hospitalization day. Pleocytosis of CSF was found although both blood and CSF cultures were negative. Despite continuation of strong antibiotics, perianal abscess and scrotal inflammation occurred, and pus culture yielded *Enterobacter cloaca.* Several days later, peritonitis developed and laparotomy found disseminated fungal granuloma over whole abdomen and culture of ascites yielded *Candida pseudotropicalis.* As his condition continued to get worse even after addition of amphotericin B, he was referred to our hospital. Treatment with vancomycin, moxalactum and amphotericin B, total parenteral nutrition and other supportive measures were instituted after admission. The fever was controlled and general condition was improved 3 weeks later, although the wound of laparotomy healed poorly. High fever recurred 1 week after discontinuation of systemic antibiotics. His condition then went downhill quickly. Chest X-ray revealed cotton ball appearance and disseminated intravascular coagulation occurred. The patient died 2 months after admission. Autopsy showed systemic fungal infection.

Immunological studies revealed increased serum immunoglobulins (IgG 1,400 mg/dl, IgA 150 mg/dl, IgM 422 mg/dl, IgE 121 IU/ml), increased C3 (112 mg/dl) and C4 (34.2 mg/dl) and normal CH_{50} (34 U/ml). T-cell function in terms of T-cell subpopulations and mitogen responses to Con A and PHA was normal. Chemotaxis and phagocytosis were normal. Myeloperoxidase stain of PMN was also normal. Erythrocyte G-6-PD activity was within normal limit (12.6 U/gHb).

Review of family history disclosed that the patient had two brothers and both of them died in early infancy. The eldest brother died at 1 wk of age because of fever and abdominal distension. The elder brother developed fever and generalized petechiae at 17 days of age and died 2 weeks later. Pneumonia, hepatomegaly, leukocytosis and increased PT and PTT were found during hospitalization. The immune function of his parents was completely normal except for a slightly higher percentage of NBT-negative phagocytes in slide NBT test of mother.

METHODS

NBT slide test

The NBT slide test was done according to the method of Ochs and Igo,[7] with slight modification. Briefly, 10 μl of 0.05 mg/ml stimulant (endotoxin extracted from *Escherichia coli* and *Pseudomonus aeruginosa,* Lot no. 108C68302, Sigma, St. Louis, MO, U.S.A.) were allowed to dry on a glass slide. Ten small drops of whole blood, obtained by venipuncture, were placed over an uncoated or stimulant-coated glass slide and incubated at 37° C for 45 min in a moist chamber. The blood clot was picked off and the slides were then washed with 37° C isotonic saline to remove red blood cells and excessive saline was absorbed with filter paper. The glass slides were immediately covered with freshly prepared NBT suspension (Sigma) and incubated again at 37° C for 20 min in a moist chamber. Finally the glass slides were washed, air dried, stained and examined under a light microscope. The deformed NBT-positive cells contained distinct blue-black granules or stippled dye precipitates and could easily be recognized. Three-hundred cells were counted, and the percentage of NBT-positive cells was calculated. In each experiment, one normal subject was included as control, and both stimulated and unstimulated NBT tests were done simultaneously for each person.

Preparation of polymorphonuclear leukocytes (PMNs)

Peripheral blood mononuclear cells (MNCs) and polymorphonuclear cells (PMNs) were prepared by the method of Boyum,[8] using Ficoll/Hypaque density gradient and dextran sedimentation. After removal of the

mononuclear cells at the interface layer, the packed cells at the bottom of the centrifuge tube were mixed with an equal volume of Hanks' balanced salt solution (HBSS) and 6% dextran in normal saline (0.2 ml/ml of cell suspension) was added. After standing at room temperature for one hour, the leukocyte-rich supernatant was collected and the contaminating RBCs were lysed hypotonically twice. The PMNs were washed three times with HBSS and then adjusted by cell counter (Sysmex CC-150, Toa Medical Electronics Co., Ltd., Kobe, Japan) to a concentration of 2×10^6 cells/ml. The purity of PMNs in the final cell suspension was more than 96% and the viability was over 98% by trypan blue exclusion test.

Spectrophotometric NBT test

This quantitative NBT test was done according to the method of Wenger and Bole.[9] Briefly, 0.5 ml of 2×10^6/ml PMNs in HBSS were mixed with 0.5 ml of autologous plasma in the presence or absence of stimulant in a siliconized test tube and incubated in 37° C for 30 min. NBT solution (0.5 ml) was added to each test tube and incubated at 37° C with shaking at 90 strokes per min (Eyelashaker, Rikakikai Co., Ltd., Tokyo, Japan). The reaction was stopped with 1.5 ml of 0.5 N HCl and centrifuged at 1,000 × g at room temperature for 10 min. The supernatant was decanted and the cell pellet washed with 1 ml of physiological saline. The cells were extracted for 10 min with 1 ml pyridine in a boiling water bath. After centrifugation at 1,000 × g for 10 min, a second extraction with 1 ml pyridine was performed. The extracting solutions were mixed and the optical density (OD) was read in a Beckman DU-65 spectrophotometer at 560 nm against a pyridine blank. The OD of a reagent blank of cells and that of NBT dye at zero time was subtracted. The OD values obtained with or without stimulant preincubation were calculated for 10^6 PMNs/30 min. All determinations were done in duplicate.

***In vitro* study of effect of isoniazine (INH) on NBT test**

One INH tablet containing 100 mg isoniazide, 75 mg starch and 5 mg talc was ground to powder and then dissolved in 20 ml of distilled water. The solution was filtered through a 0.2 μ pore membrane to obtain a clear final solution containing 5 mg INH/ml. One-tenth mg of INH was added to 0.5 ml of 2×10^6 PMNs/ml in HBSS and then mixed with 0.5 ml autologous plasma. The mixture was incubated at 37° C for 2 hours, 1 hour, 30 min and 0 min, respectively. Finally, 0.5 ml of NBT solution were added and quantitative NBT test was done. In one experiment, 1 mg of INH was added to see if a higher concentration of INH had greater enhancing effect on NBT function of PMNs.

***In vitro* phagocytosis and bactericidal tests**

The procedures were done according to the method of Wilkinson.[10] *Staphylococcus aureus* was obtained from ATCC (strain 25923). *S. aureus* was cultured overnight in BHI broth (Difco) and then suspended in 0.9% saline to give a transmittance of 90% at 500 nm (the transmittance of 0.5 Mcfarland turbidity standards) in a Perkin-Elmer, Junior III spectrophotometer. This optically adjusted suspension contained 3-9 × 10^7 bacteria per ml. After centrifugation, the bacteria were then resuspended in HBSS containing 20% fetal bovine serum (FBS) and stored at 0° C for later use.

Phagocytic tests were done in 13 × 100 mm plastic test tubes. Each tube contained 0.5 ml of 2×10^6 PMNs/ml in HBSS and 0.5 ml of bacteria suspension. To check the viability of bacteria, a control tube which contained 0.5 ml HBSS and 0.5 ml of bacterial suspension was prepared and viable bacteria count was performed directly. The test tubes were incubated at 37° C in a shaking water bath for 15 min to allow phagocytosis to occur. At the end of incubation, smears were made and the number of PMN with internalized bacteria per 100 cells was counted. For bactericidal test, the tubes were centrifuged for 5 min at 400 × g. Supernatants were decanted and viable bacteria were counted directly. The pellets were resuspended in 1 ml HBSS containing 10% FBS, 200 U/ml penicillin and 200 μg/ml streptomycin to kill extracellular bacteria. The test tubes were incubated further at 37° C for 30, 60 and 120 min, respectively. After incubation, the tubes were centrifuged for 5 min at 400 × g. The pellets were washed twice with HBSS and finally resuspended in 1 ml distilled water and pipetted vigorously for 1 min to lyse the PMN and release any surviving bacteria. Serial 10-fold dilution of lysates up to 1 to 1,000,000 was made. One hundred μl of each diluted lysate was then applied evenly onto blood agar plates with a T-shaped glass bar. The plates were incubated overnight and bacterial colonies were counted.

RESULTS

Phagocytosis

Phagocytosis was normal in patients and their parents. The percentage of PMNs with phagocytosed bacteria was 93%, 87% and 88% for patient 1, his mother and father, respectively; 82%, 89% and 90% for patient 2, his mother and father, respectively. The data for normal control was $82.73 \pm 6.75\%$. Over 90% of the bacteria was ingested in each subject studied.

Bactericidal test

The result of bactericidal test of patient 1 is depicted in Fig. 1. Ninety-two percent of ingested bacteria remained viable at 30 min after

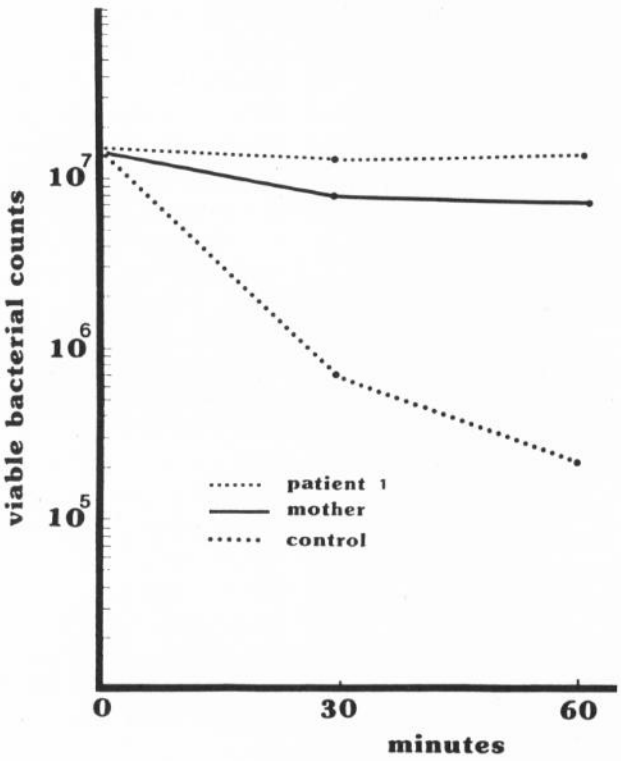

Fig. 1 Results of bactericidal test of patient 1, his mother and normal control. PMN from mother showed partial bactericidal defect.

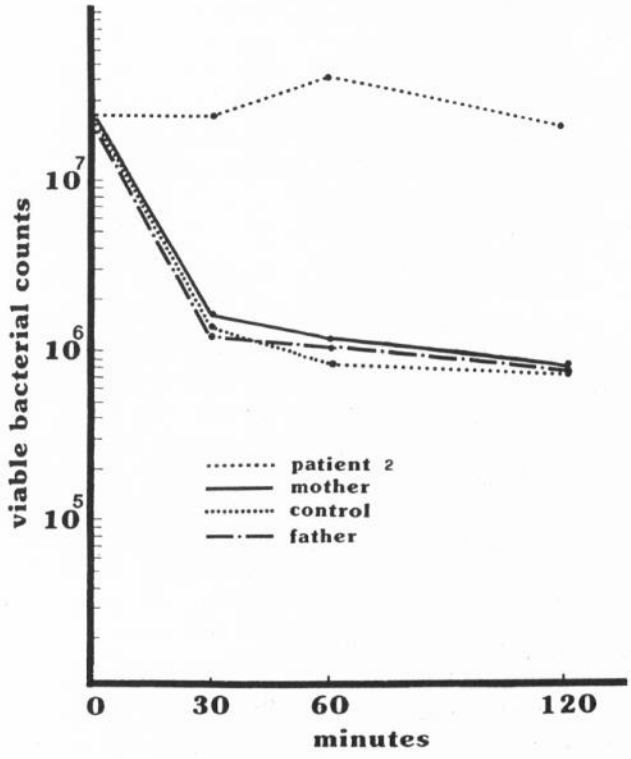

Fig. 2 Results of bactericidal test of patient 2, his parents and normal control. PMN from parents showed normal bactericidal activity.

being phagocytosed. The figure for his mother was 55%, and 19% for normal control. At 60 min,the figures for patient and his mother remained nearly the same, i.e., 92% and 47%, respectively, however, the figure decreased to 4% in normals. Thus, the bactericidal test was markedly impaired in patient 1 and moderately impaired in his mother. The result of bactericidal test of patient 2 is depicted in Fig 2. Ninety-one percent of ingested bacteria remained viable at 30 min after being phagocytosed. The figures for his father, mother and control were 22%, 34% and 23%, respectively; at 60 min, the figures for patient, father, mother and control were 148%, 21%, 23% and 11%, respectively; at 120 min, the figures were 75%, 8%, 8% and 6%, respectively. Therefore, the bacterial killing was markedly impaired in patient 2.

NBT tests

Figs. 3 and 4 show the morphological pictures of the NBT test by the slide method. In contrast to PMNs of parents and normals, all PMNs from both patients failed to reduce NBT, even after being preincubated with different concentrations of stimulant, and this finding still persisted even when the clinical condition became improved after treatment.

Table 1 summarizes the results of NBT tests by both slide and spectrophotometric methods. Again, NBT tests were negative in both patients, and there was a good correlation between slide method and spectrophotometric method in all subjects studied. It is interesting to note that the NBT-positive PMNs in the mother of patient 1 varied from 12% to 73% during the course of follow-up and the results correlated with the data obtained by the spectrophotometric method. Thus both tests indicated that mother of patient 1 was a carrier. The mother of patient 2 had slightly lower percentage of NBT-positive cells which

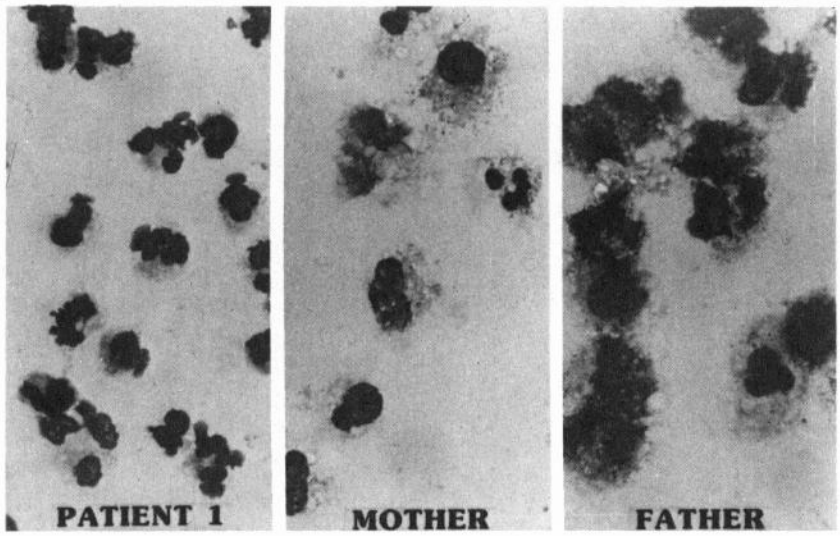

Fig. 3 Slide NBT tests of patient 1 and his parents. No NBT–positive cells were found on patient's slide. Both NBT–positive and–negative cells were present on mother's slide. All cells on father's slide were NBT – positive.

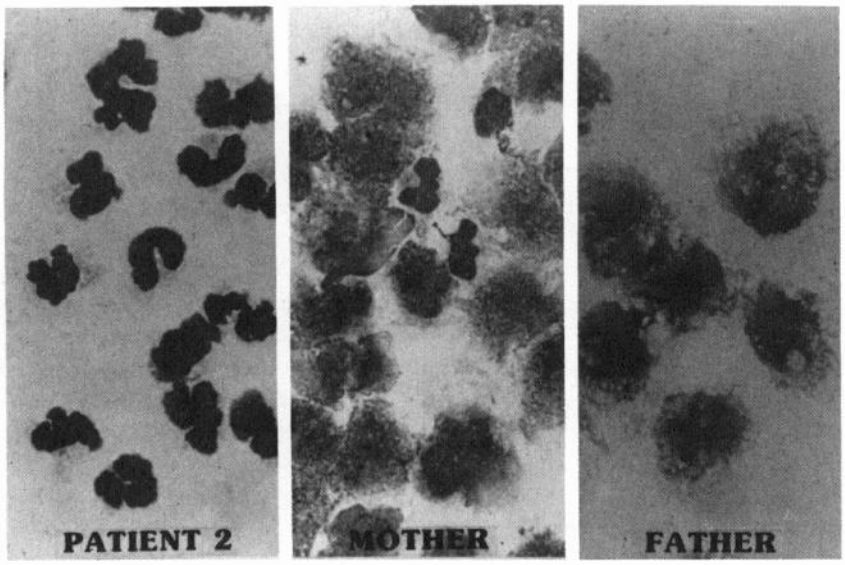

Fig. 4. Slide NBT tests of patient 2 and his parents. No NBT–positive cells were found on patient's slide. Several NBT–negative cells were found on mother's slide. All cells on father's slide were NBT–positive.

ranged from 86%-94% (Normal 96 ± 2.36%) but showed normal spectrophotometric test. The effect of INH on the NBT test was studied. As shown in Table 2, no improvement in the NBT test could be seen.

DISCUSSION

CGD in childhood is a rare syndrome characterized by extreme susceptibility to bacterial infection.[11] Initially, CGD was considered to be a distinct disease entity on the basis of characteristic pathological changes and typical clinical course.[12,13] Later on, it was defined as a syndrome of congenital disorders caused by a variety of biochemical lesions, with emphasis on phagocyte oxidase deficiencies.[14] CGD has been rarely reported in Orientals,[4] and up to now no Chinese patient was described. The clinical histories, and the negative NBT tests and impaired bactericidal activity of PMNs strongly support the diagnosis of CGD in these two patients described in this report.

The inheritance pattern of CGD in these two patients was studied. The mother of patient 1 was shown to be a carrier as evidenced by intermediate NBT test (by both slide and spectrophotometric methods) and moderately impaired bactericidal capacity. The mother of patient 2 might also be a carrier as she had NBT-positive cells slightly lower than normal (Table 1). Therefore, the inheritance pattern in these two patients seems to be X-linked. As postulated by Lyon[15] and Beutler *et al.*,[16] if inactivation of the X-chromosome occurs as a random event early in fetal development when precursors of phagocytic cells comprise relative few cells, one may expect to find heterozygous carriers of X-linked CGD whose leukocytes show functional capability lying between normals and homozygotes of CGD. The NBT test of maternal grandmother of patient 1 was found to be normal. This might be explained by: 1) an extreme lyonization of X-chromosome of the grandmother with leukocyte function close to normal, or 2) a new mutation of an X-chromosome occurring early in the mother's fetal development as proposed by Biggar *et al.*,[17] Johnston *et al.*[18] also reported a CGD carrier girl with no demonstrable carrier evidence noted in her mother and grandmother. Repine *et al.*[19] noted a wide spectrum of function of neutrophils from carriers of X-linked CGD, and most carrier mothers were healthy and did not show any tendency toward recurrent infections, even though a variable number of their phagocytes did function abnormally. In this study, both mothers were healthy carriers and this finding is consistent with the report of Repine *et al.*[19] However, exceptions did exist, *e.g.*, arthral-

Table 1. Results of slide NBT test and spectrophotometric NBT test

	Date	No. of experiment	% NBT–positive* phagocytes with stimulant‡	OD value at 560 nm† without stimulant‡	OD value at 560 nm† with stimulant‡
Patient 1		4	0	0.000	0.000
Mother	1–22, '88		12	nd	nd
	1–23, '88		20	nd	nd
	1–26, '88		14	0.012	0.032
	1–29, '88		42	0.032	0.072
	2–2, '88		73	0.160	0.169
	2–5, '88		nd	0.094	0.074
	2–12, '88		nd	0.061	0.094
	3–21, '88		24	nd	nd
Father		1	99	0.166	0.196
Aunt		1	98	0.226	0.197
Uncle		1	98	0.212	0.132
Grandmother		2	97, 98	0.212	0.173
Patient 2		4	0	0.000	0.000
Mother		3	86, 88, 94	0.218	0.239
Father		2	99, 98	0.165	0.186
Normals		16	96 ± 2.36§	0.179 ± 0.054§	0.188 ± 0.084§

* 300 cells were counted in each experiment
\+ 10^6 PMNs/30 min
‡ Endotoxin, 1.5×10^{-3} mg/ml
§ Mean ± S.D.
nd = not done

Table 2. Effect of isoniazide on spectrophotometric NBT test of PMNs.

	Incubation period (min)					
	0+	0	30	30*	60	120
Patient 2	0.007	0.002	0.000	0.001	0.000	0.003
Control	0.114	0.168	0.126	nd	0.140	0.116

The final concentration of INH was 0.1 mg/ml not specified.
\+ No INH was added.
* Final concentration of INH 1 mg/ml
nd = not done

gia, mild arthritis, recurrent fever, boils, discoid lupus, or frequent oral ulcers occurred in some carriers.[20-22]

The fluctuation of the NBT test in the mother of patient 1 is worth mentioning. During the follow-up period of just 2 months, the NBT-positive cells varied from 12% to 73% and the results correlated very well with those of the spectrophotometric method. A similar phenomenon had been found by Johnston *et al.*,[18] who reported a CGD carrier with percentage of NBT-positive cells varing from 4% to 44% on different occasions over 4 years, and he could not find any relationship with intercurrent infections.

The underlying microbicidal defect in CGD is due to a malfunction of the enzyme(s) responsible for H_2O_2 and O_2 production in phagocytosing cells. Nine different molecular defects involving the NADPH oxidase system of the phagocyte have been indentified up to now.[23] The NADPH oxidase system of the phagocytes consists of three parts, an activation apparatus, a respiratory chain (with at least two components, a flavoprotein and a cytochrome b), and several enzymes for NADPH supply (the hexose-monophosphate shunt). Defect of one of the elements results in failure of the oxidase system and leads to the whole spectrum of CGD. The introduction of an exogenous peroxide producing system can correct the microbicidal defect. After ingestion of H_2O_2 releasing bacteria (*Streptococci, Pneumococci, Lactobacilli* and others), the H_2O_2 released by the bacteria results in destruction of the microbes.[2]

Up to now there is no medication capable of correcting the underlying defect in the phagocyte respiratory burst. Attempts have been made to restore active oxygen metabolites in CGD neutrophils by using sulfamethoxazole trimethoprim but without success.[24,25] Treatment of our patients with sulfamethoxazole trimethoprim also failed to correct NBT tests. In 1986 Megyeri and Endreffy[26] reported improvement

of defective bactericidal capacity of PMN by INH in a case of CGD. However, in this study, *in vitro* incubation of PMNs from patient 2 with INH could not improve the NBT result. We tried intravenous ascorbic acid in patient 1 for 3 weeks to stimulate the respiratory burst and inhibit catalase but the follow-up NBT studies remained unchanged. Similar results were found by Anderson[27] and Orr.[28] Bone marrow transplantation offers the only potential cure for this inherited error of phagocyte function. Improvement in the ability to fight against infections has been reported.[29,30] However, this therapeutic approach cannot be unequivocally recommended, even in patients having histocompatible donors, until: (1) the natural history of this disease is better defined, (2) engraftment is assured by more effective techniques for myeloablation and immunosuppression, and (3) chronic graft-versus-host disease can be effectively prevented and ceases to be a frequent complication of marrow transplantation.[30]

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