SIMILAR PROPORTION OF SPORADIC CASES IN CYTOCHROME b_{558} NEGATIVE CHRONIC GRANULOMATOUS DISEASE AND DUCHENNE MUSCULAR DYSTROPHY

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Summary We studied 12 Japanese families of cytochrome b_{558} (b) negative chronic granulomatous disease (13 patients and 23 family members) and 15 controls for cytochrome b content, O_2^- , and H_2O_2 production. Cytochrome b content (nmol/ 10^8 cells), O_2^- production (nmol/ 10^7 cells/min), and percentage of H_2O_2 generating cells (%) were 1) 0.45–1.19, 64.1–174.2, and 85.8–100.0 in controls (mean $\pm$ 2 S.D.), 2) 0.13–0.38, 22.6–50.9, and 20.0–62.4 in healthy carriers, and 3) undetectable, undetectable, and 0 in patients, respectively. These findings indicate that healthy carriers and normal homozygotes are separable by these three parameters, and that 4 of the 12 families studied represent fresh gene mutation. Pooling of data by Segal *et al.*, Ohno *et al.*, and ours yielded the overall incidence of a fresh gene mutation to be 19.4% (7 of 36 cases). The lower fresh mutation rate than predicted from Haldane's formula suggests a higher mutation rate in males than in females, as previously suggested in Duchenne muscular dystrophy.

Key Words chronic granulomatous disease, fresh gene mutation, low temperature absorption spectroscopy

INTRODUCTION

A substantial number of individuals with a lethal form of X-linked recessive disorder are believed to represent a fresh gene mutation arising from unaffected parents, as formulated by Haldane (1935). For classical cytochrome b_{558} (b) negative X-linked CGD, however, there have been two conflicting reports: the incidence of sporadic occurrence being 16% (3 in 19 subjects) as studied by Segal *et al.* (1983), and 0% (none in 8 subjects) as studied by Ohno *et al.* (1986). We measured cyto-

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chrome *b* content in polymorphonuclear cells (PMNs) from 13 CGD patients and 23 family members from 12 CGD families using low temperature absorption spectroscopy. This method enabled us to obtain accurate quantitative results based on fine absolute spectra of cytochrome *b* in intact cells *in situ*. In addition, O_2^- and H_2O_2 production were measured in all the patients and selected families. By these methods, asymptomatic family members were separable into healthy carriers and normal homozygotes.

SUBJECTS

The subjects consist of 13 patients (10 males and 3 females, 2–22 years of age) and the 23 family members (9 mothers, 6 fathers, 5 sisters, and 3 brothers, 6–50 years of age) from 12 families (Fig. 1), and 15 controls (11 males, 4 females, 24–35

Fig. 1. Family trees of 12 CGD families studied. ■, affected male; ▨, affected male by clinical history; □, unaffected male; ☐, male who died in infancy from unknown cause; ⊙, heterozygous female in X-linked recessive inheritance; ▣, heterozygous male in autosomal recessive inheritance; ☒, died; ☐, tested; ↗, proband.

years of age). Of the 12 families, there are 10 with one or two male patients and 2 with one or two female patients. All the patients had severe recurrent bacterial infections from early infancy and were diagnosed as having CGD by impaired bacterial killing and defective O_2^- production in their infancy in Keio University Hospital during a period of 1963–1988. All the family members were asymptomatic.

METHODS

Cytochrome *b* was measured in PMNs in 13 patients and 23 family members (9 mothers, 6 fathers, 5 sisters, and 3 brothers), O_2^- production and H_2O_2 production by PMNs were measured in 13 patients and 16 family members (7 mothers, 4 fathers, 4 sisters, and 1 brother), and in 13 patients and 8 family members (5 mothers, 1 father, and 2 sisters), respectively.

1) *Isolation of PMNs.* PMNs were isolated with ficoll/dextran method (Iizuka *et al.*, 1985) from 10 ml of venous blood anticoagulated with heparin and suspended in Hanks' balanced solution, pH 7.4. More than 90% of the isolated cells were identified to be neutrophils.

2) *Measurement of cytochrome *b* in PMNs.* PMNs were suspended in Hanks' balanced solution containing 30% glycerol at $2-8 \times 10^8$ cells/ml. Absorption

Fig. 2. Absolute spectra of neutrophils at 77K (liquid nitrogen temperature). A: The spectrum of dithionite-reduced neutrophils (3×10^8 cells/ml) from a normal subject shows a sharp Soret peak (at 426 nm) and two characteristic bands (at 528 and 558 nm) in the visible region for cytochrome b_{558} . B: No definite peak of cytochrome b_{558} was detected in the spectrum of neutrophils (2.4×10^8 cells/ml) from a CGD patient.

spectrum of the cells was recorded at 77K (liquid nitrogen temperature) according to the method of Iizuka *et al.* (1985) (Fig. 2). The extinction coefficient $\epsilon_{558-575}$ was determined as $45.2 \text{ mm}^{-1}\text{cm}^{-1}$ by using solubilized cytochrome *b* under experimental condition.

3) *Assay of O_2^- and H_2O_2 production.* PMNs ($1-3 \times 10^6$ cells/ml) were stimulated by phorbol myristate acetate (PMA, 10^{-7} mM) at 37°C and O_2^- production was determined from superoxide dismutase inhibitable reduction of cytochrome *c* as described previously (Bulter *et al.*, 1975). H_2O_2 production was assayed by flow cytometry as described Bass *et al.* (1983).

RESULTS

1) *Cytochrome b content in PMNs*

Cytochrome *b* content ranged from 0.45 to $1.19 \text{ nmol}/10^8$ cells (mean ± 2 S.D.) in 15 controls, and was undetectable in all the 13 patients. In the 23 family members studied, 4 mothers (family A, B, C, D), 1 father (family K), 2 sisters (family A, B), 1 brother (family K) yielded values of $0.13-0.38 \text{ nmol}/10^8$ cells, and 5 mothers (family E, F, G, H, L), 5 fathers (family A, B, C, I, L), 3 sisters (family G, K, L), 2 brothers (family C, K) yielded values of $0.44-1.02 \text{ nmol}/10^8$ cells (Table 1).

2) *O_2^- production*

O_2^- production ranged from 64.1 to $174.2 \text{ nmol}/10^7$ cells/min (mean ± 2 S.D.) in 11 controls studied, and was undetectable in all the 13 patients. In the 16 family members studied, 3 mothers (family A, B, C) and 2 sisters (family A, B) yielded values of $22.6-50.9 \text{ nmol}/10^7$ cells/min, and 4 mothers (family E, F, G, L), 4 fathers (family A, B, I, L), 2 sisters (family G, L), 1 brother (family C) yielded values of $71.7-186.7 \text{ nmol}/10^7$ cells/min (Table 1).

3) *H_2O_2 production*

H_2O_2 production ranged from 85.8 to 100.0% (mean ± 2 S.D.) in 15 controls, and was 0% in all the 13 patients. In the 8 family members studied, 1 mother (family B) and 2 sisters (family A, B) yielded values of 20.0–62.4%, and 4 mothers (family E, G, H, L) and 1 father (family B) yielded values of 90.1–100.0% (Table 1). Three family members with low H_2O_2 production showed two peaks of H_2O_2 productive and non-productive cells in their charts and were identified as obligate heterozygotes (Fig. 3).

4) *Correlation between cytochrome b content and O_2^- production (Fig. 4)*

In all the members of 7 families (family A, B, C, E, F, G, I, $n=20$), both cytochrome *b* and O_2^- production were measured. There was a close correlation between the two parameters ($r=0.94$, $p<0.01$).

Table 1. Characteristics of the patients and family members studied.

Family	A	B	C	D	E	F	G	H	I	J	K	L
Inheritance	XR	XR	XR	probable XR	sp	sp	sp	sp	XR or sp	?	AR	?
Patient sex	M	M	M	M	M	M	M	M	M	M	F	F
age	2	8	3	2	14	14	4	8	2	2	11	22
(years)												
b_{558}	0	0	0	0	0	0	0	0	0	0	0	0
O_2^-	0	0	0	0	0	0	0	0	0	0	0	0
H_2O_2	0	0	0	0	0	0	0	0	0	0	0	0
Mother b_{558}	0.35	0.31	0.20	0.13	0.89	0.69	0.86	0.80	died	—	died	0.44
O_2^-	50.9	45.5	39.8	—	124.5	72.5	93.0	—	—	—	—	109.0
H_2O_2	—	20.0	—	—	100.0	—	97.5	100.0	—	—	—	95.4
Father b_{558}	1.02	1.00	0.62	—	—	—	—	—	0.66	—	0.38	0.73
O_2^-	173.1	159.2	—	—	—	—	—	—	90.7	—	—	125.1
H_2O_2	—	99.1	—	—	—	—	—	—	—	—	—	—
Sister b_{558}	0.27	0.16	—	—	—	—	0.55	—	—	—	0.89	0.53
O_2^-	41.0	22.6	—	—	—	—	129.2	—	—	—	—	186.7
H_2O_2	53.3	62.4	—	—	—	—	—	—	—	—	—	—
Brother b_{558}	—	—	0.77	—	—	—	—	—	—	0.29	0.55	—
O_2^-	—	—	71.6	—	—	—	—	—	—	—	—	—
H_2O_2	—	—	—	—	—	—	—	—	—	—	—	—

XR, X-linked recessive; sp, sporadic case; AR, autosomal recessive; b_{558} , cytochrome b_{558} content (control: 0.45–1.19 nmol/10⁸ cells); O_2^- , O_2^- production (control: 64.1–174.2 nmol/10⁷ cells/min); H_2O_2 , H_2O_2 production (control: 85.8–100.0%). —, not tested.

Fig. 3. Assay for H_2O_2 production by flow cytometry. Fluorescence distribution of resting and stimulated PMNs from a normal subject, a cytochrome b_{558} negative CGD patient and an obligate heterozygote. A, resting stage; B, stimulated stage by PMA (100 ng/ml). The histograms represent numbers of cells (on the ordinate) as a function of fluorescence intensity (on the abscissa).

DISCUSSION

There are two hereditary forms of cytochrome b negative CGD: autosomal recessive and X-linked recessive inheritance (Curnutte, 1988). Based on the results of the present study utilizing 3 parameters, cytochrome b content, O_2^- production, and H_2O_2 production, the mode of inheritance in our 12 families was assigned as autosomal recessive (1 family, K), X-linked recessive (3 families, A through C), probable X-linked recessive (1 family, D), sporadic (4 families, E through H), X-linked recessive or sporadic (1 family, I) and undetermined (2 families, J and L). Excluding 1 family with autosomal recessive inheritance and 2 uninformative families, the members of the 9 families (A through I) were unequivocally separable into 2 groups: 6 healthy carriers (4 mothers and 2 sisters) and 10 normal homozygotes (4 mothers, 4 fathers, 1 sister, and 1 brother). Since only 8 autosomal recessive cases of cytochrome b negative CGD were reported to date in the world literature (Weening *et al.*, 1985; Lomax *et al.*, 1988; Okamura *et al.*, 1988; Parkos *et al.*, 1989), sporadic cases of cytochrome b negative CGD are most likely due to a fresh mutation in cytochrome b gene on the X chromosome. Thus, we suspect that such a mutation occurred in at least 44% of our subjects (4/9).

Fig. 4. Relationship between cytochrome b_{558} content and O_2^- production of PMNs from 7 classical CGD families. There is a close correlation between the two parameters ($r=0.94$, $p<0.01$), and normal homozygotes ($n=8$) were clearly separable from healthy carriers ($n=5$) and CGD patients ($n=7$). Shaded area indicates normal ranges for cytochrome b_{558} content (0.45–1.19 nmol/ 10^8 cells) and O_2^- production (64.1–174.2 nmol/ 10^7 cells/min).

Regarding the proportion of sporadic cases in cytochrome b negative CGD, 2 conflicting studies have been reported. Segal *et al.* (1983) reported 3 sporadic cases in 19 families studied, utilizing cytochrome b assay and PMA-stimulated nitroblue tetrazolium (NBT) slide test for carrier detection. In these 19 families with exclusively male patients (5 families with 2 patients and 14 families with a single patient), 16 mothers were carriers and 3 mothers were normal homozygotes. Ohno *et al.* (1986) failed to identify any sporadic cases in 8 families (2 families with 2 patients and 6 families with a single patient), using PMA-stimulated NBT slide test for carrier detection. In our 9 families, there were 2 families with 2 patients and 7 families with a single patient. Since the proportion of isolated cases was nearly identical in the three reports and all of these cases had early onset X-linked cytochrome b negative CGD, we presume that ascertainment bias inherent to small number of cases studied may be a major factor in the wide disparity among the proportion of sporadic cases (0, 16, 44%). In order to minimize this bias, we pooled the data from these three reports and obtained 19.4% (7/36) as the proportion of sporadic cases.

The figure of 19.4% is significantly lower than 33%, as expected by Haldane's formula. We speculate that the discrepancy may be due to higher mutation rate in male gametes than in female gametes (Vogel and Rathenberg, 1975). According to Haldane's formula (Haldane, 1935) for the proportion of sporadic cases in X-linked recessive disorder; $m = (1-f)u/(2u+v)$ (m =proportion of sporadic cases, u =mutation rate in female gametes, v =mutation rate in male gametes, f =fertility), two factors, fertility and differential mutation rates in both sexes, influence the proportion of sporadic cases. Because classical CGD was a lethal disease during the parents' generation of our patients, m is simplified to $u/(2u+v)$ ($f=0$). Thus, to yield the figure of 19.4%, sex ratio of mutation rates (v/u) is calculated to be 3.2 for the 36 CGD patients of the three reports. Similarly, Barbuji *et al.* (1990) described that the proportion of sporadic cases in a lethal sex-linked recessive disease, Duchenne muscular dystrophy (DMD), was 23% and ascribed the lower proportion of sporadic cases to higher mutation rate in male gametes as compared with those in female gametes.

Germline mosaicism has been documented in some X-linked recessive diseases, including DMD (Bakker *et al.*, 1987), hemophilia A (Levinson *et al.*, 1990), and ornithine transcarbamylase deficiency (Brusilow and Valle, 1987). It has been proposed that in DMD, 5–7% of apparent fresh gene mutations were indeed due to germline mosaicism (Hall, 1988; Bakker *et al.*, 1989). Although the relative incidence of germline mosaicism in cytochrome *b* negative CGD has not been estimated, we presume that such a mosaicism may also be relevant to this entity. Therefore, recurrent risk should be considered even in a family with apparent fresh mutation in the genetic counseling of CGD.

Further, we have to admit that our family studies are incomplete and that the distinction between healthy carriers and normal homozygotes by the biochemical methods is not absolutely reliable. Nonetheless, we conclude that the proportion of sporadic cases in cytochrome *b* negative X-linked CGD is significantly lower than that expected by Haldane's formula and is similar to that of DMD.

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REFERENCES

- Bakker, E., Van Broeckhoven, C., Bonten, E.J., Van de Vooren, M.J., Veenema, H., Van Hul, W., Van Ommen, G.J.B., Vandenberghe, A. and Pearson, P.L. 1987. Germline mosaicism and Duchenne muscular dystrophy mutations. *Nature* **329**: 554–556.
- Bakker, E., Veenema, H., Dunnen, J.T., Van Broeckhoven, C., Grootsholten, P.M., Bonten, E.J., Van Ommen, G.J.B. and Pearson, P.L. 1989. Germinal mosaicism increases the recurrent risk for 'new' Duchenne muscular dystrophy mutations. *J. Med. Genet.* **26**: 553–559.
- Barbuji, G., Russo, A., Danieli, G.A., Spiegler, A.W.J., Borkowska, J. and Hausmanova Petruse-

- wicz, I. 1990. Segregation analysis of 1885 DMD families: significant departure from the expected proportion of sporadic cases. *Hum. Genet.* **84**: 522-526.
- Bass, D.A., Parce, J.W., Dechatelet, L.R., Szejda, P., Seeds, M.C. and Thomas, M. 1983. Flow cytometric studies of oxidative product formation by neutrophils: A graded response to membrane stimulation. *J. Immunol.* **130**: 1910-1917.
- Brusilow, S. and Valle, D. 1987. Allopurinol induced ornithine transcarbamylase (OTC) deficiency. *Pediatr. Res.* **21**: 289A.
- Bulter, J., Jayson, G.G. and Swallow, A.J. 1975. The reaction between the superoxide anion radicals and cytochrome c. *Biochim. Biophys. Acta* **488**: 215-222.
- Curnutte, J.T. 1988. Classification of chronic granulomatous disease. *Hematol./Oncol. Clin. North Am.* **2**: 241-252.
- Haldane, J.B.S. 1935. The rate of spontaneous mutation of a human gene. *J. Genet.* **31**: 317-326.
- Hall, J.D. 1988. Review and hypotheses: somatic mosaicism: Observations related to clinical genetics. *Am. J. Hum. Genet.* **43**: 355-363.
- Iizuka, T., Kanegasaki, S., Makino, R., Tanaka, T. and Ishimura, Y. 1985. Studies on neutrophil b-type cytochrome *in situ* by low temperature absorption spectroscopy. *J. Biol. Chem.* **260**: 12049-12053.
- Levinson, B., Lehesjoki, A.E., Chapelle, A. and Gitschier, J. 1990. Molecular analysis of hemophilia A mutations in the Finnish population. *Am. J. Hum. Genet.* **46**: 53-62.
- Lomax, K.J., Whitman, C.B., Tiffany, H.L., Gallin, H.L. and Malech, H.L. 1988. Analysis of chronic granulomatous disease kindreds reveals distinct genetic lesions affecting the same gene product. *Clin. Res.* **36**: 413A.
- Ohno, Y., Buescher, E.S., Roberts, R., Metcalf, J.A. and Gallin, J.I. 1986. Reevaluation of cytochrome b and flavin adenine dinucleotide in neutrophils from patients with chronic granulomatous disease and description of a family with probable autosomal recessive inheritance of cytochrome b deficiency. *Blood* **67**: 1132-1138.
- Okamura, N., Malawista, S.E., Roberts, R.L., Rosen, H., Ochs, H.D. and Curnutte, J.T. 1988. Phosphorylation of the oxidase-related 48K phosphoprotein family in the unusual autosomal cytochrome-negative and X-linked cytochrome-positive types of chronic granulomatous disease. *Blood* **72**: 811-816.
- Parkos, C., Diauer, M.C., Jesaitis, A.J., Orkin, S.H. and Curnutte, J.T. 1989. Absence of both the 91kD and 22kD subunits of human neutrophil cytochrome b in two genetic forms of chronic granulomatous disease. *Blood* **73**: 1416-1420.
- Segal, A.W., Cross, A.R., Garcia, R.C., Borrengaard, N., Valerius, N.H., Soothill, J.F. and Jones, O.G.T. 1983. Absence of cytochrome b-245 in chronic granulomatous disease, A multicenter European evaluation of its incidence and relevance. *N. Engl. J. Med.* **308**: 245-251.
- Vogel, F. and Rathenberg, R. 1975. Spontaneous mutation in men. In *Advances in Human Genetics* Harris, H., Hirschorn, K., eds., Plenum Press, New York, Vol. 5, pp. 223-318.
- Weening, R.S., Corbeel, L., Boer, M., Lutter, M., Zwieten, R., Hamers, M.N. and Roos, D. 1985. Cytochrome b deficiency in an autosomal form of chronic granulomatous disease, A third form of chronic granulomatous disease recognized by monocyte hybridization. *J. Clin. Invest.* **75**: 915-920.