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HUMAN PERIPHERAL EOSINOPHILS HAVE A SPECIFIC MECHANISM TO EXPRESS gp91-*phox*, THE LARGE SUBUNIT OF CYTOCHROME b_{558}

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Eosinophils as well as neutrophils, monocytes and B lymphocytes are noted for lacking normal cytochrome b558 in patients with X-linked chronic granulomatous disease. The eosinophils of an X-linked male patient, however, fully expressed surface cytochrome b558, generated superoxide anion to a normal extent and definitely expressed the large subunit of cytochrome b558 (gp91-*phox*). His mononuclear leukocytes contained a diminished amount of gp91-*phox* mRNA with normal coding sequences. All the coding sequences and a putative poly (A) signal of his gp91-*phox* gene were normal. These results indicate that eosinophils have a specific mechanism to express gp91-*phox* and suggest that the mechanism lies at the transcriptional step of the gene. © 1995 Academic Press, Inc.

If phagocytes are stimulated with microbes or soluble reagents such as TPA , the cells integrate NADPH oxidase system on cytoplasmic membrane and generate superoxide anion from which other active oxygens derive (1-4). This system is found in eosinophils as well as in neutrophils, monocytes and B lymphocytes of human peripheral blood (5, 6), and composed of cytochrome b_{558} (p22-*phox* and gp91-*phox*) in membrane and a few soluble proteins (7-9). These cells of patients with CGD cannot generate superoxide anion (10, 11) because of abnormal genes for these proteins (12, 13). The deficiency of

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Abbreviations: TPA, 12-O-tetradecanoylphorbol 13-acetate; CGD, chronic granulomatous disease; FITC, fluorescein isothiocyanate; PE, phycoerythrin; MoAb, monoclonal antibody; CLA, *cypridina* luciferin analog; CL, chemiluminescence; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

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cytochrome b_{558} is most common in CGD and found mainly in males because the gene for its large subunit, gp91-*phox*, is encoded on X chromosome (14). Whenever gp91-*phox* was deficient in neutrophils, monocytes and B lymphocytes of CGD patients, it was also deficient in eosinophils (15), suggesting a common mechanism for the expression of gp91-*phox* in eosinophils as well as in the other leukocytes. We reported here that all peripheral eosinophils of an X-inked CGD patient have a normal superoxide generating activity and definitely express gp91-*phox* in contrast to the remaining leukocytes. This particular case indicates an eosinophil-specific mechanism for the expression of gp91-*phox*. A selective expression of gp91-*phox* was observed in a small subset of neutrophils of three X-linked CGD patients (16), and a reporter gene fused with the 5'-flanking sequences of gp91-*phox* gene was expressed only in monocyte/ macrophage lineage of transgenic mice (17). Our observation is apparently similar to those findings but different from them in the cell type with gp91-*phox*.

Materials and Methods

Patient : He was diagnosed with X-linked CGD because his leukocytes had low CL response to TPA and his daughter's neutrophils were mosaic in the expression of surface cytochrome b_{558} (T. Matsumoto et al., manuscript in preparation).
Antibodies : FITC-conjugated and unconjugated MoAb 7D5 (IgG_1) raised against human cytochrome b_{558} (18). FITC-conjugated goat $F(ab')_2$ against mouse IgG (Tago, Inc., Burligame, CA). PE-conjugated anti-CD14 (Dakopatts, Denmark). FITC-conjugated or PE-conjugated MoAb against CD16, and control monoclonal IgG_1 (Becton Dickinson, San Jose, CA). Polyclonal rabbit antibodies against gp91-*phox* (anti-Lc) and p22-*phox* (anti-Sc) supplied by S. Imajoh-Ohmi (University of Tokyo). MoAb anti-actin (Amersham International, Tokyo). Alkaline phosphatase-conjugated goat antibodies against rabbit IgG and mouse IgG (Promega Corp. Madison, WI).
Reagents : Lucigenin and TPA (Sigma, St. Louis, MO). CLA (Tokyo Kasei, Tokyo). Eagle's minimum essential medium without a pH indicator (Nissui Pharmaceutical CO., Ltd., Tokyo). Other reagents are all in an analytical grade.
Cell Preparation : Heparinized (20U/ml) peripheral blood was withdrawn from healthy volunteers and the patient with prior informed consents. Fractions of granulocytes and mononuclear leukocytes were prepared from the blood as described previously (18). Neutrophils and eosinophils were sorted as $CD16^+$ cells and $CD16^-$ cells, respectively, from the granulocyte fraction on a FACStar PLUS II (Becton Dickinson, La Jolla, CA). Monocytes were sorted from mononuclear leukocyte fractions as $CD14^+$ cells. Separated neutrophils, eosinophils and monocytes were pure more than 99.7 %, 99.7% and 95.0 %, respectively, in morphology on a prestained glass, Blutstan (Dai-ichi Pharmaceuticals, Osaka).
Flow Cytometry : Each sample of whole blood (0.1 ml) was washed once with 1 ml phosphate-buffered saline at room temperature and stained with 7D5 and FITC-conjugated anti-mouse IgG_1 as reported previously (19). Fluorescence was detected from the cells of each region of lymphocytes, monocytes or granulocytes on a FACScan (Becton Dickinson, La Jolla, SA). For two-color analyses, the cells of granulocyte fractions were stained with FITC-conjugated 7D5 and PE-conjugated anti-CD16.
CL Assays : Cells ($1x10^4$) in 1.0 ml of an incubation buffer (dye-free Eagle's minimum essential medium, 20 mM Hepes,100 μM lucigenin or CLA, and 0.03% bovine serum albumin, pH 7.4) were stimulated with 0.3 μg of TPA and assayed for CL on a Multi-Biolumat (Berthold Japan, Tokyo) at 37 ° C (20).
Western Blots: The blots were carried out as reported previously (21). Antigen bands were located by alkaline phosphatase-linked second antibodies.
Northern Blots: Total cell RNA was extracted from mononuclear leukocytes by the single-step isolation method (22). The RNA (1-5μg/ lane) electrophoresed in 1% agarose gel was transblotted to Hybond N^+ nylon membrane (Amersham K. K., Tokyo) and

hybridized with [^{32}P]-labeled probes. As the probes, we used the cDNA for human gp91-*phox*, a kind gift from Dr. S. Imajoh-Ohmi (The University of Tokyo), and the cDNA for human p22-*phox* synthesized by PCR using a reported sequence (23).

Results and Discussion

We analyzed the surface expression of cytochrome b_{558} in the peripheral blood of the patient and a normal control (Fig. 1). In contrast to normal granulocytes (**A**, upper open histogram), most granulocytes of the patient do not express the cytochrome (**A**, lower open histogram with low FITC intensity). However, the minor population of his granulocytes does express it (**A**, lower open histogram with high FITC intensity), as if he were a mosaic CGD carrier female with a few cytochrome b_{558}^+ granulocytes. No cells of his lymphocyte fraction (Fig. 1B) have surface cytochrome b_{558} (Fig. 1B). B lymphocytes of his peripheral blood are then lacking the cytochrome. His monocytes express the cytochrome weakly even if they really do (Fig. 1C).

For defining the type of surface cytochrome b_{558}^+ cells in patient's granulocytes, we simultaneously stained surface cytochrome b_{558} and CD16 of the cells (Fig. 2) . All the surface cytochrome b_{558}^+ granulocytes are CD 16^-, and all the surface cytochrome b_{558}^-

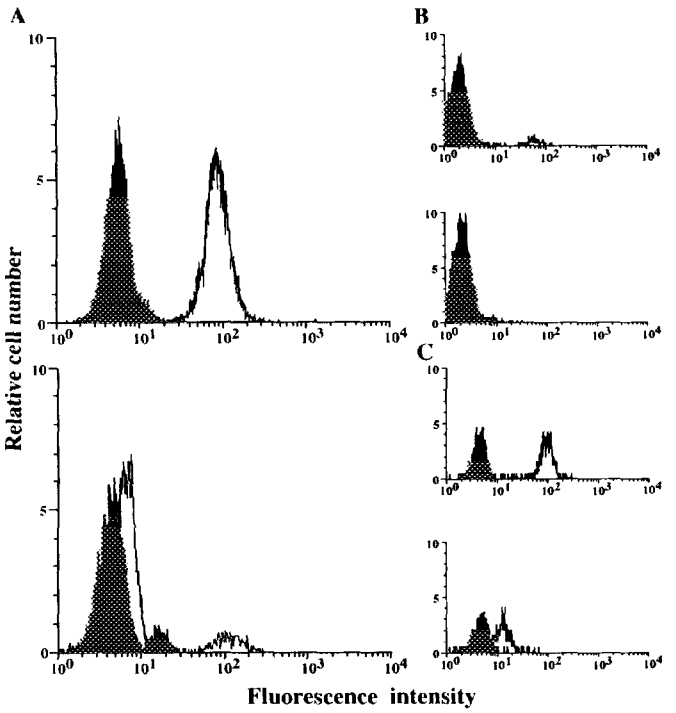

Fig. 1. Surface cytochrome b_{558} on peripheral leukocytes.
Whole bloods of a healthy control (each upper panel) and the patient (each lower panel) incubated with control IgG_1 (closed histograms) or 7D5 (open histograms) were labeled with FITC-conjugated anti-mouse IgG as desribed in the "Materials and Methods." **A**; granulocytes, **B**; lymphocytes, **C**; monocytes. Negative regions of open histograms are mostly hidden behind those of closed ones in **B**.

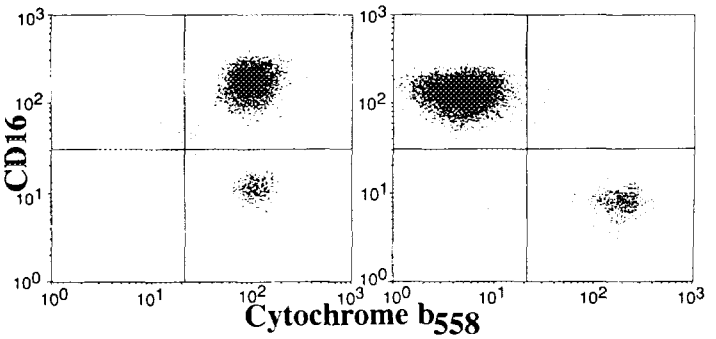

Fig. 2. Two-color stainings of peripheral granulocytes with anti-CD16 and anti-cytochrome b_{558}.
Granulocytes of a healthy control (left panel) and the patient (right panel) were stained with FITC-conjugated anti-cytochrome b_{558} [7D5] and PE-conjugated anti-CD16 and analyzed on a flow cytometer as described in the "Materials and Methods".

cells are CD 16$^+$. We microscopically examined more than 500 cells each of cytochrome b_{558}^+ granulocytes and cytochrome b_{558}^- ones, and found them eosinophils and neutrophils, respectively, without any exceptions (data not shown). Eosinophils but not neutrophils therefore have surface cytochrome b_{558} in patient's granulocytes. The fluorescence intensity of the labeled cytochrome b_{558} of patient's eosinophils is equivalent to that of normal ones (Fig. 2). These results indicate that eosinophils are the only cells with surface cytochrome b_{558} in the peripheral leukocytes of the patient, and that all of the eosinophils are expressing normal amounts of surface cytochrome b_{558}.

We examined the expression level of gp91-*phox* in peripheral leukocytes by western blots (Fig. 3A). Patient's eosinophils (Eo), but not neutrophils (Ne) and monocytes (Mo), exhibit the specific band of gp91-*phox* that is electrophoresed to the normal position for the protein. Three times more neutrophils still failed in showing a gp91-*phox* band (data not shown). Therefore eosinophils are the only cells that definitely have gp91-*phox* in the patient's peripheral blood, and the protein of his eosinophils seems normal in size. The content of gp91-*phox* in patient's eosinophils is about one-third of that in control cells (lanes 1&2 of Eo), which may be caused by the same genetic defect that makes the other cells lack the protein.

We assayed superoxide-generating activities of peripheral leukocytes (Table I). Neither neutrophils nor monocytes of the patient show CL response upon stimulation with TPA as expected from the deficiencies of gp91-*phox* and surface cytochrome b_{558} in these cells. His eosinophils, however, exhibit the response to a normal extent regardless the enhancer for CL response is lucigenin (*Experiment I*) or CLA (*Experiment II*). Therefore gp91-*phox* of his eosinophils is functionally normal and that the expression level of gp91-*phox* is enough to show a normal superoxide-generating activity in the cells.

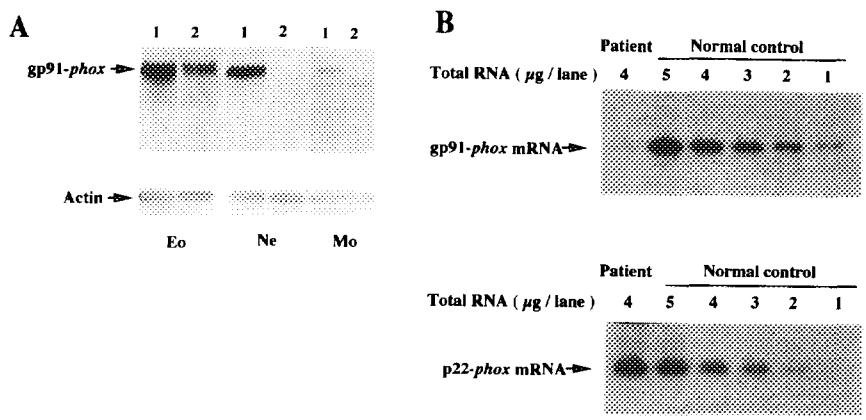

Fig. 3. Western blots of gp91-*phox* (**A**) and northern blots of gp91-*phox* mRNA (**B**) of peripheral leukocytes.
(**A**) Whole cell extracts were obtained from eosinophils (Eo), neutrophils (Ne) and monocytes (Mo) of a healthy control (1) and the patient (2) and subjected to SDS-PAGE (1 x 10^5 cells/lane) as described in "Materials and Methods". Indirectly immunostained gp91-*phox* and actin are indicated by arrows. (**B**) RNAs extracted from the mononuclear leukocyte fractions of a normal control and the patient were applied to lanes at the amounts (1-5 μg/lane) specified by the numbers. Blotted RNAs were hybridized with either one of cDNAs for human gp91-*phox* (upper panel) or p22-*phox* (lower panel).

To find out the basis of this gp91-*phox* deficiency, we have compared the content and the size of gp91-*phox* mRNA in patient's leukocytes with those in control ones by northern blots. The content of the mRNA in patient's mononuclear leukocytes is less than 10% of that of control cells (Fig. 3B), but the size of it seems identical to that in control cells. As monocytes are the major source of the RNA in mononuclear leukocytes, this result indicates that his monocytes contain quite low amounts of gp91-*phox* mRNA. We also observed the decrease of the mRNA in the neutrophils and EB-transformed B lymphoblasts of the patient (data not shown). Therefore the low content of mRNA has primarily caused the deficiency of gp91-*phox* in neutrophils, monocytes and B lymphocytes of the patient. We did not observe the content of the mRNA in patient's eosinophils because of limited number of the cells. We sequenced coding regions and a putative poly (A) tail signal (24) of his gp91-*phox* gene, and the coding regions of the gp91-*phox* mRNA of his leukocytes, and found no abnormalities (data not shown). A normal amount of p22-*phox* mRNA of normal size in patient's leukocytes supports our diagnosis of his disease as an X-linked CGD (lower panel of Fig. 3B).

This particular case of CGD indicates that eosinophils can have normal superoxide generating system even under a genetic defect which results in the lacks of gp91-*phox* and NADPH oxidase activity in monocytes, neutrophils and B lymphocytes. Therefore eosinophils should have their own regulatory mechanism for gp91-*phox* expression. A transcriptional event may be critical for the expression because of the following two

Table I. CL responses of phagocytes to TPA

	Normal		Patient	
	Peak ($x10^{-6}$)	Integral ($x10^{-7}$)	Peak ($x10^{-6}$)	Integral ($x10^{-7}$)
Experiment I				
Eosinophils	30.4	73.2	66.0	74.3
Neutrophils	12.3	40.3	<0.1	<0.1
Monocytes	11.0	15.4	<0.1	<0.1
Experiment II				
Eosinophils	20.0	35.9	19.2	36.0

Purified cells ($1x10^4$/tube) suspended in 1 ml of assay mixture were stimulated with 0.3 μg TPA at 37 °C as described in "Materials and Methods". Peak and integral counts are expressed by cpm , and cpm/30 min (*Experiment I*) or cpm/25 min (*Experiment II*), respectively. CL was enhanced by lucigenin and CLA in *Experiment I* and *II*, respectively.

reasons. 1. The content of gp91-*phox* mRNA was quite low, but the size and the coding region sequences of it were normal in the affected leukocytes of the patient. 2. A putative poly(A) tail signal that is related to the stability of mRNAs was normal in his gp91-*phox* gene. Molecular analyses are now in progress in our laboratory for elucidating the mechanism which permits the distinct expression of gp91-*phox* gene in eosinophils.

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