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## Abstract

The O<sub>2</sub><sup>-</sup> production by neutrophils was examined in 4 cases of refractory anemia with excess of blasts (RAEB) in order to evaluate the possible causes of enhanced susceptibility to infection and to gain some informations on the differentiation of neutrophils in this hematological disorder. In three of the four RAEB cases there was little O<sub>2</sub><sup>-</sup> production by neutrophils, in addition to there being morphological anomalies of the neutrophils such as a Pelger-Huet-like anomaly, granular deficiency and binucleated cells. These results suggest that the impairment of O<sub>2</sub><sup>-</sup> production by neutrophils in RAEB is one of the possible causes of susceptibility to infection and also suggest that the differentiation of neutrophils in this hematological disorder is faulty. The estimation of O<sub>2</sub><sup>-</sup> production by neutrophils may be a useful diagnostic method for preleukemia.

**KEYWORDS:** superoxide anion, refractory anemia with excess of blasts (RAEB), preleukemia

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## SUPEROXIDE ANION (O<sub>2</sub><sup>-</sup>) PRODUCTION BY NEUTROPHILS IN REFRACTORY ANEMIA WITH EXCESS OF BLASTS

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*Abstract.* The O<sub>2</sub><sup>-</sup> production by neutrophils was examined in 4 cases of refractory anemia with excess of blasts (RAEB) in order to evaluate the possible causes of enhanced susceptibility to infection and to gain some informations on the differentiation of neutrophils in this hematological disorder. In three of the four RAEB cases there was little O<sub>2</sub><sup>-</sup> production by neutrophils, in addition to there being morphological anomalies of the neutrophils such as a Pelger-Huet-like anomaly, granular deficiency and binucleated cells. These results suggest that the impairment of O<sub>2</sub><sup>-</sup> production by neutrophils in RAEB is one of the possible causes of susceptibility to infection and also suggest that the differentiation of neutrophils in this hematological disorder is faulty. The estimation of O<sub>2</sub><sup>-</sup> production by neutrophils may be a useful diagnostic method for preleukemia.

*Key words :* superoxide anion, refractory anemia with excess of blasts (RAEB), preleukemia.

Refractory anemia with excess of blasts (RAEB) is a myelodysplastic syndrome discussed with keen interest in relation to preleukemia and acute leukemia (1-3). RAEB is characterized by refractory anemia, a slight to moderate increase in blasts in the peripheral blood and bone marrow, and patients occasionally die from infection in the absence of severe neutropenia prior to overt leukemia.

The superoxide anion (O<sub>2</sub><sup>-</sup>) is related to the intracellular bactericidal activity of neutrophils, particularly in an oxygen dependent system (4, 5). Recently, the measurement of O<sub>2</sub><sup>-</sup> production by neutrophils has been widely applied to the clinical diagnosis of chronic granulomatous disease (CGD), in which neutrophils are defective in their intracellular killing activities in spite of having normal phagocytic capability. CGD patients suffer from severe and recurrent bacterial infections caused by *Staphylococcus aureus* and gram-negative rods (6, 7).

These facts led us to examine the O<sub>2</sub><sup>-</sup> production by RAEB neutrophils in order to evaluate the possible causes of enhanced susceptibility to infection and also to gain some informations on the functional defects of neutrophils in this hematological disorder.

## PATIENTS AND METHODS

*Patients.* Two male and 2 female patients with RAEB were studied. Their ages varied from 50 to 78, with a median of 75 years. The diagnosis of RAEB was made from hematological findings.

*Neutrophil preparation.* Neutrophils were isolated from heparinized venous blood. One volume of 6 % dextran in physiological saline was mixed with 5 volumes of blood and allowed to settle at room temperature. After sedimentation of the majority of the red blood cells, neutrophils were further isolated by the Ficoll-Hypaque gradient method and the remaining red blood cells were hemolyzed in hypotonic solution. After two centrifugations (4°C, 10 min at 150 g) and washings with Krebs-Ringer phosphate (KRP ; pH 7.4) solution, a differential cell count was made. The neutrophils in the KRP solution were stored in a ice box until use. More than 95 % of the cells were viable as assessed by the trypan blue dye exclusion method.

*Measurement of O<sub>2</sub> production.* Release of O<sub>2</sub> from neutrophils was measured by essentially the same method as that described by Nakagawara (6). The reaction mixture consisted of 0.05 mM glucose, 65 μM ferricytochrome C and the cells (approximately 2 × 10<sup>5</sup> cells) in 2.0 ml of KRP solution. Concanavalin A and cytochalasin D were added simultaneously to the reaction mixture at final concentration of 100 μg/ml and 20 μg/ml, respectively. The reduction of cytochrome C was measured continuously by a double beam spectrophotometer UV-210 A (Shimadzu Co. Ltd. Tokyo) at 550 nm. The rate of O<sub>2</sub> production expressed in terms of nmol cytochrome C reduced/min/10<sup>6</sup> neutrophils (band + segmented).

*In vitro assay of bactericidal activity.* Bactericidal activity of neutrophils was examined according to Quie's method (8) using *Streptococcus faecalis* and *Staphylococcus aureus*. Neutrophils obtained from a healthy 32-year-old male were used as a control.

## RESULTS AND DISCUSSION

The rate of O<sub>2</sub> production by neutrophils and hematological findings are summarized in Table 1. All patients showed anemia and thrombocytopenia. Leukocytopenia was found in 2 of the 4 cases. Neutrophils in the peripheral blood showed some morphological anomalies : binucleated cells and granular deficiency in Case 1, a Pelger-Hüet-like anomaly and granular deficiency in Case 3 and binucleated cells in Case 4.

In the bone marrow, a slight increase in myeloblasts, ranging from 3.0 to 14.8 %, was found. Sideroblasts were also increased in number and ringed sideroblasts were found in Case 3. Myeloperoxidase (MPO) satining was done in Cases 1, 2 and 3, and 15 % of the neutrophils were found to be MPO negative in Case 1 and 18 % in Case 3. The neutrophil alkaline phosphatase (NAP) score was low in Case 1.

The O<sub>2</sub> production by neutrophils was 6.45 ± 1.33 nmol/min/10<sup>6</sup> neutrophils in healthy individuals (12). On the other hand, little O<sub>2</sub> production by neutrophils was detected in Cases 1, 3 and 4, in which there were also morphological anomalies of the neutrophils. The bactericidal activity of neutrophils in Case 1 was impaired (Fig. 1).

The production of active oxygen, such as O<sub>2</sub>, hydroxyl radical (OH●), singlet

TABLE I. RATE OF O<sub>2</sub><sup>-</sup> PRODUCTION BY NEUTROPHILS AND HEMATOLOGICAL FINDINGS

	Case 1 (50, F) <sup>a</sup>	Case 2 (72, M)	Case 3 (78, F)	Case 4 (78, M)
Peripheral blood				
RBC (× 10 <sup>4</sup> /mm <sup>3</sup> )	233	223	190	233
WBC (/mm <sup>3</sup> )	2,000	3,600	5,900	9,300
Thr (× 10 <sup>4</sup> /mm <sup>3</sup> )	6.8	3.6	2.7	1.6
Blast (%)	1.0	0	5.0	2.0
Bone marrow				
NCC (× 10 <sup>4</sup> /mm <sup>3</sup> )	22.0	7.6	10.5	18.0
Erythroblast (%)	2.0	48.0	51.4	5.2
Blast (%)	8.8	14.8	3.0	8.4
Sideroblast (%)	92.0	68.0	100.0 (Ringed : 3%)	100.0
Chromosome analysis	t(9p <sup>-</sup> ,16q <sup>+</sup> )	n.d	n.d	n.d
Morphological anomalies of neutrophil	Binucleated Granular deficiency	(-)	Pelger-Huet-like Granular deficiency	Binucleated
MPO negative neutrophils (%)	15.0	0	18.0	n.d
NAP score	4	414	413	n.d
O <sub>2</sub> <sup>-</sup> production (nmol/min/10 <sup>6</sup> neutrophils)				
1st Exp	0.13	5.91	0	0
2nd Exp	0.35	8.30	0	0

<sup>a</sup>: Number and alphabet in parentheses indicate age and sex in the order of the case.

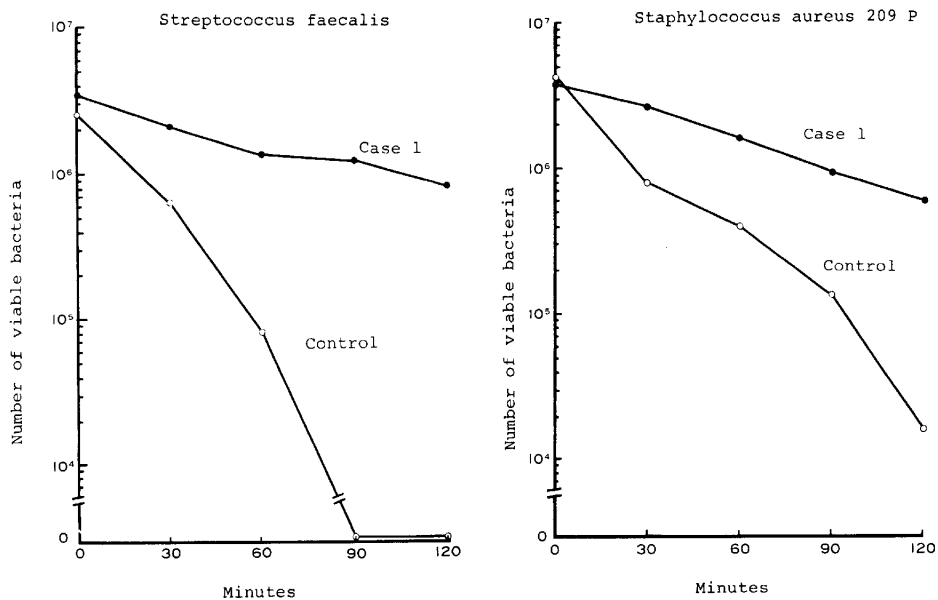


Fig. 1. Bactericidal Activity of Neutrophils *in vitro*.

oxygen ( $^1\text{O}_2$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), has been shown to be one metabolic event in phagocytosing neutrophils (4, 5, 9, 10). Neutrophils have been found to exhibit microbicidal activities independently or in the form of  $\text{H}_2\text{O}_2$ -MPO-halides (11). The release of  $\text{O}_2^-$  from phagocytosing neutrophils was first demonstrated by Babior *et al.* and it was found to be low in patients with CGD, who suffer from severe and recurrent infection. The impairment of neutrophil  $\text{O}_2^-$  production in RAEB is thought to be one of the possible causes of the inability of patients to prevent and combat infection.

The mechanism by which  $\text{O}_2^-$  production was impaired in RAEB was not clearly disclosed. However, previous reports from our laboratory have shown that blasts in acute leukemia released little  $\text{O}_2^-$  by the stimulation of concanavalin A and cytochalasin D (12). Furthermore, from electron microscopic findings, the presence of two populations of neutrophils, derived from normal and leukemic clones, was suggested in acute myelocytic leukemia (13). Therefore, there being little  $\text{O}_2^-$  production by neutrophils in RAEB may be due to a faulty differentiation of neutrophils, probably from a leukemic clone.

Schreiner *et al.* (14, 15) reported that bactericidal activities were impaired in the preleukemic or early stage of acute leukemia. Studies of neutrophils function, including the production of active oxygen, will be useful as diagnostic procedures for the preleukemic stage, and such studies are also expected to yield some information on the functional differentiation of neutrophils in RAEB.

One of the 4 RAEB cases was normal in regard to  $\text{O}_2^-$  production by neutrophils. Studies of whether impairment of  $\text{O}_2^-$  production differ among patients with RAEB, as seen in chronic myelocytic leukemia (16), whether RAEB is a heterogenous entity and whether  $\text{O}_2^-$  production differs during the clinical course are under way.

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