Host Defense Activity in Various Hosts*
Human Neutrophil NADPH Oxidase Activity
Shigenobu Umeki, M.D., F.C.C.P; and Rinzo Soejima, M.D., F.C.C.P

The superoxide generation of neutrophil NADPH oxidase from healthy subjects, patients with respiratory infections, and patients receiving effective therapy with antibiotics or steroids was investigated. In young healthy nonsmokers the mean oxidase activity of neutrophils in women was significantly lower than that in men. In healthy women the mean oxidase activity was significantly lower in young nonsmokers than in young smokers or the elderly. In young nonsmokers, oxidase activity significantly increased during respiratory infections; however, in elderly nonsmokers, no significant increase in oxidase activity was observed during respiratory infections. The mean oxidase activity in patients receiving steroids was very low. In vitro experiments using cell-free activation systems of NADPH oxidase, steroids were found to injure the membrane-bound components of the oxidase enzyme. These results suggest that decreased superoxide generation in patients receiving steroids may result from steroid-induced damage in the membrane-bound components of the NADPH oxidase system. The inhibitory effect of steroids on superoxide production may reduce bactericidal action of neutrophils; i.e., one defense mechanism of the body against many kinds of pathogens. Therefore, long-term therapy with steroids in the elderly should be avoided at all costs.  (Chest 1992; 102:1780-86)

As the respiratory tract is continuously exposed to organisms or particles in inhaled air, there is a need for appropriate defense mechanisms to prevent injurious and infectious processes. The phagocytic system plays an important role in the clearance of small inhaled particulate materials and microorganisms reaching the periphery of the lung. The contribution of phagocytes to the lung host defense is well illustrated by the high frequency of pneumonia observed in severely neutropenic patients or in cases of lung macrophage dysfunction.1 Neutrophils, the predominant phagocytes of circulating blood, are the first cells to arrive at sites of infection and have a bactericidal action in their generation of oxygen free radicals,2 including O2-, H2O2, and OH-. Superoxide is produced primarily through the activation of plasma membrane-bound NADPH oxidase (respiratory burst oxidase [EC 1.6.99.6]) by stimulation from phagocytizable particles or soluble agents.3 The superoxide production of neutrophils has been shown to be modified by various conditions, such as infections,4 steroid treatment,7 and a compromised host, eg, chronic granulomatous disease.8 We previously reported an imbalance between the levels of superoxide and its scavenger (superoxide dismutase [SOD]) in immunocompromised hosts; however, little information is available about the host defense mechanisms involved in neutrophil NADPH oxidase in various hosts, including immunocompromised hosts.

In the present study the superoxide generation of neutrophil NADPH oxidase from healthy subjects, patients with respiratory infections, and patients receiving effective therapy with antibiotics or steroids was investigated. In addition, in vitro experiments using cell-free activation systems of NADPH oxidase, steroid-induced modification of the oxidase was studied.

MATERIALS AND METHODS

Subjects
Table 1 shows the profiles of 50 healthy subjects and 34 patients. The healthy subjects included 37 young adults (22 nonsmokers and 15 smokers) aged less than 40 years and 13 elderly adults (all nonsmokers including ex-smokers) aged 70 years or more. "Ex-smoker" means a person who has not smoked for 1 year or more. The groups of patients included 15 young patients (seven male and eight female patients) aged less than 40 years, 13 elderly patients aged 70 years or more, and six steroid-treated patients.

Materials
Equine cardiac cytochrome c (type 3), bovine erythrocyte SOD, β-NADPH (type I), sodium deoxycholate, diisopropyl fluorophosphate (DFP), ATP, EGTA, PIPES, phorbol 12-myristate 13-acetate (PMA), Dulbecco's calcium-free and magnesium-free phosphate-buffered saline (PBS), Hanks' balanced salt solution (HBSS), glyceral, sucrose, and dextran (average mol wt, 78,000) were obtained (Sigma). Sodium deoxycholate was recrystallized from ethanol before use. Ficoll-Hypaque and Percoll were obtained (Pharmacia P-L Biochemicals). Other chemicals were of the highest purity available from commercial sources.

Cell Isolation and Subfractionation
Samples of venous blood were drawn from all subjects under stable (resting) and fasting conditions in the morning and were immediately employed. Human neutrophils of 90 percent purity or

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greater were prepared from healthy subjects and patients as previously described.2-18 Isolated neutrophils were treated with DFP by the method of Crowley et al.2 using acid citrate dextrose as the anticoagulant, dextran to sediment erythrocytes, and Ficoll-Hypaque to separate mononuclear cells and lymphocytes from neutrophils. Isolated neutrophils were treated with DFP by the method of Crowley et al.2 with slight modification: i.e., phosphate was omitted from the buffer, and the DFP concentration was reduced to 2.5 mM. Cells treated with DFP were washed two times with Dulbecco's PBS. The resulting cells were homogenized by nitrogen cavitation and fractionated on a discontinuous Percoll gradient by a modification of the method of Borregaard et al.19 In experiments for in vitro treatment, neutrophils (5 × 10⁶/ml in PBS; total volume of 8 ml) isolated from normal subjects were treated with a final concentration of 0.1 mM hydrocortisone sodium succinate for 20 min at 37°C prior to DFP treatment. Hydrocortisone-treated cells were washed two times with the buffer and immediately treated with DFP. Solubilized membranes and cytosolic fractions were prepared from resting and steroid-treated neutrophils as previously described.19-22 The protein concentration was determined according to the method of Lowry et al.23 with bovine serum albumin as the standard. The protein concentrations were as follows: solubilized membranes from resting neutrophils, 31.0 μg/10⁶ cells ± 1.8 μg/10⁶ cells (mean ± SD; n = 3); cytosolic fractions from resting neutrophils, 155 μg/10⁶ cells ± 15 μg/10⁶ cells (n = 3); solubilized membranes from steroid-treated neutrophils, 28.9 μg/10⁶ cells ± 2.1 μg/10⁶ cells (N = 3); and cytosolic fractions from steroid-treated neutrophils, 148 μg/10⁶ cells ± 12 μg/10⁶ cells (N = 3).

### Table 1—Profiles of Patients

<table>
<thead>
<tr>
<th>Subjects</th>
<th>No. of Subjects</th>
<th>Mean Age, yr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy subjects</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young men</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonsmokers</td>
<td>8</td>
<td>28</td>
</tr>
<tr>
<td>Smokers</td>
<td>9</td>
<td>30</td>
</tr>
<tr>
<td>Young women</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonsmokers</td>
<td>14</td>
<td>25</td>
</tr>
<tr>
<td>Smokers</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td>Elderly nonsmokers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>7</td>
<td>76</td>
</tr>
<tr>
<td>Women</td>
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<td>74</td>
</tr>
<tr>
<td>Patients</td>
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<td></td>
</tr>
<tr>
<td>Young men</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonsmokers</td>
<td>7</td>
<td>31</td>
</tr>
<tr>
<td>Young women</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonsmokers</td>
<td>6</td>
<td>28</td>
</tr>
<tr>
<td>Smokers</td>
<td>2</td>
<td>28</td>
</tr>
<tr>
<td>Elderly nonsmokers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steroid-treated</td>
<td>6</td>
<td>55</td>
</tr>
<tr>
<td>Total</td>
<td>84</td>
<td></td>
</tr>
</tbody>
</table>

*Includes ex-smokers.

NADPH Oxidase Activity in Whole-Cell Systems

Superoxide production by intact neutrophils stimulated by PMA was measured following SOD-inhibitable reduction of cytochrome c at 550 nm.2-18 Neutrophils (2 × 10⁷ cells per cuvette) were incubated in an assay HBSS medium containing 0.12 mM cytochrome c for 2 min at 37°C before the reactions were initiated by adding PMA (0.3 μg per cuvette). Assay mixtures were incubated for 4 min at 37°C in a total volume of 1.0 ml. The reference cuvette also received 20 μg of SOD.

Activation of NADPH Oxidase in Cell-Free Systems

Superoxide production in cell-free systems was assayed as previously described.2,7,10-18 Assay mixtures contained 0.1 mM cytochrome c, 3.6 mM MgCl₂, 80 mM KCl, 2.7 mM NaCl, 0.5 mM PIPES (pH 7.3), 0.9 mM ATP, 1.2 mM EGTA, 0.5 μM flavin adenine dinucleotide, 6 × 10⁶ cells of cytosolic fractions, 1.5 × 10⁶ cells, and 20 μM hydrocortisone sodium succinate (pH 7.3). PMA (0.3 μg) was added to start the reaction (pH 7.3) and was terminated by addition of ethanol.

Figure 1. Effects of smoking on superoxide generation of neutrophils from young healthy subjects. Each value is the mean of two different experiments. Open circles, PMA-stimulated NADPH oxidase activity; closed circles, resting NADPH oxidase activity.
cells of membranes solubilized in deoxycholate (0.94 mM), 0.04 mM sodium dodecyl sulfate (SDS), and 0.16 mM NADPH, in a total volume of 0.75 ml. The reference cuvette contained 40 μg of SOD. Basically, all of the constituents except NADPH were mixed in the cuvette and then were placed in the reference and sample cuvettes. Absorbance at 550 nm was followed for 3 min at room temperature (23°C to 24°C). Then the reactions were started by adding 25 μl of NADPH solution to each cuvette, and the change in absorbance at 550 nm was followed for 3 to 5 min on a double-beam spectrophotometer (Cary model 118). Superoxide production was calculated using an extinction coefficient from Yonetani, as follows:

\[ E_{\text{ext}}^{550\text{ nm}} = 19.6 \text{mM}^{-1} \text{ cm}^{-1}. \]

**Statistical Analysis**

Comparison of variables was made with Student’s t-test. Statistical analyses were made by calculating the mean (± SD) in each group and the standard error of the differences between means. All results were considered statistically significant when two-tailed p<0.05.

**RESULTS**

Figure 1 shows the effects of smoking on the superoxide generation of neutrophils exposed to PMA (whole-cell system) in young healthy subjects. The mean NADPH oxidase activity was significantly lower in female nonsmokers than in male nonsmokers or female smokers; however, there was no significant difference between the mean oxidase activities in male nonsmokers and male smokers. There were no significant changes in the oxidase activities of resting neutrophils of any of the young healthy subjects.

Figure 2 shows NADPH oxidase activities in neutrophils from elderly healthy nonsmokers aged 70 years or more. The mean oxidase activity of neutrophils with or without exposure to PMA in elderly men was almost similar to that in elderly women.

Figure 3 shows the effects of aging on the superoxide generation of neutrophil NADPH oxidase in the whole-cell system from healthy nonsmokers and ex-smokers. In healthy women, the mean oxidase activity was significantly lower in the young than in the elderly. There was no significant difference between oxidase activities in young and elderly men, but the oxidase activities in elderly men tended to be higher.

Figure 4 shows the influences of respiratory infections and antibiotic therapy on the superoxide production of neutrophil NADPH oxidase in the whole-cell system from healthy subjects and patients. In young patients with respiratory infections, the oxidase activities of neutrophils exposed to PMA significantly increased as compared with those in young healthy subjects. In young male patients, the oxidase activities of resting neutrophils also significantly increased as compared with those in young healthy men; however,
in elderly patients, the oxidase activities of neutrophils with and without exposure to PMA did not significantly increase after their respiratory infections. The oxidase activities of neutrophils from patients receiving effective therapy with antibiotics significantly decreased as compared with those from elderly healthy subjects and elderly patients with respiratory infections.

Table 2 shows three inflammatory factors in patients with respiratory infections before and after effective chemotherapy. Although white blood cell counts, neutrophil counts, and C-reactive protein levels were very high in both young and elderly patients, after each round of chemotherapy with effective antibiotics, the levels returned to within normal limits.

Figure 5 shows changes in the NADPH oxidase activities in the whole-cell system of patients receiving steroid therapy before and after therapy. The mean oxidase activity (8.5 nmol/10^6 cells per minute) of neutrophils from patients receiving steroids (30 mg of prednisolone per day for at least 2 wk) greatly decreased as compared with the value before treatment (22.5 nmol/10^6 cells per minute). There were no significant differences between the oxidase activities of resting neutrophils from patients before and after steroid therapy, but those in patients receiving steroids tended to be higher than those in patients before steroid therapy.

Table 3 shows the superoxide production of resting (control) or steroid-treated neutrophil membrane-bound enzyme in an in vitro cell-free system in which both cytosolic fractions were mixed together. Although the superoxide production of control neutrophils in a complete cell-free system was 21.4 ± 2.9 nmol/10^7 cells per minute (mean ± SD), that of steroid-treated neutrophils in the cell-free system containing steroid-treated solubilized membranes and cytosolic fractions

Table 2—Laboratory Data of Patients with Respiratory Infections*

<table>
<thead>
<tr>
<th>Data</th>
<th>Before Treatment</th>
<th>After Treatment†</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBC†</td>
<td>13,200 ± 2,810</td>
<td>6,710 ± 1,560</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Neutrophils†</td>
<td>10,890 ± 2,355</td>
<td>4,160 ± 1,113</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C-reactive protein, mg/dl</td>
<td>16.2 ± 10.4</td>
<td>0.8 ± 0.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Elderly patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBC†</td>
<td>11,150 ± 2,402</td>
<td>7,207 ± 1,474</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Neutrophils†</td>
<td>8,617 ± 2,272</td>
<td>4,766 ± 1,360</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C-reactive protein, mg/dl</td>
<td>11.5 ± 9.4</td>
<td>1.0 ± 0.5</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Table data are means ± SD.
†Effective antibiotics were used.
‡Per cubic millimeter.
was greatly diminished (3.2 ± 0.9 nmol/10⁶ cells per minute, or 15 percent of the control). This defect in oxidase activation was not due to any abnormality in the cytosolic fractions, as the steroid-treated neutrophil membrane-bound enzyme was not activated by adding control neutrophil cytosolic fractions, but the steroid-treated neutrophil cytosolic fractions were capable of producing superoxide at a nearly normal rate when activated in the presence of control neutrophil membrane fractions. Instead, there was a marked defect in the membrane-bound enzyme of the steroid-treated neutrophils.

**DISCUSSION**

Considerable progress has recently been made in defining the cellular and molecular mechanisms involved in host resistance to infection. Virtually every decision related to antibiotic therapy is influenced by an assessment of the integrity of these resistance mechanisms. Defects in each major aspect of host defense (that is, neutrophil defense, humoral immunity, and cell-mediated immunity) increase the risk of infection caused by specific groups of microorganisms. The first line of defense against potential pathogens is provided by circulating neutrophils, but neutrophils are also important in the pathogenesis of inflammation or tissue damage in certain postinfectious or noninfectious diseases. These cells are activated by phagocytic stimuli or high amounts of microbial oxidants. This process, called the phagocytic respiratory burst, results in the reduction of molecular oxygen to superoxide. The respiratory burst requires a series of electron transfers using NADPH as the electron donor, and it involves a flavin-adenine dinucleotide-containing flavoprotein and a unique cytochrome b⁵₅₆ on neutrophil plasma membranes. Recently, Volpp et al. reported that activation of the membrane-bound oxidase enzyme requires at least two cytosolic factors, each shown to be missing in chronic granulomatous disease. The superoxide is rapidly converted to hydroxyl peroxide and hydroxyl radicals, which provide most of the microbial oxidative activity within the phagosome and extracellular environment. The superoxide production of neutrophils has been shown to be modified by a wide variety of conditions, including bacterial infections, steroid treatment, and immunocompromised hosts, e.g., chronic granulomatous disease.

The results obtained here suggest that the oxidase activity in young female nonsmokers is the lowest and that in healthy female nonsmokers the oxidase activity is increased by advancing age or smoking. Because NADPH oxidase is very sensitive to many kinds of stress, these results tempt us to conclude that in young healthy female nonsmokers, circulating neutrophils receive little stress endogenously and exogenously, and their intracellular and extracellular environments in the body are very comfortable. This suggests that oxidase activity increases with advancing age or due to cigarette smoking. These results also suggest that aging and smoking are stressor factors in the extracellular environment of neutrophils. Aging is a ubiquitous biologic phenomenon associated with histologic, biochemical, metabolic, and functional

Table 3—Cell-Free Activation of NADPH Oxidase in Steroid-Treated Neutrophils

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Membranes</th>
<th>Cytosol</th>
<th>O₂⁻ Generation, nmol/10⁶ cells/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Control</td>
<td>21.4 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>Steroid-treated</td>
<td>Steroid-treated</td>
<td>3.2 ± 0.9†</td>
<td></td>
</tr>
<tr>
<td>Steroid-treated</td>
<td>Control</td>
<td>3.9 ± 1.2†</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Steroid-treated</td>
<td>20.5 ± 4.2</td>
<td></td>
</tr>
</tbody>
</table>

*Results are means (± SD) of three different experiments. p < 0.001 compared with value, 21.4 ± 2.9.
alterations. A decline in functional capacity can be
ascribed to a progressive loss with the aging of
functional tissue cells and a deterioration of the
extracellular environments. In humans, increasing age
has been associated with a high incidence of hematol-
logic abnormalities. Aging may lead to a preliminary
condition of disease as a result of an increase in
cholesterol and arachidonic acid (C20:4) levels in the
plasma. In addition, oxygen free radicals are also
implicated in the aging process. Aging can be viewed as
a process of irreversible changes related to accumu-
lization of this oxidative damage in and out of the
cell. On the other hand, cigarette smoking causes a
peripheral leukocytosis, increased influx of phago-
cytes into the lungs, and metabolic hyperreactivity
of these cells in both the bloodstream and the lungs.
Anderson et al found that relative to nonsmokers' neutrophils, the cells from smokers are hyperreactive
to N-formyl-methionyl-leucyl-phenylalanine stimulation,
with increased generation of both extracellular
and intracellular oxygen free radicals. Gillespie et al demonstrated the presence of chemotaxis and
superoxide production by neutrophils in nicotine-treated
and smoke-exposed rats. Alveolar macrophages in
smokers also exhibit numerous morphologic and func-
tional differences as compared with nonsmokers' cells,
including increased phagolysosomes and increased
production of oxygen free radicals. In the present
study the superoxide production of NADPH oxidase
also tended to be enhanced with advancing age and
by cigarette smoking in healthy male subjects; how-
ever, Holt reported that cigarette smoking produces a
decrease in complement-mediated phagocytosis. In
studies on the effects of female sex hormones on
superoxide generation of neutrophils, Buyon et al reported that female sex hormones inhibit in vitro the
superoxide generation of neutrophils and that neutro-
phils isolated from women during various phases of
the menstrual cycle and during the third trimester of
pregnancy do not differ with respect to chemotactic
peptide-stimulated superoxide generation. In the pre-
sent study cigarette smoking may diminish the inhibi-
tory effect of female sex hormones on the superoxide
production of neutrophils from young women and may
enhance the superoxide production.

The results also showed that in elderly nonsmokers
with respiratory infections, no significant increase in
oxidase activity occurred during these infections,
possibly due to the high activity levels of neutrophils
in these nonsmokers. This indicates that in elderly
subjects the NADPH oxidase activity of neutrophils
exposed to bacterial stimuli is similar to that of
neutrophils not exposed to bacterial stimuli. The
reason why the oxidase activity in the elderly patients
did not increase during respiratory infections remains
unclear; however, in these patients, laboratory data
during the period before treatment revealed acute
inflammatory reactions, including increases in neutro-
phil counts and C-reactive protein levels. Effective
antibiotic therapy produced a great decrease in oxidase
activity, with improvement of other inflammatory
factors, indicating that the levels of oxidase activity of
the elderly might be sufficient to kill invading bacteria
during respiratory infections. The decreased activity
of neutrophils in patients receiving treatment was
considered to be due to a reducible effect of antibiotics
on NADPH oxidase; however, in in vitro experiments,
several antibiotics, such as imipenem, cefazidime,
and cefoperazone, at each therapeutic dose did not
inhibit the superoxide generation of the oxidase in the
whole-cell system (data not shown). These results
suggest that these antibiotics may normalize neutro-
phil NADPH oxidase activity, resulting from their
bactericidal action and a possible biologic action by
which they normalize any of perineutrophil environ-
ment of the body. It is clinically a big problem that
the increased rates of the oxidase activity and other
inflammatory factors in elderly patients with respira-
tory infections were much lower than those in young
patients with respiratory infections. Our previous
study indicated that the superoxide-scavenger system
is reduced with advancing age and is further injured
by respiratory infections and that among all subjects,
this system functions most poorly in elderly patients
with respiratory infections.

It is well known that steroids modify the biochemical
events associated with phagocytosis. A number of
phagocytic mechanisms are inhibited by steroids, and
there is good evidence that the clearance of particulate
substances by the reticuloendothelial system in ster-
oid-treatment animals is impaired. Superoxide genera-
tion by phagocytes, such as neutrophils and
macrophages, depends on lipoxygenase activity, and
steroids inhibit phospholipase activity and the pro-
duction of prostaglandins and leukotriene B4 via
the synthesis and release of lipoctin. Buyon et al and
Goldstein et al reported that steroids inhibit the
superoxide production of stimulated neutrophils in
whole-cell systems. We recently reported that steroids
inhibit the reconstitution (activation) of NADPH oxida-
ase by SDS in a cell-free system and that they do not
alter the affinity to the substrate NADPH of the
oxidase enzyme. In the present study, long-term
steroid therapy produced a great decrease in NADPH
oxidase activity, due to the injurious effect of the
steroids on the membrane-bound components of the
oxidase. Oxygen free radicals derived from superoxide
generated by neutrophils are essential to kill exoge-
nous bacteria and maintain homeostasis of the body.
The inhibitory effect of steroids on superoxide pro-
duction may reduce the bactericidal action of neutro-
phils, i.e., one defense mechanism of the body against

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many kinds of pathogens. In particular, long-term therapy with steroids in the elderly should be avoided at all costs.

Human neutrophils play a crucial role in the host defense mechanisms against a wide variety of invading microorganisms. We consider the measurement of the NADPH oxidase activity of neutrophils from various hosts (or patients) to be potentially useful and valuable for estimating the host defense activity of immunocompromised hosts; however, for a better understanding of the relationship between NADPH oxidase activity and the host defense activity of immunocompromised hosts, further studies regarding the activation mechanisms of the NADPH oxidase of neutrophils from immunocompromised hosts in cell-free systems should be attempted. These studies are under way in our division.

ACKNOWLEDGMENT: Hydrocortisone sodium succinate was supplied by Nikken Chemicals Co., Ltd.

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