Oxidative burden and antioxidant defense system in polymorphonuclear leukocytes of human lung diseases

Rashmi N Sharma, A Bhardwaj, D Behera$ and K L Khanduja*
Department of Biophysics, Department of Pulmonary Medicine,$
Postgraduate Institute of Medical Education & Research, Chandigarh 160 012, India
Received 4 May 2001; revised and accepted 12 October 2001

Superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) production was significantly higher in blood neutrophils (PMNs) of patients with lung cancer and non-malignant lung diseases when compared to the controls (p<0.001). Superoxide dismutase (SOD) activity was significantly decreased in PMNs of patients with lung cancer (p<0.001). Similarly, catalase and glutathione peroxidase (GPx) activities were lower in PMNs of lung cancer patients as compared to non-malignant lung diseases and controls. There was an increase in HMP shunt activity as measured by rate of formation of ^14CO_2 from [1-^14C]glucose in PMNs of lung cancer patients. Modifications in the antioxidant defense system in PMNs of malignant and non-malignant lung diseases, the changes being more in the malignancy are indicated.

Polymorphonuclear neutrophils (PMNs) play an important role as a mediator of tissue destructive events in inflammatory diseases, ranging from rheumatoid arthritis and myocardial reperfusion injury to respiratory distress syndrome^1. Oxygen free radicals, proteinases and phospholipid-derived products released by the activated PMNs cause organ damage that often results in multiple failure of vital organs^2. Additionally, these mediators affect the hydration status of the cell, leading to abnormalities in protein catabolism in these patients^3. The first pathway in organ damage and injury to vascular endothelial cells is mediated by the toxic oxygen metabolites of activated PMNs^4.

When exposed to appropriate stimuli, PMNs change their pattern of oxygen uptake sharply while releasing large amounts of superoxide anion into the cell environment. The key reaction in this respiratory burst is 1-electron reduction of oxygen to superoxide anion using NADP, catalyzed by the membrane bound NADP oxidase^5. NAD^+ is then reconverted to NADP by the pentose phosphate pathway by breakdown of glucose. Thus, respiratory burst activity of PMNs depends on glucose metabolism. Activated leukocytes can cause DNA strand breaks^6, formation of base modifications in DNA^7 leading to mutagenesis^8 and carcinogenesis^9. However, the self-limiting nature of inflammatory reactions and the ability of innate antioxidant defenses to neutralize oxidants ensure that the tissue damage is minimal.

Intracellular oxygen radicals are detoxified by cytoplasmic CuZn superoxide dismutase (SOD) to form H_2O_2 which is further detoxified to H_2O by catalase and GPx. Reduced glutathione (GSH) is essential for detoxification of H_2O_2 by GPx in glutathione redox cycle. GSH can be regenerated at the expense of NADP which is generated through the HMP shunt pathway^10.

In view of the important role of neutrophils in inflammation, which is associated with tumorigenesis, the present study was undertaken to examine the ROS and antioxidant defense system in blood neutrophils of lung cancer, of non-malignant lung diseases and healthy subjects.

Materials and Methods

Chemicals
Phorbolester-12-myristate13-acetate (PMA), dextran, Ficoll-hypaque, nitroblue tetrazolium (NBT), phosphate buffered saline (PBS), glutathione reductase, NADP, GSH, hyamine hydroxide, 2,5-diphenyl oxazole (PPO), 1,4-bis 2-(5 phenyl oxazole) (POPOP) were procured from Sigma Chemical Co.,
(St. Louis, MO). [1-14C] Glucose (sp. activity, 4.07 mCi/mM) was purchased from the Board of Radioisotope Technology, Bhabha Atomic Research Centre, Mumbai, India. All other reagents were of analytical grade and purchased from SISCO Laboratories, Mumbai.

Subjects

The study was conducted on patients admitted in the medical wards of the Nehru Hospital, Postgraduate Institute of Medical Education & Research, Chandigarh, India or attending the Out Patients Department (OPD) of the hospital. Twenty-five histologically proven lung malignant patients were studied. Of these, twenty-two were smokers and three were non-smokers. Of the smokers, two had smoked only "hukka" for a period of more than 25 years; and the rest were smoking either "beedi" and/or cigarettes. All the three non-smoker were females. Nineteen of the smokers were heavy smokers (smoking index: >300 or "hukka" smoking >25 years) and three were moderate ones (smoking index: 101-300). The smoking index was defined as the number of cigarettes and/or "beedi" smoked per day × duration of smoking in years.

Twelve patients with non-malignant lung diseases were also enrolled in the study. Of these patients, three had hemoptysis and there was one for each of the disorders viz. bronchopneumonia, tuberculosis, lung inflammation, chronic obstructive pulmonary disease, transbronchial, silicosis and sarcoidosis. The remaining two patients had interstitial lung diseases. Subjects with lower respiratory tract infection in the preceding four weeks were excluded from the study. Similarly, those who had received specific therapy for lung malignancy, in the form of chemotherapy and/or radiotherapy were also excluded from the study. Fifteen healthy volunteers were included as controls. All the subjects gave their informed consent for sampling.

Isolation of polymorphonuclear leukocytes

Venous blood was drawn and PMNs were separated from heparinized blood by blood dextran sedimentation technique. Leukocyte rich plasma was aspirated and layered on the Ficoll-hypaque gradient. PMNs were washed twice with normal saline and suspended finally in phosphate-buffered saline (pH 7.4). Cell viability was estimated by the exclusion of trypan blue which was greater than 95%.

The number of cells was measured with hemocytometer (Neubauer Improved Double Chamber, Fein-Optics Rad Blamkenburg).

Superoxide and hydrogen peroxide assay

The procedure to assess superoxide anion production by PMNs was a modification of that described by Park et al. in which NBT in presence of superoxide anion was reduced to an insoluble cytoplasmic blue formazan precipitate and visualized by light microscopy. PMNs (1x10⁶) were incubated with NBT (0.05%) and 0.1 μg PMA in a final volume of 0.5 ml at 37°C in 5% CO₂ atmosphere (CO₂ incubator Model CO27, New Brunswick Scientific) for 1 hr. The reaction mixture without PMA served as blank. In all the assays a test sample was treated with SOD (0.25 mg/ml) during in vitro NBT treatment to assess the superoxide anion radical specific reduction of NBT. All the results are expressed as number of formazan positive PMNs/250 cells.

The amount of H₂O₂ released by PMNs with or without PMA stimulation was estimated by the horse-radish peroxidase method. To the PMNs, 0.5 ml of phenol red solution (1%) was added and incubated at 37°C for 30 min with or without 0.1 μg PMA. The reaction was terminated by adding calatase (150 units/assay) and the optical density was recorded at 560 nm after making the assay mixture alkaline by adding 10 μl of 1N NaOH. The cell protein was determined by covering the PMNs with 1 ml 1N NaOH, and allowing them to stand overnight at 37°C and then assaying the protein content of the digest by Lowry's method.

Antioxidant activity of polymorphonuclear leukocytes

PMNs were washed three times with sterile phosphate buffered saline (PBS) at pH 7.4. The cells were lysed by three freeze-thaw cycles followed by sonication for 1 min. The cell debris was removed by centrifugation for 1 min in Beckman's table-top microcentrifuge.

SOD activity was measured in an aliquot of the supernatant according to the method of Kono. The rate of NBT reduction by superoxide anion radical generated by photoactivation of hydroxylamine hydrochloride was recorded at 560 nm, in the absence of cellular supernatant. Following this, small aliquots of cellular supernatant were added to the reaction mixture containing 50 mM Na₂CO₃, 0.1 mM EDTA pH 10.0, 96 μM NBT, 0.6% Triton X-100 and 20 mM...
NH₂OH·HCl. The decline in the rate of NBT reduction by SOD present in the supernatant was analysed. One unit of SOD was defined as protein in mg that inhibited 50% of the reduction of NBT.

Catalase activity was measured in the supernatant by the method of Luck. The final reaction mixture (3 ml) included 0.06 M phosphate buffer, pH 7.0 and 1.25 x 10⁻⁹ M H₂O₂. The cellular supernatant (50 or 100 µl) was added to the mixture. The rate of elimination of H₂O₂ by catalase was measured by recording the time (in sec) required for 0.05A decline in OD at 240 nm.

GPx was determined by the method of Paglia and Valentine. The final reaction mixture (3 ml) included 50 mM phosphate buffer (pH 7.4), 5 mM EDTA, 1.25 M sodium azide, 0.15 M glutathione, 2.4 units of glutathione reductase and 8.4 mM NADPH. An aliquot of supernatant was added to the reaction mixture and allowed to equilibrate for 10 min at 22°C. The reaction was initiated by adding 0.1 ml of 2.2 mM H₂O₂. Absorbance at 340 nm was recorded for 3 to 4 min. Concentration of the enzyme was calculated in terms of nmol NADP oxidized to NAD⁺ by using extinction coefficient of 6.22 x 10⁻³ M⁻¹ cm⁻¹.

Activity of hexose monophosphate shunt pathway

HMP shunt pathway was determined as the amount of ¹⁴CO₂ liberated from samples incubated with D-[¹⁴C]glucose in 25 ml Erlenmeyer flask by the method of Przybykowski and Averill-Bates. PMNs (1 x 10⁶/ml) were suspended in a Krebs Ringer phosphate buffer (pH 7.4) containing 12.5 mM glucose. A quantity of 1 µCi of D-[¹⁴C]-glucose (4.07 mCi/mmol) per flask was added to the reaction mixture in the presence or absence of stimuli (1 µg PMA in 50 µl of DMSO). The flasks were immediately sealed with rubber stoppers and incubated for 30 min at 37°C with shaking. A glass tube containing 0.2 ml of hyamine hydroxide was attached inside to the rubber stopper of each flask. After 30 min, 0.2 ml of 5N H₂SO₄ was injected into the flask and the reaction mixture was incubated again at 37°C under agitation for additional 30 min. The tubes were placed to scintillation fluid. Radioactivity was determined by liquid scintillation counter (LKB Model No. 1219).

All data were expressed as the mean ± SD. Different groups were compared by the use of one way analysis of variance, ANOVA.

Results

Table 1 summarizes the clinical data of the three groups. All groups had a proportionally larger number of males.

Reactive oxygen species generation from neutrophils

Neutrophils generated O₂⁻ both with and without PMA as depicted in Fig. 1. There was a significant increase in O₂⁻ generation by PMNs of lung cancer patients as compared to non-malignant lung diseases and healthy controls (p<0.001, ANOVA). The number of F(+) cells in lung cancer, nonmalignant lung diseases and healthy controls was 8.20 ± 1.20, 4.80 ± 1.31 and 1.53 ± 0.51 respectively. On stimulation with PMA, the F(+) cells were increased to 22.0 ± 3.0, 13.7 ± 1.70 and 5.06 ± 0.76 in the three groups respectively.

Fig. 2 compares the in vitro production of H₂O₂ by PMA-treated PMNs from lung cancer, non-malignant lung diseases and healthy controls. The results revealed a significant increase in H₂O₂ formation by

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Lung cancer (n=25)</th>
<th>Non-malignant lung diseases (n=12)</th>
<th>Healthy controls (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, M/F</td>
<td>21/4</td>
<td>8/4</td>
<td>10/5</td>
</tr>
<tr>
<td>Age (years)</td>
<td>58.7 ± 7.51</td>
<td>45.3 ± 4.16</td>
<td>31.4 ± 5.60</td>
</tr>
<tr>
<td>Smoker</td>
<td>22</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Non-smoker</td>
<td>3</td>
<td>8</td>
<td>15</td>
</tr>
</tbody>
</table>

Fig. 1—PMA-induced in vitro generation of superoxide anion (expressed as number of formazan positive PMNs/ 250 cells) in peripheral blood neutrophils [1, without PMA; 2, with PMA. *p < 0.001 as compared to non-malignant lung diseases and controls; a, p < 0.001 as compared to control]
24% in the lung cancer patients. However, in the non-malignant lung diseases it was increased by 54%.

**Antioxidant activities**

SOD, catalase and GPx activities were detected in PMNs of all the three study groups (Table 2). Significant decrease in SOD activity was observed in PMNs of lung cancer patients and non-malignant lung diseases as compared with those of healthy controls. The activity of SOD in PMNs of lung cancer patients was decreased by 72%, whereas it was decreased by 49% in non-malignant lung diseases. Lung cancer patients showed a significant decrease in catalase activity by 40% and in non-malignant lung diseases it was decreased by 57%. The activities of GPx in PMNs of lung cancer patients were comparable with non-malignant lung diseases. However, a significant decrease in GPx activity by 41% in lung cancer patients and 46% in non-malignant lung diseases was observed.

**Status of hexose monophosphate shunt pathway**

Fig. 3 depicts the status of HMP shunt in PMNs with or without stimulation by PMA in various groups of subjects. The release of $^{14}$CO$_2$ per 30 min was taken as an index of HMP shunt activity. The results indicate a significant increase in HMP shunt activity in PMNs of lung cancer patients and non-malignant lung diseases when compared to healthy controls ($p<0.05$).

**Discussion**

Neutrophils play a prominent role in non-specific immune responses by phagocytosing bacteria and other pathogens. They are usually the first leukocyte to arrive at sites of infection/tissue injury, and are a major cellular component of acute inflammatory responses. Alterations in neutrophil function could result in an impaired immune defense against pathogens and/ or inappropriate inflammatory responses which may lead to tissue injury. Ludwig and Hoidal had shown that in cigarette smokers the PMN oxidative metabolism was altered as documented by the increased superoxide production by neutrophils. Another study showed an elevated superoxide release by pulmonary alveolar macrophages in subjects habitual to smoking, indicating a parallel behavior of PMNs and alveolar macrophages to oxidative stress.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Healthy controls (n=15)</th>
<th>Non-malignant lung tumour (n=12)</th>
<th>Lung cancer (n=25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD (IU/mg protein)</td>
<td>11.5 ± 1.70</td>
<td>5.67 ± 0.48</td>
<td>8.25 ± 0.95</td>
</tr>
<tr>
<td>Catalase (IU/mg protein)</td>
<td>270 ± 65.2</td>
<td>156 ± 32.9</td>
<td>110 ± 11.6*</td>
</tr>
<tr>
<td>GPx (nmoles NADPH/min/mg protein)</td>
<td>80.5 ± 18.7</td>
<td>37.8 ± 6.85</td>
<td>33.5 ± 7.16*</td>
</tr>
</tbody>
</table>

+ $p < 0.05$ wrt non-malignant lung diseases; * $p < 0.001$ wrt controls
our data (Table reports also on is well known. However, there are contradictory equilibria. An increase in circulating of lung cancer patients found in the present study may depletion of cellular thiols. The conditions, including cancer26. Systemic defense be due to excessive oxidant burden in lower respiratory enhanced free radical generation has been shown to antioxidant enzymes. GSH is a strong endogenous detoxification reactions is involved in DNA, steroid and lipid synthesis, and in maintenance of GSH/GSSG and NADP/NAD+ redox equilibria. An increase in HMP shunt activity in PMNs of lung cancer patients found in the present study may be due to excessive oxidant burden in lower respiratory tract of patients. Stimulation of HMP pathway in circulating PMNs may provide GSH to the alveolar fluid to prevent the injurious oxidative reactions initiated by the ROS.

The importance of SOD as an antioxidant defense is well known. However, there are contradictory reports also on SOD activity in human tumours27. But our data (Table 2) show that SOD activity in PMNs was decreased significantly in patients with lung tumour, possibly due to the inactivation of the enzyme. SOD is known to protect epithelial cells against malignant transformation in vitro28, and alteration in SOD possibly plays a significant role in the pathogenesis of lung cancer. Similarly, the catalase and GPx activities in PMNs were found to be decreased, which possibly led to increased H2O2 generation by PMNs in pathogenesis.

Oxygen radicals derived from inflammatory cells can induce DNA damage29 and can cause transformation of cells in culture. Endogenous production of ROS by blood neutrophils might be a mechanism, whereby tumours enhance their own genetic instability. We have earlier reported enhancement of ROS in macrophages obtained from the lobe which already had tumour. Rise in ROS in blood neutrophils and alveolar macrophages may augment both genetic instability of a tumour and its capacity to injure and penetrate the host tissues.

References
3 Haussinger D, Roth E, Lang F & Gerok W (1993) Lancet 341, 1330-1332
16 Kono Y (1978) Arch Biochem Biophys 186, 189-195

Fig. 3—Hexose monophosphate shunt activity in peripheral blood neutrophils of lung cancer patients, patients with other lung diseases, and healthy controls (1, without PMA; 2, with PMA. Values, n moles 14 CO2/30 min/106 cells, are mean ± SD. *p<0.001 as compared to control)

28 Borek C & Troll W (1983) Proc Natl Acad Sci USA 80, 1304-1307
29 Dutton D & Broden G (1985) Carcinogenesis 6, 219-228