Antioxidant Defense in Erythrocytes and Plasma of Patients with Active and Quiescent Crohn Disease and Ulcerative Colitis: A Chemiluminescent Study, Anna Blázovics,3 Ágota Kovács,2 Andrea Lugasi,3 Krisztina Haguáné,1 Lajos Bíró,7 and János Fehér† (1 Second Department of Medicine, Semmelweis University Medical School, Szentkirályi St. 46, Budapest H-1088, Hungary; 2 Elizabeth County Hospital, Alsó erdős St. 7, Budapest H-1074, Hungary; 3 National Institute of Food-Hygiene and Nutrition, Gyáli Road 3/a, Budapest H-1097, Hungary; †author for correspondence: e-mail Blaz@bel2.SOTE.hu)

Inflammatory bowel disease (IBD) can be considered multifactorial in genetic background and with regard to neuro-endocrine regulation of immune reactivity to antigens of alimentary and bacterial origin (1). The roles of free radicals and the antioxidant defense mechanisms of the intestine are also well documented (2, 3). Aminosalicylates such as 5-aminosalicylic acid (5-ASA), when used in therapy, exhibit superoxide and hydroxyl-radical scavenger properties (4). This medication can partially restore the depleted mucosal antioxidant protective function that develops in IBD.

We used a simple luminol-dependent chemiluminescence method to study the antioxidant protective mechanisms in patients with IBD. Luminol-dependent chemiluminescence, as an indicator of a free radicals, is suitable for detection of the antioxidant status of tissue. The H₂O₂/OH-microperoxidase-luminol system can be used to measure the total scavenger capacity (TSC) of the plasma and erythrocytes. We also determined the H⁺-donor activity and reducing power in the plasma.

1,1-Diphenyl-2-picryl-hydrazaZ, stable radical; 5,5'-dithiobis-2-nitrobenzoic acid; luminol; and microperoxidase were obtained from Sigma. All other reagents were purchased from Reanal.

Twenty-six Caucasian volunteers (10 males, ages 26.6 ± 11.3 years; 16 females, ages 34.2 ± 13.6 years) served as a control group. Patients were divided into three groups—inactive, moderate, and severe—based on the severity of their disease according to the Crohn disease activity index (CDAI) and the ulcerative colitis (UC) activity index. Routine laboratory data and numerical values for pain, stool characteristics, well-being, and other factors were used to calculate the CDAI and the UC activity index (5).

The UC activity index classifications for 22 patients were as follows: inactive, 1 male, age 19 years, and 3 females, ages 48.0 ± 23.0 years; moderate, 3 males, ages 36.3 ± 16.5 years, and 7 females, ages 39.5 ± 14.7 years; severe, 4 males, ages 37.7 ± 6.8 years, and 2 females, ages 30.0 ± 5.6 years. Two other male patients (ages 54.0 ± 35.3 years) had extremely severe forms of the disease. The CDAI classifications for 22 other patients were as follows: inactive, 4 males, ages 33.5 ± 13.0 years, and 3 females, ages 36.5 ± 9.2 years; moderate, 7 males, ages 33.4 ± 11.3 years, and 3 females, ages 50.3 ± 6.8; severe, 2 males, ages 38.0 ± 12.7 years, and 3 females, ages 33.3 ± 8.7 years. All patients received the same standard therapy recommended by WHO.

Patients with inactive UC and CD were treated with 5-ASA and 5-ASA plus the immunosuppressor azathioprine, respectively. Patients with moderate UC and CD were treated with 5-ASA plus a local steroid and 5-ASA plus azathioprine plus the local antibiotic metronidazole, respectively. Patients with severe UC were treated with 5-ASA plus a local steroid and/or systemic steroid. Patients with severe CD received combined therapy with steroid (local and/or systemic) plus antibiotics (metronidazole or ciprofloxacin) plus elementary diet. The study was approved by the Regional Committee of Science and Research Ethics, Semmelweis University of Medicine (Permission number, TUKKEB 24/1996.)

Plasma and erythrocytes were separated, and the hemoglobin content of samples was adjusted to 10 g/L for the measurements.

The reducing power of the sample was determined at 700 nm according to the method of Oyaizu (6), which is based on the chemical reaction Fe(III) → Fe(II). Increased absorbance indicated increased reducing power, which was also expressed as the ascorbic acid equivalent (mmol/L eqAS).

The H⁺-donating ability of the sample was estimated in the presence of 1,1-diphenyl-2-picryl-hydrazyl radical at 517 nm according to the method of Hatano et al. (7).

We determined TSC with a chemiluminescence assay adapted to a Berthold Lumat 9501 manual instrument to assess antioxidant deficiency in patients with IBD (8). This luminometer is designed to determine single photons of light emitted in the course of a chemical reaction between luminol and free radicals. The spectral range of the instrument is 390–620 nm. The entire spectrum was used by scanning, and the “raw data” operation mode was used. The built-in microprocessor performed the data processing and printed out the final results in relative light units (RLU); one RLU corresponds to the count recorded at the anode of the photomultiplier, divided by 10 and multiplied by the RLU factor, which allows compensation for the inevitable individual fluctuations in the sensitivity of the photomultiplier cathode as determined by the Berthold Company. The sensitivity of the instrument allows detection limits of <0.1 pg of material. During measurement, the program gives the integrated value of the light reaction.

The first trigger solution, H₂O₂ (0.30 mL, 1:10 000 dilution), was aspirated from the tank and placed in the first position just before the measuring chamber; the second trigger solution, 0.30 mL of 1 mmol/L microperoxidase, which is the catalyst, was aspirated and placed in the second position. Light emission was initiated by the addition of 0.050 mL of 70 μmol/L alkaline luminol solution, pH 9.8, into a polystyrol tube, and the mixing was started in front of the photomultiplier. The photon output was measured for 30 s. The solutions were protected from direct light, and the system was stable at 22–25 °C for at least 1 h. The volumes of the plasma and erythrocyte samples were 0.15 and 0.05 mL, respectively. The biological samples were added to the luminol solu-
tion and vortex-mixed (10 s) before the tube was placed in a holder.

One-way ANOVA statistical analysis was used to evaluate the significance between patient groups.

The chemiluminescence results (mean ± SD) are shown in Table 1. Each measuring point represented five parallel data points from luminol-dependent chemiluminescence experiments for which the CV was <5%. Significance was determined at P < 0.05. Mathematical analysis of the chemiluminescence according to log-log transformation between luminol concentration and chemiluminescence intensity showed a linear correlation: \( y = 1E + 06x + 3E + 06 \); \( r^2 = 0.9737; P < 0.05 \).

The specific activities or concentrations of antioxidants (enzymes, vitamins, or functional groups) do not represent the total antioxidant status of the plasma and erythrocytes. Therefore, a variety of studies are required to evaluate the reduction-oxidation status of the biological samples. The assays are very expensive and time-consuming; in addition, the results are sometimes contradictory. The present chemiluminescent method was developed to determine the TSC in a minute volume of plasma or erythrocyte suspension.

The TSC of the erythrocytes indicated serious disease despite antioxidant therapy (5-ASA or adequate medicine) in all phases of CD and in severe UC. There was a nonsignificant decrease of chemiluminescence in the moderate form of UC, indicating a larger TSC compared with the severe phase. In the inactive form of UC, the measured TSC approached the control value. Similar chemiluminescent phenomena were observed for the TSC of the plasma in the severe forms of both types of IBD. The reducing power of plasma was not significantly different in CD or UC. The value of the H⁺-donor activity of the plasma showed a significantly decreased tendency in severe IBD compared with the control.

The plasma of two patients with UC had extremely high chemiluminescence intensity values (RLU% ~70) in the applied medium. The above finding may be explained by the fact that these two patients received therapy only a few days before their plasma samples were analyzed.

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### References


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**Table 1. Chemiluminescence intensity of erythrocytes and plasma in H₂O₂/-OH-luminol-microperoxidase system and reduction-oxidation of plasma.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Erythrocyte CL intensity, a RLU%</th>
<th>Plasma CL intensity, RLU%</th>
<th>Plasma H⁺-donor activity, %</th>
<th>Plasma reducing power, mmol/L eqAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 26)</td>
<td>31.19 ± 6.08b</td>
<td>2.00 ± 1.27b</td>
<td>60.62 ± 1.98b</td>
<td>0.873 ± 0.111b</td>
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<tr>
<td>CD (n = 22)</td>
<td></td>
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<tr>
<td>Inactive (n = 7)</td>
<td>107.73 ± 14.01c</td>
<td>6.34 ± 2.11</td>
<td>58.67 ± 10.55</td>
<td>1.011 ± 0.127</td>
</tr>
<tr>
<td>Moderate (n = 10)</td>
<td>95.22 ± 7.47c</td>
<td>16.09 ± 6.69c</td>
<td>47.34 ± 10.26</td>
<td>1.017 ± 0.121</td>
</tr>
<tr>
<td>Severe (n = 5)</td>
<td>110.95 ± 20.43c</td>
<td>49.74 ± 25.46c</td>
<td>42.86 ± 4.52c</td>
<td>0.841 ± 0.082</td>
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<tr>
<td>UC (n = 22)</td>
<td></td>
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<tr>
<td>Inactive (n = 4)</td>
<td>40.49 ± 6.25</td>
<td>4.25 ± 1.34</td>
<td>45.88 ± 3.04c</td>
<td>1.001 ± 0.044</td>
</tr>
<tr>
<td>Moderate (n = 10)</td>
<td>61.33 ± 6.62c</td>
<td>15.50 ± 2.67c</td>
<td>52.01 ± 11.73</td>
<td>0.915 ± 0.185</td>
</tr>
<tr>
<td>Severe (n = 8)</td>
<td>110.68 ± 25.62c</td>
<td>33.62 ± 8.60c</td>
<td>45.44 ± 3.00c</td>
<td>0.966 ± 0.092</td>
</tr>
</tbody>
</table>

a CL, chemiluminescence.
b Mean ± SD.
c Significance vs control, P < 0.05.
d n = 6.