The prognostic potential of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) was evaluated in sepsis. Enzyme concentrations were determined in samples obtained from septic patients at time of diagnosis. Statistically significant increases in activities of total plasma SOD ($P < 0.003$, $n = 32$), erythrocyte (RBC) SOD ($P < 0.007$, $n = 16$), plasma CAT ($P < 0.0001$, $n = 32$), and RBC CAT ($P < 0.005$, $n = 16$) were found in septic patients when compared with healthy adult controls ($n = 7$). Further, within the group of septic patients, statistically significant differences were found for total plasma SOD ($P < 0.05$) and plasma CAT ($P < 0.009$) but not for RBC determinations when survivors ($n = 15$) were compared with nonsurvivors ($n = 17$). No significant differences were found for either plasma or RBC enzyme concentrations when patients who developed adult respiratory distress syndrome were compared with those who did not. The most striking finding was that plasma total SOD values of $> 10$ kU/L were found in 7 of 21 (30%) patients who did not survive their sepsis and that these values did not overlap with any surviving patients or controls. However, while high total plasma SOD activity appears to have some potential as a prognostic indicator, lower values (0.0–8.8 kU/L) do not. For plasma CAT, despite finding statistically significant differences between survivors and nonsurvivors, the substantial overlap in the values obtained for the two groups limits the practical prognostic potential of this enzyme.

Indexing Terms: superoxide dismutase/catalase/erythrocytes

The production of active oxygen species (oxygen-derived free radicals) is a universal accompaniment to cellular pathology, proposed to occur as a result of a tilt in the biochemical balance of the cell toward the oxidative side, with the result being increased production and (or) decreased removal of free radicals, leading to tissue damage (1). Septic patients are exposed to increased amounts of free radicals by two mechanisms, activated phagocytes and reperfusion. Activated phagocytes produce more free radicals in response to infection (2). Transitory circulatory insufficiencies to the heart, lungs, brain, and other organs often accompany sepsis, and during reperfusion/reoxygenation free radicals are produced. An example of a specific injury proposed to be caused by accelerated oxidant generation and having a high incidence in sepsis is adult respiratory distress syndrome (ARDS) (3).

A major protective mechanism for coping with free radicals is provided by the enzymes superoxide dismutase (SOD; EC 1.15.1.1) (4) and catalase (CAT; EC 1.11.1.6) (5). SOD catalyzes the formation of hydrogen peroxide from superoxide anions, and CAT acts to decompose hydrogen peroxide to oxygen and water. Both enzymes are present in erythrocytes (RBCs) and endothelial cells (6). SOD has several isoforms, including SOD1 (copper–zinc SOD), which is found in RBCs and in the cytosol of tissues; SOD2 (manganese SOD), which is found in cell mitochondria; and the extracellular form, SOD3, which is found in plasma.

This study addresses the hypothesis that, since oxidative injury is a likely contributor to morbidity and mortality in septic patients, the patients who survive may be those who are best able to cope with oxidative stress through inactivation of free radicals. Therefore, we studied the activities of SOD and CAT in the plasma and RBCs of septic patients to see if there were any significant differences in activity between septic patients and healthy controls. The differences in enzyme activities between different groups of septic patients, for example, survivors vs nonsurvivors and patients with and without ARDS, were also studied.

Materials and Methods

The study protocol was approved by the University of Cincinnati Medical Center Institutional Review Board. Informed consent was not required since the blood used was that obtained for clinical purposes.

Patients

The study population was a group of 32 patients (15 survivors and 17 nonsurvivors) with clinically defined sepsis ($n = 14$) or septic shock ($n = 18$) who were consecutively admitted to the medical intensive care unit of the University of Cincinnati Hospital (Table 1). Patients with the following diseases and (or) conditions were excluded: acquired immunodeficiency syndrome, pregnancy, and uncontrolled hemorrhage. Also excluded were patients taking immunosuppressive and nonsteroidal antiinflammatory drugs. The group comprised 15 men and 17 women, ages 23 to 79 years. Of the 19 patients presenting in septic shock, 11 were ultimately survivors and 8 were nonsurvivors. A subset of 16 patients for whom heparinized specimens were

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$^6$ Nonstandard abbreviations: ARDS, adult respiratory distress syndrome; SOD, superoxide dismutase; CAT, catalase; and RBC, erythrocyte.

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available (9 survivors and 7 nonsurvivors) was also evaluated for RBC enzyme activities.

Initial diagnosis of sepsis was made if two or more of the following criteria were met: (a) hyperthermia >38°C or hypothermia <36°C; (b) heart rate >90 beats/min; (c) leukocyte count <4 × 10⁹/L or >12 × 10⁹/L; (d) laboratory evidence of infection (gram stain, culture). The stage of sepsis in each patient was determined by the criteria described by Bone et al. (7).

Patients’ Samples

Heparinized blood samples and EDTA plasma used for analyte measurements were those obtained within 24 h of the time the patient was diagnosed with sepsis, and were processed within 1–2 h of collection from the patient. Samples were centrifuged for 10 min at 1200g and the plasma removed. The RBCs from the heparinized samples were washed three times with 0.154 mol/L NaCl (8). Washed RBCs and EDTA plasma samples were aliquoted and stored at −70°C until analysis. The samples from the seven healthy adults were handled in the same manner as the patients’ specimens. All analyses were performed in duplicate. Since EDTA specimens were routinely available, plasma enzyme activities were obtained for all 32 patients, but RBC enzyme activities, which required heparinized samples, were obtained for only 16 of the patients.

Preparation of RBC Samples

On the day of analysis, one volume of RBCs was hemolyzed with 1.5 volumes of cold deionized water. From this hemolysate, 100 μL was saved for hemoglobin analysis. To a 500-μL aliquot the following were added: (a) 3.5 mL of cold deionized water; (b) 1 mL of ethanol; and (c) 0.6 mL of chloroform. The mixture was shaken for 1 min and centrifuged for 10 min at 1100–2000g. The supernate was used to measure SOD and CAT activities.

Hemoglobin Assay

Hemoglobin content of RBC samples was determined by using an assay kit (Sigma Chemical Co., St. Louis, MO) according to the manufacturer’s directions.

SOD Assay

Through serial evaluation of several control samples, SOD was determined to be stable for the storage times used in the study (4 weeks). The SOD assay was the method of Joseph et al. (9) and is based on the SOD inhibition of cytochrome c reduction by xanthine oxidase. The method does not differentiate between the three isoforms of SOD. Briefly, the procedure was as follows. To a cuvette were added: 675 μL of 20 mmol/L Na₂CO₃ in a 0.1 mmol/L EDTA buffer at pH 10.0, 100 μL of 50 mmol/L xanthine, 100 μL of 5 mmol/L cytochrome c (Sigma), and the sample to be analyzed. The sample was either 100 μL of buffer (control), 100 μL of each SOD calibrator (Sigma), or a patient’s sample. Patients’ samples were either 100 μL of EDTA plasma or 25–50 μL of RBC hemolysate. When RBC hemolysate was used, the sample volume was adjusted to 100 μL by adding buffer. The contents of the cuvette were mixed gently and incubated for 5 min at 25°C, followed by addition of 25 μL of 1 kU/L xanthine oxidase stock (Sigma). The change in absorbance (ΔA) was measured for 5 min at 550 nm. One unit of SOD was defined as that amount that caused a 50% inhibition of the rate of cytochrome c reduction. A calibration curve was constructed with SOD calibrators in a range from 0.1 to 1.0 kU/L as follows: (a) The rate of reduction of cytochrome c (ΔA/t, t = 5 min) was first determined in the absence of SOD, and was designated the control rate; (b) the rate of reduction of cytochrome c was then determined in the presence of various amounts of SOD calibrators; (c) for each calibrator, the rate obtained was divided by the control rate and this value was plotted vs the number of SOD units present in the calibrator, thus generating a calibration curve; (d) by using the Sigma definition that one SOD unit (50% inhibition of the rate of reduction of cytochrome c), a curve correction was calculated; (e) the percentage rate was then determined for each patient’s specimen and the value of SOD activity was obtained from the corrected calibration curve. The range of calibrators used was verified as linear. All patients’ specimens were diluted as necessary to give values within this linear range. Patients’ results were expressed either per liter (plasma samples) or per gram of hemoglobin (RBC samples).

CAT

Through serial evaluation of several control samples, CAT was determined to be stable for the storage times used in the study (6 weeks). The Purpald® (Aldrich, Milwaukee, WI) catalase assay developed by Johansson et al. (10) was used to measure CAT activity. Since the CAT reaction is a first-order reaction, enzyme concentration is proportional to enzyme activity, and results can be reported in either activity or concentration units. Briefly, the procedure used was as follows. To a sample of either 100 μL of calibrator (Sigma) or patient’s specimen, the following were added: 40 μL of methanol and 10 μL of H₂O₂. Patients’ specimens were either plasma (25–50 μL) or RBC hemolysate (25 μL). Sufficient 0.25 mol/L KH₂PO₄ buffer, pH 7.0, was then added to give a uniform volume of 210 μL/tube. A blank was prepared by substituting buffer for sample. After incubation on a shaker at room temperature for 20

Table 1. Characteristics of study patients.

<table>
<thead>
<tr>
<th></th>
<th>Survivors</th>
<th>Nonsurvivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. patients</td>
<td>15</td>
<td>17</td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>7/8</td>
<td>10/7°</td>
</tr>
<tr>
<td>Age (years)*</td>
<td>60.8 ± 11.9</td>
<td>62.3 ± 16.4b</td>
</tr>
<tr>
<td>APACHE II</td>
<td>24.5 ± 5.6</td>
<td>28.5 ± 6.8 b</td>
</tr>
<tr>
<td>ARDS</td>
<td>10</td>
<td>8°</td>
</tr>
<tr>
<td>Sepsis/septic shock</td>
<td>4/11</td>
<td>9/9°</td>
</tr>
</tbody>
</table>

* Mean ± SD.
 b Mann–Whitney statistics: not significantly different from survivors.
 APACHE II, acute physiologic and chronic health evaluation.
min, the enzymatic reaction was terminated by adding 50 μL of 7.8 mol/L KOH and 100 μL of 34.2 mmol/L purpured in 0.48 mol/L HCl. Incubation was continued for 10 min, followed by addition of 50 μL of 0.0652 mol/L KIO₄ and 500 μL of 0.25 mol/L KH₂PO₄ buffer. Absorbance was measured at 550 nm and CAT concentration calculated from a calibration curve prepared from calibrators ranging from 182 to 909 ng. This range was linear, and patients' specimens were diluted as necessary to fall within the linearity of the method. CAT concentrations in patients' specimens were expressed in mg/L (plasma samples) or mg/g of hemoglobin (RBC samples).

Statistical Analysis

Mean ± SD values were calculated for all the analytes for both the patient and control groups (Table 2). Differences between septic patients and controls and between the survivor and nonsurvivor groups and the ARDS and non-ARDS groups of septic patients were tested for statistical significance by using the Mann–Whitney test (nonpaired data) (Tables 2 and 3).

Results

Compared with healthy adults, septic patients had statistically significantly higher concentrations of both plasma and RBC CAT and SOD (Table 2). More overlap was evident between controls and septic patients for RBC than for plasma enzyme activities, with the most impressive increases being in the plasma enzymes, particularly SOD (Fig. 1). Comparing survivors with nonsurvivors, differences were statistically significant for plasma SOD (P < 0.05) and plasma CAT (P < 0.009), but not for the RBC enzyme activities (Table 3).

When the septic patient group was divided on the basis of the presence (n = 18) or absence (n = 14) of ARDS, no statistically significant differences were found in the plasma or RBC enzyme activities of the two groups (data not shown).

Discussion

The search for prognostic factors in sepsis has engaged several investigators, particularly in recent years, perhaps because of the expected introduction of new sepsis therapies. Many novel therapies are under investigation and, if introduced, are expected to be very expensive. Therefore, hospitals will be looking for means to identify patients most likely to benefit and to curb use in patients who likely will not benefit. To help the hospital prepare for this eventuality, we began the search for sepsis prognostic factors.

Two major biochemical features of sepsis are the cytokine cascade, which results in increases in concentrations of several cytokines and increased free radical generation. We focused on the free radical aspect of sepsis, evaluating the prognostic potential of two enzymes, SOD and CAT, which play key roles in scavenging free radicals and thus limit oxidative injury. The finding of increased concentrations of plasma SOD and plasma CAT in septic patients compared with healthy controls was expected, since this had been reported previously (3, 6). Statistically significant differences between patients with and without ARDS, as reported by Leff et al. (3), however, were not found. Since the present study was designed to predict outcome as quickly as possible, the samples studied were those obtained as soon as possible after the diagnosis of sepsis was made. Leff et al., on the other hand, analyzed samples obtained at 6, 12, 24, and 48 h after initial diagnosis and evaluated the mean of the values obtained. Thus it is not surprising, given the different design of the two studies, that different results were obtained.

At present the most reasonable explanation of the increased plasma concentrations of SOD and CAT in sepsis is that these increases are a result of tissue damage, as is true in several other clinical situations. For example, increases of serum transaminases indicate liver damage, whereas increases of enzymes such as creatine kinase may indicate cardiac tissue damage (11). Leff et al. discuss several other possible mechanisms for the increases in plasma activities of antioxidant enzymes in sepsis, including hemolysis, increased enzyme production, or decreased clearance (3). Confirmation that higher plasma concentrations are indicative of damage rather than a response to an increased oxidative challenge awaits further study, since it is not clear whether the higher values found in nonsurvivors are due to increased damage or result from an increased marshalling of antioxidant forces by the more severely compromised patient. Data from the present study do suggest, however, that increased plasma enzyme activities do confer protection on the septic patient, since the highest values were found in nonsurvivors.

In spite of a better statistical correlation for CAT than SOD (Table 3), the overlap in plasma CAT values between survivors and nonsurvivors is substantial enough that this analyte may have little practical

Table 2. Antioxidant enzyme activities in septic patients vs controls.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Septic (n = 32)</th>
<th>Control (n = 7)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma SOD (kU/L)</td>
<td>5.5 ± 5.8</td>
<td>1.0 ± 2.5</td>
<td>P &lt; 0.003</td>
</tr>
<tr>
<td>Plasma CAT (mg/L)</td>
<td>40.2 ± 18.8</td>
<td>4.7 ± 4.6</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>RBC SOD (kU/g Hb)</td>
<td>19.5 ± 4.6</td>
<td>14.8 ± 1.4</td>
<td>P &lt; 0.007</td>
</tr>
<tr>
<td>RBC CAT (mg/g Hb)</td>
<td>25.3 ± 5.4</td>
<td>16.5 ± 4.7</td>
<td>P &lt; 0.005</td>
</tr>
</tbody>
</table>

Values given are mean ± SD. Hb, hemoglobin.

Table 3. Antioxidant enzyme activities in survivors and nonsurvivors.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Survivors (n = 15)</th>
<th>Nonsurvivors (n = 17)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma SOD (kU/L)</td>
<td>3.0 ± 2.9</td>
<td>8.1 ± 6.8</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Plasma CAT (mg/L)</td>
<td>25.8 ± 6.8</td>
<td>40.1 ± 18.7</td>
<td>P &lt; 0.009</td>
</tr>
<tr>
<td>RBC SOD (kU/g Hb)</td>
<td>20.7 ± 5.6</td>
<td>18.1 ± 2.3</td>
<td>ns</td>
</tr>
<tr>
<td>RBC CAT (mg/g Hb)</td>
<td>23.7 ± 5.3</td>
<td>26.4 ± 6.0</td>
<td>ns</td>
</tr>
</tbody>
</table>

Values given are mean ± SD; ns, not significant; Hb, hemoglobin.
prognostic potential. SOD measurements in plasma may have some utility since 7 of the 21 (30%) nonsurviving patients had total SOD values higher (>10 kU/L) than any of the controls or the surviving septic patients. However, surprisingly, 6 of the nonsurvivors (28%) had no demonstrable SOD activity in their plasma. This was also true for 5 of 7 of the controls and 2 of 13 of the surviving patients. Therefore, while very high SOD activities in plasma appear to have prognostic potential, lower values (0.0–8.8 kU/L) appear to have no value. Clearly the practical application of this potential will need to be studied further in a larger group of patients.

The finding of significantly higher enzyme concentrations in RBCs from septic patients compared with healthy controls was unexpected. The possibility that the increased RBC values were due to plasma contamination was considered but was thought to be highly unlikely, given the preparation steps carried out on the RBCs. SOD and CAT are normally present in large amounts in RBCs, and we hypothesized that the activities of these enzymes in RBCs may be indicative of the patient’s ability to cope with oxidative stress, with low concentrations correlating with a poor outcome. The constituents of the RBC are not expected to change once the RBC has formed and has reached the anuclear state; therefore, the fact that septic patients had higher RBC enzyme activities than did controls is difficult to explain. RBC SOD and CAT also display different patterns of enzyme activity. Although in both cases the septic patients as a group had higher concentrations of enzymes than did controls, surviving patients tended to have lower RBC CAT and higher RBC SOD concentrations than did nonsurviving patients (Fig. 1C and D, respectively).

In conclusion, although the number of patients in this study was small, the data provide some prelimi-
nary indications of the expected values for SOD and CAT in healthy controls vs septic patients and in septic patients divided into survivor and nonsurvivor groups. Different patterns appear for RBC SOD compared with RBC CAT (Fig. 1, C and D), but similar patterns are seen for the two enzymes when analyzed in plasma (Fig. 1, A and B). Of the four analytes measured, total plasma SOD appears to have the greatest prognostic potential because of the lesser amount of overlap seen when survivors are compared with nonsurvivors.

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References