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Elevated serum bleomycin-detectable iron concentrations in patients with sepsis syndrome

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Abstract *Objective:* To determine serum bleomycin-detectable 'free' iron in patients with septic shock and to relate these findings to both outcome and a marker of free radical damage.
Design: A prospective observational study.
Setting: A nine-bed intensive care unit in a university teaching hospital.
Patients: Sixteen consecutive patients with septic shock, defined as: (1) Clinical evidence of acute infection; (2) hypo- or hyperthermia ($< 35.6\,^{\circ}$ or $> 38.3\,^{\circ}$C); (3) tachypnoea (> 20 breaths/min or ventilated); (4) tachycardia (> 90 beats min); (5) shock (systolic pressure < 90 mmHg) or on inotropes. Fourteen patients also had secondary organ dysfunction.
Measurements and results: Bleomycin-detectable iron concentrations were elevated in all patients (37.2 ± 11.0 μmol/l vs 5.1 ± 3.3 μmol/l in healthy subjects, $P < 0.0001$), but there was no difference between patients who died and those who survived (39.2 ± 9.3 and 36.2 ± 12.3 μmol/l, respectively). Thiobarbituric acid reactive substances (an index of lipid peroxidation) were higher in those who died (3.33 ± 2.29 μmol/l) than in the surviving patients (0.99 ± 0.14 μmol/l, $P < 0.01$) or healthy subjects (0.92 ± 0.39 μmol/l, $P < 0.01$). Free iron did not correlate with thiobarbituric acid-reactive substances. However, a significant correlation was found between lipid peroxidation and clinical severity (APACHE II) score ($r = 0.54$, $P < 0.05$).
Conclusions: The present study provides evidence of lipid peroxidation in patients who die with septic shock. The data suggest that iron-catalysed hydroxyl radical generation does not form an important contribution to this lipid peroxidation in patients with sepsis.

Key words Septicaemia · Free radicals · Lipid peroxide · Catalytic iron

Introduction

Patients with severe infections receiving intensive care have been shown to have abnormally low concentrations of protective antioxidants [1–3] and high levels of the metabolic products of free radical attack [1, 2]. In addition, patients with sepsis and acute respiratory distress syndrome have elevated expired [4] and urine [5] hydrogen peroxide concentrations. This would

suggest that such patients have high concentrations of free radicals released endogenously which may in part cause the pathophysiological features of the disease process. It has been assumed that such free radicals are mainly released from activated leucocytes. It is well known that in biological systems the catalytic ions of transition metals such as copper and iron are responsible for the formation of oxygen-derived free radicals by the Haber-Weiss and Fenton reactions [6]. The potentially toxic nature of such transition metal ions dictates that they are usually found in the circulation bound to transferrin, lactoferrin and ferritin, rendering them unable to participate in such reactions.

There has been no previous study of the concentrations of free transition metal in patients with sepsis in relation to mortality. Using a highly specific assay we have, therefore, measured total bleomycin detectable free iron concentration in the serum of patients with sepsis syndrome and compared this with both a marker of lipid peroxidation and clinical outcome.

Patients and methods

The study was approved by the local clinical research (ethics) committee. Sixteen patients (9 men) aged 16–79 (median age: 58) with septic shock as defined by Bone et al. [7] were studied. Blood samples were taken from indwelling arterial lines into plain tubes for free iron estimation and thiobarbituric acid reactive substances (TBA-RS) assay as soon as the patients fulfilled the defined criteria. At the same time, a mean acute physiological and chronic health evaluation score (APACHE II) was recorded that was used to estimate the severity of disease and to compare this with the outcome of intensive care treatment [8]. Discharge from ITU or death on ITU was also noted. Biochemical results were compared with those from venous blood samples from ten healthy subjects (six men) in an age range similar to that of the patients. All samples were immediately centrifuged and the serum was stored at −20 °C until analysis.

Serum bleomycin-detectable iron was measured using the method of Gutteridge et al. [9] Bleomycin in the presence of ferrous iron degrades DNA to form a thiobarbituric acid-reactive product. Degradation by bleomycin is absolutely dependent on the concentration of total chelatable redox-active loosely bound 'free' iron. Therefore, the rate of degradation of DNA by bleomycin can be used to measure the concentration of free iron in biological fluids. Briefly, 0.1 ml serum was incubated for 2 h at 37 °C with 0.5 ml calf thymus DNA, 1 mg/ml (Sigma Chemical, Poole, Dorset, UK), 0.05 ml bleomycin sulphate, 1 mg/ml (Sigma) and 0.1 ml ascorbic acid solution. Ascorbic acid solution was freshly prepared by dissolving 0.7 g ascorbic acid (Sigma) in 10 ml water, shaking with 0.4 g Chelex-100 resin (Bio Rad Laboratories, St Alban's Herts, UK) and diluting the supernatant obtained after centrifugation at the ratio of 1:50. The reaction was stopped by the addition of 0.1 M EDTA (Sigma). The degradation of DNA by bleomycin in the presence of free iron leads to the formation of a product that reacts with thiobarbituric acid to produce a chromagen. Measurement of the absorbance at 532 nm was, therefore, used to quantitate the amount of free iron present. Ferric chloride (Sigma) was used as a calibration standard. Pyrogen-free water was used throughout, and reagents were pretreated with chelex resin to remove contaminating iron.

TBA-RS concentration was determined by the Yagi method [10] in which serum is heated at 95 °C for 60 min with thiobarbituric acid and the coloured reaction product extracted into n-butanol. The fluorescent product is read against 1,1,3,3-tetraethoxypropanol as standard at 555 nm excitation and 515 nm emission. This method provides improved specificity over spectrophotometric techniques [11].

Statistical analysis

Data are expressed as mean ± standard deviation. Data sets for patients who died were compared with those from patients who survived, and in addition both groups were compared with results from healthy subjects using the Mann Whitney U-test or Kruskall Wallis as appropriate. Pearson correlation coefficients were determined for 'free' iron versus both APACHE II score and TBA-RS, and TBA-RS versus APACHE II score.

Results

The mean APACHE II score in the patients studied was 16.6 (range: 5–31) and 14 patients had secondary organ dysfunction (87.5%). Ten of the 16 patients (62.5%) died whilst in the ICU. Serum free iron concentrations were elevated in all patients (37.2 ± 11.0 μmol/l compared to 5.1 ± 3.3 μmol/l in healthy subjects $P < 0.0001$), but there was no difference between those patients who died and those who survived. Patients who died had a free iron concentration of 39.1 ± 9.3 μmol/l whilst those who survived

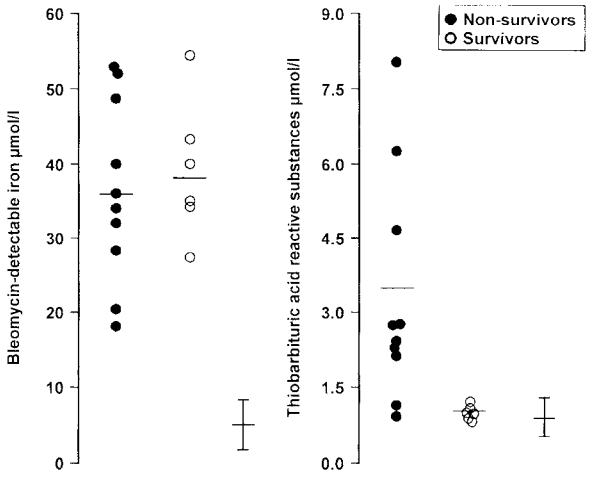

Fig. 1 Bleomycin-detectable iron and thiobarbituric acid reactive substances in non-surviving (*closed circles*) and surviving (*open circles*) patients with sepsis. *Vertical bar* is mean ± SD for healthy subjects. Iron levels in survivors and non-survivors were significantly higher than in healthy subjects ($P < 0.002$). Thiobarbituric acid-reactive substances were significantly higher in non-survivors than in both survivors and healthy subjects ($P < 0.004$)

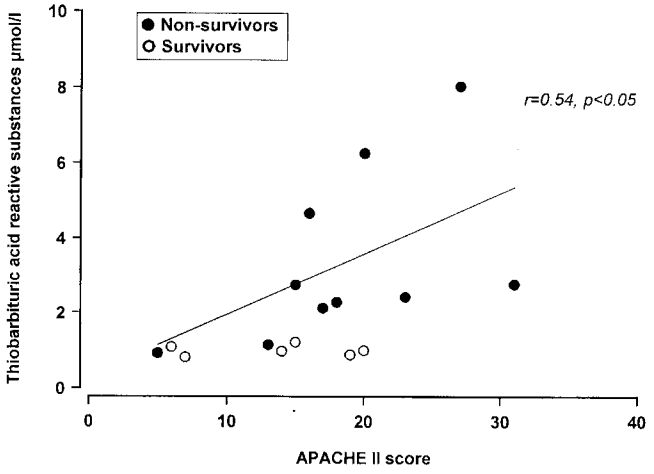

Fig. 2 Relationship between thiobarbituric acid-reactive substances and APACHE II score in non-surviving (*closed circles*) and surviving (*open circles*) patients with sepsis ($P < 0.05$)

had a free iron concentration of 36.2 ± 12.3 µmol/l (Fig. 1).

Thiobarbituric acid-reactive substances were significantly higher in those who died (3.33 ± 2.29 µmol/l) than in the surviving patients (0.99 ± 0.14 µmol/l, $P < 0.01$; Fig. 1). Results were significantly higher in those who died than in healthy subjects (0.92 ± 0.39 µmol/l, $P < 0.01$). Serum free iron did not correlate with TBA-RS.

The mean APACHE II score was 18.5 ± 7.3 in patients who died and 13.5 ± 5.9 in those who survived. There was no correlation between free iron and the APACHE II score. However, a significant correlation was found between TBA-RS and the APACHE II score ($r = 0.54$, $P < 0.05$; Fig. 2).

Discussion

The present study provides evidence of oxidant stress in patients who die with sepsis syndrome. Serum free iron concentrations were markedly elevated in all patients, both survivors and non-survivors. The level of oxidant stress as assessed by TBA-RS correlated with disease severity, as shown by the correlation between TBA-RS and the APACHE II score. Since there was, however, no correlation between free iron and TBA-RS, this study would suggest that hydroxyl radical generation via iron catalysed Haber-Weiss and Fenton reactions may not contribute to the lipid peroxidation found in patients with sepsis.

The method used in the present study for the measurement of bleomycin-detectable free iron has been shown to be specific for non-protein bound iron and is unaffected by other physiological iron-containing transport proteins and enzymes, such as haemoglobin, ferritin, transferrin, catalase and lactoferrin [9]. Gutteridge et al. failed to detect any bleomycin-detectable iron in plasma from healthy subjects [9]. Transferrin has a very high binding affinity for iron, and under normal circumstances only 20–30% of its binding capacity is occupied by iron. The free iron concentration is therefore very low, preventing participation of free iron in peroxidative damage. In the present study, blood samples were collected into routine blood tubes, and not sterile containers, as in the Gutteridge study, and control experiments showed basal contaminating iron levels. However, the same type, source and lot number of the tubes were used for both patients and healthy subjects, such that the relative results presented remain valid in the context of the study.

In healthy subjects very little redox-reactive iron is found in the plasma, and the sequestration of these metal ions form an important extracellular defence system [12, 13]. Such sequestration helps to prevent the formation of the highly reactive hydroxyl radical from superoxide anion and hydrogen peroxide. Although these oxygen-derived free radicals are generated normally during several metabolic processes, it is thought that the activated phagocyte is a major source of superoxide anion in septic patients [14, 15]. Such radicals could then interact with redox-reactive iron to cause severe cell and tissue damage. It is important to note that patients with sepsis syndrome have reduced individual antioxidant concentrations [1–3], which would render the possibility of damage more likely.

The origin of the increased bleomycin-detectable iron is not known, but may be released from damaged cells, particularly erythrocytes. The liver is responsible for taking up some of these ions, whilst others are bound to proteins made in the liver. It is therefore possible that minor derangement of liver function may result in an inadequate sequestration in the presence of excessive iron release. The data presented show that the high levels of free iron found in the patients with sepsis appear not to be responsible for the increased lipid peroxidation observed in these patients. The TBA-RS method is known not to be specific for lipid peroxide, but accepting these limitations, the technique is helpful in giving an indication of peroxidative damage [11].

The technique to measure free iron involves reduction of all non-protein-bound iron with ascorbic acid, including ferric ions bound to low-molecular-weight chelators such as citrate which, although not catalytically active, can be reduced to ferrous ions that are more soluble and can exist in ionic form at neutral pH,

enabling reaction with hydrogen peroxide to generate hydroxyl radicals. Thus bleomycin-detectable iron is not redox-reactive iron per se, and this may explain why increased bleomycin-detectable iron may not correlate with increased lipid peroxidation.

It has been argued that although an hydroxyl radical in chemical systems is the most toxic of oxygen-derived free radicals in biological systems, it reacts with any molecule that is in the near vicinity, and the relative toxicity of hydroxyl radicals is dependent upon the site of production [13]. The impact of any damage is relative to the first-line target. For example, hydroxyl radicals produced close to DNA may cause modification to purine and pyrimidine bases and DNA strand breakage, whereas damage to lactate dehydrogenase or another enzyme present in excess in a cell would be unlikely to have any major biological consequences. The concept of important and non-important sites of free radical attack may explain the relative lack of toxicity of hydroxyl radicals produced in vivo in biological compared with chemical systems. Such a concept may add to the explanation for the apparent lack of correlation of catalytic iron concentration with the degree of lipid peroxidation as assessed by TBA-RS.

In summary, the present study demonstrates increased lipid peroxidation in patients who died with sepsis syndrome, which was correlated with a severity of disease score. Markedly elevated free iron levels were also found in these patients, which did not correlate with lipid peroxide levels or APACHE II score. Taken in consideration with decreased antioxidant protection mechanisms, antioxidant therapy may be warranted in such patients.

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