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Brief Communication

ASCORBYL RADICAL FORMATION IN PATIENTS WITH SEPSIS: EFFECT OF ASCORBATE LOADING

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Abstract—Patients with sepsis have low concentrations of antioxidants, including ascorbic acid, and also have increased concentrations of markers of free radical damage. Although ascorbic acid is a potent antioxidant, it can act as a prooxidant by promoting iron-catalysed reactions. We measured baseline total vitamin C and bleomycin-detectable "free" iron levels and ascorbyl radical concentrations before and after intravenous infusion of 1 g ascorbic acid in patients with sepsis and healthy control subjects. Vitamin C concentrations were decreased in patients compared to healthy subjects ($p < 0.0001$), and "free" iron was increased ($p < 0.002$). Preinfusion ascorbyl radical concentrations were not different in patients and controls. Postinfusion ascorbyl radical levels increased in both controls and patients, with larger increases in healthy subjects ($p < 0.0001$), suggesting suboptimal basal vitamin C levels and increased scavenging of a constant oxidant pool by ascorbate in the controls. In the patients, who were all vitamin C deficient, infused ascorbate was rapidly consumed, either via the promotion of redox cycling of iron or as a result of radical scavenging. This study demonstrates markedly different handling of infused ascorbate in patients with sepsis and healthy subjects, and further studies are needed to elucidate the relative anti- and pro-antioxidant mechanisms of ascorbate in patients with raised "free" iron levels.

Keywords—Ascorbic acid, Free radicals, Iron, Electron paramagnetic resonance spectroscopy, Septicemia, Spin traps, Vitamin C

INTRODUCTION

Intensive care patients with sepsis have been shown to have abnormally low concentrations of protective antioxidants[1–3] and high levels of the products of free radical attack[1,2] associated with clinical disease severity.[1] These observations suggest that these patients are exposed to increased levels of oxidative stress and endogenous free radical production, which may be arising from activated phagocytic cells, thought to be involved in the pathophysiology of the disease process.[4] Activated phagocytes generate a wide range of reactive oxygen species including superoxide radicals, hydrogen peroxide, singlet oxygen, hypochlorous acid, and nitric oxide. Though some of these species are inherently reactive and damaging, others such as hydrogen peroxide require the presence of other cofactors to exert maximum damage. The toxicity of hydrogen peroxide is known to be exacerbated by the presence of certain low-valency transition metal ions such as copper and iron, which can catalyse the generation of the highly damaging hydroxyl radical through metal-ion catalysed Haber–Weiss and Fenton reactions.[5] The potentially toxic nature of such transition metal ions dictates that in normal circumstances they are found in the circulation in a bound form, rendering them unable to participate in such reactions. The amount of unbound "catalytic" metal ions present in the circulation may therefore play a key role in determining the levels of free radicals produced and, hence, the degree of oxidant stress.[6] Elevated levels of catalytic metal ions have been demonstrated in a number of diseases including sepsis.[7] In addition, activation of the complement system as part of the inflammatory response mediates the endotoxin-induced inhibition of ascorbic acid transport, suggesting that inadequate tissue concentrations of vitamin C may result even when circulating levels appear normal.[8]

To combat such increased oxidant stress resulting from radical generation, antioxidant therapy, including ascorbic acid administration, has been advocated. However, although ascorbic acid is a potent antioxi-

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dant,[9] it is also a powerful reducing agent and can act as a prooxidant by maintaining iron as Fe^{2+} and thus available for participation in these radical-generating reactions. Both of these actions result in the generation of ascorbyl radicals, which can be used to monitor the extent of oxidative stress. We have therefore investigated the effect of intravenous infusion of ascorbic acid on free radical concentrations using electron paramagnetic resonance (EPR) spectroscopy in patients with sepsis. We also investigated the relationship between preexisting "free" iron and total vitamin C levels on the levels of free radicals found following infusion of ascorbic acid.

METHODS

All reagents were of analytical grade and were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, UK, unless otherwise stated.

Subjects studied

Eight patients (four women) admitted onto the Intensive Care Unit with sepsis syndrome defined according to the criteria specified by Bone et al.[10] were studied. All patients were receiving a standard regimen total parenteral nutrition formula providing 100 mg ascorbic acid daily. Written informed consent was obtained from the patients' relatives, and the study protocol was approved by the local Clinical Research (Ethics) Committee. Blood samples were obtained pre- and postascorbic acid infusion, from indwelling arterial lines. Nine healthy control subjects (four women) were also studied.

Ascorbic acid infusion

Preinfusion blood samples were immediately centrifuged for 5 min at 1000 g, and serum and heparinized plasma samples were frozen at −20°C for free iron and total vitamin C assays, respectively. One milliliter of plasma from EDTA anticoagulated blood was added to an equal volume of 50 mM *N*-t-butyl-α-phenylnitrone (PBN) in phosphate buffered saline, pH 7.4, in a cryotube and incubated for 1 h at room temperature. Tubes were then snap-frozen in liquid nitrogen and stored at this temperature until EPR analysis.

Ascorbic acid (1 g in 10 ml saline) was infused intravenously over a period of 10 min through existing indwelling lines in patients and through butterfly cannulae in healthy subjects. After a further 5 min, postinfusion EDTA blood samples were obtained and treated as above for EPR studies.

Total vitamin C measurement

Plasma ascorbic acid was measured spectrophotometrically as total vitamin C (ascorbic and dehydroascorbic acids).[11] Plasma (0.5 ml) was added to 1 ml 0.75 g/ml trichloroacetic acid and centrifuged. The protein-free supernatant (0.33 ml) was mixed with 0.1 ml of 20 mg/ml dinitrophenylhydrazine in 5 M sulphuric acid and containing 2.3 mg/ml thiourea and 2.7 mg/ml copper sulphate, and incubated at 37°C for 4 h. Following the addition of 0.5 ml 75% (v/v) sulphuric acid the absorbance was recorded at 520 nm against L-ascorbic acid as standard.

Bleomycin detectable "free" iron

Serum bleomycin-detectable "free" iron was measured using the method of Gutteridge et al.[12] Briefly, 0.1 ml serum was incubated for 2 h at 37°C with 0.5 ml calf thymus DNA (1 mg/ml), 0.05 ml bleomycin sulphate (1 mg/ml) and 0.1 ml ascorbic acid solution. Ascorbic acid solution was freshly prepared by dissolving 0.7 g ascorbic acid 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 (1 in 50). The reaction was stopped by the addition of 0.1 M EDTA. 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 was used as a calibration standard. Pyrogen free water was used throughout, and all reagents were pretreated with chelex resin to remove contaminating iron.

Electron paramagnetic resonance (EPR) spectroscopy

EPR spectra were recorded at room temperature on thawed samples using a standard aqueous solution cell inserted into the cavity of a Bruker ESP300 EPR spectrometer equipped with 100 kHz modulation and a ER035M Gaussmeter for field calibration. Hyperfine coupling constants were measured directly from the experimental spectra and compared with literature values. Relative radical concentrations (in arbitrary units) were determined from measurements of line intensities (signal heights; which are directly proportional to absolute radical concentrations) on spectra recorded with identical spectrometer settings.

Statistical methods

Data are expressed as mean and standard deviation. Data sets were compared using Student's *t*-test for

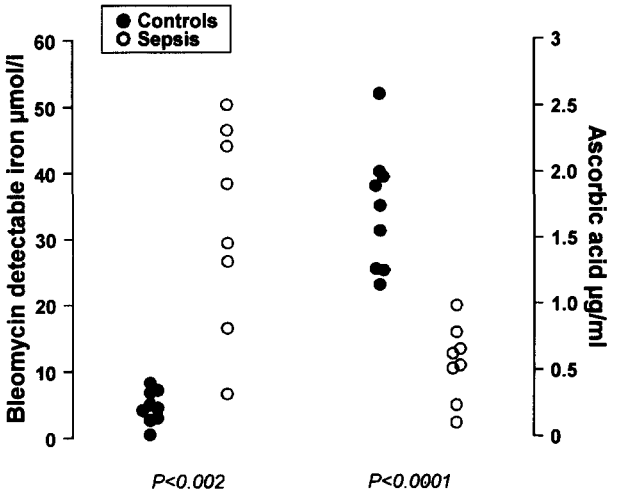

Fig. 1. Serum bleomycin-detectable "free" iron and plasma total vitamin C in patients with sepsis (open circles) and healthy control subjects (solid circles).

equal or unequal variances as appropriate, following *F*-testing. Pre- and postinfusion ascorbyl radical concentrations were compared using a paired *t*-test. Pearson's correlation coefficients were determined for postinfusion ascorbyl radical concentration versus both preinfusion total vitamin C concentration and preinfusion free iron, and preinfusion vitamin C versus preinfusion free iron.

RESULTS

The ascorbic acid infusion was well tolerated by all patients and controls. Preinfusion total vitamin C concentrations in patients with sepsis were significantly lower than in healthy subjects (0.55 ± 0.28 µg/ml compared to 1.71 ± 0.46 µg/ml, $p < 0.0001$, Fig. 1). Preinfusion bleomycin-detectable iron was increased in the patients, with a mean value of 32.3 ± 15.3 µmol/l compared to 4.7 ± 2.5 µmol/l in the control group ($p < 0.002$, Fig. 1).

Examination of plasma samples obtained prior to ascorbate infusion, by EPR spectroscopy, resulted in the detection of a relatively weak doublet signal from the well-characterised ascorbyl radical; the concentration of this species (measured in arbitrary units) was not significantly different between patients and controls (1.9 ± 1.6 units and 1.1 ± 0.3 units, respectively, NS). Following ascorbic acid administration, the ascorbyl radical concentration was found to be increased in patients to 3.2 ± 0.73 units ($p < 0.001$ vs. preinfusion samples) and healthy subjects 14.6 ± 4.3 units ($p < 0.0001$ vs. preinfusion samples). The concentration in healthy subjects was significantly higher than in patients with sepsis ($p < 0.0001$, Fig. 2).

A highly significant linear correlation was found between preinfusion total vitamin C levels and postinfusion concentrations of ascorbyl radicals in both patients with sepsis and control subjects ($r^2 = 0.854$, $p < 0.0001$, Fig. 3). A significant exponential relationship between ascorbyl radical levels postinfusion, and preinfusion bleomycin-detectable iron levels was also observed, ($r^2 = 0.868$, $p < 0.0001$, Fig. 4). However, no correlation was found between basal bleomycin-detectable iron and vitamin C concentrations prior to infusion.

DISCUSSION

This study has demonstrated that patients with sepsis syndrome have markedly decreased levels of total vitamin C compared to normal controls, in agreement with a previous study that found low levels of ascorbic acid in a heterogenous group of critically ill patients, in conjunction with a decreased ratio of ascorbic to dehydroascorbic acids.[3] Concentrations of other antioxidants, including tocopherol, retinol, and the carotenoids, are also decreased in such patients,[1,2] in addition to increased levels of lipid peroxides[1,2] and associated with clinical disease severity.[1] "Free" (bleomycin-detectable) iron concentrations were also found to be raised in this study, confirming our previous findings in patients with sepsis.[7]

The low concentrations of vitamin C in sepsis pa-

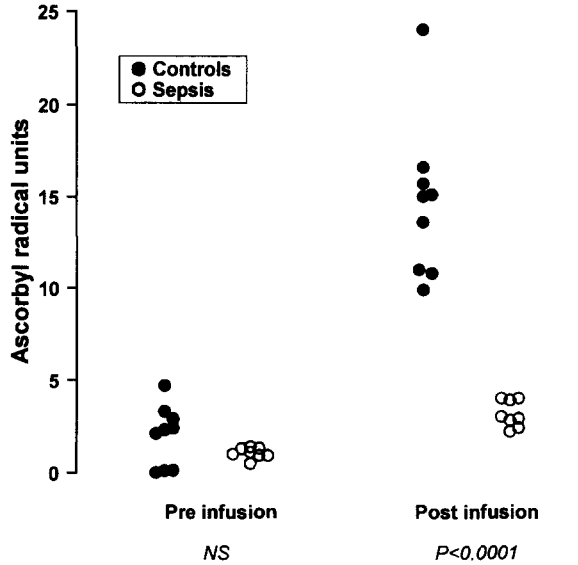

Fig. 2. Ascorbyl radical concentrations in patients with sepsis (open circles) and healthy controls (solid circles), pre- and postinfusion of 1 g ascorbic acid.

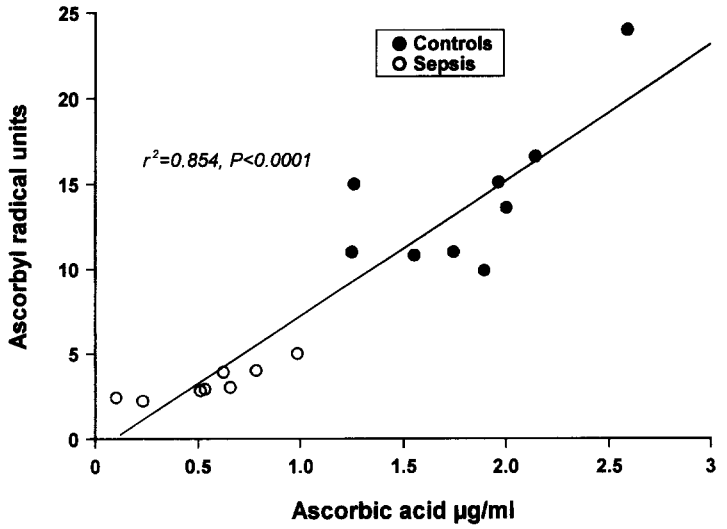

Fig. 3. Relationship between preascorbic acid infusion total vitamin C concentrations and postinfusion ascorbyl radical levels. Open circles are patients with sepsis and solid circles are control subjects.

tients suggests that these subjects are likely to be susceptible to oxidant stress. Low levels of ascorbate may result from either (i) the generation of ascorbyl radicals (and hence oxidation of reduced ascorbate to dehydroascorbate) during the reduction of "free" iron, that is, Fe(III) to Fe(II); (ii) from the scavenging of aqueous free radicals by ascorbate; (iii) the regeneration of vitamin E from vitamin E phenoxyl radicals formed as a result of membrane or low density lipoprotein (LDL) oxidation.[13] The low levels of total vitamin C, found in this study, suggest that in patients with sepsis, there is inadequate regeneration and ultimate destruction of dehydroascorbic acid. The data prove that in the presence of high concentrations of "free" iron, total levels of vitamin C are depleted. Whether this depletion in the sepsis patients (relative to healthy subjects) is due to the consumption of ascorbate via reduction of iron or via radical scavenging as a result of iron-catalysed radical generation is unclear and cannot be determined from the current data. The overall effect, however, is that vitamin C is depleted and is thus unavailable to act as an antioxidant and prevent further tissue damage.

Infusion of reduced ascorbic acid resulted in an increased level of EPR-detectable ascorbyl radicals in both the healthy subjects and the sepsis patients, with much larger increases than in the control subjects. This

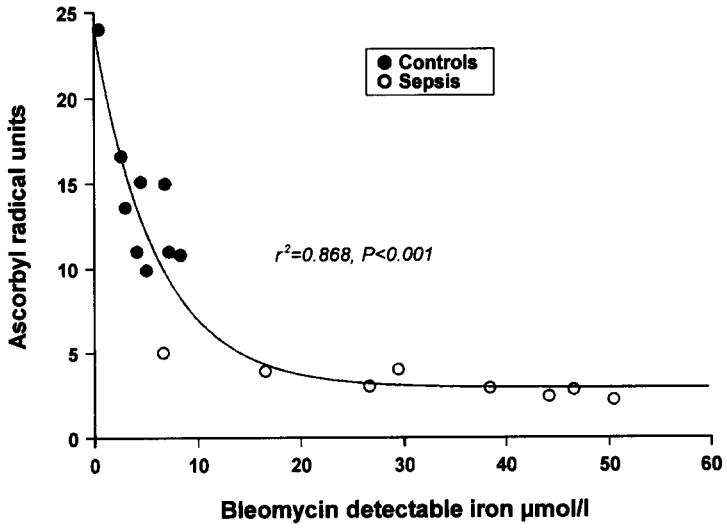

Fig. 4. Relationship between preascorbic acid infusion bleomycin-detectable "free" iron concentrations and postinfusion ascorbyl radical levels. Open circles are patients with sepsis and solid circles are control subjects.

increase in ascorbyl radical concentration in controls, who have very low levels of bleomycin-detectable iron, is believed to be due not to an increased level of radical generation, but to an increased level of scavenging of a (relatively) constant oxidant pool by the ascorbate (i.e., ascorbate acting as a sacrificial target, and protecting molecules that would otherwise be damaged). This infers that if ascorbate is indeed the terminal protective agent, as has been often suggested, the levels of ascorbate in the control subjects were not optimal, and that raising the levels of ascorbate in these subjects could be beneficial, as it would presumably protect other (nonterminal) antioxidants and target molecules.

The infusion of ascorbate into patients with sepsis might be expected to have two effects—it could either increase the extent of oxidant generation by promoting the redox cycling of "free" iron (which presumably is limited initially by the very low levels of ascorbate in these patients) and hence exacerbate tissue damage, or it might increase the antioxidant capacity of the patient and hence confer protection. The increased levels of ascorbyl radicals detected by EPR spectroscopy postascorbate infusion would be consistent with either hypothesis. The observation that the levels of ascorbyl radicals in the patients with sepsis are well below those of the normal subjects would suggest that there is either (i) a much lower level of oxidant stress in these patients postinfusion as a result of the protective effects of ascorbate, which is unlikely in view of their levels of bleomycin-detectable iron, or (ii) that the ascorbate that has been infused is rapidly being consumed (to give dehydroascorbate or further oxidation products that do not give ascorbyl radicals) and that, as with the normal subjects, the ascorbate levels achieved via this infusion protocol are still far from optimal in terms of protection against oxidants. Further studies with varying levels of infused ascorbate are required to confirm this hypothesis.

Antioxidant administration, both singly and in combination, has been shown to increase survival in animal models of sepsis and injury (reviews 4,14). A single report (in abstract form) of a clinical study of combination antioxidant therapy consisting of large intravenous doses of ascorbic acid, tocopherol, selenium and *N*-acetylcysteine, showed a striking 50% reduction in mortality in patients with acute respiratory distress syndrome.[15] This study demonstrates markedly different handling of infused ascorbate in patients with sepsis syndrome and healthy subjects, and, clearly, further detailed studies are needed to elucidate the relative anti- and proantioxidant mechanisms of ascorbate metabolism in patients with raised "free" iron levels. It is also not known whether reactive copper levels, which may contribute to the prooxidant potential, are increased in these patients. However, it is widely accepted that the major role of ascorbate in vivo is as an antioxidant,[16] and not a prooxidant, and if this is indeed the case, then infusion of ascorbate might be expected to provide protection.

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