Adjuvant treatment of patients with antineutrophil cytoplasmic antibody-associated vasculitis with vitamins E and C reduces superoxide production by neutrophils


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Abstract

Objectives. Neutrophils when activated generate a respiratory burst which has been implicated in the pathogenesis of primary systemic vasculitis. Neutrophils from patients with vasculitis have a greater respiratory burst than normal healthy donors. The aim of this study was to assess the effects of antioxidant treatment (vitamins E and C) on the generation of a respiratory burst from neutrophils isolated from patients with antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis.

Methods. Neutrophils were isolated from patients with systemic vasculitis and healthy donors. Spontaneous superoxide generation was measured by the reduction of ferricytochrome c. The patients were treated with antioxidants, vitamins E and C, and spontaneous superoxide generation, vitamin C and total antioxidant capacity were measured before and after treatment.

Results. The treatment of the patients with antioxidants resulted in a reduction in spontaneous superoxide generation (pre-treatment 8.41 ± 0.7 nmol/10⁶ cells; post-treatment 5.64 ± 0.6 nmol/10⁶ cells; P < 0.05). There was no significant difference in the superoxide generation from normal controls who did not receive treatment, measured prior to commencement of the study and 10 days later (first reading 4.81 ± 0.5 nmol/10⁶ cells; second reading 5.32 ± 0.4 nmol/10⁶ cells; P > 0.05). Total antioxidant capacity increased significantly following treatment with vitamins C and E (555.4 ± 142 vs 668.6 ± 186 μmol/l trolox equivalent; P = 0.01) as did vitamin C concentrations (56.5 ± 27 vs 137.7 ± 64 μmol/l; P = 0.002).

Conclusions. In this preliminary study, the treatment of patients with antioxidants, vitamins E and C, reduced neutrophil generation of superoxide and suggests that antioxidants may have an important role as adjuvant therapy. The evidence presented should form the basis of a larger randomized placebo-controlled trial of vitamins E and C as adjuvant therapy in patients with ANCA-associated systemic vasculitis.

Key words: Neutrophils, Vitamin E, Vitamin C, Antioxidant, Superoxide, Systemic vasculitis.

Antineutrophil cytoplasmic antibody (ANCA)-associated systemic vasculitis includes Wegener’s granulomatosis (WG), microscopic polyangiitis (MPA) and Churg–Strauss syndrome. ANCA are able to activate cytokine-primed neutrophils (PMN) to generate a respiratory burst [1, 2], degranulate and accelerate polymorphonuclear leucocyte (PMN) apoptosis using a mechanism dependent on the generation of reactive oxygen species [3]. In vivo, this has important implications for inflammation, as the generation of reactive oxygen species is a potential cause of endothelial cell damage. Our studies have shown that lysis of endothelial cells by ANCA-activated PMN was enhanced when the endothelial cells had been pre-treated with 1,3-N,N'-bis(2-chloroethyl)-N-nitrosourea (BCNU) [4]. BCNU inhibits glutathione reductase, an antioxidant, hence reducing the ability of the cells to withstand oxidant injury. Alteration in the redox balance in patients with systemic vasculitis has been shown [5]. We and others have shown that PMN isolated from patients with ANCA-associated vasculitis generate greater quantities of superoxide than PMN isolated from healthy donors [6, 7].

Reactive oxygen species play a key role in the recruitment of inflammatory cells and the subsequent
Antioxidant therapy in systemic vasculitis

Microvascular dysfunction. Oxygen radicals released from activated PMN are directly toxic to endothelial cells. They exert tissue damage by peroxidative attack on fatty acids, denaturation of proteins and DNA damage. Powerful chemotactic inflammatory mediators are generated and adhesion molecule expression on leucocytes and endothelial cells is up-regulated following activation by reactive oxygen species [8–10]. Reactive oxygen species have previously been documented to cause renal injury in animal models. Antioxidant enzymes and/or oxygen radical scavengers protect animals with antiglomerular basement membrane glomerulonephritis from proteinuria and glomerular damage [11].

A variety of natural antioxidants exist to scavenge free radicals and prevent oxidative damage. The primary defence against oxidative stress in extracellular fluids rests with several low molecular weight antioxidant molecules, including vitamin C, vitamin E, urate, thiols and bilirubin [12]. These so-called ‘chain-breaking’ antioxidants are powerful reductants that scavenge free radical species (and are consumed in the process) to prevent further oxidation of more important molecules, such as lipids or proteins. Therefore, the levels of these antioxidants indicate not only the state of protection against oxidation, but may also reflect consumption of acute oxidative status.

The aims of this study were to assess if PMN isolated from patients with ANCA-associated vasculitis produced greater amounts of superoxide than those from healthy individuals and whether treatment with antioxidants, vitamins E (α-tocopherol) and C (ascorbic acid), would reduce this.

Methods

Patients and healthy volunteers

Patients were selected who had a diagnosis of acute pauci-immune necrotizing crescentic glomerulonephritis by renal biopsy and who fulfilled the Chapel Hill Consensus Conference definitions for WG or MPA [13]. All the patients were in disease remission and receiving maintenance therapy of prednisolone (10 mg) and azathioprine (2 mg/kg). Any patient with evidence of a rising ANCA titre or clinical relapse was excluded from the study. Ethical permission was obtained from the local ethics committee of the University Hospital Birmingham National Health Service Trust and written informed consent according to the Declaration of Helsinki was obtained from the patients and from healthy age- and sex-matched individuals.

The patients were given a 10-day course of vitamin C (1 g/day) and vitamin E (800 mg/day). The antioxidants were given in addition to the patients’ normal treatment and no change in the patients’ normal immunosuppressive regime or other drug treatments was made. The patients were advised to avoid intake of citrus fruits and alcohol during the study period, but otherwise to maintain their normal dietary pattern. Blood samples were taken from patients at the commencement of the study, after 10 days of treatment with vitamins C and E, and after a 10-day washout period, for determination of PMN superoxide release and for measurement of antioxidant status (total antioxidant capacity and vitamin C concentrations). The patients acted as their own controls for the treatment. To ensure that variations in superoxide measurements were due to treatment effects and not other influences, the healthy donors were untreated and superoxide production was assayed from their PMN at commencement of the study and again 10 days later.

PMN isolation

Immediately following venesection, PMN were isolated and assayed for production of superoxide. PMN were isolated using Percoll gradients as previously reported [14]. This gave a cell purity of 95% PMN with 98% viability as assessed by trypan blue exclusion. The cell pellet was resuspended at a concentration of 2 × 10⁶ PMN/ml and used immediately.

Superoxide release measured by the reduction of ferricytochrome c

Superoxide anion production was determined by the superoxide dismutase inhibitable reduction of ferricytochrome c as previously described [15]. The experiments were carried out in at least quadruplicate.

Measurement of antioxidant status

Venous blood was sampled into plain tubes, allowed to clot for 5–10 min, then centrifuged at 3500 g, at 4°C, for 15 min. Serum was aliquotted and stored at −80°C until analysis of total antioxidant capacity. To stabilize the vitamin C, the serum was treated with an equal volume of freshly prepared 10% metaphosphoric acid and centrifuged for a further 5 min. The supernatant was stored in aliquots at −80°C until analysis.

Serum total antioxidant capacity, a marker of the overall ability to resist free radical-mediated oxidation, was determined by enhanced chemiluminescence, as described previously [16]. Briefly, this method relies on the continuous production of a free radical-mediated luminescent light signal. When a sample containing antioxidants is added, the light signal is temporarily stopped by its free radical-scavenging action. The time taken for the light signal to return is then directly proportional to the amount of antioxidant in the sample. The major individual antioxidants that contribute to the serum total antioxidant capacity include urate (70.3%), vitamin C (10.3%), vitamin E (8.6%) and vitamin A (10.7%); thiols and bilirubin contribute to a lesser degree [17]. Serum vitamin C was determined using reverse-phase high-performance liquid chromatography, with ultraviolet detection at a wavelength of 254 nm, adapted from the method by Nagy and Degrell [18]. The within- and between-batch coefficients of variation for both sets of analysis were <2.5 and <3.5%, respectively.
Statistical analysis
Data are presented as the mean and standard error of the mean. Statistical significance was determined using ANOVA for multiple comparisons and the Bonferroni test for pairs was used to compare groups. A level of $P < 0.05$ was considered to be statistically significant.

Results

Patients
Twelve patients, all of whom were in disease remission, were treated with vitamins E (800 mg/day) and C (1 g/day) for 10 days (see Table 1 for patient characteristics). The median time of follow-up was 40 weeks from the time of initial diagnosis (range 17–216 weeks). All were on maintenance therapy of prednisolone (10 mg) and azathioprine (2 mg/kg). None of the patients had evidence of active disease or infection. However, in seven patients the C-reactive protein (CRP) was greater than the normal range of 10 $\mu$mol/l. Patient 4 was the only smoker. Side-effects of vitamin E are minor, comprising mainly abdominal cramps and diarrhoea; only one patient reported mild diarrhoea, which did not necessitate discontinuation of treatment. No symptomatic side-effects were reported with vitamin C. All patients completed the 10-day course of tablets.

Spontaneous superoxide generation is reduced by antioxidants
Freshly isolated PMN from patients produced significantly greater basal superoxide than PMN from healthy donors, as has been previously shown [6]. Patients, but not healthy donors, were treated with vitamins E and C. Following treatment there was a small but significant reduction in the generation of superoxide from isolated patient PMN. Pre-treatment, the mean superoxide generation was 8.41 ± 0.7 nmol/10^6 cells and post-treatment the mean superoxide generation was 5.64 ± 0.6 nmol/10^6 cells; $P < 0.05$ (Fig. 1). There was no difference in superoxide levels pre-treatment compared with the post-washout period (pre-treatment 8.41 ± 0.7 nmol/10^6 cells, post-washout 7.97 ± 0.7 nmol/10^6 cells). There was no significant difference in the superoxide generation from normal controls who did not receive treatment, measured prior to commencement of the study and 10 days later (first reading 4.81 ± 0.5 nmol/10^6 cells, second reading 5.32 ± 0.4 nmol/10^6 cells; $P > 0.05$). Basal superoxide from patients pre-treatment was significantly greater than superoxide generation from normal healthy donors (patients 8.41 ± 0.7 nmol/10^6 cells; healthy controls 4.81 ± 0.5 nmol/10^6 cells; $P < 0.05$). However, post-treatment there was no difference in the generation of superoxide production between normal donors and patients. Only one patient showed an increase in superoxide production following treatment with antioxidants; this patient was the only one who smoked.

Measurements of antioxidant status
Baseline total antioxidant capacity was within the normal range for otherwise healthy adults (350–550 $\mu$mol/l trolox equivalent) [16]. Total antioxidant capacity increased following treatment with vitamins C and E, post-treatment.

![FIG. 1. Superoxide production from patients before and after 10 days of treatment with the antioxidants vitamins E and C.](image)

**Table 1. Patient characteristics**

<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>Sex</th>
<th>Disease</th>
<th>Organ affected at diagnosis</th>
<th>Relapses</th>
<th>Follow-up</th>
<th>Serum Cr (µmol/l)</th>
<th>CRP (µmol/l)</th>
<th>Current ANCA</th>
<th>Urate (µmol/l)</th>
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R, renal; J, joints; CNS, central nervous system; S, skin; E, eyes; L, lungs; PN, peripheral nervous system; ENT, ear, nose and/or throat; M, muscles; serum Cr, serum creatinine at the time of the study; neg, negative; p, positive; follow-up, time from initial diagnosis (weeks).
although this did not reach significance (555.4 ± 142 vs 668.6 ± 186 μmol/l trolox equivalent; P = 0.19). Vitamin C concentrations were significantly increased post-treatment (56.5 ± 27 vs 137.7 ± 64 μmol/l; P < 0.001). Total antioxidant capacity and vitamin C were both reduced following the 10-day washout period, returning to baseline (543.0 ± 450 μmol/l trolox equivalent; 61.4 ± 22 μmol/l). Vitamin C levels were significantly reduced following the washout period compared with treatment levels (P < 0.001).

Discussion

In this study we have shown that spontaneous generation of superoxide in vitro, which is increased in patients with quiescent disease compared with normal healthy donors, can be reduced with the administration of a combination of the antioxidants vitamins E and C in pharmacological doses. This is associated with an increase in the total antioxidant capacity and vitamin C concentrations, increasing the body’s ability to prevent free radical-mediated oxidative damage. Free oxygen radicals have been shown in vitro to be important in the pathogenesis of ANCA-associated vasculitis [3], and therefore the administration of antioxidants may ameliorate PMN-mediated damage and subsequent tissue injury.

PMN isolated from patients with non-ischaemic congestive heart failure have also been shown to produce greater quantities of superoxide anions than those from normal healthy donors. In agreement with our study, patients treated with vitamin C showed a significant reduction in PMN superoxide generation [19].

Other inflammatory conditions, including rheumatoid synovitis, have demonstrated low levels of antioxidants [20]. In the study by Fairburn et al. [20], it was suggested that low levels of antioxidants such as z-tocopherol were due to consumption and that damage might be reduced by administration of vitamins E and C. Our pilot study was too small to provide useful comparisons of levels of vitamins E and C with the general population. It is possible, however, that low level inflammation due to vasculitis may have been persistent in some of our patients, as seven of the 12 patients had mildly elevated CRP despite an absence of clinical symptoms.

Glucocorticoids are commonly used as immunosuppressive treatment in patients with ANCA-associated small vessel vasculitis. One of the actions of steroids is to suppress PMN production of superoxide [21]. There is increased expression of the gene encoding for manganese superoxide dismutase enzyme, which is a potent antioxidant enzyme [7]. A reduction in the production of reactive oxygen species may reduce the damage to endothelial cells and the stimuli for propagation of the inflammatory lesion. It has also been suggested that glucocorticoids produce a change in membrane phospholipid composition which may interfere with the assembly of the NADPH oxidase enzyme [22]. z-tocopherol is a membrane-bound antioxidant which acts to inhibit lipid peroxidation once initiated. It is regenerated by ascorbic acid, another potent inhibitor of lipid peroxidation [23]. By administering vitamins C and E, the antioxidant effect of glucocorticoids may be enhanced through regeneration of z-tocopherol, hence reducing the generation of superoxide, as seen in this study.

The use of vitamins E and C is safe with no serious side-effects at the doses administered in this study. Although ascorbic acid has been reported as being able to switch to having pro-oxidant activity in a study by Frei et al. [23], it was shown that in plasma, even at very high concentrations, ascorbate maintains its antioxidant activity. There is growing evidence that the use of antioxidants can prevent cardiovascular disease and cancer and have an anti-inflammatory effect [24]. Reactive oxygen radicals increase oxidation of low density lipoproteins (LDL) which may lead to accelerated atherosclerosis in patients with ANCA-associated vasculitis. Oxidative modification of LDL induces immunogenic epitopes in the LDL molecule, which results in the formation of autoantibodies against oxidized LDL [25]. Antibodies to oxidized LDL are present in patients with ANCA-associated vasculitis, particularly during active disease [26]. The administration of vitamins E and C can reduce oxidative damage in patients with moderate renal impairment due to various causes [27].

The use of vitamins E and C may have an important role as adjuvant therapy and a potential role in reducing disease morbidity. The evidence presented should form the basis of a larger randomized placebo-controlled trial of vitamins E and C as adjuvant therapy in patients with ANCA-associated systemic vasculitis.

Acknowledgement

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References


