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Reduced Free-Radical–Trapping Capacity and Altered Plasma Antioxidant Status in Cystic Fibrosis

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ABSTRACT. Plasma antioxidant status and total radical-trapping antioxidant potential (TRAP) of children ($n = 24$) with cystic fibrosis (CF) were compared with those of children ($n = 21$) without the disease. Children with CF were found to have elevated plasma concentrations of ascorbic acid (94.6 ± 58.2 μmol/L), with respect to normal children (65.6 ± 18.8 μmol/L). Plasma uric acid (330.8 ± 84 *versus* 198.0 ± 31 μmol/L $p < 0.01$) and sulfhydryl group (518 ± 43 *versus* 363 ± 31 μmol/L $p < 0.01$) concentrations were also elevated in CF. Vitamin E levels (16.9 ± 1.8 *versus* 18.4 ± 1.3 μmol/L) were at the low end of the normal range. Despite an overall increased antioxidant array, CF patients had a reduced TRAP capacity (488 ± 34 *versus* 580 ± 79 μmol/L, $p < 0.05$). TRAP measurements in CF patients showed a strong negative correlation ($r = 0.80$, $p < 0.001$) with high ascorbic acid concentration, suggesting a prooxidant effect of ascorbic acid. Oral administration of ascorbic acid to adults was found to diminish TRAP activity. Concentrations of ascorbic acid similar to those seen in CF patients were attained in ascorbate-supplemented individuals, with substantial decreases in TRAP capacity. These studies suggest that high plasma ascorbic acid levels in children with CF may have a prooxidant effect. This appears to reduce the extracellular antioxidant defense of these children and may increase susceptibility to oxidative stress. (*Pediatr Res* 33: 247–250, 1993)

Abbreviations

ABAP, 2-azobis (2-amidinopropane) hydrochloride
CF, cystic fibrosis
OH, hydroxyl radical
TRAP, total radical-trapping antioxidant potential
$TRAP_{calc}$, theoretically determined total radical-trapping antioxidant potential
$TRAP_{meas}$, experimentally determined total radical-trapping antioxidant potential
$TRAP_{diff}$, difference between $TRAP_{meas}$ and $TRAP_{calc}$
T_{Trolox}, length of induction period during which the Trolox reduces the rate of oxygen loss
T_{plasma}, length of induction period during which endogenous antioxidants reduce the rate of oxygen loss

Free radical species have been implicated in the etiology of a wide variety of human disease states ranging from atherosclerosis (1), cancer (2), and arthritis (3) to bronchopulmonary dysplasia of the premature infant (4). The ability of an individual to withstand the damaging effects of free radicals depends upon the antioxidant capacity of the body. Antioxidants fall primarily into three classes: enzymic antioxidants (superoxide dismutase, catalase, glutathione peroxidase) (5); nonenzymic antioxidants, such as vitamins C and E, uric acid, glutathione, albumin, and albumin-bound bilirubin (6–11); and metal-binding compounds such as ferritin and ceruloplasmin.

Patients with CF have an impaired ability to absorb fats from the gastrointestinal tract, as a consequence of their reduced secretion of pancreatic enzymes (12). A repercussion of this is a deficiency of the principal fat-soluble antioxidant vitamin E (13, 14). In addition, deficiencies of other circulating antioxidants, such as ferritin and serum albumin, have been reported in CF patients (15, 16). Children with CF are also deficient in selenium (14, 17), which is essential for the activity of the antioxidant enzyme glutathione peroxidase (18). Glutathione peroxidase activities in CF patients have, however, been reported to be normal (19). It has been suggested that combined selenium and vitamin E deficiencies in CF patients attaining adulthood may contribute to the development of cancers (14). Activity of superoxide dismutase and catalase in erythrocytes of CF patients has been shown to be elevated, indicating that these individuals may be undergoing an intracellular oxidative stress (20). This hypothesis is supported by studies of lipid peroxidation markers in CF patients (21–23). Evidence for an extracellular oxidant/antioxidant imbalance has been observed in the lungs of young adults with CF (24, 25).

The transient existence of free-radical species makes it impractical to measure them *in vivo*, so most studies focus on their damaging effects on cellular components, notably the peroxidation of polyunsaturated fatty acids (5). In 1985, Wayner *et al.* (26) developed the total TRAP assay. This assay requires only a small sample of plasma and measures the subject's capacity to buffer a sample of linoleic acid against peroxidation, using a heat-labile initiator ABAP as a source of free radicals. The peroxidation of linoleic acid is followed as a decrease in the free oxygen content of the sample in a Clark cell.

In the present study, the TRAP assay was used to investigate the proposal that CF patients may have a decreased capacity to withstand free-radical stress as a consequence of their reported antioxidant deficiencies.

SUBJECTS AND METHODS

Chemicals. All chemicals used were obtained from Sigma Chemical Co. (Poole, UK) or BDH Pharmaceuticals Ltd. (Poole, UK), with the exception of ABAP (Polysciences, Warrington, UK) and Trolox C (Aldrich, Gillingham, UK).

Subjects. Venous blood samples were drawn from either

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healthy children or patients with CF. CF patients were attending the CF clinic at Southampton General Hospital for a regular checkup and were not presenting with specific problems. The mean age of the CF group was 8 y 4 mo ($n = 24$, age range 2–18 y). The control group used in the study was a group of healthy children attending Southampton General Hospital or outpatient clinics for minor surgical procedures (Table 1) with a mean age of 7 y and 11 mo ($n = 21$, age range 2–17 y). Ethical permission for this study was obtained from the Joint Ethical Subcommittee, Southampton and South West Hampshire Health Authority.

Sample handling. Heparinized blood samples from CF patients and controls were centrifuged at 2000 rpm, 4°C, for 10 min. Plasma was stored at −20°C for up to 6 wk before assays. TRAP activity and antioxidant concentrations were stable over this period.

Determination of TRAP activity. TRAP was determined using a modification of the method of Wayner *et al.* (26). One hundred μL of plasma were mixed with 10 μL of linoleic acid in a glass vial by vortexing for approximately 30 s. Fifty μL of the plasma-linoleic acid mixture were added to a Perspex oxygen electrode cell (Rank Brothers, Bottisham, UK), containing 3 mL of PBS, pH 7.5, and 30 μL of 0.4 M ABAP, at 37°C. Light was excluded from the cell to prevent photodecomposition of the ABAP. Oxygen loss from the cell, representing oxygen uptake during the peroxidation of the linoleic acid, was followed until the rate of loss was maximal. At this point, 25 μL of 0.4 mM Trolox C were added.

The experimentally measured value of TRAP ($TRAP_{meas}$) was calculated from the equation:

$$TRAP_{meas} = Ri \times T_{plasma}$$
$$Ri = 2(\text{Trolox C})/T_{Trolox}$$

where Ri is the rate of peroxyl radical generation from the decomposition of ABAP at 37°C and T_{plasma} and T_{Trolox} are the respective lengths of the induction periods during which the plasma antioxidants or Trolox C buffers against the maximal rate of oxygen loss (Fig. 1).

Determination of vitamin E. One hundred μL of plasma were extracted with 100 μL of anhydrous HPLC grade ethanol and 400 μL of HPLC grade hexane. α-Tocopherol acetate was added as an internal standard. The hexane layer was removed and evaporated to dryness under a stream of nitrogen. The extract was redissolved in 400 μL of HPLC grade methanol, and 100 μL aliquots were analyzed by HPLC. A 7 × 100 mm, 5-μm C18 column (Jones Chromatography, Hengoed, UK) was eluted with methanol:water (39:1 vol/vol), at a flow rate of 0.8 mL/min. Detection was by UV absorbance at 292 nm (27).

Determination of vitamin C and uric acid. The HPLC determination of uric acid and ascorbate in plasma was based upon the method of Iriyama *et al.* (28). One hundred μL of plasma were extracted with 400 μL 2% metaphosphoric acid and 100 μL HPLC grade heptane. Samples were vortex-mixed for 40 s and then centrifuged at 13 000 × *g* for 5 min. Twenty-μL aliquots were injected for HPLC analysis. A 10 × 300 mm, 5-μm C18 column (Jones Chromatography) was eluted with a 0.2 M K_2HPO_4-H_3PO_4 buffer, pH 1.5, containing 0.25 mM octane sulfonic acid, at a flow rate of 1.8 mL/min. An EG & G electrochemical detector (Jones Chromatography) was used to detect ascorbate and uric acid, with E = 810 mV, time constant = 5 s, cathodic output, and a sensitivity of 2 μA.

Table 1. *Attendance of control children at Southampton General Hospital*

Procedure	No. of patients
Nonsurgical outpatients	8
Orthopedics	3
Osteotomy	2
Anal biopsy	2
Removal of soft tissue	1
Inguinal hernia	1
Removal of ingrowing toenail	1
Excision of ganglion	1
Atherogram	1
Unspecified	1

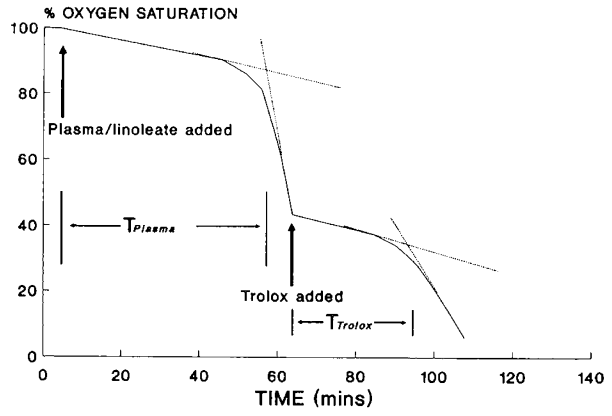

Fig. 1. Schematic representation of TRAP assay T_{plasma} is taken as the length of time between the addition of the sample to the electrode and the intersection of the slopes of the induction phase and the maximal period of oxygen uptake by the substrate. Trolox C is added to standardize the assay. T_{Trolox} is taken as the length of time between the addition of Trolox C and the intersection of the slopes of the Trolox induction phase and the maximal rate of oxygen uptake by the substrate after consumption of the Trolox C.

Determination of total plasma sulfhydryls. Plasma sulfhydryls were assayed by the method of Ellman (29). Twenty-five μL of plasma were added to 975 μL of 0.2 M Na_2PO_4, 2 mM EDTA, pH 9.0, and 20 μL of 5,5′-dithio-bis(2-nitrobenzoic) acid (10 mM in 0.05 M phosphate buffer, pH 7.0). Absorbance at 412 nm was determined against an appropriate blank.

Calculation of theoretical TRAP values. $TRAP_{calc}$ were calculated from the concentrations of individual antioxidant components of plasma, using stoichiometric values corresponding to the number of peroxyl radicals trapped by each antioxidant molecule (30, 31). In the current study, the following equation was used (30):

$$TRAP_{calc} = 1.3(\text{urate}) + x(\text{ascorbate}) + 2(\text{vitamin E}) + 0.2(\text{sulfhydryl})$$

The stoichiometric factor for the interaction of ascorbate with free radicals has been shown to vary with the concentration of ascorbate (32–34). Accordingly, the value of x was taken from a sliding scale, in which x = 2 at an ascorbate concentration of 1 μmol/L and x = 0.3 at 100 μmol/L (32).

In vivo ascorbate loading experiment. Ten normal, healthy adult volunteers provided a sample of 5–10 mL of venous blood 5 min before drinking a 10-mL solution containing 1.5 g of ascorbic acid or sucrose (control). Three h later, a second blood sample was taken. TRAP activity and plasma ascorbate status were determined in both baseline and postloading samples.

Statistical analysis. Data are presented as median values with ranges. Statistical analysis was performed using the Mann-Whitney U test for nonparametric data. Calculation of correlation coefficients were made using a linear regression analysis program.

RESULTS

Table 2 shows the plasma concentrations of the four antioxidants studied, in children with CF and in age-matched controls. Ascorbic acid concentrations in the control children ranged from 11.4 to 328.9 μmol/L, with 16 of the 21 individuals within the reported normal range of 11 to 100 μmol/L (35). Plasma ascorbate concentrations were significantly higher ($p < 0.05$) in chil-

Table 2. *Concentrations of antioxidants in plasma**

Antioxidant	Control	CF
Ascorbic acid	65.6 (11.4–328.9)	94.6 (33.7–850.5)†
Uric acid	198.0 (41.7–502.0)	330.8 (59.0–1494.0)†
Vitamin E	18.3 (14.2–24.5)	16.9 (8.6–35.6)
Sulfhydryls	363.2 (270.6–577.2)	518.4 (319.1–1162.0)†

* Antioxidant concentrations (μmol/L) in plasma from patients with CF (n = 18–24) or age-matched controls (n = 12–21) were compared. Values shown are medians with ranges.

† Statistically different between groups ($p < 0.05$ or better). Statistical analysis by Mann-Whitney U test.

dren with CF. They ranged from 33.7 to 850.5 μmol/L, with 11 of the 24 patients having plasma ascorbate levels greater than the normal reported range (>100 μmol/L).

Plasma uric acid concentrations (Table 2) were similarly elevated in patients with CF. Nineteen of 21 control children had plasma urate levels within the reported range of 40 to 440 μmol/L (35), the levels of the other two individuals being only marginally higher (502 and 547 μmol/L). CF patients had plasma urate levels ranging from 59 to 1494 μmol/L, and although the median concentration was within normal ranges, 10 of 22 individuals had higher than normal urate concentrations.

Vitamin E concentrations (Table 2) were similar in control children and CF patients. In controls, vitamin E levels ranged from 14.16 to 24.46 μmol/L, whereas levels of CF patients were more widely distributed from 8.55 to 35.60 μmol/L. A number of the CF patients (9 of 18) were found to have vitamin E levels at the lower end of the normal range.

Plasma sulfhydryl concentrations were significantly increased in children with CF (Table 2). Control children had sulfhydryl levels ranging from 270.6 to 577.2 μmol/L, whereas in CF patients the range was 319.1 to 1162.0 μmol/L.

$TRAP_{meas}$ activities are shown in Table 3. $TRAP_{meas}$ in CF patients was significantly lower (16%) than in controls. $TRAP_{calc}$ were, however, greater in CF patients than in controls (not significant). $TRAP_{meas}$ and $TRAP_{calc}$ values were similar in controls, giving a low value for $TRAP_{diff}$. In CF patients, the discrepancy between $TRAP_{meas}$ and $TRAP_{calc}$ was greater; hence $TRAP_{diff}$ was significantly higher in this group than in controls. $TRAP_{diff}$ was found to correlate strongly with plasma ascorbate concentrations among the CF patients ($r = 0.801$, $p < 0.00001$) but not in the age-matched control group ($r = -0.160$, NS).

To further investigate the influence of ascorbate on $TRAP_{meas}$, a series of eight healthy adult volunteers were orally administered 1.5 g of ascorbic acid, 5 min after a blood sample was taken. Three h later, a second blood sample was taken and the $TRAP_{meas}$ and plasma ascorbate levels in the two blood samples were compared (Table 4). Two control adults who were administered a solution of sucrose showed no change in $TRAP_{meas}$ over the 3-h period. In this period, the mean plasma ascorbate concentration of those individuals receiving ascorbate increased from 42.5 ± 14.7 (range 25–63) μmol/L to 108 ± 87.2 (range 37–280) μmol/L. Six of the eight individuals showed a 20% or greater decrease in $TRAP_{meas}$ after ascorbate administration, and five of these were observed to lose more than 45% of TRAP activity. Overall, the decrease in $TRAP_{meas}$ for all eight subjects was not significant (991 ± 409 to 633 ± 436 μmol/L). The two individuals not showing decreased $TRAP_{meas}$ after ascorbate dosing had increases of TRAP activity in excess of 30%.

Table 3. *Experimentally derived plasma TRAP activity and theoretical TRAP parameters**

Measurement	Control	CF
$TRAP_{meas}$	580 (254–1640)	488 (182–692)†
$TRAP_{calc}$	411 (232–829)	623 (286–2240)
$TRAP_{diff}$	−46 (−579–382)	480 (−330–1796)†

* TRAP capacity (μmol/L) in plasma from patients with CF or age-matched controls was compared. $TRAP_{meas}$ indicates experimentally derived TRAP; $TRAP_{calc}$ indicates theoretical TRAP values determined from the equation shown in Subjects and Methods. $TRAP_{diff}$ is the difference between measured and theoretical TRAP ($TRAP_{calc} - TRAP_{meas}$). For $TRAP_{meas}$ control, n = 20; for CF, n = 22. For $TRAP_{calc}$ and $TRAP_{diff}$ control, n = 10; for CF, n = 18. Values shown are medians with ranges.

† Statistically different between groups ($p < 0.05$ or better). Statistical analysis by Mann-Whitney U test.

Table 4. *Effect of ascorbate on measured TRAP activity in vivo*

Subject	Treatment	Initial $TRAP_{meas}$ (μmol/L)	Final $TRAP_{meas}$ (μmol/L)	Change (%)
1	Ascorbate	564	126	−78
2	Ascorbate	760	581	−24
3	Ascorbate	246	353	+43
4	Ascorbate	867	459	−47
5	Ascorbate	1026	607	−41
6	Ascorbate	1200	1599	+33
7	Ascorbate	1599	780	−51
8	Ascorbate	1029	564	−45
9	Control	900	828	−8
10	Control	499	499	0

* Normal, healthy adult volunteers were administered 1.5 g of ascorbic acid orally 5 min after giving a 5-mL venous blood sample. Three h later, a second blood sample was taken. TRAP activity was determined in both samples.

DISCUSSION

The data obtained in this study indicate that some of children with CF have elevated concentrations of three plasma antioxidants: ascorbic acid, uric acid, and sulfhydryl groups. Plasma vitamin E concentrations in the majority of CF patients were similar to those of age-matched controls. This particular finding was not surprising, as vitamin E status of CF patients attending the outpatients clinic at Southampton has been monitored over the past 5 y and supplements have been recommended where necessary. This approach has led to a normalization of plasma vitamin E concentrations in CF patients over this period.

The major surprise of this study was the elevated levels of the other antioxidants assessed. Plasma uric acid concentrations of 59 to 1494 μmol/L were observed; these were significantly higher than urate concentrations in controls. Normal plasma urate levels in children have previously been reported to be in the range of 60 to 440 μmol/L (36), and a similar range is reported for adults (35). The median plasma urate value for CF patients (330.8 μmol/L) was slightly above the mean value (270 μmol/L) previously observed in CF patients (37). In the present study, some CF patients had urate levels markedly greater than the highest values reported by Davidson *et al.* (650–740 μmol/L) (37). Elevated urate levels in urine and plasma have been previously reported in children with CF (38, 39) and have been attributed to the high purine content of the pancreatic enzyme supplements these children receive. In the current study, all the CF patients were receiving supplements of the pancreatic enzyme preparation Creon (Reid-Rowell, Marietta, GA). Increased plasma urate levels have also been indicated in children who are below normal height and weight for their age and who suffer repeated infections (36), as is the case with CF children.

Mean plasma ascorbate levels in CF patients were 44% higher than in age-matched controls and were distributed over a range of 33 to 850 μmol/L. Eleven of the 24 CF patients had plasma concentrations above the normal reported range, 11 to 100 μmol/L (35). This was also true of some (five of 20) of the controls. Two of the control subjects with high ascorbate concentrations were vegetarian. Dietary and vitamin supplement information was not available from control children, but was obtained retrospectively for six CF patients with the highest concentrations of ascorbate (data not shown). None of these patients was taking supplements or had dietary ascorbate intakes sufficient to explain his or her elevated plasma concentrations. Indeed, no clear relationship between ascorbate intake and plasma ascorbate levels was observed in these patients. The explanation for the high ascorbate levels in CF patients is thus obscure but may result from a reduced rate of clearance, an increased release, or slow uptake by the tissues, which are known to have higher ascorbate concentrations than plasma (34).

Despite this increased antioxidant capacity, CF patients were found to have decreased $TRAP_{meas}$ compared with age-matched controls. This finding suggests that CF patients have a reduced capacity to withstand oxidative stress, such as that which they experience during periods of infection when activated phagocytes release free radicals as part of the respiratory burst. Indeed, there is already evidence that CF patients undergo oxidative stress. These include elevated markers of lipid peroxidation, an increase in intracellular antioxidant defenses (20–23), and a 100- to 1000-fold elevation in the number of neutrophils in the airways of the lung (25). This increase in neutrophil numbers in the respiratory tract is likely to subject the airway to an extracellular oxidant stress (25). Depressed extracellular glutathione concentrations in the lung have been linked to a number of pulmonary pathologies, including CF, idiopathic pulmonary fibrosis, smoking-related lung injury, and HIV infection (24, 40, 41).

In summary, children with CF were found to have increased plasma concentrations of urate, sulfhydryl compounds and ascorbate, with vitamin E levels at the low end of the normal range. In spite of an increased array of extracellular scavenging antioxidants, CF patients were found to have a reduced total peroxyl trapping antioxidant potential. This appeared to be linked to their high ascorbate levels and may be attributable to the prooxidant activity of ascorbate. The study suggests that children with CF may have a reduced ability to withstand oxidative damage.

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