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THE DISTURBANCES IN IRON TRANSPORT AND STORES AND TOTAL FREE RADICAL TRAPPING ABILITY OF BLOOD PLASMA IN BABIES WITH MITOCHONDRIAL ENCEPHALOMYOPATHIES

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ABSTRACT

Babies with mitochondrial encephalomyopathies had higher ferritin levels than controls. Although plasma iron levels were similar in both groups, babies with mitochondrial encephalomyopathies had lower transferrin levels. Thiobarbituric acid reactive substances in plasma of babies with mitochondrial encephalomyopathies were higher than in controls suggesting increased lipid oxidation. We studied the total radical trapping capacity of the antioxidants in plasma (TRAP) and compared the TRAP level in children with mitochondrial encephalomyopathies with that in healthy controls. In addition, the concentrations of various known antioxidants were measured and the theoretical contribution of these antioxidants to the TRAP calculated. The measured and calculated TRAP were higher in the control group than the group of children with mitochondrial encephalomyopathies. Uric acid, vitamins E and C and sulphides concentrations were lower in children with mitochondrial encephalomyopathies compared with controls. The main conclusion from this work is that forchildren with mitochondrial encephalomyopathies, plasma contents of vitamin E, vitamin C, and cysteine-rich proteins which are all essential nutrients are too low for optimal activity of antioxidant systems.

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INTRODUCTION

A steadily increasing number of children with mitochondrial encephalomyopathies (EM) have been recognized during the past few years. The clinical presentation is variable and the symptoms are often nonspecific. Lactate is the product of anaerobic metabolism, and accumulates in body fluids when the aerobic metabolism in mitochondria is impaired. During normal mitochondrial respiration, 1-2% of the consumed oxygen is thought to generate oxygen radicals.[4] Reactive oxygen species, including peroxidized lipids may be generated in excess, especially under anaerobic conditions within the mitochondria. Oxygen radical formation in excess appears to be one of the fundamental mechanisms by which tissues are injured. Although highly reactive oxygen radicals result from normal metabolism, efficient endogenous systems of removal normally prevent injury. The total antioxidant capacity in man is dependent on the synergistic action of

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Table I. Clinical symptoms of 19 patients suspicious of having mitochondrial encephalomyopathy.

Major clinical symptoms	% of patients	no. of patients
Progressive psychomotor retardation (loss of previously acquitted motor functions and intellectual capacity	100.0	19
Impaired somatic development (short stature)	89.5	17
Recurrent seizures, resistant to treatment	68.4	13
Blindness, optic atrophy, ptosis, ophthalmoplegia, nystagmus	84.2	16
Spasticity, hemiparesis	31.6	6
Muscle weakness, hypotonia	94.7	18
Peripheral neuropathy	15.8	3
Ataxia	15.8	3
Hyperlactacidemia	47.4	9
Accompanying clinical findings	**% of patients**	**no. of patients**
Profuse sweating	36.8	7
Liver dysfunction	63.2	12
Episodic vomiting	68.4	13
Hearing loss	10.5	2
Macrocephaly	5.3	1

various antioxidants, i.e. enzymes such as glutathione peroxidase and agents such as vitamin E and C, uric acid and bilirubin.[6,7] This potentially important interaction between the various antioxidants has not been explored in clinical studies. A test has been developed for measuring the total radical trapping capacity (TRAP) in plasma.[19] TRAP has been measured in healthy adults and the contributions of vitamins E and C, uric acid and sulphydryl groups to the TRAP were estimated.[19] Subsequently, Lindemann et al.[12] studied preterm and term babies and found abnormal radical trapping capacities in preterm babies.

In children with EM the protective role of these antioxidants and their possible interactions should also be considered. We therefore measured the concentration of various plasma antioxidants and the TRAP of babies with EM.

MATERIAL AND METHODS

Patient group

19 children (6 girls and 13 boys) 8 months to 10 years of age suspicious of having EM were subjected to biochemical investigation. About 50% of the examined population consisted of infants and young children up to 2 years of age. These patients more or less showed the clinical symptoms shown in Table I.

The disorders were progressive, and periods of clinical deterioration were often associated with infection. During the clinical observation, inherited disorders of aminoacid metabolism and lysosomal storage disease were excluded. The electron microscopic examination of peripheral lymphocytes suggested the presence of ultrastructurally abnormal mitochondria.

Table II. Plasma concentrations of iron, plasma proteins, antioxidants, and peroxidation products in babies with mitochondrial encephalomyopathy (EM) and in controls.

	EM (n=19)	Control (n=22)	P
Ferritin (mcg/L)	133.7 (12.6)	89.6 (9.0)	< 0.02
Iron (mcmol/L)	33.6 (2.7)	29.8 (4.1)	< 0.05
Transferrin (g/L)	1.42 (0.22)	2.03 (0.29)	< 0.05
LIBC (mcmol/L)	6.2 (0.2)	11.8 (2.7)	< 0.01
Uric acid (mcmol/L)	237.7 (18.8)	288.6 (11.7)	< 0.02
Sulphydryl (mcmol/L)	312.0 (34.7)	420.6 (34.9)	< 0.02
Disulphides (mcmol/L)	67.8 (12.2)	ND	
Vitamin E (mcmol/L)	56.6 (5.2)	98.4 (6.0)	< 0.01
Vitamin C (mcmol/L)	87.0 (4.9)	132.8 (23.9)	< 0.01
Total bilirubin (mcmol/L)	45.7 (7.8)	30.6 (4.7)	NS

Values are means (SD) LIBC= latent iron binding capacity
n= no. of subjects ND= not detectable
P= Statistical significance NS= not significant

Control group

Twenty-two children (14 girls and 8 boys) 8 months to 10 years of age without any symptoms and signs of neurologic disorders or family history of neurological disorders, ischemic heart disease, or hypertension were included in the control group. As in the patient group, about 50% of the examined children in the control group consisted of infants and young children up to 2 years of age. These children were normal on anthropometry and physical examination.

Venous blood (2 ml) was collected into a sterile heparinized vial from each child. None of the subjects in the patient or control groups had been fed for a mean period of 4 h prior to venopuncture. Plasma was separated and the vials coded and analysed 10-15 min. after collection.

Parameters of iron metabolism

Total plasma iron was measured by a ferrozine method and transferrin was analysed by rate nephelometry using commercially available kits (Boehringer Mannheim, Germany). Latent iron binding capacity (LIBC) was calculated. Ferritin was measured by radioimmunoassay.

Plasma antioxidants

Uric acid and total bilirubin were measured by an SMA I automatic analyser (Technicon Instruments) according to the manufacturer's recommendations.[8] Vitamin E (alpha-tocopherol) and total vitamin C concentrations were measured by the HPLC method of Cham et al.[5] and the colorimetric method of Kyaw,[10] respectively. The plasma sulphydryl and disulphide concentrations were measured by the method of Adams et al.[2]

Measurement of total antioxidant capacity (TRAP)

The peroxidation of linoleic acid was induced by a water soluble thermolabile free radical initiator (2, 2'-azo-bis- (2-amidinopropane)-HCl). Peroxidation was initially inhibited (induction phase) by the plasma antioxidants and then rapidly propagated (propagation phase). The duration of the induction phase (an index of the total radical trapping capacity of the plasma) was quantified using the duration of the induction phase produced by a known quantity of an antioxidant (2 mcg of alpha-tocopherol) with a known stoichiometric factor (2). This value (mcmol/l) was the TRAPmeas.[19] In addition to the direct measurement of TRAP, this parameter was also calculated stoichiometrically (TRAPcalc). Calculated total capacity of antioxidants (uric acid, vitamin C, vitamin E, and sulphydryl groups) to trap free radicals (TRAPcalc) was calculated from concentrations and the stoichiometric factors (an index of the efficiency of each antioxidant to trap free radicals) by the formula of Wayner et al:[19]

TRAPcalc = 1.3 (uric acid) + 1.7 (vit C) + 2 (vit E) + 0.2 (total plasma sulphydryl groups) + 2(bilirubin)

Although these antioxidants are considered to be the major plasma antioxidants, other compounds may also contribute to TRAP. These unidentified antioxidants (UNID) were calculated as TRAPmeas minus TRAPcalc.

Thiobarbituric acid reactive substances (TBARS) in the plasma were determined as an index of lipid peroxidation by the spectrofluorimetric method of Yagi.[20]

Statistics

Results were calculated as means ± S D. The differences between the results in the two groups were tested by ANOVA. When significant, pair-wise comparisons were carried out using the independent t-test, except for the ferritin measurements in which a nonparametric test (Kruskal-Wallis) was used. Values of $P<0.05$ were regarded as

Table III. The measured total radical trapping antioxidant parameter (TRAPmeas), the calculated total radical trapping antioxidant parameter (TRAPcalc), and the concentration of substances reactive with thiobarbituric acid (TBARS) in babies with mitochondrial encephalomyopathy (EM) and in controls.

	EMs (n=19)	Control (n=22)	P
TRAPcalc (mcmol/L)	632.5 (30.8)	881.9 (105.6)	< 0.02
TRAPmeas (mcmol/L)	856.0 (35.5)	1085.0 (113.9)	< 0.02
TBARS (mcmol/L)	145.0 (23.5)	57.8 (12.0)	< 0.01

Values are means (SD)
n = no. of subjects
P = statistical significance

significant.

The study was reviewed and approved by an institutional committee and the ethics committee.

RESULTS

Babies with EM had higher ferritin levels than controls (Table II). Although plasma iron levels were similar in both groups, babies with EM had lower transferrin levels and therefore a lower LIBC. Concentrations of the antioxidants (uric acid, vitamin E, and total sulphydryl groups) were significantly lower in babies with EM. GSSG is not detectable in the plasma of normal children. In children with EM, plasma GSSG levels were significantly increased.

The TRAPmeas and TRAPcalc were higher in control babies than in the EM group (Table III). The TBARS levels were higher in the plasma of babies with EM.

The iron, transferrin and total bilirubin levels in both groups of babies correlated with TRAPmeas (r=0.6748; r = -0.4532 and r=0.7544 for babies with EM and r=0.7690; r= - 0.6500 and r=0.8562 for healthy babies, respectively). Ferritin, LIBC and uric acid correlated with TRAPmeas only in healthy babies (r=0.4211; r=0.7443 and r=0.8864, respectively). There was a weak negative correlation between TRAPmeas and vitamin E in babies with EM (r=-0.5332), whereas this correlation was positive in healthy babies (r=0.5620). The UNID in both groups of babies correlated with TRAPmeas (r=0.8755 and r=0.8996 for babies with EM and for healthy children, respectively).

DISCUSSION

Thiobarbituric acid reactive substances in plasma of babies with EM were higher than in controls, suggesting increased lipid oxidation. The concentration of these substances reflect the extent of lipid peroxidation but are a crude measure and are subject to interference by many factors.[6,20] The finding of lower plasma vitamin C and E levels in babies with EM is consistent with peroxidation of lipids. Vitamin C is the first plasma antioxidant to be depleted when lipid peroxidation is induced *in vitro*.[7] Sulphides, mainly glutathione, are ubiquitous, largely intracellular antioxidants, believed to play a key role in the defense against oxygen-derived free radicals.[13] In the presence of oxygen radicals, reduced sulphides are oxidized to disulphides. In normal plasma, levels of disulphides are very low or undetectable by enzymatic assay.[1] Thus, the plasma determinations of disulphides may represent a sensitive means of measuring what may be a more subtle oxidant stress.[9]

What is the evidence in our study that increased peroxidation is due to iron toxicity? Iron is mainly transported in plasma bound to transferrin and stored intracellularly bound to ferritin. However, non-specifically-bound iron (i.e. "free" iron) is found when the binding capacity of transferrin is exceded or ferritin-bound iron is released. Sullivan has suggested that iron-induced free-radical damage of various organelles is important in diseases of babies.[17] The group of children with EM had high ferritin values consistent with large iron stores, yet ferritin levels were much lower than those reported in older children in whom iron overload is believed to induce peroxidation.[3] Latent iron binding capacity (LIBC) is lower in normal babies than in children,[15,17] and is even lower in babies with EM. Increased ferritin iron and limited transferrin binding capacity could increase the production of free radicals.

The values of the calculated total capacity of antioxidants to trap free radicals (TRAPcalc) were considerably lower than the TRAPmeas in both groups. Two possible explanations for this finding are that either all the plasma antioxidants have not been identified or that the stoichiometric values used to calculate their free radical trapping capacity are too low. Recently, bilirubin has been recognized as being an important antioxidant.[16] It has a high plasma concentration and a powerful radical trapping capacity, but including its contribution in the TRAPcalc still left a large unidentified component. However, plasma proteins are present in high concentrations and only their

sulphydryl groups are used in the formula estimating the TRAPcalc;[19] the other aminoacids, such as tryptophan, tyrosine and histidine can also act as antioxidants.[14] The other explanation for the lower TRAPcalc is that the stoichiometric values nay be too low. Different workers use different stoichiometric values for the antioxidants, e.g. 0.33 and 2.0 instead of 0.2 and 1.3 for the sulphydryl groups and uric acid respectively.[14,19] Furthermore, Wayner et al.[18] have shown that the value for vitamin C is concentration dependent. However, even if all the contributing antioxidants are identified and their correct stoichiometric values used, the TRAPcalc does not take into account the very important interactions that occur between antioxidants when they work in unison.[19]

The correlations between the TRAPmeas and the TRAPcalc and the individual antioxidants were different in adults,[19] and babies. Possibly the inhibition of antioxidant activity, e.g. cation binding of urate,[11] or increased pro-oxidant activity may explain these findings. Pro-oxidant activity due to iron may be particularly important in babies, as described above. Unidentified antioxidants showed the strongest correlation with the TRAPmeas in both groups of babies, emphasizing the need to identify its components.

Our findings suggest that iron toxicity occurs in babies with EM. These changes in iron metabolism are associated with increases in *in vivo* lipid peroxidation. The potential clinical use of this approach has great appeal. Further experimental and clinical studies are needed to confirm this preliminary investigation.

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