Resistance of erythrocytes to lipid peroxidation in alcoholic patients

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Abstract
The ability of erythrocytes to resist lipid peroxidation may be a useful marker of antioxidant status in alcoholic patients, in whom depletion of dietary antioxidants may combine with increased production of free radicals to produce liver damage. There are conflicting reports, however, on the resistance of erythrocytes from alcoholic patients to lipid peroxidation. This study examined the relation between the degree of alcohol induced liver disease and the resistance of erythrocytes to chemically induced lipid peroxidation, measuring lipid peroxidation as malondialdehyde production. Erythrocytes from alcoholic patients with Child’s C cirrhosis had significantly increased resistance to lipid peroxidation compared with both controls (p<0.001) and alcoholic patients with moderate liver disease (p<0.001). There was no difference between alcoholic patients with moderate liver disease and controls. Increased resistance to free radical initiated lipid peroxidation in alcoholic patients is related to liver damage rather than to alcohol abuse alone. This could arise from changes in the lipid composition of the erythrocyte membranes resulting from abnormal liver function. Tests of antioxidant status based upon the resistance of erythrocytes to free radical stress in vitro may therefore be flawed when such changes in membrane lipid composition can occur.

(Gut 1994; 35: 1753–1756)

The generation of toxic free radicals from the metabolism of ethanol by hepatic cytochrome P450 E1 and increased lipid peroxidation have been implicated in alcoholic liver disease. In addition, the inadequate nutritional intake of alcoholic patients may lower the concentrations of the nutritionally derived components of the antioxidant defences, such as vitamins E and C, zinc, and selenium, thereby increasing the vulnerability of the liver to injury.

A simpler and perhaps more relevant alternative to the measurement of individual antioxidants is to measure the sum of their activity by assessing the functional capability of the cellular antioxidant defences. The resistance of erythrocytes to chemically induced lipid peroxidation has been suggested to reflect the resistance of other tissues, to be a measure of whole body vitamin E status, and to be of use in determining antioxidant deficiencies in alcoholic liver disease.

Methods
SUBJECTS
Twenty patients with alcoholic liver disease in St Thomas’ Hospital who were not receiving any treatment were investigated. None had consumed alcohol within the last 24 hours. Patients were divided into those with moderate liver damage (n=10), either fatty liver (n=4; serum albumin=44.7 (3.1) g/l; mean (SD)), hepatitis (n=1; serum albumin 43 g/l) or Child-Pugh Class A or B cirrhosis (n=5; serum albumin 33.8 (8.1) g/l), and severe (C cirrhosis) disease (n=10; serum albumin 25.6 (2.7) g/l). Cirrhosis was confirmed histologically.

None had markers of hepatitis B infection. Tests for hepatitis C were not performed. The control group were 10 healthy laboratory subjects with no history of liver diseases or alcohol abuse, currently consuming less than 10 g alcohol per week, and who had not taken any drugs or consumed any alcohol within the previous 24 hours. The ratio of men to women was 7:3 in each group. The age range for the alcoholic group was 26–65 and that for controls 22–62. Approval for this study was obtained from the ethical committee of St Thomas’ Hospital, and all patients gave informed consent.
PREPARATION OF ERYTHROCYTES

Ten ml heparinised venous blood was centrifuged at 4°C for 15 minutes, the plasma and buffy coat discarded, and the erythrocytes washed three times by resuspending them in phosphate buffered saline (15 mmol 1⁻¹ phosphate buffer, 139 mmol 1⁻¹ sodium chloride, pH 7.4 containing 1 mmol 1⁻¹ sodium azide) (PBS), centrifuging at 1400 g for 15 minutes at 4°C and discarding the supernatant. A 1 in 10 (vol/vol) dilution of the packed erythrocytes was prepared in PBS and 0-1 ml aliquots added to 5 ml Drabkin’s reagent, incubated at room temperature for 15 minutes, and the absorbance read at 540 nm against appropriate blanks and standards (Sigma, Poole, Dorset) to give the haemoglobin concentration. To avoid changes in cell density in alcoholic patients affecting the number of erythrocytes and hence peroxidisable substrate in each incubation, incubations were standardised to contain the same amount of haemoglobin. A dilution of the packed erythrocytes was then prepared (approximately 1 in 40 vol/vol) in PBS to give 7.5 mg haemoglobin/ml.

MEASUREMENT OF LIPID PEROXIDES

Lipid peroxidation from erythrocytes from alcoholic patients and controls was measured as malondialdehyde production as used in previous studies on erythrocytes from alcoholic patients, assessed as thiobarbituric acid reactive material, described previously. Either 2 ml of stressed erythrocyte suspension or 1 ml of a standard solution of tetra-ethoxypropane (Sigma Chemical, Poole, Dorset) was added to an equal volume of 0-61 mol 1⁻¹ trichloroacetic acid, mixed, and centrifuged at 1400 g for 10 minutes. Two ml supernatant was added to 1 ml 52 mmol 1⁻¹ thiobarbituric acid solution in 0-1 mol 1⁻¹ hydrochloric acid. Tubes were capped, mixed, and heated for 20 minutes at 95°C, cooled, and the absorbance of the product of the malondialdehyde-thiobarbituric acid reaction at 532 nm recorded. The concentration of malondialdehyde in the assays was calculated using tetraethoxypropane derived malondialdehyde.

All reagents used were from BDH, Dagenham, Essex, unless otherwise stated.

Figure 1: Time course of malondialdehyde production in t-BHP stressed erythrocytes from controls and alcoholic patients with severe liver disease. Data shown as mean (SD). *p<0.02; †p<0.001.

Figure 2: Lipid peroxidation in t-BHP stressed erythrocytes incubated for 180 minutes related to severity of alcoholic liver disease. Control group: alcoholic patients with moderate liver disease; hepatitis (Δ); fatty liver (○); class A or B cirrhosis (Ο): alcoholic patients with severe liver disease (●). Bars are means (SD).

STATISTICAL ANALYSIS

Analysis of data was performed using Student’s t test for unpaired data, expressed as mean (SD) where appropriate.
Results
After 60 minutes of incubation erythrocytes from patients with severe liver disease had a significantly (p<0.02) increased resistance to lipid peroxidation measured as lower malondialdehyde production, compared with those from healthy subjects (Fig 1). The magnitude and statistical significance of this difference increased thereafter (p<0.001) (Fig 1).

The increase of lipid peroxidation with time was similar in incubations of erythrocytes from both controls and patients with moderate liver disease even after incubating for 180 minutes (Fig 2). The resistance to lipid peroxidation of erythrocytes from patients with severe liver disease was thus also significantly increased when compared with those patients with moderate liver disease after 60 minutes' incubation (216 (31) vs 243 (34) ml/gHb; severe vs moderate; p<0.02), after 90 minutes (299 (17) vs 367 (30); p<0.001), 120 minutes (351 (37) vs 444 (22); p<0.001), and at 180 minutes (p<0.001) (Fig 2).

Discussion
The resistance of erythrocytes to chemically induced lipid peroxidation was significantly increased in alcoholic patients with severe liver disease compared with those from healthy controls or patients with moderate liver disease. Apart from this difference, the production of lipid peroxides in response to t-BHP stress with time was similar in incubations of both control and alcoholic erythrocytes and comparable with that in previous studies.21 22 This increased resistance in peroxidation is in agreement with previous studies performed on alcoholic patients with cirrhosis in which lipid peroxidation was assessed as pentane production or haemolysis.16 19 Moreover, although malondialdehyde production may be a poor absolute measurement of lipid peroxidation, as a relative measurement it compares well with other markers.23-26 Thus this increased resistance is not a result of the form of measurement of lipid peroxidation used.

As in other studies,19 the erythrocytes from patients with mild liver disease had a similar resistance to those from controls, suggesting that the observed difference is a result of severe liver damage rather than alcohol abuse alone. It is thus unlikely that the increased resistance arises from some protective change in vivo, and more probable that it is caused by changes in the composition of the erythrocyte rather than changes in antioxidant concentrations. Lipid peroxidation, measured as malondialdehyde, correlates well with loss of the substrate fatty acids arachidonic acid and docosahexaenoic acid,26 concentrations of which are lower in erythrocytes from alcoholic patients with cirrhosis.19 27 Thus this change in membrane fatty acids could produce the increased resistance. In conjunction with the decrease in these fatty acids, the cholesterol concentrations in the erythrocytes are increased.19 27 It is this increase in cholesterol, rather than the changes in fatty acids, that produces a decrease in the membrane fluidity of erythrocytes from patients with alcoholic liver disease.28

Alcohol consumption in the absence of liver disease, however, does not change erythrocyte concentrations of arachidonic acid or docosahexaenoic acid29-31 or cholesterol.29 30 32 Moreover, lower concentrations of arachidonic acid and an increased resistance to lipid peroxidation are found in rats with liver disease unrelated to alcohol.31 Similarly, in humans, increased erythrocyte cholesterol occurs in a wide variety of human liver diseases.28 Thus the resistance to lipid peroxidation in alcoholic patients may result from changes in erythrocyte membrane composition secondary to liver malfunction than from alcohol abuse alone.

Consumption of alcohol in the absence of severe liver disease can lower erythrocyte antioxidants3-5 and this could have caused the reduced resistance of erythrocytes to lipid peroxidation in studies performed on alcoholic patients with no13 or only mild41 liver disease. In both of these studies the patients were starting attendance at clinics for detoxification and so it is possible that recent, heavy alcohol consumption is required before the reduced resistance becomes apparent. Only in extreme situations in alcoholic patients, such as in the rare Zieve's syndrome, may the reduction in antioxidants be sufficient to overcome the high cholesterol and low polyunsaturated fatty acid concentrations caused by the liver disease and thus reduce resistance to lipid peroxidation.14

Thus the resistance of erythrocyte to lipid peroxidation measured in vitro may be affected by two separate events, namely changes in the cell membrane composition in response to liver disease and changes in the antioxidants in the erythrocyte in response to recent, chronic heavy intake of alcohol. These changes affect the interpretation of results of measurement of lipid peroxidation in erythrocytes.

We are grateful to the Special Trustees of St Thomas' Hospital for their continuing support. Part of this work was presented at the meeting of the Medical Research Society, January 1990, and published in abstract form (Gut Sci 1990; 17: 13).


