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Studies on the Glutathione S-Transferase of Human Platelets

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The glutathione S-transferases of human platelets have been compared with those of erythrocytes. Although wide variations in activity were found, in individual subjects, the activity in these cell types was significantly correlated. The enzymes demonstrated similar isoelectric points and electrophoretic mobilities and it appears that the platelet enzyme is also a product of the GST3 locus. There was no correlation between platelet enzyme activity and plasma concentrations of retinol and cholesterol, but in men, the relationship between activity and carotene was significant. It is suggested that GST3 isoenzyme activity depends on vitamin A.

The glutathione S-transferases (EC 2.5.1.18) are dimeric enzymes that in humans appear to be the products of three autosomal loci, GST1, GST2 and GST3 [1,2]. They catalyse the conjugation of reduced glutathione with various hydrophobic substrates [3] and, on the basis of evidence obtained mainly from experimental animals, may be important in reducing tissue susceptibility to carcinogens [4]. In humans, individuals with certain phenotypes such as the null frequently found at the GST1 locus, may be particularly susceptible [5]. Although such studies are circumstantial they could indicate that either the level of glutathione S-transferase activity or the enzyme phenotype is important in determining an individual's risk of developing cancer.

In humans, this hypothesis is more easily examined in blood cells but such studies have been limited and have been concerned mainly with describing the range of activities in cells such as erythrocytes from healthy individuals. Although erythrocytes contain only the GST3 isoenzyme [6,7], extensive variations in activity (about 10-fold) were found. Most individuals demonstrate two forms of the enzyme, one of which appears to be a post-synthetic modification of the other [8].

Simultaneous with interest in detoxicating enzymes has been the study of pro-vitamin A (carotene) and vitamin A (retinol) in cancer prevention with recent work showing an inverse relationship between risk of developing some human cancers and the plasma concentrations of carotene and retinol (as well

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as cholesterol) [9-11] . Reports therefore, of a relationship between vitamin A deficiency and glutathione S-transferase activity in some rat tissues [12,13] are important since they suggest a link between the putative anti-carcinogenic activity of vitamin A and the enzyme.

We now describe studies of the relationship between carotene, retinol and cholesterol and glutathione S-transferase activity. Although previously studied, we did not use erythrocytes; firstly, because of their long life-span and the unknown origin and variable amounts of the two enzyme forms. Secondly, only the GST3 locus is expressed and thirdly in our experience the enzyme cannot be assayed in the presence of haemoglobin, removal of which is time-consuming. Platelets are a homogeneous cell type with a short life-span and we have determined whether this enzyme demonstrates the same wide variation in activity and gene products as erythrocytes and also whether activity in these cells is related to the plasma concentrations of the putative indicators of cancer risk.

MATERIALS AND METHODS

Venous blood (5 ml) was obtained between 9.00 a.m. and 11.00 a.m. from 124 female and 82 male subjects (age ranges 18-80 years). None were known to be suffering from any illness, were pregnant, receiving drugs or hospital in-patients. All had a normal full blood count measured on the Coulter S plus. Blood was also taken from 10 patients with polycythaemia rubra vera who were undergoing venesection as their clinical management. None had received chemotherapy during the previous 10 weeks.

Blood was collected into glass bottles containing E.D.T.A. (10 mg) and centrifuged (700 g , 4°C, 6 min). The platelet-rich plasma was pipetted into sterile plastic bottles (10 ml) and the platelets sedimented by centrifugation (2000 g , 4°C, 20 min). The plasma was put back into the original blood bottles and mixed, by gentle inversion, with the cells. The centrifugation steps were repeated once more, the plasma removed and stored at -70°C. The combined platelet button was washed by resuspension in Tris/HCl buffer (10 ml, 20 mmol/l: pH 7.4) and centrifuged (2000 g, 4°C, 20 min). The clear supernatant was removed and washing repeated once more. The final platelet button was resuspended in 0.3 ml Tris-HCl buffer (20 mmol/l pH 7.4). Since the platelet enzyme appeared to be present in low concentration, the starch-gel and chromatofocussing experiments required large volumes of blood. Platelet lysates were prepared as above from about 400 ml blood obtained from 10 patients with polycythaemia and 15 platelet concentrates (about 4 days old) intended for clinical purposes but not used. The platelet-rich plasma was examined for other blood cells by placing one drop onto a Testsimplet pre-stained slide (Boehringer Mannheim) and viewing with a light microscope at 400 x magnification. Both this and the Coulter S plus failed to detect lymphocytes, polymorphs or erythrocytes. Disruption of platelet membranes was achieved using an ultrasonic bath (50KHz, 30s). Enzyme activity was measured in the supernatant after centrifugation (20000 g , 4°C, 60 min). Activity was unstable; over 24h, it decreased by an average of 45% at 4°C and 20% at -70°C. Consequently lysates were stored on ice for no more than 1h before assay.

Erythrocytes were washed three times with NaCl (154 mmol/l), lysed with distilled water (1:2 v/v, 4°C) and after standing (1h, 4°C) centrifuged (1200 g , 4°C, 10 min). About 150 µl supernatant was taken for electrophoresis. Before glutathione S-transferase activity in haemolysates was measured, platelets, lymphocytes and haemoglobin were removed [7] .

Horizontal starch-gel electrophoresis was performed using 100 mmol/l Tris-citrate electrode buffer (pH 7.50). About 150 µl lysate was added to the gels (length 19 cm., width 11 cm., depth 1 cm.) and electrophoresis carried out at 7 mA/gel (voltage about

2.5 V/cm) for 16h at 4°C. The gels were stained using an agarose overlay containing 1-chloro-2, 4-dinitrobenzene and GSH followed by an overlay containing I_2 in KI [6,8].

Chromatofocussing gel (PBE 94, Pharmacia Ltd.) was packed in a column (length 26 cm, diameter 1 cm) and equilibrated with 25 mmol/l imadazole -HCl. (pH 6.2) buffer. Erythrocyte or platelet lysate (10 ml) was pumped (15 ml/h) onto the column and followed by about 15 ml of the buffer. Glutathione S-transferase was eluted using a pH gradient between 6.3 and 4.0 obtained using Polybuffer 74 -HCl (pH 4.0). Fractions (2.5 ml) were collected and pH and enzyme activity measured.

Glutathione S-transferase activity was measured using 1-chloro-2, 4-dinitrobenzene and GSH, protein concentrations using an automated Lowry method [14] and haemoglobin concentrations using a cyanmethaemoglobin method. The coefficient of variation of the enzyme assay was 7%. Activities were expressed as µmol product/min per g protein or per g haemoglobin. Carotene concentrations were determined at 440 nm after extraction with light petroleum, cholesterol concentrations using a cholesterol oxidase method and retinol concentrations by high pressure liquid chromatography using a micropak MCH-1 0 reverse phase column after extraction with a mixture of isopropanol and toluene.

RESULTS

Figure 1 shows that in males the distribution of glutathione S-transferase activity is Gaussian but in females positively skewed. While the spread is wider in females (mean activity 35.0 ± 21.0 (S.D.) µmol/min per g protein), mean activity was significantly greater in males (39.0 ± 16.0 (S.D.)µmol/min per g protein ; $p<0.05$; Mann-Whitney U-test). To determine the contribution of intra-individual differences, platelet activities were measured, again between 2 and 12 weeks after the first measurement, in 19 subjects. The means of the two measurements were 38.4 ± 14.8 (S.D.) and 34.8 ± 14.1 (S.D.) µmol/min per g protein respectively. Analysis of variance showed within-subject variation accounted for less than 1% of the variation

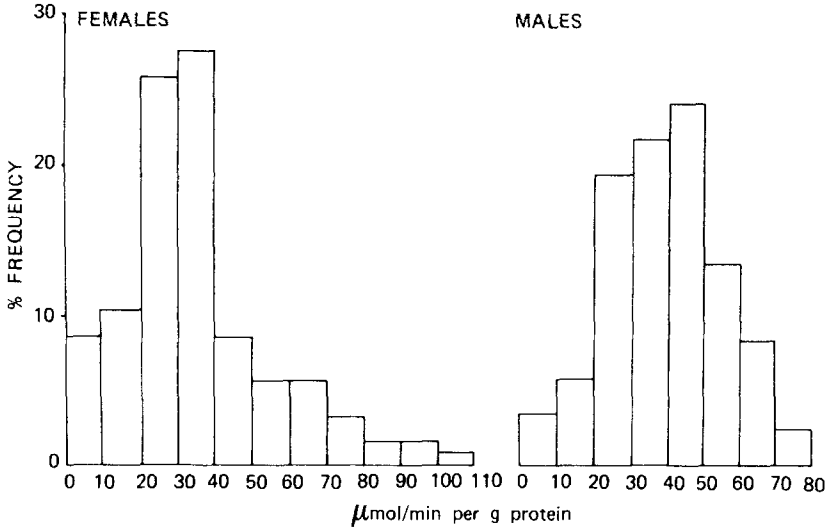

Figure 1. The distribution of glutathione S-transferase activity in platelets from 124 female and 82 male subjects.

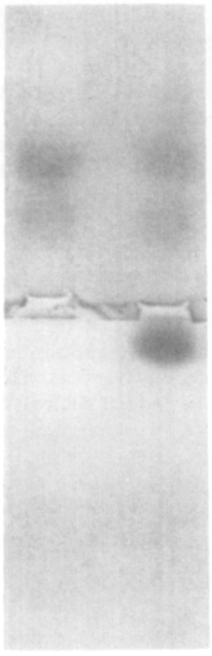

Figure 2. Starch gel electrophoresis of platelet and erythrocyte glutathione S-transferase from the same individual. A haemoglobin band can also be seen on the cathodal side of the gel.

in the subjects studied. There was no significant relationship between enzyme activity and patient age.

To determine the isoenzyme in platelets, platelet and erythrocyte lysates from patients with polycythaemia were examined using starch gel electrophoresis. In the 10 patients the patterns obtained from the two cell types were identical (Figure 2). Usually samples demonstrated two anodal bands, the most mobile was always the most densely stained and in some specimens the slower band was only just visible. The patterns of both cell types were unchanged by storage of lysates (3 days, 4°C) and as reported for the erythrocyte enzyme, platelet activity was lost if lysates were incubated (30 min, 25°C) with N-ethylmaleimide (5 mmol/l) before electrophoresis [8]. Stored platelets from 15 individuals demonstrated similar patterns to those of fresh cells. In this limited survey there was no evidence of polymorphism of the platelet enzyme or of GST1 and GST2 isoenzymes.

To confirm that the enzyme bands on starch gel were not electrophoretic artefacts, the elution profile, from chromatofocussing columns, of platelet and erythrocyte glutathione S-transferase was compared. These were similar; enzyme activity eluted either as two partially resolved peaks at pH values of about 4.35 and 4.25, or as a broad peak between pH values of 4.55 and 4.10. Maximum

activity eluted at about pH 4.35 although a shoulder of activity was often seen at a slightly lower pH.

In 19 subjects erythrocyte and platelet activities were assayed simultaneously and found to be significantly correlated ($r = 0.543$; $p<0.02$).

The relationship between plasma cholesterol concentrations and enzyme activity was studied in 46 male and 67 female subjects (mean cholesterol 5.8 ± 1.4 and 5.8 ± 1.1 (S.D.)mmol/l respectively). No significant relationship was found even when the subjects were divided into low (3.0 - 5.0 mmol/l), medium (5.1 - 5.8 mmol/l) and high (5.9 - 8.5 mmol/l) cholesterol groups. There was no significant correlation between platelet activity and plasma retinol in 22 male and 16 female subjects (mean 403 ± 120 (S.D.) and 349 ± 126 (S.D.) $\mu g/l$ respectively). The relationship between activity and plasma carotene was studied in 24 male and 18 female subjects (mean concentratic 1.95 ± 0.67 (S.D.) and 1.90 ± 0.78 (S.D.) $\mu mol/l$ respectively). Using Kendall's coefficient of rank correlation there was no relationship in female subjects but the relationship was significant in males ($t = 2.8$; $p<0.05$).

DISCUSSION

Glutathione S-tranferase activity has been measured in platelets from apparently healthy subjects. Wide variations in activity similar to those in erythrocytes were found and, while wide distributions can conceal different phenotypes, the limited electrophoretic studies described do not indicate that the platelet enzyme is polymorphic. Because of the difficulty in obtaining samples it was necessary to use cells from subjects with polycythaemia. Although this is a proliferative process that results from the emergence of mutant cell lines, no unusual isoenzyme forms were detected and patterns were identical to those of healthy subjects. The similar electrophoretic and chromatofocussing patterns suggest that the platelet and erythrocyte enzymes are both products of the GST3 locus. As previously argued [8] a genetical explanation for the starch-gel patterns is unlikely and we propose that a similar post-synthetic modification to the parent enzyme occurs in both cell types. The nature of the modification is unknown but since most such alterations result in increased anodal mobility the slower form may be the parent. Electrophoresis and chromatofocussing experiments showed that the relative amounts of the two forms varied in different individuals.

The cause and significance of these wide distributions in activity remains unknown. In both cell types intra-individual variation is small and since the activities in these cells were

significantly correlated they may be regulated by a common mechanism. The studies of Siddik et al [12] showing that in male rats, vitamin A deficiency results in an increase in enzyme activity in liver and kidney and by Dogra et al [13] showing increased activity in liver but decreased activity in lung raises the possibility that the vitamin is involved in regulation of enzyme activity. It is not known which of the presumed several loci in rats were affected by vitamin A deficiency but if these results are extrapolated to humans it is possible that the GST3 isoenzymes would be affected since although weakly expressed in liver they appear to be present in other tissues [2] including lung [15]. Our studies found no relationship between the activity of the GST3 isoenzyme and plasma retinol or cholesterol. No relationship with cholesterol was found even after subjects were divided into three groups on the basis of plasma cholesterol concentrations. Such divisions have shown significant inverse relationships between cholesterol and cancer in Maoris[10] and colon cancer in men [11]. In men however the relationship between carotene and enzyme activity was significant raising the possiblity of a relationship between the GST3 isoenzyme and vitamin A. That a relationship was found only with carotene is not surprising since the relationship between cholesterol and cancer is thought to be indirect and in well-nourished populations, retinol intake does not correlate with blood retinol concentrations. It is not know whether carotene has a direct protective effect or whether its relationship with cancer risk is also indirect. Our preliminary studies suggest a relationship between carotene and the detoxifying enzyme. The possible importance of this relationship in terms of cancer susceptibility has yet to be assessed.

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