Erythrocyte glutathione S-transferase

Electrophoretic identification of two enzyme forms

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Starch-gel electrophoresis was used to demonstrate two forms of glutathione S-transferase in human erythrocytes. Whereas considerable inter-individual differences in enzyme activity and electrophoretic patterns were detected, intra-individual differences were small.

The possibility that genetical differences in particular detoxifying enzymes may partly determine individual susceptibility to carcinogens has attracted much recent interest (Nebert, 1980). One such group of enzymes are the glutathione S-transferases (EC 2.5.1.18). These dimeric enzymes (M_r about 45000) catalyse the conjugation of reduced glutathione with a variety of hydrophobic substrates, including carcinogens (Chasseaud, 1979). It appears that three autosomal loci code for the human enzymes, and the products of at least one locus are present in all tissues (Board, 1981; Strange *et al.*, 1983).

The erythrocyte apparently contains a single form of the enzyme (Marcus *et al.*, 1978), which is the product of the GST3 locus (Board, 1981). Starch-gel electrophoresis of haemolysates supports the suggestion of only one enzyme form and indicates that the enzyme does not demonstrate polymorphism (Scott & Wright, 1980; Board, 1981). The possibility, however, that more than one form of the GST3 enzyme exists is suggested by the finding, in several human tissues, of at least two enzyme forms with the characteristically fast anodal mobilities of the erythrocyte form (Strange *et al.*, 1983). Furthermore, recent studies have shown an extensive but unexplained variation (about 10-fold) in activity in erythrocytes from healthy individuals (Scott & Wright, 1980; Strange *et al.*, 1982), and also in the stability of the enzyme at 52°C: $t_{1/2} = 13.4 \pm 5.1$ (s.d.) min, $n = 67$ (R. C. Strange, J. A. Moulton & W. Cotton, unpublished work). Since enzyme activities in individuals of the same phenotype generally differ by no more than 2-fold, the wide variation in activity may indicate undetected genetic variation. The thermostability studies support this conclusion, as

this technique can often uncover unsuspected enzyme variation (Wurzinger & Mohrenweiser, 1982).

We now describe experiments in erythrocytes re-examining variation at the GST3 locus. Firstly, starch-gel electrophoresis was used to identify two electrophoretic forms, and, secondly, the contribution of intra-individual variations in enzyme activity to the large variation in a reference population was determined.

Materials and methods

Preparation of lysates

Blood (about 5 ml) from healthy subjects was collected into tubes containing heparin and centrifuged (at 1200g for 10 min at 4°C), and the plasma and buffy layer were discarded. The erythrocytes were washed three times with 154 mM-NaCl, lysed with distilled water (1:2, v/v, at 4°C) and after standing (for 1 h at 4°C) centrifuged. When freezing was used to prepare lysates, erythrocytes were stored at -40°C for 30 min, thawed and centrifuged. In some experiments lysates were prepared with the use of EDTA as anticoagulant.

Where indicated, platelets and lymphocytes were removed from blood samples by using cellulose columns, and haemoglobin was removed by using CM-Sephadex (Strange *et al.*, 1982).

Starch-gel electrophoresis

Horizontal starch-gel electrophoresis was performed with 100 mM-Tris/citrate electrode buffer, pH 7.50. The gels (length 19 cm, width 11 cm, depth 1 cm) contained 13% starch and were prepared in a 1:10 dilution of the electrode buffer after adjustment of the pH to 7.50. The gels were used about 4 h after preparation.

Two Whatman 17 filter papers (1 cm × 0.6 cm) were soaked in lysate, the excess of lysate was drained off and the papers were inserted into slits in the gel that were sealed with starch cooled to about 40°C. About 150 µl of lysate was added. Electrophoresis was performed at 7 mA per gel (voltage approx. 2.5 V/cm) for 16 h at 4°C. The gels were sliced and then stained as follows. Agarose (0.75 g) was added to 100 ml of 100 mM-sodium phosphate buffer, pH 7.0, and heated at about 96°C until dissolved, the solution was cooled to about 55°C and 1 ml of a solution of 1-chloro-2,4-dinitrobenzene in acetone was added with thorough mixing, until dissolved, to give a 1-chloro-2,4-dinitrobenzene concentration of 2 mM. The agarose solution was then cooled to about 45°C, and reduced glutathione was added to a final concentration of 0.8 mM. This solution (200 ml) was poured on to each half-gel and incubated firstly for 1.75 h at 37°C and then, to facilitate removal of the agarose layer, at 4°C for 15 min. After removal of this layer, a solution of agarose (0.75 g) in 100 ml of 100 mM-Tris/HCl buffer, pH 8.0, was prepared and, immediately before addition to the starch gel, a solution of 80 mM-I₂ in 120 mM-KI was added with mixing. In the experiments described, the volume of the I₂ solution required for optimum demonstration of enzyme activity varied between 0.7 and 1.0 ml per 100 ml of the agarose solution, and was therefore determined for each experiment.

Analytical

Glutathione *S*-transferase activity and haemoglobin concentration were assayed as described previously (Strange *et al.*, 1982).

Incubation with thiol-blocking reagents

Erythrocyte lysates were incubated at 37°C for 30 min with 20 mM-*N*-ethylmaleimide, -maleate, -iodoacetate, -iodoacetamide, -oxidized glutathione, -cystamine or -dithiothreitol (Hopkinson & Harris, 1969).

Isopycnic centrifugation of erythrocytes

Erythrocytes were separated on the basis of age by using continuous gradients of Percoll as previously described (Strange *et al.*, 1982).

Results

An example of the electrophoretic patterns of erythrocyte lysates is shown in Fig. 1. All of the 194 specimens studied demonstrated the two-anodal-band pattern shown. Usually the faster band was the more intensely stained, and in a few specimens the slow band was only just visible. The bands were not due to a separate glutathione *S*-transferase from contaminating platelets or lymphocytes, since re-

Fig. 1. Starch-gel-electrophoretic pattern of glutathione *S*-transferase of erythrocyte lysate

(1) Lysate; (2) lysate after elution through a cellulose column; (3) lysate after elution through CM-Sephadex. For experimental details see the text.

moval of these cells by elution through cellulose columns did not alter the electrophoretic patterns (Fig. 1). Removal of haemoglobin also did not affect the mobilities of the bands. Similar patterns were found in lysates prepared from ten cord-blood samples obtained after full-term delivery (39–41 weeks). There was no apparent relationship between the relative intensity of the two bands and the total lysate activity.

Effects of cell age, storage, anticoagulant and temperature on electrophoretic mobility

The electrophoretic patterns of cells taken from different parts of the Percoll gradients were similar, indicating that the patterns are similar throughout the life of the cell.

Ten lysates were examined by electrophoresis and the patterns compared with those obtained after storage (at 4°C for 8 days) either as lysate or as intact erythrocytes. No changes in the patterns of the anodal bands were noted. Further, incubation of lysates with 10 mM-2-mercaptoethanol at 4°C for 3 days or at 37°C for 30 min did not affect the mobility of the bands. A comparison made with lysates from

eight subjects showed that the electrophoretic patterns obtained by freezing of erythrocytes were identical with those obtained when the same erythrocytes were lysed with distilled water. When blood was collected into tubes containing heparin or EDTA, the electrophoretic patterns were identical.

To study the thermostabilities of the enzyme bands, lysates were incubated at 52°C for 20 min, cooled to 0°C and examined by electrophoresis. Whereas the band with slower mobility appeared to be largely unaffected by this treatment, activity of the faster band was almost completely abolished.

Effects of thiol-blocking reagents

Incubation of lysates with dithiothreitol, oxidized glutathione and maleate appeared to have no effect on the mobility of either enzyme band. However, incubation with *N*-ethylmaleimide, iodoacetamide and cystamine resulted in loss of both enzyme bands, and treatment with iodoacetate was followed by the loss of the slower band and increased staining of the fast band.

Intra-individual variation of electrophoretic patterns

Similar results were obtained when electrophoretic patterns and enzyme activities were studied twice in 15 individuals after an interval of between 6 and 12 weeks. Analysis of variance showed that there were no significant differences in activity within each individual on the two occasions ($F = 0.15$), and that more than 95% ($F = 17.5$; $P < 0.001$) of the wide variation in activity found in a previously described population (Strange *et al.*, 1982) as well as in the 15 individuals [mean activities: 2.28 ± 0.9 (s.d.) and 2.40 ± 1.1 (s.d.) $\mu\text{mol/min per g}$ of haemoglobin respectively] resulted from differences between individuals.

Discussion

On the basis of previous electrophoretic studies demonstrating one major glutathione *S*-transferase band in erythrocytes it has been assumed that the GST3 locus is not polymorphic. Our findings show that different forms of the enzyme do exist. Typically lysates demonstrated two anodal bands, although some samples with a predominantly fast band were identified. We believe that the differences between our study and previous ones result from the use of different electrophoretic conditions. Because the concentration of the enzymes in erythrocytes is low, the staining system is at its detection limits, and in our experience small changes in technique can lead to different electrophoretic patterns.

It is difficult to provide a genetical explanation for the enzyme bands observed, and it is likely that they reflect a post-synthetic modification of the enzyme. Since many modifications result in increased anodal

mobility (Hopkinson & Harris, 1969), the enzyme band with fastest mobility could be a modified form. The slower band would be expected therefore to predominate in Percoll fractions containing the youngest erythrocytes. However, we found no difference between the electrophoretic patterns of cells throughout the gradients, indicating that the putative modification occurs before release into blood. This conclusion is supported by the finding of similar electrophoretic patterns in platelets (K. S. Rogerson, W. Cotton & R. C. Strange, unpublished work). Interestingly, although foetal enzyme patterns are often simpler than adult patterns, erythrocytes from cord blood also demonstrated two anodal bands.

Several erythrocyte enzymes have been shown to form mixed disulphides with oxidized glutathione (Hopkinson & Harris, 1969). Consequently the possibility that the electrophoretic patterns represent a parent enzyme and a mixed disulphide was investigated. Interpretation of the results is difficult, as the lysate is a complex protein mixture and some of the reagents are not specific for thiol groups. Since iodoacetate was the only reagent that modified the banding pattern, it is possible that the fast band differs from the slow band by reactions not involving a reactive thiol group, such as deamidation or proteolytic digestion.

The number of enzyme forms, their relative concentrations and erythrocyte activity appear to be constant in both foetal (Strange *et al.*, 1980) and adult life. The two enzyme forms appear to have similar specific activities but different thermostabilities. This probably accounts for our previous observation of a wide variation in thermal stability at 52°C (R. C. Strange, J. A. Moulton & W. Cotton, unpublished work).

Since the two enzyme forms in the erythrocyte have similar electrophoretic mobilities to those found in other human tissues, it is likely that the post-synthetic modification of the product of the GST3 locus occurs in other cell types and accounts for the bands with fast anodal mobility.

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