Human erythrocyte acetylcholinesterase in relation to cell age

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Acetylcholinesterase was studied in human red cells that had been fractionated on Ficoll/Triosil density gradients into classes representing different ages *in vivo*. Reticulocytes have negligible acetylcholinesterase activity; this is rapidly acquired on maturation to the erythrocyte. The activity per cell reaches a maximum and then, after a constant period, declines again towards the end of cell life. The maximum activity and the rates of activity gain and loss per cell are quantitatively different in adults and children. Kinetic studies showed that $V_{\max}$ follows the same age/activity profile but K_m is unaffected by cell age. The acetylcholinesterase protein content, determined by quantitative crossed immunoelectrophoresis, also shows a profile of increase and then decrease with cell age but the specific activity calculated from the protein estimate shows a reverse picture in which there is a slight decrease from young to mid-age cells followed by an increase again in older cells. These results are interpreted to indicate a complex developmental picture in which the overall cell age against enzyme activity profile is determined partly by the amount of enzyme protein present and partly from the modifying effect on the enzyme activity, of interactions with an aging cell membrane.

Key (1921) was the first to observe that the density of red cells from whole blood increases with the age of the cells *in vivo*. By exploiting this principle Sabine (1955) was able to separate red cells, by simple centrifugation, into two classes representing 'younger' and 'older' cells. He found that acetylcholinesterase (EC 3.1.1.7) activity was lower in the second group. By the same method Kadlubowski & Agutter (1977) were able to further separate the red cells into eight classes. They also concluded that acetylcholinesterase activity decreased with cell age. The disadvantage of this approach is that resolution of the cells into different age classes is poor so that only a qualitative assessment of the age-dependent changes can be made. The present work was undertaken to obtain a more quantitative evaluation made possible by fractionating the cells on a Ficoll/Triosil discontinuous gradient (Galbraith & Watts, 1980). By coupling this technique with that of quantitative crossed immunoelectrophoresis it was possible to estimate the relative amount of enzyme protein per

red cell and hence relative specific activity as well as total activity per cell as a function of cell age. The results indicate a complex pattern of maturation reflecting interactions between the enzyme and the erythrocyte membrane as well as changes in the absolute amount of protein present. As part of an ongoing investigation into the nature and development of hereditary myopathies the difference in these properties between normal adults and children was also investigated to provide a background for studying changes in the diseased state.

Materials and methods

Blood samples were collected into acid/citrate/dextrose anticoagulant from donors with informed consent and with the ethical permission of Guy's Hospital Medical School. All samples were immediately cooled in ice and processed within 18 h. The cells were separated from the plasma, the buffy coat removed and the remaining reticulocytes and erythrocytes then fractionated on a Ficoll/Triosil discontinuous gradient by the procedure and with the materials described by Galbraith & Watts (1980). For each sample a normal distribution of cells throughout the density gradient was obtained

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with a mean ($\pm$ S.D.) specific gravity in the range $1.1133 \pm 0.0003 \text{ g/cm}^3$ (Galbraith & Watts, 1980). It should be noted that this value is not an absolute property of human erythrocytes but reflects the anticoagulant and buffer system used for the isolation procedure. To allow for the small density differences between the cells of different individuals the distribution of cells in the gradient is expressed in terms of the mean values and standard deviations (σ) from the mean. The young cells have a negative σ and old cells a positive σ (Galbraith & Watts, 1980).

For the preparation of stroma the washed erythrocytes were lysed as described by Galbraith & Watts (1980). The haemolysate was centrifuged at $30000g$ for 30 min at 4°C and the sedimented membranes were washed in the same way with 40–60 vol. of cold 5 mM-phosphate buffer, pH 7.4, three or more times until white or very pale pink.

Acetylcholinesterase activity was measured at 30°C by the method of Ellman *et al.* (1961) using the unsolubilized stroma from the washed cells as the source of enzyme unless otherwise stated. Acetylthiocholine iodide was present at a final concentration of 0.4 mM and 5,5'-dithiobis-(2-nitrobenzoic acid) at a final concentration of 0.5 mM dissolved in 50 mM-sodium phosphate buffer, pH 7.4. A molar extinction coefficient of $1.36 \times 10^4 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ was used for the thionitrobenzoate ion at 412 nm (Ellman *et al.*, 1961). Activities were expressed per 10^9 red cells, the term 'red cells' being used to include both erythrocytes and reticulocytes, which were not discriminated between by the Coulter counter used (Galbraith & Watts, 1980). The acetylcholinesterase activity of fresh red-cell stroma was found to be unaffected by incubation for 2 h at 4°C in the gradient medium.

Solubilized acetylcholinesterase was obtained by treating a suspension of stroma with an equal volume of 2% Lubrol WX (Imperial Chemical Industries, High Holborn, London, U.K.) in 5 mM-sodium phosphate buffer, pH 7.4, for 1 h at approx. 4°C with stirring. Unsolubilized material was removed by centrifugation for 1 h at $40000g$ at 4°C . There was no acetylcholinesterase activity in the pellet and the enzyme solution was not retained by a $0.45 \mu\text{m}$ Millipore filter.

Crossed immunoelectrophoresis

Stroma were solubilized by mixing with an equal volume of 1% (w/v) Triton X-100 in 76 mM-Tris/40 mM-glycine buffer, pH 9.2, at 20°C . They were shaken for 1.5 h at approx. 4°C and centrifuged at $40000g$ for 1.5 h to remove any unsolubilized material.

Glass plates ($50 \text{ mm} \times 50 \text{ mm} \times 1 \text{ mm}$; Hoechst U.K. Ltd.) were primed with 1% (w/v) agar (electrophoresis-grade). Litex HSA Agarose (International Enzymes, Windsor, Berks., U.K.) (1%,

w/v) in a buffer containing 9 mM-sodium barbital, 1.4 mM-barbital, 94 mM-glycine, 46.5 mM-Tris base and 3.5 mM- NaN_3 , pH 8.8, was equilibrated to 54°C . Triton X-100 was added to give a final concentration of 1% (w/v).

Gels, 1.5 mm thick, were poured on the prepared glass plates. Appropriate samples of solubilized membranes were loaded into 2 mm diameter holes cut in agarose gels (one sample per gel; see Fig. 4). The plates were electrophoresed for 75 min at 5 V/cm at 16°C in 46.5 mM-Tris/93.5 mM-glycine/10.4 mM-barbital buffer, pH 8.8. The agarose gel was then cut away, leaving only a 1.5 cm strip along which the membrane proteins had been separated. This was replaced with agarose gel of the same composition containing $5 \mu\text{l/cm}$ of rabbit anti-(human erythrocyte membrane) globulins (Mercia Diagnostics, Watford, Herts., U.K.). The gels were then electrophoresed at right angles to the previous direction at 2 V/cm for 18 h at 16°C . Gels were dried under light pressure between layers of Whatman No. 1 filter paper at room temperature and then washed, with frequent changes of fluid, in 75 mM-NaCl for 1.5 h and then in water for a further $\frac{1}{2}$ h. Gels were finally dried thoroughly with a cool-air drier.

Visual detection of acetylcholinesterase activity

The method of Galbraith & Watts (1978) was followed. *N*-Methyl-1H-indol-3-yl acetate (*N*-methylindoxyl acetate) (6.4 mM) in 0.2 M-sodium phosphate buffer, pH 7.4, was prepared by first dissolving the *N*-methylindoxyl acetate in a small volume of methanol (not exceeding 4% of the final volume) and then mixing thoroughly with the buffer. Fast Blue RR salt (Edward Gurr, London, U.K.) was then dissolved in this solution to give a final concentration of $50 \text{ mg}/100 \text{ cm}^3$. Plates were incubated in the freshly prepared solution for 5 min at 30°C , with gentle agitation, dried and photographed.

Visual detection of protein

Plates were stained in 0.1% (w/v) Coomassie Brilliant Blue R in an aqueous solution containing 29% (v/v) methanol, 11.5% (w/v) trichloroacetic acid and 3.5% (w/v) sulphosalicylic acid for 15 min at 50°C . They were then destained for 15 min in a 25.5% (v/v) ethanol and 8% (v/v) acetic acid mixture at room temperature with one change of solution. The plates were dried and photographed.

Results

Acetylcholinesterase distribution with cell age

In normal adults and children the acetylcholinesterase activity in young cells increased linearly with cell age; maximum activity was achieved more

rapidly in children than in adults (Fig. 1). For adults the rate of increase was 258 nmol/min per 10^9 red cells/ σ (correlation coefficient, $r=0.87$); for children the value was 366 nmol/min per 10^9 red cells/ σ ($r=0.81$). The mean maximum value for adults (850 ± 110 nmol/min per 10^9 red cells) was higher than that for children (640 ± 90 nmol/min per 10^9 red cells) and this was followed by a linear decline in activity, which continued until the erythrocyte was removed from the circulation. For adults the rate of decline in activity was 283 nmol/min per 10^9 red cells/ σ ($r=0.93$) and for children it was much slower at 100 nmol/min per 10^9 red cells/ σ ($r=0.84$).

From the points at which the regression lines for the increase and decrease in activity intersect the mean maximum value the part of the life span over which the cells retain the mean maximum activity may be calculated. This was found to range approximately from -0.7σ to 0σ for adults and -1.3σ to -0.5σ for children. From Fig. 1 the enzyme activity of the oldest cells, corresponding to 3σ in the gradient, was also calculated by extra-

Fig. 1. Distribution of acetylcholinesterase activity in the stroma from human red cells fractionated into age classes in discontinuous Ficoll/Triosil density gradients

The results are for six adults (a) and for six children (b). The position of the cells in the gradient is expressed as the mean of the distribution $\pm$ s.d. Cell age increases non-linearly from left to right. The lines through the points are regressed.

polation of the linear decay rate. This gave zero for cells from adults and 43% of the mean maximum value for cells from children.

Spontaneous acetylcholinesterase release

An investigation was made to check whether the enzyme distribution pattern observed after fractionation resulted from release of acetylcholinesterase during the washing procedure. No activity could be detected in the supernatant after lysis either by the Ellman *et al.* (1961) method or by a pH-stat assay (Krupka, 1966) used to overcome the problem of a high background density at 412 nm caused by the presence of haemoglobin. Similarly, there was no significant activity in any of the stroma wash solutions. Nor could activity be detected after separation of haemolysate and the washings by thin-layer gel filtration on Sephadex G-150 carried out in 5 mM-sodium phosphate buffer, pH 7.4, as described by the manufacturers. Solubilized acetylcholinesterase was used as an enzyme standard and location of activity was achieved either visually by means of a filter paper overlay using the dye-coupled assay (Galbraith & Watts, 1978) or spectrophotometrically (Ellman *et al.*, 1961) after elution of the appropriate areas of the gel thin layer.

Activation of stromal fractions by Lubrol

Solubilization of total red-cell stroma with Lubrol WX as described previously results in a consistent small increase in acetylcholinesterase activity. For

Fig. 2. Acetylcholinesterase activity of age-fractionated red-cell membranes before and after solubilization with Lubrol WX detergent

The shaded area shows the increase in activity after treatment with detergent as described in the text. The percentage increase in activity is given in parentheses above the columns.

23 adult donors this increase was $14.6 \pm 3.2\%$ (mean $\pm$ s.e.m.). Fig. 2 shows for one donor that this increase is associated more with younger cells and decreases sharply towards the end of life *in vivo*. All samples tested showed activation on solubilization after fractionation; the extent of the decrease in activation of older cells was more variable.

Effect of cell age on K_m and V_{max} .

The K_m and V_{max} for acetylthiocholine iodide at 25°C were determined over the substrate range $10\text{--}400\ \mu\text{M}$. Fig. 3(a) shows double-reciprocal plots obtained with the different stromal fractions obtained after age fractionation of the sample from one donor. Five such experiments indicated that K_m was independent of cell age. For the experiment shown in Fig. 3 K_m values, computed by the method of Porter & Trager (1977), were (μM): 65, 60, 60, 62, 43 and 71 [mean value ($\pm$ s.d.) 60 ± 9] corresponding to σ positions in the gradient of -3 , -1.8 , -0.8 , -0.13 , 0.6 and 2.1 respectively. The corresponding V_{max} values are shown in Fig. 3(b); the general shape of the profile may be compared with that for the activity obtained under standard assay conditions in Fig. 1.

Effect of cell age on the enzyme protein content per erythrocyte

For this investigation stroma was solubilized in Triton X-100 as described by Bjerrum & Lundahl (1973). For 25 donors $98.2 \pm 2.5\%$ of the acetylcholinesterase was released after removal of any debris by centrifugation for 1 h at $40\,000g$ at 4°C . Because of the small size of the samples and the presence of contaminating proteins absolute quantification of the enzyme protein was not possible. Instead the relative concentration of immunogenically precipitable protein was determined by crossed immunoelectrophoresis. Fig. 4(a) compares the acetylcholinesterase band obtained by specific staining after electrophoresis with the picture for total protein (Fig. 4b) by subsequent staining of the same plate with Coomassie Blue. By using a series of samples from one donor and absolutely standard electrophoresis conditions it was found that the area under the acetylcholinesterase precipitin curve was proportional to the number of cells, and therefore enzyme protein, applied to the gel (Fig. 5). Areas were measured by the method of Baker *et al.* (1966).

The relative specific activity of the enzyme is defined as

$$\text{Relative specific activity} = \frac{\text{Enzyme activity (nmol/min per } 10^9 \text{ red cells)}}{\text{Precipitin area (mm}^2 \text{ corresponding to } 10^9 \text{ red cells)}}$$

Fig. 3. *Acetylcholinesterase kinetics of stroma from adult human red cells of differing mean age*

(a) Double-reciprocal plots of activity as a function of acetylthiocholine concentration. The values in parentheses indicate the position in the gradient. For clarity, results for only four of the six fractions estimated are shown. (b) Profile of V_{max} with cell age from the same experiment. Activity was measured at pH 7.4 and 25°C . Velocities are expressed as nmol/min per 10^9 red cells.

Fig. 4. Quantitative crossed immunoelectrophoresis of red-cell-membrane proteins

After analysis as described in the Materials and methods section the plate was first stained for acetylcholinesterase activity (a) and then for total protein (b). This example is taken from near the mean density range (σ 2 0.8) after fractionation.

The areas and relative specific activities were first determined on unfractionated samples from five children and four adults. The areas were (per 10^9 red cells): $383 \pm 82 \text{ mm}^2$ (children); $725 \pm 112 \text{ mm}^2$ (adults). These were significantly different on the basis of the unpaired Student's *t* test ($P < 0.002$). The relative specific activities were also significantly different ($P < 0.02$), being 1.316 ± 0.267 (children) and 0.811 ± 0.115 (adults), although there was no significant difference between the activities per 10^9

Fig. 5. Relationship between the amount of stroma used for analysis by crossed immunoelectrophoresis and the area under the acetylcholinesterase peak as indicated in Fig. 4

The samples were from unfractionated cells from one donor processed as described in the Materials and methods section.

Table 1. The relative specific activity of human red-cell acetylcholinesterase as a function of cell age in vivo
Cells were first fractionated on a Ficoll/Triosil gradient and the washed stroma was analysed spectrophotometrically for acetylcholinesterase activity and by quantitative crossed immunoelectrophoresis for determination of the amount of enzyme protein present. This is expressed in terms of area under the precipitation curve as described in the Materials and methods section. The relative specific activity is defined in the Results section.

Sample	Age of donor (years)	Enzyme activity (nmol/min per 10^7 red cells)	Position of fraction in gradient (σ)	Area under precipitation curve per 10^7 red cells (mm^2)	$10^3 \times$ Relative specific activity
1	13	6.66	-1.53	12.86	5.2
		6.71	-0.82	21.92	3.1
		5.07	1.15	5.56	9.1
		3.38	2.60	3.04	11.2
2	19	7.94	-1.9	10.47	7.6
		8.90	-0.85	12.71	7.0
		6.77	0.5	9.67	7.0
		5.29	2.6	5.74	9.2
3	16	7.33	-1.53	16.06	4.6
		4.53	0.78	7.40	6.1
4	6	5.29	-2.0	30.67	1.7
		4.33	0.6	15.63	2.8

red cells, which were 621 nmol/min and 585 nmol/min for children and adults respectively.

Table 1 shows for three subjects how the amount of immunogenically active acetylcholinesterase and relative specific activity varies with cell age. For all three the area under the precipitin curve decreases sharply in older cells, although the extent of this decrease exceeds that expected on the basis of enzyme activity per 10^9 red cells, as a result of which the relative specific activity actually shows a variable but distinct increase in the older cells.

Discussion

The present work confirms previous findings that acetylcholinesterase activity decreases in the older cells (Key, 1921; Kadlubowski & Agutter, 1977; Rennie *et al.*, 1979), but permits a more quantitative evaluation of the changes that occur.

In both adults and children the newly formed erythrocyte contains very little acetylcholinesterase activity. Back extrapolation of the early part of the curves in Fig. 1 indicates that there is zero activity at the theoretical position in the gradient (-4.35σ) corresponding to 100% reticulocytes (Galbraith & Watts, 1980). Hence it would seem that the acetylcholinesterase activity of the reticulocyte membrane is negligible and the activity appears after maturation to the erythrocyte. Skaer (1973) has identified histochemically an acetylcholinesterase within red cell precursors, which he suggests is an isoenzyme and precursor of the form found in the erythrocyte membrane. On the other hand Zajicek (1956, 1957) concluded that the activity is picked up from the plasma rather than originating within the erythrocyte. Rennie *et al.* (1979), by using heparinized cells and a Percoll density gradient, reported, in contrast with our results, a high activity in young cells and a continuous decline in activity with age. The reason for this difference may relate to the anticoagulant used but remains to be determined.

The acetylcholinesterase activity of young cells rapidly achieves a saturating value in the membrane, which occurs faster in children than adults. Herz *et al.* (1972) separated blood from children into young and old cells by simple centrifugation in haematocrit tubes and concluded that young cells have greater activity than old cells. This probably reflects the fact that it is much easier to prepare enriched samples of old cells than of very young cells. The data from Fig. 1 indicate that in children the increase to maximum occurs over the first 10% of the cell life span, whereas the decrease in activity occurs over the final 70%. For adults the corresponding values are 25% of the life span for the increase and 50% for the decrease. If the source of the acetylcholinesterase is exogenous, then the greater rate of accumulation in children is com-

patible with the greater turnover rate of young muscle compared with adults, which would lead to a greater release of the enzyme from the neuromuscular junction. Likewise the loss of acetylcholinesterase activity, although it onsets later in adults, occurs much more rapidly, so that the oldest adult cells have almost no activity, whereas those of children still have nearly half the maximum value. It should be stressed, however, that these are mean values; considerable variation was found between individuals, which would be expected if a 'wear and tear' factor is involved.

For convenience of assay activity measurements were carried out using stroma rather than whole cells. Hence it was important to establish that the results were not unduly influenced by the manipulations used. No activity was found to be released from the stroma during isolation that might explain the profiles obtained. It is also well established that acetylcholinesterase is located on the outside of the membrane, so that selective resealing inside out of the younger and older cells during stroma formation could influence the results by preventing access to substrates in the subsequent enzyme assay. Fig. 2 shows that there is an increase in activity on solubilization with Lubrol WX; the percentage activation is similar in young and mature cells but decreases in older cells so that the general shape of the activity-age profile is not significantly altered. This increase in activity may result from a direct effect of the detergent on the activity of the enzyme or by the release of enzyme from sealed inside-out vesicles. Little is known about the latter but the activation of acetylcholinesterase by treatment with low concentrations of Triton X-100, Tween 20, sodium dodecyl sulphate or deoxycholate is well documented (Miller, 1970; Heller & Hanahan, 1972; Paniker *et al.*, 1973; Sihotang, 1976). As we have also found with Lubrol (D. A. Galbraith & D. C. Watts, unpublished work), higher detergent concentrations are inhibitory and Sihotang (1976) has found that inhibition by deoxycholate is associated with the stripping of phospholipid from the enzyme. This phenomenon may also explain the loss of activity observed by Wright & Plummer (1972) when the stroma is treated with sufficient Triton X-100 and KCl to cause complete solubilization as judged by the failure to sediment enzyme by centrifugation for 1 h at 100 000 g. By this criterion our preparation would not be considered completely soluble. We have found that prolonged centrifugation does cause some sedimentation, suggesting that there is still some association with other membrane components, although these particles are not sufficiently large to be retained by a $0.45\mu\text{m}$ Millipore filter.

Saturating substrate concentrations inhibit acetylcholinesterase so that a non-saturating concen-

tration is used in the standard assay. There is no significant change in K_m with cell age (Fig. 3a) and the V_{max} -cell-age profile (Fig. 3b) is similar to that obtained under the standard assay conditions. Thus it may reasonably be inferred that the observed profile accurately reflects the changes occurring *in vivo*. Few detailed kinetic studies have been performed on enzymes from age-fractionated cells and none before on a membrane enzyme. However, it is instructive to compare the present results with glucose 6-phosphate dehydrogenase in which the progressive decrease in enzyme activity with cell age, expressed in terms of cell haemoglobin content, was accompanied by an increase in K_m and an increase in sensitivity to heat inactivation (Fornaini *et al.*, 1969). This may reflect protein conformational changes after mixed disulphide formation between enzyme thiols and glutathione, which Fornaini *et al.* (1969) point out has been suggested to occur in the aging of the haemoglobin molecule. It is significant that such oxidative changes occur even though the cellular activity of glutathione reductase does not alter throughout cell life (Galbraith & Watts, 1980). Acetylcholinesterase, on the other hand, is an outer-membrane enzyme and is not noticeably sensitive to oxidative changes. For this enzyme the influence of changes in membrane composition may be of greater significance.

The measurement of enzyme activity alone leaves unanswered the question of whether such measurements also indicate the amount of enzyme protein present. This knowledge is required to evaluate how enzyme-membrane interaction may affect enzyme activity. It is convenient to determine small amounts of a specific enzyme in an impure system by immunoassay but it must be recognized that the results will be affected if the number of combining sites per molecule change with the age of the enzyme or as a result of conformational changes produced by enzyme interaction with a membrane, itself undergoing change. Three considerations suggest that this is not a problem with the acetylcholinesterase system. First, the enzyme was analysed after detergent solubilization so that enzyme-membrane interaction effects should be eliminated. Secondly, there was no change in the electrophoretic mobility of the solubilized enzyme with cell age or in the location of the precipitin band that might reflect molecular reorganisation of the enzyme. Third, the solubilized enzyme was reasonably stable and retained its activity in both the solubilized form and in the solubilized-enzyme-antibody complex, so that if the number of antibody-combining sites had altered it must have done so without materially affecting the functional organization of the enzyme. Complex formation between enzyme and antibody without loss of catalytic activity was a discovery that greatly

facilitated the unambiguous and remarkably reproducible quantification of acetylcholinesterase by immunoelectrophoresis (Fig. 5). Table 1 shows that the immunogenic acetylcholinesterase protein content of the red cell, measured as the area under the precipitin curve, shows a similar profile with cell age to that found for enzyme activity but decreases sharply in older cells. In consequence the relative specific activity is not the same for all fractions but actually increases towards the end of cell life. The difference between young and old cells is statistically significant at the 0.1% confidence level for the four samples analysed in Table 1, in spite of the considerable individual variation between samples. It may be concluded that the observed change in acetylcholinesterase activity, as indicated by Fig. 1, reflects a complex sequence of events in which the general shape of the profile is determined by changes in the amount of enzyme protein present but the activity level reflects secondary modulation imposed by the enzyme-membrane interaction. Both factors may be involved in determining the different rate parameters for uptake and loss of enzyme found when adults and children are compared.

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