Breakdown of Aberrant Protein in Rabbit Reticulocytes decreases with Cell Age

Malcolm J. McKAY, Rodney S. DANIELS* and Alan R. HIPKISS
Department of Biochemistry, King's College, Strand, London WC2R 2LS, U.K.

(Received 21 December 1979)

Rabbit reticulocytes, separated into ten cell age groups by bovine serum albumin density-gradient sedimentation, were pulse-labelled with [¹⁴C]leucine in the presence of either puromycin dihydrochloride or aminoethyl-L-cysteine (a lysine analogue). Intracellular proteolysis of both the puromyciny-peptides and analogue-containing protein decreased with cell maturity.

Erythroid cells are suitable models for studying the factors regulating mammalian development and maturation, as a variety of structural and biochemical changes are known to occur, such as the onset of haemoglobin synthesis, the loss of the ability to synthesize DNA, the loss of the ability to divide, the extrusion of the nucleus and, in the final stages of maturation, the cessation of protein synthesis. The differentiation of the reticulocyte into the erythrocyte, in the last stage of erythroid-cell development, may serve as a model for aging, albeit of a specialized nature.

The reticulocyte has both protein-synthetic machinery and the ability to degrade aberrant protein rapidly and selectively (Etlinger & Goldberg, 1977; Chandler & Ballard, 1978; Daniels & Hipkiss, 1978, 1979; Ciehanover *et al.*, 1978; Botbol & Scornik, 1979). The loss of the ribosomal fraction has been identified as being largely responsible for the decline in protein synthesis with reticulocyte maturation (Glowacki & Millette, 1965). The erythrocyte has no protein-synthetic ability, and cell extracts appear to be unable to degrade aberrant proteins (Etlinger & Goldberg, 1977). As a result, any protein within the erythrocyte that is denatured by mechanical or chemical means would accumulate and possibly precipitate. Such accumulations are sometimes in the form of very-high-molecular-weight aggregates termed Heinz Bodies, which are derived from denatured haemoglobin through a sequence of oxidative steps (Carrell *et al.*, 1975; Winterbourn, 1979). Of particular interest is that early and intermediate stages of Heinz-Body formation have been observed in the relatively mature reticulocyte, but not in younger cells (Rifkind & Danon, 1965), and larger condensed forms are not observed in any but the

mature erythrocyte (Rifkind & Danon, 1965). Thus, when intracellular haemoglobin is denatured it would appear that there is an onset of Heinz-Body formation in the more mature reticulocytes and the erythrocytes. The reasons for this differential accumulation of cellular inclusions are not clear, but it could conceivably be due, at least in part, to loss of the ability to degrade aberrant or denatured haemoglobin in the more mature cells. With the knowledge that reticulocytes have the ability to degrade aberrant protein, whereas erythrocyte cell-free extracts apparently do not, and that Heinz Bodies are seen only in the more mature reticulocyte and erythrocytes, it would appear reasonable to suggest that the reticulocyte loses some of its proteolytic ability with age. The present study was therefore undertaken to examine the ability of the reticulocytes to degrade aberrant protein as they matured. Two types of aberrant protein were chosen for study; puromyciny-peptides (normal amino acid composition but shortened chain length) and protein containing the lysine analogue aminoethylcysteine. Of the variety of techniques available to separate reticulocytes on the basis of their age, the separation of cells on gradients made up of different densities of bovine serum albumin was chosen, as there is evidence, both cytological and biochemical, to support the supposition that the albumin gradient separates the cells according to their degree of maturation (Glowacki & Millette, 1965). Evidence is presented which supports the proposal that rabbit reticulocytes lose intracellular proteolytic activity, particularly against aberrant proteins, as they mature.

Experimental

Materials

Blood containing 80–90% reticulocytes was obtained by cardiac puncture from anaemic New

* Present address: Division of Virology, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K.

Zealand White rabbits, after pretreatment with phenylhydrazine (Denton & Arnstein, 1973). Bovine serum albumin powder (fraction V) was obtained from Sigma Chemical Co., as were puromycin dihydrochloride and *S*-2-aminoethylcysteine. L-[U-¹⁴C]leucine (330 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Radioactive samples were counted for radioactivity with PCS as a scintillant (Amersham Corp., Arlington Heights, IL, U.S.A.) in a Packard scintillation spectrometer.

Methods

Blood cells were centrifuged (600 *g* for 10 min) out of the plasma and washed twice with Borsook saline (0.13 M-NaCl/0.005 M-KCl/0.005 M-MgCl₂·6H₂O) (Lingrel & Borsook, 1963). Nine different concentrations of albumin, ranging from 21 to 30% (w/w), were made up in buffer (0.13 M-NaHCO₃/0.005 M-KCl/0.005 M-MgCl₂·6H₂O). The nine albumin solutions were layered with a syringe into a centrifuge tube, 2 ml in each band. A 3 ml cell sample, diluted 2:1 (cells:albumin buffer) was layered on top of the 18 ml albumin gradient. Three identical tubes were spun in a Beckman SW 25.1 rotor at 32000 *g* for 90 min at 5°C in a Beckman Spinco model L2 ultracentrifuge. After centrifugation, ten cell fractions were collected from each tube with a syringe (nine cell fractions were cells accumulated at the interfaces between bands and the tenth cell fraction was the pellet at the bottom of the tube resuspended in 2 ml of albumin buffer). The fractions were diluted with albumin buffer and centrifuged (600 *g* for 10 min) to free them of albumin. The cells were then washed twice, the last wash with Borsook saline.

The collected cell types were each incubated in the standard medium of Lingrel & Borsook (1963) for 100 min at 37°C in a shaking water bath to allow recovery from the initial centrifugation step. Then each cell type was resuspended in fresh standard medium as a 33% (v/v) cell suspension with the omission of the amino acid mixture (Lingrel & Borsook, 1963) and preincubated at 37°C for 10 min in a shaking water bath. The amino acid mixture was then added (0.01 ml/ml of cell suspension), followed by either lysine (1.8 mM), or aminoethylcysteine (1.8 mM) or puromycin dihydrochloride (5 µg/ml) plus lysine (1.8 mM). [U-¹⁴C]-Leucine (1.5 µCi/ml of cell suspension) was then added, making the final cell suspension 25% (v/v). The reticulocytes were pulse-labelled with [U-¹⁴C]leucine for 5 min. Then 5 vol. of Borsook saline supplemented with 50 mM unlabelled leucine were added, the cells centrifuged at 600 *g* for 10 min and finally resuspended as a 25% (v/v) cell suspension in preheated (37°C) standard medium of Lingrel &

Borsook (1963) supplemented with 20 mM unlabelled leucine.

Samples (50 µl) were taken at various times and added to 2 ml of 5% (w/v) trichloroacetic acid, left overnight at 4°C and the precipitated protein was filtered by using Whatman GFC filter discs. A 1 ml portion of the filtrate (acid-soluble fraction) was added to 2 ml of PCS and radioactivity determined. The total radioactivity present was determined by adding a 50 µl sample to 1 ml of 0.5 M-NaOH, and bleaching with two drops of 20-vol. H₂O₂ for 18 h. Protein breakdown is expressed as the increase in acid-soluble radioactivity detected as a percentage of the total radioactivity originally in protein (acid-precipitable fraction). This procedure was carried out with each cell fraction recovered from the albumin gradient.

Results and Discussion

It is well established that rabbit reticulocytes may be fractionated according to their degree of physiological maturity by means of buoyant-density centrifugation in an albumin gradient (Glowacki & Millette, 1965; Borsook *et al.*, 1962). The data in Table 1 are consistent with this claim. The RNA content, rate of amino acid incorporation and fumarase activity, which are cited as biochemical markers of reticulocyte maturation (Glowacki & Millette, 1965; Rubenstein, 1956), decreased with increasing cellular buoyant density.

The incorporation of the lysine analogue aminoethylcysteine into reticulocyte protein promotes proteolysis of the protein synthesized, as does incubation in the presence of puromycin dihydrochloride (Etlinger & Goldberg, 1977; Daniels & Hipkiss, 1978, 1979). When the ability to break down the protein synthesized in the presence of aminoethylcysteine was examined in the reticulocyte fractions recovered from an albumin gradient, it was found that the younger cells, banding towards the top of the gradient, were more active in their ability to degrade the aberrant protein than were the more mature cells recovered from the bottom of the gradient (Fig. 1*a*). Both the initial rate and extent of proteolysis fell as the buoyant density of the cells increased.

When the above procedure was repeated with fractionated cells pulse-labelled in the presence of puromycin dihydrochloride (5 µg/ml), a similar phenomenon was observed (Fig. 1*b*). Again, the initial rate and extent of proteolysis in the cells fell as their cellular buoyant density increased.

Proteolysis in fractionated cells incubated and pulse-labelled under control conditions (i.e. in the presence of lysine) similarly decreased with increasing buoyant density (Fig. 2).

The present results show that there is a significant age-related decrease in cellular degradative ability

Table 1. *Characteristics of albumin-gradient-fractionated rabbit reticulocytes*

Ten cell fractions were obtained from a nine-step albumin gradient and washed free of albumin. RNA was assayed as described by Schneider (1957), ¹⁴C-labelled amino acid incorporation as described under 'Methods', except that the cells were incubated with the [¹⁴C]leucine for 10 min, fumarase as described by Racker (1950), cathepsin C as described by Johnstone *et al.* (1950) and cathepsin G as described by Starkey (1976). Cell numbers were determined with a Coulter Counter (Coulter Electronics, Dunstable, Beds., U.K.).

Cell fraction	RNA ($\mu\text{g}/10^6$ cells)	$10^{-4} \times$ ¹⁴ C-labelled amino acid incorporation (c.p.m./ 10^6 cells)	Activity (μmol of product formed/min per 10^6 cells)		
			Fumarase	Cathepsin C	Cathepsin G
1	105	13.38	138	180	2520
2	90.4	8.63	119	165	2400
3	77.9	5.08	65.7	137	2350
4	82.1	3.66	57.4	102	1950
5	42.9	2.05	49.4	104	1840
6	40.3	1.74	48	81	1750
7	35.8	1.69	45.3	53	1720
8	34.7	1.21	19.5	38	1220
9	33.5	0.94	15.7	24	1100
10	28.7	0.61	16.2	21	564

Fig. 1. *Proteolysis in rabbit reticulocytes after separation on an albumin density gradient*
Ten cell fractions obtained from a nine-step albumin gradient were washed free of albumin and pulse-labelled with [¹⁴C]leucine in the presence of (a) aminoethylcysteine or (b) puromycin dihydrochloride as described under 'Methods'. Proteolysis was then determined in the absence of analogue and the antibiotic. Cell fractions numbered 1–10 indicate increasing cellular buoyant density.

after synthesis of three different protein substrates. Experiments with fractionated cells pulse-labelled under control conditions (i.e. with a full complement of amino acids in the pulse-labelling incubation mixture) produced small amounts of rapidly degradable protein, which may well represent abnormal haemoglobin. This putative aberrant protein may be formed as a result of spontaneous errors in protein synthesis, or as a result of post-synthetic denaturation/modification, possibly by the action of residual phenylhydrazine, since it has been shown in this laboratory that phenyl-

hydrazine can stimulate proteolysis in control cells (R. S. Daniels & K. Buckler, unpublished work). The exact nature of this substrate, its various forms and extent of denaturation is unclear. However, of the total amount of [¹⁴C]leucine incorporated into protein during the pulse-labelling procedure, up to 6% was degraded to acid-soluble material (Fig. 2). This control activity falls with cell maturity when both the initial rate and the extent of the degradative process are significantly decreased.
When fractionated cells were pulse-labelled in the presence of puromycin dihydrochloride they pre-

Fig. 2. Proteolysis in rabbit reticulocytes after separation on an albumin density gradient

Ten cell fractions obtained from a nine-step albumin gradient were washed free of albumin and pulse-labelled with [U - ^{14}C]leucine in the presence of lysine. Proteolysis was then determined. Cell fractions numbered 1–10 indicate increasing cellular buoyant density.

sumably synthesized small polypeptides, which were rapidly degraded (Fig. 1b). Again, there was a considerable decrease in the initial rate and extent of degradation as the reticulocytes matured (Fig. 1b). Younger cells had 5–10 times more degradative activity than the most mature cells. The activity decreased gradually, except in the region of bands 6 and 7, where there was a large fall. Similarly, with cells incubated in the presence of the lysine analogue aminoethylcysteine (Fig. 1a), there is a significant decrease in degradative activity, with the youngest cells having 4–10 times more degradative activity than the most mature cells.

The decrease in degradative activity does not appear to follow any set pattern, although the breakdown curves in Figs. 1(a), 1(b) and 2 fall roughly into three groups, with the cells in the first three or four bands of the albumin gradient consistently appearing to show little change in degradative activity. Further down the gradient, bands 4–7 show an almost linear decrease in activity, with an especially large fall in activity between bands 7 and 8. The last three bands generally have very low degradative activity.

Synthesis of aberrant protein in rabbit reticulocytes is accompanied by the appearance of a high-molecular-weight multi-peptide aggregate which has the properties of a degradative intermediate (Daniels & Hipkiss, 1979). Preliminary studies show that, although both young and mature

reticulocytes may synthesize this material, the old cells appear to be defective in its catabolism (M. J. McKay & A. R. Hipkiss, unpublished work). The observed decrease in degradative activity is thought to represent a loss or deterioration of the proteolytic system in the reticulocyte as it matures to the erythrocyte (Hershko *et al.*, 1978, 1979). The exact nature of the proteolytic system that is capable of rapidly and selectively degrading aberrant protein in the rabbit reticulocyte is not completely understood, although it is believed that there is a requirement for ATP (Etlinger & Goldberg, 1977; Daniels & Hipkiss, 1978; Ciechanover *et al.*, 1978).

However, cathepsins C and G, both neutral proteinases, but with no known ATP requirement, were also found to decrease markedly with cellular buoyant density (Table 1). The roles, if any, of these enzymes in the degradative process studied here are unknown, although evidence for ATP-independent proteolysis of certain aberrant proteins has been found (R. S. Daniels, V. C. Worthington & A. R. Hipkiss, unpublished work).

Many features in the maturing cells (apart from removal by proteolysis of proteolytic-enzyme molecules) may bring about a loss of proteolytic activity on aberrant proteins; accumulation of proteinase inhibitors, competition for the proteolytic enzyme by normally turning-over unlabelled protein, or the conversion of aberrant proteins into undegradable forms in the most mature cells.

The loss of proteolytic activity is not thought to be an artifact of the experimental procedure, such as some effect of the different concentrations of albumin in each fraction collected. Unfractionated cells have been incubated for 90 min at 5°C in different concentrations of albumin to mimic the fractionation procedure, and no differences in degradative ability were observed.

The loss or deterioration of the proteolytic activity in the rabbit reticulocytes fits a pattern observed in β -thalassaemic erythroid-cell maturation, where bone-marrow erythroid cells of increasing maturity seem to display decreasing ability to degrade excess α -globin chains (Clegg & Weatherall, 1972; Wood & Stamatogannopoulos, 1975). It would be reasonable to extend these observations to the reticulocyte's proteolytic system and postulate that the reticulocyte may continue to lose proteolytic activity with maturation. Although the substrate used by the cell in the experiments described here is not the same as that in the β -thalassaemic erythroid cells, the results with the maturing rabbit reticulocyte do fit the general pattern of decline of proteolytic activity with increasing cellular maturity.

M. J. McK. and R. S. D. thank the Science Research Council for postgraduate studentships.

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