RIBONUCLEASE ACTIVITY DURING ERYTHROID CELL MATURATION

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1. Introduction

A progressive decrease in the soluble and ribosomal RNA content due to an enzyme system that degrades RNA has been shown to occur during the late stages of erythroid cell maturation [1,2]. There is substantial evidence that ribonuclease activity also declines in the same period [3,4]. The information available on RNase activity in erythroid cells is, however, still fragmentary and concerned only with those cells at the final stage of erythroid development, namely reticulocytes.

Taking advantage of a technique recently developed in this laboratory for the fractionation of the immature cells from rabbit bone marrow by velocity sedimentation in a Ficoll gradient [5], we have investigated the changes of RNase* activity during erythroid cell development in the bone marrow. From the results of this study it appears that RNase activity is related to other biochemical and morphological changes occurring during cell maturation. Thus, the enzyme activity decreases markedly after cessation of RNA synthesis and even further after extrusion of the nucleus from the orthochromatic cells. In contrast, an examination of RNase activity by polyacrylamide gel electrophoresis of erythroid cell lysates demonstrated that the RNase electrophoretic pattern showed only slight changes as the cells move from the dividing into the nondividing cell compartment.

2. Materials and methods

2.1. *Materials*

Yeast RNA was purchased from B.D.H. Ltd., (Poole, Dorset, UK). Ficoll was a product of Pharmacia (75 Uxbridge Road, Ealing, London W.5., UK). Typhoid vaccine was obtained from the Wellcome Foundation Ltd., (Temple Hill, Dartford, Kent, UK). All other chemicals were reagent grade.

2.2. *Fractionation of bone marrow cells*

White New Zealand rabbits were made anaemic by five daily intraperitoneal injections of 2.5% neutralized phenylhydrazine hydrochloride (0.35 ml per kg body weight) and killed two days after the last injection. The white cell population in the marrow was decreased by injecting the rabbits intravenously 2 hr before death with 1 ml typhoid vaccine. The bone marrow cells were collected, washed and fractionated by velocity sedimentation in a Ficoll gradient as described elsewhere [5]. The technique is based on the fact that at unit gravity the sedimentation velocity of a cell is proportional to its size. As the cell size decreases continually during the maturation process a very good resolution is obtained between different types of cells, particularly between dividing and nondividing cells. The cells were collected from gradient fractions by centrifugation at 800 *g* for 15 min. All enzyme assays were carried out on the supernatant obtained by lysing the cells in an equal volume of 10 mM Tris–HCl buffer pH 7.4 and sedimenting the cell debris at 800 *g* for 10 min.

2.3. *Assay of RNase activity*

The enzyme activity was determined by measuring the production of acid-soluble digestion products from yeast RNA at 260 nm by a slight modification of the procedure of Egami et al. [6]. Two buffers were used. Buffer A: 0.2 M Na_2HPO_4–0.1 M citric acid for estimations at pH 5.4 or 6.5 and buffer B: 0.2 M Tris–HCl for pH 7.5. The reaction mixture

* *Abbreviations*: RNase–ribonuclease; RNA–ribonucleic acid; DNA–deoxyribonucleic acid.

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contained: 0.4 ml buffer, 0.2 ml H_2O, 0.1 ml enzyme solution and 0.3 ml 1% RNA which had been extensively dialysed against distilled water before use. After incubation at 37°C for the appropriate period, the reaction was stopped by adding 0.25 ml of 0.75% uranyl acetate in 25% perchloric acid. The mixture was left at 0°C for 20 min., then the precipitate was removed by centrifugation at 2000 *g* for 10 min. The clear supernatant (0.2 ml), was diluted to 5 ml with distilled water and the absorbance read at 260 nm. One enzyme unit is defined as the increase in the absorbance of 1.0 after incubation at 37°C for 15 min.

2.4. *Detection of RNase activity after polyacrylamide gel electrophoresis*

Detection of RNase activity after polyacrylamide gel electrophoresis was carried out generally according to Vasu and Radulescu [7]. Electrophoresis was performed in a horizontal system [8] in 7.5% polyacrylamide gel. The gel plate was 15 X 11 cm allowing six samples to be run simultaneously. The buffer was 0.012 M Tris–0.095 M glycine pH 8.4 and had the same concentration in the gel and in the electrode vessels. A current of 0.8 mA/cm at 10 V/cm was applied for 4–5 hr. The gel plate was subsequently soaked in 0.4% RNA in buffer A or B for 45 min, then incubated in a humidified chamber at 37°C for 12–16 hr. The undegraded RNA was precipitated by soaking the gel in 25% ethanol– 5% acetic acid for 2 hr. The gel plate was finally stained with 0.2% toluidine blue in 1% acetic acid for 10 min. The RNase bands were detected by washing the gel with 2% acetic acid and distilled water.

2.5. *Protein assay*

Protein concentration was assayed according to Lowry et al. [9], using bovine serum albumin as standard.

3. Results

The sedimentation profile of the bone marrow cells fractionated by velocity sedimentation and the RNase activity of each fraction of the gradient are shown in fig.1. The four main regions of the gradient contained morphologically distinct types of immature erythroid cells. Basophilic and polychromatic cells

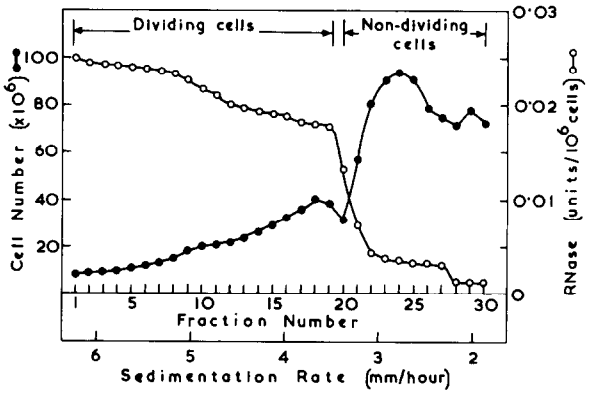


Fig.1. Cell sedimentation profile and RNase activity in erythroid cell lysates. The four regions of the gradient contained: basophilic cells (fractions 1–10), polychromatic cells (fractions 11–19), orthochromatic cells (fractions 20–27), reticulocytes (fractions 28–30). RNase activity was assayed at pH 5.4 in each fraction of the gradient as described under Methods. (•—•) cell sedimentation profile, (○—○) RNase activity.

sedimenting in fractions 1–19 constitute the dividing cell compartment, and orthochromatic and reticulocytes sedimenting in fractions 20–30 the nondividing cell compartment. The orthochromatic erythroblasts (fractions 20–27) comprise all non-dividing nucleated cells, including those previously termed 'small polychromatic cells' [5].

During the early stages of maturation (fractions 1–18) the RNase activity per cell decreases, though only to a limited extent. Between fractions 19 and 22, however, there is a marked loss of RNase activity, which coincides with cessation of cell division since fraction 22 contains cells which are no longer active in DNA synthesis [5]. Following extrusion of the nucleus from the orthochromatic erythroblasts there is a further loss of enzyme activity (fractions 27–28), which is, however, quantitatively less significant. Determinations of the RNase activity of gradient fractions at pH 6.5 and 7.5 gave essentially comparable profiles of enzyme activity.

A quantitative comparison of the RNase activities at three pH values is shown in table 1, the results for the different cell fractions being expressed in enzyme units per cell rather than mg of protein because of the marked increase in cellular protein during the later stages of development as a result of haemoglobin synthesis.

Table 1
RNase activity in the different classes of erythroid cells separated by velocity sedimentation
RNase units/10^6 cells

	Bone marrow erythroid cells				Peripheral erythroid cells
pH	Basophilic	Polychromatic	Orthochromatic	Reticulocytes	Blood reticulocytes
5.4	0.023	0.018	0.0034	0.0010	0.0007
6.5	0.021	0.016	0.0036	0.0014	0.0011
7.5	0.020	0.015	0.0035	0.0013	0.0010

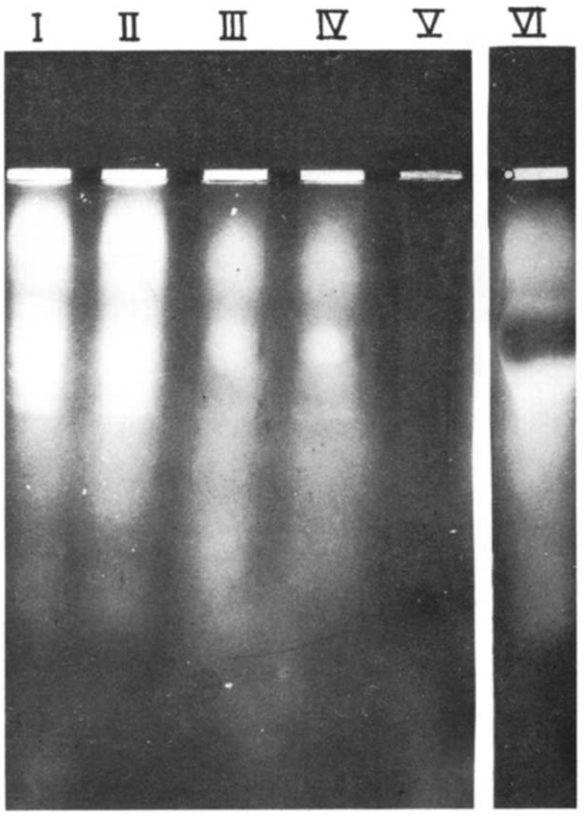
Fractions of the gradient (fig.1) corresponding to the four main classes of bone marrow erythroid cells (basophilic erythroblasts, fractions 1–8; polychromatic erythroblasts, fractions 11–19; orthochromatic erythroblasts, fractions 22–27; reticulocytes, fractions 28–30) were pooled, the cells were lysed and the RNase activity determined in the 800 *g* supernatant as described in Materials and methods.

The amount of RNase activity per cell was similar at the three different pH values, although there was a slightly higher level of acid ribonuclease activity in the less mature cells. The RNase activity of polychromatic erythroblasts is approx. 75% of that of the basophilic cells, whereas orthochromatic cells and bone marrow reticulocytes contain only approx. 15% and 4–7%, respectively, of the activity of basophilic cells. During maturation of reticulocytes after being released into the circulation, their RNase content decreases still further to approx. 3 – 5% of the level originally present in the basophilic erythroblasts. This developmental process is, however, accompanied by an approximately two-fold reduction in cell volume during each successive cell division, as well as by a similar decrease in cell size resulting from nuclear condensation and extrusion. As a result, the final reticulocyte has only approx. 1/8 of the cell volume of that of the basophilic erythroblast and on the basis of changes in cell size alone one would expect an eight-fold reduction in enzyme activity per cell. Similarly, if due allowance is made for the relevant decreases in cell volume the observed enzyme activity in the polychromatic erythroblast would correspond to an approx. 50% higher enzyme activity than that in the basophilic cell, whereas the orthochromatic erythroblast appears to contain a 40% lower RNase activity than that of the basophilic cells. The subsequent changes in enzyme activity as condensation and loss of nucleus give rise to the formation of reticulocytes appear to be relatively small. Thus, the major biochemical event occurs as the polychromatic erythroblast enters the non-dividing compartment and consists of an approx. 2.5-fold decrease in the activity of ribonuclease as calculated from the enzyme activities per cell.

It is noteworthy that during cell development there is a somewhat greater change in the activity of acid (pH 5.4) ribonuclease per cell than in that of the alkaline (pH 7.5) enzyme, the former declining by a factor of 33 whereas the latter decreases only 20-fold. These results suggested that the RNA-degrading enzyme system in erythroid cells might be more complex than has been thought hitherto. An investigation of the ribonucleases present in different erythroid cells by polyacrylamide gel electrophoresis revealed the presence of multiple enzymes (fig.2). As can be seen from the results, there is only one qualitative difference in the enzyme patterns of the dividing and non-dividing cells, namely the absence in the non-dividing compartment of the RNase band that travels towards the cathode.

4. Discussion

Separation of rabbit bone marrow erythroid cells by velocity sedimentation at unit gravity has been found to be a useful technique for investigating biochemical events associated with erythroid cell development and maturation. Thus, changes in a number of enzyme activities, some increasing and



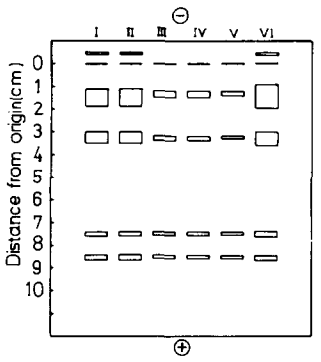


Fig.2. RNase electrophoresis pattern in different stages of erythroid cell development. The cell lysate (800 *g* supernatant) was subjected to electrophoresis in 7.5% polyacrylamide gel and the RNase activity was detected as shown under Methods. a) Photograph and b) Diagram of enzyme pattern of erythroid cells: I Basophilic cells, II Polychromatic cells, III Orthochromatic cells, IV Bone marrow reticulocytes, V Blood reticulocytes, VI Total bone marrow cell lysate.

others decreasing, have been reported recently and the most marked re-arrangement of enzyme pattern was found to coincide with cessation of DNA synthesis and cell division [10]. The results presented here show that RNase activity likewise decreases markedly at this time when other major biochemical and morphological events, including enhanced haemoglobin synthesis and condensation of the nucleus, occur within the cell. The fact that haemoglobin synthesis is still active in orthochromatic erythroblasts which no longer synthesize RNA suggests the existence of a mechanism for protecting haemoglobin-synthesizing polysomes against degradation by nucleases. In rat reticulocytes, a complex between the alkaline RNase and an inhibitor has been described and it has been suggested that this complex may play a role in preventing degradation of polysomes [11]. Burka [4] has shown that most of the RNase activity in rabbit reticulocytes is found in a latent form associated with the cell membrane. The somewhat faster decrease of acid RNase activity in comparison with the alkaline enzyme which we have found in the present work may also be a factor contributing to the relative stability of haemoglobin synthesis.

Studies in this laboratory have indicated that during erythroid cell development in the bone marrow changes also take place in the subcellular distribution of RNases (S. Hulea, unpublished observations). On the other hand, there is still uncertainty about the exact number and mode of action of the nucleases involved in RNA breakdown in the erythroid cells. Some of the experiments presented here have been designed primarily to focus attention on the heterogeneity of the RNA-degrading enzyme system in these cells and the complex pattern of RNases, as revealed by polyacrylamide gel electrophoresis, suggests that different nucleases may have specific functions in the degradation of different classes of RNA during erythroid cell maturation.

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