Studies of erythroblast function in congenital dyserythropoietic anaemia, type I: evidence of impaired DNA, RNA, and protein synthesis and unbalanced globin chain synthesis in ultrastructurally abnormal cells

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SUMMARY Two patients with congenital dyserythropoietic anaemia, type I (CDA) were studied. Their blood reticulocytes showed unbalanced globin chain synthesis with increased $\alpha:\beta$ globin chain synthesis ratios. A high proportion of the erythroblasts displayed the characteristic "Swiss cheese" abnormality of the nuclear chromatin and some also showed cytoplasmic intrusions lined with nuclear membrane within the nucleus. Occasional erythroblast profiles contained intracytoplasmic inclusions that were ultrastructurally indistinguishable from precipitated α chains. The technique of combined Feulgen microspectrophotometry and ^3H -thymidine autoradiography showed gross abnormalities of proliferation in the early polychromatic erythroblasts. The proliferative abnormalities included an arrest of DNA synthesis after the progress of cells through part of the S phase and the formation of several mononucleate and binucleate cells with hypertetraploid total DNA contents. The bone marrow cells gave a normal deoxyuridine suppressed value, indicating that there was no impairment of the methylation of deoxyuridylylate. Electron microscope autoradiographic studies showed that a high proportion of the erythroblasts with the "Swiss cheese" nuclear abnormality suffered from a severe impairment, or arrest of DNA, RNA, and protein synthesis.

Congenital dyserythropoietic anaemia, type I (CDA type I) is a rare disorder of unknown aetiology characterised by a congenital macrocytic anaemia, megaloblastoid erythroid hyperplasia, the occurrence of intererythroblastic nuclear chromatin bridges, the development of secondary haemochromatosis in later life, and, probably, an autosomal recessive inheritance.^{1,2} Multinucleate erythroblasts, although present in increased numbers, are not a prominent feature of the marrow and the acidified serum lysis test is negative. Ultrastructural studies have shown the presence of nuclei with a "Swiss cheese" appearance in a high proportion of the mononucleate early and late polychromatic erythroblasts; these nuclei have multiple rounded electron lucent areas within abnormally electron dense heterochromatin. In this paper we report the results of studies into erythro-

blast function in two new patients with CDA type I. The functions investigated included the efficiency of the methylation of deoxyuridylylate to thymidylylate in bone marrow cells; proliferative behaviour as indicated by the cell cycle distribution of the erythroblasts; and the capacity of the ultrastructurally abnormal erythroblasts to synthesise macromolecules.

Material and methods

CASES STUDIED

Table 1 summarises the essential clinical and laboratory data. In both patients the haematological findings were unexpected. Case 1 was first found to be anaemic at the age of 32 years when he had infectious mononucleosis. In case 2 persistent mild jaundice and splenomegaly led to a diagnosis of unexplained "haemolytic anaemia" at the age of 24 years (haemoglobin

Table 1 Essential clinical and laboratory data in both male patients studied

	Case 1 (34 years)	Case 2 (59 years)		Normal values
		(a)	(b)	
Splenomegaly	4 cm		2 cm	
Hepatomegaly	4 cm		4 cm	
Haemoglobin (g/dl)	10.1		11.1	13.4-17.0
Red bloods cells ($\times 10^{12}/l$)	2.91		3.41	4.4-5.8
Mean cell volume (fl)	101.9		97.8	80-92
Retic ($\times 10^9/l$)	92		75	18-158
White cells ($\times 10^9/l$)	3.5		5.3	4-11
Platelets ($\times 10^9/l$)	189		178	140-400
Red cell folate ($\mu g/l$)	340		2353	145-450
HbA ₂ (%)	3.1		2.9	1.5-3.4
HbF (%)	3.7		1.6	<2.0
$\alpha:\beta$ globin chain synthesis ratio (blood retics; one hour incubation)				
Radioactivity ratio	1.8		1.5	0.9-1.2
Specific activity ratio	1.6		1.3	0.9-1.2
Marrow erythroid:myeloid ratio	4.1	3.2	3.0	0.12-0.5
Serum iron ($\mu mol/l$)	42	48	14	12-27
Serum total iron binding capacity ($\mu mol/l$)	48	49	69	40-75
Plasma iron turnover ($\mu mol/l$ whole blood/24 hours)	1103	996		104-152
Red cell ^{59}Fe utilisation (%) at 14d		19		74-86
^{51}Cr -labelled red cell survival— $T_{1/2}$ ^{51}Cr (d)	18	21		25-33
Mean cell life-span (d)	52	50		110
Serum bilirubin ($\mu mol/l$)	28	99	75	1-15
Serum ferritin ($\mu g/l$)	3869	3695	212	17-230
Liver iron (% of dry weight)	3.0	2.6		<0.1%

(a) Values obtained at age 56 years before treatment for iron overload; (b) values obtained at the time of this study.

Conversion: SI to traditional units: serum iron, total iron binding capacity and plasma iron turnover $1 \mu mol/l \approx 5.59 \mu g/100 ml$; serum bilirubin $1 \mu mol/l \approx 0.058 mg/100 ml$.

11 g/dl, mean cell volume 106 fl). Severe iron overload developed in both patients (Table 1), providing evidence for lifelong erythroid hyperplasia associated with excessive iron absorption: neither had ever received any blood transfusions. On reinvestigation at the age of 56 years, case 2 had hepatic cirrhosis and his iron overload (about 20 g) was subsequently completely removed with a combination of regular phlebotomy and desferrioxamine infusions before the present studies were conducted. Neither patient had any family history of anaemia or jaundice.

In both cases the light microscopic appearances of the blood and bone marrow were typical of congenital dyserythropoietic anaemia, type I.¹⁻⁴ The peripheral blood film showed severe poikilocytosis and anisocytosis with obvious macrocytes, as well as some poorly haemoglobinised cells, microcytes, and red cells with basophilic stippling. The marrow smears showed severe erythroid hyperplasia. Polychromatic erythroblasts, many showing a megaloblastic chromatin pattern, dominated the marrow (56% and 53% of nucleated cells in cases 1 and 2, respectively). Internuclear fine chromatin bridges were seen in 1.0% (case 1) and 0.3% (case 2) of polychromatic and orthochromatic erythroblasts. In addition, 4.2% (case 1) and 5.8% (case 2) of these cells were binucleate; the two nuclei were often of different size and chromatin structure and were sometimes incompletely separated. Marrow smears stained for

iron showed moderately increased numbers of erythroblasts with abnormally coarse iron containing granules (abnormal sideroblasts) but no ringed sideroblasts: in case 2 an earlier marrow had shown a small proportion of ringed sideroblasts before treatment for iron overload. The presence of a gross degree of expansion of the erythroid marrow was indicated not only by the erythroid:myeloid ratios but also by the increased plasma iron turnover (Table 1).⁵ That this was related to ineffective erythropoiesis rather than haemolysis was confirmed by the normal absolute reticulocyte counts and only moderately reduced autologous red cell survival,⁶ as well as the severe reduction in the use of ^{59}Fe by red cells (Table 1).⁶

GLOBIN CHAIN SYNTHESIS

Globin chain synthesis was measured by incubating 1.0 ml of washed reticulocyte rich red cells with 100 μCi of [3H] leucine for 60 minutes at 37°C.⁷ After incubation the cells were washed, lysed, and converted to globin by acid-acetone precipitation. The globin chains were separated by chromatography on CM cellulose in 8M urea/0.05M mercaptoethanol,⁸ and the radioactivity and optical density of the fractions containing each chain were measured.

SPECIAL STUDIES ON MARROW CELLS

Freshly aspirated marrow was mixed with 2 ml

Hanks's solution containing 50 units of preservative free heparin. An aliquot of the mixture was used to prepare a red cell deficient single cell suspension of marrow cells by forcing it through 21 and 25 gauge needles, as previously described.⁹

DEOXYURIDINE SUPPRESSION TEST

This test was performed on the single cell suspension, using the method of Wickramasinghe.¹⁰

CELL CYCLE DISTRIBUTION

One ml of the single cell suspension was incubated with 20 μ Ci [methyl-³H] thymidine (specific activity 50 Ci/mmol; Amersham International Ltd) for 30 minutes and the labelled cells smeared on glass slides. The radioactive smears were used to determine the distribution of various classes of erythroblasts and neutrophil precursors in the three stages of interphase, G₁, S, and G₂ by a combination of Feulgen microspectrophotometry and ³H-thymidine autoradiography.^{9,11}

ELECTRON MICROSCOPY

Marrow fragments were removed from the aliquot of undispersed heparinised marrow, fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) for one and a quarter hours at room temperature, and processed for transmission electron microscopy as described previously.¹²

ELECTRON MICROSCOPE AUTORADIOGRAPHY

Aliquots of the single cell suspension were mixed with 25% (v/v) group AB serum and separately incubated with 40 μ Ci [methyl-³H] thymidine (specific activity 50 Ci/mmol), 50 μ Ci [5-³H] uridine (specific activity 29 Ci/mmol), and 50 μ Ci L-[4,5-³H] leucine (specific activity 56 Ci/mmol) per ml for one hour; the radio-labelled chemicals were obtained from Amersham International Ltd. The radioactive cells were used for the preparation of electron microscope autoradiographs, as described previously,⁹ except that the cells were stained en bloc with 2% aqueous uranyl acetate. One set of autoradiographs was exposed for one and another for six weeks.

Results

GLOBIN CHAIN SYNTHESIS

Alpha:beta globin chain synthesis ratios were increased in both patients. Table 1 gives the radio-activity and specific activity ratios.

DEOXYURIDINE (dU) SUPPRESSION TEST

The dU suppressed value given by the bone marrow cells was 3.4% in case 1 and 4.8% in case 2 (normal range 1.4–8.6%).¹⁰

CELL CYCLE DISTRIBUTION

Table 2 gives the cell cycle distribution of the basophilic erythropoietic cells (proerythroblasts and basophilic erythroblasts) in both cases studied. There was an increased percentage of cells in the S phase with a corresponding decrease in the percentage in the G₁ phase. There was also a slight increase in the proportion in the G₂ phase and some reduction in the ratio of the number of cells in S to that in G₂ (S:G₂ ratio).

Figs. 1 and 2 show the distribution of the total Feulgen absorbance values (relative DNA contents) given by individual mononucleate and binucleate polychromatic or orthochromatic erythroblasts of cases 1 and 2, respectively: most of the mononucleate cells had DNA contents around and between the 2 and 4c values and most of the remainder had DNA contents between the 4 and 8c values. Most of the binucleate cells had total DNA contents between the 4 and 8c values. The percentages of DNA synthesising mononucleate polychromatic and orthochromatic erythroblasts in cases 1 and 2 were 28 and 24, respectively.

In both cases studied the distribution of promyelocytes plus myelocytes in the different stages of interphase was essentially normal, the average values for the percentages of cells in G₁, S, G₂, and U (Table 2) being 58.9, 34.7, 5.7 and 1.0 (normal ranges 59–70, 20–34, 3–5, and 0–1.2, respectively).

ELECTRON MICROSCOPY

No ultrastructural abnormalities were found in the basophilic erythropoietic cells of either of the cases

Table 2 Distribution of basophilic erythropoietic cells in different stages of interphase

Case No	Percentages				S/G ₂	Total No of cells studied
	G ₁	S	G ₂	U*		
Case 1	11.7	76.7	11.7	0	6.57	120
Case 2	14.2	74.3	11.5	0	6.46	113
Normal range†	19–36	58–72	3–9	0–0.9	8.2–25.5	

*Cells that were not synthesising DNA, as judged by their failure to incorporate ³H-thymidine but which had DNA contents between the G₁ and G₂ values.

†Includes both normal and haematologically normal adults.¹¹

Fig. 1 Distribution of relative DNA contents of mononucleate (upper histogram) and binucleate (lower histogram) polychromatic and orthochromatic erythroblasts of case 1. Open bars represent cells that were unlabelled with ^3H -TdR and stippled bars cells that were labelled with ^3H -TdR. Hatched and solid bars show pairs of erythroblasts joined by internuclear chromatin bridges in which nuclei were unlabelled and labelled with ^3H -TdR, respectively. In the case of binucleate erythroblasts and cells with internuclear chromatin bridges DNA contents shown are total DNA contents of both nuclei.

Fig. 2 Distribution of relative DNA contents of mononucleate (upper histogram) and binucleate (lower histogram) polychromatic and orthochromatic erythroblasts of case 2. Open bars represent cells that were unlabelled with ^3H -TdR and stippled bars cells that were labelled with ^3H -TdR. Hatched and solid bars show pairs of erythroblasts joined by internuclear chromatin bridges in which nuclei were unlabelled and labelled with ^3H -TdR, respectively. In the case of binucleate erythroblasts and cells with internuclear chromatin bridges DNA contents shown are total DNA contents of both nuclei.

Fig. 3 Part of a late polychromatic erythroblast with spongy heterochromatin showing multiple intracytoplasmic inclusions resembling precipitated α globin chains. Case 1. $\times 46\,500$.

Fig. 4 Electron microscopic autoradiograph of marrow cells that had been incubated with ^3H -thymidine for one hour. Two erythroblasts in centre with normal looking nuclei are heavily labelled. Cells on either side show multiple electron lucent areas within heterochromatin and are either unlabelled (left) or associated with very few autoradiographic grains (right). Autoradiograph was exposed for 3d. Case 1. $\times 6575$.

Fig. 5 (a) and (b) High resolution autoradiographs of two ^3H -thymidine-labelled early polychromatic erythroblasts from case 2. Both cells show multiple electron lucent areas within heterochromatin. They also show some increase in electron density of heterochromatin. More heavily labelled cell in (a) shows lesser degree of abnormality than more weakly labelled cell in (b). Autoradiograph was exposed for 3d. $\times 9850$.

studied. Qualitatively similar abnormalities were found in the polychromatic erythroblasts of both cases. In cases 1 and 2 62% and 79%, respectively, of the profiles of the mononucleate early and late polychromatic erythroblasts contained abnormal nuclei. These were characterised by the presence of multiple electron lucent areas within abnormally electron dense heterochromatin that gave the heterochromatin a spongy or moth eaten appearance (Swiss cheese like nuclei). Some of the affected nuclei also contained nuclear membrane lined masses of cytoplasm that had invaginated through small or large defects in the nuclear membrane associated heterochromatin. In some cells these cytoplasmic intrusions filled the areas between adjacent masses of heterochromatin so that the invaginating nuclear membrane was closely apposed to the surfaces of the heterochromatin. In occasional cells cytoplasmic organelles were seen within nuclear territory. Cell profiles containing abnormal nuclei sometimes displayed various cytoplasmic abnormalities such as autophagic vacuoles, myelin figures, and iron laden mitochondria. Such profiles occasionally contained many small, rounded, or irregular masses of amorphous electron dense material in the cytoplasmic matrix (Fig. 3), or around the centrioles. These masses tended to become confluent and were indistinguishable from the α chain precipitates found in the β thalassaemia syndromes.^{13 14}

Some large erythroblast profiles contained two nuclear masses, both of which showed the Swiss cheese appearance. In such profiles the two nuclei were either partially fused together over a wide area or linked together by a bridge of chromatin; occasionally, one of the nuclear masses was ultrastructurally considerably more abnormal than the other.

Erythroblasts with a spongy appearance of the heterochromatin were recognisable at various stages of degradation within the cytoplasm of some of the bone marrow macrophages.

ELECTRON MICROSCOPE AUTORADIOGRAPHY

After exposure of the autoradiographs for three to seven days the percentages of polychromatic erythroblasts with ultrastructurally normal nuclei which incorporated ^3H -thymidine in cases 1 and 2 were 55.6 and 65.6, respectively. In case 1, however, only 12% of the mononucleate early and late polychromatic erythroblasts with the Swiss cheese nuclear abnormality were either clearly labelled, or, more usually, weakly labelled with ^3H -thymidine, as were a similar proportion of the binucleate cells showing this ultrastructural abnormality; the remainder were unlabelled (Fig. 4). In case 2 only 5.4% of these cells were labelled (Fig. 5). In both cases the majority of the mononucleate and binucleate erythroblasts displaying the Swiss cheese abnormality, including many

Fig. 6 Electron microscopic autoradiograph of marrow cells that had been incubated with ^3H -uridine for one hour. One erythroblast has a normal looking nucleus and is heavily labelled. All three remaining cells show "Swiss cheese" nuclear defect; two of these are unlabelled. Autoradiograph was exposed for six weeks. Case 1. $\times 6575$.

early polychromatic erythroblasts, remained totally unlabelled after exposure of the autoradiographs for as long as six weeks.

An average of 96.4% of mononucleate early and late polychromatic erythroblasts with ultrastructurally normal nuclei incorporated ^3H -uridine. By contrast, 25% of the mononucleate erythroblasts with Swiss cheese like nuclei in case 1 and 6.7% of such cells in case 2 were labelled moderately well with ^3H -uridine. There was either a total absence of labelling with ^3H -uridine or very weak labelling (six autoradiographic grains per cell or less) in most mononucleate and binucleate cells with this nuclear abnormality in the autoradiographs exposed for both one and six weeks (Fig. 6).

In those exposed for one week 94.1% of polychromatic erythroblasts with ultrastructurally normal nuclei were labelled with ^3H -leucine. In these autoradiographs substantial labelling was observed in only 25% of the mononucleate erythroblasts with Swiss cheese like nuclei in case 1 and 8.9% in case 2; the remaining cells were unlabelled or very weakly labelled (Fig. 7). Many completely unlabelled erythroblasts with this ultrastructural abnormality persisted in the autoradiographs exposed for six weeks; in such autoradiographs the ultrastructurally normal erythropoietic cells, including reticulocytes, were extremely heavily labelled.

Those cells with the Swiss cheese abnormality, which showed a moderate degree of labelling with ^3H -thymidine, ^3H -uridine, or ^3H -leucine, tended to display a lesser degree of nuclear abnormality than those that were completely unlabelled or very weakly labelled (Fig. 4). There seemed to be no abnormality in the distribution of autoradiographic grains over such labelled erythroblasts. Autoradiographic grains caused by the incorporation of ^3H -thymidine into DNA were more or less confined to the area over the nucleus and located mainly at or near the junction between heterochromatin and euchromatin. Those caused by the incorporation of ^3H -uridine into RNA were located largely over the nucleus, mainly at or near the junction between heterochromatin and euchromatin, but also over the cytoplasm. Autoradiographic grains caused by the incorporation of ^3H -leucine into protein were situated both over the cytoplasm and over the nucleus: most of those located over the nucleus were located close to the junction between heterochromatin and euchromatin.

Discussion

Light and electron microscopic features characteristic of congenital dyserythropoietic anaemia, type I were seen in the erythroblasts of both cases studied. These features included the presence of occasional pairs of

Fig. 7 High resolution autoradiograph of marrow cells that had been incubated with ^3H -leucine for one hour. Three erythroblasts are labelled; one shows well developed "Swiss cheese" abnormality of heterochromatin and another a mild degree of this abnormality. There are two unlabelled erythroblasts, both of which show a severe degree of abnormality. Autoradiograph was exposed for 7d. Case 1. $\times 6575$.

erythroblasts that were joined together by internuclear chromatin bridges; Swiss cheese like nuclei in over 60% of the profiles of mononucleate early and late polychromatic and orthochromatic erythroblasts^{3,4,15,16}; and large cytoplasmic intrusions lined by nuclear membrane in some of the abnormal nuclei.^{17,18} Like the cases described by Dell'orbo *et al*¹⁷ and Hiraoka *et al*,¹⁸ the erythroblasts of our two cases did not show widening of nuclear pores or loss of parts of the nuclear membrane.

In a recent study of two patients with CDA type I the blood reticulocytes of both patients displayed unbalanced globin chain synthesis with an increase in the α :non- α chain radioactivity ratio.¹⁹ Our finding that the blood reticulocytes of both cases showed a similar abnormality (Table 1) is therefore of interest. Furthermore, as we found an occasional erythroblast profile which contained intracytoplasmic inclusions that were morphologically indistinguishable from precipitated α chains^{13,14} in both cases it seems that at least some of the erythroblasts also suffered from unbalanced globin chain synthesis. An increase in the blood reticulocyte α : β chain synthesis ratio has been reported not only in the cases of CDA type I men-

tioned above but also in some cases of congenital dyserythropoietic anaemia that could not be allocated to one of the three classical types, and in some patients whose erythroblasts showed the morphological features of CDA type II but whose red cells did not express the HEMPAS antigen.²⁰⁻²²

Previous studies of the cell cycle distribution of the erythroblasts in two cases of CDA type I have shown that DNA synthesis was virtually confined to the basophilic erythropoietic cells.²³ By contrast, in the two cases reported here a substantial proportion of the polychromatic and orthochromatic erythroblasts incorporated ^3H -TdR. Many of the non-DNA synthesising mononucleate polychromatic and orthochromatic erythroblasts of our cases, however, had DNA contents corresponding to and in between the G_1 and G_2 values (Figs. 1 and 2), suggesting that a high proportion of the mononucleate polychromatic and orthochromatic erythroblasts become arrested during their progress through the S phase of the cell cycle. Our cell cycle data also showed that some mononucleate erythroblasts, as well as the binucleate erythroblasts and the cells linked by internuclear chromatin bridges, had total DNA contents greater

than 4c and usually between 4 and 8c. The normal results with the dU suppression test indicate that neither the arrest of DNA synthesis nor the mild megaloblastic changes in the erythroblasts could be attributed to any abnormality of the methylation of deoxyuridylate.

Our electron microscopic autoradiographic data show that a high proportion of the erythroblasts with the Swiss cheese nuclear abnormality fail to synthesise DNA, RNA, or protein. As some of the erythroblasts with this nuclear abnormality, including some with a mild degree of abnormality, were capable of synthesising these macromolecules, it seems that the Swiss cheese defect begins to develop before the failure of synthesis of macromolecules.

The primary biochemical defect affecting the erythroblasts of patients with CDA type I remains unknown. On the basis of the characteristic Swiss cheese appearance of many of the erythroblast nuclei it has been suggested that the primary lesion may be an abnormally high degree of condensation of nuclear chromatin.¹⁵ This possibility is corroborated by quantitative cytochemical studies of individual erythroblasts from a patient with CDA type I that have shown an increase in the mean value for the fast green:Feulgen absorbance ratio—that is, histone:DNA ratio—due to an increase in the average fast green value, thus suggesting that there may be either an increase in the histone content per nucleus or a change of the staining quality of the nuclear histones.²⁴ A change in the nuclear histones could possibly lead to an abnormal degree of chromatin condensation. Our own finding that the failure of macromolecular synthesis follows rather than precedes the development of the Swiss cheese nuclear defect also supports the view that the primary biochemical lesion may be in the nucleoproteins of the cell. We consider that the primary lesion is likely to be either the production of a changed nucleoprotein molecule (perhaps due to a mutation affecting a single amino acid), or the production of reduced quantities of a specific nucleoprotein. The abnormality in nucleoprotein synthesis may lead to various secondary effects, including the impairment of erythroblast proliferation; the failure of DNA, RNA, and protein synthesis; and unbalanced globin chain synthesis with precipitation of excess α chains. Although it has been suggested that the primary defect may lie in the nuclear membrane,¹⁸ it is equally possible that the ultrastructural abnormalities affecting this membrane also arise as a consequence of a nucleoprotein abnormality, which may lead to a change in the normal interaction between nucleoproteins and the nuclear membrane.

In both cases studied erythroblasts containing Swiss cheese like nuclei were found within the cyto-

plasm of macrophages, providing direct morphological evidence of increased ineffective erythropoiesis. Presumably, some secondary abnormality of the biochemical composition of the cell membrane of the ultrastructurally abnormal erythroblasts resulted in their interaction with and phagocytosis by macrophages. As dimethylsulphoxide induced Friend leukaemia erythroblasts treated with puromycin or cycloheximide have been shown to interact with mouse peritoneal macrophages to a greater extent than untreated erythroblasts,²⁵ the arrest of protein synthesis in the erythroblasts of our patients may have been indirectly responsible for the ineffectiveness of erythropoiesis, perhaps by causing changes in the structure or configuration of the cell membrane.

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