Cytochemical and Radioautographic Identification of Cells Induced to Synthesize Hemoglobin

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In order to facilitate the identification of bone marrow cells in which hemoglobin synthesis is initiated, erythropoiesis was first suppressed in guinea pigs through the induction of posthypoxic polycythemia, and then it was restimulated by bleeding and reexposure to hypoxia. Hemoglobin synthesis was detected with $^{59}$Fe incorporation on radioautographs, and its presence was demonstrated in the light microscope with the benzidine reaction and absorption of monochromatic light at $\lambda 4046$ Å. In the electron microscope, hemoglobin was detected in the cytoplasm by a general increase in electron density after treating the tissue with diaminobenzidine (DAB) and OsO$_4$. Density measurements were carried out on electronmicroscopic negatives, using reticular cell cytoplasm as a base line. In normal marrow, proerythroblasts were the earliest cells in which hemoglobin could be detected, but during the early phase of erythropoietic stimulation, hemoglobin was demonstrated in transitional cells with all the methods employed. Without the specific demonstration of hemoglobin, these cells could not be recognized morphologically as erythroblasts nor could they be distinguished from the precursors of bone marrow small lymphocytes. Transitional cells were numerous in the marrow at the time of stimulation, and 40 hr later a small number of them were labeled with $^{59}$Fe and synthesized hemoglobin in detectable amounts. Proerythroblasts were absent at the time of the stimulus, and when they reappeared the majority were benzidine or DAB positive and had incorporated $^{59}$Fe. The findings suggest that progenitor cells of erythroblasts are among the basophilic members of the transitional cell population, and erythropoietic stimulation induces hemoglobin synthesis in them. The relationship of these cells to the progenitors of other hemopoietic cells, as well as to the pluripotent stem cell, is discussed.

It is well established that erythropoietic activity can be partially or completely suppressed in a number of species, and, subsequently, it can be reestablished by appropriate stimuli. The aim of the experiments reported in this paper was to identify, in morphologic terms, those cells in which erythroid differentiation was initiated by an erythropoietic stimulus.

The following methods were employed for the demonstration of hemoglobin or its synthesis: In the light microscope, the benzidine reaction and light absorption at $\lambda 4046$ Å; in the electron microscope, the diaminobenzidine (DAB) reaction; and in both light and electron microscope, radioautography with $^{59}$Fe.

In order to facilitate the identification of cells in which hemoglobin synthesis had been initiated, an erythropoietic stimulus was applied to animals whose
erythropoiesis had previously been suppressed; thus, the demand for circulating erythrocytes created by the stimulus had to be met predominantly by an increased rate of differentiation of erythroblasts from the precursor-cell pool.

**MATERIALS AND METHODS**

**Suppression and Restimulation of Erythropoiesis**

Male albino guinea pigs (Hartley strain, body weight 300-400 g) were exposed to hypoxia at 1 atmosphere pressure in a decompression chamber for 2 wk in order to induce polycythemia. The polycythemia persisted after return to ambient air, and erythroblasts had practically disappeared from the bone marrow by day 7. On this day the animals were bled by cardiac puncture. The amount of blood to be withdrawn was calculated (from each animal's hematocrit and blood volume) to result in a PCV of approximately 40% after bleeding and reestablishing the total blood volume with 6% dextrose saline injected into the peritoneal cavity. The animals were then returned to the decompression chamber and were maintained again at 1 atmosphere pressure until the time of sacrifice. Under similar experimental conditions, the increase in erythroblasts was observed to commence around 48 hr. Guinea pigs were sacrificed 36-64 hr following restimulation, and their bone marrow was processed as described below. Polycythemic animals that were not bled or stimulated, as well as normal guinea pigs, served as controls.

**Sampling of Bone Marrow**

For electron microscopy, small pieces of bone marrow were scooped out of the proximal part of the tibial medullary cavity of animals under ether anesthesia and were fixed and processed as described below. For light microscopy, smears were prepared of bone marrow cells obtained from the opposite tibia. The cells were suspended in homologous serum, air dried, and fixed in methanol.

**Labeling with 55Fe**

$^{55}$Fe [ferrous citrate; specific activity 16.9-17.7 mCi/mg (New England Nuclear); dose 0.5 or 1 μCi/g body weight] was injected into the peritoneal cavity at 4 hr and again 2 hr before sampling the marrow. Fixed bone marrow smears for light microscopy were dipped once in Kodak NTB2 emulsion and developed in Dektol after 10 days of exposure. The radioautographs were stained with MacNeal's tetrachrome stain. For the electron microscopic study of bone marrow, the same procedures of fixation, radioautography, and recording were followed as we described in detail elsewhere.

**The Benzidine Reaction**

Like peroxidases, hemoglobin oxidizes benzidine, resulting in a compound which gives a golden yellow color in the light microscope and, after treatment with OsO₄, appears as an amorphous electron-dense material in the electron microscope.

For light microscopy, the benzidine reaction was carried out by applying a modified LePehne's reagent to methanol-fixed bone marrow smears for 5 min. Two components of the reagent (A and B) were mixed immediately before use in proportions 2A : 3B. Component A consisted of 0.6% benzidine in 95% ethanol and component B of 3 parts of 30% H₂O₂ and 7 parts of 70% ethanol. The reagent was washed off in running tap water, and the slides were rinsed with phosphate buffer, pH 6.8, before applying the tetrachrome stain.

Cells with doubtful or weak reaction were also examined and photographed in monochromatic light at a wavelength of 4046 Å, isolated from the mercury arc of a Zeiss Ultraphot II with an interference filter.

For the detection of hemoglobin-containing cells in the electron microscope, the blocks of tissue were reacted with diaminobenzidine (DAB; Aldrich Chemical Co., Inc., Milwaukee, Wis. 53233) followed by OsO₄ treatment. The procedure is presented in detail elsewhere. The sections were examined without staining.

The benzidine reaction was also carried out on blocks and on smears in combination with $^{55}$Fe radioautography. In order to clear the gelatin, radioautographs of sections were treated with 0.02 N NaOH following development and fixation.
The rationale and criteria for detecting a positive reaction with DAB in electron micrographs are presented in a separate report. In the present study, the same criteria were adopted and equipment, and optical and photographic procedures were standardized in the same way. Minimal increases in general cytoplasmic electron density, due to the reaction of hemoglobin with DAB, were determined on electron microscopic negatives with the aid of a densitometer (Weston Photographic Analyser). In each negative the average cytoplasmic density of a cell was compared with that of a reticular cell present in the same field. The latter was chosen as a baseline reference and is represented along the abscissa in Fig. 1. In the same figure, A represents the range of cytoplasmic light density of all recognizable, differentiated, nonerythroid cells (e.g., neutrophils), and B represents the same range for cells possessing morphological features compatible with those attributed to early erythroblasts. In differentiated, nonerythroid cells of reacted tissue, the maximum density decrement from the baseline was ~0.02 densitometric units. Only those cells were regarded as positive to the reaction which, on the negatives, showed at least twice as great a density decrement from the base line. Thus, the cutoff value for a positive reaction corresponded to ~0.04 densitometric units, and is indicated in Fig. 1 by an interrupted line. This procedure permitted the identification of reacting cells at a high level of confidence, but it may have placed some cells into the negative range which had, in fact, reacted minimally.

In photographic positives, the density differences shown in Fig. 1 are, of course, represented by an increase, rather than a decrease, in cytoplasmic density in comparison with reticular cells. The prints of Figs. 9-14 were made using a standardized procedure to ensure that differences in contrast of the figures represent, as well as possible, the differences in contrast between the negatives.

Fig. 1. The ranges of cytoplasmic light density are shown for various cells as measured on electron microscopic negatives (× 3000-6000) of sections of diaminobenidine- (DAB) reacted and unreacted bone marrow. Reticular cell cytoplasm, chosen in each negative as base-line reference, is shown along the axis of the abscissae and light density, in units measured on the Weston Photographic Analyser, is shown along the axis of the ordinates. The A represents the range of cytoplasmic light density of recognizable, differentiated, nonerythroid cells (e.g., neutrophils); B represents that of cells possessing morphologic features compatible with those attributed to early erythroblasts. The minimum light density decrement from reticular cell cytoplasm, chosen on the negatives as the denominator of a positive reaction, is indicated by an interrupted line. The C represents the range of cytoplasmic density of "positive" early erythroblasts, and D represents that of more mature erythroid cells. The DA is a reticulocyte or the cytoplasm of a late erythroblast in unreacted tissue on this density scale. The dots designate the general cytoplasmic density of cells illustrated in subsequent figures bearing the corresponding numbers (i.e., in Figs. 9-14).
Figs. 2–8. Bone marrow cells on smears obtained from guinea pigs 40–64 hr after restimulation of erythropoiesis. (× 1800.) See individual legends on facing page.
RESULTS

Cells With Minimum Reaction to Benzidine or DAB

In the light microscope, the characteristic golden color of benzidine, after its oxidation by hemoglobin, is evident only in those cells which contain relatively large quantities of the protein. All cells showing a general cytoplasmic reaction with benzidine extinguished monochromatic light at the absorption maximum of hemoglobin (4046 Å). Small amounts of hemoglobin, present in early erythroid cells, do not produce a clearly discernible golden color. Nevertheless, the reaction changes the staining characteristics of these cells. The pure purple and blue tones seen in negative or nonreacted cells are altered to more muddy colors (Fig. 2). We confirmed the presence of hemoglobin in cells which exhibited such color changes by examining them in monochromatic light at λ 4046 Å (Fig. 3).

In normal marrow, all cells with minimum amounts of detectable hemoglobin conformed morphologically to classic descriptions of proerythroblasts and basophilic erythroblasts. Also, in erythropoietically restimulated animals, the majority of minimally reacting cells were of this type, while such cells could not be found in the marrow of polycythemic controls.

In the electron microscope, DAB-reacted cells, which showed minimal but significant increases in cytoplasmic density, corresponded morphologically to the cells identified in the light microscope as proerythroblasts and basophilic erythroblasts (Fig. 9).

In the marrow of erythropoietically stimulated guinea pigs, a second type of...
Fig. 9. Radioautograph of an erythroblast with minimum detectable hemoglobin, obtained from a guinea pig 54 hr after restimulation. The animal received $^{55}$Fe (1 μCi/g body weight) at 4 hr and again at 2 hr prior to sampling. The tissue was reacted with DAB, but the section is otherwise unstained. The position of the cell on the cytoplasmic density scale is shown in Fig. 1. The cell possesses a moderate amount of cytoplasm, in which the reaction with DAB gives a finely mottled appearance that is distributed uniformly but for the area containing the Golgi complex. The perinuclear cisterna, the cisternae of the Golgi, and mitochondria are negative to the reaction. Mitochondria appear swollen, and silver grains are associated with a number of them. Two large and one small nucleolar complexes are evident. Heterochromatin is associated with the nuclear membrane and nucleolar complexes, but hardly any of it is evident independent of these structures. Fine cytoplasmic processes of a reticular cell almost completely surround the erythroblast. Exposed for 4 mo, × 13,500; marker, 1 μ.

cell was found to contain minimum but demonstrable quantities of hemoglobin. These cells were morphologically quite distinct from proerythroblasts. They are illustrated in Figs. 4 and 6. Such cells exhibited a degree of benzidine reactivity similar to that of proerythroblasts (cf. Fig. 2), and they also extinguished monochromatic light at the absorption maximum of hemoglobin (Figs. 5 and 7).

The same type of cell was also identified with the electron microscope in the marrow of stimulated animals (Figs. 10 and 11). In DAB-reacted tissue such cells exhibited a significant increase in cytoplasmic density compared to the reticular cell. Although these cells contained similar quantities of hemoglobin as proerythroblasts and basophilic erythroblasts seen in the light microscope, or as “erythroblasts with minimum demonstrable hemoglobin” seen in the electron microscope, without light absorption at λ 4046 Å or without the benzidine and DAB reactions, they could not have been identified as belonging to the erythroid series. Morphologically they were identical with large, baso-
phlic lymphoid cells of the bone marrow previously designated as transitional cells. Transitional cells are illustrated in Fig. 8 as they appear on a smear not treated with the benzidine reagent. In Figs. 15 and 16, the ultrastructural features of a transitional cell are contrasted with that of an early erythroid cell, as they appear after staining with uranyl acetate and lead citrate.

In concurrence with previous reports, transitional cells were numerous in the bone marrow of polycythemic controls, but none of them reacted with benzidine or with DAB, nor was a positive reaction seen in such cells in the marrow of normal animals. Hemoglobin was first detected in transitional cells 40 hr after erythropoietic stimulation. However, even in stimulated animals, only 3%–5% of transitional cells showed demonstrable hemoglobin, being 10–20 times less numerous than benzidine-positive proerythroblasts and basophilic erythroblasts.

Incorporation of 55Fe

On autoradiographs, background labeling was not quantitated, but it was clearly so low that it did not interfere with the identification of cells labeled due to the incorporation of 55Fe.
Fig. 11. A stronger DAB reaction is present in the transitional-type cell than in the cell shown in Fig. 10. In Fig. 1 its position on the cytoplasmic density scale is indicated by 11. The cell to the left is an intermediate erythroblast; a portion of an erythroblast with minimum detectable hemoglobin is marked with an asterisk and is also shown in Fig. 1. Its extensive cytoplasm and its well developed Golgi apparatus contrast with the transitional type cell. Reacted with DAB but otherwise unstained; × 11,500; marker, 1 μ.

One hundred per cent of nucleated cells with a generalized cytoplasmic reaction positive to benzidine were found to be labeled with 55Fe. The same was true for DAB-positive cells in sections. These statements are valid for early erythroid cells with minimum demonstrable hemoglobin (Fig. 9) and also for transitional cells positive with benzidine or DAB (Fig. 10). With the exception of transitional cells, labeled benzidine or DAB-positive cells were morphologically recognizable erythroblasts of different maturation stages. Some of the red blood cells, presumably reticulocytes, were also labeled.

Both in smears and sections, there were a number of cells similar to early erythroid cells and to transitional cells which incorporated 55Fe but were negative with benzidine or with DAB (Figs. 12 and 13). In view of their morphologic similarity to cells with minimum amounts of hemoglobin, these cells are regarded as having incorporated iron, but not yet synthesized hemoglobin in quantities detectable by the benzidine or DAB reaction. Such cells in both morphologic categories are precursors to erythroblasts.

Transitional cells labeled with 55Fe were found only in stimulated animals, where they represented 2%–3% of all labeled cells. However, even in these animals, the great majority of transitional cells were unlabeled (Fig. 14). On the other hand, only 1%–2% of cells with morphologic characteristics of pro-erythroblasts and basophilic erythroblasts did not incorporate 55Fe.
In electron microscopic radioautographs, the granules of neutrophils and eosinophils, as well as the endoplasmic reticulum of some monocytes, were positive with DAB, but did not incorporate $^{59}$Fe. In smears, 0.1%-0.2% of all labeled cells were monocytes and fewer than 0.1% were neutrophilic or eosinophilic granulocytes. Two per cent of the labeled cells could not be recognized, due to damage. Thus, in smears, 99.7% of all intact bone marrow cells (or 97.7% of all cells) labeled with $^{59}$Fe either contained hemoglobin in quantities demonstrable by the benzidine reaction, or, though negative to benzidine, were in other respects morphologically similar to those cells in which minimum amounts of hemoglobin were detected by the reaction. Of the latter type, transitional cells were the only cells which did not conform to the morphology of erythroid cells of some recognized maturation stage.
Fig. 14. The tissue in this autoradiograph was reacted with DAB, but it is otherwise unstained. The transitional cell is negative to the reaction as shown on the cytoplasmic density scale (Fig. 1), and it is also unlabeled with 59Fe. The comparison of this cell to those illustrated in Figs. 10, 11, and 13 demonstrates the morphologic similarity but functional heterogeneity among transitional cells. Fine cytoplasmic processes of a reticular cell embrace the transitional cell along its upper border. Unstained, x 9500; marker, 1 μ.

DISCUSSION

The benzidine and DAB reactions, as employed in the present experiments for the demonstration of hemoglobin, may be regarded as reliable indicators of this protein at the cellular level. This conclusion is warranted by the following findings: (1) a general cytoplasmic reaction to benzidine or DAB was present in all morphologically identifiable erythroid cells, (2) such a reaction was absent in all identifiable nonerythroid hemopoietic cells, (3) all types of cells with a general cytoplasmic reaction positive to benzidine showed absorption of monochromatic light at λ 4046 Å.

While labeling of blood leucocytes has been reported several days after the administration of radioiron in large doses,10,11 with dosages similar to that employed in the present experiments, no uptake of 59Fe was found in bone marrow cells other than recognizable erythroblasts, in vivo12 or in vitro.13 In the present studies, only 0.3% of intact cells labeled with 55Fe were distinctly not erythroid. The iron contained in cytochrome C or in various other enzymes of granulocytes, monocytes, and lymphocytes evidently has such a low rate of turnover that, under the experimental conditions employed, labeling with 55Fe may be regarded with confidence as an index of hemoglobin synthesis.

The findings reported here establish the morphologic identity of cells in which erythroid differentiation was initiated in terms of hemoglobin synthesis.
This differentiation was induced by endogenous erythropoietin, as both bleeding\textsuperscript{4} and hypoxia\textsuperscript{15} have been shown to stimulate the production of this hormone.

The evidence presented by the light and electron microscopic observations clearly establishes that in normal guinea pig bone marrow, hemoglobin synthesis commences in cells which, on the basis of their morphology, have been classically regarded as proerythroblasts.\textsuperscript{4,7} This is at variance with early spectrophotometric studies,\textsuperscript{16} but concurs with a number of reports of heme synthesis in proerythroblasts.\textsuperscript{12,13,17,18}

When erythropoiesis was restimulated in animals in which the nucleated erythron was practically eliminated, hemoglobin synthesis was detected not only in proerythroblasts but also in transitional cells. Thus, some of these cells are also linked to erythroid differentiation.

While the present studies do not provide direct evidence for the identification of the erythropoietin-responsive cell, the following evidence suggests that cells in the transitional cell population respond to erythropoietic stimulation by commencing erythroid differentiation:

(1) Iron incorporation and hemoglobin were detected in transitional cells only when the demand for red blood cell production had to be met by an increased rate of differentiation of erythropoietic progenitor cells. The pattern of \textsuperscript{55}Fe labeling in bone marrow cells lends support to the interpretation that \textsuperscript{55}Fe incorporation in transitional cells negative with benzidine or DAB occurred in preparation for hemoglobin synthesis. The majority of cells responsive to the stimulus acquired the morphology of proerythroblasts by the time detectable quantities of hemoglobin were synthesized. It is consistent with the known stimulatory effects of erythropoietin on hemoglobin synthesis\textsuperscript{19} that erythroid progenitor cells should begin to synthesize hemoglobin at a maturation stage earlier in morphologic terms than under normal circumstances. The two types of cells labeled with \textsuperscript{55}Fe are compared in Figs. 15 & 16 as they appear on conventional electron micrographs.

(2) Our previous quantitative radioautographic studies showed that, during the initial phase of erythropoietic restimulation, basophilic transitional cells were rapidly removed from their own compartment and the \textsuperscript{3}H-thymidine activity contained within them appeared, in terms of grain count, in newly formed erythroblasts.\textsuperscript{20} During the same period, basophilic transitional cells incorporated \textsuperscript{3}H-uridine at a rapid rate.\textsuperscript{20}

(3) Cells similar to transitional cells have been shown, in mouse fetal liver, to synthesize mRNA specific for globin, in response to erythropoietin.\textsuperscript{21}

(4) On the basis of \textsuperscript{3}H-thymidine incorporation, similar cells have been proposed in spleen colonies as progenitors of erythroblasts,\textsuperscript{22} although this parameter is not specific for erythropoietin-responsive cells.

It is established that transitional cells function as the immediate precursors of bone marrow small lymphocytes\textsuperscript{23,24} Progenitor cells for granulocytes and macrophages in the in vitro agar colony culture system also fall into the transitional cell category.\textsuperscript{25} No morphologic criteria could be found, even at the ultrastructural level, which would distinguish potential lymphocyte or granulocyte progenitor cells from those transitional cells which had the capacity
Figs. 15 and 16. The cells in these autoradiographs have not been treated with DAB but were stained with uranyl acetate and lead citrate to reveal morphologic features more clearly. On a morphologic basis, the cell in Fig. 15 may be identified as an early member of the erythroid series, while, on the same basis, the cell in Fig. 16 is a transitional cell. Both cells incorporated $^{55}\text{Fe}$, but without the DAB reaction it is not possible to determine whether they contain hemoglobin. The extensive cytoplasm of the blast cell is dense with polyribosomes, and in its lower half it contains a well-developed Golgi apparatus. The transitional cell has only a narrow rim of cytoplasm which also contains numerous polyribosomes. The Golgi apparatus is not evident. The mitochondria are numerous and large in both cells, and endoplasmic reticulum is sparse. The chromatin in both cells is predominantly in the extended form; marginal heterochromatin is present in the blast cell in slightly greater amount than in the transitional cell. A large and complex nucleolus is seen in the blast cell in Fig. 15, whereas only two small masses adjacent to the nuclear envelope might be designated as nucleolar material in the transitional cell. In the light microscope both cells would show deeply basophilic cytoplasm, and the cell in Fig. 15 would correspond to a proerythroblast, while the cell in Fig. 16 would be identified as a basophilic transitional cell. x 6500; marker, 1 μ.

to respond to erythropoietic stimulation. With the techniques available at present, it is not possible to determine whether, in conformity with the operational definition of a pluripotent stem cell, a single cell in this population may be induced to differentiate in one direction or in another. The available evidence strongly supports the contention that “committed stem cells” or specific progenitor cells of erythroblasts, granulocytes, macrophages, and of bone marrow small lymphocytes are contained in the transitional cell population. They are probably represented by the more basophilic cells which are known to have a high rate of turnover.26 Although cells similar to monocytes (monocytoid cells) have recently been proposed as candidates for the pluripotent stem cell,27,28 there is increasing evidence for stem cell function in transitional cells.29-32 The morphologic characteristics of stem cells recently described by two independent groups of workers33,34 are in good agreement with the
morbidity of the smaller, pale-staining members of the transitional cell population. In addition to morphology, the relatively slow rate of turnover of pale-staining transitional cells is in accord with the known proliferative behavior of colony-forming units, commonly equated with the pluripotent stem cell. The high rate of turnover of basophilic transitional cells, on the other hand, is in agreement with the more rapid proliferation of “committed stem cells.”

REFERENCES

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