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Fetal hemoglobin accumulation in vitro. Effect of adherent mononuclear cells.

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Research Article

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Fetal Hemoglobin Accumulation In Vitro

EFFECT OF ADHERENT MONONUCLEAR CELLS

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ABSTRACT In clonal cultures of erythroid burst-forming units (BFU-E) obtained from blood, the accumulation of fetal and adult hemoglobins (Hb F and Hb A) was measured by radioligand immunoassay. Inclusion of adherent mononuclear cells in the culture promoted a striking increase in the relative amount of Hb F in each of 44 experiments with 14 donors. In two-thirds of the instances, this was accounted for by a selective increase in the absolute amount of Hb F. The differential effect on Hb F and Hb A accumulation was achieved without altering the maturity of the erythroid cells, their mean hemoglobin content, or the asynchrony of the production of the two hemoglobins. Virtually all bursts produced Hb F, and the population of BFU-E as a whole, rather than a selected subset, appeared to be the target of adherent cell action. When the adherent cells were excluded from the culture input, the base-line value of Hb F was reproducible for each donor over a period of several months, and correlated with the number of in vivo circulating F cells.

INTRODUCTION

A spectrum of increasingly mature progenitors committed to erythroid differentiation is found in normal adult blood and bone marrow. At least three stages of development have been defined (1, 2) and are designated the primitive erythroid burst-forming unit (BFU-E),[1] the mature BFU-E, and the erythroid colony-forming unit (CFU-E). These cells can be detected in clonal cultures by the characteristic morphology and the sequence of appearance of the erythroid colonies they produce. The synthesis of fetal and adult hemoglobins by these clones has been extensively studied (3). According to the model proposed by Papayannopoulou and her colleagues (4–6) the genetic programs for the synthesis both of adult hemoglobin (Hb A) and of fetal hemoglobin (Hb F) are intact in the early BFU-E, but the Hb F program is lost on a stochastic basis as the progenitor divides repeatedly prior to terminal differentiation. Most mature erythrocytes are derived in vivo from the latter, mature class of progenitors and contain Hb A, but no detectable Hb F. Only a fraction of erythrocytes, called F cells (7), contain both hemoglobins. They account for the ~1% Hb F found in the blood of the normal adult.

Hb F synthesis in culture is consistently increased, presumably because terminal differentiation occurs in less mature progenitors, at a time when the suppression of the Hb F program is incomplete (5, 8). Factors that stimulate early BFU-E differentiation promote increased Hb F synthesis. Such action has been reported for burst-promoting activity (BPA) (8, 9), leukocyte-conditioned medium (10), and adherent mononuclear cells (11).

With the exception of a few studies where the actual amount of Hb F (10) or of both Hb F and Hb A (8, 12) accumulated in culture have been reported, published data have dealt only with the relative synthesis of the two hemoglobins, measured as the ratio of radiolabeled amino acids incorporated into γ- and β-chains of globin, usually over a single 24-h period. Strictly quantitative information on the amounts of the hemoglobins accumulated in culture cannot be derived from these experiments. Moreover, the relative production of Hb F in vitro decreases progressively with time in culture (13, 14), so that the results obtained depend on the time at which observations are made.

The present study on hemoglobin accumulation in clonal cultures of blood BFU-E has a threefold purpose: First, to describe the use of a quantitative approach that obviates some of the technical problems noted above. Second, to examine the effect of adherent mononuclear cells on the differential expression of the

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[1] *Abbreviations used in this paper:* BFU-E, erythroid burst-forming unit; BPA, burst-promoting activity; CFU-E, erythroid colony-forming unit; Ep, erythropoietin; Hb A, adult hemoglobin; Hb F, fetal hemoglobin; MCH, mean corpuscular hemoglobin; MNC, mononuclear cell fraction of blood; NAMNC, nonadherent MNC.

Hb A and Hb F programs. Third, to suggest the participation of donor-determined factors in the production of the two hemoglobins.

METHODS

Reagents. Partially purified human urinary erythropoietin (Ep) was provided by the Division of Blood Resources, the National Institutes of Health. Anemic sheep plasma Ep was purchased from the Connaught Laboratories, Toronto, Canada. Several batches of each Ep were used with equivalent results.

All culture components were obtained from the Grand Island Biological Co., Grand Island, NY, except for fetal calf serum (FCS), which was purchased from the Reheis Chemical Co., New York, bovine serum albumin (BSA) from Sigma Chemical Co., St. Louis, MO, and methylcellulose from the Dow Chemical Co., Midland, MI.

Human subjects. Blood for clonal erythroid cultures was obtained from 14 adult subjects. Five donors were studied repeatedly over a period of several months; they are identified in Results by the letters A through E. Four of these five were hematologically normal. Donor D was an adult nonanemic woman with β-thalassemia trait; she had microcytic erythrocytes, normal levels of serum iron and transferrin, Hb A_2 3.6% by chromatography (15), Hb F 1.9% by alkali denaturation (16), and non-α to α-globin chain synthetic ratio of 0.6 by the method of Alter et al. (17). The donors examined on a single occasion, shown in the right hand panel of Fig. 2, were hematologically normal except for one individual who had sickle cell trait and two patients who had polycythemia vera.

All blood was obtained with informed consent of the donors, as approved by the Institutional Board of Research Associates, New York University Medical Center.

Cell fractions. Heparinized blood was diluted with an equal volume of Dulbecco's phosphate-buffered saline (PBS) and was centrifuged over a cushion of Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) at 400 *g*, 14°C for 45 min. Mononuclear cells (MNC) were recovered from the interface, washed three times in PBS and suspended in RPMI 1640 medium containing 20% FCS. Nonadherent mononuclear cells (NAMNC) were recovered after the adherent cells were removed by two consecutive 1-h incubations in dishes (18). The adherent cells were then released from the plastic dishes by treatment with lidocaine (18).

The macrophage content of the cell fractions was determined as the percentage of cells capable of ingesting 0.3-μm latex particles (Dow Chemical Co.) and by conventional morphologic criteria on Wright-stained cytocentrifuge preparations. There were 26±15% macrophages in MNC, 3.7±2% in NAMNC and >85% in adherent cell fractions.

Culture method. Erythroid clones were grown in semisolid methylcellulose medium according to published methods (19) with some modifications. The culture medium contained final concentrations of 0.85–1.0% methylcellulose, 1% BSA, 30% FCS, 100 μM 2-mercaptoethanol, 1 mM $FeCl_3$, 2.7 mM $NaHCO_3$, 100 μg/ml gentamicin, and 1.5 U/ml Ep, in Iscove's modification of α-medium. MNC or NAMNC at a concentration of 5×10^5 cells/ml culture medium were plated in 35-mm diam plastic culture dishes. Incubation was carried out at 37°C in a humidified atmosphere of 4% CO_2 in air. The Ep and cell concentrations were optimal, as determined by dose-response experiments performed periodically in the course of this study.

Erythroid bursts were identified by their content of hemoglobinized cells and by their characteristic multicolony morphology. They were counted either under a dissecting microscope or from photographic enlargements when the bursts were macroscopically visible, as was usually the case.

Processing and analysis of cultured cells. After specified times in culture, the cells were collected and washed free of methylcellulose. Measured aliquots of the cell suspension were taken for cell counts, cytocentrifuge slide preparations, and quantitative assays. Cell counts were done in duplicate in a hemocytometer. Cytocentrifuge slides were prepared from $\sim 2 \times 10^5$ cells suspended in 200 μl type AB serum and 200 μl PBS. Air-dried slides were stained with Wright stain and the cell types were identified according to standard morphologic criteria. Throughout the study, microscopic identification of the cells was done by the same observer. 90±7% of the cells were recognizable erythroid precursors. The remainder consisted predominantly of small mature lymphocytes. Eosinophilic granulocytes were rarely encountered.

F cells in peripheral blood were enumerated by microscopic single cell immunodiffusion (20); this determination was kindly performed for us by Dr. George J. Dover, Johns Hopkins University School of Medicine.

Preparation of cell lysates. A measured aliquot of the cells was pelleted and lysed by sonication in 300–400 μl of carrier sheep hemoglobin (0.5 mg/ml). Cell debris was removed by centrifugation and the clear supernate was collected in preweighed tubes. The volume of the sample was determined from the weight. These samples were stored at 4°C and assayed within 3 d for Hb A and Hb F by the radioligand immunoassay method. Cells from a total of 2–4-ml culture were used for each datum point.

For the assay of hemoglobin in individual bursts, the harvesting procedure was modified as follows: Under a dissecting microscope at ×25, each burst was aspirated into a capillary tube charged with 10 μl PBS and was then rinsed into a 0.5-ml centrifuge tube containing 125 μg carrier sheep hemoglobin in 250 μl 0.05% Triton X-100. Each sample was twice frozen at −80°C and thawed to complete the hemolysis. Cell debris was removed by centrifugation; the supernatant was recovered for assay of fetal and adult hemoglobins. The hemoglobin content of individual bursts was well within the range of the assay employed.

Radioligand immunoassay of Hb A and Hb F was performed as previously described (21). The basis of this assay is as follows: Several dilutions of each sample containing Hb A, Hb F, and carrier sheep hemoglobin were incubated with an excess of antibody of desired specificity. The immune complexes formed with the corresponding hemoglobin were, in the next stage of the procedure, bound to a protein A-bearing strain of *Staphylococcus aureus* (22). Unreactive hemoglobins were removed by washing the bacterial pellet. The hemoglobin specifically retained was then measured on the basis of its ability to bind a standard sample of ^{125}I-labeled Hp 1-1. The quantity of hemoglobin was determined by reference to standard curves constructed in a similar fashion using a range of 2 to 300 ng of adult or fetal hemoglobin. These standards were included in each day's assay. Data obtained are presented as absolute amounts of Hb A and of Hb F produced in 1 ml culture, or as percent Hb F calculated from these values. The sensitivity of the assay, as applied to the culture system, was validated by the recovery of hemoglobins from complete culture media that contained known input of fetal or adult erythrocytes, as outlined in a previous report (8).

The mean corpuscular hemoglobin (MCH) for each sample was calculated from the total hemoglobin for the sample, the total cell count, and the percent erythroid cells.

The mononuclear cells plated in culture contained a variable number of erythrocytes. The latter evidently lysed in the first 3–4 d of culture since no intracellular hemoglobin could be recovered from cultures harvested at this time. Thus, all hemoglobin assayed subsequently represented that accumulated in vitro.

Statistical methods. Standard statistical procedures (23) were used to evaluate data. Means of populations were compared by the *t* test. In the analyses, if variances of populations differed significantly, the degrees of freedom were appropriately reduced (24) with consequent reduction in significance.

The data presented in Table II were evaluated as follows: For each donor the values of percent Hb F were shown by the goodness of fit test (23) not to differ significantly from a normal distribution. The difference between pairs of donors and among the entire group were tested by *t* statistics and by one-way analysis of variance, respectively. Similar results were obtained whether the percent Hb F for each donor was expressed as the mean of the values for different experiments, or as the slope of the regression line of Hb F on total hemoglobin. The correlation between percent Hb F and percent F cells was based on mean values weighted (W) for standard deviation (S) and the number of experiments (n), where $W = n/S^2$. Without this adjustment, the calculated correlation was higher ($P = 0.0026$) than the more conservative value given in Results.

RESULTS

Overall characteristics of the cultures. The course of erythroid cell differentiation and of hemoglobin accumulation in a representative NAMNC-derived culture is depicted in Fig. 1. Replicate plates were harvested and analyzed on consecutive days. The accumulation of hemoglobin began with the first appearance of recognizable erythroid precursors on day 6. In the particular experiment shown, peak values for hemoglobin were recorded between days 11 and 12 (panel a). In most cultures the peak was reached on days 13–14. The colonies degenerated after this time; more than half of the erythroid cells were lysed by day 20 of culture. During the period of net culture growth, the accumulation of Hb A and Hb F proceeded asynchronously; the relative amount of Hb F was highest at the beginning of measurable hemoglobin synthesis and steadily declined thereafter (Panel b). The developing erythroid precursors showed progressive overall maturation paralleled by increasing MCH content as shown in panels c and d of Fig. 1.

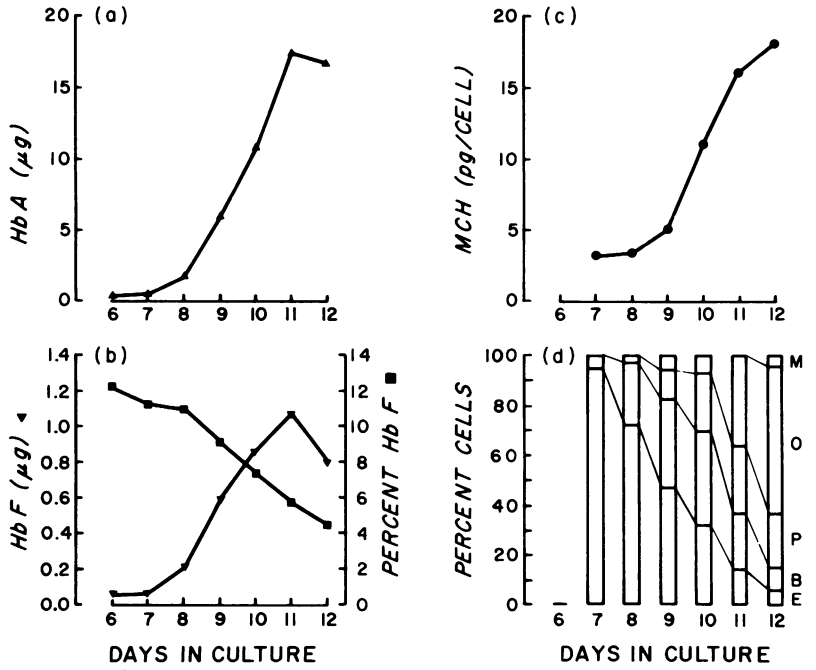

FIGURE 1 Time course of erythropoiesis in culture. Replicate cultures were harvested on consecutive days. Hemoglobin values refer to the content of cells in 1 ml of culture. (a) Accumulation of Hb A, first noted on day 6, accelerates until colony degeneration becomes a significant factor, in this case, on day 11. (b) Accumulation of Hb F (▼) does not keep pace with that of Hb A, with consequent gradual decline in percentage of Hb F (■). (c) MCH rises with increasing maturity of the erythroid precursors, shown in d. The bar graphs represent erythroblasts (E), basophilic normoblasts (B), polychromatophilic normoblasts (P), orthochromic normoblasts (O) and mature erythrocytes (M), as percentage of the total erythroid population. An accurate cell count and MCH determination could not be done on day 6.

In 11 cultures for which requisite data were available, the plating efficiency was 125±22.3 erythroid bursts per 5×10^5 cell input, and the average burst contained $22.3 \pm 12.1 \times 10^3$ erythroid cells (mean and SD). In 30 separate experiments, the MCH measured on day 13 or 14 was 17.5±7.6 pg hemoglobin/erythroid cell with a range of 5.1 to 34 pg/cell.

Hb F and Hb A in paired MNC- and NAMNC-derived cultures. Fig. 2 shows the results of 44 separate consecutive experiments with 14 donors (four examined several times each) in which MNC and NAMNC were cultured under otherwise identical conditions. Significantly different patterns of hemoglobin production were observed in the two groups. In all cases, the proportion of Hb F accumulated was higher, often strikingly so, when the adherent cells were retained in culture.

Whether the absolute amount of Hb F was increased depended largely on how well the cultures grew. As shown in Table I, in 28 of the 44 experiments (group 1) the absolute amount of Hb F was significantly greater in the MNC-derived cultures ($P = 0.0007$); accumulation of Hb A was not affected. These cultures grew well; in 25 of the 28 the overall hemoglobin content was >10 μg/ml in both the NAMNC and MNC sets. By contrast, in the cultures where the increase in Hb F was only relative (group 2), growth in the MNC set was inhibited ($P = 0.022$), hemoglobin accumulation was <5 μg/ml in 12 of the 16 experiments. In this group, the relative increase of Hb F in the MNC cultures was accounted for primarily by the selective reduction of Hb A as compared to the corresponding NAMNC cultures. Although the amount of Hb F appears relatively unaffected, comparison of the MNC-derived cultures in groups 1 and 2 showed that in fact the absolute quantities of both Hb A and Hb F were significantly lower in the second group ($P = 0.005$ and $P = 0.008$, respectively).

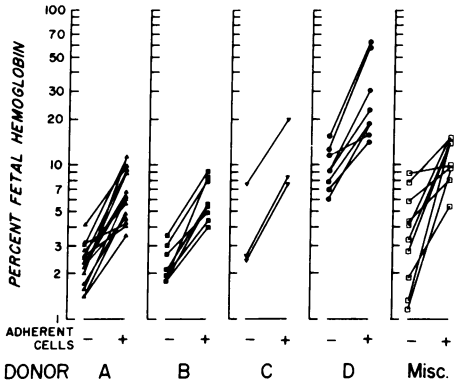

FIGURE 2 Effect of adherent cells on Hb F accumulation in paired cultures of NAMNC (adherent cell −) and MNC (adherent cell +). Each of the first four panels shows the results of separate experiments with a single donor. The last panel shows data from 10 other donors, each examined once. In every case, the relative amount of Hb F is greater in the MNC-derived cultures. Note that donor D is the individual with thalassemia trait.

Effect on burst proliferation. Data (mean and SE) on the number of bursts and their average size (cell content) were available for 15 of the paired experiments described above. In group 1 (10 experiments) the MNC- and NAMNC-derived cultures did not differ in the number of bursts (139±26 vs. 136±26 bursts/ml) or in their size (14,700±2,180 vs. 18,300±2,710 erythrocytes [RBC]/burst). In group 2 (five experiments), the MNC and NAMNC sets had 65±15 vs. 66±21 bursts/ml and contained 9,860±3,940 vs. 14,600±6,862 RBC/burst, respectively. Thus the presence of adherent cells did not affect the number of bursts within each group. As compared to group 1, the cultures in group 2 had fewer bursts, and the cell content was lower in the presence of adherent cells. These differences, however, did not attain statistical significance.

Dose-response relations. The dose dependence of the Hb F response was studied by altering the adherent cell content of the cultures in three different ways. The results are shown in Fig. 3. For the experiment depicted in panel a, increasing numbers of adherent cells, released from plastic dishes by lidocaine, were added to the NAMNC at the time of plating. For panel b, NAMNC were cultured in dishes previously coated with various numbers of adherent cells, as follows: Increasing numbers of MNC were seeded on 35-mm culture dishes and were incubated for 1 h to allow attachment. Loose cells were removed by washing; the number of adherent cells remaining on the dishes was roughly estimated from the macrophage content of the MNC used for seeding minus that of the cells recovered by washing. The wash medium was carefully removed, and autologous NAMNC in complete culture medium was layered atop the attached cells. For panel c, different adherent cell concentrations in the cell input were obtained by 0, 1, or 2 consecutive depleting incubations of MNC prior to plating.

In each of the three types of experiment, the number of adherent cells and the percentage of Hb F produced were directly related. This relative increase occurred regardless of whether overall hemoglobin accumulation was decreased or unaffected. When adherent cells alone were cultured, the quantities of hemoglobin produced were insignificant, amount to <1% of that produced in other cultures. This minimal activity probably represented the contamination of adherent cells with a few BFU-E.

Relation of adherent cells to the overall maturity of the precursor population in culture. Because the

TABLE I

Absolute Amounts of Hb A and Hb F in Paired NAMNC and MNC Cultures

		NAMNC		MNC		
Group	*n*	Hb A	Hb F	Hb A	Hb F	*P*
1	28	33,513±5,554		29,660±5,313		0.62
			867±135		2,280±353	0.007
2	16	23,453±6,501		7,411±3,052		0.022
			1,128±269		875±230	0.48
	P	0.26	0.34	0.005	0.008	

Group 1 consists of experiments in which the absolute as well as the relative amounts of Hb F were increased in the MNC set. Group 2 contains those experiments where the increase in Hb F was only relative. The amounts of hemoglobin made, in ng/ml culture, are given as mean and standard error. The *P* values show the significance of the difference between pairs of data. *n* is the number of experiments in each group.

bulk of Hb F synthesis occurs in the younger nucleated erythrocytes (14), factors delaying cell maturation may be expected to lead to a relative increase in Hb F accumulation. Comparison of the differential counts of the erythroid precursors in paired cultures of MNC and NAMNC showed no striking or consistent differences in the maturity of the cells. However, an objective evaluation of small differences cannot be made from such data. Therefore, the MCH of the entire cell population, a measure of how far hemoglobin synthesis had progressed in the culture, was used as an additional numerical estimate of mean erythroid cell maturity. In adopting this criterion it is tacitly assumed that total hemoglobin formation progresses pari passu with cell maturation.

In 20 experiments the MCH of erythroid cells in paired MNC- and NAMNC-derived cultures were related as shown in Fig. 4. There was no tendency toward a higher MCH in the NAMNC-derived cells. Furthermore, the percentage Hb F in repeated cultures from each donor was unrelated to MCH. In one donor data

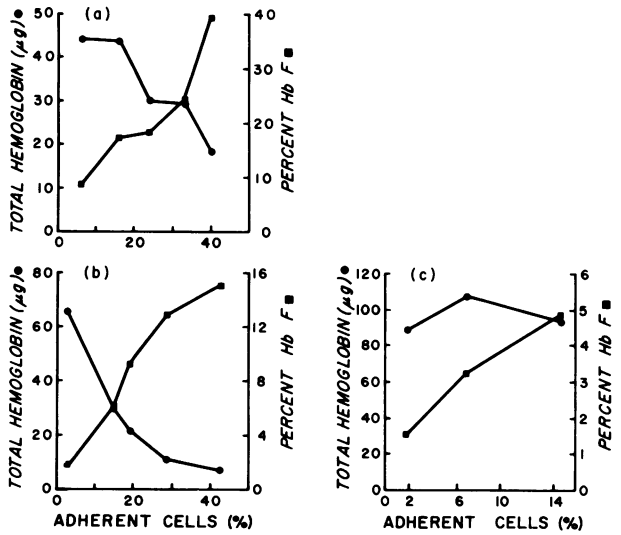

FIGURE 3 Dose response of total hemoglobin and Hb F to adherent cells. The content of the latter in the culture medium was varied as follows: Panel (a) Lidocaine-released adherent cells added to NAMNC. Panel (b) Adherent cell-coated dishes used for culture of NAMNC. Panel (c) Adherent cells removed from MNC by successive incubations. Data shown are representative in terms of the effect on Hb F. The inhibition of overall hemoglobin accumulation was not consistently seen in other, similar experiments.

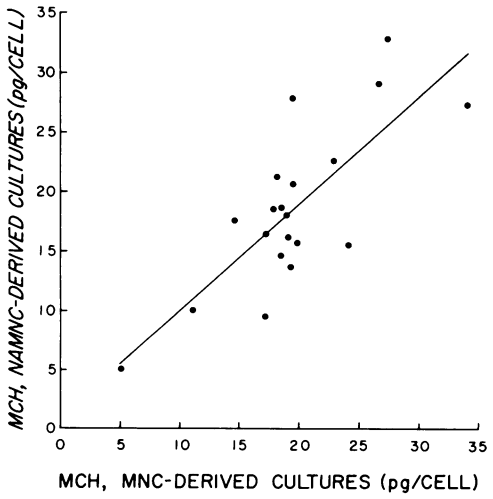

FIGURE 4 Effect of adherent cells on MCH in paired cultures. The regression line was derived by the least square method. The mean ratio of the MCH in NAMNC-derived cultures to that in the corresponding MNC-derived one was 1.03±0.22.

available for 12 experiments showed that MCH and percent Hb F varied independently ($r = 0.24$, $P = 0.45$). These analyses indicate that under the specified conditions neither the range of percent Hb F in a single donor nor its response to adherent cells can be ascribed to changes in the overall hemoglobin content, and hence the maturity, of the erythroid cells growing in culture.

Effect of adherent cells on F/A asynchrony. To determine if the adherent cells alter the asynchronous pattern of Hb F accumulation, hemoglobin assays were performed in replicate MNC- and NAMNC-derived cultures on days 10, 12, and 14. Fig. 5 shows the results of two separate experiments. In each case, the asynchrony was evident in both the MNC- and the NAMNC-derived cultures. The percentage of Hb F in MNC-derived bursts started and remained at higher levels. The slopes of the decline in percentage of Hb F with time, plotted on semilogarithmic scale, were similar in the presence and absence of adherent cells, indicating that equal fractional changes occurred in the two types of culture. By these criteria, the adherent cells did not affect the asynchronous accumulation of fetal and adult hemoglobins.

Effect of adherent cells on the distribution of Hb F among individual bursts. The foregoing data provide mean values for the entire population of erythroid cells in culture. The averaging process blurs the heterogeneity of the bursts, which may be relevant to the effect of adherent cells. Thus a question not answered by these experiments is: Do adherent cells affect all the BFU-E, or is their action exerted on a limited subpopulation, either by recruiting additional BFU-E capable of forming high Hb F bursts, or by suppressing low Hb F bursts? To evaluate these alternatives, every burst in paired MNC- and NAMNC-derived cultures was individually plucked and assayed for Hb A and Hb F. Fig. 6 shows the results of experiments with two donors. The quantitative parameters of each set are given in the figure legend. With a single exception, all bursts contained Hb F. In donor A (hematologically normal), the presence of adherent cells induced a modest (1.3-fold) increase in mean percent Hb F, accomplished by an overall shift of the population as shown by the increase in modal Hb F value from 3 to 5%. The same changes, but much more pronounced, were observed with donor D (thalassemia minor) where a 3.8-fold increase in percent Hb F and marked shift of the population were noted. In neither donor did the presence of adherent cells change the distribution of the population to a bimodal one. The plating efficiency was unchanged (donor A) or minimally reduced (donor D).

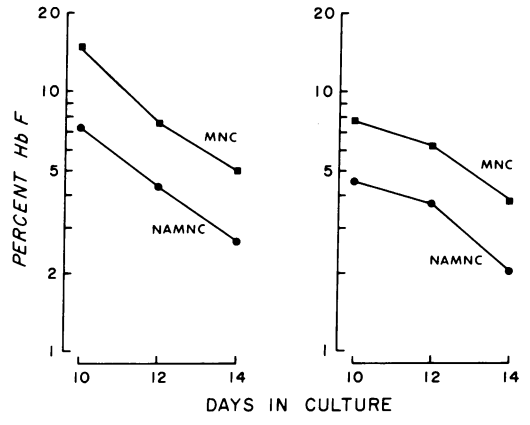

FIGURE 5 Effect of adherent cells on the asynchronous accumulation of Hb A and Hb F. Paired MNC- and NAMNC-derived cultures were analyzed 10, 12, and 14 d after plating. In each of two experiments, adherent cells did not alter the fractional rate of fall in percent Hb F, as shown by the parallel courses on a semilogarithmic scale.

Donor-determined modulation of Hb F production in vitro. Variable numbers of adherent cells are present in MNC obtained at different times from a given donor. We considered the possibility that in comparing the results of repeated cultures the dose-dependent effect of the adherent cells may mask a stable level of relative Hb F accumulation characteristic of each donor. The concentration of adherent cells in NAMNC, on the other hand, is small and varies little. The percent Hb F in NAMNC-derived colonies may therefore more closely reflect donor-specific differences.

Accordingly, NAMNC from five adults were cultured on multiple occasions over periods ranging from 9 to 29 mo. Quantitative assays for Hb A and Hb F accumulation were performed on day 13 or 14. The statistical parameters of in vitro Hb F production for the five donors are shown in the left-hand columns of Table II. Each donor accumulated a proportion of Hb F that fell within a narrow range. Analysis of variance for the entire group yielded $P = 2 \times 10^{-12}$. The data for donors A and B were very similar, as were those for donors C and E. By t test, the two pairs differed significantly from one another ($P = 1.6 \times 10^{-7}$) and from donor D ($P = 2.5 \times 10^{-10}$ for A + B vs. D, and $P = 9.3 \times 10^{-3}$ for C + E vs. D). These data strongly suggest that the proportion of Hb F in culture is determined by factors that are characteristic of the donor and that are stable over a period of several months.

To ascertain if these factors are related to the genetic locus that controls the production of F cells in vivo (25), the F cell numbers in the blood of each of the five donors was determined. The results, also presented in Table II, show a close correlation between the weighted mean percent Hb F accumulated in NAMNC-derived cultures and the relative number of circulating F cells ($r = 0.93$, $P = 0.02$).

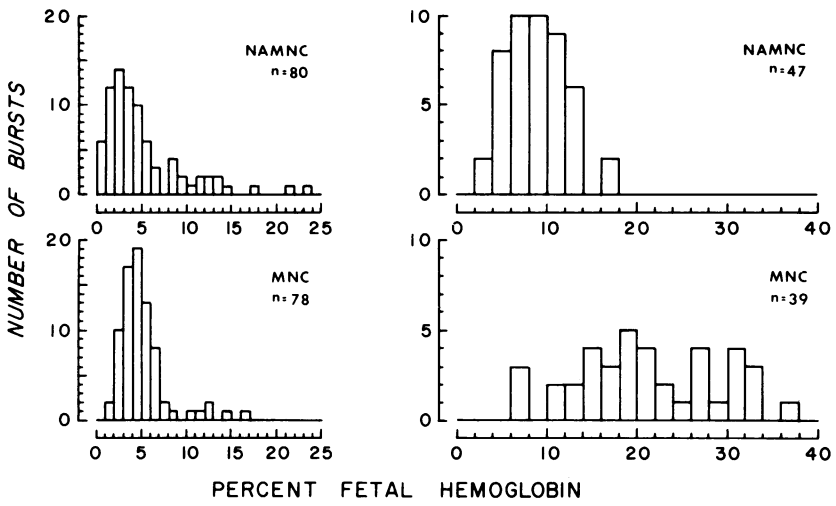

FIGURE 6 The distribution of percent Hb F among bursts derived from NAMNC and MNC cultures. Left hand panels (scaled at 1% Hb F intervals): Donor A, normal adult. The mean (±SD) content of the bursts was 829±585 ng total hemoglobin and 32±23 ng Hb F in the MNC set, and 829±585 ng total hemoglobin and 38±22 ng Hb F in the NAMNC set. Right-hand panels (scaled at 2% Hb F intervals): Donor D, nonanemic adult with β-thalassemia trait. The bursts contained 260±179 ng total hemoglobin and 50±31 ng Hb F in the MNC set, and 944±533 ng total hemoglobin and 86±55 ng Hb F in the NAMNC set.

The degree to which the presence of adherent cells in cultures obscures this donor individuality was evaluated as follows. The data for donors A, B, C, and D as presented in Fig. 2 were analyzed to see if the difference among donors is as evident from the MNC culture data as it is from the comparable number of NAMNC cultures. Analysis of variance showed that donor-determined differences are still clearly present in the MNC-derived cultures, but with reduced confidence ($P = 3.5 \times 10^{-5}$ for MNC; $P = 6 \times 10^{-10}$ for NAMNC).

TABLE II
Donor-specific Control of Hb F Synthesis In Vitro

	Culture			Peripheral blood	
Donor	*n*	Mean % Hb F	SD	% F cell	SD
A	34	2.60	1.60	0.44	0.38
B	12	2.92	1.59	0.25	0.30
C	13	5.40	2.30	2.81	0.86
E	14	5.75	1.96	3.92	1.29
D	11	8.54	3.20	5.96	1.17

The culture data show mean and standard deviation of percentage Hb F on day 13 or 14 in NAMNC-derived cultures; *n* is the number of separate experiments conducted over a period ranging from 9 to 29 mo. F cell determinations are the mean and SD of six measurements using single blood specimens. The donor designation (A–D) is the same as in Fig. 3.

DISCUSSION

The general characteristics of the cultures reported here, namely the optimal growth requirements, plating efficiency, size of the bursts, and the asynchrony of hemoglobin accumulation are similar to those published in the literature. Additional information is provided by the measurement of the absolute amounts of Hb A and Hb F that are not available from the determination of relative synthetic rates alone. The accumulation of hemoglobin may more closely reflect the final expression of the hemoglobin genome in mature erythrocytes, since it represents the production of hemoglobin over a longer course of precursor maturation than is examined in biosynthetic experiments. This is a significant consideration in view of the asynchronous synthesis of the two hemoglobins.

As studied by this approach, adherent MNC were found to increase the relative amount of Hb F in each of 44 experiments involving 14 donors. This confirms the observations made by Darbre et al. (11), but in addition shows that in most cases the effect was due to significant and selective increase in the absolute amount of Hb F accumulated. In these cultures (group 1) the MNC-derived bursts on the average contained slightly less Hb A but 2.6 times larger amounts of Hb F than the corresponding NAMNC set. In the experiments where the increase in Hb F was only relative (group 2), the presence of adherent cells was associated with decreased total hemoglobin production, predominantly at the expense of Hb A. In these cultures the

adherent cells may have selectively inhibited the clones with less active Hb F programs. Alternately, there may have been a nonselective suppression of overall hemoglobin production, partially offset by a preferential stimulation of Hb F synthesis; the net result would be the observed relative increase in the proportion of Hb F. Our data on pooled bursts do not permit a conclusive choice between these two alternatives, but the pattern of shift in the distribution of Hb F among isolated bursts is more compatible with the second hypothesis.

The mechanism of the occasional inhibition of erythropoiesis by the adherent cells is not clear. Other laboratories have reported that macrophages (adherent cells) suppress (18, 26), stimulate (27, 28), or have no effect (29) on erythropoiesis. Some studies suggest that macrophages at low concentration enhance and at high concentrations inhibit erythroid burst formation (30). In our experiments, adherent cells suppressed hemoglobin accumulation in approximately one-third of the cultures (group 2). There was an associated reduction in the number and size of the bursts that, because of the large variances, was not statistically significant.

The effect of adherent cells on relative Hb F accumulation was observed in every experiment. A dose-response relation was demonstrated with three different methods of attaining graded adherent cell concentrations. These findings make it unlikely that the observed effects are spurious or independent of interaction between adherent and nonadherent MNC. Furthermore, it is improbable that during the removal of adherent cells the progenitors of bursts with high Hb F synthesis rates were selectively removed as well; the adherent cells, cultured by themselves in complete medium failed to generate significant amounts of hemoglobin, whereas they induced Hb F synthesis when added to NAMNC.

The data suggest that adherent cells promote an increase in the absolute amounts of Hb F without having a similar effect on Hb A. These observations may be considered in terms of possible changes on the cellular or on the clonal level that may affect the final hemoglobin composition of the cultured erythroid cells.

First, the maturation of erythroid precursors in the MNC-derived cultures may be delayed, allowing continued Hb F synthesis at the high initial rate characteristic of the younger normoblasts. This notion is not supported by the finding that both the overall differential erythroid cell count and the MCH are similar in the MNC- and NAMNC-derived cultures.

Second, despite parallel morphologic maturation and total hemoglobin accumulation, it is possible that the asynchrony in the synthesis of Hb A and Hb F may be abrogated in the presence of adherent cells. This event would also lead to the accumulation of larger amounts of Hb F. This possibility is ruled out by the direct demonstration that asynchrony persists in the presence of adherent cells. The rate of Hb F synthesis in cultured cells is known to be proportional to the amount of γ-chain mRNA present in the cell (31). This correlation, coupled with our finding of unaltered asychrony, suggests that adherent MNC promote the formation of cells with a high initial rate of γ-chain mRNA transcription.

Third, macrophages are one source of BPA (9, 32, 33). Possibly, a subpopulation of more primitive BFU-E may be induced (recruited) to differentiate when adherent cells are present, yielding an additional number of bursts with high Hb F content. This model would be supported by two findings: an increase in plating efficiency, and the appearance of new bursts with high content of Hb F superimposed on an otherwise unchanged burst distribution. Neither of these predictions was met by the findings on isolated bursts.

On balance, the data favor the conclusion that the action of adherent cells is exerted, to more or less degree, on all the BFU-E differentiating in culture. The lack of discrete responding and nonresponding populations, however, may merely reflect the limited heterogeneity of the blood BFU-E population. Indeed, the widening of the distribution seen with one donor probably represents the graded response of the spectrum of BFU-E falling within the narrow range of maturity in this population. It will be important to examine, in similar experiments, the response of the more diverse erythroid progenitors found in the marrow. It may thus be possible to correlate the magnitude of the effect of adherent cells with the degree of maturity of the various progenitor classes.

Although this study does not address the issue of abnormal globin genomes, the hematologic status of donor D should be considered. It is unlikely that this individual has a deletion type $\delta\beta$-thalassemia where chromosome segments possibly involved in the modulation of Hb F synthesis may be missing. According to the criteria summarized by Weatherall and Clegg (34) the levels of Hb A_2, Hb F, and the non-α to α-globin synthetic ratio in this donor (see Human subjects) are suggestive of nondeletion β^+-thalassemia. It is evident from data presented in Fig. 2 that external signals provided by adherent cells consistently affect in vitro Hb F synthesis by this thalassemic individual, as they do in normal subjects. The pattern of distribution of Hb F among the bursts also suggests that the response in at least this donor, although amplified, may be qualitatively similar to that in a normal subject.

The influence of donor-determined factors on in vitro Hb F synthesis has not been systematically studied. Darbre et al. (11) found considerable variability in repeated examinations of individual donors. The

small number of experiments and the inclusion of adherent cells in their cultures may explain their findings. In the five donors studied here, the level of Hb F accumulated in NAMNC-derived cultures was relatively stable over a period of several months and its mean value correlated significantly with the number of F cells in the donor's circulation. There is evidence that the F cell number in vivo is under the genetic control (25) of a locus closely linked to the $\gamma\delta\beta$-loci (35). Dover and Ogawa (36) have reported that the augmentation of Hb F synthesis in culture is attained by increasing the number of F cells rather than the independently controlled content of Hb F per F cell, but they did not observe a correlation between in vivo and in vitro F cell numbers. Differences in experimental conditions, including the presence of adherent cells in their cultures, may account for this latter point. The correlation we have described suggests that genetic pathways involved in the regulation of F cell production in vivo also function under the conditions of in vitro clonal culture.

It appears, then, that in culture of peripheral blood BFU-E the genetic endowment of the individual donor regulates the base-line levels of fetal hemoglobin synthesis on which signals provided by nonerythroid elements, here exemplified by adherent cells, exert their influence. Virtually the entire population of bursts is capable of Hb F production and responds to these stimuli. The consequent increase in percent Hb F is due, in most if not all cases, to an increase in its absolute amount accumulated in the cells.

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