CONCISE REPORT

Treatment of Baboon With Vinblastine: Insights Into the Mechanisms of Pharmacologic Stimulation of Hb F in the Adult

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Vinblastine was administered to anemic baboons to test whether stimulation of Hb F takes place following distortion of erythropoiesis by an M-stage–specific compound. The treatments elicited erythroid cell cytoreduction followed by regeneration. During the phase of reticulocyte reduction, $\gamma/\gamma + \beta$ biosynthetic ratios increased without increment in F reticulocytes, suggesting that there was increased production of Hb F per F cell. The phase of reticulocyte regeneration was associated with sharp increments in relative (percentage) and absolute F reticulocytes. These data suggest that perturbations of erythropoiesis underlie the stimulation of Hb F synthesis by vinblastine. Accelerated or abnormal precursor maturation may account for the release of shift F reticulocytes with higher Hb F content, during the reduction phase. Accelerated total erythroid differentiation/maturation may account for the increment in F reticulocyte numbers during the phase of regeneration.

STIMULATION of Hb F synthesis has been achieved in anemic primates and patients with Cooley's anemia or hemoglobin S (Hb S) disease treated with various S-stage–specific compounds.[1-8] In the case of 5-azacytidine, the augmentation of Hb F synthesis has been attributed to demethylation of γ genes[1,2,8] or to the perturbations of erythroid progenitor kinetics secondary to cytoreduction[4] or to both mechanisms acting synergistically.[4] It has been speculated that distortion of the kinetics of erythropoiesis underlie the stimulation of Hb F following treatment with other S-stage compounds such as cytosine arabinoside (Ara-C).[5]

To further test whether pharmacologically induced kinetic perturbations of erythropoiesis can stimulate F cell production, we used single-dose treatments of baboons (*Papio cynocephalus*) with an M-stage–specific agent, vinblastine. This compound inhibits formation of mitotic spindles by binding to tubulin[9] preventing polymerization, and thus arresting cells in mitosis. The relatively specific mechanism whereby vinblastine produces cytotoxicity allows one to assess whether a compound that lacks a direct effect on DNA replication stimulates formation of F cells.

MATERIALS AND METHODS

Blood and bone marrow samples were obtained under general anesthesia. One animal was kept anemic by removal of up to 15% of its blood volume on a near-daily basis to maintain a hematocrit of 20% to 25%. Supplementation of iron, folate, and vitamins were provided throughout treatment. Hematocrits were performed each day a phlebotomy was done, to determine the volume of blood to be removed. Vinblastine was administered as a single-dose bolus infusion over 30 minutes, 1 mg/kg on two occasions and 2 mg/kg on two occasions, separated by a minimum of 35 days. Hematologic studies included measurements of Hb, hematocrit, WBC, and reticulocytes. F cells, F reticulocytes, and globin chain biosynthesis were done as previously described.[4]

Bone marrow mononuclear cells were separated by density centrifugation over Lymphoprep (Accurate, Westbury, NY) and cells were plated (at 4×10^5 per milliliter) in plasma clot and in methylcellulose media as described before.[4] Cultures were performed in the absence of erythropoietin and in media supplemented with 0.2 (plasma clot) or 0.5 (methylcellulose) IU of erythropoietin per milliliter. Benzidine-positive colonies appearing in day 3 plasma clots were counted as CFU-e (when they consisted of eight to 64 cells) or as erythroid clusters (when they consisted of three to seven cells). BFU-e were enumerated in day 9 methylcellulose cultures; they were identified as large red colonies composed of 100 or more cells or as colonies having multiple subcolonies.

RESULTS

The effects of the various manipulations on Hb F expression are shown in Fig 1. Phlebotomy alone produced a sharp wave of F reticulocytes peaking at 67% (treatment A, Fig 1). F reticulocytes subsequently decreased while the animal was kept chronically anemic. Administration of vinblastine (1 mg/kg) resulted in no increment in the percentage of F reticulocytes on one occasion (treatment B, Fig 1) and a 1.5-fold increase on the second (treatment E, Fig 1). Administration of 2 mg/kg produced sharp waves of F reticulocytes (Fig 1C and D).

All treatments with vinblastine resulted in drastic decreases in reticulocytes that were followed by sharp increments (Fig 1). During the reticulocyte reduction phase, the absolute number of total reticulocytes, F reticulocytes, and A reticulocytes decreased proportionally (Fig 2). During the regeneration phase, there was a disproportional increment of absolute F reticulocytes compared to A reticulocytes (Fig 2).

A sharp transient increment in $\gamma/\gamma + \beta$ ratios took place following the treatments (Fig 3). The increment in $\gamma/\gamma + \beta$ ratios preceded the increments in the percentage of F reticulocytes and took place at the time the absolute F and A reticulocytes were decreasing proportionally (compare Fig 3 with Figs 1 and 2). Increment of $\gamma/\gamma + \beta$ ratios in the absence of increment in F reticulocytes suggests that there is higher Hb F synthesis per F reticulocyte during the stage of cytoreduction immediately following treatment. The "shift" F reticulocytes released during cytoreduction may have

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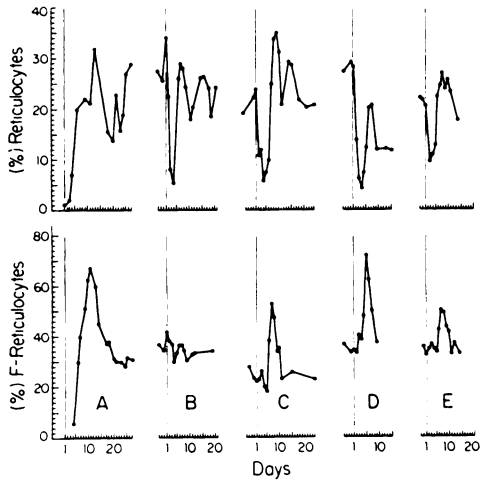

Fig 1. Changes in reticulocytes (upper panel) and F reticulocytes (lower panel) in an animal treated with vinblastine. Long vertical lines show the day the drug was given (B to E) or the day phlebotomy started (A); B and E, 1 mg/Kg; C and D, 2 mg/kg. Values of hematologic parameters and Hb F measurements at day 1 were obtained on samples drawn before the administration of the drug.

higher γ mRNA content, perhaps a reflection of faster maturation kinetics.

Erythroid cultures were performed before the administration of the 1 mg/kg dose as well as 24 and 48 hours later (Fig 4). At 24 hours, there was a small decrease of erythroid clusters but not of CFU-e. At 48 hours, all late progenitor pools showed substantial increment. In the treatment with 2 mg/kg, cultures were done before treatment as well as 24 and 72 hours later (Fig 4). A decrease in e-clusters and CFU-e was observed at 24 hours; e-clusters and CFU-e were increased at 72 hours. Early progenitors (BFU-e) were not decreased by either dose (data not shown); an increment in the size of BFU-e pool was noted in each posttreatment culture. The increase in BFU-e frequency is most likely a relative one, attributable to the drug-induced reduction in marrow precursors, which also may mask or underestimate the reduction in CFU-e.

Administration of vinblastine to a nonanemic animal (1 mg/kg/d three times) resulted in a sharp decrement in

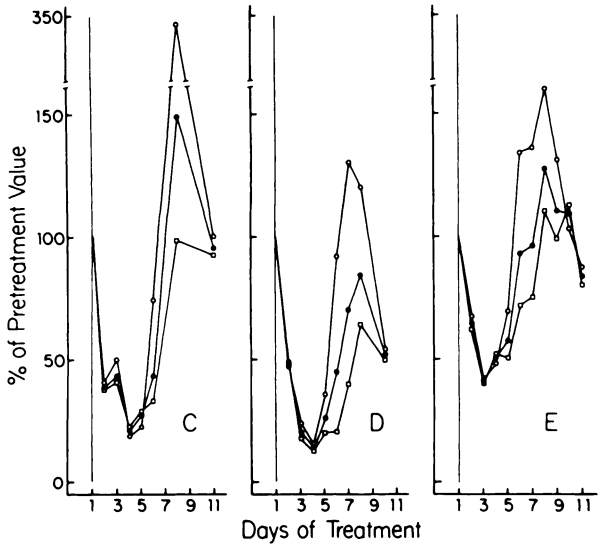

Fig 2. Changes in absolute total reticulocytes (●——●), absolute F reticulocytes (○——○), and absolute A reticulocytes (□——□) following treatment with vinblastine. Data from treatments C, D, and E of Fig 1 are presented. In order to compare the changes in total, F, and A reticulocytes, we expressed the actual values of these cells as percentages of the pretreatment value. Note the proportional decrease in total, F, and A reticulocytes immediately following treatment and the disproportional increase in F reticulocytes during the regeneration phase.

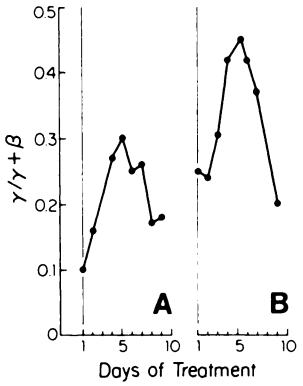

Fig 3. Changes in $\gamma/\gamma + \beta$ ratios following treatment with 1 mg/kg (A) or 2 mg/kg (B) of vinblastine. (A) corresponds to treatment E, while (B) corresponds to treatment D of Figs 1 and 2.

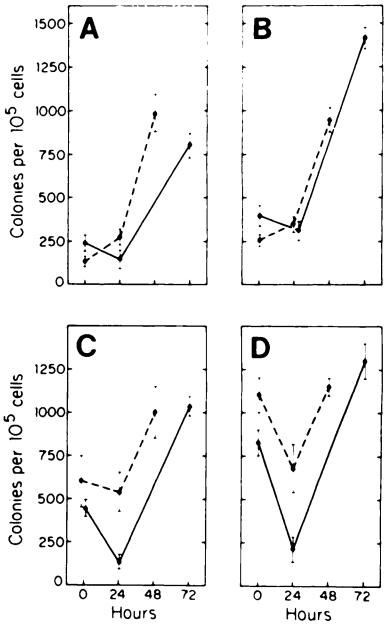

Fig 4. Counts of erythroid progenitors done immediately before treatment with vinblastine and at the indicated posttreatment times. ●- - -●, data with, 1 mg/kg; ●——●, data with, 2 mg/kg. Upper panel: Measurements of CFU-e grown in the absence (A) or presence (B) of 0.2 IU/mL of erythropoietin. Lower panel: Measurements of erythroid clusters grown in the absence (C) or in the presence (D) of 0.2 IU/mL of erythropoietin.

reticulocytes followed by regeneration six to seven days from the end of treatment. F cell numbers increased twofold over the pretreatment values (data not shown). Studies of F reticulocytes were not done in this animal because of low total reticulocyte counts.

DISCUSSION

These experiments were done to assess whether a compound that is expected to produce cytotoxicity but has no known differentiating effect on dividing cells can stimulate fetal Hb production. Stimulation of Hb F under these conditions would support a causative relationship between F cell formation and the kinetic perturbations triggered by cytoreduction. We found that vinblastine elicits a rapid and sharp decrease in reticulocytes, expected if there was cytoreduction in the precursor pool. Reduction of erythroid cluster pool was also present 24 hours following treatment. Regeneration of erythroid progenitor pools appeared as early as 48 hours posttreatment and it was followed by a brisk rebound in reticulocytes two to three days later. In parallel to these perturbations in erythropoiesis, we observed the following changes in Hb F or F cell formation.

1. During the sharp reticulocyte reduction stage (and while the percent of F reticulocytes and A reticulocytes did not change) we observed sharp increments in $\gamma/\gamma + \beta$ ratios. Since the proportion of F reticulocytes did not increase at this time, we interpret these data to suggest that the immediate effect of treatment with vinblastine is an increment of Hb F in cells which already had active γ genes (F erythroblasts). It is known that the γ chain synthesis decreases while the β chain synthesis increases during the maturation of erythroblasts[10] and that these changes reflect changes in proportion of γ and β mRNA accumulated during erythroblast maturation.[11] It is possible that vinblastine arrests this maturation process, resulting in release of reticulocytes from earlier erythroblasts that have not accumulated the normal levels of β mRNA. Alternatively, the acute cytoreduction produced by vinblastine may stimulate fast maturation of erythroblasts, resulting in the production of reticulocytes which contain more γ and less β mRNA per cell, either because of decrease in total maturation time or because of a skipped late division. Hence, we suggest that the first effect of the perturbation produced by vinblastine is an increment of the relative synthesis of γ chains per F-programmed cell either because of arrest in maturation or because of accelerated maturation kinetics of the precursor compartment.

2. During the reticulocyte regeneration phase, we observed an increment in the percentage as well as in absolute F reticulocytes (Figs 1 and 2). These results agree well with previous observations which suggest that F cell production is stimulated during fast erythroid regeneration.[12] We suggest that the stimulation of F cell production during the regeneration stage which follows treatment with vinblastine is of the same nature as the stimulation of F cells produced by a non-pharmacologic manipulation such as acute bleeding. We speculate that in both instances the cause of stimulated F cell production is the fast differentiation kinetics prevailing in the erythron during regeneration.

How kinetic perturbations of erythropoiesis result in formation of F cells remains a matter of speculation. We have previously suggested that F cells may be generated through "premature commitment" of progenitors.[13] Premature commitment during fast regeneration may result in F cell formation in various ways. Critical divisions in which the γ genes are turned off might be skipped; or shortening of total differentiation time in the erythroid compartment (from progenitor to erythroblast) may not allow completion of changes in the γ globin chromatin which normally inactivate the γ genes. Another plausible cause of F cell formation during regeneration is shortening of the erythroid cell cycle per se; for instance, shortening of cell cycle of progenitors below a critical time may increase the chance that they will form F cells.

REFERENCES

1. DeSimone J, Heller P, Hall L, Zwiers D: 5-Azacytidine stimulates fetal hemoglobin synthesis in anemic baboons. Proc Natl Acad Sci USA 79:4428, 1982

2. Ley TJ, DeSimone J, Anagnou NP, Keller GH, Humphries RK, Turner PH, Young NS, Heller P, Nienhuis AW: 5-Azacytidine selectively increases γ globin synthesis in a patient with $\beta+$ thalassemia. N Engl J Med 307:1469, 1982

3. Charache S, Dover G, Smith K, Talbot CC, Moyer M, Boyer S: Treatment of sickle cell anemia with 5-azacytidine results in increased fetal hemoglobin production and is associated with non random hypomethylation around the γ-δ-β-globin gene complex. Proc Natl Acad Sci USA 80:4842, 1983

4. Torrealba de Ron AT, Papayannopoulou Th, Knapp MS, Fu MF, Knitter G, Stamatoyannopoulos G: Perturbations in the erythroid marrow progenitor cell pools may play a role in the augmentation of Hb F by 5-azacytidine. Blood 63:201, 1984

5. Papayannopoulou Th, Torrealba de Ron A, Veith R, Knitter G, Stamatoyannopoulos G: Arabinosylcytosine induces fetal hemoglobin in baboons by perturbing erythroid cell differentiation kinetics. Science 224:617, 1984

6. Letvin NL, Linch DC, Beardsley GP, McIntyre KW, Nathan DG: Augmentation of fetal hemoglobin production in anemic monkeys by hydroxyurea. N Engl J Med 310:869, 1984

7. Platt OS, Orkin SH, Dover G, Beardsley GP, Miller B, Nathan DG: Hydroxyurea enhances fetal hemoglobin production in sickle cell anemia. J Clin Invest 74:652, 1984

8. Humphries RK, Dover G, Young NS, Moore JG, Charache S, Ley T, Nienhuis AW: 5-Azacytidine acts directly on both erythroid precursors and progenitors to increase production of fetal hemoglobin. J Clin Invest 75:547, 1985

9. Owellen RJ, Hartke CA, Dickerson RM, Harris FO: Inhibition of tubulin-microtubule polymerization by drugs of the vinca alkaloid class. Cancer Res 36:1499, 1976

10. Papayannopoulou Th, Kalmantis T, Stamatoyannopoulos G: Cellular regulation of hemoglobin switching: Evidence for inverse relationship between fetal hemoglobin synthesis and degree of maturity of human erythroid cells. Proc Natl Acad Sci USA 76:6420, 1979

11. Farquhar MN, Turner PA, Papayannopoulou Th, Brice M, Nienhuis AW, Stamatoyannopoulos G: The asynchrony of γ- and β-chain synthesis during human erythroid cell maturation. III. γ- and β-mRNA in immature and mature erythroid clones. Dev Biol 85:403, 1981

12. Papayannopoulou Th, Vichinsky E, Stamatoyannopoulos G: Fetal Hb production during acute erythroid expansion. I. Observations in patients with transient erythroblastopenia and post-phlebotomy. Br J Haematol 44:535, 1980

13. Stamatoyannopoulos G, Papayannopoulou Th: Fetal hemoglobin and the erythroid stem cell differentiation process, in Stamatoyannopoulos G, Nienhuis AW (eds): Cellular and Molecular Regulation of Hemoglobin Switching. Orlando, Fla, Grune & Stratton, 1979, p 323