PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 147, 740-743 (1974)

Studies on an Inhibitor of Erythropoiesis
I. Effects of Sera from Normal and Polycythemic Rabbits on Heme Synthesis in Rabbit Bone Marrow Cultures[1] (38429)

Y. MORIYAMA, J. J. L. LERTORA, AND J. W. FISHER

Department of Pharmacology, Tulane University School of Medicine, New Orleans, Louisiana 70112

Several investigators have reported that plasma or serum obtained from polycythemic animals, as well as from patients with polycythemia vera, caused a suppression of erythropoiesis *in vivo* (1–4). Kivilaako and Rytomaa (5) have recently reported that polycythemic rat serum significantly inhibited ^{3}H-thymidine incorporation in normal rat bone marrow cells *in vitro*. We have also reported recently the presence of an inhibitor of heme synthesis in the plasma and sera from patients with anemia associated with renal insufficiency and azotemic anephric rabbits (6). Other workers have been unable to demonstrate the presence of an inhibitor of erythropoiesis in serum from polycythemic animals when assayed in an *in vivo* system in mice and rats (7). The chemical nature, site of formation, and role of this inhibitor in the control of erythropoiesis are not known.

The present studies report the presence of an erythropoietic inhibitory substance in sera of normal and polycythemic rabbits. The inhibitory activity of these sera on heme synthesis increases exponentially with increasing concentrations when studied in rabbit bone marrow cultures. In addition, it was found that the increase in serum levels of this inhibitor of heme synthesis was directly proportional to the increase in hematocrit.

Materials and Methods. Polycythemia was induced in New Zealand albino rabbits (2.5 kg) with an iv injection of heparinized packed homologous red cell suspensions (Hct 50–60%) on three successive days to increase the hematocrit values of the recipient rabbits to 49–73%. Four days following the last blood transfusion, blood was collected by cardiac puncture and the serum separated. Sera collected from normal rabbits (Hct 36–42%) served as controls. These sera were stored at −18° until used.

The culture technique used was a modification of the method of Krantz *et al.* (8). The assay method involved culturing normal rabbit bone marrow cells in the presence of normal or polycythemic rabbit sera, respectively, and determining ^{59}Fe incorporation into heme in the cultures. The marrow cells were obtained from the rabbits as follows: The femoral bone marrows were removed and suspended in cold NCTC-109 solution (Microbiological Associates, Inc., Bethesda, MD). The cultures contained 1×10^{6} washed marrow cells per ml of culture fluid which consisted of 0, 2.5, 5, 10, 15, or 20% normal or polycythemic rabbit serum and 20, 17.5, 15, 10, 5, or 0% newborn calf sera (Microbiological Associates, Inc.), respectively, 80% NCTC-109 solution, penicillin (100 units/ml), streptomycin (50 μg/ml), and heparin (10 units/ml). Two milliliters of the marrow cell suspension were placed in 35 × 10-mm tissue culture dishes (Falcon Plastic Division of B-D Laboratories, Inc., Los Angeles, CA). Three culture plates were used in each group. The cultures were incubated with or without erythropoietin (ESF)[2] (specific activity of 5.29 units/mg protein) at 37° in an atmosphere of 5% CO_2 in a humidified incubator. After 29 hr of incubation, ^{59}Fe bound to homologous transferrin was added (0.5 μCi/plate), and the incuba-

[1] Supported by USPHS Contract No. NIAMD 70-2112 and Grant No. AM-13211.

[2] The erythropoietin was collected and concentrated by the Department of Physiology, University of The Northeast, Corrientes, Argentina, further processed and assayed by the Hematology Research Laboratories, Childrens Hospital of Los Angeles, under Research Grant No. HE-10880 (National Heart and Lung Institute).

tion continued for an additional 16 hr. The cells were then harvested, acidified, and the heme extracted into cyclohexanone. Radioactivity of the cyclohexanone extracts was determined with a Model 3320 Packard Scintillation Counter.

Results. The effects of the polycythemic rabbit sera (obtained from hypertransfused rabbits with hematocrits over 61%) on ^{59}Fe incorporation into heme in the normal rabbit bone marrow cells was studied in five different experiments (Table I). The results indicate that the polycythemic serum significantly ($P < 0.001$) inhibited ^{59}Fe incorporation into heme *in vitro* in bone marrow cells when compared with that of newborn calf serum controls. It is interesting to note that the normal serum also significantly ($P < 0.01$) inhibited heme synthesis in the cultures when compared with that of newborn calf serum controls. Iron incorporation into heme was also significantly ($P < 0.005$) less in the bone marrows treated with polycythemic serum than that seen with normal serum. The mean serum iron level in five normal rabbit sera was 136.4 ± 43.6 mg/100 ml, was not significantly different from that of five polycythemic rabbit sera which was 148.2 ± 38.0 mg/100 ml.

Figure 1 shows the dose–response curves for heme synthesis with increasing doses of ESF in the cultures with 20% polycythemic, 20% normal, and 20% newborn calf sera. The cultures treated with normal or polycythemic serum showed significantly ($P < 0.01$) less ^{59}Fe incorporation into heme at all dosages of ESF than that seen in the cultures with newborn calf serum, reaching peak levels of ^{59}Fe incorporation into heme at a dose of 0.04–0.06 units ESF. The most marked inhibitory effect was seen with polycythemic rabbit serum. The inhibitory effects of the sera on heme synthesis did not disappear even in the high-dosage range of ESF when normal or polycythemic serum (Fig. 1) was added to the cultures.

The relationship between heme synthesis, expressed as percent inhibition, and increasing concentrations of test sera in the cultures is shown in Fig. 2. Heme synthesis in the bone marrow cultures treated with normal or polycythemic serum plus 0.02 unit ESF was significantly ($P < 0.01$) decreased exponentially with increasing concentrations of the normal or polycythemic sera in the cultures when compared with that of newborn calf serum plus 0.02 unit ESF. The slope of the dose–response regression line of polycythemic serum was significantly ($P < 0.05$) greater than that of normal serum.

The regression line of best fit plotted by the method of least squares (9), showing the relationship between heme synthesis *in vitro* and hematocrit of the normal and polycythemic rabbit sera is shown in Fig. 3. The increased inhibitory activity in the cultures treated with the polycythemic sera plus ESF (0.02 unit) was directly proportional to the increase in hematocrit in the polycythemic rabbits.

Discussion. In the present study the changes in radioactive iron incorporation into heme in normal rabbit bone marrow erythroid cells *in vitro* treated with normal or polycythemic rabbit serum with and without ESF has been measured. The results indicate that polycythemic serum as well as normal serum does indeed inhibit heme synthesis in normal rabbit bone marrow cells in cultures with and without ESF when compared

TABLE I. Inhibitory Effects of Normal and Polycythemic Rabbit Sera on Heme Synthesis in Normal Rabbit Bone Marrow Cultures.

Treatment of culture medium	No. of experiments	% ^{59}Fe incorporation[a] into heme ± SEM
20% newborn calf serum + 80% TCNCl-109	(5)	7.68 ± 0.49
20% normal rabbit serum + 80% TCNC-109	(5)	4.91 ± 0.11[c]
20% polycythemic rabbit serum[b] + 80% TCNC-109	(5)	1.98 ± 0.14[d]

[a] Three culture plates/experiment.
[b] Serum from rabbits with hematocrit value of 61% or above.
[c] Mean value significantly ($P < 0.01$) less than that of newborn calf serum controls.
[d] Mean value is significantly ($P < 0.001$) less than that of newborn calf serum controls.

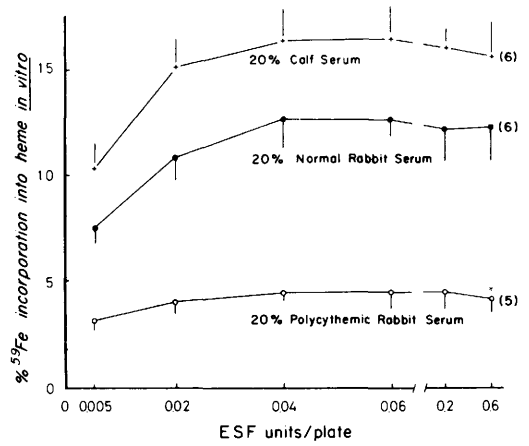

FIG. 1. Effects of erythropoietin on heme synthesis in normal rabbit bone marrow cultures treated with 20% newborn calf, 20% normal, and 20% polycythemic rabbit sera. *indicates significantly ($P < 0.001$) different when 20% normal rabbit sera were compared at each dose of erythropoietin. The vertical line at each point indicates standard error of mean.

with that of newborn calf serum controls. In addition, this inhibitory activity was increased exponentially with increasing concentrations of test sera in the cultures and was directly proportional to the increase in hematocrit in the polycythemic rabbits. This inhibitory effect of polycythemic or normal serum on heme synthesis in the bone marrow cultures could be due to the presence of cytotoxic factors or inhibitor(s) of ESF, such as anti-ESF in the test sera. These inhibitors could also be acting on a rate-limiting step in heme synthesis. Cytotoxic factors can be ruled out as a possible mechanism because (a) the number of viable nucleated cells in the cultures with the test sera did not decrease significantly before and after incubation (6), (b) nonspecific cytotoxic effects would be expected to distort the shape of the iron incorporation curve, but no distortion was seen in our studies, and (c) the inhibition in heme synthesis seen with the test sera disappeared completely after 72 hr incubation as indicated by the cumulative heme synthesis in the cultures (10). Differences in the iron pools of the rabbit bone marrow cultures with normal and polycythemic rabbit sera were considered as a possible reason for the reduced radioiron incorporation into heme in the marrows incubated with polycythemic sera. However, the serum iron values in the normal rabbit sera measured in these studies were not significantly different from that of the polycythemic sera, which clearly indicates that changes in the unlabeled iron pools in the cultures did not affect our results. The existence of antagonists to ESF in the test sera can also be excluded by the fact that the inhibition was observed in the cultures without detectable ESF (Table I) and, in addition, heme synthesis remained inhibited even when high dosages of ESF were added to the cultures with the test sera (Fig. 1). We have found in previous studies (10) that peak heme in bone marrow cultures synthesis was delayed by 24 hr by the addition of polycythemic rabbit serum when compared with that of controls. Therefore, the most likely explanation for this delay in heme synthesis produced by the serum inhibitor may be to prevent the erythroid cells from entering the generative cell cycle. It is not known whether this postulated action of the inhibitor on the cell cycle is due to inhibition of a step in heme synthesis.

The nature of the above-mentioned inhibitor is not completely clear in this study; however, we have examined some of its properties. This inhibitor, as well as an inhibitor in uremic serum, were found to be a low-molecular-weight substance between 1000 and 50,000 using an amicon membrane filtration system (6). This inhibitor is tissue-specific since the inhibitory activity was not only increased exponentially with increasing concentrations of test sera in the cultures (Fig. 2) but was also directly proportional to the increase in hematocrit in the polycythemic

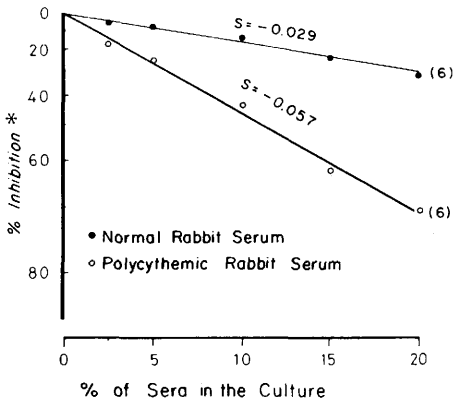

FIG. 2. Relationship between inhibitory effects of test sera on heme synthesis and concentration of test sera in the cultures.

$$* = \left(1 - \frac{\text{heme synthesis of test serum + ESF (0.02 unit)}}{\text{heme synthesis of calf serum + ESF (0.02 unit)}}\right) \times 100.$$

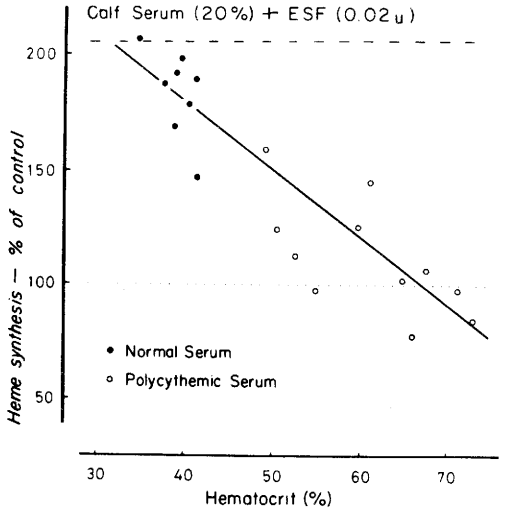

FIG. 3. Relationship between inhibitory effects of sera from normal and polycythemic rabbits on heme synthesis *in vitro* and hematocrit values. Each point indicates heme synthesis ESF (0.02U) + 10% test sera in the cultures. Correlation coefficient of regression line is − 0.895 with $P < 0.001$.

rabbits (Fig. 3). The pattern of the curves of inhibitory activity on heme synthesis in the cultures with normal or polycythemic rabbit sera were very similar. It is quite possible that the inhibitors in polycythemic and normal rabbit sera are the same.

There are several earlier reports of an erythropoietic inhibitor in association with experimental polycythemia in human subjects. These workers (1–4, 11) have reported factor(s) in serum and plasma which depress erythropoiesis *in vivo* when injected into experimental animals. The failure of some investigators (7) to demonstrate an inhibitor of erythropoiesis in serum from polycythemic rabbits may be related to the differences in the assay systems used or the time interval between the transfusion to induce the polycythemia and the removal of the serum for the detection of the inhibitor. The relationship between these inhibitors and our present findings is not clear. Although there are not studies designed to reveal the inhibitory effects of normal and polycythemic sera on the rate of heme synthesis in erythroid cells *in vitro*, the serum inhibitor observed in our system may be related to the inhibitor (erythrocyte chalone) reported by Kivilaakso and Rytomaa (5). These investigators demonstrated that the serum obtained from polycythemic rats inhibited ^{3}H-thymidine incorporation in normal rat bone marrow cells *in vitro* when compared with normal serum.

Summary. The effects of sera from normal and polycythemic rabbits on the rate of heme synthesis in erythroid cells were studied *in vitro* in rabbit bone marrow cultures. Sera from normal or hypertransfused rabbits significantly inhibited ^{59}Fe incorporation into heme in normal rabbit bone marrow cultures when incubated alone or with erythropoietin (ESF) when compared with the response in controls with newborn calf serum alone or with ESF. A significantly greater inhibitory effect was seen with sera from polycythemic than from normal animals. This inhibitory activity increased exponentially with increasing concentrations of the test sera in the cultures, and was also directly proportional to the increase seen in the hematocrit of the polycythemic rabbits. These results suggest that the inhibitor in both normal and polycythemic sera is tissue-specific and may control the rate of heme synthesis and erythroid cell proliferation. This inhibitor is a low-molecular-weight substance.

1. Krumbhaar, E. B., and Krzymowska, H., Blood **19,** 38 (1962).

2. Reynafarje, C., Ramos, J., Faura, J., and Villavicencio, D., Proc. Soc. Exp. Biol. Med. **116,** 649 (1964).

3. Whitcomb, W. H., and Moore, M. Z., J. Lab. Clin. Med. **66,** 641 (1965).

4. Moriyama, Y., Jap. J. Clin. Hematol. **10,** 701 (1969).

5. Kivilaakso, E., and Rytomaa, T., Cell Tissue Kinet. **3,** 385 (1970).

6. Fisher, J. W., Lertora, J. J. L., Lindholm, D. D., Tornyos, K., and Moriyama, Y., Proc. 3rd Ann. Clin. Dialysis Transplant Forum Washington, DC **3,** 22 (1973).

7. Erslev, A. J., and Thorling, E. B., Ann. N.Y. Acad. Sci. **149,** 173 (1968).

8. Krantz, S. B., Gallien-Lartigue, O., and Goldwasser, E., J. Biol. Chem. **238,** 4085 (1964).

9. Dixon, W. J., and Massey, F. J., Jr., "Introduction to Statistical Analysis," 3rd ed. McGraw-Hill, New York (1969).

10. Moriyama, Y., Lertora, J. J. L., and Fisher, J. W., XVI Annual Meeting, Amer. Soc. Hematol. Chicago, (Abst.). (1973).

11. Whitcomb, W. H., Moore, M. Z., and Rhoads, J. P., J. Lab. Clin. Med. **73,** 584 (1966).

Received May 7, 1974. P.S.E.B.M., 1974, Vol. 147.