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Erythroid Accelerating Activity of Rat Serum in Early Stage of Drug Induced Hemolysis

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UMENAI, T. and YOKOYAMA, M. *Erythroid Accelerating Activity of Rat Serum in Early Stage of Drug Induced Hemolysis.* Tohoku J. Exp. Med. 1998, **186** (3), 181-191 —— An increase in the number of erythroblasts can be seen to some extent in the bone marrow of rats in the early stage of experimentally induced hemolytic anemia prior to any elevation in the plasma erythropoietin (Epo) level. This observation suggests that there is another erythroid stimulating factor present other than Epo. We studied the enhancing effect of serum, taken sequentially during experimentally induced hemolysis in rats, on erythroid proliferation, differentiation and maturation in vitro. Single intraperitoneal injection of 60 mg/kg of acetylphenylhydrazine (APH) induced self-limited hemolytic anemia in rats, in which the hematocrit dropped rapidly with a nadir at day 4 after APH injection, followed by a gradual increase with return to normal level by day 8. Serum obtained consecutively every day after APH injection from day 1 to day 7 was applied to an in vitro culturing system of erythroid progenitors. Addition of day 1 serum, in which an elevation of Epo level had not occurred, to a conventional methyl-cellulose culture of rat bone marrow mononuclear cells (BM-MNCs) resulted in a significant increase in the number of colonies derived from colony forming unit erythroid, but not in burst forming unit erythroid. This erythropoietic activity of the serum was particularly evident in the presence of Epo. In the liquid culture of BM-MNCs, day 1 serum also showed some enhancing effect on erythroblast formation. We were able to see significant differences in these erythroid enhancing activities induced by serum drawn on day 1 in comparison to the serum drawn on subsequent days. These results suggest that an unknown erythroid enhancing factor besides Epo stimulates erythropoiesis in the early stage of hemolytic anemia or sudden hypoxia before there is a measurable rise in the serum Epo level. We propose that this factor be termed erythroid accelerating factor (EAF). ——
rat serum; experimentally induced hemolysis; colony forming unit erythroid; acceleration of erythropoiesis

In general, hypoxia increases renal secretion of erythropoietin (Epo) which stiumlates the production of new red blood cells (RBC). Therefore, hemolysis is followed by active erythropoiesis that shows a marked erythroid hyperplasia in bone marrow and also predominant reticulocytosis in peripheral blood. Drug-induced hemolytic anemia is a good method to observe the essential changes that

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occur in various parameters in the erythropoietic process. The hemolytic-anemia model devised for this study is a useful tool to obtain precise information about sequential changes that develop during the course of erythropoiesis, because it provides uniform and reproducible changes in the hematologic variables following acetylphenyl-hydrazine (APH) injection. Using this hemolytic-anemia model, we have previously observed that a distinct erythroblastosis occurred in rat bone marrow at day 1 of hemolysis, before there was any measurable elevation in the plasma Epo level (Itoh and Yokoyama 1988). This finding strongly suggests the presence of an additional specific enhancing factor for erythropoiesis besides Epo.

To elucidate the cause of this increase of erythroblasts observed in rat bone marrow in the early stage of hemolysis, we assayed the enhancing effects of consecutive-day serum samples taken from rats with APH-induced hemolytic anemia on erythroid progenitors, erythroblast formation and Hb synthesis of rat bone marrow mononuclear cells (BM-MNCs).

We conducted our research on the hypothesis that an unknown erythroid enhancing factor is developed that facilitates Epo activity in the early stage of sudden anemia and that this factor plays an important role to accelerate RBC production.

The stimulating factors to late erythroid progenitors in normal and anemic mice (Blanchet et al. 1984; Arnaud et al. 1989), proerythroblast stimulating activity (PSA) (Udupa and Lipschitz 1988), erythroblast enhancing factor (EEF) (Krystal 1983), B-lymphocyte derived burst promoting activity (BPA) (Feldman and Dainiak 1989; Feldman et al. 1992), erythroid potentiating activity (EPA) (Golde et al. 1980) have all been previously as erythroid enhancing factors. In addition, many kinds of cytokines, such as stem cell factor (SCF), interleukin 3 (IL-3), IL-6 etc., act on erythroid progenitors in concert with Epo and contribute to the increase in RBC production (McNiece 1992). However little is known in detail about how those factors and cytokines, whether alone or in combination, work in vivo to regulate erythropoiesis. We have undertaken to confirm the presence of erythroid enhancing activity in rat serum at an early stage of anemia and to determine its role in erythropoietic regulation.

Materials and Methods

Treatment of rats

Hemolytic anemia was induced in Wister rats weighing 180–200 g by intraperitoneal injection of 60 mg/kg APH dissolved at a concentration of 20 mg/ml in 20% ethanol on the first experimental day (day 0). Peripheral and bone marrow blood was taken for the following examinations on consecutive days after APH injection.

Hematologic examination

Peripheral blood was obtained from abdominal aorta exposed surgically

under ether anesthesia. Ht was measured using a hematocrit meter (Compur 1110, Sankyo Co., Tokyo), and the reticulocyte count was determined in a standard fashion with brilliant cresyl blue.

Epo levels in serum were assayed according to radioimmunoassay, using a conventional assay kit (Recombigen Epo kit, Japan DPC., Tokyo).

Bone marrow blood was flushed from extirpated femoral bone with 5 ml of cooling heparinized RPMI 1640 (GIBCO, Grand Island, NY, USA) medium using a syringe. An aliquot (0.3 ml) of it was used for differential count of nucleated cells on cytospine preparation stained with Giemsa.

MNCs were recovered from the remaining sample by Percoll density gradient centrifugation (SG: 1.044, 400 *g*, 30 minutes) and washed twice in the medium.

Sera

Blood was taken from the abdominal aorta on consecutive days. After clotting, the serum was separated by centrifugation and dialyzed against 0.05 M Tris buffer (pH 7.4) overnight. It was stored at $-20^{\circ}C$ and heat-inactivated ($56^{\circ}C$), and was sterilized through millipore filter before use.

CFU-E and BFU-E assay

We cultured BM-MNCs according to a previously described method (Iscove and Sieber 1975) with slight modification. The mixture containing 0.8% methylcellulose in α-minimal essential medium (GIBCO), 30% heat-inactivated fetal calf serum (FCS) (GIBCO), 1% deionized bovine serum albumin (BSA) (GIBCO), 10^{-6} M 2-mercaptoethanol (2-Me) (SIGMA, St. Louis, MO, USA), 1～2 U/ml of Epo (SIGMA), 8% each testing serum and 10^5 MNCs/ml were incubated at $37^{\circ}C$ in humidified air with 5% CO_2. On day 4 after culturing, colonies derived from colony forming unit erythroid (CFU-E) which contained more than 8 cells positively stained with benzidine were counted using an inverted microscope.

In the culture of burst forming unit erythroid (BFU-E), 2 U/ml of Epo was used, but no IL-3 or any other condition medium was added to any serum samples to see BPA activity. Colonies derived from BFU-E were counted on day 7.

Liquid culture

BM-MNCs were suspended in liquid culture at a cell concentration of 1×10^6/ml in RPMI 1640 medium supplemented with 15% FCS, 1% BSA, 10^{-6} M 2- Me, 1 U/ml of Epo and 8% testing serum, and incubated at $37^{\circ}C$ in humidified air with 5% CO_2. After 24 hours in culture, the differential counts of the cells were performed using a cytospin preparation with Giemsa stain.

Hb synthesis in vitro

Hb synthetic activity of BM-MNCs was estimated by ^{59}Fe uptake. Rat BM-MNCs (10^6/ml) were preincubated for 18 hours in medium containing the

same constituents as described above for liquid culture, followed by incubation with 0.2 μCi/ml of $^{59}FeCl_3$ for 6 hours. After that the cells were harvested and washed 2 times with RPMI 1640 medium. Radioactivity of the cells was counted by γ-counter.

Statistical analysis

Statistical comparisons were made using Student's t-test and one way analysis of variance (ANOVA) when appropriated.

Results

Changes in hematologic parameters in the course of APH induced hemolysis

Consistent with the prior experience of many workers who have used this hemolytic-anemia model to induce self-limited hemolytic anemia, the single dose of 60 mg/kg APH given intraperitoneally provided a reproducible and uniform pattern of hematologic changes in the rats in this study.

As shown in Fig. 1, APH injection resulted in a marked decrease of hematocrit (Ht) reaching a nadir on day 4, which was then followed by a gradual increase back to normal level on day 7. In contrast to Ht, plasma Epo showed a striking increase with peak value on day 4. The number of colonies derived from CFU-E also increased in parallel with Epo. The reticulocye ratio rose incrementally starting from day 3 (Fig. 2).

Histologic study of the bone marrows performed on consecutive days throughout the experiment (Itoh and Yokoyama 1988) revealed that erythroblastosis in response to hemolysis occurred more rapidly than would be expected.

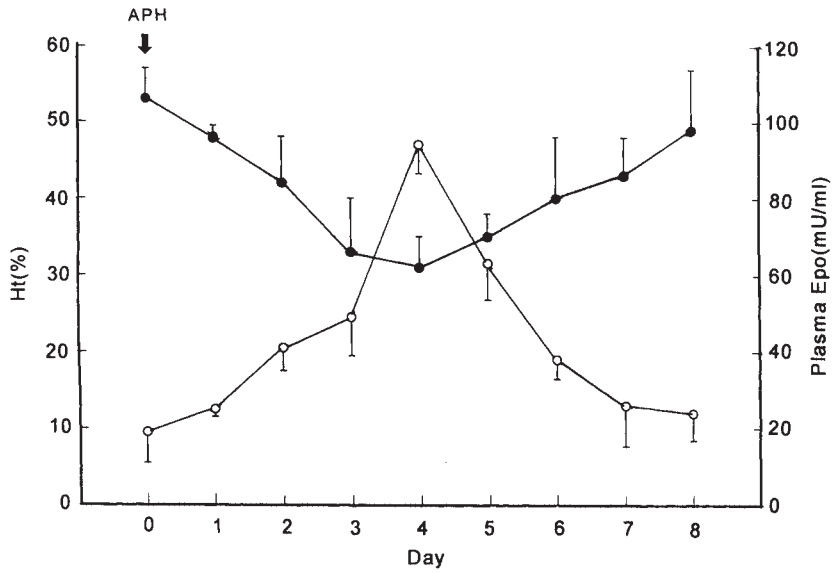

Fig. 1. Changes in Ht and Plasma Epo in the time course of APH induced hemolytic anemia of rats.
Values are mean of 7 rats. Vertical lines show standard deviation.
●, Ht; ○, Epo.

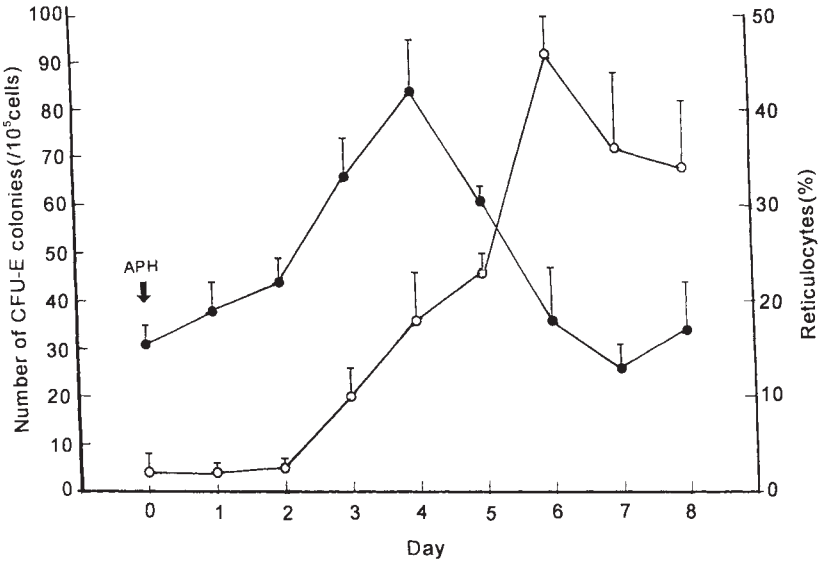

Fig. 2. Changes in number of CFU-E colony and ratio of reticulocyte in the time course of APH induced hemolytic anemia of rat.
Values are mean of 7 rats. Vertical lines show standard deviation.
●, CFU-E; ○, reticulocyte.

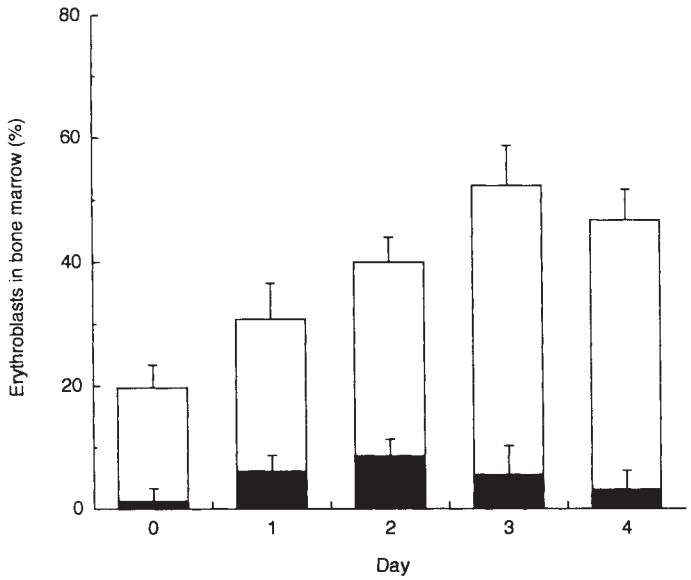

Fig. 3. Changes in ratio of BM-erythroblast induced by APH injection.
Values are mean of 5 rats. Vertical lines show standard deviation.
□, total erythroblasts; ■, basophylic erythroblasts.

As shown in Fig. 3, the ratios of mature and immature erythroblasts increased shortly after starting hemolysis (on day 1), before the serum Epo level was significantly elevated. The peak ratio of erythroblasts was found on day 3.

Although the procedure of using flushed samples from different bones of different size made it difficult to compare the absolute number of erythroblasts from different marrow-sample sites, an increase of erythroblast ratio is indicative of enhanced erythropoiesis.

Erythroid enhancing effect of serum obtained from hemolytic rats

The effects on erythroid progenitors. Serum was taken from the hemolytic rats on consecutive day from day 0 to day 4. We examined the enhancing effects of each day's serum on the colonies derived from BFU-E and CFU-E from normal rats. In this experiment, a high concentration (2 U/ml) of Epo was employed to see BPA of the serum and also to minimize the influence of the intrinsic increase of plasma Epo due to anemia. As shown in Fig. 4, the largest increase in the number of CFU-E colonies was seen in day 1 serum, which showed a significantly higher activity compared to the other sera. On the other hand, no serum samples showed any significant increase in the number of BFU-E colonies. This indicates that a stimulating factor for late erythroid progenitors (CFU-E), but not for early erythroid progenitors (BFU-E), was present in day 1 serum.

In another experiment, the ability of day 1 serum to increase CFU-E colonies only occurred in the presence of Epo. Synergistic effect of day 1 serum and Epo on CFU-E proliferation was found (Fig. 5).

The effect on erythroblast formation in vitro. In vitro formation of erythroblasts was examined in liquid culture with or without adding the serum obtained from rats on each consecutive day after hemolysis.

Erythroblasts were identified morphologically with Giemsa stain of the

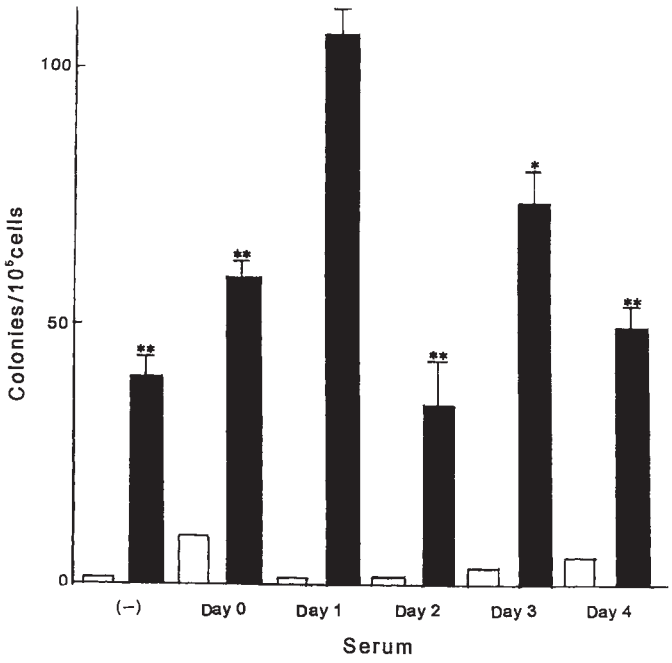

Fig. 4. Erythroid enhancing effects of serum obtained consecutively from APH induced hemolytic rats.
Values are mean of 4 cultures. Vertical lines show standard deviation.
In this study, 2 U/ml of EPO were used to see burst promoting activity (BPA). □, BFU-E; ■, CFU-E. $^{*}p<0.05$, $^{**}p<0.01$.

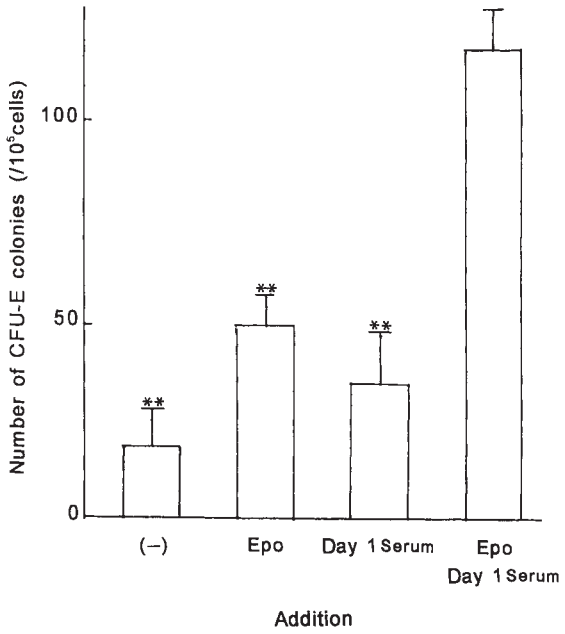

Fig. 5. Synergistic effect of day 1 and Epo serum on CFU-E proliferation in the culture of normal rat BM-MNCs.
Values are mean of 4 cultures. Vertical lines show standard deviation.
$^{**}p<0.01$.

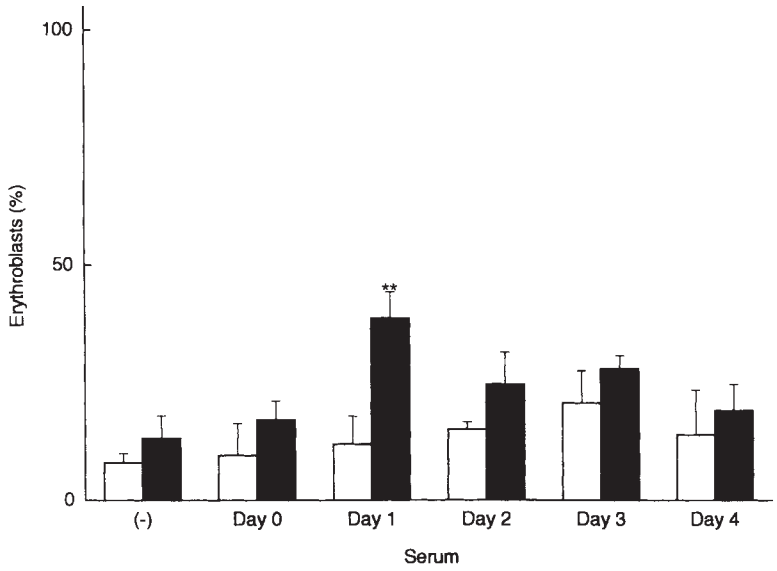

Fig. 6. Enhancing effect of day 1 serum and Epo on erythroblast formation in the liquid culture of normal rat BM-MNCs for 24 hours.
Values are mean of 5 rats. Vertical lines show standard deviation.
$^{**}p<0.01$ (vs. others).
□, Epo(−); ■, Epo(+).

cytospin preparation of cultured cells. Young erythroblasts increased markedly with colony-like appearance after incubation with day 1 serum in culture for 24 hours. Fig. 6 shows that the most significant increase in the ratio of erythroblasts was obtained in day 1 serum, which needed the presence of Epo to exhibit an

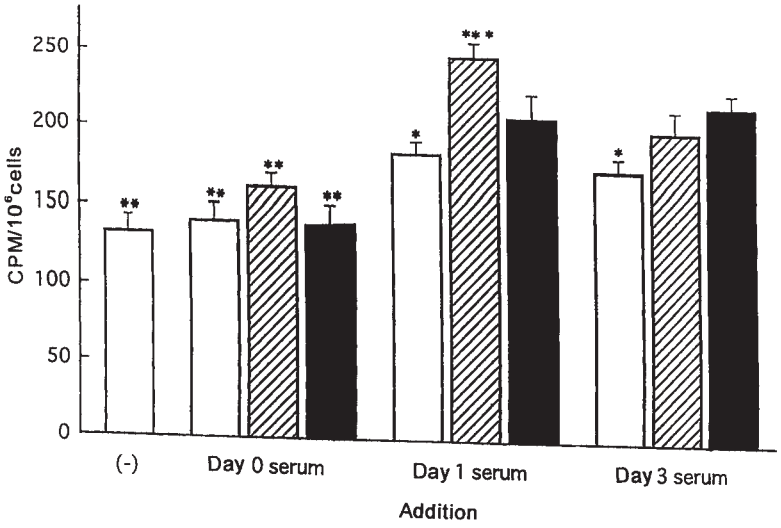

Fig. 7. Effect of serum obtained consecutively from APH induced hemolytic rats on Hb synthesis.
Results are shown as ^{59}Fe incorporation in the liquid culture of normal rat BM-MNCs.
Values are mean of 5 rats. Vertical lines show standard deviation.
$*p<0.05$, $**p<0.01$ (vs. day 1 serum with Epo 0.125 U/ml).
□, Epo(−); ▨, Epo, 0.125 U/ml; ■, Epo, 0.25 U/ml.

enhancing activity for erythroblast.

The effect on Hb synthesis. Concurrent addition of day 1 serum and 0.125 U/ml of Epo to the culture medium enhanced ^{59}Fe incorporation into normal rat BM-MNCs to a significant degree, compared with the addition of day 0 serum both with and without addition of Epo. However, no significant differences were found between the enhancing activities of day 1 and day 3 serum with Epo added. Also, there was no evidence of Epo dependency in any of the serum samples on any day at Epo concentrations from 0.125 U/ml to 0.25 U/ml (Fig. 7).

Discussion

The studies reported in this article demonstrate that rat serum in an early stage of drug-induced hemolysis exhibits distinct erythroid stimulating activity. The day 1 serum, which was obtained on 1 day after starting hemolysis, had the effects of increasing CFU-E colonies in semisolid cultures and enhancing erythroblast formation in liquid culture of rat BM-MNCs.

Although no significant differences were found between day 1 and day 3 serum, Hb synthesis was stimulated to some extent by the addition of day 1 serum.

The stimulating activity to both CFU-E and erythroblasts in day 1 serum were remarkably dependent on the presence of Epo.

This stimulating activity found in day 1 serum suggests the possible presence of a cofactor of Epo for stimulating erythropoiesis. Blanchet et al. (1984) and Arnaud and Blanchet (1986) have detected a stimulating factor to late erythroid

precursors in normal mouse serum that was termed erythropoietic stimulating cofactor (ESCF) because of its Epo dependency. A similar line of study (Arnaud et al. 1989) revealed the presence of another facilitating activity for erythropoiesis in anemic mouse serum (AMS) when anemia was induced by phenylhydrazin administration. Molecular weights of these factors were determined to be 50 kDa in ESCF and 110 kDa in AMS respectively. It is interesting to consider whether or not those factors reported in mice are similar to the erythroid enhancing activity found in day 1 serum in this study.

The hemolytic-anemia model in the rat caused by a single intraperitioneal injection of APH (60 mg/kg) is considered to be a useful tool to assess the chronological changes of various hematologic parameters in the erythropoietic process. We have extensively studied the sequential changes that occur in metabolism, various enzymes (Toki et al. 1989; Arai and Yokoyama 1991; Toki and Yokoyama 1991) and erythropoietic parameters (Yokoyama et al. 1987; Itoh and Yokoyama 1988) in the course of hemolysis and red cell regeneration using this hemolytic-anemia model.

Through those previous studies, we have suspected that is a factor with erythroid-enhancing activity in the early stage of hemolysis which is a different from Epo but which may be an activity facilitating Epo action.

The hemolytic-anemia model provides a lag period of more than 24 hours before there is a definite rise in the plasma Epo level. Therefore, the erythroid enhancing activity of day 1 serum has been successfully detected and is considered to be distinguishable from Epo. This activity in day 1 serum was not detectable when anemia was induced by blood drawing. That might be due to a prompt increase of Epo induced by bleeding with shorter lag time before the Epo level rises.

Concurrent addition of day 1 serum with Epo to BM-MNCs caused a significant increase in the number of erythroblasts as well as CFU-E colonies.

Udupa and Lipschitz (1988) reported a partially purified proerythroblast stimulating factor (PSA) in normal mouse serum, which is considered to be similar to the activity of day 1 serum described in this article. However, it might be different from PSA because it develops only in serum in an early stage of hemolysis, but not in normal serum.

Also, Krystal (1983) described an EEF in human and mouse serum that significantly stimulated ^{59}Fe incorporation in cultures of mouse bone marrow cells. It acts synergistically rather than additively with Epo. Consequently, the author hypothesized that this stimulator acts late in erythroid differentiation, especially on hemoglobin-producing erythroblasts. The activity of day 1 serum in this study also mimics EEF because it had an enhancing effect on erythroblast formation in the culture of rat BM-MNCs. However, the day 1 serum showed no significant synergism in ^{59}Fe uptake with Epo. These findings suggest that the activity of day 1 serum is different from EEF and seems to act on a broader

spectrum of erythroid differentiation. The day 1 serum is considered to accelerate differentiation both from BFU-E to CFU-E and from CFU-E to young erythroblasts in the erythropoietic process.

In this study, we employed unpurified serum which must contain many kinds of stimulating factors for erythropoiesis, including various cytokines. Therefore, the possibility may exist that the activities of day 1 serum are complicated by other stimulating factors.

Up to the present, notable stimulating activities has been known to be associated with many cytokines, such as stem cell factor (SCF) (Witte 1990), IL-3 (Sunderland and Roodman 1991), IL-4, IL-6, IL-9 and IL-11 (Musashi et al. 1991; McNiece 1992), which all need the coexistence of Epo to achieve erythroid differentiation. Moreover, B-lymphocyte-derived burst promoting activity (Feldman and Dainiak 1989; Feldman et al. 1992), T-lymphocyte erythroid potentiating activity (EPA) (Golde et al. 1980), activin (EDF) (Nakao et al. 1991) and insulin like growth factor (IGF) (Sanders 1993) have been reported to have erythroid enhancing activities. However, little has been known in detail about their physiological and biological mechanisms of action in regulating erythropoiesis. One possibility may be that erythropoiesis in vivo is ordinarily regulated by the net effect of their functions in association with various erythropoietic inhibitors. (Axelrad et al. 1990). Another possibility is that many kinds of degradation products of red blood cells which are abundant in day 1 serum can stimulate erythropoiesis. In another study (not shown in this article), the hemolysate prepared with APH in vitro and hemin showed no stimulating activity for colonies of CFU-E or erythroblast formation in cultures of normal rat BM-MNCs.

The erythroid enhancing activity of the day 1 serum seemed to be peculiar, because this activity was definitely exhibited in an earlier-than expected time frame, at an early stage of hemolysis. This suggests that the activity may contribute to a rapid erythrocyte production in response to sudden hypoxia before the Epo level has had time to rise. It may act as a cofactor for Epo and accelerate erythropoiesis especially when there is an acute and critical reduction of red blood cells.

We propose that this factor be termed erythroid accelerating factor (EAF). Further research is in progress to purify and to determine the precise biochemical and biological properties of this factor.

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