The Effect of Erythropoietin on Human Bone Marrow Cells In Vitro. I. Studies of Nine Cases of Bone Marrow Failure

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The in vitro response to erythropoietin of bone marrow from nine patients with bone marrow failure were studied. Two types of patients were observed, those in which the marrow was responsive to erythropoietin and those which were nonresponsive. Ferrokinetic data corresponded well with the response to erythropoietin in vitro. In the nonresponsive group, a recovery of sensitivity to erythropoietin was observed when the patients improved clinically. The nature of the bone marrow failure was discussed in relation to erythropoietin and stem cells.

SEVERE PANCYTOPENIA, refractory to the usual hematopoietic agents, has been variously designated as aplastic anemia,[1] refractory anemia,[2] aregenerative anemia,[3] hypoplastic anemia,[4] or bone marrow failure.[5] It has been demonstrated that the level of erythropoietin is significantly higher in this condition than that found in other anemias of comparable degrees of severity.[6-9] Therefore, it has been postulated that there may exist abnormalities in the primitive stem cells,[10-13] which normally would be responsive to erythropoietin.[14-19] Since the term "bone marrow failure" seems to cover the hematological status of all the cases described in this paper, it will be used subsequently in this report.

The present work was undertaken to investigate the kinetics of erythroid stem cells and a possible relationship to the pathogenesis of bone marrow failure. In particular, the response of cultured bone marrow cells to erythropoietin was evaluated and correlated with the clinical course of the disease. Two types of response to erythropoietin were observed. In one group of patients, cultured bone marrow cells responded to the addition of erythropoietin by increasing heme synthesis. In the second group of patients with

bone marrow failure, erythropoietin did not increase heme synthesis in the cultured marrow cells.

MATERIALS AND METHODS

Nine adult hospitalized patients with bone marrow failure were studied (Table 1). The diagnosis of bone marrow failure was based on the finding of pancytopenia, refractory to the usual hematopoietic agentes (iron, vitamin B_6 or B_{12}, or folic acid). Additional evidence for the diagnosis of bone marrow failure were hypocellularity of bone marrow aspirated from the sternum or iliac crest and decreased erythropoiesis as measured by ferrokinetics utilizing ^{59}Fe.[20] Two patients (H.K. and M.K.) were found to have patchy foci of hyperplasia with normal bone marrow elements in specimens aspirated from the sternal marrow, as has been previously described in this disorder.[2,21] However, all nine patients, including these two, had hypoplasia in specimens obtained from the iliac crest. Cases, M.K. and Y.H.1, showed a normal red cell utilization of radioiron, but case M.K., who died of pulmonary hemorrhage, showed at necropsy a generally hypoplastic bone marrow except for patchy areas of hypercellularity in the sternal marrow. Patient Y.H.1, was found to have hypocellularity of both sternal and iliac crest bone marrow on histological sections. In all nine cases, gross evidence of increased blood destruction was absent and infiltrative disease of the marrow could not be demonstrated. Moreover, none of these patients had chronic infection, malignancy, malnutrition, renal disease, liver disease, or disorders involving the spleen.

Among these cases, six patients (H.K., Y.H.1, M.H., H.Y., Y.H.2 and T.W.) had histories of contact with organic solvents, insecticide, chloramphenicol, or rauwolfia alkaloids. However, considering the chronological relationships or dosage used, it is unlikely that these drugs caused the disease, except possibly in two cases (H.K. and Y.H.2). Case H.K. had been working as a painter for 5 yr in a factory where he was suspected to have been exposed to benzol up until the time he noticed anemia. Case Y.H.2 had

Table 1.—Hematological Data From Nine Patients With Bone Marrow Failure at Time of Admission

Name	H.K.	M.K.	Y.H.1	N.Y.	F.N.	M.H.	H.Y.	Y.H.2	T.W.	Normal Range
Sex	M	F	M	F	M	F	M	M	M	
Age, years	30	22	48	56	24	46	38	39	53	
Peripheral blood										
RBC/cu mm. × 10⁴	132	142	304	314	204	174	326	115	125	
Hemoglobin, g/dl	4.1	3.5	8.7	7.6	6.6	5.7	9.0	4.0	4.6	
WBC/cu mm.	4100	3800	3200	2250	2100	2350	2600	1800	2100	
Thrombocyte/cu mm. × 10⁴	0.5	1.6	0.8	1.8	2.2	1.2	0.3	1.5	0.1	
Reticulocyte %	2.2	2.3	1.8	0.4	1.5	1.2	0.3	0.1	2.4	
Bone marrow*										
Nucleated cell count/ cu mm. × 10⁴	50.1	26.3	10.0	2.1	1.3	9.6	4.8	5.5	12.0	
Megakaryocyte count/ cu mm. × 3	15	2	0	0	1	0	0	4	0	
Lymphocyte + reticulum cell %	18.4	14.8	36.0	62.8	65.5	26.4	59.2	30.0	40.0	
Erythroblast %	19.2	45.0	29.6	11.4	9.0	26.4	6.0	26.8	32.2	
M:E	3.9	0.9	1.3	2.3	2.9	1.0	5.0	1.6	0.6	
Ferrokinetics										
PID, min	270	148	135	172	114	120	420	255	290	70–140
RCU %	63	100	76	9	59	45	5	16	44	80–100
Serum iron, µg/dl	271	65	98	198	190	204	226	235	135	
Erythropoietin in plasma,† U/ml	4.0			1.0	5.5	6.6	1.0	6.7	4.8	
Response to erythropoietin in vitro	+	+	+	+	+	+	−	−	−	

*Marrow findings from sternal puncture, at time of culture.
†Erythropoietin activity in plasma, assayed by starved rat using a modification of Fried's method.[22]
PID, plasma iron disappearance rate; RCU, red cell utilization of iron.

been injected with 1 g/day of chloramphenicol for 2 months prior to the onset of the disease.

The bone marrow cells were cultured in the presence of erythropoietin by the method of Krantz.[18] In brief, human bone marrow cells aspirated by sternal puncture were washed twice with cold NCTC 109 (Difco) solution and suspended in an incubation medium consisting of 60% NCTC 109 solution, 40% heat-inactivated human plasma of AB type, plus 50 U/ml of penicillin. The plasma was obtained from a hematologically normal volunteer.

Eight tenths of an ml of a bone marrow cell suspension which had been previously adjusted with media to a cellularity of 2000–4000 nucleated cells per cu mm, was placed into a 35 × 10 mm plastic tissue culture dish (Falcon Plastics) and then incubated in the presence of erythropoietin in an atmosphere consisting of 5% CO_2 in air at 37°C for a period of 72 hr. The erythropoietin was obtained from the urine of a patient with bone marrow failure by means of 80% acetone precipitation and ammonium sulfate fractionation. The lyophilized erythropoietin preparation had a potency of 14 U/mg dry weight (assayed by starved rat[22]). The erythropoietin preparation was dissolved in saline and sterilized through a Millipore filter prior to use.

Six hours prior to the termination of incubation, 3 μCi of radioactive iron ($^{59}FeCl_3$, specific activity 7.2 mCi/mg diluted in HCl), preincubated with 0.1 ml of type AB plasma, was added to each dish. At the conclusion of incubation, the cells were washed and centrifuged three times with cold buffered saline. The heme was extracted from the packed cells with acid methylethylketone as described previously.[23] The methylethylketone layer containing heme was dried on a steel planchet and its radioactivity was counted in a gas-flow Geiger counter. Each culture was done in duplicate or triplicate. The rate of heme synthesis was expressed as a percentage of that of a control culture grown without erythropoietin.

Bone marrow from case H.K., M.K., Y.H.1, N.Y., F.N., or M.H. was cultured once on their admission, while bone marrow from H.Y. was cultured four times during his course (2, 6, 11, and 13 months after treatment) and bone marrow from Y.H.2 and T.W. were cultured twice, in relapse and in remission.

RESULTS

When the marrow cells from hematologically normal volunteers were cultured, the rate of heme synthesis was increased about 4 times over control levels by adding 0.2 U/ml of erythropoietin in the 10 cases studied. Addition
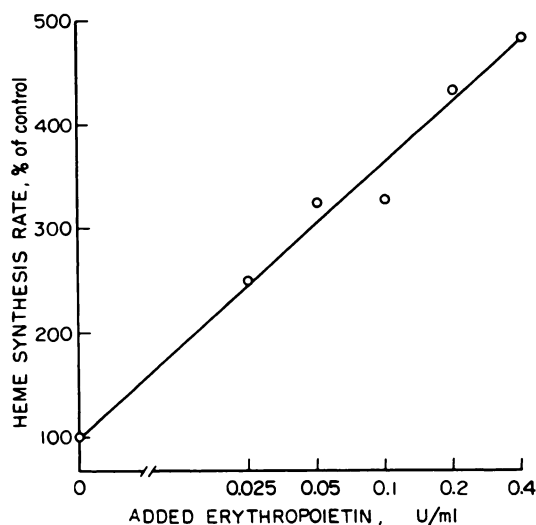

Fig. 1.—Erythropoietin dose-response curve of normal human marrow in vitro. The marrow was obtained from a 20-yr-old man (hematocrit 40%); 3600 nucleated cells/cu mm and 228,000 red blood cells/ cu mm were present in each plate with 40% homologous AB plasma. After 66 hr of incubation, 3 μCi of ^{59}Fe was added to each dish and the culture was terminated 6 hr later. Each point is the mean of duplicates. The concentration of erythropoietin added is expressed on a logarithmic scale.
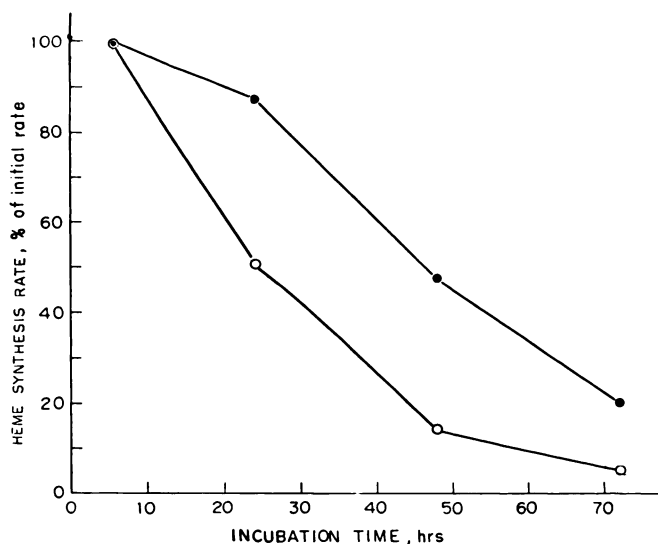
Fig. 2.—The effect of duration of in vitro incubation on normal human marrow cells with and without added erythropoietin. The marrow was a replicate of the one described in Fig. 1, and cell counts are the same. Controls (white circles) had no added erythropoietin; stimulated (black circles) had 0.2 unit per ml. Each point is the mean of duplicates and indicates the end of a 6-hr incubation period with 3 μCi ^{59}Fe per dish. The heme synthesis rate is expressed as a percentage of the value found in the first 6-hr incubation.

of erythropoietin up to 0.4 U/ml produced a linear increase in heme synthesis with a log dose of erythropoietin; inhibition of heme synthesis was observed at higher doses of erythropoietin. The inhibition may be due to impurity of erythropoietin as suggested by Krantz.[25] Figure 1 shows a typical dose response curve and Fig. 2 shows its time course.

Table 1 summarizes the hematological and ferrokinetic data in the nine cases of bone marrow failure in which the bone marrow cells were incubated in vitro with erythropoietin.

In six of the nine patients in relapse (H.K., M.K., Y.H.1, N.Y., F.N. and M.H.), the cultured bone marrow cells showed a linear relationship between heme synthesis and erythropoietin in the range of 0.025 to 0.2 U/ml of erythropoietin (Fig. 3). The inhibition of heme synthesis observed in cases M.K. and N.Y. at 0.2 to 0.4 U/ml of erythropoietin may be due to impurity of erythropoietin.

In the other three patients (H.Y., Y.H.2, and T.W.), the cultured bone marrow cells obtained in relapse did not respond to erythropoietin in doses ranging from 0.025 to 0.4 U/ml (Fig. 4). However, when in remission at a later date, all three responded in an almost normal fashion to added erythropoietin (Figs. 4, 5, and 6).

The responses of all cases to 0.2 U/ml of erythropoietin after 72 hr of incubation are summarized in Fig. 6. In both the responsive group and the initially nonresponsive group in remission, the degree of the response to
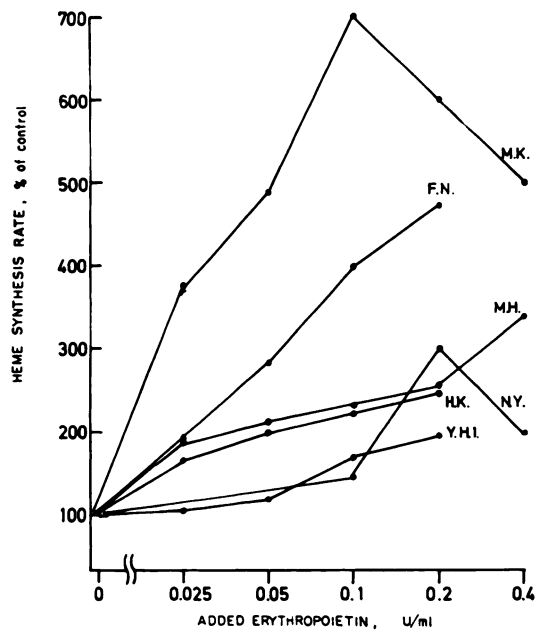
Fig. 3.—Erythropoietin dose-response curve in bone marrow failure. The marrow was obtained from the cases H.K., M.K., Y.H.1., N.Y., F.N., and M.H. Each point is the mean of duplicates or triplicates. The concentration of erythropoietin added is expressed on a logarithmic scale.
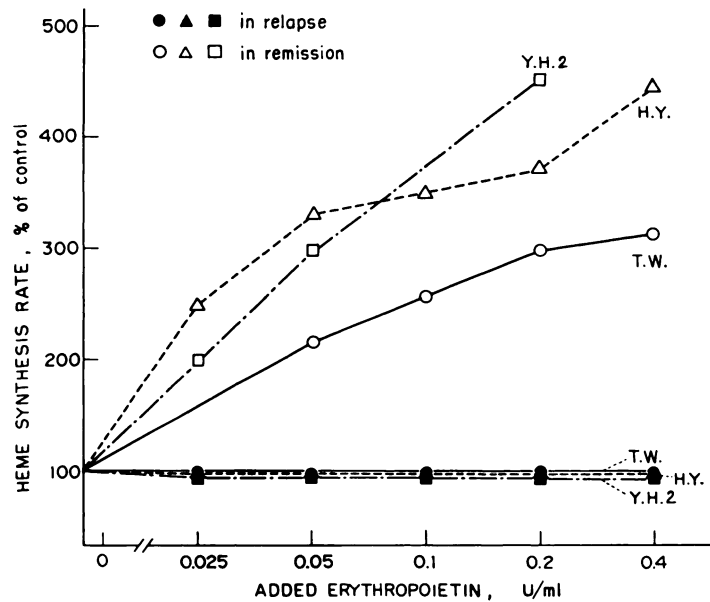

Fig. 4.—Changes in erythropoietin dose-response curves in bone marrow failure. In case H.Y., the response to erythropoietin was investigated on four occasions during his disease. The figure shows the response to erythropoietin 2 months (in relapse) and 13 months (in remission) after the beginning of the treatment. In case Y.H.2 and T.W., marrow was cultured twice, once in relapse and once in remission. The erythropoietin concentration is expressed on a logarithmic scale.
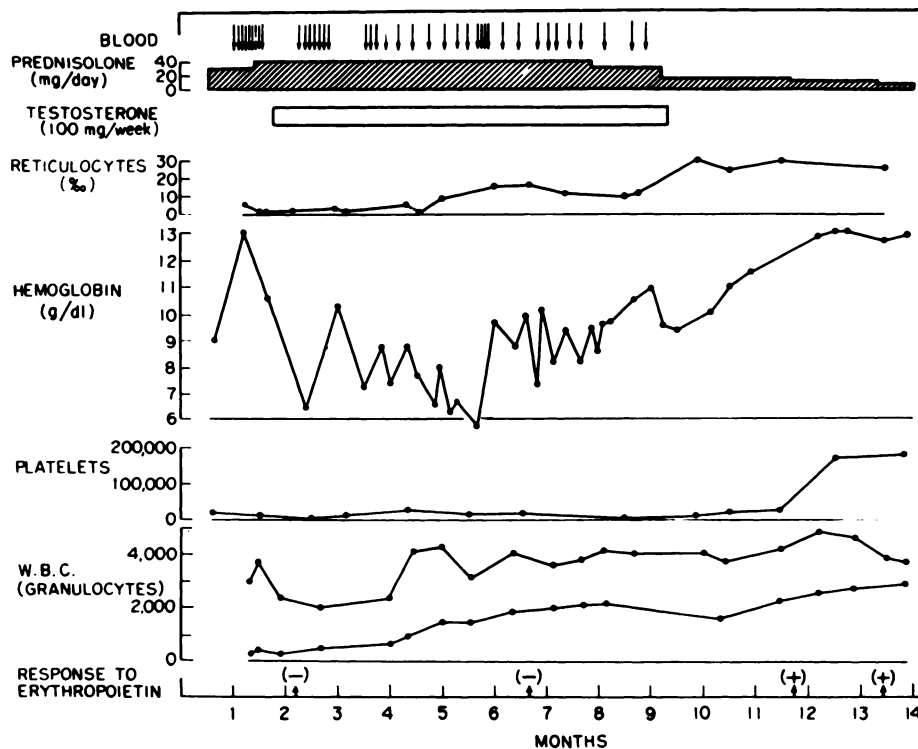
Fig. 5.—The clinical course of case H.Y. during treatment. The bone marrow of this patient was investigated four times as indicated at the bottom of the figure, the first two cultures did not respond to erythropoietin. The last two responded to erythropoietin.

erythropoietin was not significantly different from normal bone marrow cells.

Hematological data of the erythropoietin-responsive group were compared with those of nonresponsive group. As shown in Table 2, there was no significant difference between the two groups in their bone marrow morphologic findings. However, when the data on the ferrokinetic studies were compared between the two groups, plasma iron disappearance rates (T ½) were significantly prolonged in the nonresponsive group. The percent utilization of radioactive iron by peripheral red cells was also significantly decreased in the nonresponsive group.

When bone marrow cells from a normal individual were cultured in the presence of the plasma from a patient of the responsive group (case H.K.), heme synthesis was markedly increased up to 3 volumes percent of plasma, which corresponded to 0.05 U/ml of erythropoietin. Further increments of plasma inhibited heme synthesis ($p < 0.05$) (Fig. 7).

DISCUSSION

In the present study the response of cultured bone marrow cells from nine patients with bone marrow failure could be divided into erythropoietin-
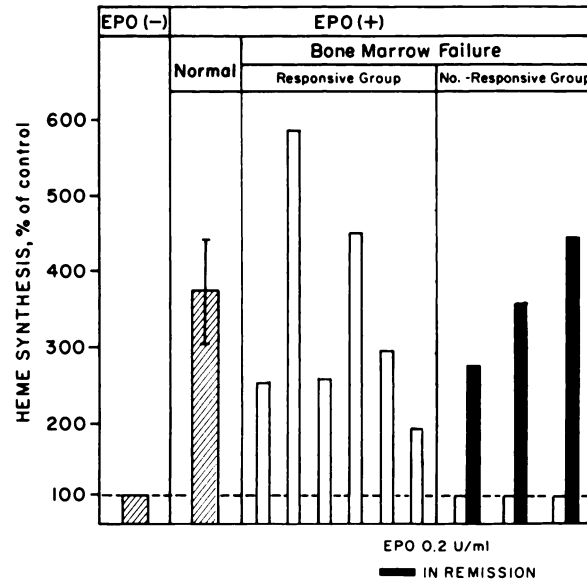
Fig. 6.—Summary of responses of marrow from normal patients and those with bone marrow failure to erythropoietin (EPO) in vitro. The figure shows the rates of heme synthesis of bone marrow in the presence of 0.2 unit of erythropoietin per milliliter of culture medium and are compared with control cultures without erythropoietin. The white columns show data from patients with active disease and the black columns show data from patients in remission. The results of normal marrow are the mean ± standard error from 10 normal volunteers.

responsive and nonresponsive groups. The most important action of erythropoietin is considered to be the induction of differentiation of primitive erythroid progenitor cells into erythroblasts.[14-19] From our findings, it can be suggested that in the nonresponsive cases of bone marrow failure, there is some disorder in the primitive stem cells' response to erythropoietin. In our studies, bone marrow cells obtained from normal individuals and patients with iron-deficiency anemia,[25] uremia,[26] or polycythemia vera[25] responded to erythro-

Table 2.—Comparison of Hematological Data and Ferrokinetics of Responsive Cases With Nonresponsive Cases

	Responsive Group	Nonresponsive Group	
Bone marrow			
Cell count × 10^4 per cu mm.	16.6 ± 7.7°	7.4 ± 2.3	NS
Erythroblast	21.8 ± 7.8%	21.7 ± 7.9%	NS
Lymphocyte			
+ reticulum cell	37.3 ± 9.0%	43.1 ± 8.5%	NS
Ferrokinetics			
PID, minutes	160.0 ± 21.0	321.0 ± 52.0	$p < 0.01$
RCU	58.7 ± 11.0%	21.6 ± 11.5%	$p < 0.05$
Serum iron, μg/dl	171.0 ± 62.1	198.7 ± 23.9	NS

° Mean value ± standard error of the mean.

PID, plasma iron disappearance rate; RCU, red cell utilization of iron; NS, not significant.
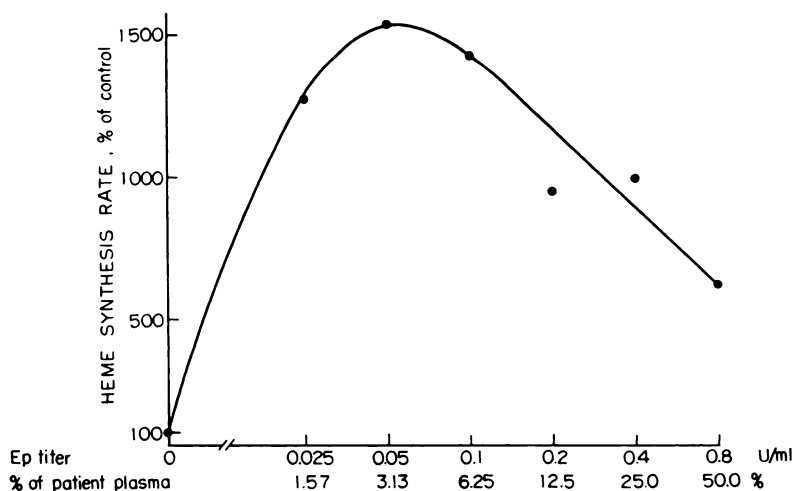
Fig. 7.—Response of normal bone marrow to the plasma from a patient with bone marrow failure. The bone marrow was obtained from a hematologically normal volunteer with the same blood type as that of the patient (H.K.). To obtain the indicated titers of erythropoietin, AB type plasma from a normal volunteer was used to dilute the patient's plasma. The amount of patient's plasma is expressed on a logarithmic scale.

poietin in this culture system. Therefore, the inability of the marrow cells from some patients with bone marrow failure to respond to erythropoietin may be specific to this disorder.

During clinical remission in the nonresponsive group, the bone marrow cells became sensitive to erythropoietin stimulation. Why these patients became sensitive to erythropoietin is not understood. It is of interest, however, to note that the response of bone marrow cells to erythropoietin in vitro seemed to reflect the clinical conditions.

It is also interesting that the patients in the nonresponsive group were clinically the most severely affected, often required blood transfusions, and had the most marked suppression of erythropoiesis as judged by ferrokinetic studies, but all of them eventually went into complete remission. In contrast, the patients in the responsive group followed rather chronic courses and none of them went into complete remission. In view of this, one might speculate that there are several different types of bone marrow failure. The mode of derangement of stem cells as well as the pathogenesis may differ amongst these groups, and the nature of these differences awaits clarification.

The question remains why the responsive cases showed marked anemia in spite of their responsiveness to erythropoietin in vitro. One possibility is that there may be an inhibitor in the plasma to erythropoietin or to erythropoietin-responsive cells as suggested by our data (Fig. 7) and those of other investigators.[27] More recently, Krantz reported an antibody to the nuclei of erythroid precursors in the plasma of patients with pure red cell aplasia.[28,29] A similar antibody may have accounted for the failure of our patients to respond to erythropoietin.

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