EFFECT OF METHENOLONE ACETATE ON ERYTHROPOIETIN RESPONSIVE CELLS IN RAT BONE MARROW

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Received July 19,1974

SUMMARY Bone marrow cells from methenolone acetate injected normal or hypertransfused polycythemic rats were cultured with erythropoietin. Heme synthesis rate in these cells was apparently increased as compared to control bone marrow cells similarly cultured. Plasma erythropoietin activity of methenolone treated rats was not detectable either by in vivo nor by in vitro assay methods. It was suggested that methenolone stimulates erythropoiesis by increasing the number and/or sensitivity of erythropoietin responsive cells.

A number of mechanisms have been proposed for the erythropoietic effects of anabolic steroids; elevation of plasma erythropoietin level (1, 2), increase in the number of erythroid precursor stem cells (3-7), and stimulation of δ-aminolevulinic acid synthetase activity (8). It has also been shown that the conversion of parent androgens to biologically active metabolites is necessary for erythropoietic stimulation (9).

In the present report, mechanism of erythropoietic effect of methenolone acetate was studied by injecting this androgen to normal or hypertransfused polycythemic rats and observing the changes in the erythropoietin responsive cells in the bone marrow of these rats by the in vitro culture method.

MATERIALS AND METHODS Virgin female rats of Wistar strain weighing 100-140g were injected intramuscularly with the methenolone acetate suspended in 0.4 ml of sesami oil (Shoering Co., West Germany) at a daily dosis of 8 mg. Control rats received 0.4 ml of sesami oil. Two rats were sacrificed at various intervals after injection. Plasma was pooled and stored at -20°C

until use. Both femora were removed aseptically from each rat and culture of the bone marrow cells was made in triplicate. In one experiment, bone marrow cells were collected from normal rats sacrificed 3 days after a single injection of methenolone or sesami oil.

Plethoric rats (initial body weight:80-90g), transfused twice intraperitoneally (a total of 15 ml of packed red cells per rat), received injection of methenolone on the second day of the last transfusion, when the reticulocyte count was 0.3 % or less. Five days after the last transfusion, rats were sacrificed and femora were resected for culture. Hematocrit was over 75 %. Erythroblasts in the bone marrow of these rats were as low as 1.5 % or less. Reticulocyte count in peripheral blood was less than 0.3 %.

Bone marrow cells were cultured by the modified method of Kranz, et al. (10). Marrow cells were flushed out from femora and washed once with NCTC 109 (Difco, Detroit, Michigan) supplemented with 10 % calf serum. Five million viable nucleated cells in 1.0 ml of culture media (inactivated calf serum,4 :NCTC 109,5 :fresh homologous serum,1) were delivered into a plastic petri dish (35×10 mm, Falcon Plastics) and incubated with or without 0.1 unit of erythropoietin at 37°C under air containing 5 % CO_2. Erythropoietin was extracted from the urine of anemic patients by alcohol acetone precipitation method. Three microcuries of [^{59}Fe] were added to the dish 6 hour prior to the termination of culture. Heme was extracted from cultured bone marrow cells with acid methylethylketone (11). The methylethylketone layer was dried and the radioactivity was counted in a gas flow counter. The rate of heme synthesis was expressed as cpm per dish.

Plasma erythropoietin activity was assayed using hypertransfused mice (12). Adult female 4CS mice were transfused intravenously with 1.0 ml of 50 % red cell suspension for 2 times. Mice with hematocrits higher than 60 % were selected and they received 1.0 ml of test plasma subcutaneously. Percent incorporation of radioactive iron into blood was determined. In the in vitro assay, 5×10^6 viable nucleated cells from normal rat bone marrow

were incubated in 1.0 ml of solution containing 20 % test plasma, 50 % NCTC 109, 20 % inactivated calf serum and 10 % fresh homologous serum. Culture was performed for 48 hours under the same condition as described above. Heme was extracted and incorporation of radioactivity was determined.

RESULTS

Effect of methenolone acetate on normal rat bone marrow. Methenolone acetate was given to rats for 1-6 days and femora were resected for culture on the 1st-6th day of the initiation of the injection. As shown in Fig. 1,

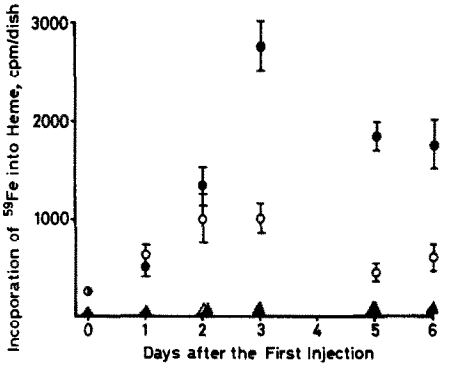

Fig.1. Effect of Methenolone Acetate (MA) on Normal Rat Bone Marrow. Incorporation of [^{59}Fe] into heme of bone marrow cells obtained from MA treated rats (Epo.(+) ●-●, Epo.(-) ▲-▲) and control rats (Epo.(+) o-o, Epo.(-) Δ-Δ) was measured after 48h incubation.

incorporation of radioactive iron into heme in the presence of 0.1 unit of erythropoietin was higher in methenolone injected rats than in controls; approximately 3 times more incorporation than control was observed. Only minimal incorporation of radio-iron occurred in cells cultured without erythropoietin both for injected and control rats.

Effect of varying the dose of erythropoietin on the incorporation of radioactive iron is depicted in Fig. 2. The response of bone marrow cells of rats injected with methenolone 3 days previously was higher than that of control, and it increased in parallel with the dose from 0.025 to 0.1 unit.

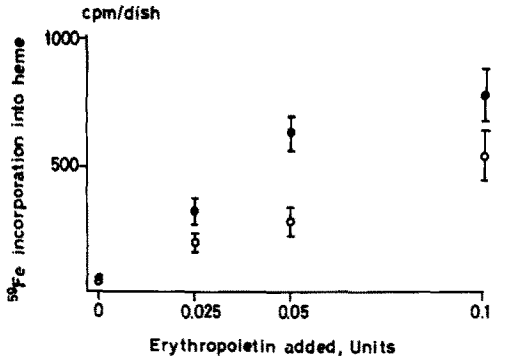

Fig.2. Effect of Erythropoietin on Incorporation of [^{59}Fe] into heme. Incorporation of [^{59}Fe] into heme in bone marrow cells of methenolone acetate treated rats (●-●) and controls (o-o) in the presence of various amount of erythropoietin was observed after 48h incubation.

Effect of methenolone on plasma erythropoietin level. The endogenous erythropoietin in plasma of methenolone treated and control rats on the third day of the injection was determined as shown in Table 1. Both in vivo and in vitro assays failed to reveal any difference between treated and control rats.

Effect of methenolone acetate on polycythemic rat bone marrow. In vitro incorporation of radioactive iron into heme in the bone marrow cells from methenolone treated and control rats at the 6th and 48th hour of the incubation with erythropoietin is shown in Table 2. Heme synthesis of methenolone treated rat bone marrow cells was about 2 times as high as that of control after 48 hours incubation. Heme synthesis of rat bone marrow cells cultured without erythropoietin for 48 hours was almost negative.

DISCUSSION

It has been reported that androgen (testosterone ester and 5α-derivatives of androgen) elevates the erythropoietin level in the plasma and increases the turnover of erythropoietin responsive cells (1, 2, 9).

In our experiment, it was demonstrated that the bone marrow cells obtained from the rats on the 3rd day of methenolone injection showed

Table 1. Assay of Plasma Erythropoietin

in vivo assay

	M. A.*	S. O.†	Saline Control
Number	8	7	4
%[^{59}Fe] Utilization	0.34±0.15	0.28±0.07	0.27±0.14
P Value	>0.1	>0.1	

in vitro assay

	M. A.*	S. O.†	Normal Rat+Epo.° 0.05U	Normal Rat
Number	13	14	6	8
[^{59}Fe] incorporation cpm/dish	174±36	157±35	774±216	190±45
P Value	>0.1	>0.1	<0.001	

Values represent mean ±S.D..

†S.O. Rats injected with 0.4ml of sesami oil.

*M.A. Rats injected with 0.8mg of methenolone acetate in 0.4ml sesami oil.

°Epo. Erythropoietin

increased response to erythropoietin in vitro, while the elevation of plasma erythropoietin level was not observed. This result suggested that methenolone increased the response of erythropoietin responsive cells through increasing the number and/or sensitivity of these cells to erythropoietin.

The discrepansy between our results and others may be due either to different method employed or the different androgen used. Methenolone acetate (1-methyl-17β-hydroxy-5α-androst-1-en-3-one acetate) used in our

Table 2. Effect of Methenolone Acetate on Polycythemic Rat Bone Marrow

		Exp. I				Exp. II			
Incubation period	Epo.°	M.A.	* mean	S.O.	† mean	M.A.	* mean	S.O.	† mean
6 hours	(-)	511		234		347		195	
		474	496	314	267	440	389	174	184
		503		252		381			
	0.1U	661		286		422		294	
		589	564	264	267	571	473	327	326
		441		251		427		352	
48 hours	(-)	66		47		81		47	
		79	72	62	58	69	75	54	50
		73		65					
	0.1U	1920		1115		1506		759	
		2817	2278	861	1113	1167	1364	834	877
		2096		1363		1419		1037	

†S.O. Rats injected with 0.4ml of sesami oil. cpm/dish

*M.A. Rats injected with 0.8mg of methenolone acetate in 0.4ml sesami oil. cpm/dish

°Epo. Erythropoietin

experiment is one of 5α derivatives but it is neither 17α-alkylated androgen nor testosterone ester, and in this point it is similar to 19-nortestosterone. This derivative was reported not to increase the erythropoietin level of plasma but to have a direct effect on erythroid stem cells (6). Some androgens may affect chiefly on stem cells but others chiefly on production of erythropoietin according to the difference in their structure.

Based on our present observations, we are inclined to consider that some androgens act directly on erythropoietin responsive cells and increase their response to erythropoietin. This view may explain a number of questions raised heretofore on the erythropoietic effect of the androgen; why the androgen does not stimulate the erythropoiesis of excessively polycythemic mice (9), why the androgen is effective in cases of aplastic anemia with high plasma erythropoietin level, and why large amounts of the androgen should be

administered for a long period in order to observe hematopoietic effect on the patients.

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