Erythropoiesis in Transfusion-Induced Polycythemic Rats Studied with H^3-Thymidine Autoradiography.* (30206)

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In the adult mammal, the factors responsible for maintaining normal peripheral erythrocyte numbers and hemoglobin content (and hence blood oxygen-carrying capacity) are not fully understood. However, it is now recognized that augmented erythropoiesis occurs through a reduced tissue oxygen-humoral mechanism(1,2). Thus decreased oxygen availability to the tissues stimulates the formation, probably by the kidney(3-5) of the hormone "erythropoietin" (erythropoiesis-stimulating-factor, ESF). ESF may then initiate erythropoiesis by promoting stem cell differentiation into erythroblasts(6-9) or by direct stimulation of proliferation of preexisting erythroblasts(10,11). It may also increase delivery of erythroid elements into the peripheral blood(12-14).

Conversely, increased oxygenation of the tissues results in depression of erythropoiesis (1,15) presumably through inhibition of ESF production. Although this suppression of red cell production is well established(1, 15,16), direct unequivocal data relating to the manner in which erythropoiesis is curtailed are lacking.

The present study was undertaken to determine if the erythropoietic depression occurring in rats, made plethoric by hypertransfusion, was due to an alteration in erythroid DNA synthesis or its temporal relation to the nuclear cycle. Using standard techniques of tritiated thymidine-autoradiography, it was found that the decreased erythropoiesis of transfusion-induced polycythemia was not associated with changes in the DNA synthesis patterns of erythroid cells.

Methods. Forty young female rats (50 g) were rendered polycythemic by a single intraperitoneal transfusion of 5 ml of isologous whole, heparinized blood obtained from ether-anesthetized 150-180 g female donor rats. Forty untreated female rats (50 g) served as controls.

Polycythemic and control rats were divided into 16 subgroups of 5 rats each. One subgroup of 5 polycythemic and one subgroup of 5 control rats were sacrificed daily for 8 days following hypertransfusions. Tail blood was taken initially and at sacrifice for red cell counts (using a Coulter electronic cell counter) and peripheral reticulocyte counts made according to Brecher's(17) technique (at least 600 reticulocytes were enumerated per count). For each rat, 200 of these reticulocytes were classified as to age using Winkert's method(18).

Both subgroups were given 0.5 μC of tritiated thymidine (H^3T)† per g body weight one hour before sacrifice. The proportion of marrow nucleated red cells labeled (*i.e.*, the labeling index) following this pulse labeling was used to estimate the length of time these cells spent in DNA synthesis (T_s)‡ in relation to their total generation time (T_c). Moreover, since the number of exposed grains in the photographic emulsion overlying a labeled nucleus is roughly proportional to the amount of DNA synthesized by the cell, grain counts were used as a semi-quantitative measure of the extent of DNA synthesis occurring in these erythroid cells. Diaphyseal marrow (from right femurs) was quantified by a method routinely used for adult rat marrow and described in detail elsewhere (19). To distinguish nucleated erythroid cells from other mononucleated elements, marrow smears were treated as follows: Before applying the photographic emulsion for autoradiography (see below) they were stained by LoBue *et al*'s(19) modification of Ralph's(20) benzidine-hemoglobin staining technique. With this procedure, the hemo-

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† Schwarz Bioresearch, Inc. (Sp. Act. 6.6 C/mM).

‡ Abbreviations are those adopted by participants of the Guinness Symposium on Cell Proliferation, Dublin, 1963. For details see Quastler(30).

TABLE I. Labeling Indices for Marrow Nucleated Erythroid Cells of Polycythemic and Control Rats. All percentages have been corrected for background.

	% Labeled nucleated red cells							
	Days post-transfusion							
	1	2	3	4	5	6	7	8
Polycythemic	26.3 ±5.9*	22.2 ±1.0	23.6 ±2.7	24.9 ±3.6	33.8 ± 3.2	33.1 ± 7.4	30.0 ±3.3	31.9 ±5.8
Control	30.6 ±5.0	35.2 ±4.9	33.7 ±2.2	21.1 ±4.1	40.7 ±11.0	46.7† ± 4.2	32.6 ±2.8	29.5 ±5.3
P values	.60	.10	.01	.60	.40	.10	.50	.70

* Mean ± standard error of mean for 5 rats. † Mean of 3 rats.

globin-containing cytoplasm of the nucleated red cells assumes a distinct golden color. Following autoradiographic processing the marrow smears were counterstained for 3 minutes using a modification§ of Gude, Upton and Odell's technique(21) to differentiate nuclei. All slides used for autoradiography were autoclaved in resin and caustic soda (Speirs *et al,* 22) to reduce background fogging. Following pre-staining with benzidine, the slides were dipped in Kodak NTB-3 liquid emulsion, exposed 28 days (in light-proof boxes at 5°C) and developed in Kodak D-19. Corrections for background were made using formulae developed by Stillström(23).

Statistical analyses for all parameters studied were carried out using "Student's" t-test (for sample size $n<30$) as outlined by Bailey (24).

Results. The hypertransfused rats were polycythemic by day 1 post-transfusion, and remained so for the entire 8-day study. Red cell counts reached a maximum on day 3 and then gradually diminished. Erythropoietic depression was demonstrated peripherally by a relative and absolute decrease in reticulocytes to less than 10% of initial values by day 5. Changes in reticulocyte age distribution with time post-transfusion indicated a definite shift toward maturity on days 4 and 5 in the polycythemic rats. No alterations in age distribution were found on days 1 to 3 or 6 to 8 or in the control rats. This phenomenon may have resulted from maturation of the transfused reticulocytes.

The effect of hypertransfusion on the absolute numbers of marrow erythroid cells is shown in Fig. 1. Although marrow erythroid depression was suggested on days 1 and 2 post-transfusion, significant decreases in the numbers of these marrow cells were not observed until day 3. This depressed state of red cell production was maintained for the entire study.

The labeling indices for polycythemic and control rats following H^3T injection are shown in Table I. It is seen that they did not differ significantly ($p \leq 0.05$) in polycythemic and controls except on day 3 when the labeling index was reduced in the polycythemic rats. This similarity in labeling indices held even though the absolute numbers of erythroid cells incorporating H^3T were considerably lower in the transfused rats' marrows (Table II) as early as 24 hours after transfusion. Decreased erythropoiesis

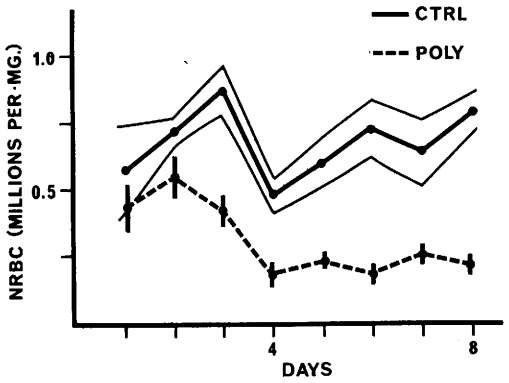

FIG. 1. Numbers of nucleated erythroid cells (NRBC) per mg of marrow. Each point on curves represents mean of 5 rats. (Ctrl: controls; Poly: polycythemic); ± one standard error is indicated by thin lines running parallel to control curve and by vertical lines in polycythemic curve.

§ Stock solution: 3 g Giesma powder, 200 ml glycerine, 200 ml methyl alcohol. Diluent: 150 ml distilled water (pH 6.2), 20 ml McIlvaine's Buffer (pH 5.6), 3 ml methyl alcohol.

For staining, 3 ml of stock solution are added to 173 ml of diluent.

TABLE II. Numbers of H^3T-Labeled Nucleated Erythroid Cells per mg of Marrow Found in Polycythemic and Control Rats. All figures have been corrected for background.

	Labeled nucleated erythroid cells (thousands)							
	Days post-transfusion							
	1	2	3	4	5	6	7	8
Exp	113 ± 27*	123 ± 18	102 ± 25	43 ± 15	42 ± 15	35 ± 4	77 ± 18	75 ± 26
Control	188 ± 72	246 ± 20	308 ± 53	104 ± 32	235† ± 59	353 ±74	214 ± 37	236 ± 64

* Mean ± standard error for 5 rats. † Mean of 3 rats.

TABLE III. Average Grain Count Distributions (in %) Obtained in Polycythemic and Control Rat Erythroid Marrow Cells. All percentages have been corrected for background.

	Net number of grains per cell				
	1–5	6–10	11–15	16–20	21–30 plus*
Polycythemic	17.60 ± 1.82†	7.23 ± .37	2.14 ± .48	.80 ± .22	.64 ± .28
Controls	18.36 ± 1.32	9.40 ±1.12	3.50 ± .65	.88 ± .20	.76 ± .31
P values	.80	.30	.10	.80	.80

* Cells with grain counts of more than 30 were rare. † Mean ± standard error of mean.

by 24 hours post-transfusion has been reported previously by Stohlman *et al*(25).

No significant differences in the average mean grain count distributions (computed by averaging the mean grain categories for all 8 days) were found between polycythemic and control rats (Table III). This was also true when grain counts obtained in polycythemic and control rats were compared at each day post-transfusion. The only exception was observed in the counts for polycythemic rats 6 days post-transfusion. In this case no erythroid cells with more than 21 grains were observed. This may have been a true difference or the result of some procedural or technical error. Furthermore, since only very small numbers of cells were found in this grain count category this difference may have been due to sampling errors.

Discussion. The data obtained in this study indicate that, excepting possibly the third day post-transfusion, the H^3T labeling index, although somewhat variable, did not differ significantly in marrow nucleated erythroid cells of both polycythemic and control rats. Assuming that erythroblasts represent asynchronously proliferating populations of cells, then the labeling index will estimate that portion of the total nuclear cycle (T_c) which a given cell spends in DNA synthesis (T_s) and these results can be interpreted in 3 ways: (a) The temporal relation between T_s and T_c was normal in erythroid cells of these polycythemic rats; (b) Both T_s and T_c were decreased proportionally; or, (c) Both T_s and T_c were increased proportionally. Since T_s in mammals has been found to be remarkably constant in cells from diverse tissues of the same animal and even when different species are compared(26), alternatives (b) and (c) appear unlikely. This conclusion is supported by the similarity in grain counts found for erythroid cells of control and polycythemic rats, a good indication that both groups were synthesizing DNA at comparable rates. In view of this, a shortened T_s would carry with it the requirement of an increased DNA synthesis rate whereas a lengthened T_s would require a decreased synthesis rate.

The significantly lower labeling index found in erythroid marrow of polycythemic rats at 3 days post-transfusion may be indicative of a relative transistory increase, in the marrow, of non-mitotable more mature nucleated erythrocytic precursors (*i.e.,* cells similar or identical to the *initially non-labeling polychromatic normoblasts* described by Odartchenko *et al,* 27). These cells would be the maturing progeny of erythroblasts differentiated prior to onset of erythropoietic suppression. It is not interpreted as representing an alteration in T_s or its relation, in time, to T_c. Such an increase in the numbers of non-mitotable erythroid precursors would be expected and would tend to decrease the labeling index(28). The data are therefore

best interpreted as indicating no differences in either DNA synthesis time, in intensity of synthesis or in the temporal relationship of T_s to T_c in nucleated erythroid cells of polycythemic and normal rat marrow.

Hence, the decreased erythropoiesis occurring in rats rendered polycythemic by transfusion is most likely not a result of alterations in maturation and proliferation rates of preexisting marrow erythroblasts. The data, in fact, support Stohlman's(16,25) contention that it results from decreases in the number of stem cells entering the erythroid compartment, this diminution being the direct effect possibly of lowered ESF titers in the blood of these animals. Further evidence against the concept that regulation of erythropoiesis involves direct control of DNA synthesis or the duration of the nuclear cycle has come from the studies of several investigators. Alpen and Cranmore(6) in dogs and Erslev(7) in rabbits demonstrated convincingly that stimulation of erythropoiesis by bleeding had no effect on erythroblast generation time. Alpen, Cranmore and Johnson (8) and Nielson *et al*(29) studying H^3T uptake in dog marrow cultures found no differences in DNA synthesis when marrows from erythropoietically-stimulated (hemorrhaged) and normal dogs were compared, thus indicating that ESF does not directly stimulate DNA synthesis.

Summary. Erythropoiesis was studied with tritiated thymidine-autoradiography in young female rats made polycythemic by hypertransfusion. Total numbers of marrow nucleated erythroid cells were markedly decreased by the third day post-transfusion, whereas absolute numbers of tritiated thymidine-incorporating nucleated red cells were depressed by day one. When labeling indices and grain count distributions were compared between erythroid marrows of polycythemic and control rats no differences were found. From this it was concluded that the suppression of erythropoiesis occurring in transfusion-induced polycythemic rats results from decreased differentiation of stem cells into erythroblasts *and not* from decreased proliferation of preexisting erythroid cells.

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Steroid Synthesis by Human Fetal Adrenals and Ovaries Maintained in Organ Culture.* (30207)

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Explants of adrenal(1) and ovarian(2) cortex from human fetuses have been maintained in organ culture for varying periods. Steroid synthesis by the adrenal explants declined rapidly with increasing incubation periods; this decline could be partially arrested by the addition of progesterone to the medium(1). The formation of steroids by the ovarian fragments was not studied.

This communication, part of a study on fetal adrenal and gonadal steroidogenesis, describes the synthesis of steroids by human fetal adrenals and ovaries maintained in a similar *in vitro* system.

Materials and methods. The intact amniotic sac of the pregnant human uterus, containing a fetus of 11 weeks gestation (7.5 cm crown-rump length), was obtained at the time of therapeutic interruption and kept on ice for one hour. The fetus was taken from the amniotic sac and the adrenals and ovaries were aseptically removed from the fetus. One gland of each pair was used as a control for chemical analysis and histological examination. The other gland was cut into segments of 2-3 mm^3. Each segment was explanted in organ culture using a siliconized lens paper raft as a modification of the Trowell technique(3). The liquid medium consisted of 70% CMRL 1066 (Connaught Medical Research Labs., Toronto) and 30% horse serum, and included 100 units/ml of a penicillin-streptomycin mixture. Six cultures were prepared from the adrenal gland and two cultures from the ovary. The cultures were incubated at 37°C in a CO_2-air (5:95) saturated atmosphere. After 48 hours incubation, the cultures were grouped and treated as follows:

Group A consisted of 2 adrenal cultures, 2 ovarian cultures and 2 control cultures composed of medium but lacking tissue. *Group B* consisted of 4 adrenal cultures. One-half of the medium of each culture in *Groups A & B* was replaced with an equal volume of medium. The media added to the cultures of *Group A* contained 10 μc (2.22×10^7 dpm) purified 7-^{3}H-progesterone (9.4 $\mu c/\mu$mole, N. E. Nuclear Corp., Boston). Two cultures of *Group B* received 13 I.U. ACTH (ACTHar, Armour & Co.); no other additions were made to the cultures of this group.

After a further 48 hours of incubation, the tissues of the cultures of *Group A* were fixed in 10% neutral buffered formalin for subsequent histological examination, and the media collected and frozen for subsequent analysis. The media of the 2 *ovarian* cultures were combined into one pool. In *Group B*, the change of medium and addition of ACTH was repeated after an additional 48 hours. After a total of 8 days in culture, the tissues were fixed and the media collected and frozen.

The analysis for conversion products of 7-^{3}H-progesterone in the culture media from *Group A* was conducted in the following

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