in tissue obtained from diabetic rats.

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Effect of Vinblastine and 4-Amino-N^{10} Methyl Pteroyl-Glutamic Acid On the Erythropoietin Responsive Cell.* (32314)

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Bruce and McCulloch(1) have postulated two kinds of stem cells: a) one capable of forming spleen colonies and reestablishing hemopoiesis in a lethally irradiated host(1), henceforth referred to as CFU, and b) cells that respond to erythropoietin, referred to here as ERU (erythropoietin responsive units) derived from the colony forming cells mentioned in a). Porteous and Lajtha, however, are of the opinion that there is no good reason to postulate two kinds of cells(2). Bruce *et al* have shown, using chemotherapeutic agents such as Vinblastine and Aminopterin, which kill cells in cycle(3), that few of CFU in marrow are in generation cycle. On the other hand, the effects of low doses of irradiation and colchicine suggest that the ERU respond to EP shortly after mitosis(4). Vinblastine has been shown by Valeriote *et al* to be useful for the study of the cell cycle *in vivo*(5). This drug kills only cells passing through mitosis(6), has a biological half life of 3.5 hours(7) and a discontinuous dose response curve with a critical dose of 5 μg for mice, below which no cells die, above which 100% mortality occurs(7). Vinblastine thus seems a valuable tool to determine whether ERU are in cycle before being exposed to EP and within what time after exposure to EP they go through mitosis. The present paper reports the results of effects of Vinblastine† (Velban) and 4-Amino-N^{10} methyl pteroyl-glutamic acid‡ (Methotrexate) a drug also known to affect cells in cycle(3), on the response to EP of mice with transfusion polycythemia.

Materials and methods. Ten-to-twelve-weeks-old 18-20 g female $B_{10}D_2$ mice, maintained in the Dept. of Biology, School of Medicine, University of Chile, were used as recipients. Each group contained at least 5 mice. They were transfused with ½ ml of washed erythrocytes, intravenously on day 0 and 1. Five units of erythropoietin were injected subcutaneously on day 6, 0.1 μc ^{59}Fe ferric citrate (specific activity >5 μc/μg) was injected on day 8. The mice were bled on day 9 and radioactivity of washed erythrocytes and micro hematocrits measured. Only mice with heart

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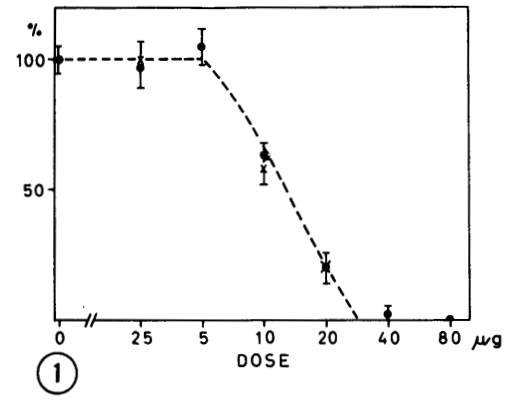

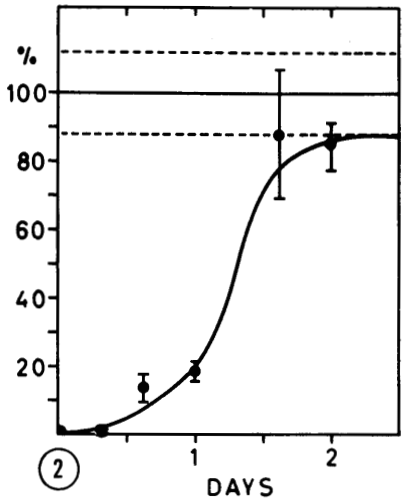

FIG. 1. Response to 5 u. erythropoietin as a function of dose of Vinblastine, drug given 7.5 hr prior to EP ×———× and 1 hr after EP injection ●———●. The line - - - is a theoretical one calculated from Valeriote and Bruce model(5), assuming T ½ for Vinblastine of 3.5 hr a threshold dose of 5 μg and assuming a cycle time for EP U of 8.5 hr.

FIG. 2. Recovery of EP response after 80 μg of Vinblastine EP 5 u. injected at varying intervals after Vinblastine.

blood hematocrits >54% were considered. The EP preparation used was a pool of plasma of irradiated phenylhydrazine treated rats(8) containing 20 U EP/ml, as determined in a 4 point parallel assay using the fasted rat and St.B§ as a reference preparation. Vinblastine dissolved in 0.9% NaCl was injected intravenously in the dosages and at times indicated in the *Results*. In other experiments the effect of methotrexate, which is known to kill cells in cycle and does not markedly reduce CFU(3), was also studied. ^{59}Fe activity was measured in a Nuclear Chicago Well Scintillation Counter with an automatic sample changer (model C-120). Each sample was counted till 10.240 counts had accumulated.

Results. Fig. 1 shows the effects of various doses of Vinblastine, injected intravenously 7.5 hours prior to and one hour after EP, on ^{59}Fe incorporation into erythrocytes. Doses below 5 μg do not affect the response, while doses of 10 and 20 μg, given either before or after EP, depress the response to 56 and 20% respectively. Methotrexate, 50 mg/kg, (Table I) given either before or after EP, completely suppresses the response even though followed at 4 hours by 3 mg of Folinic acid (Leucovorin)¶, which reverses the Methotrexate effect(9).

When a dose of Vinblastine, 80 μg/mouse, is injected at different intervals before EP the results shown in Fig. 2 are obtained: the response is 20% of normal when EP is given 24 hours after Vinblastine and complete recovery is obtained by 39 hours. The 80 μg dose used is such that effective levels of the drug should be maintained for about 12-15 hours(7).

Discussion. If one accepts the accumulated evidence that Vinblastine kills cells passing through mitosis(5,6), one is justified in concluding that ERU are in cycle before responding to EP, and go through mitosis within a few hours of being exposed to it. A similar conclusion has been reached by Stohlman(10). If one applies the model of Valeriote(5) to our data (Fig. 1) using a biological T ½ for the drug of 3.5 hours, and a threshold of dose, in accordance with our findings as well as theirs, of 5 μg, the line which fits the points corresponds to a cycle time of about 9 hours, comparable to that obtained in the rat(10).

The results of the methotrexate experiments also suggest that ERU are in cycle, presumably DNA S Phase(3) before responding to EP and continue in cycle afterwards.

§ Obtained from Division of Biological Standards, Nat. Inst. for Med. Res., London.

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TABLE I. Effect of Methotrexate (M), 50 mg/kg Intraperitoneally at Different Times in Relation to EP Injection, on RBC ^{59}Fe of Polycythemic Mice. In all cases methotrexate injection was followed 4 hr later by 3 mg/mouse of folinic acid (Leucovorin). Control polycythemic mice received normal rat plasma.

Group	Control	EP	M: −8 hr EP	M: −4 hr EP	M: 0 hr EP	M: +4 hr EP
^{59}Fe RBC, %/ml	.14 ± .023	7.1 ± .9	.05 ± .005	.25 ± .12	.06 ± .016	.05 ± .016

The maximum dose of Vinblastine and the dose of methotrexate used in our experiments are such that they reduce CFU to only about 70% of the control levels when given 24 hours before assay(3,11). Such doses, however, suppress the response to EP. This can be interpreted in one of the following ways: a) that the major portion of CFU are in a state of non-cell cycle (G_o), with a minor fraction going through cycle every 9 hours, and that only those cells in cycle, presumably those in G_1 would be able to respond to EP; b) that CFU have a long cell cycle time around 30 hours and that the capacity to respond to EP is limited to cells in G_1; c) that ERU, as Bruce and McCulloch postulate(1) are derived from CFU and are a transient population, capable of a limited number of divisions. which could act as an amplifying system by which from one CFU a few ERU could be formed. It is hard to understand how the number of CFU that have been estimated to exist in the mouse can give rise to about 10^8 RBC a day(12) and yet appear to be in a state of relative rest as far as cell cycle is concerned(3). A hypothesis such as that mentioned in c) might account for this. On the other hand, it is conceivable that the assay system for CFU, using transplantation, underestimates their number, by assaying predominantly those cells not in cycle at the time of sampling and discriminating against those in cycle(2).

Recovery of the response to EP after Vinblastine injection in mice is faster than that observed after irradiation(2) and about the same rate as that of mice given Dactinomycin (13). This fast recovery might reflect a prompt replenishing of ERU from a resting pool of CFU not affected by the drugs in the doses used.

Recovery of the ERU response in polycythemic mice after Vinblastine is faster than that of polycythemic rats after Vincristine (10). This difference may be a consequence of a longer biological half life of Vincristine in the rat.

Summary. Vinblastine given in graded doses, 10-20-40 μg, to polycythemic mice either before or after EP reduces their response to EP in proportion to log dose. Analysis of the dose response curve using Valeriote and Bruce model(5) suggests a generation time of about 9 hours for the erythropoietin responsive unit (ERU). Methotrexate 50 mg/kg given either 8 hours before, together with, or 4 hours after EP completely suppresses the response to EP even though, followed after a 4-hour interval by an injection of Folinic acid which is known to reverse the methotrexate effect on cells. It is suggested that most of ERU are in cell cycle, and respond to EP in G_1. The ERU population might be that segment of CFU in cycle, or an intermediary population, capable of limited divisions, derived from it.

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Inflammation and Tissue Injury II. Local Release of Lysosomal Enzymes During Mixed Bacterial Infection in the Skin of Rabbits.* (32315)

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It has been shown(1) that the degree of local tissue injury (*e.g.*, increased vascular permeability, hemorrhage, etc.) in response to infection was dependent upon the presence of polymorphonuclear leukocytes. Normal and leukopenic (produced with nitrogen mustard) rabbits were inoculated intradermally with "viable" and "heat-killed" human dento-gingival plaque. In normal animals, both viable and heat-killed bacterial suspensions produced marked inflammatory reactions culminating in local abscess formation. On the other hand, in leukopenic animals vascular permeability in response to viable bacterial plaque suspensions was only about half that seen in normal animals. Hemorrhage was minimal or absent. This difference between the response in normal and leukopenic rabbits was even more dramatically illustrated following injection of heat-killed plaque material. No detectable response could be seen in the skin of the leukopenic rabbits, even after 24 hours. At the ultrastructural level, infiltrating PMN-leukocytes in normal animals had phagocytosed the injected materials and subsequently underwent degranulation and lysis. Leukopenic animals showed little or no cellular activity. The process of phagocytosis, degranulation and cell lysis in PMN-leukocytes has been associated with the extracellular release of lysosomal hydrolases(2,3). Thus, it was speculated that lysosomal enzymes, especially proteases(4), played an important role in the type of tissue injury observed in the normal animal. If this reasoning was correct, then one would expect to find changes indicative of extracellular release of lysosomal enzymes in the area of tissue injury. The following experiments were designed to test this hypothesis.

Methods. Bacterial plaque adjacent to the gingival margin was obtained from humans with clinically normal and diseased gingiva. This material was pooled, weighed (wet weight), and suspended in saline to provide a concentration of 35 mg/ml. A portion was placed in a boiling water bath for one hour to provide the "heat-killed" bacterial suspension. The abdomen of 14 normal and 20 leukopenic rabbits (2-3 kg) was shaved prior to the start of each experiment. Each animal received an injection of viable plaque (0.2 ml) and heat-killed plaque (0.2 ml) on either side of the mid-line. Leukopenia was induced by administration of nitrogen mustard as outlined previously(1). Twenty-four hours after local inoculation, animals were killed with sodium pentobarbital and the injection sites were excised. An uninjected portion of abdominal skin was excised to serve as control. Each sample was minced with scissors and immediately placed into 5 ml of a tissue culture medium consisting of tyrode buffer-Eagle's medium, 500 units Penicillin, 750 μg Streptomycin, 25 μg Fungizone, 50 μg Colimycin and 10% Seitz-filtered normal rabbit serum. The tubes were stoppered and placed in a 37°C water bath for 18 hours, following which the

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