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The Effect of Parathyroid Hormone on Erythropoiesis in Serum-Free Cultures of Fetal Mouse Liver Cells (41108)

C. D. R. DUNN[1] AND D. TRENT

Department of Medical Biology/Memorial Research Center, The University of Tennessee College of Medicine/Knoxville Units, 1924 Alcoa Highway, Knoxville, Tennessee 37920

Abstract. The effects of parathyroid hormone (PTH) on erythropoiesis in serum-free cultures of fetal mouse liver cells were investigated to determine if PTH might, at least partially, be responsible for the anemias of uremia, hyperparathyroidism, and spaceflight. Both crude and pure preparations of PTH were employed with similar effects. PTH at concentrations 10–100 times "normal" produced a dose-dependent stimulation of erythropoiesis which was only observed in the complete absence of exogenous erythropoietin (Ep). Although the responses to both PTH and Ep were calcium-dependent pharmacological analysis of the PTH log–dose/log–response relationship showed the PTH effect to be markedly different from that of Ep. At concentrations approximately 240 times normal PTH inhibited both endogenous and Ep-mediated heme synthesis. These concentrations are considerably greater than those reported in uremia, hyperparathyroidism, or during spaceflight casting doubt on the hypothesis that PTH is directly responsible for the anemia seen in these disorders. Unit gravity cell separation experiments showed that PTH both stimulated and inhibited the Ep-responsive cells as a function of its concentration.

Several recent reports have implicated parathyroid hormone (PTH) as a uremic toxin at least partly responsible for erythrosuppression seen in patients with chronic renal failure. Thus, the reduced blood transfusion requirements of parathyroidectomized (PTX) patients (1), the improvement of the anemia in at least some PTX patients (2, 3) and a more severe anemia in patients with hyperparathyroidism (4) are frequently cited as supporting evidence of a toxic role for PTH. In addition, both primary (5, 6) and secondary (7) hyperparathyroidism are often associated with anemia which is ameliorated by surgical or chemical ablation of the glands (5–7). Nevertheless, few attempts have been made to directly demonstrate the erythrosuppressive effects of PTH. Levi *et al.* (8) showed, using a crude PTH preparation, that the hormone at low concentrations enhanced heme synthesis in serum-containing cultures of fetal mouse liver cells (FMLC) but was inhibitory at higher levels (2 U/culture). These authors claimed that PTH acted "by a mechanism similar to that of erythropoietin" (Ep) but their report did not include a comparison of the Ep and PTH dose/response relationships. The present studies were designed to investigate the effects of PTH on erythropoiesis in a chemically well-defined environment, i.e., serum-free cultures of FMLC.

Materials and Methods. *Cultures.* The serum-free culture system employed in these studies has been described in detail (Do, Fuhr, and Dunn, submitted). In brief, each 100 ml of M199 containing Hanks' salts, glutamine, sodium bicarbonate (all Grand Island Biological Co., Grand Island, N.Y.), penicillin, and streptomycin ("basic" medium) was supplemented with 750 mg bovine serum albumin (BSA), 28.9 mg purified human transferrin, and 3 mg egg lecithin (all Sigma Chemical Co., St. Louis, Mo.). Groups of five 1-ml cultures, each containing 5×10^5 FMLC from C3H mice of 13–15 days of gestation, were incubated at 37° ± Ep and/or ± PTH in an atmosphere of 5% CO_2 in air for 24 hr. All cultures were then centrifuged, the supernatant fluids discarded and replaced by 1.0 ml of the "basic" medium containing 0.5 μCi ^{59}Fe as ferrous citrate/ml and 28.9 mg% of purified human transferrin. The radioiron-containing mix-

[1] All correspondence should be addressed to Dr. C. D. R. Dunn at: Life Sciences Laboratory, Northrop Services, Inc., P.O. Box 34416, Houston, TX 77034.

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ture was incubated overnight at 37° before being added to the cultures. After an additional 4-hr incubation, heme was extracted into methylethylketone using minor modifications (9) of the method originally described by Teale (10). Radioactivity in aliquots of the solvent layer was determined in an automatic gamma counter. Results are reported as ^{59}Fe counts per (cp) 5 min in heme. The log–dose/log–response relationship in these serum-free cultures of FMLC is similar to that seen in cultures containing 5% fetal calf serum (9).

Culture additives. Step III sheep plasma Ep (specific activity 6.7 U/mg—obtained from Connaught Medical Research Laboratories, Swiftwater, Pa.) dissolved in calcium- and magnesium-free phosphate-buffered saline (PBS), pH 7.3, was used throughout. Routinely, four groups of cultures were established: with no Ep; with a concentration (0.002 U/ml) near to the minimal detectable dose; an intermediate concentration (0.02 U/ml); and a concentration (0.2 U/ml) producing maximal stimulation of heme synthesis.

Two preparations of PTH were employed. Except where otherwise stated a clinical material prepared from bovine glands (Eli Lilly & Co., Indianapolis, Ind.) was used. The protein content of this preparation, determined in our laboratory using the Bio-Rad Protein Assay kit, gave an estimated specific activity of 20 U/mg. Pure bovine PTH (specific activity 914 U/mg and containing all 84 amino acids) was obtained from Immunonuclear Corporation, Stillwater, Minnesota. PTH was diluted or dissolved as appropriate, in PBS.

EGTA was obtained from Sigma Chemical Company, St. Louis, Missouri, and analytical grade calcium chloride from Mallinckrodt Chemical Works, St. Louis, Missouri. Both were dissolved in normal saline and neutralized with 0.1 *M* NaOH before they were added to the cultures. The concentrations of EGTA and Ca^{2+} employed were based on those recently shown to modify CFU-E growth (11).

The "basic" medium contained <2 m*M* Ca^{2+} according to the manufacturer's specifications.

Cell separations. Unit gravity cell sedimentation was performed according to the method of Miller and Phillips (12) as described (13). FMLC ($1.75–2.5 \times 10^8$ in 40 ml of 0.3% BSA in PBS) were layered on top of a linear gradient of 1–2% BSA in PBS and sedimented for 4 hr at 4°. The cone volume was discarded and 45-ml fractions (corresponding to a chamber depth of 0.5 mm) were run from the base of the separation chamber at approximately 2 ml/min. Each fraction was centrifuged, washed twice in the supplemented medium, and counted in a Fisher autocytometer. Fractions were incubated with either 0.02 U/ml Ep, 0.625 U/ml PTH, or 0.02 U/ml Ep plus 2.5 U/ml PTH, and the cultures were processed as described above. In certain experiments some fractions were cultured without either Ep or PTH. Results are reported as percentages of the fraction containing the peak number of cells or, for the effect of Ep and/or PTH, as percentages of heme synthesis/10^6 cells observed in fractions cultured with Ep alone.

Statistical analyses. The results reported are based on at least three experiments (15 cultures/point); Student's *t* test was used to assess statistical significance. Log–dose/log–response relationships for Ep or PTH were analyzed from the data summarized in Fig. 1 using the analysis of variance technique applicable to parallel-line bioassays (14).

Results. PTH, over the concentration range of 0.02–0.625 U/ml, produced a dose-dependent stimulation of heme synthesis. This stimulation was observed only in the complete absence of Ep and reached a maximum at 185% of control values at 0.625 U/ml (Fig. 1). The log–dose/log–response relationship for PTH over this same concentration range and in the absence of exogenous Ep was defined by the formula

$$\log Y = 0.0892 \log X + 2.3275 \quad [1]$$

(where X = concentration (U/ml) and Y = response (cp 5 min in heme)). The slope of this relationship was significantly ($P < 0.02$) different from zero.

In contrast, the log–dose/log–response relationship for PTH in the presence of 0.002 U/ml Ep was defined by the formula

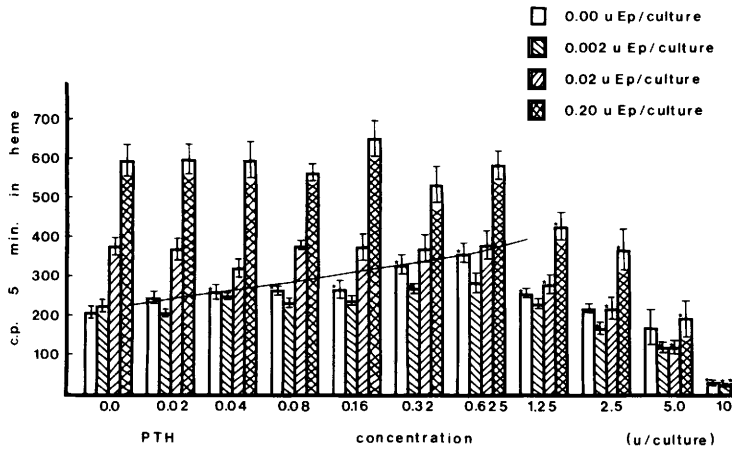

FIG. 1. The effect of parathyroid hormone (PTH), with or without graded doses of erythropoietin (Ep), on heme synthesis in serum-free cultures of fetal mouse liver cells. Vertical bars indicate ± standard error of the mean. * = $P < 0.05$ from respective controls without PTH. Solid line = regression line for the PTH response in the absence of Ep. Radiation counts corrected for machine background (approx 200 cp 5 min).

$$\log Y = 0.0324 \log X + 2.3263 \quad [2]$$

the slope of which was not significantly different ($P > 0.2$) from zero, i.e., no effect of PTH was evident.

The log–dose/log–response relationship of Ep alone over a concentration range 0.002–0.2 U/ml was characterized by the formula

$$\log Y = 0.2060 \log X + 2.3140. \quad [3]$$

The slope of this relationship was significantly ($P < 0.001$) greater than that for PTH alone. The log–dose/log–response relationship for Ep in the presence of 0.625 U/ml PTH was given by

$$\log Y = 0.1824 \log X + 2.3175, \quad [4]$$

which was not significantly different, either in slope or intercept, from the log–dose/log–response relationship of Ep alone (Formula 3).

Concentrations of PTH lower than 0.02 U/ml had no significant effect on heme synthesis while concentrations above 0.625 U/ml produced a dose-dependent inhibition of heme synthesis in both the absence and the presence of exogenous Ep (Fig. 1). At 5.0 U/ml PTH, the Ep log–dose/log–response relationship was given by

$$\log Y = 0.0766 \log X + 2.0842, \quad [5]$$

which differs significantly ($P < 0.05$), in both slope and intercept, from that of Ep alone (Formula 3). At 10 U/ml PTH, no significant effect of Ep was observed (Fig. 1).

Pure PTH produced similar results to the crude material (Fig. 1). A concentration of 0.3125 U/ml (312.5 ng/ml) gave maximum stimulation at 175% of control and, at 5.0 U/ml (5 μg/ml), 40% inhibition of heme synthesis was observed. The stimulatory effects were, once again, only observed in the complete absence of exogenous Ep while inhibitory effects were seen in both the presence and absence of Ep.

The effects of Ca^{2+}, EGTA, or Ca^{2+} plus EGTA on the Ep and PTH responses are shown in Fig. 2. The addition of 3 m*M* Ca^{2+} had little effect on either Ep- or PTH-mediated heme synthesis. EGTA (5 m*M*) completely abolished the effects of both hormones. However, both Ep and PTH responses were restored in cultures containing Ca^{2+} (3 m*M*) in addition to the EGTA (Fig. 2).

The results of the unit gravity cell sedimentation experiments are shown in Fig. 3. The majority of the nucleated cells in FMLC suspensions sedimented with a modal sedimentation velocity of 6.0–6.5 mm/hr. In contrast, the ERC- and PTH-responsive cells sedimented at 8 mm/hr.

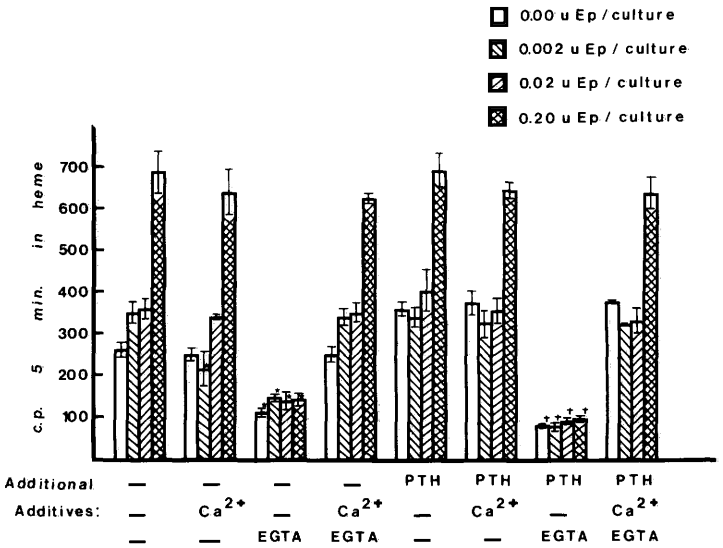

FIG. 2. The effect of Ca^{2+} (3 m*M*) and/or EGTA (5 m*M*) on the erythroid response of fetal mouse liver cells to erythropoietin (Ep) or parathyroid hormone (PTH—0.625 U/ml). Vertical bars indicate ± standard error of the mean. * = $P < 0.05$ from controls without PTH. † = $P < 0.05$ from controls with PTH. Heme synthesis in cultures with PTH but no Ep was significantly ($P < 0.02$) greater than in cultures without either Ep or PTH. Radiation counts corrected for machine background (approx 200 cp 5 min).

The ERC inhibited by a higher concentration of PTH (2.5 U/ml) also sedimented with a modal sedimentation velocity of 8 mm/hr (Fig. 3).

Discussion. PTH both stimulated or inhibited heme synthesis in serum-free cultures of FMLC, of which >90% are morphologically recognizable as erythroid (13), as a function of its concentration (Fig. 1, Formulas 1 and 5). Since similar responses were seen with both relatively crude and highly purified preparations of PTH, the effects were indeed due to PTH and not to contaminants in the crude material used for most of these studies.

The present results can be compared with those reported by Levi *et al.* (8), who used the same PTH preparation employed here but in serum-containing cultures of FMLC. Both studies showed stimulation then suppression of heme synthesis, in the absence of exogenous Ep, as the concentration of PTH was increased. However, we observed a considerably greater stimulation than reported earlier. In addition the current study: (i) demonstrated that PTH stimulation of heme synthesis occurred only in the absence of exogenous Ep; (ii) confirmed, with pure material, that the effects were indeed due to PTH; and (iii) directly compared the PTH effects with those elicited by Ep. Pharmacological analysis of the respective log–dose/log–response relationships (Formulas 1 and 3) showed that the effect of PTH was markedly different from that of Ep. However, the stimulatory effect of PTH (as with Ep) appeared dependent on the presence of Ca^{2+} (Fig. 2). Calcium ions were necessary not only for the Ep response (which confirms earlier studies using a different culture system (11)) but also for endogenous erythropoiesis which occurs in the cultures in the absence of exogenous Ep (Fig. 2).

In our studies, the effect of PTH was absolutely dependent on the complete absence of Ep—no PTH response was observed in the presence of as little as 0.002 U/ml Ep (Fig. 1). This visual impression (Fig. 1) was further investigated by an analysis of the log–dose/log–response relationships to Ep alone (Formula 3) and to Ep in the presence of a concentration of PTH which, by itself, produced its max-

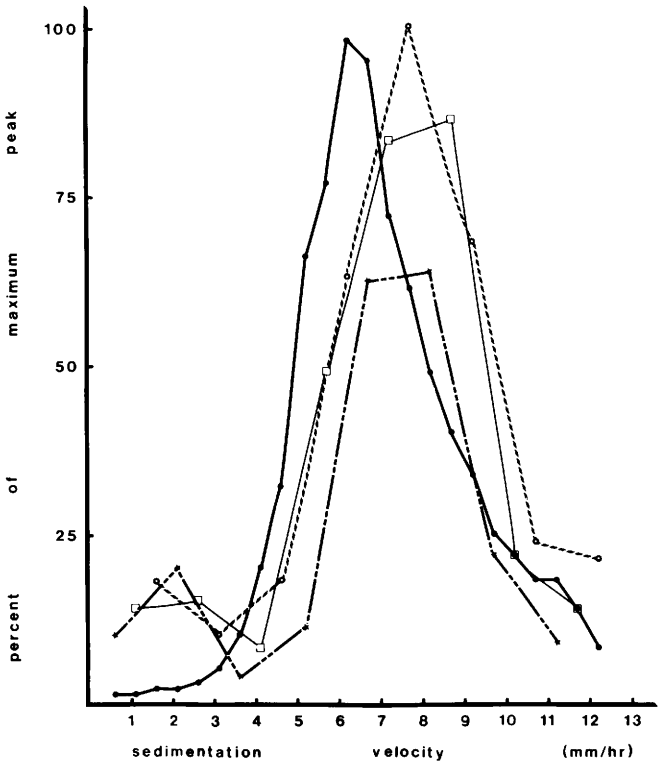

FIG. 3. Unit gravity cell sedimentation of fetal mouse liver cells. (●——●) indicates nucleated cells/fraction as percentage of maximum peak. (○- - -○) indicates erythropoietin (Ep) response to 0.02 U/ml, (□——□) indicates parathyroid hormone (PTH) response to 0.625 U/ml, (*- - -*) indicates response to 0.02 U/ml Ep + 2.5 U/ml PTH; all expressed as heme synthesis/10^6 cells as a percentage of heme synthesis in the fraction stimulated by Ep alone. Mean of four experiments.

imum stimulation (Formula 4). Since these relationships were essentially identical and because the slope of the log–dose/log–response relationship of PTH in the presence of 0.002 U/ml Ep (Formula 2) was not significantly different from zero, the absence of a PTH effect in the presence of Ep was confirmed. In fact the data suggest a degree of mutual antagonism between the stimulatory effects of PTH and Ep (Fig. 1, Formulas 2 and 3).

PTH and Ep both appeared to stimulate the same cell population—or, at least, cell populations not differing on the basis of size (Fig. 3). We believe the ERC in suspension culture to be identical to the CFU-E of semisolid cultures (13, 15). However, since subpopulations of CFU-E exist with slightly different properties (16), the possibility cannot be completely excluded that PTH and Ep stimulate different populations of cells of a similar size. It seems that PTH both stimulated and inhibited the ERC as a function of the hormone's concentration (Fig. 3, Formulas 1 and 5).

The relevance of these studies to the anemias of uremia or hyperparathyroidism is difficult to fully evaluate. Within the chemically well-defined environment of a serum-free culture system significant impairment of the Ep response by high concentrations of PTH was observed (Fig. 1). This inhibition was comparable to that seen in serum-containing cultures of FMLC (8) but there is no information available on the effects of PTH in cultures of human bone marrow cells. Another major problem is the uncertainty of physiological levels of PTH (17, 18). Present-day bioassays are not sufficiently sensitive to detect normal levels of PTH. Also, the various types of radioimmunoassays (the results of which are now

expressed as ng/ml or μl-eq/ml) detect different PTH molecular fragments not all of which may be biologically active (17, 19). We have taken as "normal," serum concentrations of 0.002–0.005 U/ml PTH, i.e., approximately 10^{-11} *M* (17, 20). At these levels PTH was without significant effects on erythropoiesis. Concentrations of PTH 10–100 times greater than normal were stimulatory by themselves but some evidence was obtained suggesting antagonism of the PTH effect by Ep and/or vice versa (Fig. 1, Formula 2). This may have some clinical significance in instances, such as uremia, where Ep titers are already low (21). Levels of PTH approximately 250 times normal were needed to produce significant suppression of erythropoiesis (Fig. 1). It is doubtful if these high values are reached clinically; a 50-fold increase in immunodetectable PTH appears to be the highest reported (4, 22–24). However, the present data do not exclude the possibility that different molecular species of PTH, which may vary with disorders of the parathyroid gland (25), might be more erythrotoxic than the native molecule. In addition, an indirect toxic effect on erythropoiesis through the induction of renal damage (26) (which might further impair the production of Ep) or marrow fibrosis (3, 26) could occur at lower PTH concentrations. Certainly, what changes occur in PTH levels during spaceflight (27) would appear to be insufficient to suppress erythropoiesis and produce the "anemia of spaceflight" (28).

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