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Effect of aminoglutethimide on murine fetal hepatic erythroid colony formation[1]

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Summary. Pretreatment of pregnant mice with aminoglutethimide phosphate, an inhibitor of glucocorticoid synthesis, increases the content of fetal liver erythroid colony-forming cells (CFU-E), as assessed by the formation of erythroid colonies in vitro by fetal liver cells in plasma clots containing exogenous erythropoietin. In addition, the inability of aminoglutethimide to influence erythroid colony formation in vitro suggests that endogenous glucocorticoids exert a suppressive effect on the number of functional CFU-E in the fetal liver.
Key words. Mice, pregnant; aminoglutethimide phosphate; mice, liver, fetal; erythroid colony-forming cells.

During fetal development, the liver is a major site of erythropoiesis in mammals[29,35]. Several studies have demonstrated that erythropoiesis in the fetal liver is regulated by erythropoietin[31,40]. Other hormones (e.g. testosterone) may also modulate erythropoiesis in utero by enhancing the production of erythropoietin[39] or increasing the sensitivity of fetal liver cells to erythropoietin[9]. In contrast, glucocorticoids appear to suppress fetal liver erythropoiesis in vivo[5,23,26,27]. In addition, fetal livers from dexamethasone-pretreated pregnant rats contained a reduced number of morphologically identifiable erythroid progenitor cells[36]. Similarly, pretreating pregnant mice with dexamethasone reduced the number of functional erythroid colony-forming cells (CFU-E)[20], the immediate progenitor of identifiable erythroblasts[16,19]. In these studies, the CFU-E can be functionally identified by its ability to form small colonies of erythroid cells in a semi-solid medium containing low concentrations of exogenous erythropoietin after 2 days of culture[25,34]. The ability of dexamethasone to inhibit erythroid colony formation in vivo suggests that inhibitors of adrenal steroid biosynthesis might reverse or abrogate the inhibitory effect of glucocorticoids on the fetal liver CFU-E. In this connection, aminoglutethimide, a compound which inhibits the formation of 20α-hydroxycholesterol from cholesterol[10,14], and therefore reduces endogenous glucocorticoid levels, was employed in an attempt to identify a potential role for endogenous glucocorticoids in the modulation of fetal liver erythropoiesis. Thus pregnant mice were injected i.v. with 1 mg of aminoglutethimide phosphate or 0.9% NaCl and, 24 h later, the formation of erythroid colonies by fetal liver erythroid progenitor cells in plasma clots containing erythropoietin (25 mU) was assessed.

Materials and methods. Chemicals. Aminoglutethimide and water-soluble aminoglutethimide phosphate were obtained from Ciba Pharmaceuticals (Summit, NJ).

Human urinary erythropoietin, processed by Dr Peter P. Dukes at the Hematology Research Laboratory, Children's Hospital of Los Angeles, was provided by the Division of Blood Diseases and Resources of the National Heart, Lung and Blood Institute, National Institutes of Health. The specific activity of this material was estimated to be 110.9 IU/mg protein.

Animals. CD-1[Crl:CD-1(ICR)BR] mice (Charles River Breeding Laboratories, Inc., Wilmington, MA), maintained on Rat Chow 5012 (Ralston Purina Co., St. Louis, MO) and water ad libitum, were mated and examined daily for vaginal plugs. The morning on which a vaginal plug was found was designated as the first day of gestation. To assess the effect of treatment with aminoglutethimide on erythroid colony formation by fetal liver cells in vitro, pregnant mice were injected i.v. with either 0.2 ml vehicle (0.9% NaCl) or 0.2 ml vehicle containing 1 mg of aminoglutethimide phosphate 24 h prior to collection of fetal livers. The mice, at 15 days of gestation, were killed by cervical dislocation on the morning of each study in order to minimize the potential effects of diurnal variations in circulating glucocorticoid levels. The individual fetuses were excised and placed in RPMI-1640 (Microbiological Associates, Inc., Walkerville, MD).

Erythroid colony assay. Erythroid colonies were grown in plasma clot cultures, and quantitated essentially as described in McLeod et al.[25] and previously reported by Leung and Gidari[21].

Statistical analysis. Statistical analysis of the data was performed by employing a one-way analysis of variance[33] to ascertain the presence of any significant difference among the group means. Subsequently, if significant differences were present, the Newman-Keuls test[33] was used to establish which pairs of means were significantly different. In some experiments, when

only two treatment groups were being compared, Student's t-test[33] was used to analyze the data.

Results. Pretreatment of pregnant mice with 1 mg of aminoglutethimide-phosphate increases the number of erythroid colony-forming cells in the fetal liver, as assessed by erythroid colony formation in response to exogenous erythropoietin (25 mU) (table 1). Simultaneously, no effect on the cellularity or the liver wet weight was detected.

To preclude the possibility that this increase in erythroid colony formation resulted from a direct action(s) of aminoglutethimide on CFU-E proliferation, fetal liver cells were cultured in the presence of aminoglutethimide (10^{-6} to 10^{-11} M) in erythropoietin-containing plasma clots. As shown in table 2, these concentrations of aminoglutethimide did not significantly influence erythroid colony formation.

In addition, aminoglutethimide was not hemolytic in the pregnant mouse, since the hematocrit was unchanged (43 ± 1 vs a control value of 44 ± 1).

Discussion. The results of this study are consistent with the hypothesis that endogenous levels of glucocorticoids exert a suppressive influence on the fetal liver CFU-E. Thus, the increase in the number of liver CFU-E in fetuses obtained from aminoglutethimide-treated mothers supports this idea. The increase in erythroid colony formation is not the result of aminoglutethimide exerting direct action(s) on the fetal liver CFU-E, as demonstrated by the inability of aminoglutethimide to enhance erythroid colony formation in vitro. The absence of any differences in the hematocrit of whole blood obtained from aminoglutethimide or untreated mothers indicates that the action(s) of aminoglutethimide is not due to stimulation of erythropoiesis by hemolysis.

On the other hand, aminoglutethimide inhibits cholesterol side chain cleavage by forming a ligand with cytochrome $P450_{scc}$[38], inhibits the cytochrome P450-mediated conversion of androstenedione to estrone[15,37], and, in the presence of ACTH, has been reported to increase serum testosterone levels in castrated male rats[8]; this latter effect may be the result of the inhibition of androstenedione conversion to estrone by the adrenal glands. Thus, it is possible that the aminoglutethimide-mediated increased in erythroid colony formation may be the result of increasing amounts of androgens and androgen derivatives in the fetus. Nevertheless, although several androgens and their derivatives are known to stimulate erythroid colony formation in vitro[32], the tibial content of CFU-E is decreased in polycythemic mice 24 h after testosterone administration[28]. The observations are in agreement with those reporting that dexamethasone administered to pregnant rats produces a reduction in the number of identifiable erythroid progenitor cells in the fetal liver[36]. Similarly, bone marrow cells obtained from adult mice pretreated with dexamethasone from a reduced number of erythroid colonies in response to exogenous erythropoietin[12]. Moreover, glucocorticoids inhibit erythroid colony formation in vitro by murine adult bone marrow[12,32], fetal liver[21] and human fetal liver[18,30] cells. These observations are at variance with those reporting that dexamethasone potentiates erythroid colony formation in vitro by murine fetal liver CFU-E[6,13].

The molecular mechanism(s) by which endogenous levels of glucocorticoids modulate fetal liver erythropoiesis in vivo is unknown. However, evidence suggesting that glucocorticoid receptor proteins may play a role in mediating the suppressive effect of glucocorticoids on erythroid colony-forming cells has been reported. For instance, 17α-methyltestosterone, a glucocorticoid antagonist in some systems[4], has been able to abrogate the inhibitory effects of glucocorticoids on erythroid colony formation by murine adult bone marrow cells[12]. In addition, glucocorticoid receptors may also be involved in the modulation of fetal liver erythropoiesis by glucocorticoids, since Billat et al.[7,24] have demonstrated that morphologically identifiable erythroid precursor cells in the fetal liver contain glucocorticoid receptors. Furthermore, the identification of a glucocorticoid receptor in transformed cells in conjunction with the inhibitory effects of glucocorticoids on clonal growth is in accord with these data[3,17]. Finally, the suppressive effect of dexamethasone on the erythroid colony-forming cells appears to be associated with a decrease in the proliferative rate of these cells[11,20,22].

In conclusion, our studies provide evidence supporting the hypothesis that endogenous levels of glucocorticoids exert a suppressive influence on the number of functional CFU-E in the fetal liver. Nevertheless, because aminoglutethimide has several pharmacological actions, further studies will be required to unequivocally substantiate this hypothesis.

Table 1. Effect of maternally administered aminoglutethimide on fetal liver CFU-E

Treatment	(Cells/ liver) × 10^{-7}	Liver wet wt (mg)	CFU-E/ 3×10^4 cells[a]	% control
Control	1.80 ± 0.14 (17)	29.20 ± 1.25 (20)	112 ± 3 (6)	(100)
Aminoglutethimide phosphate	1.70 ± 0.07 (4)	29.30 ± 1.09 (18)	178 ± 4[b] (5)	159

[a] Nucleated fetal liver cells (day 15), obtained from pregnant mice treated with 1 mg of aminoglutethimide phosphate i.v. 24 h earlier, were cultured in Epo (25 mU)-containing plasma clots (0.1 ml) for 48 h, as described in 'materials and methods'. Numbers in parantheses represent either the number of experiments or the number of fetal livers; the mean ± 1 SEM of each of the determined values is reported. [b] Significantly different from control group ($p < 0.01$).

Table 2. In vitro effect of aminoglutethimide on erythroid colony formation by murine fetal liver erythroid progenitor cells

Treatment	Erythroid colonies/ 3×10^4 cells[2]
Control	28 ± 5
+ Epo (25 mU)	117 ± 6
+ Aminoglutethimide(10^{-6} M)	116 ± 6[b]
+ Aminoglutethimide(10^{-7} M)	119 ± 3[b]
+ Aminoglutethimide(10^{-8} M)	120 ± 4[b]
+ Aminoglutethimide(10^{-9} M)	121 ± 6[b]
+ Aminoglutethimide(10^{-10} M)	105 ± 6[b]
+ Aminoglutethimide(10^{-11} M)	113 ± 10[b]

[a] Nucleated fetal liver cells obtained from fetuses on 15th day of gestation were cultured in plasma clots (0.1 ml) for 48 h in the presence (25 mU) and absence of Epo as described in 'materials and methods'. Each value represents the mean ± 1 SEM of three separate studies in triplicate. [b] Not significantly different from the Epo-treated group ($p < 0.01$).

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Distribution patterns of mammalian-like peptide immunoreactive cells in the midgut of *Aeshna cyanea* (Insecta, Odonata)

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Summary. The distribution of cells reacting with antisera to cholecystokinin, substance P, gonadoliberin, methionine-enkephalin, and vasoactive intestinal peptide, demonstrated by the indirect immunoperoxidase method, was studied along the entire midgut of an insect, *Aeshna cyanea*. For each antiserum, the number of reacting cells increased from the middle part to the end of the midgut. Only a few cells reacted to somatoliberin, leucin-enkephalin and somatostatin antisera. In the connective sheath surrounding the midgut epithelium, nerve fibers were stained by antisera to serotonin, somatostatin, cholecystokinin, vasoactive intestinal peptide and methionine-enkephalin.
Key words. Insect midgut; mammalian-like peptides; immunohistochemistry.

In insects, by means of immunohistochemical investigations or radioimmunoassays, it has recently been demonstrated that several mammalian-like neuropeptides, especially of gastro-entero-pancreatic type (GEP) occur in the nervous system, particularly in the brain. Except for the work of Iwanaga et al.[11] and our recent preliminary study[4], no report had dealt with the presence of these substances in the insect gut. Previously we identified, by an immunoperoxidase procedure, cells reactive for mammalian cholecystokinin octapeptide (CCK-8), vasoactive intestinal polypeptide (VIP), pancreatic polypeptide (PP), substance P, methionine-enkephalin (met-enkephalin), gonadoliberin (LHRH) and human pancreatic somatoliberin (hp GRF) in the midgut of *Aeshna cyanea*. With the same method, we have now undertaken a systematic investigation of the neuropeptides through the entire midgut. The results present the complete distribution of the midgut peptide endocrine cells.

Material and methods. The postembryonic development of *Aeshna cyanea* requires eleven larval instars. Larvae of the 8th instar were used in this study.

Tissue preparation. The midguts of nine larvae were rapidly dissected and immersed in a solution of picrid-acid-paraformaldehyde (PAF)[13] for 24 h. The samples were then washed overnight in phosphate buffer 0.1 M, pH 7.2, 5% sucrose, embedded in Tissue-Tek (Miles Labs.), frozen in liquid nitrogen and sectioned on a cryostate (section thickness: 15 µm).

Antisera. Twelve antisera raised against synthetic CCK-8, VIP, substance P, met-enkephalin, LHRH, hp GRF, leucine-enkephalin (leu-enkephalin), human calcitonin, neurotensin, serotonin, somatostatin-14 and ovine corticoliberin (CRF) were used. Somatostatin antiserum was a gift from Dr Dubois (Nouzilly, France). The others were produced in our laboratory by immunization of rabbits with the amine (serotonin) or the synthetic peptide linked to albumin or thyroglobulin, and emulsified with Freund's complete adjuvant. Somatostatin[8], CCK-8[9], leu- and met-enkephalin[14], CRF[15], hp GRF[16] and serotonin[17] antisera were previously employed.

Immunohistochemical staining for sections. Each serially cut section was incubated for 24 h at 4°C with one of the twelve antisera. All antisera were applied in dilutions of 1/200. After rinsing in Coons buffer pH 7.2, the sections were incubated according to the indirect immunoperoxidase method, with a 1/50-dilution of anti-Ig rabbit for 1 h at room temperature. For visualization of the peroxidase, the tissues were allowed to react with a solution of 4-chloro-1-naphthol in 0.1 M Tris-HCl buffer (pH 7.6) containing 0.01% H_2O_2.