J. steroid Biochem. Vol. 27, No. 4-6, pp. 1017-1022, 1987
Printed in Great Britain.
0022-4731/87 $3.00 + 0.00

Proceedings of the VII International Congress on Hormonal Steroids (Madrid, Spain, 1986)

THE CORTICOSTATIC (ANTI-ACTH) AND CYTOTOXIC ACTIVITY OF PEPTIDES ISOLATED FROM FETAL, ADULT AND TUMOR-BEARING LUNG

QINZHANG ZHU, AVA V. SINGH, ANDREW BATEMAN, FRED ESCH* and S. SOLOMON

Endocrine Laboratory, Royal Victoria Hospital, L-205, 687 Pine Ave. West, Montreal, Quebec H3A 1A1 and Departments of Medicine, Obstetrics and Gynecology and Biochemistry, McGill University, Montreal, Canada and the *Laboratory for Neuroendocrinology, The Salk Institute, San Diego, California, U.S.A.

Summary—The possibility that corticosteroids influence lung maturation was suggested in 1968 by Buckingham *et al.* [1] and supported by studies mounted in humans and in rabbits by Liggins and Howie [2, 3] and by a collaborative study from the N.I.H. [4]. To our knowledge no reverse process, i.e. the regulation of adrenal function by the lung, has been postulated. In attempting to isolate ACTH from fetal lung we found peptides which depressed corticosterone production. Eventually, we isolated a group of peptides from rabbit fetal lung which showed potent inhibiton of corticosterone production in rat adrenal cell suspensions. Using high-resolution reverse-phase HPLC and high-sensitivity gas-phase sequencing techniques the primary structure of one of these peptides has been determined and it was called corticostatin. The adult rabbit lung contains a large quantity of a very similar peptide, which elutes close to corticostatin but whose amino acid composition differs by a single glycine and possibly a serine. This peptide is also potently corticostatic. We have isolated homologous human peptides but these show no apparent corticostatic activity in rat adrenal cell suspensions. They do, however, show interesting effects on *in vitro* growth of lung cell lines.

INTRODUCTION

The role of glucocorticoids in the development of the fetal lung and in the prevention of the respiratory distress syndrome has been intensively studied [1–4]. Most studies on this subject focus on type II pulmonary epithelial cells which synthesise the pulmonary surfactant required for alveolar stability in the newborn (for review see Ref. [5]). Studies of cytosolic and nucleic glucocorticoid receptors by Giannopoulos *et al.* [6] showed that fetal lung is a primary target for glucocorticoids. In the past, emphasis has been placed on the role of the pituitary gland and its secretory products in regulating this process [7]. Baird *et al.* [8] reported a substance of high mol. wt (>15 kDa) from bovine adrenal medulla which was capable of inhibiting (70%) the adrenocortical response to ACTH. In addition, there are other compounds which inhibit adrenocortical function [9, 10]. The increase in glucocorticoid production prior to parturition may indicate the removal of an inhibitor as well as an increase in circulating ACTH. In the course of trying to isolate ACTH from fetal lung we found several areas of the HPLC chromatogram which depressed corticosterone production and countered ACTH stimulation. These were shown to be peptides and we set out to isolate them. We report here the isolation and biologic activity of this corticostatin.

EXPERIMENTAL

Extraction

Frozen tissue was thawed to 4°C and homogenised in an acidic medium (1 M HCl, 5% formic acid, 1% NaCl, 1% TFA), centrifuged at 3000 rpm for 15 min and the supernatant was saved. The pellet was re-extracted twice and the supernatant pooled. The pooled supernatant was then concentrated onto O.D.S. cartridges (Sep-pak, Waters, MA), and eluted in 80% acetonitrile in 0.1% TFA. In some experiments a cation exchange cartridge (Sep-pak, Waters, MA) was then used, washed with 150 mM Tris buffer, pH 7.0, and eluted with 50 mM Tris buffer containing 1 M NaCl, pH 7.0 [11].

Purification

The concentrated peptide eluate was loaded onto a Waters C_{18} μBondapak HPLC column with the aid of a pump (human peptides were loaded by injection). Peptides were eluted using gradients of 0.1 M TFA (pump A) and 80% acetonitrile in 0.1% TFA (pump B). The gradients employed are indicated in the figure legends. The partially purified material was further processed using a second gradient with 80% acetonitrile in 0.13% HFBA (pump B), and 0.13% HFBA, (pump A), followed by a third step using the 0.1% TFA–acetonitrile solvent

system [12]. Before sequence analysis the rabbit-derived peptide was taken through a final high-pressure gel-filtration step using an I-125 protein analysis column (Waters), with 40% acetonitrile in 0.1% TFA as the mobile phase [13].

Rat adrenal cell bioassay

Ten ml of washing buffer (Ham's F-12 Gibco with 0.5% BSA, Sigma) were pre-incubated at 37°C on a shaking water-bath with 95% oxygen and 5% carbon dioxide. The adrenal glands of 5–15 recently decapitated rats were quartered in the washing buffer and incubated for 60 min in dispersing buffer (10–25 mg collagenase and 2–3 mg DNA-ase in 10 ml Ham's F-12 with 0.5% BSA). The cell suspension was centrifuged at 700 rpm for 7 min at 30°C, and washed twice with 8 ml of washing buffer. The cells were resuspended in 2 ml of washing buffer and filtered through nylon gauze (100-μm pore size), and layered onto 8 ml of Ham's F-12 with 2% BSA. This was centrifuged at 800 rpm for 8 min at 30°C, and the top 6 ml removed by aspiration. The cell count of the remaining 2 ml was taken using a hemocytometer and the suspension brought to 200,000–600,000 viable cells/ml using Ham's F-12, 0.5% BSA, 7 mM Ca^{2+}. The resulting suspension was filtered a second time through nylon gauze.

A total of 0.5 ml of suspended cells was preincubated for 90 min at 37°C on a shaking water-bath and gassed as above. Samples (0.5 ml) of ACTH, ACTH + test material, or control buffer (all in incubation buffer), were added and incubated for 2 h at 37°C. Then 75 × 12 mm polystyrene tubes (Sarstedt, Federal Republic of Germany) were used for the incubation steps. After 2 h the tubes were centrifuged at 1000 rpm for 10 min and the supernatant decanted into borosilicate 16 × 100 mm culture tubes (Fisher, Pittsburgh, U.S.A.). Steroids were extracted with 2 ml of methylene chloride and the corticosterone content determined by radioimmunoassay [14, 15].

Radioimmunoassay (RIA)

Corticosterone antibody was obtained from Endocrine Sciences (Tarzana, CA.). ^{3}H-Labelled tracer was from New England Nuclear (Boston, MA), and unlabelled standard was obtained from Sigma (St Louis, MO). The protocol used for RIA was as previously described [16].

Thymidine incorporation

SK-MES-I cells were a gift from Dr Fuks, McGill University, and CCD-8LU cells were obtained from the American Type Culture Collection, Rockville, MD. [^{3}H]Thymidine (ICN Chemicals, Irvine, CA) incorporation was determined as previously described [17].

RESULTS

Figure 1 represents the results obtained from our bioassay. The plateau of the curve is at 740 pg/ml of ACTH. Figure 2 clearly shows that at least four corticostatic substances are present in rabbit fetal lung extracts as observed from the inhibition of ACTH at 150 pg/ml (the ED_{50} from Fig. 1). There is a broad region of inhibition with a retention time of 72–96 min. This major component was further purified by a second HPLC step using 0.13% HFBA as the ionic phase. The peptide eluted at 38% acetonitrile (data not shown), indicating, even at this early stage, that it was a highly basic peptide. The position of the corticostatic material was determined by the adrenal cell bioassay, and the active material was taken through a third HPLC step using the TFA–acetonitrile solvent system. Despite this extensive purification procedure, it was clear from the chromatograms that the peptide was still contaminated (Fig. 3A). Complete purification was achieved by the use of a high pressure gel-filtration method (Fig. 3B).

Amino acid analysis showed that the purified peptide was rich in arginine and cysteine. The sequence was determined by gas-phase analysis, and is identical in structure to a peptide (with antibiotic properties) recently isolated from rabbit peritoneal neutrophils [18]. A complete account of the isolation and structure of this peptide will be published elsewhere.

In subsequent experiments on rabbit tissue the purification procedure was simplified by including a preliminary step of cation-exchange prior to HPLC. Figure 4 compares the HPLC profiles of the cationic fraction of fetal rabbit lung (4a), adult rabbit lung (4b), and rabbit peritoneal neutrophils (4c), obtained as previously described [19]. Bioassay of these fractions indicated that inhibitory activity was present in all three preparations. The major peak of inhibitory activity in the adult rabbit lung elutes close to that of the fetal lung. There is, however, a slight discrepancy in the amino acid composition, the adult peptide having one fewer glycine and serine than the fetal peptide, although this has yet to be confirmed by sequence analysis. The neutrophil extract provided

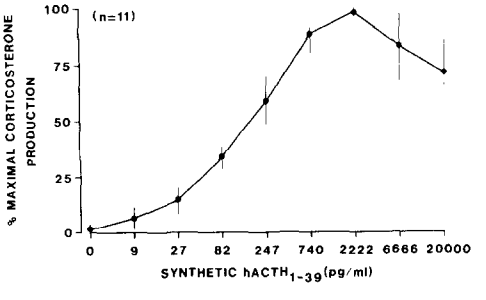

Fig. 1. Rat adrenal bioassay, dose–response curve for ACTH. See Experimental for details.

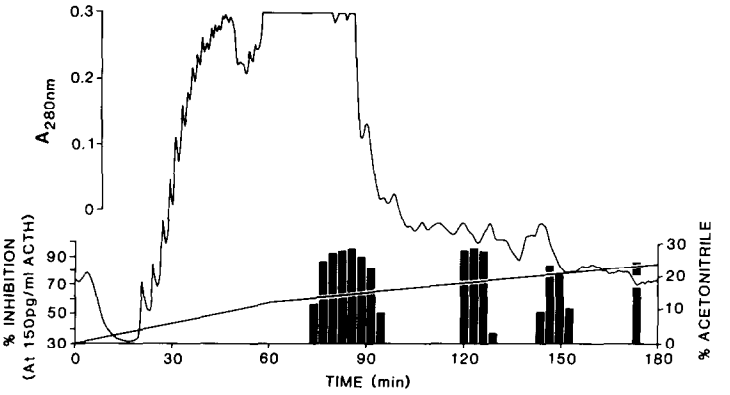

Fig. 2. HPLC purification of fetal rabbit lung peptides. One hundred fetal lungs (88 g, Pel-Freeze Biologicals, Rogers, AK, last period of gestation), were extracted, as described in Experimental, and purified on HPLC using a gradient of 0–12% acetonitrile in 1 h, followed by 12–24% acetonitrile in 2 h, all in 0.1% TFA. The dark bars indicate the per cent inhibition of ACTH by individual fractions assayed as described, and are the mean of duplicate determinations. The first peak of activity was purified through two further steps of HPLC.

four active components (31–50 mins). Peaks I, II, and IV are strongly inhibitory, whereas III is only weakly corticostatic. We are in the process of isolating these peptides for sequence analysis.

Our experiments led us to conclude that the activities of the adult and fetal peptide are identical in the rat adrenal cell suspension bioassay. Much larger quantities of the adult peptide were present, and so this material was used in all further experiments following further purification.

Adult rabbit heart, kidney, and liver were also screened by HPLC for cationic peptides but the corticostatic peptides present in the lung and neutrophil extracts were absent (Fig. 5).

Biological actions

Rabbit peptide. In order to determine the concentration of ACTH required for incubation with corticostatic peptides a dose–response curve was performed using the rat adrenal cell suspension bioassay system. An ED_{50} for ACTH of 150 pg/ml (Fig. 1) was obtained, and this was used as a fixed concentration for incubations with varying amounts of the corticostatic peptides.

The ED_{50} for the inhibition of corticostatic output by ACTH in the presence of corticostatin in Fig. 6 is 100 ng/ml. As shown in the figure, 100% inhibition can be obtained at high concentrations; however, a basal secretion of corticosterone was still evident. Preliminary experiments have shown that the cAMP-induced secretion of corticosterone is not inhibited by corticostatin (Zhu and Solomon, unpublished results). Incubations with higher concentrations of ACTH shifts the ED_{50} of inhibition to the right of the dose–response curve; thus at an ACTH concentration of 740 pg/ml the ED_{50} of corticostatin

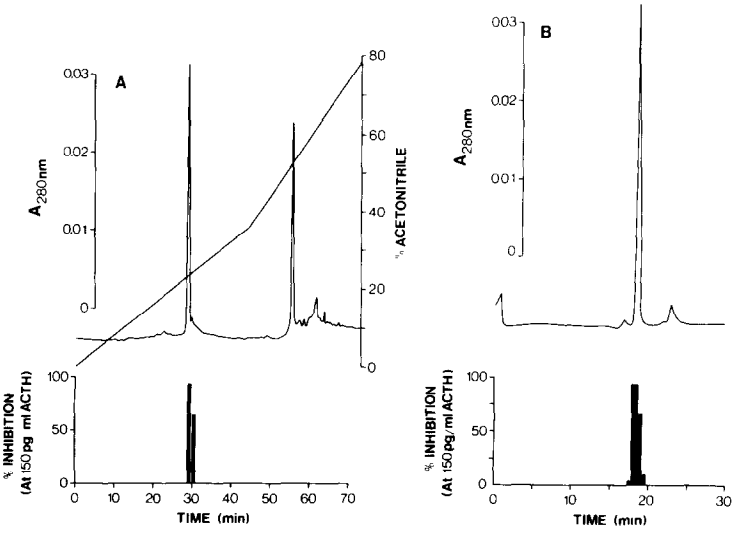

Fig. 3. Final Step of purification of corticostatin by gel-filtration HPLC. See Experimental

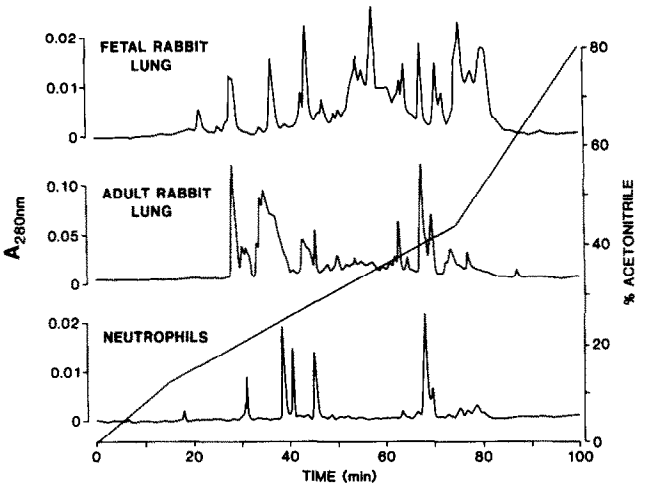

Fig. 4. HPLC purification of cationic peptides from (a) fetal rabbit lung, (b) adult rabbit lung, (c) rabbit peritoneal neutrophils. A gradient of 0–12% acetonitrile in 15 min was employed and was followed by a gradient of 12–44% acetonitrile for 1 h, then from 44 to 80% acetonitrile is 25 min, all in 0.1% TFA throughout. Corticostatic material was eluted at 28 min for fetal and adult lung and at 31 min for neutrophils.

is close to 900 ng/ml and at 27 pg/ml ACTH the ED_{50} is 50 ng/ml. The inhibitory concentrations reported here are at least two orders of magnitude lower than those reported for its antibiotic effects, suggesting the two activities are mediated by quite distinct mechanisms.

Human peptide. Fresh human lung is difficult to obtain so we have used the fluid obtained from patients with peritonitis as a source of neutrophils. A peptide similar to corticostatin, rich in arginine and cysteine, was isolated and was also tested for an inhibitory response in the rat adrenal cell assay. Despite structural similarities of this peptide to the rabbit lung corticostatic peptide we are unable to elicit an inhibitory effect on corticosterone production. We cannot, however, discount the possibility that higher concentrations, i.e. greater than 3000 ng/ml, may be inhibitory.

A number of peptides are known to stimulate proliferation of lung cells and we therefore tested for such a response using *in vitro* cell lines. Figure 7(A) shows that at concentrations greater than 1 nmol/ml an inhibitory response is obtained. In contrast, Fig. 7(B) shows a very significant proliferative effect on a second cell line, CCD-8LU. The proliferative effect is remarkably similar both in extent and potency to those reported for gastrin-releasing peptide 14–27 on human small cell carcinoma *in vitro* [20]. We have also isolated the human homologue in lung tumors (submitted for publication).

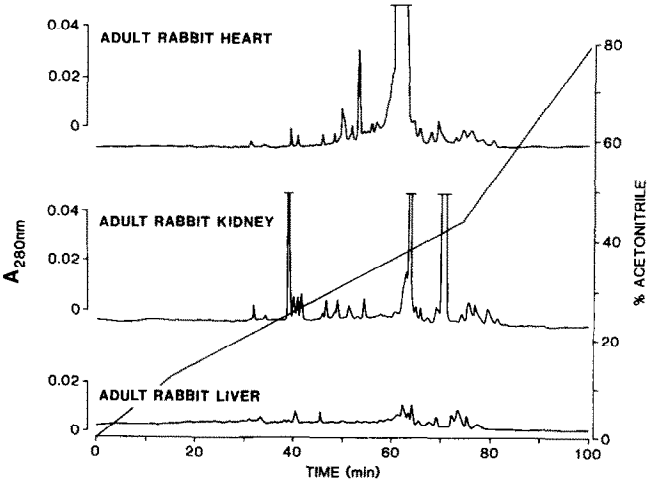

Fig. 5. HPLC purification of cationic peptides from (a) adult rabbit heart, (b) kidney, (c) and liver. The gradient used was the same as in Fig. 4.

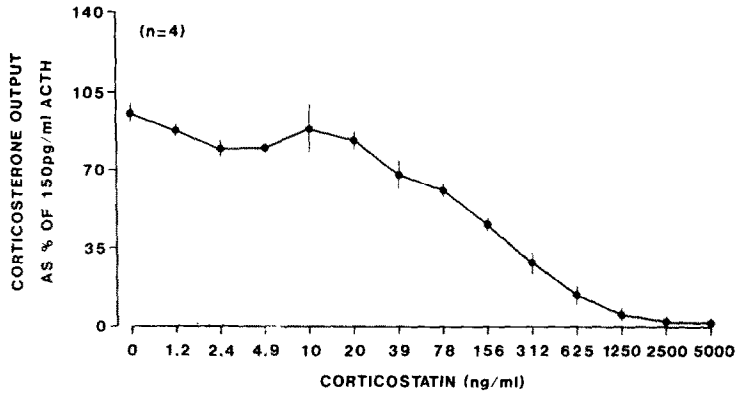

Fig. 6. Inhibition of ACTH on adrenal cell suspension by corticostatin. The ACTH concentration was fixed at 150 pg/ml throughout.

DISCUSSION

The peptides we have discussed here are known to be very potent antibiotics as well as antiviral agents [18]. Our first knowledge of these peptides came from a computer search for peptides similar to our isolated corticostatin. Following a completely independent line of inquiry we have uncovered a number of potentially very important biological effects on mammalian cells. Notable among these is the suppression of ACTH on the mammalian adrenal cortex.

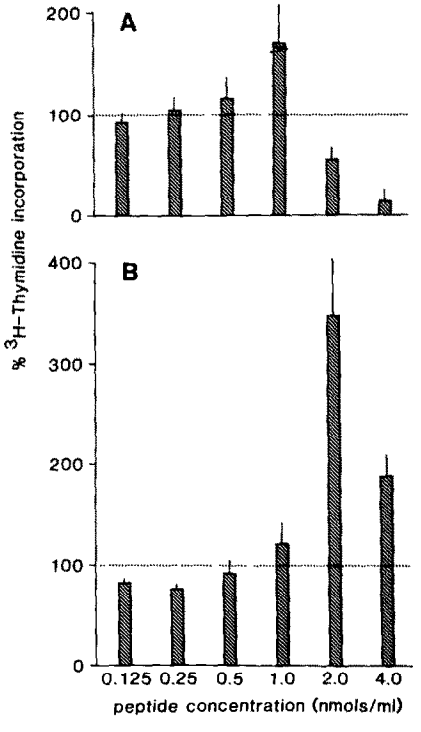

Fig. 7. The effect of the human analogue on the *in vitro* incorporation of [^{3}H]thymidine in lung cell lines. (A) SK-MES-1, an epithelial-like cell line derived from a squamous carcinoma. (B) CCD-8LU, a fibroblast-like cell line derived from a non-transformed cell line.

One of the richest sources of corticostatin in the rabbit is the lung. Since the adrenal gland plays an important role in the maturation of the lung, one can postulate a potential feedback mechanism between lung corticostatin and the adrenal gland. This proposal is now being tested experimentally. In this context it is interesting to note that the human homologue stimulates the proliferation of an *in vitro* lung cell line (CCD-8LU, Fig. 7b) at 2 μM. This is similar to the action of bombesin on small cell carcinoma of the lung [20]. The differences in potency between the action on the adrenal gland and the lung would suggest that two different modes of action of the peptide are operational.

The other major source of corticostatin-like peptides is in peritoneal neutrophils, which is clearly a corollary of their antibiotic action. Stimulated human peripheral leukocytes [21], and mouse spleen macrophages [22] synthesise immunoreactive ACTH. The function of ACTH-related peptides in immune system cells is enigmatic, but it is of great interest to note that CRF will stimulate the release of leukocyte-derived ACTH-like molecules [23]. The immunosuppressant actions of glucocorticoids are well documented. Although the relationships existing between the production of pituitary hormones in leukocytes, their stimulation by hypothalamic-releasing factors, and steroidal immunosuppression are obscure, the production of corticostatins by immune system cells strongly argues for an important link in the chain of stimulation and suppression of adrenal secretion.

We are now in the process of conducting further experiments both *in vivo* and *in vitro* to determine its mode and specificity of action. At this stage it is difficult to understand with clarity the biological role of corticostatin, and further investigation will shed light on this important problem.

REFERENCES

1. Buckingham S., McNary W. F., Sommers S. C. and Rothschild J.: Is lung an analog of Moog's developing intestine? I. Phosphotases and pulmonary differentiation in fetal rabbits. *Fedn Proc. Fedn Am. Socs exp. Biol.* **27** (1968) 638 (Abstr).
2. Liggins G. C.: Premature delivery of fetal lambs infused with glucocorticoids. *J. Endocr.* **45** (1969) 515–523.
3. Liggins G. C. and Howie R. N.: A controlled trial of antepartum glucocorticoid treatment for prevention of respiratory distress syndrome in premature infants. *Pediatrics* **50** (1972) 515–525.
4. Collaborative group on antenatal steroid therapy: effects of antenatal dexamethasone administration on the prevention of respiratory distress syndrome. *Am. J. obstet. Gynec.* **141** (1981) 276–286.
chromatography. *Analyt. Biochem.* **128** (1983) 121–129.
5. Hitchcock K. R.: Lung development and the pulmonary surfactant system: hormonal influences. *Anat. Rec.* **198** (1980) 13–34.
6. Giannopoulos G., Mulay S. and Solomon S.: Cortisol receptors in rabbit fetal lung. *Biochem. biophys. Res. Commun.* **47** (1972) 411–416.
7. Thorburn G. D. and Challis J. R. G.: Endocrine control of parturition. *Physiol. Rev.* **59** (1979) 863–918.
8. Baird A. and Guillemin R.: Adrenocorticotropic activity in bovine adrenal medulla. In Endocr. Soc. 63rd Ann. Mg (1981) Program Abstr. 181.
9. Hotta M. and Baird A.: Transforming growth factor-beta is a potent inhibitor of adrenocortical steroidogenesis. In Endocr. Soc. 63rd Ann. Mt (1981) Program Abstr. 1035.
10. Chartier L., Schiffrin E. and Thibault G.: Effect of atrial natriuretic factor (ANF)-related peptides on aldosterone secretion by adrenal glomerulosa cells; critical role of the intramolecular disulphide bond. *Biochem. biophys. Res. Commun.* **122** (1984) 171–174.
11. Bennet H. P. J.: Use of ion-exchange Sep-Pak cartridges in the batch purification of pituitary peptides. *J. Chromatogr.* **359** (1986) 383–390.
12. Bennet H. P. J., Browne C. A. and Solomon S.: Purification of the two major forms of rat pituitary corticotropin using only reversed-phase liquid chromatography. *Biochemistry* **20** (1981) 4530–4538.
13. Bennet H. P. J., Browne C. A. and Solomon S.: α-N-Acetyl-β-endorphin 1-26 from the neurointermediary lobe of the rat pituitary: isolation, purification and characterization by high performance liquid chromatography. *Analyt. Biochem.* **128** (1983) 121–129.
14. Sayers G., Swallows R. L. and Giordano N. D.: An improved technique for the preparation of isolated rat adrenal cells: a sensitive, accurate and specific method for the assay of ACTH. *Endocrinology* **88** (1971) 1063–1068.
15. Goverde H. J. M., Pesman G. J. and Benraad T. J.: Improved sensitivity to adrenocorticotrophin after purification and pro-incubation of isolated rat adrenal cells. *Acta. endocr., Copenh.* **94** (1980) 221–228.
16. Mulay S., Varma D. R. and Solomon S.: Influence of protein deficiency in rats on hormonal status and cytoplasmic glucocorticioid receptors in maternal and fetal tissues. *J. Endocr.* **95** (1982) 49–58.
17. Congote, L. F.: Similarities in structure and function of bovine serum erythrotropin and human insulin-like growth factor II: two fetal erythroid cell stimulating factors. *Biochem. biophys. Res. Commun.* **126** (1985) 653–659.
18. Selsted M. E., Brown D. M., Delange R. J., Harwig S. S. L. and Lehrer R. I.: Primary structures of six antimicrobial peptides of rabbit peritoneal neutrophils. *J. biol. Chem.* **260** (1985) 4579–4584.
19. Lehrer R. I., Ladra K. M. and Hake R. B.: Nonoxidative fungicidal mechanisms of mammalian granulocytes: demonstration of components with candidacidal activity in human, rabbit, and guinea-pig leukocytes. *Infec. Immun.* **II** (1975) 1226–1234.
20. Weber S., Zuckerman J. E., Bostwock D. G., Bensch K. G., Sikic B. I. and Raffin T. A.: Gastrin-releasing peptide is a selective mitogen for small cell lung carcinoma *in vitro*. *J. clin. Invest.* **75** (1985) 306–309.
21. Smith E. M. and Blalock S. M.: Human lymphocyte production of corticotropin and endorphin-like substances: association with leukocyte interferon. *PNAS* **78** (1981) 7530–7534.
22. Lolait S. J., Lim A. T., Toh B. H. and Funder J. W.: Immunoreactive β-endophin in a subpopulation of mouse spleen macrophases. *J. clin Invest.* **73** (1984) 227–280.
23. Smith E. M., Morrill A. C., Meyer W. J. and Blalock J. E.: Corticotropin releasing factor induction of leukocyte-derived immunoreactive ACTH and endorphins. *Nature, Lond.* **321** (1986) 881–882.