J. Steroid Biochem. Molec. Biol. Vol. 40, No. 1-3, pp. 391-398, 1991
Printed in Great Britain. 

0960-0760/91 $3.00 + 0.00


# CORTICOSTATIC PEPTIDES

S. SOLOMON,[1]* J. HU,[1] Q. ZHU,[1] D. BELCOURT,[1] H. P. J. BENNETT,[1] A. BATEMAN[1] and T. ANTAKLY[2]

Endocrine Laboratory, Royal Victoria Hospital, Departments of [1]Medicine, Obstetrics and Gynecology and [2]Anatomy, McGill University, Montreal, Quebec H3A 1A1, Canada

**Summary**—In the last four years corticostatic (anti-ACTH) peptides have been isolated from human, rabbit, guinea pig and rat tissues. These peptides do not act via the cAMP cell signalling system but rather via the inhibition of the binding of ACTH to its receptor most probably through direct competition with the 14–18 sequence of ACTH for receptor binding. ACTH has specific high affinity receptors on adrenal cells but rabbit corticostatin I (CSI) has high capacity, low affinity receptors which are competed for by unlabelled excess CSI but not by excess ACTH. This indicates the presence of specific CSI adrenal cell receptors. The rabbit pituitary, hypothalamus, thalamus, adrenals, lungs and placenta contain sizeable amounts of immunoassayable CSI. Immunochemical localization of CSI indicates that it is present in the large macrophages and in neutrophils in rabbit lung, in macrophages and "supporting" endothelial cells in the spleen and in the adrenals in the cells of the zona reticularis. We have also isolated and identified new peptides which contain 12 cysteines from immune cells of humans, rats and a teleost, the carp. The functions of these peptides are now being determined. This large family of peptides may have many other, yet unidentified functions but at present we can only describe a small number of these.

## INTRODUCTION

There are several peptides which inhibit steroid synthesis by the adrenal that need to be discussed here. It is well known that atrial natriuretic factor (ANF) inhibits aldosterone synthesis in adrenal glomerulosa cells [1–4]. Transforming growth factor $\beta$ (TGF$\beta$) inhibits many cellular functions and is a very potent inhibitor of steroid synthesis [5] as is fibroblast growth factor [6–7] which stimulates adrenal cell growth with a concomitant inhibition of steroidosynthesis. Tumor necrosis factor $\alpha$ (TNF$\alpha$) has recently been shown to be an inhibitor of steroid synthesis stimulated by ACTH [8]. A septic shock factor has been described from peritoneal exudates, which is capable of inhibiting ACTH stimulated steroid synthesis [9] and another such factor from macrophages has also been reported [10]. The latter factor may turn out to be TGF$\beta$.

Initially we isolated corticostatic (anti-ACTH) peptides from rabbit fetal and adult lung and from neutrophils [11–13] and then we went on to isolate a corticostatic peptide from human neutrophils [14]. More recently we have isolated several corticostatic peptides from the rat and guinea pig and all of these are shown in Table 1.

We have now isolated a 5th rat peptide and 2 additional guinea pig corticostatins but all of these will be described in detail elsewhere. In this paper we will discuss some of our more recent findings concerning the biological action of the corticostatins and the cellular localization of the peptides.

## EXPERIMENTAL

The methods used in this paper have all been detailed elsewhere [11–14] and any additional procedures will be referred to in the Results section.

## RESULTS

Figure 1 shows the Scatchard analysis [15] of the binding of labelled $ACTH_{1-24}$ to rat adrenal cells preincubated for 6 h without additions. The ligand used was diiodo-$Tyr^{23}$ and not the monoiodo-$Tyr^{23}$ used by others. We observed high affinity binding displaying a $K_d$

*Proceedings of the VIIIth International Congress on Hormonal Steroids*, The Hague, The Netherlands, 16–21 September 1990.

*To whom correspondence should be addressed.

Table 1. Corticostatic peptides isolated from the rabbit, rat, guinea pig and human

| Peptide | Sequence | Charge | $ED_{50}$ Corticostatic assay (nM) |
|---|---|---|---|
| CSI | G-I-C-A-C-R-R-R-F-C-P-N-S-E-R-F-S-G-Y-C-V-N-G-A-R-Y-V-R-C-C-S-R-R | +8 | 25 |
| CSII | G-R-C-V-C-R-K-Q-L-L-C-S-Y-R-E-R-R-I-G-D-C-I-R-G-V-R-F-P-F-C-C-P-R | +8 | 125 |
| CSIII | V-V-C-A-C-R-R-A-L-C-L-P-R-E-R-R-A-G-F-C-R-I-R-G-R-I-H-P-L-C-C-R-R | +9 | 375 |
| CSIV | V-V-C-A-C-R-R-A-L-C-L-P-L-E-R-R-A-G-F-C-R-R-G-R-I-H-P-L-C-C-R-R | +8 | 500 |
| HP4 | V-C-S-C-R-L-V-F-C-R-R-T-E-L-R-V-G-N-C-L-I-G-G-V-S-F-T-Y-C-C-T-R-V | +4 | 470 |
| HP1 | A-C-Y-C-R-I-P-A-C-I-A-G-E-R-R-Y-G-T-C-I-Y-Q-G-R-L-W-A-F-C-C | +3 | NA[a] |
| R1 | A-C-Y-C-R-I-G-A-C-V-S-G-E-R-L-T-G-A-C-G-L-N-G-R-I-Y-R-L-C-C | +3 | NA |
| R2 | V-T-C-S-C-R-T-S-S-C-R-F-G-E-R-L-S-G-A-C-R-L-N-G-R-I-Y-R-L-C-C | +5 | NA |
| R3 | C-S-C-R-T-S-S-C-R-F-G-E-R-L-S-G-A-C-R-L-N-G-R-I-Y-R-L-C-C | +5 | NA |
| R4 | V-T-C-Y-C-R-R-T-R-C-G-R-R-E-R-L-S-G-A-C-G-Y-R-G-R-I-Y-R-L-C-C-R-R | +9 | 50 |
| GPCS | R-R-C-I-C-T-T-R-T-C-R-F-P-Y-R-R-L-G-T-C-I-F-Q-N-R-V-Y-T-F-C-C | +7 | 400 |

[a] Not active.

of $1.7 \times 10^{-11}$ M which is lower than the $K_d$ reported by others [16] for the high affinity binding component ($K_d$ $2.5 \times 10^{-10}$ M) for rat adrenal cells. We than iodinated rabbit corticostatin I (CSI) and determined its specific binding capacity to its receptor in rat adrenal cells and the results are shown in Fig. 2.

It is obvious from this data that specific high affinity binding could be demonstrated for ACTH and for CSI only after polylysine saturation of the non-specific sites. It was found that the CSI binding capacity was 120-fold greater than the ACTH binding capacity to its receptor. We could also demonstrate that CSI binding could be readily displaced by a

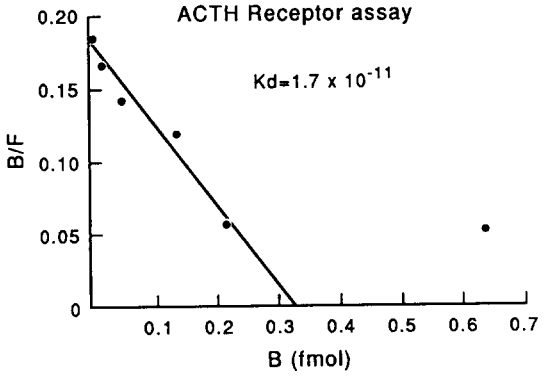


Fig. 1. Scatchard plot of the binding data of ACTH receptor. The adrenals were obtained from male Sprague–Dawley rats weighing 200–250 g and were enzymatically dispersed with collagenase and DNase as described previously [13]. The cell suspensions were pre-incubated at 37°C for 6 h in an atmosphere of 5% $CO_2$ and 95% $O_2$. At the end of incubation, the cells were washed once and 100 $\mu$l aliquots of cell suspension containing 1.2 million cells were coincubated with 150 $\mu$l of iodinated ligand or iodinated ligand and other unlabelled ligands for 2 h at 27°C in an atmosphere of 5% $CO_2$ and 95% $O_2$. The iodinated ligand used in this experiment was [2I-3, 5 $Tyr^{23}$, $Phe^2$, $Nle^4$]-$ACTH_{1-24}$ which had been purified by RP–HPLC and fully characterized after iodization by the lactoperoxidase method. The radioactivity was estimated to be 4000 Ci/mmol by using chromatography and the displacement assay. Non-specific binding was measured in the presence of 500 times excess cold ACTH. The bound and free radioactivity were separated by a gradient centrifugation at 4°C as described previously [13]. The assay was carried in triplicates for total binding and duplicates for non-specific binding. Data shown are averages for specific binding.

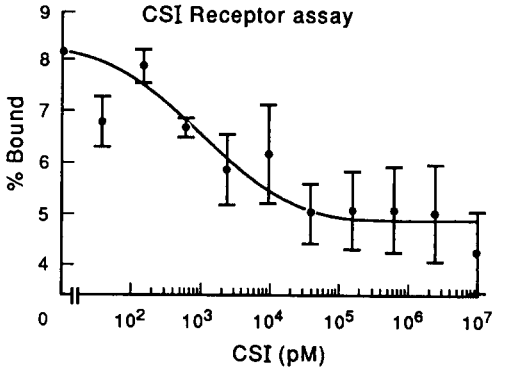


Fig. 2. Specific of CSI binding to rat adrenal cell receptors. The adrenal cells were prepared in the same way as described in Fig. 1. After pre-incubation at 37°C for 2 h in an atmosphere of 5% $CO_2$ and 95% $O_2$, the cell suspension was washed once and resuspended in the same buffer containing 10 $\mu$M of 4K polylysine (Sigma, St Louis, MO). At the end of a further 2 h incubation, 200 $\mu$l of increasing concentrations of CSI were added and coincubated for 30 min at 30°C first, and this was followed by coincubation with 50 $\mu$l of 270,000 cpm iodinated CSI (about 100 pM). The iodinated CSI used in this experiment was [31-$Tyr^{19}$, 31 $Tyr^{27}$]-CSI which had been purified by RP–HPLC after iodination by the lactoperoxidase method. The bound and free radioactivity were separated by gradient centrifugation at room temperature. The assay was carried out in quadruplicate.

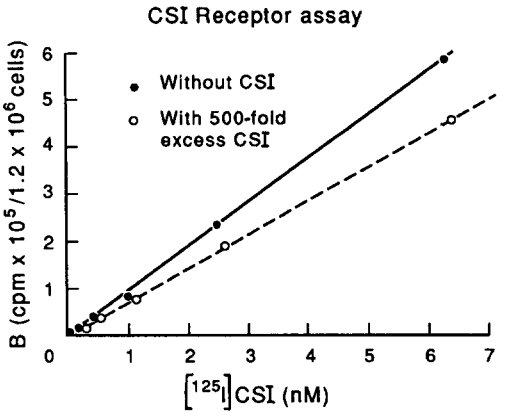


Fig. 3. The experiment was carried out as described in Fig. 2, except that polylysine was not used.

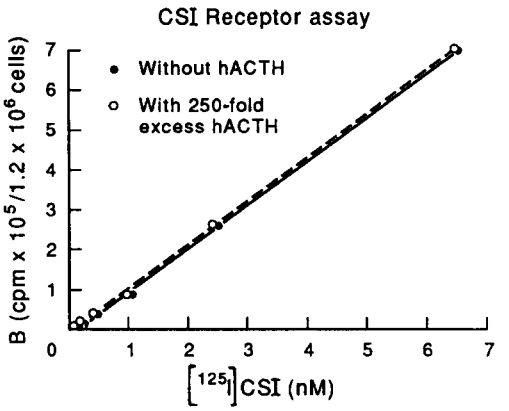


Fig. 4. The experiment was carried out as described in Fig. 2. Binding was measured in the presence of 250-fold excess hACTH and polylysine was not used.

large excess of unlabelled CSI (Fig. 3). By contrast CSI binding was not displaced by a 250-fold excess of unlabelled hACTH (Fig. 4). It therefore appears that the CSI binding receptor is specific for CSI and is not displaced by a large excess of ACTH even though we are not dealing with high affinity CSI binding sites.

We have been doing a considerable amount of work in recent months on the tissue distribution and cellular localization of CSI in a variety of fetal, maternal and adult rabbit tissues and the cell types therein. Figure 5 shows that of the brain tissues studied the pituitary, hypothalamus and thalamus of the pregnant rabbit contain large amounts of CSI

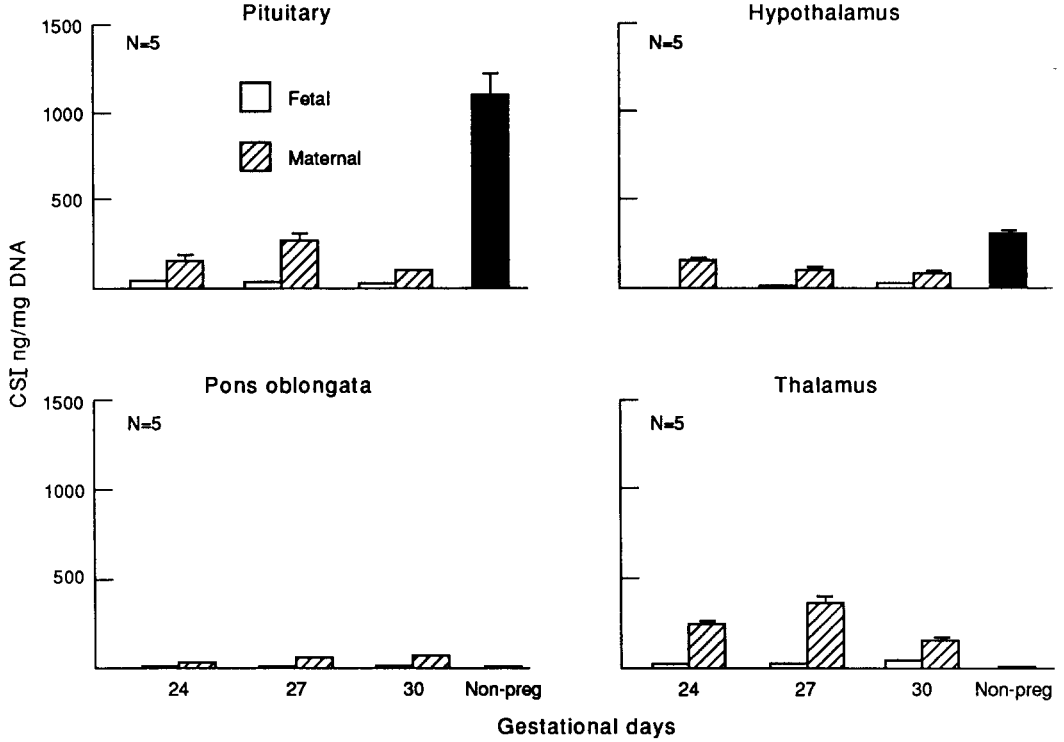


Fig. 5. Quantitation of CSI in brain tissues using a specific RIA. The pituitary, hypothalamus, pons oblongata, thalamus, cerebellum, cerebrum and corpus callosum were dissected from the brain immediately after the rabbits were sacrificed with an overdose of sodium pentobarbital (100 mg/kg given i.v. very rapidly). The tissues were frozen on dry ice and stored at −80°C. The fetal organs from 1 pregnant rabbit were pooled. The tissues were extracted twice with acidic extraction medium (1 M HCl, 5% formic acid, 1% NaCl, and 1% TFA). The peptide fractions were concentrated by using $C_{18}$ Sep-Paks, and eluted with 80% acetonitrile containing 0.1% TFA. CSI was purified partially by 1 RP–HPLC purification step prior to quantitation by RIA. Vertical bars represent the SE.

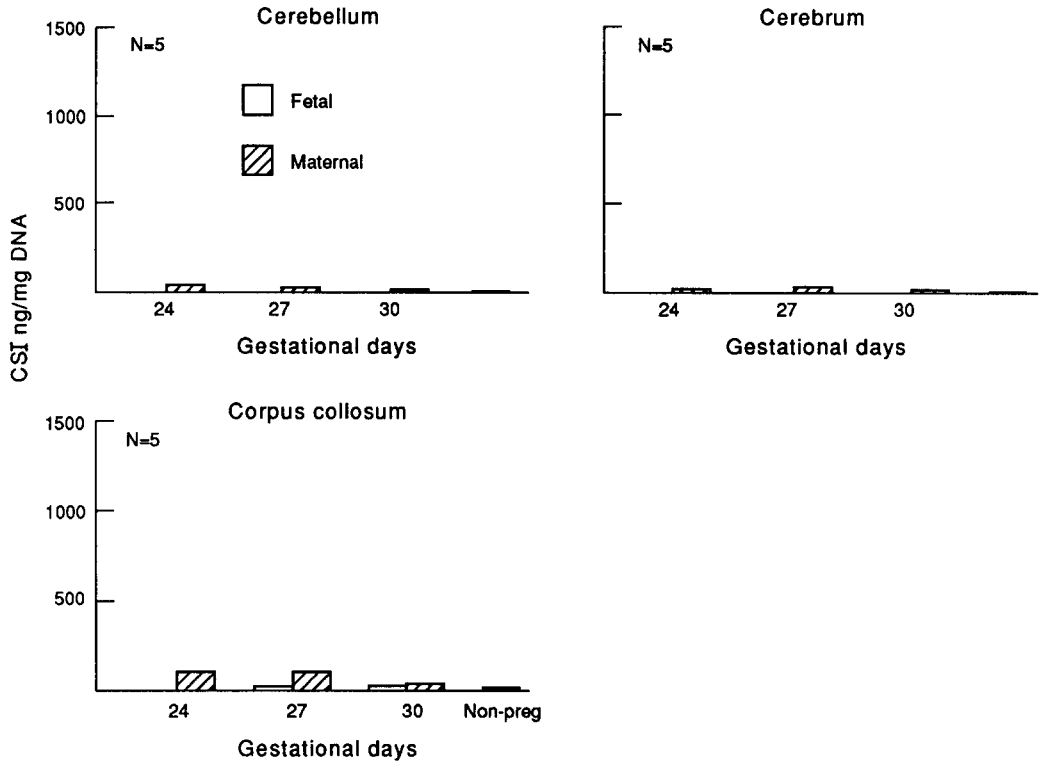


Fig. 6. See legend of Fig. 5.

on days 24, 27 and 30 of gestation. It was noted that the maternal pituitary, hypothalamus and thalamus had the highest amounts of immunoassayable CSI. The adult pituitary and hypothalamus also had very large amounts of CSI. The pons oblongata contains little CSI as did the cerebellum, cerebrum and corpus callosum (Fig. 6).

We then turned to examine the CSI content of the adrenal, lung and placenta during gestation in the rabbit (Fig. 7). It can be seen that the rabbit adrenal has large quantities of CSI

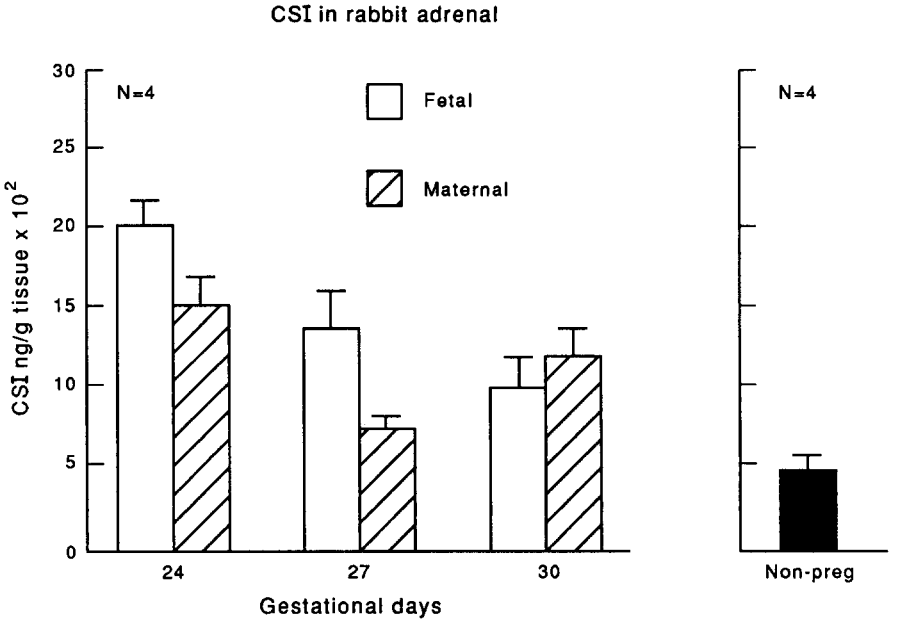


Fig. 7. The adrenals were collected and CSI was quantitated in the same way as described in the legend to Fig. 5.

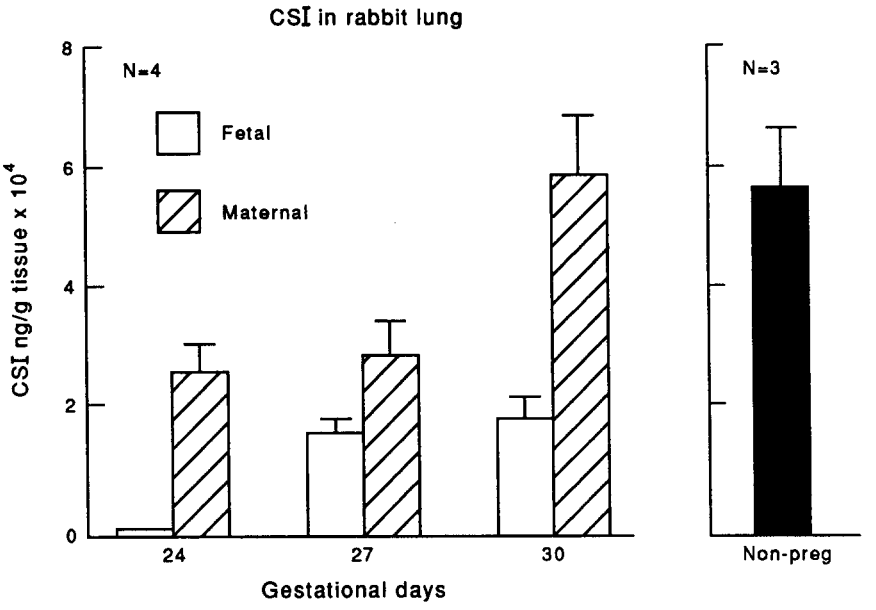


Fig. 8. The lungs were collected and CSI was quantitated by RIA in the same way as described in Fig. 5.

mainly in the fetal adrenal and these titres fall from day 24 until term while the maternal adrenal contains considerably less peptide. In contrast, the rabbit fetal lung has small amounts of CSI on day 24 of gestation but the amounts increase on days 27 and 30 (Fig. 8). In the maternal lung, much larger amounts of CSI are observed and on day 30 of gestation the values found are close to the very large titres seen in the non-pregnant adult lung (Fig. 8). The placenta contains smaller amounts of CSI (Fig. 9) with the concentration of peptide increasing towards term.

We then examined the cellular localization of CSI using an immunoperoxidase cytochemical technique [17]. The results for lung and spleen are shown in Fig. 10. Whereas tissue sections incubated with the specific CSI antiserum show numerous heavily stained cells [Figs 10(A) and (C)] no staining was observed in any of the control sections [Figs 10(B) and (D)]. It appears that CSI in the lung is abundant in large macrophages (mainly internal alveolar) and in large but not small premonocyte-like cell [Fig. 10(A)]. We have also seen CSI in lung neutrophils (data not shown). In the spleen [Fig. 10(C)] there are numerous heavily stained cells which are macrophages; there are also cells which contain CSI which seem to be larger than lymphoid cells and are presumably "supporting" epithelial and monocytic cells [Fig. 10(A)]. Using this immunocytochemical technique we were able to localize CSI in adrenal cortical cells, largely in the zona reticularis of the rabbit adrenal (manuscript in preparation). We are now examining other tissues, particularly the brain and placenta.

In addition to the corticostatic peptides isolated from the rabbit, rat, human and guinea pig shown in Table 1 we have isolated a group of related peptides shown in Table 2. They contain 12 cysteines, but unlike the corticostatins they are not basic. They are not corticostatic in that they do not inhibit ACTH stimulated corticosterone synthesis. The rat and human peptides have greatest homology. The sequence of the human peptide and the amino-terminal portion of the carp homolog are shown in Table 2. The rat peptide has been partially

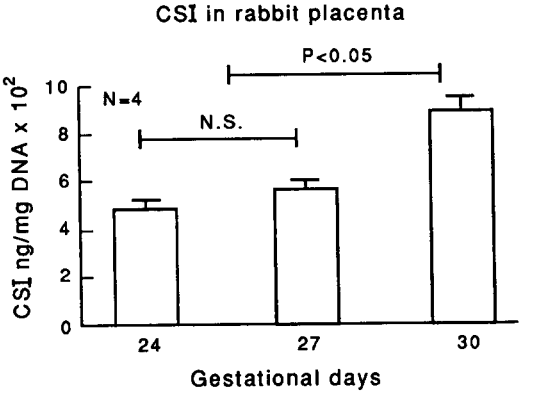


Fig. 9. CSI in the rabbit placenta on day 24, 27 and 30 of gestation was extracted and quantitated by RIA in the same way as described in Fig. 5.

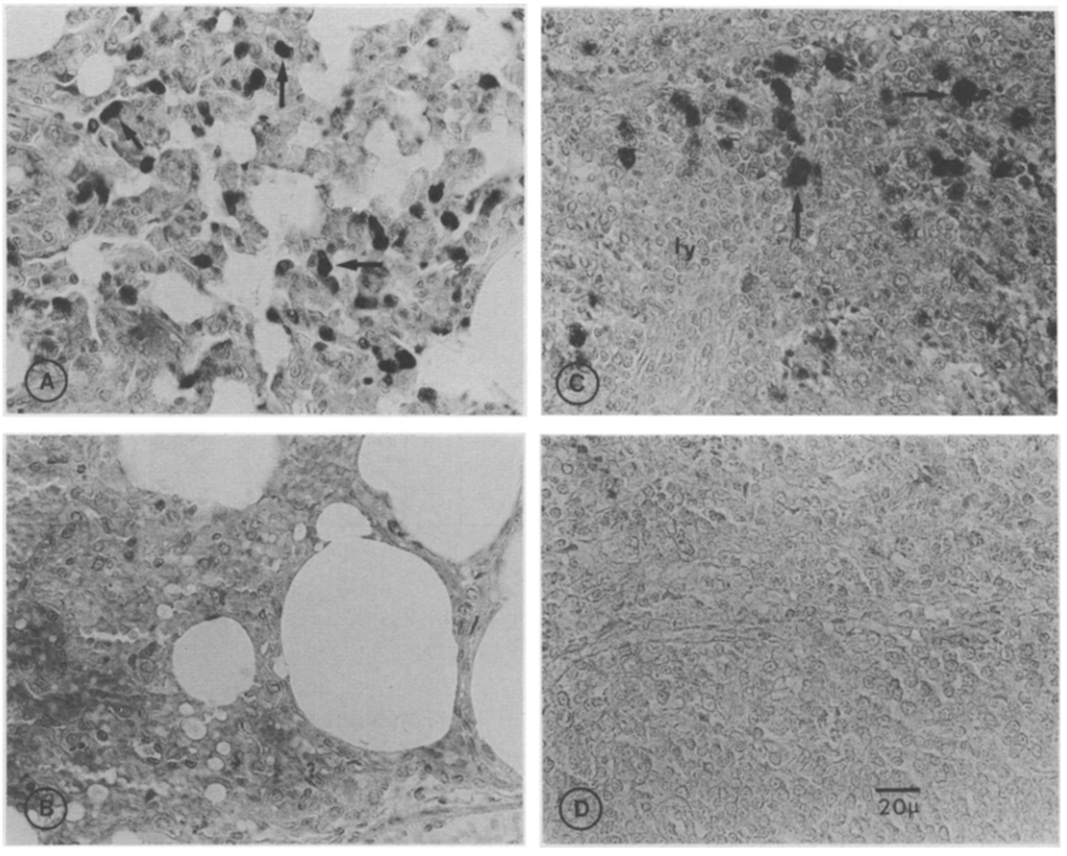


Fig. 10. Immunoperoxidase localization of CSI in tissue sections of the lung (A) and spleen (B). (A) and (B) were incubated with specific antisera to CSI [13] and the antibodies bound to the tissue sections were revealed by immunoperoxidase using the avidin–biotin system. Note in (A) numerous cells that are heavily stained (→). In the spleen (C), note heavily stained cells (→); these are generally larger than the lymphoid cells (ly) which are unstained and are presumably "supporting" epithelial cells. (B) Lung section incubated with pre-immune antisera. (D) Spleen section incubated with the CSI antisera previously absorbed with pure CSI. In both cases no specific staining is observed. All preparations were counterstained with 0.1% methylene blue to distinguish cell morphology. Bar—20 μm.

sequenced and within the first 25 residues only differs from the human peptide in having a glutamic acid at position 1, a leucine at position 6, an asparagine residue at position 20 and a threonine residue at position 21. The carp peptide was isolated from the spleen, the rat peptide from bone marrow cells and the human peptide from neutrophils. There is no doubt that these ubiquitously distributed peptides which we have named the granulins [18] and which we isolated from leukocytes are abundant in cells of the immune system.

## DISCUSSION

A large number of cysteine- and arginine-rich peptides have now been isolated from human, rabbit, rat and guinea pig tissues and immune cells (Table 1). It is clear that HP4, the human corticostatic peptide, is derived from neutrophils, but then we obtained the surprising finding that CSI is localized to the large macrophages in the lung and spleen (Fig. 10). We have previously isolated CSI from rabbit neutrophils obtained from peritoneal exudates [12]. It is evident that the possibility exists that all of these numerous peptides are formed during monocyte differentiation into granulocytes and macrophages. Lung macrophages have a 10% and spleen macrophages have a 75% content of class II major histocompatibility complex (MHC) [19]. In the lung, macrophages trap inhaled particles and may be defective in some membrane proteins. In the spleen macrophages are very heterogeneous as shown by studies using cell surface markers and here they function as antigen presenting cells (APCs) and may be critical for responses to thymus-independent antigens. How CSI and other corticostatins function in macrophages within the lung and spleen is presently

Table 2. Cys 12 peptides

| | |
|---|---|
| Human | -Asp-Val-Lys-Cys-Asp-Met-Glu-Val-Ser-Cys-Pro-Asp-Gly-Tyr- |
| Rat | -Glu- -Leu- |
| Carp | -Val-Ile-His-Cys-Asp -Ala-Ala-Thr-Ile-Cys-Pro-Asp-Gly-Thr- |
| Human | -Thr-Cys-Cys-Arg-Leu-Gln-Ser-Gly-Ala-Trp-Gly-Cys-Cys-Pro- |
| Rat | -Asn-Thr |
| Carp | -Thr-Cys-Cys-Leu-Ser -Pro-Tyr-Gly-Val- |
| Human | -Phe-Thr-Gln-Ala-Val -Cys-Cys-Glu-Asp-His-Ile-His-Cys-Cys- |
| Rat | |
| Carp | |
| Human | -Pro-Ala-Gly-Phe-Thr -Cys-Asp-Thr-Gln-Lys-Gly-Thr-Cys-Glu- |
| Rat | |
| Carp | |

not known. The possible interactions of the corticostatins in the immune–hypothalamic–adrenal system has recently been explored by us [20].

We have previously found that corticostatic peptides do not act at the level of the cAMP cell signalling system, but rather to inhibit ACTH binding [13]. The inhibition of binding was limited to amino acids 14–18 of ACTH, the so-called "address" region of the molecule [13]. We have been able to establish that our rat adrenal cells contain high affinity binding sites for ACTH (Fig. 1) that are about 10 times lower than published values [16]. This can be explained by the fact that we used diiodo-Tyr$^{23}$ labelled ACTH which gives a very high specific activity agonist for the binding assay. High affinity corticostatin I receptors can be demonstrated only after non-specific sites are saturated with polylysine (Fig. 2). The concentration of polylysine used did not increase corticosterone production by rat adrenal cells. This binding is competed for by excess CSI (Fig. 3) but not by excess unlabelled ACTH (Fig. 4), pointing to the possibility that CSI has its own specific binding protein on the adrenal cell which may be distinct from the corticotropin receptor.

It is not surprising that adult, fetal and maternal lung have the largest concentrations of immunoassayable CSI (Fig. 8) as the lung contains a lot of macrophages and neutrophils in the airways and this peptide has been localized to the particles in the lung (Fig. 10). The rabbit adrenal has CSI localized to the cells of the zona reticularis. The fetal and maternal adrenals have not as yet been examined. Neither have we localized CSI to cells in the brain. Such studies are now in progress.

The family of cystine 12 peptides are now being investigated as are their biologic functions and will be the topic of future publications.

*Acknowledgements*—This work was supported by grants from the Medical Research Council of Canada (MT-1658, MA-6733 and MT-7959), the National Institute of Child Health and Human Development (3 RO1 HD04365) and the Fonds pour la formation de chercheurs et l'aide à la recherche du Québec. Qinzhang Zhu is the recipient of a studentship of the Medical Research Council of Canada.

## REFERENCES

1. 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.
2. De Léan A., Racz K., Gutkowska J., Nguyen T.-T., Cantin M. and Genest J.: Specific receptor-mediated inhibition of synthetic atrial natriuretic factor of hormone-stimulated steroidogenesis in cultured bovine adrenal cells. *Endocrinology* **115** (1984) 1636–1638.
3. Goodfriend T. L., Elliott M. and Atlas S. A.: Action of synthetic atrial natriuretic factor on bovine adrenal glomerulosa. *Life Sci.* **35** (1984) 1675–1682.
4. Kudo T. and Baird A.: Inhibition of aldosterone production in the adrenal glomerulosa by atrial natriuretic factor. *Nature* **312** (1984) 756–757.
5. Hota M. and Baird A.: Differential effects of transforming growth factor type $\beta$ on the growth and function of adrenocortical cells *in vitro*. *Proc. Natn. Acad. Sci.* **83** (1986) 7795–7799.
6. Simmion M. H. and Gill G. N.: Regulation of deoxyribonucleic acid synthesis in bovine adrenocortical cells in culture. *Endocrinology* **104** (1979) 588–595.
7. Simmion M. H., Hornsby P. J., Ill C. R., O'Hare M. J. and Gill G. N.: Characterization of cultured bovine adrenocortical cells and derived clonal lines: regulation of steroidogenesis and culture life span. *Endocrinology* **105** (1979) 99–108.
8. Betz J. A. and Roth M.: Tumor necrosis factor inhibits ACTH stimulated corticosterone secretion by rat adrenal cortical cells. *Endocr. Soc. 71st A. Mtg*, Seattle, WA, Abstr. No. 1453 (1989).
9. Mathison J. C., Schreiber R. D., La Forest A. C. and Ulewitch R. J.: Suppression of ACTH-induced steroidogenesis by supernatants from LPS-treated peritoneal exudate macrophages. *J. Immun.* **130** (1983) 2757–2762.
10. Keri G., Parameswaran V., Trunkey P. D. and Ramachandran J.: Effect of septic shock plasma on adrenal cortical cell function. *Life Sci.* **28** (1981) 1917–1923.
11. Zhu Q., Hu J., Mulay S., Esch F., Shimasaki S. and Solomon S.: Isolation and structure of corticostatin peptides from rabbit fetal and adult lung. *Proc. Natn. Acad. Sci. U.S.A.* **85** (1988) 592–596.

12. Zhu Q., Singh A., Bateman A., Esch F. and Solomon S.: The corticostatin (anti-ACTH) and cytotoxic activity of peptides isolated from fetal, adult and tumor-bearing lung. *J. Steroid Biochem.* **27** (1987) 1017–1022.
13. Zhu Q., Bateman A., Singh A. and Solomon S.: Isolation and biological activity of corticostatic peptides (anti-ACTH). *Endocr. Res.* **15** (1989) 129–150.
14. Singh A., Bateman A., Zhu Q., Shimasaki S., Esch F. and Solomon S.: Structure of a novel granulocyte peptide with anti-ACTH activity. *Biochem. Biophys. Res. Commun.* **155** (1988) 524–529.
15. Scatchard G.: The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51** (1949) 660–672.
16. Schulster D. and Schwyzer P.: In *Cellular Receptors for Hormones and Neurotransmitters* (Edited by D. Schulster and A. Levitzki). John Wiley, New York (1980) p. 197.
17. Antakly T. and Eisen H. J.: Immunocytochemical localization of glucocorticoid receptor in target cells. *Endocrinology* (1984) 1984–1989.
18. Bateman A., Belcourt D., Bennett H., Lazure C. and Solomon S. *Biochem. Biophys. Res. Commun.* **173** (1990) 1161-1168.
19. Unanue E. R.: Macrophages, antigen-presenting cells and the phenomena of antigen handling and presentation. In *Fundamental Immunology* (Edited by W. E. Paul). Raven Press, New York (1989) pp. 95–116.
20. Bateman A., Singh A., Kral T. and Solomon S.: The immune–hypothalamic–pituitary–adrenal axis. *Endocrine Rev.* **10** (1989) 1–21.
21. Buckley D. I. and Ramachandran J.: Characterization of corticotropin receptors on adrenocortical cells. *Proc. Natn. Acad. Sci. U.S.A.* **78** (1981) 7431–7435.