

## Expression of Pro-opiomelanocortin Gene and Quantification of Adrenocorticotrophic Hormone-like Immunoreactivity in Human Normal Peripheral Mononuclear Cells and Lymphoid and Myeloid Malignancies

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### Abstract

Using Northern blotting with a human genomic DNA probe for the pro-opiomelanocortin (POMC) gene, we have shown specific mRNA in normal human peripheral mononuclear cells (PBMC); the presence of specific mRNA was also observed in a T lymphocyte cell line derived from a patient with lymphoma.

We then demonstrated that PBMC translate the message into protein. Thus, using a radioimmunoassay with an antibody for ACTH, a median of 29 pg of ACTH-like immunoreactivity (ACTH-LIR) was found in  $10^7$  PBMC. ACTH-LIR was also detected in seven different cell lines derived from patients with lymphoid and myeloid malignancies, two of them JM and U937 showing the highest values 135 and 108 pg/ $10^7$  cells, respectively. The chromatographic characterization of this ACTH-LIR showed, at least, three molecular forms of immunoreactive ACTH with molecular weights of the order of 31,000 POMC, 22,000 ACTH, and 4,500 ACTH, in addition to high-molecular-weight material (> 43,000).

We conclude that PBMC produce ACTH-LIR which may act as a paracrine immunomodulator in a similar way to lymphokines and/or may signal the adrenal gland to secrete glucocorticoids.

### Introduction

Evidence has accumulated that human peripheral blood mononuclear cells (PBMC), *in vitro*, may synthesize and release ACTH and endorphin-like peptides into the culture medium when stimulated with different substances such as Newcastle disease virus (1), lipopolysaccharide (2), or corticotrophin-releasing factor (3). The presence of this material was assessed indirectly by immunofluorescence on fixed PBMC (1, 3) and by evaluation of the steroidogenic or opioid binding activities of supernatants from cultured cells (1–3). Furthermore, it has been suggested that pro-opiomelanocortin (POMC)<sup>1</sup>-derived peptides produced by cells of the immune

system play a role in pathological conditions such as septic shock (4) and the ectopic ACTH syndrome (5, 6). Despite these data, no quantification of ACTH-like immunoreactivity (ACTH-LIR) in human PBMC has been reported. Quantitative evaluation of ACTH-LIR was achieved only in adherent macrophages purified from mouse spleen but no ACTH or  $\beta$ -endorphin-like material was detected in mouse peripheral lymphocytes (7). Using Northern blotting, expression of the POMC gene was demonstrated in mouse splenocytes (8, 9), however no such data has been reported in normal unstimulated human PBMC although, recently, transformed PBMC have been shown to produce POMC mRNA (10).

In the present study, the ACTH content of human PBMC and lymphoid and myeloid cell lines has been measured using a radioimmunoassay; Northern blotting with a human genomic DNA probe for the POMC gene and chromatographic characterization of the ACTH-LIR have also been performed.

### Methods

Human PBMC were obtained, by Lymphoprep density gradient centrifugation, from venous blood of 11 normal volunteers (three women and eight men, mean age  $30 \pm 7$  yr). At least 95% of these cells were positive for T and B markers. Several cell lines ( $n = 7$ ) derived from patients with malignancies, were also studied. They include lymphoid (JM HUT78, HFB-1, WMPT, L6) and myeloid (HL60, U937) cell lines (Table I). Cells were kept in culture with RPMI 1640 medium supplemented with 10% fetal calf serum, 100 IU penicillin/ml and 100  $\mu$ g streptomycin/ml in 5% CO<sub>2</sub>/air at 37°C until required. Before experimentation, cells were removed from tissue culture flasks and washed three times in phosphate buffered saline.

**RNA hybridization studies.** Total RNA from PBMC and lymphoid and myeloid cell lines was isolated from the cell pellet by homogenization in 4 M guanidine isothiocyanate 0.7% (vol/vol)  $\beta$ -mercaptoethanol and centrifuged through a 5.7 M cesium chloride cushion according to the method described by Chirgwin et al. (11). The RNA precipitate was resuspended in 10 mM Tris HCl (pH 7.5), plus 1 mM EDTA and extracted with chloroform/isobutanol (4:1) before ethanol precipitation. RNA was quantified by measurement of the optical density at 260 nm. Poly(A)<sup>+</sup>RNA from PBMC was prepared by oligo-dT Sepharose affinity chromatography. RNA was then separated by electrophoresis on 1.4% agarose (wt/vol) formaldehyde (6.5%) (vol/vol) gels run in 20 mM 3-*N*-morpholino propane sulphonic acid (MOPS) buffer, pH 7.0. RNA samples were denatured before loading by heating to 65°C in 33% formamide/5% formaldehyde/20 mM MOPS. After electrophoresis, RNA was stained with ethidium bromide (5  $\mu$ g/ml) in order to visualize 28S and 18S RNA bands, then transferred to nitrocellulose paper for 12–24 h and baked at 80°C for 2 h under vacuum. Prehybridization and hybridization were carried out as previously described by Thomas (12) using a 1,093-bp Sma I fragment from p $\lambda$ 26 (13) containing 780 bp of exon 3 and 313 bp of 3' flanking DNA. Probes were labeled with [<sup>32</sup>P]CTP (Amersham, Bucks,

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1. Abbreviations used in this paper: ACTH-LIR, ACTH-like immunoreactivity; nt, nucleotide(s); POMC, pro-opiomelanocortin.

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Table I. Antigen Cluster Designation of the Lymphoid and Myeloid Cell Lines Tested

Cell line	Malignancy of origin	ACTH-LIR	CD2 rosette receptor	CD3 T cells	CD4 T helper	CD15 monocyte granulocyte	CD19 B cells
		<i>pg/10<sup>7</sup> cells</i>					
JM	T leukemia	135	+	+	+	-	-
HUT78	T lymphoma	50	+	+	+	-	-
U937	"Histiocytic" lymphoma	108	-	-	-	+	-
HL60	Promyelocytic leukemia	12	-	-	-	+	-
HFB-1	Myeloma	32	-	-	-	-	-
WMPT	Waldestrom's macroglobulinemia	17	-	-	-	-	+
6L	Myeloma	26	-	-	-	-	+

England) to a high specific activity ( $10^9$  cpm/ $\mu$ g DNA) by the oligonucleotide-primed labeling technique (14). Approximately  $10^7$  cpm were used per filter. After 24 h of hybridization at  $42^\circ\text{C}$ , blots were washed twice at room temperature in  $2\times$  SSC ( $1\times$  SSC = 0.15 M NaCl, 0.015 M sodium citrate) 0.2% SDS for 30-min intervals, followed by two washes at  $55^\circ\text{C}$  in  $0.2\times$  SSC 0.1% SDS for 15-min intervals. Blots were then exposed to Kodak XAR 5 film with intensifying screens at  $-70^\circ\text{C}$ .

**Radioimmunoassay.** To assess the ACTH content of the cells, they were lysed by the addition of 2 ml of 0.01 M HCl/ $2 \times 10^7$  cells containing 2,000 KIU Trasylol at  $4^\circ\text{C}$ . One hour was required to complete cell lysis, after which the lysate was heated for 10 min at  $90^\circ\text{C}$ . The ACTH was then measured by radioimmunoassay after Vycor extraction of the neutralized cell lysate, as previously described (15). Using this system exogenously added ACTH gave a recovery  $> 95\%$ .

**Gel filtration chromatography.** The chromatographic characterization of ACTH-LIR detected in the cell lysate was performed by a modification of the technique of Ratter et al. (16) utilizing G50 and G75 Sephadex superfine columns ( $2.5 \times 100$  cm) eluted at 6 ml/h with 1% formic acid containing 1 g/liter polypep. Fractions were collected at intervals of 1 h, and after evaporation to dryness the column fractions were then reconstituted in assay buffer, and ACTH-LIR was measured by radioimmunoassay. Before processing, cell lysates were treated with 8 M urea at  $4^\circ\text{C}$  for 24 h in order to dissociate the ACTH which could have been bound to a carrier protein.

## Results

**Expression of the POMC gene.** Positive hybridization was detected with poly(A)+RNA from PBMC and total RNA from a T lymphocyte cell line (HUT78) (Fig. 1). The hybridization band in the RNA from normal PBMC was calculated to be 800 nucleotides (nt) long. By contrast, the size of the POMC mRNA of HUT78 cells was 1,200 nt long, similar to that found in the pituitary. In addition a large hybridizing band of 6–7 kb was detected in HUT78 cells (Fig. 1, lane C) and this probably represents unprocessed POMC mRNA. On reprob-ing the same blot with an intron 2-specific probe, the 1,200-nt species was not detected whereas the larger species remained (data not shown).

**ACTH-LIR evaluation.** A median of 29 pg of ACTH-LIR was found in  $10^7$  PBMC. This ACTH-LIR was not due to carryover from circulating ACTH as there was no correlation between plasma and lymphocyte ACTH-LIR concentrations (McLoughlin and Buzzetti, unpublished observations). Intracellular ACTH-LIR was also detectable in all cell lines studied, JM, U937, and HUT78 showing the highest levels, 135, 108, and 50 pg/ $10^7$  cells, respectively (Fig. 2). JM and HUT78 are

both lymphocyte cell lines, whereas U937 is a monocyte cell line (Table I).

**Chromatographic characterization.** Sephadex G50 chromatographic profile of ACTH-LIR identified the presence of 1–39 ACTH together with higher-molecular-weight material eluting after the void volume (Fig. 3). Further chromatography using Sephadex G75 superfine gel columns showed, in addition to high-molecular-weight material ( $> 43,000$ ), ACTH immunoreactive peptides eluting between 43,000 and 20,000 molecular weight markers. These may represent the precursors 31,000-mol wt POMC and 22,000-mol wt ACTH (Fig. 4).

## Discussion

Previous data have emphasized the biological property (steroidogenic activity) of supernatants of PBMC stimulated *in vitro* with different stimuli but no quantitative measurement of the ACTH-LIR content of the cells was performed. We have demonstrated that both normal PBMC and the lymphoid and myeloid cell lines tested contain ACTH-LIR. This immunoreactivity is contributed to in part by peptides with chromatographic elution characteristics of 31,000-mol wt POMC, 22,000-mol wt ACTH, and 4,500-mol wt ACTH. Additional

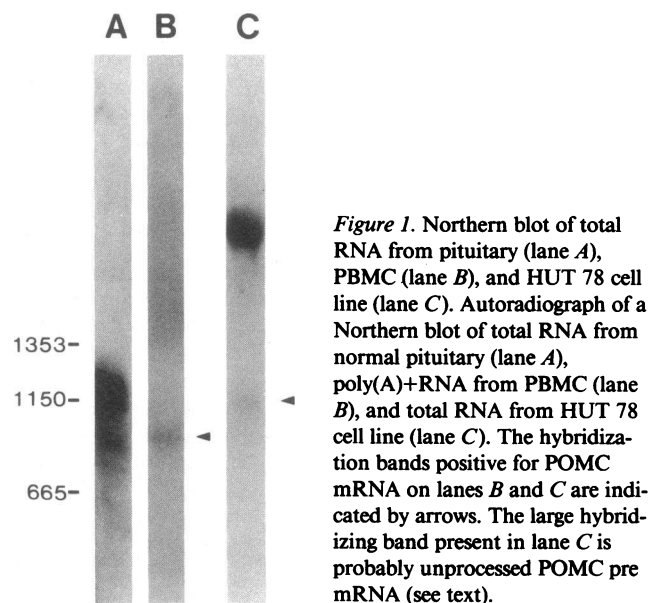


Figure 1. Northern blot of total RNA from pituitary (lane A), PBMC (lane B), and HUT 78 cell line (lane C). Autoradiograph of a Northern blot of total RNA from normal pituitary (lane A), poly(A)+RNA from PBMC (lane B), and total RNA from HUT 78 cell line (lane C). The hybridization bands positive for POMC mRNA on lanes B and C are indicated by arrows. The large hybridizing band present in lane C is probably unprocessed POMC pre mRNA (see text).

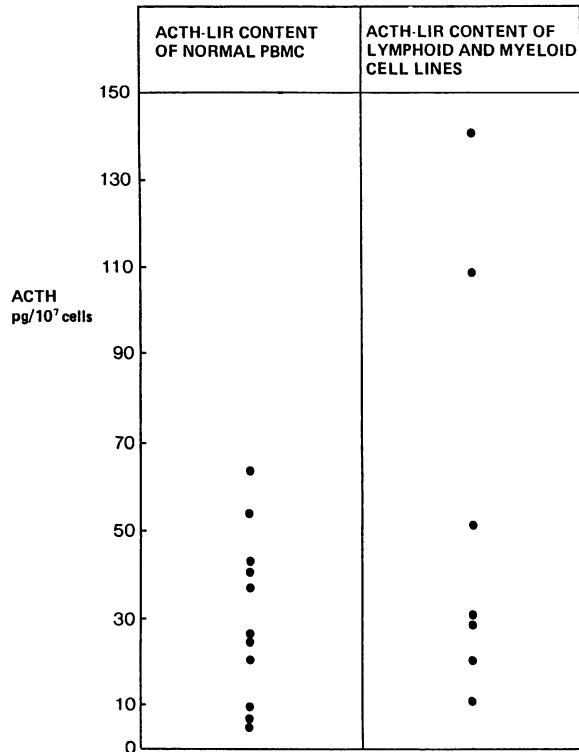


Figure 2. ACTH-LIR content of normal peripheral mononuclear cells and lymphoid and myeloid cell lines.

peaks of immunoreactivity are also present which are currently under investigation.

Here we demonstrate, for the first time, the ability of normal human unstimulated PBMC to express the mRNA for POMC. In these cells the message appears to be 800 nt long.

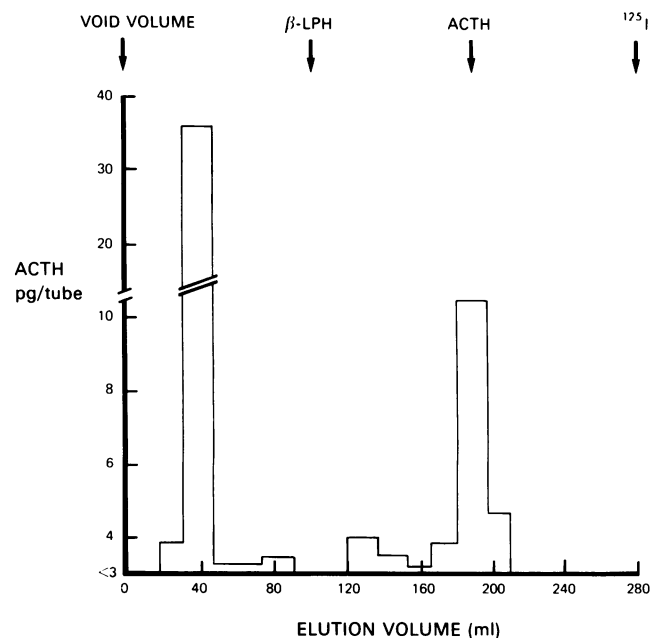


Figure 3. G50 chromatography of ACTH-LIR. Sephadex G50 chromatography of lysate obtained from peripheral mononuclear cells ( $13 \times 10^7$ ).

Oates et al. (10) recently described the presence of a "short" POMC mRNA in Epstein-Barr virus-transformed human lymphocytes, findings which are consistent with our own. This RNA species is approximately 400 nt shorter than that found in the pituitary and similar to that detected in extrapituitary tissues (17-19). A previous report (18) suggested that the 800-nt mRNA, probably representing an RNA species lacking both exon 1 and 2 sequences and part of the 5' region of exon 3, might either not be translated or translated into a truncated form of POMC which lacks a signal peptide necessary for membrane translocation and precursor processing. In this study the inability to detect the 1,200-nt mRNA in PBMC does not exclude the possibility of the existence of this message which could be present in concentrations insufficient to be detected by our method. Using a highly sensitive S1 nuclease mapping technique, the presence of 1,200-nt mRNA has been reported in human testis (18) although the major mRNA species in this tissue was 800 nt. This raises the possibility that the POMC peptide present in extrapituitary tissues might derive from efficient translation of full-length POMC mRNA present in very low concentrations. The HUT 78 cell line gave a positive signal for 1,200-nt mRNA. This may signify that this cell line is derived from a very small proportion of normal PBMC that express POMC mRNA in this form or that a switch in POMC mRNA from an 800- to a 1,200-nt follows neoplastic transformation.

The physiological significance of ACTH-LIR in human PBMC is still unknown. It has been recently demonstrated that these cells possess receptors for ACTH (20) and there are several reports indicating that ACTH may regulate the immune response; it has been shown to suppress antibody production (21), to inhibit the ability of gamma-interferon to activate macrophages (22) and to modulate B lymphocyte function (23). Therefore it is tempting to speculate that the ACTH-LIR of immune origin may be responsible for these effects and so have a paracrine function similar to that of other lymphokines. Thus, the two cell lines indicating the highest levels of ACTH-LIR may provide a model of human origin which could be utilized to study the control of the POMC gene, the regulation of processing and/or secretion of ACTH related peptides and their possible role in the etiology of these tumors.

Another possibility is that, if ACTH is both synthesized and secreted from normal PBMC, it might stimulate adrenal steroidogenesis. Evidence supporting this theory includes a patient with a paravertebral inflammatory lesion presenting with clinical features of the ectopic ACTH syndrome (24). No obvious ectopic source was found, but removal of the inflammatory tissue enriched in lymphocytes led to a regression of symptoms and production of ACTH by PBMC was proposed. Furthermore, there are reports of patients with plasmocytomas (5) and acute myeloblastic leukemia (6) in whom a diagnosis of the ectopic ACTH syndrome had been made without any identification of an alternative source of ACTH. Since the ectopic hormone production is defined as secretion by a tissue not normally engaged in the synthesis of ACTH, our data, demonstrating the production of ACTH-LIR by normal PBMC, indicates that the term "ectopic ACTH syndrome" is inappropriate in these cases. The overproduction of ACTH in some lymphoid and myeloid malignancies should be regarded as a pathological condition in which the high levels of ACTH may be due to a dysregulation in the hormone synthesis rather than to a POMC gene derepression. In some of these cases the

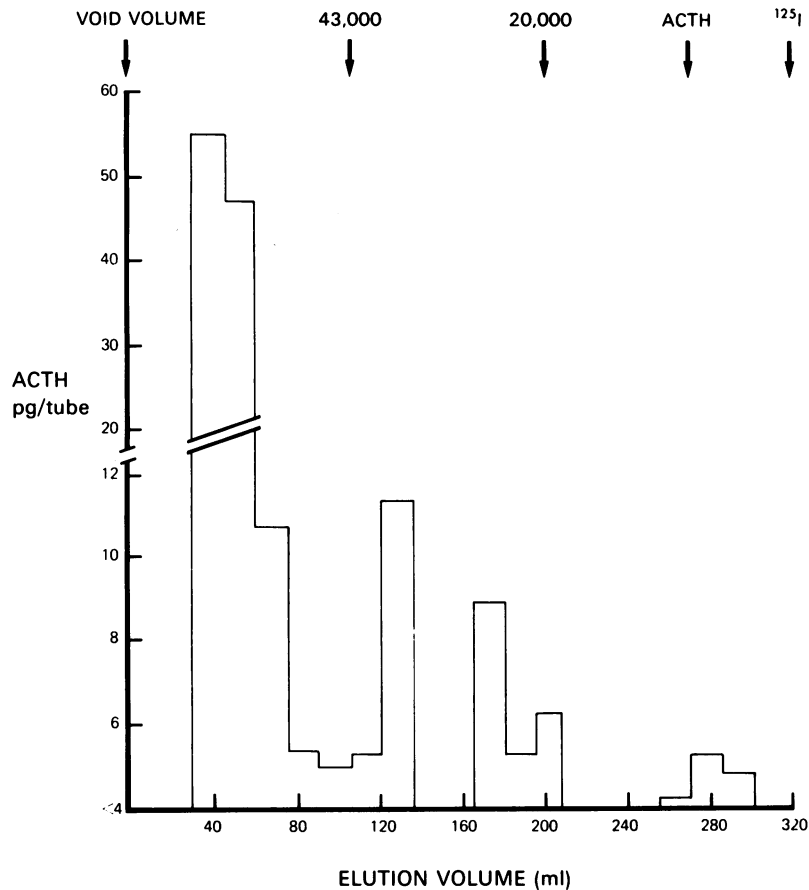


Figure 4. G75 chromatography of ACTH-LIR. Sephadex G75 chromatography of lysate obtained from peripheral mononuclear cells ( $15 \times 10^7$ ).

overproduction might lead to the appearance of clinical and laboratory features of Cushing's syndrome.

In conclusion, the demonstration of POMC gene products in human PBMC underlines the presence of a close interrelationship between the immune and endocrine systems and may explain the pathophysiology of diseases with neuroendocrine and immune components.

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