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In situ localization of ACTH receptor-like mRNA in molluscan and human immunocytes

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Abstract. Adrenocorticotropin hormone (ACTH) receptor-like messenger RNA was localized in molluscan hemocytes and human peripheral blood mononuclear cells by in situ hybridization using a digoxigenin-labelled bovine complementary DNA probe. These findings suggest that the ACTH receptor gene has been highly conserved during evolution. Moreover, these data represent further support for a relationship between the immune and neuroendocrine systems in invertebrates, as documented in our previous studies [1].

Key words. Mollusc; human; immunocyte; ACTH receptor-like mRNA; in situ hybridization.

Many studies in invertebrates and vertebrates, and particularly in mammals, suggest that the immune and neuroendocrine systems interact. Indeed, neuropeptides and hormones have been demonstrated in cells of the immune system [2, 3]. Adrenocorticotropin hormone (ACTH) was first found in the pituitary gland, but recent evidence shows that it is also synthesized in other tissue, including that from the immune system [1]. Moreover, this pro-opiomelanocortin (POMC) product has been well conserved during evolution. ACTH-like molecules have been found in the hemocytes of molluscs [4–7], insects [8] and annelids [9], as well as in lower to higher vertebrates [10, 11]. POMC mRNA is also present in rodent spleen cells and in immunocytes of molluscs, fish and frogs [6, 7, 12].

The communication between the immune and neuroendocrine systems depends on the presence of specific receptor molecules on the immunocyte plasma membrane. Relevant data, particulary in invertebrates, are only now emerging. In mammals, the presence of both high- and lower-affinity ACTH receptors on mononuclear cells from human peripheral blood has been demonstrated [13]. ACTH receptors have also been found in the murine B lymphocytic cell line BCL1 [14], and Clarke and Bost [15] report the presence of functional ACTH receptors on normal rat T and B lymphocytes. Moreover, receptors for β-endorphin have been seen in transformed human lymphocytes [16], others for vasoactive intestinal polypeptide (VIP) in lymphocytes and human monocytes [17, 18], and others again for growth hormone in human monocytes [19].

In the present study, we demonstrate that molluscan hemocytes and human peripheral blood mononuclear cells express ACTH receptor-like mRNA.

Materials and methods

Samples. Specimens of *Mytilus galloprovincialis* Lmk. were collected around Cattolica (RN) (Italy) from rocks in the Adriatic Sea and maintained in the laboratory. The hemolymph was extracted from the posterior adduc-

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tor muscle with a sterile 2 ml syringe. Hemocytes were obtained by cytocentrifugation of the hemolymph on a slide at 800 rpm for 2 min followed by air-drying.

Human peripheral blood mononuclear cells were separated by Ficoll Hypaque (Sigma, St. Louis, MO, USA) gradient centrifugation of peripheral blood obtained from healthy volunteers. The cells were removed from the interface, washed in phosphate buffered saline (PBS), resuspended in minimal essential medium (MEM) (Serva, Feinbiochemica, Heidelberg/New York) to which 0.1% bovine serum albumin (BSA) had been added and diluted to a final concentration of 1.5–2 millions/ml.

Unfixed and fixed (4% *p*-formaldehyde in Sorensen buffer, pH 7.2) molluscan hemocytes and human mononuclear leukocytes were used for in situ hybridization.

In situ hybridization reaction

Probe. Plasmid pBluescript II KS-Phagemid containing about 3000 bp bovine ACTH receptor cDNA, the characteristics of which have been previously reported [20], was linearized by digestion with restriction endonuclease Eco RI and subsequently subjected to gel electrophoresis.

Dig-labelled probe preparation. Probe labelling was carried out by incorporating digoxigenin (Dig)-labelled deoxyuridine triphosphate (Boehringer Mannheim, Germany) by random primer DNA-labelling, according to Feinberg and Vogelstein [21]. The reaction was stopped by the addition of 0.2 M EDTA, pH 8.

Hybridization procedure. Unfixed and fixed immunocytes were treated with PBS plus glycine (0.7%), permeabilized with 0.3% Triton X-100 in PBS for 15 min at room temperature, and washed with PBS and with a mixture containing 2× SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7) for 10 min. Samples were then treated for 1 h at room temperature with the prehybridization mixture (4× SSC), 40% formamide and 1× Denhardt solution). Hybridization was performed overnight at 37 °C by the addition of a denaturated Dig-labelled ACTH receptor cDNA probe (50 ng/μl) to the prehybridization mixture. The immunocytes were then rinsed once in 2× SSC for 1 h at room temperature, once in 1 × SSC for 30 min at room temperature, once in 0.5× SSC for 30 min at 37 °C, and once in 0.5× SSC for 30 min at room temperature. The reaction was visualized by immunological detection. The slides were treated with sheep anti-Dig Fab fragments conjugated to alkaline phosphatase, and the enzymatic activity was observed.

The hybridization reaction was repeated three times, and the specificity of the reaction was performed by omitting the probe.

Results and discussion

The in situ hybridization tests using a bovine ACTH receptor cDNA probe showed that molluscan hemocytes and human peripheral blood mononuclear cells express a molecule similar to ACTH receptor mRNA. The only hemocyte type present in *M. galloprovincialis* hemolymph showed a strong reaction, and about 90% of the population was positive. Similar behaviour was observed in human lymphocytes, while a less intense reaction was observed in the monocytes (figs 1a, b, c). As far as human blood cells are concerned, our data confirm the results of Smith et al. [13]. Using a radiolabelled assay, these authors demonstrated the presence of ACTH receptors on human lymphocytes and monocytes, and suggested a correlation between the structure or expression of the ACTH receptors on human peripheral blood cells and adrenal cortex cells.

The expression of ACTH receptor mRNA in molluscan hemocytes represents a key point in explaining the interrelation between immune and neuroendocrine systems in invertebrates, as we have demonstrated in previous investigations. Indeed, we found that the hemocytes from molluscs *M. galloprovincialis*, *Planorbarius corneus* and *Viviparus ater*, from the insect *Calliphora vomitoria*, and from the annelid *Eisenia foetida* contain molecules shared by both systems [1]. Using various procedures, we proved that the phagocytic hemocytes contain several molecules that are immunoreactive to neuropeptides, hormones and cytokines, such as ACTH, CRH, β-endorphin, α-MSH, IL-1α, IL-1β IL-2, IL-6 and TNF-α [1]. Morever, we also demonstrated that classical hormones or neuropeptides, such as ACTH, modulated hemocyte activity, increasing cell migration and phagocytic activity, and provoking the release of biogenic amines [1]. These latest results indicate that, as previously surmised, ACTH modulation of hemocytes is mediated by specific receptors present on hemocyte plasma membrane.

Another important result emerging from our data is that the ACTH receptor gene has been well conserved during evolution, suggesting that some sequences of the molluscan receptor are probably similar to the mammalian counterpart. However, further studies are needed to evaluate these possible similarities more fully. In previous studies using a human probe [6, 7], we also detected POMC mRNA in hemocytes from different molluscan species, including *M. galloprovincialis*, as well as in blood cells from fish and frog. In particular, mRNA was expressed by hemocytes and leukocytes with phagocytic activity from both invertebrates and vertebrates. This molecule was also found in lymphocytes from frog, but not in fish.

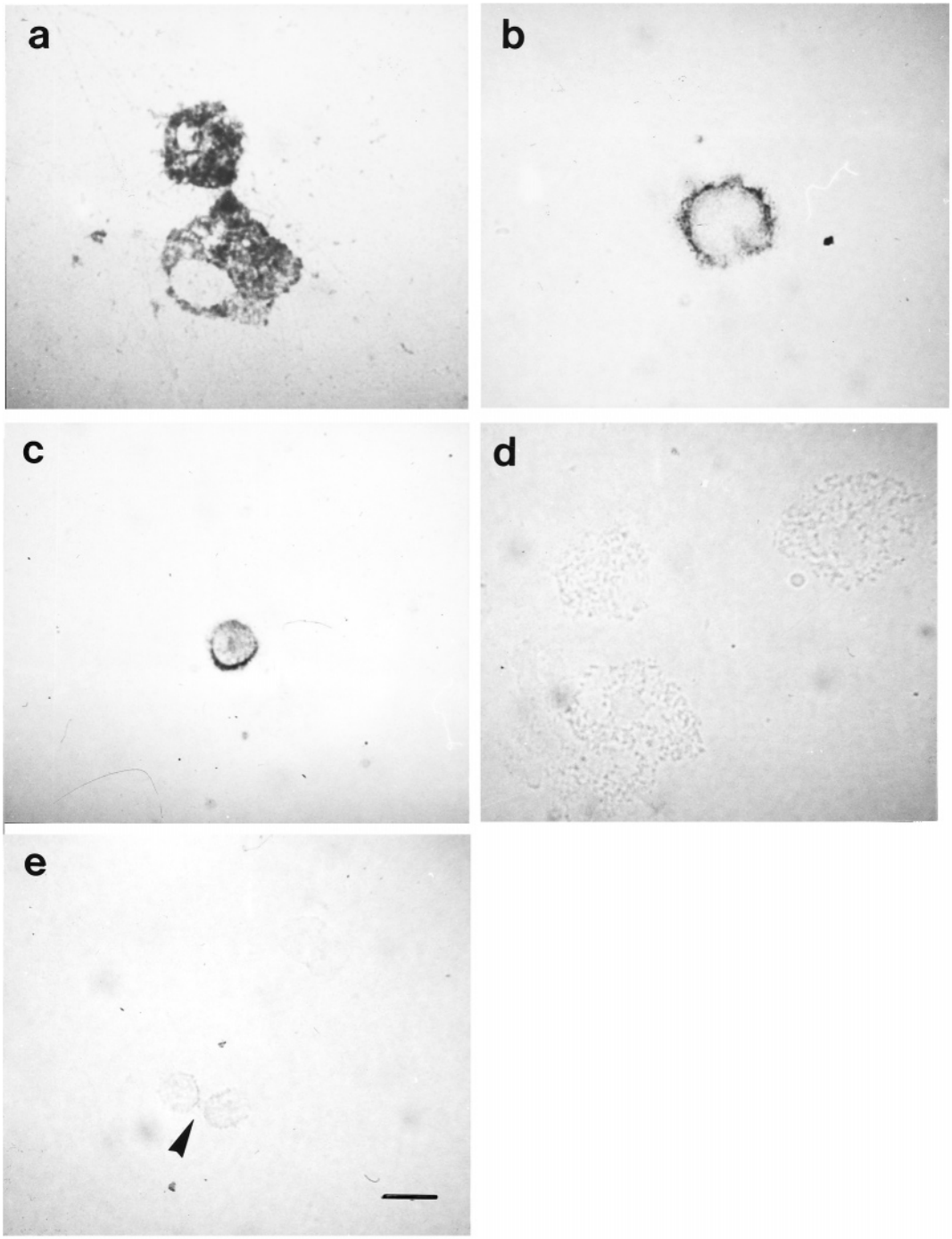

Figure 1. In situ hybridization of the expression of ACTH receptor-like mRNA in molluscan and human immunocytes: (*a*) *M. galloprovincialis* hemocytes, (*b*) human monocyte and (*c*) human lymphocyte. Negative controls: (*d*) *M. galloprovincialis* hemocytes and (*e*) human lymphocytes (arrowhead). Bar, 10 μm.

Taken together, these findings suggest that from the beginning of evolution a conservative and integrated interaction between immune and neuroendocrine systems has occurred in order to maintain body homeostasis.

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