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Immunocytochemical evidence of vertebrate bioactive peptide-like molecules in the immuno cell types of the freshwater snail *Planorbarius corneus* (L.) (Gastropoda, Pulmonata)

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An immunocytochemical investigation was carried out on round and spreading hemocytes of *Planorbarius corneus* by using 20 antisera to vertebrate bioactive peptides. The immunotests showed the presence of α_1-antichymotrypsin-bombesin-, calcitonin-, CCK-8 (INC)-, CCK-39-, gastrin-, glucagon-, Met-enkephalin-, neurotensin-, oxytocin-, somatostatin-, substance P-, VIP-, and vasopressin-immunoreactive molecules in the spreading hemocytes. The round hemocytes were only positive to anti-bombesin, anticalcitonin, anti-CCK-8 (INC), anti-CCK-39, anti-neurotensin, anti-oxytocin, anti-substance P and anti-vasopressin antibodies. No immunostaining was observed with anti-CCK-8 (Peninsula), anti-insulin, anti-prolactin, anti-thyroglobulin and anti-thyroxin (T4) antibodies. As probably in vertebrates, these bioactive peptides may modulate immuno cell function.

Planorbarius corneus; Hemocyte; Bioactive peptide; Immunocytochemistry

1. INTRODUCTION

The existence of bioactive peptides (BAPs) that function both as neurotransmitters and hormones is generally accepted; likewise, cells of both the nervous and endocrine system can produce the same BAP. These findings, that indicate a bidirectional correlation between the nervous and endocrine system, are known both in vertebrates [1] and invertebrates [2]. A further link has been demonstrated with the immune system. Considering the immune response as a homeostatic mechanism, one can view the three systems as an integral unit that contributes to maintaining the constancy and integrity of the body cells and tissues [3]. In mammals, the hypothalamus-pituitary axis controls the interactions of the neuro-endocrine-immune system by the secretion of hormones and neuropeptides. These BAPs act directly or indirectly (by means of hormonal secretion of the peripheral glands) in modulating the immune system. In turn, the immune system, via lymphokines and monokines, can modulate the functions of the neuro-endocrine system [4–6].

Little data exist in the literature on invertebrates. Previous studies have demonstrated the presence of immunoreactive (ir) ACTH and β-endorphin molecules in the immuno cells (hemocytes) of the *Planobarius corneus* by immunocytochemistry, flow cytometry and RIA tests [7]. Accordingly, this paper reports the presence of ir-BAPs in the immuno cells of *P. corneus*.

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2. MATERIALS AND METHODS

2.1. *Snails*

Adult specimens of *Planobarius corneus* (L.) maintained in dechlorinated freshwater at room temperature were used.

2.2. *Hemocytes preparation*

Hemolymph was collected from *P. corneus* as previously described [8]. The hemocytes were cytocentrifuged (Cytospin 2 cytocentrifuge, Shandon) on a slide at 1000 rpm for 10 min and air-dried. The hemocytes were then processed by an immunocytochemical method immediately or after 24 h on slides stored at 4°C.

2.3. *Immunocytochemical procedure*

The following polyclonal antibodies were used: anti-α-antichymotrypsin (1:1000) (Dakopatts, Denmark), anti-bombesin (1:250) (Miles Scientific, USA), anti-calcitonin (1:50) (Dakopatts), anti-cholecystokinin (CCK)-8 (1:500) (INC, USA), anti-CCK-8 (1:500) (Peninsula Lab., USA), anti-CCK-39 (1:500) (CRB, UK), anti-gastrin (1:400) (Dakopatts), anti-glucagon (1:1000) (Dakopatts), anti-insulin (1:500) (CRL, USA), anti-insulin (1:500) (Peninsula), anti-Met-enkephalin (1:200) (Miles), anti-neurotensin (RB) (1:500) (Miles), anti-oxytocin (1:400) (kindly provided by Prof. F. Vandesande), anti-prolactin (1:500) (Dakopatts), anti-somatostatin (1:500) (Immuno Nuclear), anti-substance P (1:500) (INC), anti-thyroglobulin (1:500) (Dakopatts), anti-thyroxin (T4) (1:50) (CRL), anti-vasoactive intestinal polypeptide (VIP) (1:500) (INC) and anti-vasopressin (1:400) (kindly provided by Prof. F. Vandesande). The unfixed hemocytes were treated with the following procedure: (i) immerse in absolute alcohol and then in 95% alcohol; (ii) inhibit endogenous peroxidase with 6% H_2O_2 in methanol for 15 min at room temperature; (iii) immerse in running tap water and rinse in PBS; (iv) incubate in goat serum (Vector Lab.) (1:5) for 30 min at room temperature (for anti-insulin incubate in rabbit

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serum); (v) treat with the above antibodies for 24 h at 4°C (for anti-insulin (CRL) incubate for 1 h at room temperature) and wash in PBS (3 × 5 min); (vi) incubate in biotinylated anti-rabbit IgG (Vector Lab.) (1:200) for 30 min (for anti-insulin incubate in biotinylated anti-guinea pig IgG) and wash in PBS (3 × 5 min); (vii) incubate in ABC (avidin-biotin-peroxidase complex) (Vector Lab.) (1:100) for 30 min at room temperature and wash in PBS (3 × 10 min); (viii) demonstration of peroxidase activity in a Petri dish: dissolve a tablet of DAB (3,3'-diaminobenzidine tetrahydrochloride) (10 mg) (Sigma) in 40 ml of 0.1 M citric acid-sodium citrate buffer, pH 5.5, add 5 μl of H_2O_2 and filter; (ix) wash in running tap water for 5 min and rinse in distilled water; (x) counterstain nuclei with hematoxylin; (xi) dehydrate through a graded alcohol series, clear and cover.

Controls were performed by substituting the primary antibody with an identical dilution of non-immune serum from the same species, or by using a primary antibody pre-adsorbed with its antigen (10 nmol/ml diluted antiserum), and no staining was observed.

It is well known that hemoglobins or erythrocruorins, but not hemocyanins, are present in the hemolymph of planorbid molluscs. Since hemocyanin is sometimes used as carrier protein for the immunization of the rabbits, the presence of false positive reactions in cells from *P. corneus* due to the presence of anti-hemocyanin antibodies can be excluded.

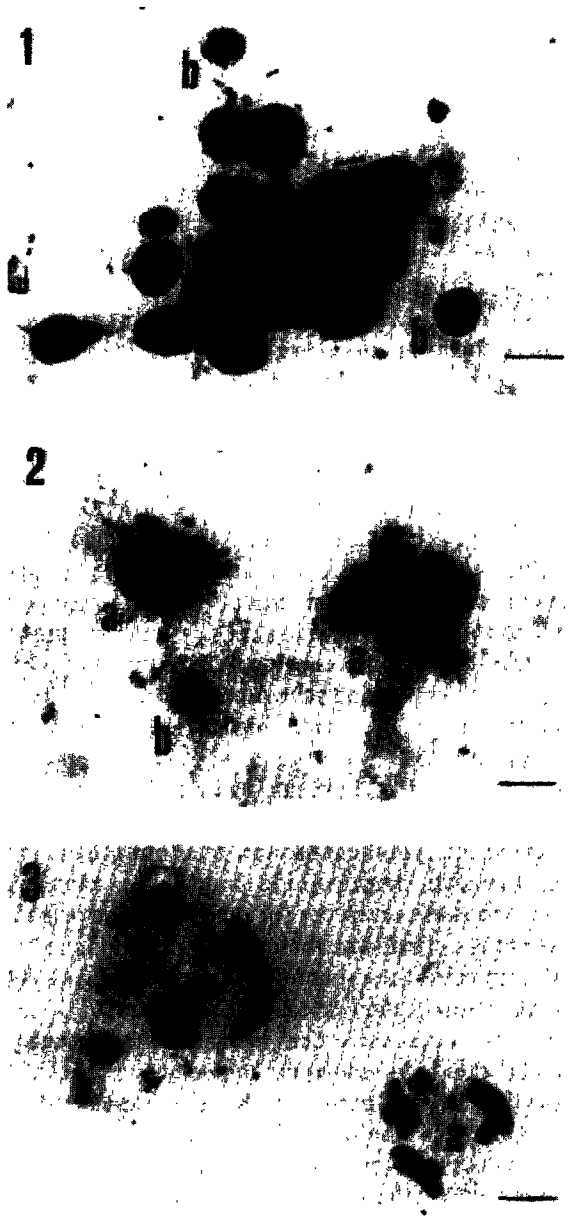
3. RESULTS

The results of the immunoperoxidase tests performed on the two cell types found in the hemolymph of *P. corneus*, round (RH) and spreading (SH) hemocytes [8,9] are reported in Table I and Figs 1–3. Negative response was found for CCK-8 (Peninsula), insulin, prolactin, thyroglobulin and thyroxin (T4) markers. SH showed immunoreactive molecules for most of the BAPs tested. These hemocytes belong to the category of macrophages. On the other hand, RH can be classified as 'proto-lymphocytes'; indeed they present some characteristics of T lymphocytes, such as responsiveness to phytohemagglutinin and cytotoxic activity (manuscript in preparation). These cells were only positive to anti-bombesin, anti-calcitonin, anti-CCK-8 (INC), anti-CCK-39, anti-oxytocin, anti-substance P and anti-vasopressin antibodies. All positive antibodies were reactive both on the cell surface and in the cytoplasm of the SH and RH.

Table I

Immunoreactive bioactive peptide (BAP) molecules in round (RH) and spreading (SH) hemocytes of *Planorbarius corneus*

BAP	RH	SH
α_1-Antichymotrypsin	−	+
Bombesin	+	+
Calcitonin	+	+
CCK-8 (INC)	+	+
CCK-8 (Peninsula)	−	−
CCK-39	+	+
Gastrin	−	+
Glucagon	−	+
Insulin (CRL)	−	−
Insulin (Peninsula)	−	−
Met-enkephalin	−	+
Neurotensin	+	+
Oxytocin	+	+
Prolactin	−	−
Somatostatin	−	+
Substance P	+	+
Thyroglobulin	−	−
Thyroxin (T4)	−	−
Vasopressin	+	+
VIP	−	+



Figs 1–3. Immunocytochemical staining of spreading hemocytes (SH) and round hemocytes (RH). Counterstaining was performed with hematoxylin. SH and RH are the two cell types present with different percentages (about 80% and 20%, respectively) in the hemolymph of *Planorbarius corneus*. Irregular shape and abundant cytoplasm are characteristics of SH, which tend to form clumps. RH possess a spherical nucleus with scanty cytoplasm which sometimes is not clearly visible. Fig. 1. SH (a) and RH (b) were stained with anti-vasopressin antibodies: both cell types were positive. Fig. 2. SH (a) and RH (b) were stained with anti-gastrin antibodies: only SH were positive. Fig. 3. SH (a) and RH (b) were stained with anti-CCK-8 (Peninsula): both cell types were negative. Scale bar = 10 μm.

Anti-CCK-8 and anti-insulin antibodies from two manufacturers were used. Anti-insulin tests were negative for both Peninsula and INC, whereas anti-CCK-8 from Peninsula gave a negative response and that from INC gave a positive response (Table I).

4. DISCUSSION

The immunocytochemical findings demonstrate an interrelationship among the neuro-endocrine and immune systems in *P. corneus*, and by extension probably in invertebrates in general, as already observed in vertebrates. Vertebrate BAPs have been reported in the nervous system of molluscs and other invertebrates [2,10–19]. Similarly, invertebrate BAPs have been found in the brain and intestine of vertebrates [13,20].

It is noteworthy that invertebrates do not have the classical endocrine organs found in vertebrates: in molluscs, their functions are in part performed by the dorsal bodies, gonad, optic tentacles and optic glands [21]. BAPs have been reported in cells from the gastrointestinal tissues and digestive gland of molluscs [22–24]. These data suggest a relationship between peptide-producing cells of invertebrates and related cell types of the APUD series in vertebrates. Accordingly, the concept of a neuroectodermic origin of BAP-producing cells known in mammals [25] may also be extended to the BAP-producing cells in invertebrates. In support of this, some representatives of the APUD series of vertebrates and neurosecretory neurons of invertebrates have the same properties, suggesting a neural origin [26].

As observed in *P. corneus*, radioimmunoassay and immunohistochemical techniques have demonstrated that vertebrate lymphocytes and macrophages contain immunoreactive neuropeptide molecules. Moreover, these cells possess receptors for many of these peptides. To date, it is not known whether these immuno cells actually synthesize these peptides or if they simply store them for a subsequent release as required [27].

Previous studies have demonstrated the presence of ir-ACTH and ir-β-endorphin molecules in SH of *P. corneus* [7]. In that paper, we have hypothesized that these hemocytes could produce ACTH-like material and were also probably targets for the same hormone, in agreement with Weigent and Blalock [28] who hypothesized an autocrine-like interaction in mammalian immunocytes. In fact, we have recently demonstrated that ACTH and β-endorphin have a chemotactic activity on hemocytes [29].

In any case, the presence of ir-BAP materials in the hemocytes of *P. corneus*, that probably modulate cell function, is a promising finding, though additional studies are required. Specifically, the possible presence of BAP receptors on the hemocyte membrane must be determined and the role that these BAPs play in modulating the immune response investigated.

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