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CYTOKINES AND EVOLUTION: *IN VITRO* EFFECTS OF IL-1α, IL-1ß, TNF-α AND TNF-ß ON AN ANCESTRAL TYPE OF STRESS RESPONSE

Enzo Ottaviani[1*], Eva Caselgrandi[2] and Claudio Franceschi[3]

[1]Department of Animal Biology, via Berengario 14,
Department of Biomedical Sciences,
[2]Section of Hygiene and [3]Section of General Pathology,
University of Modena, 41100 Modena, Italy

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Summary. Invertebrate hemocytes are immune-neuroendocrine cells which contain a variety of cytokines [Ottaviani *et al.* (1993) Biochem. Biophys. Res. Commun. 195, 984-988] and release biogenic amines when added to corticotropin-releasing factor (CRF), a phenomenon we have described as an evolutionary proto-type stress response [Ottaviani *et al.* (1991) Proc. R. Soc. Lond. B 245, 215-218]. Here we show in two molluscs, *Planorbarius corneus* and *Viviparus ater*, that this response is significantly reduced when hemocytes are pre-incubated with IL-1α, IL-1ß, TNF-α and TNF-ß before the addition of CRF. These results confirm and extend the hypothesis that a deep evolutionary relationship exists between cytokines and stress response. Moreover, these data offer an evolutionary basis for understanding the promiscuity of cytokine receptors.

In previous studies we and others have demonstrated the presence of different cytokines (IL-1α , IL-1ß, IL-2, IL-6, TNF-α and TNF-ß) in the hemocytes of the freshwater snails *Planorbarius corneus* and *Viviparus ater* (1) and of the mussel *Mytilus edulis* (2). Moreover, we found that IL-2 is involved in a proto-type stress response (3). We demonstrated that in invertebrates, the basic mechanisms of the stress response are remarkably similar to the corticotropin-releasing factor (CRF)-adrenocorticotropin hormone (ACTH)-biogenic amine axis of vertebrates. However, in these lower creatures, devoid of adrenal and pituitary glands, the target is the hemocyte itself, which is also capable of neuroendocrine activity (4, 5). Molluscan hemocytes are equipped with enzymes of the biosynthetic pathways of biogenic amines (5). The *in vitro* addition of CRF and ACTH to molluscan hemocytes eventually results in the release of norepinephrine, epinephrine and dopamine in

* To whom correspondence should be addressed. Fax:+39 59 581020.

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the supernatant. We have proposed considering this effect an evolutionary proto-type of the stress response known in mammals (6, 7). Pre-incubation of molluscan hemocytes with IL-2 or anti-IL-2 mAb reduced or completely abolished the CRF-induced release of biogenic amines (3). Taking into account that other cytokines are present in molluscan hemocytes, we asked whether cytokines other than IL-2 could exert a similar effect on the CRF-induced release of biogenic amines. The results indicate that several other cytokines are indeed involved in and capable of interfering with the proto-type of stress response we investigated in molluscs.

MATERIALS AND METHODS

Collection of hemolymph. The hemolymph from adult specimens of *Planorbarius corneus* (L.) and *Viviparus ater* (De Cristofori & Jan) maintained in dechlorinated freshwater at room temperature was collected as previously described (8).

Determination of the biogenic amines. Hemolymph of *P. corneus* and *V.ater* (a pool of several animals) was divided into six portions (3 ml each one) and placed in plastic tubes. Snail saline solution (SSS) (300 µl) (7) was added to in the first tube (control sample) and human CRF (Sigma Chem. Co., St. Louis, USA) (10^{-8} M final concentration) to the second, while the remaining tubes were pre-incubated with different cytokines for 120 min before adding CRF (10^{-8} M final concentration). The following recombinant human interleukins (IL), purchased from Sigma, Boehringer (Mannheim, Germany) and Genzyme Corp. (Cambridge, MA, USA), were used: IL-1α (1000 U/ml final concentration), IL-1ß (1000 U/ml), IL-2 (20 U/ml), TNF-α (2500 U/ml), TNF-ß (2500 U/ml). All the tubes were then incubated for 20 min at room temperature and immediately centrifuged (600 g for 15 min). After centrifugation, the supernatant (serum) was immediately analyzed by HPLC to determine the amount of biogenic amines, as described elsewhere (4, 9). Each experiment was carried out at least three times.

Statistical analysis. The Student's two-tailed *t* test was used to compare the concentrations of biogenic amines in controls, CRF and CRF plus cytokines samples.

RESULTS

As expected, the addition of CRF to molluscan hemocytes provoked a significant increase of biogenic amines in the serum (Table I). This phenomenon was strong and rapid, being quite evident 20 min after the addition of CRF. The inhibitory effect exerted on the CRF-induced release of biogenic amines by the pre-incubation of hemocytes with IL-2 was equally expected (3). An inhibitory effect was also observed when molluscan hemocytes were pre-incubated with the IL-1α, IL-1ß, TNF-α and TNF-ß before the addition of CRF. On the whole, similar results were obtained in both molluscan species, suggesting that this finding is a general phenomenon. However, the inhibitory effect of these cytokines was particularly evident in some combinations (TNF-α, TNF-β and release of NA from *P. corneus* hemocytes; IL-1β and release of A from *V. ater* hemocytes).

Table I. Concentrations of biogenic amines determined by HPLC in serum from *P. corneus* and *V. ater* following addition of 10^{-8} M CRF after pre-incubation with different cytokines

	Addition of						
	Saline	CRF	IL-2 + CRF	IL-1α + CRF	IL-1β + CRF	TNF-α + CRF	TNF-β + CRF
	$\bar{X} \pm SD$	$\bar{X} \pm SD$	$\bar{X} \pm SD$	$\bar{X} \pm SD$	$\bar{X} \pm SD$	$\bar{X} \pm SD$	$\bar{X} \pm SD$
P. corneus NA (%)	41.0 ± 15.5 (100)§	394.0 ± 28.2** (961)	115.0 ± 16.6°° (280)	317.0 ± 34.6° (773)	297.0 ± 52.5° (724)	215.0 ± 45.9°° (524)	210.0 ± 15.8°° (512)
V. ater	223.0 ± 42.5 (100)	2294.0 ± 348.4** (1029)	627 ± 28.6°° (281)	1985.0 ± 149.4 (890)	1640.0 ± 91.0° (735)	1861.0 ± 236.6 (834)	2022.0 ± 15.8 (907)
P. corneus A (%)	80.0 ± 12.2 (100)	307.0 ± 62.3** (384)	183.0 ± 17.3° (229)	222.0 ± 36.9 (277)	201.0 ± 17.1° (251)	238.0 ± 31.1 (297)	238.0 ± 26.8 (297)
V. ater	123.0 ± 30.9 (100)	1596.0 ± 297.5** (1297)	364.0 ± 36.7°° (296)	1140.0 ± 100.3° (927)	819.0 ± 34.7°° (666)	1145.0 ± 109.4 (931)	1098.0 ± 82.0° (893)
P. corneus DA (%)	274.0 ± 23.9 (100)	370.0 ± 30.7* (135)	301.0 ± 52.3 (110)	304.0 ± 30.8° (111)	296.0 ± 35.1° (108)	297.0 ± 31.8° (108)	292.0 ± 35.9° (106)
V. ater	485.0 ± 35.3 (100)	3276.0 ± 234.3** (675)	829.0 ± 78.1°° (171)	3015.0 ± 216.6 (622)	1718.0 ± 666.4° (354)	2607.0 ± 251.8° (535)	3013.0 ± 152.3 (621)

NA = Norepinephrine; A = Epinephrine; DA = Dopamine.
$^{**}p < 0.01$, $^{*}p < 0.05$ vs Control (saline) ; $^{\circ\circ}p < 0.01$, $^{\circ}p < 0.05$ vs CRF.
Statistical analysis was performed by Student *t*-test.
§ Control values are taken as 100%. Data are expressed as pg/ml.
The mean ($\bar{X}$) ± standard deviation (SD) of three experiments is shown.

DISCUSSION

The data here presented are similar to those we have previously found for IL-2, and which were reproduced in this new set of experiments. Indeed, the inhibitory effect of IL-2 on the release of biogenic amines here reported was even greater than that previously described (3). Similar variations are not unprecedented in experiments involving molluscan cells and may be due, at least in part, to seasonal effects (3, 4).

Immunocytochemical and cytofluorimetric studies have shown that IL-2 and CRF probably bind to the same receptor (3). Pre-incubation with IL-2 not only inhibits the CRF-induced release of biogenic amines, but also the binding of CRF to the membrane of hemocytes. We have hypothesized that CRF and IL-2 compete for a common receptor on the membrane of molluscan hemocytes, with CRF having a higher affinity for this receptor (3). A similar hypothesis might explain the inhibitory

effects exerted by the other cytokines studied on the CRF-induced release of biogenic amines. Accordingly, the presence on molluscan hemocytes of an ancestral and highly promiscuous receptor capable of binding several cytokines as well as CRF can be hypothesized. This would provide an evolutionary explanation to the data recently reported in mammals on the promiscuity of cytokine receptors. A characteristic of cytokines is their redundancy and great pleiotropicity. Several cytokines, such as IL-1, IL- 2, IL-6, TNF, perform various functions and some mediate the same or similar functions via the same target cells (see reference 10). Moreover, cytokine receptors have been described as multisubunit in structure, with ligand-binding (α) and affinity-converting or signal-transducing (ß) subunits. The α and ß subunits are homologous to each other, both presenting the cytokine receptor motif [see reference 11]. The molecular basis of the complex structural and functional relationships between the different cytokines has been highlighted by recent findings on IL-2 receptor. This molecule presents three subunits (see reference 12), and the γ chain is functionally involved in the IL-4 and IL-7 receptor complex (13-15). Another example is TNF, where multiple receptor types have been described. However, for the majority of cytokines, the situation is still unclear (see reference 16). The data here presented suggest that a similar situation might occur in invertebrates, and that CRF, IL-1α, IL-1ß, TNF-α and TNF-ß can bind to and compete for the same receptor.

In conclusion, our data indicate that cytokines are not only present in invertebrates but that they also play a complex modulatory role. We have shown that one target of cytokines is the neuroendocrine activity of invertebrate hemocytes. Indeed, cytokines appear to be capable of regulating important biological and neuroendocrine responses mediated by CRF, such as the release of biogenic amines. We believe that the probable promiscuity among receptors for cytokines and neuropeptides such as CRF is one of the evolutionary bases to explain at the molecular level the cross-talk between the immune and neuroendocrine systems.

The recently described CRF-mediated immunosuppressive effect of IL-1 in the rat might be an example of an evolutionary conserved interaction between cytokines and neuropeptides (17).

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