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Immunology

Interaction of immunoactive monokines (interleukin 1 and tumor necrosis factor) in the bivalve mollusc *Mytilus edulis*

(monokine cascades/comparative immunology/immunocytes/conformational alterations/neuroimmunomodulation)

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ABSTRACT **_Mytilus edulis_ has been the subject of recent studies to determine whether the relationship between the immune and neuroendocrine systems seen in vertebrates also exists in invertebrates. The effects of mammalian monokines were studied in _Mytilus_ immunocytes previously shown to produce and react to opioid peptides. These invertebrate cells respond to interleukin 1 (IL-1) and tumor necrosis factor (TNF), both _in vitro_ and _in vivo_, in a manner similar to that of human granulocytes. As in the mammalian monokine network, the effect of IL-1 on the immunocytes is brought about, at least in part, by its stimulatory effect on the formation of TNF. In addition, the presence of immunoreactive IL-1 and TNF in _Mytilus_ hemolymph was demonstrated.**

Recent data have clearly demonstrated a linkage between the immune and neuroendocrine systems of vertebrates (1, 2). Similar signal molecules are produced by, and elicit responses in, cells of either system. *Mytilus edulis*, a marine bivalve mollusc, has been used to determine whether the same relationship between these systems exists in invertebrates. Several reports have shown this to be the case for opioid peptides. These substances are present in the ganglionic cells of this invertebrate (1). Moreover, *Mytilus* immunocytes produce and respond to opioid peptides in a fashion similar to that in vertebrates (for review, see ref. 1). Stefano *et al.* (3) demonstrated that in human and invertebrate immunocytes stimulatory effects comparable to those elicited by endogenous opioids are obtained in tests with the opioid analog [D-Ala^2,Met^5]enkephalin. In both cell types, conformation and locomotory responses occurred after their exposure to this opioid. The results suggest a specific function for [Met]enkephalin in immunoregulation, which may be mediated by a subtype of δ receptor. Both cell types were shown to produce immunoactive [Met]enkephalin (1, 3).

The present study was undertaken to determine whether *Mytilus* also possess monokines and a monokine-like network. To this end, we chose to study the effects of two interacting monokines, interleukin 1 (IL-1) and tumor necrosis factor type α (TNF-α) (4, 5). We were able to show that *Mytilus* immunocytes, like human granulocytes, respond to these two factors. We further demonstrated that, as in the human system (5), the effects of IL-1 are attributable, at least in part, to its induction of TNF-α formation. Finally, we showed that *Mytilus* hemolymph contains IL-1 and TNF-α immunoreactivity.

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MATERIALS AND METHODS

Animals. *M. edulis* were obtained from the shores of Long Island Sound at Wading River. The animals were used within 3 hr from the time of collection, and hemocytes were prepared as previously noted in detail (6). Briefly, hemocytes were aspirated by syringe from the area of the posterior adductor muscle in 0.1-ml quantities from ≈40 animals, pooled, and used as noted below.

Reagents. Recombinant human TNF-α was obtained from Suntory Ltd. (Tokyo). Recombinant human IL-1α and antibodies to IL-1 and TNF-α (both polyclonal rabbit preparations against recombinant immunogen) were obtained from Genzyme. ELISA kits for IL-1 and TNF-α were obtained from Cistron Biotechnology (Pine Brook, NJ).

Determination of Immunoreactive IL-1 and TNF-α by ELISA. Hemolymph from 200 *Mytilus* was removed and concentrated by dialysis against polyethylene glycol (≈75-fold), followed by equilibrium dialysis against phosphate-buffered saline. Samples were subjected to ELISA for human TNF-α and IL-1β. ELISAs were performed by a four-stage procedure in a microtiter plate coated with monoclonal antibody specific for either IL-1 or TNF-α. Hemolymph, IL-1 or TNF-α standards, and control solutions were placed in the plate wells and incubated according to the manufacturer's instructions. The plate wells were rigorously washed between each step. After the initial incubation, polyclonal rabbit anti-IL-1 or anti-TNF-α was added to each well. Goat anti-rabbit IgG conjugated to horseradish peroxidase enzyme was then added in the next step. The substrate *o*-phenylenediamine was added for the last or indicator step. Optical densities of the indicator were then determined at 492 nm on an automated ELISA reader (Bio-Rad). All values obtained were adjusted for nonspecific binding.

Assays. Since we had previously shown that *Mytilus* and human immunocytes respond to our assays in a similar fashion (1), the following determinations were carried out. For adherence assays, 100 μl of hemolymph-containing cells plus the respective reagent were placed on a glass slide precoated with a 2% bovine serum albumin solution (6). The mixture was allowed to incubate at room temperature (23°C) for 10 min. The slide was then gently tilted, allowing excess fluid to run off, and examined by light microscopy and Zeiss-Zonax reflectance computer analysis (6). Changes in conformation of hemocytes were determined by inoculating them into the space provided by an inverted coverslip placed on a slide over a ring of Vaseline. This method allowed observation for up to 1 hr without drying of cells. The mixture was allowed to incubate at room temperature for another 10

Abbreviations: IL-1, interleukin 1; TNF-α, tumor necrosis factor type α.

min before the cell preparations were examined by use of phase contrast and Nomarski optics coupled with a Zeiss Axiophot microscope (7). Measurements were taken with a Zeiss Videoplan/Vidas image analysis system. Before they were recorded, specific images were converted to binary images after frame grabbing. Simultaneously, specific cells were photographed by an internal Sony video photography system combined with a time-lapse video synchronization system (JVC). Cellular shape ranged from rounded (inactive) to ameboid (active).

Changes in cellular conformation based on measurements of cellular area and perimeter were mathematically expressed by the use of the form-factor calculations of the Zeiss Vidas analysis system whereby the equation $\{[4 \times \pi] \times [(\text{area})/\text{perimeter}^2]\}$ provides numerical values. The lower the value, the higher the cellular perimeter and the more ameboid and active the cells.

Statistical analyses were carried out by Student's *t* test. The mean value from 10–16 separate trials was averaged with three other mean values, similarly obtained, to provide the value for each point on the various graphs.

RESULTS

The effects of two monokines, IL-1 and TNF-α, on immunoactive cells of *Mytilus*, which closely resemble the granulocyte–monocyte–macrophage lineage of vertebrates (8), were determined. Recombinant human IL-1 and TNF-α have a stimulatory effect on these cells (Fig. 1), as demonstrated by the following experiments. IL-1 can stimulate a specific population of cells to increase their area in a dose–response fashion (Fig. 2). This effect can be blocked by prior treatment

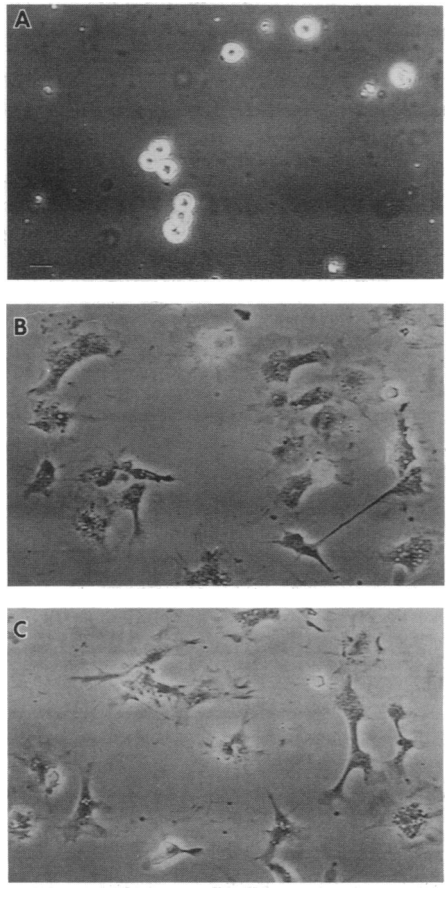

FIG. 1. Photomicrographs of *Mytilus* immunocytes. (*A*) Control. (*B*) After addition of TNF-α (10 units/ml). (*C*) After addition of IL-1 (10 units/ml). (Bar = 10 μm.)

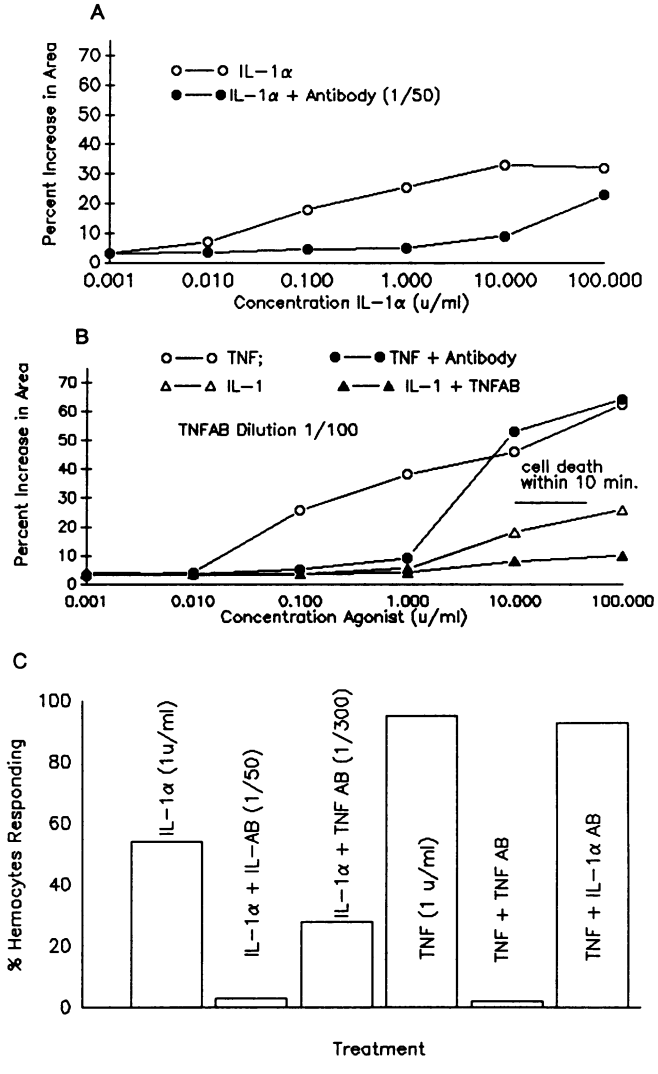

FIG. 2. Effects of recombinant human IL-1 and TNF-α on *Mytilus* immunocytes *in vitro*. (*A*) Effects of IL-1 and its blockage by anti-IL-1. (*B*) Effects of IL-1 and TNF-α and their blockage by the respective antibodies. Partial blockage of IL-1 effects by the TNF-α antibody, suggesting TNF-α induction by IL-1. (*C*) The proportion of hemocytes responding to various cytokines measuring a 30% increase in cell area, suggesting partial induction of a TNF-α-like factor.

with IL-1 antibody. TNF-α, in a dose-dependent fashion similar to IL-1, increases the relative reflectance of the cells and also causes the cells to flatten, as indicated by the measured increase in their area and their perimeter (Fig. 3 *A* and *B*). Typically, they display form-factor values of 0.40, indicating an activated state (Fig. 3*C*). As a rule, control cells during the observation period remain rounded and generally inactive (Fig. 1*A*). It is interesting to note that the responses listed are blocked by prior addition to the cells of specific antibodies to IL-1 or TNF-α (Figs. 2 and 3). Addition of normal rabbit serum as a control antibody had no effect on the responses (data not shown). We also tested the possible influence of recombinant human γ interferon on *Mytilus* cells but found no effect during our period of observation (data not shown). This is not surprising since γ interferons in general exhibit extreme species specificity in their actions (9).

It also was of interest to determine the effect of TNF-α on hemocytes when administered *in vivo*—i.e., when the TNF-α is injected into the animal. After TNF-α injection into *Mytilus*, the number of circulating hemocytes recovered by aspiration was reduced (Fig. 3*D*), in an anti-TNF-α reversible

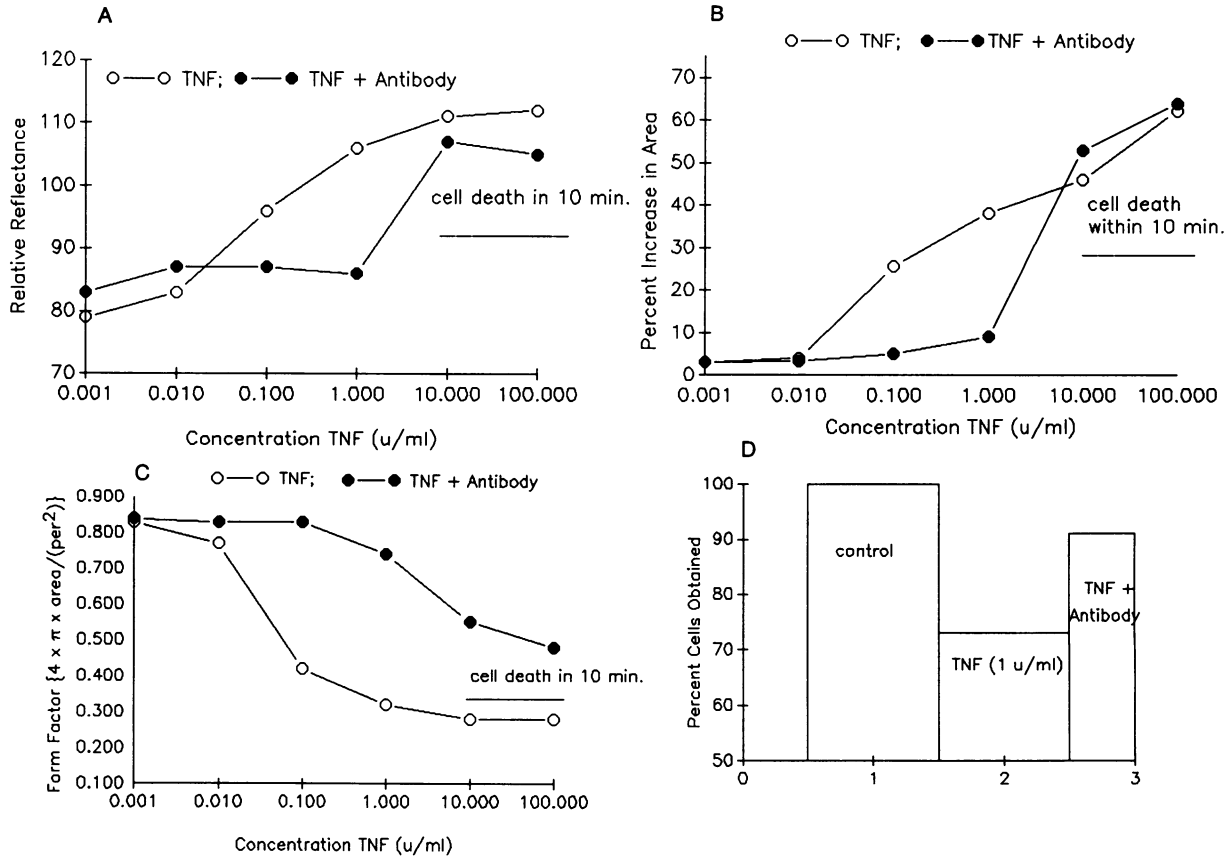

FIG. 3. Effects of recombinant human TNF-α on *M. edulis* immunocytes *in vitro*. Changes in immunocyte reflectance (*A*), area (*B*), and shape (*C*). (*D*) Effect of TNF-α on immunocyte numbers recovered from whole animal and blockage of this effect by TNF-α antibody.

manner, presumably because the activated cells migrate into various tissues (7).

In vertebrates, some of the effects of IL-1 are thought to be attributed to its induction of TNF-α formation (5). We determined whether a similar mechanism might occur in *Mytilus*. IL-1 was placed on the cells in the absence or presence of antibody to TNF-α. As shown in Fig. 2 *B* and *C*, anti-TNF-α almost completely blocked the response induced by IL-1. The remainder of the response was abolished by antibody to IL-1. Thus, it appears that IL-1 achieves part of its effect through stimulating the formation of a TNF-α-like molecule, while the rest appears to be due to IL-1 itself.

Since this experiment tends to indicate that a TNF-α-like molecule is produced by *Mytilus* cells, we wanted to determine whether this substance or an IL-1-like molecule or both can be detected in the hemolymph. Concentrated hemolymph from 200 animals was subjected to ELISA for IL-1 or TNF-α. As shown in Fig. 4, immunoreactive TNF-α and IL-1 are present in the hemolymph. These quantities are roughly equivalent to 7 fM and 15 fM, respectively, when the concentration of the hemolymph is taken into consideration (see Fig. 4 legend).

DISCUSSION

Mytilus immunocytes share a number of properties with cells of the human granulocyte–monocyte–macrophage lineage (10). They have the potential to become motile in all of the tissues examined (6). The hemolymph of *Mytilus* contains an agglutinin with opsonizing properties (11). Severance of a nerve in *Mytilus* evokes an immune-type response—i.e., the migration of immunocytes to the lesioned area and their accumulation, presumed to be due to a concentration gradient of antigenic or recognition factors (6). It has been reported that myeloperoxidase, which is present in vertebrate phagocytes, is present in some invertebrate phagocytes

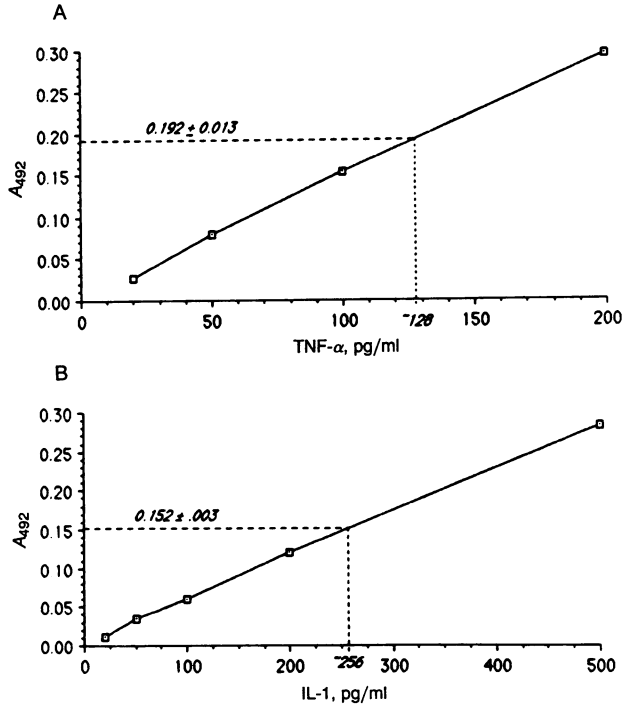

FIG. 4. Determination of TNF-α and IL-1 by ELISA. Dashed lines indicate endogenous levels of TNF-α- and IL-1-like material. Known standard concentrations of ligand are indicated as open squares. Representative of three determinations.

(12). Invertebrate immunocytes produce and respond to opioid neuropeptides (6).

The present study demonstrates that *Mytilus* hemocytes also contain and respond to two monokines, IL-1 and TNF-α. More specifically, (*i*) they respond to recombinant human IL-1 and TNF-α; (*ii*) these responses can be blocked by antibody specific for either monokine; (*iii*) the IL-1 response can be partially blocked by antibody to TNF-α, while the remainder of the response is blocked by antibody to IL-1, indicating that IL-1 exerts its effect not only directly but also through a TNF-α-like intermediate; and (*iv*) immunoreactive IL-1 and TNF-α can be detected by specific ELISA in *Mytilus* hemolymph.

Previous reports have demonstrated the presence in starfish and tunicates of factors with IL-1-like effects on vertebrate and invertebrate immunocytes (13–16). In one of these studies, Beck *et al.* (16) attempted to determine the presence of TNF-α activity in tunicates, but they were unsuccessful. Other "lymphokine-like" activities have been described in invertebrates (17). To our knowledge, however, monokine production, action, and cascade effects in invertebrates have not been reported. There is still uncertainty about the degree of homology of these immunoreactive substances in humans and *Mytilus*. In this respect, it is of interest that the application of human TNF-α at a concentration above 1 fM is lethal in *Mytilus*, even though its immunoactivity resembles that of its *Mytilus* counterpart. Perhaps in the long course of evolution, the toxicity of the human TNF-α may have markedly increased. We have yet to isolate and characterize the TNF-α of *Mytilus*, but our preliminary results tend to indicate that a substance present in *Mytilus* hemolymph exerts a toxic effect also on susceptible mammalian cells similar to that of human TNF-α.

The results of the present study strongly suggest that monokines are "ancient" signal molecules that have evolved over a period of millions of years. By the same token, the granulocyte–monocyte–macrophage cell family may be considered to be "primitive" in mammalian immune functions (10). At present, we are unsure of the forces that normally control expression of these substances in invertebrates. It is possible that naturally occurring inducers—i.e., endotoxins—could be sufficient to trigger a response but our findings open the possibility that an even larger network of soluble immune signal molecules may be operating in invertebrates such as *Mytilus*.

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1. Stefano, G. B. (1989) *Prog. Neurobiol.* **33,** 149–159.
2. Blalock, J. E. (1989) *Physiol. Rev.* **69,** 1–32.
3. Stefano, G. B., Cadet, P. & Scharrer, B. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 6307–6311.
4. Old, L. (1987) *Science* **230,** 630–632.
5. Philip, R. & Epstein, L. B. (1986) *Nature (London)* **323,** 86–89.
6. Stefano, G. B., Leung, M. K., Zhao, X. & Scharrer, B. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 626–630.
7. Stefano, G. B., Zhao, X., Bailey, D., Metlay, M. & Leung, M. K. (1989) *J. Neuroimmunol.* **21,** 67–74.
8. Renwrantz, L. (1990) in *Internal Defense System of Mytilus edulis, Neurobiology of Mytilus edulis,* ed. Stefano, G. B. (Manchester Univ. Press, Manchester, U.K.).
9. Rashidbaigi, A., Langer, J. A., Jung, V., Jones, C., Morse, H. G., Tischfield, J. A., Trill, J. J., Kung, H. F. & Pestka, S. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 384–388.
10. Millar, D. A. & Ratcliffe, N. A. (1989) *Endeavor* **13,** 72–77.
11. Renwrantz, L. & Stahmer, A. (1983) *J. Comp. Physiol.* **149,** 535–546.
12. Nakamura, M., Mori, K., Inooka, S. & Nomura, T. (1985) *Dev. Comp. Immunol.* **9,** 407–410.
13. Prendergast, R. A., Lutty, G. A. & Scott, A. L. (1983) *Dev. Comp. Immunol.* **7,** 629–632.
14. Donnelley, J. J., Vogel, S. N. & Prendergast, R. A. (1985) *Cell. Immunol.* **90,** 408–415.
15. Beck, G. & Habicht, G. S. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 7429–7433.
16. Beck, G., Vasta, G. R., Marchalonis, J. J. & Habicht, G. S. (1989) *Comp. Biochem. Physiol.* **92,** 93–98.
17. Ratcliffe, N. A. (1985) *Immunol. Lett.* **10,** 253–270.