ELSEVIER

European Journal of Pharmacology 276 (1995) 35–39

ejp

Antagonizing effect of morphine on the mobility and phagocytic activity of invertebrate immunocytes

Enzo Ottaviani [a,*], Antonella Franchini [a], Dario Sonetti [a], George B. Stefano [b]

[a] *Department of Animal Biology, University of Modena, Via Berengario 14, 41100 Modena, Italy*
[b] *Neuroscience Research Institute, State University of New York at Old Westbury, Old Westbury, NY 11568, USA*

Received 4 August 1994; revised 11 October 1994; accepted 30 December 1994

Abstract

In the present study we have demonstrated that lipopolysaccharide (LPS) acts as an activator in the immunocytes of molluscs *Planorbarius corneus, Mytilus edulis*, and the insect *Leucophaea maderae*. This stimulatory effect, demonstrated by cellular conformational changes, is concentration- and time-dependent, and is antagonized by morphine. The inhibitory effect of morphine can be counteracted by naloxone. Morphine inhibitory action on immunocyte activity is also demonstrated by a decrease in the phagocytic activity. These data suggest that the downregulation of morphine is not limited to vertebrates but is also present in invertebrates.

Keywords: Morphine; Lipopolysaccharide; Phagocytosis; Invertebrate immunocyte

1. Introduction

Recent studies in comparative neuroimmunology have demonstrated that in both vertebrates and invertebrates opioid and opiate signal molecules are used for communication among the nervous, endocrine, and immune systems, as well as in autoimmuno-regulatory processes (Stefano, 1989, 1994; Scharrer and Stefano, 1994; Stefano and Scharrer, 1994).

Concomitant comparative tests with exogenous morphine and opioid peptides have established the presence of new receptor subtypes, δ_2 receptors mediating certain opioid effects and a highly selective μ_3 receptor for opiate alkaloids that is unresponsive to opioid peptides (Stefano et al., 1992, 1993). In particular, the results of these studies have led to the conclusion that endogenous opiates downregulate certain activities of immunocytes and related cell types, i.e., microglial elements (Stefano et al., 1993; Sonetti et al., 1994; Stefano and Scharrer, 1994).

In the present study, we sought to test this hypothesis by antagonizing in various invertebrates the potent immunocyte stimulating action of lipopolysaccharide (LPS) (Hughes et al., 1991a,b) with morphine. To our knowledge, this is the first demonstration of such an antagonism in invertebrates.

2. Materials and methods

2.1. Specimens and chemical reagents

The three invertebrate species selected for this study, i.e., the molluscs *Planorbarius corneus* and *Mytilus edulis*, and the insect *Leucophaea maderae*, were obtained and maintained as previously reported (Stefano et al., 1989a,b; Ottaviani, 1983). For in vitro observation of the immunocytes, the cells of *M. edulis* and *L. maderae* were bathed in sterile saline containing antibiotics (50 mg streptomycin, 30 mg penicillin, 50 mg gentamicin in 100 ml, pH 7.5) (Sigma, St. Louis, MO, USA). The substances to be tested [LPS (0.1–100 U/ml) (Sigma), Morphine (10^{-6} M) (Salars, Camerlata, CO, Italy) and naloxone (10^{-6} M) (Wyeth Lab., PE, USA)] were used both alone and in combination. They were dissolved in snail saline solution (Ottaviani, 1983) and added directly to the hemolymph of *P. corneus*. The morphine dosage was determined from previous studies and based on the K_d of the novel μ_3

* Corresponding author. Tel. 0039-59-243-566, fax 0039-59-581-020.

SSDI 0014-2999(95)00008-9

receptor (Stefano et al., 1993). In *M. edulis* and *L. maderae* the substances were added to the cell preparation prior to that of the respective cell-free hemolymph (incubation medium); under these conditions, the cells could be maintained for up to 10 h at a time without developing any evident abnormalities. Cells from the respective animals were placed in a ring of vaseline on a slide and protected by a coverslip. Every 5 min for a period of 60 min, the changes in the shape of the hemocytes were observed and photographed under phase-contrast optics. The photographs were assessed by computer assisted microscopic image analysis (Oncor, San Diego, CA and Image Analytic Co., Hauppauge, NY, USA)

2.2. Analysis of hemocyte activity

As in past studies (Stefano et al., 1989a,b, 1992, 1993), the degree of activation of immunocytes (conformational changes and mobility) was determined by measurement of cellular area and perimeter using the Oncor (San Diego, CA) image analysis software. Briefly, cells becoming active or cells that were active would have longer perimeters since they went from a rounded shape to one that was more ameboid. Thus, this parameter has been used as an indication of immunocyte activation. A viewing diameter of 400 mm was selected for determination of cellular conformational changes and for frame grabbing. Briefly, the form-factor is equal to $AC/AT = [LT/LC]^2$ where AT is the area of a circle with the same perimeter as that of the given cell and LT is the perimeter of a circle with the same area as that of the given cell; AC and LC represent the actual area and perimeter of the cell. The percents of active versus inactive cells were determined by assigning the 'bright' inactive-rounded cells, viewed under phase contrast, a color via feature detection, whereas active-ameboid cells were assigned another color. A simple comparison of the populations over time showed either an increase or decrease in the number of active immunocytes, which could then be quantified based on their assigned color. The computer-assisted image and video analysis system (Zeiss Axiophot fitted with Normaski and phase-contrast optics) was the same as previously described in detail (Stefano et al., 1989a,b).

2.3. In vitro bacterial phagocytosis of M. edulis hemocytes

Pseudomonas stretzi were added from cell-free hemolymph, artificial sea water cultures (50:50) and diluted in an additional 100 μl volume and added to 100 μl of hemolymph containing hemocytes. Hemocytes and bacteria were obtained from the same animal and culture, respectively and compared to individual controls for each condition. This ensured that the starting numbers were uniform and avoided a preparation where the cells were killed by the bacteria. The cells were then incubated in the presence of the bacteria for 10 h at room temperature. This was repeated 5 times per animal with the effective doses of the respective pharmacological additions. The mean value plus S.E.M. is expressed in graphic form. At the end of the time period, the bacteria were counted by the feature detection function of the American Innovision commercial software (San Diego, CA). Briefly, the computer program was taught the color of the bacteria under phase-contrast optics as well as their size (< 1.5 μm in diameter) so that they could be selectively and automatically counted by color identification (Paemen et al., 1992).

Statistical analysis was performed by the Student's *t*-test. Mean values were obtained by averaging those of various trials, each representing the measurement of a single cell. For each set of experiments there was a correspondent set of controls. The averages of four such mean values provided the values for each point on the various graphs.

3. Results

LPS (4U/ml) is a potent, concentration and time dependent immunocyte activator, as indicated in *P. corneus* by conformational changes of a significant number of these cells from an inactive rounded to a more ameboid shape (Fig. 1A–D). On initial exposure to LPS (10 U/ml), *M. edulis* and *L. maderae* immunocytes exhibited form factor of 0.80 ± 0.04 (S.E.M.) and 0.83 ± 0.05, respectively. Thirty minutes later, *M. edulis* cells gave a form factors of 0.42 ± 0.05 and *L. maderae* 0.49 ± 0.03 in approximately 39% of their immunocytes, an increase from approximately 6% prior to LPS exposure in both animals (Fig. 2). *P. corneus* exhibited the same reduction in form factors (Fig. 3, inset), indicative of immunocyte activation, as well as a significant increase (61% compared to 8%) following LPS exposure in the number of activated immunocytes. These results support those from previous experiments on invertebrate immunocytes, namely, that LPS is a potent invertebrate immunocyte stimulant (Hughes et al., 1991a,b).

This stimulatory response to LPS is antagonized by morphine, a compound that has been shown to be endogenous in *M. edulis* hemolymph (Stefano et al., 1993). We know that naloxone only works if the morphinergic system is on. On the other hand, this system appears to only be on during stress and then after 24 h (Stefano et al., 1993). As a result, naloxone alone causes no apparent alterations in intact control animals. In *L. maderae* and *M. edulis* immunocytes, morphine antagonized the LPS concentration dependent

activation of immunocytes (Figs. 4 and 5). Morphine increased the form factors reduced by LPS to 0.82 ± 0.04 and 0.79 ± 0.04 for *L. maderae* and *M. edulis*, respectively. Morphine treatment also significantly reduced the number of activated immunocytes induced by LPS (Figs. 4 and 5). Morphine produced the same conformational effects in *P. corneus* following LPS exposure, including the statistical reduction of activated immunocytes from 59% to 8% (Fig. 3). The same result was obtained in tests with immunocytes from all three animals (Figs. 3–5). Furthermore, this downregulation of the opiate alkaloid can be counteracted by the addition of naloxone to the culture medium (Figs. 3–5), a result which supports the conclusion that the effect is accomplished by an opiate receptor mechanism. The downregulating action of morphine on immunocyte stimulation by LPS could be diminished with higher (100 U/ml) concentrations of LPS (Figs. 4 and 5).

An additional observation demonstrating the inhibitory effect of morphine on the activities of invertebrate immunocytes was that on their phagocytic activity. *M. edulis* immunocytes coincubated with *P. stretzi* slowly reduce the number of bacteria in the culture medium. This change, achieved by phagocytosis, was measured by computer-assisted image analysis (Fig. 6). The phagocytic process was blocked by the presence of morphine in a time and naloxone sensitive manner ($P < 0.05$ when compared to the cells alone or in the presence of both morphine and naloxone; Fig. 6). Interestingly, over time, the morphine inhibitory action on phagocytosis is lost (Fig. 6).

4. Discussion

The present study demonstrates that in three species of invertebrates representing two different phyla LPS

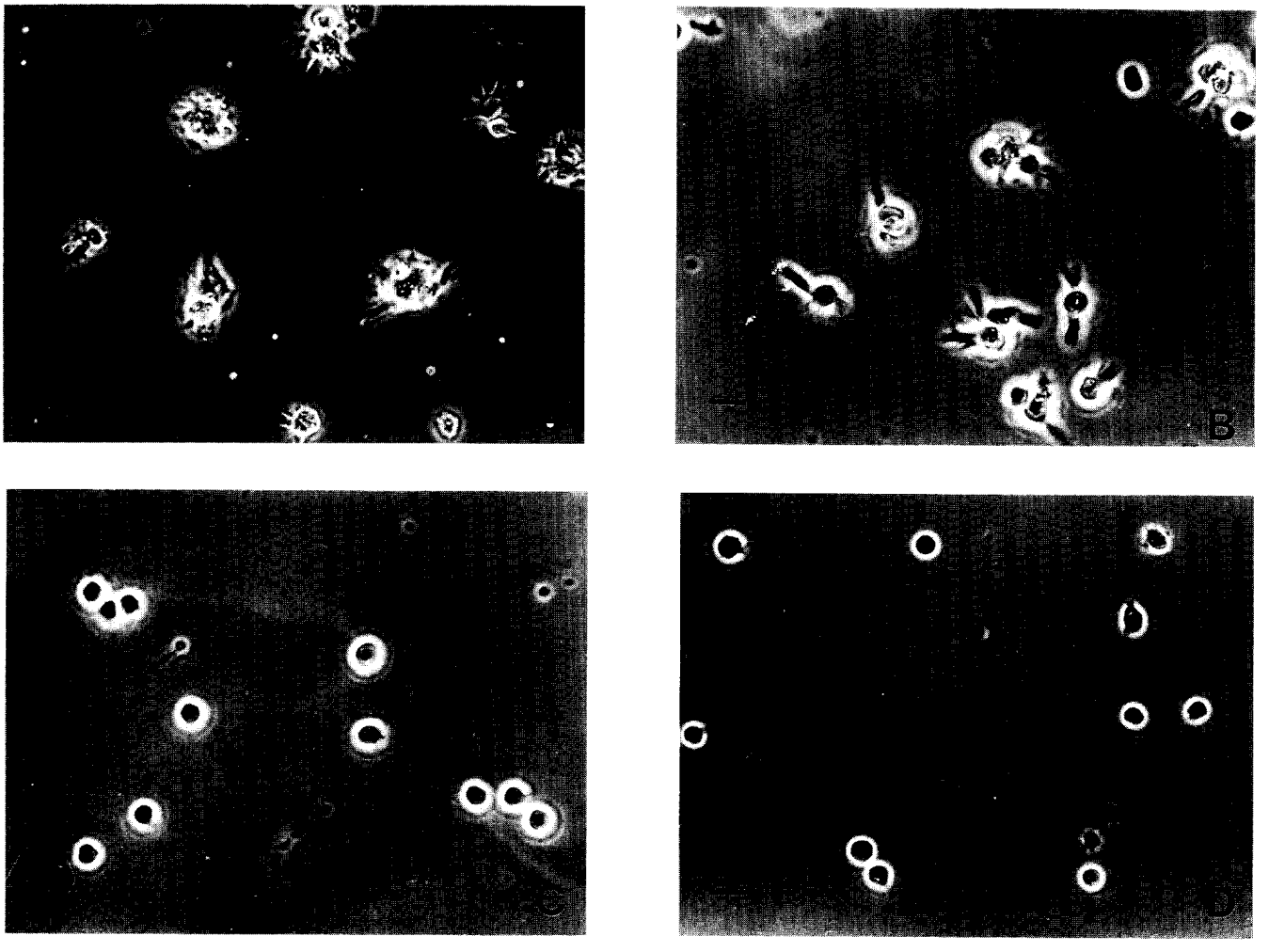

Fig. 1. Representative photomicrographs of *Planorbarius corneus* immunocytes. (A) Control: spontaneously active immunocytes; (B) incubation with LPS alone (4 Uml): note the cell activation by a more ameboid morphology; (C) incubation with morphine alone (10^{-6} M): the cells show a round conformation; (D) incubation with LPS plus morphine: the LPS activation is antagonized by morphine and round cells are seen (bar = 10 μm)

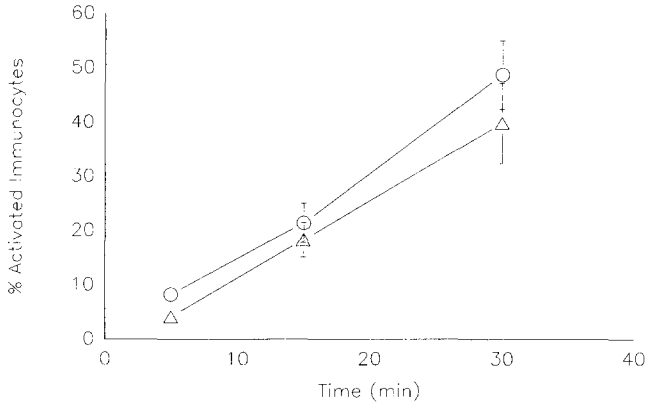

Fig. 2. LPS (10 U/ml) upregulation of invertebrate immunocytes. Control (round and inactive) cells maintain a form factor value of 0.86 ± 0.07 ($\pm$S.D.) for the entire experimental observation period, whereas those exposed to LPS have form factors below 0.5. Measurements performed as described in Material and methods. Numbers of immunocytes measured per time period varied in number from 32 to 49. Statistical significance by Student's t-test compared to the control spontaneous activation level of 6.5 ± 2.4 at the 5 min interval, and 8.6 ± 2.6 at the second and 11.3 ± 2.9 at the last ($P < 0.005$). (○) *Mytilus edulis*; (△) *Leucophaea maderae*.

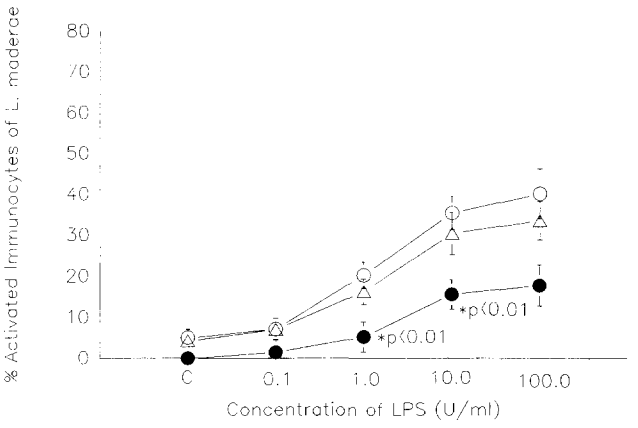

Fig. 4. Morphine antagonism of the concentration dependent LPS activation of *Leucophaea maderae* immunocytes. Morphine significantly antagonizes the LPS activation as noted by lower numbers of activated cells when compared to LPS incubation alone. Naloxone can antagonize the morphine downregulation of LPS activation at the $P < 0.05$ level of confidence when compared to morphine action on LPS stimulated immunocytes. Measurements were made as described in the text. Each value graphed represents the mean of four separate experiments consisting of the measurement of 25–42 cells ($\pm$S.D.). Statistical significance was determined by Student's t-test. (○) LPS; (●) LPS + morphine (10^{-6} M); (△) LPS + morphine + naloxone (10^{-6} M).

is a potent immunostimulatory agent, and that its effect is counteracted by the presence of morphine in a concentration dependent manner. An additional result provides evidence that the downregulating influence of morphine also applies to the immunocyte phagocytic activity.

Mammalian studies also have shown an immune downregulating action for opiates. Indeed, morphine has an immunosuppressive role both on lymphocyte activity (Bryant et al., 1987; Donahoe et al., 1987; Bayer and Flores, 1990; Weber et al., 1991) and on immune responses such as phagocytosis, chemotactic activity, cellular adherence and superoxide production in the human neutrophils and monocytes (Tubaro et al., 1985; Stefano et al., 1993) and suppresses natural

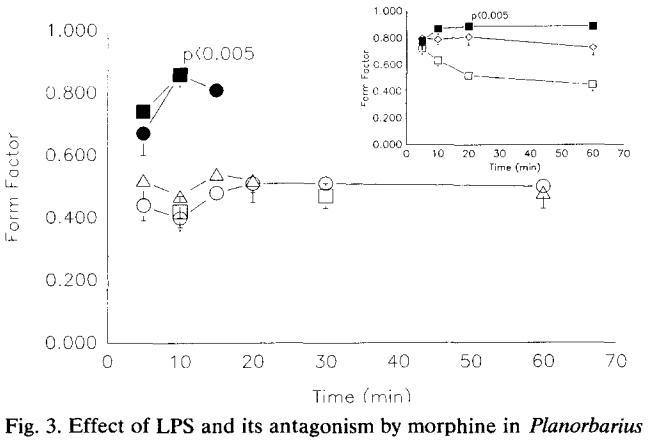

Fig. 3. Effect of LPS and its antagonism by morphine in *Planorbarius corneus* immunocytes. The number of cells varied for each test between 12–26. The mean from two to four replicates plus S.E.M. was graphed. Control cells were used that exhibited a high level of spontaneous activity to adhere to glass. *Inset*. Control cells were used that exhibited a low level of spontaneous activity. The changes in form factor, which expresses cellular shape, indicate the state of activity of immunocytes. Statistical significance was determined by Student's t-test. (○) Spontaneously active immunocytes; (◇) round, inactive control (*inset*); (●) Morphine (10^{-6} M); (△) morphine + naloxone (10^{-6} M); (□) LPS (4 U/ml); (■) LPS + morphine.

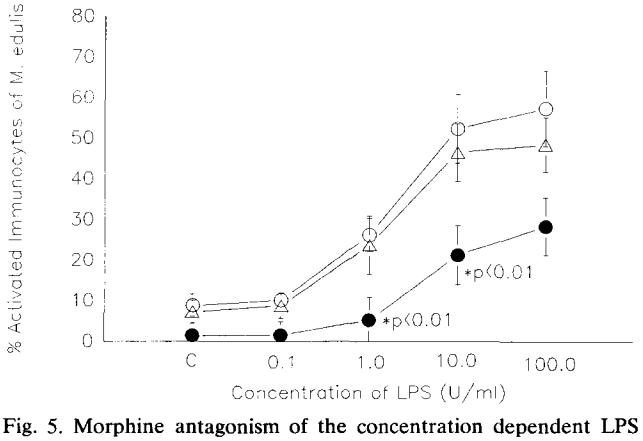

Fig. 5. Morphine antagonism of the concentration dependent LPS activation of *Mytilus edulis* immunocytes. Morphine significantly antagonizes the LPS activation, as noted by lower numbers of activated cells when compared to LPS incubation alone. Naloxone can antagonize the morphine downregulation of LPS activation at the $P < 0.05$ level of confidence when compared to morphine action on LPS stimulated immunocytes. Measurements were made as described in the text. Each value graphed represents the mean of four separate experiments consisting of the measurement of 34–44 cells ($\pm$S.D.). Statistical significance was determined by Student's t-test. (○) LPS; (●) LPS + morphine (10^{-6} M); (△) LPS + morphine + naloxone (10^{-6} M).

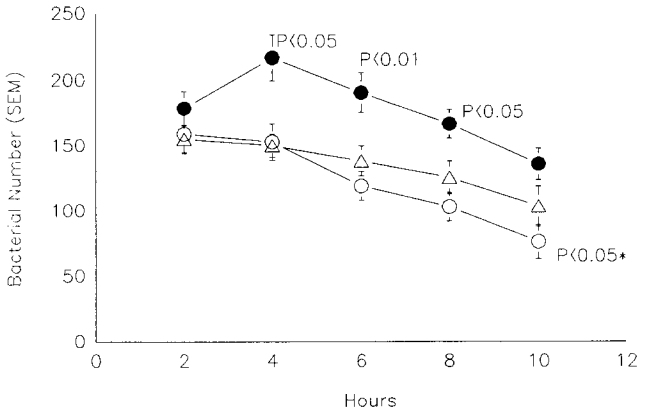

Fig. 6. Morphine inhibits the phagocytosis of *Pseudomonas stretzi*, a gram negative bacterium. Details are found in the text. Statistical significance was determined by a one tailed Students *t*-test. $P < 0.05$ and $P < 0.01$ is compared to immuncytes incubated only with the bacteria. $P < 0.05$ * is compared to the 'alone' reading, 2 h after initiation of the incubation. Cells incubated either alone or in the presence of morphine or naloxone were readily observed phagocytizing bacteria. This was rarely observed in the presence of morphine. (○) Alone; (●) morphine (10^{-6} M); (△) morphine + naloxone (10^{-6} M).

killer cell activity in rats as well (Shavit et al., 1986). Furthermore, morphine can antagonize both TNF and substance P immunocyte stimulatory actions in human immunocytes (Stefano et al., 1994).

These immunocyte immunosuppressive actions provide additional evidence for the conclusion that one of the possible functions of endogenous opiate-like compounds, previously demonstrated in invertebrates (Stefano et al., 1993) as well as vertebrates (see Stefano and Scharrer, 1994; Scharrer and Stefano, 1994; Fricchione et al., 1994) is that of downregulating immunoregulatory processes. Thus, opiate-like compounds have not only been conserved during evolution but their immunoregulatory action as well have been retained.

Acknowledgements

We acknowledge the following grant support: CNR (Italy), NIMH-NIDA-COR 17138, DA 09010, and the Research Foundation of the State University of New York (GBS) (USA).

References

Bayer, B.M. and C.M. Flores, 1990, Effects of morphine on lymphocyte function: possible mechanisms of interaction, in: Drugs of Abuse and Immune Function, ed. R.R. Watson (CRC Press, Boca Raton, FL) p. 151.

Bryant, H.U., E.W. Bernton and J.W. Holaday, 1987, Immunosuppressive effects of chronic morphine treatment in mice, Life Sci. 41, 1731.

Donahoe, R.M., C. Bueso-Ramos, F. Donahoe, J.J. Madden and A. Falek, 1987, Mechanistic implications of the findings that opiates and other drugs of abuse moderate T-cell surface receptors and antigenic markers, Ann. NY Acad. Sci. 496, 711.

Fricchione, G.L., A. Mendoza and G.B. Stefano, 1994, Morphine and its psychiatric implications, Adv. Neuroimmunol. 4, 117.

Hughes, T.K., E.M. Smith, J.A. Barnett, R. Charles and G.B. Stefano, 1991a, LPS stimulated invertebrate hemocytes: a role for immunoreactive TNF and IL-1, Dev. Comp. Immunol. 15, 117.

Hughes, T.K., E.M. Smith, J.A. Barnett, R. Charles and G.B. Stefano, 1991b, LPS and opioids activate distinct populations of *Mytilus edulis* immunocytes, Cell Tiss. Res. 264, 317.

Ottaviani, E., 1983, The blood cells of the freshwater snail *Planorbis corneus* (Gastropoda, Pulmonata), Dev. Comp. Immunol. 7, 209.

Paemen, L.R., E. Porchet-Hennere, M. Masson, M.K. Leung, T.K. Hughes, Jr. and G.B. Stefano, 1992, Glial localization of interleukin-1α in invertebrate ganglia, Cell. Mol. Neurobiol. 12, 463.

Scharrer, B. and G.B. Stefano, 1994, Neuropeptides and autoregulatory immune processes, in: Neuropeptides and Immunoregulation, eds. B. Scharrer, E.M. Smith and G.B. Stefano (Springer-Verlag, Berlin) p. 1.

Shavit, Y., A. Depaulis, F.C. Martin, G.W. Terman, R.N. Pechnick, C.J. Zane, R.P. Gale and J.C. Liebskind, 1986, Involvement of brain opiate receptors in the immunesuppressive effect of morphine, Proc. Natl. Acad. Sci. USA 83, 7114.

Sonetti, D., E. Ottaviani, F. Bianchi, M. Rodriguez, M.L. Stefano, B. Scharrer and G.B. Stefano, 1994, Microglia in invertebrate ganglia, Proc. Natl. Acad. Sci. USA 91, 9180.

Stefano, G.B., 1989, Role of opioid neuropeptides in immunoregulation. Prog. Neurobiol. 33, 149.

Stefano, G.B., 1992, Invertebrate and vertebrate immune and nervous system molecule commonalities, Cell. Mol. Neurobiol. 12, 357.

Stefano, G.B., 1994, Pharmacological and binding evidence for opioid receptors on vertebrate and invertebrate blood cells, in: Neuropeptides and Immunoregulation, eds. B. Scharrer, E.M. Smith and G.B. Stefano (Springer-Verlag, Berlin) p. 139.

Stefano, G.B. and B. Scharrer, 1994, Endogenous morphine and related opiates, a new class of chemical messengers, Adv. Neuroimmunol. 4, 57.

Stefano, G.B., P. Cadet and B. Scharrer, 1989a, Stimulatory effects of opioid neuropeptides on locomotory activity and conformational changes in invertebrate and human immunocytes: Evidence for a subtype of delta receptor, Proc. Natl. Acad. Sci. USA 86, 6307.

Stefano, G.B., M.K. Leung, X. Zhao and B. Scharrer, 1989b, Evidence for the involvement of opioid neuropeptides in the adherence and migration of immunocompetent invertebrate hemocytes, Proc. Natl. Acad. Sci. USA 86, 626.

Stefano, G.B., P. Melchiorri, L. Negri, T.K. Hughes and B. Scharrer, 1992, [D-Ala^2]-Deltorphin I binding and pharmacological evidence for a special subtype of delta opioid receptor on human and invertebrate immune cells, Proc. Natl. Acad. Sci. USA 89, 9316.

Stefano, G.B., A. Digenis, S. Spector, M.K. Leung, T.V. Bilfinger, M.H. Makman, B. Scharrer and N.N. Abumrad, 1993, Opiatelike substances in an invertebrate, a novel opiate receptor on invertebrate and human immunocytes, and a role in immunosuppression, Proc. Natl. Acad. Sci. USA 90, 11099.

Stefano, G.B., V. Kushnerik, M. Rodriquez and T.V. Bilfinger, 1994, Inhibitory effect of morphine on granulocyte stimulation by tumor necrosis factor and substance P, Int. J. Immunopharmacol. 16, 329.

Tubaro, E., U. Avico, C. Santiangeli, P. Zuccaro, G. Cavallo, R. Pacifici, C. Croce and G. Borelli, 1985, Morphine and methadoneimpact on human phagocytic physiology, Int. J. Immunopharmacol. 7, 865.

Weber, R.J., L.C. Band, B. Decosta, A. Pert and K.C. Rice, 1991, Neural control of immune function: opioids, opiod receptors and immunosuppression, NIDA Res. Monogr. 105, 96.