Evidence for the involvement of opioid neuropeptides in the adherence and migration of immunocompetent invertebrate hemocytes

(invertebrate immune reactions/endogenous opioids in hemolymph/[D-Ala²,Met⁵]enkephalinamide/high-pressure liquid chromatography/radioimmunoassay)

GEORGE B. STEFANO*[†], MICHAEL K. LEUNG*, XIAOHONG ZHAO[‡], AND BERTA SCHARRER[§]

*Multidisciplinary Center for the Study of Aging, State University of New York/College at Old Westbury, Old Westbury, NY 11568; [†]Institute of Zoology, Academia Sinica, Beijing, China; and [‡]Albert Einstein College of Medicine, Department of Anatomy and Structural Biology, Bronx, NY 10461

Contributed by Berta Scharrer, October 4, 1988

ABSTRACT Evidence for the participation of opioid neuropeptides in immunoregulatory activities, especially cellular adherence and migration, has been obtained in representatives of two phyla of invertebrates, the mollusc *Mytilus edulis* and the insect *Leucophaea maderae*. The injection of a synthetic analog of [Met]enkephalin ([D-Ala²,Met⁵]enkephalinamide, DAMA; 10⁻⁶ M) had a stimulatory, naloxone-reversible effect on the directed migration of immunocompetent hemocytes. Incubation of hemolymph in the presence of exogenous or endogenous opioid material significantly enhanced the adherence of hemocytes on albumin-coated slides as demonstrated by use of indirect Zeiss-Zonax reflectance computer analysis. Conversely, hemocyte adherence was markedly reduced by the addition of naloxone (10⁻⁸ M) to the incubation medium, either alone or in combination with DAMA. The antagonistic effects of naloxone on the stimulatory activities of opioids indicate that, like those previously reported in mammals, they are receptor-mediated. The presence of an endogenous [Met]enkephalin-like material was demonstrated in cell-free hemolymph as well as sequestered hemocytes by use of high-pressure liquid chromatography and radioimmunoassay. These results demonstrate that the capacity of immunocytes to release and respond to opioid neuropeptide messengers is not restricted to mammalian organisms but was developed early in the course of evolution.

One of the recent advances in the field of intercellular communication concerns the effective interaction among the immune, the nervous, and the endocrine systems as well as the close chemical relationship of the messenger substances used by these systems (1–4). Bioactive neuropeptides, whose multiple roles in neuroendocrine processes are well established, are known to perform additional tasks in the regulation of humoral and cellular immune responses of vertebrates. More specifically, endogenous opioid peptides have been shown to be involved in the mobilization, directed movement, and adherence of immunoreactive cells (5–8) and in several related immune processes. There is evidence for the presence of opioids in lymphoid cells and for their release into the circulation in response to stress (3, 7). Moreover, these cells possess highly specific binding sites for opiate-like agonists and naloxone-like antagonists (9).

Since the roles played by endogenous opioids and opioid receptors in neuroendocrine control mechanisms of invertebrates closely parallel those in vertebrates (10–13), the question arises whether these neuropeptides may also be engaged in receptor-mediated activities of invertebrate hemocytes comparable to those in the vertebrate immune system. This

possibility was explored in the mollusc *Mytilus edulis* and the insect *Leucophaea maderae*. Immune responses in these and other invertebrates are carried out by a class of hemocytes that have certain features in common with their counterparts in the mammalian immune system. Among these are the capacities of adherence and aggregation, chemokinesis and chemotaxis, and exocytosis and endocytosis (6, 14).

The most common immunocompetent hemocytes of invertebrates are the granulocytes (amebocytes), which ultrastructurally resemble the polymorphonuclear leukocytes of vertebrates. In lower invertebrates they are the only type of immunocyte identified thus far. These cells appear to be capable of carrying out several functions attributed to different cell types in the mammalian immune system—i.e., B and T lymphocytes and granular leukocytes (14). In *Mytilus*, the granular immunocytes (average diameter, 18 μ m) display a special fluorescence indicative of serotonin content (15). Although the functional role of this material remains to be determined, its presence serves as a convenient marker in tracing the movement of these cells.

The functional versatility of the granular hemocytes of *Leucophaea* and related insect species is reflected in the complexity of their ultrastructural organization and an apparent capacity for cellular modulation (16). The present study focuses on the participation of opioid neuropeptides in two immunoregulatory activities, cellular adherence and migration.

MATERIALS AND METHODS

Subtidal *M. edulis* were collected from the Wading River and Queens College/Caumsett State Park shore area of Long Island Sound, New York, during the months of October 1987 to January 1988, and March to May 1988 and were used for various procedures within 3 hr of collection. Adult specimens of male and female *L. maderae* were taken from stock colonies maintained in the laboratory for many years. The Student *t* test was used for statistical analysis of the results.

Hemocyte Adherence Test. For slide tests, 0.1 ml of hemolymph was withdrawn from the extracellular space in the area of the posterior adductor muscle of *Mytilus*, dispersed in a Vortex (5 sec) with either 0.1 ml of Instant Ocean or Instant Ocean plus drug, and then placed on a slide precoated with a 2% bovine serum albumin solution. Hemolymph from *Leucophaea* was collected from the abdominal region, added to an equal volume of a coagulation-inhibiting solution (0.45 M NaCl/0.1 M glucose/30 mM sodium citrate/26 mM citric acid/10 mM EDTA), and processed for tests with drugs as well as endogenous opioid material. The mixture was allowed to incubate at room

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: DAMA, [D-Ala²,Met⁵]enkephalinamide.
[†]To whom reprint requests should be addressed.

temperature (23°C) for 30 min. The slide was then gently tilted, allowing excess fluid to run off, and brought into focus for light microscopic inspection and Zeiss-Zonax reflectance computer analysis. The numbers of cells per 100× field were quantified. Reflectance is a relative measure of light reflecting off the surface of the cells during epi-illumination. The higher the value, the greater the reflectance and the greater the number of cells in the field of analysis (0.4 mm²). All estimations were taken at a gain of 1 and high voltage of 600. This indirect method proved to be quite reliable in estimating the overall number of hemocytes adhering to the slide surface.

Analysis of Hemolymph for Endogenous Opioids. The presence of opioid peptides in cell-free hemolymph and hemocytes, respectively, was demonstrated by a procedure modified from that previously reported (17). Sixty milliliters of hemolymph was aspirated from the area of the posterior adductor muscle of 160 *Mytilus*. Ten milliliters of 1.5 M NaCl was added to the hemolymph to increase the NaCl concentration by 210 mM. This ensured the dissociation of extracellular endogenous opioid peptides from the surface of the hemocytes. Hemocytes were separated from hemolymph fluid by centrifugation at 400 × *g* for 10 min. Separation was shown to be complete by microscopic examination.

For the analysis of cell-free hemolymph, 50 ml of an extraction solution containing 1 M acetic acid, 20 mM HCl, 1 μg of phenylmethylsulfonyl fluoride per ml, 1 μg of pepstatin A per ml, and 1% 2-mercaptoethanol was added to the fluid. This mixture was homogenized with a Brinkmann Polytron homogenizer with four 5-sec bursts. The cell-free hemolymph homogenate was deproteinized by the addition of 50% trichloroacetic acid to a final concentration of 10% trichloroacetic acid. This mixture was then centrifuged at 10,000 × *g* for 30 min at 4°C, and the supernatant was extracted three times with an equal volume of diethyl ether to remove lipids and the trichloroacetic acid. The aqueous portion was lyophilized and dissolved in 6 ml of 10% acetonitrile in 0.1% trifluoroacetic acid. After clarification by centrifugation, the supernatant was purified by a Sep-Pak C₁₈ (Waters) cartridge. The Sep-Pak was prewetted with 5 ml of methanol and 20 ml of H₂O. After the sample was injected into the Sep-Pak, it was rinsed with 10 ml of 1% acetic acid and eluted with 3 ml of methanol. The eluant was lyophilized and dissolved in 300 μl of 10% acetonitrile in 0.1% trifluoroacetic acid. After centrifugation, 220 μl of the supernatant was fractionated by high-pressure liquid chromatography (HPLC). For the determination of the opioid content of hemocytes, the pellet obtained by centrifugation of total hemolymph was suspended in 10 ml of the extraction solution and processed in the same manner as the cell-free hemolymph.

HPLC Fractionation. A Waters model 840 HPLC system equipped with a WISP autoinjector and a Brownlee RP-300 C₈ reverse-phase column (4.6 × 250 mm) was used for this procedure. Injection of the material was followed by elution of the column with two solvents, solvent A (0.1% trifluoroacetic acid) and solvent B (80% acetonitrile in 0.1% trifluoroacetic acid). The elution was carried out in four steps: (i) isocratically with 20% solvent B (2 min), (ii) with a gradient of 20–40% solvent B (8 min), (iii) isocratically with 40% solvent B (5 min), and (iv) with a final gradient of 40–100% solvent B (5 min). Under these chromatographic conditions the retention time of [Met]enkephalin is 10.5 min. An aliquot (220 μl) of concentrated purified extract, equivalent to the content of 44 ml of cell-free hemolymph or hemocyte suspension, respectively, was subjected to HPLC. One-milliliter fractions were collected.

Radioimmunoassay (RIA). [Met]enkephalin-like activity was determined by RIA (Immuno Nuclear, Stillwater, MN) in selected HPLC fractions. The cross-reactivity of the

[Met]enkephalin antibody used in this kit is <3% for [Met]-enkephalin-Lys, [Met]enkephalin-Lys-Arg, [Met]enkephalin-Arg-Phe, [Met]enkephalinamide, [Leu]-enkephalin, β-endorphin, or dynorphin. The results of duplicate runs were averaged. Aliquots (100 μl) of cell-free hemolymph and hemocyte samples, respectively, were used. Total [Met]-enkephalin activity in each group was quantified by assaying additional aliquots of diluted samples.

Surgical Lesions and Opioid Injections. To elicit an immune response in *Mytilus*, the branchial nerve originating from the right visceral ganglion was severed at a distance of about 8 mm from the ganglion. The presence and quantity of immunocytes in the lesioned area were determined by light microscopic examination immediately, 2 hr, and 10 hr following surgery. A second group of animals with branchial nerve severance received injections of either [D-Ala², Met⁵]enkephalinamide (DAMA; 10⁻¹¹ M) or naloxone (10⁻⁸ M) in a volume of 10 μl in close proximity to the lesion immediately after surgery. A group of unoperated *Mytilus* received injections of the same amount of DAMA. To evaluate the effect of exogenous opioid substances in comparison with those of other neuroregulators, injections were made in intact *Mytilus* of epinephrine, norepinephrine, dopamine, serotonin, substance P, insulin, octopamine, and Phe-Met-Arg-Phe-NH₂.

Indirect evidence for the existence of opioid receptors in the invertebrate immunocytes was obtained by their response to the administration of naloxone in combination with the various experimental procedures employed.

RESULTS

Severance of the branchial nerve of *Mytilus* evoked a cellular immune response, as judged by the directional migration of fluorescent immunocytes to the lesioned area (Fig. 1 *a–c*). The concentration of these cells accumulating and adhering to the lesioned tissue gradually increased over a 10-hr period of observation, a response presumed to be due to a concentration gradient of antigenic or recognition factors. The participation of opioids in these processes was ascertained by (i) tests for responses of immunocompetent hemocytes to the presence of opioids, (ii) demonstration of endogenous opio-

FIG. 1. Disposition of fluorescent immunocytes at different intervals following severance of branchial nerve of *Mytilus*. (*a*) Immediately after lesion. (*b*) Two-hour interval; beginning accumulation of immunocytes in vicinity of severed nerve (arrow, plane of severance). (*c*) Ten-hour interval; large clusters of immunocytes in area of lesioned nerve. (*d*) Injection of DAMA (10⁻⁶ M) into lesioned area immediately following nerve severance. Two-hour interval; note accumulation of immunocytes primarily at site of injection (arrow). N, severed nerve. (*e*) Same interval as in *c*; counteraction of immune response by administration of naloxone (10⁻⁸ M) immediately after lesion. Each group consisted of six animals. There was no variation in the response to the different treatments listed above. (×200.)

ids in the hemolymph, and (iii) naloxone effects indicating the operation of opioid receptors.

Effects of Exogenous Opioids. A possible chemotactic effect of opioids on *Mytilus* hemocytes was examined by injections of DAMA ($10\ \mu\text{l}$, $10^{-6}\ \text{M}$). In the unoperated animal, this exogenous stimulant elicited a directed movement of immunocytes to the site of injection and their clustering at this site. The same injection of DAMA, placed in the vicinity of a severed branchial nerve (Fig. 1*d*), showed that, after a period of 2 hr, the concentration gradient established by the injected material had taken precedence over that provided by putative endogenous antigenic messengers dispatched at the site of lesion. A possible explanation for this differential response is a critical difference between the concentrations of endogenous and injected ligands competing for opioid receptors. The specificity of the observed opioid effect became apparent from the fact that injections of epinephrine, norepinephrine, dopamine, serotonin, substance P, insulin, octopamine, and Phe-Met-Arg-Phe-NH₂ failed to elicit directed cellular movement to the site of injection.

The influence of opioids on cellular adherence was clearly demonstrated in slide tests. Incubation of hemolymph of *Mytilus* or *Leucophaea* with DAMA ($10^{-11}\ \text{M}$) for 30 min at room temperature (23°C) resulted in adherence of hemocytes to albumin-coated slides, which was significantly more pronounced than that in controls (Figs. 2 and 3). The adherence-promoting function of DAMA and its blockage by naloxone in a dose-response manner are clearly illustrated in Fig. 3. The DAMA effect is statistically significant as compared to controls, in concentrations of 10^{-2} to $10^{-8}\ \text{M}$ and 10^{-11} to $10^{-12}\ \text{M}$ (Student's *t* test: $P > 0.01$). Tests carried out with naloxone alone revealed it to act as an agonist as well as an antagonist. At higher concentrations (10^{-4} and $10^{-5}\ \text{M}$) naloxone enhanced hemocyte adherence; at lower concentrations it decreased it. The suppressive effect of naloxone on the process is also statistically significant ($P > 0.05$). Corresponding tests in *Leucophaea* gave very similar results. In both species the antagonistic activity of naloxone observed in the absence of DAMA suggests the occurrence of endogenous opioid substances in the hemolymph fluid and their role in facilitating hemocyte adherence. The antagonistic effect of naloxone at a concentration of $10^{-8}\ \text{M}$ became apparent also following its administration in combination with DAMA. The adherence-stimulating capacity of DAMA was significantly lowered by the addition of naloxone to the incubation medium. By contrast to the synthetic analog DAMA, exogenous [Met]enkephalin did not increase cellular adherence above control levels, possibly due to the presence of proteolytic enzymes in the hemolymph.

In addition to the exogenous opioid substances tested, a part of the combined fractions of endogenous [Met]enkephalin-like material obtained by HPLC of hemolymph (see below) was examined for its influence on hemocyte adherence. The effect of this material in the slide test resembled that of DAMA, in that it enhanced adherence and was blocked by naloxone. The fact that treatment of this endogenous reactive material with trypsin (1 mg/ml, 30 min) abolished its effect on hemocyte adherence indicates its proteinaceous nature. Since heat treatment (100°C , 30 min) merely reduced but did not destroy its effect, the material seems to have a molecular size in the range of [Met]enkephalin rather than a long polypeptide chain.

Additional adherence tests were performed with endogenous opioid-like material obtained from immunocytes sequestered from hemolymph fluid. For this purpose, suspensions of hemocytes collected from 70 *Mytilus* were thoroughly washed with saline and homogenized. The adherence-promoting effect of this material (relative reflectance, 92) was significantly higher than that of washed intact hemocytes (relative reflectance, 79). The addition of $10^{-8}\ \text{M}$ naloxone to the homoge-

FIG. 2. Adherence of *Mytilus* hemocytes to albumin-coated slides. (a) Control (incubation medium only). (b) Addition of $10^{-11}\ \text{M}$ DAMA to incubation medium; note clumping of larger number of cells. (c) Addition of $10^{-8}\ \text{M}$ naloxone to incubation medium; note reduction in the number of adherent cells below control level, indicative of endogenous opioid ligand in hemolymph. ($\times 300$.)

nized hemocyte preparation reduced its effect to a relative reflectance value of 83.

Endogenous Opioids in Hemolymph. The demonstration of an endogenous [Met]enkephalin-like material in the hemolymph was carried out by means of HPLC and RIA. The results of HPLC fractionation of concentrated extracts of cell-free hemolymph and hemocytes, respectively (Fig. 4), show the highest activities in fractions 5, 6, and 11 of both types of material. Moderate activity was detected in fractions 7 and 13 of the cell-free hemolymph, and very little activity was found in fractions 7 and 13 of the cellular material. Because of the presence of [Met]enkephalin-like material in a relatively large number of HPLC fractions showing more than one peak, it is within reason to assume that the hemolymph may contain more than one opioid. Moreover, the peak values for hemolymph fluid should be taken to represent minimum rather than actual levels, since they may have surpassed the upper limit of detection by the RIA

FIG. 3. Effects of DAMA, naloxone (NAL), and DAMA plus naloxone on hemocyte adherence in slide tests with *Mytilus* and *Leucophaea* hemolymph, determined by reflectance computer analysis. The control level (dashed line) represents the mean of 30 reflectance values, each obtained from 10 readings on a single slide. Readings of effects of DAMA, naloxone, and DAMA plus naloxone represent means of 10 mean reflectance values. The variability of these mean values was in the range of 1–2.6%. Note the stimulatory effect of DAMA and its suppression by naloxone, which is more pronounced at lower concentrations of DAMA.

system used (2500 pg/ml). Nevertheless, the results show a pronounced difference in the amounts of opioid content of hemolymph fluid and hemocytes.

Opioid Receptors in Hemocytes. Evidence for the presence of opioid receptors in the immunocytes studied was obtained

in the same way as in vertebrate lymphocytes (18)—i.e., by determining the effects of naloxone on the cellular activities under consideration. Naloxone injections into the area of nerve severance of *Mytilus* counteracted the cellular immune reaction observed in the absence of this drug (Fig. 1e). In the

FIG. 4. [Met]enkephalin-like activity (ME Act.) determined by RIA in selected HPLC fractions of cell-free hemolymph and hemocytes of *Mytilus*.

slide tests (Figs. 2 and 3), the application of naloxone in appropriate concentrations decreased hemocyte adherence and counteracted the stimulatory effect of DAMA as well as endogenous opioid material.

DISCUSSION

The ability to distinguish self from nonself or altered self may be considered to be critical for the survival of all living organisms. Humoral and cellular immune responses have been demonstrated in invertebrates (19, 20). Important features that their cell-mediated immune reactions have in common with those of vertebrates are the activation and directed migration of immunocytes and their accumulation at sites of antigenic challenge, as well as the capacity of cellular adherence. The results of the present study indicate that the similarities of these immune phenomena reach significantly further.

In particular, this parallelism applies to a phenomenon that in recent years has attracted considerable attention—i.e., the involvement of endogenous neuropeptides in the activities of the immune system. According to numerous mammalian studies (3, 4), neuropeptides present in the circulation may be derived from neural and endocrine tissues as well as immunocytes. They function as messengers in the bidirectional interaction between the neuroendocrine and the immune systems and also in autoregulatory communication among immunocompetent cells. As to the mode of operation of opioid peptides in internal immunoregulatory activities, a modulatory role has been proposed for the processes of cellular adherence (e.g., ref. 8) and adhesion-dependent chemotaxis (21).

The present study documents that also in invertebrates coordinated responses to antigenic challenges, especially directed movement and aggregation of hemocytes, are facilitated by receptor-mediated opioid signals. Rather comprehensive tests in representatives of two phyla have demonstrated (i) enhanced cellular adherence in the presence of opioid material; (ii) directed migration of immunocytes following the administration of a synthetic [Met]enkephalin analog (DAMA); (iii) presence of endogenous opioids (in particular, [Met]enkephalin-like material) in the hemolymph fluid and in its cellular component; and (iv) occurrence of opioid receptors, as indicated by the effects of naloxone on hemocyte activity.

The only observation in an invertebrate related to that of intercellular opioid signaling reported in the present study was made by Huxham and Lackie (22), who suggested that the recruitment of phenol oxidase-positive immunocytes, and their encapsulation of foreign material, in the insect *Schistocerca gregaria* may be enhanced by a peptide forming as a by-product in the biosynthesis of phenol oxidase.

The chemotaxis-stimulating effect of DAMA in *Mytilus* and *Leucophaea* reported here is in line with that of [Met]-enkephalin on human granulocytes via naloxone-sensitive opiate receptors, observed by Van Epps and Saland (21). The demonstration of the stimulatory effect of DAMA as well as endogenous opioid material on hemocyte adherence, and its blockage by naloxone, parallels that observed in mammalian immunocytes.

As in mammalian adherence tests (5), the response of the hemocytes of both invertebrate species examined was bimodal, a result the significance of which remains to be ascertained. No vertebrate data comparable to those on the chemical characterization by HPLC and RIA of the opioid immunoregulators in the hemolymph of the mollusc *Mytilus* are as yet available. As to the differential localization of [Met]enkephalin-like material in *Mytilus*, the amount demonstrated in the hemolymph fluid was much greater than that

in the hemocytes. This result may be accounted for in part by a rapid rate of release of material synthesized in hemocytes, in part by the fact that additional neuropeptides found in the circulation may be derived from neuroendocrine tissues.

In summary, the present study has shown that opioid peptides participate in the immune responses of invertebrates. As in vertebrates, the mode of operation of these immunoregulators appears to be modulatory and receptor-mediated. This new insight into the capacity of invertebrate immunocytes to release and respond to opioid messengers has strengthened our understanding of the long evolutionary history of neuropeptides and their multiple roles in regulatory processes.

We acknowledge the technical assistance of Mr. Juan Sinisterra, Mr. Patrick Cadet [Alcohol, Drug Abuse, Mental Health Administration—Minority Access to Research Careers (ADAMHA—MARC) Fellows], and Mr. Lynn Simmons (National Institutes of Health Minority Biomedical Research Support Assistant). We also express our gratitude to Dr. A. Gupta for information on an anticoagulation fluid. The work was in part supported by ADAMHA—MARC Grant 17138, National Institute of Mental Health Grant NIH-MH-08078 (M.K.L. and G.B.S.), National Institutes of Health Grant NS 22344-02 (B.S.), and the Research Foundation of the State University of New York. X.Z. was supported by the State University of New York/College at Old Westbury and a State University of New York/International Programs Office Grant to the Multidisciplinary Center for the Study of Aging at the State University of New York/College at Old Westbury.

1. Smith, E. M. & Blalock, J. E. (1986) in *Enkephalins and Endorphins: Stress and the Immune System*, eds. Plotnikoff, N. P., Faith, R. E., Murgo, A. J. & Good, R. A. (Plenum, New York), pp. 119–128.
2. Ballieux, R. E. & Heijnen, C. J. (1987) *Progr. Brain Res.* **72**, 71–77.
3. Jancović, B. D., Marcović, B. M. & Spector, N. H., eds. (1987) in *Neuroimmune Interactions: Proceedings of the Second International Workshop on Neuroimmunomodulation* (Ann. N.Y. Acad. Sci., New York), Vol. 496.
4. Perez-Polo, J. R., Bulloch, K., Angeletti, R. H., Hashim, G. A. & de Vellis, J. (1987) *J. Neurosci. Res.* **18**, 1–26.
5. Brown, S. L., Tokuda, S., Saland, L. C. & Van Epps, D. E. (1986) in *Enkephalins and Endorphins: Stress and the Immune System*, eds. Plotnikoff, N. P., Faith, R. E., Murgo, A. J. & Good, R. A. (Plenum, New York), pp. 367–386.
6. Fischer, E. G. & Falke, N. E. (1986) in *Enkephalins and Endorphins: Stress and the Immune System*, eds. Plotnikoff, N. P., Faith, R. E., Murgo, A. J. & Good, R. A. (Plenum, New York), pp. 263–272.
7. Plotnikoff, N. P., Faith, R. E., Murgo, A. J. & Good, R. A., eds. (1986) in *Enkephalins and Endorphins: Stress and the Immune System* (Plenum, New York), pp. 224–234.
8. Van Epps, D. E. & Kutvirt, S. L. (1987) *J. Neuroimmunol.* **15**, 214–228.
9. Sibinga, N. E. S. & Goldstein, A. (1988) *Annu. Rev. Immunol.* **6**, 219–249.
10. Stefano, G. B. (1982) *Cell. Mol. Neurobiol.* **2**, 167–178.
11. Leung, M. K. & Stefano, G. B. (1987) *Progr. Neurobiol.* **28**, 131–159.
12. Scharer, B. (1987) *Annu. Rev. Neurosci.* **10**, 1–17.
13. Scharer, B. (1987) *Annu. Rev. Entomol.* **32**, 1–16.
14. Gupta, A. P., ed. (1986) in *Hemocytic and Humoral Immunity in Arthropods* (Wiley, New York).
15. Stefano, G. B., Zhao, X., Bailey, D., Metley, M. & Leung, M. K. (1988) *J. Neuroimmunol.*, in press.
16. Scharer, B. (1972) *Z. Zellforsch. Mikrosk. Anat.* **129**, 301–319.
17. Leung, M. K. & Stefano, G. B. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 955–958.
18. Wybran, J., Appelboom, T., Famacy, J. P. & Govaerts, A. (1979) *J. Immunol.* **123**, 1068–1070.
19. Howcroft, T. K. & Karp, R. D. (1987) *Transplantation* **44**, 129–135.
20. Dimarq, J. L., Keppi, E., Dunbar, B., Lambert, J., Reichhart, J. M., Hoffmann, D., Rankine, S. M., Fothergill, J. E. & Hoffmann, J. A. (1988) *Eur. J. Biochem.* **171**, 17–22.
21. Van Epps, D. E. & Saland, L. (1984) *J. Immunol.* **132**, 3046–3053.
22. Huxham, I. M. & Lackie, A. M. (1988) *Cell Tissue Res.* **251**, 677–684.