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Microglia in invertebrate ganglia

(invertebrate glia/immunocytes/morphine/corticotropin)

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ABSTRACT **The results of this study lend strong support to the concept of the existence in insects and molluscs of a distinctive class of neuroglial cells comparable to vertebrate microglia. The evidence presented is as valid as that used in reference to the separate status of vertebrate microglia—i.e., the demonstration of a close structural and functional relationship of these cells with cells of the immune system. As in vertebrates, the excision of ganglia from three invertebrate species (the molluscs *Planorbarius corneus* and *Mytilus edulis* and the insect *Leucophaea maderae*) and their maintenance in incubation media led to an exodus of small cells and their accumulation in the culture dish. During this process, they underwent conformational changes from stellate to rounded, and then to more or less ameboid, comparable to those indicative of the process of activation in the animals' immunocytes. Functional characteristics which these translocated microglia-like cells share with immunocytes are motility, phagocytotic activity, and adherence to the culture dish. Furthermore, the two cells have certain biochemical features in common—e.g., the presence of certain cytokines and (at least in *Planorbarius*) that of corticotropin. An additional phenomenon of particular interest for the classification of microglial elements is their response to morphine. At 10^{-6} M, this drug decreases not only the number of cells emerging from the excised ganglia but also the degree of their transformation to the "active" ameboid form. This dose-dependent and naloxone-sensitive effect of morphine on microglial cells parallels that on activated immunocytes of the same species. Corresponding results demonstrating an inhibitory effect of morphine on mobilized microglial cells of the frog *Rana pipiens* indicate that this relationship between the two cell types under consideration also exists in vertebrates. Binding and displacement experiments with membrane homogenates of microglial cells as well as immunocytes of *Mytilus* have shown that the effects of morphine on both cell types are mediated by the same special opiate receptor (μ_3).**

The existence of two major classes of neuroglial elements in the central nervous system of vertebrates (macroglia and microglia) was first postulated by Hortega in 1916 (see ref. 1) on the basis of classical silver impregnation methods. Today, the special status of microglia is known to be based on its mesodermal origin, a concept that, after many years of debate, now appears to have become firmly established (see refs. 2–4). It is derived from cells of the immune system which, in the process of establishing residency in the developing nervous system as intrinsic immunoeffector cells, undergo certain structural and biochemical transformations. In response to trauma, such as experimental interference with normal neuron–glia interaction, microglia regains some of its inherent immunoregulatory attributes; the cells change shape and engage in migratory and phagocytotic activity (2, 3, 5).

By contrast, the search for the occurrence of a separate, nonneuroectodermal, class of glia among invertebrates is still at its beginning. The present study, conducted in three invertebrate species and one amphibian species, revealed structural and functional commonalities between glial cells and immunocytes comparable to those known in mammals, and these commonalities strongly support the concept of the existence of a distinctive class of microglial cells in insects and molluscs.

MATERIALS AND METHODS

The three species of invertebrates selected for this study—i.e., the molluscs *Planorbarius corneus* (a snail) and *Mytilus edulis* (a mussel) and the insect *Leucophaea maderae* (a cockroach)—were obtained and maintained as previously reported (6, 7). For *in vitro* observations of glia, the ganglionic ring of *Planorbarius* was bathed for 1 hr in sterile saline containing antibiotics (50 mg of streptomycin, 30 mg of penicillin, 50 mg of gentamicin in 100 ml, pH 7.5) and then transferred to a microwell culture dish containing modified Leibovitz 15 medium (40 mM NaCl/1.7 mM KCl/4.1 mM $CaCl_2$/0.7 mM $MgCl_2$/0.8 mM $MgSO_4$/0.45 mM Na_2HPO_4/0.44 mM KH_2PO_4). The pedal ganglia of *Mytilus* and the abdominal ganglia of *Leucophaea* were immersed in the respective physiological saline to which the respective centrifuged cell-free hemolymph or physiological saline plus antibiotics was added 50% by volume. The hemolymph was inspected light microscopically to ascertain its cell-free status and then subjected to UV radiation for 1 hr. For the examination of conformational changes, the ganglia of *Leucophaea* and *Mytilus* were placed in a ring of Vaseline on a slide and protected by a coverslip. The substances to be tested (e.g., morphine) were added to the incubation medium before the cell-free hemolymph, followed by final adjustments. After a 24-hr incubation period, the ganglionic explants of *Planorbarius*, with or without opiate substances, were fixed and stained with Mayer's hematoxylin and eosin (Sigma). The numbers of cells having moved into the incubation medium were determined by image analysis at a magnification of ×100.

The determination of the degree of activation in glial cells egressing from traumatized ganglia was based on measurements of cellular area and perimeter by use of the Oncor image analysis software (8). An area of 400 μm^2 was selected for determining the number of cells having moved into the culture dish and for "frame grabbing."

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Abbreviation: ACTH, corticotropin.
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For the immunocytochemical identification of corticotropin (ACTH), paraffin-embedded sections (10 μm thick) of the ganglionic ring of *Planorbarius* were preincubated in 5% normal goat serum for 30 min and then incubated overnight at 4°C with the primary antibody. The polyclonal antiserum used was raised in rabbits against human ACTH 1–24 (Dakopatts, Copenhagen), diluted 1:1000. After repeated washings with phosphate-buffered saline (PBS; 0.9% sodium chloride in 0.01 M sodium phosphate buffer, pH 7.4) the slides were processed according to the biotin/avidin system (BAS; Vector Laboratories). Diaminobenzidine hydrochloride (Sigma) was employed as the chromogen. The antiserum was dissolved in PBS with 0.3% Triton X-100. The specificity of the reaction was checked by omitting the primary antiserum (substituting nonimmune serum) and by preadsorbing it with the homologous synthetic antigen. The same procedure for the demonstration of ACTH was carried out in mobilized microglial cells, fixed *in vitro* with formaldehyde vapor for 10 min, and dried under reduced pressure.

Immunocytes and ganglia obtained from 500 *Mytilus* were prepared for opiate binding and pharmacological analysis described in detail elsewhere (9). The excised ganglia were incubated in cell-free hemolymph and saline for 24 hr at 23°C and then removed. This yielded "free" microglial cells to be used in binding experiments (see refs. 9 and 10).

Additional tests were carried out in a representative of the vertebrates, the freshwater frog *Rana pipiens*, with special attention given to the effects of morphine on microglial cells. The animals, obtained from Carolina Biological Supply, were maintained in a high-humidity environment for 1 or 2 days before use. Sympathetic ganglia dissected from the abdominal region were immersed in Ringer's solution plus antibiotics for 2 hr and placed into slide chambers. After incubation for 24 hr in medium with or without the addition of morphine (10^{-6} M), the explants were examined in much the same way as those of the invertebrate ganglia.

RESULTS

The response of glial cells to the excision of ganglia and their maintenance in organ culture was essentially the same in the three species of invertebrates and in the one species (*Rana pipiens*) of amphibian examined. In these ganglia, the trauma caused by excision stimulated the exodus of small cells (area = 22–26 μm^2) from the nervous tissue and their accumulation in the slide chamber, as exemplified by *Planorbarius* (Fig. 1). As previously reported in several mammalian studies (see refs. 2, 3, and 11), these cells underwent changes in morphology and engaged in migratory behavior.

This exodus had become quite pronounced within the first 24 hr (Figs. 2 and 3). The cells, considered to be of microglial nature, were spread around the ganglia and adhered to the incubation chamber slide. Upon leaving the ganglia, all the cells appeared small and round [form factor (ff) = $4 \times \pi \times$ area/perimeter^2 = 0.8–0.9]. After 24 hr, approximately 40% of the round, emerging cells had undergone a gradual morphological transformation and become more or less ameboid (ff = 0.4–0.5; Figs. 1*d* and 4). Subsequently, the proportion

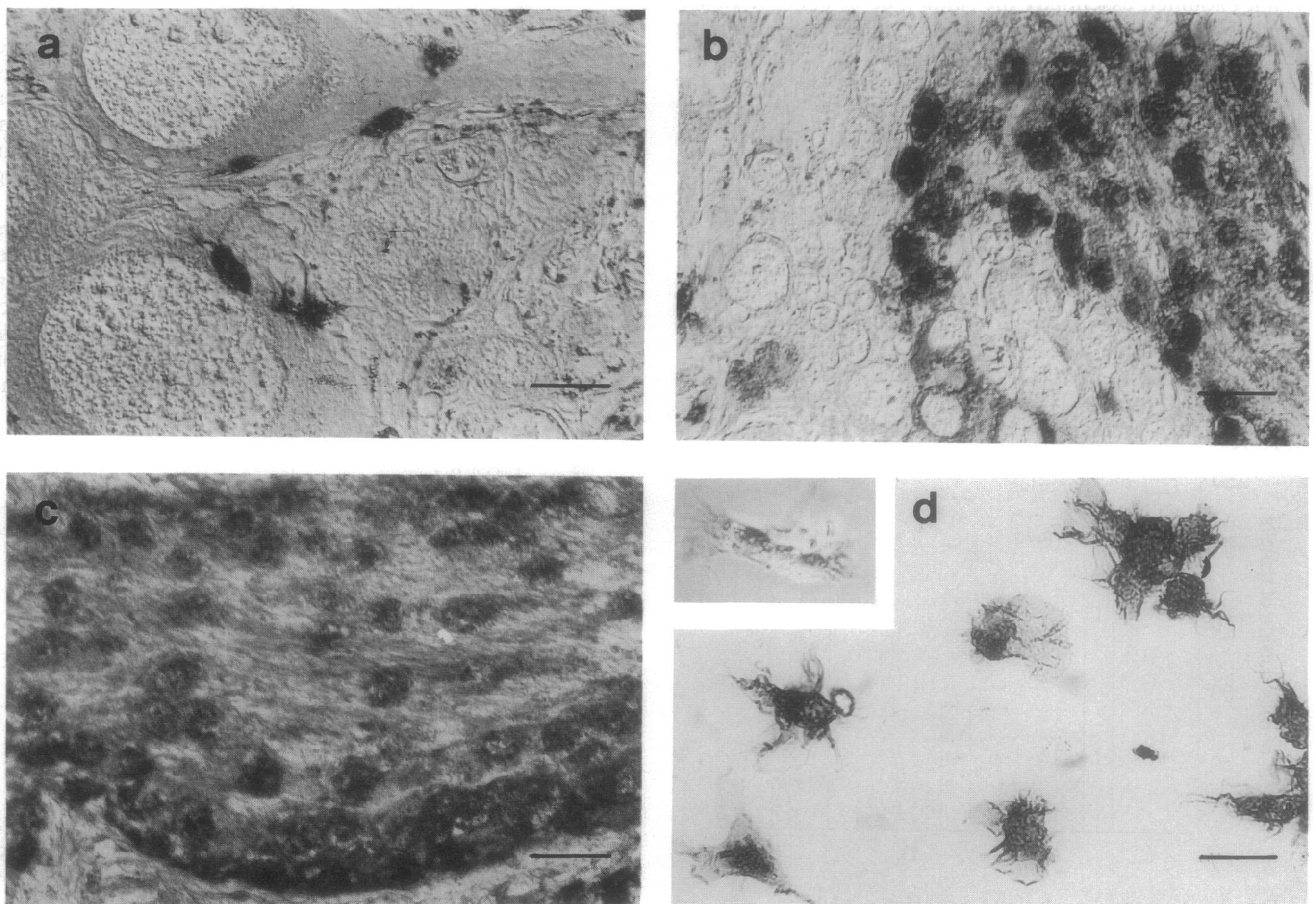

FIG. 1. Micrographs of excised visceral ganglia of *Planorbarius* demonstrating egress of microglial cells selectively immunostained for the presence of ACTH (Nomarski interference). (*a*) Microglial cells in close contact with ACTH-negative giant neurons, fixed immediately after excision. (*b*) Fixed after 24 hr of incubation in culture medium; microglia accumulating in neuropilar region. (*c*) Accumulation of migrating cells at nerve stump. (*d*) Ameboid conformation of microglial cells in extraganglionic area. (*Inset*) Abolition of immunoreaction by omission of the primary antibody. (Bars = 20 μm.)

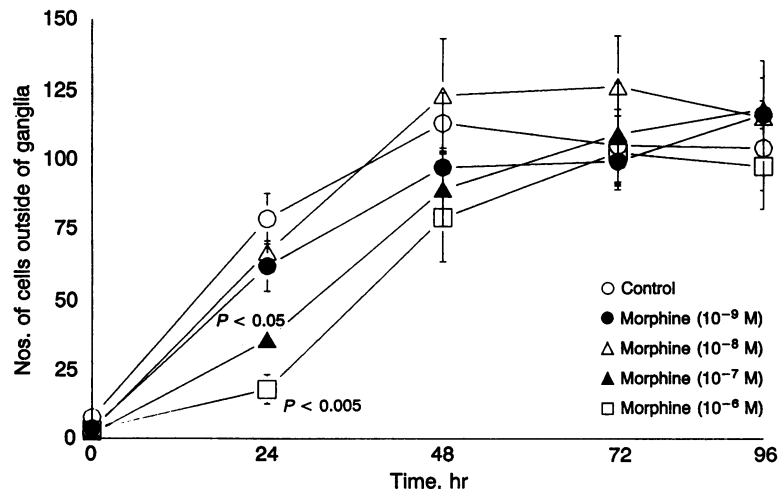

FIG. 2. Egress of microglial cells from excised pedal ganglia of *Mytilus* in the presence of morphine at various concentrations; note the inhibitory effects over the first 24-hr period. Subsequent rise of cell numbers to control levels is attributed to degradation of morphine (unpublished results). Each point represents the mean ± SEM of the mean of 4 or 5 trials. Statistical significance, indicated on the graph at the 24-hr observation period, was calculated for comparison with the control value by a one-tailed Student t test.

of ameboid activated cells increased, suggesting that a gradual transformation was in progress. Within the period of observation, 7 days for *Leucophaea* and *Mytilus*, 30 days for *Planorbarius*, the preparations maintained under these conditions showed no cellular abnormalities. The glial cells of *Rana* exhibited essentially the same kind of conformational profile—i.e., a progression of small rounded cells emerging from the ganglia to more active ameboid cells.

Since no mitotic figures could be observed within the period of observation, it could be expected that the presence of translocated cells in such numbers must be matched by a corresponding loss in intraganglionic glia. This was confirmed in micrographs of sections of *Planorbarius* ganglionic explants in which the presence of ACTH-like material in microglial elements (Fig. 1*a*) served as a marker. In specimens prepared after 24 hr of incubation in the culture medium, the number of microglial elements within the ganglionic explant was depleted, and they could be seen to accumulate on their way to the outside via the neuropile (Fig. 1*b*) and the nerve stump (Fig. 1*c*) providing access to the culture dish (Fig. 1*d*). The presence of ACTH-like material is a biochemical feature the microglial cells of *Planorbarius* have in common with its immunocytes (12). By contrast, *Mytilus* microglia did not reveal the presence of ACTH-like substances, indicating a species difference. In both molluscs, the mobility of the displaced microglial cells and their adherence to the culture slide were the same as those observed in their activated immunocytes.

An additional phenomenon of particular interest, reported here for mobilized invertebrate as well as vertebrate microglial cells, is that their activity is inhibited by morphine (Figs. 2–4). This effect expresses itself in two ways: in the numbers of microglial cells leaving the traumatized ganglia (Figs. 2 and 3) and in the numbers of mobilized glial cells undergoing conformational changes indicative of cellular activation (Fig. 4).

The inhibitory effect of morphine on the number of glial cells accumulating outside of the ganglionic explant becomes fully apparent within the first 24 hr of incubation in the culture medium (Fig. 2). On subsequent days, these values reach those of the controls, presumably due to the metabolic degradation of the morphine (unpublished results). This inhibitory effect of morphine (Fig. 3) is dose dependent,

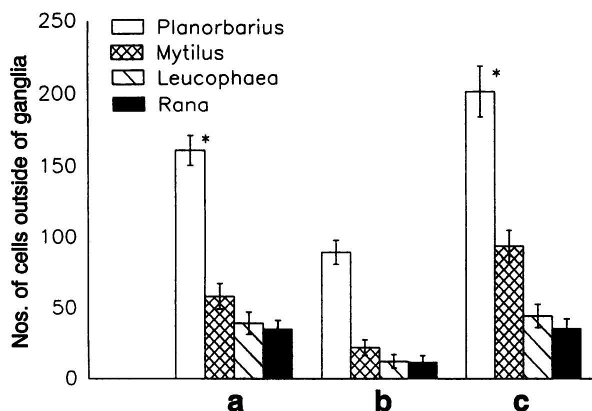

FIG. 3. Numerical analysis of morphine's inhibitory effect on exodus of microglial cells of invertebrate and vertebrate ganglia during the 24-hr period after their excision. (*a*) Without drug exposure (control). (*b*) Exposure to morphine (10^{-6} M). (*c*) Exposure to morphine (10^{-6} M) and naloxone (10^{-6} M). Each bar equals mean ± SEM for 4 to 6 trials. *, $P < 0.01$ compared with morphine values by a one-tailed Student t test.

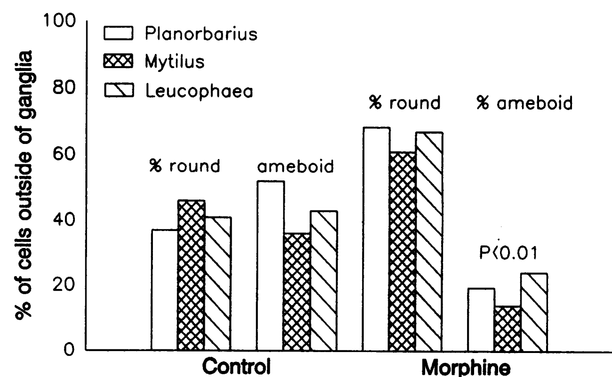

FIG. 4. Inhibitory effect of morphine (10^{-6} M) on the degree of conformational changes of microglia during the 24-hr incubation period after excision of ganglia. Form factors used for definition of round cells were 0.8–1.0, and of elongate or ameboid cells, 0.1–0.5. Values falling in between were not counted. The number of cells per animal per treatment varied from 25 to 209 due to a smaller number of translocated microglial cells in morphine-treated specimens. Each value represents the mean of 4 or 5 tests. Maximal variation was not greater than 13%. $P < 0.01$ is for comparison of control and morphine-exposed groups by a one-tailed Student t test.

Table 1. Displacement of [^{3}H]dihydromorphine by opioid and nonopioid ligands in membrane suspensions of *Mytilus* microglial cells and immunocytes

Ligand	IC$_{50}$, nM	
	Microglia	Immunocytes
δ receptor		
DPDPE	>1000	>1000
μ receptor		
DAGO	>1000	>1000
Dihydromorphine	57	59
Morphine	51	53
Antagonist		
Naloxone	310	332

Aliquots of membrane ligands were incubated with nonradioactive compounds at five concentrations for 10 min at 22°C and then with 40 nM [^{3}H]dihydromorphine for 60 min at 4°C. One hundred percent binding is defined as [^{3}H]dihydromorphine bound in the presence of 10 μM dextrorphan minus [^{3}H]dihydromorphine bound in the presence of 10 μM levorphanol. IC$_{50}$ is defined as the concentration of drug that half-maximally inhibits specific binding. DPDPE, [D-Pen^2,D-Pen^5]enkephalin (Pen, penicillamine); DAGO, [D-Ala^2, MePhe^4,Gly(ol)^5]enkephalin.

statistically significant ($P < 0.01$), and naloxone sensitive. In all species examined, morphine concentrations below 10^{-7} M were ineffective (e.g., Fig. 2). These results are in line with the previously reported observation that morphine inhibits the mobility of activated mammalian and invertebrate immunocytes (9, 13).

The second naloxone-sensitive effect of the presence of morphine in the incubation medium is that morphine antagonizes the conformational changes from rounded to ameboid-active characteristic of displaced microglial cells in a drug-free preparation (Fig. 4). The fact that the reduced cellular activity in morphine-treated specimens could be followed over a 24-hr period after explantation indicates that the displaced microglial cells retained their stereospecific morphine receptors (μ_3). The microglial cells of *Rana* subjected to the same experimental procedures proved to be equally sensitive to morphine and naloxone (Fig. 3).

Membrane homogenates of microglial cells from 500 excised pedal ganglia of *Mytilus* that had been incubated for 24 hr were used for binding experiments. Binding analysis revealed the same displacement profile in microglial membrane preparations as that observed in homogenates obtained from human monocytes and *Mytilus* immunocytes. These binding sites are selective for opiate alkaloids and inaccessible to the opioid peptides DPDPE and DAGO, the latter being an established μ-peptide ligand (Table 1). This result established that microglial cells and immunocytes have the same highly selective stereospecific affinity for morphine, mediated by the same special class of μ_3 opiate receptors (see ref. 9).

DISCUSSION

The results of the present study add considerable weight to the concept that in insects and molluscs a distinct subset of glial cells is closely related to immunoactive hemocytes (immunocytes) and, therefore, may be judged to correspond to vertebrate microglia. The existence of more than one type of invertebrate glial cells was proposed in 1883 by Vignal (14) and subsequently by a number of investigators (e.g., see refs. 15–17). However, in these studies the term "microglia" was used only rarely and primarily on the basis of morphological criteria comparable to those known in vertebrate microglia (see refs. 18 and 19).

The evidence in support of the claim made in the present study is two-fold: (*i*) In response to surgical trauma, a subset of reactive glial cells leaving the invertebrate ganglia exhibit a number of features characteristic of the animals' immunocytes. (*ii*) These structural and functional commonalities parallel those between microglia and macrophages observed in vertebrates under comparable experimental conditions (11).

The pertinent points can be summarized as follows: (*i*) In both groups of animals comparable mobile immune cells are known to enter various tissues, including the nervous system. (*ii*) In the process of transformation to resident glia, these immunocytes appear to undergo similar conformational changes and some biochemical dedifferentiation. (*iii*) During their egress from traumatized neural tissue, in excised ganglia maintained in incubation medium, the glial cells regain some of their inherent characteristics, including an ameboid conformation, adhesiveness, and motility. (*iv*) An important criterion for the immunoregulatory potential of these mobilized microglial cells and their counterpart in vertebrates is their phagocytotic activity. This capacity is made use of in the uptake and removal of degenerating neural structures, demonstrated in mammals (4, 11, 20) as well as invertebrates (18, 19). (*v*) In both groups of animals, the egress of reactivated microglial cells from traumatized nervous tissue and their conformational changes are counteracted by exogenous morphine, a process corresponding to the inhibitory effect of this drug observed in immune cells (see ref. 9). (*vi*) An additional point to be made is that a given immunoregulatory molecule may either be present in both cell types under consideration here or else be absent from both. For example, cytokines (interleukin 1 in particular) have been demonstrated in microglial and immunoreactive vertebrate cells (21) and likewise in immunocytes as well as microglial cells of an invertebrate, *Mytilus edulis* (8, 22, 23). In *Planorbarius*, both of these cell types share the presence of ACTH- and β-endorphin-like substances (12), whereas these neuropeptides could not be demonstrated in either cell type in *Mytilus*.

Two previous invertebrate studies, one in the leech *Hirudo medicinalis* (19) and another in the cockroach *Periplaneta americana* (17), reported an egress of glial cells from traumatized nervous tissue comparable to that observed in the present work. Observations in the leech (19) and in the crayfish (18) suggested that these mobilized glial cells may be engaged in clearing the damaged tissue of cellular debris. However, an understanding of the relationship of these glial cells with macrophage-like hemocytes and their microglial attributes became possible only after the new insights gained from recent investigations in comparative neuroimmunology (see refs. 7, 12, and 23–29).

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