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Generation of sensory neurons is stimulated by leukemia inhibitory factor

(neural development/neural crest/neurotrophic factors/differentiation/neurotransmitters)

MARK MURPHY, KATE REID, DOUGLAS J. HILTON, AND PERRY F. BARTLETT

The Walter and Eliza Hall Institute of Medical Research, Post Office, Royal Melbourne Hospital, Victoria 3050, Australia

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ABSTRACT **The processes that regulate the development of peripheral neurons from their precursors in the embryonic neural crest are essentially unknown. In this report, we show that leukemia inhibitory factor stimulates the generation of neurons in cultures of mouse neural crest. These neurons have the morphology of sensory neurons and contain neuropeptides found in mammalian sensory neurons. Consistent with these neurons being of the sensory lineage is the finding that they arise from nondividing precursors within the neural crest. In addition, we show that leukemia inhibitory factor supports the generation and/or maturation of sensory neurons in cultures of cells obtained from embryonic dorsal root ganglia. In cultures of postnatal dorsal root ganglia, which contain mature sensory neurons, leukemia inhibitory factor acts directly as a survival molecule on the majority of neurons.**

The sensory ganglia of the peripheral nervous system (PNS) in vertebrates are mainly derived from the neural crest, a transient population of cells that migrates from the neural tube during embryogenesis. There is evidence that individual neural crest cells are initially multipotent and that they become restricted to the sensory lineage before they migrate to form the sensory ganglia (1–4). The factors that control the development of sensory neurons from these precursor cells are ill defined, although a number of factors support the subsequent survival of these neurons (5–7). In this report we show that leukemia inhibitory factor (LIF; refs. 8 and 9), a protein with multiple activities (8–13), stimulates the generation of sensory-like neurons from a population of nondividing precursors in the mouse neural crest *in vitro*. Further, LIF supports the development and survival of sensory neurons in cultures prepared from embryonic day 12 (E12) through postnatal day 2 (P2) dorsal root ganglia (DRG), showing that it can act as a neurotrophic factor throughout sensory neuron development *in vitro*.

MATERIALS AND METHODS

Neural Crest Cultures. CBA mouse embryos at E9 were removed from the uterus and the head and tail were removed using 27-gauge needles under a dissecting microscope. The resultant cervical and thoracic segments were incubated in Hepes-buffered Eagle's medium (HEM) containing dispase (2 units/ml) at 4°C for 15 min, then incubated at 37°C for 6 min, and transferred to HEM/1% fetal bovine serum (FBS)/0.1% DNase, and the somites and surrounding tissue were carefully removed. The neural tubes were then transferred into Monomed medium (Commonwealth Serum Laboratories, Melbourne) containing 10% FBS. One tube was then placed in each well of a 24-well plate (Linbro) coated with fibronectin (5 μg/ml), and 10^4 units of LIF per ml (recombinant murine LIF, specific activity = 10^8 units/mg; refs. 8 and 9) was added after 24 hr to half of the cultures. Cultures were incubated at 37°C in 5% CO_2/95% air.

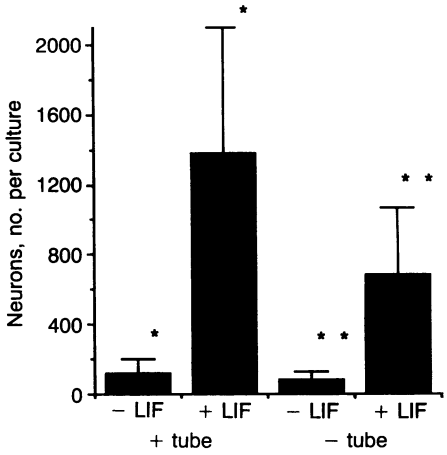

FIG. 1. Effect of LIF on neuron numbers in neural crest cultures. Neural crest cells were incubated in medium alone or in the presence of LIF for 6 days and Nissl stained (17). Neurons were counted using bright-field microscopy. In the "− tube" experiment, neural tubes were removed after 24 hr and LIF was added to the cultures. Neuron numbers could not be accurately counted at later times because of dense clustering of neurons in LIF cultures. Values are the mean and standard deviation (n = 6). *, $P < 0.005$; **, $P < 0.05$ (t test).

DRG Cultures. DRG from CBA mice of specified age were dissected free of surrounding spinal tissue, placed in HEM, finely chopped, and then incubated in HEM/0.025% (wt/vol) trypsin/0.001% DNase at 37°C (12 min for E12, 20 min for E15, and 30 min for E19 and P2). FBS was added to 20%, and the cells were centrifuged at 300 × g for 5 min, washed twice in HEM/0.01% DNase, and triturated through 18- to 25-gauge needles to obtain a single cell suspension. DRG cells were plated onto fibronectin-coated wells of HL-A plates (Nunc) at previously optimized cell numbers (3500 cells at E12, 1000 at E15, and 200 at E19 and P2). Two hours after plating, no mature neurons were observed in the E12 cultures and an average of 110, 120, and 100 neurons were present in the E15, E19, and P2 cultures, respectively. Cultures from E12 were fixed and stained for neurofilament after 5 days and neurofilament-positive neurons were counted using fluorescence microscopy. Neurons in later embryonic cultures (large, phase-bright, round cells) were counted after 2 days.

Immunohistochemistry. Neurofilament staining was as described (14) except a rabbit anti-150-kDa neurofilament antibody (Chemicon) was used. To stain for calcitonin gene-related peptide (CGRP), cultures were fixed in paraformal-

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Abbreviations: PNS, peripheral nervous system; LIF, leukemia inhibitory factor; DRG, dorsal root ganglion(ia); CGRP, calcitonin gene-related peptide; ChAT, choline acetyltransferase; E, embryonic day; P, postnatal day; FBS, fetal bovine serum.

dehyde (PFA), cleared with dimethyl sulfoxide, washed with phosphate-buffered saline, incubated with a rabbit anti-rat α-CGRP antibody [obtained from G. Olley (Monash University, Australia); the antibody shows 7% binding to β-CGRP, <0.01% binding to calcitonin, and negligible binding to substance P, neurokinin A, or enkephalins by radioimmunoassay], and washed. Antibody binding was detected using biotin-conjugated second antibodies, a biotin-avidin-horseradish peroxidase complex (Vectastain ABC; Vector Laboratories), and development with diaminobenzidine. To stain for tyrosine hydroxylase or choline acetyltransferase (ChAT), cultures were fixed in PFA (and picric acid for ChAT) and incubated with a rabbit anti-tyrosine hydroxylase antibody (Eugene Technology) or a rat antiserum prepared against porcine ChAT (15) (which recognizes ChAT in the PNS; ref. 16), respectively, and binding was detected with fluoresceinated second antibodies.

RESULTS AND DISCUSSION

To examine the effect of LIF on neural crest cells, neural tubes were dissected from the cervical and thoracic region of E9 CBA mice and plated onto fibronectin-coated wells. Neural crest cells were allowed to migrate onto the substratum for 24 hr, at which time the neural tubes were either removed or left in place and LIF was added to the cultures. After 2 days, round cells with uni- or bipolar processes, resembling sensory neurons, appeared in the cultures. In the LIF-treated cultures, there were ≈12-fold more of these cells than in controls by 6 days (Fig. 1), and they formed large clusters that increased in size up to 14 days (Fig. 2*b*). This was not dependent on the presence of the neural tube during the culture period, although, in their absence, the absolute number of neuron-like cells was smaller (Fig. 1). These neuron-like cells stained positively with the Nissl stain (ref. 18; Fig. 2 *a* and *b*) and for 150-kDa neurofilament (ref. 19; Fig. 2 *c* and

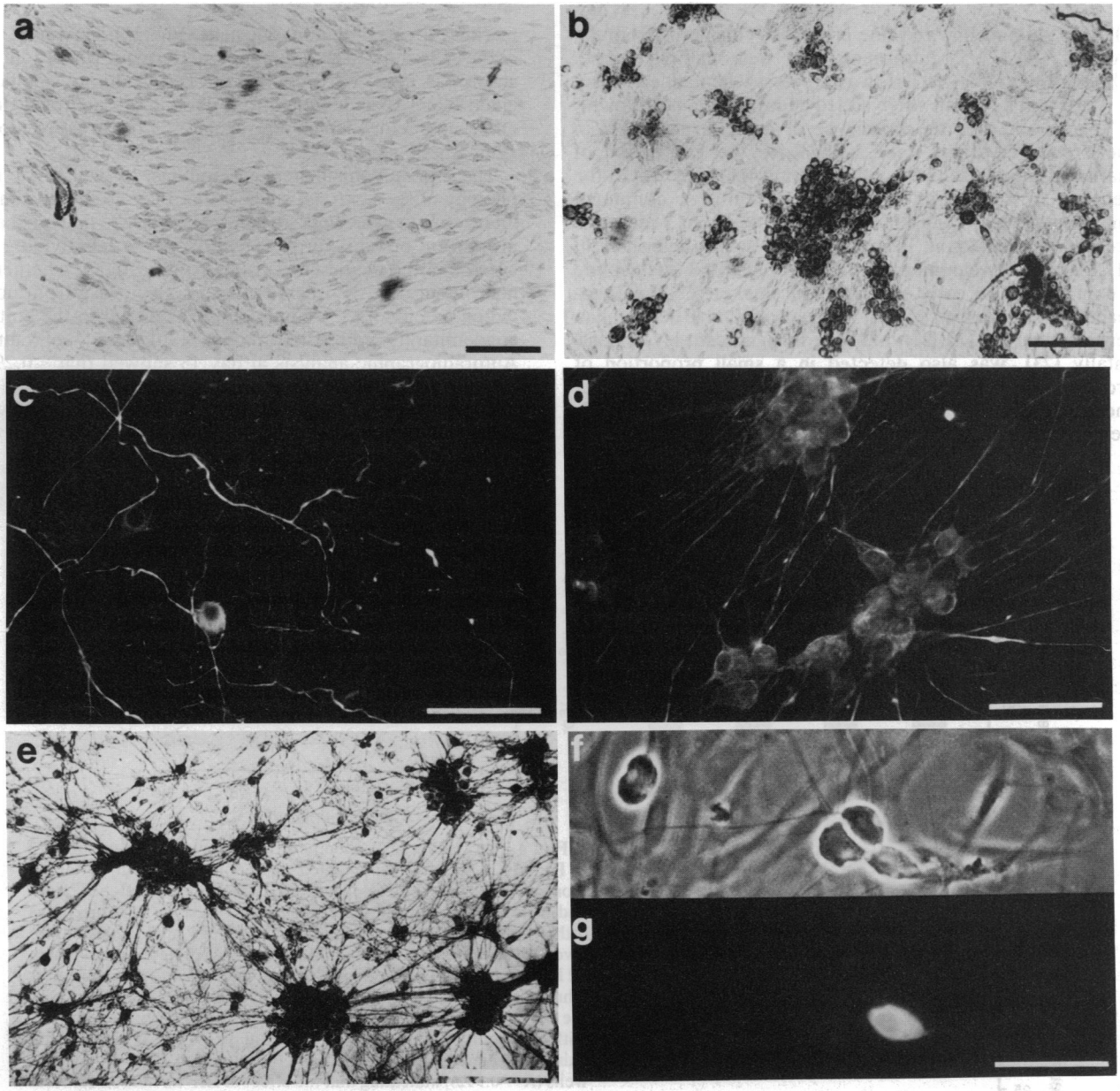

FIG. 2. Phenotype of neurons in neural crest cultures. Neural crest cultures were incubated for 13 days in the presence (*b*, *d*, and *e–g*) or absence (*a* and *c*) of LIF. (*a* and *b*) Bright-field views of Nissl-stained (18) cultures. (*c* and *d*) Fluorescence views of cultures stained for neurofilament. (*e*) Bright-field view of LIF-treated culture stained for CGRP. (*f*) Bright-field view of LIF-treated culture stained for tyrosine hydroxylase. (*g*) Fluorescence view of same field as in *f*. (*a* and *b*, bar = 200 μm; *c–g*, bar = 50 μm.)

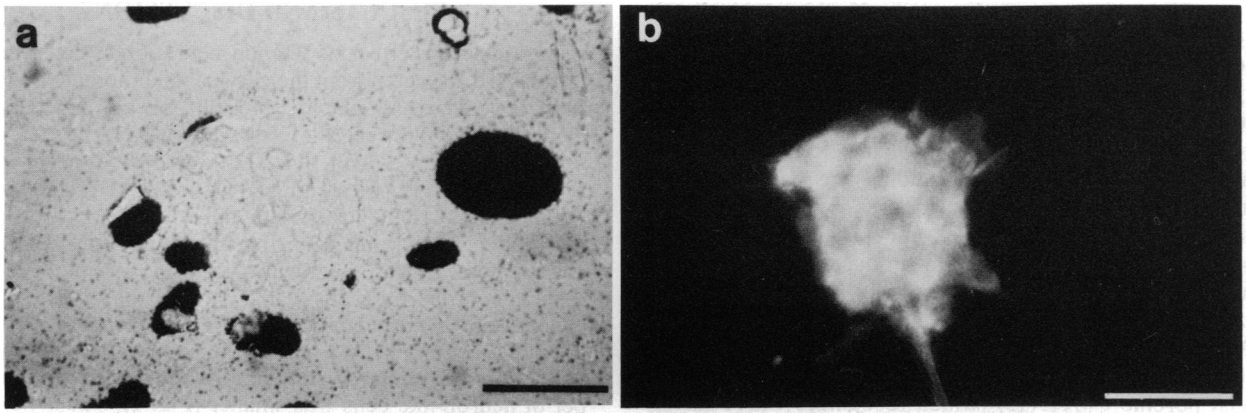

FIG. 3. [^{3}H]Thymidine incorporation into neural crest cultures. [^{3}H]Thymidine (0.03 μCi/ml; 1 Ci = 37 GBq) and LIF (10^4 units/ml) were added after 4 days of culture and incubation was continued for another 9 days, after which cultures were stained for neurofilament and autoradiographed (14). (*a*) Bright-field photomicrograph of culture. (*b*) Fluorescence view of same field. (Bar = 50 μm.)

d). This staining showed fine processes emanating from the clusters (Fig. 2*d*), confirming their neuronal phenotype. Although the effect of LIF was greatest when added at day 1, it was still apparent when added at day 7 (data not shown).

To characterize the phenotype of neurons generated in these cultures, they were stained for the expression of markers found in sensory and autonomic neurons. All of the neurons in LIF-treated and control cultures contained immunoreactivity for CGRP (Fig. 2*e*), the most widely expressed peptide found in mammalian sensory neurons (20, 21). Limited developmental studies suggest that this peptide is expressed quite early, at least in the chicken (22). Immunoreactivity for substance P, a peptide also found in mammalian sensory neurons (20, 21), but only in significant levels postnatally (23), was also detected in a small proportion of processes in LIF-treated and control cultures (data not shown). A small proportion (1–2%) of these neurons (LIF-treated and control) had tyrosine hydroxylase activity, a marker for catecholaminergic cells (Fig. 2*f*). However, none of the cells showed any immunoreactivity for ChAT (data not shown), a marker for cholinergic cells. This is significant, given that LIF is identical to a cholinergic neuronal differentiation factor, which up-regulates ChAT expression in sympathetic neurons (10).

These immunohistochemical findings, as well as the morphology of the neurons, suggest that they are in the sensory lineage. Previous work in aves has shown that at least a proportion of sensory neurons arise from nondividing precursors in the neural crest (1–3). To investigate whether the neurons in the LIF-treated cultures also arose from nondividing precursors, [^{3}H]thymidine was added to the cultures concomitantly with LIF at days 1, 4, and 7 of culture. Autoradiographic analysis at day 13 showed that <0.2% of the neurons (2 in 1100 neurons counted) that arose in the LIF cultures incorporated [^{3}H]thymidine (Fig. 3) irrespective of time of addition. These observations show that the increase

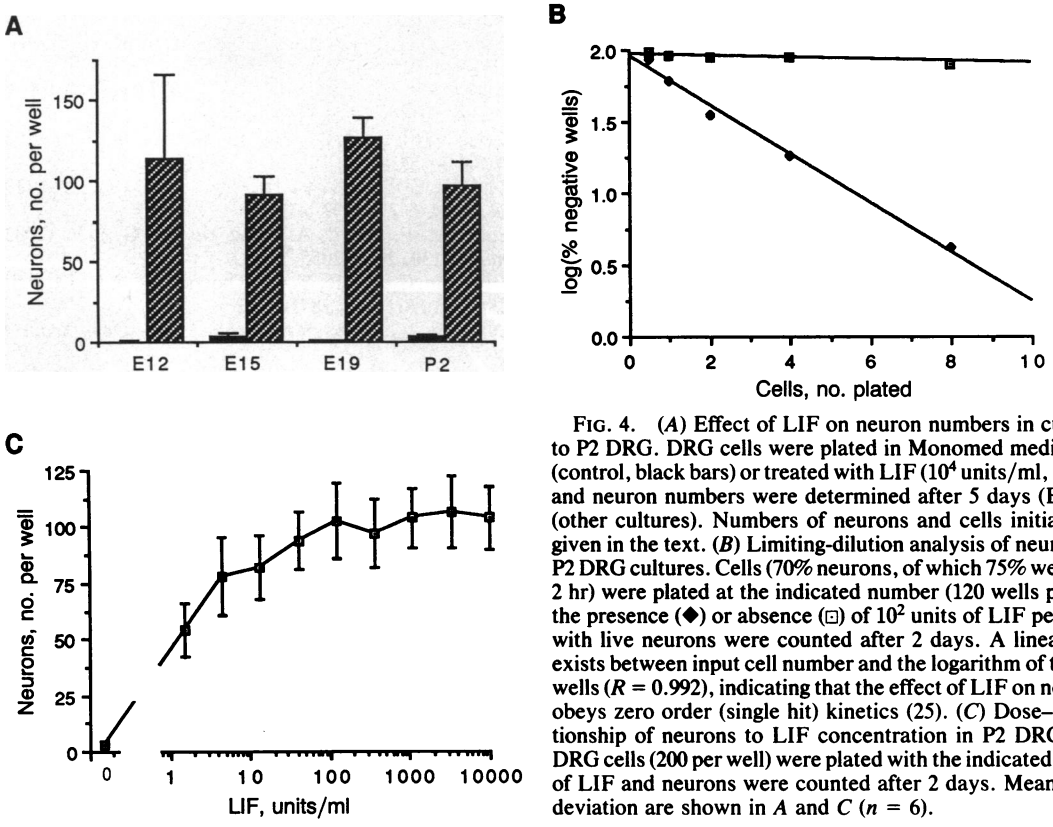

FIG. 4. (*A*) Effect of LIF on neuron numbers in cultures of E12 to P2 DRG. DRG cells were plated in Monomed medium/10% FBS (control, black bars) or treated with LIF (10^4 units/ml, hatched bars), and neuron numbers were determined after 5 days (E12) or 2 days (other cultures). Numbers of neurons and cells initially plated are given in the text. (*B*) Limiting-dilution analysis of neuron survival in P2 DRG cultures. Cells (70% neurons, of which 75% were plated after 2 hr) were plated at the indicated number (120 wells per dilution) in the presence (◆) or absence (□) of 10^2 units of LIF per ml and wells with live neurons were counted after 2 days. A linear relationship exists between input cell number and the logarithm of the % negative wells (R = 0.992), indicating that the effect of LIF on neuron survival obeys zero order (single hit) kinetics (25). (*C*) Dose–response relationship of neurons to LIF concentration in P2 DRG cultures. P2 DRG cells (200 per well) were plated with the indicated concentration of LIF and neurons were counted after 2 days. Mean and standard deviation are shown in *A* and *C* (n = 6).

in neuron numbers does not result from stimulation of precursor division. Most of the nonneuronal cells in these cultures were labeled with [^{3}H]thymidine (Fig. 3) but the presence of LIF made no significant difference to the total proportion of labeled cells: when LIF was added on day 1, 80% ± 18% of the cells were labeled compared with 78% ± 12% in control cultures, whereas at day 7, 70% ± 1% and 70% ± 6% of all cells were labeled in the presence and absence of LIF, respectively (n = 3).

As LIF stimulates an increase in sensory-like neurons in neural crest cultures, it was anticipated to have similar activity on early embryonic DRG cultures. Thus, single cell suspensions were made from E12 DRG, which contain a subpopulation of small, probably immature neurons as well as neuronal precursors (24), and were plated into wells of HL-A plates in the presence or absence of LIF. After 3 days clusters of neuron-like cells began to appear in the LIF-treated cultures but not in control cultures. After 5 days the cultures were stained for neurofilament and neurons were counted (Fig. 4*A*), showing that there were ≈100-fold more neurons in the LIF-treated cultures than in controls. Neurons were also present in cultures treated with nerve growth factor, but there were only about 10% of those seen in the LIF-treated cultures after 5 days (data not shown). Experiments on DRG cells isolated later in development (E15, E19, P2) showed a high proportion (80–100%) of neurons survived after 2 days in the presence of LIF (Fig. 4*A*).

Limiting-dilution experiments indicate that LIF acts directly on the neurons, as the rate of survival is not influenced by cell number (Fig. 4*B*). In addition, a LIF titration on the P2 DRG showed maximal activity over 10^2 units/ml and 50% activity at ≈1.5 units/ml (Fig. 4*C*), which is comparable to that observed with other neurotrophic factors (5–7).

These results indicate that LIF can act throughout embryonic sensory neuron development *in vitro*. In neural crest cultures, it may act to stimulate neuronal differentiation and/or survival of the sensory precursors. Consistent with this, we find a subpopulation of neural crest cells specifically binds ^{125}I-labeled LIF (^{125}I-LIF), indicating that they have LIF receptors (data not shown). Others have implicated brain-derived neurotrophic factor (BDNF) in the survival and/or differentiation of developing DRG cells (26, 27). One possibility is that LIF, which is produced by mesoderm-derived cells *in vitro* (17), may be produced in peripheral tissue *in vivo* and act in concert with the central nervous system-derived BDNF in the development of the DRG.

The actions of LIF on the older DRG cultures show it to be a neurotrophic factor for sensory neurons *in vitro*, like NGF. We have recently injected ^{125}I-LIF into the footpad of neonatal mice and shown retrograde transport into DRG neurons (I. Hendry, M.M., and P.F.B., unpublished observations), suggesting that LIF can act by similar mechanisms to NGF *in vivo* (28). Further experiments are necessary to clarify the relative roles of LIF and NGF in sensory neuron development.

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