In Situ Hybridization Histochemistry Localization of Interleukin-3 mRNA in Mouse Brain

By William L. Farrar, Michelle Vinocour, and Joanna M. Hill

The hematopoietic growth factor interleukin-3 (IL-3) promotes the proliferation and maturation of pluripotent myeloid progenitor cells. In the immune system, IL-3 is synthesized by mitogen or antigen-stimulated T lymphocytes. We demonstrate the expression of IL-3 mRNA in mouse brain by in situ hybridization histochemistry and Northern blot analysis. The IL-3 mRNA is localized in discrete areas of the brain and can be found in neuronal cell body and astrocytes. Northern analysis of cerebellar RNA, compared with mRNA extracted from WEHI-3 cells, showed a single hybridization band, approximately 1.2 kb, suggesting similar processing between brain and myeloid cells. The molecular evidence and previous observations of IL-3-like biologic activity found in the brain suggest a potential role for IL-3 in the neurobiology of the CNS. © 1989 by Grune & Stratton, Inc.

A large body of evidence supports a role for soluble polypeptide mediators, collectively referred to as colony-stimulating factors (CSFs) in the proliferation and differentiation of hematopoietic progenitor cells. One member of this family of hematopoietic regulatory proteins is multi-CSF or interleukin-3 (IL-3). IL-3 was initially described as a growth factor for cells of the pre-helper T lymphocyte lineage,1 and was in fact shown to be a multipotent colony stimulating factor that controls the growth and differentiation of pluripotent progenitor cells.2,3 Molecular cloning of murine IL-3 revealed a cDNA encoding a 28,000 dalton glycoprotein that stimulates the production of all the major myeloid cell types.4 Recently, molecular cloning of the gibbon IL-3-like molecule revealed a cDNA with limited homology to the murine gene showing greatest percent identity in the noncoding introns and exon organization.5 Primate IL-3 stimulates some, but not all, of the multiple spectrum of myeloid cell differentiation as the murine molecule.6

Synthesized by mitogen or antigen-stimulated T lymphocytes,4,10 the biologic action of IL-3 has been studied almost exclusively with cells of precursor myeloid or hematopoietic origins. Recently, Frei et al11 reported that astrocytes cultured from brains of newborn mice released a biological activity, when stimulated with endotoxin or phytohemagglutinin, which stimulated the growth of the IL-3-dependent murine myeloid clone 32DCL. Gel chromatography of the astrocyte culture supernatant revealed a 33,000 dalton activity which, in addition to promoting the growth of IL-3-dependent cell lines, induced the enzyme 20a-steroid dehydrogenase (20a-SDH) in nu/nu spleen cells and the growth of peritoneal macrophages.12 The activity found in astrocyte cultures demonstrated the same biochemical and biologic properties as T lymphocyte-derived IL-3.

We have used in situ hybridization histochemistry and Northern blot analysis to characterize the presence of IL-3 mRNA in mouse brain using a cDNA probe cloned from T lymphocytes. The data show that mouse cerebellum expresses of IL-3 mRNA of a 1.2 kilobase size consistent with that found in lymphocytes or the WEHI-3 cell line. Furthermore, IL-3 mRNA is localized at discrete areas of brain associated with the cytoarchitecture of the region.

**MATERIALS AND METHODS**

Reagents. All reagents unless specified were reagent grade purchased from Sigma Chemical Co, St Louis. Other reagents purchased included formamide (Fluka Chemical Corp, Hauppauge, NY), formaldehyde (Fisher Scientific, Fair Lawn, NJ), a[32P]-dCTP (New England Nuclear, Boston), polymyrid, (P & S Biochemicals, Gaithersburg, MD), salmon sperm DNA (Calbiochem, San Diego, CA), dextran sulfate (Onco, Gaithersburg, MD), nitrocellulose (Schleicher and Shuell, Keene, NH), agarose and cesium chloride (Bethesda Research Labs, Gaithersburg, MD), and [35S]-dCTP (Amerham, Arlington Heights, IL).

RNA preparation and hybridization. Mouse cerebellum was isolated immediately after the brains were removed, guanidine thiocyanate was added (25 mL/g tissue), disrupted with a Brinkman homogenizer, and stored at -80°C until use. Samples were thawed and layered over a cushion of 5.7 mol/L CsCl (25 mL sample/15 mL CsCl) and centrifuged at 20,000 g for 18 hours. The protein and DNA layers were carefully removed, and the RNA pellet resuspended in 300 µL sterile water containing 0.2% sodium dodecyl sulfate (SDS). The RNA was heated at 65°C for 15 minutes, and insoluble material (lipid) was removed by centrifugation in a microfuge. To the purified total RNA were added 50 µL of 3.0 mol/L sodium acetate, the RNA was extracted once with phenol/chloroform (1:1), once with chloroform, and precipitated with 2.5 vol of 100% ethanol. RNA samples were resuspended in sterile water, and RNA concentrations were determined by densitometer readings at 260 nm. Control RNA was isolated as described previously from WEHI-3 cells. Total RNA (20 µg) was denatured and electrophoresed on a 1% agarose formaldehyde gel according to Lehrach et al13 and modified by using 0.22 mol/L formaldehyde in the gel at 120 V for three to four hours. Northern transfer was performed as described by Thomas.14 Hybridization was performed at 42°C in...
10% dextran sulfate/50% formamide/4 x SSC with $^{32}$P-labeled restriction fragment probe. Washes were performed at 52°C with 10% dextran sulfate/0.1 x SSC.

**Cloned DNA fragments.** IL-3 probe was obtained from a cDNA plasmid (a gift from Dr. Nicola, Weiz Eliza Hall, Inst., Australia). An EcoRI digestion yielded a 1.1 kb fragment insert. Control plasmid was PBR322 (without insert). Labeling with $^{32}$P-dCTP for Northern analysis (New England Nuclear, Wilmington, DE) or with $^{35}$S-dCTP for in situ analysis (Amersham, Arlington Heights, IL), was accomplished by primer-extension (Polymeraid, P + S Biochemicals) to a specific activity of $5 \times 10^{6}$ to $1 \times 10^{8}$ cpm/μg.

**In situ hybridization histochemistry.** NIH Swiss mice were decapitated and the brains rapidly removed from the skull. Brains were briefly immersed in 2-methylbutane at -40°C, patted dry with tissue, and frozen on powdered dry ice. The fresh frozen brains were cut coronally at 12 μm on a cryostat, thaw mounted (two sections per slide) onto twice subbed (gelatin-chrome alum coated) microscope slides, and dried for two minutes on a 28 to 30°C warming tray. The slides were then kept frozen at -20°C until use.

The in situ hybridization technique was performed as described by Young et al. Before hybridization was initiated the sections were brought to room temperature, fixed in 4% formaldehyde in phosphate buffered saline (PBS) for five minutes at room temperature, dehydrated in a series of graded alcohols, and defatted with chloroform.

After drying, 45 μL of the hybridization buffer was pipetted over the sections that were then covered with a parafilm coverslip and incubated overnight at 38°C. Hybridization buffer was composed of 50% formamide, 4 x SSC, 10 mmol/L DTT, 500 μg/mL sheared single stranded DNA, 250 μg yeast tRNA, 1 x Denhardt’s, 10% dextran sulfate, and approximately $1 \times 10^6$ DPM of the interleukin-1 (IL-1) probe in 45 μL of hybridization buffer.

The following day the coverslips were removed and the sections were rinsed in four, 15-minute washes of 2 x SSC/50% formamide at 40°C, two one-hour washes in 1 x SSC at room temperature, patted dry in water, and rapidly dried in a stream of cool air. The dried sections were exposed to LKB (Uppsala, Sweden) ultrafilm for three to six days after which they were dipped into NTB3 nuclear emulsion and exposed for seven to ten days. After exposure, both film and emulsion-coated slides were developed in D19. The slides were then stained with thionin, dehydrated, cleared, and cover-slipped.

Controls were included with the in situ procedure. First, PBR322 plasmid probe less insert was labeled in a similar manner as insert and was hybridized to adjacent brain sections. Second, brain tissue sections were pretreated with 120 ng RNase for one hour at room temperature before hybridization.

**RESULTS AND DISCUSSION**

Autoradiography emerged in the late 1970s as a powerful method to examine the precise localization of specific receptors for neurotransmitters and drugs in brain. Such procedures have allowed us to examine the distribution of receptors for the monokine IL-1 in the brain, as well as receptors for radiolabeled IL-3. In situ hybridization histochemistry allows the annealing of complementary nucleotide sequences to mRNA in intact fixed tissues. Such specific hybridization in brain has previously localized corticotropin releasing factor mRNA as well as protein kinase C (PK-C) mRNA by methods used in this report. Using $^{35}$S-dCTP labeled plasmid insert cDNA for mouse IL-3, we studied the localization of IL-3 mRNA in coronal sections of mouse brain.

IL-3 mRNA is located in neurons and glia in wide regions of the mouse brain. At the level of the thalamus (Fig 1A), hybridization grains were especially evident in the medial habenula (MH) and in cells of the neuron-dense regions of the brain such as the granule cell layers of the olfactory bulb, hippocampal formation (H), and cortex (Ctx). The pyramidal cell layers of the olfactory tubercle, piriform cortex (PC), entorhinal and cingulate cortex (CC) also have abundant IL-3 mRNA positive cells. A corresponding thalamus section pre-treated with RNase did not hybridize to labelled insert (Fig 1B) nor did labeled plasmid reveal any specific hybridization (not shown). At midbrain (Fig 1C) the cells of the subiculum (Su) have very dense accumulations of grains. Moderate levels of grains were seen over cells in the Ctx, especially in the superficial and deep layers. Lower clustering of grains were seen in the striatum (S) of the thalamus (Fig 1A) and hypothalamus and mammary nuclei (MN) of midbrain (Fig 1C). Inferior colliculus and medulla (Fig 1D) showed substantial hybridization in cells in the pontine nuclei (PN) and Ctx. Microscopic evaluation of hippocampal neurons showed clustering of grains associated with the neuronal cell body (Fig 2). Cells in the deep cortical layers showed little hybridization and the grains randomly distrib-

![Fig 1.](image-url)
IL-3 IN THE BRAIN

Fig 2. Microscopic analysis of emulsion dipped hippocampal sections. (A) Cells in the deep cortical layers; (B) cells of the hippocampal formation.

uted throughout the field of view (Fig 2a). Cells of the Hippocampus showed significant grain clustering with neurons (Fig 2b). Cultured astrocytes also were positive with IL-3 cDNA probe (data not shown).

The areas of brain observed here are uniformly vascular. Therefore, hybridization to perivascular mononuclear cells would be in a diffuse pattern at all levels of brain. Two additional facts also exclude this possibility. Unstimulated lymphocytes or cells of monocytic lineage do not synthesize IL-3 mRNA and second, the microscopic analysis of hybridization grains found them to be associated with neuronal cell bodies (Fig 2b).

Total RNA was extracted from the murine cell line WEHI-3, which constitutively produces IL-3, and from freshly isolated mouse cerebellum. Northern blot analysis of IL-3 mRNA obtained from brain and the myeloleukemic cell line is shown in Fig 3. A clear 1.2 kb hybridization band is visible from RNA obtained from the WEHI-3 cell line and the same size band was found in cerebellum tissue. Exposure rates differed in the respective RNA sources since WEHI-3 produces IL-3 in large quantities, whereas cerebellum is a heterogeneous tissue. The Northern analysis confirmed the presence of IL-3 mRNA seen in brain with in situ hybridization histochemistry and suggested a message size consistent with IL-3 derived from immune or hematologic sources.

We have described the presence of IL-3 mRNA in mouse brain through in situ and Northern molecular hybridization analysis. IL-3 mRNA is present in neurons and astrocytes and not blood-borne cells of the vasculature. First, areas of brain in which discrete hybridization occurred are diffusely vascularized and the clustering of hybridization occurs primarily in cells associated with the neuroarchitecture of the region and counter stain with Nissl, a stain which delineates cell body. Second, in the immune and hematologic tissues, IL-3 is an inducible gene. In the brain, IL-3 mRNA is regulated without apparent immune stimulus. Finally, microscopic analysis of hybridization grains revealed clustering associated with neuronal cell bodies.

IL-3-like biochemical and biologic activity has been found in mouse brain astrocytes.11,12 We have confirmed that a similar transcript is seen in cerebellum tissue (Fig 3). Brain-derived IL-3 can stimulate the growth of macrophages and their representatives in brain, the microglia cells. IL-3 mRNA is also found in neurons, although a biologic role has not been tested. The role of IL-3, albeit unexplored, in brain neurobiology, development and behavior may not be unique to IL-3 as a representative of the family of CSFs.

We have shown the presence of IL-1 receptors in the brain as well as in IL-1β mRNA.16,17 We could not find evidence for IL-1α mRNA and found expression of only IL-1β mRNA.19 Like IL-3, IL-1 biologic activity has been seen in the brain and has promoted astrocyte growth.20 These observations have been supported by the recent findings of Breder et al21 who found evidence for IL-1β protein in human neurons but no expression of IL-1α protein in any areas of the brain. As an authentic monoclonal antibody against murine IL-3 become available, it will be of interest to examine whether the protein is co-expressed with mRNA. Among the many biologic effects of IL-1 studied, IL-1 has also been shown to be like hematopoietin 1, a stimulator of early progenitor cells.22 Biologically, IL-1 has been shown to synergize with IL-3 in the proliferation of multi-potent progenitors.23 In the brain, IL-3 mRNA was observed in brain areas where IL-1β mRNA and IL-1 receptors have also been localized.16,19 These are unique messages since no exon homology exists between IL-1 and IL-3 and distinct mRNA sizes are also found.

The impact of IL-3 or IL-1 on the neurobiology and pathobiology of brain as well on behavior is relatively unknown. Although IL-1 has been shown to modulate fever
and slow-wave sleep, no such activities have been reported for IL-3. The role of IL-1 in the brain, however, appears to have substantially different biologic and physiologic effects than those involved in immune and hematopoietic regulation. The finding of IL-3 in the brain would also suggest a physiologic role that may significantly differ from data and interpretations obtained from hematopoietic tissues. The physiologic regulation of IL-3 synthesis in the brain is unknown. For instance, if changes in IL-3 synthesis levels are altered during environmental or immune stress, this may indicate some level of regulation. Otherwise, constitutive synthesis may indicate some vital function of the molecule. Our initial observations may provide a basis for future studies. It is clear that substantial biochemical biologic, and molecular evidence exists that indicate that immune and hematopoietic regulatory molecules are present in neurologic tissues. These observations must now significantly broaden the potential role of these regulatory molecules in the physiology of the host and our concepts of the biologic function of these cytokine proteins.

REFERENCES

In situ hybridization histochemistry localization of interleukin-3 mRNA in mouse brain

WL Farrar, M Vinocour and JM Hill