Interferon γ Induces Retrograde Dendritic Retraction and Inhibits Synapse Formation

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The expression of interferon γ (IFN γ) increases after neural injury, and it is sustained in chronic inflammatory conditions such as multiple sclerosis and infection with human immunodeficiency virus. To understand how exposure to this proinflammatory cytokine might affect neural function, we examined its effects on cultures of neurons derived from the central and peripheral nervous systems. IFN γ inhibits initial dendritic outgrowth in cultures of embryonic rat sympathetic and hippocampal neurons, and this inhibitory effect on process growth is associated with a decrease in the rate of synapse formation. In addition, in older cultures of sympathetic neurons, IFN γ also selectively induces retraction of existing dendrites, ultimately leading to an 88% decrease in the size of the arbor. Dendritic retraction induced by IFN γ represents a specific cellular response because it occurs without affecting axonal outgrowth or cell survival, and it

is not observed with tumor necrosis factor α or other inflammatory cytokines. IFN γ -induced dendritic retraction is associated with the phosphorylation and nuclear translocation of signal transducer and activator of transcription 1 (STAT1), and expression of a dominant-negative STAT1 construct attenuates the inhibitory effect of IFN γ . Moreover, retrograde dendritic retraction is observed when distal axons are selectively exposed to IFN γ . These data imply that IFN γ -mediated STAT1 activation induces both dendritic atrophy and synaptic loss and that this occurs both at the sites of IFN γ release and at remote loci. Regressive actions of IFN γ on dendrites may contribute to the neuropathology of inflammatory diseases.

Key words: interferon γ ; bone morphogenetic protein-7; dendrites; sympathetic neurons; hippocampal neurons; STAT1; retrograde transport

Interferon γ (IFN γ) is a proinflammatory cytokine that potently stimulates cell-mediated immunity (Farrar and Schreiber, 1993; Bach et al., 1997; Boehm et al., 1997). IFN γ is made primarily by T-lymphocytes and natural killer cells; because the entry of these cells into the CNS is minimal under normal circumstances, IFN γ is usually not detectable in the brain (Traugott and Lebon, 1988; Fabry et al., 1994). However, expression of IFN γ rises rapidly in acute inflammatory reactions caused by trauma (Lau and Yu, 2001), ischemia (Li et al., 2001), or viral infection (Binder and Griffin, 2001), and elevated expression persists in chronic inflammatory diseases such as multiple sclerosis (MS) (Panitch, 1992; Popko et al., 1997) and in chronic viral infection such as that produced by human immunodeficiency virus (HIV) (Fuchs et al., 1991; Fan et al., 1993). Levels of IFN γ also are increased in murine trisomy 16, which is considered a model for Down's syndrome (Torre et al., 1995; Hallam et al., 2000). Overexpression of IFN γ causes abnormal cerebellar and hippocampal development in transgenic mice (Corbin et al., 1996; LaFerla et al., 2000), and administration of IFN γ to MS patients aggravates the disease (Panitch et al., 1987). Thus there is reason to suspect that the elevated levels of IFN γ that are associated with inflammatory

neural conditions may contribute to their pathology. However, evaluation of this hypothesis is impeded by a lack of knowledge of the direct effects of IFN γ on neurons, and, in fact, previous studies have noted primarily beneficial effects of IFN γ on the survival (Chang et al., 1990), growth (Erkman et al., 1989), and differentiation of isolated neurons (Barish et al., 1991; Jonakait et al., 1994).

Dendrites are the primary site of synapse formation in the nervous system (Purves, 1988), and dendritic atrophy has been observed in degenerative conditions such as Alzheimer's disease (Flood and Coleman, 1990) and Parkinson's disease (Patt et al., 1991), in Down's syndrome (Takashima et al., 1989), in chronic viral infections (Masliah et al., 1997; Li et al., 1999), and as part of the reaction to acute traumatic injury (Sumner and Watson, 1971; Yawo, 1987). It is, therefore, important to characterize molecules that cause regression of these processes. Previous studies have shown that leukemia inhibitory factor (LIF), ciliary neurotrophic factor, and other members of the interleukin-6 (IL-6) cytokine family induce dendritic retraction in cultured sympathetic neurons (Guo et al., 1999) and that overexpression of LIF causes dendritic atrophy in Purkinje cells (Morikawa et al., 2000). In this study we consider the possibility that IFN γ shares with IL-6-related cytokines the ability to interfere with the growth of dendritic processes. In addition, we have compared the signal transduction pathways involved in this action.

Our data indicate that IFN γ adversely affects dendritic maturation and synapse formation and that it acts by a pathway distinct from that used by members of the IL-6 family, suggesting possible synergistic interaction during inflammation. Moreover, we have found that the regressive signals generated by IFN γ can be

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retrogradely transported from distal axons to neural somata. This retrograde dystrophic interaction represents a novel method for conveying information about local injury or inflammation to distant brain loci.

MATERIALS AND METHODS

Materials. Recombinant rat IFN γ , interleukin 1- β (IL-1 β), and human tumor necrosis factor α (TNF α) were purchased from PeproTech (Rocky Hill, NJ). Rat IFN β was obtained from PBL Biomedical Laboratory (New Brunswick, NJ). Recombinant human bone morphogenetic protein-2 (BMP-2), BMP-6, and BMP-7 were generously provided by Curis (Cambridge, MA).

Cell culture. Superior cervical ganglia dissected from perinatal [embryonic day 21 (E21) or postnatal day 1–2 (P1–2)] Holtzman rats (Harlan Sprague Dawley, Rockford, IL) were dissociated after treatment with trypsin (2.5 mg/ml) and collagenase (1 mg/ml) for 40 min. Cells were plated onto poly-D-lysine-coated (100 μ g/ml) coverslips or compartmented chambers (see below) and maintained in serum-free medium containing β -nerve growth factor (NGF; 100 ng/ml) (Higgins et al., 1991). Then 1–2 d later, cytosine- β -D-arabinofuranoside (1 μ M) was added in the media for 2 d to kill non-neuronal cells. Subsequently, the cultures were allowed to recover for 1 d and experimental treatments were begun on the sixth or seventh day *in vitro*.

Embryonic hippocampal neurons (E18) were isolated by the method of Goslin et al. (1998) with the modifications of Bading and Greenberg (1991). Briefly, hippocampi were dissociated by treatment with trypsin (2 mg/ml) and deoxyribonuclease (0.6 mg/ml) for 2 min and then exposed to soybean trypsin inhibitor (1 mg/ml) for 10 min. Cells were plated onto poly-D-lysine (100 μ g/ml) and laminin (7 μ g/ml) and maintained in Neurobasal medium with B27 supplements (Invitrogen, San Diego, CA) (Brewer et al., 1993).

Compartmented cultures. Compartmented cultures (Campenot chambers) were set up as described by Senger and Campenot (1997). Briefly, 35 mm dishes were precoated with a layer of ammoniated rat tail collagen, followed by a layer of air-dried rat tail collagen supplemented with laminin (10 μ g/ml). Three-compartmented Teflon dividers (Camp 10, Tyler Research Instruments, Edmonton, Alberta, Canada) were seated on top of parallel tracks scratched in the collagen substrate with a pin rake (Tyler Research Instruments) and secured in place with silicone vacuum grease (Dow Corning, Huntington Beach, CA). Integrity of the grease seals was assessed by placing culture medium into the side chambers only and incubating the chambers overnight in a 37°C incubator. Cells were plated only into chambers that did not leak. Medium was removed from side compartments, and dissociated cells were plated in the center compartment in serum-free culture medium containing 100 ng/ml NGF. The next day the integrity of the seals between the compartments was reconfirmed by checking for leakage from the center into side compartments. Culture medium was then added to the side compartments of those cultures exhibiting intact seals between compartments, and cytosine- β -D-arabinofuranoside (1 μ M) was added to the medium in the center compartment to eliminate non-neuronal cells. After axons had extended through the grease seals into the side compartments (5–7 d after plating), NGF was withdrawn from the center compartment but was maintained in the side compartment to encourage growth of neurites into the side compartments and to eliminate neurons that had failed to extend axons into side compartments. BMP-7 (10 ng/ml) was added to medium in the center compartment when extensive axonal growth was evident in side compartments (within 10–12 d after plating); 4 d later, IFN γ (10 or 50 ng/ml) was added to medium in the right side compartment of a subset of cultures.

Morphological analyses. Cellular morphology was analyzed using previously described immunocytochemical methods (Lein et al., 1995). Briefly, cells were fixed with 4% paraformaldehyde (15 min at 20°C) and permeabilized with 0.1% Triton X-100 for 3 min. Cells were reacted with monoclonal antibodies (mAb) that recognize axons or dendrites and then with rhodamine-conjugated secondary antisera (Roche Diagnosis, Indianapolis, IN). Monoclonal antibodies to microtubule-associated protein-2 (MAP-2, SMI-52; Sternberger Immunocytochemicals, Baltimore, MD) or to nonphosphorylated forms of H and M neurofilament subunits (SMI-32; Sternberger Immunocytochemicals) were used to visualize dendrites. Previous studies have shown that immunostaining with these antibodies reveals the entire dendritic arbor (Lein et al., 1995; Bruckenstein and Higgins, 1988b). Total dendritic length was measured with SPOT software (Diagnostic Instruments, Sterling Heights, MI). A

mAb to phosphorylated forms of the H and M neurofilament subunits (SMI-31; Sternberger Immunocytochemicals) was used to identify the axons of sympathetic neurons (Osterhout et al., 1992; Bruckenstein and Higgins, 1988a).

To examine synapse formation, hippocampal neurons were plated at high density ($\sim$ 16,000/cm²) to increase the frequency of cellular contacts (Fletcher et al., 1994). After 5 d of treatment with IFN γ (30 ng/ml), the synaptic specializations that formed along dendrites were visualized by double-labeling the cultures with rabbit anti-MAP-2 IgG (a gift from Dr. Craig Garner, University of Alabama at Birmingham, Birmingham, AL) and mAb to synaptic vesicle protein-2 (SV-2; Buckley and Kelly, 1985) and then with rhodamine-conjugated antibody to rabbit IgG (Roche Diagnosis) and fluorescein-conjugated antibody to mouse IgG (Roche Diagnosis).

Cultures were also immunostained with rabbit IgG that specifically recognizes a phosphorylated form (Tyr 701) of signal transducer and activator of transcription 1 (STAT1; New England Biolabs, Beverly, MA). Fixation and permeabilization were performed according to the manufacturer's protocol.

Each experiment was performed at least three times, and data in the text and in figures are presented as the mean $\pm$ SEM. Statistical significance was assessed with a one-way ANOVA, followed by Tukey's *post hoc* test.

Western blotting. Cultures were solubilized in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1% SDS, and 2.0% β -mercaptoethanol. After centrifugation at 15,000 $\times$ g, the protein concentration was determined with the Bio-Rad protein assay (Hercules, CA). Proteins (10 μ g/lane) were separated by SDS-PAGE (7.5%) and then transferred to nitrocellulose membrane where they were probed with mAb to tau (Tau-2; Sigma, St. Louis, MO) or a rabbit anti-actin IgG fraction (a gift from Dr. John Kolega, State University of New York, Buffalo, NY). Next the membrane was reacted with an appropriate horseradish peroxidase-conjugated secondary antibody (Amersham, Piscataway, NJ), and bands were visualized with enhanced chemiluminescent reagent (Amersham).

Transfection. Cells were cotransfected with a plasmid coding the enhanced green fluorescent protein (pEGFP-N2, Clontech, Palo Alto, CA) and the expression vector pCAGGS containing HA-STAT1WT (hemagglutinin-tagged wild-type STAT1 gene), HA-STAT3WT, HA-STAT1F (Phe substitution for Tyr701), or HA-STAT3F (Phe substitution for Tyr 705) (Nakajima et al., 1996). Plasmids containing STAT1 and STAT3 were a generous gift from Dr. Toshio Hirano (Osaka University Medical School, Osaka, Japan). Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Briefly, cells were treated with 200 μ l of DMEM containing 1 μ g of DNA and 4 μ g of Lipofectamine. After incubation for 6 hr cells were washed and allowed to recover for 48 hr before experimental treatments were begun.

RESULTS

IFN γ inhibits initial dendritic outgrowth induced by BMP-7 in sympathetic neurons

Embryonic rat sympathetic neurons extend only axons (Fig. 1) when grown in serum-free medium in the absence of non-neuronal cells (Bruckenstein and Higgins, 1988a). In contrast, exposure to BMP-7, which is produced by glial cells within sympathetic ganglia (H. Beck and P. Lein, unpublished observation), causes them to also form dendrites (Fig. 1). With moderate doses (5 ng/ml) of BMP-7, neurons typically extend two to three dendrites within the first week of treatment (Fig. 2). With maximally effective doses ($\geq$ 50 ng/ml) there is a greater increase in both the number of dendrites (Fig. 3) and total process length, and the neurons eventually form an arbor that is equivalent in size to that observed *in vivo* (Lein et al., 1995).

IFN γ did not affect the morphological development of sympathetic neurons grown under control conditions (Fig. 1). However, it profoundly depressed the initial dendritic outgrowth that occurs in the presence of BMP-7 (Fig. 1). In cultures that were treated with a maximally effective dose of BMP-7 (50 ng/ml), there was a 53% decrease in the number of dendrites per cell in the presence of IFN γ (Fig. 3). In cultures treated with lower doses of

Figure 1. Effect of BMP-7 and IFN γ on the morphology of cultured sympathetic neurons. Shown are phase-contrast (*A, C, E, G*) and fluorescence (*B, D, F, H*) micrographs of neurons immunostained with a monoclonal antibody (mAb) to MAP-2 after 5 d of treatment. Neurons grown in control medium lacked dendrites (*A, B*). Exposure to BMP-7 (5 ng/ml) caused the neurons to form multiple dendrites (*C, D*). IFN γ (10 ng/ml) alone had no effect on dendritic outgrowth (*E, F*), but it inhibited the growth of these processes in cultures that were exposed to BMP-7 (*G, H*). Scale bar, 30 μ m.

BMP-7 (5 ng/ml), the effect was even greater (78% inhibition). Under these conditions total dendritic length also was reduced (Fig. 2). Inhibition was observed with concentrations of IFN γ as low as 0.1 ng/ml; the ID₅₀ (dose causing half-maximal inhibition) was $\sim$ 1 ng/ml (Fig. 3). IFN γ -induced inhibition of initial dendritic growth was long lasting, with no sign of reversal even after 2 weeks of continuous exposure (Fig. 2), and IFN γ also blocked the effects of BMP-2 and BMP-6 (data not shown), suggesting that it interacts with a common component of the BMP signaling pathway.

IFN γ inhibits synapse formation and dendritic growth in hippocampal neurons

To determine whether IFN γ also inhibits dendritic growth in CNS neurons, we examined its effects on cultured embryonic hippocampal neurons. These neurons differ from sympathetic neurons in that they spontaneously form dendrites in the absence

Figure 2. IFN γ inhibits BMP-7-induced dendritic outgrowth. Sympathetic neurons were treated with BMP-7 (5 ng/ml), IFN γ (10 ng/ml), or BMP-7 and IFN γ . On days 8 and 15, cellular morphology ($n \geq 60$ cells/condition) was visualized by immunostaining with mAb to MAP-2. *A*, Number of dendrites per cell. *B*, Total dendritic length. *C*, Number of MAP-2-positive cells per culture. * $p < 0.05$ versus BMP-7.

of exogenous BMPs. Exposure to IFN γ reduced hippocampal dendritic growth by 36% (Fig. 4*B*). In addition, treatment with IFN γ caused a 37% decrease in the number of SV-2-positive (Fig. 4*C*) and synaptophysin-positive (data not shown) varicosities associated with MAP-2-positive dendrites. Thus in CNS neurons the IFN γ -induced inhibition of dendritic growth is associated with a proportionate decrease in the formation of synapses.

IFN γ induces retraction of existing dendritic process

In addition to examining the effects of IFN γ on initial process outgrowth, we also wanted to know whether it affected existing processes. Therefore, dendritic growth was initially induced in cultures of sympathetic neurons by a 5 d exposure to BMP-7; this was followed by treatment for 6 d with either BMP-7 alone or BMP-7 with IFN γ (Fig. 5). In cells treated with an intermediate

Figure 3. Dose-dependent inhibition of dendritic outgrowth by IFN γ . The effects of varying concentrations of IFN γ were assessed in cultures treated with either a maximally effective dose of BMP-7 (50 ng/ml; open circles) or one close to the ED₅₀ (5 ng/ml; open triangles). After 5 d of cotreatment with BMP-7 and IFN γ cultures were immunostained with mAb to MAP-2.

dose of BMP-7 (5 ng/ml), IFN γ caused an ~90% decrease in both the number of dendrites per cell and in total dendritic length. Dendritic retraction was also prominent in cultures exposed to a maximally effective dose of BMP-7, with ~80% of the processes being eliminated in the presence of IFN γ . Thus the effect of IFN γ on dendritic retraction is at least as great as that on initial extension. Time course studies revealed that dendritic retraction was a slow process that began 24–48 hr after initial IFN γ exposure and required at least 4 d for completion (data not shown).

IFN γ does not affect axonal growth or cell number

To assess the specificity of action of IFN γ , we first examined its effect on the survival of sympathetic neurons. In all experiments neuronal number remained essentially constant (Figs. 2, 5), indicating that IFN γ was not acting by promoting the survival of a subpopulation of neurons. To assess specificity further, we examined the effects of IFN γ on axonal growth. To allow induction of IFN γ -sensitive genes, explants of sympathetic ganglion were initially maintained for 2 d in the presence or absence of IFN γ . After this pretreatment ganglia were dissociated, and the cells were plated onto coverslips; treatment with either control medium or IFN γ was resumed. Axonal length was assessed after 12 hr of growth and found to be unaltered by exposure to IFN γ (Fig. 6).

To explore further the possibility that IFN γ selectively affects dendritic processes, Western blot analysis was used to compare the expression of cytoskeletal proteins (Fig. 6). The expression of tau, which is found primarily in axons, was not affected by IFN γ . The expression of actin, which is involved in both dendritic and axonal development, was elevated by treatment with BMP-7 and only slightly decreased by IFN γ , as was the expression of the dendritic protein MAP-2 (data not shown). Moreover, the total protein content of control and BMP-7-treated cultures was unaffected by IFN γ exposure (data not shown), indicating that overall cellular health is not affected by IFN γ . These results suggest that IFN γ selectively affects dendritic outgrowth.

Figure 4. IFN γ inhibits dendritic growth and synaptogenesis in hippocampal neurons. Hippocampal neurons were grown in the absence or presence of IFN γ (30 ng/ml) for 5 d. *A*, Fluorescence micrograph of neuron grown in control medium. Dendritic morphology ($n > 25$ cells/condition) and presynaptic specializations were analyzed by double-labeled immunostaining with antibodies to MAP-2 (red) and SV-2 (green). Cells were examined with a Bio-Rad confocal microscope, using 1 μ m optical sections. SV-2-positive puncta that are associated with dendrites have been shown previously to represent sites of synaptic contact (Fletcher et al., 1991). Scale bar, 10 μ m. *B*, Total dendritic length. *C*, Synapses per neuron. * $p < 0.05$ versus control.

Comparison of the effects of IFN γ and other cytokines

Like IFN γ , TNF α , IL-1 β , and IFN β have been detected at sites of inflammatory reactions in the nervous system (Taupin et al., 1993; Rothwell and Hopkins, 1995; Ensoli et al., 1999). We therefore compared their effects on dendritic growth. Neither inhibition of initial dendritic outgrowth (Table 1) nor dendritic retraction (Fig. 7) was observed with TNF α , IL-1 β , or IFN β . In addition, we found no evidence for potentiation of IFN γ action by TNF α or IL-1 β .

Involvement of STAT1 in dendritic retraction

Many cellular responses to IFN γ are mediated by activation of the Janus kinase pathway causing the phosphorylation of STAT1, a transcription factor (Farrar and Schreiber, 1993; Bach et al., 1997; Schindler, 1999). To determine whether the effects of IFN γ on dendrites required the activation of STAT1, we examined the

Figure 5. IFN γ induces dendritic retraction. Sympathetic neurons were treated initially with 5 ng/ml BMP-7 (*A*, *B*, *D*) or 20 ng/ml (*C*) for 5 d. Subsequently, they were divided into two groups. One was treated continuously with BMP-7, and the other was treated with both IFN γ (10 ng/ml) and BMP7 for an additional 6 d. Dendritic morphology was assessed by immunostaining with mAb to MAP-2 ($n \geq 60$ cells/condition). *A*, *C*, Number of dendrites per cell. *B*, Total dendritic length. *D*, Number of MAP-2-positive cells per culture. * $p < 0.05$ versus BMP-7 on day 5.

distribution of STAT1 in sympathetic neurons. Immunostaining with an antibody that recognizes phosphorylated STAT1 revealed that IFN γ induced the phosphorylation and nuclear translocation of STAT1 (Fig. 8).

We next determined whether transfection with a dominant-negative mutant of STAT1 that cannot be phosphorylated on Tyrosine 701 (Y701F) (Nakajima et al., 1996) reversed the inhibitory effects of IFN γ on dendritic outgrowth. Wild-type STAT1 or mutant STAT1 was cotransfected with EGFP to identify the transfected cells. Forty-eight hours after transfection cells were treated with BMP-7 (10 ng/ml) in the presence or absence of IFN γ (20 ng/ml). Consistent with the previous observation (Fig. 3), IFN γ decreased BMP-7-induced dendritic outgrowth by ~80% in cultures transfected with control vector, indicating that transfection by itself did not change the response of sympathetic neurons to either BMP-7 or IFN γ (Fig. 9*A*). Overexpression of wild-type STAT1 potentiated the inhibition by IFN γ and also slightly decreased dendritic outgrowth that was induced by BMP-7. In contrast, transfection of mutant STAT1 itself did not change the response of BMP-7 but significantly reduced the inhibitory effect of IFN γ on dendritic sprouting.

Although IFN γ predominantly activates STAT1, it also affects STAT3 in some cells (Stephens et al., 1998). Moreover, we observed previously that the activation of STAT3 by LIF blocks dendritic growth in sympathetic neurons (Guo et al., 1999). We therefore determined whether STAT3 plays a role in IFN γ -induced dendritic inhibition. Overexpression of mutant STAT3

did not affect the dendritic inhibition caused by IFN γ (Fig. 9*B*). In addition, IFN γ did not induce nuclear translocation of STAT3 in sympathetic neurons (data not shown). These data strongly suggest that the inhibitory effects of IFN γ on dendritic growth require the STAT1 activation and thus transcriptional activity.

Dendrites retract in response to IFN γ applied selectively to distal axons

Because many inflammatory reactions occur in white matter, elevated levels of IFN γ often are found in the vicinity of axons at a substantial distance from neuronal somata. Under these conditions IFN γ signaling must be retrogradely conveyed to the cell body if it is to affect dendritic morphology. To examine this possibility, we used compartmented cultures originally developed by Campenot (Campenot, 1977; Senger and Campenot, 1997). This culture system allows physical separation of the medium that is bathing cell bodies and proximal processes from the medium that is bathing distal axons and axon terminals (Fig. 10*A*). Thus IFN γ can be applied selectively to distal axons and terminals.

Neurons plated into the center compartment extend axons into the left and right compartments within 5–7 d, and extensive axonal growth is evident in the side chambers within 10–12 d after plating. Neurons maintained in control medium do not extend dendrites; in contrast, neurons whose cell bodies are exposed to BMP-7 (10 ng/ml) exhibit significant dendritic growth (Fig. 10*C*). Subsequent addition of IFN γ (10 or 50 ng/ml) to the medium of the right compartment, which contains distal axons

Figure 6. Lack of effect of IFN γ on axonal growth. *A*, Explants of superior cervical ganglia were maintained for 2 d in the presence or absence of IFN γ (10 ng/ml). Subsequently, the ganglia were dissociated, and cells were plated onto coverslips coated with laminin (2 μ g/ml). Cells then were exposed continuously to control medium or IFN γ for 12 hr. Axons were immunostained with mAb that recognizes phosphorylated forms of H and M neurofilament subunits. Cellular morphology was analyzed using Image software ($n \geq 60$ cells/condition). *B*, Cultures were treated with BMP-7 (5 ng/ml) for 8 d; during the last 3 d of treatment some were also exposed to IFN γ (10 ng/ml). Expression of cytoskeletal proteins was detected by Western blotting with mAb to tau and a polyclonal antibody to actin. Equal amounts of protein were loaded into each lane.

Table 1. Effect of inflammatory cytokines on initial dendritic outgrowth

Cytokines	Dendrites/cell
BMP-7	2.02 $\pm$ 0.18
BMP-7 + IFN γ	0.62 $\pm$ 0.12*
BMP-7 + IFN β	2.20 $\pm$ 0.20
BMP-7 + TNF α	1.98 $\pm$ 0.17
BMP-7 + IL-1 β	1.95 $\pm$ 0.19

Cultures of sympathetic neurons were treated with BMP-7 (5 ng/ml) either alone or in combination with one of the following agents for 5 d: IFN γ (10 ng/ml), TNF α (10 ng/ml), IL-1 β (10 ng/ml), or IFN β (10 ng/ml). Dendritic morphology was analyzed after immunostaining with mAb to MAP2. * $p < 0.05$ versus BMP-7.

and axon terminals (Fig. 10*B*), elicits a dose-dependent reduction in the total dendritic length of those neurons that extend axons into the right compartment (Fig. 10*C*). Neurons that extended axons into the right compartment were identified by the uptake and retrograde transport of fluorescent beads (Fluorobeads, F8795, 8 μ l/ml; Molecular Probes, Eugene, OR) added to the right chamber 12–16 hr before the cultures were fixed and immunostained for dendritic growth. Comparative analyses of dendritic growth in fluorescently labeled neurons versus nonlabeled

Figure 7. TNF α does not potentiate the effect of IFN γ on dendritic retraction. Sympathetic neurons were exposed to BMP-7 (5 ng/ml) for 9 d. Beginning on day 5, some of them were treated additionally with IFN γ (10 ng/ml), TNF α (10 ng/ml), or the combination of both of the agents. On day 9, dendritic morphology was assessed by immunostaining with mAb to MAP-2. * $p < 0.05$ versus BMP-7 on day 5.

Figure 8. IFN γ induces phosphorylation and nuclear translocation of STAT1. Sympathetic neurons were treated with BMP-7 (5 ng/ml) for 5 d. Subsequently, IFN γ (10 ng/ml) was added for 1 hr to the medium of some cultures (*C*, *D*), whereas others were treated with BMP-7 alone (*A*, *B*). The cellular location of STAT1 was detected by immunostaining with an antibody that reacts with a phosphorylated form of STAT1 (Tyr 701); the cells were examined with a Bio-Rad confocal microscope, using 1 μ m optical sections. Shown are phase-contrast (*A*, *C*) and fluorescence (*B*, *D*) micrographs.

neurons (e.g., neurons that extend axons only into the left compartment) indicated that dendritic growth in the nonlabeled neurons was not decreased in response to the addition of IFN γ to the right compartment (data not shown). This observation confirms

Figure 9. Dominant-negative STAT1 overcomes the inhibitory effect of IFN γ on dendrites. Sympathetic neurons were cotransfected with plasmids containing the genes for EGFP and (*A*) wild-type (*STAT1 WT*) or mutant STAT1 (*STAT1 F*) or (*B*) wild-type or mutant STAT3 or mutant STAT1 by Lipofectamine reagent. After 48 hr the cells were treated with BMP-7 (10 ng/ml), IFN γ (20 ng/ml), or BMP-7 and IFN γ . On day 6, cellular morphology was visualized by immunostaining with mAb to MAP-2. Transfected cells were assessed by the expression of EGFP. * $p < 0.05$ versus BMP-7 and IFN γ in the culture transfected with the control vector.

that the effect of IFN γ on labeled neurons was not mediated by IFN γ leaking into the center compartment.

DISCUSSION

Expression of IFN γ is elevated in murine trisomy 16, a model of human Down's syndrome (Torre et al., 1995; Hallam et al., 2000), and overexpression of IFN γ induces cerebellar and hippocampal malformations in transgenic mice (Corbin et al., 1996; LaFerla et al., 2000). IFN γ is known to regulate the maturation of glia (Merrill et al., 1993; Popko et al., 1997; Benn et al., 2001), and so it is possible that the developmental defects induced by IFN γ exposure arise secondarily from its actions on these cells. Our results indicate, however, that IFN γ also has direct inhibitory effects on neurons and adversely affects their growth in the absence of non-neuronal cells. These observations suggest a novel mechanism by which IFN γ interferes with neural development, and they contrast with previous studies that have reported primarily beneficial effects of IFN γ on the growth and differentiation of isolated neural cell populations (Erkman et al., 1989; Barish et al., 1991; Jonakait et al., 1994).

Exposure to IFN γ produced a profound and long-lasting inhibition of BMP-7-induced dendritic growth in cultures of sympathetic neurons. This effect did not reflect compromised cellular viability or health because it was not associated with changes in

Figure 10. Dendritic retraction in response to retrograde signaling by IFN γ . *A*, Schematic representation of the three-compartment culture of sympathetic neurons. Dissociated neurons were plated in the *Center* compartment, and axons extended into the *Left* and *Right* compartments. *B*, Negative control cultures were maintained in control medium (C₂) in the absence of BMP-7 or IFN γ ; positive control and experimental cultures were exposed to BMP-7 (10 ng/ml) added to C₂ in the *Center* compartment for 7 d. Three days after the addition of BMP-7, IFN γ (10 or 50 ng/ml) was added to the C₂ in the *Right* compartment of experimental cultures (indicated by the arrow in *C*). Then 4 d later, dendritic growth was quantified in cultures immunostained with mAb to MAP-2 ($n \geq 60$ cells/condition). Addition of IFN γ to the distal axons and axon terminals caused a dose-dependent reduction in total dendritic length (*C*). * $p < 0.05$ versus BMP-7 on day 7.

cell number, axon elongation, or the expression of tau, a protein found primarily in axons (Binder et al., 1985; Brion et al., 1988). Rather, IFN γ had a selective morphogenic effect on the development of a restricted cellular compartment. Although smaller in

magnitude, a qualitatively similar effect of IFN γ was observed on the growth of dendrites in cultures of hippocampal neurons, a population of neurons that was not exposed to BMP-7 and spontaneously forms dendrites in its absence (Banker and Waxman, 1988). Thus it appears that IFN γ inhibits dendritic growth in cells derived from both the central and peripheral nervous systems and that it affects both BMP-7-induced and spontaneous dendritic growth. It is, therefore, likely that IFN γ has widespread effects on dendritic growth in the nervous system.

Dendrites are the primary site of synapse formation in the mammalian nervous system (Purves, 1988), and so agents that inhibit dendritic growth would be expected to reduce synapse formation. Although sympathetic neurons form functional synapses with each other in tissue culture, they do not make such connections with each other *in vivo* (O'Laigue et al., 1974; Higgins et al., 1981). We therefore did not think it appropriate to examine the effect of cytokines on synapse formation in the cultured sympathetic neurons. On the other hand, hippocampal neurons are known to form synaptic contacts with one another *in vivo* (Swanson et al., 1987) and they also do so *in vitro* (Bartlett and Banker, 1984; Fletcher et al., 1991). In such cultures, treatment with IFN γ caused a reduction in the number of SV-2-positive and of synaptophysin-positive varicosities that were associated with MAP-2-positive dendrites. Because such varicosities have been found previously to represent sites of transmitter uptake and release (Fletcher et al., 1991; Matteoli et al., 1992; Pyle et al., 1999), these observations indicate that IFN γ reduces synapse formation, at least in neurons from the CNS. In addition, Vikman et al. (2001) have reported recently that, in mixed neural glial cultures, IFN γ exposure also causes a decrease in AMPA receptor clustering, suggesting that IFN γ -induced decreases in synapse number may be aggravated by other functional changes.

In addition to having effects on initial process outgrowth, prolonged exposure to IFN γ also causes sympathetic neurons to retract most of their existing dendrites, leading to an ~90% decrease in the size of the dendritic arbor. These effects were observed with concentrations of IFN γ similar to those detected after viral infection in humans and mice (Lebon et al., 1988; Weller et al., 1991; Rockstroh et al., 1998). IFN γ plays a critical role in viral clearance from the CNS (Binder and Griffin, 2001) and chronic viral infections, such as those associated with HIV, lead to long-term increases in the expression of IFN γ (Fuchs et al., 1991; Fan et al., 1993). Such chronic infections also induce dendritic atrophy (Masliah et al., 1997; Everall et al., 1999; Montgomery et al., 1999). It is, therefore, possible that IFN γ has a dual-edged effect in the response to viral infection: it promotes clearance of virions but also contributes to the pathology, and especially the dementia, by inducing dendritic retraction. In addition, it is important to note that elevated IFN γ expression (Kiefer et al., 1991; Kristensson et al., 1994; Lau and Yu, 2001; Li et al., 2001) occurs coincident with dendritic retraction (Sumner and Watson, 1971; Purves, 1975; Yawo, 1987; Kudo et al., 1993; Park et al., 1996) in many types of acute inflammatory reactions, including those triggered by trauma, stroke, and axotomy.

Many of the effects of IFN γ are mediated by activation of the STAT1 signaling pathway (Farrar and Schreiber, 1993; Bach et al., 1997; Schindler, 1999). However, there is also evidence for the involvement of STAT3 in some IFN γ actions (Stephens et al., 1998) as well as for STAT-independent effects (Ramana et al., 2000; Gil et al., 2001). Our examination of the pathways involved in IFN γ -induced dendritic retraction revealed that IFN γ stimulated the phosphorylation and nuclear translocation of STAT1,

but not STAT3, in cultures of sympathetic neurons. In addition, dendritic retraction was blocked by expression of a dominant-negative STAT1 construct, but not by a similar STAT3 construct, strongly suggesting that the former is the primary signaling pathway involved in process elimination. We reported previously that members of the IL-6 family of cytokines induce dendritic retraction via activation of STAT3 (Guo et al., 1999). Thus there appear to be independent routes by which these two classes of cytokines can cause dendritic atrophy, and this raises the possibility of synergistic interactions. In this respect it is important to note that there is frequent coexpression of IFN γ with IL-6-related cytokines in inflammatory reactions. For example, astrocytes and Schwann cells release IFN γ and IL-6 after traumatic injury or ischemia (Banner and Patterson, 1994; Curtis et al., 1994; Sun et al., 1996; Lau and Yu, 2001). The pathways leading from STAT activation to dendritic retraction are unclear, but there is reason to suspect the involvement of the paralogous transcriptional regulators p300 and CREB binding protein (CBP). BMP-induced dendritic growth requires the phosphorylation and nuclear translocation of SMAD1 (Guo et al., 2001), and activated SMADs are known to bind to p300 (Janknecht et al., 1998; Kawabata et al., 1999). Activated STATs also bind p300, and the expression of type I collagen that is induced by SMAD3 is negatively regulated by STAT1 activation (Ghosh et al., 2001). Thus competition between activated STATs and SMADs for interaction with limiting amounts of cellular p300/CBP may account for some of the effects of IFN γ on dendritic growth in sympathetic neurons.

Target-derived growth factors regulate the development of many, if not most, classes of neurons (Oppenheim, 1991; Korsching, 1993). Such agents typically signal in a retrograde manner, with information being conveyed from distal axons to neural somata. Previous studies have focused on the beneficial nature of retrograde signals and have emphasized their role in promoting neuronal survival (Hendry and Campbell, 1976; Chun and Patterson, 1977) and differentiation (Hendry, 1977) and in stimulating initial process extension (Campenot, 1977, 1987) and regeneration (Smith and Skene, 1997; Cafferty et al., 2001). In contrast, observations of sympathetic neurons grown in compartmented Campenot chambers indicate that, when IFN γ was applied to compartments containing distal axons, it led to dendritic retraction in compartments containing cell bodies and proximal processes. This is a novel variant of retrograde interaction in which cytokine-induced signals that are generated in axon terminals cause atrophy of other parts of the cell. It implies that retrograde interactions with proinflammatory cytokines can be "dystrophic" and can lead to elimination of distant processes and their associated synaptic contacts.

Retrograde dystrophic interactions could play an important role in neural disease because they convey information about injury or inflammation in one part of the brain to another, thereby eliciting reactive changes in distal regions. For example, axotomy induces the expression of IFN γ (Kiefer et al., 1991; Kristensson et al., 1994) and also causes dendritic retraction (Purves, 1975; Yawo, 1987). Because retrograde effects of IFN γ induce dendritic retraction *in vitro*, it seems likely to contribute to this process *in vivo*. Similarly, in MS, IFN γ is highly expressed in demyelinating lesions in white matter (Traugott and Lebon, 1988; Popko et al., 1997), whereas dendritic atrophy has been observed in gray matter (Peterson et al., 2001). Retrograde effects of IFN γ acting on distal axons provide a potential mechanism for linking these phenomena and for explaining some of the cognitive deficits that are manifest in this disease (Rao et al., 1991; Ron et al.,

1991). The mechanisms underlying such retrograde interactions are currently unclear, and it will be important to determine whether they involve retrograde transport of IFN γ , its receptor, STAT1, or some combinations of these signaling elements (Ahn et al., 2000).

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