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Interferon-γ induces the expression of immediate early genes *c-fos* and *c-jun* in astrocytes

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SUMMARY

The expression of the proto-oncogenes, *c-fos* and *c-jun*, in cultured mouse astrocytes and its induction by the potent astrocyte activator interferon-γ (IFN-γ), were examined by Northern blot and flow cytometry. Both proto-oncogenes were induced in a dose-dependent manner, peaking around 100 U/ml of IFN-γ. The kinetics of expression is very transient for *c-fos*, reaching a maximum at 30 min and decreasing rapidly thereafter. The *c-jun* remained high throughout the stages analysed. Cycloheximide superinduced *c-fos* and *c-jun* induction by IFN-γ, thus indicating that both act as immediate early genes. The products of *c-fos* and *c-jun*, proteins FOS and JUN, that act in conjunction forming the regulatory factor AP-1, were detected 1 hr after stimulation in virtually all cells, using flow cytometry. The induction in astrocytes of both proto-oncogenes could be the first stage of immunological activation of these central nervous system cells by immune interferon.

INTRODUCTION

Interferon-γ (IFN-γ) is a potent activator of astrocytes. A key role for this pleiotropic lymphokine is the induction in such central nervous system (CNS) cells of the expression of major histocompatibility complex (MHC) class II antigens,[1–3] allowing them to present antigens such as myelin basic protein (MBP) to T lymphocytes.[4,5] Thus, IFN-γ becomes directly implicated in the development of inflammatory diseases in the CNS, such as multiple sclerosis.[6] The receptors for IFN-γ have been previously characterized on both murine astrocytes[7] and oligodendrocytes.[8]

The proto-oncogenes *c-fos* and *c-jun* code for growth-associated proteins that act in conjunction forming the regulatory factor AP-1.[9,10] The proto-oncogene *c-jun* is essential for normal mouse development[11] and the continuous expression of *c-fos* precedes apoptosis in the CNS[12] suggesting a cardinal role for both proto-oncogenes in cell growth, differentiation and development. Basal expression of both transcription factors was located in rat hippocampus[13] and cerebral cortex.[14] Transient expression of *c-fos* was demonstrated during development of cortex[15] and both immediate early genes were induced by nerve transection,[16] inflammation[17] and electrical stimulation.[18]

In the astrocytes, the induction of the early response gene *c-fos* by neurotransmitters,[19] as well as of the immediate early gene *crg-2* by IFN-γ[20] has been demonstrated. Furthermore, previous reports demonstrated the activation of such proto-oncogenes in fibroblasts by cytokines, such as tumour necrosis factor-α (TNF-α)[21,22] and interleukin-1 (IL-1)[22] and in B lymphocytes cell lines by IL-2.[23]

We investigated here whether *c-fos* and *c-jun* were induced by IFN-γ in astrocytes, demonstrating that this is part of the immediate early response of such glial cells to the signal produced upon binding of the cytokine to its membrane receptor.

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MATERIALS AND METHODS

Astrocyte cultures

Astrocyte cultures were prepared by mechanical dissociation of the cerebral cortex from newborn BALB/c mice.[24] The cortex was isolated under a dissecting microscope and cleaned of choroid plexus and meninges. Cell suspensions were filtered through 80-μm pore size mesh into Dulbecco's modified Eagle's essential medium (DMEM) containing 10% fetal calf serum (FCS) and gentamicin (Flow Laboratories, Irvine, UK). After centrifugation, cells were filtered through a 20-μm mesh sieve, plated in 75 cm^2 tissue culture flasks (Costar, Cambridge, MA) and cultured at 37°. The medium was changed after 4 days of culture and, subsequently, three times a week for the entire culture period. Cultures were enriched in astrocytes by removal of the less adherent oligodendrocytes by shaking for 18 hr at 37° and 250 r.p.m. in a G24 environmental shaker (New Brunswick Scientific, Edison, NJ). Cellular confluence was observed 10 days after plating, with a polygonal flat cell morphology. A mean of 98·4% astrocytes was confirmed by indirect immunofluorescence staining of methanol-fixed cultures using rabbit anti-glial fibrillar acidic protein (GFAP) antiserum (Dakopatts, Glostrup, Denmark) and fluorescein-

labelled goat anti-rabbit IgG (Miles Laboratores Inc., Elkhart, IN). The lack of noticeable oligodendrocytes and microglial/macrophage cells was determined using a guinea-pig anti-MBP antiserum prepared as described elsewhere[25] and monoclonal anti-Mac-1 antibody (Serotec, Oxford, UK). Secondary fluorescein-labelled antibodies against guinea-pig and rat IgG were purchased from Sigma Chemical Co. (St Louis, MO).

Stimulation of the cells

Confluent monolayers of astrocytes in 75 cm^2 tissue culture flasks were washed with phosphate-buffered saline (PBS), replenished with DMEM containing 0·25% FCS and incubated for 48 h in order to let the cells become quiescent. Quiescent astrocytes were stimulated with different concentrations of IFN-γ (0, 5, 10, 100 and 1000 U/ml) in medium containing 0·25% FCS for various periods of time, as indicated. Cycloheximide (Sigma) treatment was carried out at a concentration of 20 μg/ml in the same medium.

Northern blot analysis

Total RNA from astrocyte cultures was isolated by the guanidium thiocyanate–caesium chloride method: 10 μg/lane was electrophoresed on a formaldehyde–agarose gel and transferred to nylon membranes (Zeta-Probe, Bio Rad Laboratories, Hercules, CA).

The coding region of *v-fos* DNA [1·1 kilobase (kb)], isolated by *Pst*I digestion from *pvfos-1*[26] (gift of Dr A. Pellicer, New York University Medical Center, New York, NY) and a 1·0 kb *v-jun* probe (purchased from Clontech, Palo Acto, CA) were used as specific probes. A human β-actin cDNA probe was also purchased from Clontech, and used as an internal control for RNA loading.

Probes were labelled with [^{32}P]dCTP using the random primed method (Boehringer Mannheim, Germany). Unincorporated radiolabel was removed with S-300 MicroSpin columns (Pharmacia Biotech, Sweden). The blots were exposed to Kodak X-OMAT film with an intensifying screen at −75°. Signals were quantified with a computing densitometer Model 300A (Molecular Dynamics, Sevenoaks, Kent, UK).

Flow cytometry

Control and stimulated cultures were immediately fixed by exposing the cells to 4% formaldehyde in PBS for 30 min at 4°. After washing the cells with PBS, their membranes were permeabilized with 0·1% Triton-X-100 for 30 min at room temperature, washed and incubated with the primary antibodies diluted 1:500 in PBS containing 0·1% bovine serum albumin (BSA) and 0·1% Triton, for another 30 min at 4°. Cells were washed and incubated for 1 h with their respective secondary fluorescein isothiocyanate (FITC)-conjugated antibodies diluted 1:500 and exhaustively rinsed with PBS. Cells were then harvested in trypsin–ethylenediaminetetraacetic acid (EDTA) (Flow), washed twice with PBS–1% FCS containing 0·02% sodium azide and analysed by one colour flow cytometry with an EPICS-CS flow cytometer (Coulter Cientifica, Spain) equipped with a 488-nm argon laser. Data from 10 000 to 50 000 cells were collected and analysed. Residual dead cells were gated out using side-scatter channels and the fluorescence intensity of individual cells was measured as relative fluorescence units. For the negative controls cells stained with an irrelevant antibody (a monoclonal antibody against β-galactosidase, from Sigma) was used. Establishment of the gate was based on the staining profiles of negative controls.

Reagents and antibodies

Recombinant IFN-γ of murine origin (10×10^6 U/mg) was from Holland Biotechnology, Leiden, the Netherlands and reference murine IFN-γ (Catalogue number Gg 02-901-533) was provided by the National Institute of Health (NIH; Bethesda, MD).

Sheep antibodies to a c-JUN sequence common for both human and mouse,[27] prepared against a synthetic peptide, were obtained from Cambridge Research Biochemicals, Northwich, UK. Sheep anti-c-FOS oncoprotein antibodies were from the same source.

RESULTS

Induction of *c-fos* and *c-jun* mRNA in astrocytes by IFN-γ

The *c-fos* mRNA is undetectable in confluent astrocyte cultures rendered quiescent by FCS starvation (Fig. 1). However, *c-fos* mRNA levels were increased after exposure to IFN-γ in a dose-dependent way, peaking at a concentration of 100 U/ml (Fig. 1). The same results were obtained when an NIH reference murine IFN-γ standard was used. The relative levels of mRNA were illustrated when quantified with a Model 300A computing densitometer (Molecular Dynamics, Sevenoaks, Kent, UK), showing that the band obtained after a treatment with 100 U/ml is 190% more dense than those obtained at 5 U/ml.

The lack of noticeable *c-jun* mRNA is also demonstrated in confluent unstimulated astrocytes. No clear dose–response pattern was found for the IFN-γ doses tested, with almost no differences between mRNA levels (Fig. 2).

Time–course induction of *c-fos* and *c-jun*

The *c-fos* mRNA induction was transient and rapid showing maximal induction at 30 min after stimulation with 100 U/ml of IFN-γ. It then decreased at 1 hr and 2 hr, with virtually no *c-fos* mRNA being detectable by 4 hr (Fig. 3).

Induction of *c-jun* reached almost the same levels between 30 min and 2 hr, followed by a slow decline by 4 hr that

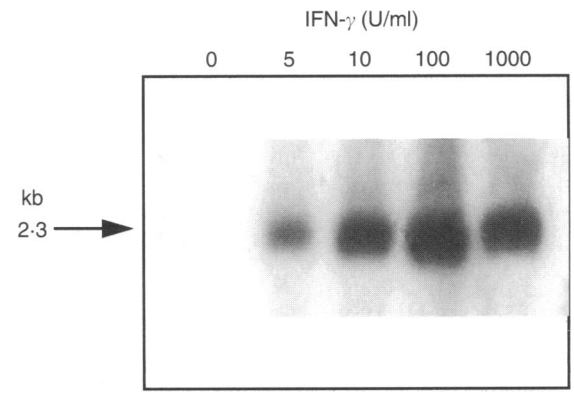

Figure 1. c-fos mRNA levels in astrocytes untreated (0) or treated with 5, 10, 100 or 1000 U of IFN-γ/ml, for 60 min. The size of the transcript is indicated on the left.

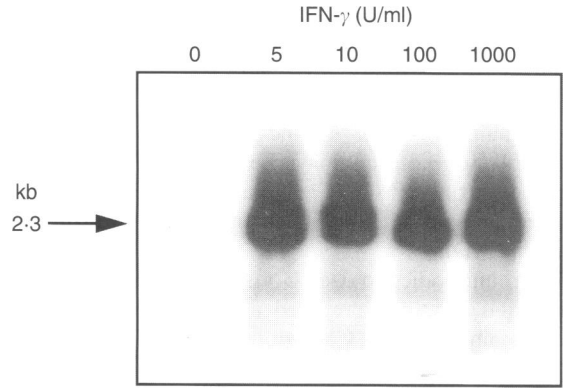

Figure 2. Dose-response induction of c-jun mRNA in astrocytes by IFN-γ. Cells were treated with 0 to 1000 U/ml for a 60-min period. Transcript size is indicated on the left.

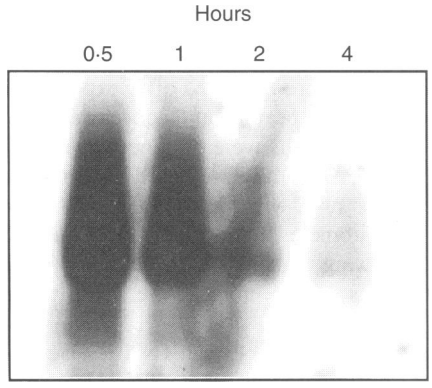

Figure 3. Time–course induction of c-fos mRNA in astrocytes by 100 U/ml of IFN-γ. Cells were treated by 0·5, 1, 2 or 4 hr. Total RNA was isolated and analysed by Northern blot.

returned to basal level at 24 hr (not shown). As a rule, *c-jun* remained high at all doses and stages analysed.

Induction of *c-fos* and *c-jun* mRNA does not require new protein synthesis

The pattern of induction of both mRNA was essentially unchanged in the presence of 20 μg/ml cycloheximide, at which dose protein synthesis was completely blocked in astrocytes. Figure 4 shows the pattern of induction of *c-fos* by 100 U/ml of IFN-γ alone (Fig. 4a) or in the presence of cycloheximide (Fig. 4b). Exactly the same results were obtained for *c-jun* (data not shown). These results demonstrate that both proto-oncogenes were activated as immediate early genes without requirement of intermediary proteins. Furthermore, cycloheximide alone slightly superinduced *c-fos* and *c-jun*, over basal constitutive expression, probably by stabilizing mRNA.

Induction of FOS and JUN proteins

Due to the nuclear location of both antigens studied,[28] we tested in previous experiments our permeability conditions with an anti-histone monoclonal antibody, which stained nuclei of cells of diverse origin, including mouse cells.[29] This positive control was performed in all experiments. We conclude

Figure 4. Requirements for new protein synthesis in IFN-γ-induced c-fos expression. Cells were treated with 100 U/ml of IFN-γ alone (a), or together with 20 μg/ml cycloheximide (b) for 1 hr.

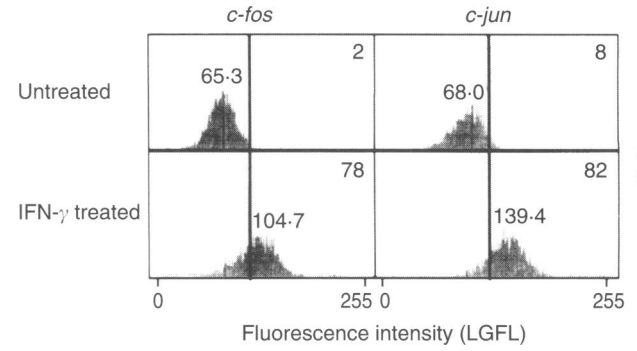

Figure 5. Fluorescence analysis of the induction FOS and JUN proteins by cytofluorometry. Astrocytes were grown in 6-cm-diameter plastic petri dishes and treated with medium alone or with medium containing 100 U/ml of IFN-γ for 1 hr. After staining as stated in the Materials and Methods, cells were detached and fluorescence was determined in the flow cytometer. Figures in the right corner indicate the percentage of positive cells. Figures on each peak indicate its mean fluorescence. The cursor is set to the right of the background expression values.

that our fixation and permeabilization conditions retain antigenic integrity in the nuclei with no significant increase in non-specific background fluorescence.

IFN-γ-stimulated astrocytes showed a clear increase in immunoreactivity when stained with both anti-FOS and anti-JUN specific antibodies, when compared with untreated cells (Fig. 5). Around 80% of the cells become positive for FOS and JUN upon induction. This was an expected figure as 98% of cells in our astrocyte cultures were GFAP-positive (see the Materials and Methods).

The effect described was saturable, as no further augmentation in fluorescence intensity nor in positive cell number resulted when treating astrocytes with amounts of IFN-γ above 100 U/ml for periods of time longer than 1 hr.

DISCUSSION

The dynamics of expression and the localization of *c-fos* and *c-jun* have been reviewed by Morgan and Curran.[28] Expression

of *c-fos* can be detected in the nuclei of neurons but not of glial cells, although heat stress and brain lesion induces massive FOS immunoreactivity in glial cells.[30,31] Overexpression of both *c-fos* and *c-jun*, but not of *ras*, has been recently reported to be induced in the glioma cell line C-6 by Theiler's murine encephalomyelitis virus infection.[32]

In this study, we have investigated the induction of both early response genes in cultured astrocytes treated with the immunological mediator IFN-γ that is secreted mainly by T lymphocytes in inflammatory processes. The treatment with the above pro-inflammatory cytokine induces MHC class II molecules in astrocytes,[1–3] in a way strictly controlled by neurons.[33]

Previous studies demonstrated that a specific and saturable binding of IFN-γ molecules takes place at the 11 100 high-affinity receptors present in each astrocyte.[7] Binding of IFN-γ to its specific receptor leads to activation of cytoplasmic tyrosine kinases which phosphorylate the family of transcription factors called STATS (signal transducer and activator of transcription). Such factors undergo homo- or heterodimerization and translocate to the nucleus.[34]

We demonstrated that astrocytes can be induced to express *c-fos* and *c-jun* mRNA by incubation with the astrocyte stimulator IFN-γ, the optimal dose being the same as the optimal dose reported to be needed for MHC class II expression (100 U/ml).[33] The addition of different amounts of such cytokine to cultures of mouse cortical astrocytes, resulted in *c-fos* and *c-jun* mRNA accumulation that reached a maximum at 30 min (Fig. 3). A rapid decline in the case of *c-fos*, and a slower one for *c-jun*, followed. The rapid kinetics of proto-oncogene stimulation, shows that the stimulatory effect was induced early upon binding of IFN-γ to its cellular receptor, as saturation of the binding capacity of 1×10^5 astrocytes needs 1 h to be accomplished at 37°.[7]

The expression of mRNA was dose-dependent, the optimal concentration being 100 U IFN-γ per ml in the case of *c-fos* (Fig. 1). In addition, the induction of both proto-oncogenes in the presence of cycloheximide indicated its immediate early gene nature in this system (Fig. 4). Their induction by cycloheximide alone may be due to an increase of the stability of the mRNA.[35]

The FOS and JUN proteins are also actively transcribed from the mRNA, as demonstrated by their detection in IFN-γ-stimulated cells with specific antibodies and cytofluorimetry (Fig. 5). Almost all cells (80%) contained the protein products, a high percentage taking into consideration that we were dealing with primary brain cultures. Changes in permeability producing an non-specific increase in the accessibility of the antibodies were ruled out as a consequence of the procedures of fixation and permeabilization used and the nuclear localization of the antigens.

A major fraction of FOS and JUN proteins is present in cells in the form of a complex named AP-1 whose role in cellular signal transduction and regulation is well documented.[9,10] IFN-γ has been postulated to be the final factor inducing inflammatory demyelination.[6] The rapid induction of *c-fos* and *c-jun* by IFN-γ in astrocytes, which express specific receptors for immune-interferon, is postulated to be an immediate early step in astrocytic immune activation.

Cytokines other than IFN-γ were previously reported to increase *c-fos* and *c-jun* expression. For instance, TNF-α and IL-1 induced overexpression of both proto-oncogenes in fibroblasts,[21,22] IL-2 in B lymphocytes,[23] or IL-6 in human B-cell lines.[36] It seems therefore that the induction of the above proto-oncogenes expression is one of the earliest responses of several cells to cytokines. As demonstrated in this article, this is also the case for astrocytes stimulated with IFN-γ.

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