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Induction and regulation of class II major histocompatibility complex mRNA expression in astrocytes by interferon-γ and tumor necrosis factor-α

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Summary

Astrocytes can function as antigen-presenting cells (APC) upon expression of class II major histocompatibility complex (MHC) antigens, which are induced by interferon-γ (IFN-γ). Previous data from this laboratory had shown that the cytokine tumor necrosis factor-α (TNF-α) enhances IFN-γ-mediated class II antigen expression on astrocytes. We have now investigated the effect of IFN-γ and TNF-α on class II MHC mRNA expression in astrocytes using Northern blot analysis. Astrocytes do not constitutively express mRNA for class II MHC. Kinetic analysis of class II MHC mRNA expression in IFN-γ-treated cells demonstrated an 8 h time lag, which was followed by an increase over the next 16 h. Optimal expression of class II mRNA was detected after a 24 h incubation with IFN-γ. This level of expression was further enhanced by the simultaneous addition of IFN-γ and TNF-α to the astrocytes, while TNF-α alone had no effect on class II mRNA expression. TNF-α does not act by increasing the stability of IFN-γ-induced class II mRNA, indicating its action is not at that specific level of post-transcriptional control. Furthermore, astrocyte class II mRNA expression was inhibited when cycloheximide (CHX) was added together with IFN-γ or IFN-γ/TNF-α, and when CHX was added up to 4 h after treatment with IFN-γ or IFN-γ/TNF-α. These results indicate that astrocyte class II mRNA expression is mediated by newly synthesized proteins induced by IFN-γ and/or IFN-γ/TNF-α. The expression of class II antigens on astrocytes, and cytokine modulation of their expression, may be important in the initiation and perpetuation of intracerebral immune responses.

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Abbreviations: APC, antigen-presenting cells; CNS, central nervous system; CHX, cycloheximide; EAE, experimental allergic encephalomyelitis; FACS, fluorescence-activated cell sorter; IFN-γ, interferon-γ; MHC, major histocompatibility complex; MS, multiple sclerosis; TNF-α, tumor necrosis factor-α.

Introduction

The non-neuronal cells of the central nervous system (CNS) are made up of the macroglia (astrocytes, oligodendrocytes and ependymal cells) and the microglia. Collectively, these glial cells perform a variety of active roles during development of the brain (Rakic, 1971; Silver and Sapiro, 1981) and subsequently in the maintenance of normal CNS physiology (Hertz, 1981; Janzer and Raff, 1987). Recent work has suggested that glial cells such as astrocytes and microglia may be involved in immunological events occurring in the brain. The astrocyte can be stimulated to secrete a number of immunoregulatory molecules, including interleukin-1 (IL-1) (Fontana et al., 1982), interleukin-3 (IL-3) (Frei et al., 1985), interleukin-6 (IL-6) (Frei et al., 1989; Benveniste et al., 1990), prostaglandins (Fontana et al., 1982), leukotriene B_4 (Hartung et al., 1988), tumor necrosis factor-α (TNF-α) (Robbins et al., 1987; Lieberman et al., 1989; Chung and Benveniste, 1990) and IFN-α/β (Tedeschi et al., 1986). The microglia can also be stimulated to secrete IL-1 (Giulian et al., 1986), IL-6 (Frei et al., 1989) and TNF-α (Frei et al., 1987), thus providing the CNS with numerous endogenous sources of cytokines necessary for immunological response.

More importantly, the astrocyte and microglia can function as antigen-presenting cells (APC) in the CNS (Fierz et al., 1985; Frei et al., 1987). These cells are able to internalize, process, express and present antigen to encephalitogenic T cells (Fontana et al., 1984). However, such a function is only possible upon expression of class II major histocompatibility complex (MHC) molecules. Indeed, astrocytes can be induced to express class II antigens both in the CNS and in vitro, following exposure to interferon-γ (IFN-γ) (Wong et al., 1984; Fierz et al., 1985) or virus (Massa et al., 1986).

MHC-encoded class II molecules are heterodimeric glycoproteins which have a central role in the regulation of immune responses (Benacerraf, 1981). The expression of class II antigens is primarily restricted to B cells, monocytes/macrophages and dendritic cells (Hammerling et al., 1975), although certain non-lymphoid cells can be induced to express class II upon exposure to IFN-γ, and function as APC. These include pancreatic beta cells (Markmann et al., 1988), keratinocytes (Gaspari et al., 1988), brain endothelial cells (McCarron et al., 1985), and most pertinent to this study, astrocytes (Fontana et al., 1984). Abnormal control in the level of expression of class II genes, and aberrant expression in cells normally class II negative have been implicated in autoimmune phenomena. Because of the importance of class II MHC antigens, many studies have been directed toward understanding the regulatory mechanisms involved in class II MHC gene expression.

It is generally accepted that induction of class II gene expression by IFN-γ occurs at the transcriptional level (Basta et al., 1987; Blanar et al., 1988; Fertsch-Ruggio et al., 1988; Rosa and Fellous, 1988; Amaldi et al., 1989), and that *trans*-acting factors interacting with *cis*-acting DNA regulatory elements are involved in the transcriptional regulation of class II MHC expression (Accolla et al., 1985; Salter et al., 1985; Sherman et al., 1987, 1989; Blanar et al., 1988; Amaldi et al., 1989; Celada et al., 1989). These *trans*-acting regulatory factors have been postulated to function positively or negatively, and to be expressed ubiquitously, or in a tissue- or stage-specific manner.

Although IFN-γ is considered the primary inducer of class II antigens, there is evidence for other cytokines contributing to class II expression. We have previously shown that TNF-α enhances IFN-γ-induced class II antigen expression on astrocytes, and that this is a synergistic interaction as TNF-α alone has no effect on class II expression (Benveniste et al., 1989). The present study was undertaken to extend these previous findings, and to examine, at the molecular level, the effect of IFN-γ and TNF-α on astrocyte class II gene expression. We report that astrocytes express class II mRNA 8 h after treatment with IFN-γ or IFN-γ/TNF-α, indicating a long lag period between exposure to the cytokines and initiation of class II gene expression. TNF-α does not act to stabilize IFN-γ-induced class II mRNA, suggesting it may act at other levels of post-transcriptional control or at the transcriptional level. Furthermore, the expression of class II MHC mRNA

was completely inhibited by cycloheximide (CHX), suggesting a role for newly synthesized proteins in astrocyte class II MHC expression.

As astrocytes can be stimulated to secrete TNF-α (Robbins et al., 1987; Lieberman et al., 1989; Chung and Benveniste, 1990), and express high affinity TNF-α receptors (Benveniste et al., 1989), TNF-α can act in an autocrine fashion to enhance class II gene expression in astrocytes. By modulating class II gene expression and thereby stimulating the APC function of astrocytes, IFN-γ and TNF-α in concert may play a pivotal role in the regulation of intracerebral immune responses.

Materials and methods

Recombinant proteins and reagents

Rat recombinant IFN-γ (specific activity: 4×10^6 U/mg) was purchased from AMGen Biologicals (Thousand Oaks, CA, U.S.A.), and human recombinant TNF-α (specific activity: 5.6×10^7 U/mg) was the generous gift of Genentech (South San Francisco, CA, U.S.A.). Monoclonal antibody to glial fibrillary acidic protein (GFAP) was obtained from Boeringher Mannheim (Indianapolis, IN, U.S.A.), and monoclonal antibody to rat class II MHC antigens (clone OX-6) was from Accurate Corporation (Westbury, NY, U.S.A.). Second antibody was affinity-purified goat anti-mouse Ig conjugated to fluorescein-isothiocyanate (FITC) from Southern Biotechnology (Birmingham, AL, U.S.A.). Cycloheximide and actinomycin-D were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Primary glial cell cultures

Primary glial cell cultures were established from neonatal rat cerebra by a modification of the McCarthy and de Vellis technique (1980) as previously described (Benveniste and Merrill, 1986). Meninges were removed from rat brains prior to glial cell dissociation and culture. Culture medium (CM) was Dulbecco's modified essential medium (DMEM), high glucose formula supplemented with glucose to a final concentration of 6 g/l, 2 mM glutamine, 0.1 mM non-essential amino acid mixture, 0.1% gentamicin, and 10% fetal bovine serum (Hyclone, Logan, UT, U.S.A.). After 10 days in primary culture, oligodendrocytes were separated from the glial cultures by mechanical dislodging, and the astrocytes were obtained by trypsinization (0.25% trypsin/0.02% EDTA) and replated at a density of $6-10 \times 10^6$ cells/100 mm^2 tissue culture plate and allowed to adhere for at least 24 h. The cells were counted using trypan blue; cell viability was 99–100%. The astrocytes were monitored for purity by immunofluorescence, and by non-specific esterase staining for contaminating microglia as previously described (Benveniste and Merrill, 1986). The primary astrocytes were plated (5.0×10^4) on 12 mm glass coverslips, incubated in culture medium for 2 days, washed twice with phosphate-buffered saline (PBS), and fixed for 10 s in cold acetone. The cells were then stained for GFAP, an intracellular antigen unique to astrocytes (Bignami et al., 1972), using a monoclonal antibody to GFAP (1 : 4) for 30 min at room temperature, followed by a 30 min incubation with goat anti-mouse Ig/FITC (1 : 20). The coverslips were then mounted in 30% glycerol, and visualized by fluorescent microscopy. Astrocyte cultures were routinely > 97% positive for GFAP, and less than 2% of the cells were microglia based on their positive staining for non-specific esterase.

RNA isolation and analysis

Total cellular RNA was isolated from confluent monolayers of astrocytes that were incubated for various intervals (0–48 h) without or with IFN-γ and/or TNF-α. In some experiments, the protein synthesis inhibitor, CHX (5 μg/ml) or the RNA synthesis inhibitor, actinomycin D (5 μg/ml), were added to the cytokine-treated astrocytes for 0–24 h. RNA isolation followed the procedure of Chomczynski (1987). The cells were collected, washed 2 times with cold PBS, and pelleted. RNA was extracted with guanidinium isothiocyanate and phenol, and precipitated with ethanol. Samples (15 μg) of total cellular RNA were denatured with formaldehyde for 15 min at 55°C, and RNA was size fractionated by electrophoresis through a 1.0% agarose gel containing ethidium bromide for visualization of 28 S and 18 S ribosomal RNA bands. The visualization of RNA bands was useful for assessing the integrity of the RNA and for varifying the amount of RNA loaded. The RNA was then transferred to nitrocellulose paper in 20 ×

standard saline citrate (SSC) (3 M NaCl and 0.3 M sodium citrate) at 4°C. After the transfer, the nitrocellulose paper was air-dried and the RNA cross-linked in a UV Stratalinker oven. Prehybridization was performed at 42°C in a solution containing 50% (v/v) formamide, 5 × SSC, 1 × Denhardt's solution, 50 μg/ml of denatured salmon sperm DNA, and 0.1% sodium dodecyl sulfate (SDS) for 8–24 h. Hybridization was carried out at 42°C for 48 h in prehybridization solution containing 10% dextran sulfate, 0.05 mM Na phosphate buffer and denatured ^{32}P-labeled murine class II E-α cDNA probe (2×10^6 cpm/ml). The blots were then washed in 2 × SSC (twice for 20 min) at room temperature, followed by 1 × SSC containing 0.1% SDS (twice for 30 min) at 42°C and finally in 0.1 × SSC for 30 min at 42°C. The blots were dried between Whatman filter paper and exposed to Kodak X-Omat AR film plus intensifying screens at −70°C. The autoradiographs were quantitated by scanning densitometry with a Bio-Rad Model 620 video densitometer. Filters were stripped to remove bound class II MHC probe, and rehybridized with a second control probe, cyclophilin.

cDNA probes

A cDNA probe (peac11) specific for mouse class II E-α (Mathis et al., 1983) was the generous gift of Dr. Jerold Woodward, University of Kentucky. The 1.08 kb *Eco*RI insert was isolated, and labeled with [α-^{32}P]deoxyCTP using an Amersham nick translation kit according to the manufacturer's instructions. A specific activity of $0.5–1 \times 10^8$ cpm/μg DNA was routinely attained. A cDNA probe for rat cyclophilin (p1B15) (Danielson et al., 1988) was the generous gift of Dr. Jim Douglass, The Oregon Health Sciences University.

Quantitative analysis of class II MHC antigen induction by immunofluorescence flow cytometry

Primary rat astrocytes were resuspended in DMEM containing 10% fetal bovine serum (FBS), and plated at $4–5 \times 10^5$ cells/well into 6-well (35 mm) plates (Costar, Cambridge, MA, U.S.A.). The plates were incubated overnight to allow recovery of the cells from trypsinization and to assure adherence of the astrocytes. After 24 h the original medium was aspirated off and fresh serum-free medium (1 ml) was added to the wells. Triplicate wells of primary rat astrocytes were treated with 100 U/ml of recombinant rat IFN-γ and/or 50 ng/ml of recombinant human TNF-α for various incubation periods (0–3 days). At each time point, the cells were trypsinized and stained for class II antigens, as previously described (Benveniste et al., 1989). Briefly, astrocytes were incubated with 30 μl of OX-6 monoclonal antibody for 60 min in the cold, washed 3 times with PBS containing 0.5% FBS and 0.02% azide (PBS-FBS-azide), and then incubated with 30 μl of goat anti-mouse Ig-FITC (1 : 20) for another 30 min in the cold. After washing 3 times with PBS-FBS-azide, the cells were fixed in a final volume of 100 μl of 1% paraformaldehyde and analyzed on the FACStar (Becton-Dickinson, Mountain View, CA, U.S.A.) for class II antigen expression. Negative controls were incubated with 30 μl of PBS-FBS-azide in place of first antibody, or with an irrelevant monoclonal antibody of the same isotype.

The gate window of forward-angle light scatter lay between channels 10 and 255; the gate window for log of green FITC fluorescence lay between channels 0 and 255. Ten thousand cells were analyzed for each sample.

Results

Induction of class II MHC mRNA in astrocytes by IFN-γ and TNF-α

The level of class II MHC mRNA was examined in astrocytes following treatment for various times with IFN-γ, TNF-α or a combination of the two cytokines. To determine the steady-state level of mRNA for class II, Northern blot analysis was performed using a cDNA probe for murine class II genes (E-α), with total RNA isolated from cultured astrocytes. As seen in Fig. 1, a 1.3 kb class II MHC mRNA transcript was present in IFN-γ treated astrocytes (lanes 2 and 4) and absent in untreated cells (lanes 1 and 3). Class II MHC mRNA expression was more pronounced when the cells were cultured with IFN-γ in serum-free medium (SFM) (Fig. 1, lane 4) as opposed to serum-containing medium (Fig. 1, lane 2), thus, all the subsequent experiments were con-

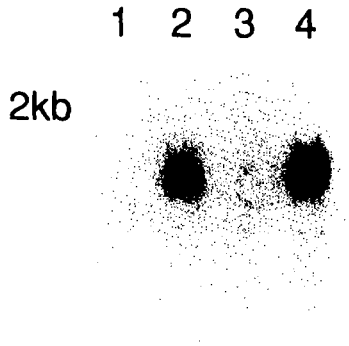

Fig. 1. The effect of IFN-γ on class II MHC mRNA expression in primary rat astrocytes. Northern blot of RNA from astrocytes that were incubated in serum containing media (lanes 1 and 2) or serum-free medium (SFM) (lanes 3 and 4) without (lanes 1 and 3) or with IFN-γ (100 U/ml) (lanes 2 and 4) for 24 h. Total RNA was extracted and size fractionated by gel electrophoresis. Hybridization was performed with a cDNA probe (E-α) specific for a murine class II MHC gene. The blot was then exposed at −70 °C for 24 h to Kodak X-Omat AR film plus two intensifying screens. kb, kilobases.

ducted in SFM. Optimal expression of class II mRNA was detected when cells were stimulated with 100–250 U/ml of IFN-γ (data not shown). Some variability in the concentration of IFN-γ required for induction of class II mRNA was noted, and this variability was dependent on the lot of IFN-γ used. Therefore, it was necessary to do a dose–response study for each lot of IFN-γ used. For this study, 100 U/ml of IFN-γ was sufficient for maximal expression of class II mRNA. The optimal time required for class II mRNA expression following treatment of astrocytes with IFN-γ is illustrated in Fig. 2. Astrocytes were incubated in SFM without or with IFN-γ for 12, 24 or 48 h prior to harvesting. A low level of class II MHC mRNA was detected at 12 h following treatment with IFN-γ, with maximal expression detected after a 24 h incubation with IFN-γ. There was a 2.7-fold increase in class II MHC mRNA expression from 12 to 24 h, and a slight reduction at 48 h.

We have previously shown that the level of class II protein expression, based on fluorescence-activated cell sorting (FACS) analysis, was enhanced when the cells were treated with both IFN-γ and TNF-α (Benveniste et al., 1989). Similarly, in this present study, the incubation of as-

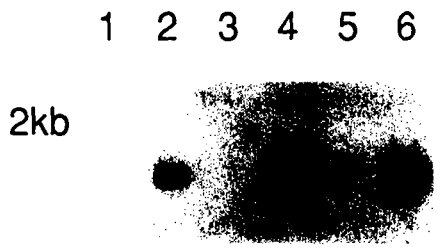

Fig. 2. Kinetic analysis of IFN-γ treatment on astrocyte class II MHC mRNA expression. Astrocytes were cultured in SFM without (lanes 1, 3, and 5) or with IFN-γ (lanes 2, 4, and 6) for 12 h (lanes 1 and 2), 24 h (lanes 3 and 4) or 48 h (lanes 5 and 6). Total RNA was extracted and analyzed for class II mRNA by Northern blot hybridization method. The blot was exposed to Kodak X-Omat AR film plus two intensifying screens at −70 °C for 24 h.

trocytes with both IFN-γ and TNF-α resulted in an enhanced expression of class II mRNA compared to IFN-γ alone (Fig. 3). Optimal enhancement of class II mRNA was demonstrated using TNF-α at 50 ng/ml (Fig. 3, lane 4), which correlates with the concentration of TNF-α used for synergistic induction of class II MHC protein (Benveniste et al., 1989). A 2.2-fold increase in class II mRNA expression in the presence of 50 ng/ml of TNF-α was detected, compared to IFN-γ alone. As expected, TNF-α alone did not induce mRNA for class II antigens (data not shown). Class II MHC mRNA expression induced by IFN-γ/TNF-α was also enhanced when experi-

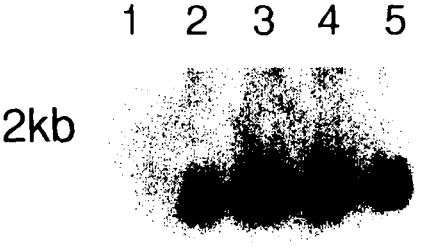

Fig. 3. TNF-α dose–response for optimal enhancement of IFN-γ induced class II mRNA expression. Primary astrocytes were treated with SFM alone (lane 1), IFN-γ (100 U/ml) (lane 2), IFN-γ plus TNF-α (5 ng/ml) (lane 3), IFN-γ plus TNF-α (50 ng/ml) (lane 4), and IFN-γ plus TNF-α (250 ng/ml) (lane 5), for 24 h. RNA was isolated for analysis by Northern blot hybridization method. The blot was probed with labeled E-α cDNA, and exposed at −70 °C for 24 h to Kodak X-Omat AR film plus two intensifying screens.

ments were performed in SFM (data not shown), indicating that a serum component(s) has a slight inhibitory effect on class II mRNA expression.

Kinetics of induction of class II MHC genes in astrocytes

In other cell types, a lag phase of approximately 6–8 h precedes the appearance of class II mRNA induced by IFN-γ (Basta et al., 1988; Blanar et al., 1988; Rosa and Fellous, 1988; Amaldi et al., 1989). We performed a more in-depth analysis of the kinetics of induction of class II mRNA by IFN-γ and IFN-γ/TNF-α in astrocytes. Analysis of mRNA was performed at different times after induction with IFN-γ (Fig. 4, lanes 2, 4, 6, and 8) and IFN-γ/TNF-α (Fig. 4, lanes 3, 5, 7, and 9). No class II mRNA was detected until 8 h following treatment with IFN-γ, with maximal expression detected 24 h after exposure to IFN-γ. Similarly, class II mRNA was not detected until 8 h in astrocytes that were stimulated with IFN-γ/TNF-α; however, the intensity of the RNA signal was increased in the presence of both cytokines, as expected. Thus, there was an 8 h time lag before class II mRNA was detected in astrocytes. At early time points (8 and 12 h), mRNA doublets are seen which ultimately merge into a diffuse,

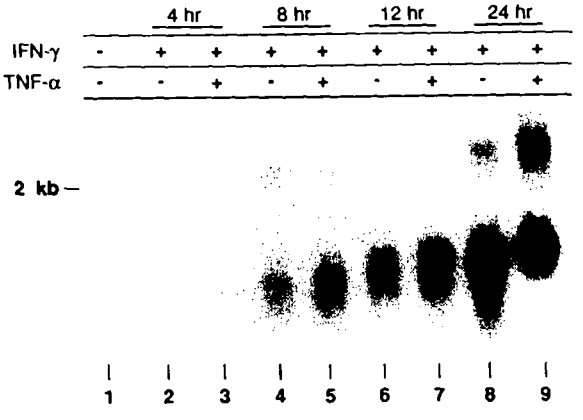

Fig. 4. Kinetic analysis of IFN-γ or IFN-γ/TNF-α treatment on astrocyte class II MHC mRNA expression. Total RNA was extracted from astrocytes treated with IFN-γ (100 U/ml) or IFN-γ plus TNF-α (50 ng/ml) for 4 h (lanes 2 and 3), 8 h (lanes 4 and 5), 12 h (lanes 6 and 7), and 24 h (lanes 8 and 9). Astrocytes in SFM alone (lane 1). The blot was exposed to Kodak X-Omat AR film plus two intensifying screens at −70 °C for 4 days.

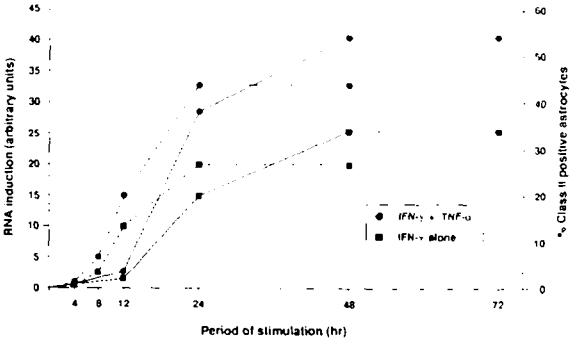

Fig. 5. Kinetics of induction of class II MHC protein and mRNA by IFN-γ and TNF-α. Astrocytes were treated with IFN-γ (100 U/ml) (■———■) or IFN-γ plus TNF-α (50 ng/ml) (●———●) for the times indicated. Induction of class II antigen protein expression was determined by FACS analysis. mRNA levels induced by IFN-γ (■-----■) or IFN-γ plus TNF-α (●-----●) were determined by Northern blot analysis. mRNA values are expressed in arbitrary units as determined from densitometric scanning of autoradiographs.

more intense 1.3 kb band at 24 h. This may be due to multiple transcription initiation sites described for the E-α gene (Mathis et al., 1983). In addition, a larger mRNA species of 2.4 kb is seen at 24 h. The significance of this band is unknown at this time. Results for the induction of class II MHC antigen expression and mRNA accumulation are summarized in Fig. 5.

Class II mRNA stability in the presence of IFN-γ and TNF-α

TNF-α increases IFN-γ-induced class II expression by increasing levels of mRNA for the class II molecule. However, it is not known whether TNF-α acts by increasing transcription or by stabilizing the mRNA. Experiments were conducted to assess class II mRNA stability in the presence of IFN-γ or IFN-γ/TNF-α. Class II mRNA was induced in astrocytes with either IFN-γ or IFN-γ/TNF-α for 24 h, then actinomycin D (a transcription inhibitor) was added for various times (1, 2, 4, 8, 16, and 24 h). Total cellular RNA was isolated and analyzed by Northern blotting. Preliminary results indicated that a decrease in class II mRNA was not detected until 8 h of actinomycin D treatment (data not shown). Subsequent experiments were performed utilizing RNA extracted after 8, 16 and 24 h of actinomycin D treatment. As shown in Fig. 6, within 16–24 h of

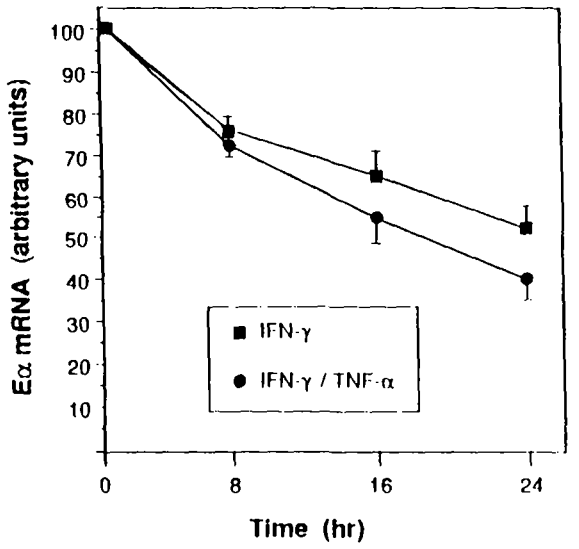

Fig. 6. The effect of IFN-γ and TNF-α on stabilization of class II mRNA. Astrocytes were incubated with either IFN-γ or IFN-γ/TNF-α for 24 h, then actinomycin D (5 μg/ml) was added for various times (8, 16, and 24 h). Total cellular RNA was isolated and analyzed by Northern blotting. RNA was hybridized with labeled E-α cDNA and the blots were exposed at −70°C to Kodak X-Omat AR film plus two intensifying screens. mRNA values are expressed in arbitrary units as determined from densitometric scanning of autoradiographs. mRNA values for cells treated with IFN-γ ± actinomycin D (■) or IFN-γ/TNF-α ± actinomycin D (●) are plotted. A value of 100 was assigned to cells treated with either IFN-γ or IFN-γ/TNF-α in the absence of actinomycin D. Each data point is the mean ± SD of two experiments.

actinomycin D treatment, TNF-α did not appreciably affect the stability of E-α mRNA compared to the stability of IFN-γ-induced E-α mRNA. In fact, it appears that TNF-α contributes to an accelerated destabilization of class II mRNA. The approximate half-life of E-α mRNA in the presence of IFN-γ/TNF-α was 19 h, compared to greater than 24 h in the presence of IFN-γ alone. These same blots were reprobed for cyclophilin mRNA to demonstrate that the integrity and quantity of RNA loaded in each lane was similar (data not shown). These data indicate that TNF-α does not act by mRNA stabilization to enhance IFN-γ-induced class II expression.

Induction of class II mRNA by IFN-γ and TNF-α is abolished by cycloheximide treatment

The 8 h delay in class II mRNA expression after IFN-γ or IFN-γ/TNF-α stimulation of astrocytes suggests that signal transmission initiated by these cytokines involves a number of intermediary steps, possibly the expression of newly synthesized gene products. To test this, we examined whether protein synthesis was required for induction of class II mRNA by IFN-γ and IFN-γ/TNF-α. CHX, an inhibitor of protein synthesis, was added to astrocytes at a concentration (5 μg/ml) that inhibited protein synthesis by more than 92%, while still maintaining cell viability (data not shown). Astrocytes were cultured for 24 h in the presence of IFN-γ, IFN-γ/TNF-α, CHX alone, IFN-γ plus CHX, IFN-γ/TNF-α plus CHX, RNA extracted, and then analyzed. Fig. 7 demonstrates the effect of CHX on the induction of class II mRNA by IFN-γ and TNF-α. No mRNA for class II was detected in cells treated with CHX alone, IFN-γ plus CHX, or IFN-γ/TNF-α plus CHX (Fig. 7, lanes 4, 5, and 6). Inhibition of protein synthesis completely abolished the induction of class II mRNA by IFN-γ and IFN-γ/TNF-α. However, there was no inhibition of cyclophilin mRNA expression (Fig. 7*B*), and no alteration in the pattern of ethidium bromide staining of RNA in all the samples treated with CHX alone or CHX plus the cytokines (Fig. 7*C*), indicating that CHX did not cause a generalized inhibition of mRNA expression in astrocytes. Cyclophilin was used as a control for these experiments as RNA levels do not change upon treatment with IFN-γ or IFN-γ/TNF-α.

Time course of protein synthesis required for induction of astrocyte class II MHC mRNA

That newly synthesized protein(s) is required for the induction of the class II MHC gene in astrocytes treated with IFN-γ or IFN-γ/TNF-α was suggested by results in Fig. 7. The duration of protein synthesis required to allow expression of the class II MHC gene in astrocytes was examined in cells that were pretreated with IFN-γ or IFN-γ/TNF-α for different lengths of time prior to the addition of CHX. Class II MHC mRNA was measured 24 h after the treatments were started. As shown in Fig. 8*A*, when CHX was added simultaneously with IFN-γ/TNF-α or 1–2 h after IFN-γ/TNF-α treatment, there was no detectable expression of class II MHC mRNA. However, when astrocytes were incubated with IFN-

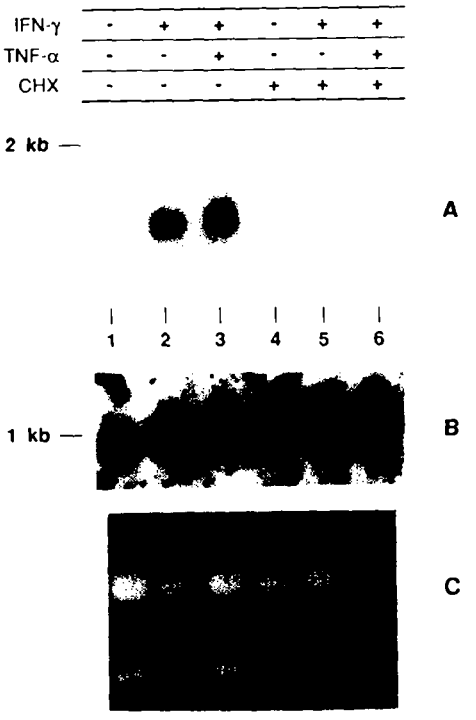

Fig. 7. Cycloheximide inhibits IFN-γ or IFN-γ/TNF-α-induced class II MHC mRNA expression. Astrocytes were incubated in SFM (lane 1), IFN-γ (100 U/ml) (lane 2), IFN-γ/TNF-α (50 ng/ml) (lane 3), CHX (5 μg/ml) (lane 4), IFN-γ/CHX (lane 5) or with IFN-γ/TNF-α/CHX (lane 6), for 24 h. Total RNA was extracted, Northern blot hybridization performed and the blot was exposed to Kodak X-Omat AR film plus two intensifying screens at −70°C for 3 days. Autoradiograph of class II mRNA (*A*). Autoradiograph for cyclophilin mRNA was obtained by stripping class II probe and rehybridizing with a second probe to detect cyclophilin mRNA expression (*B*). Photograph of the original gel stained with ethidium bromide to show that there was no alteration in the quantity or the quality of RNA in all the samples treated with CHX (*C*).

γ/TNF-α for 4 h prior to addition of CHX, and class II RNA measured 24 h later, a low level of class II RNA was detected. The increase in the level of class II MHC mRNA detected parallels the increase in the amount of time the cells were treated with IFN-γ/TNF-α before the addition of CHX, i.e., the longer the treatment with IFN-γ/TNF-α before the addition of CHX, the stronger the mRNA signal. These results, therefore, suggest that protein synthesis, initiated within 4 h of the cells encountering IFN-γ/TNF-α, is critical for subsequent class II MHC mRNA expression.

However, in samples that were treated for 12 h with IFN-γ/TNF-α before CHX was added, there was still a 25% reduction in the expression of class II MHC signal compared to the positive control of IFN-γ/TNF-α alone (Fig. 8*A*, lane 2), suggesting that continuous synthesis of protein is required for optimal expression of the class II MHC gene. CHX treatment had no effect on the expression of cyclophilin RNA (Fig. 8*B*). Similar results were seen when astrocytes were incubated with IFN-γ and CHX, except that the expression of class II MHC mRNA was detected only after cells were incubated with IFN-γ for 8 h prior to the addition

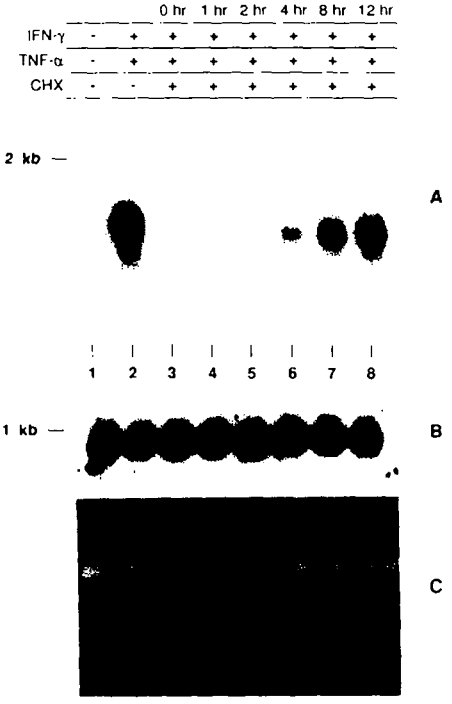

Fig. 8. Duration of protein synthesis required for IFN-γ/TNF-α induction of astrocyte class II MHC mRNA. Northern blot analysis of total cellular RNA isolated from astrocytes in SFM alone (lane 1), IFN-γ (100 U/ml)/TNF-α (50 ng/ml) (lane 2), IFN-γ/TNF-α/CHX (5 μg/ml) (lane 3) for 24 h. Astrocytes were pretreated with IFN-γ/TNF-α for 1 h (lane 4), 2 h (lane 5), 4 h (lane 6), 8 h (lane 7) or 12 h (lane 8) prior to the addition of CHX. RNA was hybridized with labeled E-α cDNA. The blot was exposed at −70°C for 3 days to Kodak X-Omat AR film plus two intensifying screens (*A*). Autoradiograph for cyclophilin mRNA obtained by stripping class II probe and rehybridizing with a second probe to detect cyclophilin mRNA (*B*). Photograph of the original gel stained with ethidium bromide (*C*).

of CHX (data not shown), indicating that 8 h of protein synthesis was critical for IFN-γ-induced class II mRNA expression.

Discussion

In this study we have shown that primary neonatal rat astrocytes, upon stimulation with IFN-γ, express mRNA transcripts for class II MHC genes, and that TNF-α enhances the expression of IFN-γ-induced class II mRNA. These results support previous findings that IFN-γ and TNF-α synergize in the induction of class II MHC protein expression in rat astrocytes (Benveniste et al., 1989). Kinetic analysis demonstrated that class II mRNA was first detected after 8 h of treatment with IFN-γ, followed by an increase in mRNA expression over the next 16 h. When astrocytes were treated with IFN-γ and TNF-α simultaneously, the kinetics of class II mRNA expression did not change; however, the overall amount of steady-state class II mRNA was increased. Optimal expression of class II mRNA was detected 24 h after incubation with IFN-γ alone or IFN-γ/TNF-α.

Although the predominant forms of gene regulation occur at the transcriptional level, a number of control mechanisms can act on RNA once its transcription has been initiated. Post-transcriptional regulatory mechanisms include (1) changes in mRNA stability, (2) alternative RNA splicing, (3) poly A addition, and (4) control of translational initiation. In our experiments, TNF-α did not increase the stability of IFN-γ-induced class II mRNA, indicating that TNF-α did not act at that level of post-transcriptional control. Preliminary results from our laboratory suggest that the increase in class II mRNA occurs primarily by an increase in transcription of the E-α gene since nuclear run-on assays detected no transcription of the class II genes without induction by IFN-γ, and enhanced transcription in the presence of IFN-γ plus TNF-α. Further experimentation is necessary to determine conclusively if TNF-α acts solely at the transcriptional level, or whether both transcriptional and post-transcriptional events result in increased class II MHC mRNA and protein.

The time required for the appearance of class II MHC mRNA following treatment with IFN-γ or IFN-γ/TNF-α (8 h) suggests that cytokine signal transmission is complex and may involve a number of intermediary steps. We examined whether protein synthesis was required for IFN-γ or IFN-γ/TNF-α-induced expression of astrocyte class II genes. The expression of class II mRNA was completely inhibited when CHX was included with IFN-γ and IFN-γ/TNF-α treatment, indicating that newly synthesized protein is required for astrocyte class II MHC gene expression. A minimum of 4 h of active protein synthesis was required for subsequent IFN-γ/TNF-α-induced class II mRNA expression, while 8 h was required for subsequent IFN-γ expression. However, in experiments where CHX was added 12 h after treatment with IFN-γ or IFN-γ/TNF-α, there was still a 45% and 25% reduction, respectively, in the expression of class II mRNA compared to astrocytes incubated with the cytokines alone. This indicates that the synthesis of novel proteins is required continuously for optimal class II gene expression in astrocytes. Other studies have shown that protein synthesis was required for up to 12 h after IFN-γ was added to murine P388D cells to detect an increase in the level of I-Aα (Boettger et al., 1988), while in peritoneal mouse macrophages, a 30% decrease in I-Aα mRNA levels was observed even when CHX was added after 12 h of IFN-γ treatment (Fertsch et al., 1987). In contrast, Celada et al. (1989) demonstrated that protein synthesis was only required for 30 min after murine macrophages were treated with IFN-γ for an increase in I-Aβ mRNA to be detected. Thus, different cell types have varying requirements for active protein synthesis to express class II mRNA in response to IFN-γ.

Other reports on IFN-γ-induced expression of class II genes have indicated that protein synthesis is not required. Induction of DRα mRNA in the human glioblastoma cell line U373-MG (Basta et al., 1988), dermal fibroblasts (Collins et al., 1986) and I-Aα in murine WEHI-3 cells (Woodward et al., 1989), occurs in the absence of protein synthesis. This suggests that the expression of class II mRNA in these cells is mediated by pre-existing *trans*-acting factors that are triggered by IFN-γ (Woodward et al., 1989). It is also important to

note that primary astrocytes (this study) and glioblastoma cells (Basta et al., 1988) differ in their requirements for protein synthesis for class II expression, illustrating fundamental differences between normal astrocytes and transformed glial cells.

TNF-α may be an important enhancer of class II expression in the CNS as it can function in an autocrine fashion on the astrocyte. In addition to responding to TNF-α and expressing specific high affinity receptors for this factor (Benveniste et al., 1989), astrocytes can also secrete TNF-α (Robbins et al., 1987; Sawada et al., 1989; Chung and Benveniste, 1990). More importantly, IFN-γ primes the astrocyte to produce TNF-α (Chung and Benveniste, 1990), thus IFN-γ can influence both TNF-α and class II gene expression in the astrocyte.

Although class II expression on astrocytes has been conclusively demonstrated in vitro, in vivo studies have generated conflicting results. Direct injection of IFN-γ into the brains of mice induced class II antigens on astrocytes, indicating that astrocytes have the potential to express these antigens in vivo (Wong et al., 1984). Many laboratories have examined whether astrocytes express class II antigens in a variety of immune-mediated disease states to better understand the possible role of the astrocyte as a local APC. Traugott et al. (1985) demonstrated class II expression on astrocytes in active chronic multiple sclerosis (MS) lesions, and then confirmed these studies by performing double-staining for both class II and GFAP (Traugott and Lebon, 1988). A study by Hofman et al. (1986) also identified class II-positive astrocytes in MS brain by double-staining. Rodriguez et al. (1987) have studied class II expression on glial cells in an animal model of CNS demyelination induced by Theiler's virus. In susceptible strains of mice (BIO.S and BIO.ASR2), the majority of class II-positive glial cells had morphological characteristics of astrocytes, while uninfected mice or resistant strains (BIO.S, (9R)) were class II negative. In SJL mice with acute or chronic relapsing experimental allergic encephalomyelitis (EAE), an animal model for MS, some class II-positive cells were identified as astrocytes (Sakai et al., 1986). However, other studies investigating the EAE model in Lewis rats failed to detect class II-positive astrocytes in the brain (Hickey et al., 1985; Matsumoto et al., 1986; Vass et al., 1986). These conflicting results may be due solely to technical problems involved with antigen fixation and staining methodologies, or may indicate that the ability of astrocytes to function as APC in vivo may only be relevant in certain diseases or specific stages of disease. Another possibility may be the loss of class II-positive astrocytes by class II MHC-restricted T cell-mediated cytotoxicity as shown by Sun and Wekerle (1986).

The disease EAE appears to be strain-specific as Brown-Norway rats and BALB/c or C57BL/6 mice are resistant, whereas Lewis rats and SJL mice are susceptible (Linthicum and Frelinger, 1982). Recent studies have demonstrated that astrocytes derived from susceptible strains express much higher levels of class II antigen upon treatment with either IFN-γ or virus compared to astrocytes prepared from EAE-resistant strains (Massa et al., 1987a, b). This hyperinduction of class II in EAE-susceptible animals was astrocyte specific as both peritoneal macrophages and microglial cells of susceptible and resistant strains showed identical patterns for class II induction. This differential expression of class II on astrocytes in response to IFN-γ compared to microglia suggests that regulation of class II expression on astrocytes may correlate with antigen-presenting capacity and ultimately, disease development in the CNS.

We have begun, at the molecular level, to dissect the regulatory mechanisms utilized by primary rat astrocytes for class II MHC gene expression. Future studies will focus on the regulation of gene expression at the transcriptional level, and IFN-γ/TNF-α-induced *trans*-acting regulatory factors required for class II gene expression.

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