Tumor necrosis factor-α enhances interferon-γ-mediated class II antigen expression on astrocytes

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Summary

Astrocytes can function as antigen-presenting cells (APC) upon expression of class II antigens, which are induced by interferon-γ (IFN-γ). Tumor necrosis factor-α (TNF-α) can act synergistically with IFN-γ with respect to class II expression on a variety of cells. As brain cells themselves can secrete TNF-like factors upon stimulation, we examined the effect of TNF-α on IFN-γ-mediated class II induction on astrocytes. TNF-α alone had no effect on class II expression, but did synergize with IFN-γ for enhanced expression of class II antigens. The specificity of TNF-α activity was demonstrated by blocking the amplifying effect of TNF-α with a polyclonal anti-TNF-α antibody. Kinetic analysis of the synergistic effect indicated that optimal TNF-α enhancement of class II expression was observed when astrocytes were pretreated with IFN-γ 12–24 h prior to TNF-α addition. A possible mechanism for the synergistic action between IFN-γ and TNF-α may be increased TNF-α receptor expression by IFN-γ. Astrocytes treated with IFN-γ for 24 h express more TNF-α receptors (3900/cell) than do untreated astrocytes (2483/cell), with no significant change in the binding affinity (K_d). These results suggest that the synergistic activity of TNF-α requires an inductive signal from IFN-γ, which in part may be increased TNF-α receptor expression. Altogether, our observations indicate that TNF-α enhances ongoing class II major histocompatibility complex gene expression in rat astrocytes, which in this system is initially induced by IFN-γ. TNF-α exerts its effect by binding to high affinity TNF-α receptors on astrocytes, whose expression is also enhanced by IFN-γ. These two cytokines work in concert to elevate class II expression on astrocytes, an event which can contribute to initiation and/or perpetuation of intracerebral immune responses.

Introduction

The central nervous system (CNS) has traditionally been considered an 'immunologically privileged site' due to its physical separation from the body's immune system (Baker and Billingham, 1977). This separation occurs in two major ways: (1) the CNS lacks for the most part a lymphatic system that drains the tissues and captures potential antigens, and (2) the CNS is protected from the blood by the blood–brain barrier (BBB), which is impermeable to many soluble substances, and restricts the migration of lymphoid cells into the CNS. Recent work has demonstrated, however, that glial cells may be involved in immunological events occurring in the brain. The astrocyte appears to function in a manner analogous to monocytes/macrophages, and has been postulated to act as an antigen-presenting cell (APC) in the CNS. The astrocyte is capable of secreting prostaglandins (Fontana et al., 1982), an interleukin-1 (IL-1)-like factor (Fontana et al., 1982), a tumor necrosis factor (TNF)-like molecule (Robbins et al., 1987), can be induced to express class II major histocompatibility complex (MHC) antigens following exposure to interferon-γ (IFN-γ) (Wong et al., 1984; Fierz et al., 1985) or virus (Massa et al., 1986, 1987), and can present antigen to T-cell clones in an MHC-restricted fashion upon expression of class II molecules (Fontana et al., 1984; Takiguchi and Frelinger, 1986).

TNF-α is a 17 kDa polypeptide synthesized by activated macrophages during host responses to microbial infections and neoplastic diseases (for reviews see Beutler and Cerami, 1987; Old, 1988). This cytokine causes hemorrhagic necrosis of certain murine tumors (Carswell et al., 1975), and is also cytostatic or cytotoxic to a variety of human tumor cell lines in vitro (Williamson et al., 1983; Sugarman et al., 1985). TNF-α elicits a variety of responses in nonmalignant cells. For example, TNF-α enhances the growth of normal fibroblasts (Vilcek et al., 1986), and induces expression of class I MHC antigens in endothelial cells and fibroblasts (Collins et al., 1986). With respect to glial cells, TNF-α increases expression of class I MHC antigens on human fetal astrocytes and oligodendrocytes (Mauerhoff et al., 1988) and mouse astrocytes (Lavi et al., 1988), while having

no effect on induction of class II MHC molecules. The action of TNF-α requires specific binding to high-affinity surface receptors which are expressed not only on most malignant cells, but also on normal cells of various tissues (Aggarwal et al., 1985; Tsujimoto et al., 1985, 1986; Ruggiero et al., 1986; Imamura et al., 1987).

IFN-γ and TNF-α have been shown to act synergistically in a number of systems including lysis of tumor cells in vitro (Williamson et al., 1983; Stone-Wolff et al., 1984), induction of monocytic differentiation (Trinchieri et al., 1986), and class II induction in human islet cells (Pujol-Borrell et al., 1987), colocarcinoma cells (Pfizenmaier et al., 1987), and human monocytes (Arenzana-Seisdedos et al., 1988).

As brain cells themselves (astrocytes and microglia) can secrete TNF-like factors upon stimulation (Frei et al., 1987; Robbins et al., 1987), we were interested in examining the effect of TNF-α on IFN-γ induction of class II MHC antigens on astrocytes. We report that recombinant human TNF-α enhances IFN-γ-mediated expression of class II molecules in a time-dependent manner, and that this enhancement, in part, may be the result of increased TNF-α receptor expression by IFN-γ. These two cytokines may play a pivotal role in the regulation of intracerebral immune responses by modulating the expression of class II MHC antigens on astrocytes.

Materials and methods

Recombinant proteins and antibodies

Rat recombinant IFN-γ and human recombinant TNF-α were purchased from AMGen Biologicals (Thousand Oaks, CA, U.S.A.), and human recombinant TNF-α was also the generous gift from Genentech (South San Francisco, CA, U.S.A.). Anti-rat IFN-γ monoclonal antibody was purchased from AMGen Biologicals, anti-human TNF-α polyclonal antibody was from Endogen (Boston, MA, 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 (clones OX-6 and OX-3) 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.).

Primary glial cell cultures

Primary glial cell cultures were established from neonatal rat cerebra by a modification of the McCarthy and de Vellis technique (McCarthy and de Vellis, 1980) as previously described (Benveniste and Merrill, 1986). Meninges were removed prior to 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 nonessential amino acid mixture, 0.1% gentamicin, and 10% fetal bovine serum (Hyclone, Logan, UT, U.S.A.). Astrocytes were obtained after 10 days in primary culture, and were used up to the age of 10 weeks (70 days). Briefly, oligodendrocytes were separated from the astrocytes by mechanical dislodging, and then the astrocytes were obtained by trypsinization (0.25% trypsin-0.02% EDTA). The cells were counted using trypan blue; cell viability was 99–100%. The astrocytes were monitored for purity by immunofluorescence, and by nonspecific esterase staining for contaminating microglia as previously described (Benveniste and Merrill, 1986). 5.0×10^4 primary astrocytes were plated on 12 mm glass coverslips, incubated in culture medium for 2 days, washed twice with 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 > 95% positive for GFAP, and less than 3% of the cells were microglia based on their positive staining for nonspecific esterase.

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 either fresh DMEM-10% FBS or DMEM-serum-free medium (1 ml) was added to the wells. Triplicate wells of primary rat astrocytes were treated with 20–80 U/ml of recombinant rat IFN-γ and/or 1–50 ng/ml of recombinant human TNF-α for various incubation periods (1–4 days). The amounts of IFN-γ and TNF-α used were selected on the basis of prior experimentation. At each time point, the cells were trypsinized and stained for class II antigens. Preliminary studies preceded the optimization of the method. Briefly, astrocytes were incubated with 30 μl of OX-6 or OX-3 monoclonal antibody for 60 min in the cold, washed 3 times with phosphate-buffered saline (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.

Immunofluorescence staining for class II antigens

5×10^4 primary rat astrocytes were plated on 12 mm glass coverslips with or without growth factors for various time periods. At the end of the incubation period, the cells were washed twice with PBS, and stained live for class II molecules. The cells were stained for class II using a monoclonal antibody to rat class II determinants (OX-6) for 60 min in the cold, followed by a 30 min incubation with goat anti-mouse Ig-FITC (1:20). The cells were then fixed for 10 min in 3.7% paraformaldehyde, mounted in 30% glycerol, and

visualized by fluorescent microscopy. 100–200 cells were examined per coverslip.

Iodination of tumor necrosis factor

Recombinant human TNF-α was iodinated by the solid-phase lactoperoxidase procedure as previously described by Baglioni et al. (1985). 5 μg of TNF-α was incubated for 1 h at 4°C with Enzymobead iodination reagent (Bio-Rad, Richmond, CA, U.S.A.) and ^{125}I (1 mCi). The ^{125}I-TNF-α was purified by column chromatography on a Bio-Gel P-6DG column. The specific activity of the ^{125}I-TNF-α was $2.1–2.5 \times 10^4$ cpm/ng TNF-α. Retention of ^{125}I-TNF-α biological activity was confirmed by performing parallel experiments for enhancement of IFN-γ-induced class II antigen expression with unlabeled TNF-α.

Binding assays

$2–3 \times 10^5$ astrocytes/well were plated in 24-well tissue culture plates (Falcon) in 1 ml of DMEM-10% FBS, and allowed to grow to confluency. At this point, the medium was removed and duplicate cultures received either DMEM-10% FBS alone or 40–80 U/ml of IFN-γ for an additional 24 h. The concentration of IFN-γ used varied depending on the lot of IFN-γ, but routinely was between 40 and 80 U/ml. The cells were then

TABLE 1

TNF-α DOES NOT INDUCE CLASS II MHC ANTIGEN EXPRESSION ON RAT ASTROCYTES

Cell treatment [a]	Class II positive (%)
Culture medium with 10% serum	0.55 ± 0.05 [b]
IFN-γ [c], 20 U/ml	12.30 ± 1.24
TNF-α [d], 1 ng/ml	0.90 ± 0.18
TNF-α, 10 ng/ml	0.25 ± 0.03
TNF-α, 25 ng/ml	0.00 ± 0.00
TNF-α, 50 ng/ml	0.55 ± 0.07
Culture medium, serum-free	3.88 ± 1.2 [e]
IFN-γ, 80 U/ml	16.00 ± 3.2
TNF-α, 10 ng/ml	2.00 ± 0.2
TNF-α, 50 ng/ml	3.20 ± 0.05

[a] 48 h incubation.
[b] Mean ± SD of two experiments performed in triplicate.
[c] Recombinant rat IFN-γ.
[d] Recombinant human TNF-α.
[e] Mean ± SD of two experiments performed in duplicate.

washed 2 times with binding buffer (BB) (Iscove's medium with 25 mM Hepes and 2 mg/ml bovine serum albumin (BSA), pH 7.2), and incubated with the amounts of ^{125}I-TNF-α indicated in the text for 4–5 h at 4°C. The cells were then washed 3 times with cold BB, solubilized in the presence of 2 N NaOH (60 min at 37°C), and radioactivity was determined in a Beckman gamma counter. Specific binding is defined as the difference between total binding and nonspecific binding in the presence of a 100-fold excess of unlabeled TNF-α. Nonspecific binding did not exceed 8% of total binding.

Statistical analysis

Levels of significance for comparisons between samples were determined using the Student t-test distribution.

Results

TNF-α enhances IFN-γ-mediated class II MHC antigen induction on rat astrocytes

TNF-α alone induced little or no detectable class II antigens on rat astrocytes, while IFN-γ caused an induction of class II antigen synthesis (Table 1). This effect was seen in both serum-containing and serum-free media. Similar results were obtained with a 72 and 96 h incubation (data not shown). However, when added simultaneously with IFN-γ, TNF-α at concentrations ranging from 10 to 50 ng/ml significantly enhanced class II antigen expression ($p \leq 0.001$). Optimal expression was demonstrated using IFN-γ at 20 U/ml and TNF-α at 50 ng/ml for 48 h (Fig. 1). Comparable results were obtained when these same experiments were performed under serum-free conditions: 16.0% positive upon IFN-γ stimulation and 40.0% positive upon stimulation with both TNF-α and IFN-γ. These findings indicate that the presence of serum does not interfere with or modulate class II antigen expression. Immunohistochemical staining for class II antigens was also performed on nontrypsinized astrocytes to compare with the FACS data. Similar results were obtained: 7–10% positive upon stimulation with IFN-γ, and 40–47% positive with both IFN-γ and TNF-α, indicating that the trypsinization procedure did not interfere
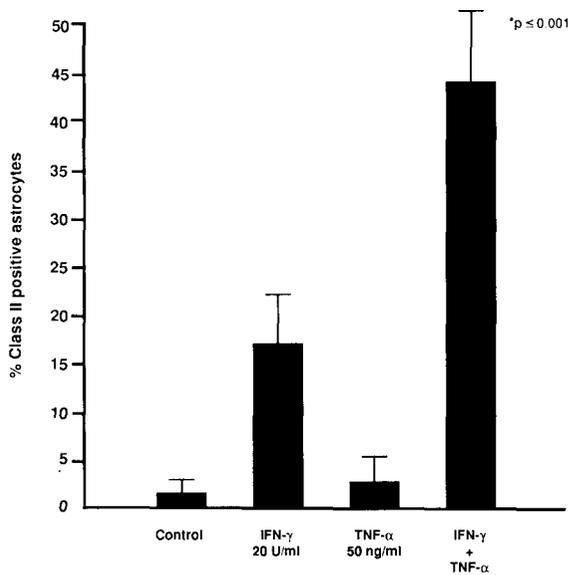
Fig. 1. TNF-α enhances IFN-γ-induced class II MHC antigen expression on rat astrocytes. Rat astrocytes were incubated with control medium, IFN-γ (20 U/ml), TNF-α (50 ng/ml), or IFN-γ + TNF-α for 48 h, trypsinized, and stained for class II antigens. The data presented are the mean ± SD of eight separate experiments. Statistical analysis was performed comparing IFN-γ alone values to IFN-γ + TNF-α.

with class II antigen expression. These results imply synergistic action of these two cytokines on rat astrocytes, especially as TNF-α alone has no effect on class II expression.

The specificity of this synergistic response was investigated using antibodies against both IFN-γ and TNF-α. To determine whether the enhancement of IFN-γ-mediated class II antigen expression was indeed due to TNF-α, we attempted to abrogate the enhancing effect with an antibody to TNF-α. Table 2 shows that the inclusion of such an antibody essentially negates the enhancing activity of TNF-α, while not influencing the inductive effect of IFN-γ alone. The polyclonal antibody to TNF-α alone had no effect on class II antigen expression compared to culture media. Table 2 also demonstrates that the addition of a monoclonal antibody to IFN-γ abrogates IFN-γ-induced class II expression as expected, and also inhibits the ability of TNF-α to amplify IFN-γ-induced class II expression. The monoclonal antibody alone had no effect on class II antigen

expression compared to culture media. The data from Table 2 suggest that TNF-α is responsible for enhancing the ability of IFN-γ to induce class II antigens.

Kinetic analysis of IFN-γ / TNF-α pretreatment on astrocyte class II antigen expression

We next determined the effect of various times of pretreatment with either TNF-α or IFN-γ on astrocyte class II expression. Rat astrocytes were pretreated for 0–48 h with one cytokine, then the other cytokine was added for an additional 48 h at which time class II antigen expression was examined by FACS. As shown in Fig. 2A, pretreatment with IFN-γ augments the synergistic effect of TNF-α. This effect is most pronounced when the cultures are pretreated with IFN-γ for 12–24 h, with an 18 h preincubation being most effective. Pretreatment with IFN-γ for 48 h, however, results in a diminution of the synergistic effect of TNF-α, and in fact, is comparable with

TABLE 2

INHIBITION OF TNF-α ENHANCEMENT OF IFN-γ-MEDIATED CLASS II MHC ANTIGEN EXPRESSION BY ANTIBODIES TO TNF-α AND IFN-γ

Cell treatment [a]	Class II positive (%)
Culture medium	1.6 ± 0.5
IFN-γ [b]	19.0 ± 2.8
TNF-α [c]	1.3 ± 0.4
IFN-γ + TNF-α	30.0 ± 1.4
[TNF-α + anti-TNF-α] [d,e] + IFN-γ	18.1 ± 3.6
[IFN-γ + anti-IFN-γ] [f,g] + TNF-α	6.1 ± 0.3
Anti-TNF-α [e]	0.0 ± 0.0
Anti-IFN-γ [g]	2.6 ± 0.1

[a] Rat astrocytes were incubated with culture medium or cytokines for 48 h, washed, and then stained for class II MHC antigens.
[b] 20 U/ml of rat recombinant IFN-γ.
[c] 50 ng/ml of human recombinant TNF-α.
[d] Antibody was preincubated for 1 h at 37°C with TNF-α, then added to cultures containing IFN-γ for an additional 48 h.
[e] 500 neutralizing units (NU)/ml of polyclonal antibody against human recombinant TNF-α.
[f] Antibody was preincubated for 1 h at 37°C with IFN-γ, then added to cultures containing TNF-α for an additional 48 h.
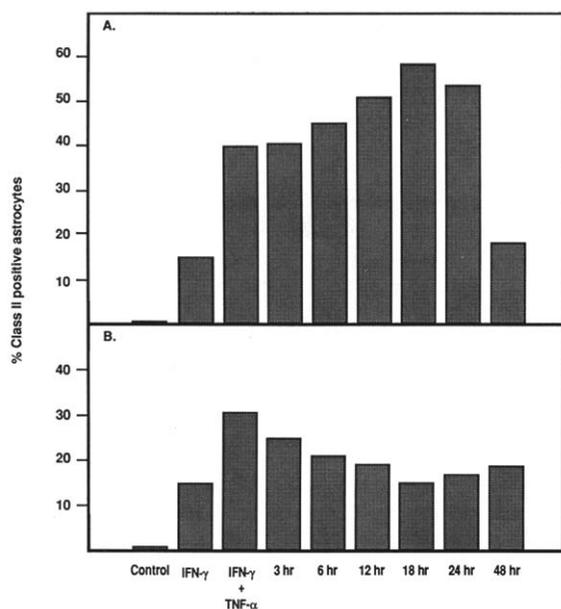[g] 40 NU/ml of monoclonal antibody against rat recombinant IFN-γ.

Fig. 2. Kinetic analysis of IFN-γ/TNF-α-pretreated astrocyte class II antigen expression. *A*: Rat astrocytes were preincubated with IFN-γ (20 U/ml) for various times (3–48 h), at which point TNF-α (50 ng/ml) was added for an additional 48 h. Control alone contained culture medium for 96 h, IFN-γ (20 U/ml) alone was incubated with culture medium for 48 h, then IFN-γ for 48 h, and IFN-γ plus TNF-α was incubated with culture medium for 48 h, then received IFN-γ and TNF-α simultaneously for an additional 48 h. At the end of the incubation, cells were trypsinized, stained for class II antigens, and analyzed by FACS. The data presented are the mean of two experiments. *B*: Rat astrocytes were preincubated with TNF-α (50 ng/ml) for various times (3–48 h), at which point IFN-γ (20 U/ml) was added for an additional 48 h. Control alone contained culture medium for 96 h, IFN-γ (20 U/ml) alone was incubated with culture medium for 48 h, then IFN-γ for 48 h, and IFN-γ plus TNF-α was incubated with culture medium for 48 h, then received IFN-γ and TNF-α simultaneously for an additional 48 h. At the end of the incubation, cells were trypsinized, stained for class II antigens, and analyzed by FACS. The data presented are the mean of two experiments.

the IFN-γ alone treatment. As shown in Fig. 2*B*, addition of TNF-α for 12–48 h prior to IFN-γ results in no synergistic effect, whereas a synergistic effect is observed only when TNF-α is added 3 and 6 h prior to IFN-γ. This effect is, however, not as pronounced as that seen when the two cytokines are added simultaneously. These results indicate that the synergistic effect is observed when treatment with IFN-γ precedes the addition

of TNF-α, or when IFN-γ and TNF-α are added together, again suggesting an inductive signal from IFN-γ.

Binding of ^{125}I-TNF-α to rat astrocytes: increased TNF-α receptor expression via IFN-γ

A possible explanation for the synergism observed between TNF-α and IFN-γ is an increase in the synthesis of TNF-α receptors by IFN-γ. The effect of a 24 h incubation with IFN-γ on the amount of ^{125}I-TNF-α specifically bound during a subsequent 4 h exposure at 4°C was compared in rat astrocytes. Treatment of astrocytes with IFN-γ increased specific binding of ^{125}I-TNF-α compared to untreated cells (Fig. 3). Scatchard analysis of this data ((Scatchard, 1949); Fig. 3, inset) indicates that incubation with IFN-γ increases the number of specific TNF-α receptors (2650 receptors/untreated cell vs. 4800 receptors/IFN-γ-treated cell), but does not change the dissociation constant (K_d) (0.83 × 10^{-9} M/untreated cells vs. 0.87 × 10^{-9} M/IFN-γ-treated cells). Table 3 contains data from three separate binding experiments, and illustrates that the increase in TNF-α receptors on IFN-γ-treated astrocytes (3900 ± 781) compared to untreated cells (2483 ± 332) is significant ($p ≤ 0.05$), with no significant change in binding affinity (K_d). Thus, astrocytes express one class of high-affinity binding sites for TNF-α.

Kinetic analysis of pretreatment periods with IFN-γ demonstrated that a 48 h incubation with IFN-γ prior to the addition of TNF-α did not result in a synergistic enhancement of class II antigen expression (Fig. 2*A*). TNF-α receptor ex-

TABLE 3

INCREASED NUMBER OF TNF-α RECEPTORS IN RAT ASTROCYTES AFTER INCUBATION WITH IFN-γ

Treatment condition	TNF receptors (Scatchard analysis) [a]	
	Receptors/cell ± SD	$K_d × 10^{-9}$ M ± SD
Untreated	2483 ± 332	0.69 ± 0.18
IFN-γ-treated [b]	3900 ± 781 ($p ≤ 0.05$) [c]	0.71 ± 0.19 (NS) [d]

[a] Based on experimental data shown in Fig. 3 and two additional experiments performed in duplicate.
[b] 24 h incubation with IFN-γ (80 U/ml).
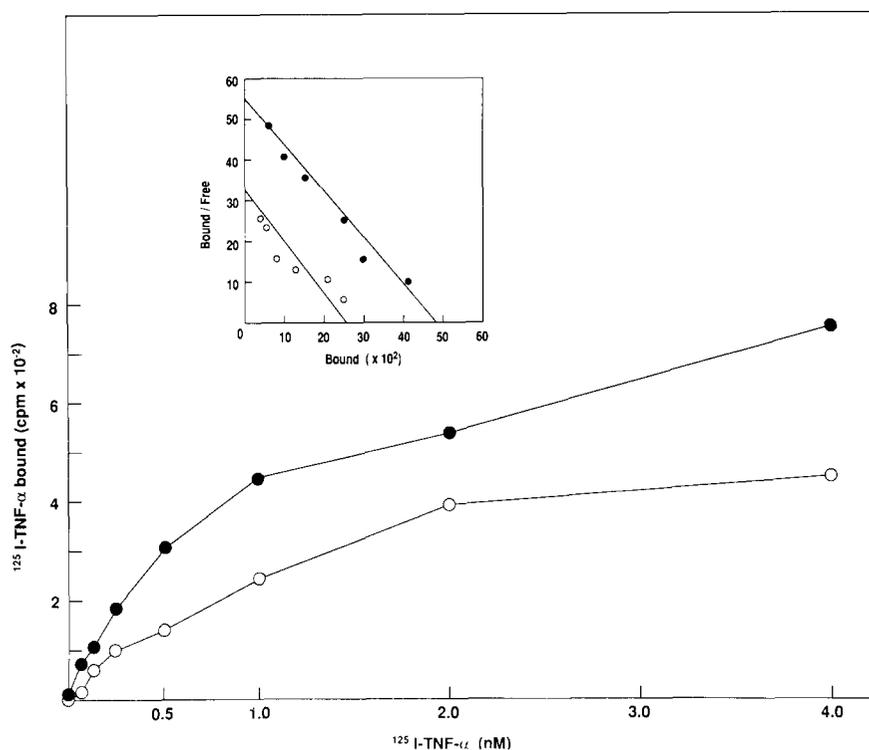[c] Student's *t*-test (two-tailed).
[d] NS = not significant.

Fig. 3. Effect of IFN-γ on the binding of ^{125}I-TNF-α to rat astrocytes. 3×10^5 cells were incubated in the presence (●) or absence (○) of 80 U/ml of rat recombinant IFN-γ for 24 h at 37°C. ^{125}I-TNF-α binding to the cells was then determined as described in Materials and Methods. Nonspecific binding was measured by adding a 100-fold excess of unlabeled TNF-α together with ^{125}I-TNF-α, and has been subtracted from the values shown. Experimental values are means of duplicate determinations. Inset, a Scatchard plot of the data.

pression may be decreased or downregulated after an extended incubation with IFN-γ. To test this possibility, rat astrocytes were treated with IFN-γ
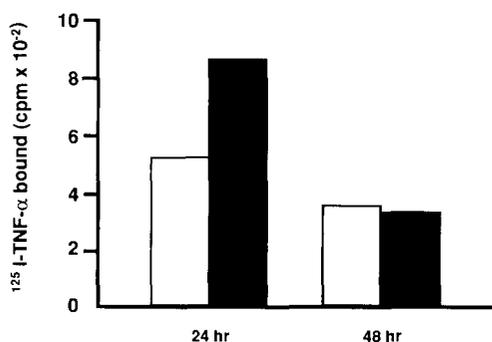

Fig. 4. Binding of ^{125}I-TNF-α to astrocytes treated with IFN-γ for 24 or 48 h. 3×10^5 astrocytes were treated with 80 U/ml of rat recombinant IFN-γ (■) or untreated (□) for 24 or 48 h. The cells were washed 2 times and incubated for 4 h at 4°C with 4 nM of ^{125}I-TNF-α. Nonspecific binding was measured in parallel incubations with a 100-fold excess of unlabeled TNF-α, and was subtracted from the data shown.

(80 U/ml) for 24 and 48 h, and specific binding assessed. As shown in Fig. 4, specific binding of ^{125}I-TNF-α to cells continuously treated with IFN-γ for 48 h returned to the level of untreated cells, in contrast to the increased binding observed in 24 h IFN-γ-treated cells. Constitutive TNF-α receptor expression is also slightly diminished in vitro after 48 h compared to 24 h untreated control cells.

Discussion

In this report we have demonstrated that two cytokines, IFN-γ and TNF-α, synergize in the induction of class II MHC antigen expression in rat astrocytes. TNF-α alone had no effect on class II MHC expression in astrocytes, which are cells that are constitutively negative for class II. TNF-α does, however, synergize with IFN-γ for enhanced class II gene expression. It appears that TNF-α

does not act as a direct inducer of class II gene expression, but instead amplifies an ongoing expression which in this case is induced by IFN-γ. The specificity of TNF-α action was confirmed by blocking the amplifying effect of TNF-α by anti-TNF-α antibody. This same type of synergistic effect between IFN-γ and TNF-α has been noted with respect to HLA-DR expression in human monocytes (Arenzana-Seisdedos et al., 1988), and human endothelial cells (Leeuwenberg et al., 1988).

The kinetic data from our study indicate that TNF-α enhancement of IFN-γ-induced MHC expression requires an inductive signal from IFN-γ. When TNF-α was added 12–48 h prior to the addition of IFN-γ, a very slight to negligible synergistic effect was observed compared to when TNF-α was added 3–6 h prior to IFN-γ, or simultaneously with IFN-γ. In contrast, an augmented synergistic effect was noted when astrocytes were preincubated with IFN-γ, then treated with TNF-α for an additional 48 h. This was particularly pronounced when the cells were preincubated with IFN-γ for 12–24 h prior to the addition of TNF-α. However, when cells were pretreated with IFN-γ 48 h prior to incubation with TNF-α, no synergistic effect was observed, and in fact, the number of class II-positive cells was comparable to that seen with IFN-γ alone. This would suggest that the synergistic activity of TNF-α requires an 'inductive signal' from IFN-γ which: (1) persists for up to 24 h, and (2) ceases to exist in cells which have been in continuous culture with IFN-γ for 48 h.

A possible mechanism for the synergy between TNF-α and IFN-γ could be increased TNF-α receptor expression induced by IFN-γ. This has been demonstrated to occur in other TNF-α-responsive cells such as human colon carcinoma cell lines, HeLa cells, melanoma cells, and human cervical carcinoma cell lines (Aggarwal et al., 1985; Ruggiero et al., 1986; Tsujimoto et al., 1986). To determine if TNF-α receptor expression was increased by IFN-γ in primary rat astrocytes, we performed binding experiments on both untreated and IFN-γ-treated astrocytes. Our data indicate that astrocytes incubated with IFN-γ for 18–24 h express more TNF-α receptors (3900 ± 781/cell) than do untreated astrocytes (2483 ± 332/cell). The apparent binding affinity (K_d) calculated

from these data was 0.71×10^{-9} M for IFN-γ-treated cells and 0.69×10^{-9} M for untreated cells. These results demonstrate that treatment with IFN-γ does not significantly change the binding affinity of TNF-α receptors, but increases their number per cell. TNF-α receptor expression is optimal after a 24 h incubation with IFN-γ, and diminishes after a 48 h incubation. These results are in agreement with the hypothesis that IFN-γ increases the sensitivity of rat astrocytes to class II antigen amplification by TNF-α by inducing the synthesis of TNF-α receptors. The kinetic data indicate that pretreatment with IFN-γ for 12–24 h results in the most pronounced synergistic effect of IFN-γ and TNF-α, while a 48 h pretreatment with IFN-γ prior to the addition of TNF-α does not culminate in synergistic activity between these two cytokines. Thus, it appears that the 'inductive signal' for TNF-α enhancement of class II antigen expression by IFN-γ is, in part, increased TNF-α receptor expression. This is the first formal demonstration of biologically active TNF-α receptors on primary rat astrocytes and their modulation by the lymphokine IFN-γ. The binding affinity of astrocyte TNF-α receptors is similar to those reported for other primary cells and cell lines, and indicates a single class of high affinity receptors. This increase in TNF-α receptors by IFN-γ may well be the signal by which TNF-α asserts its biological effect in astrocytes with respect to class II antigen expression. As TNF-α alone can increase class I MHC expression in astrocytes (Lavi et al., 1988), constitutively expressed TNF-α receptors also are biologically functional.

In addition to responding to TNF-α and expressing receptors for this factor, astrocytes have been shown to secrete a TNF-α-like molecule upon stimulation with calcium ionophore and/or lipopolysaccharide (Robbins et al., 1987). This factor, termed astrocyte cytotoxic factor (AST-CF), killed murine L929 cells, the traditional target cell for TNF-α, as well as rat oligodendrocytes, the myelin-producing cells of the CNS. Our results would also suggest that TNF-α can act in an autocrine fashion on astrocytes. It will be important to determine if IFN-γ or other cytokines induce TNF-α gene expression in astrocytes as has been demonstrated in other systems (Arenzana-Seisdedos et al., 1988).

Class II MHC gene expression is generally limited to bone marrow-derived cells such as B-lymphocytes, and cells of the macrophage/dendritic cell lineage. These class II-positive cells possess APC function, i.e., they can stimulate T-lymphocytes reactive to antigen plus MHC (Benacerraf and Germain, 1978). Several non-lymphoid cells, upon expression of class II antigens, can also function as APC. These include pancreatic beta cells (Markmann et al., 1988), keratinocytes (Gaspari et al., 1988), brain endothelial cells (McCarron et al., 1985), microglial cells (Frei et al., 1987), and astrocytes (Fontana et al., 1984). There are several mechanisms by which class II MHC expression is induced in astrocytes: (1) treatment with IFN-γ (Fierz et al., 1985), and (2) exposure to viruses (measles virus, coronavirus) (Massa et al., 1986, 1987). Induction of class II by viral particles differs from that by IFN-γ with respect to the magnitude and kinetics of class II expression (Massa et al., 1986). TNF-α has been shown to amplify class II induction by viral particles (Massa et al., 1987), again demonstrating an effect of TNF-α on ongoing class II gene expression.

In vivo, there may be several ways by which astrocytes are induced to express class II antigens. Direct induction by neurotrophic viruses would represent one mechanism, and infiltration of activated T-lymphocytes from the periphery another. Pathological events within the CNS often result in the breakdown of the BBB, which would permit cells of the immune system to enter the CNS. Patients with various neurological diseases such as viral encephalitis and multiple sclerosis (MS) have inflammatory infiltrates composed of T-lymphocytes, B-lymphocytes, and macrophages in their brains (Traugott et al., 1983; Moench and Griffin, 1984). Activated T-lymphocytes could provide an endogenous local source of IFN-γ in the CNS, and thereby initiate class II antigen expression on astrocytes. Indeed, IFN-γ has been localized in frozen sections of MS brain (Traugott and Lebon, 1988), and the synthesis of IFN-γ and IL-6 has been detected in the CNS during viral meningitis and encephalitis (Frei et al., 1988). Once astrocytes express class II, they can now function as APC and induce further expansion of T-lymphocyte clones within the CNS. TNF-α,

released by infiltrating macrophages or produced locally by activated astrocytes and/or microglia, can interact with class II-positive astrocytes previously activated by IFN-γ and enhance class II MHC expression.

By regulating class II antigen expression and thereby stimulating the APC function of astrocytes, IFN-γ and TNF-α in concert participate in the control of interactions between T-lymphocytes and the CNS. This has implications for normal as well as pathological immune responses since malfunction of this system could result in accelerated and/or aberrant immune reactions within the CNS.

Acknowledgements

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