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Interferon-beta is a potent promoter of nerve growth factor production by astrocytes

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the degree of ***MASTER OF SCIENCE***

" The small differences between his conclusions and my own ...
such divergence, by which one can push research forward,
is always useful to science ".

Camillo Golgi (1843-1926)
(reported by Jacobson, 1991)

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I. Abstract

Recent clinical evidence has suggested that interferon-β is efficacious in the treatment of multiple sclerosis, a demyelinating disease that is thought to be immune mediated. The mechanism of its efficacy remains unclear. Possible modes of action include effects of interferon-β on systemic or CNS immune or non-immune parameters. In the latter context, astrocytes are known to provide trophic support to oligodendrocytes and neurons. Since nerve growth factor (NGF) is reported to cause adult porcine oligodendrocytes to proliferate and extend processes and since these phenotypes can impact favourably on remyelination, we have examined the possibility that interferon-β could increase the astrocyte production of NGF. Recombinant mouse interferon-β (rmIFN-β) was added to confluent neonatal mouse astrocyte cultures. Analyses reveal an increase in NGF mRNA and protein elicited by rmIFN-β. This finding may be relevant to interferon-β's clinical efficacy in multiple sclerosis.

II. Résumé

Des résultats cliniques démontrent que l'interféron-β est efficace dans le traitement de la sclérose en plaques (SP); une pathologie démyélinisante que l'on croit d'origine immunitaire. Le mode de fonctionnement de l'interféron-β, dans cette pathologie, demeure inconnu. Il est possible que l'interféron-β interagit avec les systèmes immunitaire, neuroimmunitaire ou non-immunitaire. Cette dernière possibilité inclue les astrocytes qui présentent des capacités trophiques pour les neurones et les oligodendrocytes. Sachant que le facteur de croissance neuronal, soit NGF (nerve growth factor), augmente le taux de la prolifération et influence la différenciation morphologique des oligodendrocytes porcins et que ces phénotypes sont prérequis à la remyélinisation, nous avons examiné la possibilité que les astrocytes augmentent leur production d'NGF lorsqu'ils furent traités avec de l'interféron-β. Les cultures d'astrocytes confluentes, provenant de souriceaux nouveaux-nées, furent traités avec de l'interféron-β de souris recombinant. Ce traitement augmenta l'ARN et la protéine d'NGF. Ces résultats pourraient être pertinents à l'efficacité de l'interféron-β dans le traitement de la SP.

III. Acknowledgments

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1. Introduction

1.1 What my work entails

As previously mentioned and as detailed later on, there are many mechanisms that may explain the effectiveness of IFN-β in multiple sclerosis (MS). None of these mechanisms are mutually exclusive. One of these includes a direct effect on astrocytes. Taking into account that part of the astrocyte function is the synthesis of trophic factors for oligodendrocytes and neurons, it is possible that part of IFN-β's efficacy in MS is through the increased production of trophic factors by astrocytes. **It is therefore the purpose of my work to determine whether or not IFN-β modulates trophic factor production, e.g. upregulation of NGF mRNA and protein, in astrocytes.**

The upcoming sections will overview how MS, IFN-β and astrocytes relate to one another in an attempt to understand how IFN-β may improve the clinical outcome of MS via astrocytes.

1.2 Brief history of MS

It is virtually impossible to ascertain the date of the first report concerning MS. Nevertheless, there are a couple of noteworthy reports which are mentioned. The oldest of these describes a fourteenth century woman named St. Lidwina of Shiedam whose case is reported by a court physician (reported by Medaer, 1979; Francis et al. 1991). Another such report is about Sir Augustus Frederick d'Esté, a nineteenth century relation of Queen Victoria and King George III (reported by Francis et al. 1991; Poser, 1994). Despite the uncertainties of the latter report, it would be the nineteenth century that would see the birth and development of accurate descriptions of MS.

The clinical features of MS were described for the first time by two physicians Jean Cruveilhier and Robert Carswell in 1835 and in 1838 respectively (reported by Dejong, 1970; Medaer, 1979; Francis et al. 1991). A description of the neuropathological hallmarks of MS which Cruveilhier called " sclérose en taches " or " sclérose en îles " soon followed. Frerichs diagnosed, in 1849, the first living patient with MS. Rokitansky in 1850 and Rindfleisch, in 1863, provided detailed anatomical descriptions of the disease. In addition, Rindfleisch was the first to suggest that the

disease started around blood vessels (reported by Dejong, 1970; Francis et al. 1991). In 1868 the French neurologist Charcot gave this pathology its modern name, " Sclérose en plaques ", which means multiple sclerosis. This neurologist was responsible for correlating the clinical symptoms of the patients with the neuropathology (reported by Dejong, 1970; Medaer, 1979).

The idea that this pathology could be instigated by an infectious agent traced its steps back to the nineteenth century when Marie, in 1884, suggested that a central nervous system (CNS) infection might account for the pathogenesis of MS (reported by Dejong, 1970; ter Meulen and Stephenson, 1983). Although many infectious agents had been thought to be implicated in MS (Johnson, 1975), none demonstrated a causal relationship to the disease (Bell and Lathrop, 1996; Ebers, 1996; Ebers et al. 1996).

Many studies done on relatively large cohorts in the twentieth century tried to characterize the general features of MS (Adams, 1983). These studies helped to further Charcot's initial effort at correlating the neuropathology with the clinical symptoms. Other studies focused on different aspects of MS which are covered in various sections.

2. Multiple sclerosis

2.1 Anatomical and cellular description of lesions

While the exact etiology of MS is still unknown, it has been believed, for several reasons, to be an inflammatory disease that affects CNS myelin. In support of this, is the presence of inflammatory cells, such as the macrophages and all the major types of lymphocytes, in and around the periphery of CNS lesions, especially active lesions (Brosnan and Raine, 1996). Second, there is the synthesis of oligoclonal immunoglobulins within the CNS (Francis et al. 1991; Weiner et al. 1995; Brosnan and Raine, 1996; Cuzner and Norton, 1996). Third, in the blood of patients with MS alterations of T cell populations, the presence of activated T cells, an increased secretion of inflammatory cytokines, and a loss of suppressor T cell function are hallmarks of MS (Weiner et al. 1995; Brosnan and Raine, 1996; Stinissen et al. 1997).

MS is characterized by regions of demyelination and the presence of inflammation. Both active and inactive lesions can exist side-by-side. There is,

nonetheless, a greater degree of inflammation in the active lesion. One of the methods used to detect active lesions is by MRI (magnetic resonance imaging). This technology allows the monitoring of blood-brain barrier damage (Lassmann et al. 1994) which is known to enable serum proteins to enter the brain parenchyma (Brosnan and Raine, 1996; Cuzner and Norton, 1996; Lucchinetti et al. 1996). Indeed, active and inactive lesions are typified by the presence of serum proteins in the brain, although these proteins are sequestered by the local cellular population. Other proteins, including myelin proteins, can also be detected within macrophages by immunocytochemistry. Additional markers of this pathology include the upregulation of certain antigens such as MHC (major histocompatibility complex) antigens and cell adhesion molecules (Cannella and Raine, 1995; Lucchinetti et al. 1996).

Although the demyelination process is a hallmark of MS, the ongoing disability that is experienced by patients is mostly due to the destruction of axons (Lassmann et al. 1994), which can be detected by proton magnetic resonance spectroscopy or MRI (Arnold et al. 1990; Allen et al. 1991; Barnes et al. 1991; Matthews et al. 1991; Kidd et al. 1993; Gass et al. 1994; Davie et al. 1995; Davies et al. 1995; Sobel, 1995). It is perhaps ironic that the destruction of axons in MS, which has been known since Charcot's time (reported by Davie et al. 1995), is only now being accorded the attention it deserves. In light of these observations **my thesis seeks to address the following question: Can IFN-β increase the trophic factor support, given by astrocytes, to neurons and oligodendrocytes ?**

The varied anatomical and cellular components of MS are mirrored by the existence of different clinical subtypes of the disease.

2.2 Subtypes of MS

It was not long after Charcot's coining of the expression "Sclérose en plaques" that Marburg defined a subtype of this illness which he termed acute MS (Lassmann et al. 1994; Lucchinetti et al. 1996). This category of MS is characterized by a progressive or relapsing-remitting disease course. The severe neurological problems which accompany this class of MS can lead to death within 6 to 12 months. In contrast to the classic chronic MS, acute MS is often accompanied by inflammatory demyelination in the peripheral nervous system (PNS) (Lassmann et al. 1994).

Other subtypes of MS include relapsing-remitting, relapsing-remitting progressive, chronic progressive and stable MS (Weiner et al. 1995). Relapsing-remitting MS (RRMS) is characterized by an attack that takes 1 to 2 weeks to manifest itself, lasts 1 to 2 months, subsides on its own or with medication, and returns to base-line levels. During the attack, different regions of the brain may be affected which can translate into various symptoms such as sensory, motor, cerebellar or visual problems. In contrast, patients that suffer from relapsing-remitting progressive MS do not return to base-line values after an attack but are disabled in a stepwise manner. Chronic-progressive MS does not have periods of remission and there is a steady worsening of the clinical picture. This subtype can be divided into 2 categories according to the diagnosis at the onset of the disease. The first of these categories, primary progressive, is thus diagnosed from the onset, while the other, secondary progressive, develops after an initial diagnosis of RRMS. Finally, stable MS is defined by the absence of clinical disease and the lack of worsening in the patient's condition, as seen from their perspective, during an interval of twelve months (Weiner et al. 1995).

It is worth mentioning that the former nomenclature may vary somewhat according to reports (Lassmann et al. 1994; Lucchinetti et al. 1996). Moreover, the characteristics of some subtypes may vary clinically as progressive MS has been described to manifest itself, then apparently stop for a time or sometimes permanently (Weinshenker, 1994). Finally, the complexity of MS may be compounded or explained by its complexity at the genetic level.

2.3 Genetics of MS

2.31 Who is at risk ?

It is presently impossible to predict exactly who is at risk of developing MS in the general population. The exceptions are the immediate family members of an MS patient, especially in the case of a monozygotic and, to a lesser extent, a dizygotic twin (Ebers, 1994; Sadovnick and Ebers, 1995; Ebers, 1996). In addition, there seems to be a sex bias as women are reported to be more susceptible than men to become afflicted with the disease (Francis et al. 1991; Duquette et al. 1992; Duquette and Girard, 1993). It is unclear why such a difference in susceptibility may exist, since no apparent sex-

linked gene can be attributed to this disease (Duquette et al. 1992; Weinshenker, 1994). Duquette et al. (1992) have noted a higher frequency of early and late-onset cases in women, which correlate temporally with puberty and menopause (Duquette and Girard, 1993), in the familial cases and twins, suggesting a hormonal component to MS. Finally, a genetic aspect appears to pervade MS as discussed below.

2.32 MS and the HLA (human leucocyte antigen) connection

Population genetics data imply a certain role for MHC class II molecules in MS disease susceptibility (Haegert and Marrosu, 1994; Owens and Sriram, 1995; Sadovnick and Ebers, 1995). The identity of the particular HLA that correlates with MS depends on the population under consideration (Haegert and Marrosu, 1994; Owens and Sriram, 1995). The HLA alleles that are most widely agreed upon which confer susceptibility to MS in Caucasians, are the HLA-DR2 and HLA-DQw1 (Haegert and Marrosu, 1994; Owens and Sriram, 1995; Sadovnick and Ebers, 1995). Despite this general consensus, recent studies do not find a major susceptibility gene but do confirm a correlation between the HLA locus and MS (Bell and Lathrop, 1996; Ebers et al. 1996; Kuokkanen et al. 1996; Sawcer et al. 1996; The multiple sclerosis genetics group, 1996).

Other loci tested for a correlation with or a predisposition to MS, such as the loci for the immunoglobulin heavy chain (IgH), T cell receptor (α and β chains) and myelin basic protein (MBP) among others (Haegert and Marrosu, 1994; Sadovnick and Ebers, 1995; Ebers et al. 1996) are suggestive at best (Haegert and Marrosu, 1994; Ebers et al. 1996; Sawcer et al. 1996; The multiple sclerosis genetics group, 1996).

2.33 The multigene and multifactor theory in MS

Because of this want of a single gene predicting the predisposition to MS, a hypothesis relying on the existence of multiple genes for disease susceptibility has emerged (Bell and Lathrop, 1996; Ebers et al. 1996; Kuokkanen et al. 1996; Sawcer et al. 1996; The multiple sclerosis genetics group, 1996). This interesting postulate, which may account for much of the intricacies of MS, does not explain the geographical or environmental factor(s) that seem to permeate this already complex disease. Unfortunately, these environmental factors remain unknown and the link between the geographical region and the frequency of MS cases remains tenuous at best (Ebers,

1994,1996). Therefore, the resulting white and grey matter damage in MS may have its basis in multiple factors that are genetic, hormonal and environmental (Duquette et al. 1992; Duquette and Girard, 1993).

2.34 Mitochondria and MS

The greater frequency of women than men with MS that have affected children, even after taking into consideration the greater preponderance of women over men with MS, suggests a mitochondrial involvement. Moreover, some women with Leber's optic atrophy, a mitochondrial disease, develop a pathology that is indistinguishable from MS. A mitochondrial component to MS has been substantiated by some studies (Hanefeld et al. 1994; Kalman et al. 1995) while others found no reproducible link (Chalmers et al. 1995,1996). Opponents of this theory point to the observation that mitochondrial diseases are transmitted from mother to both sexes and that there is precedence, in MS, for fathers to transmit disease to their offspring (Duquette et al. 1992). Whether or not there is a link between mitochondria and MS is an issue that remains to be resolved.

The uncertainties in MS, that result from a gap in the knowledge of the etiology, genetics, hormonal and environmental factors, are mirrored by the vast array of treatments that have been tried in the clinical setting, and the lack of a definite strategy to deal with MS.

2.4 Prophylactic, symptomatic and reparative strategies

There are several approaches to MS therapy that are categorized as follows (Weiner et al. 1995): 1) prophylactic treatments designed to prevent the characteristic myelin damage in MS; 2) symptomatic treatments that try to diminish the disability that often accompanies MS and maximize the function of the CNS; and 3) therapies designed to repair the damage that is caused by MS.

A worthwhile therapy is one which can arrest the immune mediated inflammatory process, and prevent the development of MS before cumulative damage sets in (Weiner et al. 1995). Unfortunately, the therapies designed to repair the degenerative process that occurs in MS, whether at the ensheathing or axonal level, are still at the laboratory stage. Such treatments would be invaluable in accelerating the process of recovery from relapses (Weiner et al. 1995).

2.5 Various treatments for MS

Over the years, many chemical, biological and physical interventions have been used in an attempt to slow or even halt the progression of MS, with varying degrees of success (Whitaker, 1994; Weiner et al. 1995; Waubant and Goodkin, 1996; Stinissen et al. 1997).

Some of the immunomodulatory treatments, including the interferons (IFNs), have been tried in MS clinics and other trials with varying degrees of success (see below). The nomenclature, identity and usage of IFNs in the MS clinics, is dealt with in the next section. **It is how IFNs, particularly IFN-β, function within the framework of the CNS, in MS, that will be of interest throughout this thesis.**

3. Interferons: discovery and usage

3.1 A bird's eye view of interferons

These cytokines were classically thought of as being able to induce an antiviral state in cells, and indeed that was how the concept of IFN came into being. In 1957, Isaacs and Lindenmann described a phenomenon of interference, by heat-inactivated viruses, with the growth of live viruses *in vitro*. This effect was believed to be mediated by a new factor that was released from cells. At the time, the exact nature of the IFN was unknown.

The division of IFNs into the various members of their family started with the recognition of IFN-γ (gamma) as a novel kind of IFN (Wheelock, 1965). The next step was the division between a major (leucocyte) and a minor, fibroblast like (Berg et al. 1975; Havell et al. 1975), type of IFN in leucocyte (Havell et al. 1975). These cytokines comprised 3 groups: IFN-α (alpha), -β and -γ also called leucocyte, fibroblast and lymphocyte IFN, respectively. This nomenclature reflected the major cell type that synthesized them (Weinstock-Guttman et al. 1995). IFN-α/β were type 1 IFN and bound to type 1 receptor. Type 2 IFN, whose only member was IFN-γ, bound to type 2 receptor (Weissmann and Weber, 1986; Pestka et al. 1987; Flores et al. 1991). The previous nomenclature for type 1 IFN changed with time. In fact a novel type of IFN, IFN-ω (omega), was added to the ever growing list (Capon et al. 1985; Feinstein et al.

1985; Hauptmann and Swetly, 1985).

A new nomenclature has been worked out for the IFNs as new family members are being discovered. This classification is based on the gene sequence analysis of the IFNs (Weissmann and Weber, 1986). Accordingly the IFN family is presently divided into these categories: IFN-α, -β, -τ (tau), -ω, -γ. Other members of the family are hypothesized to exist as variations on the type 1 IFN theme (Lefèvre and Boulay, 1993; Liu et al. 1996). The first four IFNs (α, β, τ, ω) of the previous list are classified as type 1 IFN. All these IFNs bind to type 1 receptor (Stewart et al. 1987; Flores et al. 1991; Li and Roberts, 1994; Subramaniam et al. 1995; Usé et al. 1995; Weinstock-Guttman et al. 1995) and are related to each other through evolution (Weissmann and Weber, 1986; Roberts et al. 1992; Hughes, 1995).

Type-1 and type-2 IFNs are known to have similar properties where antiproliferative and antiviral activities are concerned (Weissmann and Weber, 1986; Pestka et al. 1987; Pontzer et al. 1988; Baron et al. 1991; Pontzer et al. 1991; Roberts et al. 1992; Soos et al. 1995; Weinstock-Guttman et al. 1995). However, they diverge in some of their immunomodulatory roles (Panitch and Bever, 1993). The IFN-α family is comprised, in humans, of over 18 nonallelic genes including four pseudogenes (Weinstock-Guttman et al. 1995). It should be noted, however, that the number of IFN-α genes varies from one species to another (Hughes, 1995). It is unknown why there are so many IFN-α genes, however the current belief is that they arose by gene duplication (Weissmann and Weber, 1986). Unlike the IFN-α genes which exist in multitudes, IFN-β is the only member of its family in most species, the exception thus far is bovine IFN-β (Weissmann and Weber, 1986; Liu et al. 1996).

The other two type 1 IFNs, IFN-τ and -ω, are the newer members of the family. In humans, IFN-ω is expressed by leucocytes (Adolf et al. 1990) and is comprised of six genes, five of which are pseudogenes (Weissmann and Weber, 1986; Weinstock-Guttman et al. 1995). It is interesting that not all species have IFN-ω, as mouse and dog are not known to have this IFN (Hughes, 1995; Liu et al. 1996).

IFN-τ's discovery was related to pregnant cows and sheep (Roberts et al. 1992) and its sequence was identified in the late 80s (Imakawa et al. 1987). In ruminants the major role played by this IFN, synthesized by the first epithelium of the conceptus (the trophectoderm), related to the maintenance of an appropriate environment for the embryo

via receptors in the endometrium (Stewart et al. 1987; Cross and Roberts, 1991). Humans on the other hand expressed an IFN resembling IFN-τ, which did not seem to play the same role as IFN-τ did in the ruminants even though it had been recently localized to the placenta (Whaley et al. 1994). Contrary to the ruminant IFN, which was synthesized for a couple of days preceding implantation (Short, 1969; Stewart et al. 1987), the human IFN was synthesized throughout the pregnancy, by the placenta and adult lymphocytes, but its function was not known (Whaley et al. 1994).

All the type 1 IFN genes are in a cluster on the short arm of the human chromosome 9. None of these genes has introns and it is hypothesized that they evolved from a single ancestral gene (Weissmann and Weber, 1986). In contrast, IFN-γ is a single gene that has three introns and is located on chromosome 12 in humans. This type 2 IFN, which is still the only member of type 2 group (Weinstock-Guttman et al. 1995), is structurally unrelated to the other members of the superfamily of IFNs (Weissmann and Weber, 1986). Its similarity to the other IFNs lies in the homology of its function, however, it has little homology in its sequence (Weissmann and Weber, 1986; Billiau, 1987; Roberts et al. 1992). The evolutionary significance of these findings, i.e. similarity in function but difference in sequence is unknown (Weissmann and Weber, 1986; Pestka et al. 1987; Mitsui et al. 1993). It is, nevertheless, the pleiotropic functions of the IFNs that has made these molecules the target of clinical trials.

3.2 Interferons in the clinical setting

While antiproliferative and antiviral activities are a some of the functions of IFNs, they are by no means the only roles that these pleiotropic proteins play in the nervous system or outside it (Panitch and Bever, 1993; Weinstock-Guttman et al. 1995). These functions have not gone unnoticed, in fact both type 1 (IFN-α/β) and type 2 (IFN-γ) IFNs have been used in clinical trials. These include infectious diseases, malignancies, autoimmune, immune and allergic diseases (Dunnick and Galasso, 1979; Baron et al. 1991; Weinstock-Guttman et al. 1995). In addition, many IFNs have been used in either MS clinics (sections 5, 7.1; Durelli et al. 1994) or have been tried in an animal model of MS, e.g. EAE (experimental allergic or autoimmune encephalomyelitis) (section 4; Abreu, 1985; Herber-Katz, 1995; Owens and Sriram, 1995; Tsunoda and Fujinami, 1996). The success or the lack thereof, in reducing the clinical signs of MS or EAE,

using IFNs, is reviewed in the coming sections, with special emphasis on IFN-β.

4. Animal model for the study of MS

EAE has been used as an animal model of MS in various species, from mice to non-human primates (Rose et al. 1994; Herber-Katz, 1995; Tsunoda and Fujinami, 1996) since the 1930's (Rivers et al. 1933). In contrast to MS, EAE is induced by a known antigen in an adjuvant. These antigens include whole brain homogenate or myelin proteins such as proteolipid protein (PLP) or myelin basic protein (MBP) (Owens and Sriram, 1995). EAE has some resemblance to MS in the clinical, immunologic, pathologic and radiologic aspects of the pathology, however, the duration of a given lesion is shorter than the ones in MS, lasting a couple of weeks instead of a couple of months (Rose et al. 1994).

The immunological aspects of EAE made it a target for the immunomodulatory IFNs (Abreu, 1982, 1985). Indeed, it had been shown that clinical signs of EAE could be delayed by the systemic administration of IFN (Abreu, 1982), and that the adoptive transfer of EAE could be inhibited by preincubating MBP-sensitive lymphocytes with murine IFN-β (Abreu, 1985). More recently, Yu et al. (1996) reported that the progression of relapsing-remitting EAE, in mice, was delayed by murine IFN-β. This group described the following characteristics for EAE: 1) a relapsing-remitting course that changed to chronic progressive phenotype with time; and 2) a CNS demyelinating phenotype. The improved outcome in the IFN-β treated EAE animals, as compared to EAE control animals, was measured by several criteria: 1) a decreased frequency of exacerbation; 2) a reduced disease burden; 3) an increased lag in the onset of the first exacerbation; 4) a decreased course to incapacity; 5) an increased lag in the delayed-type hypersensitivity; and 6) an ameliorated histopathological outline.

Another member of the type 1 IFN clan, which reared its head in the EAE circle, was IFN-τ. The latter was considered a pregnancy IFN at first (section 3.1), nonetheless, it was successfully used in the treatment of both the developmental and reactivated, via superantigen, forms of EAE (Soos et al. 1995). It also had a reduced toxicity, compared to IFN-α/β (Soos et al. 1995; Subramaniam et al. 1995), which was believed to be due to a reduced receptor affinity (Subramaniam et al. 1995).

The mechanism of IFN-β/-τ in EAE remains unclear. The existence of an animal model, however, permits a much greater flexibility in experimentation than a human disease does. Through such models it may be possible to divulge part of the mechanism by which the type 1 IFNs function in EAE and hopefully MS.

5. Clinical studies using IFNs in MS

The initial rationale for using IFNs in MS patients were: 1) the belief that the cause, or partial cause, for MS was a viral infection in the CNS which acted as a trigger mechanism for repeated exacerbations and clinical symptoms (Cook and Dowling, 1980; Johnson et al. 1981); and 2) the inherent antiviral activity of IFNs which occurred naturally in the human body (Dunnick and Galasso, 1979).

There are many IFNs that have been used in MS clinical trials, these include IFN-α (Durelli et al. 1994), -β (present section) and -γ (Panitch et al. 1987 a,b). As my work seeks to discover whether or not IFN-β can increase astrocyte derived NGF, the focus is on IFN-β. Since subtypes of MS other than RRMS are reported to by refractory to IFN-β (Camenga et al. 1986; Huber et al. 1988) the attention is centred around RRMS. The involvement of the other IFNs, in RRMS, are briefly mentioned as needed.

5.1 The use of natural IFN-β in MS clinics

The first report on natural human IFN-β being used in MS, demonstrated the efficacy of intrathecally administered IFN-β (Jacobs et al. 1981). The study was randomized, unblinded and it was deemed unjustified to include a placebo intrathecal injection as a control at that time. In addition, Jacobs et al. (1981) judged that the risks associated with the intrathecal administration of a foreign protein, for the purposes of a proper scientific control, outweighed the advantages. It should be noted, however, that this control group continued to receive the steroids that they had been taking before the study and that the IFN-β treatment group stopped their use of steroids for the duration of the study.

Despite this lack of good controls, the conclusion attained by the authors from this study was that natural IFN-β decreased the frequency of exacerbations in the recipients

as compared to their base line value. In contrast, the control group's values were not significantly different at the end of the study, as compared to their base line values.

The patients in the previous study had been followed for a time after their treatments. The effects of IFN-β on exacerbations were still seen, 4.4 to 5.3 years, after the end of the intrathecal injection treatments and usually without having to readminister IFN-β (Jacobs et al. 1985, 1987). The encouraging results of the previous study served as a basis for a multicenter, double-blinded, placebo-controlled study using fibroblast derived, human IFN-β, which was administered intrathecally. This 2-year study confirmed the effect of IFN-β on the reduction (57 %) of exacerbation frequency as compared to a placebo-induced reduction (26 %) in the rate of exacerbations in the control group (Jacobs et al. 1987).

The reports by Jacobs et al. are not the only ones that demonstrate the effect of natural IFN-β in MS. Another more recent study by Fernández et al. (1995) confirms a 38 % reduction in exacerbation frequency and a 58 % reduction in serial MRIs by using subcutaneous administration of natural IFN-β.

5.2 The use of IFN-β-1b in MS clinics

Just as natural IFN had shown effectiveness in RRMS, a bacterial (*Escherichia coli*) recombinant non-glycosylated, serine substituted IFN-β (Russell-Harde et al. 1995), termed IFN-β-1b, had been used successfully in MS trials. It should be noted that it was only with the advent of recombinant IFN that large scale studies became possible, owing to its greater availability (Panitch and Bever, 1993).

The well tolerated IFN-β-1b was the focus of a large clinical trial that stemmed from a multicenter, randomized, double-blinded, placebo-controlled endeavour. The results from this study, using RRMS patients, suggested that IFN-β: 1) decreased the number of relapses in RRMS; 2) produced a 33% reduction in the rate of exacerbations; 3) elicited a 50% reduction of severe exacerbations; and 4) reduced the number of active lesions and disease burden that were detected by MRI (Paty et al. 1993; The IFNβ multiple sclerosis study group, 1993; The IFNβ multiple sclerosis study group et al. 1995). However, the degree of disability was not affected.

The clinical and MRI results were reported in a five year study of MS treatment with IFN-β (The IFNβ multiple sclerosis study group et al. 1995). This report

underscored the beneficial effects of 8 MIU of IFN-β, as compared to placebo controls, where the rate of exacerbations was concerned. The effect was seen every year for the five year study. MRI scans on 217 of the patients were done at the 4 or 5 year time point, and they manifested a lack of advancement of the lesion burden in the 8 MIU treatment arm when compared to their base-line MRI. For the controls, the situation was different as this group demonstrated an important enlargement of lesion areas.

During this trial, there was a significant reduction in the number of hospitalizations in the group that received the 8 MIU dose. This had a beneficial outcome for the patient, not only health wise but also in terms of health care costs (The IFNβ multiple sclerosis study group, 1993). In stark contrast, this study failed to demonstrate a statistical significance where the IFN-β effect on disability was concerned (The IFNβ multiple sclerosis study group et al. 1995). IFN-β-1b was not the only recombinant form that had been used clinically, however, and another IFN-β had been more successful in clinical trials.

5.3 Just another twist on the IFN-β theme used in MS clinics

In a **1996 landmark report** by Jacobs et al., recombinant IFN-β-1a was used to reduce the accretion of clinical disability. Unlike the IFN-β-1b, the recombinant IFN-β-1a was synthesized by eucaryotic cells (Chinese hamster ovary cells) instead of bacteria, was glycosylated, and had a natural sequence. This phase III, multicenter, randomized, placebo-controlled clinical trial, was directed to answering the question: Could IFN-β-1a "slow the progressive, irreversible, neurological disability of relapsing MS" ? The results indicated that weekly intramuscular injections of IFN-β-1a was able to reduce the frequency of exacerbations, disease burden, as measured by gadolinium-enhanced MRI, and neurologic impediment (Jacobs et al. 1996).

Jacobs et al. (1996) hypothesized about the major reason why their report was able to demonstrate that the **natural course of RRMS could be altered by IFN-β-1a** and that the previous reports could not. According to this group, the reports that use IFN-β-1b (section 5.4) or natural IFN-β (section 5.3) do not demonstrate that the disease course is alterable because they are not designed to show an IFN-β effect on disease course.

In a more recent report by Pozzilli et al. (1996), gadolinium enhanced MRI scans were used to demonstrate a reduced mean number and mean volume of lesions which

only occurred after the first month of IFN-β-1a treatment. In this study the treatment arm was not compared to placebo controls, but the same patients were compared before and after IFN-β-1a treatment. This comparison was made possible by serial monthly MRI scans which were done for 6 months before treatment in order to establish a baseline value for each patient. It should be noted, however, that this study was not designed to show, and indeed did not reveal, long term effects on disability because of the brevity of the study. Overall their conclusion echoes the judgements reached from the other studies previously cited.

From Pozzilli et al. (1996), it is clear that the difference in the ability of IFN-β to alter the disease progression of MS may not lie solely on the method of deriving IFN-β, but also depends on the design of the study.

Finally, another factor that could explain the difficulties in trying to link the IFN-β effect on disease course is the observation that MS affects the spinal cord in addition to the brain, as detailed in the next section, which was not mentioned in these trials. Such a factor may also influence the outcome of the study depending on the patients overall condition.

6. Brain versus spinal MS

Many studies attempted to relate the pathological and the clinical symptoms (section 1.2; Fog, 1950; Oppenheimer, 1978) to the MRI findings (Miller et al. 1987; Honig and Sheremata, 1989; Turano et al. 1991). Unfortunately, until recent advances in technology permitted a good resolution of the spinal cord lesions in a reasonable acquisition time, some of the problems plaguing such studies were the poor resolution caused by the low signal-to-noise ratio and movement artifacts. With the improvement of technology, a shorter acquisition time (~5 min) and a higher resolution were feasible.

Using recent advances in technology, Kidd et al. (1993) demonstrated that there was no significant correlation between the lesion load in the spinal cord and the patient's disability. In fact, the authors found no correlation between the degree of incapacity and the lesion load in the spine or the brain. This lack of association might result, in this study, from a lack of evaluation of the transverse damage. The evaluation of track damage, through axial imaging, and the measure of area or volume that was involved in

the lesion would probably rectify this problem (Kidd et al. 1993). According to this group, another plausible reason for the lack of correlation was the heterogeneity of the disease. Kidd et al. (1993) divided the chronic lesion into two important categories, based on Barnes et al. (1991) classification; the gliotic lesions with preserved axons, and the axonless lesions. Kidd et al. (1993) equated the spinal atrophy they saw with demyelination and axonal loss. They believed that the low correlation that existed between the disability and the atrophy could be accounted for by the methods used for estimation of cord damage. The assessment of the global cord section was a rough estimate of damage, if one took into account the relative importance of individual fibre tracts.

As technology advances and better ways are found to determine which fibre tracts are involved in the cord lesions of MS patients, it may become possible to use MRI technology to correlate, to a greater degree of reliability, cord and brain lesions with disability.

Such a lack of correlation between lesions and disability makes it all the more difficult to evaluate the effectiveness of IFN-β in MS. Despite this obstacle many have tried to understand the mechanism by which IFN-β may function to reduce the various clinical symptoms in MS.

7. The mechanism of action of IFN-β in MS

While the effects of IFN-β in MS have been studied at length, its mechanism of action still remains unknown. It is therefore **the purpose of my thesis to examine the effect of IFN-β on one of the compartments in which it may act, the CNS, and more specifically on astrocytes.** It should be noted, however, that there are many hypotheses as to the means by which IFN-β may achieve its effect. Finally, the mechanisms described in this section are not mutually exclusive and they may function simultaneously or in concert.

7.1 The effects IFN-β on the systemic immune system

IFN-β may have, among other functions in MS or EAE, an immunomodulatory role in the systemic immune system (Panitch and Bever, 1993; Weinstock-Guttman et al.

1995). Indeed, Noronha et al. (1990, 1992, 1993) had shown that the nonspecific suppressor T cell function, which was known to be decreased in progressive MS and before or during relapses in RRMS (Antel et al. 1978, 1988), increased upon IFN-β treatment.

Other facets of IFN-β function include the *in vitro* downregulation of both preactivated T cells, $CD4^+$ and $CD8^+$, and the IFN-γ that is produced (Noronha et al. 1993). Moreover, the increased IFN-γ production in relapsing-progressive MS, as compared to normal controls, and normal IFN-γ levels are decreased by IFN-β (Noronha et al. 1993). Such a decrease in IFN-γ production is also seen in the serum levels of RRMS patients after IFN-β treatment, when compared to pretreatment (Revel et al. 1995). This downregulation is an important aspect of IFN-β immunomodulatory function, if one considers some of the functions of IFN-γ (Weinstock-Guttman et al. 1995) which include: 1) the upregulation of the MHC class II expression on microglia/macrophage (King and Jones, 1983; Kitaura et al. 1988; Yong and Antel, 1992) among other cell types (Benveniste, 1993); 2) the induction TNF-α in monocytes (Philip and Epstein, 1986); and 3) the induction of cell adhesion molecules on endothelial cells (Duijvestijn et al. 1986; Thornhill et al. 1991).

MHC class II molecules are known to have the capacity to be involved in the presentation of autoantigens (Yong and Antel, 1992; Owens and Sriram, 1995; Stinissen et al. 1997). This last observation made IFN-γ potentially hazardous for MS patients, since autoantigens might worsen the disease (Cannella and Raine, 1995; Brosnan and Raine, 1996; Navikas and Link, 1996). It is therefore believed that the resulting effect of IFN-γ is a local upregulation of immune functions (Noronha et al. 1993).

In line with the previously stated immune-related functions of IFN-γ, MS patients which were on an IFN-γ trial had increased exacerbations (Panitch et al. 1987 a,b). In addition to the former IFN-γ-mediated effects, IFN-γ and TNF-α can act synergistically to regulate many immune parameters (Collart et al. 1986; Lapierre et al. 1988; Benveniste et al. 1989; Vidovic et al. 1990; Thornhill et al. 1991). Moreover, TNF-α and lymphotoxin (TNF-β) have been implicated in the *in vitro* destruction of oligodendrocytes (Selmaj et al. 1988, 1991b; Louis et al. 1994; D'Souza et al. 1995, 1996). Furthermore, MS lesions demonstrate the presence of both TNFs (Hofman et al. 1989; Selmaj et al. 1991a) and others correlate the presence of TNF-α with clinical

severity (Sharief and Hentges, 1991) and blood-brain barrier damage (Sharief and Thompson, 1992). Finally, spontaneous MS attacks are preceded by the presence of peripheral blood cell whose mitogen stimulated IFN-γ and TNF-α production is elevated (Beck et al. 1988).

In stark contrast to these reports on the deleterious effects of these cytokines, Cheng et al. (1994) had found that TNF-α could act as a neurotrophic factor, and Vervliet et al. (1983,1984) reported on the decreased IFN-γ production by leucocytes derived from MS patients.

Another effect of IFN-β on the systemic immune response, which may relate to its antagonism of the IFN-γ response, may lie in its ability to modulate the transit of lymphocytes across the blood-brain barrier. As mentioned above, IFN-γ can modulate the adhesion molecules on endothelial cells. The adhesion to such molecules, by T cells, is part of a multistep process thought to be involved in the trafficking of T cells across the vascular endothelium (Bevilacqua, 1993; Bevilacqua et al. 1993; Springer, 1994). In keeping with IFN-β's ability to downregulate some immune functions, Stüve et al. (1996) demonstrate that pretreating T lymphocytes derived from normal donors with IFN-β reduces their migration across an artificial membrane as compared to untreated lymphocytes. Indeed, Stüve et al. (1996) demonstrate that the migration of $CD4^+$, $CD8^+$ and natural killer cells can be reduced in a paradigm that does not depend on the antiproliferative action of interferons, as IFN-γ does not diminish the migratory ability of these cells. Since lymphocyte migration across the blood-brain barrier reduces the integrity of the endothelial cell barrier (Pollard et al. 1994), a benefit from reducing this migration is a possible enhancement of the blood-brain barrier. Such an improvement in blood-brain barrier integrity is seen in patients treated with IFN-β (Stone et al. 1995). Therefore, the reduced transit of T cells across the blood-brain barrier may account, in part, for IFN-β's therapeutic efficacy in MS.

7.2 The effects IFN-β on the CNS

7.21 Immunological effects of IFN-β on the CNS

The useful effects of IFN-β in MS need not be limited to the systemic immune system. It is possible that IFN-β affects the CNS as well. There are reports that adult

derived astrocyte cell lines downregulate their IFN-γ-induced MHC class II expression, when preincubated with IFN-β (Barna et al. 1989; Ransohoff et al. 1991). McLaurin et al. (1995) reproduce these results when cell lines are used, but this regulation is not observed when primary fetal human cells are utilized. It should be noted, however, that this difference may reflect a disparity in the age as well as the primary versus cell line nature of the cultures (an example of the latter is given for NGF in section 12.51 of the discussion). Various groups demonstrate that IFN inducibility and sensitivity cannot be elicited in mouse embryonal carcinoma cell lines (Burke et al. 1978; Harada et al. 1990) or early stage embryos (Barlow et al. 1984). Indeed, Harada et al. (1990) demonstrate that some responses are developmentally regulated. Therefore, the lack of responsiveness in fetal human astrocytes, for the MHC class II genes, may be a developmentally controlled phenomenon.

The belief that the expression of MHC class II molecules on astrocytes is important, has its basis in the observation that these glial cells are found to express MHC class II in MS autopsy tissue (Hofman et al. 1986,1989; Traugott, 1987; Traugott and Lebon, 1988b; Hofman et al. 1989). In addition, Traugott and Lebon (1988 a, b and c) correlate the presence of MHC class II expressing astrocytes with the presence of IFN-γ at the expanding edge of some lesions in MS. In contrast, Bo et al. (1994) detect no MHC class II positive astrocytes in active MS lesions, while these molecules are detected on macrophage and microglia. It is not known at present, how or if this finding has any bearing on the development or progress of MS as the expression of MHC class II is much lower on astrocytes than on macrophages (Lee et al. 1990). This observation, though potentially important, does not preclude an effect of IFN-β on the CNS enshething cells, the oligodendrocytes.

7.22 The effects of IFN-β on non-immune parameters of the CNS

It was possible that IFN-β affected oligodendrocytes directly, and in this line of thought, McLaurin et al. (1995) tried to determine the effects of IFN-β on adult human oligodendrocytes. They found that IFN-β did not affect either the state of morphological differentiation or the rate of proliferation of cultured oligodendrocytes (McLaurin et al. 1995). The authors also found that IFN-β was not toxic to various cell types of the CNS, such as fetal human astrocytes or neurons as well as adult human oligodendrocytes and

microglia, even at concentration of 1000 U/ml. Such low toxicity was important for a drug that was used for extended periods of time in the clinics.

Oligodendrocytes have been the centre of attention in MS (section 7 and covered in discussion) ever since the first neuropathological descriptions of MS (section 1.2). This monopoly can best be exemplified in historical terms. Despite the involvement of the grey matter and the knowledge of axonal loss that occurs in MS, which has existed since Charcot's time (reported by Davie et al. 1995), it is interesting that this damage has taken a backseat to the demyelination that occurs in the white matter of the CNS. In trying to devise a treatment directed to help the white matter oligodendrocytes (sections 2.4 and 2.5) and putting the destruction of the grey matter on the backburner, many strategies have, for intensive purposes, neglected the short term health of the neurons and the long term effects of the course of MS on neuronal functioning and survival. Since both neurons and oligodendrocytes are affected in MS, **it is the purpose of my thesis to examine how IFN-β, one of the most successful drugs that has ever been tried in MS, affects the most numerous glial cell type in the CNS, the astrocytes (Kandel, 1991), which are known to lend trophic support to neurons and oligodendrocytes (see below).**

8. But why astrocytes ?

8.1 Astrocytes and glial function: A historical perspective

Neuroglia were first described, in 1846 by Virchow, in light of the concept or prejudice of connective tissue that must exist to fix the neurons into place. The very word neuroglia means " nerve glue ", and such was the fate of astrocytes. They were categorized in a role of nerve glue, along with the other resident glial cells. In addition, this description of function was done, in all likelihood, in the absence of their visualization. This description was followed by Golgi's discovery of astrocytes, in 1873, and the report that describes their foot-like processes, which they extend to the blood vessels. Golgi conjectured, as early as 1895, that astrocytes might mediate the transport of nutrients from blood to neurons. However, the credit of the recognition of astrocytes as a different glial subclass, as well as the name astrocytes, went to Lenhossék in 1891

(reported by Jacobson, 1991).

By the end of the nineteenth century many hypotheses surrounding the origin and function of neuroglia, of which astrocytes are a component, were summarized by Soury in 1899 (reported by Jacobson, 1991). Many of these functions were the fabric of pure conjecture and logical argument. Despite the primitive tools, by our standards, that were at the disposal of these scientists, the available technology did not prevent them from elaborating theories such as the involvement of neuroglia in: 1) the provisions of nutrition; 2) the function of support; 3) the myelination of axons; 4) the formation of the blood-brain and blood-CSF barriers; 5) the limitation of neuronal activity; 6) a proliferative reaction, among other repercussions, in response to neurodegeneration; 7) the neuronal and axonal guidance during the developmental stage; and 8) the processes of learning, memory and conscious experience (reported by Jacobson, 1991).

Though many, if not all, of these functions reflect modern theories, they are not all attributed to astrocytes. The functions that are relevant to astrocytes are described in latter subsections. Because of the number and complexity of these functions, which vary at different stages of life (Vernadakis, 1996), it is not within the scope of this thesis to describe each role in great detail that these multifunctional cells have within the CNS. Instead I will give a general overview of some of their functions and describe in detail certain tasks, that may need clarification, to help situate the importance of astrocytes in the proper functioning of the CNS.

8.2 Astrocytes during development

8.21 The guidance of neurons

During the developmental stage of the CNS, a primitive form of astrocytes called radial glia span the distance between the ventricular surface, from which they emanate, to the pial surface where their endfeet attach (Rakic, 1990; Martin-Padilla, 1995). These fibres can stretch several millimetres in length and are considered to be the prevalent mode of transit for some postmitotic mammalian neuronal cells. This migration relies on neurons that are said to be gliophilic because of the observation that the neurons in question migrate along the glial fibres while avoiding the axons they encounter along their route. Structures that are formed by such a migratory mechanism are the neocortex

and the hippocampus. Other regions that also rely on such a migration for their genesis, but to a lesser extent, are the spinal cord, the brain stem and the diencephalon (Rakic, 1990).

The importance of such a system is best demonstrated when a gyrencephalic brain, such as the human brain, develops and the gyral formation causes a shift in the cortical plate. Indeed, the existence of such fascicles allows the establishment of stable highways on which columns of neurons can migrate to their appropriate destination. This becomes crucial when one considers that the migration of neurons progresses from the inside out, i.e. the neurons destined to reside in the outer most surface of the brain are the last to migrate. These neurons must find their appropriate target regardless of how rapidly and extensively the growing cerebral wall shifts during the formation of the gyri (Rakic, 1988).

The gliophilic trek is not the only means by which the developing brain has to target its neuronal populations to the appropriate sites. Other types of migration include neurons that have a neurophilic or a biphilic propensity. The first relies on neuron-neuron interactions while the second relies on neuron-glia interactions. The neurophilic interactions consists of neurons that migrate along the axons of other neurons to arrive at their destination. The biphilic interactions can best be exemplified by granule cells, within the cerebellum, which have two classes of neurites. The ascending neurites follow the axons of previously generated granule cells, these form the parallel fibre system within the granule layer (Rakic, 1990). The second class of neurites follow the Bergmann glial cells which are the equivalent of the radial glial cells within the cerebellum (Rakic, 1990; Mittal and David, 1994).

The importance of this migratory mechanism is underscored by the pathologies that are related to migratory defects such as gross brain malformations and the more subtle synaptic circuit defects (Rakic, 1990).

8.22 Growth cone guidance

The neuronal guidance function of astrocytes, while important, is by no means the only task that these cells perform during development. In fact, astrocytes have been associated with growth cone guidance (Silver et al. 1982; Silver and Ogawa, 1983; Silver, 1994). The importance of this facet of astrocyte function can be demonstrated by

considering the formation of one of the structures that develops during embryogenesis, e.g. the corpus callosum. The latter along with the anterior commissure form the major nerve pathways which serve to unite and integrate the functions of both halves of the brain (Kupfermann, 1991). During embryogenesis, the corpus callosum depends on the presence of glial cells for the crossover of its axons (Silver et al. 1982).

In a series of experiments, Silver et al. (1982) demonstrated that genetically acallosal mutant mice and animals that have a surgically resected corpus callosum generate nerve fibres that do not cross the corpus callosum. Some of these fibres use the fornix or the anterior commissure to cross to the other side. The fibres that do not cross, which constitute the majority, tend to form a coiling mass called Probst's fibres at the altered midline (Silver et al. 1982). These whirling masses, once formed, can be redirected to the contralateral side by reintroducing embryonic glia into the cerebral fissure of postnatal acallosal animals (Silver and Ogawa, 1983).

Finally, Silver et al. (1982) theorize, based on the previous observations, that the oriented glial cells can generate specific pathways which determine axonal guidance and control, but cannot define the potential targets. Nevertheless, proper neuronal and axonal guidance are of great importance if the appropriate synapses are to have a chance to form. The astrocyte involvement does not stop at this level, as other functions assure the proper development of the brain.

8.23 The blood-brain barrier

The blood-brain barrier is believed to limit the access of molecules, that exists within the lumen of the capillaries, to the brain's parenchyma (Betz and Goldstein, 1986). This seal was thought to be achieved, in large part, by the tight junctions which interconnect the endothelial cells of brain's blood vessels (Reese and Karnovsky, 1967). The formation of such a barrier could be an intrinsic property of the endothelial cells, an extrinsic property imparted to them by their environment or a combination thereof. Stewart and Wiley (1981) partly resolved these considerations. This group demonstrated that the quail non-vascularized brain tissue that was transplanted into the embryonic chick coelomic cavity formed a blood-brain barrier, from the invading endothelial cells, that was characteristic of the brain's barrier. In contrast, the non-vascularized quail coelomic tissue that was transplanted into the embryonic chick's brain formed a leaky vasculature

from the invading endothelial cells. Therefore, Stewart and Wiley (1981) concluded that the environment dictated the properties of the endothelium.

It has been known since the nineteenth century that astrocytes extend foot-like processes to the endothelial cells (section 8.1). This observation made them a likely candidate, then as now, for the modulation or participation in the formation of the blood-brain barrier. Indeed, Janzer and Raff (1987) demonstrated that astrocytes, which were able to form vascularized aggregates, could induce a " tight " endothelium. The latter did not allow leakage of protein (albumin) or dye, within the aggregate, even when the majority of the surrounding tissue was stained or found to contain albumin. When an analogous experiment was done with meningeal cells or peripheral tissue fibroblasts, a vascularization was observed whose endothelium was sufficiently leaky to allow dye or protein to infiltrate the aggregate.

Other studies (Cancilla et al. 1993) revealed that astrocytes could modulate the function of the endothelium. Such an effect could be seen at many levels, including: 1) the modulation of protein expression on the endothelial cells; 2) the induction of a vectorially directed protein expression on the endothelial surface; 3) the stimulation of glucose uptake; and 4) the downregulation of endothelial cell proliferation. The latter function was postulated to help differentiate the endothelial cells and establish the previously mentioned characteristics.

The invaluable functions that astrocytes perform during development are a fraction of their multifaceted tasks. In fact, these glial cells continue to execute various functions throughout the life-span of the animal (see below). These tasks may be very different from those affected during the developmental stage, though they are no less crucial for the proper functioning of the CNS.

8.3 Astrocytes during normal function

8.31 Maintenance work

Under such a heading one can find a myriad of tasks that astrocytes perform as part of their typical maintenance work. These tasks are thought to maintain a homeostatic environment within the CNS (Kimelberg et al. 1993). Some of these functions include: 1) the uptake of neurotransmitters, which is a function that was suggested by Lugaro in

1907 (reported by Kimelberg et al. 1993), such as glutamate, gamma-aminobutyric acid, taurine, catecholamines and serotonin among others. Such a function prevents a constant state of stimulation or inhibition and assures proper excitability (Belin and Hardin, 1991; Kimelberg et al. 1993); 2) the uptake of ions, which include Na^+, K^+, Cl^- and H^+ among others, thereby assuring a homeostatic environment; 3) the regulation of the extracellular pH, by modifying the content of this space in acid and base equivalents (Kimelberg et al. 1993); 4) the provision of glucose from the blood (Forsyth, 1996); and 5) the storage of glycogen, which is mostly done by astrocytes (Belin and Hardin, 1991; Magistretti et al. 1993; Forsyth, 1996; Forsyth et al. 1996).

Another way astrocytes contribute to housekeeping is by the maintenance of myelin. This is thought to be mediated by the gap junctions which astrocytes share with oligodendrocytes. This postulate is buttressed by the observation that astrocyte-oligodendrocyte gap junctions are more suited to the trafficking of small metabolites, in contrast to the strong electrical interactions which occur between astrocytes via their functionally distinct gap junctions (Gard, 1993).

Apart from housekeeping, astrocytes have been implicated in processes that are, for the most part, considered the sole dominion of neurons. Indeed, such can be said of cognition. As the upcoming section reveals, however, astrocytes may contribute to the process of cognition in different ways.

8.32 Memory, learning and behaviour

Astrocytes have been implicated in a role consistent with that of modulating memory and learning. Some of these functions reflect the process of trophism, especially noticeable in the disease or injury paradigms. Other tasks mirror the day-to-day maintenance that these cells perform. It is therefore difficult to categorize this topic in terms of the normal versus the injury situation. Moreover, depending on the experimental paradigm one can make a case for either or both categories.

In the injury paradigm, Kesslak et al. (1986) found that transplants of purified astrocytes could accelerate spontaneous functional recovery from bilateral mediofrontal lobe ablation. This recovery was translated into a spatial learning time that was no different from sham-operated controls. The enhancement of recovery was attributed to several possibilities. The first was a trophic support derived from the transplanted

astrocytes or from the host brain, upon stimulation by the exogenous astrocytes. This might allow damaged neurons to survive and healthy ones to continue their function. The second was the removal of excitotoxic neurotransmitters from the extracellular milieu. The third, might be the reinstatement of the ionic balance in the extracellular compartment (Kesslak et al. 1986).

Other studies confirmed Kesslak et al.'s (1986) findings and reported additional observations such as the correlation of GFAP protein (glial fibrillary acidic protein, an intermediate filament that was specific to astrocytes within the CNS) with the improved behavioral and memory performances of rats (Wets et al. 1991; Bradbury et al. 1995). The experimental paradigm relied on an induced chemical lesion of the cholinergic projections of the forebrain and the resulting increase in GFAP protein was interpreted as astrocyte involvement (Wets et al. 1991) e.g. astrocytes becoming reactive and possibly trophic (section 8.41; Bradbury et al. 1995).

The previously mentioned functions demonstrate that astrocytes contribute to learning and behaviour. However, this cell type may also contribute to the function of memory. Indeed, visual cortical astrocytes have been found to mimic a response characteristic of neurons, which is a glutamate-induced long-term change in the intracellular calcium concentration ($[Ca^{2+}]_i$) oscillatory response. This may have an important physiological significance, since: 1) astrocytes communicate with each other via gap junctions and can therefore propagate a calcium wave. Long distance communication may thus be achieved (Cornell-Bell et al. 1990); 2) the activity in neurons can cause an oscillation of $[Ca^{2+}]_i$ in neighbouring astrocytes (Murphy et al. 1993); and 3) the $[Ca^{2+}]_i$ oscillation in astrocytes is known to cause a $[Ca^{2+}]_i$ increase in adjacent neurons through gap junctions (Nedergaard, 1994; Vernadakis, 1996) or by local glutamate release (Parpura et al. 1994).

The calcium modulation is all the more interesting in light of the theory that the phenomena of learning and memory are governed by long-term changes in the strength of synapses (Levitan and Kaczmarek, 1991). The latter is thought to be modulated by neurotransmitter mediated increase in $[Ca^{2+}]_i$. The previous observations about astrocytes and calcium fluxes together with the hypothesis of learning and memory processes in eukaryotes, at the cellular level (Pasti et al. 1995), make for interesting possibilities for the involvement of astrocytes in the processes of memory and learning.

Another way astrocytes may contribute to learning and memory, as previously stated, is via the life support they give to neurons during injury or disease. This trophic support is part of an astrocyte mechanism designed to control the destructive effects of injury.

8.4 Astrocytes during injury

8.41 The gliosis response

In response to injury, astrocytes become hypertrophied and/or proliferate, and increase their content of GFAP (Balasingam et al. 1994) among other proteins (Eddleston and Mucke, 1993; Cannella and Raine, 1995; Eng et al. 1996; Navikas and Link, 1996). This phenomenon is termed astrogliosis (Balasingam, 1995). The astrogliotic process has its proponents, which believe that it is the CNS's way of trying to repair itself, and its opponents that believe the gliotic scar is a hinderance to the axonal repair process (Balasingam, 1995). Whether or not the CNS is able to repair itself, and to what extent, is still a debated matter (Steindler, 1993; Bahr and Bonhoeffer, 1994; Balasingam, 1995; Blanco and Orkand, 1996). Interestingly, Steindler (1993) points out that the mechanisms that cause hinderance of axonal repair may be of use in the adult CNS. Such an obstacle may prevent the wrong synapses from being formed in the adult brain whose synaptic circuitry is much more complex than that of a fetus or newborn. Indeed, establishing the wrong synaptic connections may result in severe neurologic problems. Moreover, the glial scar may serve to confine the wound site in order to minimize the damage that occurs to the affected region (Steindler, 1993).

These previous considerations on astrogliosis are all the more important in light of the report by Guénard et al. (1996) which states that the *in vitro* model of axonal injury can cause astrocytes to increase their proliferation and change their morphology. Such characteristics are the hallmark of astrogliosis and may relate to pathologies in which axonal injury is found, such as MS. It should be kept in mind, however, that astrogliosis may also be caused by the degeneration of dendrites and/or synapses (Sugaya et al. 1996). As a result there may not always be a correlation between visible neuronal damage and gliosis. Given the previously mentioned observations, it is advisable to try and prevent axonal or synaptic damage in MS. One of the ways this may be achieved is

by the increased synthesis of trophic factors by astrocytes.

8.42 The trophic role

In light of the damage that is done to the white and grey matter areas of the CNS in MS (Prineas and Connell, 1978; Arnold et al. 1990; Allen et al. 1991; Barnes et al. 1991; Matthews et al. 1991; Kidd et al. 1993; Gass et al. 1994; Davie et al. 1995; Davies et al. 1995; Hartung, 1995; Broznan and Raine, 1996; Cuzner and Norton, 1996; Lucchinetti et al. 1996), a valued treatment would be one that lends trophic support to both oligodendrocytes and neurons. In that line of thought, astrocytes have been known to provide trophic support to different neuronal populations (Lindsay, 1979; Müller et al. 1995). These life support molecules are as diverse as the neuronal populations they are designed to protect and include NGF, brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), ciliary neurotrophic factor (CNTF), basic fibroblast growth factor (b-FGF), insulin like growth factor (IGF) among other trophic factors (Rudge, 1993; Müller et al. 1995; Yong, 1996). It is interesting that some of these molecules have been reported to be trophic to oligodendrocytes as well (see below).

If IFN-β does not have a direct effect on oligodendrocytes, it remains possible that this molecule may have indirect actions on oligodendrocytes, via the increased production of oligodendrocyte trophic factors by astrocytes. In support of such a hypothesis, astrocytes have been documented to increase their production of trophic factors, among other molecules (Benveniste, 1993), *in vitro* and *in vivo*, when treated with various inflammatory cytokines that include interleukin-1, TNF-α, transforming growth factor beta among others (Carswell, 1993; Rudge, 1993; Müller et al. 1995; Yong, 1996). Some of these astrocyte derived growth factors, including NT-3, PDGF (platelet derived neurotrophic factor), CNTF and b-FGF, are important for oligodendrocyte development, maturation, and survival (Richardson, 1988; Barres et al. 1994; Louis et al. 1994; D'Souza et al. 1995, 1996; Oh and Yong, 1996). Finally, NGF has been demonstrated to cause adult porcine oligodendrocytes to extend processes and to undergo proliferation (Althaus et al. 1992) and to be trophic to mature rat oligodendrocytes (Cohen et al. 1996), which are phenotypes that can impact favourably upon successful remyelination in the CNS.

9. The purpose of my thesis

The aim of my thesis is to **determine whether or not IFN-β can cause astrocytes to increase the trophic support they provide to neurons and oligodendrocytes, via their increased production of NGF.**

10. Materials and methods

10.1 Cells and their characterization

Neonatal CD1 mouse brains, from 1 day-old pups, were used in order to generate enriched astrocyte cultures. Following the removal of the meninges, the brains were pipetted in the presence of 300 μg/ml DNase in phosphate-buffered saline (PBS) and then incubated for 30 min at 37°C in 0.25% trypsin. Trituration with a disposable pipet was followed by a wash and centrifugation (2000 rpm for 10 min). The pellet was resuspended with a Pasteur pipet and retreated with 300 μg/ml DNase for 20 min at 37°C. Following dilutions with medium containing 5% fetal bovine serum (FBS) (Yong et al. 1992), the cells were plated at a density of 3 million in 6 ml medium per 25 cm^2 flask and grown in a humidified incubator at 37°C. The medium was changed after 24h and at day 3 of culture. At day 4 the serum-containing medium was substituted for a serum-free chemically defined medium (Neveu et al. 1992). This chemically defined medium, designated M2, was DMEM/F12 supplemented with 33 mM glucose, 25 U/ml penicillin, 25 μg/ml streptomycin, 25 μg/ml insulin, 100 μg/ml transferrin, 20 nM progesterone, 50 μM putrescine and 30 nM selenium. All the feeding medium components were from Gibco BRL (Burlington, Ontario, Canada). The cells were grown in this medium until day 10, with a media change every 2 to 3 days, followed by treatment with test agents.

Cell-types were characterized by immunocytochemistry using antibodies to glial fibrillary acidic protein (GFAP, for astrocytes), fibronectin (for fibroblasts), galactosylcerebroside (for oligodendrocytes) and Mac-1 (for microglia) (Yong et al. 1992). Each slide was also counter stained with nuclear propidium iodide (10 μg/ml) to label nuclei. Using an immunofluorescence microscope, the percentage of each cell type was assessed.

10.2 Treatment of cells with test agents

Unless otherwise stated, astrocytes were treated for 6 hours with recombinant murine (rm) IFN-β (see below). Other test proteins were recombinant human (rh) interleukin (IL)-1β, transforming growth factor beta 1 (rhTGFβ1) (Collaborative

Biomedical Products, Belford, MA), rmIFN-γ (Boehringer Mannheim Canada), tumor necrosis factor alpha (rhTNF-α), rhIL-2, and rhTGF-α (Upstate Biotechnology Inc., Lake Placid, NY). 4β-phorbol-12,13-dibutyrate (PDB, Sigma Chemical Co., St-Louis, USA), a phorbol ester, was used as a positive control to stimulate NGF mRNA of astrocytes, in accordance with the reports by other investigators (Neveu et al. 1992; Pshenichkin et al. 1994).

RmIFN-β was produced essentially as previously described for human IFN-β-1b (Russell-Harde et al. 1995). The specific activity was 1-1.5 x 10^6 U/mg and was determined by VSV antiviral assay using murine L929 cells. Purity of murine IFN-β was determined to be greater than 95% as assessed by SDS-PAGE analysis of the recombinant material (Fig. 1).

10.3 Northern blot analyses

To extract total RNA, cell medium was aspirated from astrocytes cultured in 25 cm^2 flasks and 1 ml of Trizol® (Gibco BRL) was then added. Total RNA was processed as described by manufacturer (Gibco BRL). For Northern blot analyses, 20-40 μg of total RNA was added per lane and electrophoressed in a 1.2% agarose gel containing formaldehyde. The total RNA was transferred by capillary action, overnight, to a Zeta-Probe (Bio-Rad Laboratories Ltd., Mississauga, Ontario) or Hybond (Amersham Oakville, Ontario) membrane, cross-linked to the membrane by UV irradiation (UV Stratalinker™ 1800, Stratagene, PDI Bioscience Inc., Aurora, Ontario), prehybridized and then hybridized with cDNA probes. The prehybridization was done in a solution containing deionized formamide 50%, SSPE 5X, Denhart's 10X, herring sperm DNA 500 μg/ml and SDS 0.5%. This step was carried out at 42°C, for 2 hours. The hybridization solution was made of deionized formamide (Gibco BRL) 50%, SET 5X, Denhart's 1X, herring sperm DNA 100 μg/ml and 0.5% SDS. The hybridization step was done at 42°C overnight. A α-^{32}P (dCTP, Dupont Canada, Mississauga, Ontario) randomly labelled cDNA probe to NGF was used for hybridization. Following hybridization, membranes were rinsed 3 times at room temperature in 2X SSC and then washed successively at 42°C in the following solutions: in 2X SSC + 0.1% SDS for 10 min (twice) and then rinsed with 2X SSC. The membranes were exposed in a PhosphorImager cassette and signals were quantified the next day using a

phosphorImager equipped with Image Quant software. The resultant membrane was subsequently reprobed for GAPDH (D-glyceraldehyde phosphate dehydrogenase), or for 18S ribosomal RNA, to normalize for differences in RNA loading.

10.4 Detection of NGF protein in astrocyte conditioned medium using a PC12 bioassay

For the NGF bioassay, $5x10^4$ (PC12) pheochromocytoma cells were seeded on 16 well glass chambers (in medium containing 10% FBS). After 24h, the adherent PC12 cells were washed twice with PBS and incubated with conditioned medium (24 h) derived from either unstimulated astrocytes or from interferon-β (1000 U/ml) treated astrocyte cultures. To eliminate the possibility that the effects of the interferon-β-astrocyte conditioned medium may be due to interferon-β itself, PC12 cells were also treated with, non-cell exposed, M2 medium where 1000 U/ml interferon-β was added. Purified NGF (Cederlane Laboratories Ltd., Hornby, Ontario) was also added to PC12 cells in M2 medium as a positive control for PC12 cell differentiation.

In all cases, PC12 cells were treated for 12h with test agents, fixed (4% paraformaldehyde, 15 min), and then stained with (1 %) Coomassie blue for 10 min in order to colour the cells for easier visualization. Using a phase inverted microscope (Nikon Diaphot), the percentage of PC12 cells with neurites of at least 1 soma diameter in length were tabulated.

10.5 NGF ELISA

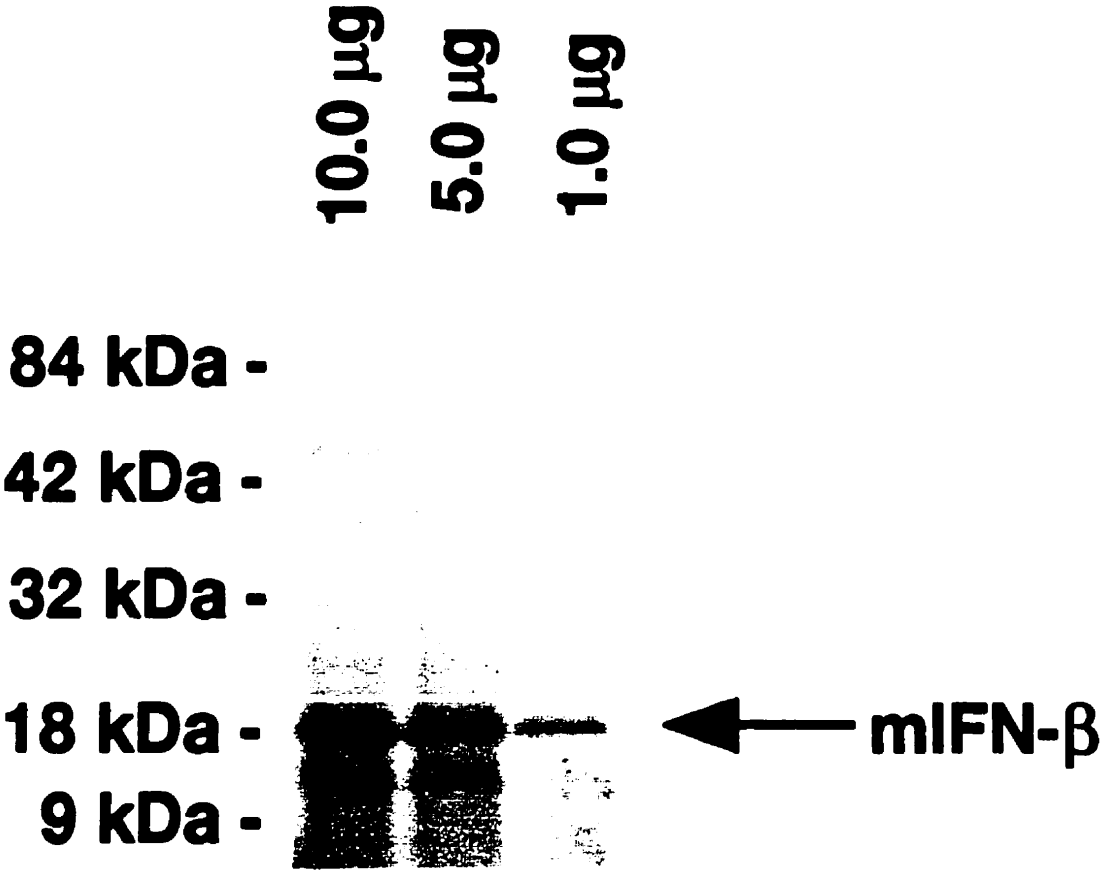
For the determination of NGF concentrations in astrocyte conditioned medium, a sandwich ELISA assay was used. Here, a 96 well microtiter plate was coated with an affinity purified sheep anti-mouse NGF antibody (kindly provided by Dr. Richard Murphy, McGill University, Montreal, Canada) (polyclonal antibody, 300 ng/ml (30 ng/well) in 1x Na_2CO_3 buffer, pH 9.6). After coating the plates with primary antibody, a buffer containing 3% FBS in PBS, was used for 1 h at room temperature to block non-specific sites. PBS containing 0.05 % tween 20, was used for each set of 5 washes which was done between each of the following steps. All incubations were done overnight at 4°C on a shaker unless otherwise stated. All subsequent dilutions were done in a PBS antibody buffer containing 3% FBS and 0.05% Tween 20. Standards and unknowns

were added to plates coated with capture antibody for 3 h at room temperature and the resultant plate washed 5x with wash buffer. The plates were coated with rabbit anti-NGF serum (1/2000 dilution) and incubated overnight at 4°C. The plates were washed 5x with wash buffer and the donkey anti-rabbit-HRP conjugated secondary antibody was then added (1/1000 dilution) for 4h at 4°C. The resultant plates were first washed 5x with wash buffer followed by addition of ABTS (Citrate-2.2'-Azino-di-[3-äthyl-benzthiazolinsulfonat (6)]-H_2O_2, Boehringer Mannheim Canada). The intensity of the colour reaction was read (at 405 nm) with a spectrophotometer (EAR 400AT) and the NGF levels were quantified against NGF standards.

10.6 Protein gel

Figure 1. SDS-PAGE analysis to determine the purity of rmIFN-β. Following electrophoresis of different amounts of rmIFN-β, the gel was stained with Coomassie blue to visualize protein. The resultant gel was scanned using a densitometric scanner and relative purity determined. Analysis of the gel demonstrated that rmIFN-β constituted over 95% of the sample. RmIFN-β was identified after SDS-PAGE by immunoblotting and N-terminal amino acid sequencing (results not shown).

Recombinant murine IFN-β



11. Results

By immunocytochemistry for cell type-specific markers (Fig. 2), the neonatal mouse astrocyte cultures were found to contain over 90% astrocytes (205 GFAP-positive cells out of 225 propidium iodide-positive cells counted) with the major contaminant being fibroblasts (~5%). Other cell types that were present included oligodendrocytes (~1%), and microglia (~1%). The percentage of contaminating cells in these cultures is in agreement with previous reports that estimate the percentage of non-astroglial cells to be in the same range (Morrison and de Vellis, 1983; Gebicke-Haerter et al. 1989).

There are many ways to establish astrocyte-enriched cultures. Perhaps one of the easiest and most straightforward is the shaking of cultures. This method, which was developed by McCarthy and de Vellis (1980), relied on two parameters: 1) a shearing force that selectively detached the overlying oligodendrocytes; and 2) a lack of viable neurons. Moreover, such a method has been reported for the removal of microglia from variously aged cultures (Giulian and Baker, 1986; Bocchini et al. 1988; Gebicke-Haerter et al. 1989).

The shaking of cultures is not the only method for purifying astrocyte cultures, however, as other methods have been used to achieve this end. Meier and Schachner (1982) had made use of antibodies and magnetic beads to isolate oligodendrocytes. In brief, this group used a primary monoclonal antibody that could bind to the surface of oligodendrocytes. This antibody bound to a secondary antibody that was coupled to magnetic beads. The resulting complex (primary antibody - secondary antibody - magnetic beads) was added to the cells in suspension and the mix was rotated for a time. Then the unbound cells were washed away and the complex attached cells were pipetted until the antibodies were detached and the cells were cultured or discarded.

This technique may be used with many cell types, provided that the appropriate antibodies are found and the cells express a cell-type specific antigen. However, this technique relies on the specificity of antibodies and the ability to remove the antibodies, once bound, without harming the cells if they are to be kept. If the contaminating cells are to be removed using this technique, then multiple antibodies must be used which may be expensive and not very specific depending on the cell surface marker that is targeted. Alternatively, one pair of antibodies can be used to purify the astrocytes, however, such

an endeavour would be costly since astrocytes are the major cell type; furthermore, there is a paucity of antibodies that are specific to surface proteins of astrocytes.

Another method that also relied, at least in part, on antibodies, was reported by Lindsay (1982). This method was used to eliminate fibroblasts from astrocyte cultures, by the use of an anti-Thy-1 antibody which recognized the Thy-1-antigen that was expressed on fibroblasts, among other cells, but not astrocytes. Lindsay (1982) used an anti-Thy-1 primary antibody and a secondary antibody (directed against the species in which the primary antibody was raised) that is chemically coupled to a plant lectin, ricin. The Thy-1 positive cells that took-up the ricin died. Using this method, Lindsay (1982) found that over 90% of the fibronectin positive cells died and there were virtually no Thy-1 positive cells.

The various methods of purification previously cited are by no means the only ones that are available and the reader is directed to seek the best one(s) for his/her needs. However, as our cultures did not reveal the presence of large amounts of contaminating oligodendrocytes or microglia, we did not resort to such techniques to purify our cultures.

The astrocyte-enriched cultures increased their production of NGF mRNA in a time-dependant manner, when exposed to recombinant murine IFN-β at a concentration of 1000 U/ml (Fig. 3). By 3 hours of treatment, the NGF mRNA was already markedly increased and this continued to elevate and peaked (40-fold) at 6 hours after treatment. Longer treatment periods with rmIFN-β produced a less pronounced increase in NGF mRNA and by 24 hours of treatment, no NGF mRNA increase was evident (Fig. 3). While it is possible that the incubation of the IFN-β with the cells downregulates the IFN-β receptor, which is located on astrocytes (Tada et al. 1994) among other cell types (Stewart, 1981; Peska et al. 1987; Navarro et al. 1996), another probable cause that no increase in NGF mRNA could be seen 24h after a single treatment with rmIFN-β is its lability, because if the cells were pulsed with another 1000 U/ml 12 h after the first treatment, a marked NGF mRNA increase was again elicited (results not shown). Because a period of 6h treatment with rmIFN-β was optimal to elicit maximal rise of NGF transcript, all subsequent treatments were carried out for 6 hours unless otherwise stated.

To determine if the 1000 U/ml of rmIFN-β was the optimal concentration to elicit

NGF increase by astrocytes, a dose response curve was done. The results demonstrate a gradual rise in NGF mRNA levels with increasing concentrations of rmIFN-β (3-fold increase at 300 U/ml) which then sharply elevates, to 40-fold increase, at a 1000 U/ml rmIFN-β (Fig. 4).

The high potency of rmIFN-β in eliciting the astroglial production of NGF mRNA became evident when we investigated the effects of other factors that have been reported to increase NGF mRNA. Among the many agents that have been described to increase the NGF transcript, none duplicated the 40-fold magnitude of increase elicited by rmIFN-β. Figure 5 shows that IL1-β elevated NGF mRNA 1.5-fold while IFN-γ increased NGF mRNA 2.5-fold. These were the highest NGF mRNA increases, that were obtained by the other cytokines tested, after a 6h treatment.

NGF is a secreted protein and it was of interest to determine whether the rmIFN-β induced increase in the NGF mRNA was represented by elevated secretion of NGF protein into the culture medium. The astrocyte conditioned medium (ACM) was tested for its content of NGF protein using two different assay systems. First, PC12 cells, which differentiate into a neuronal-like phenotype in the presence of NGF (Tischler and Perez-Polo, 1975; Luckenbill-Edds et al. 1979; Hall et al. 1988), were used to determine whether or not the ACM contained sufficient amounts of biologically active NGF protein. In Figure 6, PC12 cells extended neurites following their exposure to conditioned medium (CM) from astrocytes treated with 1000 U/ml rmIFN-β. When quantified, the CM from astrocytes treated with rmIFN-β increased morphological differentiation of PC12 cells 4-fold when compared to ACM collected from controls (Fig. 7). RmIFN-β itself, at 1000 U/ml, did not promote the PC12 cells to differentiate morphologically indicating that the effect of the IFN-β-treated ACM was not due to IFN-β itself.

We quantified the NGF protein content in ACM using a sandwich ELISA. The ACM from the rmIFN-β treated cultures contained nearly 10-fold more NGF than ACM-control (Fig. 8).

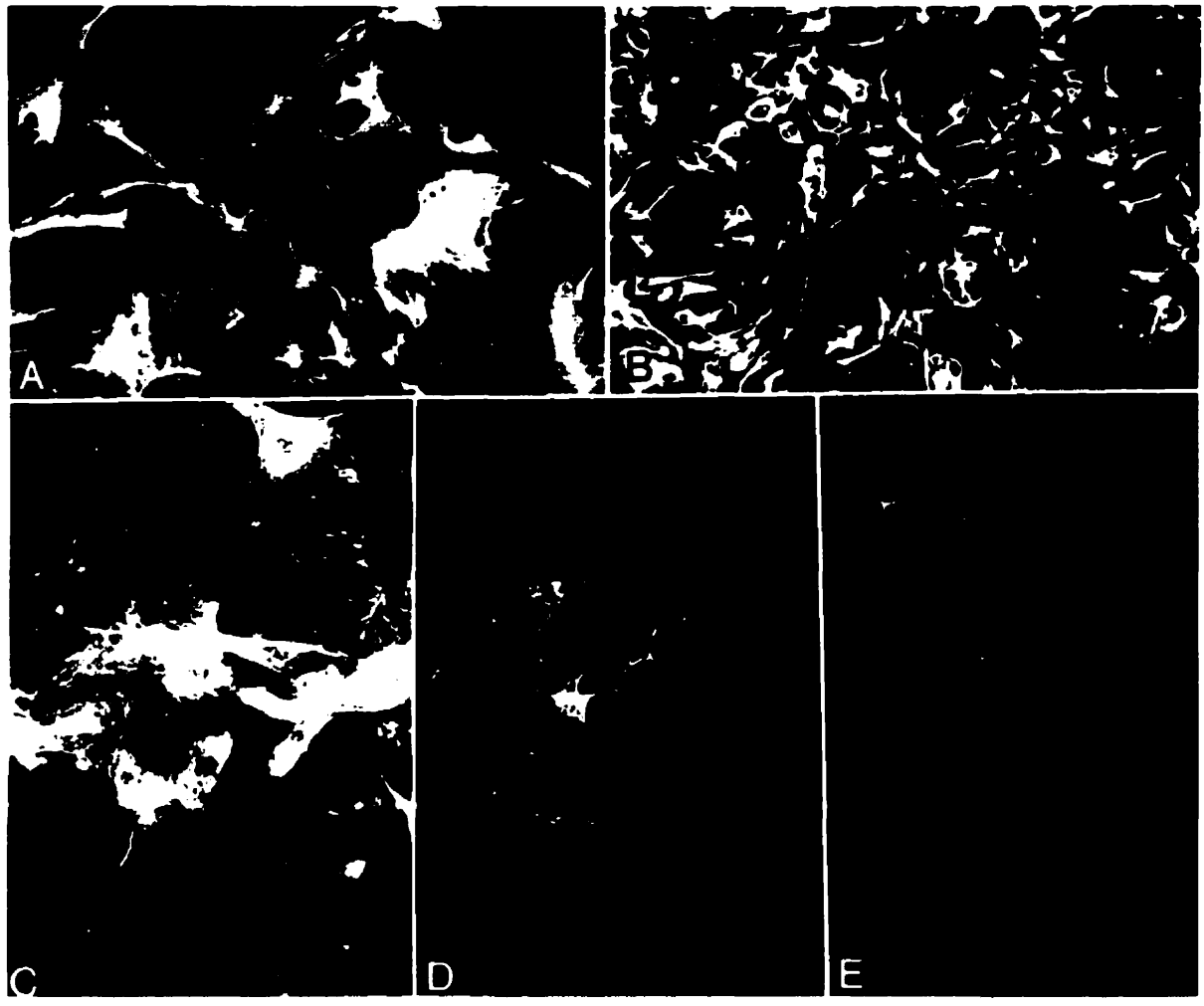
The process outgrowth from the ACM derived from cultures treated with 1000 U/ml of IFN-β, containing $\sim$250 pg/ml of NGF as determined by ELISA, was not statistically different from that of its sister culture which was treated with 5 ng/ml of murine NGF. The apparent discrepancy between the different concentrations of NGF and the process extension could be explained if the 5 ng/ml was at saturating levels for PC12

cell process extension. However, in order to formally rule out the involvement of any other factor contributing to process extension, anti-NGF neutralizing antibodies would have to be used to neutralize the NGF effect on process extension.

Finally, we did not test the ability of the ACM (e.g. NGF), from cultures treated with 1000 U/ml rmIFN-β, to reduce the proliferation rate of PC12 cells as IFN-β is known to cause a decrease in the proliferation rate in most cells (see section 3) including astrocytes (results not shown). In order to conduct such an experiment, anti-rmIFN-β antibodies would have to be preincubated in the ACM and then the PC12 cells could be treated with the ACM. Then it could be possible to evaluate the NGF effect on the reduction of proliferation (Greene and Shooter, 1980), assuming that all the IFN-β has been neutralized.

11.1 Immunocytochemistry and cell types in culture

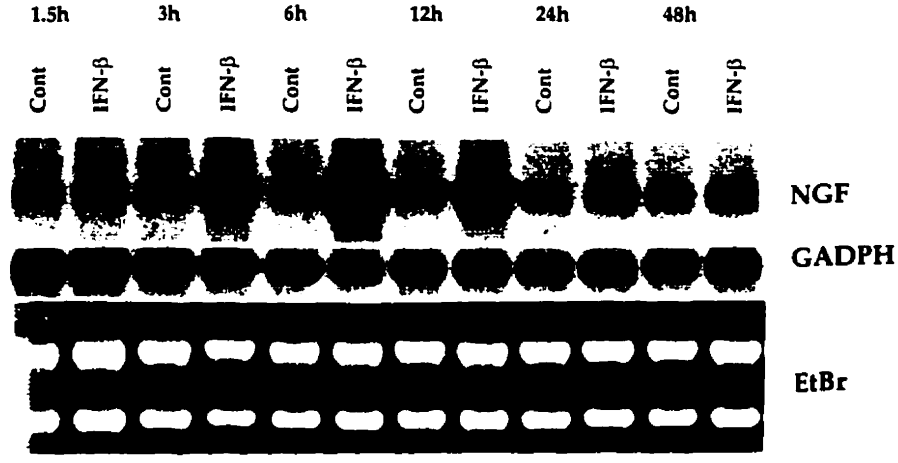
Figure 2. Determination of neural cell types by immunocytochemistry. A,B) Astrocytes were specifically labelled with anti-GFAP (A, 1000X magnification, B, 400X). C) Fibroblasts labelled with anti-fibronectin (1000X). D) A single oligodendrocyte labelled with anti-GalC (1000X). E) Microglial cells were immunoreactive for anti-Mac-1 (1000 X).

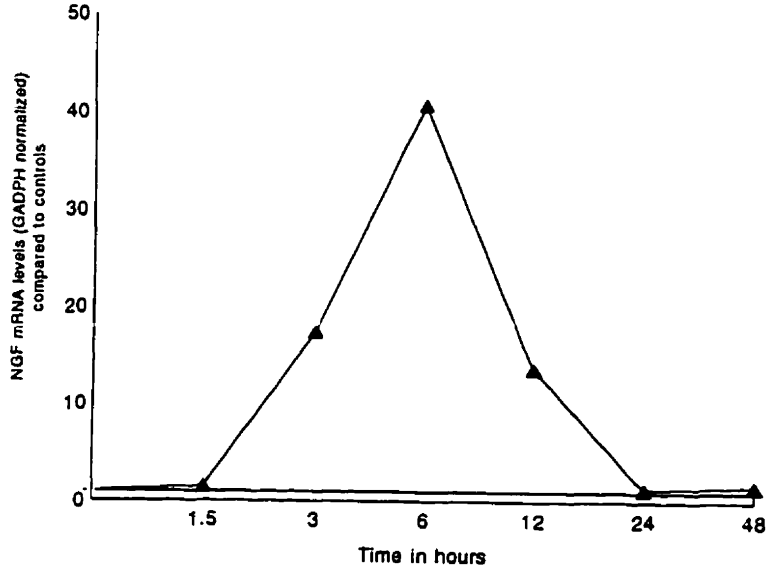

A
B
C
D
E

11.2 rmIFN-β time course

Figure 3. Time course of the increase of NGF mRNA induced by a single application of 1000 U/ml IFN-β. The Northern blot bands for NGF and GAPDH are shown for control and IFN-β-treated astrocytes at the different time points indicated, while the corresponding ethidium bromide (EtBr) gel is shown below the Northern blot bands. The bottom panel shows the phosphor Imager quantified increase in NGF mRNA levels as a function of time following treatment.

The effects of 1000 U/ml IFN-β on the production of NGF mRNA by astrocytes: Time course

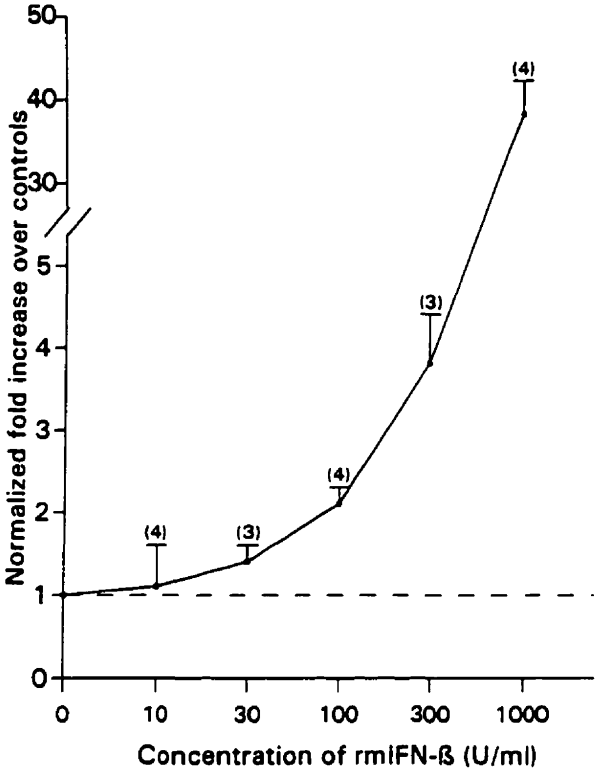




11.3 rmIFN-β dose-response

Figure 4. NGF mRNA of neonatal mouse astrocytes is significantly elevated by different concentrations rmIFN-β. Northern blot analysis of a dose-response curve was performed and pooled from 3 or 4 different experiments (values are Mean $\pm$ SEM). The corresponding NGF and GAPDH signals for each concentration of IFN-β are displayed immediately below the listed concentrations of the dose-response curve.

Recombinant Murine IFN-ß Elevates NGF mRNA Of Astrocytes



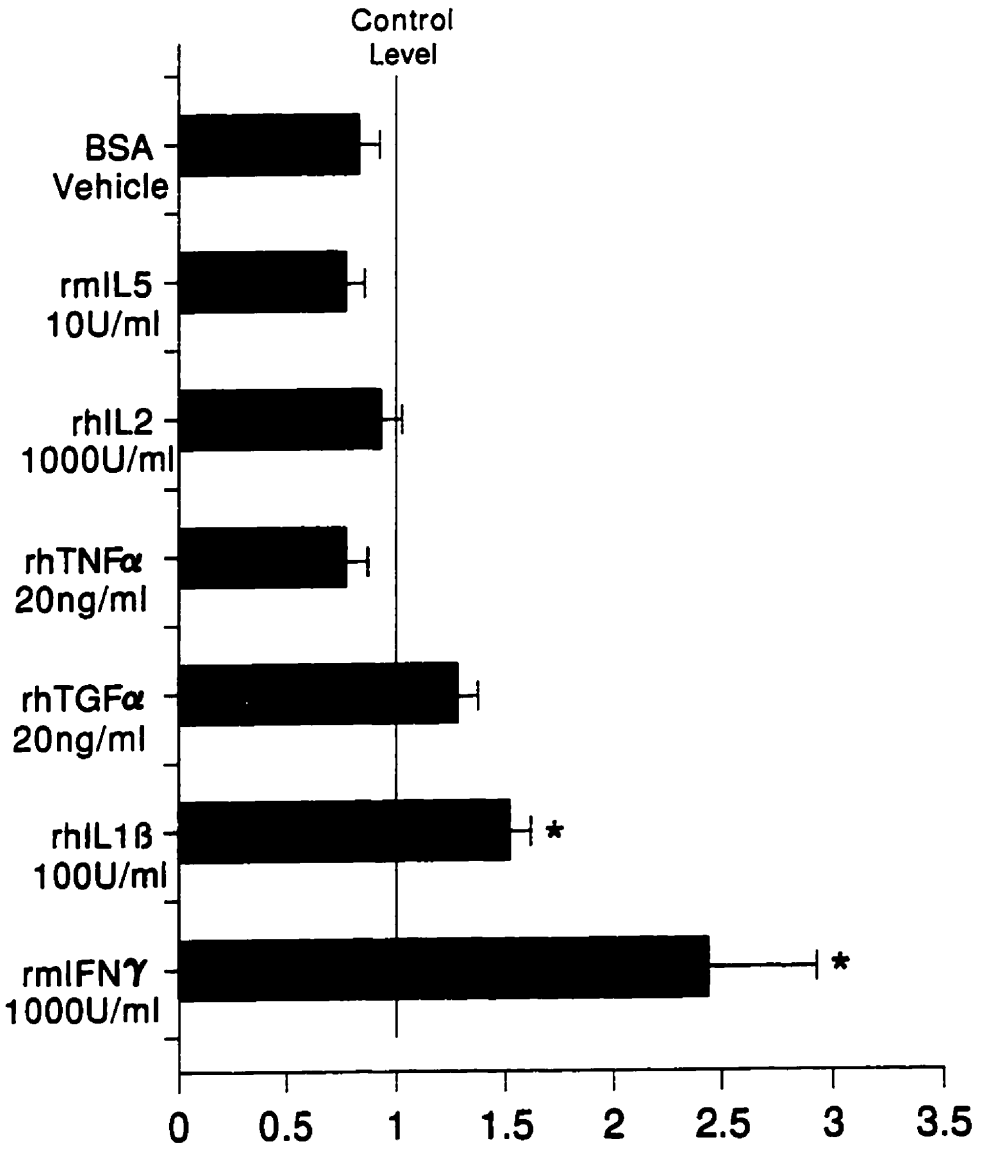
NGF

GADPH

11.4 Various cytokines

Figure 5. Among the different cytokines tested at the indicated concentrations and given to astrocytes for 6h, only rhIL1-β and rmIFN-γ statistically increased NGF mRNA; however the maximum increase was 2.5-fold. Values are the Mean $\pm$ SEM, plotted from 3 separate experiments *$p < 0.05$ compared to BSA vehicle (1 way ANOVA with Duncan's multiple comparisons).

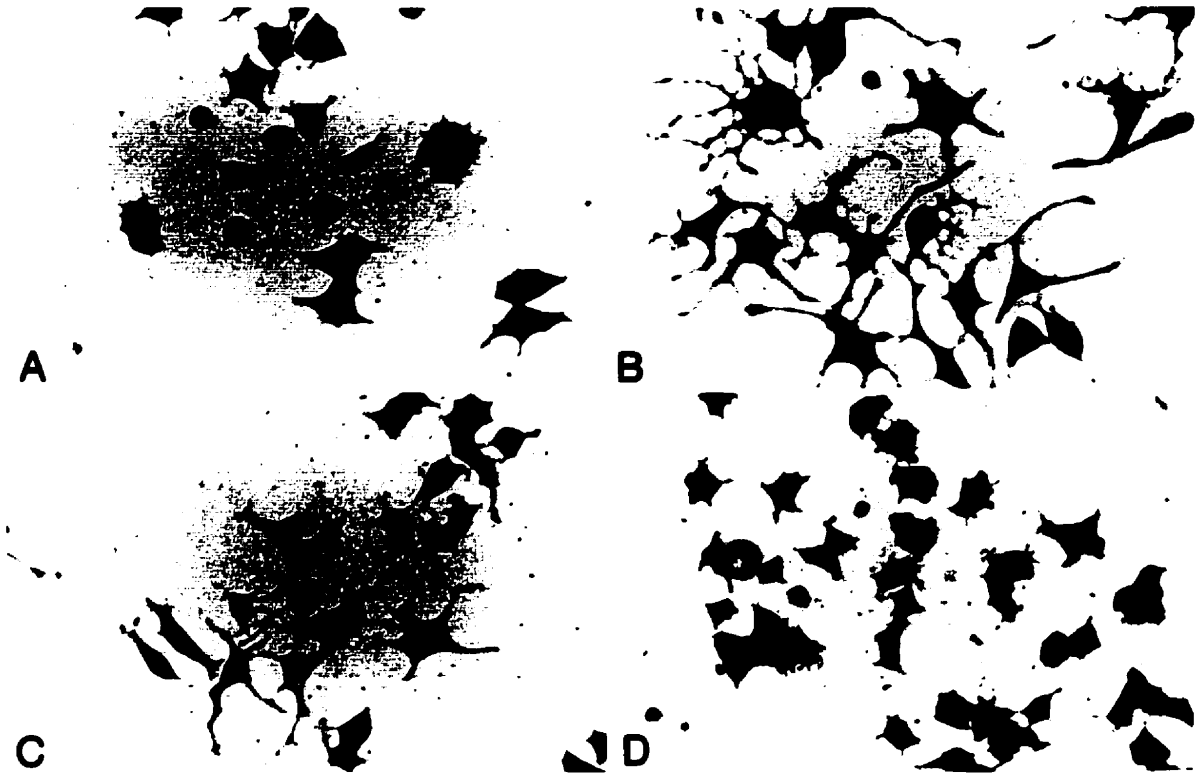
The Effects Of Other Cytokines On NGF mRNA Of Astrocytes



*p<0.05 compared to BSA vehicle

11.5 Conditioned media and process extension: qualitative result

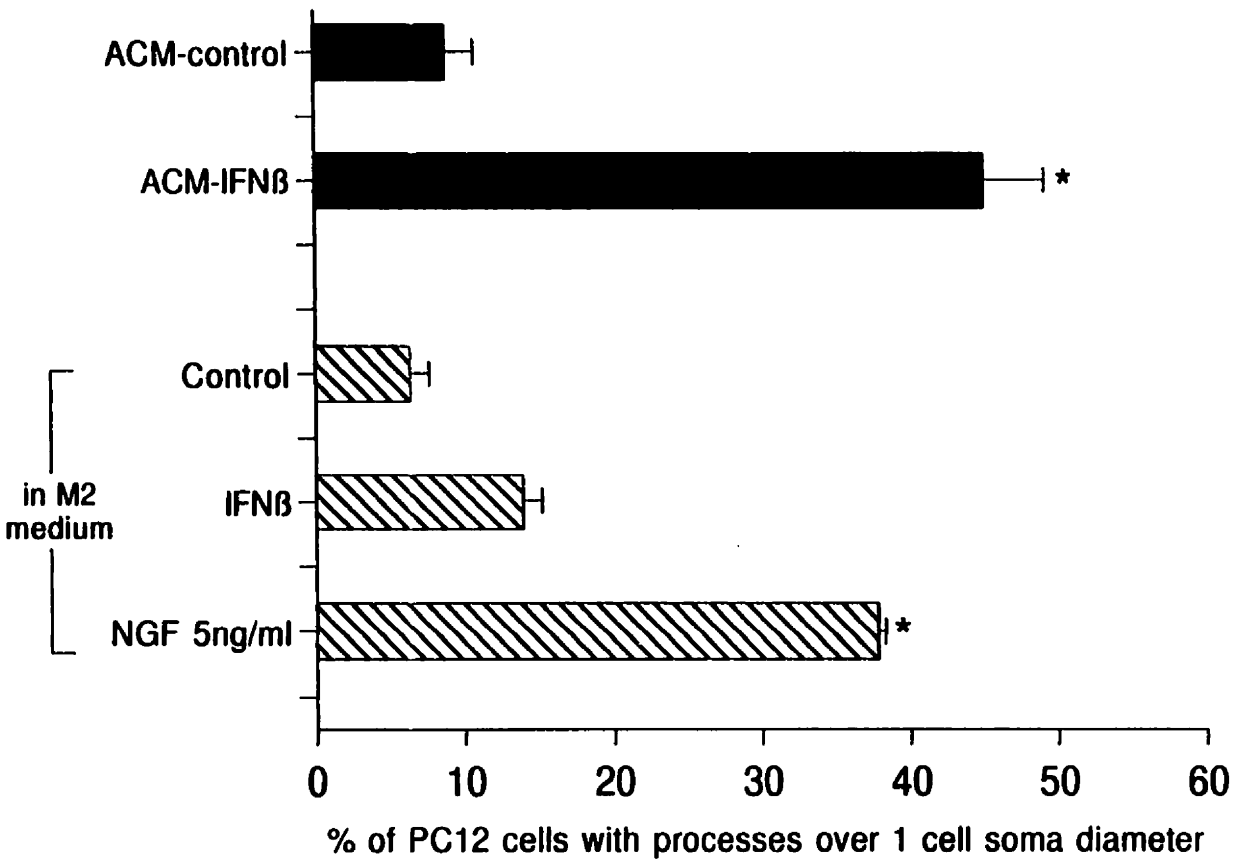
Figure **6.** Conditioned medium derived from IFN-β treated astrocytes promoted PC12 differentiation. PC12 cells were exposed to 24h conditioned medium for 12 hours, and then stained with Coomassie blue. **A)** PC12 cells exposed to conditioned medium from untreated astrocytes; **B)** PC12 cells treated with conditioned medium from rmIFN-β (1000 U/ml)-treated astrocytes; **C)** purified NGF from mouse salivary gland (5 ng/ml); and **D)** rmIFN-β (1000 U/ml) alone.


A
B
C
D

11.6 Conditioned media and process extension: quantitative result

Figure 7. Effects of astrocyte conditioned medium on PC12 process extension. Values were mean ± SEM of triplicates. Cells that had at least 1 neurite greater than 1 cell soma diameter were expressed as a ratio of total PC12 cells counted. *p <0.05 compared to ACM-controls or controls in M2 (1 way ANOVA with Duncan's multiple comparisons).

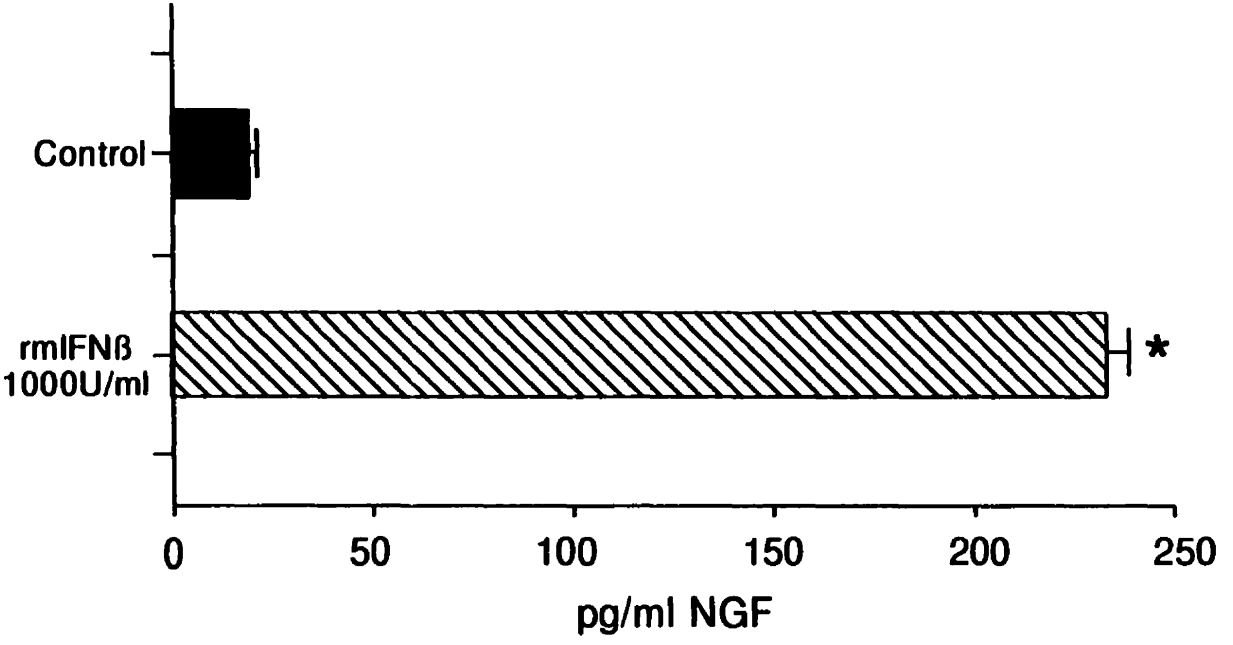
Astrocyte Conditioned Medium (ACM) From Interferon-ß Treatment Promotes PC12 Differentiation



11.7 NGF ELISA

Figure 8. Quantification of NGF protein, by sandwich ELISA, contained in the supernatant of control or recombinant murine IFN-β treated astrocytes. Values are Mean $\pm$ SEM of triplicate experiments. *$p < 0.001$ compared to control (Student's t-test).

Content Of NGF Protein In Astrocyte Conditioned Medium: ELISA Determinations



12. Discussion

It is unknown how IFN-β acts in MS. **This study has found that there is a very potent increase in NGF production by astrocytes, upon IFN-β treatment.** An analysis of the results and a review of the potential effect of NGF in the pathological state and at the cellular and molecular levels are given.

12.1 Analysis of the results

The results demonstrate that rmIFN-β has a potent effect for increasing the level of NGF mRNA in astrocytes. Within 6 hours of treatment of 1000 U/ml of IFN-β, NGF mRNA expression in astrocytes was augmented by as much as 40-fold compared to non-treated controls. This magnitude of NGF mRNA elevation was comparable to that reported by Pshenichkin and Wise (1995), who treated astrocytes with okadaic acid. In comparison, other agents that had been reported to increase NGF mRNA, such as IL-1β and IFN-γ (Carswell, 1993; Yong, 1996), were much less effective, producing only a 2.5-fold increase of astrocyte NGF mRNA at best. We did not distinguish between an IFN-β effect on increased *de novo* transcription of the NGF gene or on stabilization of the NGF mRNA, or a combination of both; these mechanisms remain to be elucidated.

Previously, Awatsuji et al. (1995) showed that murine IFN-β *downregulated* NGF mRNA in astrocytes, in contrast to our results. There exists several differences, that could account for the apparent discrepancy, including: 1) the culture conditions (DMEM containing 10% FBS in contrast to our serum-free environment); 2) the age of the animals that were used by Awatsuji et al. (1995) was 8-day-old and 3) the cultures were subjected to trypsin treatment and reculturing three times before test agents were used. Therefore, the difference between the age of the animals used the repeated trypsinization of the cultures as well as the use of 10% serum could account for the difference in the results presented here, and those reported by Awatsuji et al. (1995).

The increase in NGF mRNA produced by rmIFN-β, in our experiments, was also observed at the level of NGF protein. Thus, NGF protein as determined by ELISA was increased 10-fold by rmIFN-β when compared to controls. The 40-fold increase in NGF mRNA, accompanied by a 10-fold increase in protein, could suggest a post transcriptional control mechanism. Another possibility is that some of the NGF protein

was not exported out of the cell. A third possibility is an increase in the protein turnover rate. These mechanisms are not mutually exclusive and some or all of these could be simultaneously operational.

In the following sections, a more detailed look is taken to address how an increase in astrocyte production of NGF, in response to IFN-β, may be useful in MS. In this regard, NGF may influence MS pathogenesis at possibly three levels, including immunomodulatory effects, promotion of oligodendrocytes survival/differentiation, and the promotion of axonal protection/recovery.

The well established trophic capabilities of NGF in the CNS, for cholinergic neurons (Hagg et al. 1993; Rylett and Williams, 1994; Hayashi, 1996), and the increasing recognition that NGF is also trophic for noncholinergic neurons (Holtzman et al. 1995) and oligodendrocytes (Althaus et al. 1992; Cohen et al. 1996) make NGF, a molecule that has trophic potential for MS, a disease that affects neurons and oligodendrocytes (section 12.53). The significance of the current results, may be more easily understood if a review of the documented and potential effects of NGF *in vitro* and *in vivo* is made.

12.2 The receptors of NGF, trkA and $p75^{NTR}$

In order to appreciate the potential effect of NGF on the nervous system, especially the brain, it is important to review the distribution of the NGF receptors trkA (also called the high affinity [$K_D \sim 10^{-11}$ M] or $p140^{trk}$ or trk receptor) and $p75^{NTR}$ (also called the low affinity NGF [$K_D \sim 10^{-9}$ M] or low affinity neurotrophin receptor or LNGFR or $p75^{LNGFR}$) (Meakin and Shooter, 1992; Chao and Hempstead, 1995). The regulation of these receptors by NGF and the relative contribution of each receptor to NGF signalling are also reviewed.

12.21 Distribution of the receptors in the brain

$p75^{NTR}$ and trkA mRNAs are colocalized for some structures and have different distribution patterns for others. The NGF-responsive cholinergic structures include: the medial septum, the diagonal band of Broca, the substantia innominata, the nucleus basalis of Meynert and the caudate (Gibbs and Pfaff, 1994). Other regions also coexpress trkA and $p75^{NTR}$: the interpeduncular nucleus, the prepositus hypoglossal nucleus, the raphe,

the ventricular cochlear nucleus and the medullary reticular formation (Gibbs et al. 1989; Koh et al. 1989; Gibbs and Pfaff, 1994). These regions also correspond to the high affinity binding sites for NGF as verified by autoradiography (Richardson et al. 1986; Raivich and Kreutzberg, 1987).

The areas that are positive of trkA but negative for $p75^{NTR}$ mRNA are: the paraventricular nucleus of the thalamus, the lateral vestibular nucleus, the solitary nucleus and the principal sensory or the nucleus oralis of the trigeminal nerve. The localization of neurons that are positive for $p75^{NTR}$ and negative for trkA mRNA include the subependymal region of the lateral ventricle (Gibbs et al. 1989; Koh et al. 1989; Pioro and Cuello, 1990a; Gibbs and Pfaff, 1994), the arcuate (tanycytes) (Gibbs et al. 1989; Koh et al. 1989; Gibbs and Pfaff, 1994), the lateral hypothalamus (Gibbs et al. 1989, Gibbs and Pfaff, 1994), the superior colliculus (Pioro and Cuello, 1990b) and the mesencephalic trigeminal nucleus (Koh et al. 1989; Pioro and Cuello, 1990a; Gibbs and Pfaff, 1994).

Finally, Holtzman et al. (1995) reported on NGF-responsive noncholinergic structures within the CNS. These structures, some of which have been previously mentioned, include: 1) the paraventricular nucleus of the thalamus; 2) the prepositus hypoglossal; 3) the rostral and intermediate subnuclei of the interpeduncular nucleus; 4) the area postrema; and 5) the scattered neurons in the ventrolateral and paramedian medulla. This varied distribution of $p75^{NTR}$ and trkA may have its basis in the signalling potential of each receptor.

12.22 Signalling capabilities and binding affinities of trkA and $p75^{NTR}$

While there is a consensus on the ability of trkA to transmit an intracellular signal, there are many conflicting reports as to the role of $p75^{NTR}$ in the signalling process. Indeed, some findings have trkA receptor being able to bind NGF with high affinity (Klein et al. 1991; Chao and Hempstead, 1995), while others are reporting the necessity of $p75^{NTR}$ for trkA to bind NGF with high affinity. This high affinity site is speculated to be created via the $p75^{NTR}$ presentation of NGF to trkA, or the direct physical interaction of $p75^{NTR}$ with trkA to create a high affinity binding site for NGF (Hempstead et al. 1991; Kaplan et al. 1991; Chao and Hempstead, 1995). Despite this cooperation, $p75^{NTR}$ is apparently incapable of signalling on its own (Lee et al.

1992,1994) whether or not trkA is present on the cell (Smeyne et al. 1994). Others report that the presence of trkA is necessary for $p75^{NTR}$ mediated signalling (Volonté et al. 1993a,b). In contrast, Berg et al. (1991) believe that $p75^{NTR}$ is required for NGF-mediated tyrosine phosphorylation of trkA in a PC12 derived cell line. Finally, there are reports that $p75^{NTR}$ can transmit an intracellular signal in the absence of trkA (Represa et al. 1991; Rabizadeh et al. 1993; Anton et al. 1994; Barrett et al. 1994; Blöchl and Sirrenberg, 1996; Carter et al. 1996; Casaccia-Bonnefil et al. 1996; Cortazzo et al. 1996; Frade et al. 1996).

The low affinity $p75^{NTR}$ is also described as being able to make trk receptors more ligand selective. For example, trkA can bind NGF, NT-3 and NT-4/5 (neurotrophin-4/5) with low affinity ($K_D \sim 10^{-9}$ M) in the absence of $p75^{NTR}$; however, trkA binds NGF exclusively, and with high affinity ($K_D \sim 10^{-11}$ M), when $p75^{NTR}$ is expressed (Meakin and Shooter, 1992; Chao and Hempstead, 1995). The importance of the role that each receptor plays becomes evident when one considers that certain cell populations express $p75^{NTR}$, trkA, or both (section 12.21). The effect of NGF on such cell types may depend on the local concentration of NGF, the receptors that they express as well as the signalling capabilities of these receptors.

In an attempt to elucidate the role of each receptor as well as that of NGF, several groups have created knockout mice for $p75^{NTR}$ (Lee et al. 1992, 1994), trkA (Smeyne et al. 1994) and NGF (Crowley et al. 1994).

12.3 Are we learning something from knockout mice for NGF, trkA and $p75^{NTR}$?

An appreciation for the roles of NGF, trkA and $p75^{NTR}$, and their overall importance, can best be underlined by a review of what the corresponding knockout mice are able to unveil. Moreover, the role for each is underscored during development, or the lack thereof, of these mice.

12.31 NGF knockouts

Despite the observation that the NGF (-/-) mice (homozygous knockout or null mice) are capable of surviving *in utero*, the phenotype of these mice confirms the invaluable trophic support that this neurotrophin provides and the incapacity of other trophic factors to compensate completely for the lack of NGF. Among the myriad of

phenotypes that these mice manifest, are the following: 1) a lack of response to noxious stimuli accompanied by a prodigious cell loss in the sympathetic and sensory ganglia. This lack of response is not due to a lack of food intake, since the pinch test is done before their first feeding. It should be noted that some of the homozygous (-/-) mice do not ingest milk and therefore do not increase their weight and die within 3-days of birth, while others scarcely consume food but manage to survive for 1-4 weeks; 2) ptosis (a drooping of the upper eyelids) is a consequence of a lack of sympathetic innervation to the eye; 3) delayed eye opening; and 4) locomotion, which is characterized by a tremor and a gait (Crowley et al. 1994; Snider, 1994).

In contrast to the plethora of phenotypes manifested by these mice, the mice showed no obvious signs of organ abnormalities, on the day of birth, as could be detected by haematoxylin/eosin stained sections of the thoracic and abdominal cavities. Moreover, the brains of homozygous (-/-) animals, from day 3 to day 28, expressed markers such as mRNA for trkA, $p75^{NTR}$, choline acetyltransferase and acetylcholinesterase (AChE) activity in the basal forebrain cholinergic neurons (the medial septum, the diagonal band of Broca, and the nucleus basalis magnocellularis). These markers were expressed by the cholinergic neurons and their projections for the life of the animals. However, the cells seemed smaller and the staining was weaker for these markers (Crowley et al. 1994).

12.32 TrkA knockouts

The trkA knockout mice have a phenotype that overlaps, to a certain extent, with that of the NGF knockout mice. The trkA (-/-) mice are smaller at post-natal day 10 (P10) though they appear normal at birth. By P20 half of the mice die, and none live beyond P55. These knockout mice do not react to noxious olfactory, thermal or pain stimuli in their rear paw or whisker pads. Such results indicate that the trigeminal and the peripheral sensory systems are affected. In fact, the PNS, the trigeminal, the sympathetic and the dorsal root ganglia which express trkA, suffer over a 70% loss in neurons (Barbacid, 1994). Smeyne et al. (1994) conclude that the neuropathies, both sensory and sympathetic, observed in these mice are caused by the loss of NGF dependant neurons in these ganglia.

In the CNS, a decrease in AChE staining is observed when comparing knockout mice to the wild-type mice. Indeed, the hippocampus and the cerebral cortex receive

cholinergic projections from the affected medial septum and the nucleus basalis, respectively, which have a notable decrease in AChE staining. Smeyne et al. (1994) conclude that most CNS and PNS structures that usually express trkA are affected by the absence of this receptor, in the homozygous knockout mice.

12.33 $p75^{NTR}$ knockouts

The last of the three knockout mice that is discussed here is the $p75^{NTR}$ null mouse. Contrary to the previously mentioned knockouts, the $p75^{NTR}$ (-/-) mice are viable and fertile. They exhibit no evident organ defects (Lee et al. 1992) despite the high level of $p75^{NTR}$ expression by various organs, such as the kidneys (Sariola et al. 1991), the teeth (Byers et al. 1990), the testis (Persson et al. 1990), the ears (von Bartheld et al. 1991) and the motor neurons (Yan and Johnson, 1988), in the wild-type mice. The widespread expression of this receptor suggests that it has a role in mediating NGF (or other ligand) response during the development or function of these organs. Lee et al. (1992) hypothesize that the $p75^{NTR}$ has a redundant function and that other receptors may compensate for its absence or that the deficiencies are more subtle in these null mice than in other knockout mice.

In a subsequent publication, Lee et al. (1994) conducted a more detailed investigation of the $p75^{NTR}$ knockout's sympathetic innervation. The authors demonstrated that what appeared to be a lack of effect of the $p75^{NTR}$ null mutation, on sympathetic innervation (Lee et al. 1992), was in fact a selective effect on the sympathetic neurons, e.g. a lack of sympathetic innervation of the pineal gland and the lateral footpads (Lee et al. 1994). Based on the observation that these targets did not the cause the failure of their innervation, Lee et al. (1994) suggested that $p75^{NTR}$ might play a role in directing axons toward their target. This was based on the observation that $p75^{NTR}$ was found on the pathways on which axons grow, on axons and Schwann cells (Yan and Johnson, 1988). It was therefore possible that $p75^{NTR}$ served to sequester, present and/or increase the sensitivity of neurons to NGF.

The knockout mice presented in the past sections underscore the importance of NGF during development, and this is especially evident with NGF and trkA (-/-) mice. Unfortunately the NGF and trkA (-/-) mice did not have a sufficiently long life-span to allow the discovery of possible deleterious effects that result from a lack of NGF or trkA

receptor expression in the CNS at an adult age. Moreover, the CNS may be more adept at compensating for the lack of trophic support that is mediated by NGF or is receptors, than is the PNS, which could also explain why there is no obvious CNS-mediated phenotype.

The NGF influence may start during development, however, it does not end at this period of life. This trophic factor also influences other systems (e.g. non-neuronal) as revealed in the upcoming sections.

12.4 Effects of NGF on the immune system

The immune system is one of the compartments in which NGF has a modulatory role. This topic is dealt with in two sections. The present section deals with NGF and its modulatory action on various immune cells. The next section reveals the possible role for NGF in MS.

12.41 NGF and monocytes, macrophages and microglia

Brown et al. (1991) provided evidence that the regrowth of axons in the PNS, after Wallerian degeneration, required the presence of macrophages. This group concluded that the macrophage recruitment was a necessary step in the synthesis of NGF as well as the regrowth and maintenance of most sensory axons.

The study done by Houle (1992) extended the previous finding that NGF plays a role in axonal regeneration. However, Houle (1992) suggested that NGF's effect in axonal regeneration, might be indirect, e.g., via the non-neuronal cell population that was drawn to the bound NGF, such as the macrophages. Indeed, Houle (1992) found that a nitrocellulose strip and fetal spinal cord tissue transplanted along its sides induced an ordered array of cells in the hemisection of the spinal cord of rats only when NGF bound to the nitrocellulose. A stratified layer of macrophages, that were the closest to the bound NGF, was followed by a layer of fibroblasts and astrocytes (most likely derived from fetal tissue). The neurons, whose origin was uncertain in this paradigm, extended axons in between the fibroblast and astrocyte layer. Such a layering of cells was not seen in the untreated nitrocellulose strip, as the macrophages, fibroblasts and astrocytes were intermixed and contained fewer regenerating axons (Houle, 1992). This suggested that the presence of certain cell types was not enough to induce regrowth of axons and that

a certain cellular array was necessary to ensure axonal regeneration.

Avellino et al. (1995) find increased staining in the dorsal root axonal pathway, for $p75^{NTR}$ protein prior to macrophage infiltration into the injured PNS segment of this track. This increase in receptor immunocytochemistry is not seen in the CNS portion of the injured dorsal root pathway, which also undergoes Wallerian degeneration. Avellino et al. (1995) hypothesize that the difference detected in macrophage recruitment in the PNS versus the CNS in response to Wallerian degeneration, may be due to NGF and $p75^{NTR}$. Moreover, the number of immunocytochemically stained monocyte/macrophage/ microglia, is increased after NGF injection into the CNS versus control injection (Kliot et al. 1994).

The difference in the macrophage response in the CNS versus the PNS may not be the only factor that influences the regeneration of axons. The age of the animal, among other factors (section 8.4), can influence the healing process. This can be exemplified by results from the neonatal rat brain where an intermixed array of astrocytes and macrophages, on a nitrocellulose implant, can induce axonal repair. In contrast, a nitrocellulose implant in an adult animal has an array of astrocytes, fibroblasts and macrophages that induce minimal axonal regrowth (section 8; Rudge et al. 1989).

Finally, macrophages and microglia are known to be able to synthesize NGF (Mallat et al. 1989; Levi-Montalcini et al. 1995), this may indicate an autocrine or paracrine function of NGF depending on whether or not they express NGF receptors. Ehrhard et al. (1993) demonstrated that monocytes expressed trkA and this expression was downregulated, *in vitro*, upon maturation of monocytes to macrophages. However, this group found no expression of $p75^{NTR}$ mRNA by RT-PCR, in monocytes, when previous reports (Morgan et al. 1989; Otten et al. 1989) detected the expression of the $p75^{NTR}$ protein on the cell surface.

12.42 The effects of NGF on T and B cells

This is discussed in section 12.5.

12.43 NGF and various aspects of the immune function

NGF can influence other facets of the immune system (Levi-Montalcini et al. 1995). Among these are the modulation of the shape of platelets (Gudat et al. 1981), the

degranulation of rat peritoneal mast cell (Bruni et al. 1982; Mazurek et al. 1986; Pearce and Thompson, 1986) and an increase in vascular permeability in the skin (Otten et al. 1984). Other reports, concerning humans cells, demonstrate the influence of NGF on myeloid progenitor cells. This modulation involves colony growth and the differentiation of cells (Matsuda et al. 1988). Moreover, mature basophils respond to NGF by regulating their lipid mediators (Bischoff and Dahinden, 1992).

These observations shed light on the potential influence of NGF on the immune system. Such a plethora of potential responses and the observation that systemically administered NGF causes hyperalgesia in normal humans (Petty et al. 1994) argue for a more localized modulation of NGF and its effect. This can be achieved by manipulating the local NGF receptors in order to maximize the effect of a given concentration of NGF (Hempstead, 1993), or by modulating the local NGF synthesis, such as can be obtained by the use of a cytokine (Carswell, 1993).

12.5 Hypotheses: how NGF could be useful in MS

The previous sections have, in some detail, presented the unquestionable usefulness of NGF during the developmental stage in mice, the effects and potential effects of NGF on neuronal populations and the sphere of influence of NGF on different cell types. In addition to these functions, NGF has other immune and non-immune effects that may be of great importance to EAE and MS. This section reviews some of these functions.

12.51 Immunomodulatory function

To the above mentioned observations (section 12.4), other immunological findings are added, such as the detection of NGF in Th1, splenic B cells, B lymphoma cell line (Santambroglio et al. 1994) and Th2 cells (Ehrhard et al. 1993; Santambroglio et al. 1994). However, only Th2 clones increase their content of NGF mRNA upon specific antigen (Ag) stimulation (Ehrhard et al. 1993; Santambroglio et al. 1994). This may indicate an autocrine mechanism since NGF has been reported to cause B cells to survive (Torcia et al. 1996) and lymphocytes (T and B cells) to proliferate (Thorpe and Perez-Polo, 1987; Otten et al. 1989; Levi-Montalcini et al. 1995).

The NGF effect on lymphocytes is not restricted to proliferation as it can

stimulate the *in vitro* secretion of immunoglobulins (Ig) such as IgM and IgA (Otten et al. 1989; Kimata et al. 1991). The NGF-mediated increased synthesis and secretion of IgG4, is augmented even further when NGF and T cells synergize in the induction of IgG4 production by B cells (Kimata et al. 1991). The effect of NGF on immunoglobulin production in clones is in stark contrast to its effect on immunoglobulin synthesis and secretion by cell lines. NGF decreases the immunoglobulin production of IM-9 and AF-10 plasma cell lines and inhibits the IgG, IgA and IgM production by PCA-1^+. Finally, Brodie and Gelfand (1992) find that NGF deceases Ig secretion by B-lymphoblastoid cell lines in a dose-dependant manner.

While it is easy to understand that a reduction of antibody synthesis and secretion is beneficial in MS, it is not as obvious how an increased synthesis and secretion of antibody can help an MS patient. The increase in Ig synthesis effected by NGF may not necessarily be deleterious, as it can have indirect immunosuppressive effects, depending on the concomitant expression of the Fc receptors on the surface of lymphocytes or other immune cells. In this regard Daëron et al. (1995a) have proposed a mechanism by which a specific Fc receptor, FcγRIIB found on many immune cells, could negatively regulate the activation state of many cell types. FcγRIIB is a low affinity single chain receptor, for the Fc segment of the IgG antibodies (Hibbs et al. 1988), which conserves its homology in mice and human (Brooks et al. 1989).

The FcγRIIB receptor and the immunoglobulins may regulate an array of cell types which express this receptor. These include the myeloid, the lymphoid (Hulett and Hogarth, 1994) and other lineages, such as: mast cells, T cells, basophils, eosinophiles, neutrophils, monocytes, macrophages and Langerhans cells (Daëron et al. 1995a). If B cells are used as an example, then the antigen-immunoglobulin complex (Ag-IgG) binds to specific B cell receptors (BCR) and FcγRIIB. This binding is realized through the attachment of the Fc portion of the Ag-IgG complex, to the FcγRIIB receptor, and the antigen portion of the immune complex to the BCR. This causes the coaggregation of these two receptors. FcγRIIB does not trigger cell activation upon aggregation (Daëron et al. 1992) and in fact, it has been known since the early seventies, to inhibit the BCR-dependent B cell activation in the murine system (Sinclair and Chan, 1971). This mechanism is an FcγRIIB-dependent negative feedback regulation. It could decrease an antibody response to a specific antigen. Such regulation can also be achieved by

complexes between an antigen and the immunoglobulins (IgG, IgA and IgE). This mechanism depends on the coexpression of the FcγRIIB receptor, and the appropriate high affinity immunoglobulin receptor on the immune cells (Daëron et al. 1995a).

Daëron et al. (1995b) modeled this inhibitory mechanism via the cross-linking of the constitutively expressed FcϵRI (high affinity IgE receptor) and FcγRIIB, on mast cells and basophils. This lead to an inhibition of the IgE-induced release of mediators (serotonin) and cytokines by these cells. The inhibition is not an all or nothing proposition as the FcϵRI receptors, that are not cross-linked, can aggregate and signal normally; the FcϵRI receptors that are cross-linked can disengage and the inhibition is reversed. This implies that the inhibition is not due to a global desensitization of the cells (Daëron et al. 1995b). Such a fine tuned mechanism may allow cells to carry out other aspects of their function, such as the clearance of debris, while controlling the possible antibody attack on myelin and its constituent proteins. On the other hand, this reversible desensitization may be one of the reasons why IFN-β does not halt the relapses in RRMS, assuming that NGF is modulated in MS as it is in EAE (see below) and that IFN-β can induce an *in vitro*-like increase *in vivo*.

NGF has been shown to be upregulated during the acute phase of EAE (Micera et al. 1995; De Simone et al. 1996). De Simone et al. (1996) believe that NGF's role in EAE is one of trophism and axonal remodelling. Moreover, NGF is not likely to contribute to the inflammatory process per se (Micera et al. 1995; De Simone et al. 1996) as it can be administered in high doses without any inflammation (Banks, 1984; Aloe et al. 1992) and has been noted to have anti-inflammatory activity (Banks, 1984; Diaz-Villoslada et al. 1997). These observations point to a possibly beneficial effect of NGF in the inflammatory aspect of EAE and perhaps MS. In support of such a hypothesis, Diaz-Villoslada et al. (1997) demonstrate that human recombinant NGF decreases the pathological and clinical signs associated with EAE, when it is administered to marmosets. Such an observation lends credence to the idea that the IFN-β effect in MS may be partially mediated by an increase in NGF, assuming that the *in vitro* IFN-β-mediated NGF upregulation by astrocytes is mimicked *in vivo*.

The previously cited effects on the inflammatory process, though important, do not eliminate the possibility of a direct effect of NGF on oligodendrocytes.

12.52 NGF and oligodendrocytes

12.521 No visible effect of NGF on oligodendrocytes

Rat oligodendrocytes have been shown to express $p75^{NTR}$ (Kumar et al. 1993) which is reported to transduce an intracellular signal upon NGF binding (section 12.22). Kumar et al. (1993) discovered, however, that NGF did not modulate the mRNAs for trkB or $p75^{NTR}$ in mature oligodendrocytes.

In agreement with Kumar et al's (1993) results which suggest a lack of an obvious NGF-induced effect on oligodendrocytes, Oh and Yong (1996) do not observe a modulatory role for NGF on adult human oligodendrocytes, with respect to process extension. Indeed, NGF does not enhance process outgrowth in adult human oligodendrocytes at all concentrations tested, from 50 ng/ml to 1000 ng/ml, even when this neurotrophin is used repeatedly (Oh and Yong, 1996).

12.522 The trophic role of NGF for oligodendrocytes

NGF from mouse submaxillary gland or human recombinant NGF has been reported, as previously mentioned, to increase the proliferation and differentiation of oligodendrocytes obtained from the adult porcine brain (Althaus et al. 1992). At a concentration of 1 ng/ml of NGF, a slight but visible increase in process extension is seen. This fibre production is concentration dependent, and at 10 ng/ml of NGF or more, up to 100 ng/ml, elongation and branching are enhanced. This dose-dependent response to NGF does not extend to the speed fibre production. In addition, a small subset of oligodendrocytes are induced to increase their [^{3}H]thymidine uptake upon NGF treatment. This is also a dose-dependent phenomenon and is elicited when either mouse or human NGF is used.

The NGF-mediated increase in oligodendrocyte number and their process extension, the latter constituting a key step in myelogenesis, may be relevant with regards to remyelination. Indeed, if NGF increased the number of oligodendrocytes and/or the length of their processes, it could indicate that there are more oligodendrocyte-derived fibres that are available to myelinate the axons, since a given oligodendrocyte must extend a fibre before the latter can ensheathe the axon.

An NGF-mediated increase in fibre length or oligodendrocyte proliferation does

not eliminate the importance of protecting the myelination that is already established. In that line of thought, Diaz-Villoslada et al. (1997) demonstrate that the NGF-treated EAE marmosets have smaller lesions and reduced demyelination in contrast to the placebo (cytochrome C)-treated EAE animals.

Finally, Cohen et al. (1996) demonstrate the presence of functional trkA and trkC receptors, but only the truncated form of trkB, on developing and mature rat oligodendrocytes. They further reveal that a combination of NGF and b-FGF is a more potent mitogen for precursor oligodendrocytes than b-FGF alone. Moreover, Cohen et al. (1996) demonstrate that NGF and NT-3 are trophic for mature oligodendrocytes, in accordance with Althaus' results.

12.523 The toxic role of NGF for oligodendrocytes

In stark contrast to the trophic effect of NGF on oligodendrocytes, revealed by Althaus et al. (1992) and Cohen et al. (1996), a recent report by Casaccia-Bonnefil et al. (1996) found that NGF induced apoptosis in adult rat oligodendrocytes. The latter effect was not seen when immature oligodendrocytes or astrocytes were used. There is precedence for both ligand-induced (Frade et al. 1996) and ligand-independent (Rabizadeh et al. 1993) $p75^{NTR}$-mediated apoptosis, despite its controversial role in signal transduction (section 12.22). The contrasting results obtained between the different glial cultures may reflect the serum-free culture conditions of the mature oligodendrocytes versus the presence of the 15 % fetal bovine serum in the astrocyte culture, or the presence of B104 conditioned medium in the immature oligodendrocyte culture (Casaccia-Bonnefil et al. 1996). Unfortunately, it is unknown whether the mature oligodendrocytes would have undergone apoptosis, in the presence of NGF, in the other media. Such differences in the composition of the media make a direct comparison of the effects of NGF, on the various glial types, extremely difficult.

The other neurotrophins that had been tried by Casaccia-Bonnefil et al. (1996) were BDNF and NT-3 which bind to trkB (Klein et al. 1991; Soppet et al. 1991; Squinto et al. 1991) and trkB/trkC respectively (Klein et al. 1991; Lamballe et al. 1991; Soppet et al. 1991; Squinto et al. 1991). These neurotrophins did not elicit an apoptotic response, in oligodendrocytes, to any detectable levels (Casaccia-Bonnefil et al. 1996). This discrepancy, between the results with the various neurotrophins, could be accounted

for by several observations which involve the expression of the trk (A, B or C) and $p75^{NTR}$ receptors on oligodendrocytes.

The types of trk receptors that are expressed on oligodendrocytes seem to vary with the experimental paradigms. The truncated splice forms of trkB appears to be expressed (Kumar et al. 1993; Cohen et al. 1996), while trkA is reported to be present on oligodendrocytes by some authors (Cohen et al. 1996) and absent by others (Kumar et al. 1993; Casaccia-Bonnefil et al. 1996). The presence of trkC does not seem to be disputed, however, the splice form that is expressed does. Cohen et al. (1996) report a functional receptor while Kumar et al. (1993) are of the opinion that only the truncated form is expressed on these cells.

Since Casaccia-Bonnefil et al. (1996) did not report on an investigation of the possible presence of trk (B or C) in the cultured oligodendrocytes, it was difficult to ascertain whether the differences seen between the neurotrophins corresponded to the nature of the neurotrophin or to the presence of the high affinity receptor. In a scenario where trkB, trkC, or both are present, the high affinity receptors could sequester the neurotrophins and make these neurotrophins unavailable to bind $p75^{NTR}$. Moreover, in the absence of trkA, NGF might be free to bind to $p75^{NTR}$ and signal through this receptor since this neurotrophin was not known to bind to trkB or trkC (Klein et al. 1991; Lamballe et al. 1991; Soppet et al. 1991; Squinto et al. 1991). In addition, there had been reports on the differences in $p75^{NTR}$-mediated signalling depending on the presence or absence of trk receptors (section 12.22). Finally, to recapitulate, it is not known if this apoptotic mechanism is an NGF-specific phenomenon, or the result of a molecule that signals through $p75^{NTR}$ in the absence of its high affinity receptor.

12.524 The evidence for NGF trophism versus toxicity to oligodendrocytes, *in vitro* and *in vivo*

There was no overt NGF-induced lethal effect, reported by Althaus et al. (1992), Kumar et al. (1993), Cohen et al. (1996) or by Oh and Yong (1996) on porcine, rat or human primary oligodendrocyte cultures. In addition, and in contrast to Casaccia-Bonnefil et al.'s (1996) report, NGF has not caused cultured adult human oligodendrocytes to undergo apoptosis despite the presence of the $p75^{NTR}$ receptor and the absence of trkA in these cells (Ladiwala et al. 1997). Moreover, in the murine system

both primary mature and immature oligodendrocytes had been reported by Byravan et al. (1994) to synthesize NGF mRNA and protein. Again, no toxicity was reported by the latter group.

Despite the wide-spread use of NGF in animal models of cholinergic degeneration, whether in rats (Will and Hefti, 1985; Hefti, 1986; Williams et al. 1986; Fisher et al. 1987; Tuszynski et al. 1991; Markowska et al. 1994; Wilcox et al. 1995) or primates, both human and non-human (Tuszynski et al. 1990, 1991; Olson et al. 1992; Olson, 1993; Emerich et al. 1994; Kordower et al. 1994), none of these studies reported the degeneration of oligodendrocytes in either the exogenously administered or endogenous NGF paradigm. In addition, NGF was upregulated in the acute phase of EAE, as previously mentioned, and was believed to be trophic and tropic to neurons (De Simone et al. 1996).

In light of these observations, it is unlikely that NGF participates in a toxic effect to oligodendrocytes *in vitro* in all paradigms or *in vivo*. In fact, Diaz-Villoslada et al. (1997) report that NGF-treated EAE marmosets demonstrated little demyelination when compared to their placebo-treated counterparts.

An NGF effect on oligodendrocytes is not the only way this neurotrophin may be of use in MS. NGF has been known to be trophic to neurons, in the PNS and CNS, for several decades (Levi-Montalcini, 1987; Hagg et al. 1993; Rylett and Williams, 1994; Hayashi, 1996).

12.53 NGF and axonal loss

The finding of increased levels of NGF, by rmIFN-β, which may promote axonal rescue/repair, is all the more interesting in light of the report by Holtzman et al. (1995). This group localizes trkA to NGF-responsive noncholinergic neurons which may establish NGF as a trophic molecule for different classes of neurons (section 12.21). The upregulation of NGF and the distribution of trkA and $p75^{NTR}$, may have implications for MS where there is a 10 to 80% axonal loss, within a lesion, depending on the subtype of MS (Lassmann et al. 1994). Indeed, while oligodendrocytes and myelin have been the focus of MS (Ozawa et al. 1994; Brosnan and Raine, 1996; Cuzner and Norton, 1996; Lucchinetti et al. 1996; Steinman, 1996), other reports demonstrate evidence for accompanying axonal damage (Prineas and Connell, 1978; Arnold et al. 1990; Allen et

al. 1991; Barnes et al. 1991; Matthews et al. 1991; Kidd et al. 1993; Gass et al. 1994; Davie et al. 1995; Davies et al. 1995) which is a characteristic that has been known since Charcot's time (reported by Davie et al. 1995). In fact, Arnold et al. (1990) find that demyelination, axonal loss and gliosis, which are the pathological hallmarks of chronic plaques, represent irreversible damage to the CNS.

Because of the mounting evidence of axonal loss in MS, it is now increasingly appreciated that the persistent clinical disability seen in MS patients is probably due to axonal loss (Weller, 1985; Barnes et al. 1991; Kidd et al. 1993; Gass et al. 1994; Davie et al. 1995; Sobel, 1995). It is consequently possible that the prevention of axonal or neuronal loss may alleviate the clinical disability of MS patients. Indeed, the loss of the myelin sheath may not prevent CNS neurons from signalling (Halliday et al. 1972; Black et al. 1991; Moll et al. 1991). In this regard, the neurotrophic activity of NGF may protect axons from further damage and/or facilitate their recovery from damage.

12.54 NGF in the CSF (cerebrospinal fluid) of MS patients

The presence of NGF in the nervous system during and after MS attacks was a point of some controversy. Laudiero et al. (1992), using a double-site ELISA, found elevated NGF in the CSF of MS patients when compared to the CSF of age-matched controls. The latter group included patients with other neurological diseases, such as: headache, cerebellar neoplastic syndrome, myelopathy, polyneuropathies, cerebral ischemia, leukodystrophy and leukoencephalopathy. Laudiero et al. (1992) demonstrated that in normal humans (without inflammatory disease) the level of NGF decreased as a function of age, within the CSF, to virtually undetectable levels in adults regardless of their sex. In contrast, during an acute MS attack, the levels of NGF increased (16.2 pg/ml) well above the levels of control. The amount of NGF decreased during remission (5.6 pg/ml), though this level was still above that of control. According to Laudiero et al. (1992), the levels of NGF in MS followed closely the disease course, increasing during the inflammatory response. This increase was attributed to the presence of inflammatory cytokines such as IL-1, IL-6, and TNF-α. Moreover, this group suggests that NGF might be used as a clinical marker for the inflammatory period of MS. This indicated a possible role for NGF in MS.

Massaro et al. (1994), on the other hand, did not find elevated levels of NGF in

patients with various neuroimmune diseases, including MS, Alzheimer's disease, amyotrophic lateral sclerosis, chronic relapsing polyradiculoneuritis, Guillain-Barré syndrome, and tumours of the nervous system. Although the detection sensitivity of their ELISA was 28 pg/ml, Massaro et al. (1994) were unable to detect NGF in any of the CSFs, even after concentration, except for one patient with Guillain-Barré syndrome (0.016 ng/ml, not concentrated).

As neither group specified whether or not the patients were on medication nor did they provide detail on their patient's case history, it was difficult to account for this apparent discrepancy. Nonetheless, the finding that NGF is increased in the CSF of MS patients during an acute attack, but decreases during remission (Laudiero et al. 1992) is interesting and may suggest that NGF is being synthesized to facilitate recovery from acute inflammation as suggested for EAE (section 12.51).

12.6 The final analysis

During the evolution of this thesis, I have tried to explain how IFN-β may be useful in RRMS. More specifically, the present investigation used an *in vitro* paradigm to address the possibility that IFN-β could cause astrocytes to increase their NGF production. The potential role of this trophic factor was reviewed within the context of MS, a disease that was known to cause destructive damage to myelin and axons (section 12.5). Since there are at least two cell types that are affected in MS, oligodendrocytes and neurons, it is logical to believe that an agent that can offer life-support during repair processes and cause cells to proliferate or differentiate would be of enormous help, especially if such an agent could be of benefit to the former cell types (section 12.5). While IFN-β is known to generally downregulate the immune system, its effectiveness may not be sufficient to silence the disease (sections 3, 4, 5 and 7). Another agent that can also downregulate the inflammatory processes that are believed to participate in the initiation of the relapses in RRMS, may be of critical use.

Based on the findings that IFN-β increases the production of NGF by astrocytes, that NGF increases in EAE and the reports that NGF has all the previously mentioned criteria, i.e. possibly trophic to oligodendrocytes and neurons and may participate in the downregulation of immunoglobulin production and the downregulation of the state of activation of immune cells (section 12.5), **it is conceivable that part of the usefulness**

of IFN-β in RRMS may lie in the increased production of NGF by astrocytes.

12.7 Perspectives

It is not difficult to imagine a logical continuation to the start of this project. Indeed, there are several possible avenues one could pursue, which include among others: 1) the determination of the nature of the increase in the NGF mRNA, i.e., the stabilization of messenger RNA or *de novo* transcription or both; 2) the determination of the mechanism of upregulation of the mRNA, i.e., which second messengers and which transcription factors are involved; 3) the determination of whether or not the increase in NGF is an IFN-β specific versus a type 1 IFN specific response of astrocytes; 4) verifying whether or not the CM derived from IFN-β-treated astrocytes is trophic and/or tropic to oligodendrocytes and neurons, since Ladiwala et al. (1997) demonstrate that NGF is slightly trophic to oligodendrocytes; and 5) the determination of the IFN-β effect on astrocytes *in vivo*, i.e. do the astrocytes increase their production of NGF mRNA and protein *in vivo* upon IFN-β treatment ?

While all the previously mentioned possibilities represented worthwhile topics of research, perhaps the fifth option was best suited to help sustain the arguments that were put forward in this thesis. These hypotheses concerned the mechanism of action of IFN-β and the relevance of NGF in MS. Such conjectures could be sustained more firmly if *in vivo* results from IFN-β treated EAE animals could be gotten (section 4). Using *in situ* hybridization to locate NGF mRNA, in conjunction with anti-GFAP antibody to identify astrocytes, it could be possible to verify whether or not astrocytes upregulate their production of NGF mRNA in slices of brain tissue (Arendt et al. 1995) in response to IFN-β.

Since the production of NGF during injury is believed to be done mostly by astrocytes (Arendt et al. 1995), and since NGF is upregulated in EAE (section 12.51), it is tempting to hypothesize that IFN-β can improve upon such an increased NGF production and that this would be an astrocyte-derived effect.

In the end, it is the advancement of technology, the use of drugs in clinical trials, and the use of animal models that will see this collective struggle come to fruition as the saga of MS takes new turns and we slowly close in on this pathology.

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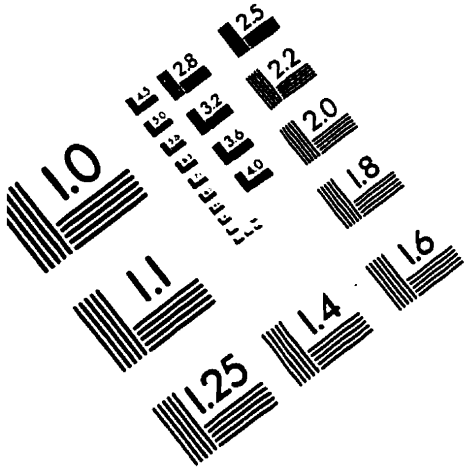
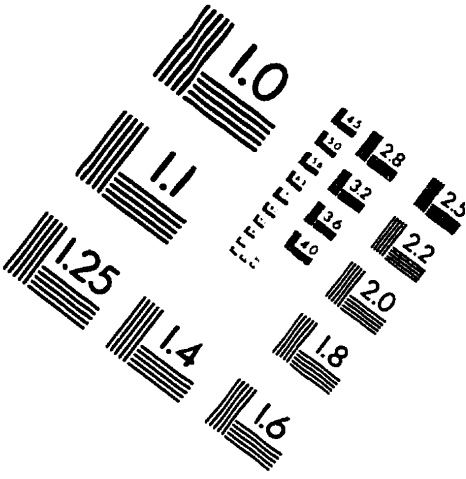
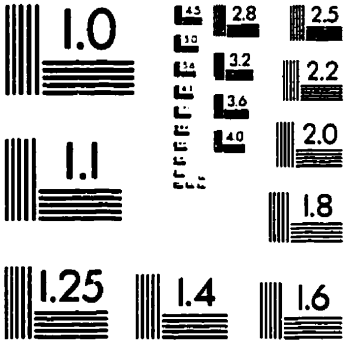
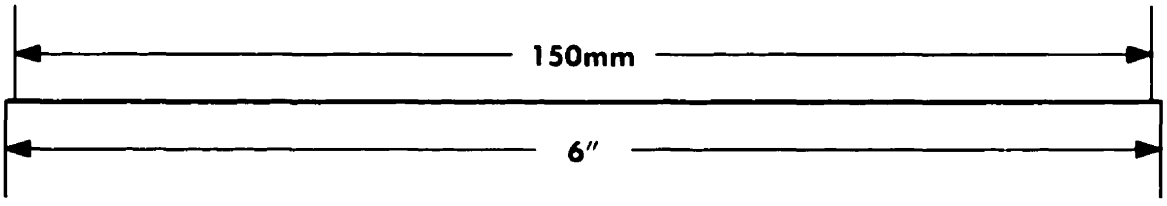
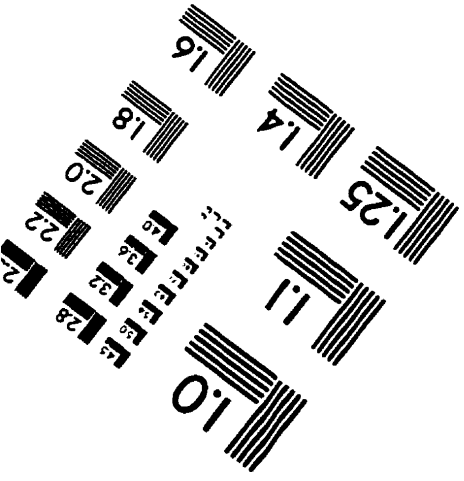
IMAGE EVALUATION
TEST TARGET (QA-3)

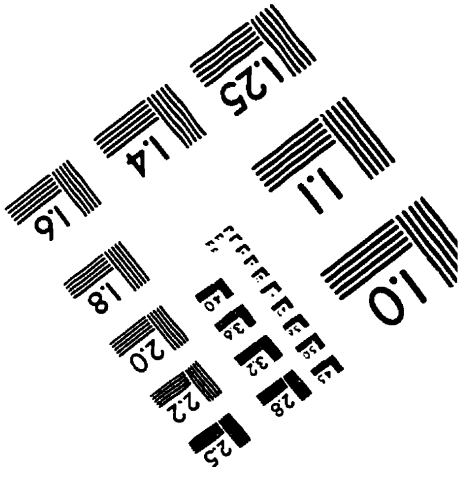












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