RESPONSE OF HUMAN OLIGODENDROCYTES TO IMMUNE-MEDIATED INJURY: SELECTIVE VULNERABILITY AND SELECTIVE PROTECTION

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This thesis is dedicated to my father, Albert, my mother, Nora, and my sister, Natasha for their inexhaustible love, support and encouragement during the course of my Ph.D studies.

In Puccini's version of Belasco's *Madama Butterfly*, the emperor has sent a sword to Butterfly's father, inscribed "Death with honor, for those who cannot live with honor." Both father and daughter know when and how they must respond to this message, and cells evidently do, too.

Puccini, G (1983). "*Madama Butterfly*," p.70. Dover Publications, New York.

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I would like to thank Dr. Mark Freedman, my initial mentor, who introduced me to the world of neuroimmunology and multiple sclerosis. I am indebted to Drs. Eugenia Wang, Alan Peterson and Philip Barker, members of my advisory committee, for their guidance in the complicated fields of cell death and neurotrophic factors. I would like to extend my gratitude to Dr. Geralyn Trudel for her friendship and for teaching me tissue culture techniques when I first joined the laboratory. I would also like to thank Dr. Eugenia Wang from whom I learned the TUNEL technique for assessing DNA fragmentation. I am indebted to all my collaborators including Drs. Bruno Bonetti and Cedric Raine at the Albert Einstein College of Medicine, USA, and Ms. Karen Alinauskas and Mr. Vijayabalan Balasingam at the MNI. I would like to thank members in Dr. Antel's laboratory for their assistance, in particular, Ms. Ellie McRea and Manon Blain.

I would like to thank my friends at the MNI and McGill University for their valued friendship, support and encouragement.

Finally, I am eternally grateful to my parents and sister, for their love, support and encouragement at every step of the way on my road to this Ph.D degree. Without them, this thesis would not have been possible. No son and brother could ask for more. The countless hours that I spent explaining every hypothesis and laboratory result to my father, an electrical engineer, served to focus my experimental designs and efficiently plan my experiments. His ability to understand some of the complex issues of cell death and multiple sclerosis during my discussions with him provided me with immense gratification and encouragement.

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ABSTRACT / RÉSUMÉ

ABSTRACT

This thesis studying the injury response of human central nervous system (CNS)-derived oligodendrocytes (OL) to immune mediated effector mechanisms and its relevance to protective strategies for OL, assessed the basis for the selective injury of OL, as occurs in the human demyelinating disease multiple sclerosis (MS). This thesis tested the postulate that selective target injury within the CNS may reflect target-cell rather than effector-cell properties. Differences in susceptibility of CNS neural cells to a common immune mediator or the cell-specific expression of a surface receptor for a putative injury mediator could result in specific target cell injury. With regard to the former possibility, OL amongst neural cells were selectively vulnerable to the cytokine tumor necrosis factor (TNF), death occurring via apoptosis. With regard to the latter possibility, the cytokines γ-interferon, TNF, and interlukin (IL)-1, selectively upregulated the expression of heat shock protein-72, a postulated ligand for cytolytic γδ-T cells, on OL in mixed glial cell cultures via a final common pathway involving IL-1 binding to its receptor on OL. In addition, OL amongst other glial cells *in vitro* selectively expressed fas, a cell surface receptor that transduces apoptotic cell death signals when ligated by agonist antibodies or by fas ligand (FasL). Fas ligation on OL resulted in OL cell death via a novel apoptosis-independent lytic mechanism. Selective upregulation of fas on OL and FasL on microglia in MS lesions compared to control CNS tissue further implicated fas signalling as a potential contributor to OL pathology in MS. Only ciliary neurotrophic factor (CNTF) amongst an array of neurotrophic factors and cytokines protected OL from TNF-mediated apoptosis. CNTF did not protect other neural cells from TNF-mediated apoptosis, nor did it protect OL from lytic injury mediated by activated $CD4^+$ T cells or by fas ligation. These data indicate that target rather than effector cell properties may determine target cell injury in the CNS. The potential protective effects of neurotrophic factors and cytokines on neural cells may be specific for both target cell type and nature of the target cell injury response.

RÉSUMÉ

Cette thèse étudie la réponse des oligodendrocytes (OD) dérivés du système nerveux central (SNC) aux lésions médiées par le système immunitaire et son implication dans des stratégies de protection des OD, et démontre les bases d'une destruction sélective des OD telle qu'elle survient dans la sclérose en plaques (SEP) chez l'homme. Cette thèse teste l'hypothèse selon laquelle des lésions sélectives au sein du SNC reflètent des propriétés liées aux cellules cibles plutôt qu'aux cellules effectrices. D'un coté, parmi les cellules nerveuses, seuls les OD sont vulnérables à la cytokine "facteur nécrosant des tumeurs" (TNF) induisant la mort cellulaire par apoptose. D'un autre coté, les cytokines interféron-γ, TNF et interleukine-1 (IL-1) augmentent l'expression de la protéine de choc thermique 72, un ligand présumé des cellules γδ-T cytosoliques, par les OD en culture mixte de cellules gliales. Cette régulation passe par une voie finale commune impliquant la liaison de l'IL-1 à son récepteur sur les OD. Par ailleurs, parmi toutes les cellules gliales in vitro, seuls les OD expriment la protéine Fas, un récepteur membranaire qui transduit le signal apoptotique lorsqu'il est activé par des anticorps agonistes ou par le ligand Fas (FasL). L'activation de Fas dans les OD entraine la mort cellulaire de ces OD via un nouveau mécanisme cytolytique indépendant de l'apoptose. De plus, une augmentation sélective de Fas dans les OD et de FasL dans la microglie dans la SEP renforce l'implication de la voie Fas dans la pathologie des OD au cours de la SEP. Parmi un éventail de facteurs neurotrophes et de cytokines, seul le facteur neurotrophe ciliaire (CNTF) protège les OD d'une apoptose médiée par le TNF. Le CNTF ne protège pas les autres cellules nerveuses d'une telle apoptose, pas plus qu'il ne protège les OD des lésions lytiques induites par les cellules T $CD4^+$ activées ou par l'activation de Fas. Ces résultats indiquent que les lésions des cellules du SNC sont déterminées par les propriétés des cellules cibles plutôt que des cellules effectrices. Les propriétés protectrices potentielles des facteurs neurtotrophes ou des cytokines sur des cellules nerveuses peut être spécifique à la fois du type de cellule cible et de la nature de la réponse à la lésion de la cellule cible.

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As stated in the *"Guidelines Concerning Thesis Preparation", Faculty of Graduate Studies and Research, McGill University:*

Elements in the thesis that are to be considered as ***contributions to original knowledge*** *must be clearly indicated in the preface or by a separate statement at the beginning or at the end of the thesis. This requirement is* ***mandatory for Ph.D. thesis.***

CHAPTER 2

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3. Fas is selectively upregulated on oligodendrocytes in multiple sclerosis lesions as compared to normal CNS white matter. In such lesions, high levels of fas ligand are expressed on microglia and infiltrating lymphocytes. These results implicate the fas:fas-ligand system as a potential contributor to the selective injury of oligodendrocytes in multiple sclerosis, and further implicate microglia as a potential contributor to oligodendrocyte destruction in multiple sclerosis.

MANUSCRIPTS AND AUTHORSHIPS

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As stated in the "Guidelines Concerning Thesis Preparation", Faculty of Graduate Studies and Research, McGill University:

"*Candidates have the option, **subject to the approval of their department**,* of including, as part of their thesis, copies of the text of a paper(s) submitted for publication, or clearly-duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis.

If this option is chosen, **connecting text providing logical bridges between the different papers are mandatory.**

The thesis must still conform to all other requirements of the "Guidelines Concerning Thesis Preparation" and should be in a literary form that is more than a mere collection of manuscripts published or to be published. **The thesis must include, as separate chapters or sections:** (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general review of the background literature to the subject of the thesis, when this review is appropriate, and (5) a final overall conclusion and/or summary.

Additional material (procedural and design data, as well as descriptions of equipment used) must be provided when appropriate and in sufficient detail (e.g. in appendices) to allow a clear and a precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent;** supervisors must attest to the

accuracy of such claims at the Ph.D. oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of the different authors of co-authored papers"

CONTRIBUTIONS OF THE AUTHORS ON CO-AUTHORED PAPERS

In the initial stages of my graduate studies in the Neuroimmunology Unit of the Montreal Neurological Institute (MNI), McGill University, Dr. Mark S. Freedman was my direct supervisor and Dr. Jack P. Antel was my co-supervisor. Studies under their guidance led to the publication of the manuscript in chapter 2. As such, they are senior authors on this manuscript. When Dr. Mark Freedman left the MNI to take up a position at the Ottawa General Hospital, Dr. Jack Antel became my direct supervisor. As such, he is senior author on all manuscripts in this thesis (chapters 3, 4 and 5).

The following individuals are co-authors on manuscripts in the indicated chapters: Drs Neil Cashman and Philip Barker, members of Ph.D. advisory committee, provided guidance in the experiments that contributed to the manuscript in chapter 5. Dr. Cindi Goodyer provided invaluable fetal CNS tissue for the studies in chapter 3, and Dr. Anthony Troutt (Immunex Corporation) kindly provided us with the anti-fas antibodies, M3 and M33, antibodies crucial to our studies detailed in chapter 5. Except for the following individuals listed below, I conducted all the experiments of this thesis.

CHAPTER 2

Cytokine induction of heat shock protein in human oligodendrocytes: an interlukin-1 mediated mechanism.

D'Souza SD, Antel JP, Freedman MS

(J Neuroimmunol 50:17-25)

CHAPTER 3

Differential susceptibility of human CNS-derived cell populations to TNF-dependent and independent immune-mediated injury.
D'Souza, SD, Alinauskas K, McRea E, Goodyer C, Antel JP
(J Neurosci 15:7293-7300, 1995)

Karen Alinauskas: As a honours microbiology and immunology student in Dr. Antel's laboratory, Ms. Alinauskas assisted in performing preliminary experiments related to the toxicity of tumour necrosis factor on U251 glioma and fetal CNS cells.

Ellie McCrea: As a technician in Dr. Antel's laboratory, Ms. McCrea provided technical support in performing the ^{51}Chromium release assays related to the T cell-mediated cytotoxicity experiments.

CHAPTER 4

Ciliary neurotrophic factor selectively protects human oligodendrocytes from tumor necrosis factor-mediated injury.
D'Souza SD, Alinauskas KA, Antel JP
(J Neurosci Res 43:289-298).

Karen Alinauskas: Ms. Alinauskas' contributions to the manuscript in this chapter are the same as those in chapter 3

CHAPTER 5

Multiple Sclerosis: potential for fas signalling in oligodendrocyte cell death
D'Souza SD, Bonetti B, Balasingam V, Cashman NR, Barker PA, Troutt AB, Raine CS, Antel JP.
(Nature Medicine, submitted)

Drs. Bruno Bonetti and Cedric Raine: As a post-doctoral fellow in Dr. Raine's laboratory, Dr. Bonetti performed the fas and fas-ligand immunohistochemistry on tissue sections from multiple sclerosis lesions.

Vijayabalan Balasingam: Mr. Balasingam provided technical assistance in confocal laser scanning microscopy.

CHAPTER 1: GENERAL INTRODUCTION

GENERAL INTRODUCTION

In health, the central nervous system (CNS) is considered to be a site of immune privilege (reviewed in Cserr and Knopf, 1992). However, in an array of CNS disorders, immune effectors gain entry into the CNS and mediate CNS tissue injury. In the human demyelinating diseases multiple sclerosis (MS), HTLV-1-associated myelopathy (HAM) and acute disseminated encephalomyelitis (ADEM), the CNS white matter is the selective target of immune-mediated injury, with damage to myelin and/or its cell of origin, the oligodendrocyte (OL)(reviewed in Francis et al., 1995).

The major issue pursued in this thesis relates to the mechanisms which could account for the relatively selective injury of the OL/myelin complex in immune-mediated diseases such as MS. The basis for this selectivity could be dependent on the interaction of OL- or myelin-specific effectors with the OL/myelin complex. However, the majority of the immune effectors in MS lesions are not OL/myelin-specific (Cross et al., 1993; reviewed in Steinman et al., 1996). In addition, OLs *in situ* do not express the major histocompatibility complex (MHC) class I and II molecules (Lee and Raine, 1989), prerequisites for interaction with antigen-specific T cells, raising the possibility, addressed in this thesis, that selective OL/myelin injury may reflect target-cell rather than effector-cell properties. A corollary, addressed in this thesis, would be that the potential protective effects of neurotrophic factors and cytokines on OL in cases of immune-mediated injury may also be dependent on target cell properties.

I. THE OLIGODENDROCYTE-MYELIN COMPLEX

Robertson (1899) using his platinum stain was the first to identify oligodendroglial cells as one of the cellular components that comprised the neuroglia of the CNS. Rio-Hortega (1919, 1921a) using his silver carbonate stain independently rediscovered these cells, naming them oligodendrocytes because

their processes were shorter and sparser than astrocytes. He made the distinction between interfascicular oligodendroglia that were situated in rows between myelinated axons of the white matter, and perineuronal satellite oligodendroglia of the grey matter.

From their anatomical position and the fact that their appearance in development corresponded with the period of myelination, it was proposed that oligodendroglia were involved in myelination in the CNS (Rio-Hortega, 1921b, 1922, 1924, 1928; Penfield 1924; Linell and Tom, 1931; Morrison, 1932). Tissue-culture studies (Lumsden and Pomerat, 1951) demonstrated that oligodendroglia were capable of elaborating myelin sheaths. Electron microscopy provided definitive proof of a continuity between the OL membrane and the multilamellar myelin sheath that wraps around axons in the CNS (Luse, 1956, 1960; Farquhar and Hartman, 1957; Schultz et al., 1957, 1964; Maturana, 1960; Peters, 1960, 1964a,b, 1966; Bunge et al., 1962; Mugnaini and Walberg, 1964; Hirano et al., 1966; Kruger and Maxwell, 1966; Knobler and Stempak, 1973; Meier, 1976). Immunohistochemical detection of OL and their myelin membranes, *in situ* and in culture, is now possible owing to the availability of monoclonal antibodies against the protein, glycoprotein and glycolipid components of myelin. Such components include, among others, myelin basic protein (MBP), galactocerebroside (GalC), proteolipid protein (PLP), 2'-3' cyclic nucleoside phosphodiesterase (CNPase), myelin associated glycoprotein (MAG) and myelin/oligodendrocyte glycoprotein (MOG).

The myelin sheaths that wrap around axons in the CNS are discontinuous because each myelin-generating OL furnishes myelin for only a segment of the axon. Between these segments, short positions of axons, called nodes of Ranvier, are left uncovered (Tourneux and LeGoff, 1875; reviewed in Peters and Vaugh, 1970; Raine, 1984a). The myelin sheath increases the resistance to ion flow through the membrane of the axon; however, ions can flow easily at the nodes

facilitating saltatory conduction, a feature that permits transmission to occur at a greater velocity, frequency and efficiency than in unmyelinated fibres.

In the CNS, mature post-mitotic OL (Skoff and Knapp, 1991), myelinate multiple axons (reviewed in Peters and Vaugh, 1970; Raine, 1984a; McLaurin and Yong, 1995). As such, loss of a relatively small number of OL could potentially result in the demyelination of a large number of axons. Charcot (1868) proposed that the neurologic deficits in MS occurred due to axonal conduction block produced by demyelination. Halliday et al. (1972) provided experimental evidence for this hypothesis by demonstrating increased latency and decreased amplitude in visual evoked potentials in optic neuritis, an inflammatory demyelinating condition of the optic nerve often associated with MS.

II. MULTIPLE SCLEROSIS

Multiple sclerosis is the most prevalent of the immune-mediated demyelinating diseases, with onset usually in early adult life. Clinically, the disease presents itself in the form of recurrent attacks of focal or multifocal neurologic deficits, reflecting lesions within the CNS. Magnetic resonance imaging (MRI) of MS patients reveals multifocal areas of white matter destruction in the CNS, i.e demyelination. The course of MS is highly variable, but may be broadly classified as relapsing-remitting (recurrent attacks of neurologic dysfunction followed by complete or partial recovery) or chronic-progressive (gradual progressive worsening without periods of remission). Current opinion implicates an autoimmune etiology for MS, perhaps triggered by a viral infection in a genetically susceptible host (reviewed in Francis et al., 1995).

(A) Evidence for the immune basis of multiple sclerosis

Evidence for involvement of immunological mechanisms in the

pathogenesis of MS is inferred by analogy from experimental disease models, particularly experimental allergic encephalomyelitis (EAE), and derived from direct studies of MS patients.

(1) Experimental models

In 1884, Pasteur developed a method for rendering dogs refractory to the neurotropic rabies virus by multiple inoculations with neural tissue from infected rabits (Vallin, 1885). Humans who had been exposed to rabies were treated in a similar manner (Pasteur, 1885; Pasteur, 1886). Although this treatment achieved the goal of inducing resistance to the virus and preventing the progression of rabies, it soon became apparent that severe neuroparalytic complications could be precipitated by this method of vaccination (Bareggi, 1889; Remlinger, 1928). Postmortem pathological examination of the CNS of afflicted patients revealed perivascular lymphocytic infiltration, edema, and focal or multifocal areas of demyelination within the CNS (Babes and Mironesco, 1908; Simon, 1913; Fielder, 1916; Adams, 1959). This uniphasic syndrome was hence called postvaccination acute disseminated encephalomyelitis (ADEM).

In the 1930s, Rivers and co-workers, after repeatedly injecting homogenates of normal rabbit brain into monkeys, induced an inflammatory encephalomyelitis in the animals that bore striking resemblance to ADEM in humans (Rivers et al., 1933, 1935), work representing the dawn of EAE. The use of adjuvants to enhance immune response times (Freund and McDermott, 1942) enabled the development of highly reproducible models of EAE in several species (Freund et al., 1947; Kabat et al., 1947; Morgan, 1947; Morrison, 1947). When preparations from brains and spinal cords of groups of rabbits of varying ages were used, it was evident that the substance inducing encephalomyelitis was absent in the brains of young animals and present only after the laying down of myelin (Kabat et al., 1948). Adams (1959) and Waksman and Adams (1962) were among the first to dissect

the sequence of inflammatory events in EAE and showed that depletion of myelin was preceded by blood-barrier breakdown, followed by invasion of the tissue by small lymphocytes ('messengers of death') which formed perivascular cuffs after which large mononuclear cells appeared and invaded the tissue. These data confirmed the postulate that as with EAE, ADEM in humans was due to immune-mediated mechanisms directed against CNS myelin.

In the 1960s, attempts at passively transferring EAE by injecting lymph node cells derived from animals with EAE were successful (Paterson, 1960; Stone, 1961). It was later shown by passive transfer studies that the encephalitogenic cells were myelin-reactive $CD4^+$ T cells (Petinelli and McFarlin, 1981; Holda and Swanborg, 1982). By analogy with EAE, it is postulated that in MS, myelin-reactive $CD4^+$ T cells, upon activation in peripheral blood, traverse the blood-brain barrier and infiltrate into the CNS. Here, in response to local antigen on antigen-presenting cells, they get re-activated, releasing soluble factors such as cytokines. These effector molecules increase the permeability of the blood-brain barrier, resulting in the recruitment of further waves of cytopathic immune effector cells and soluble molecules. As with EAE, analysis of the specificity of the cellular effectors in MS lesion sites indicates that while myelin-specific effector cells are present, the vast majority of the effector cells are non-specific (Cross et al., 1993; reviewed in Steinman, 1996). It is postulated that specific disease-producing cell infiltrates may initiate the inflammatory disease process, but actual tissue injury may be mediated by an array of non-specific inflammatory mediators recruited to lesion sites (discussed below).

(2) MS patient studies

(a) Immunogenetic studies

Presentation of antigen to T cells involves the interaction between T cell

receptor (TCR) and peptide antigens, sitting in the groove of a MHC class I (HLA A,B,C) or class II (HLA-DR, DQ, DP) molecule expressed on an antigen-presenting cell (APC). The unique structure of each MHC molecule and the processing capability of the APC determines which peptide portion of a given molecule will occupy the groove, and the combined peptide-MHC complex will determine which TCR is engaged. The immunogenetic basis of autoimmune diseases stems from the argument that if patients share distinct properties of their MHC or TCR molecules, these individuals are more likely to respond to the same peptide portions of an auto-antigen.

(i) Major histocompatibility complex

Within selected populations of MS patients, there is an overrepresentation of specific HLA antigens, particularly class II, suggesting an immunogenetic predisposition to the disease (Opelz et al., 1977; Bertram and Kuwert, 1982); the strongest association resides in the DR2 subregion of the HLA genes and specifically with the class II haplotype DRw15 DQw6 Dw2 (Cohen et al., 1984; Vartdal et al., 1989; Heard et al., 1989; Olerup et al., 1989; Spurkland et al., 1991; Martin et al., 1992). This association is greatest in white populations and is associated with a relative risk of four times normal for the development of MS. However, in families with multiple members with MS, this association is at best weak. It is equally clear that no single HLA allele can explain the susceptibility to MS, as most individuals carrying the MS-associated HLA antigens do not develop MS and, conversely, these HLA antigens are not a prerequisite for developing disease either in large populations or within families. Estimates of the maximum contribution to MS susceptibility of the HLA region are less than 10%. Current data suggest that other genes and triggering factors in addition may influence susceptibility to developing the disease (Oksenberg et al., 1993a; Ebers and Sadovnick, 1994)

(ii) T cell receptor

TCR genes were considered as potential additional genes that could have a role in MS susceptibility on the basis of the importance of such receptors in the trimolecular complex (antigen, MHC-II, and TCR) crucial to immune reactions. CNS and CSF derived MBP-specific T cells were shown to exhibit a limited heterogeneity in the rearrangement of their T cell receptors (TCR)(Oksenberg et al., 1990; Ben-Nun et al., 1991; Wucherpfennig et al., 1992a) possibly the result of enrichment of T cells of similar specificities. Further analysis of the TCR usage by MBP-specific T cells, however, produced conflicting results (Martin et al., 1990; Wucherpfennig et al., 1990; Kotzin et al., 1991). At present, there is no agreement on whether the TCR usage in MBP-specific T cells is restricted, limited or heterogenous.

(b) Immunopathologic studies of multiple sclerosis CNS tissue

In this section, the immune effectors that could potentially mediate CNS tissue destruction in MS will be described and evidence for their activation and involvement in immune effector functions will be discussed. Evidence for the ability of these effectors to mediate OL/myelin injury is largely based on *in vitro* studies and will be discussed in a later section (see section on *In vitro* models of OL/myelin injury).

i. Infiltrating inflammatory cells

In MS, acute and active chronic lesions that display recent or ongoing myelin destruction are invariably hypercellular and inflammatory. Vessels display perivascular cuffs of lymphocytes, macrophages and plasma cells that, upon traversing the blood brain barrier, occupy the Virchow-Robin space in early lesions and invade the parenchyma in more developed lesions (Adams, 1977;

Prineas and Wright, 1978; reviewed in Raine, 1994). It was not until the development of monoclonal antibodies against the constituent cells of the immune system, that the nature of the infiltrating inflammatory cells in MS could be studied (Traugott et al., 1982). In many of these studies, in addition to post-mortem MS plaque tissue, cerebrospinal fluid (CSF) was analysed to provide an approximation of the characteristics of the infiltrating inflammatory cells and their products in the CNS. As with EAE, immunohistochemical studies have identified $CD4^+$ T cells bearing the αβ TCR, $CD8^+$ αβ TCR T cells, γδ TCR T cells, macrophages, and B cells to be amongst the infiltrating mononuclear cells both in CSF and active lesion sites from MS patients (Traugott et al., 1983; reviewed in Raine, 1994). These cells are rarely found in the normal CNS (Hauser et al., 1983).

T cells from both CSF and plaques demonstrate increased Ta_1 (Hafler et al., 1985) and IL-2 receptor (Hofman et al., 1986) expression, and decreased CD45 expression (Sobel et al., 1988; Chofflon et al., 1989; Salonen et al., 1989; Zaffaroni et al., 1990; Svenningsson et al., 1993), a profile that is consistent with T cells that have been activated in response to antigen and are actively engaged in immune effector functions. Elevated frequencies of MBP, PLP, MOG and MAG-reactive T cells have been detected in the CSF of MS patients (Olsson et al., 1990; Sun et al., 1991a,b; Chou et al., 1992; Zhang et al., 1993). Analysis of TCR gene rearrangements in MS lesions have indicated the presence of TCR reactive to MBP (87-99) peptide (Oksenberg et al., 1993b; reviewed in Steinman, 1996). γδ-T cells derived from plaque tissue and CSF of MS patients express a limited TCR repetoire indicating antigen-driven clonal expansion (Wucherennig et al., 1992b; Shimonkevitz et al., 1993). Macrophages at lesion sites express elevated levels of MHC class II molecules, immunoglobulin Fc and complement receptors, activation markers that implicate them in immune effector responses (Ulvestad et al., 1994b,c; Williams et al., 1994).

B cell activation as measured by intrathecal immunoglobulin (IgG) synthesis is well documented in MS (Tourtollotte et al., 1984; Xiao et al., 1991) and is oligoclonal (Walsh et al., 1986). Specific anti-OL/myelin antibodies have been detected in the CSF of MS patients (Traugott and Raine, 1981; Link et al., 1990; Martino et al., 1991; Xiao et al., 1991; Gerritse et al., 1994). IgG purified from brain lesions reacted with the same region of MBP, p85-96, that is the immunodominant T cell epitope in MS patients who are HLA DR2b (Warren et al., 1995). It should again be noted however, that the majority (estimated at 98%) of activated T and B cells at lesion sites are not OL/myelin-specific (reviewed in Steinman, 1996).

ii. Endogenous glial cells

Microglia and astrocytes have also been implicated as effectors in MS lesions. Upregulation of Fc receptors, complement receptors, MHC class II, CD45 and B7, activation markers of microglia *in vitro* (Williams et al., 1993; Williams et al., 1994) has been demonstrated on microglia in MS lesions as compared to control CNS tissue (Ulvestad et al., 1994b,c; Williams et al., 1994). Astrocytes in MS lesion sites have been shown to exhibit reactive changes, including increased GFAP immunoreactivity (Raine, 1978; Raine and Cross, 1989). Whether increased astrocytic GFAP content represents a state of activation or a stimulated or enhanced metabolic state is presently not yet determined.

iii. Soluble factors

Proinflammatory effector soluble factors, products of an actively engaged immune response, are detected in MS tissue or CSF of MS patients (reviewed in Brosnan et al., 1995). *In situ* and *in vitro* studies have demonstrated that these soluble effectors are secreted by infiltrating inflammatory cells as well as endogenous activated glial cells at lesion sites (reviewed in Brosnan et al., 1995).

These proinflammatory effector soluble molecules include cytokine and non-cytokine soluble factors. Cytokines were initially defined as non-immunoglobulin protein molecules secreted by cells of the immune system and involved in either regulating or effecting immune responses. Cell surface-bound forms of many of these molecules, with biological activity, also exist. Cytokines detected in MS lesions include interlukin (IL)-1 (Cannella and Raine, 1995), IL-2 (Hofman et al., 1986), IL-6 (Woodroofe et al., 1993), γ-interferon (IFN)(Traugott and Lebon, 1988), tumor necrosis factor (TNF)-α and β (Hofman et al., 1989; Selmaj et al., 1991b; Cannella and Raine, 1995). Other non-cytokine soluble effector molecules include nitric oxide (Brosnan et al., 1994), terminal complement complex components such as C5b-9 (Compston et al., 1986; Sandus at al., 1986; Compston et al., 1989; Rodriguez et al., 1990; McGeer et al., 1993), proteases (Inuzuka et al., 1987; Gijbels et al., 1992; Rozniecki et al., 1995), the eicosanoids prostaglandin (PG)-E2, PGD2 and leukotriene (LT)-C4 (Dore-Duffy et al., 1991; Neu et al., 1992) and vasoactive molecules such as histamine (Tuomisto et al., 1983).

3. Summary

Evidence derived from experimental animal models, immunogenetics and immunopathology strongly indicate an immune basis for MS. It is postulated that specific disease-producing T cells gain entry into the CNS and initiate the disease process; actual tissue injury may however be mediated by an array of non-specific inflammatory mediators that are subsequently recruited to lesion sites. The immune effectors present in MS lesions appear to be activated and engaged in immune effector functions.

(B) Target injury in multiple sclerosis

While much is known about the effectors in MS, less is known about the targets. Early neuropathologic studies clearly identified the myelin membrane as the target of immune-mediated destruction in MS (Carswell, 1835; Cruveilhier, 1838; Charcot, 1868; Dawson, 1916). However, recent studies indicate that myelin membranes are not the only targets of the immune response in MS.

(1) Myelin

Early light microscope studies by Dawson (1916) indicated myelin pallor and the presence of lipid-laden cells in acute MS lesions. Ultrastructural studies have provided a more detailed picture of the patterns of myelin breakdown in MS. Observed patterns of myelin disruption include (i) increased interlamellar spacing in myelin (Gonatas, 1970; Allen, 1984; Prineas et al., 1985), (ii) vesicular dissolution of myelin (Kirk et al., 1979; Guo and Gao, 1983; Lassmann et al., 1983; Prineas et al., 1985), (iii) splitting and vacuolation of myelin sheaths (Suzuki et al., 1969; Prineas, 1975), (iv) dilatation of the periaxonal space and partial detachment from axon (Prineas and Connell, 1978; Prineas and Graham, 1981; Raine et al., 1981; Selmaj and Raine, 1988), (v) irregularly thin myelin sheaths (Suzuki et al., 1969; Gonatas, 1970; Prineas, 1975), (vi) macrophage-associated myelin thinning (Prineas, 1975; Prineas and Raine, 1976; Prineas and Connell, 1978; Allen, 1984; Prineas, 1985) and (vii) myelin phagocytosis involving active peeling of layers of myelin by infiltrating macrophages (Prineas, 1975; Prineas and Raine, 1976; Lampert, 1978; Allen, 1984; Raine, 1984b; Prineas, 1985).

Ultrastructural details of the macrophage-myelin interface in MS have shown that during the initial stages of myelin breakdown, superficial myelin lamellae are attached to receptor-rich areas on the macrophage surface (clathrin-coated pits) indicating receptor-mediated phagocytosis (reviewed in Prineas, 1985;

Raine, 1991). Putative receptors include the immunoglobulin Fc and complement C3 receptors present on macrophages in MS lesions. Consistent with these findings, Lumsden (1971) reported immunoglogulin and C3 deposition on myelin at plaque margins.

(2) Oligodendrocytes

Lumsden (1948), reasoning that any process which affected the myelin sheath would have a profound effect upon the myelinating cell, the OL, analysed seventy-five formalin-fixed plaques from fifteen MS patients using light microscopy. He reported that in all the plaques without exception, the oligodendrocytes had completely disappeared. Outside demyelinated lesions, however, they were present in normal density and were normal in appearance. Later, using fresh material from a few cases obtained within four hours of death, Lumsden (1951) confirmed his earlier observations. Lumsden (1955) proposed that myelin and OL were the *selective* targets of immune-mediated destruction in MS. Reports by Adams and Kubik (1952), Lumsden (1970), Seitelberger (1973), Guo and Gao (1983), Prineas (1984) and Raine (1984) all supported this contention, indicating that OL are lost in old inactive, sclerotic plaques of typical chronic MS.

However, a variable degree of OL preservation or even hyperplasia in early active MS plaques has been observed in different light microscopic (Friede, 1961; Ibrahim and Adams, 1963; Ibrahim and Adams, 1965), electron microscopic (Prineas, 1975; Prineas and Raine, 1976; Prineas and Connell 1978, 1979; Raine et al., 1981; Lassmann, 1983; Prineas, 1984; Moore, 1885; Prineas, 1985) and immunohistochemical studies (Prineas, 1985; Prineas et al., 1989; Prineas et al., 1993a; Bruck et al., 1994; Ozawa et al., 1994). Much confusion exists as to the extent of OL loss in the early course of MS. This has been partly due to the problem of immunohistochemical detection of OLs in demyelinating lesions as

well as the limited availability of brain tissue sections from MS patients in the early course of the disease. Using a marker (MOG) for OL that persists on the surface of OL, even when myelin is destroyed, Bruck et al. (1994) and Ozawa et al. (1994) have recently shown that there is a case-to-case variability with regard to the extent of OL cell loss in early lesions, but there is a more striking loss in late chronic lesions.

(3) Axons

Early descriptions of the pathology of MS by Carswell (1835), Cruveilhier (1838), Charcot (1868) and Dawson (1916) indicated a striking preservation of axons in MS lesions. However, in some cases, axonal injury has been documented. In a study of 125 plaques from 13 cases, Greenfield and King (1936) found that less than 10% of plaques showed axonal destruction. The axonal damage was non-specific and "consisted of thickenings and varicosities, the formation of end bulbs and loops, and vacuolation". It has been suggested that simple swelling of axons and slight vacuolation probably does not represent an irrecoverable state; "the preservation of neurofibrillae .... may be taken as an index of viability of the axon" (Lumsden, 1955). Guillain and Bertrand (1924) laid considerable stress on the importance of what they considered to be temporary axonal damage in the early phases of the evolution of the plaque. They found evidence of swelling and vacuolation in small early lesions and in the advancing margins of larger active lesions. In a more recent morphological analysis of MS plaques, it was demonstrated that while axons were preserved in early plaques, more chronic plaques sustained some axonal loss and a reduction in axonal diameter (Prineas and Connell, 1978). Axonal loss was nonetheless mild when compared with OL loss in the same lesions. Recent magnetic resonance spectroscopy imaging (MRSI) studies of MS patients have shown that there is a decrease in the resonance intensity of the neuron-specific marker, N-acetyl-aspartate (NAA), in patients with moderately severe symptoms as compared to

patients with mild symptoms or controls, indicating axonal injury in more established stages of the disease (Arnold et al., 1990; den Hollander et al., 1991; Husted et al., 1991; Matthews et al., 1991; Miller et al., 1991; Van Hecke et al., 1991; Wolinsky and Narayana, 1991; Arnold et al., 1992; Grossman et al., 1992)

4. Summary

Neuropathologic and neuroimaging studies indicate that the OL/myelin complex is the selective target of immune-mediated injury in MS. Axonal injury, albeit to a lesser extent has also been documented. The major issue pursued in this thesis relates to the immune effector mechanisms and the target response to them which could account for the relatively selective injury of the OL/myelin complex in MS. To address this issue, *in vitro* dissociated cultures of OL derived from human CNS tissue were used.

III. *IN VITRO* MODELS OF IMMUNE-MEDIATED OL/MYELIN INJURY

To assess the potential contribution of immune effector mechanisms to the destruction of the OL/myelin unit in MS, susceptibility of OL and their myelin antigen-containing membrane processes to immune-mediated injury has been studied *in vitro* using either organotypic or dissociated tissue culture systems. The former, to date, has largely involved immature rodent CNS preparations (Selmaj and Raine, 1988; Kerlero de Rosbo et al., 1990); the latter have used purified OL cultures prepared from a range of species, including humans, and from both the immature and mature CNS (Yong and Antel, 1992; Grever et al., 1995). The organotypic or explant culture systems have the advantage of containing all the components of the CNS, including compact myelin ensheathing axons, thus simulating the actual *in vivo* environment. A disadvantage is that the analysis of the injury response has, to date, largely been limited to morphologic assessments. The dissociated cultures provide a means to assess OL injury

responses at a more detailed cellular and molecular level. Such studies are, however, carried out on cells out of their natural environment. Dissociated OLs extend extensive processes which express all the expected myelin antigens (McCarthy and deVellis, 1980; Yong and Antel, 1992). However, rarely have these cells been shown to form compact myelin if cocultured with neurons. Although the various potential OL/myelin-directed immune effectors will be discussed individually, the actual extent of *in vivo* tissue injury may reflect combinations of effects.

(A) Cell-mediated immune injury of oligodendrocytes/myelin

The cellular effectors considered in this category include T cells, macrophages and endogenous glial cells. These effectors may induce OL injury via (1) cell-cell contact-dependent mechanisms, or (2) soluble factor-dependent mechanisms.

(1) Cell-cell contact-dependent OL/myelin injury

(a) T cell-mediated OL/myelin injury

As previously mentioned, T cells bearing both the αβ ($CD4^+$ and $CD8^+$) and γδ TCR are implicated in many of the events that contribute to the pathogenesis of MS (reviewed in Raine, 1991). In this section, their effects on OL cytotoxicity will be discussed.

(i) αβ $CD4^+$ T cells:

Myelin-reactive αβ $CD4^+$ T cells have been tested for their capacity to induce cytotoxicity *in vitro*. Such cells do have the capacity to induce cytotoxicity in appropriate targets in an antigen- and MHC class II restricted manner in a 5

hour ^{51}chromium release assay (Martin et al., 1990; Pette et al., 1990; Weber and Buurman, 1988; Cross et al., 1993). Most such studies have used as targets autologous or histocompatible transformed B cell lines which have been coated with a myelin antigen, usually MBP. $CD4^+$ T cells derived from human donors are not cytotoxic to autologous or histocompatible human CNS-derived OL in the conventional 5 hour cytotoxicity assay, even in the presence of exogenous MBP (Antel et al., 1994). Such findings are consistent with observations made in a number of species, including humans, that OL do not express MHC class II molecules under basal culture conditions, nor can they be induced to express these molecules by γ-IFN or endotoxin (Lee and Raine, 1989).

Several *in vitro* assays have been developed to demonstrate the capacity of $CD4^+$ T cells to mediate non-MHC-restricted target cell injury. One form of this cytotoxicity, referred to as "promiscuous" killing was initially defined using, as effectors, long-term antigen- or mitogen anti-CD3 antibody and exogenous IL-2-activated T cell lines and, as targets, natural killer (NK) cell- and TNF-resistant-cell lines (Patel et al., 1987; Weber et al., 1987). Cytotoxicity was observed using effector:target coculture assays which were more prolonged, typically 18 hours, than the 5 hour classical cytotoxicity assays. The ligand involved in mediating the effector:target cell interaction remains to be identified and may differ amongst cell types. Myelin-reactive and mitogen-activated human $CD4^+$ T cells have been shown to induce cytotoxicity of human OL in this non-MHC-restricted or promiscuous manner (Antel et al., 1994).

Short-term activated $CD4^+$ T cells have been shown to mediate non-MHC-restricted cytotoxicity if effector:target adherence is enhanced by use of lectin agglutinins such as phytohemagglutinin (PHA) or concanavilin-A, a process termed lectin-dependent cell cytotoxicity (Patel et al., 1987). Lectin-dependent cytotoxicity of human OL mediated by PHA-activated human $CD4^+$ T cells has been demonstrated (Ruijs et al., 1993; Antel et al., 1994). Whether the molecular

mechanisms underlying promiscuous and lectin-dependent killing are identical is not yet clarified. Human $CD4^+$ T cells activated short-term in the presence of IL-2 also mediate a degree of promiscuous killing of human OL (Antel et al., 1994).

(ii) αβ $CD8^+$ T cells:

These classical cytotoxic cells recognize their targets in the context of MHC class I molecules. *In vitro* studies of both human and rodent OL indicate that these cells will express MHC class I molecules and are susceptible to classic (5-hour) cytotoxicity mediated by $CD8^+$ T cells sensitized to the appropriate MHC class I molecules (Ruijs et al., 1990). Myelin peptide-reactive MHC class I-restricted $CD8^+$ T cells have been generated *in vitro* and shown to induce cytotoxicity in cell lines expressing these molecules in the presence of exogenously added peptide (Tsuchida et al., 1994). Whether myelin-reactive $CD8^+$ T cells can induce cytotoxicity of OL in the absence of added peptide remains to be established. $CD8^+$ T cells, as $CD4^+$ T cells, can also mediate promiscuous OL target cell killing *in vitro* (Ruijs et al., 1993).

(iii) γδ-T cells:

These T cells, defined on the basis of the TCR chains expressed, are shown to exert potent, rapid (5 hours), non-MHC-restricted cytotoxicity of human OL *in vitro* (Freedman et al., 1991). Other neural cell types, including astrocytes and microglia, are also susceptible to γδ-T cell-mediated injury. The precise molecule on the target cell being recognized by the γδ-T cells remains speculative. One family of candidate molecules are the heat shock or stress proteins - discussed later (Haregewoin et al., 1989; Indreshpal et al., 1993).

The actual mediators of T cell-mediated cytotoxicity remain under study.

Results of *in vitro* studies of either short-term activated or long-term T cell-lines and clones indicate that T cells may mediate cytotoxicity via three mechanisms: (1) a membranolytic one whereby T cell secreted perforin induces the formation of pores in target cell membranes, providing for the entry of T cell secreted granzymes that cause fragmentation of the target cell's DNA (apoptosis - discussed later); it is not always clear whether target cell death in this case is due to primary membrane injury (lysis - discussed later) mediated by perforin or primary nuclear injury mediated by granzymes, (ii) T cell secreted TNF that may induce membrane (lysis) or nuclear (apoptosis) injury of the target cell, and (iii) a nonsecretory receptor mediated mechanism involving the interaction of fas ligand on the T cell and fas on the target cell, resulting in transduction of apoptosis-inducing signals in the target cell and subsequent apoptotic target cell death (Kagi et al., 1994; reviewed in Berke, 1994).

(b) Microglia/Macrophage-mediated OL/myelin injury.

These cells are capable of effecting injury via an array of mechanisms involving either cell-cell contact or release of soluble factors (see below). As regards the former, microglia adhere to target cells using one or more of a series of receptors expressed on their surface. Some of these receptors are expressed constitutively, whilst expression and activity of others requires activation by a variety of cytokines such as γ-IFN (Williams et al., 1994). It is also necessary for the target cell to express the corresponding ligand, endogenously or as a result of injury. Like *in vivo*, microglia *in vitro* express Fc receptors and several complement receptors including CR1, CR2 and CR4 (Suzumura et al., 1987; Williams et al., 1993; Ulvestad et al., 1994a). Rodent macrophages are capable of phagocytosing OL coated with antibody directed at several antigens expressed on the cell surface (GalC and MOG)(Scolding and Compston, 1991), binding presumably occurring through the Fc receptor. Human microglia have been shown to phagocytose antibody-coated myelin beads (Ulvestad et al., 1994c).

Activated products of the complement system occurring on the surface of OL may act as ligands for microglial complement receptors. Zajicek et al. (1992a) showed *in vitro* that in OL-microglia cocultures, resting microglia usually had minimal contact with OL at any one time. These microglia bound myelin debris, most of which remained cell-surface associated. The addition of complement to these cultures increased MBP staining on microglia, and again this appeared to be largely cell surface associated. Microglia activated with γ-IFN or γ-IFN and lipopolysaccharide (LPS) showed a greater propensity for OL interactions; they displayed increased cell-surface binding of MBP and demonstrated phagocytosis of myelin in the presence of myelin. Human microglia have also been shown to bind and phagocytose target cells that are complement-coated (Ulvestad et al., 1994a).

Macrophages and microglial cells are capable of inducing target cell cytotoxicity via a process termed antibody-dependent cell cytotoxicity (ADCC), which involves antibody coated target cells binding to Fc receptors on effector cells, triggering the release of cytopathic molecules into the microenvironment between the plasma membranes of the juxtaposed target and effector cells. The injury-mediating potential of ADCC effected by macrophages on OL and other targets has been demonstrated *in vitro* (Griot-Wenk et al., 1991; Scolding and Compston, 1991; Zajicek et al., 1992a; Ulvestad et al., 1994c).

(2) Soluble factor dependent OL/myelin injury

(a) Cytokines

The cytokines TNFα and TNFβ have been shown to mediate injury to both myelin and OL in myelinating explant cultures *in vitro* (Selmaj and Raine, 1988) and to OL in dissociated cultures (Selmaj et al., 1991c; Louis et al., 1993). Studies in goldfish have shown that a divalent form of IL-2 generated by a

transglutaminase released from sectioned optic nerve can be toxic to OL (Eitan and Schwartz, 1993). Recently, Vartanian et al. (1995) reported that γ-IFN is toxic to rat OL derived from the CG-4 rat OL precursor cell line. Bovine OL have been shown to be resistant to cytotoxicity mediated by γ-IFN (Selmaj et al., 1991c).

(b) Non-cytokine soluble factors

In this category, one need consider mediators such as nitric oxide (NO), free radicals and proteases (reviewed in Hartung et al., 1995). Rodent OL have been shown to be susceptible to NO-dependent mechanisms (Lee et al., 1993; Merrill et al., 1993). Oxygen radicals generated from an array of sources have been shown to be cytotoxic to human (Kim and Kim, 1991) and rodent (Noble et al., 1994) OL. In dog glial cell cultures exposed to reactive oxygen radicals, a selective degeneration of OL was noted (Griot et al., 1990). Reactive oxygen species can damage myelin by lipid peroxidation and have indeed been found to degrade myelin *in vitro* (Chia et al., 1983; Konat and Offner, 1983; Konat and Wiggins, 1985).

(B) Humoral immune-mediated injury of OL/myelin

Humoral immune-mediated OL injury *in vitro* has been shown to occur either via (i) antibody-dependent complement-mediated cytotoxicity, whereby antibody binding to plasma membrane-expressed determinants on OL is followed by fixation and activation of complement, leading to membrane insertion of the MAC and subsequent membrane rupture and cell death of the OL, or (ii) antibody-independent complement-mediated cytotoxicity.

Antibody-dependent complement-mediated cytotoxicity of rodent OL has been demonstrated *in vitro* using antibodies directed at plasma membrane-expressed myelin compoments such as MOG (Piddlesden et al., 1993; Scolding

and Compston, 1995) and GalC (Lubetzki-Korn et al., 1984; Scolding and Compston, 1995).

Antibody-independent complement-mediated cytotoxicity of rodent OL is well documented (Scolding et al., 1989a-c, 1990b, 1992; Wren and Noble, 1989; Zajicek et al., 1992a). Unlike rodent OL, human OL are resistant to complement, a finding explained by the presence of the complement inhibitory protein CD59 on human but not rodent OLs (Wing et al., 1992; Zajicek et al., 1992b; Piddlesden and Morgan, 1993; Zajicek et al., 1995). Again, unlike OL, astrocytes are resistant to complement-mediated cytotoxicity (Scolding et al., 1989a).

(C) Summary

The above discussion has indicated that *in vitro*, OL and myelin are susceptible to both cell- and humoral-mediated immune effector mechanisms. OL in MS lesions do not express MHC class I and II molecules, prerequisites for interaction with antigen-specific cytotoxic T cells. Furthermore the vast majority of T cells in MS lesions are not OL/myelin-specific. Cytotoxic humoral effector mechanisms directed against the human OL/myelin complex are dependent on the presence of OL/myelin-specific antibodies. As with T cells, most antibodies in MS lesion sites are however not OL/myelin-specific. It would thus appear that non-specific immune effectors such as T cells acting in a non-MHC 'promiscuous' manner, microglia, macrophages recruited to lesion sites, and cytotoxic cytokines such as TNFα and TNFβ, could contribute perhaps more significantly to OL/myelin injury in MS lesions. However, this raises the issue as to how non-specific immune mediators may induce rather selective OL/myelin injury in MS. This thesis tests the postulate that selective target injury within the CNS may reflect target-cell rather than effector-cell properties. In this regard, differences in susceptibility of CNS neural cells to common immune mediators or the cell-specific expression of a surface receptor for a putative injury mediator may result

in specific target cell injury.

IV. MECHANISMS OF TARGET CELL INJURY - IMPLICATIONS FOR OLIGODENDROCYTE INJURY IN MS

Cell injury responses are now recognized to involve distinct biochemical pathways. Strategies aimed at protecting target cells from injury would thus require an understanding of the nature of the target cell injury response. Most data regarding mechanisms of immune-mediated injury have been generated using proliferating target cells or cell lines derived from non-neural tissue. This thesis addresses the issue of the response of neural cells, in particular, non-proliferating OL, to immune-mediated injury and its relevance to strategies aimed at protecting OL from immune-mediated injury (see later section on "Neuroprotective strategies for OL").

Mild forms of injury may result in sublethal injury, which may manifest itself by a shutdown of the cells "luxury" function or the enhanced expression of so-called "stress" or "heat shock" proteins (HSPs); in such cases, if the injury stimulus is maintained for only a short period of time, there is usually potential for recovery. Severe and sudden forms of injury usually result in cell death via necrosis which is typified by membrane rupture and cell lysis. When the injury stimulus is more modest, cell death may also occur via a more subtle process, termed apoptosis, which is typified by DNA and nuclear fragmentation in the absence of membrane rupture. Each of these forms of injury are induced, regulated and executed distinctly.

(A) Sublethal injury with loss of "luxury" function

In many organs in the body, constituent cells acquire certain "differentiation" or "luxury" functions upon terminal differentiation. "Luxury"

functions could include the production of hormones, neurotransmitters, cytokines, immunoglobulins and myelin (Oldstone et al., 1982; reviewed in de la Torre et al., 1991). When cells that possess certain luxury functions are subjected to sublethal forms of injury, cell viability is maintained. However, disturbances in the "luxury" function of the cells may ensue, leading to disruption of homeostasis and disease. Noncytopathic viruses have been particularly implicated as agents that may be capable of shutting down the luxury function of a cell. Infection of mice with lymphocytic choriomeningitis virus (LCMV) for example, which shows tropism for the anterior lobe of the pituitary gland, results in viral replication in cells that make growth hormone (GH). This results in diminished synthesis of GH with a concomitant clinical picture of retarded growth and hypoglycemia. However, pathologic examination reveals no evidence of cell loss in the anterior lobe of the pituitary gland of infected mice with clinical symptoms (Oldstone et al., 1982).

Electron microscopic evaluation of stereotaxic brain biopsy specimens that demonstrated inflammatory primary demyelination consistent with acute MS has revealed that although OL cell bodies are morphologically preserved in early lesions, morphologic abnormalities in the most distal extensions of their myelin sheaths are prominent (Rodriguez et al., 1993). The uniform widening of the inner lamellae and degeneration of the inner glial loops ("dying-back" oligodendrogliopathy) observed in these biopsies suggested a disturbance in the myelinating function of OL in early active MS lesions.

In vitro, immune-mediated sublethal injury of OL has been documented in the following cases: (i) the addition of anti-MOG antibodies and complement to rodent organotypic or explant CNS cultures has been shown to induce a highly significant decrease in MBP content and CNPase specific activity without cell death (Honeggar et al., 1989; Kerlero de Rosbo et al., 1990; Loughlin et al., 1994), (ii) antibody-independent complement-mediated injury in rodent OL indicate

initial sublethal injury owing to vesicular removal of membrane attack complexes (MAC) from the OL membrane, thus affording a limited capacity for recovery (Scolding et al., 1989b,c), (iii) rodent OL exposed to low levels of the T cell effector mediator, perforin have been shown to undergo reversible plasma membrane injury, owing to vesicular removal to membrane-associated perforin (Scolding 1990a), and (iv) TNF has been shown to cause a reduction in the content of MBP in myelinated aggregated rat brain cultures (Loughlin et al., 1994), and dysfunction of potassium channels in ovine OLs (Soliven et al., 1994) in the absence of cytotoxicity.

(B) The cellular stress response

Under normal physiological conditions, cells express 'constitutive' members of a group of proteins called heat shock proteins (HSPs). HSPs are evolutionarly highly conserved proteins categorized by molecular masses into various families (e.g. HSP60 kDa, HSP70 kDa, HSP90 kDa). They serve as chaperonins, playing a pivotal role in normal cellular protein processing, such as in protein folding or unfolding, assembly or degradation of proteins, or translocation (chaperoning) of protein molecules to sites such as the plasma membrane (Sheffield et al., 1990; Craig et al., 1990; Kang et al., 1990; Gething and Sambrook, 1992; Schlesinger, 1990). Exposure of cells to a variety of environmental conditions such as heat shock, ischemia, nutrient deprivation, metabolic disruption, viral infection, and pharmacological agents including cytokines, may result in direct damage to cellular proteins or aberrant protein synthesis in the endoplasmic reticulum. In these cases, in an effort to protect vital cellular proteins and enzymes, cells upregulate the production of the 'constitutive' HSPs, and in addition, a group of 'inducible' HSPs (reviewed in Lindquist and Craig, 1988; Ellis and van der Vies, 1991). These HSPs bind to denatured proteins and enzymes and maintain their conformation during the period of stress. The HSP response is evolutionarly designed to afford cells a limited window of protection from potentially lethal stresses. Immunohistochemical detection of the known HSPs is now possible.

The mechanisms underlying the signal transduction pathway activating the HSP response remain to be clarified. It has been hypothesized that the constitutive (or cognate) species of HSP70, HSC70, transiently associates with the monomeric form of heat shock transcription factor (HSF), ensuring its deactivation under non-stressful conditions (Craig and Gross, 1991; Morimoto, 1993). Conceivably, under conditions of stress resulting in protein denaturation, the number of substrates competing for association with HSC70 increases, thereby exhausting the pool of free HSC70, leading to the dissociation of inactive HSF-HSC70 complexes. Upon release from HSC70, the HSF monomers oligomerize into a trimeric state which attains DNA-binding capacity and translocates to the nucleus where transcription of the heat shock genes is activated. The resultant enhanced expression of HSPs, including HSP70, which aid HSC70 in salvaging the denatured proteins, serves to "free-up" the HSC70, enabling a free pool of HSC70 to reform as the latter is released from the HSP70-denatured protein complexes (reviewed in Fernandes et al., 1994; Wu et al., 1994; Morimoto et al., 1994; Craig et al., 1994).

HSPs have been observed to be one of the dominant antigens recognized by cytotoxic γδ-T cells during immune responses induced by a variety of microbial pathogens (Holoshitz et al., 1989; Janis et al., 1989; reviewed in O'Brien and Born, 1991). These γδ-T cells may not require target cell expression of MHC antigen for interaction (O'Brien et al., 1989; Holoshitz et al., 1989). HSP-responsive γδ-T cells have been implicated in many autoimmune diseases including rheumatoid arthritis, diabetes and systemic lupus erythematosis (Holoshitz, 1990). Due to the high percentage of sequence homology that exists between HSP species within a single HSP family, the proposed mechanism for the role of HSP in autoimmunity is one of molecular mimicry; HSP-reactive T cells generated in response to a HSP, expressed on a microbial agent, would be able to recognize HSP of stressed self-cells, provoking an autoimmune reaction (Cohen et al., 1990). Thus instead of responding to self versus non-self MHC plus

antigen, as in the case of traditional αβ-T cells, cytotoxic γδT-cells may prefer to recognize stressed versus non-stressed target cells as part of a 'first-line' defence system (Elliott and Young, 1989; Born et al., 1990). Since γδ-T cells are known to be indiscriminate killers (Lanier et al., 1987; Hochstenbach and Brenner, 1990), selectively of target cell damage may be regulated by differential expression of HSPs.

OL-specific expression of HSP65 has been detected in MS lesion tissue but not in control CNS tissue (Selmaj et al., 1991a; Selmaj et al., 1992). In these lesions, cytotoxic γδ-T cells are shown to be concentrated and co-localize with HSP65-expressing OL (Selmaj et al., 1991a; Selmaj et al., 1992). OL-specific expression of HSP65 in MS lesions may promote potentially deleterious interactions with cytotoxic γδ-T cells.

A number of studies have shown that under basal temperatures, rodent and human OL *in vitro* constitutively express the constitutive HSP species, HSP60 (Freedman et al., 1992; Selmaj et al., 1992; Satoh et al., 1992b). In response to thermal stress (heat shock), OL upregulate expression of the inducible HSP species, HSP72 (Freedman et al., 1992; Satoh et al., 1992a). Satoh and Kim (1995) have shown that bovine OL upregulate expression of HSP72 in response to heat shock, but not in response to oxidative stress mediated by hydrogen peroxide or the cytokines IL-1 α, IL-1β, TNFα or TNFβ.

(C) Necrotic cell death

Necrosis refers to a pathological form of cell death that results from sudden overwhelming cellular injury (reviewed in Wyllie, 1981; Walker et al., 1988). This "accidental" cell death occurs in response to a wide variety of harmful conditions and toxic substances including hyperthermia (Buckley, 1972), hypoxia (Laiho et al., 1983), ischemia (Borgers et al., 1987), complement attack (Hawkins et al., 1972),

perforin release from T cells (Scolding et al., 1990a), metabolic poisons (Trump et al., 1984) and direct cell trauma (Trump et al., 1967).

A cell undergoing necrosis typically exhibits distinct morphological and biochemical characteristics (reviewed in Trump et al., 1982). The earliest changes include swelling of the cytoplasm and organelles, especially the mitochondria, with only slight changes in the nucleus. These changes ultimately lead to organelle dissolution and rupture of the plasma membranes, allowing the cellular contents to leak out into the extracellular space, a process termed lysis. The morphological changes are due to the loss of control of selective permeability of the plasma membrane (Trump et al., 1984). These alterations are in response to the early disappearance of membrane ion-pumping activities, either directly due to membrane damage (Schanne et al., 1979) or secondary to cellular energy depletion (Jennings and Reimer, 1981). As cations move across the membrane along concentration gradients, the accompanying fluid shifts cause tremendous cellular swelling. The increase in free cytosolic calcium (Ca^{2+}) results in activation of membrane-bound phospholipases, which degrade membrane phospholipids and cause widespread disruption of membranes (Chien et al., 1978; Smith et al., 1980). Assays that measure lactate dehydrogenase (LDH) release and release of prelabelled ^{51}chromium are routinely used to quantitate necrotic death *in vitro*. *In situ*, necrosis is usually assessed by the ultrastructural detection of ruptured plasma membranes and swollen cytoplasm. The release of hydrolases from ruptured lysosomes cause a rapid acceleration of cellular disintegration in the late stages of necrosis (Hawkins et al., 1972). Rapid decreases in protein, RNA, and DNA levels occur (Trump et al., 1982). DNA exposed by proteolytic digestion of histones is cleaved by lysosomal deoxyribonuclease into fragments displaying a continuous spectrum of sizes (Afanasev et al., 1986; Duvall and Wyllie, 1986). Necrosis typically affects groups of contiguous cells, and an inflammatory reaction usually develops in the adjacent viable tissue in response to the released cellular debris.

Several studies have provided ultrastructural evidence for necrotic cell death of OL in MS lesions (Prineas, 1985; Raine, 1990). Comparison of OL at lesion sites with those in normal white matter indicated that some of the OL in lesion sites exhibited cellular swelling and disorganization and rupture of the plasma membrane and internal membranes.

Immune-mediated lysis of OL *in vitro* has been documented in the following cases: (i) antibody-dependent complement mediated lysis of rodent OL (Lubetzki-Korn et al., 1984; Piddlesden et al., 1993; Scolding and Compston, 1995), (ii) antibody-independent complement mediated lysis of rodent OL (Scolding et al., 1989a-c, 1990b, 1992; Wren and Noble, 1989; Zajicek et al., 1992a)(as mentioned previously, human OL are resistant to complement mediated lysis), (iii) non-MHC restricted $\alpha\beta$ $CD4^+$, $\alpha\beta$ $CD8^+$ and $\gamma\delta$-T cell mediated lysis of human OL (Ruijs et al., 1990; Freedman et al., 1991; Antel et al., 1994), (iv) macrophage/miroglia mediated ADCC (Griot-Wenk et al., 1991; Scolding and Compston, 1991; Zajicek et al., 1992a; Ulvestad et al., 1994c), and (v) exposure to reactive oxygen species (Griot et al., 1990; Kim and Kim, 1991; Noble et al., 1994).

(D) Apoptotic cell death

In contrast with the injury-induced death of necrosis, several investigators in the late 1960s had observed a morphologically distinct, nonpathological, spontaneous form of cell death that occurred at specific times in many different tissues during development (Klion and Schaffner, 1966; Farbman, 1968; Kerr 1969, 1971). This loss of cells, termed programmed cell death (PCD), owing to the predictability of its occurrence during development, was thought to play a number of important roles, including the removal of superfluous cells, the sculpting of the body, and the removal of vestigial tissues. Kerr et al. (1972), studying the kinetics and ultrastructural features of PCD in mammalian cells, noted that this type of cell death typically occurred under various physiological

and pathological conditions, in scattered cells and progressed so rapidlly that it was difficult to observe. They likened the kinetics of this process to the "falling off" of petals from flowers or leaves from trees in autumn, and proposed the term "Apoptosis"[1] (greek for "falling off") to describe PCD. Today, a diverse array of inducers of apoptosis have been identified; most notably, TNF, fas ligand and cytotoxic T cells have been identified as inducers of target cell apoptosis in a wide array of immune-mediated disorders (reviewed in Thompson, 1995).

In situ and *in vitro* studies have indicated that apoptosis occurs in several discrete steps (reviewed in Cohen, 1991; Tomei and Cope, 1991; Schwartzman and Cidlowski, 1993). Initially, the cells lose some volume and their cytoplasmic organelles become more tightly packed, cytoskeletal disruption occurs and nuclear clumping is evident. At this stage, membrane changes that can lead to phagocytosis are present. As this process continues, the plasma membrane of the apoptosing cells gradually becomes more ruffled and blebbed. Endonuclease activation results in degradation of nuclear DNA into oligonucleosomal fragments that are multiples of 180-200 base pairs; DNA gel electrophoresis of apoptosing cells produces a characteristic "DNA ladder", now one of the *in vitro* tests of apoptosis. DNA fragmentation is accompanied by the collapse of chromatin into crescents along the nuclear envelope, then into a "black hole", and eventual breakage into spheres. DNA fragmentation assays such as the terminal transferase (TdT)-dUTP nick end-labelling (TUNEL) technique and morphological assessments of nuclear chromatin structure by fluorescent DNA-binding dyes, are now routinely used to quantitate apoptosis *in vitro* and *in situ*.

In vivo, the intact apoptotic cell may be rapidly phagocytosed by neighbouring phagocytic cells and degraded in lysosomal compartments; in contrast to necrosis, the process does not stimulate an inflammatory response. It is thought that apoptosis was born out of an evolutionary need to dispose of superfluous or damaged cells in a coordinated fashion without provoking

inflammation (Ellis et al., 1991; Raff et al., 1993). Alternatively, the apoptotic cell may fragment into apoptotic bodies consisting of intact plasma membrane enclosing variable amounts of cytoplasm and/or condensed chromatin. Apoptotic bodies, both *in vivo* and *in vitro*, exclude vital dyes such as propidium iodide (PI) and acridine orange, and do not release their intracellular enzymes like LDH, prior to phagocytosis. Specific recognition mechanisms are now proposed for phagocyte recognition of cells undergoing apoptosis. These include, (reviewed in Savill et al., 1993): (i) interaction of lectins on the surface of the phagocyte with exposed glycoprotein sugar chains, caused by the loss of sialic acid residues, on the surface of the apoptosing cell (Morris et al., 1984); (ii) linkage of vitronectin receptors (e.g. $\alpha_v\beta_6$) and CD36 on the surface of the phagocyte, with the thrombospondin (TSP) binding moiety on the surface of the apoptosing cell, owing to their common interactions with phogocyte-secreted TSP (Pytela et al., 1985), and (iii) interaction of phosphatidylserine (PS) on the surface of the apoptosing cell (PS is normally expressed on the inner leaflet of the plasma membrane, but it gets exposed on the outer leaflet in apoptotic cells), with PS receptors on the surface of the phagocyte (Fadok et al., 1992).

In vitro, in the absence of phagocytic cells, apoptotic bodies eventually begin to non-specifically lose their membrane permeability, releasing intracellular LDH or prelabelled ^{51}Cr. The use of LDH or ^{51}Cr release assays without assessing for possible prior or concomitant apoptosis in culture systems could thus lead to erroneous conclusions regarding the mechanism of cell injury. In the cases described above regarding immune mediated lysis of OL *in vitro*, assays to screen for apoptosis prior to lysis were not performed. The nature of the injury response in these cases thus needs further clarification. In this thesis, kinetic analysis of both apoptosis and lysis were performed to prevent erroneous conclusions.

In several well-studied models of apoptosis, there is a requirement for new gene expression for both the morphological changes and death itself to occur

(reviewed in Cohen, 1991). Inhibitors of mRNA or protein synthesis in these cases prevent apoptosis (reviewed in Schwartzman and Cidlowski, 1993). This mechanism of apoptosis is referred to as induction. There are other models, however, in which apoptosis is triggered by the induction of mRNA or protein synthesis (reviewed in Schwartzman and Cidlowski, 1993). Because these cells behave as though the apoptotic program is constitutively expressed but inhibited by factors with short half lives, the mechanism is referred to as release. These observations have led to the conclusion that during apoptosis, activation of 'suicide genes' results in the cell committing suicide in response to the apoptotic signal; the cell is not 'murdered'.

It is now known that a number of evolutionary genes regulate a final common pathway in apoptosis that is conserved from worms to humans (Yuan et al., 1993; Hengartner and Horvitz, 1994). It is now widely accepted that just as the mammalian cell cycle has multiple checkpoints (Hartwell and Weinert, 1989), the apoptotic cell death cascade is also regulated by a number of decisional checkpoints. An apoptotic stimulus that is strong enough to break through these checkpoints, activates the apoptotic executioner. Activation of the executioner represents a point of no return for the cell; the cell then rapidly undergoes apoptosis. Multiple candidate apoptotic checkpoints have been described to date: the bcl-2/bax, bcl-2/bcl-x_s, bcl-2/A1, bcl-x_l/bcl-x_s, bcl-x_l/bax, Mcl-1/bax, A1/bax and ICH-1_s/ICH-1_l checkpoints (Oltvai and Korsmeyer, 1994; Sato et al., 1994; Sedlak et al., 1995). The ratio of the proteins of each pair, which bind either to themselves or to each other, determines whether a cell receiving an apoptotic stimulus will decide to undergo apoptosis or not. The former members of each of the above pairs are anti-apoptotic proteins that prevent cell death from occurring, whereas the latter members are pro-apoptotic and promote cell death. The relative ratios of these dueling dimers sets the 'apoptotic rheostat' of a cell.

Activated proteases have been implicated as central components of the

executioner. Among them are calpain I (Sarin et al., 1993; Squier et al., 1994), the Interlukin-1-β converting enzyme (ICE) and an ever-expanding family of ICE-like proteases. These include ICE_{rel}-II (TX, ICH-2), ICE_{rel}-III, ICH-1, CPP-32 (apopain, Yama), Mch2 and Mch3 (ICE-LAP3) (Kumar et al., 1992, 1994; Wang et al., 1994; Fernandes-Alnemri et al., 1994, 1995a,b; Faucheu et al., 1995; Kamens et al., 1995; Munday et al., 1995; Duan et al., 1996 in press). Selective inhibitors of the ICE-like proteases have been shown to inhibit apoptosis in several mammalian systems of apoptosis (reviewed in Martin and Green, 1995; Nicholson, 1996). It has been postulated that activation of proteases results in proteolytic cleavage of specific substrates, and this represents the effector stage of this process. For example, proteolytic cleavage of lamin B1 has been implicated to contribute to the chromatin collapse during apoptosis (Neamati et al., 1995). Proteolytic cleavage of the cytoskeletal proteins β-actin (Kayalar et al., 1995) and α-fodrin (Martin et al., 1995) during apoptosis have been implicated in the blebbing and cellular fragmentation that results in the formation of apoptotic bodies. Inability to repair endonuclease-mediated DNA nicks during apoptosis has been attributed to the cleavage of the DNA repair enzyme, poly-(ADP-ribosyl)-polymerase (PARP)(Kaufmann et al., 1993; Labeznik et al., 1994). Other substrates that have been reported to become degraded in association with the onset of apoptosis include the 70kDa protein component of the U1 small nuclear ribonucleoprotein (Casciola-Rosen et al., 1994), the catalytic subunit of the DNA repair enzyme DNA-dependent protein kinase ($DNA\text{-}PK_{cs}$)(Casciola-Rosen et al., 1996 in press) and topoisomerase I (Voelkel-Johnson et al., 1995).

Although OL pathology in MS lesions has been the subject of many intensive studies, data indicating the nature of OL injury in MS, particularly with respect to apoptosis has not been forthcoming for the following reasons: (i) the tools to study apoptosis in situ have only recently been made available, (ii) apoptosis occurs with rapid kinetics *in vivo*, making it a difficult process to detect, (iii) the apppropriate immunohistochemical techniques to identify OL in

demyelinating MS lesions have only recenly been developed, and (iv) there is a paucity of well preserved MS autopsy or biopsy tissue. Nevertheless, there are reports in the literature of instances of OL apoptosis in MS (Raine and Scheinberg, 1988; Ozawa et al., 1994). Ozawa et al., 1994, using the *in situ* TUNEL labelling technique demonstrated that apoptosis does occur in MS lesions, mainly in cells located in areas of active demyelination. The majority of the degenerating apoptotic cells were identified by double staining as OL or T lymphocytes. Rare apoptotic macrophages and astrocytes were reported. These results have not been reproduced by other laboratories yet. As mentioned before, specific mechanisms such as those involving the vitronectin receptor are now defined for phagocytosis of cells undergoing apoptosis. Within MS lesions, microglia and macrophages are readily found to be in contact with OL and their processes (Prineas, 1985). In diseased CNS tissue, these cells express elevated levels of the vitronectin receptor as compared to normal CNS tissue.

Several soluble factors, known to be present in elevated levels in MS lesions have been shown to induce apoptosis of OL in dissociated culture. These include TNFα and β (bovine and rodent OL; Selmaj et al., 1991c; Louis et al., 1993), dimeric IL-2 (goldfish OL; Eitan and Schwartz, 1993) and γ-IFN (rodent OL; Vartanian et al., 1995). As mentioned before, species variations exist, for example, γ-IFN induces apoptosis of rodent OL (Vartanian et al., 1995) but not bovine OL (Selmaj et al., 1991).

V. NEUROPROTECTIVE STRATEGIES FOR OLIGODENDROCYTES

In demyelinating diseases such as MS, cell-cell interactions, both immune-CNS and CNS-CNS, as well as soluble factors derived from immune or CNS components are considered to be primary contributors to the tissue injury. In health, CNS-CNS cell-cell interactions and CNS-derived soluble factors contribute in a positive manner to the survival and maintenance of neural elements in the

CNS. Identification of such interactions and soluble factors would be a necessary first-step towards designing therapeutic strategies aimed at promoting survival, recovery and plasticity of neural elements under pathologic conditions. A major issue of this thesis relates to the identification of neurotrophic factors or cytokines that are capable of protecting human OL from immune-mediated injury, as occurs in demyelinating diseases such as MS. The study of naturally-occurring cell death in the developing vertebrate nervous system has contributed significantly to the discovery of neuroprotective trophic factors and cytokines.

(A). Naturally-occurring neuronal cell death in the developing vertebrate nervous system -the discovery of neurotrophic factors

During development of the vertebrate nervous system, axons, guided by interactions between their growth cones and environmental cues, grow towards their appropriate targets and form synaptic connections with them (reviewed in Jacobson, 1991). Not all of the neurons produced during development survive; a significant fraction of them normally die (Ernst, 1926; Glucksmann, 1951) with a morphology characteristic of programmed cell death (apoptosis) (reviewed in Oppenheim, 1991). Pioneering studies by Levi-Montalcini, Hamburger and Cohen on normal neuronal death and neuron-target-cell interactions (Hamburger and Levi-Montalcini, 1949; Levi-Montalcini and Hamburger, 1951; Levi-Montalcini, 1952), led to the advancement of the neurotrophic theory - that the size of neuron cell pools during development is regulated by competition for limited amounts of target-derived survival factors (reviewed in Cowan et al., 1984; Purves, 1988; Jacobson, 1991; Oppenheim, 1991). Such a mechanism would ensure that the number of neurons is adjusted to the number of target cells requiring innervation. Commensurate with their proposal of the neurotrophic theory, they discovered nerve growth factor (NGF), a target-derived soluble factor that could rescue developing sympathetic and sensory neurons from normal cell death (Cohen et al., 1954).

Since the discovery of NGF, a variety of factors similar in function to NGF have been characterized. These proteins fulfill the functional definition of "neurotrophic factor", that is, proteins able to regulate survival and differentiation of nerve cells. Many of these factors can now be classified into families based on their structural homology, biological activity or use of a similar signal transducing receptor mechanism. Two of the most characterized neurotrophic factor families are the neurotrophins and the IL-6 family of cytokines. The neurotrophins NGF, brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3 and NT-4/5 share structural homology (Hallbook et al., 1991; reviewed in Bothwell, 1995) and the use of a unique signal transduction system represented by two different transmembrane receptors - the trk family of receptor tyrosine kinases (Martin-Zanca et al., 1989; Lamballe et al., 1991; reviewed in Barbacid, 1994) and the p75, or low-affinity, neurotrophin receptor (Chao et al., 1986; Radeke et al., 1987; reviewed in Chao, 1994). The IL-6 family of cytokines consist of the distantly related cytokines ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), IL-11 and oncostatin M (OSM) (Bazan, 1991; Rose and Bruce, 1991), that share the "β" signalling subunits gp 130 and/or LIFRβ. In addition to the β components, some of these cytokines (CNTF and IL-6) also require specificity-determining "α" components (Stahl and Yancopoulos, 1993). A detailed discussion on the signal transduction mechanisms employed by these neurotrophic factors is beyond the scope of this thesis; the reader is referred to the following excellent articles on the subject: Chao M, 1994; Kaplan and Stephens, 1994; Stahl and Yancopoulos, 1994; Ip and Yancopoulos, 1996.

(B). Naturally-occurring oligodendrocyte cell death in the developing vertebrate nervous system

Although the normal large-scale death of developing vertebrate neurons has been recognized for many years, the normal large-scale death of developing

OL was recognized only recently, in studies of the developing rat optic nerve (Barres et al., 1992). The rat optic nerve contains the axons of retinal ganglion neurons that project from the eye to the brain, and two major classes of glial cells: OL, which myelinate the axons, and astrocytes which, among other functions, provide a structural framework for the nerve. During development, astrocytes appear prenatally, whereas OL and their precursors first appear in the rat optic nerve at birth and increase in numbers postnatally.

When frozen sections of developing postnatal rat optic nerves were stained with PI to label nuclear DNA, about 50% of the OL nuclei were seen to be pyknotic (Barres et al., 1992), a morphology characteristic of naturally-occurring programmed cell death (apoptosis) (Kerr et al., 1972; Wyllie et al., 1980; Ellis et al., 1991; Tomei and Cope, 1991).

Why do newly formed OL die in the developing optic nerve? By analogy with developing neurons, it is possible that OL in the optic nerve require specific neurotrophic factors secreted by their target, the axons that they myelinate, to survive *in vivo*, and that not all of them get enough. The limited availability of the axon-derived survival signals could ensure that the number of OL is automatically adjusted to the number and length of axons requiring myelination. The axonal dependence of OL survival in the optic nerve has been demonstrated by Barres et al., 1993a: transection of the postnatal rat optic nerve behind the eye, such that all of the axons in the nerve rapidly degenerate, results in selective death of the OL in the nerve. It is not clear how axons promote OL survival. It is possible that they act indirectly by stimulating astrocytes to secrete survival factors. However, they may act directly on the OL to enhance survival. The use of highly purified dissociated OL culture systems has helped identify survival factors for OL.

(C). Protection of oligodendrocytes from naturally-occurring cell death *in vitro* - *in vivo* correlates

Raff (1992) has proposed, that in higher animals at least, just as a cell seems to need signals from other cells in order to proliferate (Sato, 1979; Ham, 1981; Baserga, 1985), so it needs signals from other cells in order to survive. In their absence, the cell kills itself by activating an intrinsic suicide programme. This implies that cells are programmed to kill themselves unless they are continuously signalled by other cells not to do so. This model is an extension of the well-accepted neurotrophic theory, and there is indeed growing support for this view. Culturing highly purified cells in medium lacking exogenous signalling molecules (serum-free and mitogen-free medium) results in rapid programmed cell death (apoptosis) in most cell types tested. Serum-deprivation of highly purified cells is now regarded as a classical method of inducing programmed cell death (apoptosis) *in vitro*.

As such, when either OL or their precursors are isolated from the developing rat optic nerve and are cultured in the absence of other cell types or exogenous signalling molecules (serum-free and mitogen-free medium), they rapidly die with the morphological features characteristic of cells dying by programmed cell death (apoptosis) (Barres et al., 1992). They can be saved, although only for a few days, by individual neurotrophic factors and cytokines that include platelet-derived growth factor (PDGF), insulin-like growth factors (IGFs), CNTF, and NT-3 (Barres et al., 1992; Barres et al., 1993b; Barres et al., 1994; Kahn and deVellis, 1994). Long-term survival of purified OL, however, requires a combination of at least three signalling molecules: IGF-1 (or IGF-2 or a high concentration of insulin, both of which activate IGF-1 receptors), CNTF (or the related cytokines LIF or IL-6) and NT-3 (Barres et al., 1993b).

Transplantation of COS cells, genetically engineered to secrete either PDGF

(Barres et al., 1992), IGF-1 (Barres et al., 1993a), NT-3 (Barres et al., 1994) or CNTF (Barres et al., 1993a), into the postnatal rat brain results in a striking reduction in the normal death of newly formed OL. More importantly, injection of mouse hybridoma cells secreting neutralizing anti-NT-3 monoclonal antibody results in a reduction of NT-3 levels and an increase in cell death of OL and their precursors, indicating the requirement of NT-3 for OL survival *in vivo*. No such data yet exists for the other above mentioned factors.

Each of the above-mentioned factors are normally present in the developing optic nerve and are made by astrocytes and neurons in culture (Barres et al., 1992; Barres et al., 1993a). Human OL cultured immediately following their isolation in the presence of astrocytes show enhanced survival and process extension compared to OL cultured in isolation (Oh and Yong, 1996).

(D). Protection of oligodendrocytes from immune-mediated injury *in vitro* - *in vivo* correlates

The discovery of neurotrophic factors and the emergence of the neurotrophic theory emphasized the importance of survival signals in vertebrate neuron and OL development. The theory, however, did not predict the surprising findings that delivery of exogenous neurotrophic factors or cytokines ameliorates the effects of many types of neuronal injury including ischemia, hypoglycemia, excitotoxicity, oxidative damage (Mattson et al., 1989; Cheng and Mattson, 1991; Shigeno et al., 1991; Gluckmann et al., 1992) and even genetic defects (Sendtner et al., 1992). These studies have prompted investigations into the potential protective effects of neurotrophic factors, cytokines and other glial cell-derived molecules in cases of immune-mediated OL injury. Transforming growth factor (TGF-β), a cytokine which can be derived from glial cells (Cannella and Raine, 1995) has been shown to inhibit microglia-mediated injury of rodent OL (Merrill and Zimmerman, 1991). The specific effector mechanism modulated is not yet

identified. Louis et al. (1993) have reported that CNTF, a molecule derived from astrocytes (Rudge et al., 1992), protects OL derived from the rat OL precursor cell line, CG-4, from apoptotic injury induced by the cytopathic inflammatory cytokines TNF-α and TNF-β; CNTF could not protect OLs from lytic injury mediated by exposure to complement. Astrocytes, a potent source of neurotrophic factors in culture, have been shown to protect rodent OL from the cytopathic effects of oxygen free radicals generated by the addition of epinephrine or norepinephrine to the cultures (Noble et al., 1994). In MS lesions, OL internalized within hypertrophic astrocytes are identified, raising the speculation of the latter protecting the former (Wu and Raine, 1992).

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CHAPTER 2

CHAPTER 2

CYTOKINE INDUCTION OF HEAT SHOCK PROTEIN EXPRESSION IN HUMAN OLIGODENDROCYTES: AN IL-1 MEDIATED MECHANISM.

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PREFACE

The precise basis for the selective injury of myelin and its cell of origin, the oligodendrocyte (OL), in immune-mediated demyelinating diseases such as multiple sclerosis (MS) remains to be clearly defined. One possible mechanism for this selective injury could be the selective upregulation of immune recognition molecules on OL. Cytokines, by way of upregulating the expression of immune recognition molecules in the CNS, have been implicated to be key contributors to the ongoing pathology in demyelinating diseases such as MS. The aim of the present study was to determine whether cytokines, known to be present in MS lesions, could selectively upregulate the expression of the inducible heat shock protein (hsp)-72 species in human adult CNS-derived OL. Selective upregulation of hsps in OL should be of interest in the context of selective injury of OL for the following reasons: (i) hsps have been postulated to serve as recognition molecules for γδ-T cells, (ii) γδ-T cells have been shown to be lytic to human adult OL *in vitro*, and (iii) γδ-T cells have been detected in MS lesions in association with hsp-expressing OL.

ABSTRACT

In this study, we examined the role of cytokines, known to be in elevated levels in multiple sclerosis (MS) plaques, in regulating oligodendrocyte (OL) expression of heat shock protein (hsp) in human brain-derived glial cell cultures. Using dual-stain immunohistochemistry, we initially compared the ability of a mixture of cytokines (Interlukin (IL)-1α, IL-1β, IL-2, IL-6, IL-8, tumor necrosis factor (TNF)-α, TNF-β, interferon (IFN)-β and IFN-γ) with that of physical stimuli such as heat shock and peroxide, to increase cellular expression of the mainly inducible hsp72 species in mixed glial cell cultures (containing OL, astrocytes and microglia). Similar to heat shock and peroxide, the cytokine mixture induced hsp72 expression only in OL (70 ± 5% vs. a baseline of 3±1% positive cells). When used individually however, only IL-1α (79 ± 3%), IFN-γ (70 ± 2%) and TNF-α (65 ± 5%) induced OL hsp72 expression in mixed glial cell cultures. In purified OL preparations, only IL-1α induced hsp72 expression (84 ± 4%). An IL-1 receptor antagonist (IL-1ra), abrogated hsp72 induction by IL-1α (16 ± 3%) as well as that due to IFN-γ (14 ± 1%) and TNF-α (13 ± 2%) in mixed glial cell cultures. Furthermore, OL express IL-1 receptors, detected by confocal laser scanning microscopy. Our data indicate that cytokines mediate hsp induction in OL possibly via a final common pathway involving IL-1 binding to its receptor on OL. Such interaction could enhance any putative OL-immune interactions which are dependent on hsp molecule recognition.

INTRODUCTION

Multiple Sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system (CNS) characterized by multifocal inflammatory lesions containing perivascular infiltrates of T cells, B cells and macrophages, in which destruction of the (OL)-myelin unit is seen (Prineas, 1985; Calder et al., 1989; Hafler et al., 1989), possibly as an early event (Rodriguez et al., 1993). The antigen that induces or perpetuates the presumed autoimmune response is as yet unknown. The target of the immune-mediated destruction, the OL-myelin unit, expresses neither MHC class I nor MHC class II proteins (Grenier et al., 1989; Raine, 1991), raising the possibility that the immune injury might be mediated by effector T cells that are capable of MHC unrestricted or antigen non-specific interaction, such as promiscuous cytotoxic αβ-T cells or γδ-T cells (deVries et al., 1989; Ruijs et al., 1993).

γδ-T cells have been detected within MS plaques (Selmaj et al., 1991a; Wucherpfennig et al., 1992) in association with hsp-expressing OL (Selmaj et al., 1991b). Hsp have been postulated to serve as recognition molecules for γδ-T cells (Haregewoin et al., 1989; Indreshpal et al., 1993), and as such, may serve to regulate the number and activity of γδ-T cell infiltrates in MS plaques; hsp-expressing target cells *in vitro*, including OL, have been shown to induce proliferation of γδ-T cells (Freedman et al., 1991a). γδ-T cells have been shown to be lytic to adult human-derived OL *in vitro* (Freedman et al., 1991b) suggesting that the γδ-T cells gaining entry into the brain white matter may be deleterious to the OL-myelin unit and thus may contribute to the pathogenesis of MS.

Since the expression of hsp in ODC could be the "rate-limiting step" for

elevated levels in the inflammatory milieu of MS plaque tissue (Hofmann et al., 1986; Hofmann et al., 1989; Selmaj et al., 1991c), in regulating hsp expression in human OL. We found that cytokines can mediate hsp expression in OL and that IL-1 may play a critical role in this process, probably through interaction with its receptor on OL.

MATERIALS AND METHODS

Mixed glial and purified oligodendrocyte cell cultures

Human brain tissue was obtained from 6 different epilepsy patients undergoing surgical treatment. Mixed glial cells consisting of OL, astrocytes and microglia were isolated as previously described (Yong and Antel, 1992) and suspended in DMEM (Gibco, Canada) supplemented with penicillin 2.5 U/ml, streptomycin 2.5 µg/ml, glutamine 2mM and 5% FCS (all from Gibco Canada). Mixed glial cells consisting of approximately 70% OL, 25% microglia and 5% astrocytes were plated onto poly-L-lysine (10 µg/ml; Sigma, St. Louis, MO) coated coverslips at a density of 1-5 x 10^4 cells per coverslip, placed in Nuntron petri dishes (Gibco, Canada) containing DMEM supplemented with 5% AB^+ normal human serum (NHS)(Pel-freeze, WI, USA) and maintained in a 5% CO_2 humidified 37°C incubator. Purified OL cultures (> 90% $CNPase^+$) were prepared by gently shaking mixed glial cell cultures and replating the less adherent, floating OL onto poly-L-lysine coated coverslips (Yong and Antel, 1992).

Cytokines, antibodies and other reagents.

Recombinant human (rh) interlukin (IL)-1α, IL-1β, IL-2, IL-8, tumor necrosis factor (TNF)-α and TNF-β [lymphotoxin (LT)] were obtained from Intermedico Diagnostics (Markham, Ontario); rh IL-6 from Immunex Corp.(Seattle, WA); rh

Diagnostics (Markham, Ontario); rh IL-6 from Immunex Corp.(Seattle, WA); rh IFN-β from Berlex (Alameda, CA); rh interferon (IFN)-γ from Boehringer Manheim (Laval, Quebec) and hydrogen peroxide from Fischer (Fair Lawn, NJ). The human IL-1 receptor antagonist, IL-1ra, was supplied by Synergen (Boulder, CO) and the IL-1 receptor antibody was obtained from Immunex Corp.(Seattle, WA). The following primary antibodies (Ab) were used: polyclonal rabbit anti-cow CNPase (2'3' cyclic nucleotide phosphodiesterase) Ab that cross reacts with the human protein, as a marker for OL (a gift of Dr. P. Braun; McGill University, Montreal)(Freedman et al., 1992), polyclonal rabbit anti-cow GFAP (glial fibrillary acidic protein) Ab that recognizes the human protein, as a marker for astrocytes (Dakopatts, Denmark) and mouse anti-human hsp72 IgG_1 mAb (Amersham; Oakville, Ontario) specific for the inducible hsp72 species, but not the constitutive hsc 70 species. Secondary Ab included rhodamine-conjugated goat anti-rabbit mAb (Organon-Teknika; Scarborough, Ontario) and goat anti-mouse-FITC (TAGO; Burlingame, CA).

Induction of hsp72 in oligodendrocytes

Mixed glial cells or purified OL were cultured on coverslips and placed into individual wells of 24-well Nuntron (Becton Dickinson; Mountain View, CA) plates containing culture medium alone or the indicated stimuli: IL-1α, IL-1β, IL-2, IL-6, IL-8, TNF-α, TNF-β, IFN-β and IFN-γ, individually, each at a concentration of 1 U/ml, 10 U/ml, 100 U/ml or 1000 U/ml, or as a mixture, each at a concentration of 1000 U/ml, or hydrogen peroxide (400 mM). As a positive control, some coverslips containing mixed glial cells or purified OL were heat shocked for 30min in a water bath preheated to 43°C (37°C for negative control) as previously described (Freedman et al., 1992). All treatments except heat shock, were carried out for 18 h in a 5% CO_2 humidified 37°C incubator.

Inhibition of cytokine-mediated induction of hsp72 by the IL-1 receptor antagonist, IL-1ra

In some cases, coverslips containing mixed glial cells or purified OL were pretreated for 4 h with 10μg/ml of IL-1ra (Dinarello and Thompson, 1991a) and treated as above. This concentration represents a 500-fold excess relative to 2000 U/ml of IL-1α (Dinarello and Thompson, 1991a; Dinarello, 1991b; Kent et al., 1992).

Double Immunohistochemistry

OL were identified using rabbit anti-cow CNPase Ab (1:100) followed by rhodamine-conjugated anti-rabbit IgG secondary Ab (1:100); astrocytes, using rabbit anti-cow GFAP Ab (1:100) followed by rhodamine-labelled goat anti-rabbit IgG (1:100); microglia, based on their bipolar morphology and lack of either GFAP or CNPase staining. Hsp72 expression was detected by double staining with anti-human hsp72 Ab (1:500) followed by goat anti-mouse FITC secondary Ab (1:40). Isotype control studies included use of an irrelevant IgG_1 mAb. All analyses were performed using a Reichert Polyvar 2 Leica immunofluorescence microscope.

To detect IL-1 receptors on the OL plasma membrane, purified OL as well as mixed glial cell cultures were stained as described above for CNPase followed by mouse anti-human IL-1R IgG_1 (1:50) (a generous gift from Immunex Corp., Seattle, WA) and goat anti-mouse FITC (1:40) and analysed using a confocal laser scanning microscope (Leica Lasertechnik, Heidelberg, Germany). Isotype controls were also included in these studies. Samples were scanned with a 40 X 1.3 NA oil immersion objective with a band pass filter peaking at 535 ± 7nm for FITC specificity and a 580nm high pass filter for rhodamine.

For each experiment, 600 to 1200 OL, 40 to 80 astrocytes and 100 to 200

microglia over four coverslips were counted by an observer blinded to the treatment received by the cells and the number of cells positive for hsp72 was determined and expressed as a percentage of the total number of cells counted ± SD.

RESULTS

Hsp72 expression in mixed glial cell cultures: comparison of cytokines to physical inducing agents.

In keeping with our previous data (Freedman et al., 1992), we found that at basal temperatures (37°C), few non-treated OL and no astrocytes or microglia expressed detectable hsp72 (Fig. 1; Fig. 4 a, b). After physical heat shock (43°C), hsp72 expression was observed only in the OL with little or no expression in microglia or astrocytes (Fig. 1; Fig. 4 c, d). Other physical stimuli such as hydrogen peroxide (400 mM), gave similar results (Fig. 1). Using a panel of nine cytokines (IL-1α, IL-1β, IL-2, IL-6, IL-8, TNF-α, TNF-β, IFN-β and IFN-γ), each cytokine within the mixture at a concentration of 1000 U/ml, we found that, similar to the results of physical stimuli such as heat shock and hydrogen peroxide, upregulation of hsp72 expression was observed only in the OL (Fig. 1).

Cytokine-mediated induction of hsp72 in OL of mixed glial cell cultures.

In order to determine which of the cytokines from the mixture of nine induced hsp72 in OL, we tested each cytokine individually, each at a concentration of 1000 U/ml on mixed glial cell cultures and found that only IL-1α, IFN-γ and TNF-α induced OL hsp72 expression in the OL of mixed glial cell cultures (Fig. 2). None of the cytokines tested were capable of inducing hsp72 in the astrocytes or microglia of mixed glial cell cultures (data not shown).

Cytokine-mediated induction of hsp72 in purified OL cultures.

In order to determine whether IL-1α, IFN-γ or TNF-α directly induced hsp72 in OL, we re-tested each of the cytokines on purified OL cultures at 1 U/ml, 10 U/ml, 100 U/ml and 1000 U/ml. Only IL-1α retained the ability to induce hsp72 in the OL. Treatment with IL-1α at 1000 U/ml, but not at lower concentrations (1-100 U/ml - data not shown) resulted in an increase in the number of hsp expressing OL compared to non-treated cells (Fig. 2); at higher concentrations (2000 U/ml), hsp72 induction in OL (Fig. 2; Fig. 4 e, f) quantitatively matched the results obtained by heat shock and peroxide treatment (Fig. 1). However, IL-1β did not induce hsp72 at 1000 U/ml (Fig. 2) or 2000 U/ml (data not shown).

Competitive inhibition of cytokine-mediated hsp72 induction in OL by the IL-1 receptor antagonist, IL-1ra.

To determine whether IL-1α-mediated hsp72 induction in purified OL and mixed glial cell cultures was due to the binding of IL-1α to its receptor on OL, IL-1ra, a specific IL-1 receptor antagonist was used at the recommended concentration for *in vitro* studies (Dinarello and Thompson, 1991a; Dinarello, 1991b; Kent et al., 1992). IL-1ra blocked hsp72 induction in the purified OL (Fig. 3; Fig. 4 g, h). Since IL-1α was the only cytokine that induced hsp72 in purified OL, we considered that TNF-α or IFN-γ-mediated hsp72 induction in the mixed glial cell cultures possibly operated through a final common pathway which involved IL-1α. To test this hypothesis, IL-1ra was included in the TNF-α and IFN-γ experiments on mixed glial cell cultures. Similar to the results found using purified OL, IL-1ra substantially blocked TNF-α and IFN-γ-induced OL hsp72 expression (Fig. 3).

Presence of IL-1 receptor on human brain-derived OL.

To determine whether IL-1α-mediated hsp72 induction might occur through an IL-1 receptor on OL, purified OL as well as mixed glial cell cultures were stained for the presence of IL-1 receptors. As illustrated (Fig. 5), IL-1 receptors can be demonstrated on human brain-derived OL with homogenous staining among the OL. IL-1 receptor immunoreactivity was also detected on astrocytes and microglia (data not shown).

DISCUSSION

The finding in the present study that cytokines as well as hydrogen peroxide are capable of inducing hsp72 expression preferentially in human brain-derived OL as compared to other glia, extends our earlier observations with physical heat shock (Freedman et al., 1992). Other investigators (Satoh et al., 1992a), using adult mouse glial cells, have also demonstrated OL-specific hsp72 induction with only low levels of hsp72 in astrocytes in response to heat shock. Hydrogen peroxide, as well as the neurotransmitters epinephrine and norepinephrine, which all generate reactive oxygen species, have been shown to be toxic to OL (Griot et al., 1990; Noble et al., 1992). Comparable to heat shock, hydrogen peroxide induced hsp72 only in the OL, whereas norepinephrine and epinephrine did not induce hsp72 in the OL or other glia (data not shown). TNF-α, which has also been shown to be toxic to OL by inducing them to undergo an apoptotic death (Selmaj et al., 1991d), did not directly induce hsp72 expression in purified OL. These findings suggest that all agents which are toxic to OL do not necessarily invoke hsp expression.

Elevated levels of cytokines in MS lesions may derive from activated T cells, monocytes, astrocytes or microglia (Hintzon et al., 1992). IL-1α, TNF-α and

IFN-γ in particular, have been postulated to play an active role in the ongoing disease process in MS: they have been implicated in the induction of OL death by apoptosis (TNF-α)(Noble et al., 1992); MHC II upregulation on macrophages, microglia and astrocytes (IFN-γ)(Wong et al., 1984; Fierz et al., 1985; Benveniste et al., 1989); upregulation of adhesion molecules on endothelial cells of CNS blood vessels (IFN-γ, TNF-α)(Male et al., 1990); astrogliosis, (IL-1, TNF-α)(Merrill, 1991); release of prostoglandin E_2 (IL-1, TNF-α)(Bernheim, 1986); induction of fever (IL-1)(Merrill, 1991); and stimulating cytotoxicity of macrophages and microglia (IFN-γ)(Hartung et al., 1990; Zielasek et al., 1992). We now report that IL-1α, TNF-α and IFN-γ can also upregulate OL expression of hsp through a mechanism which appears to operate through a final common pathway involving IL-1 binding to its receptor on OL.

IL-1 has been implicated to play a pathological role in several chronic inflammatory autoimmune diseases such as rheumatoid arthritis and type I diabetes by way of inducing expression of the hsp70 family of stress proteins on the target cells; *in vitro*, IL-1 has been shown to induce hsp70 in rat pancreatic islets (Helqvist et al., 1991), bovine chondrocytes (Cruz et al., 1991), as well as murine cardiac myocytes (Low-Friedrich et al., 1992). In all these diseases, part of the pathological process and chronicity has been linked to the increased number of T cells and antibodies reactive with the elevated levels of hsp. Hsp-reactive γδ-T cells have been implicated in many chronic autoimmune diseases, particularly rheumatoid arthritis, diabetes and systemic lupus erythematosis (Holoshitz, 1990). Hsp-responsive γδ-T cells have been isolated from the synovial fluid of rheumatoid arthritis patients (Holoshitz et al., 1989). With regard to MS, it is intriguing to postulate that cytokine induction of hsp in OL leads to enhanced, probably deleterious interactions between γδ-T cells and OL.

In vivo, IL-1α is for the most part, a membrane expressed cytokine (Dinarello and Wolff, 1993), which may be directionally focused onto its receptors

on target cells. This highly efficient juxtacrine signalling may thus result in very high local cytokine concentrations. The use of large doses of cytokines are therefore justified in our experiments, in order to reproduce *in vitro* effects comparable to those described *in vivo*.

Hsp72 was used as a model for studying hsp induction in OL because it is a mainly inducible species which is minimally expressed constitutively (Freedman et al., 1992). Following induction, cells become brightly immunoreactive with anti hsp antibody, facilitating the quantitation of hsp72$^+$ cells. Other hsp, e.g., hsp65 upregulate their expression following the same stimuli which induce hsp72, however, because they are constitutively expressed in OL (Satoh et al., 1992b), it would be difficult to distinguish the upregulation using the double immunohistochemistry technique described here. Flow cytometry could ideally be used for this, but is not possible owing to the paucity of glial cells. Our results with hsp72 are therefore probably applicable to other hsp.

The ability of IL-1ra to effectively block IL-1 cell surface receptors without triggering cellular responses typical of IL-1 as well as its biological inactivity (Dinarello and Thompson, 1991a) makes it an attractive agent for clinical use. Recombinant IL-1ra is presently in clinical trials for treating several inflammatory conditions including rheumatoid arthritis, septic shock, inflammatory bowel disease, asthma and graft versus host disease (Dinarello and Thompson, 1991a). The demonstration that IL-1ra blocks IL-1 mediated induction of hsp72 in OL *in vitro* suggests that if a similar effect could be achieved *in vivo*, then it might be possible to decrease hsp expression in OL of MS plaques and hence dampen putatively harmful OL-immune interactions which are dependent on hsp molecule recognition, such as those possibly involving γδ-T cells.

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FIGURE 1: Induced intracellular hsp72 expression in mixed glial cell cultures: comparison of a cytokine mixture (IL-1α, IL-1β, IL-2, IL-6, IL-8, TNF-α, TNF-β, IFN-β and IFN-γ), each at 1000 U/ml, to the physical stimuli heat shock (43°C) and peroxide (400 mM). Results represent the mean ± SD of six experiments.

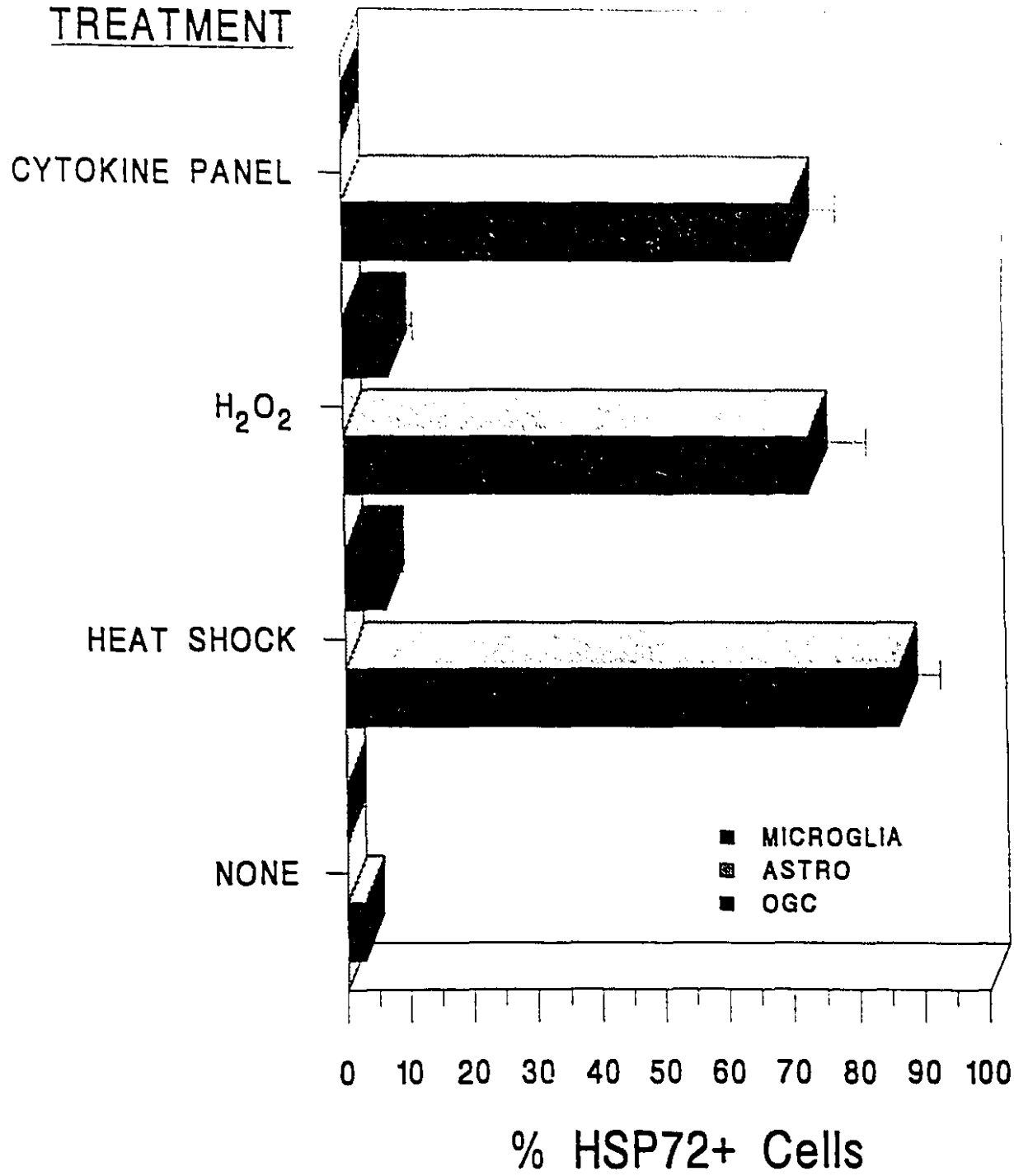

TREATMENT
CYTOKINE PANEL
H_2O_2
HEAT SHOCK
NONE
MICROGLIA
ASTRO
OGC
0 10 20 30 40 50 60 70 80 90 100
% HSP72+ Cells

FIGURE 2: Cytokine-induced intracellular hsp72 expression in both purified human OL and OL contained in mixed glial cell cultures (1000 U/ml). Results represent the mean ± SD of six experiments.

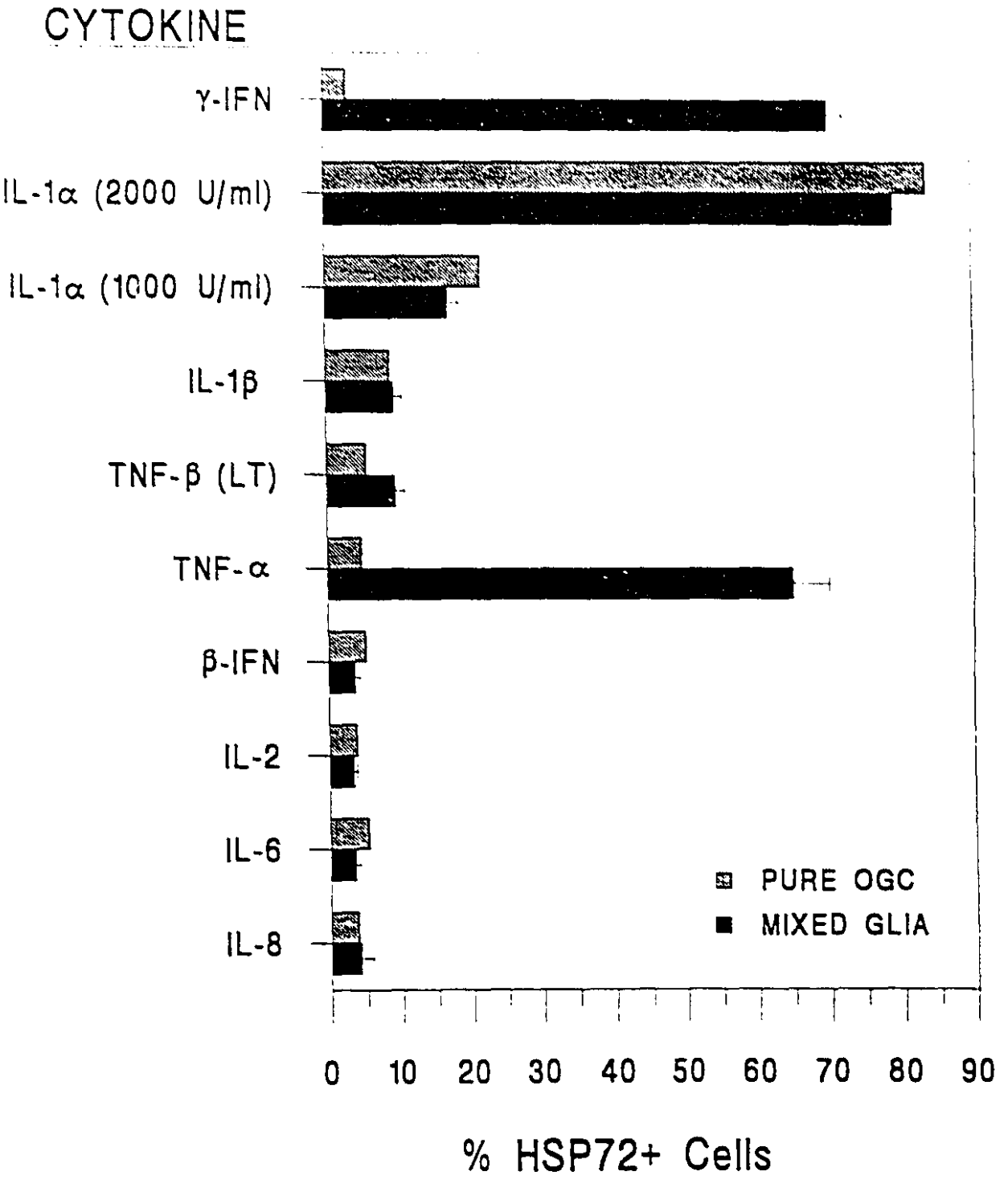

CYTOKINE
γ-IFN
IL-1α (2000 U/ml)
IL-1α (1000 U/ml)
IL-1β
TNF-β (LT)
TNF-α
β-IFN
IL-2
IL-6
IL-8
PURE OGC
MIXED GLIA
0
10
20
30
40
50
60
70
80
90
% HSP72+ Cells

FIGURE 3: Effect of IL-1ra (10 μg/ml) on IL-1 (2000 U/ml), TNFα (1000 U/ml) and IFN-γ-mediated (1000 U/ml) induction of intracellular hsp72 in both purified human OL and OL contained in mixed glial cell cultures. Results represent the mean ± SD of three experiments.

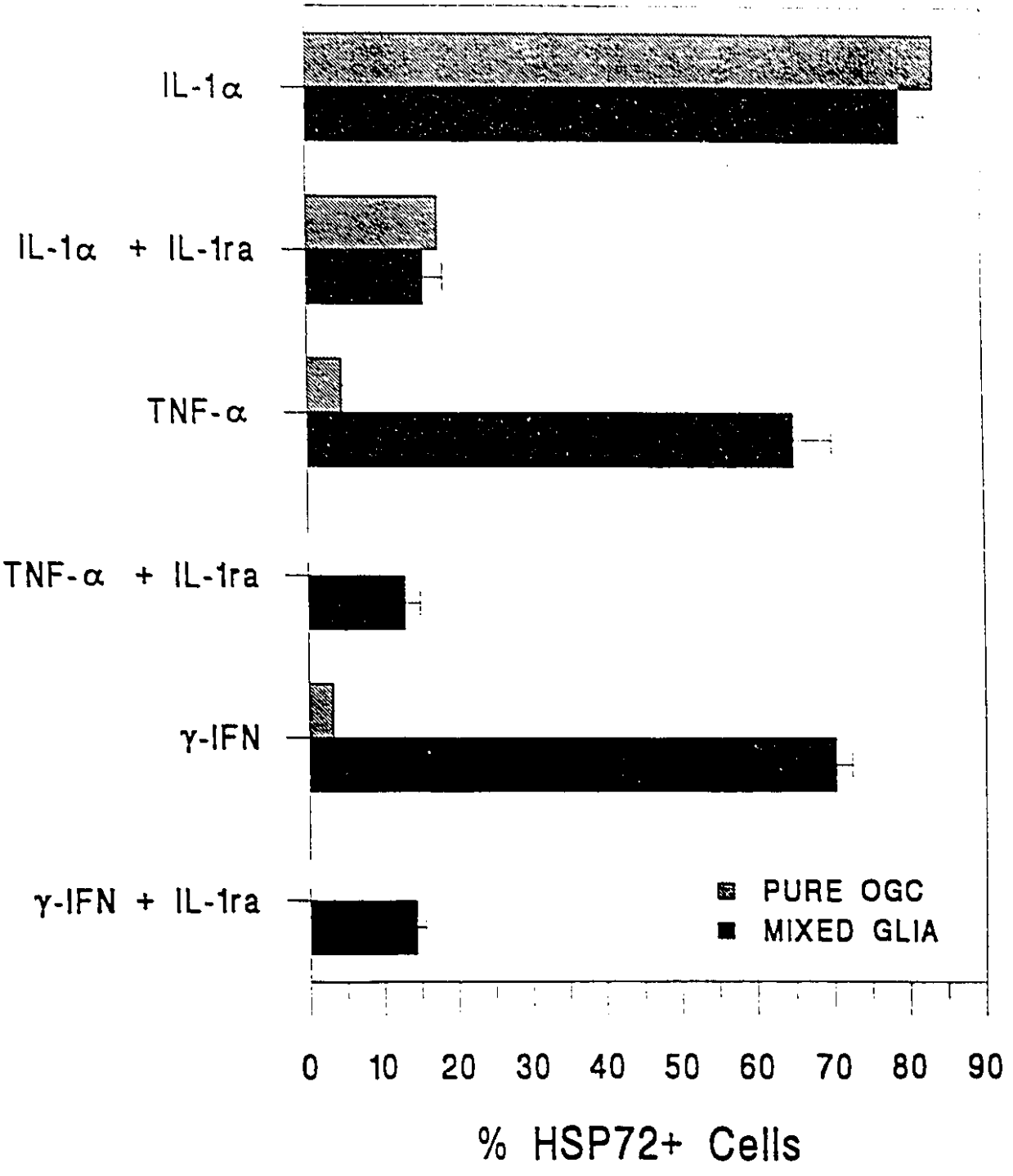
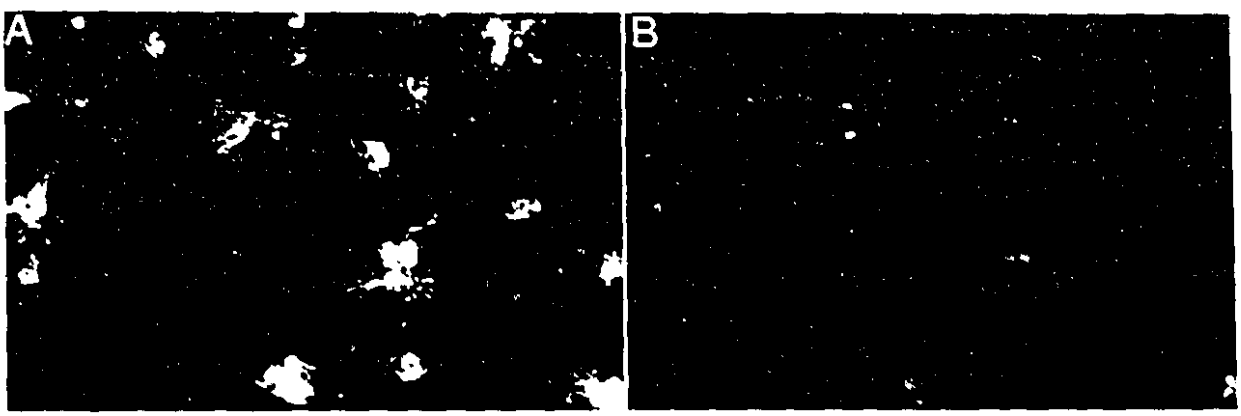
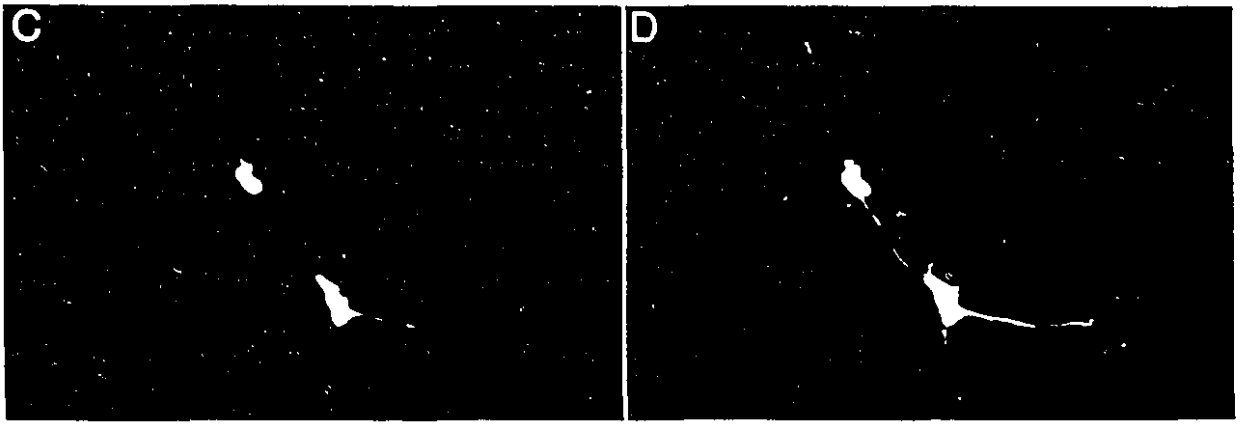
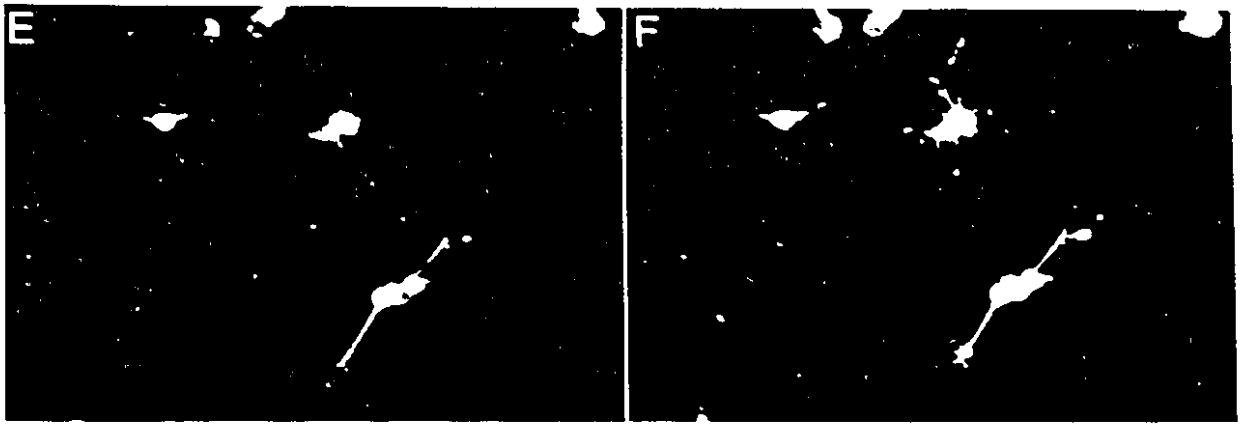

CYTOKINE
IL-1α
IL-1α + IL-1ra
TNF-α
TNF-α + IL-1ra
γ-IFN
γ-IFN + IL-1ra
PURE OGC
MIXED GLIA
0
10
20
30
40
50
60
70
80
90
% HSP72+ Cells

FIGURE 4: IL-1 mediated induction of hsp72 expression in purified OL cultures. Panels a, c, e, and g represent anti CNPase staining to identify OL, and panels b, d, f, and h represent the same fields stained for hsp72 immunoreactivity using anti hsp72 antibody. Panel b: 37°C non-heat shock; panel d: 43°C heat shock; panel f: IL-1 treatment, and panel h: IL-1 ra pretreatment followed by IL-1 treatment (800 X Mag).


A
B


C
D


E
F

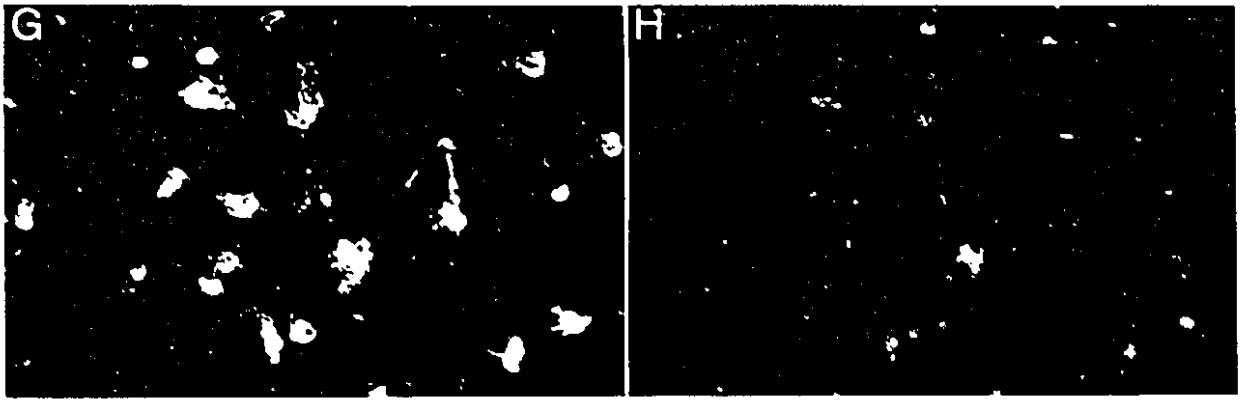
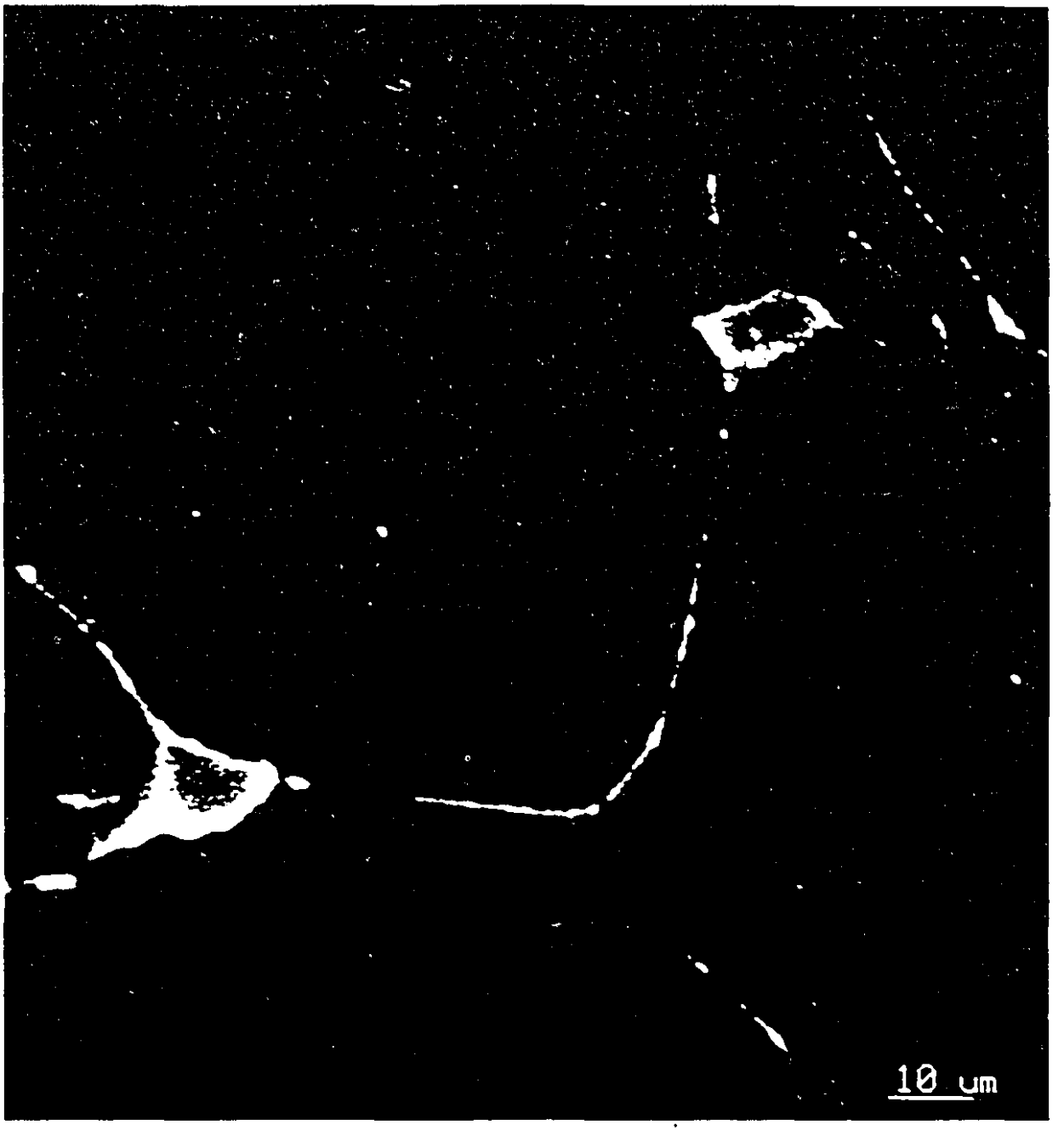

G
H

FIGURE 5: Confocal laser scanning microscope generated image of purified human brain derived OL stained for the presence of IL-1 receptors. Typical positively stained OL are depicted (identified by double staining with CNPase) with receptors noted well out onto processes.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CHAPTER 3

CHAPTER 3

DIFFERENTIAL SUSCEPTIBILITY OF HUMAN CNS-DERIVED CELL POPULATIONS TO TNF-DEPENDENT AND INDEPENDENT IMMUNE-MEDIATED INJURY

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Cindi Goodyer, Jack P. Antel

PREFACE

Having previously demonstrated that adult human oligodendrocytes (OLs) are susceptible to immune mediated injury via both tumor necrosis factor (TNF)-dependent and TNF-independent mechanisms (McLaurin et al., 1995 - refer to Appendix I for summary of this paper), we sought to determine: (i) whether different CNS cell populations are selectively vulnerable to TNF-dependent and TNF-independent immune mediated injury, and (ii) whether the nature of the injury response to a common immune mediator differs amongst CNS cell populations. Such differences may account for the selective injury of CNS cells in certain neurologic disorders; selective injury of myelin and its cell of origin, the oligodendrocyte, are implicated to be the basis of the neurologic deficits that occur in demyelinating diseases such as multiple sclerosis.

ABSTRACT

We examined whether oligodendrocytes, neurons, and astroglia derived from the human central nervous system differ in susceptibility to injury mediated by tumor necrosis factor (TNF)-α and by activated $CD4^+$ T cells acting via a TNFα-independent mechanism. Injury was assessed either as cell membrane-directed (lysis), measured by ^{51}chromium (Cr) or lactate dehydrogenase (LDH) release, or nucleus-directed (apoptosis), measured by morphologic features based on propidium iodide (PI) staining and by DNA fragmentation measured by a terminal transferase (TdT)-mediated dUTP biotin nick end labelling technique (TUNEL). TNFα did not induce LDH release in any cell targets, but did induce nuclear (66 ± 2% of cells) and DNA (68 ± 2% of cells) fragmentation selectively in the oligodendrocytes over 96 hours. At this time, there was no significant loss of oligodendrocyte cell number. Nuclear injury could be induced in neurons by serum deprivation and in malignant astrocytes by the combination of TNFα and low serum. $CD4^+$ T cells activated with phytohemagglutin (pha) or anti-CD3 plus interleukin-2 induced significant ^{51}Cr and LDH release in all target cells tested; only pha-activated $CD4^+$ T-cell co-cultures showed reduced target cell numbers. Significant nuclear fragmentation was observed only for glioma cells (22 ± 1% of cells). Differences in susceptibility to different immune effector mechanisms and in the nature of the injury response to the same effector mediator amongst human CNS-derived neural cells will need to be considered in design of therapeutic strategies aimed at protecting or limiting target cell injury consequent to disease or trauma.

INTRODUCTION

Cell-mediated immune (CMI) responses are implicated as the primary mediators of tissue injury which occur in an array of inflammatory diseases of the central nervous system (CNS). Such responses can be mediated either by cell-cell contact-dependent or soluble factor-dependent mechanisms. In multiple sclerosis, myelin and its cell of origin, the oligodendrocyte (OL), are considered the major targets of this response (Bruck et al., 1994). Pathologic and neuroimaging studies indicate that axonal injury also occurs, particularly during the progressive phase of the disease (Arnold et al., 1994). In some viral diseases, including HIV encephalopathy, immune effectors contribute to the target cell injury (Wilt et al., 1995). Immune system products are also implicated as secondary contributors to the cellular injury in neurodegenerative disorders such as Alzheimers disease (McGeer et al., 1989).

A specific issue raised with regard to immune mediators of tissue injury relates to whether different populations of CNS cells are selectively vulnerable to particular effectors. A corollary would be whether the mechanism of target cell injury to a common effector differs amongst neural cell populations. Cell injury has usually been considered in terms of primary cell membrane injury (lysis) or nuclear injury (apoptosis). In the present study, we address these issues using dissociated *in vitro* cultures of human adult CNS-derived OLs, fetal CNS-derived neurons and astrocytes, and human glioma cell lines as target cells. As effector mediators, we used tumor necrosis factor (TNF)-α and *in vitro*-activated $CD4^+$ T cells. The former, under pathologic conditions in the CNS, may be derived both from infiltrating mononuclear cells and glial cells, both microglia and astrocytes (Robbins et al., 1987; Lee et al., 1993; Williams et al., 1994). TNFα has been shown to induce DNA fragmentation of primary rodent, bovine, and human OLs

after 72-96 hr of *in vitro* exposure (Selmaj et al., 1991; Louis et al., 1993; Prabhakar et al., 1995; Wilt et al., 1995) and of the OL precursor cell line CG4, induced to mature *in vitro* (Louis et al., 1993). The related molecule TNFß is at least as toxic to OLs (Selmaj et al., 1991). Gelbard et al. (1993) reported that TNFα was toxic to cultured primary human fetal cortical neurons, as assessed morphologically.

We have previously shown that mitogen- or antigen-activated $CD4^+$ T cells, the T-cell subset used to transfer autoimmune encephalitis in animals, can induce OL cell membrane injury, measured using ^{51}Cr or LDH release, within 18 hr of effector:target co-culture (Antel et al., 1994). The effect is TNF-independent and non-MHC-restricted (Antel et al., 1994). At this time point, there is no evidence of nuclear injury, as measured by a DNA fragmentation assay (McLaurin et al., 1995). In the present study, we have assessed target cell responses to the T-cell effectors in terms of cell membrane and nuclear-directed injury over short (18-hour) and longer term (96-hour) culture. A non-MHC-restricted $CD4^+$ T-cell effector response should be of particular interest in context of CNS cell injury, in that neither neurons or OLs to express MHC class II molecules, the restricting element for antigen-specific $CD4^+$ T cells.

MATERIALS AND METHODS

Preparation of Effectors

Recombinant human TNFα used in these studies was obtained from Intermedico Diagnostics (Markham, Ontatario). Preliminary studies established 1000 units per ml as a standard dose to perform the comparative studies described in this report (Prabhakar et al., 1995).

$CD4^+$ T cells were prepared from peripheral blood samples obtained from

healthy volunteers using an initial Ficoll-hypaque (Pharmacia, Baie D'Urfe, Quebec) gradient centrifugation step (30 min. at 500 g) to obtain mononuclear cells (MNCs). T cells were isolated by a rosetting technique in which MNCs were incubated for 1 hr at 4°C with S-(2 aminoethyl) isothiouronium bromide (AET) (Aldrich, Milwaukee,WI)-treated sheep red blood cells and then centrifuged for 30 min on a Ficoll-hypaque gradient (Antel et al., 1994). The rosetted cells (T lymphocytes) were collected from the pellet and the sheep red blood cells were lysed by use of Gey's solution, a hypotonic lysis buffer, for 4 min at 4°C. The cells were then centrifuged for 30 min on a 43.5% Percoll (Pharmacia) gradient to remove large granular lymphocytes and incubated for 1 hr at 37°C with anti-CD8 monoclonal antibody (from ATCC hybridoma cell line CRL 8014) and complement (baby rabbit serum, Cedar Lane, Hamilton, Ontario). Following lysis, the remaining cells ($CD4^+$ T cells) were collected, washed x 3 in phosphate-buffered saline (PBS), and suspended at $1x10^6$ cells per ml in culture medium comprised of RPMI (GIBCO, Burlington, Ontario) supplemented with 5% human serum (Pel Freeze, Brown Deer,WI), 2.5 U per ml penicillin and 2.5 µg per ml streptomycin. Cells were placed in 25 cm^2 flasks (Falcon, Fisher Scientific, Montreal, Quebec) together with irradiated (2500 rads) autologous non-rosetting (E^-) cells (2:1 ratio) as a source of feeder cells. Cells were activated *in vitro* for 72 hr with either soluble anti-CD3 antibody (10 µg per ml; ATCC CRL 8001), anti-CD3 plus recombinant human Interlukin (IL)-2 (20 units per ml; gift of Immunex, Seattle,WA), or with phytohemagglutinin (pha) (1 µg per ml; Wellmark Diagnostics, Guelph, Ontario). Cells were then harvested and centrifuged on a Ficoll-hypaque gradient to remove dead cells. Extent of T-cell activation was assessed by determining ^3H-thymidine uptake over 5 hr by an aliquot of these cells. Purity of the $CD4^+$ T cells was determined by FACScan analysis. The enriched $CD4^+$ T cells were washed twice and resuspended in RPMI plus 10% fetal calf serum (FCS). In some experiments, anti-CD3-stimulated cells were coated with pha immediately prior to their use as effector cells.

Preparation of targets:

Adult CNS-derived cells - OLs were derived from temporal lobe tissue specimens resected from young adults undergoing surgical resection for the treatment of intractable epilepsy. The method of cell isolation, previously described in detail (Yong and Antel, 1992) utilized an initial trypsinization step, followed by Percoll gradient centrifugation. Dissociated cells were cultured in minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS), and 0.1% glucose. Subsequent separation of glial cell subtypes is based on their differential adhesion to uncoated Falcon tissue culture flasks (Fisher, Montreal, Canada) or Petri dishes. Enriched populations of OLs were harvested from the mixed glial preparation in the form of the non-adherent cell fraction after 24 hr and replated on coated Petri dishes. After a further 24 hours, the non-adherent cells were again collected and placed either into 96-well microtiter wells (5×10^4 cells per well) or onto poly-L-lysine-coated Aclar coverslips (5×10^4 cells per coverslip) which could be placed in 24-well microtiter wells. Cells were allowed to extend processes, being optimal for functional assays within 2-4 weeks from time of initial isolation.

Fetal CNS-derived cells - Neurons and astrocytes were derived from human fetal CNS tissue (cerebral hemispheres) obtained at 12-16 week gestation, using Medical Research Council of Canada approved guidelines. The cultures were prepared by dissociation of the fetal CNS tissue with 0.05% trypsin (30 min) and 50 µg per ml DNase, passing the tissue through a 132 µm nylon mesh screen, and then through a 70 µm screen. After washing with PBS, the cells were suspended in culture medium (Eagle's MEM supplemented with 5% FBS, 0.1% glucose, and 1 mM sodium pyruvate), and then placed into poly-L-lysine-coated 96-well microtiter wells or onto Aclar coverslips which were then placed in Petri dishes (5×10^4 cells per microwell or coverslip). To obtain neurons, culture dishes are first treated on day 4 *in vitro* with 1 mM 5-fluoro-2-deoxyuridine (5-FDU) for

2 days to deplete astrocytes; the treatment was repeated three times over a 2-week time period. These neurons are used in functional assays within 14 days.

To obtain astrocytes, non-treated fetal CNS-derived dissocated cultures were passaged 2 or 3 times using 0.25% trypsin for 5 min at 37C when cultures were confluent. Cells could be reseeded into either 96-well microwells or onto coverslips (2.5 x 10^4 cells per ml) 24 hr prior to use in functional assays.

Human glioma cell lines - Cell line U251 was obtained from ATCC, and maintained in serial passage in RPMI supplemented with 5% FCS. Cells were trypsinized and reseeded for functional assays, as described for fetal astrocytes. Additional studies were conducted using the A172 glioma cell line; results were comparable to those with the U251 cells.

Immunocytochemistry

The individual neural cell types were characterized by immunostaining. OLs were identified using the mouse anti-R monoclonal antibody (mAb), H8H9 (Ranscht et al., 1982), derived from hybridoma supernatant, followed by goat anti-mouse IgG conjugated with Texas Red (Jackson Immunoresearch Lab, West Grove, PA). Neurons were identified by staining with anti-MAP 2 mAb (1:1000 dilution), SMI-52, (Sternberger-Meyer Immunocytochemicals, Whitehall, MD), followed by biotinylated horse anti-mouse IgG (1:100 dilution), followed by fluorescein-conjugated streptavidin (Boehringer-Manheim, Laval, Quebec). Additional antibodies used to identify neurons included anti-neurofilament SMI33 (Sternberger-Meyer), anti-neuron-specific enolase (NSE) (Dako, Westchester, PA). and anti-Tau (Sigma #T-5530, St. Louis, MO) mAbs. Rabbit anti-glial fibrillary acidic protein (GFAP) Ab was used as the astrocyte marker. Neuron and astrocyte staining involved fixation of cells in acid alcohol (5% glacial acetic acid: 95% absolute alcohol) for 15 min. Except for fixation, which was done at -20°C,

all steps were performed at room temperature. The OL-directed immunostaining was done on live cells without fixation. Cells on coverslips were incubated with all primary and secondary antibodies for 45 min; cells were washed 3 times in PBS between incubations. After staining, previously non-fixed cells were fixed in 2% paraformaldehyde (20 min).

Assays of neural cell injury

TNFα or $CD4^+$ T cells were added to cultures of target cells contained in 24- or 96-well microtiter plates at concentrations of effector:target (E:T) ratios and for the time durations indicated in the Results section. Initial studies established that TNFα effects were concentration-dependent and that $CD4^+$ T-cell activity was dependent on E:T ratio (Antel et al., 1994). Most T-cell-mediated injury studies reported here were performed at a 10:1 E:T ratio.

Cell membrane-directed injury - These studies were conducted over either 5 or 18 hr using ^{51}Chromium (Cr) release as the standard measure of cell membrane injury. For the ^{51}Cr assay, target cells were labelled overnight with 0.1 μCi of ^{51}Cr (NEN DuPont, Mississauga, Ontario) per well of 96-well microtiter places. Cells were then washed twice in PBS before addition of TNFα or $CD4^+$ T cells at indicated concentrations or E:T ratios. After 5 or 18 hr, 100 μl of cell-free supernatant was collected from individual microwells to determine induced ^{51}Cr release (IR). One hundred microliters of 5 N sodium hydroxide (Fisher Scientific) was then added to each well for 30 min, and a further 100 μl was collected to determine residual ^{51}Cr release (RR). Non-specific ^{51}Cr release (NSR) was determined from microwells containing only target cells as follows:

$$[\text{spontaneous release (SR)} \div (\text{SR} + \text{RR})] \times 100\%.$$

Per cent specific release was calculated as:

$$[(\text{IR} \div (\text{IR} + \text{RR})) - \text{NSR}] \times 100\%$$

In some studies, an LDH release assay (as described below) was used, although in this assay the cell source (effector or target) of released LDH would be uncertain.

For the TNFα studies, the LDH release assay was used as the standard assay at both the 18 and 96 hr time points. For this assay, culture supernatants were collected and analyzed using a commercial kit (Sigma). Results are expressed as LDH units of test samples ÷ LDH units of culture medium alone X 100%. The assay is insufficiently sensitive to detect LDH in serum-free conditions.

Nuclear injury (apoptosis) - These studies were performed over a 18-96 hr time period on cells contained on coverslips placed into 24-well microtiter plates. Given that our cell targets of injury are non-dividing primary cells and are available only in relatively small cell numbers, we have utilized, as our measures of nuclear injury, propidium iodide (PI) staining to identify changes in nuclear morphology (fragmentation, condensation of chromatin) and the terminal transferase (TdT)-mediated dUTP-biotin nick end labelling (TUNEL) technique to identify DNA fragmentation. PI staining was carried out on acetone:methanol or acid alcohol fixed coverslips. After rehydration for 30 min in PBS, coverslips were stained with PI (10 µg per ml, 20 min incubation), washed in PBS, and mounted for counting. For the TUNEL assay, coverslips containing the specific cell type were incubated for 1 hr at 37°C with 50 µl of nick end labelling solution containing TdT (0.3 units per ml) and biotinylated dUTP (0.01 nmol per ml) in TdT buffer (Promega). The reaction was terminated by transferring the coverslips into microwells containing Tris buffer for 15 min at room temperature. After blocking with 2% BSA for 15 min and washing, the coverslips were incubated with Streptavidin-FITC (1:20, 30 min at 37°C) (Boehringer-Manheim).

We have used serum deprivation, a commonly used means to induce apoptosis in many cell lines, as a means to determine whether our target cells will show evidence of nuclear injury in the above assays.

TUNEL and PI quantitative data were obtained by counting coded slides. At least 200 cells per slide were counted. The number of coverslips counted and the number of individual experiments included for each test condition are indicated in the Figures. In some studies, the total number of cells present in a defined area of a coverslip was determined under control and test conditions. ^{51}Cr and LDH data are presented as means ± standard error of mean (SEM) for the number of experiments performed; each experiment was performed in triplicate.

RESULTS

Properties of target cells

The morphologic and/or immunocytochemical features of the neural cells used in this study are illustrated in Fig. 1. Purity of the OLs in enriched cultures was estimated at 80-90% during the 2- to 4-week culture period. Contaminating cells included astrocytes and fibroblasts. The latter overgrew the culture over longer time periods. The fetal cortical neurons were estimated to comprise 80-90% of cells in their cultures, with $GFAP^+$ astrocytes being the other cell type. Although only immunoreactivity with MAP 2 mAb is illustrated, these cells could be immunostained with anti-neurofilament, anti-NSE, and anti-Tau mAbs. The U251 glioma line has lost GFAP immunoreactivity. All the above cell types express TNF receptors as assessed by immunofluorescence microscopy (data not shown).

Target cell susceptibility to TNFα-mediated injury

Membrane-directed injury - Levels of LDH measured in supernatant of 5% serum-supplemented cultures of OLs, neurons, and astroglia did not differ between those which did or did not contain TNFα (Table 1).

Nucleus-directed injury - Addition of a single dose of 1000 units of TNFα to 5% serum-supplemented OL-enriched cultures resulted in nuclear fragmentation in a significant proportion of OLs at 96 hr (66 ± 3%, n=6) compared to control cultures (7 ± 0.6%, $p < 0.01$) by PI criteria (Fig. 2). A similiar proportion of OLs (68 ± 2%) showed DNA fragmentation as determined by TUNEL assay (Fig. 1, Table 2). This effect was not apparent at 24 hours. The proportion of OLs showing TNFα-induced nuclear fragmentation at 96 hr was significantly greater than that induced by serum deprivation (43 ± 3%, n=6, $p < 0.01$). The number of OLs remaining in the TNFα-treated and non-treated cultures after 96 hr did not differ significantly (Table 1).

The proportion of fragmented nuclei in neurons exposed to TNFα for 96 hr was significantly increased over control values (10 ± 1% vs. 6 ± 0.4% , n=7, $p < 0.01$). This proportion was, however, markedly lower than that for OLs and significantly lower than that induced by serum deprivation (25 ± 3%, n=7, $p < 0.001$).

The proportion of fragmented nuclei in glioma cells was not significantly increased in cultures containing 5% serum plus 1000 units per ml of TNFα (7 ± 0.9%, n=6) compared to control values (5 ± 0.6%, n=6) (Fig. 2). Serum deprivation alone augmented nuclear fragmentation only to a small extent (12 ± 2%, n=3). Fragmentation of nuclei could be more readily observed in these cells by combining reduced serum conditions (1%) and TNFα (35 ± 3%, n=5, $p <$

0.001). Similiar results were obtained with the TUNEL assay (42 ± 3%, n=3) (Table 2). The fetal astrocytes did not undergo significant nuclear fragmentation (Fig. 2) or DNA fragmentation in response to either TNFα and/or low serum conditions. The overall correlation coefficient between the results of the PI assay of nuclear fragmentation and the TUNEL assay of DNA fragmentation in the above TNF assays and the $CD4^+$ T-cell effector assays described below was 0.979, n=14.

Target cell susceptibility to $CD4^+$ T-cell-mediated injury

Membrane-directed injury - As shown in Figure 3 and Table 1, the membrane-directed lytic capacity of $CD4^+$ T cell at 24 hr was found to be dependent on the method of T-cell activation. Pha-activated $CD4^+$ T cells induced significant ^{51}Cr release from each of the neural cell targets, with release being highest in the proliferating cell targets (fetal astrocytes and glioma cells). These results were confirmed in the LDH assay (Table 1). Significant loss of both OLs and glioma cells, the only two target cells examined, was observed following their exposure to the pha-activated $CD4^+$ T cells (Table 1). Pha-activated $CD4^+$ T-cell-mediated ^{51}Cr release could be detected as early as 5 hr in effector:target co-cultures (data not shown).

$CD4^+$ T cells activated with anti-CD3 and IL-2 also induced significant ^{51}Cr release from all neural cell targets tested in the 18 hr assay, with no apparent differences amongst the targets in their relative suceptibilities (Fig. 3). Significant cell loss could not be detected for either the OLs or gliomas (Table 1). $CD4^+$ T cells activated by anti-CD3 alone did not induce significant ^{51}Cr release from neurons, astrocytes, or glioma cells (Fig. 3). A low and variable level of OL-directed cytotoxicity was found. Studies using glioma cells as targets indicated that anti-CD3-activated T cells could induce ^{51}Cr release of previously resistant targets if the effector cells were coated with pha just prior to being added to the

previously "resistant" target cells (6 ± 5% for uncoated vs. 19 ± 2% for pha-coated anti-CD3-activated effectors, n=2).

^{3}H-thymidine uptake was comparable between CD4^{+} T cells activated by pha (34226 ± 5591 cpm, n=4) or anti-CD3 (38355 cpm ± 5534, n=4); uptake by anti-CD3 and IL-2-activated cells was significantly higher (59252 ± 7788 cpm, n=4, $p < 0.05$). Purity of CD4^{+} T cells did not differ as a function of activation conditions (mean per cent CD4^{+} T cells derived from anti-CD3-activated cultures was 89 ± 3%; from anti-CD3 plus IL-2-activated cultures 87 ± 3%; and, from pha-activated cultures 92 ± 2%, n=7) as assessed using flow cytometry. There were less than 2% CD8^{+} T cells or NK cells.

Total ^{51}Cr labelling of the various cell targets used did not vary widely (for OLs: 5000-7000 cpm; for neurons: 2500-3000 cpm; for astrocytes and glioma cells: 6000-8000 cpm). Non-specific release was comparable amongst the neural cells, with a mean range of 26-33%. These values are higher than those found using a 5 hr ^{15}Cr release assay.

Nucleus-directed injury - Although all target cells released ^{51}Cr upon co-culture with pha-activated CD4^{+} T cells, only in the glioma cells was there a significant increase in the proportion of fragmented nuclei compared to control cultures, as measured either by PI staining or TUNEL technique (Fig. 3, Table 2). The proportion was increased as early as 18 hours, with a further increase by 96 hours.

DISCUSSION

We have utiiized dissociated cultures of human CNS-derived cells to examine the relative susceptibility of these cells to TNF- and non-TNF-dependent

effector mechanisms, the latter mediated by activated $CD4^+$ T cells acting in a non-MHC-restricted manner. The target response was assessed in terms of the two major categories of cell injury, namely primary cell membrane-directed injury (lysis) and nucleus-directed injury (apoptosis) (Zychlinsky et al., 1991; Taylor and Cohen, 1992; Cohen, 1993; Louis et al., 1993; Schwartzman and Cidlowski, 1993), and in terms of the time required for the response to develop. The results indicated that the target cells studied (OLs, neurons, and malignant and non-malignant astrocytes) differed in their relative susceptibilities to these effector mechanisms and in the nature of the cellular injury which occurred.

The susceptibility of human adult CNS-derived OLs to the nuclear injury-inducing effects of TNFα that we found in our study are consistent with those observed by others using rodent and bovine primary OL cultures and a rodent OL-precursor cell line (Selmaj et al., 1991; Louis et al., 1993). Over the same time period, we did not detect significant cell membrane rupture, as assessed using ^{51}Cr or LDH release, nor loss of cell numbers. The latter results are consistent with those of Zajicek et al. (1992), who found that soluble human recombinant TNFα did not induce cytotoxicity of rodent OLs, whereas cell-bound TNF was the putative mediator of microglia-induced cytotoxicity of OLs *in vitro*. In contrast to the OLs, the neurons, in our study, were relatively resistant to TNFα in terms of nuclear injury. The extent of TNFα-induced nuclear injury of OLs was greater than that induced by serum deprivation; the reverse was observed for the human fetal neurons. Giulian et al. (1993) previously observed the greater susceptibility of neurons, compared to OLs, to non-cytokine low molecular weight factors released by microglia.

Neither the malignant nor non-malignant fetal astrocytes showed significant nuclear injury in response to TNFα under our usual culture conditions. In addition, we did not observe significant nuclear or DNA fragmentation in the small proportion of astrocytes contained in our adult CNS-derived OL-enriched

cultures. These results indicate the differences in susceptibility to a common effector between glial cells of apparently shared developmental lineage (Barres et al., 1992). The glioma cells, but not the non-malignant astrocytes, showed significant nuclear and DNA fragmentation in response to the combination of serum deprivation and TNFα. The glioma cells had a higher rate of proliferation than did the fetal astrocytes; the proliferation of the latter was higher than that of the adult CNS-derived astrocytes (Yong et al., 1992). The adult astrocytes can be shown to incorporate significant levels of BrdU *in vitro* in contrast to the OLs (Grenier et al., 1989).

Nuclear injury was assessed using morphologic criteria of PI-stained cells and a fluorescence DNA fragmentation assay (TUNEL). A strong correlation was found between results in the two assays. The numbers of OLs and neurons available and difficulties in recovering these cells from cultures limited the use of other techniques to assess DNA fragmentation, such as DNA ladders or FACS analysis. The former would have limited use in the co-culture systems discussed below, containing both effector and target cells.

Target cell injury mediated by $CD4^+$ T cells depended both on the means of T-cell activation and the cell type serving as the target. Pha-activated $CD4^+$ T-cell lysis could be detected in 5 hr as well as 18 hr co-cultures, suggesting that these cells had residual surface lectin, and their effect was akin to lectin-dependent cell cytotoxicity (Bevan and Cohen, 1975; Ruijs et al., 1990). Our previous studies indicated that the effect was not mediated via TNF-dependent mechanisms, in that it could not be blocked with neutralizing anti-TNF antibody or reproduced with TNF (Antel et al., 1994). $CD4^+$ T cells have previously been demonstated to be capable of mediating both TNF-dependent and -independent cytotoxicity (Ju et al., 1990; Liu et al., 1992; Smyth et al., 1992).

$CD4^+$ T cells activated by anti-CD3 and IL-2 induce membrane injury to a

greater extent in the 18 hr than in the 5 hr cytotoxicity assays (Ruijs et al., 1990). The cytotoxic effect of anti-CD3 plus IL-2-activated $CD4^+$ T cells would seem akin to that initially termed promiscuous killing in assay systems using either antigen or anti-CD3 and IL-2 to generate T-cell lines as effectors and dividing non-neural NK cell-resistant tumor lines as targets (Patel et al., 1987; Thiele and Lipsky, 1989). The $CD4^+$ T cells activated by anti-CD3 alone did possess cytotoxic potential, as revealed by coating these cells with pha immediately prior to adding them to target cells. IL-2 augmented cytotoxicity mediated by anti-CD3-activated T cells has previously been described (Stohl et al., 1990; Nishimura et al., 1992). The lack of cytotoxicity found using $CD4^+$ T cells activated with anti-CD3 alone, compared to anti-CD3 plus IL-2, suggests that residual anti-CD3 antibody on the T cells is not serving as a ligand. The essential ligands involved in promiscuous T-cell killing of particular cell targets remains to be defined.

Extent of T-cell proliferation as a function of method of T-cell activation did not correlate with cytotoxicity, in that ^{3}H-thymidine uptake was similar between anti-CD3- and pha-activated T cells. T cells activated with anti-CD3 and IL-2 showed the highest proliferation rates. However, even at a 20:1 E:T ratio, $CD4^+$ T cells activated by anti-CD3 alone did not induce the extent of ^{51}Cr release found using anti-CD3 plus IL-2-activated $CD4^+$ T cells at a lower E:T ratio (data not shown), suggesting that differences in effector cell numbers that may have occurred during the co-culture period did not account for the functional results. Any remaining exogenous IL-2 in the anti-CD3 plus IL-2-activated cultures would have been removed at the time the T cells were washed prior to adding them to the cell targets. The T-cell populations used were highly enriched for $CD4^+$ T cells, and thus the effects induced are unlikely to be due to a small population of NK cells (Galandrini et al., 1994). Mechanisms of $CD4^+$ α/β T-cell receptor (TcR) bearing T-cell-mediated cytotoxicity may differ from those used by other T-cell populations, including $CD8^+$ T cells and γ/δ TcR-bearing T cells (Smyth, 1992).

With regard to the target cell response to pha-activated T cells, only in the glioma cells did DNA and nuclear fragmentation develop. Significant fragmentation was observed within 18 hours, a time period which precedes that required for TNFα and serum depletion-induced fragmentation to occur. Cell numbers were reduced in all cell cultures exposed to pha-treated $CD4^+$ T cells. The lack of DNA and nuclear fragmentation in the non-glioma neural cells would seem to differ from previous studies of $CD4^+$ T-cell-mediated cytotoxicity using non-neural cell targets, in that the latter suggested that nuclear injury preceded cell disintegration (reviewed in Berke, 1994; Grogg et al., 1992; Stalder et al., 1994; Duke, 1992; Thiele and Lipsky, 1989). The glioma cells are the most actively proliferating of the cell targets used in our study. Our data suggests that primary non-dividing CNS cells, OLs, and neurons, are resistant to this pathway, although they are capable of undergoing apoptosis as shown using either TNF or serum deprivation. These data would be consistent with the existence of multiple different pathways whereby nuclear injury programs are activated (Rose and Henneberry, 1993; Schwartz et al., 1993; Schwartzman and Cidlowski, 1993; Oltavi and Korsmeyer, 1994).

Cell membrane injury mediated by the anti-CD3-activated $CD4^+$ T cells was not accompanied by either the extent of DNA fragmentation (in the case of gliomas) or cell loss (found with all cell targets tested) observed using the pha-activated T cells. These findings could provide a model whereby T cells could induce membrane injury without necessarily cell body loss - i.e., sublethal injury with potential for recovery, as suggested for complement-mediated injury (Scolding et al., 1989). Our previous studies did indicate that human OLs are susceptible to promiscuous cell-mediated cytotoxicity mediated by-MBP reactive $CD4^+$ T-cell lines (Antel et al., 1994). In the case of multiple sclerosis, the issue of myelin loss, with or without OL cell loss, continues to be studied (Bruck et al., 1994).

In summary, our current *in vitro* data indicate the differing susceptibility and cellular response of human CNS neural cells to TNF-dependent and independent immune effector mechanisms. Therapeutic strategies applied to diseases or injury of the human CNS will need to consider this heterogeneity.

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FIGURE 1: Induction of DNA fragmentation in human neural cells: **(a)** Adult CNS-derived oligodendrocytes (OLs) maintained in 5% serum-supplemented culture medium (control culture) and immunostained with anti-R mAb (350 X); **(b)** OLs cultured in 5% serum-supplemented medium plus 1000 units per ml TNFα and stained with PI (450 X); arrows indicate examples of fragmented or condensed (apoptotic) nuclei; **(c)** OLs cultured as in panel B and stained with TUNEL technique to demonstrate DNA fragmentation (350 X). Inserts in panels B and C indicate OLs grown in control medium and stained with PI and TUNEL (450 X and 350 X, respectively). **(d)** Human fetal CNS-derived cortical neurons maintained in control medium and immunostained with anti-MAP2 Ab (350 X); **(e)** Neurons cultured in serum-deprived medium and stained with PI; arrows indicate apoptotic nuclei (450 X); **(f)** Neurons cultured as in panel E and stained with TUNEL technique (350 X). Inserts in panels E and F are control cultures stained with PI and TUNEL (450 X and 350 X, respectively). Arrow in panel F insert indicates an example of a TUNEL positive neuron under control culture conditions; **(g)** Phase contrast micrograph of GFAP$^-$ human glioma line U251 maintained in control medium (350 X); **(h)** Glioma cells maintained in 1% serum and 1000 units per ml TNFα and stained with PI; arrows indicate apoptotic nuclei (350 X); **(i)** Glioma cells cultured as in panel H and stained with TUNEL technique (350 X). Inserts in panels H and I are control cultures stained with PI and TUNEL.

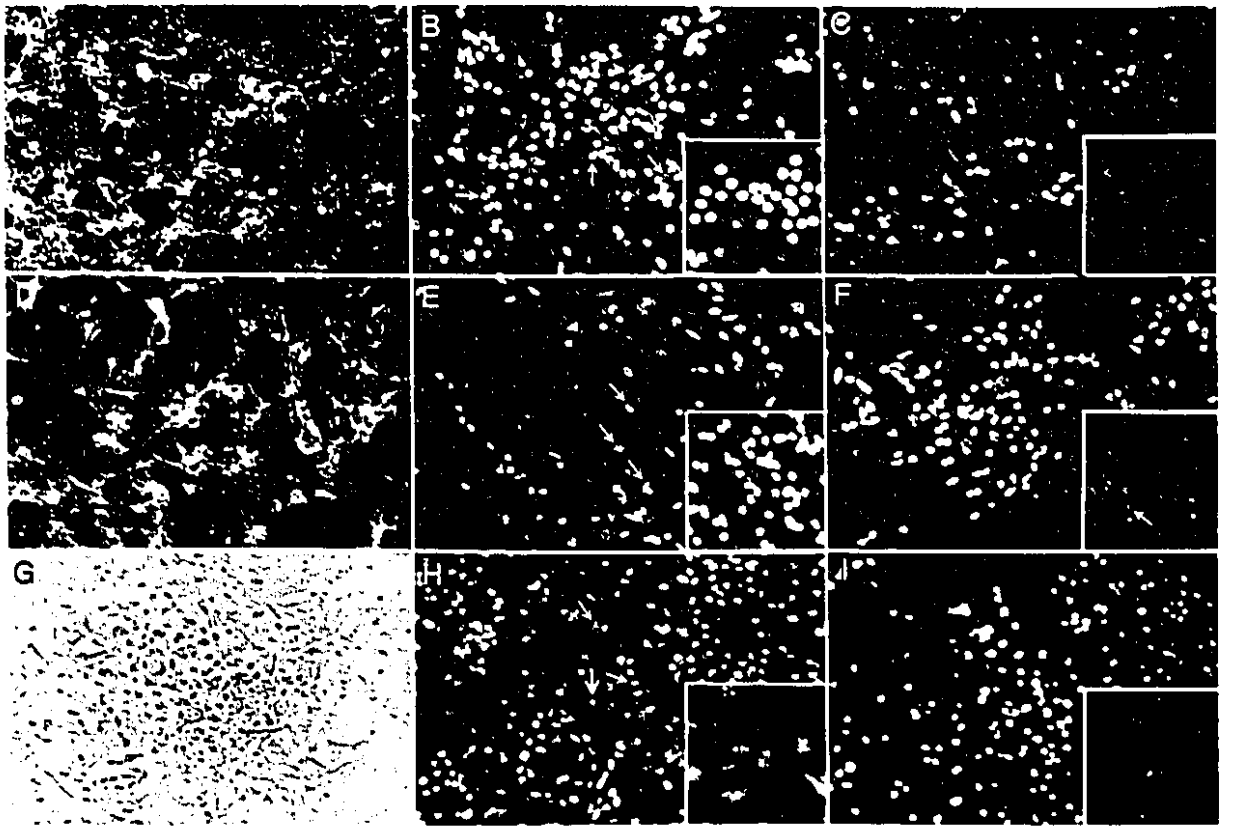
A
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TABLE 1

Effects of TNFα and activated CD4+ T cells on target cells - release of LDH and cell survival

	Target cells				
	LDH release (% of control)			Surviving cell numbers (% of control)	
Effector	OLs	neurons	glioma	OLs	glioma
TNFα	96±1% (n=3)	121±11% (n=3)	110±6 (n=3)	102±12 (n=4)	ND
CD4+ T cells plus					
- pha	172%	ND	250% (249, 250)	40±9% (n=3)	29±4% (n=3)
- anti CD3 plus IL2	185%	ND	206% (186, 225)	103±30% (n=3)	97±7% (n=3)
- anti CD3	126% (n=2)	ND	126% (135, 117)	92±19% (n=3)	91±2% (n=3)

Comparison of LDH release and number of surviving target cells in effector:target co-cultures with that found in cultures containing target cells alone. For LDH studies, data are expressed as LDH in test cultures ÷ LDH in control cultures X 100% for the number of experiments performed ± SEM, except when only single experiments (single value shown) or duplicate experiments (averaged and individual results shown) were performed. For single and duplicate experiments, each data point represents mean of triplicate cultures. Culture medium alone contains 400-600 units of LDH. LDH values of cultures containing target cells only did not differ significantly from medium alone values.

For cell survival studies, data are expressed as number of target cells in a defined coverslip region of an effector:target co-culture ÷ number of cells in a defined coverslip region of a target cell only culture X 100% ± SEM for the number of experiments performed. ND - not done.

FIGURE 2: Susceptibility of human neural cells to TNFα-induced nuclear injury - propidium iodide (PI) assay - bars indicate % ± SEM of OLs, neurons, glioma, and fetal astrocytes which show morphologic features of nuclear fragmentation after 96 hr in tissue culture medium containing either 5% FCS, 5% FCS plus 1000 units per ml TNFα, 1% FCS, 1% FCS plus TNFα, or no serum. Black portions of bars indicate % of cells with fragmented nuclei found at 18 hr. For each test condition the number of coverslips counted is indicated. At least 2 individual experiments were performed for each condition.

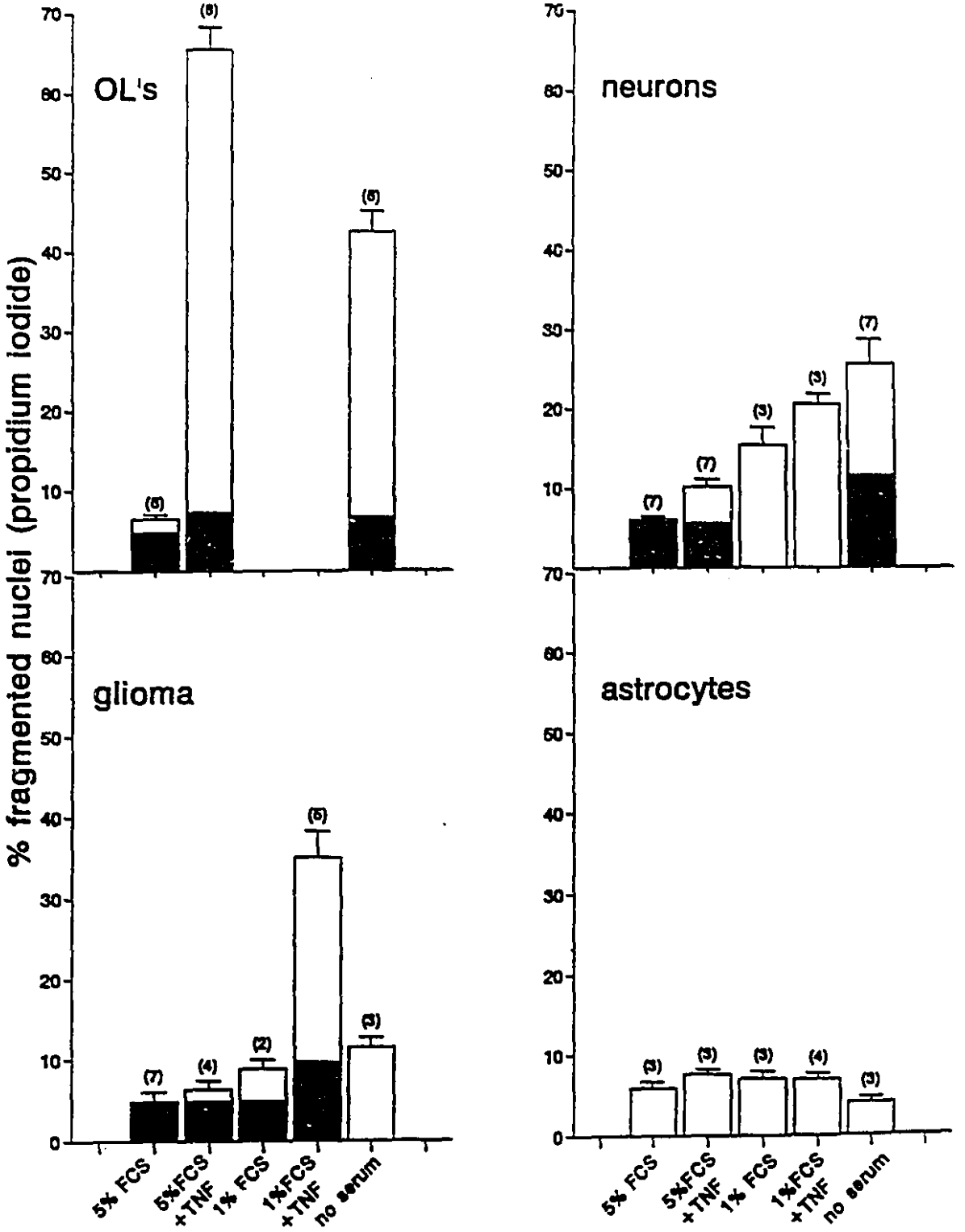

OL's
neurons
glioma
astrocytes
% fragmented nuclei (propidium iodide)
0
10
20
30
40
50
60
70
80
5% FCS
5%FCS +TNF
1% FCS
1%FCS +TNF
no serum

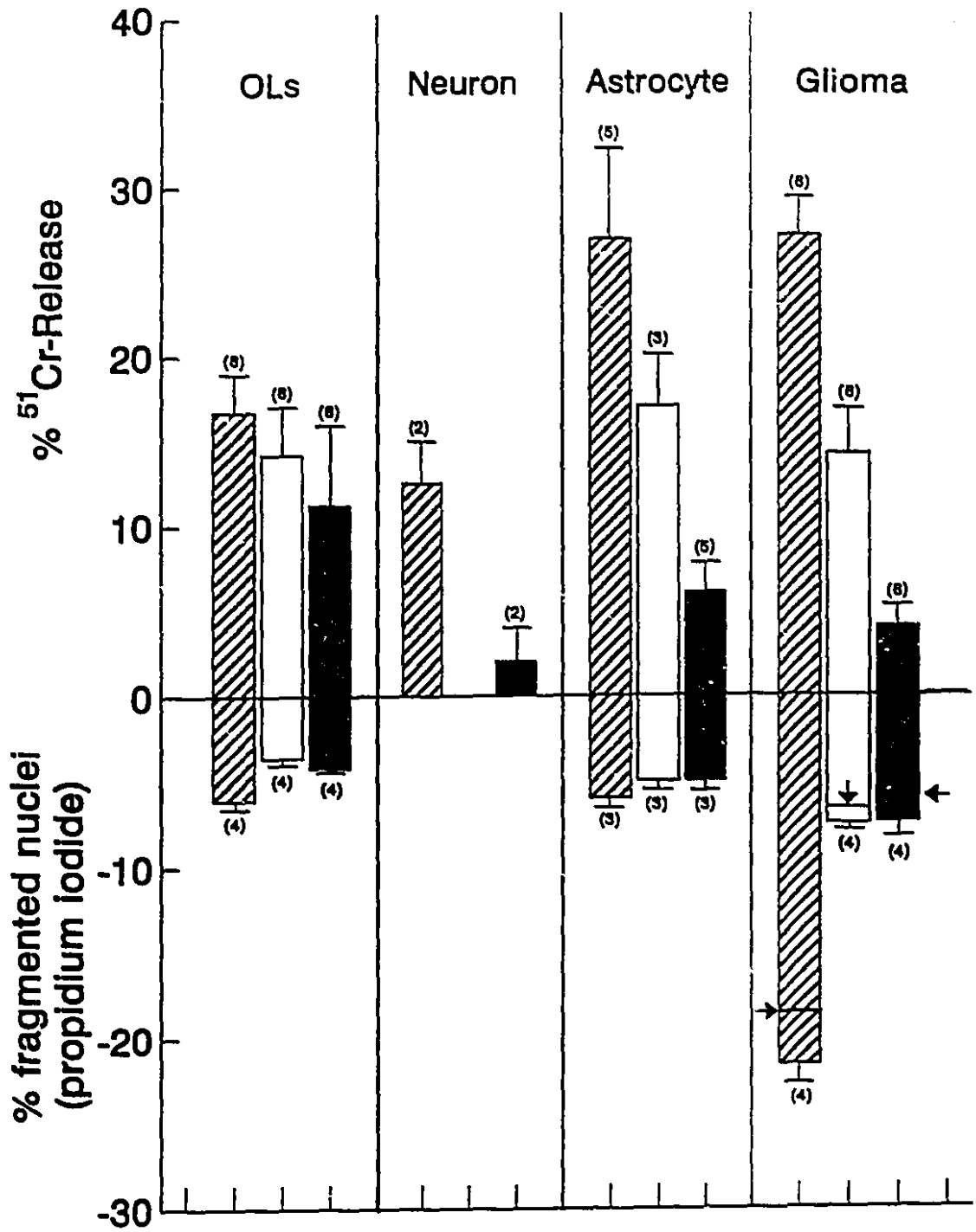
TABLE 2

Extent of DNA fragmentation (TUNEL assay) of target cells in response to effector conditions inducing significant nuclear fragmentation (PI assay)

Target cell	Effector condition	TUNEL (% positive cells)	PI (% fragmented nuclei)
OLs	TNFα	68±2% (6)	66±3% (6)
	serum deprivation	ND	43±3% (6)
Neurons	serum deprivation	32±2% (2)	25±3% (7)
Glioma	TNFα + 1% serum	42±3% (3)	35±3% (5)
	pha-activated $CD4^+$ T cells	33	22±1% (4)

Data indicate results of PI and TUNEL assays obtained for the same test conditions, in which significantly elevated values for the proportion of cells with nuclear injury were found. Data are expressed as mean ± SEM% for the number of coverslips counted. At least two experiments were done for each condition, except glioma plus pha-activated T cells where a single coverslip was counted.

FIGURE 3: Susceptibility of human neural cells to cell membrane injury as measured by ^{51}Cr release and to nuclear fragmentation as measured by PI staining, induced by $CD4^+$ T cells activated by either pha antibody (▨), anti-CD3 plus IL-2 (□), or anti-CD3 antibody (■). Studies were performed at 10:1 E:T ratios. Upper panel indicates mean % specific ^{51}Cr release ± SEM at 18 hr for the number of individual studies indicated for each target cell type. Lower panel indicates mean % ± SEM of fragmented nuclei for the corresponding target cells, as determined at 96 hr; 18 hr data is indicated by arrows. Number of coverslips or experiments used in each condition is indicated.


OLs
Neuron
Astrocyte
Glioma
% ^{51}Cr-Release
% fragmented nuclei (propidium iodide)
40
30
20
10
0
-10
-20
-30

CHAPTER 4

CHAPTER 4

CILIARY NEUROTROPHIC FACTOR SELECTIVELY PROTECTS HUMAN OLIGODENDROCYTES FROM TUMOR NECROSIS FACTOR-MEDIATED INJURY

Sameer D. D'Souza, Karen A. Alinauskas and Jack P. Antel

PREFACE

A number of molecules have recently been identified, which may rescue oligodendrocytes (OLs) from developmentally-related or experimentally-induced cell death. Ciliary neurotrophic factor (CNTF) has been shown to protect OLs derived from rodent O-2A progenitor cells and the CG4 OL cell line from TNF and serum deprivation induced death (Louis et al., 1993; Kahn and DeVellis, 1994). Having demonstrated that human adult CNS-derived OLs are vulnerable to apoptotic injury mediated by TNF, we sought to determine whether ciliary neurotrophic factor (CNTF) and other members of the neurotrophic family of molecules and anti-inflammatory cytokines could protect human adult CNS-derived OLs from injury mediated by TNF. We further wished to determine whether the protective effect of CNTF on these OLs was restricted to TNF-dependent mechanisms, whether protection conferred on OLs was selective for apoptotic injury as opposed to cell membrane injury, and whether any protective effect was restricted to OLs amongst neural cells. TNF-independent non-apoptotic injury was induced using phytohaemagglutinin (PHA)-activated $CD4^+$ T cells. Neurons and glioma cells were the other neural cells tested for CNTF-mediated protection.

ABSTRACT

Oligodendrocytes (OLs) and their myelin membranes are the apparent injury targets in the putative human autoimmune disease multiple sclerosis. The basis for this selective injury remains to be defined. OLs *in vitro* have been shown to be susceptible to both tumor necrosis factor (TNF) and non-TNF-dependent immune effector mechanisms. The former involves initial nuclear injury (apoptosis); the latter, when mediated by activated T cells, involves initial cell membrane injury (lysis). In the current study, we determined whether human adult CNS-derived OLs could be protected from the above immune effector mechanisms by selected neurotrophic factors (CNTF, BDNF, NGF, NT-3 and NT-4/5) or cytokines demonstrated to protect from human or experimental autoimmune demyelinating diseases (β-interferon (IFN), IL-10 and TGF-β). Nuclear injury was assessed in terms of DNA fragmentation using a DNA nick-end-labelling technique; cell membrane injury was assessed by lactate dehydrogenase or chromium^{51} release. MTT and cell counting assays were used to assess cell viability and cell loss, respectively. Amongst the neurotrophic factors and cytokines tested, only CNTF significantly protected the OLs from TNF-mediated injury. CNTF also protected the OLs from serum deprivation-induced apoptosis. CNTF, however, did not protect the OLs from injury induced by activated $CD4^+$ T cells. CNTF also did not protect human fetal cortical neurons from serum deprivation or TNF-induced DNA fragmentation, nor did it protect the U251 human glioma cell line from DNA fragmentation induced by a combination of TNF and reduced serum concentration in the culture media. Our results indicate that potential protective effects of neurotrophic factors or cytokines on neural cell populations can be selective both for cell type involved and mechanism of immune-mediated injury. CNTF is the protective factor selective for nuclear-directed injury of OLs.

INTRODUCTION

The mechanisms contributing to the immune-mediated multifocal areas of demyelination and destruction of the oligodendrocyte (OL) in multiple sclerosis (MS) remain to be clearly defined. By analogy with the experimentally-induced disorder in animals, experimental allergic encephalomyelitis (EAE), antigen-reactive T cells are considered a requirement for development of the disease. Lack of the requisite major histocompatibility complex (MHC) molecules on OLs (Kim, 1985; Grenier et al., 1989; Lee and Raine, 1989; Kawai and Zweiman, 1990) raises the possibility that actual target tissue injury may occur via MHC-unrestricted effector mechanisms. Such mechanisms could involve immune effector-target cell contact-dependent mechanisms mediated by α/β T cells (Antel et al., 1994), γ/δ T cells (Freedman et al., 1991) or macrophages and microglia (Merrill and Zimmerman, 1991; Zajicek et al., 1992; Merrill et al., 1993) or they may be dependent on soluble factors such as proinflammatory cytokines released into lesion sites.

Levels of an array of cytokines are elevated in the brains and cerebrospinal fluid (CSF) of MS patients (reviewed in Raine, 1994). Cytokine production in MS lesions, based on *in vivo* and *in vitro* data, has been assigned to both endogenous CNS elements as well as T-cell and macrophage infiltrates (Selmaj, 1992). Amongst the cytokines, tumor necrosis factor (TNF) is particularly implicated as a mediator of myelin-OL injury. *In vivo* intravitreal injection of TNF-α has been shown to induce demyelination of mouse optic nerve axons (Jenkins and Ikeda, 1992; Butt and Jenkins, 1994); injection into mouse spinal cord has been shown to cause an autoimmune encephalomyelitis-like response (Simmons and Willenborg, 1990). Anti-TNF-α or β antibody administration prevents the transfer of EAE and abrogates subsequent autoimmune demyelination (Ruddle et al., 1990; Selmaj et

al., 1991a). TNF-α and β have been shown to mediate injury to both myelin and OLs *in vitro* (Selmaj and Raine, 1988). TNF has also been shown to cause a reduction in the content of myelin basic protein (MBP) in myelinated aggregated rat brain cultures (Loughlin et al., 1994). Dissociated cultures of OLs undergo apoptosis in response to TNF (Selmaj et al., 1991b, Louis et al., 1993; Prabhakar et al., 1995; Wilt et al., 1995). Such OLs are also susceptible to primary membrane injury induced by complement (Scolding et al., 1989; Zajicek et al., 1992) or activated T-lymphocytes (Antel et al., 1994).

A number of molecules have now been identified, which may rescue OL from developmentally-related or experimentally-induced cell death. Ciliary neurotrophic factor (CNTF) has been shown to protect OLs derived from rodent O-2A progenitor cells and the CG4 OL cell line from TNF and serum deprivation-induced death (Louis et al., 1993; Kahn and DeVellis, 1994). CNTF and the related cytokine leukemia inhibitory factor (LIF) (Barres et al., 1992; Raff et al., 1993; Kahn and deVeillis, 1994; Mayer et al., 1994) and neurotrophin-3 (NT-3) (Barres et al., 1994), have been shown to be necessary for the long-term survival of purified rodent OLs in culture. Rodent OLs express full length, biologically active forms of *trk*B and *trk*C, high affinity receptors for brain-derived neurotrophic factor (BDNF) and NT-3 respectively, and $p75^{NGFR}$, a low affinity receptor for all the neurotrophins (Kumar et al., 1993; Lee et al., 1994).

Control of immune-mediated injury in MS and the animal model of the disease EAE may be achieved by the use of cytokines or biomodifiers that downregulate immune effector responses. The cytokines beta-interferon (β-IFN) (Paty and Li, 1993), transforming growth factor (TGF)-β (Merrill and Zimmerman, 1991; Racke et al., 1991) and interleukin-10 (IL-10) (Kennedy et al., 1992), have each been shown to reduce the immune-mediated attack directed at the CNS in MS and/or EAE. The precise site of the action of these cytokines, whether central or peripheral, as well as the mechanism of their action have yet to be determined.

β-IFN and TGF-β have been reported to have effects on OL development (McKinnon et al., 1993; Vartanian et al., 1994).

The purpose of our present study was to determine whether CNTF and other members of the neurotrophic family of molecules and anti-inflammatory cytokines could protect human adult CNS-derived OLs from injury mediated by TNF. We further wished to determine whether the protective effect of CNTF on these OLs was restricted to TNF-dependent mechanisms, whether protection conferred on OLs was selective for apoptotic injury as opposed to cell membrane injury, and whether any protective effect was restricted to OLs amongst neural cells. TNF-independent non-apoptotic injury was induced using phytohaemagglutinin (PHA)-activated $CD4^+$ T cells. Neurons and glioma cells were the other neural cells tested for CNTF-mediated protection.

MATERIALS AND METHODS

Target Cells

In this study, the following neural target cells were used: human adult OLs, human fetal neurons and the U251 human glioma cell line.

Establishment of Enriched Human Oligodendrocyte Cell Cultures

Human brain tissue was obtained from patients undergoing temporal lobe resection or callosotomy as part of a surgical therapeutic treatment for intractable epilepsy. The glial cell isolation procedure has previously been described (Yong and Antel, 1992). Briefly, the brain tissue was subjected to enzymatic dissociation using trypsin (0.25%)(Gibco, Canada) and DNase I (25 μg/ml)(Boehringer Manheim; Laval, Quebec) for 30 min at 37°C, and mechanical dissociation by

passage through a 132 µm nylon mesh (Industrial Fabrics Corporation; Minneapolis, MN). Mixed glial cells, consisting of approximately 70% OLs, 25% microglia and 5% astrocytes were obtained by separation on a 30% Percoll (Pharmacia LKB; Montreal, Quebec)-gradient (15000 rpm at 4°C for 30 min). To enrich for OLs, freshly isolated mixed glial cells were left overnight in Falcon tissue culture flasks (Nunclon, Gibco) and the less adherent OLs were removed by gentle shaking. The differential adhesion protocol was repeated 24 hrs later on this semi-enriched OL culture. This population of OLs was immunostained for 30 min at room temperature with R monoclonal antibody (mAb) (Ranscht et al., 1982) obtained as a hybridoma supernatant, followed by Texas-red-conjugated goat anti-mouse immunoglobulin (Ig)(1:100 dilution for 30 min at room temperature; Jackson Immunoresearch Lab, West Grove, PA) and was found to contain >90% OLs. The derived OLs were plated onto poly-L-lysine (10 µg/ml; Sigma, St. Louis, MO)-coated Aclar 9 mm diameter coverslips or into 96-well Nuntron (Becton Dickinson; Mountain View, CA) plates at a density of 5×10^4 cells per coverslip or microwell; coverslips were placed in Nuntron petri dishes. Microwells or petri dishes were filled with minimum essential culture medium (MEM) supplemented with 5% fetal calf serum (FCS), 2.5 U/ml penicillin, 2.5 µg/ml streptomycin, and 0.1% glucose (all from Gibco, Canada).

Establishment of Human Fetal Neuron Cell Cultures

Human fetal CNS tissue was obtained at 12-16 weeks gestation. The cerebral hemispheres were dissected apart and subjected to enzymatic dissociation using 0.05% trypsin and 50 µg/ml DNase I (15 min), followed by mechanical dissociation by serial passage through 132 µm and 70 µm nylon meshes. The derived neural cells were suspended in MEM culture medium, supplemented with 5% FCS, 0.1% glucose and 1 mM sodium pyruvate, and then seeded as described above for the OLs. To obtain enriched neuron preparations, the culture dishes containing the coverslips were treated on day 4, and subsequently once a

week over a 2-week period with 1 mM 5-fluoro-2-deoxyuridine (5-FDU) to deplete the proliferating astrocytes. These cells were immunostained with mouse anti-mitogen-activated protein (MAP-2)(SMI 52; Sternberger Monoclonals Inc., Baltimore, MD) or mouse anti-neuron-specific-enolase (NSE)(Dako; Carpinteria, CA)(1:100 dilution for 30 min at room temperature), followed by biotinylated anti-mouse Ig (1:100 dilution; Vector Laboratories Inc., Burlingame, CA), followed by Streptavidin-Fluorescein isothiocyanate (FITC)(Boehringer Manheim) (1:20 dilution for 30 min at room temperature) and found to be >85% neurons (McLaurin et al., 1995b).

The U251 Human Glioma Cell Line

The U251 cell line was obtained from ATCC, and maintained in serial passage in RPMI supplemented with 5% FCS. Cells were trypsinized using 0.25% trypsin for 5 min at 37°C when cultures were confluent and reseeded onto coverslips at a density of 2.5 X 10^4 per coverslip, 24 hr prior to use in functional assays. These cells are not glial fibrillary acidic protein (GFAP)-reactive.

Generation of Mitogen Activated $CD4^+$ T Cells

This procedure has been detailed elsewhere (Antel et al., 1994). Briefly, mononuclear cells (MNCs) from young adult healthy donors were isolated from peripheral blood on a Ficoll-Hypaque (Pharmacia, Baie D'Urfe, QC)-gradient (500*g* for 30 min). T cells were isolated from the MNCs by rosetting with S-(2 aminoethyl)-isothiouronium bromide (AET)-treated sheep red blood cells (Aldrich, Milwaukee, WI). Depletion of natural killer cells and $CD8^+$ T cells was achieved by Percoll (Pharmacia)-gradient (43.5%) centrifugation and complement-mediated lysis (baby rabbit serum, Cedar Lane, Hamilton, Ontario; and, anti-CD8 mAb ATCC CRL 8014), respectively. The resulting $CD4^+$ T cells were then cultured on a layer of autologous radiated non-rosetting feeder cells and activated for 72 hr

with PHA (1 μg/ml; Wellmark Diagnostics, Guelph, Ontario), before use in cytotoxicity assays.

Cytokines and Neurotrophic Factors

Recombinant human (rh) TNF-α, TNF-β and rabbit anti-human TNF-α- and β-neutralizing antibodies were obtained from Intermedico Diagnostics (Markham, Ontario). CNTF was generously supplied by Dr. P. Richardson (McGill University, Montreal). BDNF, NT-3, NT-4/5 and nerve growth factor (NGF) were all obtained from PeproTech Inc. (Rocky Hill, NJ). Rh β-IFN was obtained from Berlex (Alameda, CA); rh IL-10 was obtained from PeproTech Inc (Rocky Hill, NJ) and rh TGF-β was obtained from Intermedico Diagnostics (Markham, Ontario).

Induction of Cytotoxicity

To establish conditions optimal for study of toxic effects of TNF on OLs, OLs in 96-well plates or on coverslips placed in individual wells of 24-well Nuntron (Becton Dickinson; Mountain View, CA)-plates were incubated for up to 7 days, either in MEM culture medium supplemented with 5% FCS alone or in medium to which was added TNF-α or β at concentrations of 10 U/ml, 100 U/ml, 500 U/ml or 1000 U/ml. In other experiments, OLs were cultured under serum-free conditions or in serum-supplemented medium to which were added mitogen (PHA)-activated $CD4^+$ T cells at a predetermined effector:target (E:T) ratio of 10:1 (Antel et al., 1994).

For neuron-directed toxicity studies, fetal neurons on coverslips were either incubated for 4 days in 5% FCS-supplemented MEM alone or in serum-supplemented medium containing TNF-α at a concentration of 1000 U/ml, or in serum-free medium.

Preliminary studies established that U251 glioma cells incubated for 4 days in RPMI medium supplemented with 1% FCS plus TNF-α at a concentration of 1000 U/ml induced toxicity (D'Souza et al., 1995). During experiments, medium and reagents were replaced on a daily basis.

Neutralization of TNF-α or β

Mouse anti-human TNF-α or β antibodies were preincubated with TNF-α (1000 U/ml) or TNF-β (1000 U/ml) respectively, for 1 hr before testing. The pretreated TNF was then added to OLs in 96-well plates and maintained for the duration of the assay, with daily replacements of pretreated TNF.

Neurotrophic Factor and Cytokine-Mediated Protection of Oligodendrocytes, Neurons and Glioma Cells From Immune-Mediated Injury

Based on initial studies of TNF toxicity, protection studies were conducted on OLs incubated in 5% FCS-supplemented culture medium plus TNF-α (1000 U/ml). In these studies, either CNTF at titrated concentrations ranging from 0.1-100 ng/ml, the neurotrophic factors BDNF, NGF, NT-3, or NT-4/5, each at a recommended concentration of 10 ng/ml (PeproTech Inc.), or the cytokines β-IFN, IL-10, or TGF-β, each at a concentration of 1000 U/ml, were added to the OL cultures. Based on the results of the CNTF protective effects of TNF-mediated injury of OLs, studies of the effects of CNTF on protecting OLs maintained under serum-free conditions or in 18-hour co-cultures (Antel et al., 1994) with PHA-activated $CD4^+$ T cells were conducted using 100 ng/ml of CNTF.

CNTF at a concentration of 100 ng/ml was also added to cultures of human fetal cortical neurons grown either in serum-supplemented medium containing 1000 U/ml of TNF-α or in serum-free medium or to U251 glioma cells maintained in 1% FCS-supplemented medium containing 1000 U/ml of TNF-α.

Cytotoxicity Assays

MTT assay MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma; St. Louis, MO), at a final concentration of 500 µg/ml was added to the individual wells of the 96-well plates. Viable, metabolizing cells convert the tetrazolium ring in MTT into dark blue formazan crystals; non-viable cells do not. After a 4-hr incubation at 37°C, a SDS:HCl solution (10% SDS/0.01 N HCl) was added to the wells at a 1:1 dilution. After adequate mixing and incubation overnight at 37°C, the OD_{550} was read.

% viability = [OD_{550} of treated OL ÷ OD_{550} of untreated OL control] X 100%

TdT-dUTP Nick End-labelling (TUNEL) Coverslips containing target cells were fixed in acid:alcohol (5% glacial acetic acid:95% absolute alcohol) for 15 min at -20°C. After rehydration for 30 min in PBS, the OLs and neurons were identified by immunolabelling as described above. The coverslips were then incubated for 1 hr at 37°C with nick end-labelling solution containing TdT (0.3 e.u/ml)(Promega Corp; Madison, WI), biotinylated dUTP (0.01 nmol/ml)(Boehringer Manheim) in TdT 5X buffer (500mM cacodylate buffer, pH 6.8; 5 mM $CoCl_2$, 0.5 mM DTT and 0.5 mg/ml BSA) (Promega Corp). The reaction was terminated with Tris buffer (10 mM Tris-HCl, pH 6.8 for 15 min). After blocking with 2% bovine serum albumin (BSA)(15 min), the coverslips were incubated with Streptavidin-FITC (1:20 dil, 30 min at 37°C)(Boehringer Manheim). Hoechst dye 33258 (10 µg/ml, 20 min; Sigma, St. Louis, MO) was used to identify target cell nuclei. All analyses were performed using a Reichert Polyvar 2 Leica immunofluorescence microscope. For each experiment, 200-400 cells were counted per coverslip, each treatment being performed on 3 replicate coverslips.

Experiments were conducted on cells derived from at least 3 separate brain specimens and counting was done by an observer blinded to the treatment received by the cells.

$$\% \text{ apoptotic nuclei} = \frac{\text{number of TUNEL}^+ \text{ nuclei}}{\text{number of Hoechst labelled nuclei}} \times 100\%$$

The immunofluorescence ApopTag In Situ Apoptosis Detection Kit (Oncor; Cat#: S7110-KIT, Gaithersburg, MD) was also used to quantify % apoptotic target cells and yielded similar results.

LDH release assay Cell-free supernatant was collected from OL cultures exposed to TNF-α or β. Sample tubes containing 0.5 ml of 2 mg/ml NADH, 0.5 ml of 1.5 mmol/l pyruvate substrate (all from Sigma) and 100 µl of test sample were incubated for 30 min at 37°C. Pyruvate calibration curve tubes were set up. One ml of colour reagent (Sigma) was added to each tube to stop the reaction. Absorbency was read at 460 nm. Test sample LDH was calculated by comparison with a curve generated using the pyruvate standards.

^{51}Chromium (^{51}Cr) release assay OLs in 96-well plates were labelled overnight with 0.1 µCi of ^{51}Cr (NEN DuPont, Mississauga, Ontario) per well. The OLs were then washed twice and PHA-activated CD4$^+$ T cells added at an E:T ratio of 10:1. After 18 hr, 100 µl of cell-free supernatant was collected from individual wells to determine induced ^{51}Cr release (IR). One hundred µl of 5N sodium hydroxide (Fisher Scientific) was then added to each well for 30 min and a further 100 µl was collected to determine residual ^{51}Cr release (RR). Non-specific ^{51}Cr release (NSR) was determined from wells containing only target cells as follows:

$$NSR = [\text{spontaneous (SR)} \div (SR + RR)] \times 100\%$$

Percent specific release was calculated as:

$$[(IR \div (IR + RR)) - NSR] \times 100\%$$

Data Analysis

For all studies involving MTT, LDH release, and TUNEL assays, total number of data points are derived as the sum of replicate cultures (3 or more per experiment) from multiple (at least 3) individual experiments. For studies involving counting of numbers of OLs on coverslips, the number of cells along the vertical length of the center of the coverslips was examined.

RESULTS

TNF-Induced Injury of OLs and Protection by CNTF

TNF-α and TNF-β both induced a dose-dependent loss of OL viability after 4 days of OL exposure, beginning at an exposure dose of 10 U/ml and being maximal at 1000 U/ml of TNF-α or β, as measured by the MTT assay (Fig. 1a) with accompanying DNA fragmentation as measured by the TUNEL assay (Fig. 1b). There was no concomitant loss of membrane integrity as measured by the LDH-release assay (Fig. 1c). The kinetics of cell viability loss and DNA fragmentation paralleled each other (Fig. 2a). These changes preceded retraction of cell processes (Fig. 4a inset) and cell loss (Fig. 2b); these latter changes became readily apparent on day 7. Specific neutralizing antibodies inhibited TNF-mediated OL cytotoxicity in a dose-dependent manner (Fig. 2c).

The MTT and TUNEL assays were used to determine the ability of CNTF to protect OLs from TNF-mediated cytotoxicity. In the MTT assay, CNTF

conferred protection in a dose-dependent manner with a maximal effect at 100 ng/ml and an ED_{50} (median effective dose) at 1 ng/ml (Fig. 3a). At concentrations greater than 1 µg/ml, CNTF demonstrated a marked toxicity to the OLs (Fig. 3a). The protective effect effect of 100 ng/ml of CNTF persisted throughout the time period studied (6 days) (Fig. 3b). The protective effect of CNTF was confirmed using the TUNEL assay (Figs. 3c, 4).

The TUNEL assay was also used to determine whether other neurotrophic factors (BDNF, NGF, NT-3 and NT-4/5) or inhibitory cytokines (β-IFN, IL-10 or TGF-β) were capable of protecting the OLs from TNF-mediated nuclear injury. No significant effects were found (Fig. 3c). These neurotrophic factors and cytokines, themselves, did not induce any significant DNA fragmentation in the OLs (data not shown).

Protection of OLs From Serum Deprivation Induced Nuclear Injury

Serum deprivation also induced nuclear injury in the OLs by day 4 (Fig. 3d). At 100 ng/ml, CNTF also significantly protected the OLs from this injury (Fig. 3d).

Protection of Oligodendrocytes From T Cell Induced Lytic Injury

As previously reported (Antel et al., 1994), OLs were found to be susceptible to membrane injury induced by PHA-activated $CD4^+$ T cells, as measured by the ^{51}Cr-release assay (mean % ^{51}Cr release = 18.57 ± 3.61, n=3). In contrast to nuclear-directed OL injury, CNTF did not protect the OLs from injury induced by the PHA-activated $CD4^+$ T cells (mean % ^{51}Cr release = 17.73 ± 4.60, n=3).

Protection of Other CNS-Derived Neural Cells From Nuclear Injury

TNF, as well as serum deprivation on day 4, induced significant nuclear injury in human fetal cortical neurons maintained under identical culture conditions as the OLs (5% serum-supplemented medium), although the TNF effect was less than that obtained for the OLs. TNF, on day 4, also induced significant nuclear injury in the human U251 glioma cell line maintained under reduced serum (1% serum) conditions. CNTF, at a concentration of 100 ng/ml, did not protect the neurons from nuclear injury induced by either TNF or serum deprivation. CNTF also did not protect the U251 glioma cells from DNA fragmentation induced by the combined effect of 1% serum conditions plus TNF (Fig. 3d).

DISCUSSION

The current study demonstrates that human OLs, in parallel with results from rodents, are protected from TNF and serum deprivation-induced apoptosis by CNTF. Other growth factors and cytokines are not protective. The OL-directed CNTF protective effect is not observed for OL injury induced by a TNF-independent effector mechanism mediated by activated $CD4^+$ T cells. CNTF does not protect other neural cells from undergoing apoptosis induced by TNF or serum deprivation.

The susceptibility of OLs to TNF-induced apoptosis was initially described using primary dissociated cultures of bovine (Selmaj et al., 1991b) and rodent (Louis et al., 1993) OLs. Our previous studies have demonstrated a similar susceptibility of human OLs to TNF-induced apoptosis (Prabhakar et al., 1995; D'Souza et al., 1995); human fetal CNS-derived neurons exhibited only a modest level of susceptibility to TNF as compared to the OLs, whereas astrocytes were

resistant to the same TNF exposure. Wilt et al. (1995) have recently described similar responses of human OLs to TNF derived from HIV-infected microglia. In the current study, in which DNA fragmentation, MTT, and cell count assays were performed over a 7-day time period, we found that DNA fragmentation (as measured by the TUNEL technique) and mitochondrial dysfunction (as measured by the MTT assay) were apparent beginning on day 2, before any apparent loss of cell numbers or morphologic changes in the OLs (process retraction). Selmaj et al. (1991b) previously described early (within 12 hr) retraction of cell processes and depolymerization of the cytoskeletal protein F-actin in bovine OLs exposed to TNF, followed by DNA fragmentation at 72 hours. Our data is consistent with that of McLarnon et al. (1993) and Soliven et al. (1991), who found a more delayed retraction of cell processes using human and ovine OLs, respectively. Our results suggest that TNF-induced cytoskeletal disruption is not the initiating event in the apoptotic death of human OLs, but rather TNF-induced DNA fragmentation and mitochondrial shutdown may be the initiating event in the eventual demise of the OL.

Protection of OLs from TNF- and serum deprivation-induced apoptosis was initially demonstrated by Louis et al. (1993) using the CG4 OL precursor cell line which could be induced to differentiate by withdrawing PDGF and bFGF; survival of the differentiated cells was maintained by the addition of serum. Within 36 hours of serum withdrawal, 50% of these cells were reported to be MTT-negative; this effect was totally preventable by CNTF. Similar results were found using OLs derived from O2A progenitor cell cultures established from fetal rat cortex. The optimal CNTF dose was 10 ng per ml with an ED_{50} of 1 ng per ml. CNTF at the same concentration protected the CG4 cells from TNF-α and β-induced cell death, as defined by the MTT assay. Kahn and deVellis (1994) also found that CNTF and LIF, members of the IL-6 family of cytokines, protected both O-2A progenitor cells and the CG-4 OL cell line from serum deprivation-induced apoptosis whereas IL-6, which shares structural homology and the use

of a similar transducing receptor mechanism with CNTF and LIF, did not. Our data demonstrate the protective effect of CNTF for human OLs induced to undergo apoptosis, as assessed directly in a DNA fragmentation assay, either in response to serum deprivation or TNF. We have also used the MTT assay, as did Louis et al.(1993). The ED_{50} for CNTF in the MTT assay was also 1 ng per ml, in accord with the studies on OLs derived from precursor cells. The human OLs used in our study are all non-proliferating differentiated cells which all express mature myelin antigen markers (Yong and Antel, 1992). Human OLs derived from adult surgical specimens do undergo a transient dedifferentiation in culture, but have not yet been made to proliferate (Armstrong et al., 1992; Cogate et al., 1994).

CNTF did not protect human OLs from non-apoptotic (lytic) cell injury induced by activated $CD4^+$ T cells acting in a lectin-dependent cytotoxic manner. As previously shown, these T cells induce membrane injury and cell death without evidence of initial nuclear injury (D'Souza et al., 1995). These results contrast with initial apoptotic responses reported for $CD4^+$ T cell-mediated injury, when proliferating cell lines are used as targets (Berke, 1994). Louis et al. (1993) also found that CNTF did not protect the CG4 OL cell line from antibody-independent complement-mediated cell membrane lysis. Human OLs are not susceptible to this form of injury since, unlike rodent OLs, they express the complement-protective protein CD59 (Wing et al., 1992). The inability of CNTF to protect human OLs from the T cell-mediated injury suggests that the protective action of CNTF on human OLs may be restricted to the apoptotic mode of cell death. For assays involving the co-culture of T cells with OLs, primary membrane injury of OLs was measured by the ^{51}Cr-release assay and not the LDH-release assay, since the source of the LDH (effector or target cell) would have been uncertain.

CNTF receptor-α is widely expressed on neurons in the adult cerebral cortex (Ip et al., 1993). In cell culture, CNTF has been shown to protect various populations of cortical neurons, such as corticospinal neurons from spontaneous death (Magal et al., 1993). CNTF has also been shown to protect neurons from other forms of injury, such as exitotoxicity (Skaper et al., 1992). In our study, CNTF did not protect the human fetal cortical neurons from TNF-induced and serum deprivation-induced apoptosis. The neurons used were a heterogeneous population of cells prepared from total cerebral tissue. Identification of anatomic landmarks in the fetal material, which would allow isolation of different neuronal subpopulations, was not possible. Our data suggest that the ability of CNTF to protect against TNF-induced apoptosis may be cell-specific amongst CNTF receptor-bearing cells. Gliomas are reported not to express CNTF receptors (MacLennan et al., 1994). The U251 glioma cell line was used instead of primary fetal or adult astrocytes, since our initial work indicated the resistance of these primary cells to undergo apoptosis in response to either serum deprivation or TNF (D'Souza et al., 1995).

The CNTF protective effect on OLs could not be reproduced using other neurotrophic factors or with inhibitory cytokines. As mentioned, OLs do express receptors for all of the neurotrophins tested (Kumar et al., 1993; Lee et al., 1994). We did not test the other neurotrophins in our TNF-treated or serum-deprived neuron cultures. Although β-IFN did not protect our adult OLs, this cytokine has been shown to have an effect on OLs. Vartanian et al. (1994) found that IFN-α and β induced differentiation of rat OLs from precursor cells and promoted their survival *in vitro*. We previously did not find direct toxicity or growth-promoting effects of IFN-β on our human adult CNS-derived OLs (McLaurin et al., 1995a). TGF-β has been shown to inhibit microglia-mediated cytotoxicity of OLs (Merrill and Zimmerman, 1991), stimulate the differentiation of OLs from O-2A progenitor cells (McKinnon et al., 1993), inhibit EAE (Racke et al., 1991), and is in early phase 1 clinical trials for MS. Similarly, IL-10 has been shown to inhibit microglia

activation (Williams et al., in press) and inhibit EAE (Willenborg et al., 1995). Our data suggests neither a direct toxic nor a protective effect of TGF-β or IL-10 for OLs.

In summary, our data indicate the effectiveness of CNTF for protecting human adult OLs undergoing injury which induces apoptosis. CNTF is not effective in protecting OLs from non-apoptotic cell injury nor in protecting other neural cells from apoptosis. The CNTF effect cannot be reproduced with either other neurotrophins or inhibitory cytokines.

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FIGURE 1: Concentration effects of TNF-α and TNF-β on adult human OLs after 4 days of exposure in culture: **a:** Calculated % viable OLs based on OD readings obtained in the MTT assays (n=12). **b:** % DNA-fragmented OLs as measured by the TUNEL assay (n=18). **c:** LDH release by OLs exposed to indicated concentrations of TNF-α and β (n=9).

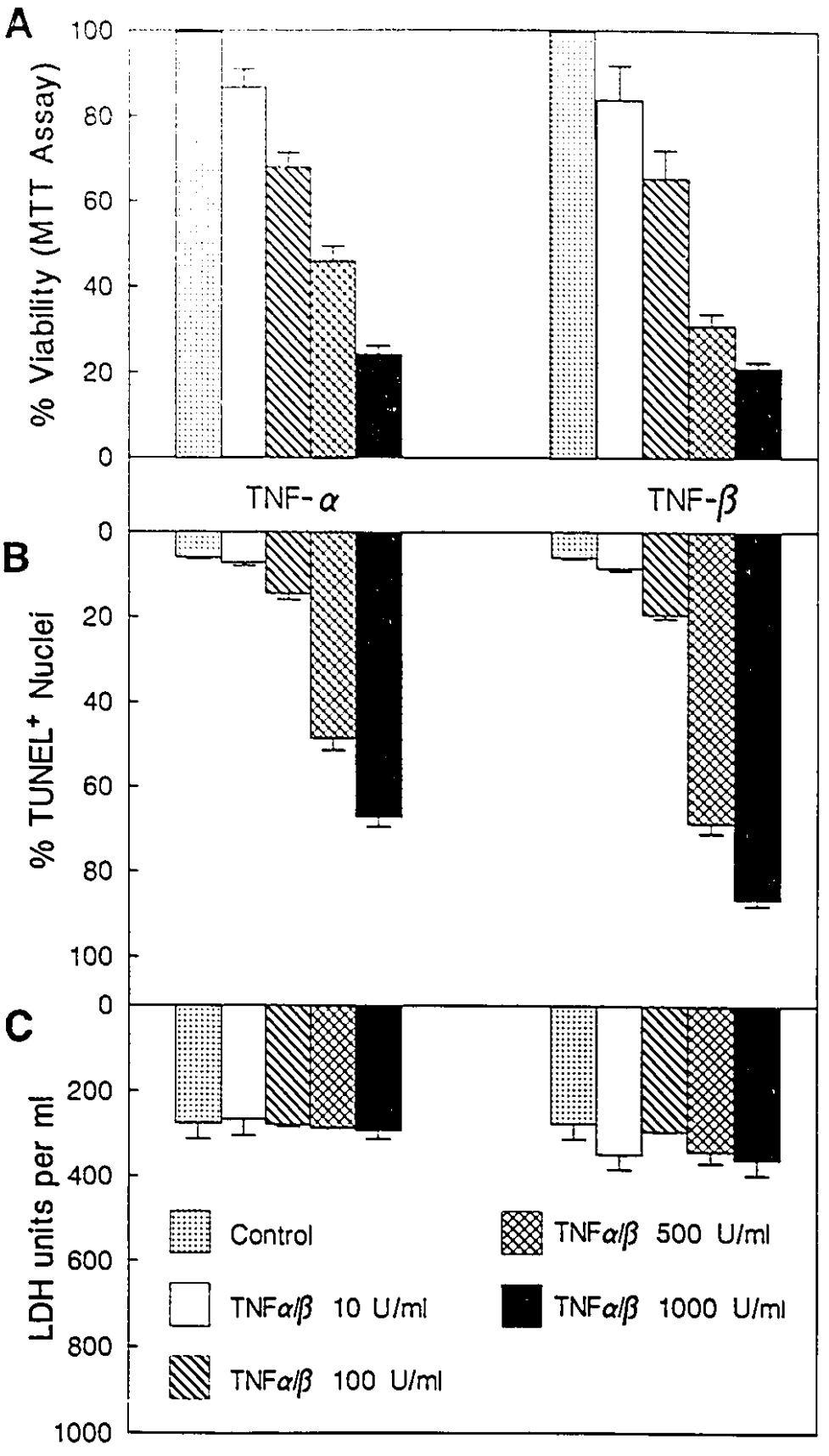

A
% Viability (MTT Assay)
100
80
60
40
20
0
TNF-α
TNF-β
B
% TUNEL+ Nuclei
0
20
40
60
80
100
C
LDH units per ml
0
200
400
600
800
1000
Control
TNFα/β 10 U/ml
TNFα/β 100 U/ml
TNFα/β 500 U/ml
TNFα/β 1000 U/ml

FIGURE 2: Kinetics of TNF-α and β (1000 U/ml)-induced injury of human OLs. **a:** Individual curves indicate either % viable OLs on each day following exposure to TNF-α or β, as measured in the MTT assay, or % of OLs showing fragmented nuclei, as measured by the TUNEL technique (n=18). **b:** Each bar represents the number of OLs surviving each day after exposure to TNF-α, calculated as a % of the number of OLs contained in control cultures on the corresponding day (n=9). Reduction in cell number on day 6 and 7 is significant at $p < .01$ level (*). There was no significant difference between cell numbers in day 0 and day 7 control cultures. Range of cell numbers counted in control cultures was 500 to 1300. **c:** Indicates the protection of OLs exposed for 4 days to TNF-α or ß (1000 U/ml) by pre-incubating the TNF-α or ß with the concentrations of neutralizing antibodies indicated on the horizontal axis (n=9).

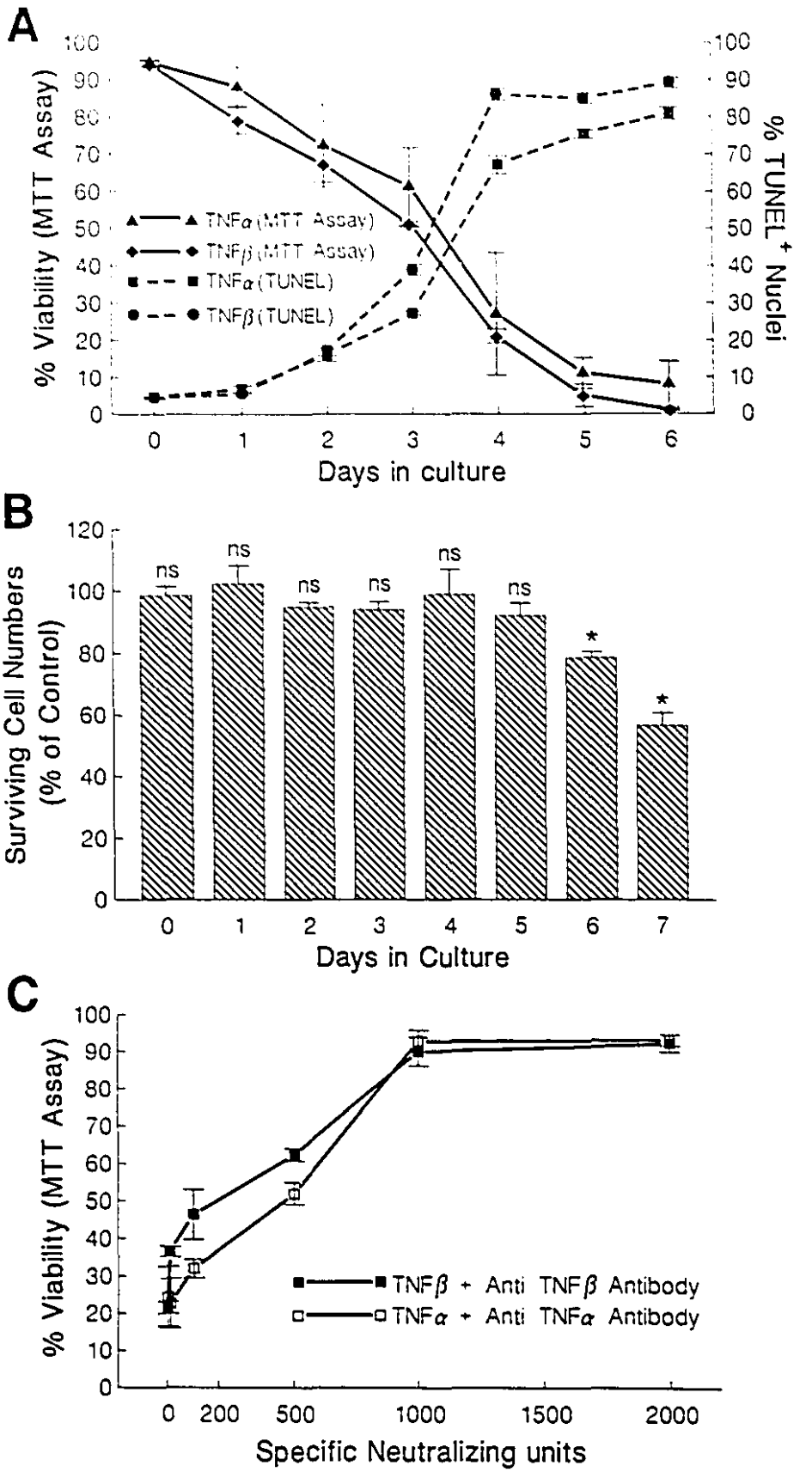

A
% Viability (MTT Assay)
% TUNEL+ Nuclei
TNFα (MTT Assay)
TNFβ (MTT Assay)
TNFα (TUNEL)
TNFβ (TUNEL)
Days in culture
B
Surviving Cell Numbers (% of Control)
ns
*
Days in Culture
C
% Viability (MTT Assay)
TNFβ + Anti TNFβ Antibody
TNFα + Anti TNFα Antibody
Specific Neutralizing units

FIGURE 3: Neurotrophic factor and cytokine-mediated protection of neural cells from immune injury: **a:** Dose-dependent CNTF protection of OLs from TNF-α-mediated toxicity, as measured by the MTT assay. Data are expressed as % viable cells, using the formula given in the text, at each dose of CNTF added to OL cultures exposed to 1000 U/ml of TNF for 4 days (n=9). CNTF alone is not toxic to OL cultures up to a concentration of 1 μg/ml, above which it is toxic (*). **b:** Kinetics of CNTF-mediated protection of OLs from TNF-α and TNF-β-induced toxicity, as measured by the MTT assay. Data are again expressed as % viable cells at each indicated day following addition of 100 ng/ml of CNTF to OL cultures containing 1000 U/ml of TNF-α or β (n=9). **c:** Prevention of DNA fragmentation, as measured by TUNEL technique, in OLs exposed for 4 days to TNF-α (1000 U/ml) by) CNTF (10 and 100 ng/ml) and other neurotrophic factor- (BDNF, NGF, NT-3 and NT-4/5) (10 ng/ml) and cytokines ß-IFN, IL-10, and TGF-ß (1000 U/ml). Data indicate % fragmented nuclei for each test and control condition (* $p < 0.01$, ** $p < 0.001$ and not significant (ns) as compared to OL cultures containing TNF-α alone) (n=9). **d:** Effect of CNTF on preventing DNA fragmentation of OLs exposed to serum-free conditions for 4 days, or human fetal neurons or glioma cells exposed for 4 days to the conditions indicated on the graph. Control represents cells grown in serum-supplement culture media. Data are expressed as % fragmented nuclei as measured by the TUNEL technique (n=9). OLs are significantly protected from serum deprivation (* $p < 0.01$). Neurons and glioma cells are not significantly (ns) protected from TNF and/or serum deprivation by CNTF.

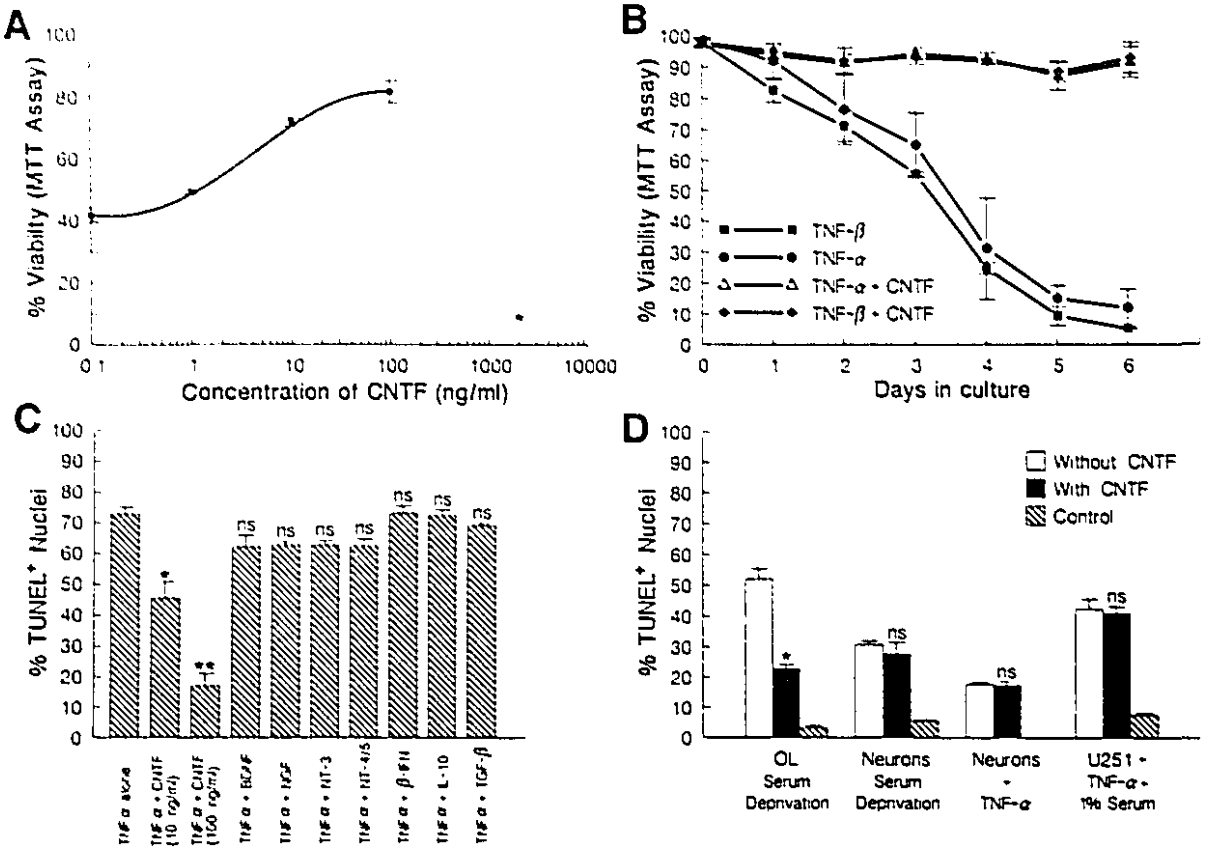
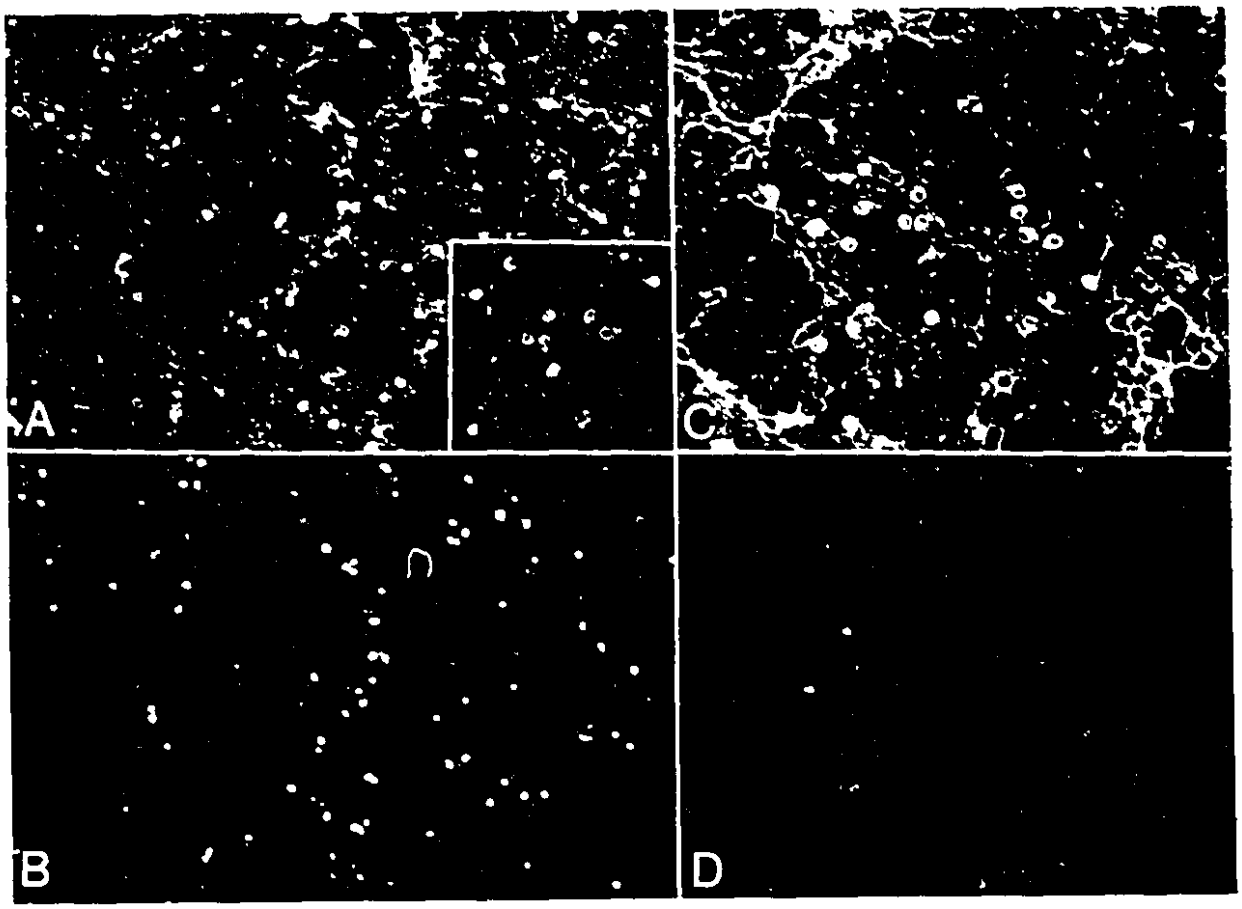

A
% Viability (MTT Assay)
Concentration of CNTF (ng/ml)
B
% Viability (MTT Assay)
TNF-β
TNF-α
TNF-α + CNTF
TNF-β + CNTF
Days in culture
C
% TUNEL+ Nuclei
ns
D
% TUNEL+ Nuclei
Without CNTF
With CNTF
Control
OL Serum Deprivation
Neurons Serum Deprivation
Neurons + TNF-α
U251 + TNF-α + 1% Serum

FIGURE 4: CNTF-mediated protection of human adult OLs exposed to TNF-α: **a and c:** OLs immunostained with anti-R mAb (X 300 magnification). These cells are also immunoreactive with anti-myelin basic protein Ab (Prabhakar et al., 1995); **b and d:** OLs assessed for DNA fragmentation using the TUNEL technique (X 300 magnification). **a** and **b** represent OLs maintained in 5% FCS MEM culture medium treated with TNF-α (1000 U/ml) for 4 days, and **c** and **d** represent OLs in 5% FCS MEM culture medium treated with TNF-α (1000 U/ml) plus CNTF (100 ng/ml) for 4 days. The inset in **a** is an anti-R mAb stain of OLs in 5% FCS MEM after being treated with TNF-α (1000 U/ml) for 7 days, indicating a marked retraction of OL processes (X 450 magnification).


A
C
B
D

CHAPTER 5

CHAPTER 5

MULTIPLE SCLEROSIS: POTENTIAL FOR FAS SIGNALLING IN OLIGODENDROCYTE CELL DEATH

Sameer D. D'Souza, Bruno Bonetti, Vijayabalan Balasingam, Neil R. Cashman, Philip A. Barker, Anthony B. Troutt, Cedric S. Raine, Jack P. Antel

PREFACE

As mentioned before, one mechanism that could account for the selective injury of oligodendrocytes (OLs) in multiple sclerosis (MS) could be the selective upregulation of immune recognition molecules on OLs. One such potential candidate molecule is fas, a cell surface receptor that belongs to the TNF receptor superfamily. Crosslinking of surface fas by agonist antibodies or by fas ligand results in the induction of apoptotic cell death in most cells. While early reports indicated that fas is not expressed in the brain, recent reports suggest that fas may be upregulated in the brain under pathologic conditions. The aim of the present study was to determine: (i) whether fas, amongst glial cells, is selectively expressed on OLs in *vitro*, (ii) whether OLs are susceptible to injury via this signalling pathway, (iii) whether selective expression of fas occurs on OLs in MS lesions, and (iv) whether fas-ligand is expressed on potential immune effector cells in MS lesions.

ABSTRACT

Fas is a cell surface receptor that transduces cell death signals when crosslinked by agonist antibodies or by fas ligand. In this study, we examined the potential of fas to contribute to oligodendrocyte (OL) injury and demyelination as occurs in the human demyelinating disease, multiple sclerosis (MS). In dissociated glial cell cultures prepared from human adult central nervous system (CNS) tissue, fas expression was restricted to OLs. Fas ligation with the anti-fas monoclonal antibody M3 induced rapid OL cell membrane lysis, assessed by LDH release and trypan blue uptake, and subsequent cell death. In contrast to the activity of fas in other cellular systems, dying OLs did not exhibit evidence of apoptosis, assessed morphologically and by TUNEL staining. Other stimuli such as C2-ceramide were capable of inducing rapid apoptosis in the OLs. Antibodies directed at other surface molecules expressed on OLs or the M33 non-activating anti-fas monoclonal antibody did not induce cytolysis of the OLs. In situ immunohistochemical studies demonstrated elevated fas expression on OLs in chronic active MS lesions compared to OLs in control CNS tissue. In such lesions, microglia and infiltrating lymphocytes displayed intense immunoreactivity to fas ligand. Our results suggest that fas-mediated signalling might contribute in a novel cytolytic manner to immune-mediated OL injury in MS.

INTRODUCTION

Multiple sclerosis (MS) is a progressive disease of the central nervous system (CNS) characterized by multifocal areas of inflammation and demyelination (Dawson, 1916; Lumsden, 1955; Prineas, 1985; Raine, 1990). The disease is considered to be immune-mediated and directed at myelin and its cell of origin, the oligodendrocyte (OL)(Raine, 1990). The precise basis for this selective injury remains to be established. Depletion of OLs is a recognized feature of MS lesions, becoming more apparent as the disease evolves (Raine, 1994). Examples of OLs undergoing lytic (Prineas, 1985; Raine, 1990) or apoptotic (Raine and Scheinberg, 1988; Ozawa et al., 1994) cell death *in situ* in MS tissue are described, although their frequency remains to be established. OLs *in situ* do not appear to express major histocompatibility (MHC) molecules, prerequisites for recognition by antigen-specific cytotoxic T cells (Lee and Raine, 1989), raising the possibility that OL/myelin injury in MS may occur via MHC-unrestricted effector mechanisms. OLs in vitro do express MHC class I (Ruijs et al., 1990) but not MHC class II molecules (Grenier et al., 1989).

OLs *in vitro* have been shown to be susceptible to MHC unrestricted injury mediated either via soluble factor-dependent mechanisms (Selmaj and Raine, 1988; Kim and Kim, 1991; Selmaj et al., 1991a; Lee et al., 1993; D'Souza et al., 1995; Wilt et al., 1995) or by cell-cell contact-dependent mechanisms (Ruijs et al., 1990; Freedman et al., 1991; Merrill and Zimmermann, 1991; Zajicek et al., 1992; Antel et al., 1994; D'Souza et al., 1995). Prolonged exposure to tumor necrosis factor (TNF) α or β induces apoptotic cell death in OLs after 72-96 hr (Selmaj et al., 1991; D'Souza et al., 1995; Wilt et al., 1995). Mitogen-activated or myelin-reactive $CD4^+$ T cells acting in a MHC unrestricted manner can induce lysis of OLs without prior apoptosis (Antel et al., 1994).

Fas is a cell surface receptor belonging to the TNF receptor superfamily, that transduces cell death signals when ligated by agonist antibodies or by fas ligand (Suda and Nagata, 1994; Nagata and Golstein, 1995). Although fas signalling usually induces apoptotic cell death, fas ligation has been shown to trigger other cellular responses including proliferation (Aggarwal et al., 1995). $CD4^+$ and $CD8^+$ T cells (Nagata and Golstein, 1995) and macrophages (Badley et al., 1996), cell types found within active MS lesions (Raine, 1990) all express fas ligand (fasL) and *in vitro*, can induce injury via engagement of fas on target cells (Kagi et al., 1994; Stalder et al., 1994; Berke, 1995; el-Khatib et al., 1995; Golstein, 1995). Although, in initial studies, fas was not detected in the uninjured brain (Watanabe-Fukunaga et al., 1992; Matsuyama et al., 1994), recent reports suggest that fas expression can be induced in pathological conditions such as cerebral ischemia (Matsuyama et al., 1994) and Alzheimer's disease (Nishimura et al., 1995). To establish the potential involvement of fas signalling in OL cell death in MS, we have assessed whether fas is expressed on OLs *in vitro* and *in situ* in MS tissue, whether OLs *in vitro* are susceptible to injury mediated via the fas agonist antibody M3 and whether fas ligand is expressed *in situ* in MS tissue.

MATERIALS AND METHODS

Establishment of human CNS-derived glial cell cultures

Human brain tissue was obtained from patients undergoing temporal lobe resection or callosotomy as part of a surgical therapeutic treatment for intractable epilepsy. The glial cell isolation procedure has previously been described (Yong and Antel, 1992). Briefly, the brain tissue was subjected to enzymatic dissociation using trypsin (0.25%)(Gibco; Canada) and DNase I (25 µg/ml)(Boehringer Mannheim; Laval, Quebec) for 30 min at 37°C, and mechanical dissociation by passage through a 132 µm nylon mesh (Industrial Fabrics Corporation;

Minneapolis, MN). Mixed glial cells, consisting of approximately 70% OLs, 25% microglia and 5% astrocytes [assessed by 2'-3' cyclic nucleotide phosphodiesterase (CNPase), LeuM5, and glial fibrillary acidic protein (GFAP) immunoreactivity, respectively] were obtained by separation on a 30% Percoll (Pharmacia LKB; Montreal, Quebec)-gradient (15000 rpm at 4°C for 30 min). To enrich for OLs, freshly isolated mixed glial cells were left overnight in Falcon tissue culture flasks (Nunclon, Gibco) and the less adherent OLs were removed by gentle shaking. The differential adhesion protocol was repeated 24 hrs later on this semi-enriched OL culture. This population of OLs was identified using the rabbit anti-CNPase polyclonal antibody, a marker for mature OLs (1 hr at 1:40 dilution) (gift from Dr. Peter Braun, McGill University, Canada), followed by goat anti-rabbit IgG conjugated with Texas red (1 hr at 1:100 dilution) (Jackson Immunoresearch Lab; West Grove, PA) and was found to contain >90% OLs. The derived OLs were plated onto poly-L-lysine (10 µg/ml; Sigma, St. Louis, MO)-coated Aclar 9 mm diameter coverslips or into 96-well Nuntron (Becton Dickinson; Mountain View, CA) plates at a density of 5×10^4 cells per coverslip or microwell; coverslips were placed in Nuntron petri dishes. Microwells or petri dishes were filled with minimum essential culture medium supplemented with 5% FCS, 2.5 U/ml penicillin, 2.5 µg/ml streptomycin, and 0.1% glucose (all from Gibco; Canada). The OLs were allowed to extend processes, and were used in functional assays 2-4 weeks from the time of initial isolation. At this time, the OL preparations lacked endothelial and fibroblast cell contamination (Yong and Antel, 1992). The remaining adherent populations containing astrocytes and microglia were trypsinized and plated as described for the OLs to give mixed astrocyte-microglia cultures (up to 30% astrocyte purity); pure microglia cultures (>95% enriched) were obtained by shaking the less adherent astrocytes off (5 hr on a rotary shaker in a humidified incubator maintained at 37°C and 5% CO_2) and trypinizing and plating the cells as described for the OLs. Astrocytes were identified using polyclonal rabbit anti-GFAP (1 hr at 1:100 dilution)(Boehringer Mannheim), followed by goat anti-rabbit conjugated with Texas red (1 hr at 1:100 dilution),

and microglia were identified using an anti-LeuM5 (1 hr, neat) (Becton Dickinson), followed by Texas red conjugated goat anti mouse IgG2b Ab (Jackson Immunoresearch Lab; West Grove, PA).

Expression of fas on human adult CNS-derived glial cells *in vitro*

To determine whether target cells expressed fas, live unfixed target cells on coverslips were incubated with either M3 or M33, activating and non-activating anti-fas IgG_1 mAbs, respectively (1 hr at 5 μg/ml) (supplied by Immunex Corp., Seattle, WA), followed by biotinylated goat anti-mouse IgG (1 hr at 1:100 dilution) (Boehringer Mannheim), followed by FITC-conjugated Streptavidin (1 hr at 1:20 dilution) (Boehringer Mannheim). The cells were then fixed in acid/alcohol (5% glacial acetic acid/95% absolute ethanol). OLs were identified by anti-CNPase immunostaining, astrocytes were identified by anti-GFAP immunostaining and microglia, after blocking with mouse serum for 30 min, were identified by anti-LeuM5 immunostaining, as described above. Glial cell fas immunoreactivity (IR) was compared to fas IR on a panel of fas-expressing (U251 glioma cells, Jurkat T cells, U937 monocytic cells) and fas-non-expressing (L929 fibroblast cells) cell lines; the cell lines used in this study were single-stained for fas IR, as described above. Immunocytochemical analysis was performed using either a Reichert Polyvar 2 Leica immunofluorescence microscope, or in the case of the OLs, a confocal laser scanning microscope (Leica Lasertechnik, Heidelberg, Germany). Negative controls included omission of the primary antibody and the use of isotype-specific, irrelevant antibody. Samples were scanned with a 40 X 1.3 NA oil immersion objective with a band pass filter peaking at 535±7 nm for FITC specificity and a 580 nm high pass filter for Texas red.

Cell death assays

A. Membrane injury: LDH release assay and trypan blue uptake

To assess LDH release, previously described (D'Souza et al., 1995, 1996), cell free supernate was collected from OL cultures exposed to fas-ligation. Sample tubes containing 0.5 ml of 2 mg/ml NADH, 0.5 ml of 1.5 mmol/l pyruvate substrate and 100 µl of test sample were incubated for 30 min at 37°C. Pyruvate calibration curve tubes were set up. 1 ml of color reagent was added to each tube to stop the reaction. Absorbency was read at 460 nm. Test sample LDH was calculated by comparison with a curve generated using the pyruvate standards.

To assess trypan blue uptake, trypan blue (Sigma; St. Louis, MO) was added at a 1:1 dilution to cell cultures previously exposed to fas ligation. Cells staining blue, indicating membrane disruption, were counted and expressed as a percentage of the total number of cells counted.

B. Nuclear injury: Propidium iodide and TUNEL labelling

Nuclear fragmentation was assessed morphologically (nuclear fragmentation and chromatin condensation) by propidium iodide (PI) staining (10µg/ml for 20 min on coverslips fixed with acetone:methanol 1:1 for 10 min at -20°C), as previously described (D'Souza et al., 1995). DNA fragmentation was assessed using the terminal transferase (TdT)-mediated dUTP-biotin nick end-labelling (TUNEL) technique, previously described (D'Souza et al., 1995, 1996). For adherent target cells (OL, mixed astrocyte-microglia, microglia, U251 glioma cells and L929 cells), cells on coverslips were fixed in acetone:methanol (1:1) for 10 min at -20°C; non-adherent target cells were cytospun onto gelatin-coated slides and then fixed in acetone:methanol as described above. After rehydration

for 30 min in PBS, cells were incubated for 1 hr at 37°C with 50 µl of nick end-labelling solution containing TdT (0.3 U/ml) and biotinylated dUTP (0.01 nmol/ml) in TdT buffer (Promega Corp; Madison, WI). The reaction was terminated by incubation in Tris buffer (10 mM Tris-HCl, pH 6.8 for 15 min). After blocking with 2% BSA for 15 min, the cells were incubated with streptavidin-FITC (1:20 dilution, 30 min at 37°C) (Boehringer Mannheim). Hoechst dye 33258 (10 µg/ml, 20 min; Sigma, St. Louis, MO) was used to identify target cell nuclei.

$$\% \text{ apoptotic nuclei} = \frac{\text{number of TUNEL}^{+} \text{ nuclei}}{\text{number of Hoechst labelled nuclei}} \times 100\%$$

For each experiment (n), 200-400 cells were counted per coverslip or slide and counting was done by an observer blinded to the treatment received by the cells. Each test condition was assessed in triplicate per experiment.

Expression of fas and related molecules in normal and MS CNS tissue

A. Tissue samples

Early postmortem (between 4 and 8 hours) CNS tissue was obtained from 4 subjects with a clinical diagnosis of chronic progressive MS. Two patients were pathologically classified as chronic active (females aged 34 and 38 with MS for 2 and 10 years, respectively); and 2 as chronic silent MS (one male 46 y.o. with MS for 15 years, and one female 68 y.o., with a 25 year history of MS). A minimum of 2 blocks were studied for each case. Normal CNS tissue came from 3 subjects (mean age, 70 years) succumbing to non-neurological conditions. All tissue was embedded in OCT medium and stored at -70°C until use.

B. Immunohistochemistry

Frozen sections were air-dried, then fixed in 4% paraformaldhyde for 10 minutes. After quenching with 0.03% hydrogen peroxide and blocking with normal serum, sections were incubated with primary antibody overnight. Monoclonal anti-fas antibodies, M3 (Immunex Corp.) or UB2 (Immunotech Inc., West Brook, MD), were incubated at room temperature at a dilution of 1:200, whereas monoclonal IgM antibody Leu-7 (Becton Dickinson; San Jose, CA) and polyclonal antisera recognizing fasL (Santa Cruz Biotechnology; Santa Cruz, CA) and CNPase were used overnight at 4°C at 1:800, 1:3200 and 1:100 dilution respectively. Appropriate secondary biotinylated antibodies were applied for 60 minutes at room temperature followed by avidin-biotin-complex *Elite* reagent (Vector Labs; Burlingame, CA) for a further 45 minutes. The chromogen used was 3,3'-diaminobenzidine. Negative controls included omission of the primary antibody and the use of isotype-specific, irrelevant antibody.

RESULTS

Expression of fas on human adult CNS-derived glial cells *in vitro*

To determine whether human adult CNS-derived glial cells were capable of expressing cell surface fas, fas IR on 2-4 week old glial cell cultures was examined using an anti-fas IgG_1 (M3) monoclonal antibody (mAb) and compared with that on known fas-expressing cells including the Jurkat T cell line (Walsh et al., 1994; Chow et al., 1995), U937 myeloid leukemia cells (Sumimoto et al., 1994), and U251 human glioma (malignant astrocyte) cells (Weller et al., 1994) and on the known fas-negative L929 mouse fibroblast cell line (Walsh et al., 1994). The majority of OLs (79±4%, n=4 as determined by CNPase/fas double-immunolabelling) expressed fas with fas IR extending well out onto processes

(Fig. 1) whereas the microglia and astrocytes were fas-negative (Fig. 2). Fas IR using the M3 mAb on OLs was confirmed using another anti-fas mAb, M33 (Immunex Corp,). Similar staining patterns were observed (data not shown).

Susceptibility of human adult CNS-derived glial cells to fas-mediated injury

We next investigated whether human OLs were susceptible to fas-mediated injury. Anti-fas IgG_1 (M3) monoclonal antibody (mAb)(Lynch, 1995)(25 μg/ml; 24 hr incubation) was added to cultures of either adult human CNS-derived OLs, microglia, mixed astrocyte-microglia or the cell targets cited above. Crosslinking with anti-IgG_1 mAb for 24 hrs induced membrane injury of the OLs to an extent comparable to other fas-susceptible cells, including the malignant astrocyte cell line (U251), as measured by lactate dehydrogenase (LDH) release; microglia and the L929 cell line were resistant to membrane injury (Fig. 3). OL membrane injury by fas ligation with M3 was confirmed by trypan blue uptake (48.7±6.7% trypan blue positive cells compared to control cultures of 1.5±0.6%, n=3). Lack of pure cultures prevented testing of astrocytes in the LDH assay; however the trypan blue uptake assay revealed no loss of membrane integrity and cell viability in mixed astrocyte-microglia cultures upon fas ligation (4.4±2.1% trypan positive cells compared to control cultures of 3.6±1.3%, n=2). In contrast to other fas-susceptible cells, no nuclear or DNA fragmentation concomitant with membrane injury was observed in the OLs after 24 hrs fas ligation with M3 mAb, as assessed morphologically by propidium iodide (PI) staining (Fig. 3) or by the TUNEL technique (5.8±1.0% compared to control conditions of 3.6±2.3%, n=3). Within the first 24 hrs after fas ligation, there was no significant change in the number of adherent OLs (92.5±3.5% of the untreated control cultures, n=4), despite the LDH release; significant OL process retraction, however, was evident. However, after 4 days, significant cell loss was apparent (25.3±7.2% of the untreated control cultures, n=4). In all target cells tested, replacement of anti-fas M3 mAb with a non-activating anti-fas (M33) mAb (Lynch, 1995), or with anti-galactocerebroside

(GalC) - an OL-specific cell surface-expressed molecule, or with anti-IgG_1 mAb alone did not induce significant injury compared to control cultures (Fig. 3 legend).

Induction of rapid and delayed apoptotic responses in human adult CNS-derived oligodendrocytes

The lack of evident apoptosis prior to, or concomitant with membrane injury in the OLs upon fas ligation, contrasts with previous findings using other cell targets in which fas ligation is associated with induction of apoptotic cell death. Our findings could reflect that OLs have an indolent apoptotic program that is not activated after 24 hrs of fas ligation. In this regard, we have previously demonstrated that TNF-α or serum deprivation induces a delayed apoptotic response in human OLs after 72-96 hrs of exposure (D'Souza et al., 1995, 1996). However, the addition of membrane soluble C2-ceramide (50 μM), an analog of the complex lipid ceramide, a molecule that has been implicated in apoptotic signalling pathways (Obeid et al., 1993; Cifone et al., 1994; Gulbins et al., 1995; Tepper et al., 1995; Verheij et al., 1996) induced rapid apoptosis of the OLs within 18 hrs, as determined by the TUNEL technique (52.6±4.4% apoptotic cells compared to control conditions of 4.6±1.3%, n=5)(Fig. 4). In addition, fas ligation of the OLs for 96 hrs did not induce apoptosis of the OLs as assessed by the TUNEL technique (6.3±2.1%, n=3). Taken together, these data suggest that while OLs have the capability of undergoing both rapid and delayed apoptotic responses upon exposure to C2-ceramide and TNF-α respectively, their response to fas ligation does not involve apoptosis, but rather, lysis.

Expression of fas and related molecules in normal and MS CNS tissue

In normal CNS white matter, by immunohistochemistry, faint fas immunoreactivity using the M3 mAb was detected on scattered glial cells. This

occurred on cells with small, round nuclei, a thin rim of cytoplasm and one or two tenous processes (Fig. 5a). Such elements were identified as OLs by their morphologic phenotype and positive staining for Leu-7 and CNPase in serial sections. Fas was also constitutively expressed on endothelial cells of small blood vessels. Other glial cells and neurons were invariably fas-negative. In tissue from all cases of MS, fas reactivity was prominent on OLs along the margin of lesions and in adjacent white matter (Figs. 5 c and d). These same cells also stained positively for CNPase and Leu-7 in serial sections (Figs 5 e and f) although the pattern of staining was different with Leu-7 and CNPase staining the cell body and M3, the cell membrane and its fine processes. Fas IR on OLs in MS lesions was confirmed using another anti-fas mAb, UB2 (Immunotech Inc.) and similar results were obtained (data not shown). Apart from endothelial cells and infiltrating lymphocytes (Fig. 5b), no other cell type showed fas reactivity. Staining for fas ligand in MS lesions (Figs 5 g, h) revealed intense positivity on microglia and scattered infiltrating lymphocytes, but not on OLs or astrocytes; weak, but significant fas ligand expression was detectable on microglia in normal postmortem CNS white matter tissue (data not shown).

DISCUSSION

In this report, we describe results from *in vitro* and *in situ* studies that support a role for fas mediated signalling in the susceptibility of OLs to immune-mediated injury, information of relevance to the pathogenesis of MS. The *in vitro* studies indicated that amongst human CNS glial cells, OLs selectively express fas, resulting in their differential susceptibility to injury via this pathway. Although human OLs are capable of undergoing apoptotic responses, either rapidly as shown in this study using C2-ceramide, or in a delayed manner in response to TNF or serum deprivation (D'Souza et al., 1995), their response to fas ligation involves a lytic, rather than an apoptotic form of cell injury. Cross-ligation of

antibodies directed against other surface molecules expressed on OLs, including anti-GalC and non-activating anti-fas mAb, M33, did not induce lysis of the OLs, excluding the possibility that the novel lytic response of OLs to fas ligation was due to complement-mediated lysis. A lytic response of OLs *in vivo* could result in the release of myelin antigens, thus provoking further inflammation. Most previous cytotoxicity studies involving fas-mediated target cell injury have involved the use of proliferating targets; mature OLs rarely, if ever, divide in culture (Prabhakar et al., 1995). It is possible that end-mitotic cells such as OLs may employ a fas signal transduction pathway that is different from proliferating targets, or alternatively, the cell cycle may play a role in shaping the cell death response triggered by fas ligation. This would be in keeping with our previous data in which $CD4^+$ αβ T cells , widely known to employ the fas pathway in effecting target cell injury (Stalder et al., 1994; el-Khatib et al., 1995) induced apoptotic lysis in proliferating U251 glioma cells, but induced lysis without prior DNA fragmentation in OLs. The existence of different cell injury mechanisms (lysis and apoptosis) of OLs have therapeutic implications. We have previously demonstrated (D'Souza et al., 1996), as reported by others (Louis et al., 1993; Kahn and DeVellis, 1994) that CNTF selectively protects OLs from nuclear-directed, but not lytic cell death (Appendix II contains data on the effect of CNTF on OLs subjected to fas-mediated lytic injury).

Our finding that OL cell loss does not occur after 24 hr of fas ligation, despite LDH release, raises the possibility that fas ligation could result in sublethal injury of OLs, affecting their capacity to maintain their myelin without actual cell body loss. The significant retraction of OL processes observed during the period of fas ligation supports this contention. We have previously shown that $CD4^+$ T cells activated with anti-CD3 mAb and interlukin-2 also induces LDH or ^{51}Chromium release from human OLs without OL cell loss (D'Souza et al., 1995). The timing of OL depletion in MS remains to be defined although the consensus of most studies is that depletion is a late event (Raine, 1994). The

delayed OL cell loss that occurred *in vitro* upon more prolonged fas ligation could thus reflect the findings in more established MS lesions.

Our demonstration of selective upregulation of fas expression on OLs and upregulation of fas-ligand expression on endogenous microglia (scattered infiltrating lymphocytes also expressed fas ligand), in active MS lesions compared to control CNS tissue, further implicates fas signalling as a potential mechanism for MHC-unrestricted OL injury in MS. Such upregulation could reflect the effects of inflammatory mediators in the MS plaque milieu (Raine, 1994) in a manner akin to cytokine [interferon (IFN)-γ]-induced upregulation of fas on lymphocytes (Yonehara et al., 1989; Moller et al., 1993). In fact, IFN-γ and lipopolysaccharide (LPS), known activators of microglia (Williams et al., 1992), in combination, upregulate fas-ligand expression on human microglia *in vitro* (B.B, S.D.D, J.P.A and C.S.R, manuscript in preparation). Mechanisms for selective OL injury in MS are of particular interest in that OLs *in situ* do not express MHC molecules, prerequisites for antigen-specific interaction with cytotoxic T cells (Lee and Raine, 1989). While MHC-unrestricted mechanisms of OL injury have been postulated, the apparent lack of specificity of these mechanisms raises the issue as to how such mechanisms could selectively injure OLs and spare other neural cells in MS. One possible explanation could be that OLs are selectively vulnerable to these immune effector mechanisms. We have previously shown that human OLs, amongst other human CNS-derived neural cells, are selectively vulnerable to TNF-mediated apoptosis (D'Souza et al., 1995). Griot et al., 1990 have shown that OLs are selectively vulnerable to reactive oxygen species. Another possible explanation could be that certain immune recognition molecules are selectively upregulated on OLs in MS lesions. In this regard, previous studies have shown that OLs in MS lesions selectively express heat shock proteins (hsps), such as hsp-60 (Selmaj et al., 1991b); heat shock proteins have been postulated as putative ligands for cytolytic γδ-T cells (Haregewoin et al., 1989; Indreshpal et al., 1993). *In vitro* studies on human OLs have shown that the cytokine, interlukin-1,

selectively upregulates hsp-72 expression on OLs (D'Souza et al., 1994) and that γδ-T cells are lytic to human OLs *in vitro* (Freedman et al., 1991). Our demonstration here that fas is selectively upregulated on OLs in MS lesions, further extends our understanding of the basis for selective OL injury in MS. The availability of soluble fas, neutralizing antibodies to fas or fas ligand, or inhibitors of fas ligand induction or fas-mediated cell death provides potential means to manipulate this signalling pathway for therapeutic applications in MS.

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FIGURE 1: Fas expression on human adult CNS-derived oligodendrocytes as assessed by confocal laser scanning microscopy. Human oligodendrocytes were maintained in 5% serum-supplemented culture medium for 3 weeks and assessed for **(a)** fas immunoreactivity (green) and **(b)** CNPase immunoreactivity (red). **(c)** double-staining is depicted by superimposing the fas (green) and CNPase (red) images, in which yellow indicates co-localization of the fas and CNPase signal. **(d)** an irrelevant IgG_1 mAb isotype control for oligodendrocyte immunostaining.

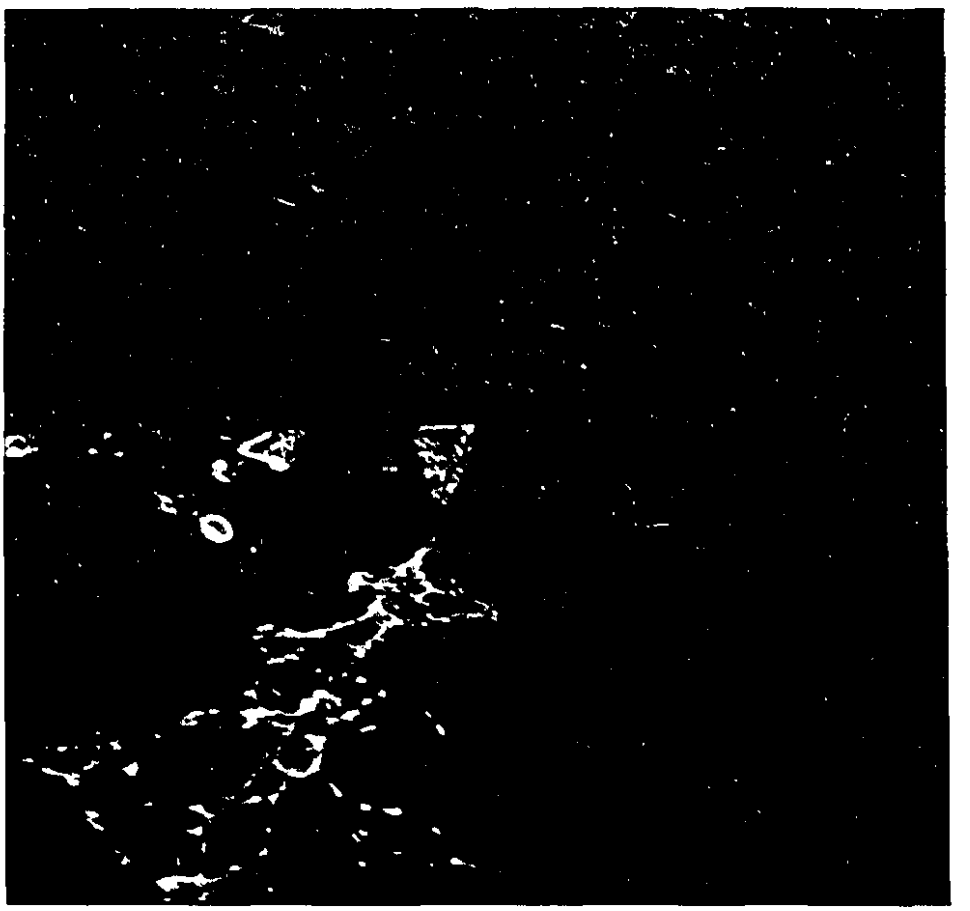

FIGURE 2: Fas expression on human adult CNS-derived astrocytes, microglial cells and known fas-expressing (U251 glioma cells, Jurkat T cells, and U937 myeloid leukemia cells) and fas-negative cells (L929 fibroblast cells). Panels **a** and **b** (Mag X 400) represent adult astrocytes: **(a)** lack of fas immunoreactivity on astrocytes and **(b)** GFAP immunoreactivity of the same field as in **(a)** to identify astrocytes. Panels **c** and **d** (Mag X 250) represent adult microglial cells. **(c)** lack of fas immunoreactivity on microglia and **(d)** Leu M5 immunoreactivity of the same field as in **(c)** to identify microglia. The remainder of the panels represent fas immunoreactivity on **(e)** U251 glioma cells (Mag X 400), **(f)** Jurkat T cells (Mag X 250), **(g)** U937 myeloid leukemia cells (Mag X 400) and **(h)** L929 fibroblast cells (Mag X 400).

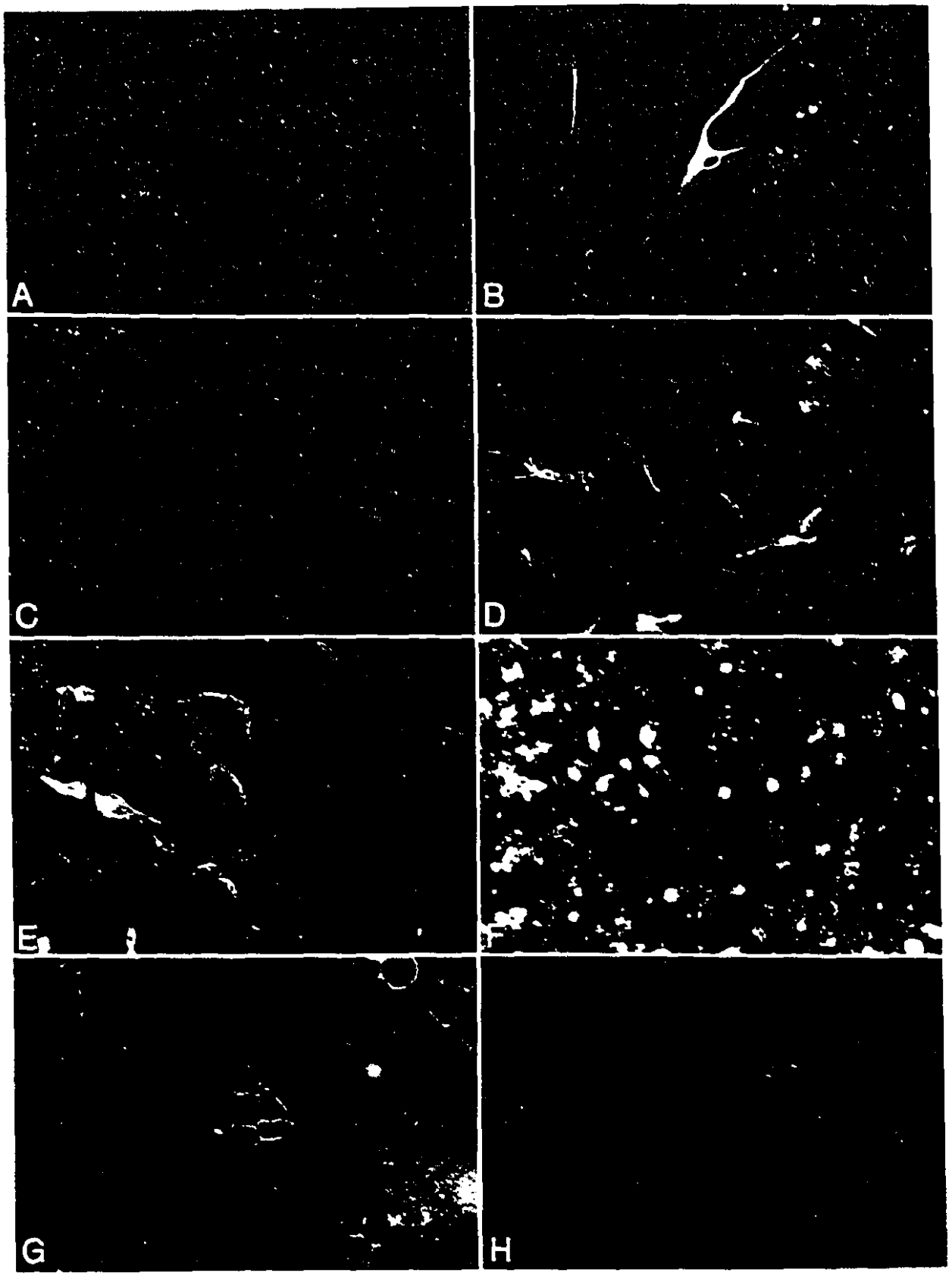
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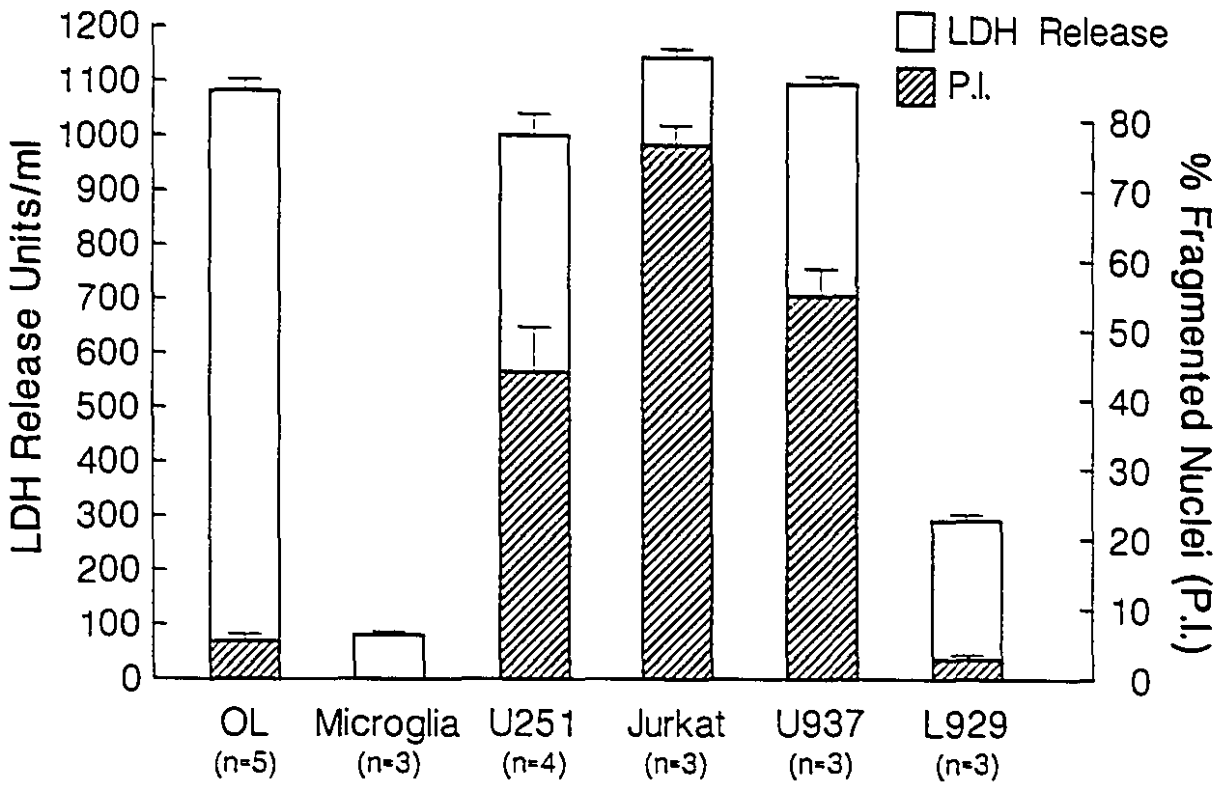
FIGURE 3: Mean LDH release in units per ml (unshaded bars) and mean % fragmented nuclei as assessed by propidium iodide (PI) staining (shaded bars) for the indicated cell targets treated with anti-fas IgG_1 (M3) (25 µg/ml; 24 hrs) mAb followed by crosslinking with anti-IgG_1 mAb (1:100 dilution; 24 hrs). Mean LDH release for control target cells, or target cells treated with anti-fas (M3) mAb alone, or non-activating fas (M33) mAb or anti-GalC followed by crosslinking with anti-IgG_1, or anti-IgG_1 alone was less than 200 units per ml. 'n' represents the number of LDH experiments performed; for PI staining studies, n=3. For LDH and PI studies, each test condition was assessed in triplicate per experiment.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Microglia
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U251
(n=4)
Jurkat
(n=3)
U937
(n=3)
L929
(n=3)

FIGURE 4: Induction of DNA fragmentation in human adult CNS-derived oligodendrocytes, as assessed by the TUNEL technique, **(a)** after exposure to C-2 ceramide (50 μM) for 18 hr (Mag X 250). **(b)** represents oligodendrocytes under control culture conditions.

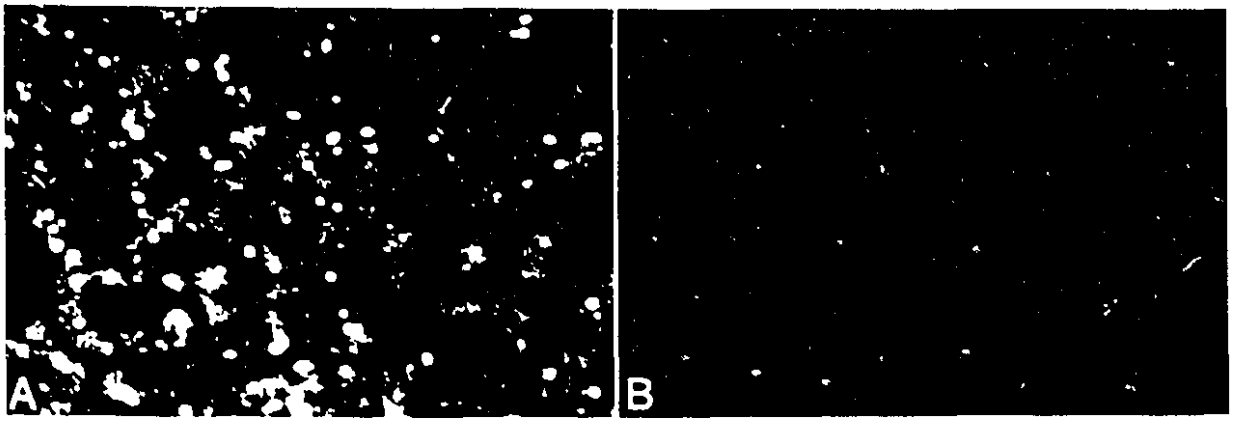
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FIGURE 5: Expression of fas and related molecules in normal and MS CNS tissue.

(a) Normal human white matter reacted with M3 anti-fas monoclonal antibody. Note the faintly positive cells (brown) which have the morphology of oligodendrocytes. Frozen section; DAB-reacted and counter-stained with hematoxylin (Mag X 750).

(b) The edge of a chronic active MS lesion shows fas-positive infiltrating cells around a blood vessel. DAB-immunoreacted, no counterstain (Mag X 750).

(c) White matter adjacent to a chronic active MS lesion shows numerous fas-positive interfascicular oligodendrocytes (brown). DAB and hematoxylin (Mag X 300).

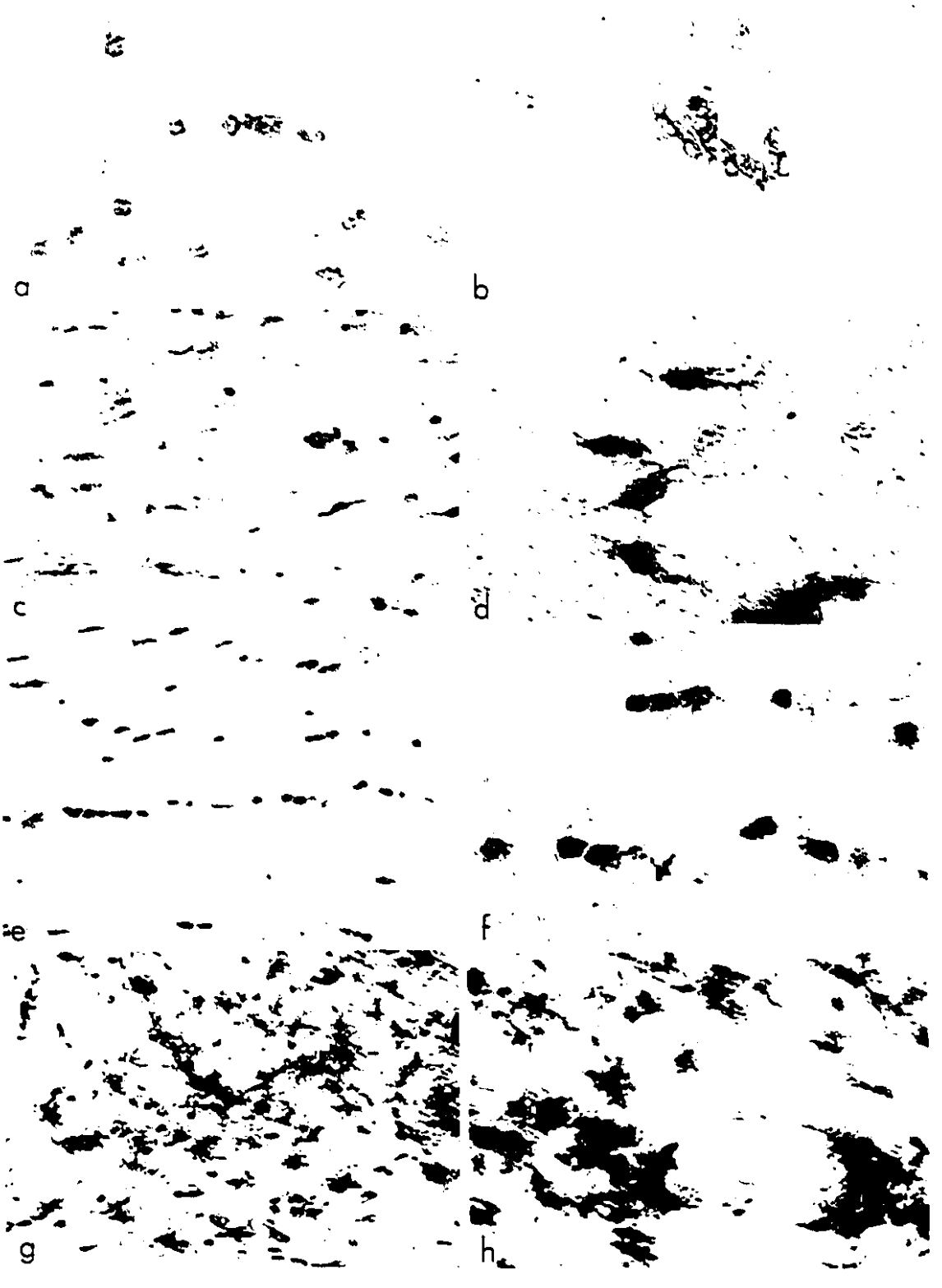
(d) Detail of fas-positive oligodendrocytes from the edge of a chronic silent MS lesion. Note the typical bipolar outline of the cells. DAB and hematoxylin (Mag X 750).

(e) Interfascicular oligodendrocytes in white matter adjacent to a chronic silent MS lesion show positive immunoreactivity for Leu-7. Similar, but less intense staining was obtained with anti-CNPase anti-serum. DAB and hematoxylin (Mag X 300).

(f) Detail of Leu-7 positive interfascicular oligodendrocytes from **(e)**. DAB and hematoxylin (Mag X 750)

(g) Microglial cells at the periphery of a chronic silent MS lesion display intense immunoreactivity for fas ligand (fasL). DAB and hematoxylin (Mag X 300).

(h) Detail of fasL positive microglia at the periphery of a chronic silent MS lesion. DAB and hematoxylin (Mag X 750).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CHAPTER 6: SUMMARY AND DISCUSSION

SUMMARY AND DISCUSSION

The experimental data presented in this thesis indicates that non-specific immune-mediators may induce oligodendrocyte (OL)-specific injury, information that may be of relevance to the pathogenesis of multiple sclerosis (MS), via the mechanisms detailed below:

(1). Selective upregulation of immune recognition molecules on oligodendrocytes

(a) In this thesis, using dissociated cultures of human OL, experimental evidence for the cytokine-mediated selective upregulation on OL of heat shock protein (HSP)-72, a putative ligand for cytolytic γδ-T cells, is presented (chapter 2). Selective upregulation of HSP on OL may enhance potentially deleterious OL-immune interactions which may be dependent on HSP recognition. Previous studies on HSP expression in MS lesions revealed the colocalization of $HSP65^{+}$-expressing OL with γδ-T cells (Selmaj et al., 1991; Selmaj et al., 1992).

Recently, another HSP molecule, the small heat shock protein αB-crystallin was detected on OL in MS but not in control CNS white matter (van Noort et al., 1995). Intense OL-specific immunoreactivity for αB-crystallin was frequent in early lesions, whereas astrocytic staining predominated in older lesions. The factors that induce upregulation of the large HSP60 are probably different from those that trigger expression of the small HSP αB-crystallin. In addition, while HSP65 may serve to trigger responses in T cells bearing the γδ-T cell receptor, αB-crystallin triggers responses in αβ- but not γδ-T cells. As such, it is possible that different pathogenetic mechanisms are operative amongst MS patients. Ozawa et al (1994) have attributed the case-to-case variability in the number of degenerating OL observed in MS lesions to the existence of different pathogenetic

mechanisms amongst patients.

(b) This thesis provides the first report of the potential involvement of a novel immune recognition molecule, fas, in OL cell death in MS (chapter 5). Fas was shown to be selectively expressed on OL amongst human glial cells *in vitro*. Cross-linking of surface-expressed fas on OL resulted in a hitherto novel fas-mediated injury response, apoptosis-independent lysis rather than apoptosis. This novel injury response was restricted to post-mitotic adult human OL; other proliferating neural and non-neural cell lines underwent apoptosis upon fas ligation. An issue that needs to be addressed is why does fas ligation on OL not activate the apoptosis cascade as it does in most other cells? It is possible that signalling components involved in cell-cycle arrest may alter the response of OL to fas activation. Experiments comparing the fas-activation response of post-mitotic OL to proliferating oligodendrogliomas may provide further insight regarding this possibility. Alternatively, OL may utilize a novel signal transduction pathway in response to fas activation. Fas activation in some cells induces responses other than apoptosis. Aggarwal et al. (1995) have shown that fas activation in mouse fibroblasts results in cell proliferation rather than apoptosis. The effects of fas are now considered to be mediated via the second messenger ceramide, generated by the hydrolysis of sphingomyelin by activated acidic sphingomyelinase (Cifone et al., 1994). C2-ceramide, a cell-permeable analog of ceramide induces apoptosis in OL. This raises the issue as to whether fas activation in OL generates intracellular ceramide. Measurement of intracellular ceramide fluxes or sphingomyelin hydrolysis in OL upon fas activation would reveal whether this signalling pathway is coupled to activation of the fas receptor in OL. As will be discussed later, the existence of different cell injury mechanisms of OL has therapeutic implications.

Our demonstration of selective upregulation of fas on OL and upregulation of fas ligand on endogenous microglia in active MS lesions compared to control

CNS tissue, further implicates fas signalling as a potential mechanism for selective injury of OL in MS. Direct verification of the fas signalling pathway in immune-mediated injury of OL could be tested in the experimental model, experimental allergic encephalomyelitis (EAE). In this case, issues that need be addressed include whether OL in demyelinating EAE lesions selectively express fas, whether microglia or lymphocytes in such lesions express fas ligand, whether intrathecal CNS administration of agonist fas antibodies or fas ligand induces severe demyelination in EAE mice, and whether intrathecal CNS administration of inhibitors such as soluble fas or neutralizing antibodies to fas or fas ligand reduce demyelination in such conditions. Experiments aimed at downregulating OL fas expression and susceptibility to fas ligation, using for example, inhibitory cytokines such as interlukin (IL)-4, IL-10, or transforming growth factor (TGF)-β, may prove to be a promising avenue of research.

(2) Selective vulnerability of OL to a common immune mediator.

Selective vulnerability of OL amongst neural cells to a common immune mediator could represent another mechanism whereby apparently non-specific immune mediators may induce selective OL injury. We have previously shown that human OL are susceptible to TNF-dependent and non-TNF-dependent immune effector mechanisms (Appendix I). The former involves initial nuclear injury (apoptosis); the latter when mediated by activated T cells, involves cell membrane injury (lysis). In this thesis, the selective vulnerability of OL amongst human CNS-derived neural cells to TNF-mediated injury is demonstrated. All these cells express TNF receptors. The findings of our study not only further implicate TNF in the pathogenesis of MS, but also complements a recent immunogenetic study conducted on MS patients. In this study, Zipp et al. (1995), reasoning that since both TNFα and TNFβ are encoded in the HLA region, the HLA association of MS (HLA-DR2) may be related to the production of these cytokines. To test this hypothesis, they investigated the production of TNFα,

TNFβ and γ-IFN by $CD4^+$ T cell lines specific for myelin basic protein (MBP) isolated from MS patients and normal controls. Antigen-stimulated $CD4^+$ T cells from $HLA\text{-}DR2^+$ donors produced significantly more TNFα and TNFβ than $CD4^+$ T cells from $DR2^-$ donors. In contrast, $HLA\text{-}DR2^+$ and $DR2^-$ $CD4^+$ T cells did not differ in the production of γ-IFN, a cytokine also produced by T cells but not encoded in the HLA region. The results suggest that the association of MS with HLA-DR2 implies a genetically determined propensity of T cells to produce increased amounts of TNF. In such individuals, increased amounts of TNF secreted by T cell infiltrates at lesion sites could lead to, as the work of this thesis indicates, selective injury of OL and myelin.

Two issues in particular that deserve further investigation are (i) the mechanisms that confer OL selective vulnerability to TNF, and (ii) the mechanisms that confer fetal astrocyte resistance to TNF. To approach these issues, one would have to consider receptor/receptor-associated protein interactions, proximal signalling events, distal signalling events and the contribution of pro-apoptotic and anti-apoptotic genes to the TNF signalling cascade. A detailed consideration of TNF signalling is beyond the scope of this discussion and the reader is referred to the following excellent articles: Baker and Reddy, 1996; Hsu et al., 1996 for receptor/receptor-associated protein interactions; Kolesnick and Golde, 1994 for proximal signalling events; Verheij et al., 1996 for distal signalling events; Oltvai and Korsmeyer, 1994; Nicholson, 1996 for contribution of pro-apoptotic and anti-apoptotic genes.

In addition to addressing the mechanisms which could account for the relatively selective injury of OL, as occurs in MS, the thesis also addressed the issue of how strategies aimed at protecting OL from immune-mediated injury would need to consider the mechanisms of such injury (Chapter 4). The work presented in this thesis showed that only ciliary neurotrophic factor (CNTF) amongst an array of neurotrophic factors and cytokines protected OL from TNF-

mediated apoptosis. CNTF also protected OL from serum-deprivation induced apoptosis. It did not protect other neural cells from TNF or serum-deprivation induced apoptosis. It also did not protect OL from lytic injury mediated by mitogen-activated $CD4^+$ T cells or by fas ligation (Appendix II). The protective effects of CNTF on OL exposed to TNF are cell-specific and mechanisms-specific. Questions that need further study include what cell signalling pathway mediates the protective effect of CNTF on apoptosis of OL induced by TNF or serum deprivation and why does CNTF protect OL in a cell-specific and mechanism-specific manner.

The IL-6 family that include CNTF, leukemia inhibitory factor (LIF) and IL-6 among others (see General Introduction -Chapter 1), utilize two receptor components, gp130 and LIFRβ, that are shared with other members of this family. CNTF however differs from its relatives in that its actions are largely limited to cells of the nervous system due to the restricted expression of one of its receptor components, CNTFRα. CNTFRα does not play a direct role in signalling, but instead forms a complex with CNTF that promotes its binding to the signal transducing "β" receptor components, gp130 and LIFRβ. *In situ* hybridization studies have shown that CNTFRα is expressed widely throughout the adult peripheral and central nervous systems, with most of the expression localized to neuronal cells; no significant hybridization signals were observed in non-neuronal cells, including OL in CNS white matter (Ip et al., 1993).

Thus the known effects of CNTF on OL and those reported in this thesis would appear difficult to explain with this simple model. Subsequent experiments showed that several cell lines expressing gp130 and LIFRβ could also respond to CNTF in the absence of CNTFRα, indicating that CNTFRα is not an absolute prerequisite for CNTF signal transduction (Davis et al., 1993; Gearing et al., 1994). A more likely possibility is that the expression of CNTFRα in the non-neuronal cells such as OL is so low that it cannot be detected by *in situ*

hybridization. Alternatively, CNTFRα might not be expressed by OL but available in soluble form from the small percentage of contaminating glial cells in the OL preparations (Davis et al., 1993), and thus be able to form a functional receptor after association with gp130 and LIFRβ on the cell surface. The use of highly purified OL preparations by fluorescence activated cell sorting (FACS) may help clarify this issue. Owing to the paucity of human adult CNS-derived OL, this may not be possible. The use of OL cell lines may be useful in this case.

To conclude, the work of this thesis suggests that selective target injury in MS may reflect target-cell rather than effector-cell properties. The potential protective effects of neurotrophic factors and cytokines on neural cells may be specific for both target cell type and nature of the target cell injury response.

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APPENDICES I AND II

APPENDIX I

Cytokines produced by infiltrating hematogenous cells or by glial cells activated during the course of central nervous system (CNS) disease or trauma are implicated as mediators of tissue injury. In the human demyelinating disease multiple sclerosis (MS), the cytokine tumor necrosis factor (TNF) is strongly implicated in contributing to the destruction of myelin and its cell of origin, the oligodendrocyte (OL). The following summary represents initial data (McLaurin et al., 1995) obtained assessing the extent and mechanism of injury of human-derived CNS oligodendrocytes (OL) *in vitro* mediated by the cytokines tumor necrosis factor (TNF)-α and β compared with the previously described tumor necrosis factor independent effects mediated by mitogen-activated $CD4^+$ T cells (Antel et al., 1994). We found that activated $CD4^+$ T cells, but not TNF-α or β (1000 U/ml) could induce significant release of lactate dehydrogenase (LDH), a measure of cell membrane lysis, from OL within 24 hr (Fig 1a,b). Neither induced DNA fragmentation (apoptosis) as measured by the fluorescence TdT-dUTP nick end-labelling (TUNEL) technique at this time point (Fig 1a,b). After a prolonged time period (96 hr), TNF-α did induce DNA fragmentation (apoptosis) in a significant proportion of OL without increased LDH release (Figs 1c, 2). TNF-α-mediated apoptosis of OL was confirmed by electron microscopy (Fig 3). Other pro-inflammatory cytokines such as interlukin-1β and γ-interferon (IFN) did not induce DNA fragmentation in the OL as assessed by TUNEL staining [6±0.3% for γ-IFN (n=2) and 4.3±0.3% for IL-1β (n=2) compared to 5.7±0.9% for control cultures (n=9)]. These results suggest that human OL are susceptible to both TNF-dependent and TNF-independent mechanisms of immune-mediated injury; the former results in OL apoptosis whereas the latter, when mediated by mitogen-activated $CD4^+$ T cells, results in OL lysis.

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FIGURE 1: Comparison of mechanisms of human OL injury mediated by $CD4^+$ T cells (10:1 effector:target ratio) and TNF-α (1000 units/ml). **(A)** Cell membrane injury as measured by LDH release (units/ml). **(B)** Nuclear injury as measured by percent of cells showing DNA fragmentation by the fluorescence TdT-dUTP nick end-labelling (TUNEL) technique. $CD4^+$ T cell effects were assayed at 24 hr (a); TNF-α effects were assayed at 24 (b) or 96 hr (c). Each bar represents the result of an individual experiment in which LDH and TUNEL assays were applied to the same target cells exposed to the indicated effector condition. Open bars in [A(a)] indicate LDH in $CD4^+$ T cell: OL co-cultures; hatched bars indicate LDH in cultures containing only $CD4^+$ T cells. LDH release by OL alone did not differ significantly from LDH values in serum-containing culture medium alone.

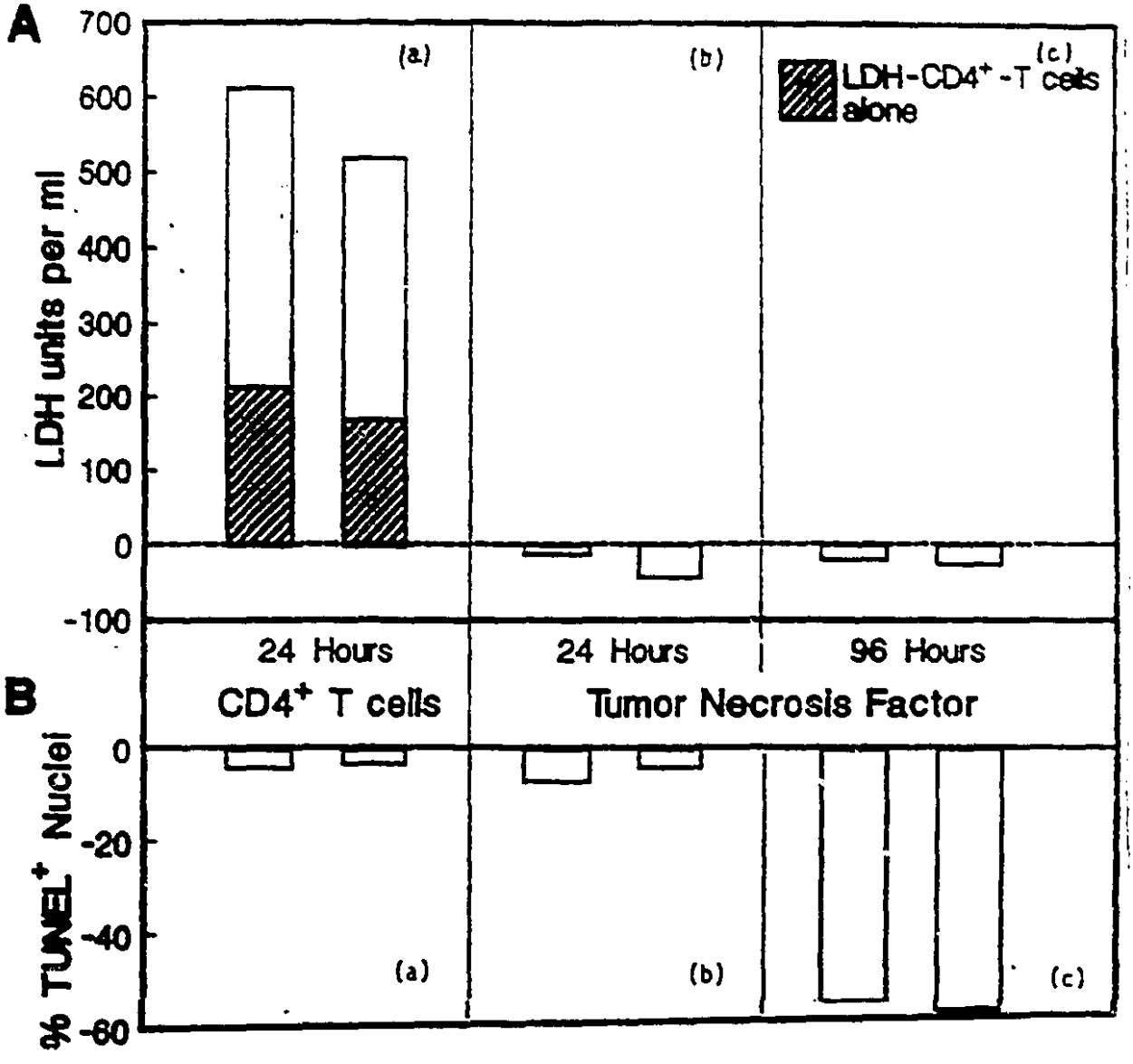
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FIGURE 2: Four-week-old human adult OL maintained in dissociated culture and then exposed to 1000 units/ml TNF-α for 96 hr. Panels A and D are TNF-treated and non-treated (control) cultures, respectively, immunostained with anti-myelin basic protein antibody to identify OL. Panels B and E are the corresponding treated and control cultures labelled with the TUNEL technique. Panels C and F are the corresponding treated and control cultures stained with Hoechst dye 33258 to identify OL nuclei. (Mag X 300)

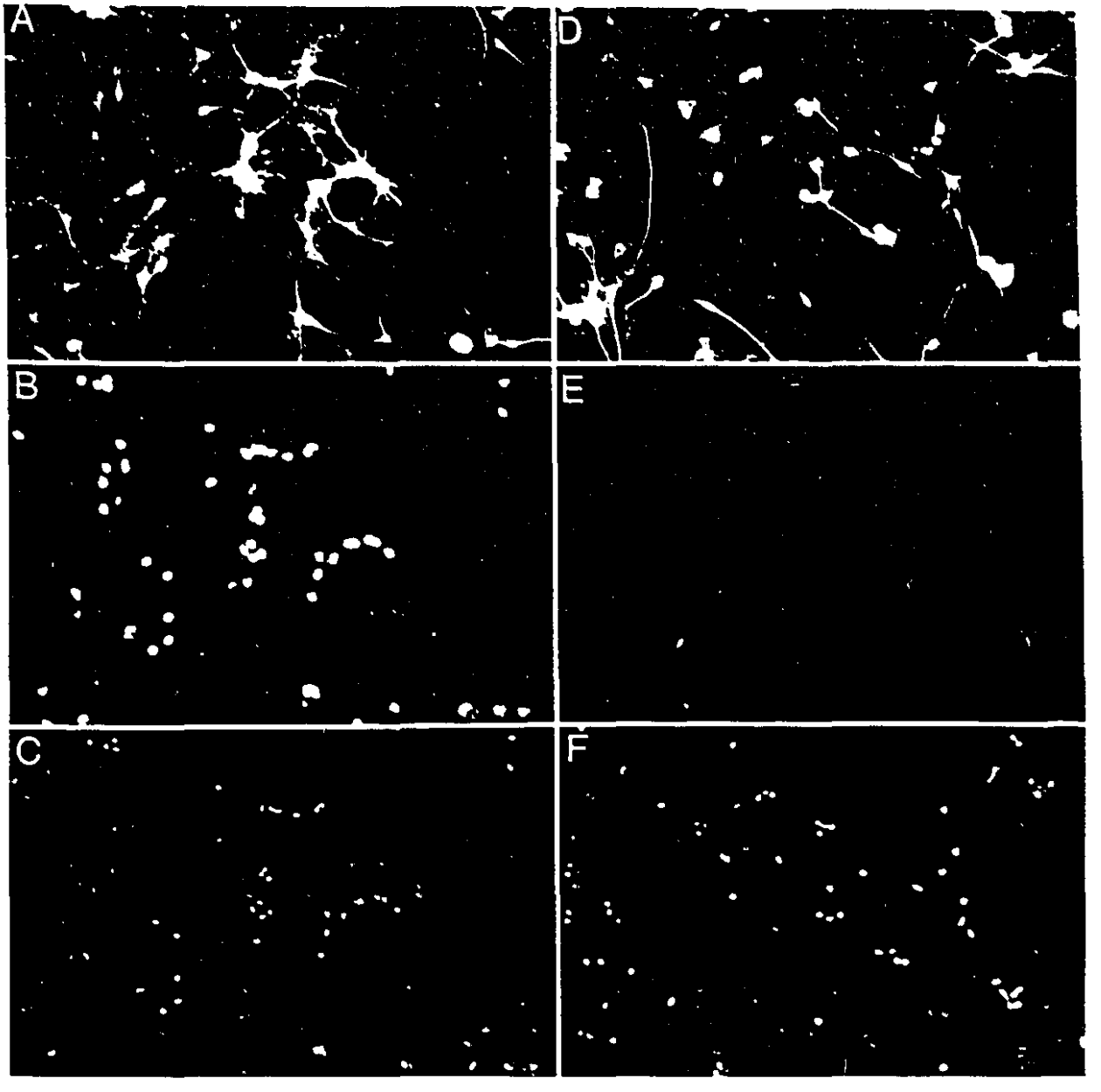
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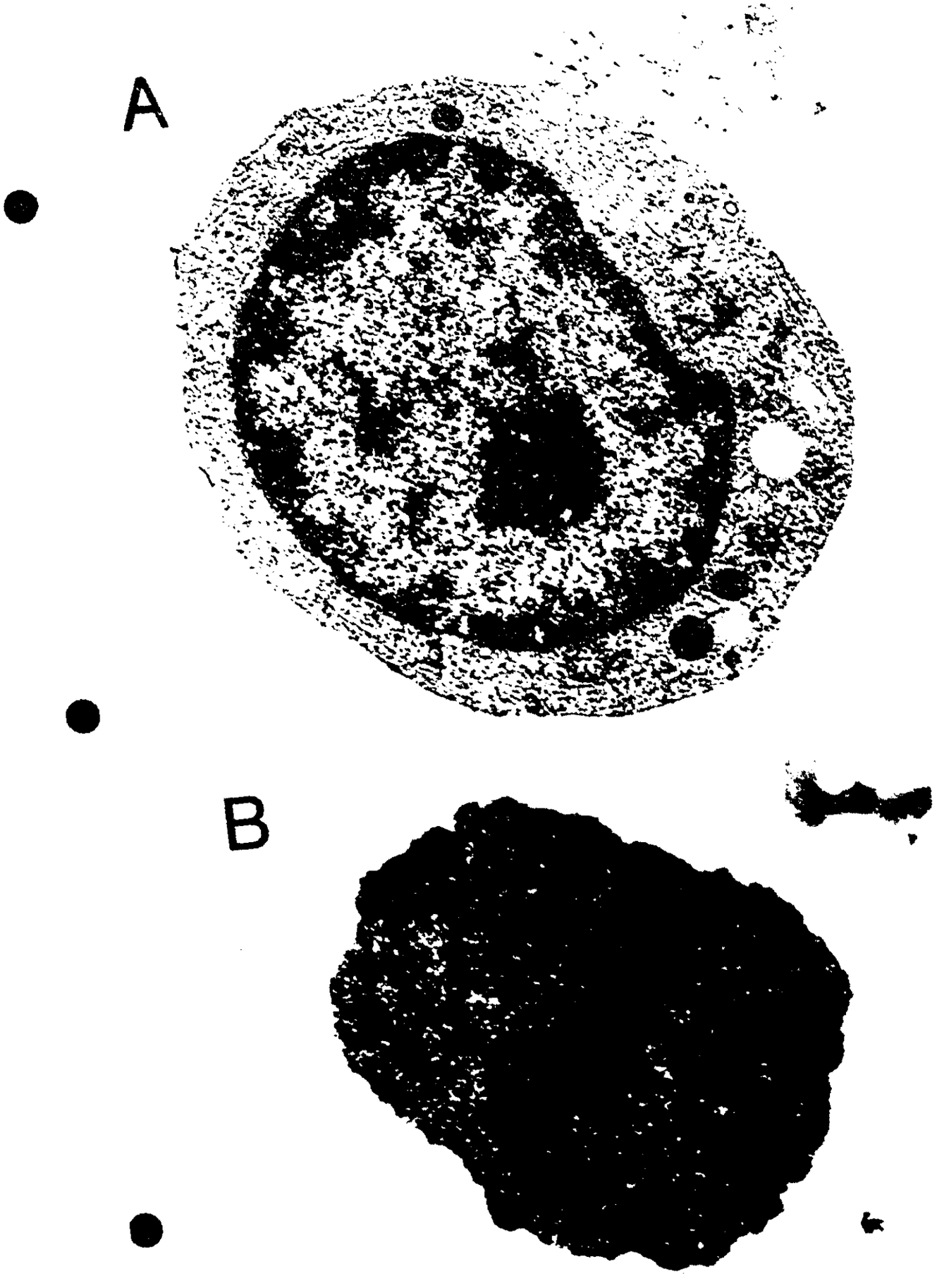
FIGURE 3: Electron micrograph of human adult OL maintained in dissociated cell culture under control conditions (A) or exposed to TNF-α 1000 units/ml (B) for 72 hr. TNF-α-exposed cells show chromatin condensation, cell volume reduction and membrane blebbing, characteristics typical of cells undergoing apoptosis. (Mag X 10000).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APPENDIX II

We have previously shown that the protective effects of ciliary neurotrophic factor (CNTF) on oligodendrocytes (OL) are mechanism-specific, in that CNTF protected OL from TNF- and serum-deprivation-induced apoptosis but not from lysis mediated by activated $CD4^+$ T cells. Subsequent to the finding that fas ligation on human CNS-derived OL induced a novel apoptosis-independent lytic death of the OL (Chapter 5), we tested whether CNTF could protect OL from fas-mediated lytic injury.

CNTF at a concentration of 100 ng/ml was added to OL cultures maintained in 5% serum-supplemented medium and exposed to anti-fas (M3)(IgG_1) antibody (25 µg/ml; 24 hr) followed by cross-ligation with anti mouse IgG_1 antibody (1:100 dilution for 24 hr). Membrane injury was assessed using the lactate dehydrogenase (LDH) release assay for the following test conditions: medium alone, OL alone, OL plus anti-fas IgG_1 plus anti-IgG_1, OL plus anti-fas IgG1 plus anti-IgG1 plus CNTF, OL plus CNTF. LDH results were expressed as LDH release for test condition minus LDH release for the medium alone condition.

CNTF did not protect OL from fas-mediated cytolysis (1254±52 LDH units per ml for OL plus anti-fas IgG_1 plus anti-IgG_1 compared to 1211±72 LDH units per ml for OL plus anti-fas IgG_1 plus anti-IgG_1 plus CNTF, n=5). LDH values for OL alone cultures was 118±45 LDH units per ml (n=5).

Our findings that CNTF does not protect OL from fas-mediated cytolysis further supports our previous findings (Chapter 4) that the protective effects of CNTF on OL are mechanism-specific.