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DIFFERENTIAL EFFECTS OF IFN-β-1b ON αβ vs. γδ T CELLS: AN INVESTIGATION OF ACTIVATION, PROLIFERATION AND ACTIVATION-INDUCED APOPTOSIS

A Thesis submitted to the

School of Graduate Studies

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In Partial Fulfillment of the Requirements for the Degree

of

Master of Science

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School of Medicine

By

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ABSTRACT

αβ and γδ T cells are both postulated to play a role in the pathogenesis of Multiple Sclerosis (MS). IFN-β-1b (Betaseron), a novel treatment for MS, can change the natural course of MS disease, though its exact mechanism of action is unknown. Since any benefit probably involves effects of IFN-β-1b on αβ or γδ T cells, I derived T cells from MS patients and healthy controls and investigated the effects of IFN-β-1b on their activation, proliferation and activation-induced apoptosis. PHA, anti-CD3 and tetanus toxoid were used as stimulants for one or both T cell types in the presence of 0-1000 IU/mL IFN-β-1b (10-100 IU/ml is close to the range measured in the serum of patients 24 hrs after subcutaneous injections). In a dose-dependent manner, IFN-β-1b suppressed the proliferation of αβ T cells stimulated with PHA, anti-CD3 or tetanus toxoid. The same was true for PHA activated γδ T cells. MS derived αβ T cells were more sensitive to PHA suppression than were controls. At 1000 IU/ml, IFN-β-1b enhanced the activation-induced apoptosis of αβ but not γδ T cells, as measured by PI stain and flow cytometric analysis and confirmed with DNA ladders. At low doses of IFN-β-1b, PHA activated αβ T cells exhibited a significant increase in CD25 expression, whereas γδ T cells exhibited a significant decrease, as measured by two colour flow cytometry. The percentage of both αβ and γδ T cells expressing CD25 and the amount of CD25 expression following PHA stimulation was reduced with 1000 IU/ml IFN-β-1b, compared to PHA alone. MS derived αβ T cells exhibited increased CD25 expression at rest and following stimulation, and did not exhibit as obvious an increase in CD25 expression at low doses of IFN-β-1b, in comparison to controls. MS γδ T cells, following expansion and PHA activation, actually showed decreased CD25 expression vs. controls. Results indicate that IFN-β-1b differentially affects αβ and γδ T cells both in terms of activation and apoptosis, and that MS cells exhibit differential sensitivity to these effects compared to controls.

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ABBREVIATIONS

$\alpha\beta$	alpha beta
Ag	antigen
AIM V	Serum-free lymphocyte medium
anti-CD3	anti-CD3 monoclonal antibody (OKT3)
APC	Antigen presenting cell
BSA	Bovine serum albumin
CD3	Cluster of differentiation marker 3
CD4	Cluster of differentiation marker 4
CD8	Cluster of differentiation marker 8
CD25 (IL-2Rα)	Cellular differentiation marker 25
CD26	Cellular differentiation marker 26
CNS	Central nervous system
CSF	Cerebral spinal fluid
DNA	Deoxyribonucleic acid
EAE	Experimental allergic encephalomyelitis
EDSS	Expanded Disability Status Scale
FITC	Fluorescein isothiocyanate

$\gamma\delta$	gamma delta
HSP	Heat shock protein
IFN-γ	Interferon-gamma
IgG	Immunolglobulin Gamma
IL-2	Interleukin-2
IL-2Rα	Interleukin-2 receptor, alpha chain
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
ICAM-1	Intercellular adhesion molecule-1
kd	Kilodaltons
L-selectin	Leukocyte adhesion molecule-1
LFA-1	Lymphocyte function associated antigen-1
mAb	Monoclonal antibody
MAG	Myelin associated glycoprotein
MBP	Myelin basic protein
MHC	Major histocompatability complex
MIU	Million international units
MOG	Myelin-oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
NRS	National Rating Scale
OGC	Oligodendrocyte

PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PFA	Paraformaldehyde
PLP	Proteolipid protein
PI	Propidium iodide
rhIL-2	recombinant human interleukin-2
RPMI 1640	Nutrient rich cell culture medium
TBE	Tris, borate, EDTA containing buffer
TE	Tris and EDTA containing buffer
TNF-α	Tumor necrosis factor-alpha
TNF-β	Tumor necrosis factor-beta
TCR	T cell receptor
VLA-4	Very late activation antigen-4

INTRODUCTION

DEFINITION

Multiple Sclerosis (MS) is a chronic, inflammatory, demyelinating disease of the central nervous system. Myelin, produced by oligodendrocytes (OGCs) surrounds and insulates axons allowing for rapid conduction of impulses. When axons are stripped of their myelin, conduction of impulses slow or stop resulting in acute attacks of neurological dysfunction. Such attacks, separated by periods of remission are characteristic of MS. Damage to myelin in MS causes physical symptoms such as optic neuritis, weakness in limbs, urinary urgency or incontinence and vertigo.

There is no classical case of MS, as patient clinical presentation and course of the disease are highly variable. Therefore, patients may be classified into one of four different groups. 1) Patients classified as having relapsing-remitting MS experience neurological attacks followed by periods of remission. 2) Benign MS involves a relapsing-remitting state which never progresses beyond a certain level. 3) A secondary progressive condition begins as a relapsing-remitting state and evolves into a progressive phase wherein relapses and remissions are no longer appreciated and just gradual progressive disability is apparent. 4) Primary progressive disease is similar to secondary progressive disease except no relapse-remitting phase is apparent. A severe sub-type of MS is characterized by a progressive state in which the disease manifests as a rapid disability, a condition that leads to severe disability or death. In a large percentage of patients, the disease becomes secondary and progressive (Weinshenker and Sibley 1992).

EPIDEMIOLOGY

The age of onset of MS can range from mid-adolescence to approximately 60 years of age, with the majority of cases beginning during the late twenties (Ransohoff, 1992). The disease is more common in women and has a relative risk (F:M) of ~ 1.8 (Ransohoff, 1992). The incidence of MS is estimated at 30-80 /100,000 in high risk regions versus 4-6/100,000 in regions of low incidence (Matthews, 1985). A recent study conducted in Nova Scotia indicates the prevalence of MS is 1/297 (Brown and Murray, 1996).

Epidemiological studies have shown that there is an uneven geographical distribution of MS. The disease distribution is regional with an obvious predilection for cooler climates and economic development. It is considered that as one increases the distance from the equator, the frequency of MS also increases (Kurtzke 1975, 1980), although there are examples that do not apply to this rule. Small heterogeneous populations close to the equator do exist. (Alter, Leibowitz et al. 1966). Based on such geographical variance, it is possible that an infectious organism may be involved in the development of MS. To date, such evidence is not available.

The environment is suggested to play a role in MS since migration can have an effect on disease prevalence. Several studies have shown that if migration takes place prior to 15 years of age, the prevalence of MS changes toward the value of the destination, either increasing or decreasing in risk (Alter and Kahana et al., 1978, Dean and Kurtske, 1971).

Research in MS has also made a strong case for the involvement of genetics in the susceptibility of the disease. It has been shown that certain racial groups are more susceptible to MS whereas others seem resistant. In general, those protected from the disease include US blacks, Amercian Indians, Eskimos, Hutterites, Hungarian Gypsies and Orientals. Those of Fenno-Scandian origin have the highest risk of developing MS (Compston, 1986). MS may be a polygenic disease. Inheritence within families is far from strong, yet a certain genetic predisposition does exist. This is made clear from twin studies in which a concordance rate of 25-30% was observed in monozygotic twins and only 2-5% of dizygotic twins were concordant (Sadovnick, 1983).

An increased risk of disease onset does exist for relatives of MS patients. The incidence of two cases of MS occurring within the same family is ~15% (Compston, 1986) with the highest risk occurring between siblings (2.8%) and the lowest for occurrence in nieces/nephews (0.2%) (Sadovnick and McLeod, 1981). In an effort to identify a genetic marker for the disease, it was found that specific MHC class II alleles (DRw15 and DQw6) are associated with an increased risk of developing MS (Olerup and Hillert 1990), however strong associations with MS do not exist..

DIAGNOSIS

In order to diagnose a patient with multiple sclerosis, the individual must have experienced at least two episodes of neurological disturbance which involve lesions in distinct areas of the central white matter (McDonald and Silberberg 1986). Progressive myelopathy without other rational explanation lasting over six months or more is also considered an equivalent diagnostic criterion. The Poser Committee's criteria (Poser et al. 1983; Poser et al. 1984) outlines the factors that should be taken into account when attempting to diagnose MS. These include age of onset, characteristics of the attack, clinical and paraclinical evidence of MS, evidence of remission of disease, laboratory evidence, as well as indications of separate lesions.

It is considered acceptable to diagnose MS anywhere between 10 and 59 years of age. Outside of this range, other possible contributing factors of symptoms can lead to a false positive diagnosis. The MS attack is also known as a relapse, exacerbation or episode. A single symptom or symptoms indicative of neurological dysfunction lasting more than 24 hours constitutes an attack. Examples of such neurological dysfunction include muscle spasticity, weakness, visual loss, incontinence and numbness. It is important that both clinical and paraclinical evidence of an MS lesion be investigated.

The demonstration of abnormal signs on physical examination constitutes clinical evidence. Examples include findings associated with changes in the optic nerve, such as that seen with optic neuritis (ie., a rapid loss of vision which may be accompanied by pain with eye movement), unilateral loss of colour vision or slits in the nerve fibre layer of the retina. Other signs or symptoms might include jerky eye movements, weakness in the limbs, vertigo, urinary urgency or incontinence. Also taken into account in the diagnosis of MS are the characteristics of a remission, i.e., a period of >24 hours in which there is an improvement of symptoms or signs of the disease. A remission is considered significant by the Poser Committee if it has lasted at least one month.

Paraclinical evidence includes measures that will determine the presence of lesions as indicated by evoked potential techniques and imaging of the CNS by magnetic resonance imaging (MRI). MRI allows for a sensitive and accurate measurement of the appearance and extent of lesions in MS. Lesions present as white areas usually within the periventricular region of the white matter on MRI scans (Paty et al. 1991).

Other indicators of MS may be detected by laboratory testing (Gallo et al. 1989). The presence of oligoclonal protein bands from the CSF of a suspected MS patient following electrophoresis, is suggestive of disease. The bands, as indicated by electophoresis and silver staining of CSF samples, are IgG and are thought to be due to clonal or polyclonal activation of B cells within the CNS. This is in contrast to the banding patterns of healthy CSF samples, in which the bands are not distinct and are smeared with one another. Positive oligoclonal bands are not specific to MS and may be seen in other inflammatory CNS diseases. However, with the right clinical picture and in the absence of other inflammatory conditions, their presence is considered supportive of the diagnosis of MS. Important as well in the diagnosis of MS is an indication of the presence of separate lesions. Such can be suggested by the clinical presentation of physical symptoms which are possible only from the presence of more than one lesion, as well as their appearance on MRI.

In order to determine what causes MS, an understanding of the underlying mechanisms of initiation and propagation is required. To date, investigations of the pathology and pathophysiology of MS have revealed some clues.

PATHOLOGY

The hallmark of MS is the plaque or lesion, located mainly in the periventricular white matter of the brain. In the healthy central nervous system, OGCs, the producers of myelin, wrap around an axon creating an insulating sheath. Along the length of the nerve, areas of such insulation are separated by gaps, called nodes of Ranvier. The presence of these gaps are necessary for rapid conduction of impulses via saltatory conduction. In MS, the myelin sheaths are destroyed and 'stripped', leaving a naked axon. Following this is scar formation, constituting a lesion that can be visualized by MRI. Without this insulation and resultant gap architecture, saltatory conduction is slowed or lost, leading to neurological deficiencies.

Lumsden (1970) and others have suggested that one of the earliest events in the development of an MS lesion is the infiltration of lymphocytes into the meninges and Virchow-Robin spaces. During the course of lesion formation, lymphocytes and plasma cells are present at the margin of the lesion and outside the perivascular compartment among demyelinated axons. In the acute lesion, one sees myelin breakdown from the nerve axon which is then phagocytosed by macrophages surrounding the area of demyelination. Once complete demyelination of the area has occurred, the axons traversing the new lesion exhibit irregular beading, large astrocytes are increased in number in the area of the lesion and gliosis, ie., glial fibre formation, commences. This fibre formation produces a scar also known as a plaque or lesion.

In the early stages of MS, oligodendrocytes have been seen to proliferate and fresh plaques can exhibit extensive remyelination. This remyelination in the act of repairing the lesion is most often incomplete. As MS demyelination proceeds to a chronic state, the number of oligodendrocytes are depleted as are the number of immune cell infiltrates (Prineas et al., 1993). In chronic lesions, rims of remyelination persist around their margins

(Raine and Cannella, 1994). Most lesions however undergo repeated episodes of demyelination, potentiating a permanent scar. Cells that are found to be increased in number in the MS lesion include astrocytes and microglia, two antigen-presenting cells of the CNS (Hartung et al., 1995a). T cells also reside within the lesions. Overall, one sees more CD8+ than CD4+ T cells in the parenchyma of active plaques and normal white matter (Traugott et al., 1983). Of the T cells shown to surround lesions, up to 30% are γδ TCR +ve, a much higher ratio than that detected in peripheral blood (1-10%) (Selmaj et al., 1991a, Wucherpfennig et al., 1992). T cells, microglia and astrocytes are in an activated state in the MS CNS (Lee et al., 1990, Ulvestad et al., 1994). The presence of these immune cells in close proximity to lesions, as well as their state of activation suggests their involvement in lesion formation.

PATHOPHYSIOLOGY

The cause of MS is not known. Evidence exists to support the involvement of environmental and genetic factors but neither can fully explain the initiation or propagation of the disease. It is believed that the myelin breakdown and resultant lesion formation is caused by activated immune cells in response to self-antigens. Considerable evidence supports an autoimmune etiopathogenesis of MS.

A series of immunological processes are suspected to be involved in the onset of lesion formation in MS. In general, these processes can be described as four main steps:

1. Activated autoreactive T cells cross the blood brain barrier in an antigen-independent manner.

2. These T cells, having entered the central nervous sytem, will recognize their corresponding antigen on resident antigen-presenting cells (APCs) such as astrocytes and microglia.

3. Upon formation of specific TCR-MHC+ag complexes, T cells will become locally activated and secrete a variety of cytokines which in turn, will stimulate other cells to upregulate MHC expression and present more antigen.

4. This activation produces a local inflammatory reaction which induces other cells (B cells, macrophages and granulocytes) to cross the blood brain barrier and participate in the immune response. As a result, increased amounts of soluble factors might enter the region, stimulating further activity.

It is therefore possible that myelin damage occurs due to the action of cytokines, autoantibodies, complement components and lymphocytes. The focus of this work involves the role of T cells in these processes.

T CELLS

T cells, long thought to be involved in MS pathogenesis, include both αβ and γδ T cells as potential candidates. Both T cell types express clonotypic antigen specific receptors that allow for the extremely large repertoire of specificity. This varied specificity is created by somatic gene rearrangements of each chain, α and β or γ and δ, due to random combinatorial joining of the various gene segments coding for each chain (Abbas et al., 1991). αβ T cells make up the majority of cells found in peripheral blood (90-99%) with γδ T cells accounting for the rest. The large majority of T cells recognize antigen when it is presented in association with class I or class II MHC on antigen presenting cells (Hohlfeld 1989).

Mature αβ T cells are either CD4+ or CD8+ which in general, are markers that dictate their function. CD4+ αβ T cells possess helper function, being either T_H1 or T_H2 cells. T_H1 cells secrete interferon-gamma (IFN-γ) and interleukin-2 (IL-2) causing delayed-type hypersensitivity reactions and macrophage activation (Mosmann and Coffman, 1989, Cher and Mosmann, 1987). T_H2 cells secrete interluekin-4 (IL-4), IL-5, IL-6 and IL-10 and may be important in activating B cells to secrete specific antibody (Mosmann and Coffman, 1989, Coffman et al., 1988). These cells become activated and participate in immune functions following formation of a trimolecular complex with another cell presenting antigen in association with MHC class II molecules. CD8+ cells possess killer function and upon formation of a trimolecular complex with MHC class I presented antigen, release perforin which forms pores in target cells followed by granzymes which enter the target cell and activate endonucleases, resulting in the demise of the target. In general, MHC class I molecules present endogenously processed antigen whereas MHC class II molecules present exogeneous antigen.

The exact functions of γδ T cells are not well understood. Mature γδ T cells are either CD4- CD8- or CD4- CD8+ and can exhibit cytotoxic activity, as shown by ^{51}-Cr release assays.. The majority of γδ T cells express neither CD4 nor CD8 and are able to act in an MHC unrestricted manner (Kabelitz et al., 1990), but have been shown to recognize MHC class I and II molecules (Bluestone et al., 1988). Although γδ T cells have been shown to possess cytotoxic activity much like CD8+ αβ T cells, their function in help and cytokine production is not well understood (Zeigler et al., 1994) These cells are able to recognize heat shock proteins and peptide antigens.

In order for these two types of T cells to initiate immune actions involved in MS pathogenesis (i.e., oligodendrocyte (OGC) damage), they need to be activated and cross the blood brain barrier. $\alpha\beta$ and $\gamma\delta$ T cells are found in an activated state in the periphery and CNS of MS patients (Golaz et al., 1983, Perrella et al., 1993). Analysis of activation markers on cells from MS vs. controls has shown an increase in the number of CD26-reactive T cells in blood of chronic progressive MS patients (Hafler et al., 1985). Also noted is an increase in the expression of IL-2R (CD25) on T cells (Bellamy et al. 1985) and elevated levels of sIL-2R in the sera of clinically stable MS patients and the same occurs during an acute relapse in chronic patients (Gallo et al., 1989, Bansil et al., 1991).

An activated state and the upregulated expression of cell adhesion molecules on endothelium and T cells would allow for T cell entry into the CNS. ICAM-1, an adhesion molecule found on endothelial cells, is upregulated and found to be increased in its soluble form in MS during exacerbation as compared to stable disease periods (Hartung et al., 1993). As well, ICAM-1 has been detected on brain macrophages, vasculature and astrocytes in active MS plaques (Sobel et al., 1990 and Raine and Cannella, 1992). Adhesion molecules expressed on T cells include ICAM-1, VLA-4, LFA-1 and L-selectin (Hartung et al., 1995a). Soluble L-selectin molecules are increased in the serum MS patients as compared to controls (Hartung et al. 1995b). Cannella and Raine (1995) suggest that VLA-4 interactions with VCAM-1, an adhesion molecule expressed on endothelial cells and macrophages, are increased in chronic MS lesions as measured immunohistochemically.

The presence of activated αβ and γδ T cells within the MS central nervous system is well documented. CD4+ αβ T cells are found in acute MS lesions (Raine, 1991). As well, activated T cells reactive to a number of self-antigens have been isolated from MS patients (Allegretta et al., 1990 and Correale et al., 1995). Such T cells however have also been isolated from control subjects, albeit in a less activated state. T cells possessing certain TCR rearrangements associated with myelin protein reactivity are present in MS lesions more so than in other CNS inflammatory lesions (Oksenberg et al., 1993). During thymic selection of T cells, T cells which express a TCR that recognizes self antigens are deleted, yet some manage to survive. It is possible that such T cells may be involved in autoimmune disease such as MS. The individual may be protected from the activity of these cells by sequestering the self-antigen, such as within the CNS. MS may be expressed once access to such myelin antigens is made available.

γδ T cells, although few in number in peripheral blood (1-10% of total T cells), comprise up to 30% of the T cells surrounding MS plaques (Selmaj et al., 1991 and Wucherpfennig et al., 1992). The frequency of γδ T cells is increased in both CSF and blood of MS patients in comparison to controls, suggesting *in vivo* activation and expansion of these cells in MS. No differences in the frequencies were noted in relapse-remitting versus chronic or primary progressive disease patients, suggesting their presence and possible involvement in more than one form of MS (Nick et al. 1995 and Stinissen et al. 1995). These cells predominantly express Vδ1 and Vδ2 gene segments in MS, which may be due to clonal expansion (Wucherpfennig et al., 1992). Such cells have been isolated from the CSF of MS patients with recent onset of the disease (Shimonkevitz et al., 1993).

Once such activated T cells cross the blood brain barrier, it is believed that they recognize an as of yet unknown antigen on resident APCs, and initiate responses toward self-proteins. Research has suggested that both αβ and γδ T cells recognize native antigen within the CNS. Is there an antigen responsible for the lesion formation in MS?

Strong support for the involvement of αβ T cells in MS pathogenesis is derived from their role in initiating the animal model of MS, experimental allergic encephalomyelitis (EAE) (Ben-Nun et al., 1981). The induction of EAE occurs in certain experimental animals following a) injection of myelin proteins with complete Freund's adjuvant or b) the transfer of activated MBP (myelin basic protein) reactive CD4+ T cells (Weller, 1985). Varying degrees of disease severity in EAE exist and are dependent upon the injection schedule, the strain and species of animal used. The chronic-relapsing form of EAE most closely resembles MS. With this form, one sees well defined lesions in the white matter and the spinal cord usually suffers damage as well. Like MS, in EAE one sees perivascular cuffing by lymphocytes, phagocytosis of myelin by macrophages and widespread demyelination in the CNS.

As such, myelin proteins, those which constitute the myelin sheaths surrounding healthy nerves, are suspected as possible target antigens in MS. Peptides derived from myelin basic protein (MBP), proteolipid protein (PLP), myelin associated glycoprotein (MAG) and myelin-oligodendrocyte protein (MOG) have all been implicated (Ota et al., 1990, Linington et al., 1993 and Trotter et al., 1991). Another potential antigen, though not of myelin origin, is S-100β, an astrocyte derived calcium-binding protein. Kojima et al. (1994) have shown that this antigen is encephalitogenic in an animal model, as are the aforementioned myelin proteins.

Of interest is the fact that an antigen such as S-100β, located outside of the CNS is able to prime encephalitogenic T cells and lead to demyelination. This suggests that OGC damage may occur by bystander effects of non-myelin T cell reactivity in the CNS. Although potentially all involved, no antigen or antigens have been confidently implicated in playing a role in MS pathogenesis, in either its onset or propagation. The search continues for the αβ T cell antigen in MS.

A second animal model of MS worthy of note is caused by Theiler's virus, a murine picorna-virus. Infection results in persistent CNS infection and demyelination (Brahic and Strop, 1981). Lipton and Al Canto (1976) showed that demyelination by Theiler's virus infection was prevented by immunosuppression, suggesting an immunological mechanism of OGC damage. Many viruses have been detected in MS CNS such as rabies, herpes, measles, scrapie, and coronaviruses yet their presence is not consistent in all patients and no causal association has been made (Weiner et al., 1991).

Heat shock proteins (hsps) are a heterogeneous group of proteins with >50% conservation of amino acid sequence between prokaryotes and eukaryotes. These proteins are classified based on molecular mass, owing to groups of 24-45, 60, 70 and 90-110 kd molecules. Some species are constitutively expressed on a variety of cells and can be upregulated by a number of factors including elevation in temperature (hence 'heat shock'), ischemia hormones, glucose deprivation, infection and various cytokines (Young, 1990).

Of interest as a possible self-antigen in MS, hsp 60 and 70 were found to be expressed on OGCs, the latter of which is upregulated following increased temperature exposure (Freedman et al., 1992a).

Human γδ T cells respond to mycobacterial heat shock proteins (Haregewoin et al., 1989). These cells have been shown to co-localize with hsp65-expressing oligodendrocytes in active chronic MS lesions (Selmaj et al., 1992) and with hsp-60-expressing OGCs and astrocytes in EAE (Gao et al., 1995). In vitro, Freedman et al. (1991) have shown that γδ T cells lyse fresh human derived OGCs. It has also been shown that the proliferative responses of CSF lymphocytes to hsp65 and hsp72 are significantly higher in MS patients than in controls with other neurological diseases (Salvetti et al., 1992 and Birnbaum et al., 1993).

Measured in situ, OGCs express no class I or II MHC which would prevent their direct interaction with CD8+ve and CD4+ve T cells (Lee et al., 1990), but still potentiate γδ T cell reactivity due to their expression of hsps. Prokaryotic hsps elicit immune responses in certain infectious diseases such as Lyme disease, tuberculosis and Legionnaire disease (Young, 1990 and Young et al., 1988). Since prokaryotic hsp60 and 70 share homology with mammalian hsps (Winfield and Jarjour, 1991 and Shinnick et al., 1988), it is possible that following infectious disease, molecular mimicry may ensue and allow for the γδ T cell-hsp response suggested to occur in MS lesion formation.

Such an interaction of γδ T cells with OGCs could provide a primary insult, and allow for the secondary involvement of MHC-restricted αβ T cells, to respond to internal OGC proteins such as MBP, as presented by macrophages and astrocytes. That γδ T cells initiate the demyelination process in the proposed sequence of events requires that they are integral in the onset and propagation of the disease.

The third step hypothesized to be involved in lesion formation is the production of cytokines following TCR-MHC+Ag engagement, in effect stimulating cells to upregulate MHC and present more antigen. Cytokines are soluble substances produced by a variety of cells and participate in orchestrating many actions of the immune system. As such, their levels have been measured in both MS and controls in an effort to define the altered immune state of the disease.

IL-2 is known to be elevated in the serum of MS patients (Gallo et al., 1989) as well as serum levels of IL-2R. Tumour necrosis factor-α (TNF-α) (Sharief and Hentges, 1991 and Tsukada et al., 1991) is also elevated in the serum of MS patients and is considered important in contributing to disease process. TNF-α has been shown to cause delayed-onset myelin dilatation and later myelin degradation in organotypic nerve tissue cultures derived from embryonic mouse spinal cord tissue (Selmaj et al., 1991b). As well, TNF-β (a member of the TNF family) and TNF-α induce OGC injury in a time and dose dependent manner (Selmaj et al., 1991b). TNF-α is mitogenic for astrocytes and therefore might enhance reactive gliosis or scar formation in the CNS. Both TNF-α and TNF-β have been observed within MS lesions upon post-mortem histochemical analysis (Hofman et al., 1990). Their presence was located within active acute and chronic lesions but not within chronic silent lesions. This implies a role for TNF-α and TNF-β in ongoing myelin damage (Selmaj et al., 1991b). TGF-β activity was noted to be elevated in MS patients recovering from a relapse compared to stable patients (Beck et al., 1991), suggesting that this cytokine may be involved in the recovery process from an attack.

Interferon-γ (IFN–γ), produced by activated lymphocytes, is a cytokine capable of upregulating MHC class II, activating macrophages and inducing adhesion molecules as well as toxic factors, together with TNF-α and IL-1 (Philip and Epstein, 1986). Its presence has been localized to perivascular inflammatory cells and on microglia and astrocytes in proximity to MS lesions and shown to upregulate the expression of MHC class II on these cells (Lee et al., 1990). IFN-γ activates macrophages to release myelin-degrading proteases (Bever, 1991) and induces ICAM-1 expression, increasing the ability of immune cells to cross the blood brain barrier (Male et al., 1990). Due to the ability of IFN-γ to upregulate the immune status of cells within the CNS, it is not surprising that administration of IFN-γ to relapsing-remitting patients in a clinical trial was aborted after its administration was found to provoke exacerbations (Panitch et al., 1987).

The fourth and final proposed process involved in MS pathogenesis is a culmination of the first three processes described, which allows for the entry of B cells, macrophages and granulocytes to the CNS across the blood brain barrier to participate in demyelination. The production of oligoclonal bands (IgG visualized following electrophoresis of CSF) in the CSF is suggestive of MS and more specifically, antibodies specific for MBP have been found in increased levels in MS patients with progressive disease (Warren and Katz, 1987). Anti-MBP antibodies were elevated in the CSF of 96% of MS patients with relapsing and progressive disease (Warren et al., 1994).

Although much is known about the immunological processes occurring in MS and EAE, the jury is still out on what the exact mechanism(s) of initiation and propagation are.

A strong case is presented for the role of both αβ and γδ T cells.

MANAGEMENT

Many theories exist with respect to the initiation and propagation of demyelination in MS, but a clear understanding is absent. A variety of therapies have been tested and prescribed for the management of MS. Management of MS by pharmacological methods is based on two modes of treatment, defined as abortive and chronic therapies. Abortive therapies treat the symptoms of an attack in an effort to alleviate the acute exacerbation, without addressing the permanent damage it may cause the patient. Chronic treatments are used in an effort to change the natural history of the disease.

Due to the fact that neither the target antigen(s) nor triggering agent(s) are definitively known in the pathogenesis of MS, therapies have been designed that provide a generalized immunosuppressive action and therefore are non-specific. Three therapies suggested to be "probably effective" by Bansil et al. (1995) that act by this method include corticosteroids, azathioprine and cladribine.

Corticosteroids are used most commonly during an acute relapse. They have been shown to exert anti-inflammatory and immunosuppressive effects. More specifically, corticosteroids have been shown to change the normal IL-2 and IFN-γ production, the antigen presentation by monocytes and IL-2R expression by immune cells (Gallo et al., 1989 and Troiano et al., 1984). They have the ability to alleviate symptoms temporarily during an acute attack, however their use in treating progressive disease is not considered effective. In such cases, azathioprine, a purine analogue, may be administered. Long term use of azathioprine is considered essential to provide a therapeutic effect, although an overall consensus for its use in MS has been difficult (Goodkin et al., 1991 and Ellison et al., 1988). This agent acts to suppress the immune system as white cell numbers are decreased over a period of use.

Cladribine (2-chlorodeoxyadenosine) is also an agent considered to be a 'probable' effective therapy for MS. This agent acts by inducing the apoptotic death of lymphocytes, resulting in a long-term state of lymphopenia. Early studies have shown that cladribine is able to significantly reduce Expanded Disability Status Scale (EDSS) scores and decrease the MRI-measured demyelinated volumes of MS patients in comparison to placebo controls (Sipe et al., 1994). Clinical trial data is still in the early stages and long term follow-up, double blind and placebo controlled data is eagerly awaited.

None of the three therapies described above have been shown to alter the natural course of MS disease. A novel agent that does appear to improve the clinical state of MS patients and change the disease course is IFN-β-1b (Betaseron). Suspected cellular activities may be investigated under the influence of chronic agents like Betaseron, to gain a further understanding of the mechanisms involved in MS pathogenesis.

INTERFERON β-1b (Betaseron)

Interferons are a group of glycoproteins produced and secreted by a variety of cells. These agents are able to induce an antiviral action in other cells, and can aid in regulating the immune response (Kuby, 1994). In 1980, the International Committee on Interferon nomenclature defined these proteins on the basis of their antiviral mechanism of action:

"To qualify as an interferon, a factor must be a protein which exerts non-specific antiviral activity at least in homologous cells through cellular metabolic processes involving synthesis of both RNA and protein." (Stewart et al., 1980).

Since 1980, the interferons and their biological effects have been more clearly defined. The interferons are products of a multigene family and are classified as α,β and γ, based on the main type of cell that produces them, leukocyte, fibroblast and a variety of immune cells respectively. At least 23 different human interferon-α genes have been located with only one for interferon-β, all located on the short arm of chromosome 9. The only gene which codes for interferon-γ is located on the long arm of chromosome 12 (De Maeyer and De Maeyer-Guignard, 1988). The IFN-αs and IFN-β share 30-60% amino acid sequence homology and less than 10% with IFN-γ. Unlike IFN-α, IFN-β and IFN-γ are glycosylated while both IFN-α and β contain internal disulphide bonds essential for biological activity retention.

An analogue of IFN-β (IFN-β-1b, Betaseron) has been tested as a treatment for MS and can change the natural course of the disease. Betaseron is a 165 amino acid, type one recombinant interferon made in Escherichia coli (The IFNB Multiple Sclerosis Study Group, 1993). This analogue differs from the native protein in that it is not glycosylated, it has a serine substitution for cysteine at position 17 and lacks the N-terminal methionine. Despite these differences, IFN-β-1b acts on the same receptor as the native protein (Thompson et al., 1985).

The receptor for IFN-β (also utilized by IFN-α) is present on almost all cell types, albeit at a rather low abundance (100-5000 molecules per cell) (Novick et al., 1995). The receptor is a 102 kd dimer protein, consisting of disulphide linked 51 kd subunits. Upon binding of its ligand, the receptor recruits cytoplasmic kinases Tyk2 and Jak1 (Novick et al., 1994). These two tyrosine kinases are likely to be implicated in the phosphorylation of latent cytoplasmic proteins called STATs (signal transducers and activators of transcription). Such phosphorylated STATs are believed to migrate to the nucleus, where they combine to form the transcription factor ISGF3 (interferon stimulating gene factor 3). This ISGF3 then interacts with an ISRE (interferon stimulating response element) present in the promoter of an interferon-inducible gene (Uze et al., 1995). The identification of such inducible genes is unclear and as a result, so are the end effects of IFN-β.

The rationale for using IFN-β–1b in the treatment of MS is based on at least four findings. Knowing that interferons have anti-viral properties, its use in the treatment of MS was rationalized by the observations of two studies, showing that intercurrent clinical viral infections triggered new attacks. Such infection was suggested to be a very powerful influence on MS progression. Suspecting that MS was due to a virus, Jacobs et al. (1982) injected MS patients intrathecally with natural IFN-β and observed a reduction in exacerbations in relapse-remitting patients. Using 69 patients with a relapsing-remitting diagnosis (they previously showed no effect of IFN-β on chronic progressive patients) a double-blind and placebo controlled study was conducted to determine the effects of natural IFN-β after two years of receiving intrathecal interferon. The exacerbation rate proved to be significantly lower in the treated group compared to controls (Jacobs et al., 1986). A clinical trial which tested the effect of IFN-γ on MS showed that this interferon actually increased exacerbation rates and was aborted prior to study completion (Panitch et al., 1987). Due to the fact that IFN-β possesses some antagonistic properties in comparison to

IFN-γ, it was thought that perhaps IFN-β would alleviate, instead of augment, the disease. Based on the above, a strong case was made for further investigation of IFN-β's role in the treatment of MS.

Two large scale clinical trial studies investigating the effect of IFN-β on MS have been completed. In the first study a multicentre, randomized, double-blind, placebo-controlled trial of interferon β-1b (Betaseron) was tested in 372 ambulatory relapse-remitting MS patients. It was administered subcutaneously since it was shown that systemic interferon could pass the blood brain barrier at areas of active damage in MS (Prineas and Right, 1978 and Sharief and Thompson, 1992) and could also initiate events at the molecular level in the brain (Smith et al., 1987).

The patients were split into three groups, one receiving placebo, one 1.6 MIU IFN-β and one 8 MIU IFN-β by self-administered subcutaneous injection every other day. Outcome was measured by yearly MRI readings, differences in exacerbation rates and proportion of patients remaining exacerbation-free. Also measured were time to first exacerbation, exacerbation duration and severity, change in Expanded Disability Status Scale score (EDSS) and Neurological Rating Scales (NRS), from baseline. After two years, the results were extremely promising. The yearly exacerbation rate was significantly lower in both treatment groups with a lower rate overall in the high-dose group. The severity of attacks was reduced and the number of exacerbation-free patients was highest in the high-dose group. Serial MRI scans also showed a decrease of 6.2% in lesion area in the high-dose group while placebo subjects showed an increase of 17.1% (The IFNB Multiple Sclerosis Study Group, 1993). The results at five years follow-up show that these effects were sustained over time (The IFNB Multiple Sclerosis Study Group, 1995).

The second study investigated the effects of weekly intramuscular injections of 6 x 10 MIU of IFN-β-1a or placebo on 301 relapsing-remitting MS patients and revealed a positive effect on disease progression (Bansil et al., 1995). There was a 31% reduction in the relapse rate in treated patients and only 22.6% of the treated patients showed disease progression over two years compared to 36.6% of placebo treated subjects. Results from both of these studies suggest that the natural course of MS can be altered, yet this is only true for relapsing-remitting patients. Further studies are awaited to determine IFN-β's effect on the chronic progressive patient.

In vitro analysis of the effects of IFN-β-1b may provide some clues about its activity *in vivo*, and in alleviating MS. IFN-β was shown to reduce the production of TNF-α and IFN-γ by mononuclear cells derived from MS and control subjects (Rudick et al., 1993), and inhibit the IFN-γ-driven HLA-DR expression on astrocyte and glioblastoma cultures (Joseph et al., 1988), potentially reducing antigen presentation in the CNS. This activity is important as IFN-γ is known to exacerbate the disease.

In vitro, IFN-β-1b reduces the number of activated T cells derived from both MS and controls (Rudick et al., 1993 and Noronha et al., 1993), as measured by cell surface expression of CD25 (IL-2Rα) and CD69, an early activation marker. It is able to suppress the Con-A and anti-CD3 antibody induced proliferation of T cells (90-99% being ab TCR +ve) derived from MS and controls (Rudick et al., 1993 and Noronha et al., 1993). By this mechanism, fewer cells would be activated and able to participate in lesion formation.

It has been shown previously that interferon-α is able to augment apoptosis in a

MOLT-16 T cell line that was activated with PHA (Dao et al. 1994). IFN-α is 30-60 % homologous to IFN-β in terms of amino acid sequence. Since T cells are activated in MS, it is also possible that IFN-β-1b could induce apoptosis in peripheral blood T cells under similar stimulatory conditions.

Apoptosis is the physiological process of cell death that occurs separately from necrosis. The term apoptosis is derived from archaic Greek and implies leaves falling off trees in autumn, a process that is beneficial to the organism (Touchette and Foggle, 1991). When a cell undergoes apoptosis, the cell shrinks due to loss of cell volume and can lose up to 30% of its cell volume (Ohyama et al., 1991). The cell's cytoskeleton is then modified such that the plasma membrane becomes blebbed, a process known as zeiosis (Sanderson, 1982). The cause of this decrease in cell volume and cytoskeletal disruption is not known but an increase in internal proteolysis may be a contributor (Amenta and Baccino, 1989). The most obvious change in a cell undergoing apoptosis is the collapse of the nucleus (Kerr et al., 1972 and Wyllie, 1980). Chromatin is condensed and locates in the margins of the nuclear envelope. After this has occurred, the cell may break up into apoptotic bodies, spheres of plasma membrane containing condensed chromatin.

During this whole process, the chromatin is cleaved into single and multiple nucleosomes (Cohen, 1989). Upon electrophoretic separation of this DNA, one can visualize a ladder pattern of bands of approximately 200, 400, 600 base pairs, etc. This is due to the cleavage occurring between nucleosomes, spaced at ~180-200 base pair intervals at the nucleosome linker region in which it is weakly associated with histone H1 (Arends et al., 1990). This cleaved DNA is also visualized by flow cytometry of permeabilized and PI stained cells. PI is a DNA stain which binds to DNA and fluoresces at a higher intensity if more DNA is present to be bound. DNA content of cells increases two-fold during the cell cycle from G1 to M. Apoptotic cells are visualized as a sub-G1 peak to the left of cells in the G1 stage of the cell cycle. This nuclear fragmentation process is extremely rapid and it is very difficult to isolate cells with partially fragmented DNA (Cohen et al., 1992) and may take only minutes.

The mechanisms involved in this type of cell death are not fully known nor are the ways in which various factors can induce it. Recently, a number of the biochemical events that contribute to apoptosis have been revealed. ICE, a mammalian interleukin-1b-converting enzyme (Yuan et al., 1993), is a cysteine protease related to the product of a key nematode pro-apoptotic gene called CED-3 (Ellis et al., 1991). When transfected into a number of cell types, ICE is able to induce apoptosis. BCL-2, a proto-oncogene known for its anti-apoptotic abilities, is able to counteract the ICE induced apoptosis to a certain degree. ICE may induce apoptosis by binding to an enzyme involved in DNA repair and maintenance, called PARP (poly (ADP-ribose) polymerase). Recently, a member of the ICE cysteine protease family has been identified and shown responsible for PARP breakdown (Nicholson et al., 1995). This ICE protease also known as CPP-32 or apopain, appears critical for apoptosis at its onset.

Clinical trial data is highly suggestive that IFN-β-1b is able to change the natural history of the disease. The critical data essential to understanding the disease is still unclear; what is the underlying pathophysiology of MS? How is the disease initiated and propagated? What are the cell types involved in this process? The answer may become clearer when the mechanism of action of IFN-β-1b is elucidated.

To date, the exact mechanism(s) by which this interferon acts in MS is not known, yet its anti-proliferative and immunomodulatory properties may be involved. Based on previous experimental data presented thus far, αβ and γδ T cells are probably involved in MS pathogenesis. If the level of activation and rate of proliferation are indices of T cell activity in MS, investigating the effect of IFN-β-1b on these measures, on the two T cell types, could deduce the agent's mechanism of action. Data may indicate not only the mode of action of IFN-β-1b, but also define the role of T cells in MS.

If activated and proliferating T cells contribute to the disease process, one would expect that an agent which reduces the severity of the disease, also reduces the state of activation and level of proliferation of the cells that cause it. Therefore, the purpose of this project was to investigate the effects of IFN-β-1b on the activation, proliferation and activation-induced apoptosis of αβ vs γδ T cells, derived from both MS and healthy control subjects.

RATIONALE

αβ and γδ T cells may be involved in the pathogenesis of MS. Myelin protein reactive, CD4+, αβ TCR+ T cells induce EAE, the animal model of MS, in susceptible animals. αβ T cells are in an activated state in MS CNS and are present within lesions. Myelin reactive αβ T cells have been isolated from both MS and controls, but in vivo, may be prevented from reacting with their targets until OGC damage has occurred, since many myelin proteins are located intracellularly. γδ T cells comprise up to 30% of the T cells surrounding MS lesions. OGCs, the target cell of damage in MS, express no MHC class I or II, preventing direct interaction with αβ T cells. γδ T cells can behave in an MHC unrestricted manner and are reactive toward heat shock proteins, which are expressed on OGCs. *In vitro*, γδ T cells lyse fresh human derived OGCs.

If γδ T cells initiate damage to OGCs by direct lysis, myelin proteins would then be available for processing and presentation by CNS APCs to MHC-restricted αβ T cells. Further OGC damage would result from bystander effects of CD4+and CD8+ αβ T cells reactivity to presented myelin Ags. Such a sequence of events might require the involvement of γδ T cells to initiate the cascade of OGC damaging processes.

IFN-β-1b has recently been shown to alter the disease course in MS. Results from controlled clinical trials indicate that IFN-β-1b reduces lesions as detected by MRI, as well as the number and severity of relapses. How IFN-β-1b alleviates MS remains elusive, but its known anti-proliferative and modulatory effects on immune cells are possible mechanisms.

If αβ and or γδ T cells play a significant role in MS pathogenesis, one would expect IFN-β-1b to exert an effect on these cells that would reduce their ability to participate in aberrant immune functions; ie., they would be rendered less activate. Such information could further implicate the role of these T cells in MS pathogenesis.

HYPOTHESIS

This work asserts perhaps a more important initial involvement of γδ T cells in the OGC damage of MS, but acknowledges the involvement of both T cell subsets in the disease propagation. As such, it is hypothesized that IFN-β-1b exerts a down-regulatory effect on the activation and proliferation of $\gamma\delta$ and $\alpha\beta$ T cells.

OBJECTIVES

1) The first objective is to determine the ability of IFN-β-1b (1-1000 IU/ml) to suppress the mitogen or antigen induced proliferation of $\alpha\beta$ and $\gamma\delta$ T cells. Proliferation can be measured by ^{3}H-thymidine incorporation. Suppression of proliferation can be calculated using the formula:

1-[(cpm with mitogen or antigen + IFN-β-1b / cpm with mitogen or antigen - IFN-β-1b)] x 100

All stimulations, ie., using PHA, anti-CD3, tetanus toxoid and hsp70, can be assessed in this manner.

2) The second objective is to determine the ability of IFN-β-1b to induce apoptosis in $\alpha\beta$ and $\gamma\delta$ T cells stimulated with mitogen or antigen. The presence of apoptotic populations can be revealed by PI staining of permeablized cell samples from each experiment followed by flow cytometric analysis. Data can be corroborated with DNA ladders produced by gel electrophoresis.

3) The final objective is to assess the induced activation state of $\alpha\beta$ and $\gamma\delta$ T cells in the presence of various concentrations of IFN-β-1b (range 1-1000 IU/ml). This is conducted only on T cells activated with PHA. CD25 (IL-2Rα) expression can be used as a measure of activation, and can be assessed using two colour flow cytometry by using antibodies directed toward $\alpha\beta/\gamma\delta$ TCR (FITC-conjugated) and CD25 (PE-conjugated).

MATERIALS AND METHODS

Human Subjects:

Peripheral blood was donated by healthy laboratory and clinic staff for use as controls (n=20, 6 males:14 females, avg. age 32, range 23-45). Blood donations from consenting MS patients (n=21, 5 males:16 females, 39, range 18-66) defined clinically as shown in Appendix A, were treatment-free for at least 6 months prior to the study. Samples were collected in 15 ml heparin coated vacutainer tubes. Patients gave informed consent and were required to sign a consent form similar to that seen in Appendix B.

Cell Isolation and Culture:

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density centrifugation, washed three times in sterile 1 x PBS and counted in trypan blue to determine the viability of the cells. Those cells unable to exclude the dye were not counted. PBMCs were cultured in RPMI 1640 (to which was added 10% fetal calf serum, 2 mM L-glutamine, 100 mg streptomycin and 100 units penicillin, constituting complete RPMI) at $1x10^6$ cells/ml, in 50 ml flasks or 24 well plates at 37°C and 5% $C0_2$.

IFN-β-1b (Betaseron) Preparation:

Interferon-β-1b, supplied lyophilized at 5.4 million international units (MIU) by Berlex Laboratories, San Francisco, was reconstituted in sterile AIM V (serum free) media at $1x10^5$ IU/mL and stored at -70°C until use.

Expansion of γδ and αβ T Cells:

The percentage of γδ T cells present in peripheral blood (1-10% of total T cells) was too small to provide large enough populations for use in experiments. For this reason, γδ T cells had to be preferentially expanded in culture in order to attain a sufficient quantity of cells. On day 0 following cell isolation, 3 x 200 ml sample ($2x10^5$ cells) per donor were pelleted, stained with 10 μL of isotype control ($\gamma_1\gamma_1$, Becton Dickinson), FITC conjugated anti-γδ TCR mAb (Becton Dickinson) and PE conjugated anti-CD3 mAb mAb (Becton Dickinson), or not stained, and analyzed by flow cytometry to determine the percentage of γδ T cells present. PBMCs were then cultured to expand γδ T cells to allow for reliable cell numbers in all experiments, using the following method. Petri dishes were coated with 10mL of 5 μg/ml sheep anti-mouse IgG1 (Intermedico) in PBS and incubated for one hour at 37°C and 5% CO_2. This step was done to allow for the Fc portion of the anti-TCRδ1 antibody to adhere to the surface of the plate. In order to remove unbound antibody and to saturate any antibody-free areas on the plate (to prevent non-specific binding), the plates were washed twice with warm PBS and once with PBS containing 1-2% fetal calf serum. 5 ml complete RPMI was added to each plate with 10 ng/ml of anti-TCRδ1 mAb (a kind gift from Dr. M. Brenner, Boston, M.A.) and incubated for 20 mins at 37°C. This antibody is directed toward the δ chain of the γδ TCR and would therefore only stimulate γδ T cells. 5 ml of $2x10^6$ PBMCs/ml were then added to the petri dishes and returned to the incubator. At 24hrs., 20 units/ml rhIL-2 (Immunex, Seattle, W.A.) was added to each dish and incubated again. On day 5, cells were harvested from the dishes, washed with 1xPBS, pelleted, counted and adjusted to 2.5×10^5 cells/ml in AIM V media. Cells were cultured in 250 ml flasks in the upright position. On day 9, cells were harvested, Ficolled, washed three times in PBS and resuspended in complete RPMI at $1x10^6$ cells/ml. $2x10^5$ cells were double stained with FITC conjugated anti-γδ TCR mAb

(Becton Dickinson) and PE conjugated anti-CD3 mAb (Becton Dickinson) and analysed by flow cytometry to determine the purity of γδ T cells. If purity was <85% γδ TCR positive, γδ T cells were negatively selected using anti-pan-αβ TCR mAb in conjunction with anti IgG1 coated Magna Beads (DYNAL) as per manufacturer's protocol. Purity was reassessed by flow cytometry as described above.

αβ Tcells were expanded in a similar manner using anti-pan-αβ (T Cell Sciences) coated plates. A six day incubation for expanded αβ T cells was conducted without a re-seeding step on day 5. Since so many cells were αβ TCR positive on day 0, sufficient expansion and blast formation was evident by day 6.

STIMULATION ASSAYS

PHA Stimulation:

PBMCs, purified expanded γδ or αβ T cells, were cultured at $1x10^6$ cells/ml in the absence or presence of purified PHA (phytohemagglutinin) HA 16/17 (Murex) at 1μg/ml. Those cells cultured in the presence of PHA were also treated with various concentrations of IFN-β-1b at 0-1,000 IU/ml and incubated at 37°C and 5% CO_2 for 72 hours.

Anti-CD3 mAb Stimulation:

PBMCs were incubated in flasks for 1 hour to allow monocytes to adhere to the surface. Unbound PBMCs were then carefully removed with a wide mouthed pipette being careful not to disturb the adherent cells. Adherent monocytes and macrophages were removed in order to purify the sample. 24 well tissue culture plates were coated with 5μg/ml of sheep anti-mouse IgG (Intermedico) for one hour, washed three times with s1xPBS, the third wash including 1-2% fetal calf serum. 30µl of a 1/500 dilution of OKT3 supernatant (a kind gift from Dr. L. Filion, University of Ottawa), was added to each well with 1ml of complete RPMI and incubated for 20 mins. 2mL of $1.5\text{x}10^6$ PBMCs/ml was then added to each well with various concentrations of IFN-β-1b (0-1,000 IU/ml). Control wells were free of anti-CD3, anti-mouse IgG and IFN-β-1b. Plates were incubated at 37°C, 5% CO_2 for 5 days, with the addition of 5 units/ml rhIL-2 at 24 hours.

Tetanus Toxoid Stimulation:

PBMCs ($1\text{x}10^6$ cells/ml) were incubated in 24 well tissue culture plates in the absence or presence of 0.1 Lf/ml tetanus toxoid and various concentrations of IFN-β-1b (0-1,000 IU/ml). Lf represents the number of floculating units present, and this is a measure of the antigenicity of the solution. After 5 days of incubation, 10 units/ml of rhIL-2 was added to each well, returned to the incubator and cells assessed 48 hours later.

Hsp70 Stimulation of Expanded γδ T cells:

Expanded and purified (>85% γδ TCR positive) γδ T cells ($1\text{x}10^6$ cells/ml) were incubated in the absence or presence of 5µg/ml hsp70 (World Health Organization, Dr. Singh, Germany) and various concentrations of IFN-β-1b (0-1,000 IU/ml) in 24 well plates. On day 4, 20 units rhIL-2/ml were added to each well. Cells were assessed 48 hours later on day 6.

Cell Proliferation:

During the last 16 hrs of each activation period, $1\text{x}10^5$ cells/treatment were plated in 96 well plates (Costar) in triplicate, pulsed with 1µCi ^{3}H-thymidine and returned to the incubator. Plates were then harvested on glass fiber filters (Canberra Packard) and counted on a Matrix 9600 beta counter (Canberra Packard). % suppression of proliferation by IFN-β-1b was calculated using the following formula:

1-[(cpm with mitogen or antigen + IFN-β-1b / cpm with mitogen or antigen - IFN-β-1b)] x 100

Measure of Activation:

At the end of each culture period, $2x10^5$ cells/treatment were pelleted, washed once in 1xPBS and double stained with FITC conjugated anti-αβ or anti-γδ TCR mAb and PE conjugated anti-CD25 mAb for 30 min. at 4°C. Cells were then washed twice in 1xPBS, fixed in PBS with 0.01% sodium azide and 0.1% BSA and analyzed by two colour flow cytometry (Coulter Epics XL). Prior to the analysis of samples, colour compensation was conducted. αβ or γδ TCR positive cells were gated on FL1 and the mean channel fluorescence (MCF) of the respective T cell population was measured on a FL2 x count histogram. MCF is a measure of the average fluorescence of bound antibody/cell. A difference of greater than or equal to 5 MCF units is considered significant on Coulter flow cytometric systems. For control purposes, an isotype stained sample was analyzed each session ($\gamma_1\gamma_1$, Becton Dickinson).

Measure of Cell Cycling/Apoptosis by Flow Cytometry:

At the end of each culture period, $1x10^6$ cells/treatment were pelleted at 200 rpm x 1 min., permeabilized with 400 µl of 0.2% Tween 20 in 1x PBS for 20 mins. at room temperature and washed twice in 1xPBS. Cells were then resuspended in 400 ml of 0.25% paraformaldehyde (PFA) and stored at 4°C overnight. Cell suspensions were then stained with 40 µl of a 100 µg/ml PI solution in the dark at room temperature for one hour and analyzed by one colour flow cytometry (Coulter) on linear and log FL3 scales x count.

Histograms were later analyzed on multicycle software (Coulter) to qualitatively determine cycling and apoptotic populations.

Apoptosis as Measured by DNA Ladders:

DNA Ladders were performed similarly as previously described (eds. Coligan et al., 1994) Ladders were not produced for each subject in each experiment but for a sample from each activation protocol. At the end of each culture period, $2x10^6$ cells/treatment were pelleted by centrifugation for 10 min. at 200 x g at 4°C. The first supernatant was removed and the remaining pellet was vortexed vigorously upon addition of 0.5 ml TTE solution (0.2% Triton X-100 in TE buffer, pH 7.4). Fragmented DNA was separated from intact chromatin by microcentrifugation at 13,000 x g for 10 min. at 4°C. This supernatant was removed and marked T (contains fragmented DNA). 0.1ml of ice-cold 5 M NaCl was added to the tube marked T, vortexed and 0.7 ml ice-cold isopropanol was added to each tube, vortexed and placed at -20°C overnight. Tubes were then microcentrifuged for 10 min. at 13,000 x g and 4°C and all supernatant aspirated. Tubes were then half filled with ice-cold ethanol (70%) and microcentrifuged at 13,000 x g for 10 min. Again, supernatant was removed and pellets air dried for 6 hr. 30 μl TE buffer was added to DNA pellets and incubated at 37°C for 72 hrs. 10 x loading buffer was then added to 1x final concentration of each tube, heated at 65°C for 10mins and loaded onto a ethidium bromide stained 1% agarose gel using bromphenol blue tracking dye. The gel was run for 3 hours at 60V and visualized under UV light source. DNA electrophoresed from tubes marked T (containing fragmented DNA) are shown in figure 14. Controls used include DNA extracted from cells in media alone, and cells exposed to 1000 and 2000 rads of γ–irradiation followed by 24hrs incubation (known to induce apoptosis). γ-irradiation is able to induce T cells to a state that is both morphologically and biochemically characteristic of apoptosis. Most obvious in irradiated cells is the appearance of ladders (Sellins and Cohen, 1987). It is for this reason that γ-irradiated peripheral blood T cells were used as a positive control in experiments measuring apoptosis.

Statistical Analysis: Where statistical analysis was performed, one or two-tailed paired and unpaired Student's t-tests were conducted, using a computer software statistical program called "Instat".

RESULTS

1. Optimization of System

1.1 Titration curves for T cell stimulations. Prior to conducting stimulations of T cells using PHA or anti-CD3 antibody, titration curves were produced for each in order to define a concentration that would produce a submaximal stimulation. The cell proliferation was measured by ^{3}H-thymidine incorporation. Figure 1 shows a titration curve of PHA, using PBMCs from two donors in separate experiments, indicating a concentration of 1μg/ml as submaximal for inducing proliferation.

Similarly, a titration curve of anti-CD3 antibody (figure 2) was conducted. The concentrations used in subsequent experiments were 10μl of a 1/500 dilution of OKT3 / supernatant/ml. Titration curves for tetanus toxoid and Hsp70 were not conducted. A concentration of 0.1 LF/ml per $1x10^6$ cells/ml for tetanus toxoid and 5μg/ml for Hsp70 were used, as previously determined (Jutel et al., 1995 and Salveti et al., 1992).

1.2 Time of IFN-β-1b Addition. Prior to conducting our experiments, the time in which IFN-β-1b was to be added to the cultures was determined, in order to observe its effects. Three separate cultures of αβ T cells were stimulated with anti-CD3 antibody and IL-2 for 5 days, and various concentrations of IFN-β-1b were added on day 0, 1, or 2 (figure 3). Results show that IFN-β-1b suppressed proliferation when added to cultures on day 0, but no suppression was noted if added at 24 or 48 hours. In all subsequent experiments, IFN-β-1b was added on day 0, at time 0.

Figure 1. PHA Standard Curve. 1 x 10^6 PBMCs from 2 control donors were cultured in triplicate with various concentrations of PHA. At 60 hours, cells were pulsed with 1 μCi ^{3}H-thymidine for 12 hours and harvested, and the radioactivity associated with each culture was determined. CPM values indicated 1 μg/mL PHA produces submaximal proliferation.

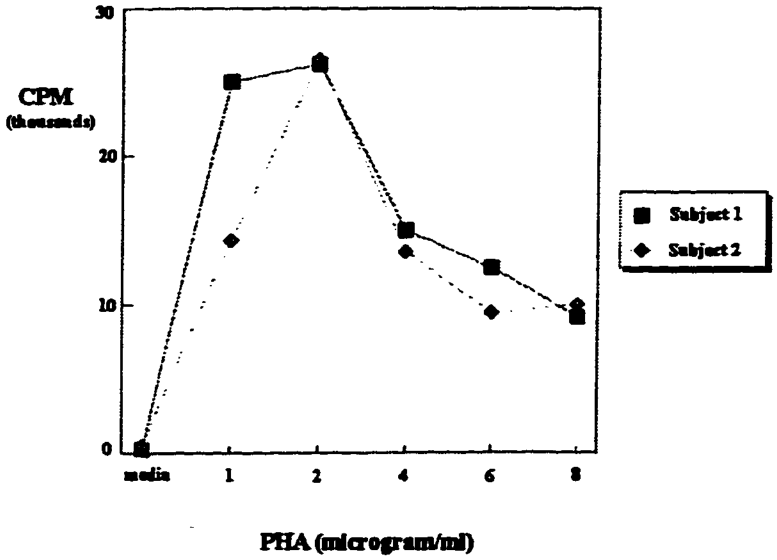

CPM
(thousands)
30
20
10
0
media
1
2
4
6
8
PHA (microgram/ml)
■ Subject 1
◆ Subject 2

Figure 2. OKT3 Supernatant Titration Curve. 1x 10^6 PBMCs from one donor were partially macrophage/monocyte depleted and cultured in triplicate for 5 days with various dilutions of OKT3 supernatant (10 µL/mL), followed by 5 units/ml IL-2 at 24 hours for a total of 5 days (120 hours), on plates initially coated with sheep anti-mouse IgG1. At 108 hours, cells were pulsed with 1 µCi ^{3}H-thymidine and harvested 12 hours later. Radioactivity of each culture was then assessed.

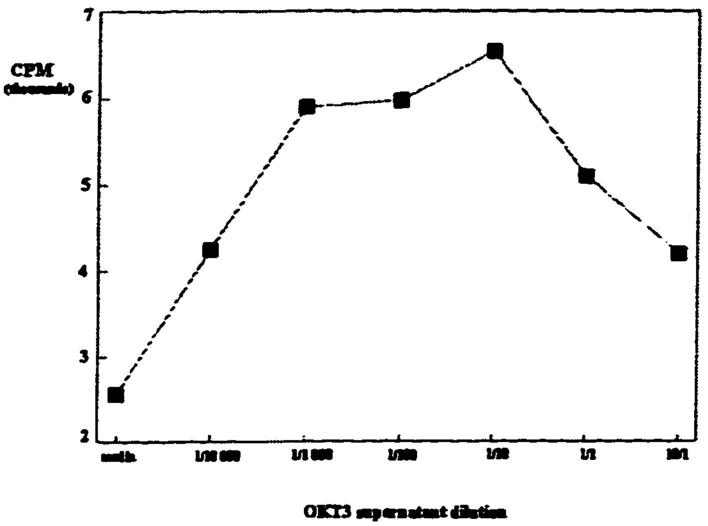
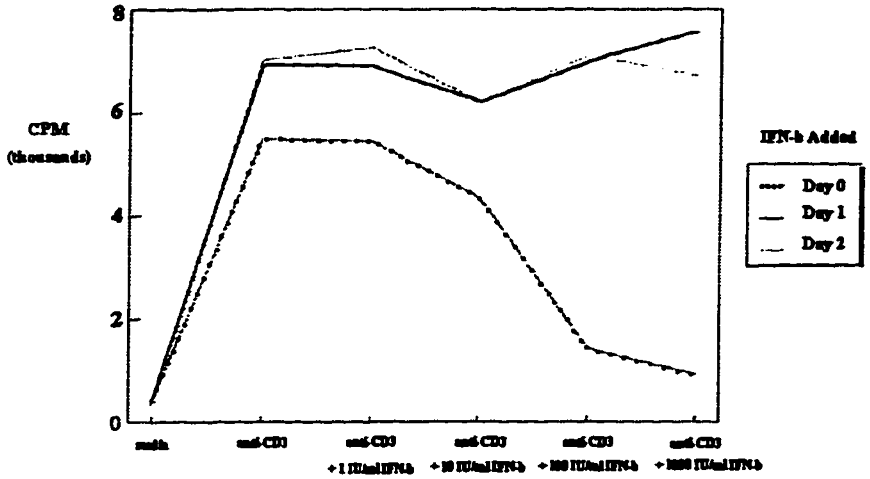

CPM
(thousands)
7
6
5
4
3
2
medium
1/10 000
1/1 000
1/100
1/10
1/1
10/1
OKT3 supernatant dilution

Figure 3. Effect of Time on Addition of IFN-β-1b on Anti-CD3 mAb Induced Proliferation. To determine the time for IFN-β-1b addition in order to observe its maximal effects, T cell proliferation by ^{3}H-thymidine incorporation was used as an example due to the known susceptibility to the anti-proliferative effects of IFN-β-1b. Various concentrations of IFN-β-1b were added to OKT3 supernantant stimulated cultures on day 0, day 1, or day 2.


8
6
4
2
0
CPM
(thousands)
media
anti-CD3
anti-CD3
+ 1 IU/ml IFN-b
anti-CD3
+ 10 IU/ml IFN-b
anti-CD3
+ 100 IU/ml IFN-b
anti-CD3
+ 1000 IU/ml IFN-b
IFN-b Added
Day 0
Day 1
Day 2

2. Suppression of Proliferation by IFN-β-1b

2.1 PHA activated αβ T cells. IFN-β-1b exerts a dose-dependent suppression of proliferation of PHA activated, αβ T cells, derived from both MS (n=7) and controls (n=11) (figure 4). At 1,000 IU/ml IFN-β-1b, the average percent suppression is higher for MS derived cells than control derived cells, both of which are obviously greater than the observed suppression by 1 IU/ml IFN-β-1b and 10 IU/ml IFN-β-1b. A difference in suppression between 1 and 10 IU/ml IFN-β-1b is obvious for control derived cells but the same is not true of MS cells. Similarly, a difference is noted between the suppression caused by 100 vs. 1000 IU/ml IFN-β-1b on control cells but not on MS derived cells. At higher doses (100 and 1000 IU/ml), MS derived cells are relatively more sensitive to suppression by IFN-β-1b than are control cells. All differences described are based on observable trends, as statistical significance was not calculated.

2.2 PHA activated expanded γδ T cells. Due to the fact that so few γδ T cells are present in peripheral blood (1-10%), γδ T cells were first preferentially expanded on anti-TCRδ1 coated plates for nine days. Such expansion, followed by purification with magnabeads allowed for populations of γδ T cells that were >85% pure (figure 5). These cell populations were used in subsequent experiments. Expanded γδ T cells from MS (n=4) and control (n=5) samples are also susceptible to a dose-dependent supression of PHA induced proliferation by IFN-β-1b (figure 6). The difference between the suppression observed at 1000 IU/ml IFN-β-1b is much higher than that seen at 1 IU/ml and 10 IU/ml in both MS and control cells. An obvious difference in suppression under each concentration

Figure 4. Dose-dependent suppression of PHA induced αβ T cell proliferation by IFN-β-1b. PBMCs from MS patients (n=7) and healthy controls (n=11) were cultured with PHA and various concentrations of IFN-β-1b for 72 hours. Proliferation was assessed by ^{3}H-thymidine incorporation and suppression by IFN-β-1b assessed relative to PHA alone.

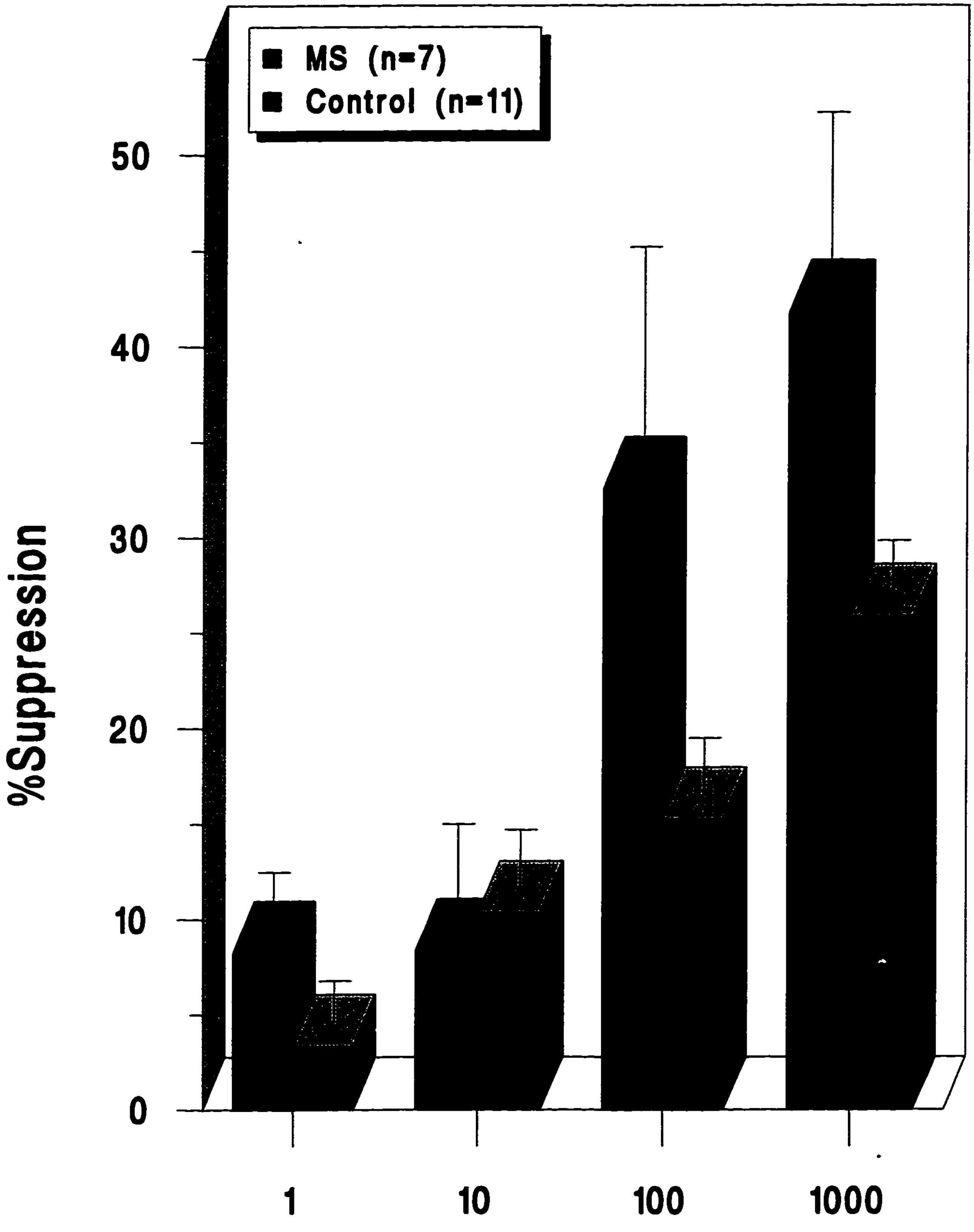
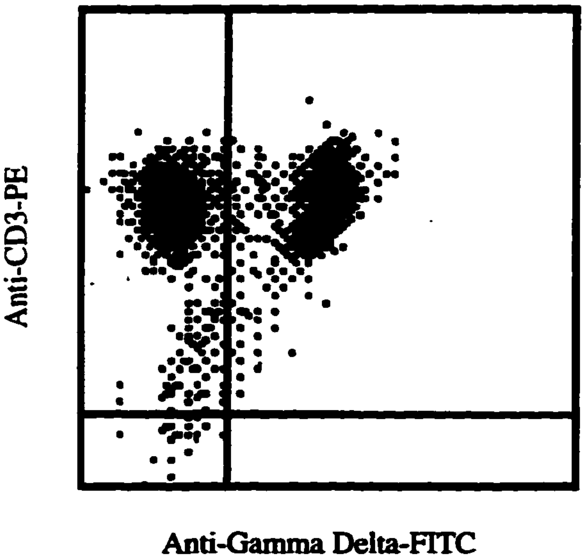
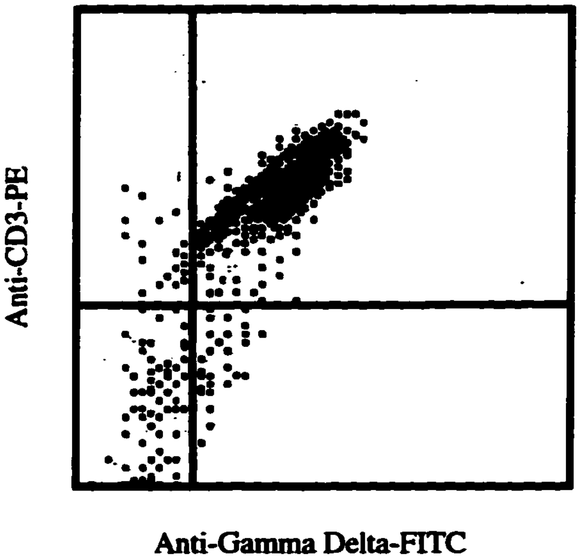

MS (n=7)
Control (n=11)
%Suppression
0
10
20
30
40
50
1
10
100
1000
IFNβ IU/ml

Figure 5. γδ T Cell Purification. Prior to use in experiments, γδ T cells were preferentially expanded for 9 days in order to increase the proprtion of γδ T cells in culture. Purification was accomplished by negative magnabead selection allowing for >85% γδ T cell populations. Histograms depict two colour flow cytometry of expanded cells before (left) and after (right) magnabead purification. Depicted is typical of the samples studied.

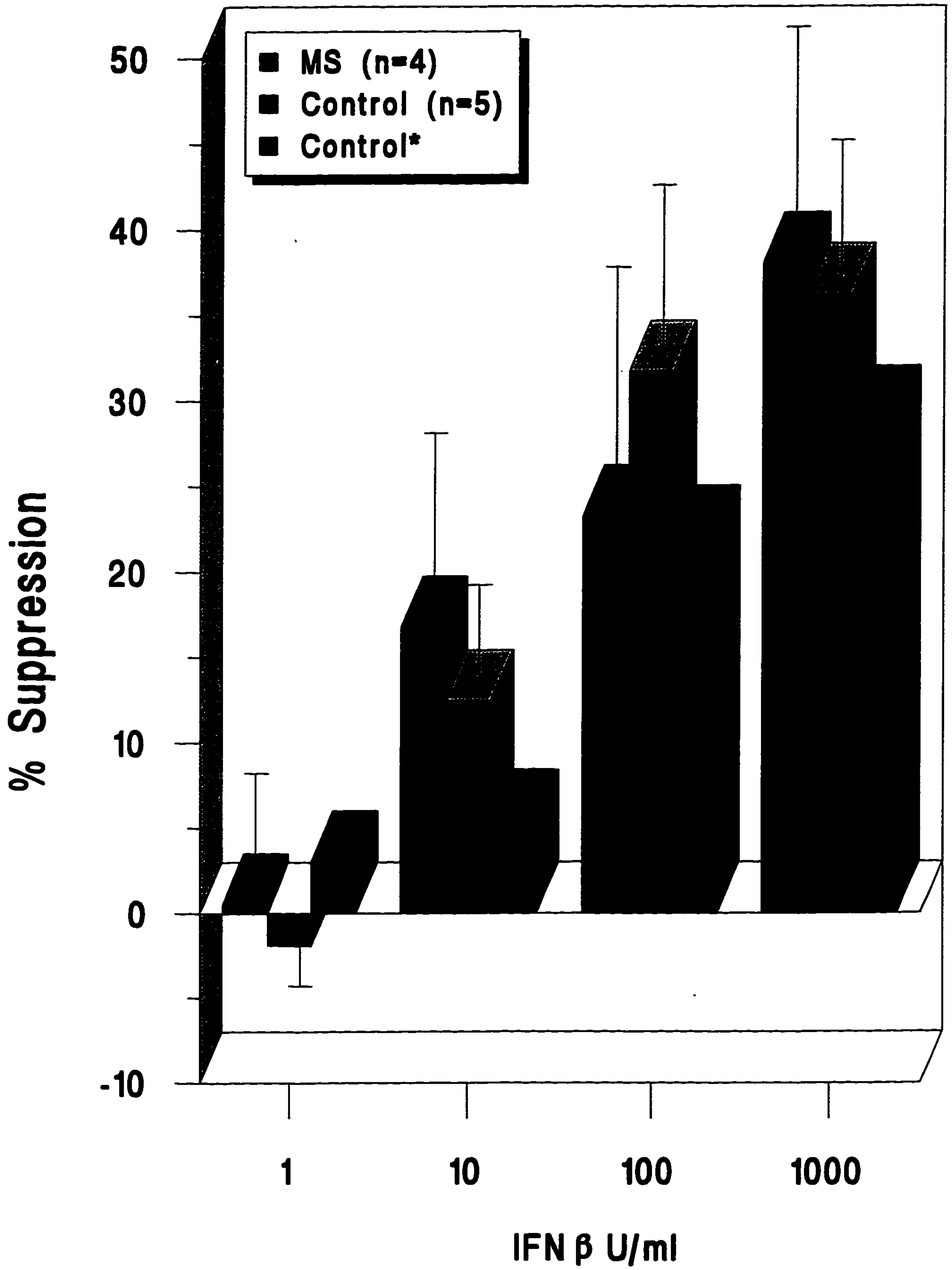




96.6% CD3+ve
47.6% Gamma-Delta TCR+ve

84.5% CD3+ve
97% Gamma-Delta TCR+ve

Figure 6. Dose-dependent suppression of PHA induced γδ T cell proliferation by IFN-β-1b. Expanded and purified γδ T cells from MS (n=4) and healthy controls (n=5) were cultured with PHA and various concentrations of IFN-β-1b for a total of 72 hours. Proliferation was assessed by ^{3}H-thymidine incorporation and suppression assessed relative to c.p.m. values with PHA alone. As a control for comparison with αβ T cell suppression, αβ T cells were expanded for 6 days and assessed in a similar manner.



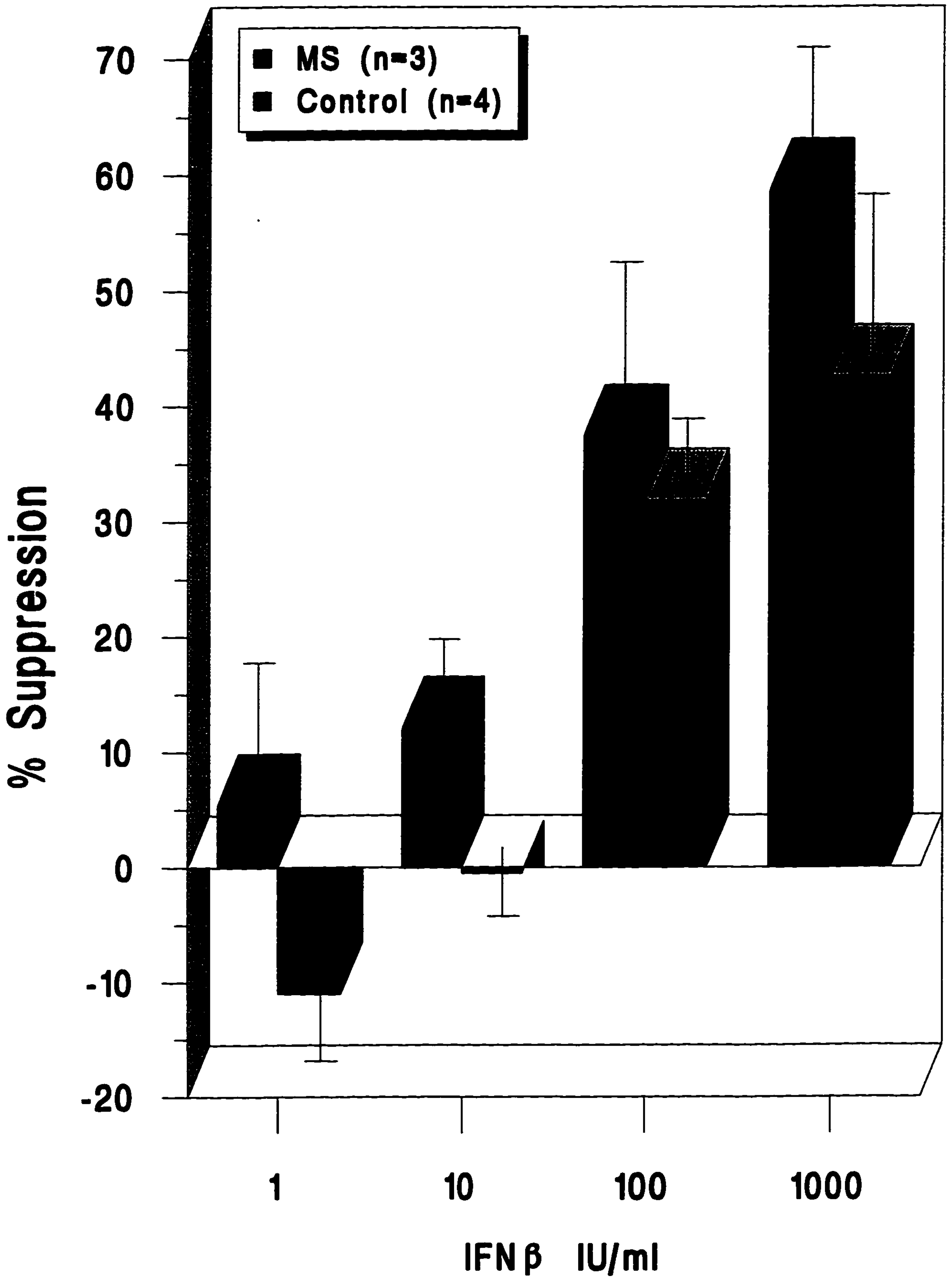
*6 day pre-expanded αβ T cells

is present for control derived γδ cells from 1-100 IU/ml however the difference is not as clear for MS derived γδ cells under the same treatment. There is no relative difference between the susceptibility of either MS or control γδ T cells to be

suppressed by IFN-β-1b as was seen with αβ T cells (figure 4). As a control, αβ T cells from one individual were also expanded prior to culture with PHA and IFN-β-1b. The dose-dependent suppression of proliferation by IFN-β-1b seen here (figure 6) is similar to that shown for non-expanded cells (figure 4), suggesting the expansion process does not alter susceptibility to IFN-β-1b-induced suppression of proliferation. The results for expanded γδ T cells might therefore be reflective of what is seen with non-expanded γδ T cells.

2.3 Anti-CD3 Antibody Activated αβ T cells. In an effort to mimic the in vivo activation state of T cells, stimuli that involve more direct interaction through the TCR were used in subsequent experiments measuring suppression of proliferation (i.e., first anti-CD3 mAb, then antigen (tetanus toxoid)). αβ T cells were stimulated with anti-CD3 mAb and IL-2 (5 units/ml) and cultured with various concentrations of IFN-β-1b for five days. Again, a dose-dependent suppression of proliferation by IFN-β-1b is observed (figure 7), similar to PHA (figure 4). Suppression seen at 1,000 IU/ml for both MS and control cells was much greater than that seen at 1 and 10 IU/ml. Differences between groups (MS and control) are evident only at 10 IU/ml, with MS cells appearing more sensitive to suppression. Again, stated differences are based on observed trends as statistics were not

Figure 7. Dose-dependent suppression of anti-CD3 mAb induced αβ T cell proliferation by IFN-β-1b. MS (n=3) and control (n=4) αβ T cells were cultured in the presence of anti-CD3 mAb and IL-2 (added at 24 hours) and various concentrations of IFN-β-1b for 5 days. Results were collected following ^{3}H-thymidine incorporation and comparison to c.p.m. values with anti-CD3 mAb and IL-2 alone.



■ MS (n=3)
■ Control (n=4)
70
60
50
40
30
20
10
0
-10
-20
% Suppression
1
10
100
1000
IFN β IU/ml

performed.

2.4 Tetanus toxoid activated αβ T cells. Cells stimulated with tetanus toxoid and IL-2 for 7 days exhibit a dose-dependent suppression of proliferation by IFN-β-1b as well. Obvious differences were observed between 1 IU/ml vs. 100 and 1,000 IU/ml for and 10 IU/ml vs. 100 and 1,000 IU/ml. MS derived T cells are also susceptible to suppression of proliferation by IFN-β-1b as measured in two experiments (figure 8).

Together, data indicate that IFN-β-1b suppresses the proliferation of T cells stimulated with potent mitogens and more specific antigens such as tetanus toxoid. Several stimulations of γδ T cells with hsp70 were attempted in order to determine the ability of IFN-β-1b to suppress TCR-mediated proliferation of γδ T cells, but all were unsuccessful, probably owing to too few hsp70 specific γδ T cells in expanded populations.

3. Augmentation of Apoptosis by IFN-β-1b

The suppression of cell proliferation was measured by ^{3}H-thymidine incorporation. The decreased incorporation of thymidine at higher doses of IFN-β-1b could have been due to a reduction in the proliferation of the cells or an increase in cell death. Figure 9 suggests that either mechanism is possible; as cells are cultured with increasing doses of IFN-β-1b, the number of viable cells is reduced in both MS and controls (as determined by counts in trypan blue). Dao et al. (1993) showed that IFN-α augments PHA-activated apoptosis in T cells. Since IFN-α and IFN-β act on the same receptor, αβ and γδ T cells following the aforementioned cultures were analyzed by flow cytometry to determine if an apoptotic mechanism could account for the reduction in thymidine incorporation.

Figure 10 shows the absence of apoptosis in αβ T cells incubated in media alone or with PHA (negative controls), and obvious apoptotic populations to the left of the G1 peak in αβ T cells treated with 1000 and 2000 rads of γ-irradiation (positive controls). PHA has been shown to induce apoptosis in T cells, which was observed in some but not all αβ T cell samples analyzed.

Figure 8. IFN-β-1b suppresses antigen induced proliferation of αβ T cells. αβ T cells from MS (n=2) and controls (n=4) were cultured with 0.1 Lf/ml tetanus toxoid and various concentrations of IFN-β-1b for 5 days. 10 units/ml IL-2 was then added and incubated for a further 48 hours. Results were measured by ^{3}H-thymidine incorporation and comparison to tetanus toxoid and IL-2 alone.

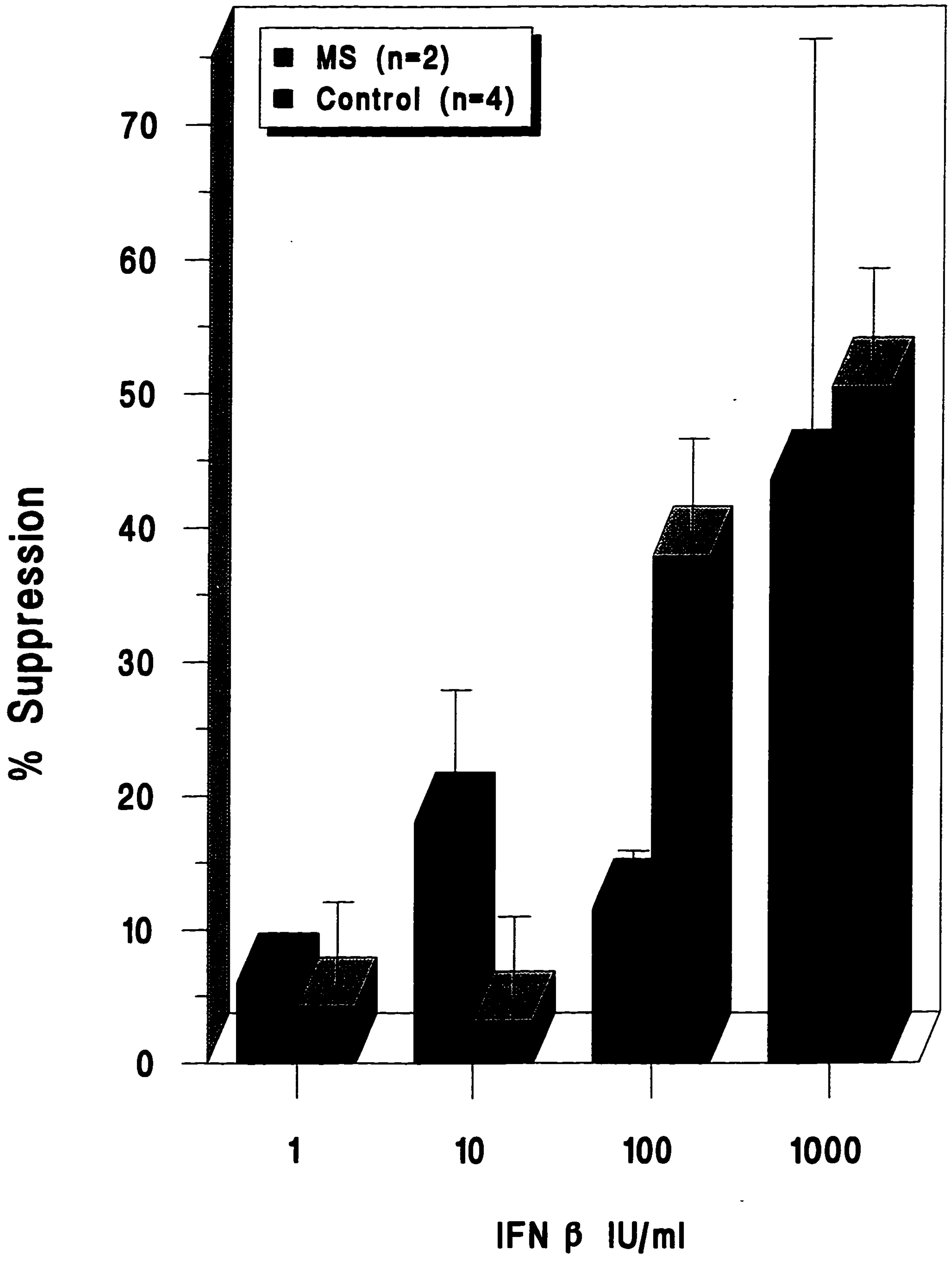

■ MS (n=2)
■ Control (n=4)
% Suppression
0
10
20
30
40
50
60
70
1
10
100
1000
IFN β IU/ml

Figure 9. The viability of αβ T cells stimulated with PHA and various IFN-β-1b concentrations for 72 hours was assessed by counts in trypan blue. Here, two representative samples of MS and control cells indicate that at 1000 IU/ml, the viability of cells is reduced, especially those that are MS derived.

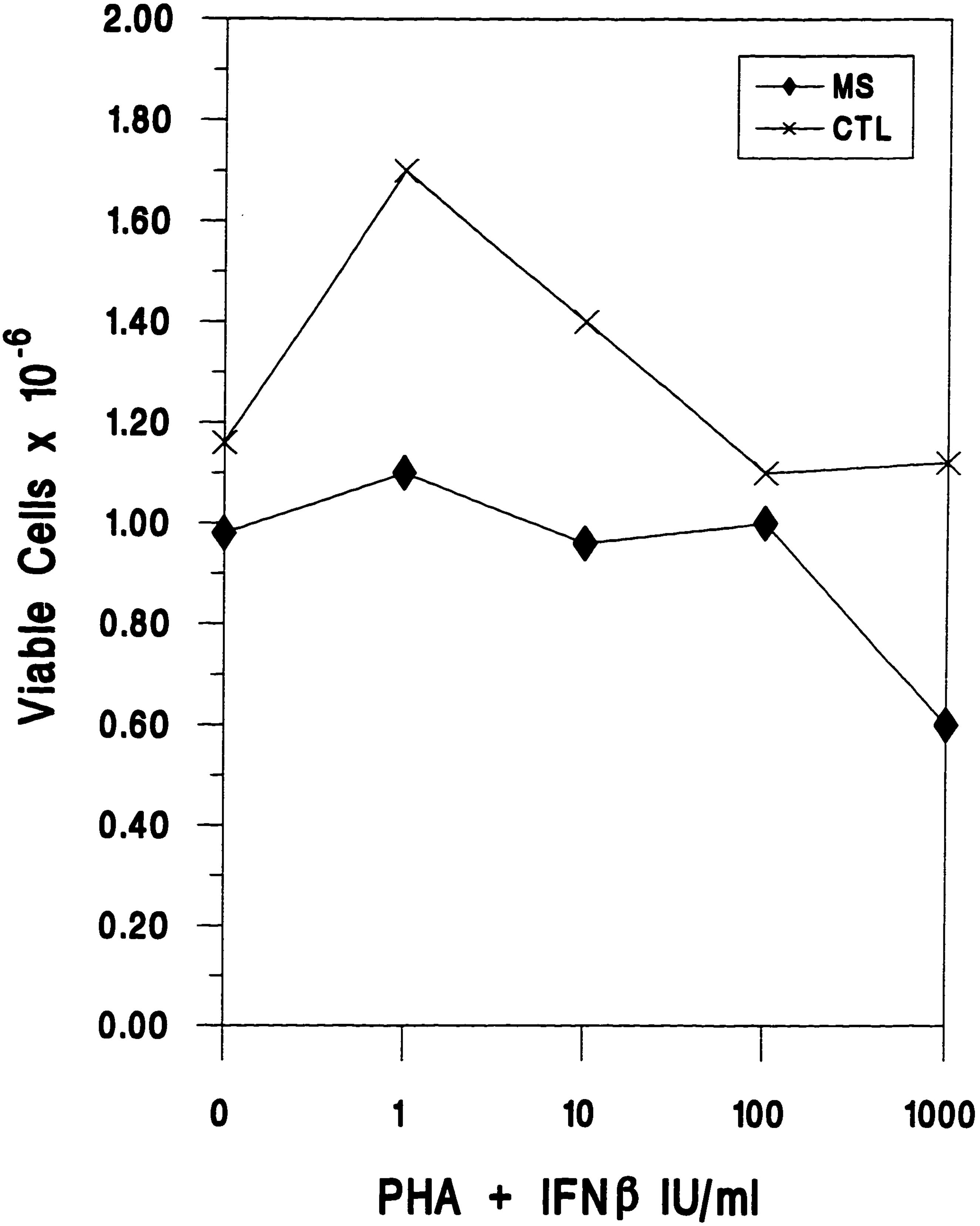
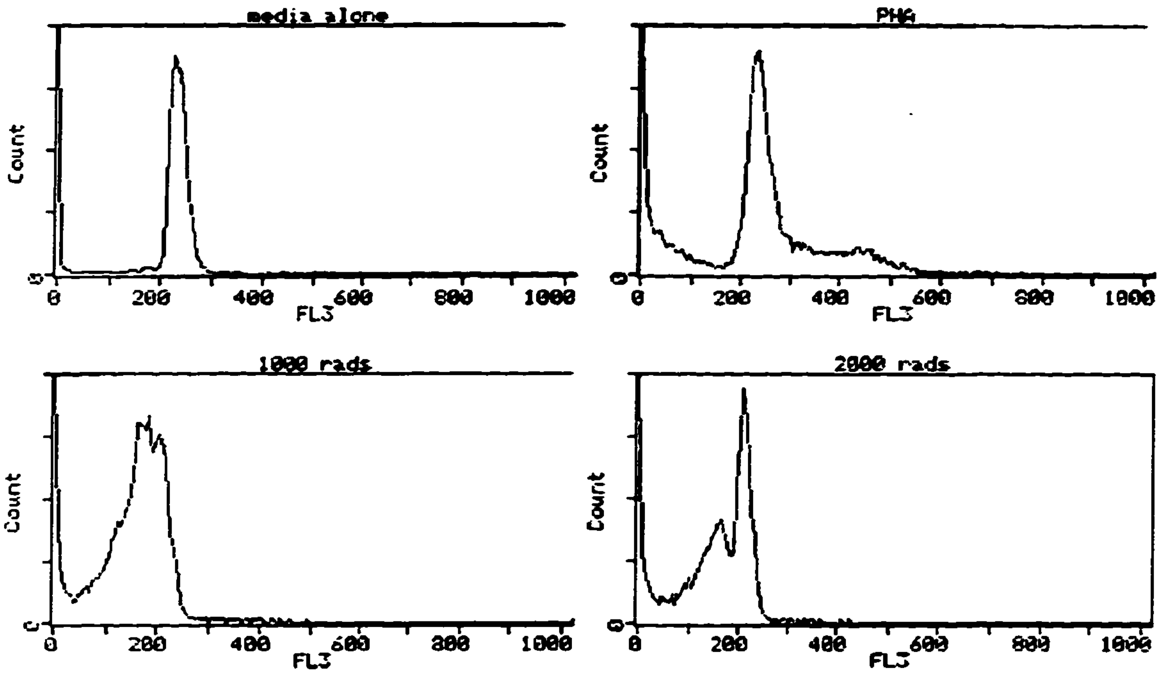

Viable Cells x 10^{-6}
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
MS
CTL
0
1
10
100
1000
PHA + IFNβ IU/ml

Figure 10. Controls for apoptosis detection by flow cytometry. Four flow cytometry histograms of permeabilized and PI stained αβ T cells analyzed on count vs. FL3 scales serve as controls for the measurement of apoptosis. An apoptotic hypodiploid peak left of cells in stage G1 of the cell cycle is absent in cells incubated for 72 hours in media alone or PHA, and therefore serve as negative controls. Apoptotic populations are evident in T cells treated with 1000 and 2000 rads of γ-irradiation prior to 72 hours incubation, and serve as positive controls.


media alone
PHA
1000 rads
2000 rads
Count
FL3
0
200
400
600
800
1000

Under the stimulation of all mitogens/antigens used, an enhancement of apoptosis by IFN-β-1b was observed at 1,000 IU/ml on αβ T cells, as measured by flow cytometry, on both MS and control cells. Apoptotic populations, those located to the left of the G1 peak, are obvious in samples cultured with 1000 IU/ml IFN-β-1b and PHA (figure 11), anti-CD3 mAb (figure 12), and tetanus toxoid (figure 13), more so than is evident from samples stimulated without IFN-β-1b. This augmentation was not noted on every sample tested; apoptosis was observed in 8 of 17 samples investigated. Following the analysis of 10 separate samples, I was unable to detect apoptosis in γδ T cells cultured with PHA +/- 1000 IU/ml IFN-β-1b (data not shown), by either flow cytometry or DNA electrophoresis.

DNA gel electrophoresis confirmed these results (figure 14). Apoptosis leads to ordered enzyme cleavage of DNA into multiples of 200 base pairs, which when depicted on gels, gives the appearance of a step 'ladder'. These ladders are absent in samples extracted from αβ T cells cultured in media alone (lane 1), while γ-irradiated cells exhibited obvious ladders (lane 3). αβ T cells treated with PHA + 1,000 IU/ml IFN-β-1b (lane 4) and PHA alone (lane 5) after 72 hours exhibited ladder formation. αβ T cells not activated but incubated in the presence of 1,000 IU/ml IFN-β-1b for 3 days exhibited no DNA fragmentation by electrophoresis (lane 6). Ladders are faint but present with DNA extracted from αβ T cells incubated with anti-CD3 mAb for 5 days (lane 7) and with anti-CD3 in the presence of 1,000 IU/ml IFN-β-1b (lane 8). Ladders are not evident from αβ T cells stimulated with tetanus toxoid in the presence (lane 10) or absence of IFN-β-1b (lane 9), although in a different subject sample, apoptosis was evident by flow cytometry

Figure 11. IFN-β-1b augments apoptosis in PHA activated αβ T cells. αβ T cells incubated with PHA +/- 1000 IU/ml IFN-β-1b for 72 hours were permeabilized, stained with propidium iodide and analyzed by flow cytometry on count x FL3 log scales. As is evident in the upper histogram, an apoptotic population is present in αβ T cells incubated for 72 hours with PHA alone. With the addition of 1000 IU/mL IFN-β-1b, the lower histogram indicates that the apoptotic population is augmented. Presented is one representative sample.

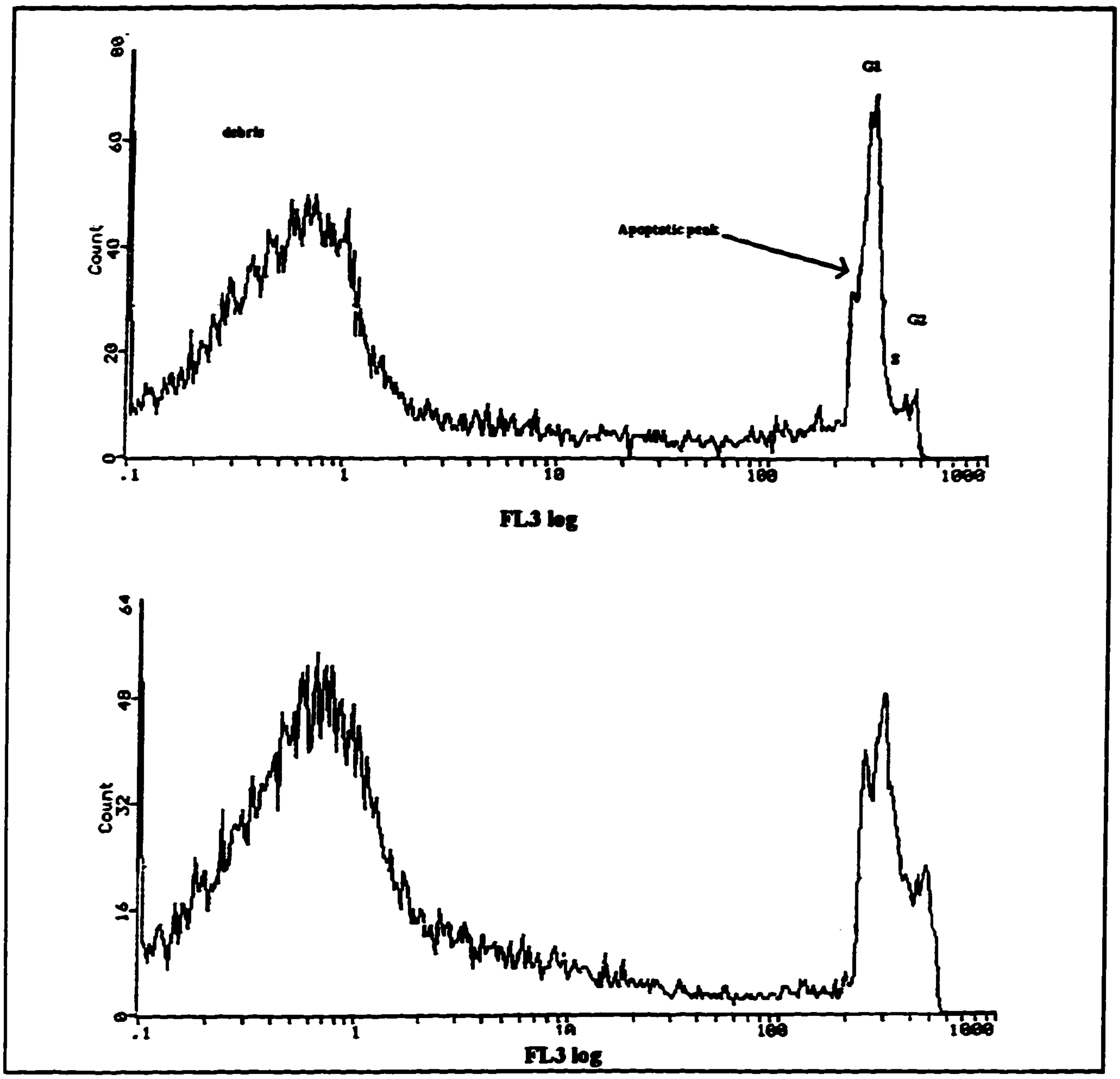

80
60
40
20
0
Count
debris
Apoptotic peak
G1
S
G2
.1
1
10
100
1000
FL3 log
64
48
32
16
0
Count
.1
1
10
100
1000
FL3 log

Figure 12. IFN-β-1b induces apoptosis in anti-CD3 mAb+IL-2 activated αβ T cells. Following 5 day culture with anti-CD3 and IL-2 +/- 1000 IU/ml IFN-β-1b, αβ T cells were permeabilized, stained with PI and analyzed by flow cytometry on count x FL3 log histograms. Apoptosis is absent in αβ T cells stimulated with anti-CD3 and IL-2 for 5 days (A) and is small yet obvious upon similar stimuation in the presence of 1000 IU/ml IFN-β-1b (B). Presented is one representative sample.

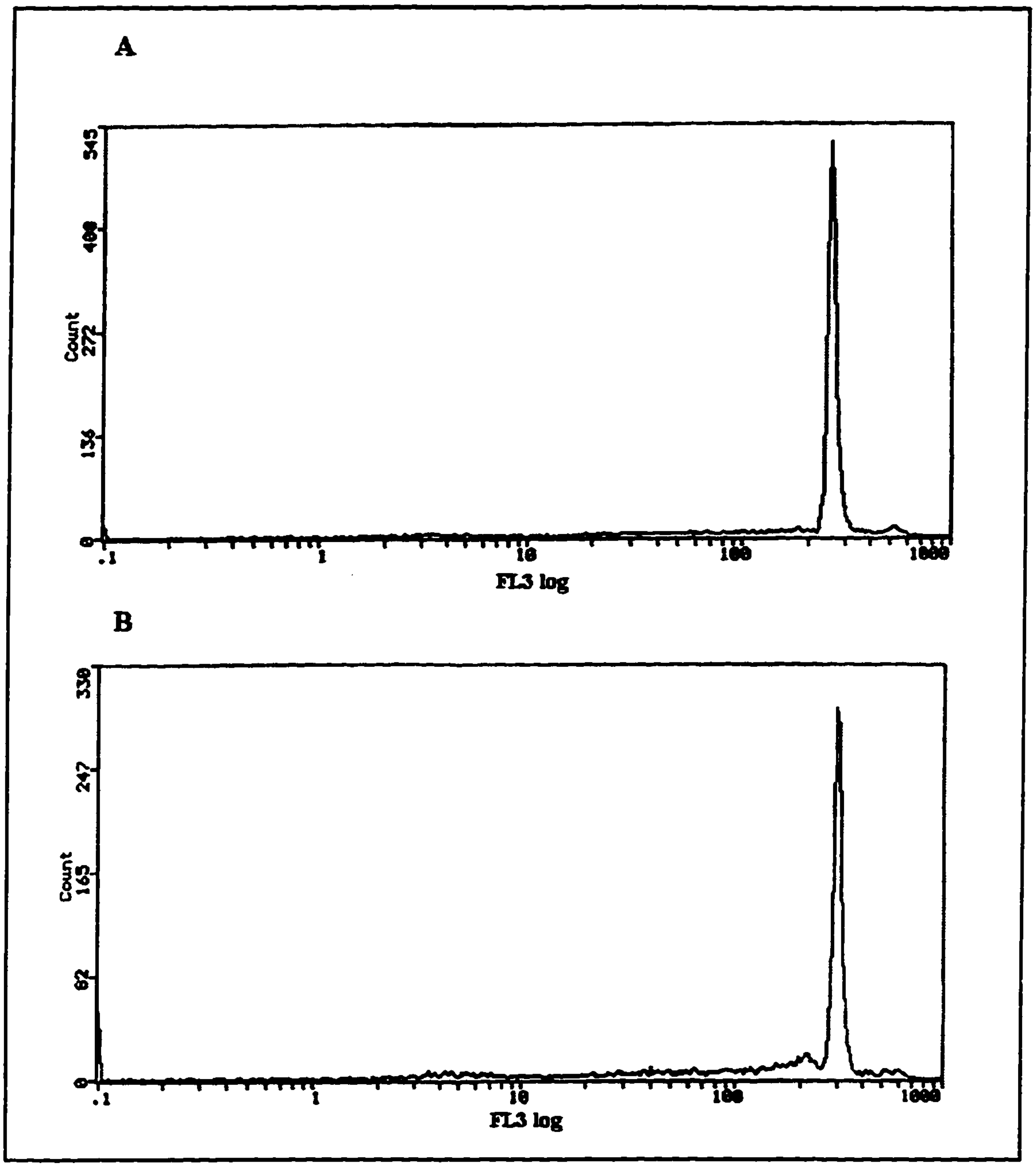

A
545
408
272
136
0
Count
.1
1
10
100
1000
FL3 log
B
330
247
165
82
0
Count
.1
1
10
100
1000
FL3 log

Figure 13. IFN-β-1b induces apoptosis in tetanus toxoid and IL-2 stimulated αβ T cells. Assessment of αβ T cells following 7 days of culture in the presence of tetanus toxoid and IL-2 (final 48 hours) +/- 1000 IU/ml IFN-β-1b by permeabilization, PI stain and flow cytometry reveals (A) an absence of apoptosis in cells cultured without IFN whereas an apoptotic peak is obvious in cells stimulated in its presence (B). One representative experiment is shown.

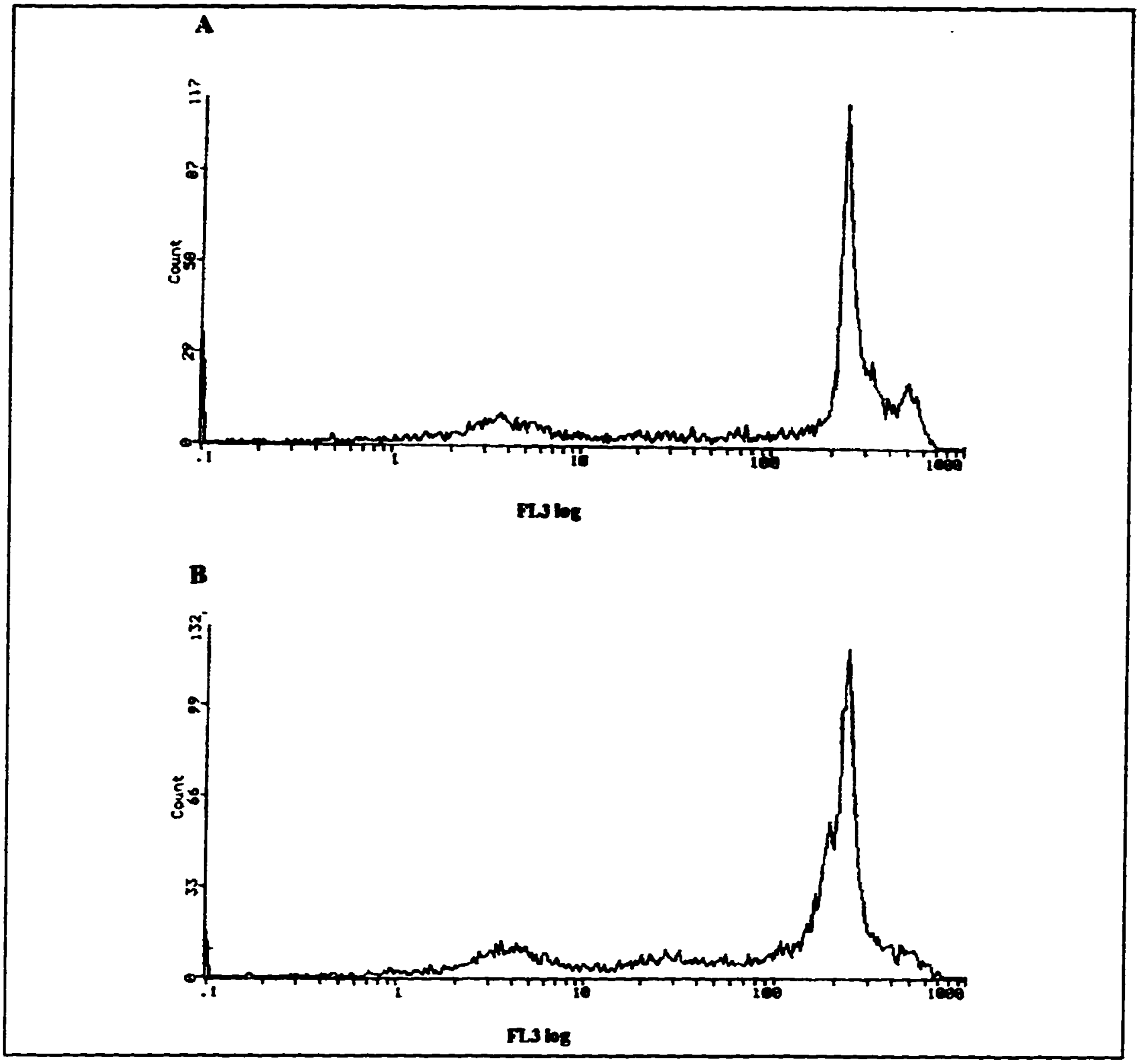
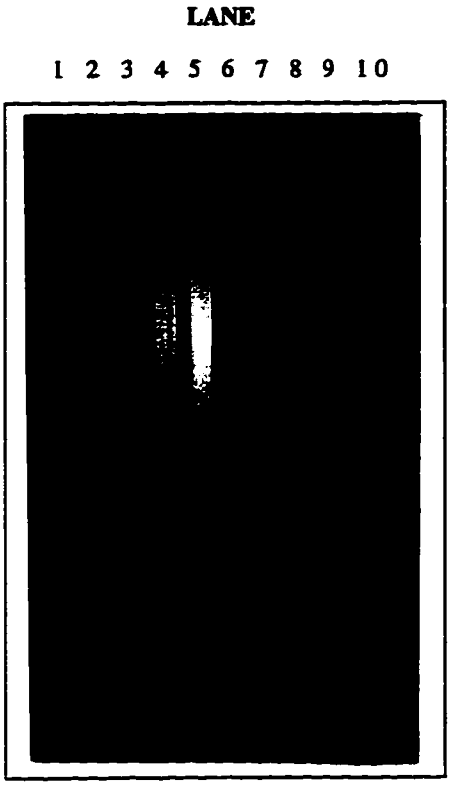

A
117
87
58
29
0
Count
.1
1
10
100
1000
FL3 log
B
132
99
66
33
0
Count
.1
1
10
100
1000
FL3 log

Figure 14. Confirmation of apoptosis by DNA electrophoresis. The presence of DNA ladders, produced by gel electrophoresis of low molecular weight DNA extracted from cultured cells, confirms the presence of apoptosis. No ladders are seen in cells cultured in media alone (lane 1) and in one sample cultured with tetanus toxoid and IL-2 alone (lane 10) or + 1000 IU/ml IFN-β-1b (lane 9). Ladders are present from cells incubated following 1000 rads γ-irradiation (lane3), 72 hours culture with PHA alone (lane 5) and PHA + 1000 IU/ml IFN-β-1b (lane 4), and following anti-CD3 +IL-2 stimualtion with (lane 8) or without (lane 7) 1000 IU/ml IFN-β-1b. Lanes 2 and 6 are empty.



LANE
1 2 3 4 5 6 7 8 9 10

(figure 13).

These results suggest that IFN-β-1b induces apoptosis that is detectable at high concentrations, particularly in αβ T cells. The lack of detection of any apoptosis with lower concentrations indicates that not all of the IFN-β-1b induced suppression of proliferation observed with these concentrations may be accounted for by an apoptotic mechanism.

4. Effect of IFN–β-1b on the Activation of T cells as Measured by CD25 Expression.

4.1 CD25 expression on MS and control T cells. The final objective addressed by this work involved an analysis of the effects of IFN-β-1b on the T cell expression of CD25 (IL-2R), an activation marker. Specifically, the mean channel fluorescence (MCF) of CD25 was assessed. It is widely reported that CD25 expression is upregulated on MS total T cells vs. controls. Here, I defined such expression on both T cell subsets, αβ and γδ (figure 15). Following 72 hours of incubation in media alone, MS fresh αβ and expanded γδ T cells express significantly higher CD25 MCF than control derived counterparts ($p<0.03$ and $p<0.02$, respectively). Following stimulation with PHA for 72 hours, MS derived αβ T cells also express significantly higher CD25 MCF units, in comparison to controls, yet differences between MS and control γδ T cells are not significant ($p<0.02$ and $p<0.3$, respectively).

4.2 Effect of IFN-β-1b on the CD25 expression of Control αβ and γδ T cells.

Figure16 indicates results from one representative experiment (total n=8) on control derived cells. Freshly isolated T cells were cultured and analyzed at 72 hours by two colour flow cytometry. For this experiment, T cells were not expanded prior to culture with IFN-β-1b. At 72 hours, CD25 expression is greatly increased on both αβ and γδ T cells following stimulation with PHA. When cultured in the presence of 10 IU/ml IFN-β-1b, αβ T cells show a significant increase in CD25 expression. Conversely, under the same conditions, γδ T cells exhibit a significant decrease in CD25

Figure 15. CD25 expression on MS vs. control αβ and expanded γδ T cells with and without PHA activation. Following 72 hours in culture in media alone or with PHA, non-expanded αβ T cells from MS (n=5) and control (n=5) were assessed by flow cytometry to determine MCF of CD25 expression. Similarly, MS (n=5) and control (n=5) expanded γδ T cells were cultured a further 72 hours in media or with PHA and activation, indicated by CD25 MCF, was assessed by flow cytometry.

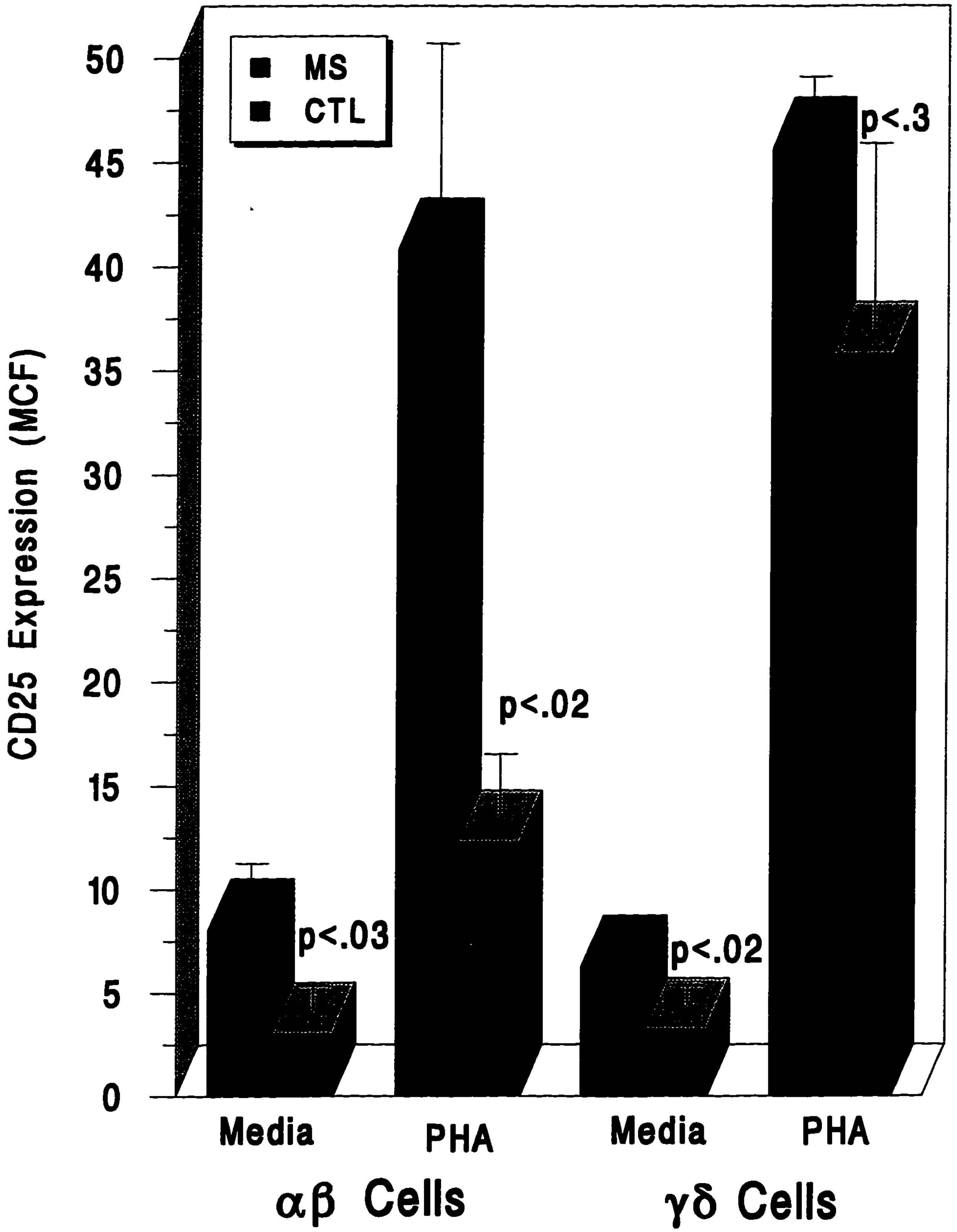
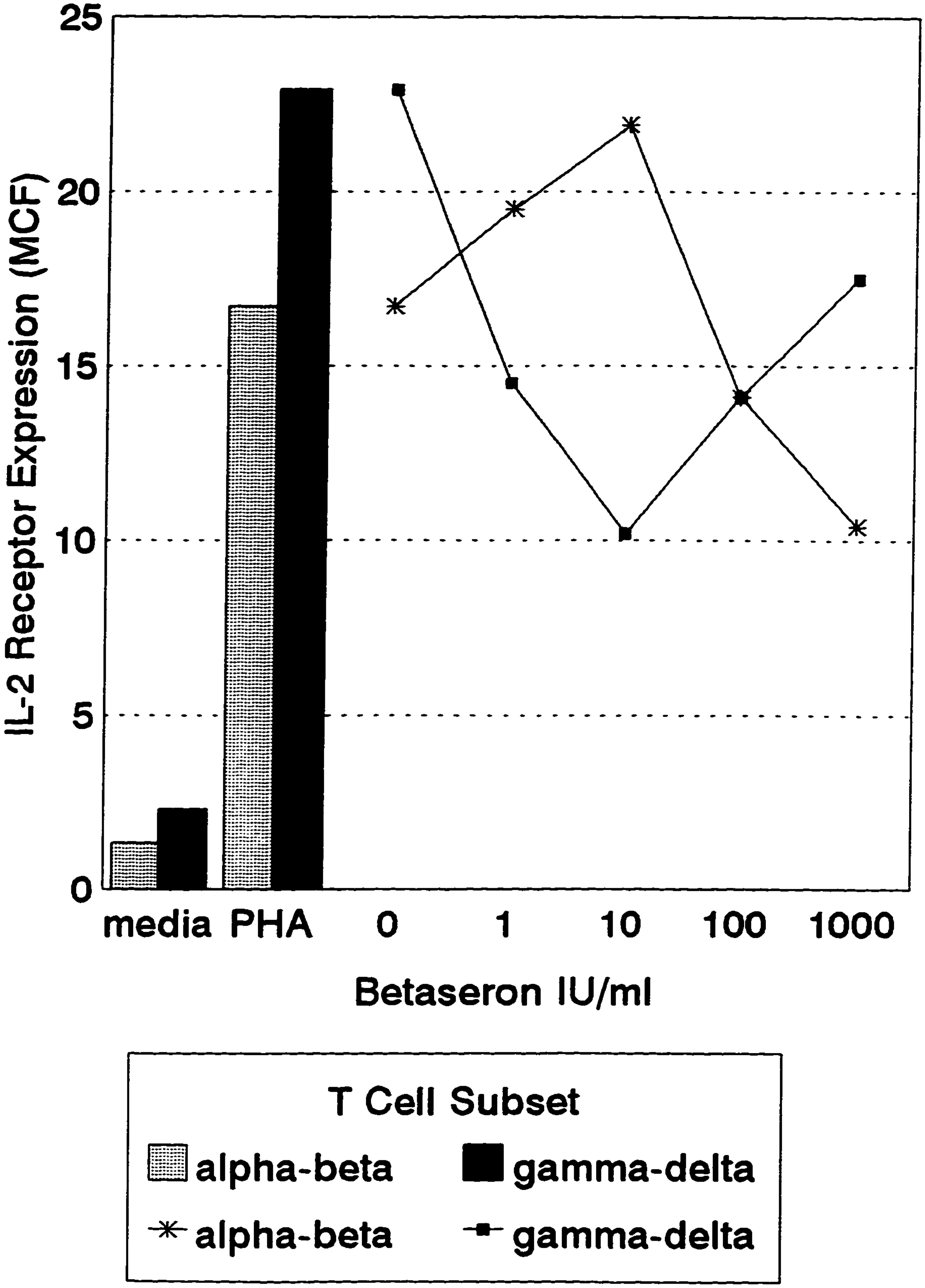

CD25 Expression (MCF)
0
5
10
15
20
25
30
35
40
45
50
■ MS
■ CTL
p<.03
p<.02
p<.02
p<.3
Media
PHA
Media
PHA
αβ Cells
γδ Cells

Figure 16. IFN-β-1b Differentially Effects CD25 Expression on αβ vs. γδ T cells. Control derived, non-expanded PBMCs were incubated for 72 hours with PHA and various concentrations of IFN-β-1b and CD25 expression was determined. This expression was assessed on αβ and γδ T cells individually, by two colour flow cytometry. Shown is one representative sample of 8.



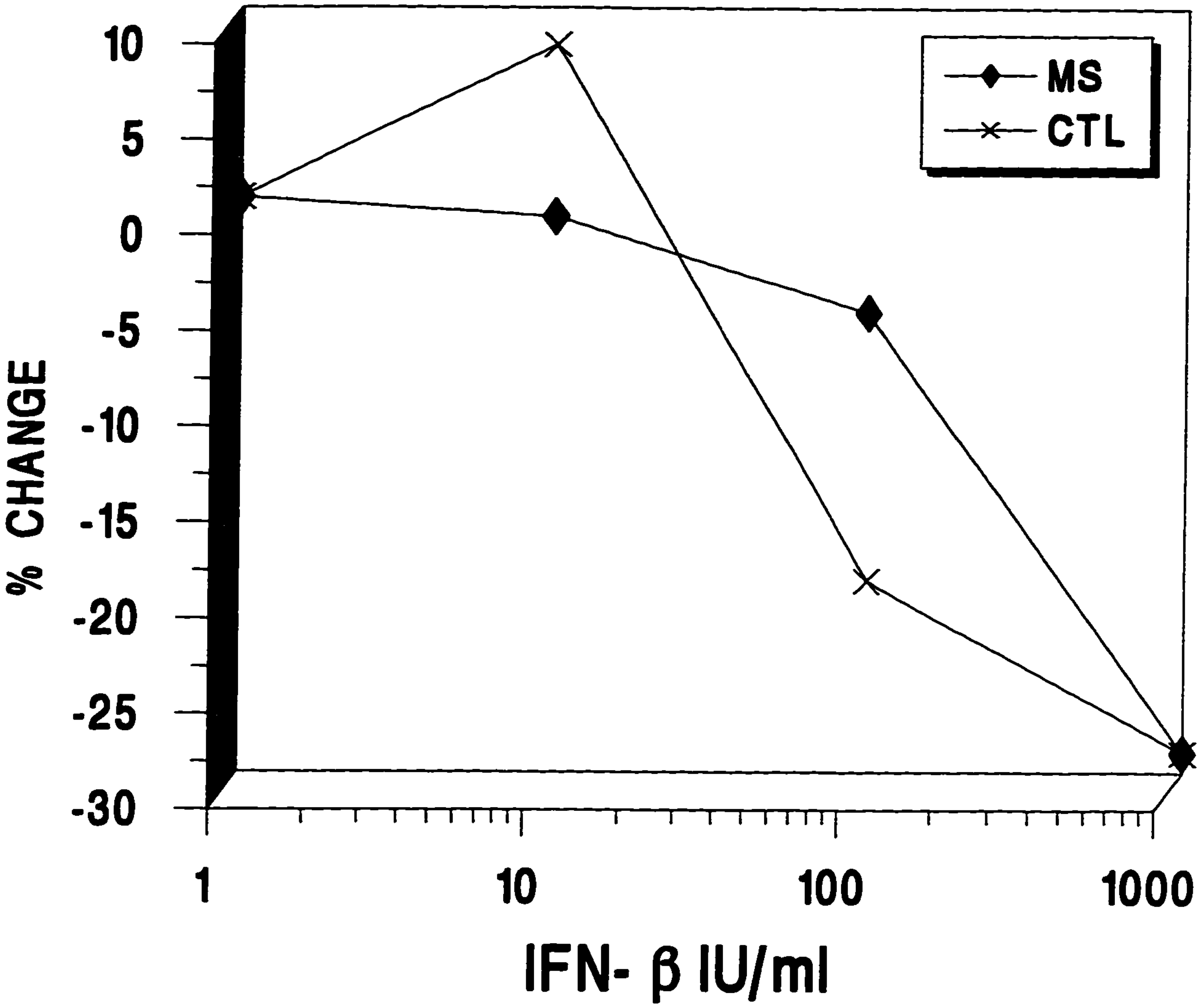
25
20
15
10
5
0
IL-2 Receptor Expression (MCF)
media
PHA
0
1
10
100
1000
Betaseron IU/ml
T Cell Subset
alpha-beta
gamma-delta
alpha-beta
gamma-delta

expression. Upon culture with 1000 IU/ml IFN-β-1b, both αβ and γδ T cells show a significant reduction in CD25 expression as compared to levels with PHA alone.

4.3 Effect of IFN-β-1b on the CD25 expression of MS and Control αβ and expanded γδ T cells. CD25 expression on freshly derived and expanded MS PBMCs could not be assessed as above due to too few γδ T cells present in peripheral blood samples. For this reason, γδ cells were first expanded for nine days prior to culture, and these expanded γδ T cells from control and MS subjects show overall reduction in the expression of CD25 following culture with PHA and IFN-β-1b (figure 18). At 10 IU/ml however, MS γδ T cells showed a more obvious decrease in CD25 expression, similar to that observed with non-expanded control γδ T cells (figure 16). Fresh αβ T cells from MS (n=5) and controls (n=4), following 72 hours culture with PHA and various IFN-β-1b concentrations, exhibited a dose-dependent decrease in CD25 expression (figure 17). Only control derived cells however, exhibited an increase in CD25 expression at 10 IU/ml, similar to that seen with the initial CD25 experiment (figure 16). Signifance with respect to differences cannot be made here as statistics were not conducted.

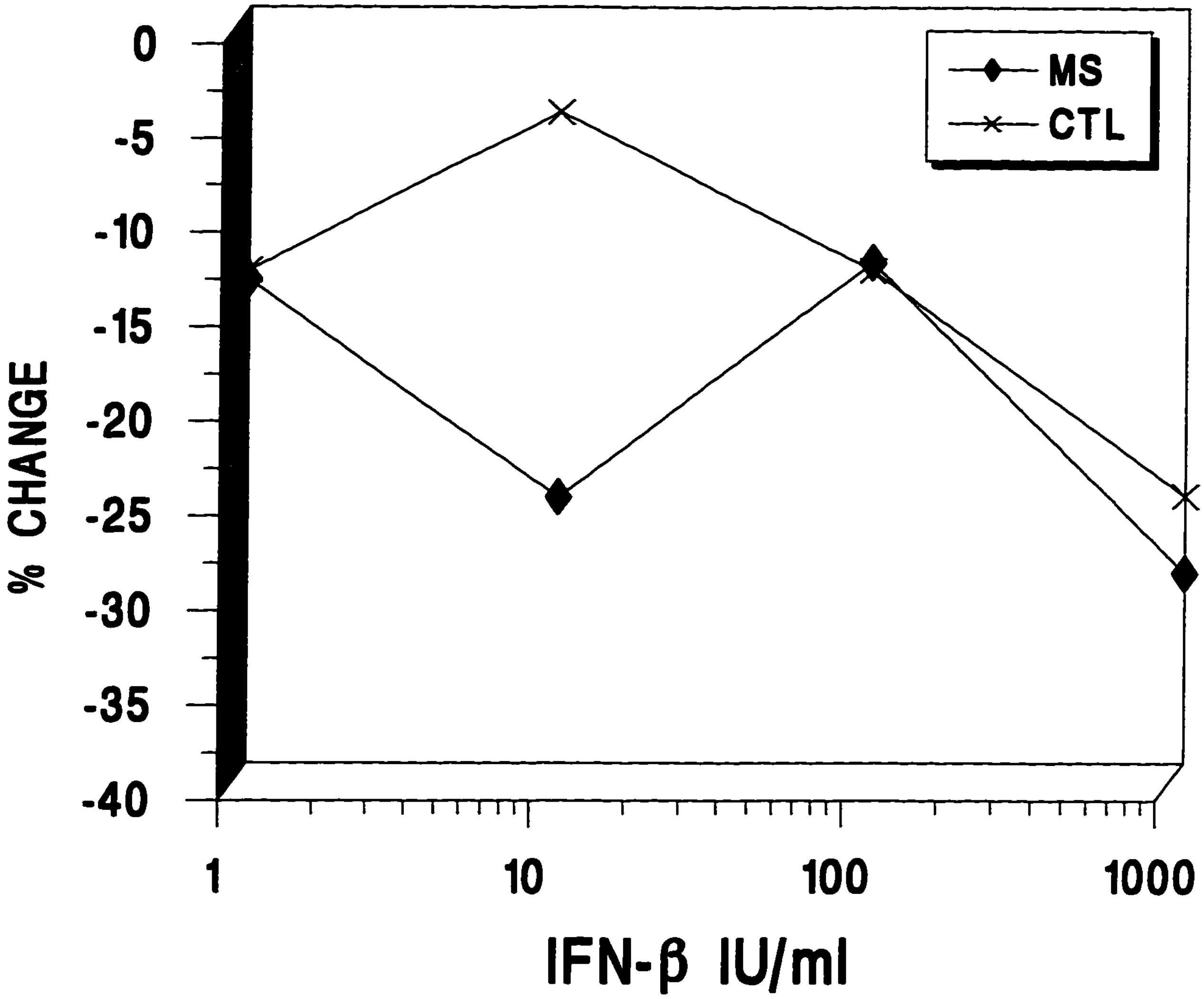
As a control for investigating the effect of IFN-β-1b on PHA activated and expanded γδ T cells, control αβ T cells were also expanded for 6 days prior to the 72 hour culture with and without PHA and various IFN-β-1b concentrations. A six day culture was felt to be appropriate as a comparative measure since αβ T cells comprise a larger proportion of peripheral blood T cells and would therefore proliferate in less time on coated plates, as more cells are available for both autocrine and paracrine stimulation. These cells also exhibit a similar increase in CD25 expression under the therapeutic dose range (1-100

Figure 17. % change in CD25 expression on PHA activated αβ T cells from MS (n=5) and controls (n=5) induced by IFN-β-1b. Following incubation with PHA and various concentrations of IFN-b-1b for 72 hrs., flow cytometric analysis revealed an upregulation of CD25 on control cells at low doses, and at higher doses, this expression decreased. CD25 expression decreased at all concentrations on MS αβ T cells.



10
5
0
-5
-10
-15
-20
-25
-30
% CHANGE
1
10
100
1000
IFN- β IU/ml
MS
CTL

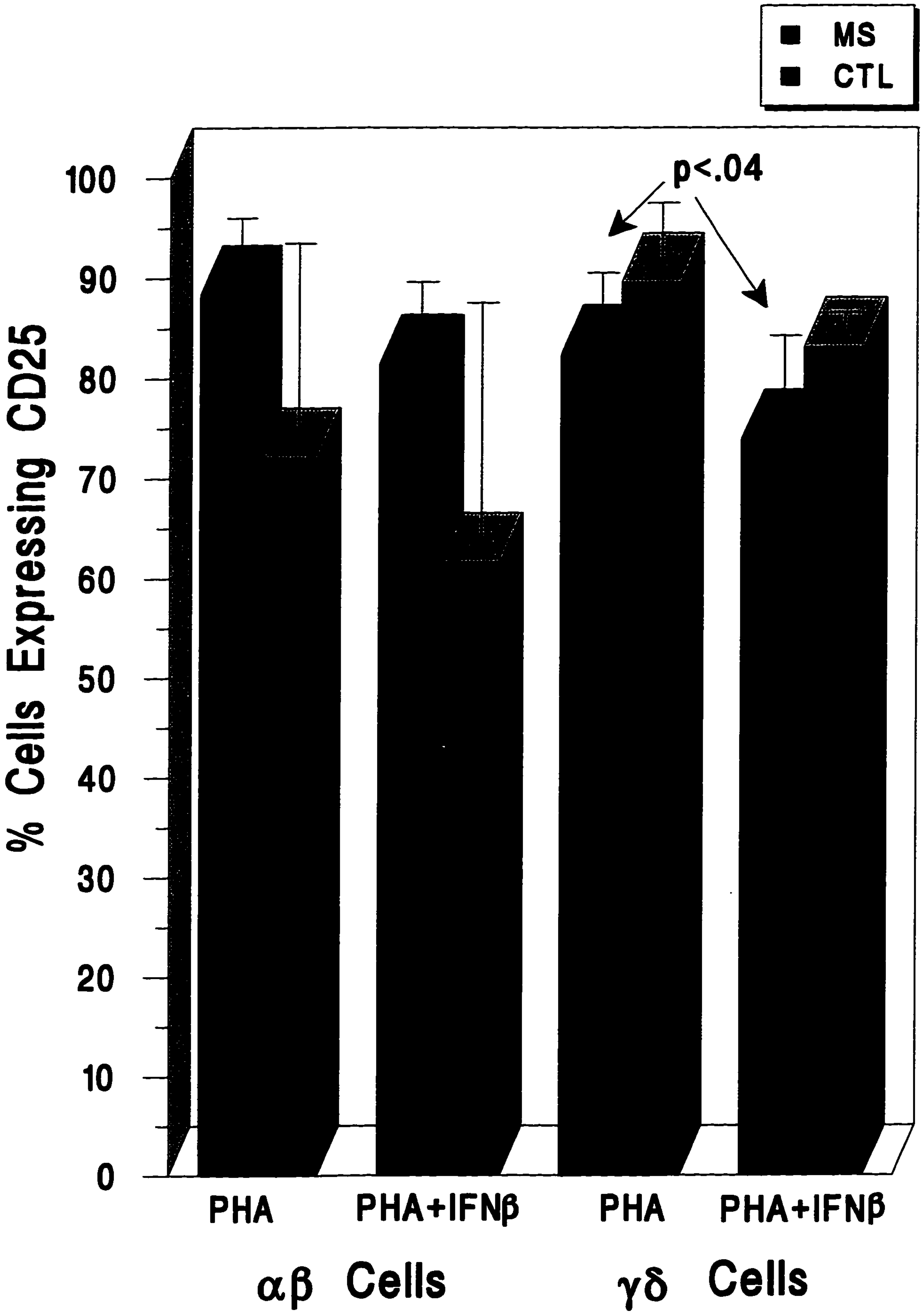
Figure 18. % change in CD25 expression on PHA activated MS (n=5) and Control (n=5) expanded γδ T cells. Following expansion, purification and culture with PHA for 72 hours, IFN-β-1b, at all doses exerts a negative % change in CD25 expression on both MS and control derived cells.


0
-5
-10
-15
-20
-25
-30
-35
-40
% CHANGE
1
10
100
1000
IFN-β IU/ml
MS
CTL

IU/ml, control n=2) compared to non-expanded αβ T cells (results not shown). These results suggest that culturing and expanding αβ or γδ T cells does not alter the characteristic response of CD25 expression to IFN-β-1b.

4.4 Effect of IFN-β-1b on % of T cells expressing CD25. Previous reports have shown that IFN-β-1b is able to reduce the % of T cells (the majority of which were αβ TCR positive) expressing CD25. Using flow cytometry, we were able to measure not only MCF of CD25 on cells, but also the % of total cells expressing CD25. Figure 19 indicates that at high doses (1000 IU/ml), IFN-β-1b is able to decrease the percentage of PHA activated and expanded γδ T cells expressing CD25, being significantly different only in MS samples ($p<0.04$). MS and control PHA activated αβ T cells in the presence of high doses of IFN-β-1b exhibited a similar trend in the reduction of the overall number of CD25 +ve cells, yet these decreases were not statistically significant (figure 18), (paired t-test).

Figure 19. Effect of IFN-β-1b on % of CD25 expressing T cells. αβ and γδ T cells from MS (n=5,5) and controls (n=5,5) were incubated for 72 hours with PHA alone or with PHA + 1000 IU/mL IFN-β-1b. Two colour flow cytometric analysis allowed for the % of cells expressing CD25 to be determined. An insignificant trend suggests a decrease in the number of αβ CD25 expressing T cells in MS and controls, whereas 1000 IU/mL IFN-β-1b significantly reduced the number of γδ T cells expressing CD25 in MS samples, yet not in controls.


■ MS
■ CTL
% Cells Expressing CD25
100
90
80
70
60
50
40
30
20
10
0
p<.04
PHA
PHA+IFNβ
PHA
PHA+IFNβ
αβ Cells
γδ Cells

DISCUSSION

The mechanism by which IFN-β-1b alters the natural history of MS is unknown but could involve interactions with T cells, the purported orchestrators of the autoimmune response, through anti-proliferative or immunomodulatory effects. Given that at least two types of T cells may be involved in lesion pathogenesis, we investigated whether IFN-β-1b exerted differential effects on either αβ or γδ T cells, perhaps implicating a particular T cell subset in disease pathogenesis.

T cells present in the CNS and periphery are found in an activated state in MS patients vs. controls. For this reason, the effects of IFN-β-1b on T cells were investigated on cells that were simultaneously activated by a variety of substances, including mitogen and antigen, allowing for global and specific modes of stimulation. Such stimulations might mimic the elevated activation state of T cells *in vivo*. Titration curves of PHA and anti-CD3 were produced, measuring T cell proliferation prior to their use in experiments. This was done to select a concentration of each stimulant that produced a submaximal level of proliferation, allowing the potential effects of Betaseron to be observed.

The time in which IFN-β-1b was to be added to culture to allow for observable effects was determined. Rudick et al. (1993) previously showed that pretreatment of T cells with IFN-β-1b prior to stimulation with Concanavalin A resulted in diminished proliferation rates. The addition of IFN-β-1b to culture following one or two days of the induction of stimulation with anti-CD3 mAb had no effect on proliferation rates (figure 3). This suggests that IFN-β-1b is unable to suppress the proliferation of cells that are activated *a priori*. If so, these results might explain why IFN-β-1b does not exhibit its clinical effects until after at least one month following initiation of therapy (Dr. M. Freedman, personal communication).

Rudick et al. (1993) and Noronha et al. (1993) have both shown that IFN-β-1b suppresses the proliferation of Con A stimulated total T cells. Here, the suppressive effect of IFN-β-1b was investigated separately on αβ and γδ T cells, in an effort to differentiate the activity of the drug on these two potentially pathogenic cells. In the case of αβ T cells, IFN-β-1b exerted a dose dependent suppression of PHA induced proliferation on T cells derived from both MS and controls. This effect was more obvious on MS derived cells, suggesting that *in vivo*, MS αβ T cells may be more effectively downregulated. MS derived T cells were also in a more activated state than those from healthy controls, as demonstrated by CD25 expression (figure 15). Such an increased activation state might allow for enhanced expression of cytokine receptors, for example, the IFN-α/βR. This could render the MS cells more sensitive to the suppressive effects of IFN-β-1b and explain the difference between MS and control data.

IFN-β-1b also suppressed the proliferation of PHA activated MS and control γδ T cells in a dose-dependent manner (figure 6). A difference in suppression between MS and control γδ T cells was not noted. Regardless, IFN-β-1b did significantly suppress the proliferation of γδ T cells, similar to αβ T cells. Taken together, these data suggest that IFN-β-1b is a strong inhibitor of αβ and γδ T cell proliferation, as it down-regulates the proliferative effects of PHA, a potent T cell mitogen.

A dose-dependent suppression of proliferation by IFN-β-1b was also observed on αβ T cells stimulated with immobilized anti-CD3 + IL-2 (figure 7) and with tetanus toxoid and IL-2 (figure 8). Although proliferation experiments conducted with γδ T cells, hsp70 and IFN-β-1b were unsuccessful, (due to lack of proliferative response to hsp70), it is possible that IFN-β-1b can interfere with such responses. Proliferative responses to tetanus toxoid vs. hsps are more reliable due to active immunization of subjects with the toxoid, whereas variable responses to hsps have been noted by γδ T cells (Salvetti et al., 1992). Future experiments to determine the suppressive effects of IFN-β-1b on antigen activated γδ T cells are needed, and this may be accomplished by conducting more experiments with hsps, being either soluble or cell surface as they exist on Daudi cells, a B cell line.

These results suggest that IFN-β-1b not only suppresses the proliferation of cells stimulated by a global mitogen but also by stimulation through the T cell receptor, a response more likey to occur *in vivo*. By suppressing proliferation, IFN-β-1b may act to alleviate MS by reducing the number of αβ and γδ T cells that can cross the blood brain barrier and participate in lesion formation, or reduce their number following entry to the CNS. The mechanism by which IFN-β-1b suppresses T cell proliferation is still unclear, but may involve this agent's ability to induce apoptosis.

Since IFN-α and IFN-β act on the same cell surface receptor and IFN-α augments PHA induced apoptosis (Dao et al., 1994), it was rationalized that IFN-β-1b may act in a similar fashion, accounting for the suppressed proliferation. Prior to testing for the presence of apoptotic death in cultures, viable cell counts were conducted on αβ T cells to estimate the number of live cells present following treatment with PHA and Betaseron. As figure 9 demonstrated, the number of viable T cells decreased as the dose of IFN-β-1b increased, especially in MS γδ T cells. This reduction in the number of viable cells may have been due to cell death via apoptosis. Further investigation revealed that at 1000 IU/ml, IFN-β-1b was observed to either induce or augment apoptosis.

At high concentrations of IFN-β-1b (1000 IU/ml), apoptotic populations in αβ T cell samples were augmented following activation with PHA(figure 11), anti-CD3 (figure 12) or tetanus toxoid (figure 13), in comparison to mitogen or antigen alone. Although induction of apoptosis was not observed in every sample (as evident from the presence of apoptosis in one tetanus toxoid stimulation (figure 13) by flow cytometry and absence in another sample as measured by DNA ladders (figure 14)), these results do provide a potential mechanism for at least some but not all of the observed proliferation suppression data. Future experiments to quantitate the amount of apoptosis and the cell cycle stage in which it occurs may provide more insight into whether suppression of proliferation and apoptosis are clearly linked.

The detection of apoptotic populations in at least 10 separate samples of γδ T cells following expansion and 72 hours incubation with PHA +/- 0-1000 IU/mL IFN-β-1b was not made. Measurements by flow cytometry and DNA electrophoresis were all negative (data not shown). Our inability to detect apoptosis in these cells does not negate the possibility that it did occur. γδ T cells are able to undergo apoptosis following steroid treatment (Spinozzi et al. 1995) and ionizing radiation (Seki et al. 1994) and appear as equally susceptible as αβ T cells (Seki et al., 1994). The fact that apoptosis was not observed in γδ T cells treated with IFN-β-1b may be due to the nine day expansion process of γδ T cells, which may have 'weeded out' those cells that would have apoptosed under a non-expanded 72 hour culture. The window for observing apoptosis is narrow, being only minutes long, thus by analyzing cells on day 12, the time at which cells underwent apoptosis was missed. Seki et al., (1994) have shown that irradiation-induced apoptosis can be inhibited by IL-2, so perhaps the pre-expansion with IL-2 addition at 24 hours reduces the sensitivity of these cells to apoptose. Further experiments measuring the presence of apoptotic cells over the total 12 day culture for γδ T cells might indicate a window period in which such mechanisms took place, or such induced cell death may be observable if non-expanded γδ T cells are used.

The variability in working with different human samples could account for the inability to detect apoptosis or its augmentation by IFN-β-1b in all samples tested. Different samples might exhibit an increased or decreased sensitivity to apoptosis induction and therfore be undetected at the 72 hour post-PHA culture analysis. Novelli et al. (1994) have shown that apoptosis is enhanced in cells expressing increased IFN-γR, following treatment with IFN-γ. It is possible that T cells from various human samples express different levels of IFN-βR, rendering them variably susceptible to the induction of apoptosis in intensity and time, following IFN-β-1b treatment. Flow cytometric analysis of heterogeneous T cells for the IFNβR might clarify this.

The viability of PHA activated MS αβ T cells in the presence of IFN-β-1b was reduced (figure 9). This corresponds to the increase in apoptotic cells under the influence of IFN-β–1b. Since cells undergoing apoptosis that exhibit DNA fragmentation still have intact membranes prior to ultimate membrane degradation, these data suggest that before the end of the 72 hour culture period, more cells under high doses of IFN-β-1b have already died. Since MS αβ T cells were more sensitive to IFN-β-1b induced suppression of proliferation at all doses vs. γδ T cells, compared to controls, and also fewer viable cells were noted at all doses, perhaps at least a portion of IFN-β-1b induced supression involves non-apoptotic induced cell death. This non-apoptotic death may account for the observed γδ T cell data. Whether either type of Betaseron-induced cell death occurs *in vivo* remains to be seen.

IFN-β-1b clearly affected the activation state of T cells, both αβ and γδ, in both MS and controls. IFN-β-1b exerted a differential effect on CD25 expression on the two T cell types, as measured in non-expanded control cells. CD25 expression on γδ T was downregulated by IFN-β-1b both in terms of the observed reduction in the MCF (increased % change) and in the % of cells expressing CD25 (figures 16 and 18), in MS and controls. If an enhanced activation state of T cells and subsequent cell adhesion molecule upregulation is required to cross the blood brain barrier to allow for participation in CNS damage, IFN-β-1b appears to reduce the ability of γδ T cells to be involved in lesion formation. The dose-dependent down-regulation of CD25 on γδ T cells by IFN-β-1b may happen peripherally or centrally *in vivo*, and prevent such cells from contributing to further MS pathogenesis. In addition, their ability to proliferate is impaired under the influence of IFN-β-1b.

In contrast to the effects on γδ T cells, CD25 expression on αβ T cells was upregulated by IFN-β-1b on control cells, at physiological concentrations of IFN-β-1b (i.e., 10-100 IU/ml) (figures 16 and 17). This was especially true for pre-expanded αβ T cells, in which an increased CD25 expression was even seen with the highest dose of IFN-β-1b (1,000 IU/Ml) (data not shown). MS derived cells did not appear to appreciably upregulate

CD25 expression under the influence of Betaseron, in comparison to controls, as indicated by percent change (figure 17). However, recent *in vivo* studies demonstrate that IFN-β-1b increased the expression of CD69, an early activation marker, on αβ T cells one month following treatment initiation (Weinstock-Guttman et al., 1995). The exaggerated activation state induced by PHA treatment in my experiments might therefore account for the inability of IFN-β-1b to enhance an already maximal CD25 expressing state on MS derived T cells. Investigations using a weaker mitogen or antigen might clarify this hypothesis.

The results obtained may also be confirmed by measuring the concentration of soluble IL-2R or IL-2 by ELISA, at 72 hours of culture and comparing the results to CD25 expression. This was not performed due to time constraints, however when sIL-2R levels were measured in the serum of patients taking IFN-β-1b, a dose dependent increase in soluble IL-2R levels was seen with low and high dose therapy (Freedman et al. 1992b). This would suggest that *in vivo*, increased CD25 expression may be seen.

The effect of Betaseron on the CD25 expression of αβ T cells was somewhat unexpected. It has long been thought that these T cells are important in the demyelination process due to their large percentage of total T cells and their activity in animal models of the disease. An agent that actually increased their activation state might contribute to their involvement in continued or enhanced pathogenic processes. Perhaps this explains why there appears to be a transient increase in symptoms and flu-like side effects which are most prominent when patients first start injecting the drug. Lublin et al. (1995) have suggested this is due to a transient increase in IFN-γ, which has been shown to exacerbate

MS. The findings in the short term cultures described here (i.e., an increase in CD25 expression) might well reflect what occurs *in vivo* when patients first start therapy. It would therefore be of interest to compare results on cells obtained from patients soon after starting therapy and after six months to see if the effects are maintained.

Rudick et al (1993) and Noronha et al. (1993) measured the effect of IFN-β-1b on the total number of T cells expressing CD25, in MS and control subjects, following incubation with Concanavalin A. They reported that at high concentrations of IFN-β-1b (100 IU/ml), the number of T cells expressing CD25 was significantly reduced. This was similar to the findings reported here (figure 19), using PHA stimulation of either αβ or γδ T cells and 1000 IU/ml IFN-β-1b. The differences in the concentrations of IFN-β-1b needed to produce the reduction in CD25 expressing cells might well be due to the more potent stimulus of PHA used here, compared to the less potent Con A response.

At high doses, IFN-β-1b exerts the same effect on both T cell subsets; i.e. reduction in the average number of CD25 molecules per cell and the total number of T cells expressing CD25 at 1000 IU/ml. At lower doses however, IFN-β-1b differentially modulated the expression of CD25, upregulating it on αβ T cells while downregulating it on γδ T cells. CD25 expression did not however, correlate with a dose-dependent consistent suppression of proliferation by IFN-β-1b on αβ T cells in response to PHA. These results were somewhat different than was expected, i.e., as a T cell is activated, both the production of and receptors for IL-2 are increased, initiating autocrine and paracrine stimulation and proliferation, not its suppression.

In recalling the enhanced CD25 expression of αβ T cells at 10 IU/mL IFN-β-1b, one would predict that subsequent proliferation would occur, but did not. Perhaps enhanced activation and proliferation is offset by a concomitant increase in activation-induced cells such as that due to apoptosis. This may explain why αβ T cells were observed to undergo apoptosis as opposed to γδ T cells. αβ T cells seem to increase CD25 expression whereas γδ T cells decrease it. An activated state may be a prerequisite for the onset of apoptosis, therefore αβ T cells would be more sensitive to IFN-β-1b's apoptotic induction.

Serum levels of IFN-β-1b in MS patients on therapy were found to be up to 1000 IU/ml (Kahn et al., 1996), therefore it is possible to propose that down-regulatory effects on CD25 expression, on both T cell types, would happen *in vivo*. At 120-420 IU/ml, IFN-β-1b would be expected to downregulate the activation state of both αβ and γδ T cells, rendering them less capable of participating in CNS pathogenesis.

A number of future experiments might define more clearly the ability of Betaseron to interrupt the T cell activities suspected to be involved in MS. γδ T cells are suspected to react with heat shock proteins expressed in MS lesions (Gao et al., 1995). *In vitro*, γδ T cells lyse Daudi cells, a B cell line known to express increased levels of hsps, by a ^{51}Cr-release assay. Co-incubation of γδ T cells with hsp-expressing Daudi cells in the presence or absence of IFN-β-1b might reveal this agent's ability to reduce or inhibit γδ T cell reactivity with hsps, and potentially decrease self-reactive tissue damage *in vivo*.

The mere presence of γδ T cells had been shown to enhance hsp expression on mouse macrophages previously immunized with Toxoplasma homogenate, an obligate intracellular parasite known to induce hsp expression (Nagasawa et al., 1994). This provides more targets to which γδ T cells can react and it can increase their activity. Perhaps such an autostimulatory effect happens between OGCs and γδ T cells, the latter of which are found in increased numbers in MS lesions for reasons that are as of yet unclear. Flow cytometric analysis of hsp expression on Daudi cells following incubation with γδ T cells +/- IFN-β-1b could determine if hsp upregulation occurs and if IFN-β-1b can interfere with the stimulation.

A variety of activated myelin protein specific αβ T cells have been isolated from MS patients and are suspected to play a role in MS pathogenesis. The adoptive transfer of similar cells in animals produces EAE. If it can be shown that IFN-β-1b is able to suppress the proliferation of such specific αβ T cells in response to myelin proteins, it could implicate this agent's activity in suppressing a potentially pathogenic process in MS.

IFN-β-1b had wide acting effects and may ultimately down-regulate the immune response in MS such that further CNS damage does not occur. It appears as though the presence of CD4+ and CD8+ T cells within demyelinating lesions in rodents actually hinders the remyelinating process as described by Rodriguez and Miller (1994) since mice depleted of such T cells exhibited enhanced remyelination. The anti-proliferative and activation-induced apoptotic properties that IFN-β-1b has on T cells, as described by this project, may reduce the number of T cells in MS lesions and not only prevent further damage, but allow for remyelination to occur.

Other reported effects of IFN-β-1b that would downregulate the inflammatory response in MS include the observation that IFN-β-1b inhibits mononuclear cell proliferation (Shalaby et al., 1984). Therefore, immune cells other than T cells are affected.

Noronha et al. (1992) found that IFN-β-1b reduces the secretion of IFN-γ and TNF-α secretion from mononuclear cells, two cytokines known to enhance immune responses. IFN-β-1b is also able to inhibit the IFN-γ -driven surface expression of HLA-DR, HLA-DRa and DRb mRNA accumulation and HLA DRa gene transcription in a human astrocytoma cell line (Ransohoff et al., 1991). Therefore, IFN-β-1b may have a therapeutic effect by indirectly decreasing the concentration of class II MHC in MS brain tissue, preventing further myelin damage.

So, what do all of these results mean in terms of IFN-β-1b's mechanism of alleviating MS? Based on the assumption that both γδ and αβ T cells are involved in the inflammation process leading to demyelination, our results suggest that IFN-β-1b modulates the activity of these cells by reducing their activation state (γδ T cells at all doses, and αβ T cells at high doses), reducing their proliferative ability, and augmenting activation-induced apoptosis (αβ T cells only). These three processes will decrease the number of cells capable of participating in MS pathogenesis.

SUMMARY

γδ **T cells.** Considering all of the results produced by this work concerning the effects of IFN-β-1b on γδ T cells, it is possible to propose that the following events occur in MS (figure 20). IFN-β-1b may prevent γδ T cells from participating in oligodendrocyte damage by down-regulating their activation state at all concentrations, thereby reducing the cells ability to upregulate the necessary adhesion molecules which would allow its entry into the central nervous system, as well as reducing its ability to produce cytokines which might orchestrate other pathogenic immune functions. IFN-β-1b also suppresses the proliferation of γδ T cells at all concentrations, as was shown using PHA, a potent T cells mitogen, as the stimulating factor. This indicates that in the presence of the drug, fewer cells will be present that may participate in immune responses contributing to MS pathogenesis. It is unclear from the data presented whether IFN-β-1b causes activation induced apoptosis in this type of T cell.

αβ T cells. Results presented by this work suggest a slightly different mechanism of action of IFN-β-1b on αβ T cells in MS (figure 21). At high doses (100-1000 IU/ml), IFN-β-1b down-regulates the activation state of αβ T cells. Similar to what was observed with γδ T cells, the proliferation of such treated cells is also suppressed. Together, this action of IFN-β-1b would reneder these cells less able to enter the central nervous system or be activated to the extent that allows for the production cytokines which can contribute to oligodendrocyte damage. Alternatively however, at low doses of IFN-β-1b (1-10 IU/ml), the activation state of these cells is enhanced. This might allow for the upregulation of adhesion molecules and production of cytokines which contribute to MS damage. Such potentially harmful cells may be deleted by activation induced apoptosis with IFN-β-1b treatment, as was demonstrated in this work.

It is unclear as to whether IFN-β-1b actually crosses the blood brain barrier and enters the central nervous system. It is known however that T cells are able to cross the blood brain barrier to and from the periphery and central nervous system, a number of times. Supposing that IFN-β-1b has the ability to act only in the periphery, αβ T cells which become activated at low doses of the drug and enter the central nervous system, may be deleted by apoptosis or be exposed to the drug once again upon re-entrance to the periphery. Multiple exposures of these cells to the drug might ultimately render these cells incapable of re-entry to the central nervous system by a down-regulated activation state or death by apoptosis.

Figure 20. Summary depiction of the action of IFN-β-1b on γδ T cells in MS. IFN-β-1b may act to prevent γδ T cells from participating in oligodendrocyte damage by down-regulating their activation state, thereby preventing adhsion molecule expression required for crossing the blood brain barrier. As well, the antiproliferative effect IFN-β-1b has on this subset may reduce the overall number of pathogenic cells normally present in MS, again leading to the alleviation of CNS damage and its symptoms.

Summary of Action of IFN-β-1b on γδ T cells

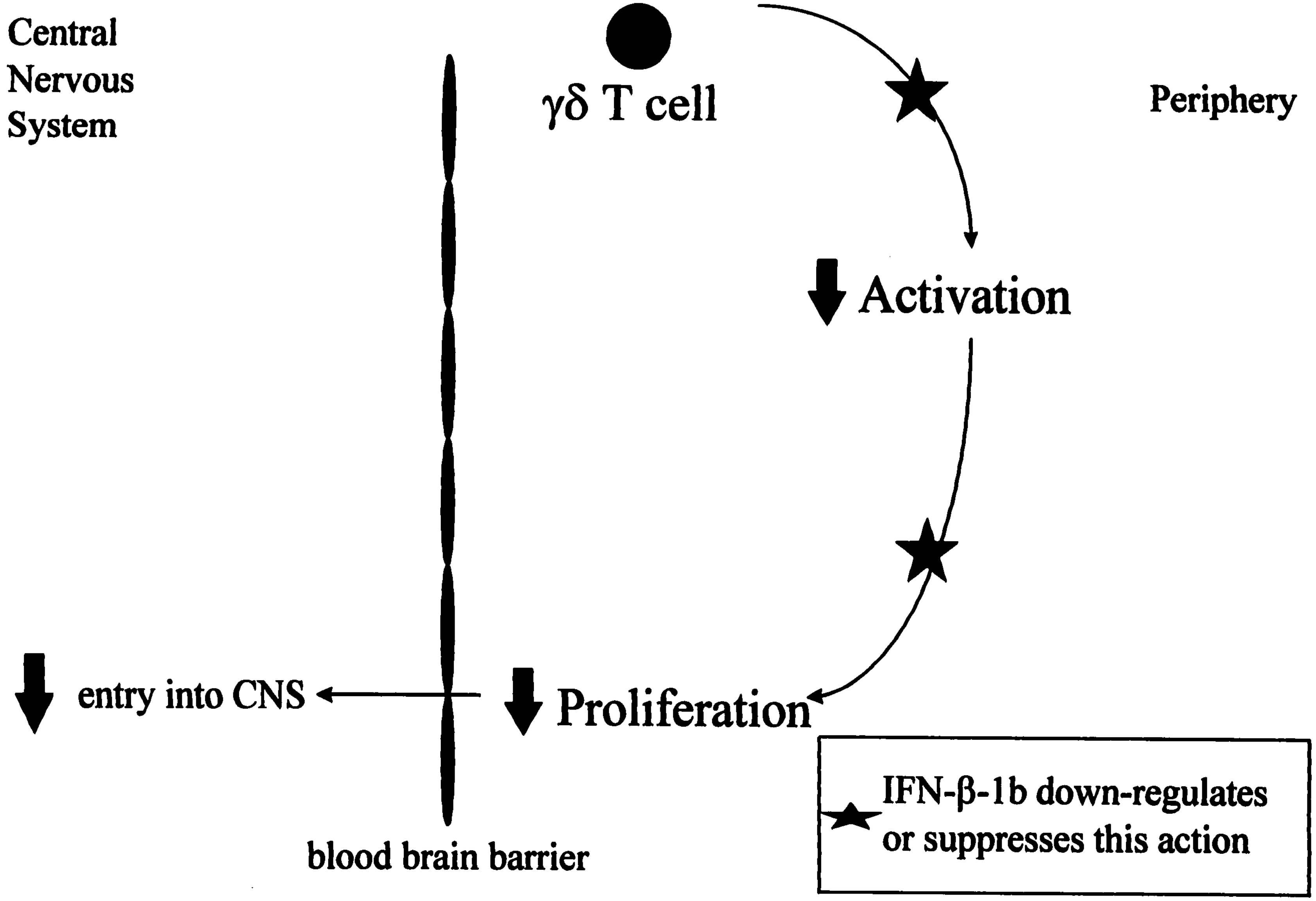
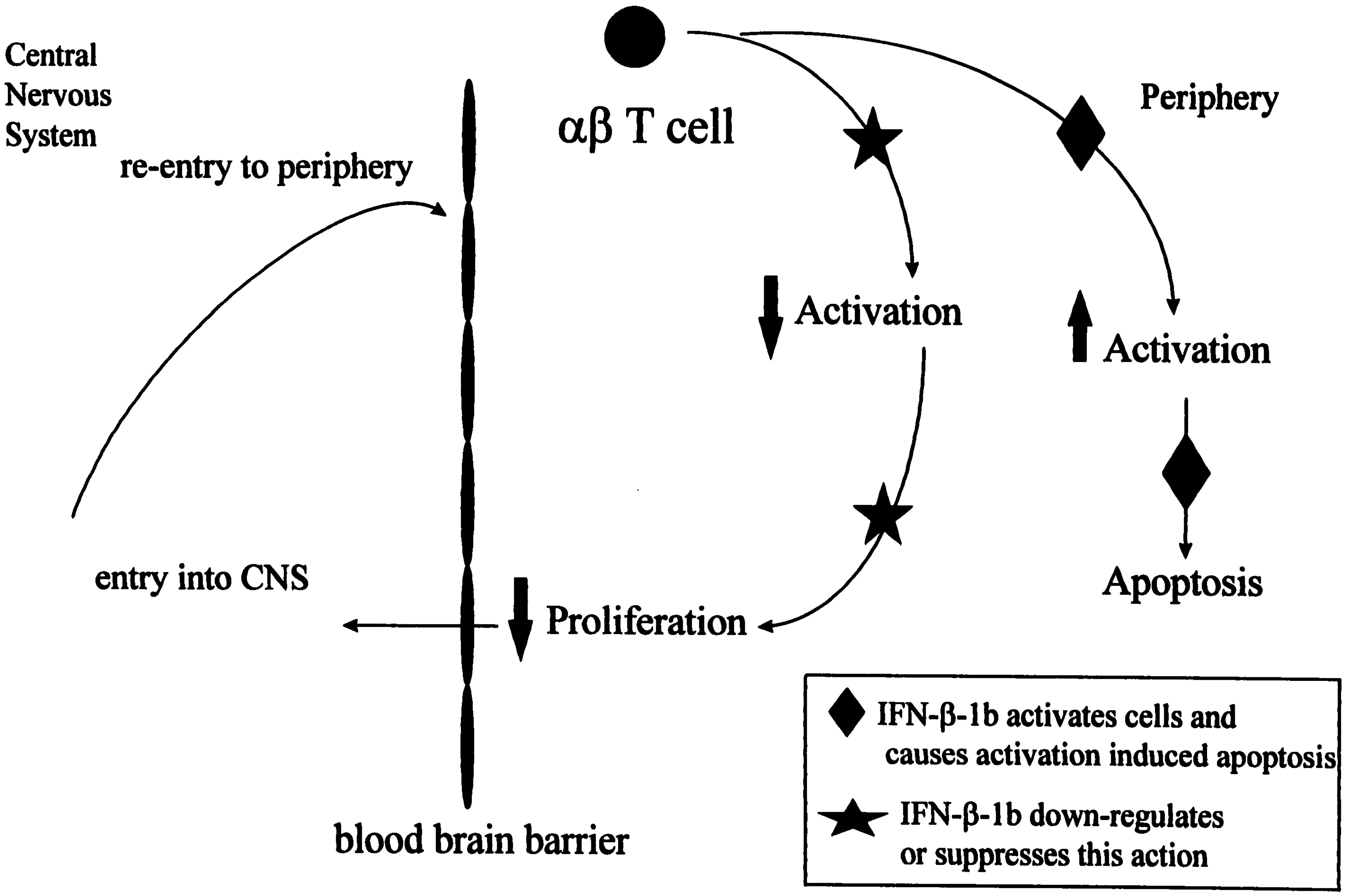


Figure 21. Summary depiction of the action of IFN-β-1b on αβ T cells in MS. IFN-β-1b may act to alleviate MS by preventing αβ T cells from entering the CNS and cause damage. It may do so by reducing the activation state of these cells, reducing their ability to express cell adhesion molecules which prevents access to the CNS via the blood brain barrier (at high doses). Its antiproliferative effect will also result in fewer cells present that can participate in CNS damage. Alternatively, at low doses IFN-β-1b may activate αβ T cells. This might allow for access to the CNS by upregulation of cell adhesion molecules. Because T cells can traverse the blood brain barrier relatively freely, such cells may be exposed to drug more than once and end up 1) down-regulated in terms of their activation state (at high dose of drug) or 2) undergoing activation induced apoptosis. Such apoptosis may occur prior to entering the CNS, deleting the cell and preventing its involvement in MS pathogenesis.

Summary of Action of IFN-β-1b on αβ T cells



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Appendix A

MS Patient Donor Data

n	**Disease Type**	**Duration of Disease** (average years)	**EDSS** (average)	**Age** (average)
5	Benign	9	0.8	40.8
6	Relapsing-remitting	4.8	2.3	31.5
10	Secondary-progressive	18.8	5.8	51.5

HÔPITAL GÉNÉRAL D'OTTAWA
OTTAWA GENERAL HOSPITAL
501 Smyth. Ottawa. Ontario K1H 8L6

CONSENTEMENT À UNE INTERVENTION CHIRURGICALE
CONSENT FOR OPERATIVE PROCEDURE

J'autorise les médecins et le personnel médical de l'Hôpital général d'Ottawa incluant mais ne se limitant pas au docteur :

I authorize the physicians and the medical personnel of the Ottawa General Hospital including but not restricted to doctor:

Mark S. Freedman

à effectuer ou à aider à effectuer l'(les) intervention(s) chirurgicale(s) suivante(s) :
to perform or assist in carrying out the following operative procedure(s) :

		Gauche Left	Droit Right	Aucun Neither
1	Phlébotomie de 50 cc au maximum de sang entier pour les besoins de l'isolement et de la culture in vitro à court terme de lymphocytes.	☐	☐	☐
2	Phlebotomy of not more than 50 cc of whole blood for the purpose of isolation and in vitro growth of lymphocytes in short term cultures.	☐	☐	☐
3		☐	☐	☐
4		☐	☐	☐

sur le patient:

on the patient:

à l'Hôpital général d'Ottawa.

at the Ottawa General Hospital.

Le médecin m'a expliqué l'opération ou l'intervention et j'en comprends la nature, les risques et les effets probables. On m'a donné l'opportunité de poser des questions.

The doctor has given me an explanation of the operation or procedure and I understand its nature, risks and probable effects. I have been given an opportunity to ask questions.

Au cas où l'on découvrirait pendant l'opération ou l'intervention un état quelconque non soupçonné auparavant, j'autorise le médecin à prendre toutes les mesures qu'il jugera nécessaires ou opportunes en plus ou en dehors des dispositions prévues antérieurement à l'intervention. Je consens à recevoir des transfusions de sang ou produits sanguins. Je consens à recevoir une anesthésie et les anesthésiques qui seront jugés utiles.

In the event that a condition not previously apparent is discovered during the course of the operation or procedure, I authorize the doctor to take whatever measures considered necessary or desirable in addition to or different from the operation or procedure initially contemplated. I consent to the administration of blood transfusions or blood products. I consent to the administration of anaesthesia and to the use of such anaesthetics as may be deemed advisable.

Je consens à ce que les autorités de l'Hôpital général d'Ottawa disposent de tout tissu ou organe enlevé au cours de l'opération ou de l'intervention.

I consent to the disposal by the Ottawa General Hospital authorities of any tissues or organs removed in the course of the operation or procedure.

Nom (en lettres moulées) - Name (print)	Signature	Lien de parenté- Relationship

(PATIENT OU PERSONNE LÉGALEMENT RESPONSABLE - PATIENT OR PERSON LEGALLY RESPONSIBLE)

1. Nom du témoin (en lettres moulées) - Name of witness (print)	Signature	Date (j/d-m/m-a/y)

Un deuxième témoin est requis si le consentement est obtenu par téléphone :

A second witness is required if consent is obtained by telephone:

2 Nom du témoin (en lettres moulées) - Name of witness (print)	Signature	Date (j/d-m/m-a/y)

Heure - Time

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