FUNCTIONAL AND CELLULAR STUDIES IN HTLV-I-ASSOCIATED MYELOPATHY AND MULTIPLE SCLEROSIS

by

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ABSTRACT

Multiple sclerosis (MS) and human T-lymphotropic virus type I (HTLV-I) associated myelopathy (HAM) are inflammatory demyelinating diseases of the central nervous system (CNS). Current opinion implicates immune mediated factors, particularly T cells in the pathogenesis of both diseases. Histopathological studies in HAM and MS show perivascular mononuclear cell (MNC) infiltration into the central nervous system (CNS). The mechanism by which MNC gain access the CNS involves adhesion of peripheral blood MNC to cerebral endothelial cells that constitute the blood-brain barrier (BBB).

The objective of this thesis was to investigate, first, the phenotype of lymphocytes of HAM patients with a focus on T cell activation and adhesion related antigens; second, the adhesion and mechanism of adhesion of blood MNC of HAM and MS patients to endothelial cells; and third, the effects of immunomodulating drugs on lymphocyte subsets and function in MS.

We utilized direct two-color flow cytometry to study lymphocyte subsets in a group of patients with HAM and compared the results with those of HTLV-I asymptomatic carriers and seronegative controls. We found that in HTLV-I carriers, lymphocytes are activated and that activation is even more profound in HAM patients.

To investigate the factors regulating the entry of blood MNC into the CNS, we used human umbilical vein endothelial cells (HUVEC) as a model for endothelial function and, after growing them to confluence, studied the adhesion of ^{51}Cr-labeled MNCs to the monolayers. Adhesion experiments indicated that MNC from HAM and

from clinically active (secondary progressive) MS patients adhered significantly more to HUVEC monolayers than MNC from controls. This supports the view that infiltration of MNC across the BBB into the CNS in HAM and MS is due to increased interaction between blood MNC and endothelium.

Monoclonal antibody blocking studies indicated that the adhesion molecules LFA-1/ICAM-1 pathway plays a pivotal role in adhesion both under inflammatory and non-inflammatory conditions, while the VLA-4/VCAM-1 pathway contributes to MNC-HUVEC adhesion only when HUVEC are stimulated and therefore, might be important in recruiting immune cells under inflammatory conditions as in HAM and MS.

Studies of IgG secretion by peripheral blood MNC in stable relapsing-remitting (sRR) MS and healthy controls after Pokeweed mitogen (PWM) stimulation indicated that sRR-MS patients produced more immunoglobulin (Ig) G and had a higher percentage of "high responders" compared with controls. This increase in IgG secretion was significantly inhibited by interferon beta (IFN-β). This inhibition was not equivalent among three commercially available preparations of IFN-β (Avonex™, Betaseron®, and Rebif®). We found that Avonex™ had the highest inhibitory effect followed by Rebif® and Betaseron® respectively.

In this study, we also examined the effects of IFN-β on MNC-HUVEC adhesion and demonstrated that IFN-β pretreatment of MNC, but not HUVEC results in significant reduction in MNC-HUVEC adhesion. This might partially explain the beneficial effects of IFN-β in MS.

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A 405 nm	Absorbance at 405 nm
ADCC	Antibody-dependent cell-mediated cytotoxicity
ANOVA	Analysis of variance
APC	Antigen-presenting cells
ATCC	American type culture collection
BBB	Blood-brain-barrier
BSA	Bovine serum albumin
CD	Cluster of differentiation
CHO	Chinese hamster ovary
CNS	Central nervous system
Con. A	Concavalin A
^{51}Cr	Radio-isotope chromium-51
CSF	Cerebrospinal fluid
CTL	Cytotoxic T-lymphocytes
EAE	Experimental allergic/autoimmune encephalomyelitis
EC	Endothelial cells
ECM	Extracellular matrix
ELISA	Enzyme linked immunosorbent assay
ESL-1	E-selectin ligand-1
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum

FITC	Fluorescein isothiocyanate
FSc	Forward scatter
GlyCAM-1	Glycosylation-dependent cell adhesion molecule-1
HAM	HTLV-I-associated myelopathy
HBSS	Hanks' balanced salt solution
HLA	Human leukocyte antigen
HRP	Horse radish peroxidase
HS	Horse serum
HTLV-I	Human T lymphotropic virus type I
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intracellular adhesion molecule-1
IFN-γ	Human recombinant interferon-gamma
IFNs	Interferons
IFN-ß	Human recombinant interferon-beta
IgG	Immunoglobulin G isotype
IGSF	Immunoglobulin supergene family
IL-10	Interleukin-10
IL-2	Interleukin-2
IL-2R	Interleukin-2 receptor
ILs	Interleukins
IV	Intravenous
kD	Kilodalton
kg	Kilogram

LAD	Leukocyte adhesion deficiency
LFA-1	Leukocyte function-associated antigen-1
mAb	Monoclonal antibody
MAC	Membrane attack complex
MadCAM-1	Mucosal addressin cell adhesion molecule-1
MAG	Myelin-associated glycoprotein
MBP	Myelin basic protein
mg	Milligram
MHC	Major histocompatiblity complex
MIU	Million international units
MNC	Mononuclear cells
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
NK	Natural killer cells
NOD	Non-obese diabetic
PBS	Phosphate buffered saline
PE	Phycoerthyrin
PLP	Proteolipid protein
PMA	Phorbol myristate acetate
PP-MS	Primary progressive multiple sclerosis
PSGL-1	P-selectin glycoprotein ligand-1

PWM	Pokeweed mitogen
rhu-	Recombinant and humananized
RR-MS	Relapsing-remitting multiple sclerosis
s	Stable
SD	Standard deviation
SEM	Standard error of the mean
SP-MS	Secondary progressive multiple sclerosis
SSc	Side scatter
TAC	T cell activation antigen
TCR	T cell receptor
TGF-ß	Tumor growth factor-beta
Th1	T helper-1 subtype
Th2	T helpler-2 subtype
TNF-α	Tumor necrosis factor-alpha
TNF-β	Tumor necrosis factor-beta
TSP	Tropical spastic paraparesis
TSP	Tropical spastic paraparesis
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very late antigen-4

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CHAPTER ONE
INTRODUCTION

INTRODUCTION

1.1 HTLV-I-ASSOCIATED MYELOPATHY AND MULTIPLE SCLEROSIS: AN OVERVIEW OF THEIR IMMUNOPATHOLOGY

1.1.1 HTLV-I-associated myelopathy

The human T cell lymphotropic virus type I (HTLV-I) can be associated with a slowly progressive neurological disease called HTLV-I-associated myelopathy (HAM) (Osame et al., 1986) or Tropical spastic paraparesis (TSP) (Gessain et al., 1985). These two conditions have been shown by subsequent comparative studies to be identical to each other, but are endemic in different geographical locations (Roman and Osame, 1988). HAM usually begins in adulthood and affects more women than men. The disease has usually been reported in high HTLV-I endemic areas and occurs mainly in persons of African or Asian origin but can also be observed in whites (Gessain 1996). Despite the fact that HTLV-I has been established as the etiologic agent in HAM, its pathogenic mechanisms remain unknown. The neuropathology of HAM provides evidence that immunological processes in association with HTLV-I infection may play a significant role in the pathogenesis of the disease. Pathologically, HAM is characterized by perivascular cuffing by mononuclear cells and demyelination in the central nervous system (CNS), predominantly in the thoracic region of the spinal cord (Iwasaki et al., 1992, Itoyama et al., 1988 a,b; Jacobson et al., 1988). Early in the disease, lymphocytes are shown to be abundant and consist of equal numbers of CD4+ and CD8+ T cells as well as some B cells (Moore et al., 1989). However, some reports indicate the

preponderance of CD4+ T cells in very early stages of the spinal cord lesions (Iwasaki et al., 1992). In patients with chronic HAM, lymphocytic infiltrates are less abundant and consist mostly of CD8+ T cells (Umehara et al., 1993). Immune abnormalities, including an increase in activated T cells and spontaneous T cell proliferation have also been demonstrated in peripheral blood of HAM patients (Itoyama et al., 1988, Jacobson et al., 1988). Although these abnormalities are believed to result from the active replication of HTLV-1 genome, the exact mechanism of these activated T cells involvement in the pathogenesis of HAM is still unclear (Oger and Dekaban, 1995; Yoshida et al., 1989). It also remains unclear why only a small percentage of HTLV-I infected individuals develop HAM and what determines the progression from the carrier state to clinical disease (Kaplan et al., 1990).

Analysis of cerebrospinal fluid (CSF) in HAM patients shows activated T cells, mild lymphocytic pleocytosis, protein elevation, elevated IgG synthesis, and oligoclonal bands (Jacobson et al., 1990; Ceroni et al., 1988; Link et al., 1989; Ijichi et al., 1989). Some of the oligoclonal bands are directed to HTLV-I proteins (Levin and Jacobson, 1997). Magnetic resonance imaging (MRI) of the spinal cord may also reveal atrophy and MRI of the brain shows periventricular white matter lesions in large number of HAM patients (Nakagawa et al., 1995).

There are two major hypotheses that have been proposed to explain the immunopathogenesis of HAM. In the first hypothesis, HTLV-I infects the glial cells in the CNS, and a subsequent cytotoxic immune response against the infected cells results in demyelination (Moore et al., 1989, Levin and Jacobson, 1997). In the second hypothesis, HTLV-I infection leads to the random activation of autoreactive T cells and the induction

of an autoimmune process (Oger et al., 1995). Recent evidence that the frequency of HLA class I restricted and HTLV-I tax-specific CD8+ cytotoxic T-lymphocytes (CTL) is high in blood mononuclear cells and in CSF of HAM patients but not in carriers or in patients with adult T-cell leukemia favors the first hypothesis (Jacobson et al., 1992; Elovaara et al., 1993). Demyelination mediated by cytotoxic T cells could occur either by direct killing of proposed HTLV-I-infected glial cells in a manner restricted by MHC class I antigen, or by secretion of cytokines from cytotoxic T cells that could adversely affect the function of uninfected neurons and glial cells within the CNS (Giraudon et al., 1996). Whereas the presence of CD8+ T lymphocytes in HAM lesions is well documented, it is controversial whether HTLV-I infects the resident cells of the CNS. The direct demonstration of HTLV-I antigens in glial cells is difficult, because of the close association with potentially infected T cells. It is possible that CD8+ cytotoxic T cells are recognizing viral products presented by HTLV-I-infected T cells. This might result in the activation of cytotoxic T cells and the subsequent secretion of pro-inflammatory cytokines such as IFN-γ and TNF-α.

An autoimmune attack on CNS in HAM could be explained by at least two different mechanisms. In the first scenario, CD4+ T cells displaying cross-reactivity between viral antigens and CNS antigens could mediate autoimmune reactions. This is supported by findings that T cell receptors are much more cross-reactive than had been previously thought (Oldstone 1987, Wucherpfennig and Strominger 1996). Furthermore, Nagai et al., have recently characterized a T cell clone that displays reactivity to both HTLV-I-infected cells and a yet unknown spinal cord antigen (Nagai et al., 1996). An alternative mechanism for an autoimmune pathogenesis in HAM relies on random

infection of CD4+ T cells. It is known that T cells with specificity for self-antigens including myelin basic proteins are part of the normal T cell repertoire. The presence of activated T cells with specificity for myelin basic protein can induce experimental autoimmune encephalomyelitis (EAE) in mice, indicating that the control of the activation state of autoreactive T cells is critical in avoiding self-reactivity. According to this scenario, in HAM, autoreactive CD4+ T cells are infected by HTLV-I in the periphery, and become activated and migrate to the CNS where they recognize a CNS autoantigen, resulting in a specific immune response and subsequent demyelination. However, the analysis of HTLV-I *in vivo* infection of autoreactive T cells is hampered by the HTLV-I-mediated T-cell activation. Therefore, it is impossible to discriminate between antigen reactivity and virally mediated spontaneous proliferation. Thus, it is unknown whether a subset of HTLV-I-infected T cells cross reacts with self-autoantigens.

1.1.2 Multiple sclerosis

Multiple sclerosis (MS) is the most common neurological disease of young and middle-aged adults of Northern European decent. MS affects women more commonly than men (Duquette et al., 1992; Sadovnick et al., 1993). MS is a chronic inflammatory disease of the CNS. The clinical course of MS is highly unpredictable. In the majority of cases, MS starts with a relapsing-remitting course (RR-MS) that eventually changes to a secondary progressive (SP-MS) course; less commonly the course is progressive from the onset (primary progressive, PP-MS) (Lublin and Reingold, 1996). While the etiology of MS is still unknown, immunological factors are believed to play an important role. The

histopathology of the lesion in MS is characterized by multifocal and periventicular infiltration of the white matter by inflammatory immune cells in the CNS, and a selective destruction of myelin and myelin-forming oligodendrocytes (Raine, 1994; Cannella and Raine, 1995). Immune cells consist mostly of T-cells and macrophages. Both CD4 positive and CD8 positive T-cells are present in MS lesions. It is believed that macrophages play a primary role as effector cells in the destruction and removal of CNS myelin, while the lesion progression is driven by the activity of CD4+ T cells (Scholding et al., 1994).

The main immune abnormality in MS probably involves T cell-mediated immune function, but one of the hallmarks of MS disease is the presence of immunoglobulin (Ig) G of restricted heterogeneity in the cerebrospinal fluid in majority of patients (Johnson and Nelson, 1977) also referred to as "oligoclonal bands". Since oligoclonal bands are only occasionally found in the serum of MS patients, it is conceived that a few clones of plasma cells are activated intrathecally. Recently it was reported that oligoclonal band negative MS patients were significantly less disabled compared with matched oligoclonal band-positive MS patients (Zeman et al., 1996). A dysregulation of IgG synthesis in the peripheral blood has also been described by *in vitro* studies. Analyzing IgG production in lymphocyte cultures stimulated by a T cell-dependent B cell activator like Pokeweed mitogen (PWM, Fauci et al., 1980), it was found that MS patients synthesize larger amount of IgGs (Goust et al., 1982; O'Gorman et al., 1987). Furthermore, B cells isolated from CSF of MS patients have been shown to produce antibodies targeted against myelin components such as: proteolipid protein (PLP), myelin basic protein (MBP), and myelin-associated glycoprotein (MAG) (Olsson et al., 1990; Sun et al, 1991; Baig et al., 1991).

Studies in animal models of MS have shown that autoreactive T cells are not sufficient to provoke EAE, since IgG-deficient rats fail to develop EAE (Willenberg and Prowse, 1983) and, in *Callithrix Jacchus* primate model of MS, enchephalitogenic T cells only cause full demyelination in the presence of anti-myelin antibodies (Genain et al., 1995). Recently, it was reported that IgG, including IgG directed against peptides of MBP and MOG, was localized within acute MS lesions (Raine et al., 1999). These data indicate that the role and abnormalities of B cells are also an integral component of MS pathogenesis (Levinson et al., 1983; Oger et al., 1981 and 1988; Raine et al., 1999). B cells can be involved either as antigen presenting cells (APCs) or as antibody-secreting cells. They can interfere directly with the mechanism of demyelination or act thereafter (Glynn and Linington, 1989). B cells may also damage the myelin sheath and clear them with aid of complement and/or the microglial cells (Goldenberg et al., 1989; Mosley and Cuzner, 1996; Ulvestad et al., 1994).

The combined actions of the cellular and humoral immune components in MS lesions, the association of MS with specific MHC genes and the failure to detect a specific infectious agent support the notion that MS could be an autoimmune disease (Bertram and Kuwert, 1982; Marrosu et al., 1988; Gran et al., 1999). Further evidence to support the possible autoimmune nature of MS has been derived based on the analogy with an experimental animal model, experimental autoimmune encephalomyelitis (EAE) (Bernard et al., 1992). Additional evidence to support an autoimmune origin of MS came from the detection of myelin-reactive T cells in the blood and CSF of MS patients. Further studies however, revealed that the autoreactive T cells also form part of the normal T-cell repertoire in healthy donors. Despite an extensive search for an autoantigen

that elicits a self-reactive immune response in MS, none of the candidates proved to be causative. Most of the candidates studied are myelin proteins that have been shown to be encephalitogenic in EAE. The antigenic target in the CNS is unlikely to be found in a discovery of a single antigen. This is because of the phenomenon of epitope spreading (Lehmann et al., 1992) that also may operate in MS pathogenesis. According to this scenario, inflammatory process initiated by T cell recognition of one protein epitope can subsequently lead to activation of T cells recognizing other epitopes of the same protein. In time, there might also be activation of T cells that recognize other proteins that presumably get degraded and then presented by local antigen-presenting cells (APC) in association with MHC. Data showing myelin reactive T cells activated against both myelin basic protein and proteolipid protein in the same MS patient support this concept (Zhang et al., 1994).

Imbalances in the cytokine network have also been implicated in MS pathology. It has been suggested that MS pathology is due to a Th1 cell-mediated immune response, driven by a specific antigen that triggers the production of proinflammatory cytokines and secondary immune cells recruitment and activation. Cytokines regulate immune responses by modulating lymphocyte and monocyte function, and may directly cause demyelination and gliosis. Studies in murine models have revealed that upon activation, CD4+ T lymphocytes differentiate into two main types of effector cells that can be separated based on their cytokine secretion profile: Th1 cells that secrete cytokines interleukin-2 (IL-2), tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) and Th2 subset that produce IL-4, IL-5, IL-6, IL-10, and IL-13. The Th1 subset regulates proinflammatory effector mechanisms involved in cell-mediated immunity such as

delayed type hypersensitivity response and macrophage activation, while the Th2 subset regulates humoral immunity and also downregulates local inflammation (Romagnani, 1997). The cytokines that are produced by Th1 or Th2 can also affect each other's development. For example IFN-γ produced by Th1 cells promotes the differentiation of Th1 cells and inhibits the development of the Th2 response. Alternatively, IL-4 and IL-10 favor the development of Th2 cells and inhibit the Th1 response. The following observations support this view point: susceptibility to EAE correlates with a predominant Th1 response to myelin antigens, and resistance to disease induction correlates with a predominant Th2 immune response (Smeltz and Swanborg, 1998), myelin-reactive T cell clones capable of transferring resistance to other animals in EAE models secrete IL-4 and IL-10 (Chen et al., 1994), the recovery from EAE is associated with an increase in Th2 type cytokines in CNS, and administration of IL-10 suppresses the development of EAE (Rott et al., 1994). In MS, cells isolated from the CSF during active disease expressed a Th1 pattern of cytokine production. Increased levels of TNF-α are detected only during active disease and not in inactive MS (Drulovic et al., 1997). MS relapses precede the increased IFN-γ and TNF-β secretion by blood mononuclear cells. (Link et al., 1994). Proteolipid protein (PLP)-specific T-cell clones generated from MS patients during relapse secreted mostly IFN–γ and TNF-α, but clones isolated during remission secreted high levels of IL-10 (Correale et al., 1995). Studies of MS lesions based on composition of the inflammatory cells expressing adhesion molecules, and histocompatibility antigen also support the role of Th1 response in MS pathology (Woodroofe and Cuzner, 1993; Schluesener H, Meyermann, 1993; Traugott et al., 1983). However, it should be noted that no clear bias toward Th1 or Th2 profile has been found in MS, as both pro-

inflammatory as well as regulatory cytokines are present in MS lesions (Canella and Raine, 1995). Furthermore, the Th1/Th2 dichotomy is less clear in the human immune system and represents the extreme of a range of cytokine production profiles. Many instances exist in humans of cells that secrete combinations of Th1 and Th2 cytokines. For example, cytokines such as IL-10, can be produced by both Th1 and Th2 cell subsets (Romagnani et al, 1997). Thus, the descriptions of cytokines with pro- or anti-inflammatory properties may be more appropriate in classifying their functional properties.

Some scientists do not favor the autoimmune hypothesis of MS; they favor an infectious aetiology. According to this hypothesis a neurotropic virus infects the CNS in MS, and T cells infiltrate the brain to target the foreign viral antigens. The neural tissue is then damaged, either directly or via a bystander effect of the ongoing inflammatory response. However, no causative virus has been reproducibly identified yet (Karpas et al., 1986). There is indirect evidence that viruses may play a role in initiating MS. For example, infections with measles, rubella or mumps virus predisposes an individual to an increased risk of developing MS (Waksman, 1989, Martin et al., 1996; Monteyne, 1998). Furthermore, several cases have been documented of brain virus infection leading to MS (Challoner et al., 1995; Sanders et al., 1996). The experimental viral model best characterized is Theiler's virus-induced encephalomyelitis. Theiler's virus is a naturally occurring pathogen in mice that produces a chronic persistent CNS infection resulting in inflammatory demyelination similar pathologically and clinically to the chronic progressive form of MS (Roos, 1983; Dal Canto et al., 1995).

1.2 ROLE OF T CELLS IN THE IMMUNOPATHOGENESIS OF HAM AND MS

Although different mechanisms of pathology have been proposed, there is a general agreement that the pathogenesis of both HAM and MS is immune mediated and that T lymphocytes play a central role in both disease processes (Moore et al., 1989; Hafler and Weiner, 1989; Chang et al., 1992; Utz and McFarland, 1994). HTLV-I has a preferential tropism for CD4+CD45+ T lymphocytes in patients with HAM *in vivo* (Richardson et al., 1990) and T lymphocytes are the predominant cell type in the cerebrospinal fluid (CSF) of HAM patients (Moore et al., 1989). HAM patients have also been shown to have high levels of activated T lymphocytes in their peripheral blood (Itoyama et al., 1988a) and CSF (Mori et al., 1988). This is highlighted by an increase in the number of large CD3+ cells that also express markers of activation, such as HLA-DR and IL-2 receptor molecules. In addition, peripheral blood lymphocytes of HAM patients show spontaneous proliferation in the absence of any exogenous antigen/mitogen *in vitro* (Itoyama et al., 1988b). Furthermore, HAM patients have a high frequency of HTLV-I specific CD8+ CTL in their circulating blood (Jacobson et al, 1990; Elovaara et al., 1993) and CSF (Elovaara et al., 1993).

T lymphocytes are also the predominant cell type in the cerebrospinal fluid (CSF) of MS (Hafler and Weiner, 1989), and activated T cells have been localized in the MS plaques (Hofman et al., 1986; Bellamy et al., 1985), CSF (Noronha et al, 1980; Hafler et al., 1985) and circulating blood (Hartung et al., 1990). Patients with acute relapsing MS have also been found to have increase T cell adherence to their brain endothelial cells

during exacerbation (Tsukada et al., 1993a). Furthermore, experimental autoimmune encephalomyelitis (EAE), an animal model for MS, is T-cell dependent and can be induced through the transfer of myelin basic protein (MBP)-sensitized T lymphocytes (Mokhtarian et al., 1984).

1.3 LEUKOCYTE-ENDOTHELIAL CELL ADHESION AND ITS IMPLICATION IN HAM AND MS IMMUNOPATHOLOGY

The recruitment of circulating leukocytes into inflammatory lesions requires adhesion to vascular endothelium, followed by migration between endothelial cells into the underlying tissue. The recruitment of leukocytes into the central nervous system is complicated by the existence of a specialized microvasculature, characterized by the presence of a continuous network of high resistance and complex tight junctions. Under the control of surrounding astrocytes, this microvasculature constitutes the blood-brain barrier (BBB) that limits the exchanges between the blood and brain of soluble substances such as growth factors, cytokines and immunoglobulins as well as immune cells (Goldstein and Betz, 1983; Pardridge, 1988; Joo, 1993). On the basis of the existence of the BBB and of low levels of major histocompatibility complex (MHC) molecules on brain cells, the CNS has often been considered an "immunologically privileged" site and not normally accessible to leukocyte traffic (Baker and Billingham, 1977). However, this viewpoint has been challenged. Recent evidence indicates that leukocytes can invade the brain parenchyma at very low levels under normal conditions and at much higher levels when T cells are activated (Wekerle et al, 1986; Raine et al.,

1990; Hickey et al., 1991). Furthermore, there is an emerging view that there is a definite connection between the CNS and the peripheral blood through draining lymphatic channels to the cervical lymph nodes (Cserr and Knopf, 1992; Weller et al., 1996).

Histopathological and MRI studies of the CNS in HAM and in MS indicate that the breakdown of the BBB and infiltration of leukocytes are early events in the formation of HAM and MS lesions (Kermode et al., 1990, Umehara et al., 1993). A prerequisite for the passage of lymphocyte across the BBB into the CNS parenchyma is binding to endothelial cells. The molecular mechanisms that governs leukocytes infiltration into the CNS involves selective and sequential adherence between cell surface molecules on both leukocytes and endothelium. Adhesion molecules also mediate the subsequent migration of leukocytes into the surrounding tissue. While adhesion molecules also participate in T cell costimulation (Davignon et al., 1981), helper function for B cell immunoglobulin production (Miedema et al., 1985), antibody dependent cell mediated cytotoxicity (Capron et al., 1987), and cytotoxic T cell mediated cytolysis (Krensky et al., 1983), in the following sections the general role of these cell surface molecules in adhesion of leukocyte to endothelial cells are emphasized and discussed.

1.4 ADHESION MOLECULE CLASSIFICATION, CASCADE AND REGULATION

1.4.1 Classification of adhesion molecules

Based on their structure adhesion molecules have been classified into three major groups: selectin, integrin, and immunoglobulin supergene family (IGSF) members

(Osborn, 1990S; Springer, 1990, 1994) (Table 1.1). Selectins are expressed on leukocytes, platelets, and endothelial cells, and their common structural component is a N-terminal lectin-binding domain. Selectins have been subclassified according to the cell type on which they were first identified: L-selectin (lymphocyte), P-selectin (platelets/endothelium), and E-selectin (endothelium) (Bevilacqua and Nelson, 1993; Tedder et al., 1995).

L-selectin is expressed constitutively on all leukocytes and has a critical role in the adhesion of lymphocytes to peripheral lymph node cells and activated endothelium. (Tedder et al., 1995) Upon activation, L-selectin is lost rapidly from the surface of leukocytes (Tedder et al., 1990). In fact, L-selectin and ß2 integrin Mac-1 (CD11b/CD18) expression appear to be regulated inversely (Kishimoto et al, 1989). P-selectin, found on platelets and in Weibel-Palade bodies of endothelial cells (EC) synergizes with cytokines to upregulate leukocyte integrin expression (Bevilacqua and Nelson, 1993). E-selectin, expressed on endothelium, is upregulated after exposure to tumor necrosis factor alpha (TNF-α), and shed rapidly after loss of cytokine stimulation (Doukas and Pober; 1990). E-selectin binding of leukocyte triggers more stable adherence by integrin receptors (Lawrence and Springer, 1991). All selectins bind in a Ca^{++}-dependent manner to sialylated carbohydrate structures. The main counter-receptors for L-selectin that have been characterized so far are: glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1), the glycosylated and sulphated form of CD34 expressed by endothelial cells, and mucosal addressin cell adhesion molecule-1 (MadCAM-1), and P-selectin. P-selectin binds to P-selectin glycoprotein ligand-1 (PSGL-1) and E-selectin. E-selectin interacts with E-selectin ligand-1 (ESL-1) (Varki, 1997). Binding of E-selectin to CD66

(Kuijpers et al., 1992), CD11/CD18 (Kotovuori et al., 1993), and L-selectin (Picker et al., 1991) has also been reported. Despite their short intracellular regions, selectins are able to generate costimulatory signals that contribute to leukocyte activation after interaction with their counter-receptors. The physiological importance of selectins in inflammatory responses is seen in leukocyte adhesion deficiency (LAD) type II, in which the congentical absence of selectin ligands produces significant adhesion defects and recurrent life-threatening infections (Etzioni et al., 1992).

Integrin adhesion molecules are heterodimeric structures composed of noncovalently linked α heavy chain and ß light subunits. Subunit combinations form functionally different receptors (Larson and Springer, 1990). The name integrin was based on the function of these transmembrane molecules to "integrate" extracellular information into the cytoskeleton. Integrins are arranged in subfamilies according to the ß subunits and each ß subunit may have from one to eight different α subunits associated with it. It is also possible for individual α subunits to be associated with different ß chain. Within the integrin family of adhesion receptors so far only five members have been shown to be involved in leukocyte adhesion to endothelium: The $ß_2$ leukocyte integrins (CD11a/CD18, CD11b/CD18, and CD11c/CD18), the $ß_1$ integrin VLA-4 ($\alpha_4ß_1$, CD49d/CD29), and $\alpha_4ß_7$. The ß2 integrins share a common ß chain (CD18) and can be noncovalently associated with any of the three CD11a (LFA-1), CD11b (Mac-1), and CD11c (p150, 96) α subunits. Peripheral blood lymphocytes mainly express CD11a/CD18 (LFA-1) whereas neutrophil, monocytes, and NK cells express all three ß2 integrins. Ligands for $ß_2$ integrins include immunoglobulin supergene family (IGSF) protein family, which are ICAM-1 for CD11a/CD18, CD11b/CD18 and ICAM-2, -3 for

CD11a/CD18. $ß_2$ integrins also bind soluble proteins such as fibrinogen, and factor X (Larson and Springer, 1990). The $ß_1$ integrins share CD29 as their common ß subunit. $\alpha_4ß_1$ (VLA-4, CD49d/CD29) a prototypical $ß_1$ integrin is mostly prominent on cells of the hematopoietic system. Ligands for $\alpha_4ß_1$ include VCAM-1 and extracellular matrix (ECM) proteins fibronectin, vitronectin, laminin, collagen, von Willebrand factor, and fibrinogen (Larson and Springer, 1990) and the ligands for $\alpha_4ß_7$ are VCAM-1 and MAdCAM-1 (Springer, 1994).

Integrins exhibit important functional features such as their ability to increase the avidity for their counter-receptors (Clark and Brugge, 1995; Aplin et al., 1998). The enhancement of integrin avidity is due to intracellular signals that are generated through other cell surface receptors. Integrins are also linked through the cytoskeleton, to molecules involved in the generation of intracellular signals such as focal adhesion kinase or the PI 3-kinase. Thus, the interaction of integrins with their ligands induces costimulatory signals that contribute to cell activation and differentiation (Clark and Brugge, 1995).

The cell adhesion molecules that belong to IGSF have one or more domains homologous to those found in immunoglobulin and therefore are named and classified together (Springer, 1990, 1994; Carlos and Harlan, 1994). Members of this superfamily are expressed by endothelial cells (e.g. MadCAM-1 and vascular cell adhesion molecule-1 (VCAM-1), or by both endothelial cells and leukocytes [e.g. intracellular adhesion molecule-1 and –2 (ICAM-1 and –2)]. ICAM-1 and VCAM-1 are detected on activated endothelial cells, whereas ICAM-2 is expressed by both resting and activated endothelial cells. An additional member of this family, PECAM-1 (CD31) plays a role in homotypic

adhesion of leukocytes and promotes adhesion between the endothelial cells and leukocytes. ISGF cell adhesion molecules may interact among themselves in a heterotypic or homotypic fashion or with cell adhesion molecules from the integrin families. Additional receptors in this family that function as adhesion molecules include LFA-2 (CD2) and LFA-3 (CD58) (Dustin and Springer, 1991).

There are additional intercellular adhesion molecules that also participate in inflammatory phenomenon. Cadherins are calcium-dependent adhesion proteins that mainly interact homotypically (Takeichi, 1995; Yap et al., 1997). Members of this superfamily are expressed by, and are responsible for the integrity of endothelial and epithelial cells. Cadherins found at intercellular endothelial junctions seem to play a key role in the extravasation of inflammatory cells. Lastly, other molecules mainly involved in signal transduction such as the chemokine/chemokine receptor may also function as cell adhesion receptors (Imai et al., 1997).

1.4.2 Adhesion cascade (leukocyte-endothelial adhesion)

Leukocyte adhesion and migration is a complex phenomenon regulated by a cascade of molecular events that take place in an ordered series of steps involving close interactions between adhesion receptors expressed by migrating leukocytes and endothelial cells (EC). A consensus model of leukocyte migration in four sequential steps is now generally accepted (figure 1.1) (Butcher, 1991; Shimizu et al., 1992; Springer, 1994). In the first step (tethering/rolling), some of the flowing leukocytes come into brief contact with the vessel wall, slow their movement, and roll on the endothelium. This step is transient, reversible and mediated by constitutively expressed selectin molecules and

their cognate oligosaccharide ligands (Bevilacqua and Nelson, 1993). In addition to selectins, it has been found that VLA-4 integrin is also able to sustain the rolling of leukocytes both *in vivo* (Johnson et al., 1996) and *in vitro* (Berlin et al., 1995). In the second step (triggering/activation), rolling leukocytes are exposed to the local endothelial microenvironment in the presence of inflammatory mediators such as chemoattractant/cytokines that further deliver activating signals to the leukocytes resulting in upregulation of adhesion molecules and leading to adhesion arrest (Schall and Bacon, 1994, Campbell et al., 1998). The third step (firm adhesion) is primarily mediated by activated $ß_1$ (VLA-4) and $ß_2$ (LFA-1 and Mac-1) integrins, which bind to their counter receptors VCAM-1 and ICAMs, respectively (Hogg and Landis; 1993). The activation of leukocytes induces a rapid shedding of L-selectin caused by cleavage of the extracellular portion of L-selectin by an unidentified endogenous protease (Kansas, 1996). The activation of leukocytes also results in an increase in avidity of integrins for their ligands due to the conformational changes in integrin heterodimer. The increased avidity of the leukocytes integrins results in the firm adhesion of leukocytes to EC. During this phase, leukocytes change shape and acquire a flattened morphology. Then leukocytes transmigrate between EC (diapedesis), or through them following the chemotactic gradient generated by inflammatory foci (fourth step or extravasation). The molecular interactions that are involved in the extravasation of leukocytes are those that mediate the firm adhesion (LFA-1/ICAM-1, -2 and VLA-4/VCAM-1), but adhesion receptors located at the EC junctions such as CD31 and VE-cadherin also have an important role in leukocyte extravasation (Piali et al., 1995; Bianchi et al., 1997). The migration of leukocytes from the blood vessel wall toward the inflammatory foci also involves

interactions of the leukocyte receptors mainly β_1 integrins with the components of the extracellular matrix such as collagen, fibronectin, and laminin.

1.4.3 Regulation of adhesion

Regulation of adhesion occurs through increased avidity of existing adhesion molecules or increased expression of molecules on the cell surface. For example, activation of T cells with the phorbol myristate acetate (PMA) has been demonstrated to increase the affinity of LFA-1 and VLA-4 for their counter receptor ICAM-1 and VCAM-1, respectively, without changing the levels of integrins cell surface expression (Wilkins et al., 1991; Shimizu et al., 1991; Dustin and Springer 1989). Cross-linking of T-cell receptors also increases LFA-1 avidity; however, T cell receptor-induced changes in LFA-1 avidity are transient (Dustin and Springer, 1989). Cell-cell adhesion is generally more efficient when an appropriate antigen: MHC complex is recognized (Martz, 1987). Antigen-independent adhesion requires prior activation of T lymphocytes, because spontaneous adhesion is low or absent in resting T lymphocytes (Dustin and Springer, 1989). Furthermore, binding of cell adhesion molecules with their respective ligands causes alteration in the expression and affinity of adhesion molecules (Frelinger et al., 1988; Lou et al., 1996).

Activation of cells not only induces changes in avidity but also can upregulate adhesion receptor expression (Springer, 1994). For example, cytokines such IL-4, IL-1ß, TNF-α, and IFN-γ have been shown to promote the adhesiveness of T cells for endothelial cells by increasing the expression of VCAM-1, ICAM-1 and ELAM-1 on endothelial cells (Hughes et al, 1988; Minovsky et al., 1990; Thornhill et al., 1991;

Shimizu et al., 1991). Differential regulation of adhesion molecule expression determines homing of lymphocytes to organs and sites of inflammation. Adhesion receptor expression on leukocytes, vascular endothelium, and other cell types may be constitutive or regulated (Springer, 1994, Imhof and Dunon, 1995). For example, selectin adhesion is mediated by the presence or absence of receptor expression on the cell surface. Selectin adhesion is transient and unstable under intravascular conditions but slows leukocyte circulations dramatically (Springer, 1994; Bevilacqua and Nelson, 1993). TNF-α or IL-1 induces endothelial expression of E-selectin, but expression is typically short lived. Concomitant stimulation with interferon gamma (IFN-γ) enhances and prolongs expression of E-selectin (Doukas and Pober, 1990). If chemoattractant or additional adhesion mechanisms are not present at a microvascular site, transiently adherent leukocytes are released back into circulation. If secondary adhesion molecules (integrins, IGSF receptors) are expressed, leukocyte adherence to endothelium becomes more stable, leading to infiltration of leukocytes into the ECM and tissue (Springer, 1994; Tedder, 1995).

Besides changes in avidity and cell surface expression, differential distribution and expression of adhesion molecules on T lymphocyte subsets may modulate immune/autoimmune response patterns. On the one hand, CD8+ T cells have been reported to have higher LFA-1 expression compared with CD4+ T cells (Pardia et al., 1989). On the other hand, it has been shown that mAb against VLA-4 inhibited CD4+ but not CD8+ T cell infiltration of the pancreas in the non-obese, diabetic (NOD) mouse model of diabetes (Baron et al., 1994). This finding implies that differential adhesion

molecule expression occurs on lymphocyte subsets and determines transendothelial migration patterns for CD4+ and CD8+ T cells.

In addition to the broadly expressed proinflammatory cytokines such as TNF-α, IFN-γ, IL-1, IL-4, and IL-6, chemokines are also important soluble mediators of inflammation (Bacon, 1994; Baggiolini, 1998). Once released by immune cells, they strongly modulate adhesion molecules expression and affinity on both endothelial cells and lymphocytes (Wong and Dorovini-Zis, 1996; Merrill and Benveniste, 1996). Important chemokines include RANTES, IL-8, MIP-1ß, MIP-1α MCP-1, colony stimulating factors (G-CSF, GM-CSF), chemoatractant peptides (C5a, FMLP) and neuropeptides (Luster, 1998; Baggiolini, 1998). Binding of chemokines, cytokines or chemoattractants to leukocyte expressing complimentary receptors transduces signals that can augment ß1 or ß2 integrin-dependent adhesion. Some chemoattractant factors influence narrow population of cells. For example, RANTES acts primarily on memory T cells (Schall et al., 1990). MIP-α attracts monocytes and CD8+ T cells, whereas the closely related MIP-1ß acts on CD4+ T cells (Ming Wang et al., 1993; Taub et al., 1993). Furthermore, MIP-α but not MIP-β increases the adherence of T cells to endothelial cell (Tanaka et al., 1993). Most "classic" cytokines (e.g. IL-1, IL-4, TGF-ß) exert their adhesion modulating effects on a variety of cells, but they may show different effect on different cells. For example, TGF-ß induces migration of T cells and monocyte but not granulocytes and IL-4 induces adhesion molecules on HUVEC but inhibits monocyte adhesion.

1.5 ADHESION MOLECULES IN HAM AND MS

Analysis of the spinal cord lesions of HAM patients has shown enhanced expression of VCAM-1 and E-selectin on endothelium, and high levels of VLA-4, LFA-1 and Mac-1 expression on infiltrating mononuclear cells (Umehara et al., 1996). Increased levels of soluble ICAM-1 and VCAM-1 have been detected in the serum and CSF of HAM patients (Mainnolfi and Rothlein, 1992; Tsukada et al., 1993b; Matsuda, 1995a). Significant elevation of soluble L-selectin has also recently been reported in the sera of HAM patients (Tsujino et al., 1998). Expression of adhesion molecules on freshly isolated lymphocytes from HAM patients have not been studied. However, enhanced expression of VLA-4 and VLA-5 integrins has been shown in peripheral blood lymphocytes of healthy controls that were infected with HTLV-I *in vitro* (Dhawan et al., 1993). Enhanced expression of ICAM-1 and LFA-3 has also been reported in T cell lines carrying HTLV-I (Fukodome et al., 1992; Imai et al., 1993)

Most of the studies of the role of adhesion molecules during MS have relied on immunochemical analyses of the expression of adhesion molecules during the different stages of disease on autopsy CNS material or on blood or CSF-derived lymphocytes from patients with MS. In typical MS lesions, upregulation of ICAM-1, VCAM-1 and E-selectin on the endothelium, and VLA-4, and LFA-1 on the infiltrating mononuclear cells have been described (Washington et al., 1994; Brosnan et al., 1995; Cannella and Raine, 1995). In addition, in MS lesions some resident cells of the CNS, such as astrocytes or microglia, show increased cell surface expression of ICAM-1 and LFA-1 (Bo et al., 1996; Cannella and Raine, 1995). Lymphocytes from either CSF or the blood of MS patients

also express increased levels of adhesion molecules such as LFA-1, LFA-3, CD2, and CD44 on their surface (Svenningsson et al., 1993). Cultured brain microvascular endothelial cells derived from MS patients were shown also to constitutively express high levels of ICAM-1 and were demonstrated to have a high capacity in adhering to isolated leukocytes (Lou et al., 1997). A further clue that lends support to the importance of adhesion molecules in MS is derived from measurement of circulating soluble adhesion molecules during MS. Investigators found that circulating forms of ICAM-1, ICAM-3, VCAM-1, and L-selectin were increased in serum and CSF from most MS patients (Rieckmann et al., 1994b, Mobner et al., 1996, Droogan et al., 1996). Circulating levels of ICAM-1, VCAM-1, E-, and L-selectin are also correlated with clinical relapse (Sharief et al., 1993; Rieckmann et al., 1994b, Hartung et al, 1995; Dore-Duffy et al., 1995) and the appearance of new gadolinium-enhancing lesions in MRI (Mobner et al., 1996) (this is a parameter of BBB breakdown and disease activity).

1.6 IMMUNOTHERAPY IN MS

Proinflammatory cytokines, such as IFN-γ and TNF-α, have been shown to be associated with exacerbation in patients with MS (Panitch et al, 1987). Furthermore, increase in TNF-α mRNA expression and decrease in IL-10 mRNA expression positively correlates to exacerbation of MS (Rieckmann et al, 1994a). Thus, cytokine-based strategies for the treatment of MS have focused on anti-inflammatory cytokines such as IL-10 and IFN-β.

The adhesion and subsequent migration of circulating leukocytes across the BBB into the CNS in inflammatory conditions, such as MS, involve a complex series of adhesion molecules expressed on leukocytes and endothelium. An approach to immunotherapy in MS has also been to inhibit this interaction using anti-adhesion molecules monoclonal antibodies. An important and central adhesion molecule in leukocyte-endothelium interaction is LFA-1 integrin.

1.6.1 Interleukin-10

Interleukin-10 (IL-10) is believed to be produced by Th-2 cells and to inhibit the function of Th1 cells (Mosmann and Moore, 1991). IL-10 is also thought to participate in recovery from the inflammatory events by down-regulating the activated state of endothelial cells and macrophages (Olsson, 1995). In MS, levels of IL-10 mRNA were reported to be higher in stable MS compared with relapsing MS and the levels of IL-10 were shown to decline prior to relapse in MS (Rieckmann et al., 1994a). These observations prompted Schering-Plough Corporation to develop recombinant human interleukin-10 (rhuIL-10) for potential therapeutic use in multiple sclerosis. RhuIL-10 was produced in a strain of *Escherichia coli* bearing a genetically engineered plasmid that contains a rhuIL-10 gene. With the exception of methionine residue at the amino-terminus, rhuIL-10 is identical to endogenous human IL-10 protein. The effect of rhuIL-10 has been examined in several rodent models of EAE. In one study where TNF-α was used to induce relapses of EAE in SJL mice that had recovered from acute EAE, rhuIL-10 given with TNF-α provided complete protection against relapses (Crissi et al., 1995). In another study where acute EAE in the Lewis rat was induced by MBP, administration

of rhuIL-10 during the initial phase of the disease suppressed the subsequent induction of EAE (Rott et a., 1994). In a different study in which an acute EAE was induced in mice, conflicting results were obtained. A single dose of rhuIL-10 given immediately after MBP injection showed no effect (Smith, 1994). However, a similar dose of rhuIL-10 given at the onset of EAE symptoms showed a trend toward improvement (Schering-Plough, Data on file as D-27219). However, repeated doses of rhu-IL-10 at 7 and 14 days during the initial phase of the disease resulted in an exacerbation of the disease (Schering-Plough, Data on file as P-5806). The safety studies in multiple dose pilots clinical trials with healthy volunteers, patients with Crohn's disease, Ulcerative Colitis, and Rheumatoid Arthritis indicated that the rhuIL-10 is safe with minimal side affects up to 25 μg/kg dose levels. In a multi-center, randomized, double blind, placebo-controlled study, the subcutaneous injection of single dose of rhuIL-10 was tested in clinical trial of relapsing-remitting form of MS with MRI evidence of disease activity. The results of study indicated that there was no significant benefit for the use of rhuIL-10 in RR-MS.

1.6.2 Interferon β

Interferons are a family of proteins that inhibit viral activities and cellular proliferation and modify the immune response (Becker et al., 1995). One member of the IFN family, IFN-β has been shown to be of benefit in relapsing-remitting MS. The mechanisms underlying the efficacy of IFN-β in MS patients are still not completely known. However, there are several proposed mechanisms that may play a role in the efficacy of IFN-β in MS (Yong et al., 1998). These include: down-regulation of the IFN-γ activity; induction of T suppressor cell function; augmentation of IL-10 production;

inhibition of T cells migration into CNS and antiviral effect. Interferon β has received regulatory approval in the United States, Canada, Europe, and Australia for the treatment of MS. Currently there are three preparation of interferon β in use. These are, Avonex™ (IFN-β-1a, Biogen), Betaseron® (IFN-β-1b, Berlex), and Rebif® (IFN-β-1a, Serono). The similarities and differences between the three preparations of IFN-β are summarized in Table 1.2.

Betaseron® (IFN-β-1b) was initially tested in a multicentre trial involving 372 patients with relapsing-remitting MS and mild to moderate disability. Treatment consisted of either 8 MIU (250 μg) or 1.6 MIU (50 μg) or placebo given by subcutaneous injection on alternate days. The high dose was set on the basis of patient tolerance to a single injection. Compared with placebo, treatment with the higher dose reduced the relapse rate by 31%, increased the time to first relapse and the proportion of patients who were relapse free (The IFN-β Multiple Sclerosis Study Group, 1993, 1995). In addition, there was a significant reduction in disease activity as measured by the analysis of new or enlarging lesions on serial MRI (Paty and Li and the UBC MS/MRI Study group, 1993).

A second multicentre trial of Betaseron® was recently completed in Europe, comprising 718 patients with secondary progressive MS who had been clinically active in the 2 years preceding the study (European Study Group on interferon β-1b, 1998). Treatment consisted of either 8 MIU or placebo subcutaneously on alternate days over 3 years. Treatment with Betaseron® resulted in the significant delay of disease progression and reduced the disease activity. Furthermore, it increased the time to first relapse and the proportion of patients who were relapse free.

Avonex™ (IFN-β-1a) was tested in a trial involving 301 patients with relapsing form of MS and mild to moderate neurological impairment. Treatment consisted of weekly intramuscular injections with 6 MIU (30 μg) Avonex or placebo. An 18% reduction in exacerbation rate was seen for the treated group (Jacobs et al., 1996). The treatment was also accompanied by a reduction of gadolinium enhancement and of new or enlarging lesions on annual MRI (Simon et al., 1998).

Rebif® (IFN-β-1a) was investigated in a large study involving 560 patients with active relapsing-remitting MS and mild to moderate disability who were randomized to treatment with IFN-β-1a at 6 MIU (22 μg) or 12 MIU (44 μg) or placebo, given subcutaneously three times a week for 2 years. The result showed that, compared with placebo, Rebif® significantly decreased the number and severity of exacerbation by 27% and 33% in the 22 μg and 44 μg groups, respectively, (PRISMS study group, 1998). Rebif® also increased the time to first and second relapse, and increased the percentage of patients who were relapse free during the study. Furthermore, there was a significant reduction in the disease activity on MRI, as defined by new or enlarging lesions (PRISMS study group, 1998).

1.6.3 Anti-LFA-1 monoclonal antibody

The importance and beneficial effects of anti-LFA-1monoclonal antibody (mAb) in inflammation has been demonstrated in animal models in bacterial meningitis (Tuomanen et al., 1989) and acute lung injury (Mulligan et al., 1992). In rodent EAE, mAb against LFA-1 had either no effect (Canella et al., 1992) or a deleterious effect (Welsh et al., 1993). However, mAbs against Mac-1 suppressed EAE, but only if given

prior to the disease onset (Huitanga et al., 1993). Thus, the role of LFA-1 in the pathogenesis of EAE remains not well defined.

The presence of brain inflammation in EAE and the anti-inflammatory effects of antibodies against LFA-1 adhesion molecule led ICOS Corporation to develop and test a humanized anti-LFA-1 named Hu23F2G. Hu23F2G is produced by Chinese hamster ovary (CHO) as γ4 immunoglobulin. This mAb recognizes both LFA-1 and Mac-1. As expected, *in vitro* Hu23F2G has the following properties: inhibits LFA-1 dependent cell aggregation; blocks binding of LFA-1 bearing cells to purified ICAM-1 and blocks LFA-1-dependent leukocyte transmigration through endothelial cells monolayers. Furthermore, ICOS tested the effects of Hu23F2G in nonhuman primate *Macaca fascicularis* EAE. In this EAE model animals were treated with intravenous injection of either Hu23F2G (2 mg/kg for 7 days) or dexamethasone (4 mg/kg for 3 days). The result indicated less severity of EAE, more resolution of brain MRI abnormalities, and longer survival than animals given dexamethasone alone. These *in vivo* and *vitro* observations indicate that anti-LFA-1 might also have a favorable effect on MS. This led ICOS to determine the safety and efficacy of Hu23F2G in the treatment of acute exacerbations of MS. A multi-center, randomized, double-blind, placebo-controlled trial was conducted. A total of 169 patients were enrolled within seven days of the onset of symptoms recurrence to one of four treatment groups: Placebo (n=43), methylprednisolone at 1 gram intravenously (IV) for 3 days (n=41), Hu23F2G at 1 mg/kg IV (n=44) or Hu23F2G at 2 mg/kg IV (n=41). The result indicated that while Hu23F2G was safe and well tolerated at the doses used, it was ineffective at either dose as compared to placebo (Lublin, 1999).

1.7 CHALLENGES IN STUDYING MS AND HAM

Variability in disease progression is a prominent clinical feature of MS. Some patients have initial attacks, complete recovery, and no further symptoms, while others progress rapidly within months of initial involvement. Most patients have exacerbation and remission that follow an unpredictable course. Immunopathological studies of lesions in a single MS patient reveal a fairly uniform pattern of inflammation, demyelination, axonal loss, and remyelination. However, profound heterogeneity exists in lesional structure between different patients (Lassmann et al., 1998). To further dissect which specific mechanisms are involved in MS pathogenesis, Lucchinetti et al. (1996) have analyzed the MS lesions in a large number of early biopsy and autopsy of lesions. Based on this study, the MS lesions were categorized to five different types; demyelination with minimal oligodendrocyte damage; demyelination associated with extensive oligodendrocyte loss; primary demyelination with a gradient of oligodendrocyte loss toward the inactive plaque center; demyelination paralleled with nonselective destruction of oligodendrocytes, axons and astrocytes; and primary oligodendrocyte damage in the periplaque white matter with secondary demyelination. In addition to structural differences in MS lesions, the predominant immunopathological contribution to lesions formation was also seen. The cases were segregated into those with antibody and complement involvement, those with a dominant T cells/macrophage reaction and those with primary oligodendrocyte involvement. The diverse clinical course and pathological findings might imply the diversity in MS pathogenesis (Lucchinetti et al., 1996; Lassmann et al., 1998). Based on these studies it was suggested that MS might represent

a neurological syndrome with several different immunopathological mechanisms that lead to a final common trail of CNS injury rather than a single disease with a single cause (Lucchinetti et al., 1996; Lassmann et al., 1998). These diverse clinical and pathogenic findings in MS have contributed to ambiguity and uncertainty in understanding MS pathology and have slowed the course of development of effective therapy in MS.

To elucidate the mechanism of pathogenesis of MS, investigators have relied on studies of experimental autoimmune encephalomyelitis (EAE), considered to be an animal model of MS. Acute and chronic variants of this T-cell driven autoimmune disorder of the CNS have been established in rodents (Swanborg, 1995) and non-human primates (Massacaesi et al., 1995). Generally EAE is induced by sensitization with myelin antigens such as myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG) or S-100 (an intracellular protein present in astrocytes). An additional approach to inducing EAE in an animal is by injection of myelin-specific T cells that have been activated *in vitro*. Frequently used animals include mice (strains SJL/J, PL/J, Biozzi), rats (strain Lewis and DA), guinea pigs (strain 13, Hartley) and more recently marmosets. Depending on the species and the agents used to elicit EAE, distinct histological and clinical features result, each resembling MS to a variable degree (Brochet and Dousset, 1999). Many animal models of MS feature mostly acute attacks and full recovery often not associated with destruction of myelin sheath. MS, in contrast to EAE is chronic, lasting a lifetime, and commonly manifests episodes of inflammation in the white matter, leading to permanent disabilities. Furthermore, MS is a spontaneous chronic disorder of the CNS within a heterogeneous population in contrast to EAE, which is an experimentally induced acute inflammatory reaction, mostly

in inbred rodents. These observations indicate that, while helpful in the study of the basic mechanisms involved in the immune attack on the CNS, EAE does not adequately represent MS pathogenesis and lacks the clinical complexity of MS.

Studies of MS are further complicated by unidentified environmental influences and their interactions with individual immuno-genetic backgrounds. One approach to advance our understanding of the MS pathophysiology is to study immune-mediated diseases of the CNS associated with well-known etiology. An example of such a disease is HAM. The presence of activated T cells associated with proinflammatory cytokines as well as elevated levels of IgG and the presence of oligoclonal bands in the CSF of both HAM and MS patients indicate that demyelinating factors in both diseases might be similar. While there are clear clinical and histopathological differences between HAM and MS, there are subsets of MS patients such as those with progressive MS that are clinically similar to HAM (Kira et al., 1993, Godoy et al., 1995). Magnetic resonance imaging studies of the brain and spinal cord may also be indistinguishable. In general, HAM and MS share the common findings of demyelination and axonal damage associated with inflammatory cell infiltrates in the CNS (Moore et al., 1989).

On the one hand, multiple sclerosis is a common neurological disease among people of European descent in British Columbia. On the other hand, the number of HAM patients is very few and confined mostly to West Coast Amerindian populations. In fact, the total number of known HAM patients in British Columbia, Canada is believed to be 15 cases (Oger, personal communication). Furthermore, unlike MS patients who live mostly in urban areas, the HAM patients mostly live in remote regions. Therefore, the low number of patients combined with inaccessibility makes phenotypic and functional

immunological studies that require fresh blood in HAM patients a particularly challenging task.

1.8 RATIONALE, HYPOTHESES AND OBJECTIVES

1.8.1 Studies of lymphocyte subsets in HAM

The technique of flow cytometry allows for large number of cells to be analyzed and divided into subsets based on expression of surface antigens (Tragnos, 1984) and is particularly suitable for quantification of immune cells (Landay, 1988). The development of fluorochrome-conjugated monoclonal antibodies with emission wavelengths, which can be separated spectrally, has contributed a great deal in assessing diseases. Fluorescein isothiocyanate (FITC) was the first fluorochrome to be developed for use in biology (Coon et al., 1950) and continues in common use today. The development of antibodies conjugated to the phycoerthyrin (PE) dye allowed simultaneous analysis of two different molecules with a single laser line (Oi et al., 1982), thus providing more information than single color analyses.

Flow cytometry has also been used to study lymphocyte subsets in peripheral blood of HAM patients. However, to date, most lymphocyte subset studies in HAM have relied on single color analysis of lymphocyte expressing markers of activations confined to HLA-DR, or CD25. In the last few years due to increased development and availability of monoclonal antibodies against lymphocyte surface antigen, much has been learned regarding the immunoregulatory roles that various lymphocyte subsets play in both health and diseases. Some of the lymphocyte subsets characterized by monoclonal antibodies

have been assigned to participate in a defined immunological function(s). T lymphocytes are believed to play a critical role in the immunopathogenesis of HAM. Therefore to further distinguish and characterize immunoregulatory lymphocytes subsets, we investigated lymphocyte subsets employing two-color flow cytometry using a panel of well defined monoclonal antibodies against T lymphocyte markers of activation and adhesion related antigens (CD molecules) in a group of patients with HAM, and compared the results with those of HTLV-I asymptomatic carriers, and seronegative controls. The Principal features of the CD molecules referenced in this thesis are indicated in Table 1.3.

One of the immunological hallmarks of HTLV-I infection is spontaneous T cell proliferation in culture and this phenomenon has been reported to be more intense in HAM than in carriers (Itoyama et al., 1988). To investigate whether this difference in T cell spontaneous mitogenesis is accompanied by changes in cell surface phenotypes, we also studied lymphocyte subsets after 2 days in culture without mitogen. This timing selection was based on our laboratory observations that spontaneous MNC proliferation initiates after 2 days in culture.

1.8.2 Studies of Lymphocyte-endothelial cell adhesion in HAM and MS

To understand the pathology of HAM and MS it is also important to define the influences that permit the circulating immune cells to enter the CNS, and also the factors that downregulate the inflammatory cell invasion of the CNS. A prerequisite to the passage of lymphocyte across blood vessel walls is binding to endothelial cells. *In vitro* studies have indicated that adhesion of lymphocytes to endothelium is increased by

activation of lymphocytes (Brown et al., 1993) and it is these cells rather than quiescent lymphocytes that are more likely to penetrate the blood-brain barrier (Wekerle et al., 1986). As circulating lymphocytes are activated in patients with HAM and MS (Hafler et al., 1985; Jacobson et al., 1988), this current study was undertaken to determine whether adhesion of HAM and MS lymphocyte to endothelium *in vitro* is augmented as well. We studied this interaction in an *in vitro* model that measures the binding of ^{51}Cr-labeled human lymphocyte to endothelial cell monolayers (Brown et al., 1993). Using this system, the contribution of different well-known adhesion molecules in lymphocyte binding to endothelium was also investigated by monoclonal antibody blocking.

Due to difficulties in obtaining and growing endothelial cells from human sources, several laboratories have relied extensively on the recent availability of endothelial cell lines to investigate adhesion, transmigration and the responsiveness of the endothelium to pro- and anti-inflammatory agents. ECV-304 was reported to be a spontaneously transformed and immortalized human umbilical vein endothelial cell line. ECV-304 cells do not require special growth factors, can be maintained in culture indefinitely, and have been described to display and retain most of the characteristic markers of endothelial cells such as *Ulex europaeus* agglutunin-1 binding and secretion of von Willebrand's factor (Takahashi et al, 1990; Bowie et al., 1995; Hughes, 1996; Dolman et al., 1997). ECV-304 has also been previously used in adhesion as well as transmigration assays (Romanic and Madri, 1994; Bowie et al., 1995; Romanic et al., 1997; Kuchler-Bopp et al., 1999). In a part of this project, we also utilized this cell line as a source of endothelial cells for adhesion and inhibition of adhesion assays. However, once this project was initiated, we were informed (ATCC official letter) that ECV-304 is

a subclone of the epithelial bladder cancer cell line T-24 (Dirks et al., 1999), rather than an endothelial cell line. Since ECV-304 has been used both by us and other investigators, we further wanted to compare it to HUVEC and assess its relevance in adhesion assays. Thus experiments were undertaken in which the adherent properties of ECV-304 cell line for lymphocytes were compared with those of HUVEC.

1.8.3 Studies of immune modulating agents in MS

Three different preparations of IFN-β, namely Avonex™, Betaseron® and Rebif®, are currently in use for the treatment of relapsing and remitting MS patients. These drugs were shown in controled trials to be effective in reducing the frequency of relapses, the number of demeylinating plaques and suppression toward disability. The beneficial effect of IFN-β in MS is likely due to immuno-modulating properties of IFN-β and not due to suppression of viral infections (Panitch, 1994). Nevertheless, the relative units of these IFN-β are all measured by a viral plaque assay and represent the antiviral potency of the drugs, which may not correlate with their beneficial effect in MS.

In trials, evaluating the safety and efficacy of the three IFN-β, different doses, dosing regimen, and application routes were used. Due to differences in study design and populations, no direct comparison can be made on efficacy of these three IFN-β in use. Therefore, the optimal dose for treatment of MS with each of the IFN-β in use is still under debate. In an approach to resolving this issue, we directly compared the effects of each of the three preparations of IFN-β in use in an *in vitro* assay representing an immunomodulating model that relies on inhibition of T-cell dependent B cell activation.

More specifically, the potency of the three IFN-β was calculated from their ability to suppress PWM induced IgG secretion in the culture supernatant.

The potential roles of LFA-1 adhesion molecules and IL-10 in MS are addressed in the above paragraphs. During the course of this thesis, two separate double blind, placebo controlled, multicenter trials were initiated by Schering-Plough and ICOS Corporation to respectively, test the safety and efficacy of IL-10 and anti-LFA-1 in relapsing-remitting MS. The University of British Columbia multiple sclerosis clinic was one of the trial sites. Therefore, we had an opportunity to study the potential *in vivo* effects of IL-10 and Anti-LFA-1 on lymphocytes surface activation and adhesion related antigens using flow cytometry.

IFN-β has been shown to favorably alter the disease course of relapsing-remitting multiple sclerosis patients. This clinical efficacy is accompanied by a more profound reduction in the number and size of lesions as measured by gadolinium enhanced MRI (Paty et al., 1993). Thus, it is possible that IFN-β may mediate its effect in MS at least in part by affecting the interaction of blood MNC with the BBB and inhibiting the migration of MNC into the CNS. However, the mechanisms by which IFN-β works in MS remain unclear. Since an important pathologic feature of MS is the transmigration of leukocytes across the BBB into the CNS, we proposed that a potential mechanism of action of IFN-β could be due to the ability of the IFN-β to inhibit adhesion of circulating lymphocytes to endothelium. Therefore, we also studied the *in vitro* effect of IFN-β on lymphocyte-endothelial adhesion.

Table 1.1. Adhesion Molecules Involved in Leukocyte-Endothelial Cell Adhesion

Adhesion receptor	Family	Main ligand	Expressed on
L-selectin (LAM-1, CD62L)	Selectins	GlyCAM-1, CD34, MadCAM-1, P-selectin	lymphocytes, monocytes, neutrophils
E-selectin (ELAM-1, CD62E)	Selectins	ESL-1, L-selectin	Activated endothelial cells
P-selectin (PADGEM, CD62P)	Selectins	PSGL-1, L-selectin, PSL	Activated platelets and endothelial cells
VLA-4 ($\alpha 4\beta 1$, CD49d/CD29)	$\beta 1$ integrin	VCAM-1	Lymphocytes, monocytes, eosinophils
LFA-1 ($\alpha L\beta 2$, CD11a/CD18)	$\beta 2$ integrin	ICAM-1, -2, -3	All leukocytes
Mac-1 ($\alpha M\beta 2$, CD11b/CD18)	$\beta 2$ integrin	ICAM-1, iC3b	Lymphocyte subsets
$\alpha 4\beta 7$ integrin (CD49d/---)	$\beta 7$ integrin	MadCAM-1, VCAM-1	Lymphocyte subsets
ICAM-1 (CD54)	Ig superfamily	LFA-1, Mac-1	Leukocytes, endothelial cells
ICAM-2 (CD102)	Ig superfamily	LFA-1	Leukocytes, endothelial cells
PECAM-1 (CD31)	Ig superfamily	PECAM-1 (homophilic)	Leukocytes, endothelial cells, platelets
VCAM-1 (CD106)	Ig superfamily	VLA-4	Activated endothelial cells.

ESL, E-selectin ligand; ICAM, intercellular adhesion molecule; LFA, leukocyte function antigen; MAdCAM, mucosal address in cell adhesion molecule; PECAM, platelet-endothelial cell adhesion molecule; PSGL, P-selectin glycoprotein ligand; PSL, P-selectin ligand, VCAM, vascular cell adhesion molecule; VLA, very late antigen.

Table 1.2. Similarities and Differences Between Avonex™, Betaseron®, and Rebif®

	Avonex™ (IFNß-1a)	**Rebif® (IFNß-1a)**	**Betaseron®(IFNß-1b)**
Company	Biogen	Serono	Berlex
Site of Production	Chinese Hamster Ovary	Chinese Hamster Ovary	*E. coli* bacteria
Amino acid sequence	Identical to natural IFN-ß	Identical to natural IFN-ß	Cystine mutation at position 17
N-terminal methionine	Yes	Yes	No
Glycosylation state	Glycosylated	Glycosylated	Not glycosylated
Specific activity (IU/mg)	200×10^6	273×10^6	32×10^6
Weekly dose in MS (µg)	30	132 (high dose)	875
Weekly dose in MS (IU)	6×10^6	36×10^6	28×10^6
Frequency of administration	Once a week	Three times a week	Alternate days
Route of administration	Intramuscularly	Subcutaneous	Subcutaneous

Table 1.3. Principal Features of the CD Molecules Referenced in This Thesis

CD Designation	Common Synonym(s)	Molecular Structure	Main Cellular Expression	Known or Proposed Function(s)
CD3	T3; Leu-4	Composed of five chains	Thymocytes, T cells	Associated with TCR, signal transdcuction as a result of antigen recognition by T cells
CD4	T4; Leu-3	55 kD	Class II MHC-restricted T cells, monocytes, macrophages	Co-receptor for MHC II, receptor for HIV-1 & HIV-2 gp-120, signal transduction
CD8	T8; Leu-2	Composed of two 34 kD chains; expressed as αα or αβ dimmer	Class I MHC-restricted T cells (cytotoxic T cells), thymocytes	Co-receptor for MHC I, signal transduction
CD11a	LFA-1 α chain	180 kD	Lymphocytes, granulocytes, monocytes, macrophages	αL subunit of integrin LFA-1 (associated with CD18), binds to CD54 (ICAM-1), CD102 (ICAM-2), CD50 (ICAM-3)
CD11b	Mac-1; CR3 α chain	170 kD	Granulocytes, monocytes, NK cells	αM subunit of CR3 (associated with CD18), binds CD54, iC3b, extracellular matri
CD11c	p150,95; CR4 α chain	150 kD	granulocytes, monocytes, NK cells	αX subunit of CR4, (associated with CD18), binds fibrinogen
CD14	Mo2	55 kD; PI-linked	Monocytes	Receptor for complex of Lipopolysaccharide

Table 1.3-(continued). Principal Features of the CD Molecules Referenced in This Thesis

CD Designation	Common Synonym(s)	Molecular Structure	Main Cellular Expression	Known or Proposed Function(s)
CD16	FcRIII	50-70 kD; PI-linked and transmembrane	NK cells, granulocytes, macrophages	Low-affinity Fcγ receptor: ADCC, activation of NK cells
CD18	β chain of LFA-1 family (β2 integrins)	95 kD; noncovalently linked to CD11a, CD11b, or CD11c	Leukocytes	
CD19	B4	90 kD	Most B cells	possible role in B cell activation
CD25	TAC	55 kD	Activated T cells, B cells, macrophages	IL-2 a receptor, T cell growth
CD26	-	120 kD	Activated T and B cell; macrophages	Serine peptidase
CD27	TNF receptor	55 kD	Most T cells, NK cells, some B cells, medullary thymocytes	Binds CD70, can function as a costimulator for T and B cells
CD28	Tp44	Homodimer of 44 kD Chains	T cells (most CD4+, some CD8+), activated B cells	T cell receptor for costimulatory molecule(s)
CD29	β chain of VLA-4 antigen (β1 integrins)	130 kD; noncovalently associated with VLA-4 α chains	Leukocytes	Adhesion to extracellular matrix proteins, cell-cell adhesion

Table 1.3-(continued). Principal Features of the CD Molecules Referenced in This Thesis

CD Designation	Common Synonym(s)	Molecular Structure	Main Cellular Expression	Known or Proposed Function(s)
CD30	Ki-1	105 kD	Activated T, B cells, NK cell, monocytes	Binds CD30L (CD153), T and B cells proliferation
CD38	T10	45 kD	Activated T cells, plasma cells, thymocytes	B cell proliferation
CD45	T200, leukocyte common antigen	180-220 kD (multiple isoform)	Leukocytes	Role in signal transduction
CD45RA	-	220 kD	B cell, T cell subsets (naïve cells), monocytes	
CD49d	VLA-4 α chain	150 kD; associates with CD29 to form VLA-4 (β1 integrin)	T cells, monocytes, B cells	Peyer's patch homing receptor, binds to VCAM-1; adhesion to fibronectin
CD54	ICAM-1	80-114 kD	Broad, many activated cells (cytokine-inducible)	Binds LFA-1 and Mac-1, receptor for rhinovirus
CD56	Leu-19	Heterodimer of 135 and 220 kD chains	NK cells	Homotypic adhesion, isoform of neural cell, adhesion molecule (N-CAM)
CD57	HNK, Leu-7	110 kD	NK cells, subset of T, B cells	
CD62L	LAM-1, LECAM-1, L-selectin	150 kD	T, B, and NK cells, monocytes	mediates rolling with endothelium

Table 1.3-(continued). Principal Features of the CD Molecules Referenced in This Thesis

CD Designation	Common Synonym(s)	Molecular Structure	Main Cellular Expression	Known or Proposed Function(s)
CD62P	PADGEM, P-selectin	140 kD	Platelets, endothelium, megakaryocytes	mediates rolling with endothelium
CD69	AIM	Homodimer of 28-34 kD chains, phosphorylated glycoprotein	Activated T and B cells, macrophages, NK cells	Early activation antigen

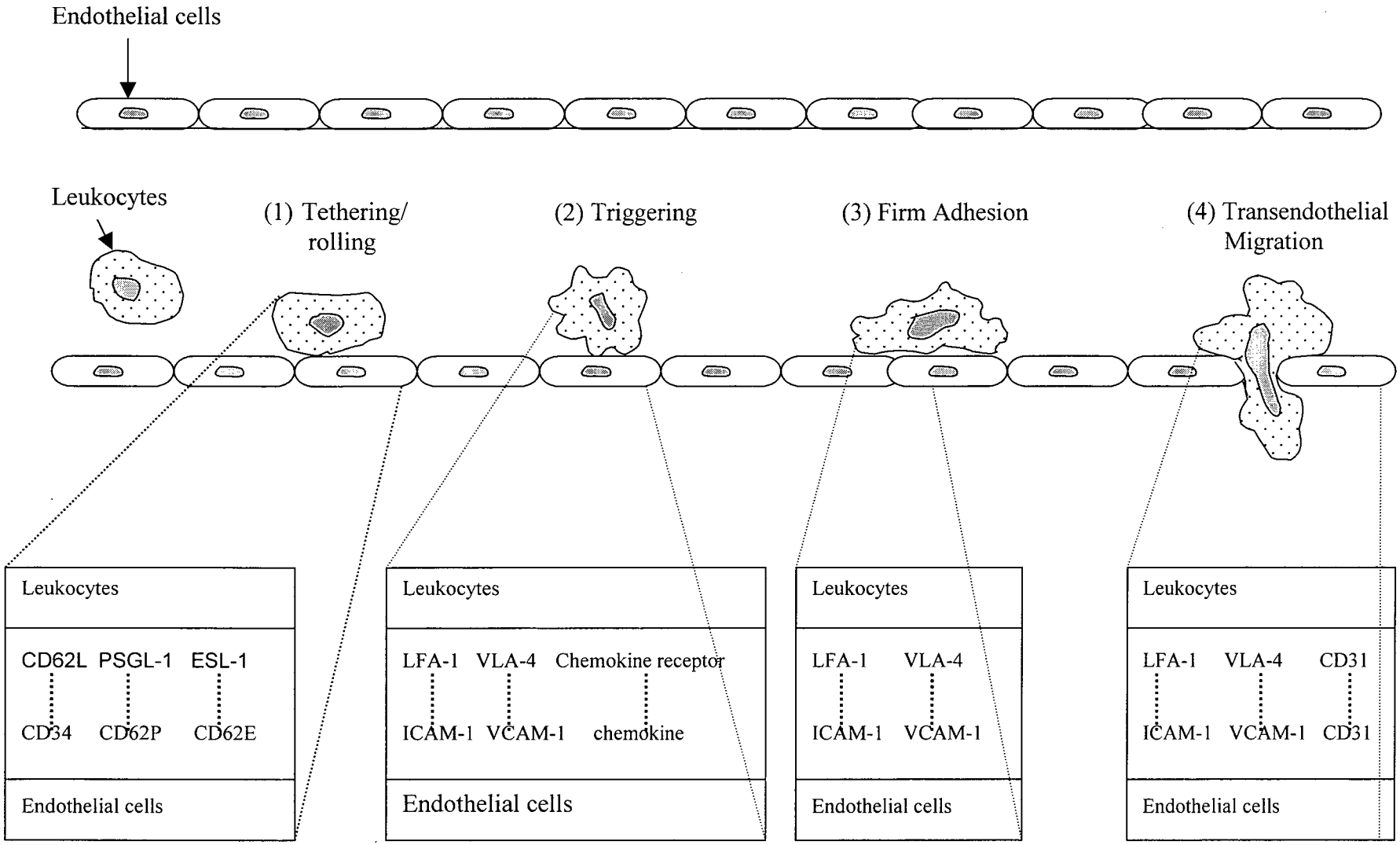

Figure 1.1. Sequential steps in simplified model of Leukocyte-endothelial cells adhesion. The cascade starts with (1) tethering of leukocytes on endothelium and is mediated mainly by selectins. Subsequently, several interaction pathways have the potential to produce a trigger signal (2), which results in strong adhesion (3) mediated by functionally upregulated leukocyte receptors. Adhering cells then migrate through the endothelium into the surrounding tissue (4).

CHAPTER TWO
MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 STUDY SUBJECTS

The human T cell lymphotropic virus type I (HTLV-I) associated myelopathy (HAM) patients and HTLV-I carriers involved in this study were seen at the Vancouver Hospital and Healthy Sciences Center, University of British Columbia Site (VH&HSC/UBC) outpatient medical clinic. The diagnosis of HAM was based on clinical criteria (Osame et al., 1987; Oger et al., 1993). This included patients with gradual and progressive spasticity that had the following characteristics: 1) antibody titers to HTLV-I in serum and CSF; 2) predominantly upper motor neuron disorder, symmetrical, sensory and bladder disturbance; and 3) presence of adult T-cell leukemia-like cells (cells with lobulated nuclei) in both peripheral blood and CSF. The presence of HTLV-I in the investigated HAM and HTLV-I carriers was previously confirmed by PCR on blood mononuclear cells (Dekaban et al., 1993). For comparing lymphocyte subsets in HAM, HTLV-I carriers and healthy controls by flow cytometry, blood samples were obtained from 7 HAM patients, 3 males and 4 females whose age ranged from 37 to 79 years (mean ± S.D., 56.4 ± 15.9 years), 9 HTLV-I carriers, 3 males and 6 females (age range 33-88, 52.2 ± 17.4 years) and 10 healthy subjects, 5 males and 5 females (age range 23-67, 41.1 ± 13.8 years). For flow cytometric analysis of lymphocytes after 2 days in culture, blood samples of 7 of the 7 HAM patients, 7 of the 9 HTLV-I carriers, 3 males and 4 females (age range 34-69, 48.5 ± 11.2 years) and 8 of the 10 healthy subjects, 4 males and 4 females (age range 25-67, 43.7 ± 13.7 years) were used. For the adhesion assays, comparing HAM and non-HAM, blood was obtained from

8 patients with HAM (4 males, and 4 females) whose ages ranged from 43 to 79 years (56.8 ± 13.8 years). Blood was also obtained from 8 non-HAM (4 HTLV-I seropositive carriers, 46.8 ± 10.2 years and 4 healthy control subjects, 40.5 ± 9.7). These were 3 males and 5 females (age range 33-57, 43.6 ± 9.8 years). Seven of the HAM patients, all the HTLV-I carriers and 4 of the healthy controls were Coastal Amerindians from British Columbia, Canada.

The multiple sclerosis (MS) patients who participated in this study were seen at the VH&HSC/UBC MS clinic. All MS patients had clinically definite MS as were diagnosed according to Poser's criteria (Poser et al., 1983). This means patients qualified if they had two attacks and clinical evidence of two separate lesions or two attacks and clinical evidence of one lesion and paraclinical evidence of another separate lesion. Furthermore, the attacks should involve different parts of the central nervous system (CNS) and be separated by a period of at least one month. An attack in MS is defined as the occurrence of one or more symptoms of neurological dysfunction that lasts more than 24 hours and which is not due to temporary factors such as fevers. None of the MS patients had other chronic diseases and they had not received anti-inflammatory or immunoregulatory drugs for at least 2 months preceding the tests. The relapsing-remitting (RR) MS patients who were free of clinical attacks during the 2 months prior to the study were classified as stable (s) RR-MS. Clinically active secondary progressive (SP) MS patients were those who had lost at least 1 point on Extended disability Status Scale (EDSS) (Kurzke, 1984) during the last 6 months preceding the assays. For the adhesion assays using human umbilical vein endothelial cells (HUVEC) as a source of endothelial cells in comparing multiple sclerosis (MS) and healthy controls, subjects

included 12 SP-MS, 4 males and 8 females (age range 32-69, 54 ± 11.3 years), 14 sRR-MS disease, 3 male and 11 female (age range 18-58, 40 ± 9.7 years), and 18 healthy controls, 11 male and 7 female (age range 27-65, 44.9 ± 12.2 years). The adhesion assays were also done using ECV-304 cell line as substrate in comparing MS and healthy controls, subjects were 12 sRR-MS, 2 male and 10 female (age range 32-56, 43.2 ± 8.8 years) and 12 healthy controls, 2 male and 10 female (age range 21-54, 38.7 ± 9.4 years).

For comparing pokeweed mitogen-induced IgG secretion, subjects were 39 sRR-MS, 9 male and 30 female (age range 22-57, 41.8 ± 9.6) and 23 healthy controls, 6 male and 17 female (age range 21-60, 38.6 ± 8.9).

Healthy controls included in this study were patients' relatives/companions and VH&HSC personnel who were free of chronic infection and inflammation. All blood samples from patients and healthy controls were obtained with written informed consent from the donors.

The characteristics of individual MS patients participating in the trial with recombinant anti-LFA-1 (Hu23F2G, ICOS CORPORATION, Bothel, WA) are stated in Table 2.1. Two-color flow cytometric analysis of lymphocyte subsets were performed at screening and 5 days post treatment. Flow cytometric analysis of lymphocyte subsets were also done at screening and at 2 and 7 days post treatment for 1 RR-MS patient participating in a separate trial with recombinant human interleukin-10 (rhuIL-10, SCH 5200, Schering-Plough). However, this trial was still blinded.

2.2 PREPARATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS

Peripheral blood was collected in heparinized (50 U/ml) Vacutainer tubes (Beckton Dickinson, Mountain View, CA). Blood mononuclear cells (MNC) were isolated by density-gradient centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) at 400g at room temperature for 30 min. Interface cells were washed twice in Ca^{+2}- and Mg^{+2}-free Hanks' balanced salt solution (Gibco, Grand Island, NY) at 300g at 4°C for 10 min. The viability of MNC was greater than 95% as measured by Trypan blue exclusion. In some experiments blood MNC were cultured as follows: After suspending at 1 X 10^6 cells/ml in medium consisting of RPMI-1640 (Gibco) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 25 mM HEPES, 100 μg/ml streptomycin, 100 U/ml penicillin (Gibco) (complete RPMI-1640), cells were cultured for 2 days at 37°C in a 5% CO_2-humidified atmosphere and 95% air in a 25-cm^2 tissue culture flask (Corning, Cambridge, MA) without mitogen.

2.3 MONOCLONAL ANTIBODIES AND TWO-COLOR FLOW CYTOMETRY

Fluorochrome-labelled monoclonal antibodies (mAbs) to the following cell surface antigens were used as outlined in Table 2:2: CD45, CD14, CD19, CD16, CD56, CD3, CD4, CD8, HLA-DR, CD25, CD57, CD26, CD27, CD69, CD49d, CD62L, CD54 (Beckton Dickinson, San Jose, CA) and CD45RA, CD29, CD38, CD28, CD30 (Coulter

Immunology, Hialeah, FL). Controls included nonspecific IgG2a and nonspecific IgG1 (Beckton Dickinson).

After washing, MNC were divided into aliquots each containing 3-5 X 10^5 cells and stained with specific mAbs at dilutions recommended by the manufacturer. After incubation for 30 min at 4°C, the cells were washed twice with phosphate-buffered saline (PBS) supplemented with 1% FCS and 0.1% NaN_3 and fixed in 500*ul* of 1% paraformaldehyde in PBS, 1% FCS, 0.1% NaN_3. Two color immunofluorescence was recorded using a FACStar® plus (Becton Dickinson, Mountain View, Ca). Lymphocytes were gated on their forward scatter (FSc) and side scatter (SSc) characteristics resulting in more than 95% of them being $CD45^{bright}CD14^{-}$. The FITC and PE gains were optimized by using FITC-nonspecific IgG2a and PE-nonspecific IgG1. Data analysis was performed using CELLQuest™ software. The operation of the FACStar® equipments and analysis of data was carried out in the Department of Medicine, Division of Neurology at Vancouver Hospital and Health Sciences Center by Abdulaziz Al-Fahim.

2.4 CULTURE OF HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

Primary and secondary cultures of human umbilical vein endothelial cells (HUVEC) were obtained from Dr. Doronini-Zis laboratory and established as described by slightly modified methods of Jaffe et. al. (Jaffe et al., 1973). In brief, umbilical cords obtained at normal deliveries were perfused with HBSS. The umbilical veins were then treated with 0.1% collagenase (Sigma Chemical Co., St. Louis, MO) in M199 medium

for 15 min at 37°C water-bath. Subsequently, the collagenase suspension was harvested and HUVEC were obtained by centrifugation. The pelleted cells were suspended and maintained in Medium 199 (StemCell Technologies Inc., Vancouver, BC) supplemented with 10% heat-inactivated horse serum (Gibco), 25 mM HEPES (Gibco), 20 μg/ml endothelial cell growth supplement (Sigma Chemical Co.), 100 μg/ml heparin (Sigma Chemical Co.), 100 μg/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B (Gibco). The endothelial nature of isolated cells was previously confirmed by Dr. Dorovini-Zis laboratory personnel according to their binding for factor VIII-related antigen and of *Ulex Europaeus Agglutinin I* (UEA-I) lectin as described (Dorovini-Zis et al., 1991). For demonstration of factor VIII related antigen, cultured monolayers were incubated with a 1:100 dilution of polyclonal antibodies to rabbit anti-factor VIII antigen (Dakopatts). To demonstrate the binding of UEA-I lectin by endothelial-specific receptors, the cultured monolayers were incubated with 1:400 dilution of UEA-I lectin (Vector, Mississauga, Ontario). After washing, they were incubated with a 1:100 dilution of rabbit antiserum to UEA-I. Subsequently the monolayers were incubated with a 1:400 dilution of HRP-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories). After further washing, cultured monolayers were incubated with amino-ethyl-carbazol, and counterstained with hematoxylin. Stained monolayers were then examined under a light microscope (Nikon, Labphot).

HUVEC were grown to confluence on fibronectin (Sigma Chemical Co., 100 μg/ml) coated 96-well flat bottom microtitre plates (Falcon, Beckton Dickinson, Franklin Lakes, NJ. Culture media were changed every 2-3 days.

To examine the binding of MNC to cytokine-treated HUVEC, monolayers were treated for 48 h with 100 U/ml of human recombinant interferon-gamma (IFN-γ) (Chemicon International Inc., Temecula, CA) or 50% filtered (0.2 μm, Gelman Sciences, Ann Arbor, MI) murine L-929 fibrosarcoma supernatant prior to adhesion assay. Confluent monolayers of HUVEC were obtained after 7-11 days of culture at 37°C in a 5% CO_2-humidified atmosphere and 95% air.

2.5 CULTURE OF ECV-304 CELL LINE

ECV-304 cells (American Type Culture Collection, Rockville, MD) were cultured in M-199 medium containing 100 μg/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B (Gibco) and 10% heat-inactivated fetal bovine serum (Gibco) (culture medium). ECV-304 were grown to confluence on 0.5% (w/v) gelatin (Sigma) coated 25mm tissue culture flasks (Corning) at 37°C in a 5% CO_2-humidified atmosphere and 95% air. When confluent monolayers of ECV-304 had formed, the cells were detached from the culture flasks by brief (1-2 min) treatment with 0.025% trypsin (Sigma) in PBS. The enzyme digestion was arrested by addition of 10 ml ECV-304 culture medium. ECV-304 was pelleted at 400g for 10 min, resuspended in culture medium to 1 X 10^5 cells/ml and 100 μl added to each well of fibronectin coated 96-well flat bottom microtitre plates (Falcon). Culture media were changed every 2-3 days and confluent monolayers were obtained after 4-6 days of culture.

2.6 ADHESION ASSAY

Cultured MNC were pelleted by centrifugation and 200 μCi of $Na_2{}^{51}CrO_4$ (ICN Biomedicals Inc, Costa Mesa, CA) were added to 5 $X10^6$ cells in a total volume of 200 μl of complete RPMI-1640 medium. After incubation for 90 min at 37 °C, cells were washed three times and resuspended at 1 X 10^6 cells/ml.

Confluent endothelial cells or ECV-304 monolayers in 96-well flat-bottom microtitre plates were washed twice with pre-warmed RPMI 1640 supplemented with 10% heat inactivated fetal calf serum medium, and 100 μl of fresh medium was added to each well. Then 1 X 10^5 ^{51}Cr-labelled MNC were added in a further 100 μl volume of medium. After a 1 h incubation at 37°C, non-adherent cells were removed by gently washing the monolayers five times with 200 μl of pre-warmed medium. Wells were examined by phase-contrast microscopy before and after washing to determine the evenness of cell settling and potential damage to the endothelial/ECV-304 cells monolayers during washing. The HUVEC or ECV-304 monolayers and remaining adherent cells were lysed by the addition of 200 μl of 0.1 M HCl. The lysate was collected and counted in a γ counter (Beckman gamma 5500). Each assay was performed using four to six replicate wells. The percentage of adhesion to the HUVEC or ECV-304 monolayer was calculated as follows:

$$\% \text{ adherence} = \frac{\text{CPM in 100 μl lysate}}{\text{CPM in 100 μl original cell suspension}} \times 100$$

2.7 MONOCLONAL ANTIBODY BLOCKING STUDIES

Antibodies directed against VLA-4 (CD49d), ICAM-1 (CD54), and L-selectin (CD62L) (all from Beckton Dickinson) and LFA-1 (CD11/CD18, ICOS, Bothel, WA), were diluted in medium to a final concentration of either 2.5 μg/ml or 0.5 μg/ml and were added to HUVEC or ECV-304 monolayers. Then, 1 X 10^5 ^{51}Cr-labelled cells were added. Each assay was performed using four to six replicate wells. Control mAb included was mouse IgG2b isotype anti-dansyl, (monoclonal antibody specific for hapten dansyl (5-[dimethyl amino naphthalene-1 sulfonyl) (PharMinogen Canada, Mississauga, ON). Percent inhibition was calculated as:

$$\% \text{ inhibition} = \left[1 - \left(\frac{\text{CPM in 100 μl lysate with inhibitor}}{\text{CPM in 100 μl lysate without inhibitor}}\right)\right] \times 100$$

2.8 POKEWEED MITOGEN-INDUCED IgG SECRETION

Pokeweed-mitogen-induced IgG secretion was carried out *in vitro* as previously described (O'Gorman et al., 1988). Briefly, mononuclear cells (MNC) isolated by Ficoll-hypaque (Pharmacia) density gradient centrifugation from peripheral blood were washed four times in Ca^{+2} and Mg^{+2} free Hanks' balanced salt solution (Gibco) and one time with the culture medium consisting of RPMI 1640 with 10% fetal calf serum, 2 mM L-glutamine and 2 mg of genitmycine per 100 ml. Afterward, the blood MNC were

suspended at 10^6 MNC in 1 ml of the culture medium with pokeweed mitogen (PWM, Gibco) at the optimal final dilution of 1:300 in 12 x 75 mm plastic capped tubes (Falcon). Control cultures, consisting of unstimulated blood MNC, were set up in parallel for each sample. The effects of different preparations of IFN-ß on PWM-induced IgG secretion was tested by adding varying doses (ranging from 0.000384 to 312 ng/ml) of human recombinant interferon ß to the culture. The three interferons currently available for use in RR-MS that were also used in this assay are Betaseron® (IFNß-1b, Berlex Laboratories), Avonex™ (IFNß-1a, Biogen Inc.), and Rebif® (IFNß-1a, Serono). After 7 days at 37°C and 5% CO_2 in air, the cultures were centrifuged at 400 X g for 10 min and the cell free supernatants were harvested for IgG content.

2.9 DETERMINATION OF IgG CONTENT BY ELISA

IgG content of the supernatant was measured by an enzyme-linked immunosorbent assay (ELISA). Microtitre plates (Maxisorb, Nunc, Rosklide, Denmark) were coated with 100 µl of goat anti-human IgG (Cappel, West Chester, PA) at 10 µg/ml diluted in 0.05 M carbonate-bicarbonate buffer (pH 9.6), incubated overnight at 4°C. All washes were done with PBS containing 0.05% (v/v) Tween-20. The plates were washed 3 times then blocked with PBS containing 0.1% bovine serum albumin (BSA) for 1 h at room temperature. Serial dilution of known concentration of human IgG (NIH, Betheda, MD) were used as standard in parallel with samples to be tested and added to the wells for 1 h at room temperature. The plates were washed as above and 1:1000 dilution of alkaline phosphatase conjugated goat anti-human IgG (Biosource, Camarillo, CA) was

added. After 1 h at room temperature, the plates were washed and then developed by adding 100 µl of the phosphatase substrate *p*-nitrophenyl phosphate (Sigma) at 1 mg/ml in diethanolamine buffer. After 30 min, the absorbance at 405 nm was read on a specrophotometer (MRX microplate reader, Dynex Technologies) and the results were expressed as ng/ml. In each experiment, a 10-point standard curve was generated from a standard human serum containing known concentration of IgG. The level of IgG in the unknown samples was then derived from the standard curve using Revelation™ software. All samples were run in triplicates.

2.10 STATISTICAL ANALYSIS

Data are reported as mean ± SEM or mean ± S.D where indicated. Statistical analysis utilized paired and unpaired Student *t*-test as indicated with $p<0.05$ accepted as statistically significant. The differences between the inhibitory effects of Avonex™, Betaseron®, and Rebif® β-interferon on PWM-induced IgG secretion were assessed using two-way analysis of variance (ANOVA). If the differences between the three β-interferon treatments were significant, Tukey multiple comparison test was used to rule out the probability of type I error (erroneously declaring something by chance). Student *t*-tests were performed using StatWorks™ software. Two-way ANOVA and Tukey analyses were done in S-Plus™ software.

Table 2.1

Individual MS Patient Characteristics and Treatment Participating in ICOS Trial

Patient No.	Age (years)	Sex	Treatment
DES #0801	34	Male	Hu23F2G (2.0 mg/kg)
MCE #0802	36	Female	Hu23F2G (1.0 mg/kg)
MOS #0803	36	Female	Placebo
TES #0804	48	Female	Methylprednisolone

Table 2.2

Monoclonal Antibody Pairs Used for Lymphocyte Subset Analysis

Phycoerythrin-conjugated	Fluorescein-conjugated
Nonspecific IgG1	Nonspecific IgG2a
Anti-CD14 (Leu M3)	Anti-CD45 (LCA)
Anti-CD19 (Leu 12)	Anti-CD3 (Leu 4)
Anti-CD4 (Leu 3a)	Anti-CD3
Anti-CD8 (Leu 2a)	Anti-CD3
Anti-CD56 (NKH-1)	Anti-CD16 ($F_{c\gamma}$RIII)
Anti-CD4	Anti-CD49d(VLA-4α)
Anti-CD8	Anti-CD49d
Anti-CD4	Anti-CD62L (LECAM-1)
Anti-CD8	Anti-CD62L
Anti-CD54 (ICAM-1)	Anti-CD3
Anti-CD4	Anti-CD45RA
Anti-CD4	Anti-CD29
Anti-CD8	Anti-CD28
Anti-CD8	Anti-CD57 (Leu 7)
Anti-CD27	Anti-CD3
Anti-CD4	Anti-HLA-DR (HLA-II)
Anti-CD8	Anti-HLA-DR
Anti-CD4	Anti-CD25 (Tac)
Anti-CD8	Anti-CD25
Anti-CD4	Anti-CD38
Anti-CD8	Anti-CD38
Anti-CD3	Anti-CD26
Anti-CD3	Anti-CD30
Anti-CD3	Anti-CD69 (AIM)

CHAPTER THREE
RESULTS

RESULTS

3.1 HTLV-I INFECTION

The human T lymphotropic virus type I (HTLV-I) has been identified as the etiological agent of the inflammatory and chronic progressive demyelinating disease called HTLV-I-associated myelopathy (HAM). The major clinical features of HAM consist of spasticity and hyper-reflexia of the lower extremities, bladder disturbance, lower extremity muscle weakness, and sensory disturbance (Osame et al., 1986). HAM appears one or more decades following infection with HTLV-I. However, the onset of HAM is substantially shorter in patients infected by transfusion of HTLV-I-contaminated blood than in patients who acquire the infection by breast-feeding or by the venereal route (Osame et al., 1990). The mean age of onset is 43 years and the male: female ratio of occurrence is 1:2.9. The lifetime risk of development of HAM among HTLV-I carriers is estimated to be less than 5% and most (~95%) individuals chronically infected with HTLV-I remain clinically asymptomatic (Kaplan et al., 1990; Maloney et al., 1998).

3.2 LYMPHOCYTE SUBSETS IN HAM, HTLV-I CARRIERS AND HEALTHY CONTROLS

Lymphocytes were analyzed at isolation and after 2 days in culture without mitogen. The mean percentage of each lymphocyte subset for the patients with HAM, the HTLV-1 carriers and controls are presented in Tables 3.1 to 3.4. Statistically

significant differences ($p<0.05$) between HAM, carriers, and controls are identified in Tables 3.1 to 3.4.

3.2.1 Double staining for CD3+, CD4+, and CD8+ T cells

The mean percentage of CD3+, CD4+ and CD8+ T cells were not significantly different between HAM patients, HTLV-1 carriers and seronegative controls (Table 3.1).

3.2.2 Double staining for putative markers of function

The mean percentage of CD4+CD29+ (memory/helper inducer) cells was higher in both HAM and carriers compared with controls ($p<0.05$) at isolation (Table 3.2). Furthermore, HAM patients had a significantly higher percentage of CD8+CD57+ (cytotoxic) cells compared with HTLV-I carriers and seronegative controls. At isolation, the percentage of CD3+CD27- (primed T cells) was significantly higher in HAM patients compared with controls. No significant difference was observed between HTLV-I carriers and controls in the percentage of CD3+CD27-.

3.2.3 Double staining for markers of activation

Table 3.3 shows markers of activation on CD3+, CD4+, and CD8+ subsets. The percentage of CD4+ cells coexpressing activation markers HLA-DR or CD25, and of CD8+ cells expressing HLA-DR were significantly higher in HAM patients and HTLV-I carriers than controls ($p<0.05$). The percentage of CD8+ cells coexpressing CD25 was low; however, after 2 days in culture without any mitogenic stimulation, a significant increase in the percentage of CD8+CD25+ population was observed in HAM patients but

not in carriers or controls. We also assessed CD4+ and CD8+ cells for the expression of the "activation" antigen CD38. There was no significant difference between HAM patients, carriers, and controls in percentage of CD4+CD38+ or CD8+CD38+ subpopulations at isolation. However, after 2 days in culture, the mean percentage of CD8+CD38+ cells increased significantly in HAM patients and carriers compared with controls ($p<0.05$). Interestingly, a significantly higher percentage of CD8+CD38+ cells were observed in patients with HAM compared with carriers. With respect to T cells coexpressing the early activation marker, CD69, we only observed significant increases in the percentage CD3+CD69+ in HAM and carriers after 2 days in culture.

3.2.4 Double staining for markers of adhesion

The percentage of CD4+ and CD8+ cells co-expressing CD49d (α-chain of VLA-4) was generally higher in patients with HAM and in HTLV-I carriers (Table 3.4) than in controls, but this difference reached significance only in carriers. There were significantly lower numbers of CD4+ and CD8+ cells co-expressing CD62L (L-Selectin) in HAM patients compared with healthy controls. There were also a significantly fewer double staining CD4+CD62L+ cells in HAM patients compared with HTLV-I carriers. Table 3.4 also reveals that, compared with controls, the percentage of mature T cells expressing adhesion molecule CD54 (ICAM-1) was higher in patients with HAM and HTLV-I carriers than in controls, but this difference reached significance only in HAM; however, after 2 days in culture this difference reached to a statistically significant level for both HAM patients and HTLV-I carriers.

3.3 BLOOD MNC-HUVEC ADHESION IN HAM

3.3.1 Adhesion of blood MNC to IFN-γ and L-929 supernatant treated HUVEC

Table 3.5a shows that treatment of HUVEC with IFN-γ (100 U/ml) or L-929 supernatant (50%) for 48 h resulted in significant increase in adhesion of MNC from two controls, two RR-MS and two HAM patients ($p<.02$). Similar results were obtained using cryopreserved MNC from two controls, two HTLV-1 carriers and two HAM patients (Table 3.5b). Thus, it is likely that MNC maintain their binding characteristics to endothelial cells after cryopreservation.

3.3.2 Adhesion of HAM and non-HAM blood MNC to L-929 supernatant treated HUVEC

Figure 3.1 summarizes the results of 8 paired assays comparing HAM and non-HAM (4 HTLV-I carriers and 4 healthy) controls blood MNC to L-929 supernatant treated HUVEC. The HAM patients' MNC adhered significantly more to activated HUVEC (mean 30%) than non-HAM cells (mean 19.1%) in 7 out of 8 assays.

3.3.3 Effects of anti-adhesion molecule antibodies on the adhesion of HAM patients blood MNC to activated HUVEC

To assess the contribution of ICAM-1, VLA-4 and L-selectin in promoting the binding of HAM derived MNC to endothelium, MNC where co-incubated with HUVEC in the continued presence of mAbs against each of theses adhesion molecules. Figure 3.2 show that the binding of MNC from HAM patients to L-929 supernatant activated

HUVEC was reduced by antibodies directed against VLA-4 (mean 31% inhibition; $p<0.001$), ICAM-1 (mean 43% inhibition; $p<0.001$), and L-selectin (mean 38% inhibition; $p<0.001$) at 2.5 μg/ml. At 0.5 μg/ml of antibodies, significant inhibition only occurred with anti-ICAM-1 (mean 16% inhibition), and anti-L-selectin (mean 9% inhibition).

3.4 BLOOD MNC-HUVEC ADHESION IN MS

3.4.1 Adhesion of healthy, sRR-MS and SP-MS blood MNC to HUVEC

Table 3.6 shows the results of adhesion comparing sRR-MS, SP-MS and healthy controls blood MNC to untreated HUVEC. The SP-MS patients' MNC adhered significantly more to HUVEC than healthy controls MNC ($p<0.02$). The adhesion of sRR-MS blood MNC were generally higher than that of healthy subjects. However, with the number of patients that we tested, this difference was not statistically significant.

3.4.2 Effects of anti-adhesion molecule antibodies on the adhesion of RR-MS and health blood MNC to HUVEC

Several specific antibodies directed against adhesion molecules were used in order to determine the contribution of adhesion molecules in the adherence of RR-MS and healthy blood MNC to HUVEC. MNC where co-incubated with HUVEC in the continued presence of mAbs against each of LFA-1, ICAM-1, VLA-4 and L-selectin or combinations of all of theses adhesion molecules. Irrelevant mAb against dansyl hapten was also used. Anti-dansyl mAb was selected because it does not react to any know

human leukocyte antigen. Results from six experiments using MNC from RR-MS are shown in Figure 3.3. At 2.5 μg/ml, the anti-LFA-1 and anti-ICAM-1 produced a mean 54% and 28% inhibition of adhesion, respectively ($p<0.01$). At 0.5 μg/ml of antibodies, inhibition with anti-LFA-1 (mean 47% inhibition) and anti-ICAM-1 (mean 16% inhibition) were still significant ($p<0.02$). Antibodies against VLA-4 and L-selectin did not significantly inhibit MS MNC binding to HUVEC. Inclusion of a combination of mAb to LFA-1, ICAM-1, VLA-4, and L-selectin in the adhesion assay significantly increased the inhibition. However, the level of inhibition was not much higher than when mAb to LFA-1 was used alone. Irrelevant mAb against dansyl hapten also did not influence the adhesion of blood MNC to HUVEC. Figure 3.4 shows results from 3 experiments measuring the inhibition of adhesion of healthy blood MNC to HUVEC. Similar results were obtained when MNC of healthy control subjects were used. These results indicate that the binding pathways of both RR-MS and control subjects MNC to resting HUVEC are similar and mainly utilize LFA-1/ICAM-1.

3.4.3 Effects of anti-adhesion molecule antibodies on the adhesion of RR-MS blood MNC to untreated and IFN-γ treated HUVEC

To compare the contribution of different adhesion molecules under both stimulatory and unstimulatory conditions in MNC-HUVEC adhesion, HUVEC was left untreated or treated with 100 U/ml of IFN-γ for 48 h and the monoclonal antibody-blocking assays were subsequently performed. The results from three separate paired experiments using RR-MS blood MNC are shown in Figure 3.5. When HUVEC were untreated, antibodies directed against LFA-1 and to a lesser degree ICAM-1 produced

significant inhibition of adhesion, whereas anti-VLA-4 or anti-L-selectin mAb had no significant effect. When HUVEC were stimulated with IFN-γ, anti-VLA-4 also significantly inhibited MNC-HUVEC interactions. These data indicate that adhesion of MNC to HUVEC mainly involve LFA-1/ ICAM-1 when HUVEC are untreated. However, when HUVEC are treated with IFN-γ, in addition to LFA-1/ICAM-1, VLA-4/VCAM-1 pathway also mediates adhesion.

3.5 BLOOD MNC-ECV-304 ADHESION IN MS

3.5.1 Adhesion of healthy and sRR-MS blood MNC to ECV-304

Table 3.7 shows the results comparing adhesion of sRR-MS and controls blood MNC to untreated ECV-304. In 12 paired assays, no significant differences were found between sRR-MS and healthy controls blood MNC in binding to ECV-304.

3.5.2 Effects of anti-adhesion molecule antibodies on the adhesion of sRR-MS and healthy blood MNC to ECV-304

Contributions of different adhesion molecules to the adherence of sRR-MS and healthy blood MNC to ECV-304 was also determined using monoclonal antibody-blocking functional assays. MNC where co-incubated with ECV-304 in the continued presence of mAbs against each of LFA-1, ICAM-1, VLA-4 and L-selectin or combinations of all monoclonal antibodies to theses adhesion molecules. Results from eight experiments using MNC from sRR-MS and eight experiments using MNC from healthy subjects are shown in Figures 3.6 and 3.7, respectively. At both 2.5 and 0.5

μg/ml, the anti-LFA-1 and anti-ICAM-1 significantly reduced adhesion of blood MNC in sRR-MS and healthy subjects to untreated ECV-304. Antibodies against VLA-4 and L-selectin did not significantly inhibit binding of sRR-MS or healthy blood MNC to ECV-304. Using a combination of mAb to LFA-1, ICAM-1, VLA-4, and L-selectin also significantly increased the inhibition; however, the level of inhibition was not much higher than when mAb to LFA-1 was used alone. These results indicate that the binding pathways of both sRR-MS and healthy control subjects MNC to uninduced ECV-304 are similar and mainly involve LFA-1/ICAM-1 pathway.

3.5.3 Adhesion of blood MNC to IFN-γ and L-929 supernatant treated HUVEC and ECV-304

In the next series of experiments, we directly compared adhesion properties of HUVEC and ECV-304 for MNC in parallel assays. Figure 3.8 shows the results from two separate experiments using blood MNC from two healthy, two sRR-MS and two HAM patients. In these experiments, HUVEC and ECV-304 were left untreated or treated with IFN-γ (100 U/ml) or L-929 (50%) supernatant for 48 h prior to the adhesion assay. The results indicate that treatment of HUVEC with either IFN-γ or L-929 supernatant significantly increases their adherence for blood MNC. However, similar treatment in ECV-304 fails to increase their adherence for MNC.

3.5.4 Effects of anti-adhesion molecule antibodies on the adhesion of RR-MS blood MNC to untreated and IFN-γ treated ECV-304

To further compare the adhesion properties of ECV-304 to those of HUVEC for MNC, we determined the contribution of different adhesion molecules in binding to untreated or IFN–γ treated ECV-304. ECV-304 monolayers were untreated or treated with 100 U/ml of IFN-γ for 48 h prior to the monoclonal antibody-blocking assays. For direct comparison, the MNC used in these experiments (Figure 3.9) are the same as those used in experiments of figure 3.5. Furthermore, the assays were also performed in parallel to that of the experiments of Figure 3.5. When ECV-304 monolayers were untreated, antibodies directed against LFA-1 and to a lesser degree ICAM-1 produced significant inhibition, whereas anti-VLA-4 or anti-L-selectin mAb had no significant effect on MNC-ECV-304 interaction (Figure 3.9). This is similar to what was seen when HUVEC was used as substrate in binding MNC (figure 3.5). However, unlike the results obtained using HUVEC (Figure 3.5), when ECV-304 monolayers were activated with IFN-γ, anti-VLA-4 did not inhibit MNC adhesion (figure 3.9). These data indicate that adhesion of MNC to ECV-304 involve LFA-1/ ICAM-1 whether or not ECV-304 are stimulated with IFN-γ.

3.6 *IN VITRO* EFFECTS OF IFN-β ON PWM-INDUCED IgG SECRETION AND MNC-HUVEC ADHESION

3.6.1 In vitro IgG secretion in healthy and sRR-MS

Table 3.8 shows that IgG concentration in unstimulated cultures was comparable in 39 stable relapse and remitting multiple sclerosis (sRR-MS) and in 24 healthy control

subjects (95 ± 52 ng/ml vs 116 ± 80 ng/ml, respectively). It increased significantly after pokeweed (PWM) stimulation in both sRR-MS and healthy controls. However, after PWM stimulation, the amount of IgG concentration was significantly higher in sRR-MS (2173 ± 1432 ng/ml) compared with healthy controls (1159 ± 913 ng/ml, $p<0.02$). Based on their response to PWM stimulation, sRR-MS and healthy subjects were divided to two distinct populations: one population of "low responder" subjects (producing <900 ng/ml IgG) and one population of "high responder" subjects (producing ≥900 ng/ml IgG). Table 3.8 further shows that the percentage of high responders was larger in sRR-MS patients (66%) than in healthy controls (38%).

3.6.2 In vitro effects of IFN-β on PWM-induced IgG secretion

The *in vitro* effects of different preparations of IFN-β were studied on blood MNC of six sRR-MS and five healthy subjects whom were categorized as "high responders" in response to PWM stimulation (producing >900 ng/ml IgG). In the absence of IFN-β, the concentration of IgG was similar for sRR-MS (2519 ± 629 ng/ml) and healthy (2476 ± 936 ng/ml). Figure 3.10a, and 3.10b show that Avonex™, Betaseron® and Rebif® used at concentration ranging from 30 ng/ml to 0.194 pg/ml all inhibited PWM-induced IgG secretion in a dose-related manner in both healthy (3.10a, 69% to 20% inhibition) and sRR-MS (3.10b 76% to 21% inhibition) subjects. Comparing Figure 3.10a and b also indicates that IFN-β at different dilutions, tended to suppress PWM-induced IgG secretion more in sRR-MS than in healthy subjects. However, this difference did not reach a statistically significant level. The comparative inhibition of PWM-induced

IgG secretion in sRR-MS and healthy controls by Avonex™, Rebif® and Betaseron® are shown in Figures 3.10c, 3.10d, and 3.10e.

3.6.3 Comparing the in vitro effects of Avonex™, Betaseron® and Rebif® on PWM-induced IgG secretion

Although all three preparations of IFN-β are available for clinical use in RR-MS, few data have been reported to directly compare their immunomodulatory effects. Therefore, we expanded this study to include an additional 16 "high responding" sRR-MS patients and compared the *in vitro* effects of Avonex™, Betaseron® and Rebif® in parallel for their ability to inhibit PWM-induced IgG secretion. This high responding group of 16 sRR-MS patients produced 91 ± 56 ng/ml of IgG (mean ± SEM) spontaneously. However, when MNC were stimulated with PWM and in the absence of IFN-β, they produced 3070 ± 1507 ng/ml of IgG (mean ± sem). Figure 3.11 shows inhibition of PWM-induced IgG secretion using the same dose (ng/ml) of different IFN-β preparation. At concentration of 312 ng/ml, no significant differences were found between Avonex™, Betaseron® and Rebif® in inhibiting PWM-induced IgG secretion, perhaps reaching a saturation point. However, at 31.2 ng/ml and 3.12 ng/ml, Avonex™ inhibited IgG secretion significantly more than Betaseron® ($p<0.05$). Moreover at 31.2 and 3.12 ng/ml, Rebif® also inhibited IgG secretion significantly more than Betaseron® ($p<0.05$).

When we took into consideration the amount of IFN-ß used *in vitro* as a approximate proportion of the daily recommended dose of each of these IFN-ßs (this is 300 μg, 44 μg, and 30 μg for Betaseron®, Rebif® and Avonex, respectively), Avonex™

still had generally higher *in vitro* inhibitory effects on IgG synthesis compared with Betaseron® and Rebif® (Figure 3.12). However, these differences did not reach a statistically significant level. Furthermore, when we calculated the amount of IFN-ß used *in vitro* as a fraction of weekly administration using their antiviral activity in Units (Log) Betaseron®, Avonex™ and Rebif® had equivalent activity with respect to their influence on PWM-induced IgG secretion (Figure 3.13).

3.6.4 Effects of IFN-β on MNC-HUVEC adhesion

To determine the role of IFN-β in MNC-HUVEC adhesion, HUVEC and/or MNC were cultured in the absence or continued presence of IFN-β-1b (Betaseron®, 1000 U/ml) for 48 h. The IFN-β-1b was then washed and adhesion assays were carried out as in Materials and Methods. Table 3.9 shows that treatment of MNC with 1000 U/ml of IFN-β-1b for 48 h significantly reduced MNC adhesion to HUVEC in both RR-MS and healthy controls ($p<0.001$). In contrast, treatment of HUVEC with the same dose of IFN-β-1b had no significant effect on MNC adhesion. The combined treatment of HUVEC and MNC of RR-MS patients with IFN-β-1b did not result in further reduction of adhesion beyond what was seen with MNC treatment alone. On the contrary, it resulted in slight augmentation of this adhesion.

Table 3.1

T Cell Subsets of Fresh and Cultured Peripheral Blood Lymphocytes From Controls, HAM, and Carriers

	Fresh			48 h cultured		
Subset	Controls (n=10)	HAM (n=7)	Carriers (n=9)	Controls (n=8)	HAM (n=7)	Carriers (n=7)
CD3+	79.7 ± 2.5	70.0 ± 7.6	73.1 ± 2.7	82.5 ± 2.1	68.0± 8.0	76.1 ± 2.3
CD3+CD4+	48.2 ± 2.9	44.8 ± 7.8	47.4 ± 2.2	49.9 ± 2.1	44.9 ± 7.3	49.4 ± 1.9
CD3+CD8+	25.8 ± 2.0	23.2 ± 6.7	22.3 ± 2.4	27.5 ± 1.9	23.7 ± 6.6	26.5 ± 2.5

Note. Data represent the mean percentage of lymphocytes expressing the indicated marker ± 1 SEM. Subsets are given as percentage of the total number of lymphocyte ($CD45^{bright}$CD14-). Number of subjects studied are given in parentheses.

Table 3.2

Percentages of CD3+, CD4+, and CD8+ Cells Bearing Putative Functional Markers in Fresh and Cultured Peripheral Blood From Controls, HAM, and Carriers

	Fresh			48 h cultured		
Subset	Controls (*n*=10)	HAM (*n*=7)	Carriers (*n*=9)	Controls (*n*=8)	HAM (*n*=7)	Carriers (*n*=7)
CD4+CD45RA+	42.8 ± 5.2	28.5 ± 6.0	35.0 ± 5.1	35.8 ± 1.8	31.9 ± 6.4	30.5 ± 4.5
CD4+CD29+	47.6 ± 3.3	**64.4 ± 3.6***	**62.7 ± 4.4***	51.8 ± 3.8	55.0 ± 9.8	60.7 ± 6.4
CD8+CD28+	10.7 ± 4.1	10.2 ± 2.6	8.1 ± 2.6	4.3 ± 1.8	7.1 ± 2.0	5.4 ± 1.6
CD8+CD57+	27.8 ± 4.0	**52.0 ± 6.8*, ‡**	37.4 ± 3.2	29.4 ± 4.9	**54.8 ± 6.6*, ‡**	36.7 ± 4.6
CD3+CD27-	18.8 ± 3.1	**32.2 ± 6.0***	23.9 ± 3.8	26.8 ± 3.4	37.7 ± 6.2	31.6 ± 4.1

Note. Data represent the percentage of group mean ± 1 SEM. The proportion of cells expressing a given second marker was calculated by using the equation: [% dual positive/(% dual positive + single positive only)] x 100. Number of subjects studied are given in parentheses.

*$p<0.05$ *versus* controls.
‡ $p<0.05$ *versus* carriers.

Table 3.3

Percentages of CD3+, CD4+, and CD8+ Cells Expressing Activation-Related Antigens in Fresh and Cultured Peripheral Blood From Controls, HAM, and Carriers

	Fresh			48 h cultured		
Subset	Controls (n=10)	HAM (n=7)	Carriers (n=9)	Controls (n=8)	HAM (n=7)	Carrier (n=7)
CD4+HLA-DR+	4.7 ± 0.8	**24.4 ± 4.4***	**18.2 ± 3.9***	7.5 ± 1.6	**27.4 ± 5.9***	17.5 ± 5.6
CD8+HLA-DR+	7.5 ± 2.1	**30.7 ± 3.9***	**22.4 ± 2.5***	11.2 ± 3.2	**53.6 ± 8.0*, ‡**	**27.0 ± 3.5***
CD4+CD25+	2.1 ± 0.5	**12.0 ± 3.8***	**9.7 ± 3.7***	4.7 ± 0.5	**17.7 ± 4.1***	12.8 ± 5.0
CD8+CD25+	0.4 ± 0.3	1.9 ± 0.7	0.6 ± 0.3	0.3 ± 0.1	**6.9 ± 2.4*, ‡**	1.0 ± 0.3
CD4+CD38+	11.8 ± 2.6	7.3 ± 0.8	11.8 ± 2.6	7.1 ± 1.1	10.2 ± 1.5	6.9 ± 1.4
CD8+CD38+	10.7 ± 1.6	16.8 ± 4.2	17.1 ± 3.8	8.6 ± 1.1	**41.5 ± 7.5*, ‡**	**20.4 ± 3.8***
CD3+CD26+	49.2 ± 3.3	37.8 ± 6.7	44.2 ± 5.6	38.4 ± 3.6	40.1 ± 6.3	39.8 ± 5.1
CD3+CD30+	0.2 ± 0.1	1.0 ± 0.2	1.0 ± 0.5	0.1 ± 0.1	1.3 ± 0.5	1.3 ± 1.0
CD3+CD69+	1.0 ± 0.2	2.7 ± 1.3	1.6 ± 0.5	1.0 ± 0.2	**8.8 ± 2.4***	**8.2 ± 3.7***

Note. The proportion of cells expressing a given second marker was calculated by using the equation: [% dual positive/(% dual positive + single positive only)] x 100. Number of subjects studied are given in parentheses.

*$p<0.05$ *versus* controls
‡ $p<0.05$ *versus* carriers

Table 3.4

Surface Phenotypes of Fresh and Cultured Lymphocytes From Controls, HAM, and Carriers

	Fresh			48 h cultured		
Subset	Controls (n=10)	HAM (n=7)	Carriers (n=9)	Controls (n=8)	HAM (n=7)	Carriers (n=7)
CD4+CD49d+	23.9 ± 3.5	30.2 ± 2.2	**38.2 ± 3.6***	28.5 ± 4.2	29.7 ± 3.3	36.8 ± 4.2
CD8+CD49d+	44.9 ± 3.9	52.9 ± 4.7	**58.8 ± 4.4***	41.5 ± 5.1	39.6 ± 5.7	42.3 ± 3.1
CD4+CD62L+	68.9 ± 4.7	**49.0 ± 6.8*, ‡**	65.2 ± 3.9	81.3 ± 1.7	**64.1 ± 6.7***	**74.0 ± 3.1***
CD8+CD62L+	49.8 ± 5.6	**24.7 ± 7.5***	35.8 ± 3.0	48.1 ± 4.0	**26.2 ± 7.3***	37.4 ± 3.6
CD3+CD54+	3.8 ± 0.8	**8.3 ± 1.9***	11.7 ± 4.7	4.5 ± 1.2	**24.0 ± 5.1***	**16.4 ± 3.5***

Note. The proportion of cells expressing a given second marker was calculated by using the equation: [% dual positive/(% dual positive + single positive only)] x 100. Number of subjects studied are given in parentheses.

*$p<0.05$ versus controls.
‡$p<0.05$ versus carriers

Table 3.5a

Adhesion of Blood MNC to Untreated and IFN-γ or L-929 Treated HUVEC

	% Adhesion		
Subject	No treatment mean ± SEM	IFN–γ (100 U/ml) mean ± SEM	L-929 (50%) mean ± SEM
Healthy	12.7 ± 2.3	15.6 ± 2.5*	16.1 ± 2.1*
MS	12.9 ± 0.7	14.7 ± 0.9*	15.2 ± 1.1*
HAM	15.3 ± 1.7	18.0 ± 1.7*	18.7 ± 1.4*

Table 3.5b

	% Adhesion		
Subject	No treatment mean ± SEM	IFN-γ (100 U/ml) mean ± SEM	L-929 (50%) mean ± SEM
Healthy	11.7 ± 2.2	14.4 ± 2.2*	15.5 ± 1.8*
Carrier	13.0 ± 0.5	15.2 ± 1.3*	16.3 ± 1.2*
HAM	21.7 ± 2.2	28.0 ± 2.8*	25.9 ± 2.6*

Note. In experiments of Table 5a, MNC were from two healthy controls, two multiple sclerosis (MS) and two HAM patients. In experiment of Table 5b, MNC were from two healthy controls, two HTLV-1 carriers and two HAM patients that were previously cyopreserved at –70 °C. In each experiment, MNC were added to HUVEC monolayers that were untreated or pretreated with IFN–γ (100 U/ml) or conditioned medium containing L-929 (50%) supernatant for 48 h. Each number represents the mean ± SEM percentage of quadruplicates in two separate experiments.

*$p<0.02$ compared with untreated monolayers.

Table 3.6

Adhesion of sRR-MS, SP-MS, and Healthy Subjects Blood MNC to HUVEC

% Adhesion (mean ± SEM)		
Healthy (n=18)	sRR-MS (n=14)	SP-MS (n=12)
12.4 ± 3.4	14.5 ± 3.0	16.1 ± 2.9 *

Blood MNCs from healthy, stable relapsing-remitting multiple sclerosis (sRR-MS), and secondary progressive multiple sclerosis (SP-MS) were incubated with untreated HUVEC for 1 hr. In these experiments the binding of MNC to HUVEC was expressed as the percentage of total binding. The MNC from SP-MS adhered significantly more to HUVEC than MNC from healthy subjects. Each assay was performed in four replicates. The numbers of subjects studied in each category are indicated in the parentheses.

$p<.02$ *versus* healthy controls

Table 3.7

Comparative Adhesion of Healthy and RR-MS Blood MNC to ECV-304

	% Adhesion (mean ± SEM)
Healthy (n=12)	17.1 ± 5.9
sRR-MS (n=12)	19.6 ± 5.1

Blood MNCs from stable relapsing-remitting (RR-MS) and healthy subjects were incubated with untreated ECV-304. Each assay consisted of adding MNC from one sRR-MS and one healthy subject to ECV-304 monolayers. The number indicates the mean ± SEM from 12 paired assays. Each assay was performed using four replicate wells. No significance differences were seen between sRR-MS and healthy subjects in binding to ECV-304.

Table 3.8

In Vitro IgG Secretion in sRR-MS and Healthy Subjects

	sRR-MS (n=39)	Healthy (n=24)
PWM - (ng/ml IgG)	95 ± 52	116 ± 80
PWM + (ng/ml IgG)	2173 ± 1432*	1159 ± 913
High responders (>900 ng/ml)	66%	38%

The number of subjects for each category is indicated in parentheses. In these experiments blood MNCs were incubated without (PWM-) or with pokeweed mitogen (PWM+) for 7 days at 37°C. The IgG content of supernatant was measured by an ELISA and expressed as mean ± SEM in ng/ml. The results indicate that stable relapsing and remitting MS (sRR-MS) patients produce significantly ($p<0.02$) higher amount of IgG in response to PWM compared with healthy controls. Furthermore, the percentage of individuals producing greater than 900 ng/ml of IgG in response to PWM ("high responders") are higher in sRR-MS than in healthy controls.

*$p<0.02$ *versus* healthy controls

Table 3.9

Effects of IFN-β-1b on MNC-HUVEC Adhesion

		IFN-β-1b treatment of:		
	Untreated	MNC	HUVEC	MNC& HUVEC
RR-MS (n=8)	13.2 ± 1.3	8.4 ± 1.3*	14.6 ± 2.9	9.5 ± 1.9*
Healthy (n=7)	12.6 ± 1.8	7.8 ± 0.9*	nd	nd

Blood MNC and/or HUVEC were pretreated with 1000 U/ml of IFN-β-1b (Betaseron®) for 48 hr prior to doing the adhesion assay. The results indicate that pretreatment of MNC with IFN-β-1b results in a significant reduction of their bindings to HUVEC in both healthy and RR-MS subjects. However, treatment of HUVEC with IFN-β does not influence MNC-HUVEC adhesion. Each assay was performed in four replicates and the number of subjects studied is indicated in parentheses. Results are expressed as mean ± SEM.

*$p<0.03$ *versus* untreated HUVEC monolayers

Adhesion of HAM and Non-HAM Blood MNC to Activated HUVEC

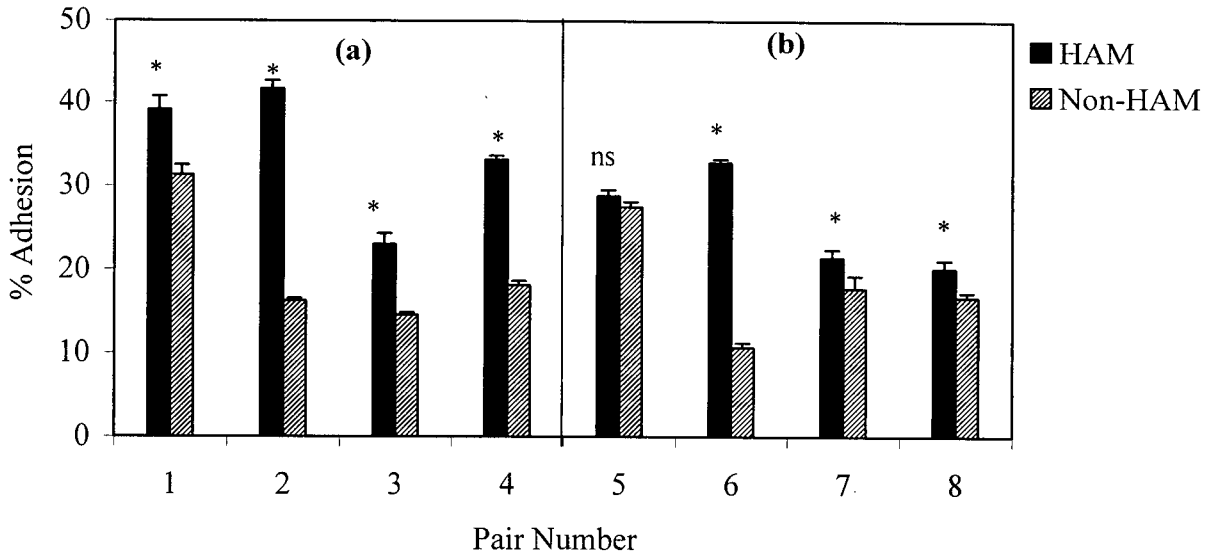

Figure 3.1. MNC from eight HAM patients and eight non-HAM controls (four HTLV-I carriers and four healthy) were incubated with L-929 supernatant activated HUVEC. Each assay consisted of adding MNC from one HAM and one non-HAM subject to HUVEC derived from the same umbilical cord vein. Each assay was performed using six replicate wells. The adhesion of HAM patients' MNC to HUVEC was significantly greater than that observed with non-HAM controls in seven out of eight paired assays. In panel (a) controls were HTLV-I carriers, while in panel (b) controls included were healthy subjects. Vertical bars represent SEM.

*$p<0.01$ compared with non-HAM.

Effect of Anti-adhesion Molecule Antibodies on the Adhesion of MNC of HAM Patients to Activated HUVEC

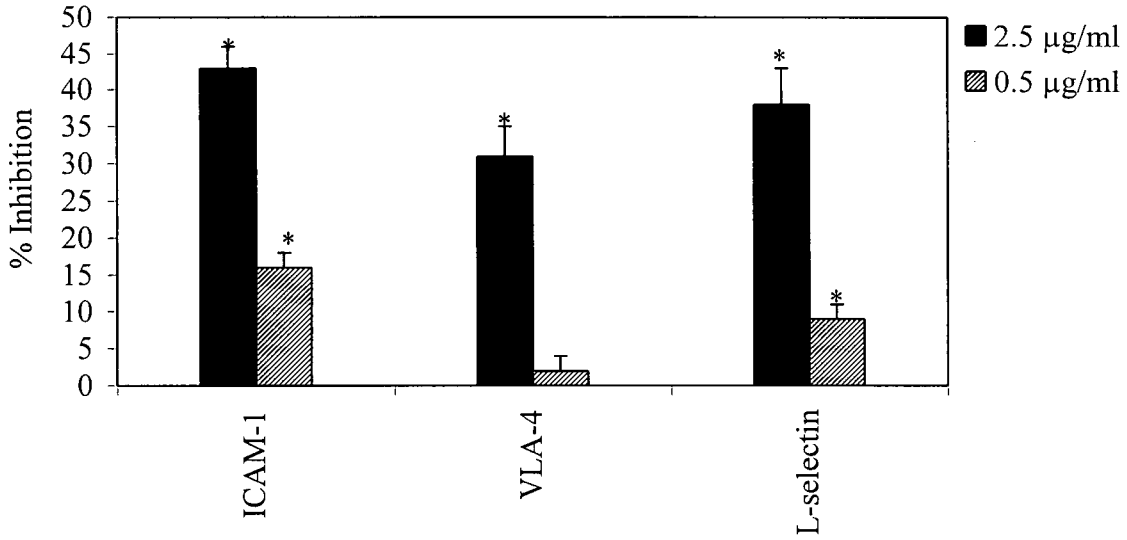

Figure 3.2. MNC from four different HAM patients were coincubated with anti-ICAM-1 (CD54), anti-VLA-4 (CD49d), and anti-L-selectin (CD62L) antibodies at concentration of 2.5, 0.5 µg/ml or medium alone, during the assay. In these experiments, HUVEC monolayers were previously treated with conditioned medium containing L-929 (50%) supernatant. Each assay was performed using six replicate wells. Results are representative of four separate experiments and are expressed as mean ± SEM percentage inhibition of adhesion.

*$p < 0.05$ compared with MNC incubated with medium.

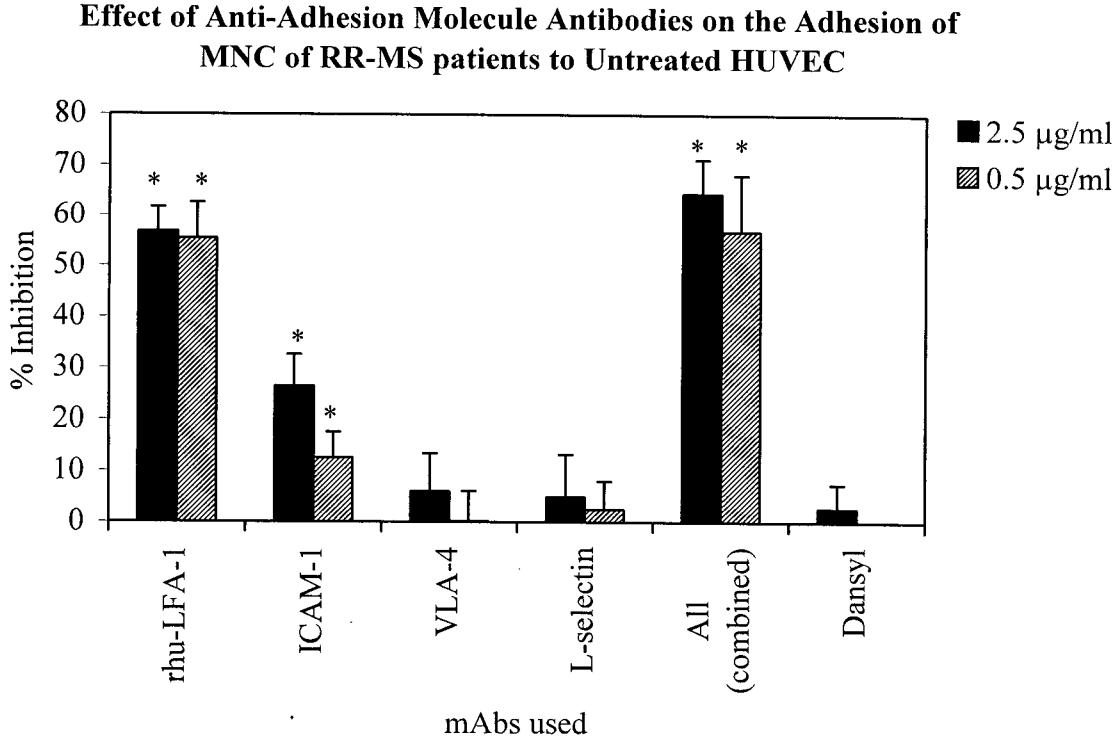

Figure 3.3. MNC from six different RR-MS patients were co-incubated with recombinant and humanized (rhu) anti-LFA-1 (CD11/CD18), anti-ICAM-1 (CD54), anti-VLA-4 (CD49d), anti-L-selectin (CD62L), or combination of all anti-adhesion antibodies during the assay. Control included was anti-dansyl mAb. Each assay was performed using four replicate wells. Results are from six separate experiments and are expressed as mean ± SEM percentage inhibition of adhesion.

*$p<0.03$ compared with MNC incubated with medium.

Effect of Anti-Adhesion Molecule Antibodies on the Adhesion of MNC of Healthy Subjects to Untreated HUVEC

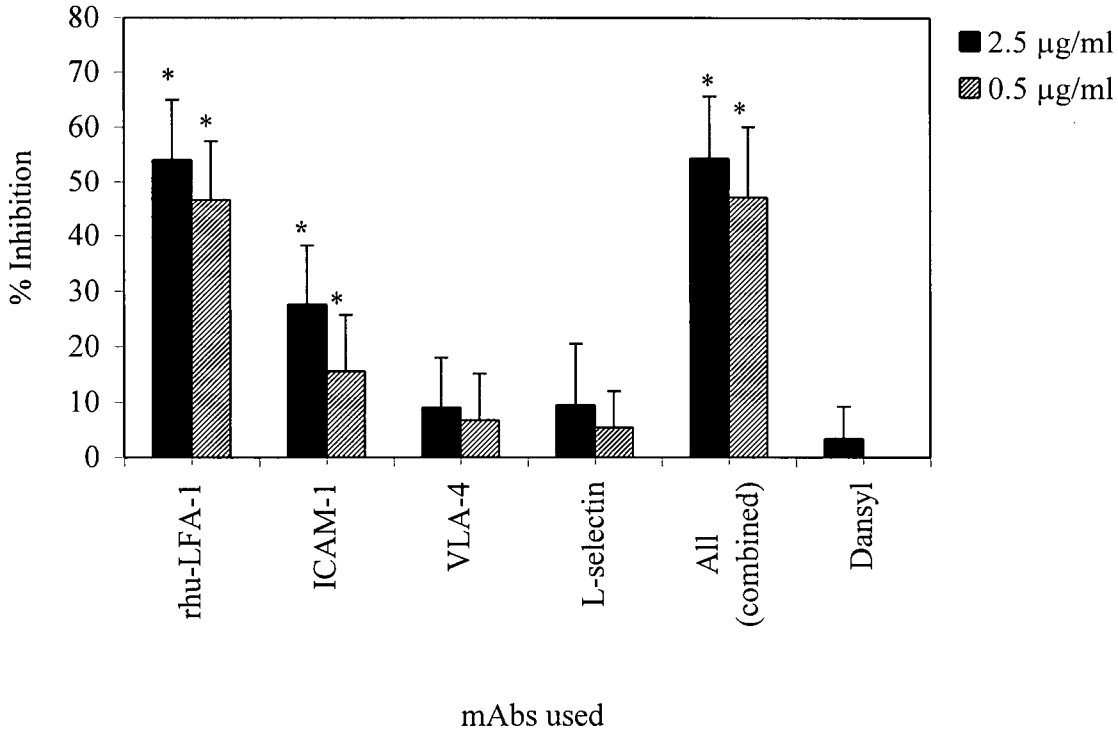

Figure 3.4. MNC from three different healthy subjects were co-incubated with recombinant and humanized (rhu) anti-LFA-1 (CD11/CD18), anti-ICAM-1 (CD54), anti-VLA-4 (CD49d), anti-L-selectin (CD62L) or combination of all antibodies during the assay. Control included was anti-dansyl mAb. Each assay was performed using four replicate wells. Results are from three separate experiments and are expressed as mean ± SEM percentage inhibition of adhesion.

*$p<0.02$ compared with MNC incubated with medium

Effects of Anti-Adhesion Molecules antibodies on the Adhesion of MNC of RR-MS Patients to Untreated and IFN-γ Treated HUVEC

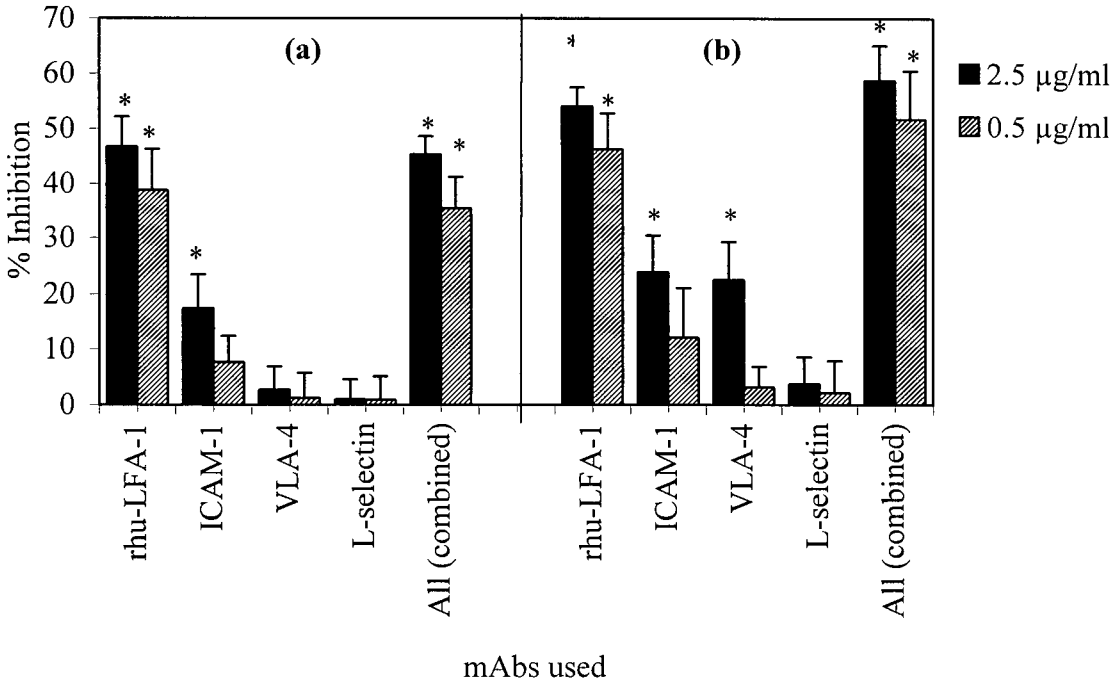

Figure 3.5. MNC from three different RR-MS patients were coincubated with recombinant and humanized (rhu) anti-LFA-1 (CD11a/CD18), anti-ICAM-1 (CD54), VLA-4 (CD49d), anti-L-selectin (CD62L) or combination of all antibodies during the assay. HUVEC monolayers were untreated (a) or treated with 100 U/ml of IFN-γ for 48 h prior to doing the assay (b). Each assay was performed using four replicate wells. Results are from three separate experiments and are expressed as mean ± SEM percentage inhibition of adhesion.

*$p < 0.001$ compared with MNC incubated with medium.

Effect of Anti-adhesion Molecule Antibodies on the Adhesion of MNC of RR-MS Patients to untreated ECV-304

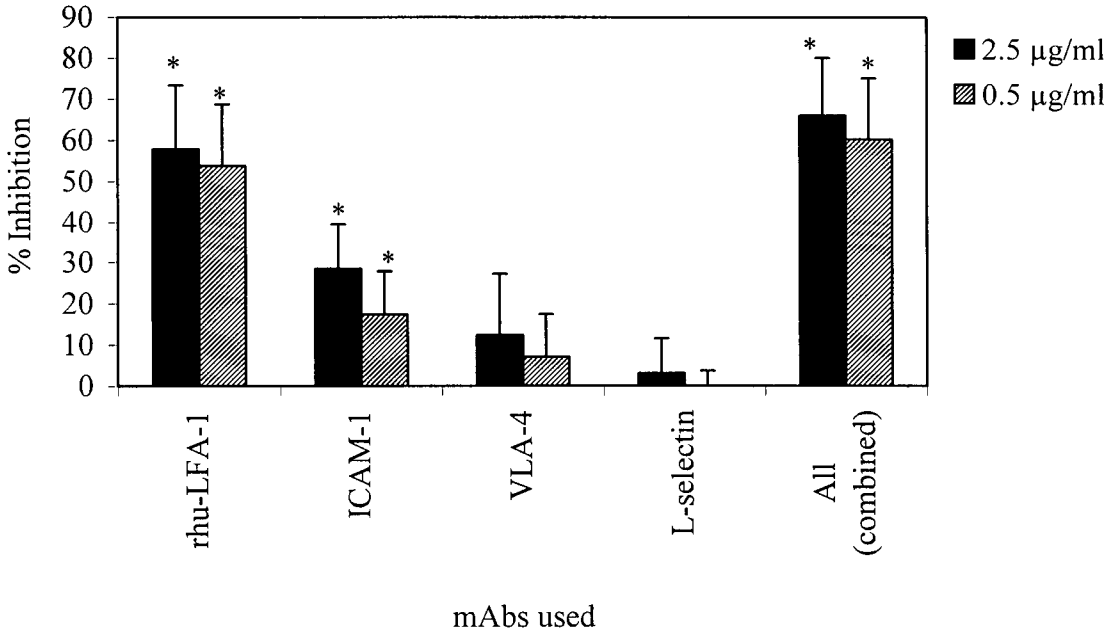

Figure 3.6 MNC from eight different RR-MS patients were coincubated with recombinant and humanized (rhu) anti-LFA-1 (CD11/CD18), anti-VLA-4 (CD49d), anti-ICAM-1 (CD54), anti-L-selectin (CD62L) or combination of all antibodies during the assay. Each assay was performed using four replicate wells. Results are from eight separate experiments and are expressed as mean ± SEM percentage inhibition of adhesion.

*$p < 0.01$ compared with MNC incubated with medium.

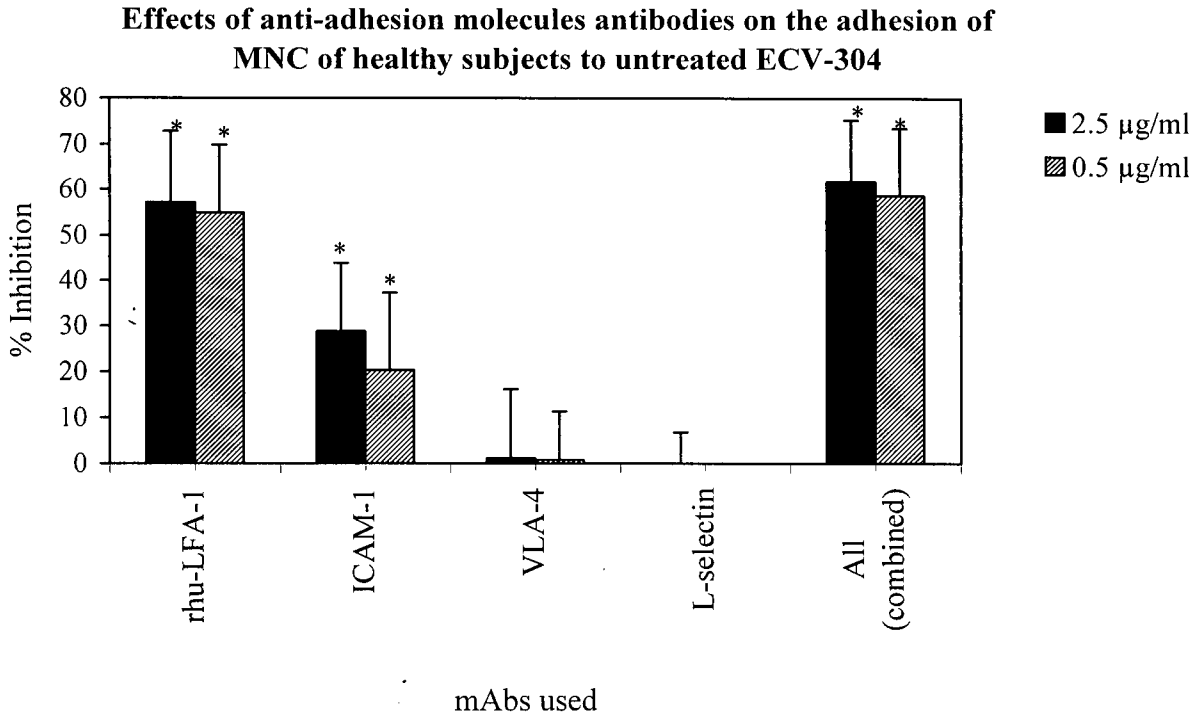

Figure 3.7 MNC from eight different healthy subjects were coincubated with recombinant and humanized (rhu) anti-LFA-1 (CD11/CD18), anti-VLA-4 (CD49d), anti-ICAM-1 (CD54), anti-L-selectin (CD62L) or combination of all antibodies during the assay. Each assay was performed using four replicate wells. Results are from eight separate experiments and are expressed as mean ± SEM percentage inhibition of adhesion.

*$p < 0.01$ compared with MNC incubated with medium.

Adhesion of Blood MNC to Untreated or IFN-γ and L-929 Supernatant Treated HUVEC and ECV-304

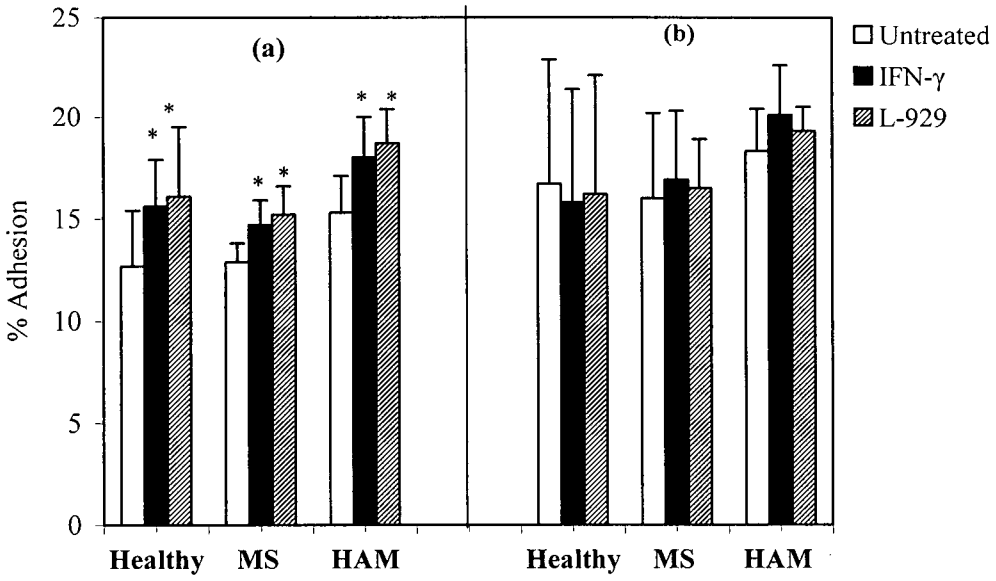

Figure 3.8. In these experiments, MNC were from two healthy controls, two multiple sclerosis (MS) and two HAM patients. In each experiment, MNC were added to HUVEC (a) or ECV-304 (b) monolayers that were untreated or pretreated with IFN-γ (100 U/ml) or conditioned medium containing L-929 (50%) supernatant for 48 h. Each column represents the mean ± SD percentage of quadruplicates of two separate experiments.

*$p<0.01$ compared with untreated HUVEC monolayers.

Effects of Anti-adhesion Molecules Antibodies on the Adhesion of MNC of RR-MS Patients to Untreated and IFN-γ Treated ECV-304

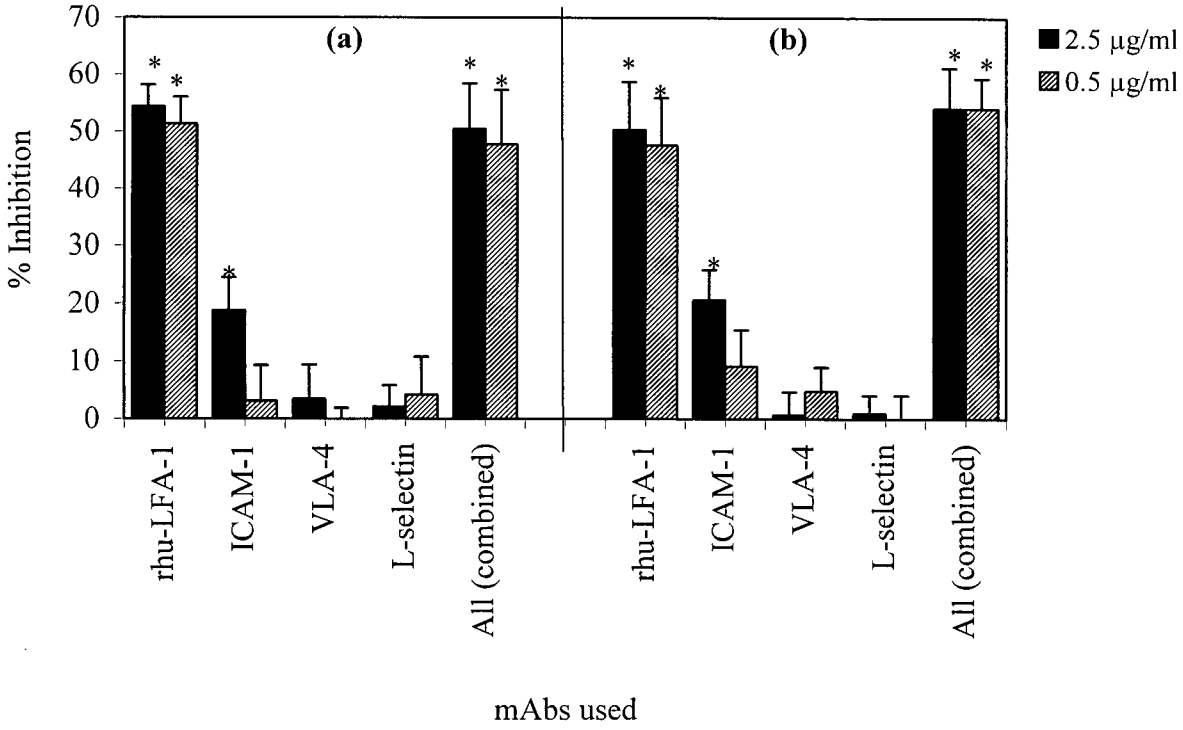

Figure 3.9. MNC from three different RR-MS patients were coincubated with anti-LFA-1 (CD11/CD18), anti-VLA-4 (CD49d), anti-ICAM-1 (CD54), anti-L-selectin (CD62L) or combination of all antibodies during the assay. ECV-304 monolayers were untreated (a) or treated with 100 U/ml of IFN-γ for 48-h prior to the assays (b). Each assay was performed using four replicate wells. Results are from three separate experiments and are expressed as mean ± SEM percentage inhibition of adhesion.

*$p < 0.005$ compared with MNC incubated with medium.

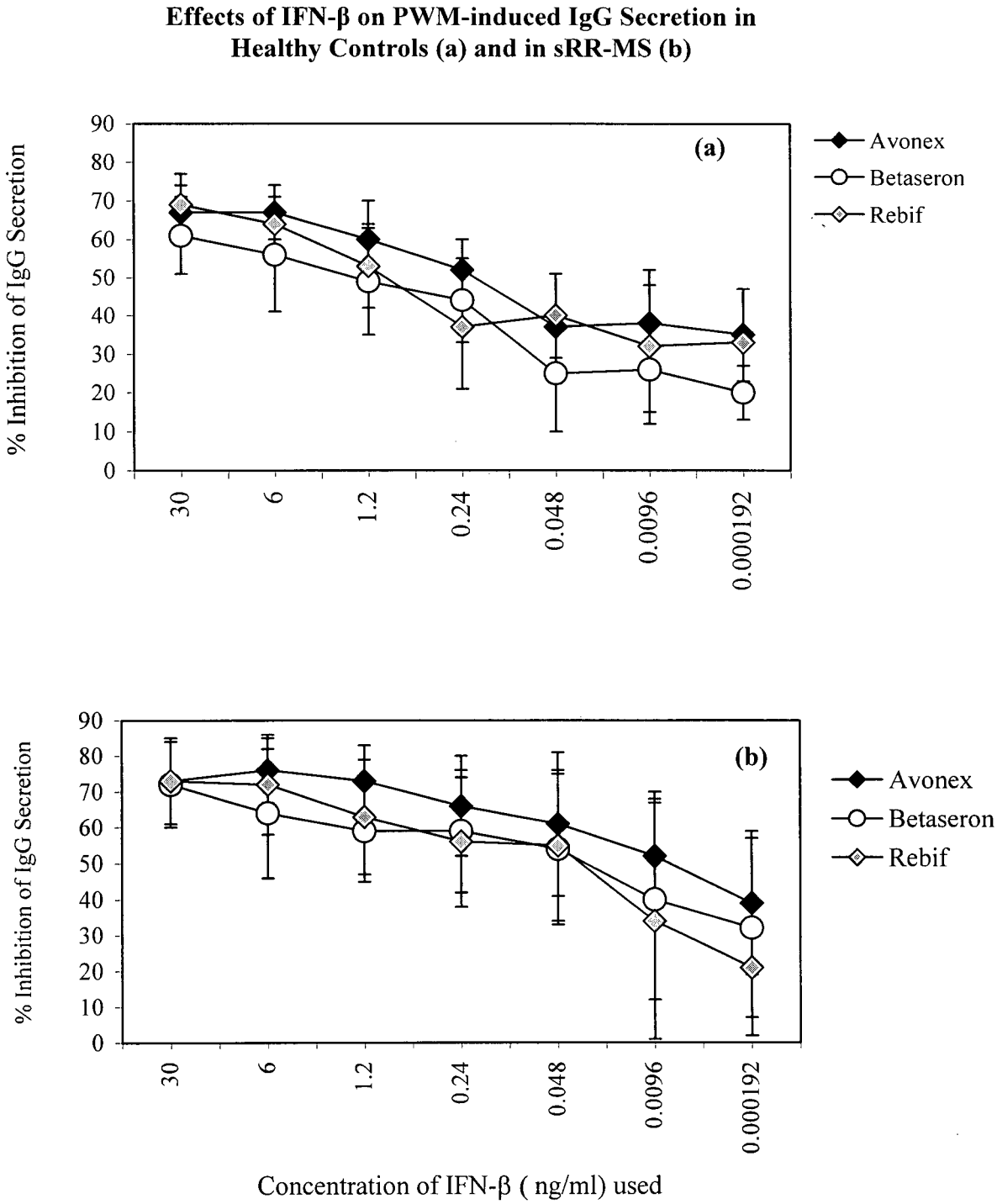

Figure 3.10a and b. Suppression of PWM-induced IgG secretion by IFN-β. Blood MNC were stimulated with PWM and co-incubated without and with indicated concentrations (ng/ml) of IFN-β from Avonex™, Betaseron® and Rebif®. After 7 days at 37°C, the IgG content of the supernatants were measured by an ELISA. These figures show a dose-dependent inhibition (mean ± SEM) of PWM-induced IgG secretion by different preparations of IFN-β in 5 high responding healthy individuals (a) and 6 high responding sRR-MS patients (b).

Inhibition of PWM-induced IgG Secretion by Avonex™ (c), Betaseron® (d), and Rebif® (e) in Multiple sclerosis and Healthy controls

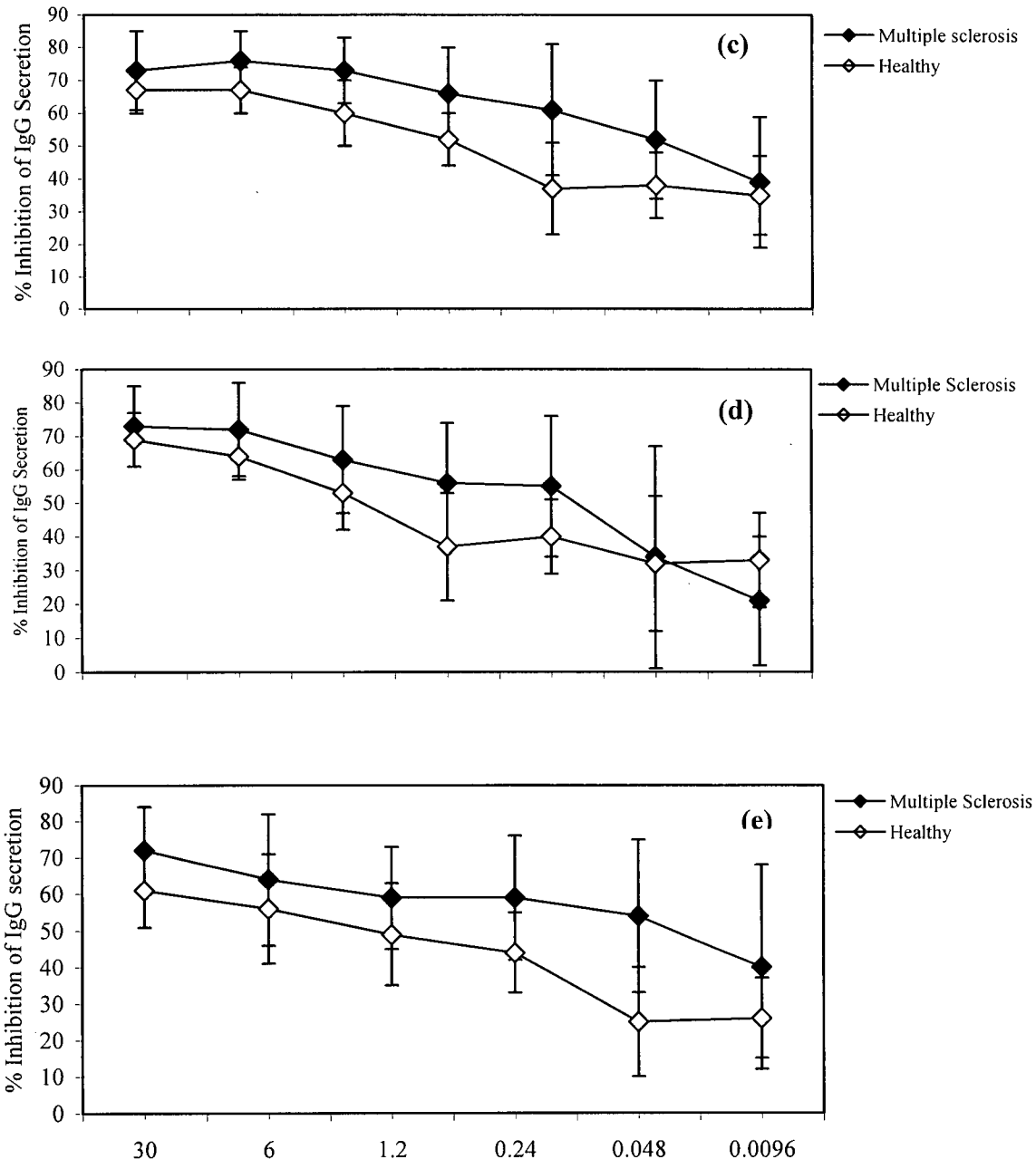

Figure 3.10 c, d, and e. Inhibition (mean ± SEM) of PWM-induced IgG secretion by Avonex™ (c), Betaseron® (d), and Rebif® (e) in 5 high responding healthy individuals and 6 high responding stable RR-MS patients.

Same Dose Comparison of Avonex™, Betaseron® and Rebif® on Inhibition of PWM-induced IgG Secretion

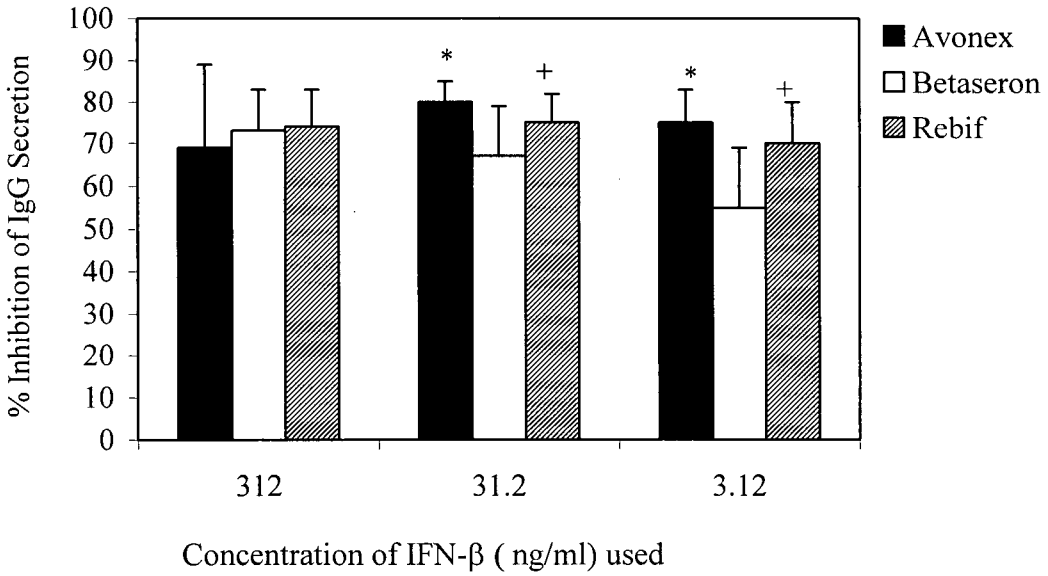

Figure 3.11. In these experiments blood MNC from 16 high responding sRR-MS were stimulated with PWM and co-incubated without and with indicated doses (ng/ml) of IFN-β from Avonex™, Betaseron® and Rebif®. After 7 days at 37°C, the IgG content of the supernatants were measured by an ELISA. Each column indicates mean ± SEM inhibition of PWM-induced IgG secretion by different preparation of IFN-β. At 312 ng/ml, no significant differences were seen between different IFN-β in inhibition of PWM-induced IgG secretion. At 31.2 and 3.12 ng/ml, Avonex™ or Rebif® inhibited significantly more IgG secretion than that of Betaseron® ($p<0.05$).

Significance $p<0.05$

* Avonex™ *versus* Betaseron®
+ Rebif® *versus* Betaseron®

Per Dose Comparison of Avonex™, Betaseron® and Rebif® on Inhibition of PWM-induced IgG Secretion

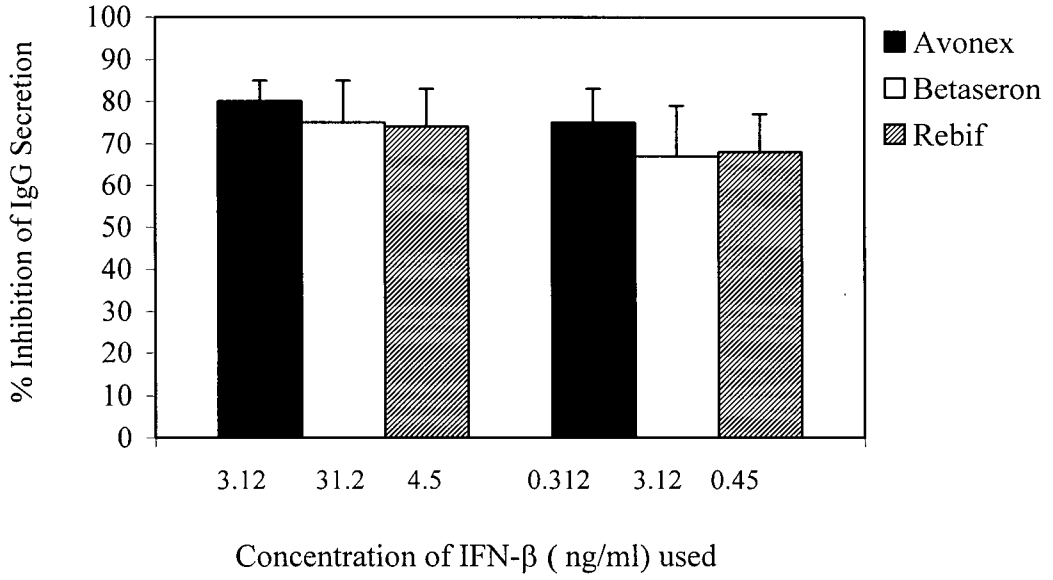

Figure 3.12. Effects of Avonex™, Betaseron® and Rebif® on inhibition of PWM-induced IgG secretion were compared according to their proportion of daily recommended doses. Each column indicates mean ± SEM inhibition of PWM-induced IgG secretion by different preparation of IFN-β. No significant differences was found between the three treatment groups.

Fraction of Weekly Dose Comparison of Avonex™, Betaseron®, and Rebif® on Inhibition of PWM-induced IgG Secretion

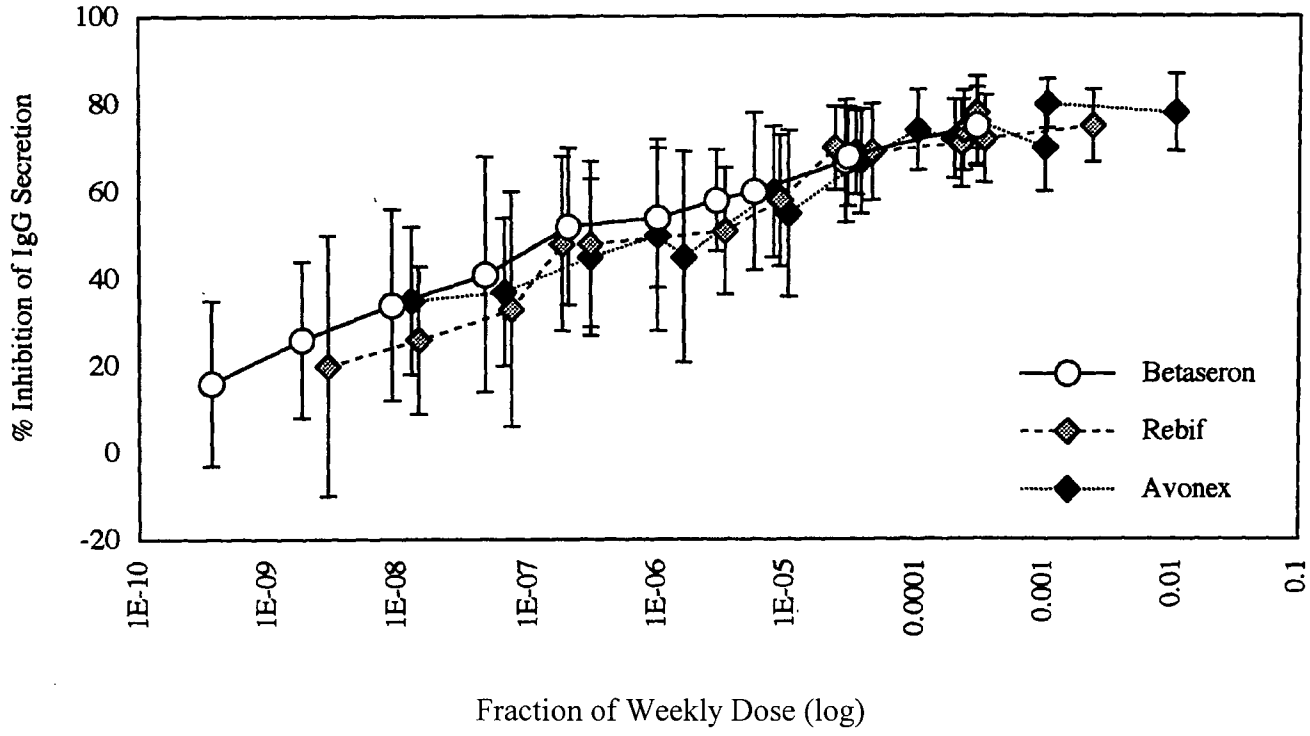

Figure 3.13. Effects of Avonex™, Betaseron®, and Rebif® on inhibition of PWM-induced IgG secretion were compared according to their fraction of weekly dose using their antiviral activity (units). The concentration of IFN-β used ranged from 312 ng/ml to 0.384 pg/ml

CHAPTER FOUR
DISCUSSION AND CONCLUSION

DISCUSSION AND CONCLUSION

4.1 LYMPHOCYTE SUBSETS IN HAM, HTLV-I CARRIER AND HEALTHY CONTROLS

In this study, we demonstrated that lymphocyte subsets are altered in patients with HAM and in asymptomatic HTLV-I carriers. We did not find significant differences among major lymphocyte subsets including CD3+, CD4+, and CD8+ T cells between HAM, HTLV-I carriers and controls. This is in contrast with some (Itoyama et al., 1988) but also in agreement with other (Yasuda et al., 1986; Prince, 1990; Mukae et al., 1994) previous published reports on major lymphocyte subsets in HAM and HTLV-I carriers.

We furthermore found that the percentage of CD4+CD29+ was significantly higher in HAM patients and carriers compared with controls. CD4+CD29+ lymphocytes represent "memory " cells (Sanders et al., 1988; Akbar et al., 1988). After encounter with an antigen, activated T cells acquire CD29 expression, which is paralleled by a downregulation of CD45RA expression (Akbar et al., 1988). CD4+CD29+ cells have also been called helper-inducer since they produce a variety of cytokines including IL-2, IL-4, IL-5, and IFN-γ and provide help for immunoglobulin production (Morimoto et al., 1989). Therefore, the increase in CD4+CD29+ cells may account for high HTLV-I antibody titers and polyclonal B cell activation observed in the serum and cerebrospinal fluid of HAM and HTLV-1 carriers (Itoyama et al., 1988; Yasuda et al., 1986; Geroni et al., 1988; Link et al., 1989; Osame et al., 1987; Mori et al., 1988).

We observed significantly higher levels of CD8+CD57+ cells in HAM compared with both carriers and controls. Increased number of CD8+CD57+ cells have been

reported to be associated with a number of clinical disorders including human immunodeficiency virus (HIV) infection (Lewis et al., 1985; Borthwick et al., 1994), rheumatoid arthritis (Burns et al., 1992), Crohn's disease (James et al., 1984), and in recipients of cardiac (Maher et al., 1985) and bone morrow (Leroy et al., 1986) transplants. It is difficult to propose a specific role for CD8+CD57+ subset and its role in HAM pathogenesis, since a broad range of functions has been proposed for CD8+CD57+ cells. Some of the proposed functions are: lectin-dependent and antibody-directed cytotoxicity (Phillips and Lanier, 1986), cytotoxic responses (Joly et al., 1989; Autran et al., 1991), and suppression of the generation of cytotoxic T lymphocytes (Wang et al., 1994). If we assume a cytotoxic role for CD8+CD57+ cells, increased CD8+CD57+ could explain the high levels of the virus-specific cytotoxic T lymphocytes observed in HAM but not in carriers (Jacobson et al., 1990; Elovaara et al., 1993).

We found high levels of CD3+CD27- cells only in HAM patients and not in carriers. The CD27 molecule is a member of the tumor necrosis factor superfamily (Goodwin et al., 1993). T cell activation studies *in vitro* have shown that CD27- cells arise from CD45RA-, CD45RO+, CD27+ T cells after prolonged restimulation (De Jong et al., 1992; Hintzen et al., 1993). An increased percentage of CD4+CD27- cells have also been reported in peripheral blood, synovial fluid, and synovial tissue of patients with rheumatoid arthritis, and these cells exhibit an enhanced capacity for transendothelial migration (Kohem et al., 1996). This could also explain the enhanced binding of lymphocytes to endothelial cells seen in HAM patients (Ichinose et al., 1992). This is also consistent with recent findings of high levels of the soluble form of CD27 in the cerebrospinal fluid of patients with HAM and multiple sclerosis (Hintzen et al., 1999).

Our study revealed that the percentage of both CD4+ and CD8+ cells which expressed HLA-DR was significantly higher in HAM and carriers. HLA-DR is the class II MHC antigen and acts as a restricting element required to mediate T cell activation (Corley et al., 1985). The activation molecule CD25, is an α-chain of the receptor for interleukin-2 (Uchiyama et al., 1981) and here we showed that it is upregulated on CD4+ cells of both HAM and carriers. These findings are consistent with previous observations that showed HAM patients and HTLV-I carriers have high levels of CD25+ and HLA-DR+ cells in their PBL (Itoyama et al., 1988). It is known that lymphocytes in peripheral blood from HAM and HTLV-I carriers show an enhanced spontaneous proliferation *in vitro* (Itoyama et al., 1988). An IL-2 autocrine mechanism may operate in this phenomenon because a unique transregulatory protein Tax which is encoded by the pX region of the HTLV-I proviral genome has been shown to induce the expression of host cellular genes IL-2 and IL-2 receptor α chain (Tender et al., 1990). Therefore, increase of CD4+CD25+ cells in peripheral blood in HAM and HTLV-I carriers may result from the activation of IL-2 and its receptor gene by this transregulatory protein. However, the enhanced expression of HLA-DR on CD4+ and CD8+ cells and upregulation of CD25 molecules on CD4+ cells have not been observed in American asymptomatic HTLV-I carriers (Prince, 1990). We also found a high percentage of activation markers CD38, HLA-DR, and CD25 on CD8+ cells of HAM patients after 2 days in culture. This is of particular interest and potentially relevant to the pathological findings of HAM patients in whom lymphocytes infiltrating the central nervous system were predominantly CD8+ (Jacobson et al., 1992). The levels of CD8+CD38+ cells have been reported to have prognostic value for HIV disease progression (Ho et al., 1993). High levels of

CD8+CD25+, CD8+CD38+, and CD8+HLA-DR+ cells in HAM patients may be due to chronic antigenic stimulation in response to HTLV-I infection and may be indicative of progression of the infectious process or disease development. The levels of early activation antigen CD69 expressed on CD3+ (Hara et al., 1986) cells was low, but after 2 days in culture increased significantly in both HAM and in HTLV-I carriers. This may indicate that the circulating T lymphocytes of HTLV-I infected individuals have not been activated recently. It is possible, however, that recently activated lymphocytes expressing CD69 may be sequestered in lymph node or other organs.

4.2 BLOOD MNC-HUVEC ADHESION IN HAM

In this study, we used primary and secondary culture of human umbilical vein endothelial cells (HUVEC) monolayers as a source of endothelial cells, which are more readily available than cerebral endothelial cells. Cerebral endothelium that constitutes the BBB is different in many ways from extracerebral endothelium particularly in the structural features. For example cerebral endothelium is further supported by astrocytic processes and contain tight junctions. However, endothelial cells from HUVEC also share many similarities with cerebral endothelial cells (Pober, 1988). For example stimulation of both umbilical and cerebral endothelial cells with cytokines increases their adherence for leukocytes (Pober, 1988; Tsukada et al., 1994). Furthermore, TNF-α increases the expression of ICAM-1 and VCAM-1 on both cerebral endothelial cells and HUVEC (Stins et al., 1997). As lymphocytes were subjected to extensive manipulation, we performed our adhesion assays after 48 h in culture. We also speculate that

lymphocytes in culture could partially mimic the CNS microenvironment: secreted lymphokines and cytokines accumulate in culture, a situation that probably occurs at the blood brain barrier in CNS inflammation. In the past oligoclonal bands were generated *in vitro* using this strategy (Oger et al., 1981).

The adhesion of lymphocytes to the brain microvascular endothelium, which form the blood-brain barrier, is a critical step in the initiation of the inflammatory response in the CNS and therefore probably plays an essential role in the pathogenesis of many neurological diseases (Raine et al., 1990; Martin et al., 1992). Adhesion is mediated by multiple receptor-ligand systems including cell adhesion molecules selectins, integrins and immunoglobulins expressed on lymphocytes and endothelial cells (Bevilacqua, 1993). The expression of adhesion molecules on endothelial cells is regulated by several cytokines (Yu et al., 1985; Carley et al., 1988; Pober, 1988). Indeed, we have verified here that treatment with IFN-γ enhanced the MNC-HUVEC adhesion. We have also used conditioned medium containing L-929 supernatant to stimulate HUVEC in some of our experiments as it contains many different cytokines (Oger et al., 1974; Tonetti et al., 1997), thus mimicking more closely the situation in inflammatory conditions in CNS. Unknown soluble factors in L-929 supernatant were previously shown to induce the release of TNF-α from a macrophage cell line (Tonetti et al., 1997). L-929 supernatant could have similar effects on HUVEC, as HUVEC is also capable of producing TNF-α (Nilsen et al., 1998), and thus enhancing MNC-HUVEC adhesion.

We have confirmed earlier observations (Ichinose et al., 1992) that MNC obtained from HAM patients adhere more readily to HUVEC than those of HTLV-I carriers or healthy controls, and this is highly relevant to the pathogenesis of HAM. The

greater binding of HAM patients' blood MNC to activated HUVEC could be due to their greater recognition of ligands on activated HUVEC. The activation of MNC is important in this regard, and it is the highly activated, rather than the quiescent MNC, that are more likely to bind endothelial cells (Brown et al., 1993; Oen et al., 1994; Vora et al., 1995). It has been shown that the activation of T cells with PMA increases the affinity of LFA-1 and VLA-4 integrins for their counter ligands ICAM-1 and VCAM-1 on endothelial cells without changing the levels of cell surface expression (Dustin and Springer, 1989; Wilkins et al., 1991). Pro-inflammatory cytokines such as TNF-α, IL-1 and IFN-γ are secreted by activated Th1-lymphocytes and these cytokines have been demonstrated to increase both the affinity and the induction of adhesion molecules on lymphocytes (Yu et al., 1985; Pober and Cotran, 1990). In HAM, blood MNC produce large amounts of IFN-γ in culture (Nishiura et al., 1994) and therefore, could function in an autocrine fashion in promoting their binding to endothelial monolayers.

4.2.1 Mechanism of adhesion of HAM blood MNC to activated HUVEC

Using monoclonal antibody blocking studies, we have shown the respective role of VLA-4α, ICAM-1, and L-selectin molecules in HAM blood MNC adhesion to activated HUVEC. These adhesion molecules may also play an important role in directing T cells to inflammatory sites in patients with HAM. In our studies, antibody to ICAM-1 was the most effective in inhibiting the binding of HAM cells to HUVEC. This is in agreement with previous findings that effective blocking of lymphocyte-endothelial cell interactions could be achieved both *in vivo* and *in vitro* by antibody to ICAM-1 (Whitcup et al., 1993; Greenwood et al., 1995; Wong et al., 1999). Antibody to VLA-4

was the least effective in inhibiting the binding of cultured HAM lymphocytes to activated HUVEC; it is probably because T cells are fully activated and activated cells preferentially use LFA-1/ICAM-1 interactions rather than VLA-4/VCAM-1 (Van Kooyk et al., 1993). We also have shown that antibodies to L-selectin block the binding of HAM patients' lymphocytes to HUVEC. This inhibition was unexpected, since an inhibitory effect with anti L-selectin antibody has been demonstrated only under flow but not static conditions (Spertini et al., 1991). This difference could be because our experimental system resulted in activation of both lymphocytes and HUVEC. This inhibition could be attributed to L-selectin recognition of its ligand on EC with higher affinity or binding to an alternative ligand on activated EC. An inhibitory role by E-selectin has been reported when both HUVEC and CD4+ T cells are activated (Shimizu et al., 1991). A more recent study (Wong et al., 1999), indirectly supporting the role of L-selectin other than rolling, has shown that E-selectin, a ligand for L-selectin inhibits transmigration of T cells across TNF-α activated human brain microvessel endothelial cells. Furthermore, its has been shown that E-selectin mediates a major role for adhesion of Adult T-cell Leukemia (also caused by infection with HTLV-1) cells to HUVEC (Ishikawa et al., 1993). It is therefore possible that L-selectin plays a role not only in rolling but also in binding when lymphocyte and endothelial cells are activated. In our antibody blocking experiments, control mouse serum also slightly inhibited MNC attachment to EC when used at 2.5 μg/ml of immunoglobulin concentration (data not shown). We also used mouse IgG2b mAb isotype control anti-dansyl, (monoclonal specific for hapten dansyl (5-[dimethylamino] naphthalene-1-sulonyl), normal human serum, and normal mouse serum to inhibit adhesion of MNC of healthy controls and

multiple sclerosis patients to EC and found that both normal mouse as well as pooled human serum occasionally inhibit MNC-EC adhesion, whereas anti-dansyl mAb does not. Therefore, anti-dansyl mAb serves as a more appropriate control.

4.2.2 Expression of adhesion molecules on HAM and non-HAM lymphocytes

To investigate whether the increase in binding of lymphocytes from HAM patients was mediated by specific receptor-ligand interactions, we analyzed the expression of adhesion molecules on lymphocytes after two days in culture. We found that the expression of L-selectin on CD4+ and CD8+ cells decreased in HTLV-I carriers and even more in HAM. L-selectin is an adhesion molecule of the selectin family that mediates the initial step of lymphocyte attachment to vascular endothelium ("rolling") (Lawrence and Springer, 1991). Upon extensive and prolonged cellular activation, this molecule is shed (Jung and Dailey, 1990). Elevated serum levels of soluble L-selectin have been reported during the period of active disease in patients with multiple sclerosis (Hartung et al., 1995) and adult T-cell leukemia (Tatewaki et al., 1995). In addition, significant elevation of soluble L-selectin has recently been reported in the sera of HAM patients (Tsujino et al., 1998). The reduced expression of L-selectin on lymphocytes thus probably best represents chronic cell activation. Furthermore, as activation of lymphocytes following receptor engagement results in lymphocyte shedding of L-selectin, this may allow the leukocyte to break its tight bonds with the vascular endothelium and proceed with emigration into the underlying tissue into the CNS. We further showed high levels of expression of ICAM-1 in T cells from HTLV-I carriers and at even higher levels in patients with HAM following two days in culture. This fits well

with the fact that ICAM-1 is upregulated in T cell lines carrying HTLV-I and in lymphocytes of patients with adult T cell leukemia (ATL) (Yamamoto et al., 1982; Yamamoto and Hinuma, 1985). It is known that cell-free HTLV-I exhibits very low infectivity and cell-to-cell infection is regarded as the major route of HTLV-I transmission both *in vitro* and *in vivo* (Weiss et al., 1985; Yamamoto and Hinuma, 1985). Therefore, constitutive expression of ICAM-1 in HTLV-1-infected T cells might be important for cell-mediated transmission by prompting cell adhesion between HTLV-I-positive T cells and uninfected T cells. Increased levels of soluble ICAM-1 have also been reported in the sera of multiple sclerosis and HAM patients (Sharief et al., 1993; Tsukada et al., 1993b).

Analysis of adhesion molecules on lymphocytes at isolation showed that the percentage of CD4+ and CD8+ cells expressing VLA-4 was generally higher in both patients with HAM and carriers than in controls. Therefore, it is likely that VLA-4 does not contribute much to the increased binding of lymphocytes to HUVEC seen in HAM patients. Antibody blocking experiments further demonstrated that VLA-4 was the molecule least involved in this adhesion.

4.3 BLOOD MNC-HUVEC ADHESION IN MS

In this study we also investigated the adhesion properties of peripheral blood MNC of sRR-MS and SP-MS to HUVEC. Blood MNC from SP-MS patients exhibited significantly higher adhesion capacity than MNC from normal donors. This is consistent with the previous published report indicating increased adhesion of chronic progressive

MS blood MNC to cultured cerebral endothelium (Lou et al., 1997; Tsukada et al., 1993a). This increase in adhesion was attributed in part to higher expression of LFA-1 on circulating blood MNC than on those of normal controls (Lou et al., 1997). Increased adhesion of SP-MS blood MNC to HUVEC is also probably due to increased levels of activated lymphocytes seen in circulating peripheral blood of SP-MS patients (Hafler et al., 1985) and it is activated lymphocytes that are highly adherent to endothelial monolayers (Brown et al., 1993). Our data are also in agreement with a recent report indicating enhanced transmigration of secondary progressive MS lymphocytes across fibronectin-coated membranes (Prat et al., 1999). In previous studies, increased IFN-γ and TNF-α production capacity were found in cultured MNC from patients with SP-MS (Beck et al., 1988; Chofflon et al., 1992). Since we performed the adhesion assay after two days in culture, IFN-γ and TNF-α, the well known inducers of adhesion molecules (Yu et al., 1985; Pober and Cotran, 1990) also might have contributed in an autocrine fashion to the enhanced SP-MS blood MNC adhesion observed in this study. The adhesion of clinically stable RR-MS blood MNC was generally higher than that of healthy subjects (although not statistically significant). This is probably because a certain proportion of stable RR-MS in this study had biological activity that could have been recognized by MRI. MRI was not done for this study. Our data might also explain the reason for significant impairment of blood brain barrier from RR-MS to secondary progressive MS (McLean et al., 1993). Increased binding of RR-MS blood MNC to endothelial monolayers have been previously reported, but only during the clinically active phase of the disease (Tsukada et al., 1993a). This might also imply that the expression of some adhesion molecules on active RR-MS and SP-MS differ from that on

stable RR-MS. In another study of heterogeneous populations of MS it was found that MS blood MNC adhered to HUVEC more than did healthy, but only after IFN-γ, TNF-α, and IL-1 cytokine activation of HUVEC (Vora et al., 1996). Such differences were not seen with resting HUVEC. However, in studies of Tsukada et al. (1993a), no significant differences were found between adhesion of MNC from clinically active RR-MS and healthy control after TNF-α treatment of cerebral endothelium.

4.3.1 Mechanism of adhesion of RR-MS and healthy blood MNC to HUVEC

We also compared the relative contribution of major adhesion molecules in binding blood MNC of RR-MS and of normal subjects to HUVEC by monoclonal antibody blocking studies. The adhesion of MNC from both RR-MS and healthy subjects to untreated HUVEC was significantly reduced by mAbs. to LFA-1 and ICAM-1. This is consistent with previous observation that inhibition of MNC adhesion to untreated HUVEC can be achieved with monoclonal antibodies to LFA-1 and ICAM-1 (Shimizu et al., 1991; Oppenheimer-Marks et al., 1991; Watson et al., 1996). The mAb to ICAM-1 was not as effective as LFA-1 in inhibition of adhesion. This is perhaps due to LFA-1 binding to ICAM-2 in addition to ICAM-1. ICAM-2 is constitutively expressed on both resting and activated HUVEC (Staunton et al., 1989). In fact, ICAM-2 is expressed at greater levels than ICAM-1 on untreated HUVEC (Staunton et al., 1989). Antibody directed against VLA-4 or L-selectin had no inhibitory effects. This is probably because untreated HUVEC do not express VCAM-1 or E-selectin, the main ligands for VLA-4 and L-selectin, respectively (Shimizu et al., 1991; van Kooyk et al., 1993), although there is some dispute (Hughes, 1996). Previous studies has shown that adhesion of

lymphocytes to resting cerebral endothelium cells also involve VLA-4 adhesion molecule (Matsuda et al., 1995b). Perhaps, this is because resting cerebral endothelium expresses low levels of VCAM-1, the main ligand for VLA-4 (Wong and Dorovini-Zis, 1995). In a study using retinal endothelial cells, it was found that antibody to VLA-4 does not inhibit resting lymphocyte adhesion to untreated retinal endothelial cells. However, when lymphocytes were activated by ConA, antibody to VLA-4 significantly inhibited adhesion to untreated retinal endothelial cells (Greenwood et al., 1995). As retinal endothelial cells were shown not to express VCAM-1, this inhibition was attributed to the ability of the antibody to VLA-4 to induce aggregation of ConA-activated lymphocytes *in vitro*. Lymphocyte aggregates are then easier to remove during the washing stage of the assay, thus producing an apparent reduction in binding. Combinations of LFA-1, ICAM-1, VLA-4 and L-selectin mAbs failed to show greater inhibition than seen with the LFA-1 mAb alone, indicating that the LFA-1 pathway predominates in mediating adhesion of RR-MS and healthy subjects to untreated HUVEC. We further have shown that the major adhesion molecules investigated in this study are equivalently involved in adhesion of both RR-MS and healthy subjects to resting HUVEC with LFA-1/ICAM-1 pathway mediating a predominant role. Our data do not support the previous report that indicates the involvement of LFA-1 adhesion pathway in binding of RR-MS, and not in binding of healthy subjects blood MNC to cerebral endothelium (Tsukada et al., 1993a).

4.3.2 Mechanism of adhesion of RR-MS blood MNC to unstimulated and IFN-γ-stimulated HUVEC

To characterize further the adhesion molecule-ligand pairs involved in inflammation, we extended this study to test antibodies directed against various adhesion molecules to block the adhesion of RR-MS blood MNC to HUVEC stimulated with IFN-γ. Monoclonal antibodies directed against LFA-1 and ICAM-1 strongly inhibited RR-MS blood MNC-HUVEC interaction. Our results are in agreement with a previous study showing significant role for LFA-1 and ICAM-1 in adhesion of MNC to both resting and IL-6 activated HUVEC (Watson et al., 1996). Contrary to our finding is a report indicating significant involvement of LFA-1 and ICAM-1 on binding of MNC to resting but not to IL-1 activated HUVEC (Oppenheimer-Marks et al., 1991). The reasons for this disparity are not clear. In addition to LFA-1 and ICAM-1, the adhesion of RR-MS blood MNC to IFN-γ activated HUVEC was shown to also significantly involve VLA-4 adhesion molecule. These results are in agreement with previous published observation on the inhibitory role of antibody to VLA-4 on MNC adhesion to activated endothelial cells (Oppenheimer-Marks et al., 1991; Watson et al., 1996). In our study, it is likely that IFN-γ might have induced the expression of VCAM-1, the main ligand for VLA-4 on HUVEC. Indeed IFN-γ has been previously shown to enhance the expression of VCAM-1 on HUVEC (Lindington et al., 1999). An increase in levels of expression of VLA-4 on lymphocytes (Svenningsson et al., 1993) and VCAM-1 on brain microvessel endothelial cells (Washington et al., 1994) have been found in MS. Therefore, VLA-4 might play an important role in mediating MNC adhesion and subsequently transmigration in MS. Our result could also partly explain the previous observation that administration of anti-VLA-

4 antibody alleviated the clinical and pathological symptoms of EAE (Yednock et al., 1992). In the present study antibody to VLA-4 inhibited MNC adhesion to activated HUVEC but to a lesser extend than did anti-LFA-1. This may be explained by differences in the distribution of these two adhesion molecules. Unlike LFA-1, which is present on all lymphocytes, VLA-4 expression is confined to a subpopulation of the cells (Shimizu et al., 1990). We were not able to completely inhibit MNC adhesion to HUVEC using a combination of the studied mAbs. This indicates the potential involvement of other receptor/ligand interactions. Some other candidate that might be involved in adhesion include CD44, which has been implicated in the adhesion of activated T cell to activated HUVEC (Oppenheimer-Marks et al., 1990) and CD2 which mediates T cell adhesion to other cell types by binding to its ligand, LFA-3 (Makgoba et al., 1989).

4.4 COMPARING ADHESION PROPERTIES OF HUVEC AND ECV-304 FOR BLOOD MNC

In a part of this project, we also assessed the suitability of ECV-304 cell lines as a substitute for HUVEC for the adhesion of blood MNC. The adhesion of sRR-MS and healthy controls blood MNC to ECV-304 monolayers were not significantly different. This is consistent with our observations using HUVEC to compare sRR-MS and healthy control MNC. However, we cannot conclude with absolute certainty that the adhesion pathways for sRR-MS and healthy controls MNC to untreated HUVEC and to ECV-304 monolayers are similar as we did not use MNC from the same individuals and the assays

were not done in parallel. We furthermore found significant differences between ECV-304 and HUVEC in their ability to respond to IFN-γ and L-929 supernatant. Treatment of HUVEC with IFN-γ and L-929 supernatant resulted in significantly enhancing their adhesion for MNC. However, similar treatment of ECV-304 failed to enhance their adhesion for MNC. It is possible that unlike in HUVEC, IFN-γ and L-929 supernatant does not result in upregulation of adhesion molecules expression and subsequent functional enhancement for MNC adhesion to ECV-304.

Monoclonal antibody blocking studies demonstrated that adhesion pathways of RR-MS and healthy controls to untreated HUVEC and ECV-304 are similar and predominantly involve LFA-1/ICAM-1 pathway. In a parallel study, we found that when both monolayers were treated with proinflammatory IFN-γ cytokine, VLA-4 was involved in MNC adhesion to HUVEC but not to ECV-304. Our results agree with recent observations that indicate ECV-304 constitutively express ICAM-1 but not VCAM-1 (Dobbie et al., 1999). Moreover, our results are also in agreement with the findings that unlike in HUVEC, the treatment of ECV-304 with TNF-α does not result in the induction of VCAM-1 expression (Lindington et al., 1999). Our data however, conflicts with a report that demonstrated constitutive expression of VCAM-1 on ECV-304 which was upregulated following activation with LPS (Hughes, 1996). The exact reason (s) for the discrepancy between these studies is unclear but could be due to variations in adhesion molecule expression in response to different activators, differences in antibodies utilized and in the sensitivity of the detection methods employed.

4.5 THE EFFECTS OF IMMUNOMODULATORY DRUGS ON LYMPHOCYTE SUBSET AND FUNCTION IN MS

4.5.1 Lymphocyte subsets in a RR-MS patient participating in Schering-Plough trial

Analyzing lymphocytes subset of a RR-MS patient who participated in a double-blind and placebo controlled trial of Schering-Plough, we observed a major shift in lymphocyte subsets at both 2 and 7 days post treatment. However, at present we do not know whether the patient received placebo or rIL-10. Therefore, we cannot conclude whether the observed shifts in lymphocyte subsets are due to rIL-10 treatment or spontaneous.

4.5.2 *Lymphocyte subsets in RR-MS patients participating in ICOS trial*

We also were interested in verifying whether a short-term treatment with Hu23F2G (recombinant and humanized anti-LFA-1 antibody) is able to modify peripheral blood lymphocyte subsets in RR-MS. We observed a major shift in some lymphocyte subset in 4 patients who participated in this study at the VH&HSC/UBC site Multiple Sclerosis Clinic. Due to the limited number of participants, statistical analysis was not possible and all changes in lymphocyte subsets cannot be discussed rationally. Hu23F2G treatment had no major effect on the number of circulating CD3+, CD4+ or CD8+ T cells. Focusing only on few lymphocyte subsets, we observed a major reduction of CD3+CD26+ (activated T) cell and CD4+ and CD8+ cells expressing adhesion molecules VLA-4 (CD49d) and L-selectin (CD62L) following *in vivo* treatment with high dose (2 mg/kg) Hu23F2G. At lower dose (1 mg/kg), HU23F2G effects were less

profound, and resulted only in a large reduction in the percentage of CD4+ and CD8+ cells expressing VLA-4 adhesion molecules. On the contrary, in a placebo treated patient the percentage of CD4+ and CD8+ cells expressing VLA-4 and L-selectin increased while that of CD3+CD26+ remained unchanged. Furthermore, in a RR-MS patient treated with intravenous methylprednisolone no major reduction of the discussed adhesion molecules was observed. In contrast to high dose Hu23F2G treated patient, the percentage of CD4+ and CD8+ cells expressing L-selectin increased. These results, with some degree of confidence, indicated that the observed shifts with respect to the discussed lymphocyte subsets are likely due to immunotherapy with Hu23F2G rather than spontaneous. Moreover, our data particularly in the RR-MS patient treated with high dose Hu23F2G indicates that the immunotherpay with Hu23F2G achieved its desired objectives of reducing activation and adhesion related antigens on peripheral blood lymphocytes. The reduction of activation and adhesion related antigen on peripheral blood lymphocytes is relevant in CNS inflammatory cell infiltration in MS since it is these cells that are likely to be involved in the process of endothelial adhesion and extravasation to sites of inflammation (Wekerle et al., 1986; Estess et al., 1999). The significance of adhesion molecules in MS pathology has already been addressed in the Introduction. Furthermore, in peripheral blood of active MS patients' lymphocytes were found to have higher expression of the activation marker CD26 compared to patients with inactive MS, patients with other neurological diseases, or healthy controls (Hafler et al., 1985). Therefore, it is reasonable to expect a beneficial therapeutic effect in Hu23F2G treated MS patients. The reason for clinical inefficacy of Hu23F2G in RR-MS patients is not clear (Lublin 1999). However, we have to bear in mind that the treatment

schedule with Hu23F2G is for relapses of MS and this might be too late once the inflammation is irretrievably established in the CNS. Therefore, Hu23F2G might achieve its desired effects if given at very early stage of the MS relapse. A note of caution should also be raised and stressed here in the interpretation of fluctuations of lymphocyte subsets in the studied patients, as the number of participant in this study was highly limited.

4.5.3 PWM-induced IgG secretion in sRR-MS and healthy controls and the effects of IFN-β on this function

We found that PWM-induced IgG secretion by blood MNC was significantly increased in sRR-MS patients compared to the normal healthy populations, as has already been reported (Levitt et al., 1980; Oger et al., 1988, Antel et al., 1984). In agreement with other reports (Antel et al., 1984; Rosenkoetter et al., 1984), we also found that the proportion of high responders to PWM stimulation is higher in RR-MS than in healthy subjects. The main reason for the high *in vitro* IgG secretion in MS is believed to be defective function of T-suppressor lymphocytes (Antel et al., 1984, 1986). It should also be noted that elevated PWM-induced IgG secretion has not always been found in MS patients (Kelley et al., 1981; Hauser et al., 1985).

In our study, all different preparation of IFN-β caused a dose-related inhibition of IgG secretion induced by PWM in peripheral blood MNC of high responders in both sRR-MS and healthy controls. Similar inhibitory effects with IFN-β and closely related IFN-α have been previously reported in both *in vivo* and *in vitro* studies (Siegel et al., 1986; O'Gorman et al., 1987; Bratt et al., 1996). It has also been shown that in contrast to

their suppressive action on IgG production in unseparated MNC, IFN-β enhanced IgG production in purified B cells (Siegel et al., 1986). The inhibitory action of IFN-β on PWM-induced IgG production in unseparated MNC is believed to be mediated by IFN-β effect on a non-B cell population and in part related to the inhibitory effect of IFN-β on PWM-induced MNC proliferation (Siegel et al., 1986).

4.5.4 Comparing the effects of different preparations of IFN-β on PWM-induced IgG secretion

We also directly compared the *in vitro* biological activity of IFN-β from Avonex™, Betaseron®, and Rebif® utilizing inhibitory effects of IFN-β on PWM-induced IgG secretion in high responding sRR-MS patients. Our results demonstrated that Avonex™ and Rebif® had higher biologic activity compared with Betaseron® when used at a similar concentration. The difference was particularly evident when we compared Avonex™ (IFNβ-1a) with Betaseron® (IFNβ–1b). Avonex™ and Rebif® amino-acid sequence and glycosylation pattern are identical to those of endogenous human IFN-β. By contrast, in Betaseron® serine is substituted for cysteine at position 17, the N-terminal methionine is missing and the glycosylation of the natural product is lacking. There is evidence that carbohydrate plays a vital role in stabilizing the IFN-β molecules, and its absence from Betaseron® may explain why this molecule in our assay as well as in standard antiviral assay has much less biological activity per milligram of protein compared with Avonex™ and Rebif® (Runkel et al., 1998). We did not expect generally a higher inhibitory effects for Avonex™ compared with Rebif® on PWM-induced IgG secretion as these two preparations of IFN-β are similar and are produced by

inserting the natural human gene for IFN-β into Chinese hamster ovary cells. Thus, it is unlikely that there is a structural difference between Avonex™ and Rebif®. We do not have a clear explanation to our observation. The differences in formulation might explain this phenomenon. Avonex™ is formulated in a higher concentration of albumin (15 mg/ml after reconstitution *versus* 9 mg/ml for Rebif®), at a different pH (7.2 *versus* 3.8) and in a different buffer (phosphate *versus* acetate). It is possible that Avonex™ is more stable in its formulation or is better absorbed by IFN-β receptor on MNC than Rebif®. This higher *in vitro* activity of Avonex™ compared to Rebif® is also consistent with their *in vivo* pharmacodynamic activity (Alam et al., 1997). It has been shown that when an equal dose (6 MIU) of Avonex™ and Rebif® was administrated intramuscularly to healthy volunteers, the serum neoptrin concentration was higher in Avonex™ treated individuals ((Alam et al., 1997). When we corrected for specific activity (MIU) and weekly dose and expressed concentration as a fraction of the MIU of IFN-β activity injected per week, Betaseron®, Avonex™ and Rebif® had similar activity with respect to their influence on inhibition of PWM-induced IgG secretion.

4.5.5 Effects of IFN-β on MNC-HUVEC adhesion

In this study we demonstrated that IFNβ-1b treatment of resting HUVEC has no significant effect on MNC adhesion. This is in agreement with a previous report (Dhib-Jalbut et al., 1996). We did not study the effects of IFNβ-1b on activated HUVEC in binding to MNC; however, others have shown that the effects of IFNβ-1b on ICAM-1, VCAM-1 and E-selectin adhesion molecules expression induced by IFN-γ, IL-1β, or TNF-α on HUVEC is slightly additive, and is associated with significant augmentation of

MNC-HUVEC adhesion (Dhib-Jalbut et al., 1996). Contrary to their effects on HUVEC, we demonstrated that the pretreatment of MNC with IFNβ-1b results in significant reduction of MNC-HUVEC adhesion. This effect was not a result of a cytotoxic effect because IFN-β–1b had no effect on cell viability as was determined by trypan blue exclusion dye. It is therefore possible that the effects of IFNβ-1b on adhesion may be cell-specific. This explanation is supported by the finding that IFN-β is capable of downregulating IFN-γ-induced expression of HLA-DR on cerebral endothelial cells (Huynh et al., 1995), but not on monocytes (Soilu-Hanninen et al, 1995). Our findings are also consistent with a previous report showing significant reduction of MNC-HUVEC adhesion after *in vivo* IFN-β–1b treatment in RR-MS patients (Corsini et al., 1997, Gelati et al., 1999). In addition, it has been shown that pretreatment of epidermal carcinoma cell line with closely related IFN-α results in a significant reduction of their binding to HUVEC (Dao et al., 1995). Our results showing the effects of IFNβ-1b on MNC adhesion to HUVEC is also in agreement with a recent report showing that pretreatment of MNC with IFN-β results in significant reduction in migration of MNC through cultured cerebral endothelial cells (Lou et al., 1999). *In vitro*, IFN-β–1b treatment has also been shown to inhibit the transmigration of activated T cells through fibronectin, by acting on a matrix metalloproteinase (MMP-9) produced by the lymphocytes (Stuve et al., 1996). Recently it has also been shown that the migration across fibronectin-coated membranes of lymphocytes from RR-MS patients receiving IFNβ-1b were significantly reduced compared with untreated RR-MS patients (Prat et al., 1999). One of the possible mechanisms for the inhibitory effects of IFNβ-1b on MNC-HUVEC binding is that IFNβ-1b could alter the level of adhesion molecule expression on MNC. We did not

address this question in our study; however, it has been previously shown that pretreatment of MNC with IFN-β results in lower basal and IFN-γ-induced expression of VLA-4 (Soilu-Hanninen et al., 1995). Downregulated expression of VLA-4 on lymphocytes has also been shown in RR-MS patients after treatment with IFNβ (Calabresi et al., 1997). This does not satisfactorily explain our observation, as we did not activate HUVEC and demonstrated that VLA-4 is not significantly involved in MNC adhesion to untreated HUVEC. However, it is possible that IFNβ-1b might also alter the level of expression or function of other adhesion molecules involved in MNC-HUVEC binding. In fact, a decrease in expression of CD18 (β chain of LFA-1) on MNC of IFNβ-1b treated MS patients has been previously reported (Corsini et al., 1997, Gelati et al., 1999). The effects of IFNβ-1b on the functional activity of adhesion molecules might also be responsible for the decreased adhesiveness of MNC to HUVEC since quantitative changes in the expression of cell surface adhesion molecules are not always consistent with the level of cell adhesion (Piela and Korn, 1990; Gamble and Vdas, 1991). *In vitro* treatment with IFNβ-1b has also been shown to downregulate the expression of IL-2 receptor α chain on T cells (Leppert et al., 1996). Thus, it is possible for IFNβ-1b to lower the T cell state of activation and thereby inhibit their adhesion to HUVEC.

4.6 SUMMARY AND CONCLUSIONS

We have shown that patients with HAM exhibit highly activated and differentiated lymphocyte subsets. It is likely that highly activated and differentiated lymphocyte subsets play a critical role in the pathogenesis of HAM and therefore the

assessment of these lymphocyte subsets may be of value in detecting or evaluating inflammatory diseases and monitoring treatment. We have also demonstrated increased adhesion of HAM and clinically active MS blood MNC to HUVEC. Our data and that of others lend support to the view that infiltration of MNC across the BBB into the CNS in HAM and MS is due to increased interaction between blood MNC and endothelium. We also speculate that in both HAM and MS chronic and systemic activation of immune cells result in increased adhesion and initiate events, which lead to central nervous system inflammation.

We have shown that pretreatment of MNC with IFN-β significantly inhibits blood MNC-HUVEC adhesion. Therefore, our data supports the postulate that IFN-β might influence the evolution of the MS lesions at the level of BBB by influencing the circulating MNC and inhibiting MNC-endothelial adhesion and the subsequent migration of inflammatory MNC into the CNS.

We also investigated mechanism of adhesion of blood MNC in HAM, RR-MS, and healthy controls to HUVEC under different experimental conditions. Our results demonstrate that adhesion of HAM blood MNC to activated HUVEC in addition to ICAM-1 and VLA-4 is also mediated by L-selectin. Adhesion pathways in RR-MS and healthy controls to untreated HUVEC were similar and were mainly mediated by LFA-1/ICAM-1. In addition to LFA-1/ICAM-1 pathways, VLA-4 was also involved in adhesion only after stimulation of HUVEC. These findings are relevant to better understanding the mechanisms of adhesion under both inflammatory and non-inflammatory conditions.

We have also compared adhesion properties of HUVEC and ECV-304 for adhesion to MNC and demonstrated that when untreated, HUVEC and ECV-304 utilize shared adhesion pathways for binding to MNCs that predominantly involve LFA-1/ICAM-1; however, when treated with IFN-γ, ECV-304 unlike HUVEC does not utilize VLA-4/VCAM-1 pathway. Furthermore, unlike HUVEC, ECV-304 does not respond to INF-γ and L-929 supernatant for enhancing their adhesion to MNC adhesion. Therefore, ECV-304 may not be useful for some adhesion assays studies.

We demonstrated increased IgG secretion in stable RR-MS in response to PWM. This increase in T cell dependent B cell activity further adds to a spectrum of immune abnormality that has already been reported in this disease. This also supports the view that clinical stability in MS does not necessarily translate into normal functional immune responses. We further demonstrated that IFN-β is capable of significantly inhibiting mitogen-induced IgG secretion. This indicates that another beneficial mechanism of actions of IFN-β in MS might be due to the capacity of IFN-β to downregulate B cell activity and IgG secretion.

We also compared the biological activity of different preparations of IFN-β according to their capacity in inhibiting PWM-induced IgG secretion. The results indicated that Avonex™ had the highest *in vitro* activity followed by Rebif® and Betaseron®, respectively, when used at the same mass dose or proportion of their daily recommended dose. However, when we calculated the amount of different IFN-β used *in vitro* as a fraction of their weekly injection using their antiviral activity in units, these differences disappeared. These *in vitro* effects of Avonex™ Rebif® and Betaseron® were more consistent with their antiviral activity and therefore may be used as an

alternative assay in evaluating the biological activities of IFN-β. Direct comparative clinical trials have yet to be conducted, but if *in vitro* biologic activity of each of these preparations of IFN-β is representative of their physiologic activity *in vivo*, there might not be significant differences on the clinical outcome when they are used at the recommended dosage and frequency of administration.

4.7 FUTURE EXPERIMENTAL CONSIDERATIONS

In the current project, we found that a number of lymphocyte subsets were significantly altered in HAM patients. In order to further elucidate the role of the altered lymphocyte subsets in immunopathology of HAM and examine a potential association between the lymphocyte subsets and disease progression, it would be of significant interest to perform a long follow-up study of early HAM patients. Our understanding of the pathomechanisms of HAM could be further enhanced by defining the functional properties of the altered lymphocyte subsets in terms of determining specificity to HTLV-I antigens, ability to release cytokines, and cytotoxic activity.

Immunological studies on CSF cells maybe more relevant to pathogenesis of HAM than cells of the peripheral blood. Therefore, a comprehensive analysis of lymphocyte subsets in the CSF of HAM patients is another avenue that needs to be explored. Longitudinal analysis of lymphocyte subsets in both the peripheral blood and CSF with a focus on T cell adhesion and activation related antigens could also be expanded to MS patients comparing different stages of the disease.

In this project endothelial cells from umbilical veins were used to support the binding of MNC. Ideally more appropriate endothelial monolayers would be from human cerebral microvessels, which are more difficult to obtain. To better understand the phenomenon of increased MNC adhesion to endothelial monolayers in HAM and secondary progressive MS patients, flow cytometric techniques could be utilized to characterize the surface phenotype markers of the adhesion and activation related antigens of the MNC that are adherent to the endothelial monolayers.

We observed that the adhesion of blood MNC in clinically active MS (SP-MS) was generally higher compared to that of clinically stable MS (sRR-MS). This might indicate that the increased adhesion of blood MNC to endothelial cells in MS is correlated with disease activity. Longitudinal adhesion assays done in parallel with MRI evaluation of the disease activity in RR-MS patients during both relapse and remission be better in delineating the precise sequence of events.

We have also shown that *in vitro* treatment of MNC with IFN-β results in significant reduction in MNC-endothelial adhesion. To examine whether this phenomenon can also be achieved following *in vivo* treatment, longitudinal analysis of MNC adhesion to endothelial monolayers could be carried out in RR-MS patients before and during treatment with IFN-β. Furthermore, the mechanism by which IFN-β may influence MNC to alter their adhesion to endothelial monolayers is not fully known. It is possible that that IFN-β may act on MNC by decreasing their activation state. Studies could be conducted to address this possibility by direct *in vitro* treatment of MNC with IFN-β and subsequent analysis of the expression of markers of activation. Another area of study would be to examine the effects of IFN-β on HAM blood MNC adhesion to the

endothelial monolayers. We have shown that HAM blood MNC are highly activated. Therefore, the potential action of IFN-β in downregulation of activated MNC might be more easily observed in HAM.

We have evaluated the *in vitro* biological activity of the three preparations of IFN-β, namely Avonex™, Betaseron®, and Rebif® and found that Avonex™ could suppress PWM-induced IgG secretion the most when used at the same mass concentration. This study could be extended to evaluate the *in vivo* biological activity of the different preparations of IFN-β. For example, longitudinal measurement of PWM-induced IgG secretion by blood MNC could be performed in RR-MS patients before and after treatment with Avonex™, Betaseron® or Rebif® at the current recommended frequencies, doses, and routes of administration. Using the same assay, *in vivo* biological activity of the different IFN-β preparations could be further compared in MS patients or healthy volunteers when used at the similar specific activity, frequency and route of administration.

REFERENCES:

Akbar A, Terry L, Timms A, Beverley P, Janossy G. Loss of CD45RA and gain of UCHL1 reactivity is a feature of primed T cells. J. Immunol. 140: 2171-2178; 1988.

Alam J, Goelz S, Rioux P, Scaramucci J, Jones W, McAllister A, Campion M, Rogge M. Comparative pharmacokinetics and pharmacodynamics of two recombinant human interferon beta-1a (IFNβ-1a) products administered intramuscularly in healthy male and female volunteers. Pharm. Res. 14: 546-549; 1997.

Antel J, Bania M, Noronha A, Neely S. Defective suppressor cell function mediated by T8+ cell lines from patients with progressive multiple sclerosis. J. Immunol. 137: 3436-3439; 1986.

Antel J, Rosenkoetter M, Reder A, Oger J, Arnason G. Multiple sclerosis: relation of in vitro IgG secretion to T suppressor cell number and function. Neurology. 34: 1155-1160; 1984.

Aplin A, Howe A, Alhari S, Juliano R. Signal transduction and signal modulation by cell adhesion receptors: the role of integrins, cadherins, immunoglobulin molecules, and selectins. Pharm. Rev. 50: 197-263; 1998.

Autran B, Leblond V, Sadat-Sowti B, Lefrance E, Got P, Sutton L, Binet J, Debre P. A soluble factor released by CD8+, CD57+ lymphocytes from bone morrow transplant patients inhibit cell mediated cytolysis. Blood. 77: 2237-2241; 1991.

Bacon K. Chemokine, leukocyte trafficking, and inflammation. Curr. Opin. Immunol. 6: 865-873; 1994.

Baggiolini M. Chemokines and leukocyte traffic. Nature 392: 565-568; 1998.

Baig S, Olsson T, Yu-ping J, Hojeberg B, Cruz M, Link H. Multiple sclerosis: cells secreting antibodies against myelin-associated glycoprotien are present in cerebrospinal fluid. Scan.. J. Immunol. 33: 73-79; 1991

Baker C, Billingham R. Immunologically priviledged sites. Adv. Immunol. 25: 1-54; 1977.

Baron J, Reich E, Visintin I Janeway C. The pathogenesis of adoptive murine autoimmune diabetes requires an interaction between α4 integrins and vascular cell adhesion molecule-1. J. Clin. Invest. 93: 1700-1708; 1994.

Beck J, Rondot P, Catinot L, Falcoff E, Kirchner H, Wietzerbin J. Increased production of interferon gamma and tumor necrosis factor precedes clinical manifestation in multiple sclerosis: do cytokines trigger off exacerbations? Acta Neruol. Scand. 78: 318-323; 1988.

Becker C, Gidal B, Fleming J. Immunotherapy in multiple sclerosis, part 2. Am. J. Health-Syst. Parm. 52: 2105-2120; 1995.

Bellamy A, Cadler V, Feldmann M, Davison A. The distribution of interleukin-2 receptor bearing lymphocytes in multiple sclerosis: evidence for a key role of activated lymphocytes. Clin. Exp. Immunol. 61: 248-256; 1985.

Berlin C, Bargatz R, Campbell J, von Andrian U, Szabo M, Hasslen S, Nelson R, Berg E, Erlandsen S, Butcher E. α4 integrins mediate lymphocyte attachment and rolling under physiologic flow. Cell. 80: 413-422; 1995.

Bernard C, Mndel T, Mackay I. Experimental models of human autoimmune disease: Overview and prototypes. In: Rose N, Mackay I. The autoimmune diseases II. San Diego: Academic press, 47-106; 1992.

Bertram J, Kuwert E. HLA antigen frequencies in multiple sclerosis. Eur. J. Neurol. 7: 74-791 ; 1982.

Bevilacqua M, Nelson R. Selectins. J. Clin. Invest. 91: 379-387; 1993.

Bianchi E, Bender J, Biasi F, Pardi R. Through and beyond the wall: late steps in leukocyte transendothelial migration. Immunol. Today. 18: 586-591; 1997.

Bo L, Peterson J, Mork S, Hoffman P, Gallatin W, Ransohoff R, Trapp B. Distribution of immunoglobulin superfamily members ICAM-1, -2, -3, and the beta 2 integrin LFA-1 in multiple sclerosis lesions. J. of Neuropath. Exp. Neurol. 55: 1060-72; 1996

Borthwick N, Bofill M, Gombert W, Akbar A, Medina E, Sagawa K, Lipman M, Johnson M, Janossy G. Lymphocyte activation in HIV-I infection. II. Functional defects of CD28- T cells. AIDS. 8: 431-441; 1994.

Bowie A, Moynagh P, O'Neill L. The human endothelial cell line ECV-304 as a model of endothelial cell activation by interleukin-1. Biochem. Soc. Trans. 23: S109; 1995.

Bratt K, Guthikonda P, Goodman A, Mattson D. The effects of in vitro and in vivo interferon-b-1b on immunoglobulin G synthesis by peripheral blood lymphocytes. Neruology. 46: A136-A137; 1996.

Brochet B, Dousset V. Pathological correlates of magnetization transfer imaging abnormalities in animal models and human with multiple sclerosis. Neurology. 53: S12-S17; 1999.

Brosnan C, Cannella B, Battistini L, Raine C. Cytokine localization in multiple sclerosis lesions: correlation with adhesion molecules expression and reactive nitrogen species. Neurology. 45: S16-S21; 1995.

Brown K, Vora A, Biggerstaff J, Edgell J, Oikle S, Mazure G, Taub N, Meager A, Hill T, Watson C. Application of an immortalized human endothelial cell line to the leukocyte: endothelial adherence assay. J. Immunol. Meth. 163: 13-22; 1993.

Burns C, Tsai V, Zvaifler N. High percentage of CD8+, Leu-7+ cells in rheumatoid arthritis synovial fluid. Arthritis Rheum. 35: 865-873, 1992.

Butcher E. Leukocyte-endothelial cell recognition: three (or more) steps in specificity and diversity. Cell: 67: 1033-1036; 1991

Calabresi P, Pelfrey C, Tanquill L, Maloni H, McFarland H. VLA-4 expression on peripheral blood lymphocytes is downregulated after treatment of multiple sclerosis with interferon beta. Neurology. 49: 1111-1116; 1997.

Campbell J, Hedrick J, Zlotnik A, Siani M, Thmpson D, Butcher E. Chemokines and the arrest of lymphocytes rolling under flow conditions. Science. 279: 381-384; 1998.

Cannella B, Cross A, Raine C. Anti-adhesion molecule therapy in experimental autoimmune encephalomyelitis. J. Neuroimm. 46: 43-55; 1993.

Canella B, Raine C. The adhesion molecule and cytokine profile of multiple sclerosis. Ann. Neurol. 37: 424-435; 1995.

Capron M, Kazatchkine M, Fischer E, Butterworth A, Joseph M, Kusnierz J, Prin L, Papin J, Capron A. Functional role of the a-chain of complement receptor type-3 in human eosinophil-dependent antibody mediated cytotoxicity against schistosomes. J. Immunol. 139: 2059-2065; 1987.

Carley W, Milici A, Madri A. Extracellular matrix specificity for the differentiation of capillary endothelial cells. Exp. Cell Res. 178: 422-434; 1988.

Carlos T, Harlan J. Leukocyte-endothelial adhesion molecules. Blood. 84: 2068-2101; 1994.

Ceroni M, Piccardo P, Rogers-Johnson P, Mora C, Asher D, Gajdusek C, Gibbs C. Interathecal synthesis of IgG antibodies to HTLV-I supports an etiological role for HTLV-I in tropical spastic paraparesis. Ann. Neurol. 23: S188-S191; 1988.

Challoner P, Smith K, Parker J, et al. Plaque-associated expression of human herpesvirus 6 in multiple sclerosis. Porc. Natl. Acad. Sci. USA. 92: 7440-7444; 1995.

Chang J, Weiner H, Hafler D. Autoreactive T cells in multiple sclerosis. Int. Rev. Immunol. 9: 183-201; 1992.

Chen Y, Kuchroo V, Inobe J, Hafler D, Weiner H. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. Science. 265: 1237-1240; 1994.

Chofflon M, Juillard C, Juillard P, Gauthier G, Grau G. Tumor necrosis factor alpha production as a possible predictor of relapse in patients with multiple sclerosis.Euro. Cytokine Netw. 3: 523-531; 1992.

Clark E, Brugge J. Integrins and signal transduction pathways: the road taken. Science. 268: 233-239; 1995.

Coons A, Kaplan M. Localization of antigen in tissue cells: Improvement in a method for the detection of antigen by means of fluorescent antibody. J. Exp. Med. 91: 1-10; 1950.

Corley R, LoCascio N, Ovnic M, Haughton G. Two separate functions of class II Ia molecules: T cell stimulation and B cell excitation. Proc. Natl. Acad. Sci. USA. 82: 516-520, 1985.

Correale J, Gilmore W, McMillan M, Li S, McCarthy K, Le T, Weiner LP. Patterns of cytokine secretion by autoreactive proteiolipid proteins-specific T cell clones during the course of multiple sclerosis. J. Immunol. 154: 2959-2968; 1995.

Corsini E, Gelati M, Dufour A, Massa G, Nespolo A, Ciusani E, Milanese C, La Manita L, Salmaggi A. Effects of β-IFN-1b treatment in MS patients on adhesion between PBMNC, HUVEC and MS-HBEC: and in vivo and in vitro study. J. Neuroimmunol. 79: 76-83; 1997.

Cserr H, Knopf P. Cervical lymphatics, the blood-brain barrier and the immunoreactivity of the brain: a new view. Immunol Today. 13: 507-12; 1992.

Dal Canto M, Melvolv R, Kim B, Miller S. Two models of multiple sclerosis: Experimental allergic encephalomyelitis (EAE) and Theiler's murine encephalomyelitis virus (TEMV) infection. A pathological and immunological comparison. Micro. Res. Tech. 32: 215-229; 1995.

Dao T, Iwaki K, Taeuchi M, Oahshi K, Fukuda S, Kurimoto M. Natural human interferon-α inhibits the adhesion of human carcinoma cell line to human vascular endothelium. J. Interferon. Cytokine Res. 15: 869-876; 1995.

Davignon D, Martz E, Reynolds T, Kurzinger K, Springer T. Monoclonal antibody to a novel lymphocyte function-associated antigen (LFA-1): mechanism of blocking of T lymphocyte-mediated killing and effects on other T and B lymphocyte function. J. Immunol. 127: 590-595; 1981.

De Jong R, Brouwer M, Hooibrik B, Pouw-Kraan T, Miedema F, van Lier R. The CD27- subset of peripheral blood memory CD4+ lymphocytes contains functionally differentiated T lymphocytes that develop by persistent antigenic stimulation in vivo *Eur.* J. Immunol. 22: 993-999; 1992.

Dekaban G, Oger J, Foti D, King E, Waters D, Picard F, Arp J, Werker D, Rice G. HTLV-1 associated with disease in Aboriginal Indians from British Columbia: a serological and PCR analysis. Clin. Diagn. Virol. 2:67-78; 1993.

Dhawan S, Weeks S, Abbasi S, Gralnick H, Notkins A, Klotman M, Yamada K, Klotman P. Increased expression of α4β1 and α5β1 integrins on HTLV-I-infected lymphocytes. Virology. 197: 778-781; 1993.

Dhib-Jalbut S, Jiang H, Williams G. The effect of interferon-β-1b on lymphocyte-endothelial cells adhesion. J. Neuroimmunol. 71: 215-222; 1996.

Dirks W, MacLeod R, Drexler H. ECV-304 (endothelial) is really T24 (bladder carcinoma): cell line crosscontamination at source. In vitro cell. Dev. Biol.-Animal. 35: 558-559; 1999.

Dobbie M, Hurst R, Klein N, Surtees R. Upregulation of intercellular adhesion molecule-1 expression on human endothelial cells by tumor necrosis factor-α in an in vitro model of the blood-brain barrier. Brain research. 830: 330-336; 1999.

Dolman D, Anderson P, Rollinson C, Abbott N. Characterization of a new in vitro model of the blood-brain barrier (BBB). J. Physiol. 505: 56-57; 1997.

Dore-Duffy P, Newman W, Balabanov R. Lisak R, Mainolfi E, Rothlein R, Peterson M. Circulating, soluble adhesion proteins in cerebrospinal fluid and serum of patients with multiple sclerosis: correlation with clinical activity. Ann. Neurol. 37: 55-62; 1995.

Dorovini-Zis K, Prameya R, Bowman P. Culture and characterization of microvascular endothelial cells derived from human brain. Lab. Invest. 64: 425-436; 1991.

Doukas J, Pober J. IFN-γ enhances endothelial activation induced by TNF-α but not IL-1. J. Immunol. 145: 1727-1733; 1990.

Droogan A, McMillan S, Douglas J, Hawkins S. Serum and cerebrospinal fluid levels of soluble adhesion molecules in multiple sclerosis: predominant intrathecal release of vascular adhesion molecules-1. J. Neuroimmunol. 64: 185-191; 1996.

Drulovic J, Mostarica-Stojkovic M, Levic Z, Stojsavljevic N, Pravica V, Mesaros S. Interleukin-12 and tumor necrosis factor-a levels in cerebrospinal fluid of multiple sclerosis patients. J. Neurol. Sci. 147: 145-150; 1997.

Duquette P, Pleines J, Girard M, Charest L, Senecal-Quevillon M, Masse C. The increased susceptibility in women to multiple sclerosis. . Can. J. Neurol. Sci. 19: 466-471; 1992.

Dustin M, Springer T. T cell receptor crosslinking transiently stimulates adhesiveness through LFA-1. Nature. 341: 619-624; 1989.

Dustin ML, Springer TA. Role of lymphocyte adhesion receptors in transient interactions and cell locomotion. Annu Rev Immunol. 9:27-66; 1991.

Elovaara, I., Koenig, A., Brewah, R., Woods, R., Lehky, T. Jacobson, S. High human T-cell lymphotropic virus type I (HTLV-I)-specific precursor cytotoxic T lymphocyte frequencies in patients with HTLV-I-associated neurological disease. J. Exp. Med. 177: 1567-1573, 1993.

Estess P, Nandi A, Mohmadzadeh M, Siegelman M. Interleukin 15 induces endothelial hyaluronan expression in vitro and promotes activated T cell extravasation through a CD44-dependent pathway in vivo. J. Exp. Med. 190: 9-19; 1999.

Etzioni A, Frydman M, Pollack S, Avidor I, Phillips M, Paulson J, Gershoni-Baruch R. Recurrent severe infections caused by a novel leukocyte adhesion deficiency. N. Engl. J. Med. 327: 1789-1792; 1992.

European Study Group on interferon b-1b in secondary progressive MS. Placebo-controlled multicentre randomized trial of interferon b-1b in treatment of secondary progressive multiple sclerosis. Lancet. 352: 1491-1497; 1998.

Fauci A, Whalen G, Burch C. Activation of human B lymphocytes. Cellular requirements, interactions and immunoregulation of pokeweed-induced total-immunoglobulin producing plaque-forming cells in peripheral blood. Cell. Immunol. 54: 230-240; 1980.

Frelinger A, Lam S, Plow E, Smith M, Loftus J, Ginsberg M. Occupancy of an adhesive glycoprotein receptor modulates expression of an antigenic site involved in cell adhesion. J Biol Chem. 263:12397-12402; 1988.

Fukodome K, Furuse M, Fukuhara N, Orita S, Imai T, Takagi S, Nagira N, Hinuma Y, Yoshie O. Strong induction of ICAM-1 in human T cells transformed by human T-cell-leukemia virus type-1 and exression of ICAM-1 or LFA-1 in adult T cell leukemia-derived cell lines. Int. J. Cancer. 52: 418-427; 1992.

Gamble J, Vdas M. Endothelial cell adhesiveness for human T lymphocytes in inhibited by transforming growth factor-β. J. Immunol. 146: 1149-1154; 1991.

Gessain A, Barin F, Vernant J, Gout O, Calender A. Antibodies to human T lymphotropic virus type-1 in patients with tropical spastic paraparesis. Lancet. 2: 407-409; 1985.

Gessain A. Epidemiology of HTLV-I and associated disease. IN: Hollsberg P, Hafler D. ed. Human T cell lymphotropic virus type I. Chichester: Wiley & Sons, 33-64; 1996.

Gelati M, Corsini E, Dufour A, Massa G, La Manita L, Milanese C, Nespolo A, Salmaggi A. Immunological effects of in vivo interferon-b-1b treatment in ten patients with multiple sclerosis: a 1-year follow up. J. Neurol. 246: 569-573; 1999.

Genain C, Nguyen M, Letvin N, Pearl R, Davis R, Adelman M, Lees M, Linington C, Hauser S. Antibody facilitation of multiple sclerosis-like lesions in a nonhuman primate. J. Clin. Invest. 96: 2966-2974; 1995.

Geroni M, Piccardo P, Rodgers-Johnson P, Mora C, Asher D, Gajdusek D, Gibbs C. Ann. Neurol. Intrathecal synthesis of IgG antibodies to HTLV-I supports an etiological role for HTLV-I in tropical spastic paraparesis. 23: S188-S191; 1988.

Giraudon P, Buart S, Bernard A, Thomasset N, Belin M. Extracellular matrix-remodeling metalloproteinases and infection of the central nervous system with retrovirus human T-lymphotropic virus type I (HTLV-I). Prog. Neurobiol. 49: 169-84; 1996.

Glynn P, Linington C. Cellular and molecular mechanisms of autoimmune demyelination in the central nervous system. Crit. Rev. Neurobiol. 4: 367-387; 1989.

Godoy A, Kira J, Hasuo K, Goto I. Characterization of cerebral white matter lesions of HTLV-I-associated Myelopathy/tropical spastic paraparesis in comparison with multiple sclerosis and collagen-vasculitis: a smiquantative MRI study. J. Neurol. Sci. 133: 102-111; 1995.

Goldenberg P, Kwon E, Benjamins J, Whitaker J, Quarles R, Prineas J. Opsonization of normal myelin by anti-myelin antibodies and normal serum. J. Neuroimmunol. 23: 157-:; 1989.

Goldstein G, Betz A. Recent advances in understanding brain capillary function. Ann. Neurol. 14: 389-395; 1983.

Goodwin R, Alderson M, Smith C, Armitage R, VandenBos T, Jerzy R, Tough T, Shoenborn M, Davis-Smith T, Hennen K. Molecular and biological characterization of a ligand for CD27 defines a new family of cytokines with homology to tumor necrosis factor. Cell. 73: 447-456; 1993.

Goust J, Hogan E, Arnaud P. Abnormal regulation of IgG production in multiple sclerosis. Neurology. 32: 228-234; 1982.

Gran B, Hemmer B, Vergelli M, McFarland HF, Martin R Molecular mimicry and multiple sclerosis: degenerate T-cell recognition and the induction of autoimmunity. Ann Neurol. 45: 559-67; 1999.

Greenwood J, Wang Y, Calder V. Lymphocyte adhesion and transendothelial migration in the central nervous system: the role of LFA-1, ICAM-1, VLA-4 and VCAM-1. Immunology. 86: 408-415; 1995.

Hafler D, Fox D, Manning M, Schlossman S, Reinherz E, Weiner H. In vivo activated T lymphocytes in the peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. N. Eng. J. Med. 312: 1405-1411; 1985.

Hafler D, Weiner H. MS: a CNS and systemic autoimmune disease. Immunol. Today. 10: 104-107; 1989.

Hara T, Jung L, Bjorndahl J, Fu S. Rapid induction of a phosphorylated 28 kD/32 kD disulfide-linked early activation antigen (EA 1) by 12-o-tetradeanoyl phorbol-13-acetate, mitogens, and antigens. J. Exp. Med. 164: 1988-2005; 1986.

Hartung H, Hughes R, Taylor W, Heininger K, Reiners K, Toyka K. T cell activation in Guillain-Barré syndrome and in MS: elevated serum levels of soluble IL-2 receptors. Neurology, 40: 215-218; 1990.

Hartung H, Reiners K, Archelos J, Michels M, Seeldrayers P, Heidenreich F, Pflungupt K, Toyka K. Circulating adhesion molecules and tumor necrosis factor receptor in multiple sclerosis: correlations with magnetic resonance imaging. Ann. Neurol. 38: 186-193; 1995.

Hauser S, Ault K, Johnson D, Hoban C, Weiner H. Increased IgG secretion by unstimulated mononuclear cells in active multiple sclerosis and functional assessment of the T8 subset. Clin. Immunol. Immunopathol. 37: 312-323; 1985.

Hess D, Bhutwala T, Sheppard J, Zhao W, Smith J. ICAM-1 expression on human brain microvascular endothelial cells. Neurosci. Lett. 168: 201-204; 1994.

Hickey W, Hsu B, Kimura H. T lymphocyte entry into the central nervous system. J. Neurosci. Res. 28: 254-260; 1991.

Hintzen R, de Jong R, Lens S, Brouwer M, Baars P, van Lier R. Regulation of CD27 expression on subsets of mature T-lymphocytes. J. Immunol. 151: 2426-2435, 1993.

Hintzen R, Paty D, Oger J. Cerebrospinal fluid concentrations of soluble CD27 in HTLV-I associated myelopathy and multiple sclerosis. J Neurol Neurosurg Psychiatry. 66:791-793; 1999.

Ho H, Hultin L, Mitsuyasu R, Matud J, Hausner M, Bockstoce D, Chou C, O'Rouke S, Taylor J, Giorgi J. Circulating HIV-specific CD8 cytotoxic T cells express CD38 and HLA-DR antigens. J. Immunol. 150: 3070-3079; 1993.

Hofman F, von Hanwehr R, Dinrello C, Mizel S, Hinton D, Merrill J. Immunoregulatory molecules and IL-2 receptors identified in multiple sclerosis brain. J. Immunol. 136: 3239-3245; 1986.

Hogg N, Landis R. Adhesion molecules in cell interactions. Curr. Opin. Immunol. 5: 383-390; 1993.

Hughes S. Functional characterization of the spontaneously transformed human umbilical vein endothelial cell line ECV-304: use in an in vitro model of angiogenesis. Exp. Cell Res. 225: 171-185; 1996.

Hughes C, Male D, Lantos L. Adhesion of lymphocytes to cerebral microvascular cells: effects of interferon-gamma, tumour necrosis factor and interleukin-1. Immunology. 64: 677-81; 1988.

Huitanga I, Damoiseaux J, Dopp E, and Dijkstra C. Treatment with anti-CR3 antibodies ED7 and ED8 suppresses experimental allergic encephalomyelitis in Lewis rats. Eur. J. Immunol. 23: 709-715; 1993.

Huynh H, Oger J, Dorovini-Zis K. Interferon-β downregulates interferon-γ-induced class II MHC molecules expression and morphological changes in primary culture of human brain microvessel endothelial cells. J. Neuroimmunol. 60: 63-73; 1995.

Ichinose K, Nakamura T, Kawakami A, Egyuchi K, Nagasato K, Shibayama K, Tsujihata M, Nagataki M. Increased adherence of T cells to human endothelial cells in patients with human T-cell lymphotropic virus type I-associated myelopathy. Arch. Neurol. 49: 74-76; 1992.

Ijichi S, Eiraku N, Osame M, Izumo S, Kubota R, Maruyama I, Matsumoto M, Niimura T, Sonoda S. Activated T lymphocytes in cerebrospinal fluid of patients with HTLV-I-associated myelopathy (HAM/TSP). J. Neuroimmunol. 25: 251-254; 1989.

Imai T, Hieshima K, Haskell C, Baba M, Nagira M, Nishimura M, Kakizaki M, Takagi S, Nomiyama H, Schall T, Yoshie O. Identification and molecular characterization of fractalkine receptor CXCR1, which mediates both leukocyte migration and adhesion. Cell. 91: 521-530; 1997.

Imai T, Tanaka Y, Fukudome K, Takagi S, Araki K, Yoshie O. Enhanced expression of LFA-3 on human T cell lines and leukemic cells carrying human T-cell leukemia virus type-1. Int. J. Cancer. 55: 811-816; 1993.

Imhof B, Dunon D. Leukocyte migration and adhesion. Adv Immunol. 58: 345-415; 1995.

Ishikawa T, Imura A, Tanaka K, Shirane H, Okuma M, Uchiyama T. E-selectin and vascular cell adhesion molecule-1 mediate adult T-cell leukemia cell adhesion to endothelial cells. Blood. 82: 1590-1598; 1993.

Itoyama Y, Minato S, Kira J, Goto I, Sato H, Okochi K, Yamamoto N. Altered subset of peripheral blood lymphocytes in patients with HTLV-1-associated myelopathy (HAM). *Neurology*. 38: 816-818; 1988a.

Itoyama Y, Minato S, Kira J, Goto I, Sato H, Okochi K, Yamamoto N. Spontaneous proliferation of peripheral blood lymphocytes increased in patients with HTLV-1-associated myelopathy. Neurology. 38: 1302-1307; 1988b.

Iwasaki Y, Ohara Y, Kobayashi I, and Akizuki I. Infiltration of helper/inducer T lymphocytes heralds central nervous system damage in human T –cell leukemia virus infections. Am. J. Pahol. 140: 1003-1008; 1992.

Jacobs L, Cookfair D, Rudick R, Herndon RM, Richert JR, Salazar AM, Fischer JS, Goodkin DE, Granger CV, Simon JH, Alam JJ, Bartoszak DM, Bourdette DN, Braiman J, Brownscheidle CM, Coats ME, Cohan SL, Dougherty DS, Kinkel RP, Mass MK, Munschauer FE 3rd, Priore RL, Pullicino PM, Scherokman BJ, Whitham RH., Intramuscular interferon β-1a for disease progression in relapsing multiple sclerosis. Ann. Neurol. 39: 285-294; 1996.

Jacobson S, Zainovic V, Mora C, Rodger-Johnson P, Sheremato W, Gibbs C, Gadjusek C, McFarlin D. Immunological findings in neurological diseases associated with antibodies to HTLV-1: activated lymphocytes in tropical spastic paraparesis. *Ann. Neurology*. 23: S196-200; 1988.

Jacobson S, Shida H, McFarlin D, Fauci A, Koeing S. Circulating CD8+ cytotoxic T lymphocytes specific for HTLV-I pX in patients with HTLV-I associated neurological disease. Nature. 348: 245-248, 1990a.

Jacobson S, Gupta A, Mattson D, Mingioli E, McFarlin D. Immunological studies in tropical spastic paraparesis. Ann. Neurol. 27: 149-156; 1990b.

Jacobson S, McFarlin D, Robinson S, Voskuhl R, Martin R, Brewah A, Newell A, Koenig S. Demonstration of HTLV-I specific cytotoxic T-lymphocytes in the cerebrospinal fluid of patients with HTLV-I-associated neurological disease. Ann. Neurol. 32: 651-657; 1992.

Jaffe E, Nachman R, Becker C. Minick R. Culture of human endothelial cells derived from umbilical veins: identification by morphologic and immunological criteria. J. Clin. Invest. 52: 2735-2756; 1973.

James S, Neckers L, Graeff A, Cossman J, Balch C, Strober W. Suppression of immunoglobulin synthesis by lymphocyte subpopulations in patients with Crohn's disease. Gastroenterology. 86: 1510-1518; 1984.

Johnson B, Issekutz T, Kubes P. The alpha 4-integrin supports leukocyte rolling and adhesion in chronically inflamed postcapillary venules in vivo. J. Exp. Med. 183: 1995-2006; 1996.

Johnson K, Nelson B. Multiple sclerosis: diagnostic usefulness of cerebrospinal fluid. Ann Neurol. 2: 425-431; 1977.

Joly P, Guillon J, Mayaud C, Plata F, Theodorou I, Denis M, Debre P, Autrant B. Cell-mediated suppression of HIV-specific cytotoxic T lymphocytes. J. Immunol. 143: 2193-2001; 1989.

Joo F. The blood-brain barrier in vitro: the second decade. Neurochem. Int. 23: 499-521; 1993.

Jung T, Dailey M. Rapid modulation of homing receptors (gp90Mel-14) induced by activation of protein kinase C. Receptors shedding due to accelerated proteolytic cleavage at the cell surface. J. Immunol. 144: 3130-3136; 1990.

Kansas G. Selectins and their ligands: current concept and controversies. Blood. 88: 3259-3287; 1996.

Kaplan J, Osame M, Kubota H. The risk of development of HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP) among persons infected with HTLV-I. J. Acquir. Immune. Defic. Syndr. 3: 1096-1001; 1990.

Karpas A, Kampf U, Siden A, Koch M, Poser S. Lack of evidence for involvements of know human retroviruses in multiple sclerosis. Nature. 322: 177-178; 1986.

Kelly R, Ellison G, Myers L, Goymerac V, Tarrick S, Kelly C. Abnormal regulation of in vitro IgG production in multiple sclerosis. Ann. Neurol. 9: 267-272; 1981.

Kermode A, Thompson A, Tofts P, et al., Breakdown of the blood-brain barrier precedes symptoms and other MRI signs of new lesions in multiple sclerosis. Pathogenetic and clinical implications. Brain. 113: 1477-1489; 1990.

Kira J, Tobimatsu S, Goto I, Hasuo. Primary progressive versus relapsing remitting multiple sclerosis patients in Japanese patients: a combined clinical, magnetic resonance imaging and multimodality evoked potential study. J. Neurol. Sci. 117: 179-185; 1993.

Kishimoto T, Jutila M, Berg E, et al. The neutrophil Mac-1 and MEL-14 glycoproteins are inversely regulated by chemotactic factors. Science 245: 1238-1241; 1989.

Kohem C, Brezinschek R, Wisbey H, Tortorella C, Lipsky P. Enrichment of differentiated CD45RB, CD27- memory T cells in the peripheral blood, synovial fluid, and synovial tissue of patients with rheumatoid arthritis. Arthr. Rheum. 139: 844-854, 1996.

Kotovuori P, Tontti E, Pigott R, Shepherd M, Kiso M, Hasegawa A, Renkonen R, Nortamo P, Altieri DC, Gahmberg CG.. The vascular E-selectin binds to the leukocyte integrins CD11/CD18. Glycobiology. 3: 131-136; 1991.

Krensky A, Sanchez-Madrid F, Robbins E, Nagy J, Springer T, Burakoff S. The functional significance, distribution, and structure of LFA-1, LFA-2, and LFA-3: cell surface antigens associated with CTL-target interactions. J. Immunol. 131: 611-616; 1983.

Kuchler-Bopp S, Delaunoy J, Artault J, Zaepfel, Dietrich J. Astrocytes induce several blood-brain barrier properties in non-neural endothelial cells. NeruroReport. 10: 1347-1353; 1999.

Kuijpers T, Hoogerwerf M, Van der Laan L, Nagel G, van der Schoot C, Grunert F, Roos D. CD66 nonspecific cross-reacting antigens are involved in neutrophil adherence to cytokine-activated endothelial cells. J. Cell Biol. 118: 457-466; 1992.

Kurtzke J. Disability rating scales in multiple sclerosis. Ann. NY. Acad. Sci. 346: 347-360; 1984.

Landay A, Bauer K. Flow cytometric analysis of cells of the immune system. J. Immunol. Immunopharmacol. 8: 166-171; 1988.

Larson R, Springer T. Structure and function of leukocyte integrins. Immunol. Rev. 114: 181-217; 1990.

Lassmann H, Raine C, Antel J, Prineas J. Immunopathology of multiple sclerosis: Report on an international meeting held at the institute of neurology of the university of Vienna. J. Neuroimmunol. 86: 213-217; 1998.

Lawrence M, Springer T. Leukocyte roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. Cell. 65: 859-873; 1991.

Lehmann P, Forsthuber T, Miller A, Sercarz E. Spreading of T-cell autoimmunity to cryptic determinants of autoantigen. Nature. 558: 155-157; 1992.

Leppert D, Waubant E, Burk M, Oksenberg J, Hauser S. Interferon Beta-1b inhibits gelatinase secretion and in vitro migration of human T cells: a possible mechanism for treatment efficacy in multiple sclerosis. Ann. Neurol. 40: 846-852; 1996.

Leroy, E., Calvo, C., Divine, M., Gourdin, M., Baujean, F., Ben Ariba, M., Mishal, Z., Vernant, J., Farcet, J., and Senik, S. Persistence of surviving patients after allogeneic bone morrow transplantation. J. Immunol. 137: 2180-2189; 1986.

Levin M, Jacobson S. HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP): a chronic progressive neurological disease associated with immunologically mediated damage to the central nervous system. J. Neurovirol. 3: 126-140; 1997.

Levinson A, Sandberg-Wollheim M, Lisak R, Zweiman B, Sjogren K, Laramore C, Moskovitz A, Hedstrom S. Analysis of B-cell activation of cerebrospinal fluid lymphocytes in multiple sclerosis. Neurology. 33:1305-10; 1983.

Levitt D, Griffin N, Egan M. Mitogen-induced plasma cell differentiation in patients with multiple sclerosis. J. Immunol. 124: 2117-2121; 1980.

Lewis D, Puck J, Babock G, Rich R. Disproportionate expansion of a minor T cell subset in patients with lymphoadenopathy syndrome and acquired immunodeficiency syndrome. J. Infect. Dis. 151: 555-559; 1985.

Lindington E, Moyes L, McCormack A, Rose M. A comparison of primary endothelial cells and endothelial cell lines for studies of immune interactions. Transpl Immunol. 7: 239-246; 1999.

Link H, Cruz M, Gessain A, Gout O, Kam-Hansen S. Chronic progressive Myelopathy associated with HTLV-I: Oligoclonal IgG and anti-HTLV-I IgG antibodies in the cerebrospinal fluid and serum. Neurology. 39: 1566-1572; 1989.

Link J, Soderstorm M, Olsson T, Hojeberg B, Ljungdahl A, Link H. Increased transforming growth factor b, increased IL-4, and interferon-g in multiple sclerosis. Ann. Neurol. 36: 379-386; 1994.

Lou J, Dayer M, Grau E, Burger D. Direct cell/cell contact with stimulated T lymphocytes induces the expression of cells adhesion molecules and cytokines by human brain microvascular endothelial cells. Eur. J. Immunol. 26: 3107-3113; 1996.

Lou J, Chofflon M, Juillard C, Donati Y, Mili N, Siergrist C, Grau G. Brain microvascular endothelial cells and leukocytes derived from patients with multiple sclerosis exhibit increased adhesion capacity. NeuroReport. 8: 629-633; 1997.

Lou J, Yvan G, Zheng L, Giroud C, Morel P, Clements J, Ythier A, Grau G. Interferon-β inhibits activated leukocyte migration through human brain microvascular endothelial cell monolayer. Lab. Invest. 79: 1015-1025; 1999.

Lublin F, Reingold S. Defining the clinical course of multiple sclerosis: Results of an international survey. Neurology, 46: 907-911; 1996.

Lublin F. A phase II trial of anti-CD11/CD18 monoclonal antibody in acute exacerbation of multiple sclerosis. Neurology. 52(suppl 2): A290; 1999.

Lucchinetti C, Bruck W, Rodriguez M, Lassmann H. Distinct patterns of multiple sclerosis pathology indicates heterogeneity in pathogenesis. Brain Pathol. 6: 259-274; 1996.

Luster A. Chemokines-chemotactic cytokines that mediate inflammation. New Engl. J. Med. 338: 436-445; 1998.

Maher P, O'Toole C, Wreghitt T, Spiegelhalter D, English T. Cytomegalovirus infection in cardiac transplant recipients associated with chronic T cell subset ratio inversion with expansion of a Leu-7+ TS-C+ subset. Clin. Exp. Immunol. 62: 515-524; 1985.

Mainolfi L, Rothlein R. Elevated levels of sICAM-1 in patients with human T-cell leukemia virus type I associated myelopathy and adult T-cell leukemia. Blood. 80: 2434-2435; 1992.

Makgoba M, Sanders M, Shaw S. The CD2-LFA-3 and LFA-1-ICAM-1 pathways: relevance to T-cell recognition. Immunol. Today. 10: 417-422; 1989.

Male K, Pryce G. Kinetics of MHC gene expression and mRNA synthesis in brain endothelium. Immunology. 63: 37-42; 1988.

Maloney E, Cleghorn F, Morgan O. Incidence of HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP) in Jamaica and Trinidad. J Acquir Immune Defi Syndr Human Retrovirol. 17: 167-170; 1998

Mantovani A, Bussolino F, Dejana E. Cytokine regulation of endothelial cell function. FASEB J. 6: 2591-2599; 1992.

Marrosu M, Muntoni F, Murru M Spinicci G, Pischedda MP, Goddi F, Cossu P, Pirastu M. Sardinian multiple sclerosis is associated with HLA-DR4. Neurology, 38: 1749-1753; 1988.

Martin R., Hohlfeld R., McFarland H. Infections and inflammatory diseases: multiple sclerosis. In: Brandt T, Caplan R, Dichgans J, eds. Neurologic disorder course and treatment. New York: Academic Press, 483-505; 1996.

Martin R, McFarland H, McFarlin D. Immuological aspects of demyelinating diseases. Ann. Rev. Immunol. 10: 153-187; 1992.

Martz E. LFA-1and other accessory molecules functioning in adhesions of T and B lymphocytes. Hum. Immunol. 18: 3-10; 1987.

Massacesi L, Genain C, Lee-Parritz D, Canfield D, Hauser S. Active and passively induced experimental autoimmune encephalomyelitis in common marmosets: a new model for multiple sclerosis. Ann. Neurol. 37: 519-530; 1995.

Matsuda M, Tsukada N, Miyagi K, Yanagisawa N. Increased levels of soluble vascular cell adhesion molecule-1 (VCAM-1) in the cerebrospinal fluid and sera of patients with multiple sclerosis and human T lymphotropic virus type-1-associated myelopathy. J. Neuroimmunol. 59: 35-40; 1995a.

Matsuda M, Tsukada N, Miyagi K, Yanagisawa N. Adhesion of lymphocytes to endothelial cells in experimental allergic encephalomyelitis before and after treatment with endotoxin lipopolysaccharide. Int. Arch. Allergy Immunol. 106: 335-344; 1995b.

McFarland H. The multiple sclerosis lesion. Ann. Neurol. 77: 419-420; 1995.

McLean B, Zeman A, Barnes D, Thompson E. Pattern of blood-brain barrier impairment and clinical features in multiple sclerosis. J. Neurol. Nerurosurg. Psychiatry. 56: 356-360; 1993.

Merrill J, Benveniste E. Cytokines in inflammatory brain lesions: helpful and harmful. Trends Neurosci. 19: 331-338; 1996.

Miedema F, Tetteroo P, Terpstra F, Kiezer G, Roos M, Weemomg R, Weemacs C, Roos D, Melief C. Immunological studies of LFA-1 and Mo1-deficient lymphocytes from patients with recurrent bacterial infections. J. Immunol. 34: 3075-3081; 1985.

Ming Wang J, McVicar D, Oppenheim J, Kelvin D. Identification of RANTES receptors on human monocytic cells: competition for binding and characterization by homologous chemotactic cytokines. J. Exp. Med. 177: 669-705; 1993.

Mobner R, Fassbender K, Kuhnen J, Schwartz A, Hennerici M. Circulating L-selectin in multiple sclerosis patients with active, gadolinium-enhancing brain plaques. J. Neuroimmunol. 65: 61-65; 1996.

Mokhtarian F, McFarlin D, Raine C. Adoptive transfer of myelin basic protein-sensitized T cells produces chronic relapsing demyelinating disease in mice. Nature. 309: 356-358; 1984.

Monteyne P, Bureau J, Brabic T. Viruses and multiple sclerosis. Curr. Opin. Neurol. 11: 287-291; 1998.

Moore G, Traugott U, Scheinberg L, Raine C. Tropical spastic paraparesis: a model of virus induced, cytotoxic mediated demyelination ? Ann Neurol. 26: 523-530; 1989.

Mori M, Kinoshita K, Ban N, Yamada Y, Shiku H. Activated T lymphocytes with polyclonal gammopathy in patients with human T-lymphotropic virus type I-associated myelopathy. Ann. Neurol. 24: 280-282; 1988.

Morimoto C, Letvin N, Boyd A, Hagan M, Brown H, Kornacki M, Schlossman S. The isolation and characterization of the human suppressor-inducer T cell subset. J. Immunol. 134: 3762-3769, 1989.

Mosley K, Cuzner M. Receptor-mediated phagocytosis of myelin by macrophages and microglia: effects of opsonization and receptor blocking agents. Neurochem. Res. 21: 481-487; 1996.

Mosmann T, Moore K. The role of IL-10 in cross-regulation of Th1 and Th2 responses. Immunol. Today. 12: A49-A53; 1991.

Mukae H, Kohno S, Nobuo M, Morikawa N, Kadota J, Matsukura S, Hara K. Increase in T-cells bearing CD25 in bronchoalveolar lavage fluid from HAM/TSP patients and HTLV-I carriers. Microb. Immunol. 38: 55-62; 1994.

Mulligan M, Varani J, Warren J, Till G, Smith C, Anderson D, Todd R, Ward P. Roles of β2 integrins of rat neutrophils in complement-and oxygen radical-mediated acute inflammatory injury. J. Immunol. 148: 1847-1857; 1992.

Nagai M, Yashiki S, Fujiyoshi T, Fujiyama C, Kitze B, Izumo S, Osame M, Sonaoda S. Characterization of a unique T-cell clone established from a patient with HAM/TSP which recognized HTLV-I-infected T-cell antigens as well as spinal cord tissue antigens. J. Neuroimmunol. 65: 97-106; 1996.

Nakagawa M, Izumo S, Ijichi S, Kubota J, Arimura K, Kawabata M, Osame M. HTLV-I associated Myelopathy: Analysis of 213 patients on clinical features and laboratory findings. J. Neurovirol. 1: 50-61; 1995.

Nilsen E, Johansen F, Jahnsen F, Lundin K, Scholz T, Brandtzaeg P, Haraldsen G. Cytokine profiles of cultured mircovascular endothelial cells from the human intestine. Gut. 42: 635-642; 1998.

Nishiura Y, Nakamura T, Ichinose K, Nagasato K, Ohishi R, Watanabe H, Tsujihata M, Nagataki M. Antiproliferative factor against the human glioblastoma cell line T98G identified in culture supernatant of CD4+ cells from patients with HTLV-1-associated myelopathy. J. Neurol. Sci. 122: 214-219; 1994.

Noronha A, Richman D, Arnason B. Detection of in vivo stimulated cerebrospinal fluid lymphocytes by flow cytometry in patients with multiple sclerosis. N. Eng. J. Med. 303: 713-717; 1980.

O'Gorman M, Oger J, Kastrukoff L. Reduction of immunoglobulin G secretion in vitro following long term lymphoblastoid interferon (Wellferon) treatment in multiple sclerosis patients. Clin. Exp. Immunol. 67: 66-75; 1987.

Oen K, Danell G, Stewart S, Wilkins J, Tazumi K, Jacobson K. Adhesion of peripheral blood lymphocytes of children with arthritis to human umbilical vein endothelial cells. Clin. Exp. Immunol. 95: 415-423; 1994.

Oger J, Dekaban G. HTLV-I associated myelopathy: A case of viral-induced autoimmunity. Autoimmunity. 21: 151-159; 1995.

Oger J, Arnason B, Pantazis N, Lehrich J, Young M. Synthesis of nerve growth factor by L and 3T3 cells in culture. Proc. Natl. Acad. Sci. USA. 71: 1554-1558; 1974.

Oger J, Mattson D, Roos R, Antel J, Arnason B. Isoelectric focusing of IgG secreted by human peripheral blood lymphocytes in Multiple Sclerosis and controls. Neurol. 31: 144; 1981.

Oger J, O'Gorman M, Willoughby E, Li D, Paty D. Changes in immune function in relapsing multiple sclerosis correlate with disease activity as assessed by magnetic resonance imaging. Ann. N. Y. Acad Sci. 540:597-601; 1988

Oger J, Werker D, Foti D, Dekaban G. HTLV-1-associated myelopathy: an endemic disease of Canadian Aboriginal of the Northwest Pacific. Can. J. Neuro. Sci. 20: 302-306; 1993.

Oi V, Glazer A, Stryer L. Fluorescent phycobiliprotein conjugates for analyses of cells and molecules. J Cell Biol. 93: 981-986; 1982.

Oldstone M. Molecular mimicry and autoimmune disease. Cell., 50: 819-820; 1987
Olsson T, Baig S, Hojeberg B, Link H. Animyelin basic protein and antimyelin antibody-producing cells in multiple sclerosis. Ann. Neurol. 27: 132-136; 1990.

Olsson T. Critical influences of the cytokine orchestration on the outcome of myelin antigen-specific T-cell autoimmunity in experimental autoimmune encephalomyelitis and multiple sclerosis. Immunol. Rev. 144: 245-268; 1995.

Oppenheimer-Marks N, Davis L, Boque D, Ramberg J, Lipsky P. Differential utilization of ICAM-1 and VCAM-1 during the adhesion and transendothelial migration of human T lymphocytes. J. Immunol. 147: 2913-2921; 1991.

Oppenheimer-Marks N, Davis L, Lipsky P. Human T lymphocyte adhesion to endothelial cells and transendothelial migration: alteration of receptor use relates to the activation status of both the T cell and the endothelial cell. J. Immunol. 145: 140-148; 1990.

Osame M, Matsumoto M, Usuku K, Izumo S, Ijichi N, Amitani H, Tara M, Igata A. Chronic progressive myelopathy associated with elevated antibodies to human T-lymphotropic virus type I and adult T-cell leukemialike cells. Ann. Neurol. 21: 117-122; 1987.

Osame M, Usuku K, Izumo S, Ijichi N, Amitani H, Tara M, Igata A. HTLV-I associated myelopathy, a new clinical entity. Lancet. 1: 1031-1032, 1986.

Osame M, Izumo S, Igata A. Blood transfusion and HTLV-I-associated myelopathy. Lancet. 2: 104-105; 1990.

Osborn L. Leukocyte adhesion to endothelium in inflammation. Cell 62: 3-6; 1990.

Panitch H, Hirch R, Schindler J, et al. Treatment of multiple sclerosis with g interferon: exacerbation associated with activation of the immune system. Neurology. 37: 1097-1120; 1987.

Panitch H. Influence of infection on exacerbations of multiple sclerosis. Ann. Neurol. 36: S25-S28; 1994.

Pardia R, Bender J, Dettori C, et al. Herterogeneous distribution and trnasmembrane signaling properties of lymphocyte function associated antigen (LFA-1) in human lymphocyte subsets. J. Immunol. 143: 3157-3166; 1989.

Pardridge W. Recent advances in blood-brain barrier transport. Ann. Rev. Pharmacol. Toxicol. 28: 25-39; 1988.

Paty D, Li K, The UBC MS/MRI Study group, and the IFNβ Multiple Sclerosis Study Group. Interferon β-1b is effective in relapsing-remitting multiple sclerosis. II. MRI analysis. Neurology. 43: 662-667; 1993.

Phillips J, Lanier L. Lectin-dependent and anti-CD3 induced cytotoxicity are preferentially mediated by peripheral blood cytotoxic T lymphocytes expressing Leu-7 antigen. J. Immunol. 136: 1579-1585; 1986.

Piali L, Hammel P, Uherek C, Bachmann F, Gisler R, Dunon D, Imhof B. CD31/PECAM-1 is a ligand for avb3 integrin involved in adhesion of leukocytes to endothelium. J. Cell. Biol. 130: 451-460; 1995.

Picker L, Warnock R, Burns A, Doerschuk C, Berg E, Butcher E. The neutrophil selectin LECAM-1 presents carbohydrate ligands to the vascular selectins ELAM-1 and GMP-140. Cell, 66: 921-933; 1991.

Piela T, Korn J. ICAM-1-dependent fibroblast-lymphocyte adhesion: discordance between surface expression and function of ICAM-1. Cell. Immunol. 129: 125-137; 1990.

Pober J, Cotran R. Cytokine-mediated activation of vascular endothelium Physiol. Rev. 70: 427-451; 1990.

Pober J. Cytokine-mediated activation of vascular endothelium. Am. J. Pathol. 133: 426-433; 1988.

Poser's CM, Paty D, Scheinberg L, McDonald W, Davis F, Ebers G, Johnson K, Sibley W, Silberberg D, Tourtellotte W. New diagnostic criteria for multiple sclerosis: guidelines for research protocols. Ann Nerurol. 13: 227-231; 1983.

Prat A, Al-Asmi A, Duquette P, Antel J. Lymphocyte migration and multiple sclerosis: relation with disease course and therapy. Ann. Neurol. 46: 253-256; 1999.

Prince H. American blood donor seropositive for human T-lymphotropic virus type I/II exhibit normal lymphocyte subsets. Transfusion. 30: 787-790; 1990.

PRISMS Study Group. Randomised double-blind placebo-controlled study of interferon b-1a on neurological disability in relapsing/remitting multiple sclerosis. Lancet. 352: 1498-1504; 1998.

Raine C, Canella B, Duyvesteyn A, Cross A. Homing to central nervous system vasculature by antigen specific lymphocytes. II Lymphocyte endothelial cell adhesion during the initial stages of autoimmune demyelination. Lab. Invest. 63: 476-489; 1990.

Raine C, Canella B, Hauser S, Genain C. Demyelination in primate autoimmune encephalomyelitis and acute multiple sclerosis lesions: a case for antigen-specific antibody mediation. Ann. Neurol. 46: 144-160; 1999.

Raine C. The Dale E. McFarlin Memorial Lecture: the immunology of the multiple sclerosis lesion. Ann. Neurol. 36(suppl): S61-S72; 1994.

Raine C, Canella B, Duyvesteyn A, Cross A. Homing to central nervous system vasculature by antigen specific lymphocytes. II Lymphocyte endothelial cell adhesion during initial stages of autoimmue demyelination. Lab. Invest. 63: 476-489; 1990.

Richardson J, Edwards A, Cruickshank J, Rudge P, Dalgleish A. In vivo cellular tropism of human T-cell leukemia virus type I. J. Virol. 64: 5682-5687, 1990.

Rieckmann P, Albrecht M, Kitze B. Cytokine mRNA levels in mononuclear blood cells from patients with multiple sclerosis. Neurology. 44: 1523-1526; 1994a.

Rieckmann P, Martin S, Wechselbraun I, Albrecht M, Kitze B, Weber T, Tumani H, Broocks A, Luer W, Helwig A, Poser S. Serial analysis of circulating adhesion molecules and TNS receptor in serum from patients with multiple sclerosis: Cicam-1 is an indicator for relapse. Neurology, 44: 2367-2372; 1994b.

Romagnani S. The Th1/Th2 paradigm. Immunol. Today. 18: 263-265; 1997.

Roman G, Osame M. Identity of HTLV-I-associated tropical spastic paraparesis and HTLV-I-associated Myelopathy. Lancet 1: 651; 1988.

Romanic A, Madri J. The induction of 72-kD gelatinase in T cells upon adhesion to endothelial cells is VCAM-1 dependent. J. Cell Biol. 125:1165-78; 1994.

Romanic A, Graesser D, Baron JL, Visintin I, Janeway CA Jr, Madri JA. T cell adhesion to endothelial cells and extracellular matrix is modulated upon transendothelial cell migration. Lab Invest.76:11-23; 1997.

Roos R. Viruses and demyelinating disease of the central nervous system. Neurol. Clin 1: 681-700; 1983.

Rosenkoetter M, Reder A, Oger J, Antel J. T cell regulation of polyclonal induced immunoglobulin secretion in humans. J. Immunol. 132: 1779-1783; 1984.

Rott O, Fleischer B, Cash E. Interleukin-10 prevent the development of EAE in mice. Eur. J. Immunol. 24: 1434-1440; 1994.

Runkel L, Meier W, Pepinsky R, Karpusas M, Whitty A, Kimball K, Brickelmaier M, Muldowney C, Jones W, Goelz S. Structural and functional differences between glycosylated and non-glycosylated forms of human interferon b (IFN-β). Pharm. Res. 15(4): 641-649; 1998.

Sadovnick A, Ebers G. Epidemiology of multiple sclerosis: A critical overview. Can. J. Neurol. Sci. 20: 17-29; 1993.

Sanders V, Waddell A, Felisan S, Li X, Conard A. Herpes simplex virus in postmortem multiple sclerosis brain tissue. Arch. Neurol. 53: 123-124; 1996.

Sanders M, Makgoba M, Shaw S. Human naive and memory T cells. Reinterpretation of helper-inducer and suppressor-inducer subsets Immunol. Today. 9: 195-199; 1988.

Schall T, Bacon K, Toy K, Goeddel D. Selective attraction of monocytes and T lymphocyte of the memory phenotype by cytokine RANTES. Nature. 347: 669-671; 1990.

Schall T, Bacon K. Chemokine, leukocyte trafficking, and inflammation. Curr. Opin. Immunol. 6: 865-873; 1994.

Schluesener H, Meyermann R. Intercrines in brain pathology: expression of intercrines in multiple sclerosis and Morbus Creutzfeldt-Jacob lesion. Acta Neruopathol. 86: 393-396; 1993.

Scholding N, Zajicek J, Wood N, Compston D. The pathogenesis of demyelinating disease. Prog. Neurobiol. 43: 143-173; 1994.

Sharief M, Noori M, Ciardi M, Cirelli A, Thompson E. Increased levels of circulating ICAM-1 in serum and cerebrospinal fluid of patients with active multiple sclerosis. Correlation with TNF-α and blood-brain barrier damage. J. Neuroimmunol. 43: 15-21; 1993.

Schering-Plough. Further studies on the biological properties of interleukin-10. Data on file as P-5806.

Schering-Plough. Single dose therapy with IL-10 in mice with experimental allergic encephalomyelitis (EAE). Data on file as D-27219.

Shimizu Y, van Seventer G, Horgan K, Shaw S. Roles of adhesion molecules in T-cell recognition: fundamental similarities between four integrins on resting human T cells (LFA-1, VLA-4, VLA-5, VLA-6) in expression, binding, and costimulation. Immunol. Rev. 114:109-43; 1990.

Shimizu Y, Newman W, Gopal V, Horgan K, Graber N, Beall D, van Seventer G, Shaw S. Four molecular pathways of T cell adhesion to endothelial cells: role of LFA-1, VCAM-1, and ELAM-1, and changes in pathway hierarchy under different activation conditions. J. Cell. Biol. 113: 1203-1212; 1991.

Shimizu Y, Newman W, Tanaka Y, Shaw S. Lymphocyte interactions with endothelial cells. Immunol. Today. 13: 106-112; 1992.

Siegel D, Le J, Vilcek J. Modulation of lymphocyte proliferation an immunoglobulin synthesis by interferon-γ and type-I interferons. Cell. Immunol. 101: 380-390; 1986.

Simon J, Jacobs L, Campion M, Wende K, Simonian N, Cookfair D, Rudick R, Herndon R, Richert J, Salazar A, Alam J, Fischer J, Goodkin D, Granger C, Lajaunie M, Martens-Davidson A, Meyer M, Sheeder J, Choi K, Scherzinger A, Bartoszak D, Bourdette D, Braiman J, Brownscheidle C, Whitham R. Mangetic resonance studies of intramuscular interferon β-1a for relapsing multiple sclerosis. Ann. Neurol. 43: 79-87; 1998.

Smeltz R, Swanborg R. Concordance and contradiction concerning cytokines and chemokines in experimental demyelinating disease. J. Neursci. Res. 51: 147-153; 1998.

Smith S. IL-10 does not prevent the development of EAE in mice. FASEB. 8: A994; 1994.

Sobel R, Mitchell M, Fondren G. Intercellular adhesion molecule-1 (ICAM-1) in cellular immune reactions in the human central nervous system. Am. J. Pathol. 136: 1309-1316; 1990.

Soilu-Hanninen M, Salmi A, Salonen R. Interferon-β downregulates expression of VLA-4 antigen and antagonizes interferon-g-induced expression of HLA-DQ in human peripheral blood monocytes. J. Neuroimmunol. 60: 99-106; 1995.

Spertini O, Luscinskas F, Kansas G, Munro J, Griffin J, Gimbrone M, Tedder T. Leukocyte adhesion molecule-1 (LAM-1, L-selectin) interacts with an inducible endothelial cell ligand to support leukocyte adhesion. J. Immunol. 147: 2565-2573; 1991.

Springer T. Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. A. Rev. Physiol. 57: 827-872; 1995.

Springer T. Adhesion receptors of the immune system. Nature. 346: 425-434; 1990.

Springer T. Traffic signals for lymphocyte recirculation and leukocyte emigration: The multistep paradigm Cell. 76: 301-314; 1994.

Staunton D, Dustin M, Springer T. Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1. Nature. 339: 61-64; 1989.

Stins M, Gilles F, Kim K. Selective expression of adhesion molecules on human brain microvascular endothelial cells. J. Neuroimmunol. 76: 81-90; 1997.

Stuve O, Dooley N, Hum J, Antel J, Francis G, Williams G, Yong V. Interferon-β-1b decreases the migration of T-lymphocytes in vitro: effects on matrix metalloproteinase-9. Ann. Neurol. 40: 853-863; 1996.

Sun J, Olsson T, Wang W, et al., Autoreactive T and B cells responding to myelin proteolipid protein in multiple sclerosis and controls. Eur. J. Immunol. 21: 1461-1468; 1991.

Svenningsson A, Hansson GK, Andersen O, Andersson R, Patarroyo M, Stemme S. Adhesion molecule expression on cerebrospinal fluid T lymphocytes: evidence for common recruitment mechanisms in multiple sclerosis, asceptic meningitis, and normal controls. Ann. Neurol. 34: 155-161; 1993.

Swanborg R. Experimental autoimmune encephalomyelitis in rodents as a model for human demyelinating disease. Clin. Immunol. Immunopathol. 77: 4-13; 1995.

Takahashi K, Sawasaki Y, Hata J, Mukai K, Goto T. Spontaneous transformation and immortalization of human endothelial cells. In Vitro Cell. Dev. Biol. 25: 265-274; 1990.

Takeichi M. Morophogenic roles of classic cadherins. Curr. Opin. Cell Biol. 7: 619-627; 1995.

Tanaka Y, Adams D, Huscher S, Hirano H, Siebenlist U, Shaw S. T-cell adhesion induced by proteoglycan-immobolized cytokine MIP-1 beta. Nature. 361: 79-82; 1993.

Tatewaki, M., Yamaguchi, K., Matsuoka, M. Ishii, T., Miyasaka, M., Mori, S., Takatsuki, K. and Watanbe, T. Constitutive overexpression of the L-selectin gene in fresh leukemic cells of adult T-cell leukemia that can be transactivated by human T-cell lymphotropic virus type 1 Tax. Blood. 86: 3109-3117; 1995.

Taub D, Conlon K, Lioyd A, Oppenheim J, Kelvin D. Preferential migration of activated CD4+ and CD8+ T cells in response to MIP-1 alpha and MIP-1 beta. Science. 260: 355-358; 1993.

Tedder T, Penta A, Levine B, Freedman A. Expression of the human leukocytes adhesion molecule LAM-1: Identity with TQ1 and Leu 8 differentiation antigens. J. Immunol. 144: 532-540, 1990.

Tedder T, Steeber D, Chen A, Engel P. The selectins: vascular adhesion molecules. FASEB. 9: 866-873; 1995.

Tender C, Greenberg S, Blattner W, Manns A, Murphy E, Fleisher T, Hanchard B, Morgan O, Burton J, Nelson D, Waldmann T. Transactivation of interleukin 2 and its receptor induces immune activation in human T-cell lymphotropic virus type I-associated myelopathy: pathogenic implications and a rationale for immunotherapy. Proc. Natl. Acad. Sci. U.S.A. 87: 5218-5222; 1990.

The IFNβ Multiple Sclerosis Study Group and the UBC MS/MRI Analysis Group. Interferon β-1b in the treatment of multiple sclerosis: final outcome of the randomized controlled trial. Neurology. 45: 1277-1285; 1995.

The IFNβ Multiple Sclerosis Study Group: Interferon β-1b is effective in relapsing-remitting multiple sclerosis. I. Clinical results. Neurology. 43: 655-661; 1993.

Thornhill M. Wellicome S. Mahiouz D. Lanchbury J. Kyan-Aung U. Haskard D. Tumor necrosis factor combines with IL-4 or IFN-gamma to selectively enhance endothelial cell adhesiveness for T cells. The contribution of vascular cell adhesion molecule-1-dependent and -independent binding mechanisms. J. Immunol. 146: 592-598; 1991.

Tonetti M, Millo E, Sturla L, Bisso A, De Flora A. Effects of the murine L929 and L1210 cell lines on nitric oxide and TNF-a production by RAW 264.7 murine macrophages. Biochem. Biophy. Res. Commun. 230: 636-640; 1997.

Traganos F. Flow cytometry: Principle and applications. Cancer Invest. 2: 149-163; 1984.

Traugott U, Reinherz E, Raine C. Multiple sclerosis: Distribution of T cells, T cell subsets, and Ia-positive macrophages in lesions of different ages. J. Neuroimmunol. 4: 201-221; 1983.

Tsujino A, Nakamura T, Furuya T, Goto H, Nishiura Y, Shirabe S, Nakane S, Motomura M, Nagataki S. Elevated serum levels of soluble E- and L-selectin in patients with human T-cell lymphotropic virus type I-associated myelopathy. J. Neurol. Sci. 155: 76-79; 1998.

Tsukada N, Matsuda M, Miyagi K, Yanagisawa N. In vitro intercellular adhesion molecule-1 expression on brain endothelial cells in multiple sclerosis. J. Neuroimmnol. 49: 181-187; 1994

Tsukada N, Matsuda M, Miyagi K, Yanagisawa N. Adhesion of cerebral endothelial cells to lymphocytes from patents with multiple sclerosis. Autoimmunity. 14: 329-333; 1993a.

Tsukada N, Miyagi K, Matsuda M, Yanagisawa N. Increased levels of circulating intercellular adhesion molecule-1 in multiple sclerosis and human T-lymphotropic virus type I-associated myelopathy. Ann. Neurol. 33(6): 646-9; 1993b.

Tuomanen E, Saukkonen K, Sande S, Cioffe C, Wright S. Reduction of inflammation, tissue damage, and mortality in bacterial meningitis in rabbits treated with monoclonal antibodies against adhesion-promoting receptors of leukocyte. J. Exp. Med. 170: 959-969; 1989.

Uchiyama T, Nelson D, Fleisher T, Waldmann T. A monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T cells. II. Expression of Tac antigen on activated cytotoxic killer T cells, suppressor cells, and on one of two types of helper T cells. J. Immunol. 126: 1398-1403; 1981.

Ulvestad E, Williams K, Wedeler C, Antel J, Nyland H, Mork S, Matre R. Reactive microglia in multiple sclerosis lesions have an increased expression of receptors for the Fc part of IgG. J. Neurol. Sci. 121: 125-131; 1994.

Umehara F, Izumo S, Takeya M, Takahashi K, Sato E, Osame M. Expression of adhesion molecules and monocyte chemoattractant protein –1 (MCP-1) in the spinal cord lesions in HTLV-I-associated myelopathy. Acta Neuropathol. 91:343-350; 1996.

Umehara F, Izumu S, Nakagawa M, Ronquillo A, Takahashi K, Matsumoro K, Sato E, Osame M. Immunocytochemical analysis of the cellular infiltrates in the spinal cord lesions in HTLV-I associated myelopathy. J. Neuropathol. Exp. Neurol. 52: 424-430; 1993.

Utz U, McFarland HF. The role of T cells in multiple sclerosis: implications for therapies targeting the T cell receptor. J. Neruopathol. Exp. Neurol. 53: 351-358; 1994.

Van Kooyk Y, Kemenade E, Weder P, Van de Wiel-van Kemenad E, Huijbens R, Figdor C. Lymphocyte function-associated antigen dominates very late antigen 4 in binding of activated T cells to endothelium. J. Exp. Med. 177: 185-190; 1993.

Varki A. Selectin ligands: will the real ones pleas stand up ? J. Clin. Invest. 100: S31-S35; 1997.

Vora A, Perkin G, McCoy T, Dumonde D, Brown K. Enhanced binding of lymphocytes from patients with multiple sclerosis to tumor necrosis factor-alpha-treated endothelial monolayers: associations with clinical relapse and adhesion molecule. Clin. Exp. Immunol. 105: 155-162; 1996.

Vora A, Perry M, Hobbs C, Dumonde D, Brown K. Selective binding of peripheral blood lymphocytes to the walls of cerebral vessels in frozen sections of human brain. J. Immunol. Meth. 180: 165-180; 1995.

Waksman B. Multiple Sclerosis. Curr. Opin. Immunol. 1: 733-739; 1989.

Wang E, Lehner P, Graham S, Borysiewicz L. CD8 (C57+) T cells in normal, healthy individuals specifically suppress the generation of cytotoxic T cells to Epstein Barr virus-transformed B cell lines. Eur. J. Immunol. 24: 2903-2909; 1994.

Washington R, Burton J, Todd R, Newman W, Dragovic L, Dore-Duffy P. Expression of immunologically relevant endothelial cell activation antigens on isolated central nervous system microvessels from patients with multiple sclerosis. Ann. Neurol. 35: 89-97; 1994.

Watson C, Whittaker S, Smith N, Vora A, Dumonde D, Brown K. IL-6 acts on endothelial cells to preferentially increase their adherence for lymphocytes. Clin. Exp. Immunol. 105: 112-119; 1996.

Weiss R, Clapham P, Nagy K, Hoshino H. Envelope properties of human T-cell-leukemia viruses. Curr. Topics Microbiol. Immunol. 115: 235-246; 1985.

Wekerle H, Linington C, Lassmann H, Meyermann R. Cellular reactivity within the CNS. Trends Neurol. Sci. 4: 271-277; 1986.

Weller R, Englehardt B, Phillips M. Lymphocyte targeting of the central nervous system: a review of afferent and efferent CNS-immune pathways. Brain Pathol. 6: 275-288; 1996.

Welsh C, Rose J, Hill K, Townsend J. Augmentation of adoptively transferred experimental allergic encephalomyelitis by administration of a monoclonal antibody specific for LFA-1. J. Neuroimmunol. 43: 161-167; 1993.

Whitcup S, DeBarge R, Caspi R, Harning R, Nussenblatt R, Chan C. Monoclonal antibodies against ICAM-1 (CD54) and LFA-1 (CD11a/CD18) inhibit experimental autoimmune uveitis. Clin. Immunol. Immunopathol. 67: 143-150; 1993.

Wilkins J, Stupack D, Stewart S, Ciaxia S. β1 integrin-mediated lymphocyte adherence to extracellular matrix is enhanced by phorbol ester treatment. Eur. J. Immunol. 21: 517-522; 1991.

Willenborg D, Prowse S. Immunoglobulin-deficient rats fail to develop experimental allergic enchephlomyelitis. J. Neuroimmunol. 5: 99-109; 1983.

Wong D, Dorovini-Zis K. Expression of vascular cell adhesion molecule-1 (VCAM-1) by human brain microvessel endothelial cells in primary culture. Microvasc. Res. 49: 325-339; 1995.

Wong D, Dorovini-Zis K. Regulation by cytokines and lipopolysaccharide of E-selectin expression by human brain microvessel endothelial cells in primary culture. J. Neuropathol. Exp. Neurol. 55: 225-235; 1996.

Wong D, Prameya R, Dorovini-Zis K. J. Neuropathol. In vitro adhesion and migration of T lymphocytes across monolayers of human brain microvessel endothelial cells: regulation by ICAM-1, VCAM-1, E-selectin and PECAM-1. Exp. Neurol. 58: 138-152; 1999.

Woodroofe M, Cuzner M. Cytokine mRNA expression in inflammatory multiple sclerosis lesions: Detection by nonradioactive in situ hybridization. Cytokine. 5: 583-588; 1993.

Wucherpfennig K, Strominger J. Molecular mimicry in T-cell mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. Cell. 80: 695-705; 1996.

Yamamoto N, Hinuma Y. Viral aetiology of adult T-cell leukemia. J. Gen. Virol. 66: 1641-1660; 1985.

Yamamoto N, Okada M, Koyanagi Y, Kannagi Y, Hiunuma Y. Transformation of human leukocytes by co-cultivating with an adult T-cell leukemia-virus produce cell line. Science. 217: 737-739; 1982.

Yap A, Brieher W, Gumbiner B. Molecular and functional analysis of cadherin-based adherens junctions. Ann. Rev. Cell. Dev. Biol. 13: 119-146; 1997.

Yasuda Y, Yoshitatsu S, Mitchel M, Sei Y, Yokoyama M, Tanaka K, Hara A. Healthy HTLV-I carriers in Japan: the haematological and immunological characteristics. Br. J. Haemat. 64: 195-203; 1986.

Yednock T, Cannon C, Fritz L, Sanchez-Madrid F, Steinman L, Karin N. Prevention of experimental autoimmune encephalomyelitis by antibodies against a4b1 integrin. Nature. 356: 63-66; 1992.

Yong W, Chabot S, Suve O, Williams G. Interferon beta in the treatment of multiple sclerosis: mechanisms of action. Neurology. 51: 682-689; 1998.

Yoshida M, Osame M, Kawi H. Increased replication of HTLV-1 in HTLV-1-associated myelopathy. *Ann. Neurol.* 26: 331-335; 1989.

Yu C, Haskard D, Cavender D, Johnson, Ziff M. Human gamma interferon increases the binding of T lymphocytes to endothelial cells. Clin. Exp. Immunol. 62: 554; 1985.

Yu M, Nishiyama A, Trapp B, Tuohy V. Interferon-β inhibits progression of relapsing-remitting experimental autoimmune enchephaloyelitis. J. Neuroimmunol. 64: 91-100; 1996.

Yu C, Haskard D, Cavender D, Johnson A, Ziff M. Human gamma interferon increases the binding of T lymphocytes to endothelial cells. Clin. Exp. Immunol. 62: 554-560; 1985.

Zeman A, Kidd D, McLean B, Kelly M, Francis D, Miller D, Kendall B, Rudge P, Thompson E, McDonald W. A study of oligoclonal band negative multiple sclerosis. J. Neurol. Neurosurg. Psychiatry. 60: 27-30; 1996.

Zhang J, Maarkovic S, Raus J, Weiner H, Hafler D. Increased frequency of IL-2 responsive T cells specific for myelin basic protein and proteolipid protein in peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. J. Exp. Med. 179: 973-984; 1994.

APPENDIX A

A.1 THE EFFECTS OF IMMUNOMODULATORY DRUGS ON LYMPHOCYTE PHENOTYPE IN MS

A.1.1 Lymphocyte subsets in a RR-MS patient participating in Schering-Plough® trial

Table A.1 shows lymphocyte subsets of a RR-MS patient who participated in Schering-Plough® trial at pretreatment, and at 2 and 7 days post therapy. The nature of treatment is still unclear as to whether the patient received placebo or rhuIL-10. Major shifts in lymphocyte subsets are observed. However, we do not know if the observed shifts in lymphocyte subsets are due to immunotherapy with rhuIL-10 or spontaneous. Following are some of the highlighted change in lymphocyte subsets that occurred either at 2 and/or 7 days post-treatment. Major reduction is seen in the percentage of CD19+ (B cells), CD4+CD29+ (helper induced), CD3+CD26 (activated T cells), CD4+CD62L+ (selectin expression on CD4+), CD8+CD62L+ (L-selectin expression on CD8+), and CD4+CD49d+ (VLA-4 expression on CD4+) cells. Of the lymphocyte subsets that were highly increased after treatment CD8+C57+ (cytotoxic cells) can be pointed out.

A.1.2 Lymphocyte subsets in RR-MS patients participating in ICOS trial

The results in Table A.2 through A.5 indicate lymphocyte subsets of RR-MS patients participating in ICOS Corporation trial. Lymphocyte subsets were analyzed before treatment and 5 days post treatment. This double blind study on the effect of rhu-anti-LFA-1 on the clinical course of RR-MS is now unblinded, therefore, the nature of treatment is known.

Table A.2 shows lymphocyte subsets in a RR-MS patient who received a single injection of 2 mg/kg of recombinant and humanized anti-LFA-1 mAb. Some of the major highlighted shifts in lymphocyte subsets are as following. Table A.2 shows a major reduction in the percentage of CD3+CD26+, CD4+CD49d+, CD8+CD49d+, CD4+CD62L+, and CD8+CD62L+ cells five days after treatment.

Table A.3 shows lymphocyte subsets for a RR-MS patient who received 1 mg/kg of anti-LFA-1 mAb. After a single injection of 1 mg/kg of anti-LFA-1, there was a major reduction in the percentage of CD4+CD49d+ and CD8+CD49+ cells. However, unlike the patient who was treated with 2 mg/kg (Table A.2), this patient showed no major reduction in her percentage of CD3+CD26+, or CD4+CD62L+ and CD8+CD62L+ cells after the treatment.

Table A.4 shows the lymphocyte subsets of a RR-MS patient in ICOS trial that received placebo. Unlike the RR-MS patients who were treated with anti-LFA-1 (Table A.2 and A.3), this patient showed an increase in the percentage of CD4+CD49d+ and CD8+CD49+ after placebo treatment. The percentage of CD4+CD62L+ and CD8+CD62L+ cells were also moderately increased. Moreover, the percentage of CD3+CD26+ cells remained unchanged.

Table A.5 shows the lymphocyte subsets of a RR-MS patient in ICOS Corp. trial that received intravenous methylprenisolone. Similar to the patient who received placebo (Table A.4), the percentages of CD4+CD62L+ and CD8+CD62L+ cells were moderately increased while the percentage of CD3+CD26+ remained almost unchanged after treatment. The percentage of CD4+CD49d+ was also unchanged.

Table A.1

Lymphocyte Subsets of a RR-MS Patient Enrolled in Schering Plough Trial

Subsets	pre-treatment	2 days Post treatment	7 days Post treatment
CD3+	76.4	83.0	72.3
CD19+	16.7	**5.0** ↓	18.8
CD16+CD56+	4.6	5.0	6.0
CD3+CD4+	55.3	63.4	50
CD3+CD8+	19.4	18.1	19.4
CD4+CD45RA+	48.6	51.5	46.2
CD4+CD29+	39.6	46.7	28.2
CD8+CD28+	4.2	**2.8** ↓	0.4
CD8+CD57+	24.6	**42.9**↑	32.8
CD3+CD27-	86.4	82.3	82.8
CD4+HLA-DR+	2.4	2.8	1.7
CD8+HLA-DR+	3.7	4.3	2.2
CD4+CD25+	0.6	0.8	0.4
CD8+CD25+	0	0	0.1
CD4+CD38+	6.0	**10.3**↑	3.7
CD8+CD38+	2.8	**5.0**↑	2.4
CD3+CD26+	40.8	**17.0**↓	55.2
CD3+CD30+	0.1	0	0
CD3+CD69+	0.5	0	1
CD4+CD49d+	20.4	23.5	**10.8**↓
CD8+CD49d+	42.4	54.6	31.4
CD4+CD62L+	86.4	90.1	**58.4**↓
CD8+CD62L+	57.5	44.1	**29.4**↓
CD8+CD11b+	14.3	14.5	10.8
CD3+CD54+	2.4	1.1	3.1

Note. CD3+, CD19+, CD16+CD56+, CD3+CD4+, and CD3+CD8+ lymphocyte subsets are given as percentage of the total number of lymphocyte ($CD45^{bright}CD14-$). Other lymphocyte subsets were calculated as the proportion of cells expressing a given second marker by using the equation: [% dual positive/(% dual positive + single positive only)] x 100. ↑ and ↓ indicate major (>30%) increase and decrease, respectively, *versus* pre-treatment.

Table A.2

Lymphocyte Subsets of a RR-MS Patient (DES #0801) Enrolled in ICOS Trial Who Received 2 mg/kg of Anti-rhu-LFA (Hu23F2G)

Subsets	Pre-treatment	5 days post-treatment
CD3+	74.8	73.6
CD19+	8.9	**5.8**↓
CD16+CD56+	8.3	**11.3**↑
CD3+CD4+	56.3	51.5
CD3+CD8+	15.7	15.9
CD4+CD45RA+	53.2	**34.8**↓
CD4+CD29+	39	**24.5**↓
CD8+CD28+	11.9	**3.8**↓
CD8+CD57+	18.8	14.4
CD3+CD27-	7.1	**3.5**↓
CD4+HLA-DR+	5	**2.7**↓
CD8+HLA-DR+	7.3	**4.2**↓
CD4+CD25+	3.2	**1.7**↓
CD8+CD25+	0.1	0
CD4+CD38+	17	**5.4**↓
CD8+CD38+	19.4	14.4
CD3+CD26+	71.1	**29.4**↓
CD3+CD30+	0.4	0
CD3+CD69+	0.6	1.5
CD4+CD49d+	15.1	**5.4**↓
CD8+CD49d+	34.1	**12**↓
CD4+CD62L+	84.1	**42.8**↓
CD8+CD62L+	54.1	**31.5**↓
CD8+CD11b+	25.8	30.9
CD3+CD54+	3.6	2.5

Note. CD3+, CD19+, CD16+CD56+, CD3+CD4+, and CD3+CD8+ lymphocyte subsets are given as percentage of the total number of lymphocyte ($CD45^{bright}$CD14-). Other lymphocyte subsets were calculated as the proportion of cells expressing a given second marker by using the equation: [% dual positive/(% dual positive + single positive only)] x 100. ↑ and ↓ indicate major (>30%) increase and decrease, respectively, *versus* pre-treatment.

Table A.3

Lymphocyte Subsets of a RR-MS Patient (MCE #0802) Enrolled in ICOS Trial Who Received 1 mg/kg of Anti-rhu-LFA (Hu23F2G)

Subsets	Pre-treatment	5 days post-treatment
CD3+	79	78.6
CD19+	13.2	10.9
CD16+CD56+	4.5	**8.2**↑
CD3+CD4+	49.8	49.7
CD3+CD8+	27.8	26.8
CD4+CD45RA+	23.6	**57.7**↑
CD4+CD29+	39.7	40.5
CD8+CD28+	6.4	**12.9**↑
CD8+CD57+	12.8	15.6
CD3+CD27-	5.4	4.3
CD4+HLA-DR+	5.6	**3.4**↓
CD8+HLA-DR+	9.2	**6**↓
CD4+CD25+	2.5	2.6
CD8+CD25+	0.1	0.2
CD4+CD38+	19.2	14.3
CD8+CD38+	10.3	14.3
CD3+CD26+	49.6	45.3
CD3+CD30+	0.2	0.2
CD3+CD69+	0.9	1.1
CD4+CD49d+	25.8	**11.1**↓
CD8+CD49d+	26.1	**13**↓
CD4+CD62L+	65.9	64.2
CD8+CD62L+	50.6	49.3
CD8+CD11b+	13.9	**20.9**↑
CD3+CD54+	1.8	**11.1**↑

Note. CD3+, CD19+, CD16+CD56+, CD3+CD4+, and CD3+CD8+ lymphocyte subsets are given as percentage of the total number of lymphocyte ($CD45^{bright}$CD14-). Other lymphocyte subsets were calculated as the proportion of cells expressing a given second marker by using the equation: [% dual positive/(% dual positive + single positive only)] x 100. ↑ and ↓ indicate major (>30%) increase and decrease, respectively, *versus* pre-treatment.

Table A.4

Lymphocyte Subsets of a RR-MS Patient (MOS #0803) Enrolled in ICOS Trial Who Received Placebo

Subsets	Pre-treatment	5 days post-treatment
CD3+	78.4	75.4
CD19+	4.9	**8.4**↑
CD16+CD56+	7	**14.9**↑
CD3+CD4+	37.4	32.7
CD3+CD8+	33	33.9
CD4+CD45RA+	53.3	62.2
CD4+CD29+	44.8	57.9
CD8+CD28+	5.4	nd
CD8+CD57+	23.9	29.4
CD3+CD27-	27.8	24.5
CD4+HLA-DR+	4.1	3.2
CD8+HLA-DR+	3.1	2.9
CD4+CD25+	1.4	**3.3**↑
CD8+CD25+	0.2	0.1
CD4+CD38+	24.8	**47.2**↑
CD8+CD38+	12.7	**21**↑
CD3+CD26+	35.9	35.9
CD3+CD30+	0.1	0.1
CD3+CD69+	1.5	0.7
CD4+CD49d+	18.3	29.1
CD8+CD49d+	27.5	**59.9**↑
CD4+CD62L+	75.2	84.6
CD8+CD62L+	44.1	56.9
CD8+CD11b+	22.7	29.5
CD3+CD54+	1.3	1.1

Note. CD3+, CD19+, CD16+CD56+, CD3+CD4+, and CD3+CD8+ lymphocyte subsets are given as percentage of the total number of lymphocyte ($CD45^{bright}CD14-$). Other lymphocyte subsets were calculated as the proportion of cells expressing a given second marker by using the equation: [% dual positive/(% dual positive + single positive only)] x 100. ↑ and ↓ indicate major (>30%) increase and decrease, respectively, *versus* pre-treatment.

Table A.5

Lymphocyte Subsets of a RR-MS Patient (TES #0804) Enrolled in ICOS Trial Who Received Intravenous Methylprednisolone

Subsets	Pre-treatment	5 days post-treatment
CD3+	79.1	76.1
CD19+	14.6	**20.1**↑
CD16+CD56+	3.7	3.8
CD3+CD4+	56	59.2
CD3+CD8+	21.2	17.4
CD4+CD45RA+	24.8	**50**↑
CD4+CD29+	36	36.4
CD8+CD28+	6.5	**3.3**↓
CD8+CD57+	10.8	10.1
CD3+CD27-	7.3	**3.9**↑
CD4+HLA-DR+	1.7	1.3
CD8+HLA-DR+	5.8	5.4
CD4+CD25+	1.1	0.1
CD8+CD25+	0.2	0.3
CD4+CD38+	8.9	7.7
CD8+CD38+	8.1	10.4
CD3+CD26+	57.2	62.3
CD3+CD30+	0.1	0
CD3+CD69+	0.5	1
CD4+CD49d+	14.5	12
CD8+CD49d+	nd	33
CD4+CD62L+	77.8	90.7
CD8+CD62L+	59.7	74.7
CD8+CD11b+	16.8	12.2
CD3+CD54+	3.1	2.7

Note. CD3+, CD19+, CD16+CD56+, CD3+CD4+, and CD3+CD8+ lymphocyte subsets are given as percentage of the total number of lymphocyte ($CD45^{bright}$CD14-). Other lymphocyte subsets were calculated as the proportion of cells expressing a given second marker by using the equation: [% dual positive/(% dual positive + single positive only)] x 100. ↑ and ↓ indicate major (>30%) increase and decrease, respectively, *versus* pre-treatment