DYSFUNCTION OF THE BLOOD-BRAIN BARRIER IN THE PATHOGENESIS OF MULTIPLE SCLEROSIS
PROCEEDINGS OF A 2001 CHICAGO INTERNATIONAL CONFERENCE SPONSORED BY SERONO SYMPOSIA INTERNATIONAL

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Early pathological studies in multiple sclerosis (MS) demonstrated growth of plaques from venules (Dawson's fingers). Perivenular distribution of lesions suggested a pathological process occurring around blood vessels. A century later, serial magnetic resonance imaging with contrast enhancement has demonstrated that inflammatory damage to blood vessels is an early event in the pathogenesis of MS.[1] In both experimental animals and in MS patients, accumulation of fibrin and alterations in the extracellular matrix around the blood vessels are found. Disruption of the blood-brain barrier (BBB) suggests that an interaction of blood factors with neural elements occurs during the neuroinflammatory response.

The symposium entitled *Dysfunction of the Blood-Brain Barrier in the Pathogenesis of Multiple Sclerosis* focused on basic and clinical aspects of BBB alterations pertinent to MS. This symposium provided a solid knowledge base on structure and function of the BBB. Future research directions include signal transduction mechanisms, the role of soluble mediators, including cytokines, chemokines and matrix metalloproteinases, the interactions of viruses, bacteria and parasites, structural and angiogenesis mechanisms, and molecular mechanisms controlling BBB development.

As Hippocrates of Cos, and then Maimonides[2] wrote in one of their famous aphorisms -- "*The life is short, the task is long"* -- an integrative, multidisciplinary approach is necessary to gather useful information of the BBB in MS, so that new therapeutic approaches can be swiftly developed.

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LEARNING OBJECTIVES AND ACCREDITATION INFORMATION

Learning Objectives

Upon completion of this program, the participant will be able to:

- Discuss the protective effects of interferon beta.
- Identify the structure and function of the blood brain barrier.
- Discuss the transport mechanisms of the blood brain barrier.
- Describe the initiation of inflammatory responses in the central nervous system (CNS).
- Identify the importance of chemokines in the pathogenesis of CNS inflammation.
- Discuss EAE as a model for neuroinflammation.

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ACKNOWLEDGEMENT

Serono Symposia International would like to acknowledge the contributions of the faculty who presented at the **Dysfunction of the Blood Brain Barrier in the Pathogenesis Of Multiple Sclerosis** symposium held in Chicago, IL on September 29, 2001.

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INTRODUCTION

For the past 20 years brain endothelial cells have been recognized not just to form a passive barrier between the blood and the extravascular tissues, but to participate in crucial physiologic and pathologic mechanisms, including inflammation. Endothelial cells from various vascular beds and species differ in their morphology, function, permeability, and immunological properties. These concepts have provided the impetus for organ and species-specific endothelial research.

Increased permeability of the BBB is a feature of many diseases of the central nervous system (CNS), among them MS.[3] BBB increased permeability is an early and critical event, often preceding symptoms, and, in most cases, being present in chronic lesions. Endothelial cells, under the influence of cytokines, chemokines and other putative inflammatory molecules may become activated and undergo morphologic and phenotypic changes that either initiate or contribute to the development of MS.[4] Understanding the structure and permeability properties of BBB and the role of endothelial cells in CNS inflammation may lead to new therapeutic considerations in MS and in other inflammatory diseases of the CNS.[5]

New areas of BBB research include signal transduction mechanisms operating at tight junctions, disruption and reassembling of the BBB under pathological conditions, responses to cytokines, to bacterial and viral products, interactions with the extracellular matrix, molecular mechanisms that control normal development and hematogenous spread of tumor cells.

STRUCTURE OF THE NORMAL BLOOD BRAIN BARRIER (BBB)

The primary function of the normal BBB is to establish and to maintain homeostasis in the CNS. Anatomically, CNS blood vessels, tight junctions, basal lamina, composed among others of laminin and fibronectin, endothelial and glial cells are the components of the BBB which separate blood plasma from the CNS interstitial fluid. Viewed as static in the past, current knowledge demonstrates that the BBB is dynamic and plastic, rather than immutable.[6]

Intravenous horseradish peroxidase has been used for studying the BBB. By using this dye, areas of BBB vulnerability have been identified in the median eminence of the hypothalamus, the third ventricle, the area postrema and the lamina terminalis.

Negative charges in the lumen and successive in-tandem junctions seal capillaries. Infusion of the ion lanthanum, which has the size of a hydrated sodium ion marks the beginning of the tight junction and defines the rest of the paracellular cleft and the basal lamina. This anatomical arrangement produces sluggish, if any traffic at the tight junction level. Tight junctions are arranged in complex rows of anastomotic, interconnected strands. The complexity and depth of the strands have been roughly related to the degree of impermeability or electrical resistance. The molecular structure of the tight junction has just been elucidated. Twenty variants of the structural protein *claudein* have been identified. The zonula occludens has 4 transmembranous loops which connect with tight junction proteins. Zonula occludens proteins are in turn linked to the cytoskeleton. Protein-protein changes in moieties modify tight junction permeability. More recently, the junction adhesion molecule (JAM) protein has been identified. JAM is not connected to the ancillary proteins in the cytoplasm, but it affects the passage of cells when endothelium or mononuclear cells are activated. This interaction then opens the tight junction, permitting the passage of mononuclear cells. This is the case of leukocytorrachia in acute bacterial meningitis and of lymphocytorrachia in MS.

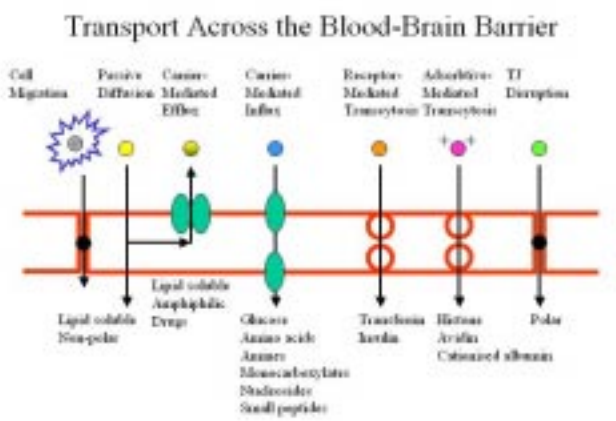
Typically, BBB capillaries have 4-6 nM fenestrations, preventing protein permeability. Protein and amino acids get across by active transport, carriers, channels, vesicular transport, receptor-mediated endocytosis or through open junctions. In vitro models of BBB have been developed with endothelial and co-cultured astrocytes. In areas in which the BBB is open, such as the neurohypophysis, median eminence, area postrema, etc. substances penetrate the BBB faster, but the total interchange in this restrictive area is less than 0.2%. In contrast with the BBB, the blood-skeletal muscle barrier is 100-1000 times more permeable because of the low resistance of capillaries, which leak because of discontinuous junctions. Blood-muscle increased permeability

might represent the way by which proteins and viruses might produce disease within the CNS when entering through the muscle end-plate.

In the BBB, matrix-metalloproteinases, lymphokines and chemokines are necessary for mononuclear cell invasion and penetration. It is known that tumor necrosis factor alpha (TNF-alpha) stimulates production of collagenase type IV, which then digests the basal lamina.[7]

TRANSPORT MECHANISMS OF THE BLOOD BRAIN BARRIER

The complex structure of the CNS protects the neuron, which always neighbors energy sources. It is estimated that no neuron is more than 2 seconds away from a molecule of glucose, due to the large vascular surface of diffusion. In humans there are 12,000-18,000 square centimeters of blood brain barrier (BBB) surface area. As aforementioned, the areas that lack BBB, like those of the circumventricular organs, represent only about 0.2% of the total surface area. Thus, those leaky areas are insignificant when drug delivery into the CNS is considered. The much tighter, large surface area is the real barrier to be overcome. Materials can diffuse passively across the BBB in direct relation to its lipid solubility. Lipid-soluble, non-polar substances enter the brain more easily by this route. Many of these lipid-soluble substances are picked up by efflux transporters which are carrier mediated, and many lipid-soluble amphophilic drugs are pumped actively out of the brain via these efflux transporters. Polar substances require carrier-mediated influx. Among others, glucose, amino acids, amines, monocarboxinates, nucleosides, and small peptides are all transported across the endothelium by carrier-mediated influx. Vesicular transport is either receptor-mediated or absorptive-mediated transcytosis via cationic proteins. Ultimately, this active system is dependent upon the ATP - sodium-potassium pump, which pumps sodium out of the cells, taking potassium into the cells and is counterbalanced by number of exchanges -- a sodium-proton exchanger, sodium, potassium, and chloride co-transporter, and a bicarbonate-chloride exchanger. These are in turn balanced by cell pores. Active and passive mechanisms set up an osmotic gradient. Secretion of CSF produces a “sink action”, which dilutes substances introduced within the BBB. The mechanism of Gadolinium permeability through the BBB is still unclear, though one presumption assumes the modification of a tight junction.



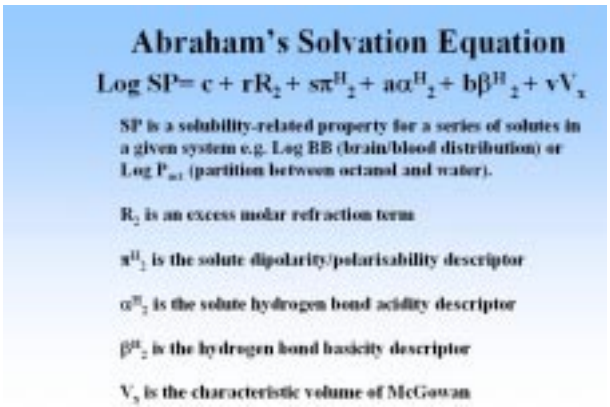
Slide presented by David J. Begley, PhD

The dilution in the CSF is higher than that in the brain extracellular fluid. For substances coming in the blood circulation, brain and CSF concentrations will always be lower than those in blood, no matter how well they penetrate the BBB. The faster a substance penetrates the BBB, the smaller these differences become. Strategies to speed the movement of drugs into the brain include:

- chemical modifications, to make the substances more lipid soluble;
- design drugs to mimic transporters for substrates which normally go into the brain;
- decrease of effluxes.

A classic example of lipidization is that of the opioids -- morphine, codeine, and heroin. In heroin, hydroxyl groups are replaced by acetyl groups, greatly increasing its lipophilicity and its uptake into the brain by 68%. Within the brain, heroin is converted back into morphine by enzymes before it finds the receptors, so morphine is still the principal active component, although it is heroin which goes through. This paradigm represents an effective pro-drug technology. The lipid-soluble heroin is being converted into to the more polar morphine and it is getting locked into the brain. It now cannot diffuse out again. More sophisticated attempts to maximize drug within the BBB have used medicinal chemistry and predictive formulae by the solvation equation, which include knowledge of size, polarity, hydrogen bonds, pH, and other chemical properties of the molecule. Empiric

constants which predict BBB permeability are then obtained.



Slide presented by David J. Begley, PhD

This, the Abraham method, is rather lengthy because many of these measurements need to be obtained independently. Simpler methods just measure the polar surface area of a molecule. Estimation of polar surface gives a very good guide for BBB permeability.

Most CNS drugs which are active are small, their molecular weight is lower than 400-500 Dalton, they are lipophilic, with pH relatively neutral, and are relatively slim like a torpedo. Amino acid transporters demand energy consumption and are highly stereospecific. Many medications, such as gabapentin, L-dopa and baclofen use the L-system amino acid transporter. Efflux mechanisms pump out drugs. One of them, p. glycoprotein is a large efflux molecule on the luminal membrane of the BBB. It contains 12 transmembrane domains, with 3 glycosylation sites in one of the external loops, and 2 cytoplasmic ATP-binding sites. This molecule hydrolizes ATP and uses the energy derived to actually push drugs out of the cell. It is constitutively expressed in the BBB. In humans, there are drug-pumping gene product from chromosome 7, named MDR-1a and MDR-1b. These gene products are responsible for multidrug resistance and are located in the luminal endothelial membrane of the BBB. Pumping of drugs as fast as they diffuse is a mechanism by which new antihistamines do not produce somnolence. Some kinds of cytokines are PGP substrates, certainly, and lymphocytes have PGP simply to secrete these cytokines. The implications are that some may be substrates in the blood-brain barrier.

In summary, the brain endothelium poses a formidable obstacle to drugs. This is one of the major challenges in the future for development of new CNS therapies. The BBB is not simply an anatomical entity, but a biochemical, physiological, and pharmacological entity as well. It is very reactive, complex, and highly regulated.

INITIATION OF INFLAMMATORY RESPONSES IN THE CNS

The CNS is isolated in an immunologically privileged area within the BBB. CNS inflammation occurs in a myriad of disorders, including not only MS, but HIV-associated phenomena, Alzheimer's disease, ALS, and others.

Multiple sclerosis is in itself proteiform. At least 4 histopathological sub-variants have been recently redefined.[8] In addition, immunological processes in the relapsing-remitting form differ from those of chronic-progressive phases. Acute plaques are infiltrated by T-cells, macrophages and B-cells. Structural proteins associated with myelin, including myelin-basic protein, proteolipid protein and myelin-oligodendrocyte glycoprotein play substantial immunogenic roles. Apoptosis of oligodendrocytes and axonal degeneration add complexity to the equation. Death of oligodendrocyte precursors and axonal damage are both responsible for disability, whereas axonal remyelination might potentially restore function. Immunoglobulins, especially anti-myelin IgGs, complement activation and the membrane attack complex formation are part of the humoral immunity in MS. Expression of specific myelin proteins in the CNS, molecular mimicry among antigens and myelin proteins and production of superantigens are part of the abnormal immune response in MS.

Animal models of experimental allergic encephalomyelitis (EAE) have permitted the study of the cascade of immunological and molecular mechanisms of neuroinflammation. EAE is not multiple sclerosis, but a model for neuroinflammation and delayed-type hypersensitivity. EAE is an autoimmune disease manifested by T-cell and macrophage CNS infiltrates, with or without demyelination. Some models are monophasic while others are relapsing. EAE starts when a processed peptide of a myelin antigen binds to an appropriate MHC class II molecule that is recognized by the T-cell

signaling apparatus on the surface of a CD-4-positive Th-1 type T-cell. T-cells can exist in the circulation either in a resting form or in a fully activated one. Likewise, the CNS endothelial bed can be either in a resting state, or in a state of activation by lymphokines, chemokines, cell infiltration or trauma. It is assumed that in health, resting T-cells pass by a normal resting endothelium. A different phenomenon is the passage of non-activated T-cells through a fully activated endothelium, such as high endothelial venules in the lymph nodes or endothelium that exists in areas that have already become inflamed. A third paradigm involves activated cells passing a resting endothelium. Resting cells are excluded from passing resting endothelial beds, while activated cells may pass those barriers. However, not all tissues have equal abilities in attracting various cells, even in the activated state. Indeed, the CNS has immunologic privilege. In inflammed CNS, even resting cells will cross over that particular endothelial bed and into the CNS. Thus, significant changes occur when the vascular bed is resting or activated, and when the T-cell is resting or activated. Changes in BBB permeability may even occur in areas of CNS where there is not much inflammation. Soluble factors, such as lymphokines and chemokines may explain this phenomenon and also why few lesions may predispose to the development of new lesions in normal adjacent areas.

EAE AS A MODEL FOR NEUROINFLAMMATION

Injection of myelin proteins-activated T-cells produces EAE, which has served as a model for the study of neuroinflammation. Phases of the experimental disorders include T-cell entry, antigen recognition, commitment, recruitment, clinical illness development and histopathological inflammation.[9] Initially, T-cells come earliest on, followed by NK cells and macrophages as the endothelial bed changes, with final development of full-blown disease. The initial lymphocyte entry phase is antigen-specific. Subsequent phases are antigen-independent.

Passage of activated T-cells through the BBB activates perivascular microglia in specific areas, changing the expression of endothelial adhesion molecules, among them the integrins, notably VLA-4. Interferon beta down regulates the expression of VLA-4, possibly accounting for a decrease in enhancing lesions. Starting at about 24 hrs, up to about 60 hrs, natural killer (NK) cells become detectable in the perivascular space and the CNS parenchyma. The recruitment phase, which occurs before the animal gets sick, involves the secretion of pro-inflammatory lymphokines and chemokines at 48-72 hours, with infiltration of the parenchyma by activated T-cells and macrophages. The BBB undergoes significant change, from resting into activated stages, with high expression of adhesion molecules and leakiness. In addition, reactive B-cells, for example, to myelin-oligodendrocyte glycoprotein (MOG) and other relevant antigens penetrate the CNS parenchyma in similar fashion to that of T-cells. These B-cell lymphoblasts switch into oligoclonal plasma cells, which then secrete the appropriate antibody. B-cells also have the ability to cross the BBB looking for antigen if they are in the activated state. Oligoclonal bands can be found in the CSF of these animals. Subspecies of B-cells that had migrated into the CNS and begun producing antibody were specific for the antigen but of a different type than those appearing in the peripheral blood. CNS macrophages are abundant and proteiform. They originate from circulating monocytes and dendritic cells. Once they cross the BBB, they assume distinct morphological and immune phenotypes. Parenchymal microglia and meningeal macrophages can also take place in inflammation, but they become activated in a considerably lesser percentage.

The vast majority of activated macrophages derives from the circulation. Perivascular cells lie beyond the basement membrane of the endothelium, by the glia limitans but in the perivascular space, ideally situated to encounter any type of cell that is crossing into the CNS parenchyma. Perivascular cells are CNS-antigen-presenting cells, are normally present in the CNS and enter the CNS with high turnover; about 30% of them turn over every 2-3 months. With this facility for penetration and antigen presentation, perivascular cells are the main carriers of the HIV inside the CNS. Interestingly, the perivascular cell has also the ability to leave the CNS, back to the lymphopoietic system. Perivascular cells share many functions with dendritic cells, in terms of antigen presentation. In some EAE models and in Devic's neuromyelitis optica eosinophils also infiltrate the CNS.

In addition to these specific cell types, a site-specific sensitivity to CNS inflammation is fundamental. Not the whole CNS suffers inflammation in EAE. In rats, the lower half of the spinal cord, cerebellum, pons, and medulla become markedly inflammed, whereas the mesencephalon and cerebrum suffer no inflammation. This selectivity is quite curious, because T-cells go everywhere in the CNS. Also, myelin basic protein is ubiquitous. In MS, plaques tend to form around the ventricles or in particular areas where white matter touches a CSF-containing space. When axons are damaged, CNS areas previously immune to developing EAE become susceptible. In the case of optic nerve damage, changes in susceptibility occur within 24 hours. TGF-beta mRNA decreases to zero in the affected colliculus. TGF-beta is constitutively anti-inflammatory. Perhaps one mechanism by which trauma might trigger attacks of MS is by decreasing the expression of normal TGF-beta. Future research will determine whether neurotrophic factors protect animals from trauma-mediated EAE induction. Whether distribution of blood vessels plays a role in EAE susceptibility remains to be clarified.

CHEMOKINES IN THE PATHOGENESIS OF CNS INFLAMMATION

Chemokines are relatively newly discovered molecules. About 50 members of the chemokine group have been cloned and identified. Four family subgroups can be recognized. The CX3C family member, alternatively called fractokine or neurotactin, has two cystines, separated by 3 amino acids and then 2 more conserved cystines in the sequence. The second family, with approximately 15 members, is called CXC. These molecules have 2 cystines separated by one amino acid and the characteristic 2 conserved cystines in the carboxy terminus of the molecule. There are approximately 15 members in this family. The largest family member is the CC chemokine, also termed the beta chemokine subfamily. These members have two cystines juxtaposed and then the conserved cystines later on in the molecular sequence. The final fourth subfamily is called C. It has only one cystine in the immuno terminus and only one conserved cystine in the carboxy terminus. There are 2 members of this family that are splice variants of the same gene product.[7]

As opposed to large cytokine molecules like gamma-interferon or TNF, chemokines are small molecular weight proteins of 5-10 kD, originally described as chemotactic peptides, because they induced migration of leukocytes across diffusion chambers. When the first characterization of these molecules was done, conditioned media containing these molecules was demonstrated to induce the migration of lymphocytes or macrophages or neutrophils across a membrane. Chemokines such as CCL21 are involved in the formation of lymphoid tissue and in homeostatic lymphoid trafficking. T-cells and B-cells that bear the receptor for that chemokine go to lymphoid structures that are expressing that chemokine, which is involved in the normal maintenance of the immune system. Transcriptionally regulated chemokines are produced in early inflammatory events. These molecules are ligands for 7 transmembrane spanning receptors.

The nomenclature and classification of chemokines is extremely complex. The CXC chemokines were initially thought to promote neutrophil trafficking, but it is clear now that members of this family are also involved in lymphocyte trafficking and NK cell trafficking. There is now a standard nomenclature indicating whether a certain chemokine belongs to the CXC, CC, C or CX3C family. The largest CC family contains at least 26 chemokines and includes inducible and transcriptional chemokines. The CXC chemokines bind to CXC receptors found on leuko and lymphocytes. For instance, CXCR1 is found in neutrophils, monocytes, T-cells, NK cells, basophils, mass cells, and endothelial cells. CXCR2 is found on a similar subpopulation, and also on astrocytes. CCR1 is expressed on neutrophils, monocytes, T-cells, NK cells, B-cells, mass cells, astrocytes, and neurons. RANTES binds to CCR1, CCR3, and CCR5. The Duffy antigen receptor for chemokines is expressed on endothelial cells, red blood cells, and T-cells. It is a nonfunctioning, non-signaling receptor which behaves as a chemokine sink.

When a chemokine binds its seven transmembrane receptors, it initiates a number of signaling pathways through G proteins and stimulation of protein kinase C. Adhesion molecules become expressed and activated and permit the cell to flatten out and enter the tissue. Cells form uripods, processes that permit that

migration. Chemokine receptors concentrate on the leading edges of these cells as they are moving through tissue or even as they are moving through in vitro assays.

In MS, chemokines play important roles by regulating CNS mononuclear cell infiltration, leukocyte trafficking, and the movement of microglia or other cell types within the CNS. In relapsing EAE models, T-cells are activated in the peripheral lymphoid tissue. They traffic into the bloodstream, and once in the bloodstream they get into the CNS. Once inside, T-cells may or may not be restimulated. If they are stimulated T-cells produce inflammatory cytokines, chemokines and gamma-interferon, which induce the accumulation of other mononuclear cells and they activate endogenous macrophages and microglial cells, which produce these oxygen radicals and proteolytic enzymes. Tissue can be presented by either tissue-specific antigen-presenting cells or external antigen presenting cells, and reactivate T-cells for these newly released neoantigens, a process termed epitope spreading.

This new wave of CNS inflammation and T cell accumulation results in clinical disease progression. In experimental models MCP-1, MIP-1 alpha, MIP-1 beta, RANTES, and other chemokines are rapidly expressed in day 1. As the disease progresses there are increases in the expression of chemokines which bind to proteoglycans and extracellular matrix and provides a highway for which leukocytes can move along to get to their destination. Chemokine expression varies, as MIP-1 alpha is highly expressed, MIP-1 beta is barely expressed, RANTES is somewhat expressed, and MCP-1 does not become fully expressed until the relapsing phase of disease. Also, MIP-1 alpha correlates with increasing disease severity. T-cells express MIP-1 alpha, MIP-1 beta, TCA-3 RANTES, and IP10. The monocytes and the macrophages that infiltrate from the periphery express MIP-1 alpha, MIP-1 beta, MCP-1. Astrocytes express IP10, MCP-1, RANTES, STF-1, and fractokine in later disease phases. Cerebrovascular endothelial cells express fractokine and it is not clear if they express any other chemokine. Anti-MIP-1 alpha and anti-IP10 inhibit acute EAE development, even though all these other proteins were expressed at the time of disease development.

It is unknown why so many chemokines are expressed and only a small subset are functionally related to disease development. Plausibly, CCL3 or MIP-1 alpha and CXCL10 or IP10 are functionally important for acute EAE by controlling the accumulation of CNS disease-inducing T-cells, whereas CCL2 or MCP-1 control monocyte accumulation in the relapsing phase of disease. The functions of CCL4, CCL5, CXCL2, CXCL9 are unknown.

All of the chemokine receptors are expressed by normal, primed, and reactivated T cells except CCR4 and CCR10, which are only expressed by in vitro reactivated antigen-specific T-cells. CCR1 is expressed by only CNS-infiltrating T-cells, either donor or host origin, but not T-cells isolated from the spleen. CXCR3 is only expressed by the T-cells infiltrating or derived from the central nervous system and not peripheral T-cells in the spleen.

In summary, all chemokine receptors are expressed by activated T-cells. However, CNS-infiltrating T-cells express CCR1 and CCR3 compared to peripheral T-cells. The combination of MIP-1 alpha and IP10 expression, along with expression of the receptors on the disease-inducing T-cells leads to accumulation of those cells in the CNS and the induction of the inflammatory events that precipitate clinical disease.

METALLOPROTEINASES IN THE PATHOGENESIS OF CNS INFLAMMATION

Proteolytic enzymes have been detected in the CSF and brain of patients with MS. Neutral proteases are increased during acute exacerbations of MS. Matrix metalloproteinases (MMPs) are neutral proteases that attack the extracellular matrix. In patients with MS, elevated levels of the MMP-9 (gelatinase-B) have been found in the CSF and also in serum, before an acute attack. It is known that intracerebral injection of gelatinase A (MMP-2) opens the BBB. T lymphocytes use MMP-9 to attack the capillary basal lamina, allowing them to cross the BBB. EAE brings about an increase of MMP-9 in rodent brains, and inhibitors of MMPs block the manifestations of the disease in animals. Hence, considerable evidence in experimental animals and humans implicates MMPs in the pathophysiology of MS.[10]

MMPs are a family of 20 members that comprise 4 major groups differing in protein structure and substrate specificity. The major MMPs in the brain include gelatinase A (MMP-2), stromelysin (MMP-3), matrilysin (MMP-7), gelatinase B (MMP-9) and membrane-type metalloproteinases. MMP-2 and MMP-9 attack the basal lamina around the blood vessels, altering the permeability. MMP-2 is a constitutive enzyme normally found in the CSF. MMP-9, which is induced during the neuroinflammatory response, is increased in the CSF of MS patients with gadolinium-enhancing lesions on MRI. Treatment with high-dose steroids returned the MMP-9 levels to normal. In contrast, patients with Devic's neuromyelitis optica had no elevations of CSF MMP-9. MS patient subanalysis disclosed significant differences in MMP-9 CSF levels in relapsing/remitting (RR) and in secondary progressive (SP) MS patients. CSF-MMP-9 levels were markedly elevated in RR, but not in SP cases. In patients with RR disorder, CSF levels of tissue-inhibitors of metalloproteinases (TIMP-1) were decreased, whereas in patients with SP disease these levels were normal, as in patients with Devic's syndrome. These results suggest an excess of MMP-related proteolytic activity in brains of patients with MS. MMPs are produced by all cell types in the brain, including neurons, glia and invading leucocytes and macrophages. The enzymes attack all components of the extracellular matrix, and participate in the opening of the BBB by disrupting the basal lamina around the blood vessels.[10]

IFN-beta blocks the activation of MMP-9, and is synergistic with the action of high-dose steroids. IFN-beta-1a supressed MMP-9 and MMP-7 mRNA in R/R patients, but not in SP patients, as predicted from the previous study. The IFN-beta mediated decrease in MMP-9 expression may contribute to suppressed migratory capacity and reduced transavasation of immune cells into the CNS. In vitro data has demonstrated that exposing activated T cells to IFN-beta resulted in a significant decrease in MMP-9 expression associated with inhibited T-cell migratory capacity, likely at the transcriptional level, or through expression of lymphokines or chemokines. Changes in MMP and TIMP correlated with clinical reduction of disease activity induced by IFN-beta. A shift in the MMP/TIMP ratio from a "pro-proteolytic" toward an "anti-proteolytic" profile appears to occur with IFN-beta-1A. This shift occurs in parallel to the IFN-beta-mediated cytokine deviation from Th1 to Th2 profile. The inhibition of MMP-s proteolytic activities resulting from IFN-beta1 treatments may decrease the autoiimune cells' migratory capacity and the invasiveness through the BBB into the CNS, and reduce MMP's-mediated damage to myelin.[11]

IN VITRO MODELS OF CELL TRAFFICKING ACROSS THE HUMAN BBB

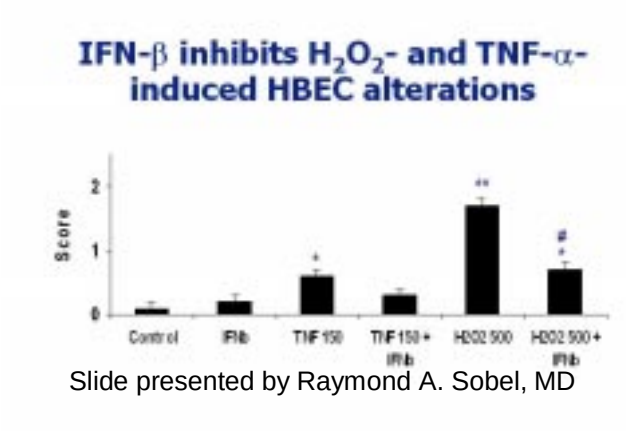
CNS inflammation is characterized by migration of leukocytes across the BBB, which normally excludes their entry into the CNS and the compromised in BBB permeability due to the inflammation. In vitro systems of human brain endothelial cells have been developed from temporal lobectomy specimens. Well organized cell monolayers express endothelial and coagulation system markers for at least 12 days in primary culture. They share features characteristic of their in vivo counterparts, such as tight junctions with high electrical resistance. Physiologically, these cell layers exclude large molecules such as horse-radish peroxidase from passing between endothelial cells.

These confluent monolayers express TNF alpha, interleukin-1 beta, interferon gamma, LPS, p-selectin, and P-protein. Adhesion molecules preferentially localize on the apical surface of endothelial cells, and particularly along well-developed finger-like projections.

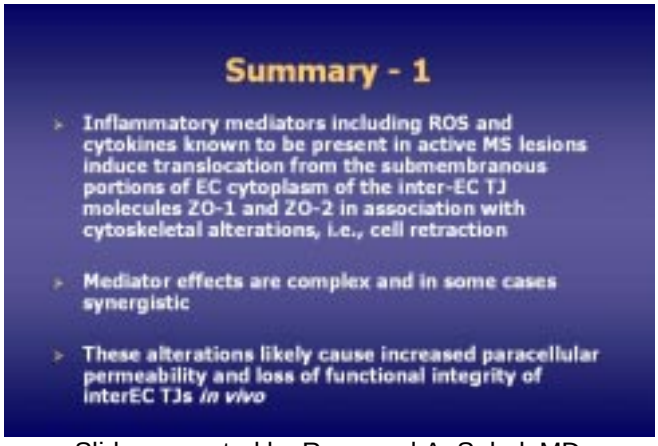
HUMAN BRAIN ENDOTHELIAL CELL JUNCTION MOLECULES: PROTECTIVE EFFECTS OF INTERFERON-BETA

Inter-endothelial cell tight junctions constitute major structural and functional components of the BBB, which regulate paracellular permeability and leukocyte transmigration. The major molecular components include occludins, which are transmembranous and structural proteins, junctional adhesion molecules, claudins and zonula occludens-submembranous-proteins. Zonula occludens molecules are members of the family of membrane-associated guanylate-kinase homologues, which mediate tyrosine phosphorylation and cyclooxygenase pathways. Normally, these proteins concentrate in tight junction areas.

Zonula occludens proteins are essential for targeting tight junction structures, and they link to actin cytoskeleton and related signal transducing mechanisms, critical for tight junction function. Tight junctions and zonula occludens are highly sensitive to the microenvironment. In vitro they respond to inflammatory cytokines, VEGF and reactive oxygen species, which alter the subcellular localization, dissociating the occluding/zonula occludens complex. In situ loss and fragmentation of the zonula occludens in HIV is associated with an impaired BBB.



Slide presented by Raymond A. Sobel, MD



Slide presented by Raymond A. Sobel, MD

Interferon beta reduces the BBB injury in MS. During the acute phase, damage of the BBB in MS is notorious and, perhaps, a primary mechanism of neuroinflammation. An impaired BBB and morphologically abnormal endothelial cells persist in chronic lesions.[12] BBB damage also produces alterations in the normally appearing white matter as detected by MRI, in relation to abnormal extracellular matrix and axonal degeneration.

Interferon beta reduces gadolinium-enhancing lesions, supporting its effects on BBB permeability mechanisms, which are not fully understood yet. Potential biological targets include monocytes, T, glia, endothelial cells and basal lamina. Interferon beta also produces direct effects on human brain endothelial cells, including the down regulation of interferon-gamma-induced class II-MHC expression.

Among the beneficial actions on the BBB in MS patients, interferon beta counteracts soluble mediator effects on tight junction integrity through the effects on CNS endothelial cell-tight junction molecules. The effects of beta-interferon were tested on a semi-quantitative in vitro assay for induction of reversible soluble inflammatory mediator effects on CNS tight junction molecules by using an endothelial cell line which expresses factor VIII and glutamate receptor activity. This cell line was maintained in media with astrocytoma-conditioned supernatants. Cells were then aliquoted onto collagen-coated cover slips growth in petri dishes and in vitro treatments were initiated when the degree of confluency reached 60%. After treatments cells were rinsed, fixed, and immunostained with anti-ZO-1 and ZO-2 antibodies. The soluble inflammatory mediators tested included tumor necrosis factor-alpha (TNF-α), IL-1, IL-6, TGFbeta-1, interferon (IFN) gamma and interferon-beta, the last one at doses of 5,000-10,000 U/ml.

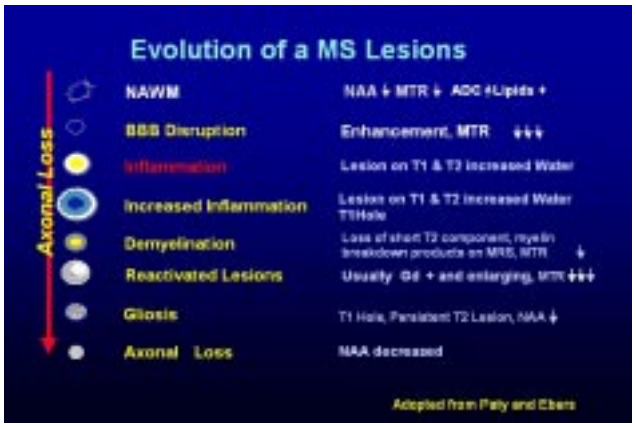
TNF-alpha induced dose-dependent human brain endothelial cell (HBEC) alterations in junction molecules. IL-1 also induced HBEC alterations and enhanced the damaging effects of hydrogen peroxide, whereas IFN gamma alone did not induce HBEC altrerations, though enhanced the effects of hydrogen peroxide. IL-6 did not induce HEBC alterations, nor enhanced hydrogen peroxide damaging effects. Finally, the protective effects of interferon beta were analyzed. Interferon beta inhibited hydrogen peroxide and TNF-alpha induced HBEC alterations.

In summary, inflammatory mediators, including hydrogen peroxide and cytokines, known to be present pathologically in active MS lesions induced translocation from the submembranous portions of the endothelial cell cytoplasm of the

inter-endothelial cell tight junction molecules ZO-1 and ZO-2 in association with cytoskeletal alterations such as cell retraction. The mediator effects were complex and in some cases synergistic. These alterations likely caused increased paracellular permeability and loss of fuctional integrity of interendothelial cell tight junctions in vivo. Similar alterations in tight junction molecules were found in active inflammatory/demyelinating lesions in situ, at sites of focal BBB breakdown. Finally, soluble mediator-induced alterations were reduced by interferon beta. Among the mechanisms of the beneficial effects of interferon beta in MS patients may be direct effects on intracellular localization of CNS endothelial cell tight junction molecules.

MAGNETIC RESONANCE IMAGING (MRI) OF THE BLOOD BRAIN BARRIER IN MS

The identification of pathological-radiological and clinical correlates of enhancing lesions in MS might provide an understanding of the disease mechanisms. In addition to the traditional measurements of T1, contrast-enhanced T1, and T2 signals, new MRI techniques in MS include FLAIR (fluid-attenuated inversion recovery), magnetization transfer (to study the normally-appearing white matter), magnetic spectroscopy and volumetric and atrophic indexes. Thus, the brain MRI can be used as “a window” in the pathology of MS. Circumscript white matter anomalies might result from edema, inflammation, demyelination and remyelination. Black holes result from axonal loss. Incomplete ring enhancing lesions are often encountered in MS.



Slide presented by Joseph A. Frank, MD

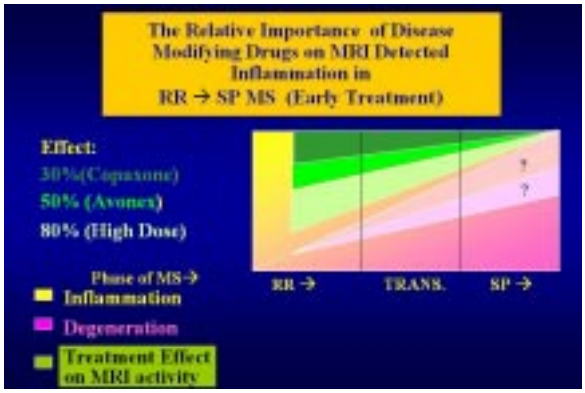
MS lesion evolution was studied by Paty.[3] This evolution is not linear. Firstly, in the normally appearing white matter the NAA peak, a marker for viable neurons by MR spectroscopy, decreases, with a decrease of the magnetization transfer ratio due to abnormal increase in lipids and ADC. With BBB disruption enhancement with gadolinium occurs with significant decrease in magnetization transfer ratios. During the inflammatory phase T1 and T2 lesions reflect increased water. As inflammation worsens T1 and T2 lesions expand and T1 holes develop, signaling initial imaging evidence for axonal loss. During the period of demyelination there is a loss of the short T2 component and myelin breakdown products become reflected on spectroscopy and magnetization transfer. In reactivated lesions enlarged gadolinium-enhanced lesions appear, with decrease in magnetization transfer ratios. When gliosis occurs, T1 holes become more evident, T2 lesions persist, and NAA decrease. Finally, at more advanced phases of axonal loss NAA decreases.

Differences in contrast enhancement occur in various types of acute lesions. The detection of areas of BBB disruption depends upon physiological parameters of the permeability surface area and leakage space of the lesion. BBB permeability depends on the fraction of lesion tissue which the leakage space occupies, the lesion tissue volume, the permeability coefficient, the surface area of the leaking membrane and the tracer concentration in the leakage space. On the other hand, contrast-enhanced MRI does not detect and open BBB in chronic MS lesions, though neuropathological and immunocytochemical studies found variable degrees of BBB leakage in many types of MS lesions, including inactive demyelinated plaques.

In acute MS lesions the BBB remains open 3-7 days, possibly as short as 6 hours. About 75% of lesions enhanced on the first monthly, contrast-enhanced MR, whereas 22% enhanced at the second month, and 5-10% of enhancing lesions reenhance within 2-11 months. About 56% of enhancing lesions are present in the deep white matter, 24% in the gray-white junction, and 20% periventricularly. Eighty percent of enhancing lesions are hypointense on T1-weighted imaging. In a study by Kappos, contrast-enhanced lesions predicted relapses but not future disability, whereas measures of

global atrophy were better correlated with disability.

Natural history studies in MS revealed that enhancing lesions reflected acute BBB disruption. By imaging, 5-10 times more activity was detected over clinical attacks. The burst of imaging activity was correlated to clinical exacerbations. Finally, about 30-70% of relapsing-remitting MS had enhancing lesions.



Slide presented by Donald W. Paty, MD, FRCPC

The effect of interferon beta treatment on relapsing-remitting MS and the expression of gadolinium-enhanced lesions has been extensively studied at the National Institutes of Health. Interferon beta markedly and significantly reduced gadolinium-enhancing lesions in relapsing-remitting MS.[13] Remarkably, longitudinal studies have also shown month-to-month fluctuations (30-70%) of enhancing lesions in MS patients before interferon treatment. The fact that interferon beta markedly suppresses enhancing lesions suggest that early treatment stabilizes the BBB and may delay further disease progression.

In summary, by using MRI the detection of areas of BBB disruption depend upon physiological parameters of the permeability surface area and leakage space of the lesion. MRI is used frequently by clinicians as a major criterion to define lesion activity, and is thought to reflect BBB damage. Although this is considered to be one of the earliest changes in observed MS lesions, neuropathological and immunocytochemical studies reveal that BBB leakage can be found to variable degrees in every MS lesion, including inactive demyelinated plaques (luchinetti)). In any event, MRI has been very helpful in correlating acute exacerbations with contrast-enhanced abnormalities. Changes in MRI contrast-enhanced abnormalities have been used clinical trials. With both IFN beta-1B and IFN beta-1A at higher doses significant reductions in gadolinium-enhanced lesions were found. These reductions were dose-dependent.

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