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Handbook of Neurochemistry and Molecular Neurobiology Neuroimmunology

Volume Editors: Armen Galoyan and Hugo Besedovsky

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Preface

Neuroimmunology is one of the most rapidly developing branches of Neurobiology, prompted by novel neurochemical, neuroendocrinological, and neurophysiological investigations of the central and peripheral nervous system including neuroendocrine systems. Neuroimmunology can be considered as an interdisciplinary science that covers relevant aspects of how the peripheral immune system can influence brain physiology and elicit neuroendocrine immunoregulatory responses and also how local interactions between immune and neuronal mediators of the brain influence the occurrence and course of neuropathologic diseases. Therefore, we have in these volume chapters, focused on immune–neuro–endocrine interactions underlying the control and regulation of processes involved in both immune and brain physiology and in the pathogenesis of different nervous diseases. Among such diseases are: schizophrenia, HIV-associated dementia, rheumatoid arthritis, several experimental pathologies, multiple sclerosis, autoimmune encephalomyelitis, Theilers virus infection, nervous system demyelination diseases, the primary degenerative disorders such as Alzheimer's and Parkinson's as well as brain injuries resulting from stroke and trauma, the neuroimmunology of gene therapy, amyotrophic lateral sclerosis, Prion disease, and all theoretical questions covering these pathologies. All of the above-mentioned involve autoimmune processes.

It is difficult, indeed, to imagine fundamental neurobiological processes, autoimmune, neuroendocrine, and infectious diseases, where immune factors are not of prime importance. The elucidation of the intimate molecular-biological problems of immunopathologies requires deep knowledge of the intricate connection between immunomodulators, immune competent cells of blood, brain, and other organs.

This volume contains data on multiple immunomodulators, many of which are also the products of hypothalamic brain cell neurosecretion. Interleukins (IL-1 α , IL-1 β , IL-2, IL-4, IL-6, TNF α), immunophyllin and ubiquitin as well as proline-rich peptides, comprised of 10–15 amino acids are being produced in Nucleus supraopticus and Nucleus paraventricularis and then secreted into neurohypophysis. Along the neurosecretion of the mentioned cytokines, there are other immunomodulators, the primary structure of which had been completely deciphered such as: Immunophyllins, intracellular receptors of immunosuppressors FK506, cyclosporine A, rapamicin. They are peptidyl-prolyl-*cis-trans*-isomerases. There are novel immunological hypothalamic factors such as ubiquitin, macrophage migration inhibitory factor (MIF), as well as Thymosin β_4 (1–39). The latter is the activator of Ca²⁺-calmodulin dependent enzymes without Ca²⁺ and calmodulin participation. These data allowed us to propose the concept of neuroendocrine immune system of the brain (A. Galoyan, Chapter 7).

The new compounds participating in immune response are poly (ADP-ribose) polymerase (C. Szabo, Chapter 20) and immunoproteasome in the nervous system (M. Rinaudo and M. Piccinini, Chapter 9). Polymerase (PARP-1) is a DNA-binding protein and is involved in the control of DNA metabolism. PARP-1 is involved in the upregulation of numerous proinflammatory genes that play a pathogenic role in the later stage of central nervous system (CNS) diseases. Immunoproteasome is a nonlysosomal ubiquitin-dependent protease. The proteasome activity is essential in a wide range of vital cellular processes extending from cell division and differentiation, DNA repairing, transcription factor and regulatory protein processing, membrane receptor internalization, inflammatory response and antigen presentation.

The intimate interaction of neurons and immune cells is of fundamental theoretical importance, bringing attention to the neural and immune activity of the same brain cells. The discovery that “brain-born” cytokines are induced during hippocampal long-term potentiation was important to prove that these mediators, when released at low levels as a consequence of increased neural activity, are involved in physiologic functions of the CNS such as synaptic plasticity and memory consolidation. Data documenting

this aspect, which is incorporated to the conceptual framework that a network of immune–neuro–endocrine interactions operates during health and disease, is the main subject of Chapter 1: “Brain Cytokines as Integrators of the Immune–Neuroendocrine Network” (H. Besedovsky and A. del Rey). In Chapter 6: “Neuro-immune associative learning” (Niemi M.B et al) the idea of afferent and efferent pathway of brain-immune communications is further developed, focusing on the immune response change under the stimuli of conditioned and unconditioned reflexes. Also the fact that certain immune processes can be behaviorally conditioned is indicative of an active exchange of information between the immune and nervous systems (Chapter 6). The influence of the CNS mediators on immunocompetent cells is also illustrated for example by effects of the endogenous purine nucleoside adenosine that express four subtypes of adenosine receptors (A_1 , A_2 , A_{2b} , and A_3). These receptors are expressed on cells of the brain immune system (G. Hasko and E.S. Vizi, Chapter 12).

There is clear information documenting effects of neurotransmitters on the immune system. Serotonin regulates the hematopoiesis immunocompetent cells and that of bone marrow. The serotonin in blood is stored in the dense granules of platelets. Serotonin has marked activities on inflammation and immunity, affecting almost all the types of mature blood cells. Of interest are reports on the cytokine effects, produced in peripheral immunocompetent cells on brain neurochemistry (A. Dunn, Chapter 3). The most studied effect is the capacity of this cytokine to activate the hypothalamo-pituitary-adrenocortical (HPA) axis. Interrelationship between peripheral and brain immune system, including innervation of immune organs and tissues, are examined. Of interest is the innate immune system of brain that is commonly affected by mediators and transmitters derived from both neurons and immune cells of the brain and the similarities between neuronal and immune synapses. There are clear data on peripheral cytokines, hormones, together with mediators of the immune system on the brain, can affect specifically neuroendocrine and behavioral mechanisms and responses (G. Juhasz, Chapter 13). These data further reinforce the relevance of the neuroimmune cross talk. A. Rostami (Chapter 16), discusses experimental autoimmune encephalomyelitis (EAE) (animal model of multiple sclerosis), where antigen-presenting cells, primed autoreactive CD4+ T cells, react against myelin components and migrate in the brain (through the blood-brain barrier, which is reactivated by microglia and present myelin peptides), triggering in this way processes that cause autoimmune demyelination. The understanding of the origin of autoimmune diseases in the brain as well as the treatment of those diseases, represent an important task for Neuroimmunology (D. Jessop, Chapter 2). EAE is a well-characterized disease of CNS (C. Welsh and C. Young, Chapter 15). One chapter is dedicated to the treatment of the autoimmune diseases “Drugs and target sympathetic-immune pathways for treatment of autoimmune diseases” (D. Lorton, Chapter 5), characterized by immune system dysregulation of nervous stress pathways, the sympathetic nervous system, and the hypothalamic-pituitary-adrenal axis. This chapter summarizes changes that occur in sympathetic to immune signaling in autoimmune diseases using rheumatoid arthritis as a specific example and presenting similarities of other autoimmune diseases. The potential drugs for the treatment of autoimmune diseases targeting sympathetic nervous system are considered. Among the diseases considered as autoimmune are: encephalomyelitis, paraneoplastic encephalomyelitis, autoimmune neurological disorders. Autoimmune diseases can be induced by intracellular vesicle-associated proteins, multiple sclerosis, several neurodegenerative diseases, etc. They are all subjects for the brain immune system studies, each one of them has specific mechanism. A few chapters are dedicated to the inflammatory components in different brain pathologies and infection. Inflammation is a key component in the immunological defense of organism against health-threatening pathogens. Dysregulation of inflammatory response can lead in turn to tissue damage and subsequently to disease. An immunosuppressive environment is mainly responsible for the characteristic features of the inflammatory responses in CNS. Inflammation is now known to have toxic and protective effects in neurodegenerative diseases such as Parkinson’s, Alzheimer’s, and Prion diseases; microglial activation appears as a common feature to all those pathologies (F. Pitossi, Chapter 18). Neuroinflammation is a pathological condition of the CNS that happens in response to invasion of a multitude of infectious pathogens and also in autoimmune diseases. There is a great variation in susceptibility to specific infectious agents in human and mouse CNS reflecting genetic predispositions. The CNS is not dependent on resident tissue leukocytes or infiltrating macrophages or neutrophils to recognize and clear pathogens. Instead, the CNS has established its own immune system, based on activation of resident innate immune cells such as microglia or astrocytes

(W. Stenzel and G. Alber, Chapter 10; N. Bhat, Chapter 14). Inflammation takes place in HIV associated dementia (J. Tan, Chapter 19). Immunological aspects of CNS demyelination are discussed in Chapter 17 (S. Sriram and S. Pawate).

Special chapters review our understanding of how normal aging alters the cross talk between the autonomic nervous system and the immune system to alter immune functions with age (D. Bellinger et al., Chapter 4) and the neuroimmunology of gene therapy using the virus vectors for the treatment of neurological diseases (P. Lowenstein et al., Chapter 11). Numerous preclinical and clinical gene transfer studies have been carried out using viral vectors modified from pathogenic viruses or artificial nonviral liposome-based approaches. In this volume, we discuss immune response during Schizophrenia (F. Gaughran and J. Welsh, Chapter 21). However, at present time it is very difficult to define the interrelationship between the brain immune system and the pathogenesis of complicated systems.

For the first time the neuroimmunological, neurophysiologic, and neurochemical aspects of neuro-immune interactions are broadly discussed by worlds leading neurobiologists. We hope that this unusual multidimensional association would serve to increase the understanding of the relevance of neuro-endocrine-immune networks during health and disease.

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Table of Contents

Preface	v
Contributors	xi
Part 1: Neuro-Endocrine Immunoregulation	
1 Brain Cytokines as Integrators of the Immune–Neuroendocrine Network	1
<i>H. O. Besedovsky · A. del Rey</i>	
2 Neuropeptides in the Immune System: Mediators of Stress and Inflammation	19
<i>D. S. Jessop</i>	
3 Effects of the Immune System on Brain Neurochemistry	37
<i>A. J. Dunn</i>	
4 Age-Related Alterations in Autonomic Nervous System Innervation of Lymphoid Tissue	61
<i>D. L. Bellinger · C. L. Lubahn · A. B. Millar · J. L. Carter · S. Vyas · S. D. Perez · D. Lorton</i>	
5 Drugs that Target Sympathetic–Immune Pathways for Treatment of Autoimmune Diseases	83
<i>D. Lorton · C. Lubahn · D. Bellinger</i>	
6 Neuro-Immune Associative Learning	123
<i>M.-B. Niemi · G. Pacheco-López · H. Engler · C. Riether · R. Doenlen · M. Schedlowski</i>	
Part 2: The Brain’s Immune System	
7 The Brain Immune System: Chemistry and Biology of the Signal Molecules	151
<i>A. Galoyan</i>	
8 The Dialect of Immune System in the CNS: The Nervous Tissue as an Immune Compartment for T Cells and Dendritic Cells	197
<i>Z. Fabry · E. Reinke · A. Zozulya · M. Sandor · I. Bechmann</i>	

9	Immunoproteasome Activity in the Nervous System	223
	<i>M. T. Rinaudo · M. Piccinini</i>	
10	Regulation of the Inflammatory Response in Brain	235
	<i>W. Stenzel · G. Alber</i>	
11	Evolutionary Origins of the Brain's Immune Privilege. Implications for Novel Therapeutic Approaches: Gene Therapy	263
	<i>P. R. Lowenstein · K. Kroeger · C. Barcia · J. Zirger · D. Larocque · M. G. Castro</i>	
12	Adenosine: An Endogenous Regulator of the Brain Immune System	283
	<i>G. Haskó · E. S. Vizi</i>	
13	Neuroimmune Cross Talk	293
	<i>G. Juhász</i>	
14	Role of Glia in CNS Inflammation	309
	<i>S. Pawate · N. R. Bhat</i>	
Part 3: Neuro-Immune Mechanisms of Brain Diseases		
15	Autoimmune Processes in the Central Nervous System	331
	<i>C. J. Welsh · C. R. Young</i>	
16	Experimental Autoimmune Encephalomyelitis	355
	<i>B. Gran · K. O'Brien · D. Fitzgerald · A. Rostami</i>	
17	Immunological Aspects of Central Nervous System Demyelination	379
	<i>S. Pawate · S. Sriram</i>	
18	The Inflammatory Component of Neurodegenerative Diseases	395
	<i>C. C. Ferrari · F. J. Pitossi</i>	
19	Mechanisms of Inflammation in HIV-associated Dementia	407
	<i>B. Giunta · F. Fernandez · J. Tan</i>	
20	Role of Poly(ADP-ribose) Polymerase in Brain Inflammation and Neuroinjury	427
	<i>G. S. Scott · K. Komjáti · V. C. Besson · C. Szabó</i>	
21	Schizophrenia and Immune Responses	467
	<i>F. Gaughran · J. Welch</i>	
	Index	489

1 Brain Cytokines as Integrators of the Immune–Neuroendocrine Network

H. O. Besedovsky · A. del Rey

1	<i>Introduction</i>	4
2	<i>The Immune–Neuroendocrine Network</i>	4
3	<i>Cytokine Receptors and Production in the Brain</i>	6
4	<i>Effect of Peripheral or Central Administration of Cytokines on Brain Functions</i>	6
4.1	Effect of Cytokines on the Production of Hypothalamic Releasing Factors and Neurotransmitters, and on Neuronal Activity	6
4.2	Effect of Cytokines on Thermoregulation, Nociception, Food Intake, Sleep, and Behavior	7
5	<i>Resetting Brain-Controlled Homeostatic Mechanisms by Cytokines</i>	7
6	<i>Induction and Relevance of Endogenous Cytokines for Brain Functions</i>	9
6.1	Peripheral Immune Signals Trigger Cytokine Production in the CNS	9
6.2	Neuronal Signals Induce Cytokine Production in the Brain	10
6.3	Relevance of Brain-Borne Cytokines for Brain Functions, Particularly Synaptic Plasticity, Learning, and Memory	10
7	<i>Overview and Proposal</i>	12

List of Abbreviations: BBB, blood–brain barrier; KO, knockout; LPS, lipopolysaccharide; LTP, long-term potentiation; MHPG, 3-methoxy-4-hydroxyphenylethylene glycol; NA, noradrenaline; REM, rapid eye movement

1 Introduction

It has been repetitively acknowledged that the immune and the central nervous systems have several features in common. Particularly, the capacity of both systems to process an enormous amount of information from inside and outside the organism, and their intrinsic plasticity, which form the basis of memory and learning, allows them to perform better when confronted with the same stimulus after a previous experience. Even the term “immunological synapse” (Bromley et al., 2001) to describe the transmission of signals between immune cells that are in close contact has recently become popular among immunologists. However, these analogies are only operational, because the information acquired is transmitted and propagated in a different way in each of the two systems. In fact, the brain receives information from well-defined external and internal sensorial systems, and neurons communicate using electrochemical signals released, in general, by preestablished neuronal contacts. In contrast, immune cells are mobile, express highly discriminative receptors for antigens, and establish *de novo* functional contacts between different cell types during the immune response.

Although it has been reported that lymphoid cells can produce certain “classical” hormones and neurotransmitters, there is no evidence that these products can play a role in immune–brain communication, and it seems more likely that they would rather act as “paracrine” messengers within the immune system. On the other hand, it is now known that neural cells can produce several cytokines originally described as immune-derived products. Furthermore, it is clearly established that immune-derived cytokines serve as afferent messengers from the immune system to the CNS. This evidence supports our proposal that the immune system itself is also a sensorial organ that can inform the brain about changes in body composition, such as those triggered by the intrusion of foreign molecules or the appearance of modified self-antigens (Besedovsky et al., 1983). We shall briefly refer here to the immune–neuroendocrine network to introduce the conceptual framework of the main subject of this chapter: the physiological function of brain-borne cytokines in the absence of CNS pathologies. It is important to point out that the purpose of this chapter is not to make an exhaustive review of how cytokines act and are produced in the CNS, but rather to stress their physiological relevance for brain functions and neuroendocrine adjustments during immune processes.

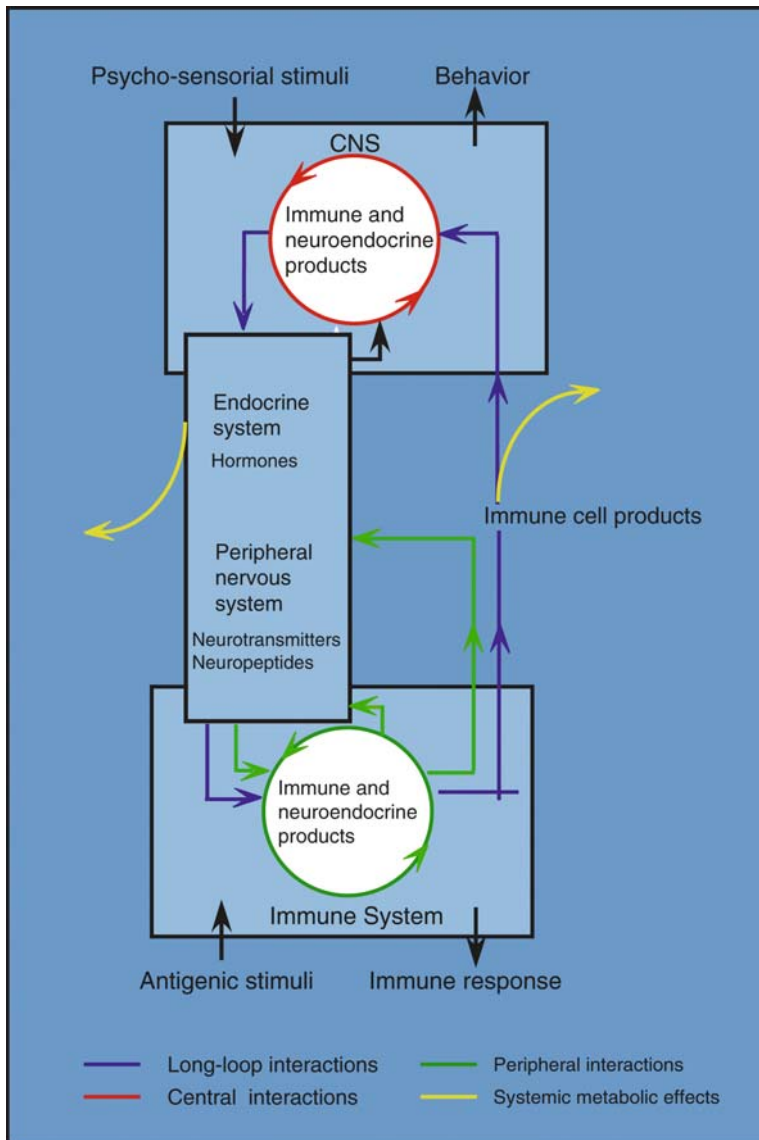
2 The Immune–Neuroendocrine Network

It is now well accepted that there is a cross talk between the immune system and the brain. This cross talk is based on afferent signals conveyed by cytokines and other immune-derived products and efferent signals provided by hormones and neurotransmitters that are, directly or indirectly, under brain control. Evidence indicating that the brain receives information from the immune system derives from studies showing that peripheral immune responses to innocuous T-dependent and -independent antigens, such as sheep red blood cells or TNP-hemocyanin, result in increased neuronal activity in defined hypothalamic nuclei (for review Besedovsky and del Rey, 1996). More recently, it has been shown that different types of immune responses can affect different brain areas. For example, when the immune response is induced by allogeneic antigens that do not cause an overt disease, neurons of the piriform and frontal cortex are activated, while those of most hypothalamic nuclei are not (Furukawa et al., 2004). The evidence that changes in brain activity occur during the immune response implies that signals released by immune cells mediate these effects. In this respect it is clearly established that products derived from the immune system, particularly cytokines, affect neuroendocrine functions (see below). Because the communication between the immune system and the brain occurs at multiple levels, this communication is now defined as a “network of

interactions” (Besedovsky and del Rey, 2001). The operation of this immune–neuroendocrine network, which is affected by immune and neurosensory inputs, has consequences for immunoregulation, CNS functions, and general homeostasis (► [Figure 1-1](#)).

■ **Figure 1-1**

The immune–neuroendocrine network. An active exchange of signals between the immune system, the brain, and neuroendocrine structures under brain control establish a network of interactions that result in reciprocal modulatory effects. This network can be affected by immunologically derived and psychosensorial stimuli. Within this network, there are long-loop (*dark gray*) and short-loop interactions that can occur either at peripheral (*lighter gray*) or at central (*upper circle*) levels. The *branching arrows* indicate effects of immune and neuroendocrine signals on general homeostasis



3 Cytokine Receptors and Production in the Brain

Without attempting to review this subject exhaustively, this section provides selected evidence that receptors for cytokines and their ligands are expressed in the brain.

Different techniques such as autoradiography, *in situ* immunohistochemistry, and hybridization histochemistry, occasionally combined with reverse transcriptase–polymerase chain reaction, have been used to study whether receptors for cytokines are present in the brain. As a whole, the evidence indicates that receptors (or of the corresponding mRNA) for interleukin (IL)-1 α and - β , IL-2, IL-4, IL-6, IL-10, IL-12, IL-18, TNF- α , IFN- γ , M-CSF, and SCF are found in the brain, either under basal conditions or following induction (for review see Van Wagoner and Benveniste, 1999; Szelenyi, 2001; Marques-Deak et al., 2005).

Probably the best-studied cytokine receptors on brain cells are those for IL-1. Although not all studies completely agree in the localization of these receptors in the brain, there is a general consensus that, in adult mice and rats, IL-1 receptors are mainly located or more concentrated in the dentate gyrus of the hippocampus. Some authors have reported that these receptors are present constitutively, whereas others have detected them following endotoxin administration (for review see Besedovsky and del Rey, 1996). More recently, the effect of glucocorticoids and antiinflammatory cytokines, such as IL-10, on IL-1 receptor expression in the brain has been addressed (Lledo et al., 1999; Pousset et al., 2001; Strle et al., 2001).

Several immune-derived cytokines are produced within the CNS during brain pathologies, for example encephalomyelitis, brain trauma, infection with neurotropic viruses, and multiple sclerosis. This important issue will not be covered here (for review see Rothwell and Hopkins, 1995; Merrill and Benveniste, 1996; Allan and Rothwell, 2001). Astrocytes and microglial cells are the brain cells that were first shown to produce several cytokines. It has been also reported that certain neurons can also produce cytokines such as IL-1. Several cytokines, such as IL-1, its natural receptor antagonist (IL-1ra), IL-2, IL-3, IL-6, IL-8, IL-10, IL-12, IL-18, and IFN- γ have been found constitutively in the CNS (for review see Besedovsky and del Rey, 1996; Safieh-Garabedian et al., 2004). Interestingly, recent works from Galoyan and coworkers have shown IL-1 α , IL-1 β , IL-2, IL-6, and TNF- α bioactivity in isolated neurosecretory granules from the paraventricular and supraoptic nucleus of the hypothalamus (for review Galoyan, 2004). Furthermore, they have also identified new hypothalamic proline-rich peptides with cytokine like activity that can affect myelopoiesis and leukocyte functions (Galoyan and Aprikyan, 2002; Davtyan et al., 2005).

4 Effect of Peripheral or Central Administration of Cytokines on Brain Functions

4.1 Effect of Cytokines on the Production of Hypothalamic Releasing Factors and Neurotransmitters, and on Neuronal Activity

CRH was the first hypothalamic releasing factor whose production was shown to be affected by a cytokine (Berkenbosch et al., 1987; Sapolsky et al., 1987; Uehara et al., 1987). IL-1, either administered systemically, *i.c.v.*, or applied *in vitro* (Tsagarakis et al., 1989; Cambronerio et al., 1992), stimulates CRH production in the hypothalamus. Later, it was shown that cytokines can also exert inhibitory effects, for example on TSH and LHRH release, and dual effects on GHRH (Pawlikowski et al., 1994; McCann et al., 2000; Ferri and Ferguson, 2003).

The first indication that cytokines can affect brain neurotransmitters was that administration of immune cell-conditioned media containing lymphokines and monokines decreases noradrenaline (NA) content in the brain (Besedovsky et al., 1983). Later, it was shown that catecholaminergic and serotonergic neurons could be affected by different cytokines. For example, IL-1 reduces NA content and increases the 3-methoxy-4-hydroxyphenylethylene glycol (MHPG)/NA ratio, which reflects increased NA metabolism (Dunn, 1988, 2002). This effect is observed in the hypothalamus, hippocampus, brain stem, and spinal cord. The fact that IL-1 stimulates catecholaminergic fibers in the spinal cord may indicate one neural pathway for the effect of this cytokine in the CNS. The stimulation of noradrenergic neurons in the CNS by IL-1 is consistent with other evidence indicating that the stimulation of CRH production by catecholaminergic neurons is involved

in the response of the HPA axis to IL-1 (Weidenfeld et al., 1989). This is further supported by studies showing that the response of the HPA axis to IL-1 is blocked by surgical interruption of noradrenergic innervation of CRH-producing neurons in the paraventricular nucleus of the hypothalamus or by NA antagonists Ovadia et al., 1989; Matta et al., 1990; Chuluyan et al., 1992). There are selected effects of cytokines on CNS neurotransmitters in different brain regions. For example, IL-1 enhances NA turnover in the hypothalamus and hippocampus, serotonin turnover in the hippocampus and prefrontal cortex, and dopamine utilization in the prefrontal cortex. IL-6 also increases serotonin and dopamine activity in the hippocampus and prefrontal cortex, but does not affect central noradrenergic activity. IL-2 increases NA turnover in the hypothalamus and dopamine turnover in the prefrontal cortex, but does not influence central serotonergic activity (Zalcman et al., 1994). However IL-1 causes a general accumulation of tryptophan in the CNS (Kabiersch et al., 1988; Dunn, 1988, 2002). IL-3, IFN- γ , G-CSF, GM-CSF, and IL-2 augment choline-acetylcholine transferase activity in septal neurons in vitro (Konishi et al., 1993; Tabira et al., 1995; Jonakait et al., 1996; Mennicken and Quirion, 1997). Neuronal–glial interactions mediated by IL-1 enhance neuronal acetylcholinesterase activity and mRNA expression (Li et al., 2000).

Early evidence that cytokines can affect the electrical activity of defined neuronal populations was previously reviewed (Besedovsky and del Rey, 1996). Thus, we shall mention here some more recent publications addressing this issue. Interleukin-1 β depolarizes parvocellular neurons of the paraventricular nucleus, and studies performed in vivo show that IL-1 administration stimulates neurons of the paraventricular and supraoptic nucleus of the hypothalamus and of the stria terminalis (Ferri and Ferguson, 2003; Ferri et al., 2005). IFN- β differentially augments neocortical neuronal activity and excitability (Hadjilambrea et al., 2005). In contrast, IL-1 and IL-6 exert an inhibitory action on neurons of the anterior hypothalamus (Alam et al., 2004). There is also evidence that cytokines affect long-term potentiation (LTP) in the hippocampus. This issue is discussed below with more detail.

4.2 Effect of Cytokines on Thermoregulation, Nociception, Food Intake, Sleep, and Behavior

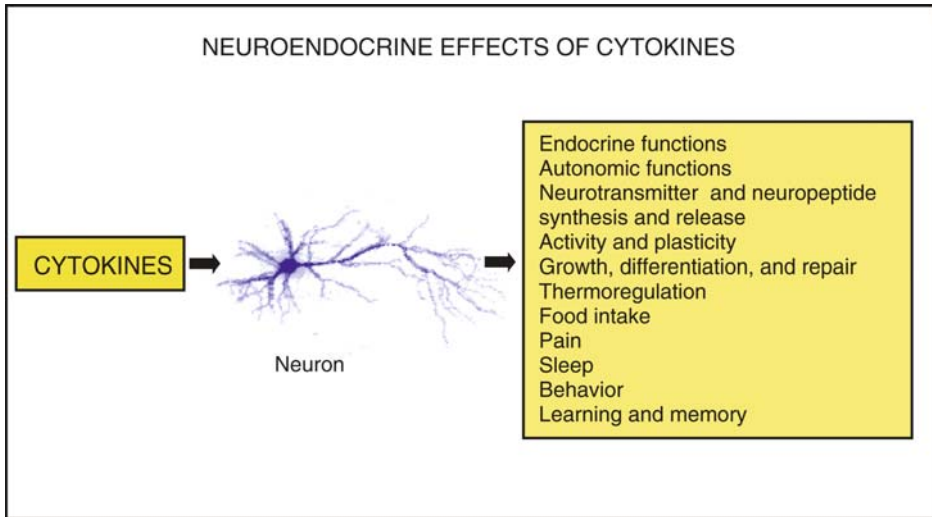
Immune cytokines can influence complex mechanisms that involve a variety of neuronal circuits such as thermoregulation, food intake, sleeping patterns, and behavior. As stated in *Sect. 1*, these effects will not be discussed in detail since recent reviews on these subjects have been published. We shall mention here only a few examples. It is now well established that IL-1, IL-6, IL-8, IFN- γ , IFN- β , and GM-CSF can induce fever, acting as endogenous pyrogens (Kluger, 1991; Rothwell, 1991; Blatteis, 1992). Several cytokines, among which are IL-1, IL-6, IL-8, and TNF, can inhibit food intake (Plata-Salaman, 1989; Rothwell, 1991). The capacity of increasing slow-wave sleep is also shared by different cytokines such as IL-1, IL-2, IFN- γ , and TNF (Nistico and De Sarro, 1991; Opp et al., 1992). A dual role of cytokines in the sensitivity to pain has been described. IL-1 and IL-6, acting at sites of inflammation, sensitize afferent fibers by stimulating the release of prostaglandins (Dray and Bevan, 1993; Kidd et al., 2004). However, when IL-1 is given systemically, it mediates an antinociceptive response (Kita et al., 1993). Several cytokines can exert profound effects on behavior, for example, on learning and explorative and avoidance behavior (Dyck and Greenberg, 1991; Kent et al., 1992b) following lipopolysaccharide (LPS) administration. Some of these actions are likely to occur at CNS levels since i.c.v. administration of IL-1ra blocks such effects (Kent et al., 1992a). Cytokines are also involved in the central mechanisms of learning and memory (see below). Taken together, the evidence available shows that cytokines can affect central and peripheral neuronal activity and influence many brain functions (🔗 *Figure 1-2*).

5 Resetting Brain-Controlled Homeostatic Mechanisms by Cytokines

The effects of cytokines on mechanisms under brain control could be just a transient perturbation that dissipates as soon as cytokines induced in the periphery disappear from the circulation. However, this is generally not the case, despite the fact that the neuroendocrine and metabolic processes affected by cytokines are tightly controlled by feedback systems that tend to normalize the variable affected around

■ Figure 1-2

Cytokines can affect neuronal functions. Multiple mechanisms under brain control can be targets of cytokine effects



a fixed set point. Adjustments based on complex neuroendocrine responses are necessary during diseases that involve the activation of the immune system. Such adjustments are needed because immune mechanisms are based on processes that demand large amounts of energy (Sellmeyer and Grunfeld, 1996; Powanda and Beisel, 2003) such as cell turnover, traffic and homing, clonal expansion, generation of cytotoxic cells, phagocytosis, endo and exocytosis, and synthesis of different mediators and effector molecules. Neuroendocrine adjustments, together with reduced physical activity and cytokine-mediated increase of blood flow in lymphoid organs (Rogausch et al., 1997), would result in redistribution of energy supply to support immune cell functions. In the following, evidence indicating that cytokines released during the activation of immune cells can “move” homeostatic set-points, most likely by acting at brain levels, is discussed.

Fever is defined as a change in the set point of centrally integrated thermoregulatory mechanisms. This implies that the balance between neuroendocrine mechanisms involved in thermogenesis and thermolysis are transiently operating at a level different from that operating under basal conditions. As mentioned, several immune-derived cytokines have been identified as “endogenous pyrogens” (Dinarello, 1999; Cartmell et al., 2001).

Glucose is the main source of energy for the brain and for most peripheral tissues. Thus, the maintenance of appropriate levels of glucose in blood and tissues is essential for survival and, as known, glucose homeostasis is controlled at central levels. We have shown that low, subpyrogenic doses of IL-1 induce a profound, long lasting hypoglycemia that is not related to possible insulin secretagogue effects of the cytokine (del Rey and Besedovsky, 1987, 1989, 1992). This effect, which is also observed in insulin-resistant animals, develops in mice against increased levels of counterregulatory hormones such as catecholamines, glucocorticoids, and glucagon. There is also evidence that the hypoglycemic effect of IL-1 can be triggered at central levels since intracerebroventricular administration of the cytokine induces a reduction in blood glucose levels (del Rey et al., 1998). However, the most surprising effect is observed when mice and rats are challenged with a glucose load several hours after a single intraperitoneal injection of IL-1 (del Rey et al., 1996). In this situation, it is clearly seen that, following a transient elevation of glucose levels in blood, its concentration returns to the previously reduced levels and the animals remain hypoglycemic for several hours more. These findings strongly indicate that IL-1 changes the rigid set point that characterizes glucose homeostasis.

An appropriate control of osmolarity is essential for an adequate distribution of water and electrolytes in different body compartments. Since a tendency to deshidration is commonly observed during infections, osmolarity is another vital variable in homeostasis that must be adjusted. Aldosterone is a crucial hormone in osmoregulation because it controls sodium reabsorption in the kidney and therefore the concentration of this electrolyte in blood. IL-1 β and TNF- α induce an increase in aldosterone blood levels through their effects on ACTH release and the increase in plasma renin activity (Bataillard et al., 1992; Barmeyer et al., 2004). These effects are indicative that immune cell products can also affect the set point for osmoregulation.

Adjustments of cardiovascular functions can be exerted by cytokines released during infections. For example, IL-1 produced in lymphoid organs selectively increases blood flow by interfering locally with the sympathetic tonus and causing a redistribution of blood flow (Rogauch et al., 1997, 2000). This effect results in a preferential supply of blood to lymphoid organs (Rogauch et al., 2000) and increases the probability of antigen and cell trapping, a process that is essential for an appropriated immune response (Starzl and Zinkernagel, 1998). Another example is provided by LPS that, most likely via induction of cytokine production, causes an increase in the sensitivity of baroreceptor reflexes, indicating that a resetting of the central mechanisms that control blood pressure may occur during certain infections (Rogauch et al., 2000).

The release of glucocorticoids is a well-controlled endocrine response subject to feedback mechanisms integrated at the level of the pituitary, the hypothalamus, and the limbic system. As mentioned, several cytokines can stimulate the HPA axis and, in the case of IL-1, administration of a single dose decreases the threshold for activation of the HPA axis for several weeks (Schmidt et al., 2003). This effect suggests that the cytokine can affect the set point of the control of glucocorticoid output.

An association between immune and reproductive functions is also observed. Reproductive functions are controlled at brain levels by mechanisms that set the dynamics of sexual hormone production. Cytokines such as IL-1, TNF, and IL-6 inhibit the hypothalamus-gonadal axis and female sexual cycle (for review see Besedovsky and del Rey, 1996). Interestingly, female birds do not choose for reproduction males that are undergoing an immune response, even when the response has been triggered by innocuous antigens (Faivre et al., 2003). Taken together, the evidence suggests that the complex, well programmed, regulatory mechanisms involved in the control of reproduction are downregulated during the immune response, an effect that may serve to limit the eventual transmission of microorganisms to the progeny.

An overall appraisal of the work discussed above indicates that cytokines not only affect essential homeostatic mechanisms but also their central regulatory set points, thus mediating adjustments necessary to support immune processes.

6 Induction and Relevance of Endogenous Cytokines for Brain Functions

The question that we want to analyze is whether cytokines synthesized by glial cells and certain neurons play a role in the “healthy” brain and in physiological neuroendocrine responses. Here we shall discuss evidence supporting the hypothesis that cytokines play a physiological neuromodulatory role in the brain and integrate neural and peripheral immune signals at central levels.

6.1 Peripheral Immune Signals Trigger Cytokine Production in the CNS

Cytokines are not released only during inflammatory and infectious diseases within the brain. Most reports agree that also peripheral administration of LPS from gram negative bacteria results in increased expression of cytokines in the central nervous system (Gatti and Bartfai, 1993; Laye et al., 1994; Quan et al., 1994; Buttini and Boddeke, 1995; Gabellec et al., 1995; Van Dam et al., 1995). However, because the doses of LPS administered in most of these studies were rather high and LPS can disturb the blood–brain barrier (BBB) (Lustig et al., 1992), the results obtained may indicate a direct effect of the endotoxin in the brain.

Furthermore, elevated doses of LPS can induce a septic shock, and cardiovascular and respiratory derangements might induce the expression of cytokines in the CNS nonspecifically. To overcome these objections, we have studied the expression of cytokines in defined areas of the mouse brain following systemic injection of a low dose of LPS that does not disrupt the BBB and is well below those that can induce a septic shock (Pitossi et al., 1997). Constitutive expression of IL-1 β , IL-6, and TNF- α (but not of IFN- γ) is detected in the brain, but a three- to four-fold variation in cytokine mRNA expression between different regions is noticed. The gene expression of the four cytokines studied is increased following peripheral administration of LPS. The onset of transcription and the peak of mRNA accumulation are dependent on the cytokine and the brain region. IL-1 β and IL-6 expression is preferentially increased in the hypothalamus and hippocampus, whereas TNF- α expression is more marked in the thalamus-striatum. These cytokines are less inducible in the brain cortex. No correlation between cytokine gene expression and the density of vascular structures in a given brain area was detected, neither was a preferential cytokine expression in brain areas that include circumventricular organs. This suggests that even if some LPS would have crossed the BBB at these sites, it did not significantly contribute to the induction of cytokines in regions near to the circumventricular organs. Taken together, the results show that stimulation of peripheral immune cells induces cytokine expression in the brain. The particular pattern of regional expression of cytokines in the hypothalamus and hippocampus suggests that, during activation of the immune system, brain-borne cytokines may affect neuroendocrine mechanisms controlled in these areas.

6.2 Neuronal Signals Induce Cytokine Production in the Brain

There is evidence that massive stimulation of neuronal activity with convulsion-inducing agents, such as kainic acid (Eriksson et al., 1999; Vezzani et al., 1999; Lehtimäki et al., 2003), can induce the production of IL-1 and other cytokines in the brain. Although this effect is of clear relevance for the pathogenesis of diseases like epilepsy, they do not indicate a physiologic induction of cytokines in the brain. In addition, the seizures produced by kainic acid may induce peripheral effects that can secondarily result in cytokine production in the brain. Cytokines can also be induced in the brain during stress (Plata-Salaman, 1989; Nguyen et al., 1998; Goshen et al., 2003) and their concentration correlate with sleep–wake activity (Krueger et al., 2001; Obal and Krueger, 2003). However, these data constitute only indirect evidence that changes in neuronal activity can result in cytokine induction in the brain since, for example, peripheral endocrine signals and circadian rhythms can influence the production of these mediators in the CNS. Direct evidence derives from the demonstration that presynaptic stimulation of defined neurons, as it happens during physiologic conditions, can control the local production of cytokines by glial cells and neurons. LTP of synaptic activity in the hippocampus, a process characterized by a sustained enhancement in synaptic transmission and postsynaptic neuronal activity following a high frequency stimulation of afferent fibers, has served as a model to approach this issue. LTP induction allows exploring whether a long lasting increase in the activity of a defined population of neurons affects the production of a given cytokine, and whether, in turn, this cytokine can affect these neurons. A clear increase in IL-1 β gene expression, triggered by glutaminergic neurons via NMDA receptors, was observed in hippocampal slices and in freely moving rats during the course of LTP (Schneider et al., 1998). More recently, we have observed that the IL-6 gene is also over expressed during *in vivo* and *in vitro* LTP (Besedovsky et al., 1999; Balschun et al., 2004). Other laboratories have confirmed these results (Jankowsky et al., 2000). These data constitute the first evidence that cytokine gene expression in the brain can be triggered by a presynaptically induced increase in the activity of a discrete population of neurons.

6.3 Relevance of Brain-Borne Cytokines for Brain Functions, Particularly Synaptic Plasticity, Learning, and Memory

There is now considerably evidence that brain-borne cytokines can affect CNS mechanisms. Cytokines have been shown to be “sleep factors” and to affect both rapid eye movement (REM) and non-REM sleep. Brain

levels of IL-1 and TNF correlate with sleep propensity; for example, their levels increase after sleep deprivation. Furthermore, immune neutralization of IL-1 or blockade of its receptors in the brain affect slow-wave sleep (Krueger et al., 2001; Obal and Krueger, 2003), indicating that endogenous IL-1 and TNF are part of a complex biochemical cascade regulating sleep. There is also evidence that some of the symptoms of “sickness behavior” are integrated by cytokines produced in the brain. Sickness behavior refers to a coordinated set of subjective, behavioral, and physiological changes that develop in sick individuals during the course of an acute infection. These changes are caused by effects of IL-1 and other proinflammatory cytokines on brain cellular targets. Indeed, interference with the effects of these cytokines in the brain abolishes the expression of certain symptoms of sickness behavior. This evidence indicates the role of proinflammatory cytokines in orchestrating sickness behavior during acute diseases, and is summarized in a recent review (Dantzer, 2004).

Complex mechanisms such as thermoregulation, sleep, food intake, pain, and stress are integrated at brain levels but are also modulated by endocrine and autonomic effects linked to such mechanisms and by cytokines produced at peripheral levels. This is not expected to occur during LTP, which is initiated in the brain. Therefore, we and others have studied to what extent cytokines produced in the brain during LTP can affect synaptic plasticity and performance. At this stage, it is necessary to distinguish between studies based on exogenous administration of cytokines and those that focus on the effects of cytokines endogenously produced by brain cells. There is a vast literature showing that exogenous *in vivo* and *in vitro* administration of cytokines can affect LTP induction and synaptic plasticity. Administration of IL-1 (Katsuki et al., 1990; Bellinger et al., 1993), IL-2 (Tancredi et al., 1990), IFN- α and IFN- γ (Tancredi et al., 1990; D’Arcangelo et al., 1991; Mendoza-Fernandez et al., 2000), TNF (Tancredi et al., 1992; Albensi and Mattson, 2000), IL-6 (Li et al., 1997), and IL-18 (Curran and O’Connor, 2001) inhibit LTP. These studies, although important from the pharmacological point of view, cannot reliably reveal the effect of cytokines produced in the brain under natural conditions. In fact, LTP is a complex phenomenon that involves a number of receptors and mediators that influence its inducibility, establishment, and maintenance in different ways. In particular, the maintenance of LTP is protein synthesis-dependent and involves the activation of genes in a given sequence and the release of their products in certain quantity. It is almost impossible to mimic these conditions by the exogenous application of cytokines. For example, as it is discussed below, IL-1 (a cytokine that inhibits LTP) contributes to the consolidation and maintenance of this process when it is produced endogenously.

Using the specific IL-1 receptor antagonist (IL-1ra), we found that blockade of IL-1 receptors, both *in vivo* and in hippocampal slices, results in the inhibition of LTP maintenance. This effect is reversible and occurs only when the antagonist is administered after LTP is triggered, i.e., at a time when, according to the studies mentioned above, increased IL-1 levels are expected. Studies in type 1 IL-1 receptor knockout (KO) mice are in line with this finding (Avital et al., 2003). These mice exhibit enhanced paired-pulse inhibition in response to perforant path stimulation and no LTP in the dentate gyrus. Decreased paired-pulse responses and complete absence of LTP were observed in the CA1 region of hippocampal slices obtained from type 1 IL-1 receptor KO mice. We have recently found that, in contrast to the supportive effect of IL-1, IL-6 contributes to the extinction of a well-consolidated LTP (Balschun et al., 2004). Collectively, these results strongly suggest that IL-1 β and IL-6 can control the maintenance of LTP in the brain, a process that is assigned a role in memory formation and in certain types of learning. Furthermore, these studies provide evidence for a physiologic, neuromodulatory role of cytokines originally described as immune mediators.

As in the case of LTP, the effects of cytokine administration on learning, memory, and behavior in general have been extensively investigated (for review Muller and Ackenheil, 1998; Anisman et al., 2005). Again, these studies that are undoubtedly of pharmacological relevance may not reflect the effect of cytokines in the “normal” brain, which is the main scope of this chapter. Thus, only possible physiologic effects of endogenous cytokines on memory and learning are discussed below. As mentioned above, a transient blockade of endogenous IL-1 in hippocampal slices and in the brain of freely moving rats results in the inhibition of LTP maintenance. Considering that it is currently accepted that LTP underlies certain forms of memory, it was predicted that this process would be inhibited in animals in which IL-1 effects cannot be manifested. This is the case of type I IL-1 receptor KO mice. Several paradigms of memory

function and hippocampal plasticity have been studied in these animals (Yirmiya et al., 2002; Avital et al., 2003). Compared to wild-type controls, type 1 IL-1 receptor KO mice display significantly longer latency to reach a hidden platform in the spatial version of the water maze test. Furthermore, type 1 IL-1 receptor KO mice exhibit diminished contextual fear conditioning, but behave similar to control animals in hippocampal-independent memory tasks, i.e., their performance in the visually guided task of the water maze and the auditory-cued fear conditioning is normal. Blockade of IL-1 receptors in the brain of normal animals with IL-1ra administered in the brain and following a learning task (Morris water maze) causes hippocampal-dependent memory impairment, but does not influence the hippocampal-independent, nonspatial version of this test. IL-1ra caused memory impairment in the passive avoidance response, which also depends on hippocampal functioning. These results suggest that IL-1 signaling within the hippocampus plays a critical role in learning and memory processes (Avital et al., 2003). It is worth noting that in the previously mentioned studies, the impediment in IL-1 signaling was induced after the training procedure. In contrast, there is evidence that blockade of IL-1 effects prior training by using an adenovector expressing IL-1ra, which causes an improvement of both short-term and long-term memory retention scores (Depino et al., 2004). These results are in line with the inhibitory effects in LTP and memory formation observed when IL-1 was administered prior to the learning sessions. However, as mentioned, endogenously produced IL-1 during learning significantly contributes to memorize an established task.

The role of IL-6 endogenously produced in the brain has also been studied. As discussed above, IL-6 is produced during LTP. Neutralization of the cytokine after tetanization results in a clear prolongation of LTP. In agreement with these results, blockade of endogenous IL-6 after hippocampus-dependent spatial alternation learning resulted in significant improvement of long-term memory (Balschun et al., 2004). Furthermore, IL-6 KO mice exhibited a facilitation of radial maze learning over 30 days, in terms of lower number of working memory errors when compared with wild type mice (Braidá et al., 2004).

Taking these results together, it appears clear that, although having an opposite role, endogenous IL-1 and IL-6 produced in the “healthy” brain are important in the control of synaptic plasticity and in the hippocampal processing of memory. The mechanism underlying the relevant role of IL-1 and IL-6 on these processes is still unknown, but recent data indicate the involvement of NF- κ B, a transcription factor that mediates the production and effects of multiple cytokines (Meffert et al., 2003; Meffert and Baltimore, 2005). Indeed, basal synaptic input activates the p65/p50 NF- κ B form, which is selectively localized in synapses, through cCa^{2+} /calmodulin-dependent kinase and local submembranous Ca^{2+} elevation. p65-deficient mice have no detectable synaptic NF- κ B and show a selective learning deficit in the spatial version of the radial arm maze.

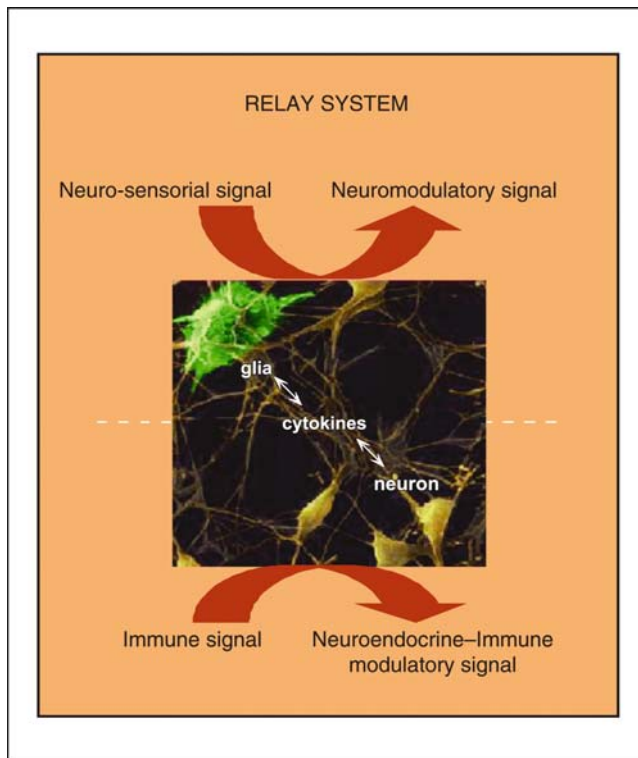
7 Overview and Proposal

The exchange of information between the immune and the nervous systems, which results in mutual effects, indicates the operation of a network of immune–neuroendocrine circuits. It was originally assumed that such a network is based on neuroendocrine effects of cytokines produced in the periphery by activated immune cells. However, cytokines produced in the brain could also play a role in mediating interactions between the immune and the central nervous systems. It is worth noting that effects of cytokines, when injected or induced either peripherally or centrally, are, in many cases, remarkably similar. In our opinion, these similarities indicate that the production of both peripheral and central cytokines underlie well-programmed steps of responses integrated at brain levels. Under basal conditions, the release of low amounts of cytokines by brain cells could be one of the various inputs that control the activity of neurons involved in the regulation of adaptive functions, such as neuroendocrine and metabolic mechanisms, synaptic plasticity, and behavior. Under pathological conditions, during which changes in the activity of the immune system occur, peripheral cytokines and other mediators could trigger the initial step of homeostatic adaptive adjustments. The quick neuroendocrine response observed when certain cytokines

are administered peripherally may indicate that this initial step does not involve the *de novo* synthesis of cytokines in the brain. However, peripheral immune mediators and neurons activated during this initial step could trigger an increased expression of cytokines in the brain. This confluence of signals may contribute to determine a defined pattern of central cytokine expression during an immune response. As shown during LTP, these cytokines are expected to feedback on the neurons whose activation has induced the production of such cytokines. In this way, *de novo* produced cytokines would influence the neural, behavioral, or endocrine mechanisms controlled by the neurons that trigger their production. Cytokine-producing cells (glial cells, neurons) located in brain regions where neuroendocrine mechanisms capable of affecting immune functions are controlled may also be influenced by neurons stimulated by other sensorial inputs, for example during different types of stress. On the other direction, immune signals capable of inducing cytokine production in the brain could affect CNS functions. We therefore postulate a “relay system” based on interactions between neurons and cytokine-producing brain cells that could integrate peripheral immune and central neural signals and modulate neuroendocrine and behavioral responses to these stimuli (● [Figure 1-3](#)).

■ **Figure 1-3**

Integrative role of cytokines in the brain. A relay system based on interactions between cytokine-producing brain cells and neurons located in their loose vicinity would integrate peripheral immune and neurosensorial signals. When the production of cytokines in the brain is increased as a consequence of immune and/or neuronal signals, a resetting of homeostatic functions would occur. This resetting is expected to be especially relevant for neuroendocrine adjustments during conditions in which primarily the immune system (e.g., infections) or the CNS (e.g., stress) is affected



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2 **Neuropeptides in the Immune System: Mediators of Stress and Inflammation**

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<i>1</i>	<i>Introduction</i>	<i>20</i>
<i>2</i>	<i>CRF Family of Peptides</i>	<i>21</i>
<i>3</i>	<i>Arginine Vasopressin</i>	<i>23</i>
<i>4</i>	<i>Proopiomelanocortin Peptides</i>	<i>23</i>
<i>5</i>	<i>Non-POMC-Derived Opioid Peptides</i>	<i>26</i>
<i>6</i>	<i>Variant Forms of Peptides in Immune Tissues</i>	<i>28</i>
<i>7</i>	<i>Conclusion</i>	<i>29</i>

Abstract: Neuropeptides and cytokines expressed within the brain and the immune system can act in association with circulating cytokines, catecholamines, and glucocorticoids to form an elegant bidirectional symmetry of brain–immune system communications. Interactions between these compounds are critical for the maintenance of homeostasis during immune system activation and during chronic inflammatory stress. Now that many fundamental principles governing the general anatomy of this network have been established, a major research challenge lies in elucidation of the functional roles of cytokines in the brain and neuropeptides in immune tissues. This chapter will focus on immunoneuropeptides, expression of many of which is altered in autoimmune diseases and also in models of chronic stress associated with inflammation. This has led to the proposition that immunoneuropeptides may be involved in underlying processes in the development of, and the responses to, acute and chronic inflammation. In support of this concept, potential for modulation of inflammation *in vivo* and *in vitro* has been demonstrated for many neuropeptides synthesized within immune cells. The purpose of this chapter is to summarize the evidence for involvement of immunoneuropeptides in disease processes and to discuss potential antiinflammatory therapies that may arise from our increased understanding of the roles these compounds play in immune responses.

List of Abbreviations: AA, adjuvant arthritis; AVP, arginine vasopressin; CNS, central nervous system; CRF, corticotropin-releasing factor; EAE, experimental allergic encephalomyelitis; EMs, endomorphins; HPA, hypothalamo-pituitary-adrenal; IL, interleukin; MC, melanocortin; OFQ, orphanin FQ; PAN, peripheral afferent nociceptive; PBMC, peripheral blood mononuclear cells; POMC, proopiomelanocortin; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SNS, sympathetic nervous system; SP, substance P; Ucn, Urocortin 1

1 Introduction

The general principle is now well established that the central nervous system (CNS) and the immune system are closely integrated in a bidirectional network of communication to maintain homeostatic control when one or the other is challenged. The immune system is activated in response to life-threatening foreign pathogens while activation of specific pathways within the CNS protects against stressful challenges from the environment. An overreaction of inflammatory immune responses to pathogenic invasion is prevented and controlled by the CNS, alerted by elevated levels of circulating cytokines, through the production of glucocorticoids and catecholamines, which act as antiinflammatory agents. In addition to systemic and neuronal control, immune responses are also under paracrine regulation by neuropeptides released at sites of inflammation. Expression and release of neuropeptides from neurons or leukocytes within inflamed tissue is increased in many acute and chronic inflammatory conditions as disparate as transient infections or autoimmune diseases. Expression of neuropeptides within inflamed tissues may be stimulated by locally secreted immune signals such as chemokines or cytokines, by neuronal input such as noradrenergic release of catecholamines from sympathetic nerve terminals, or by systemic compounds such as glucocorticoids. A wide range of paracrine immunomodulatory functions has been ascribed to neuropeptides synthesized within immune cells. Therefore the magnitude of the immune response to antigenic challenge is under multiple, complex, and integrated regulation by the CNS, hormones, and immunoneuropeptides to permit appropriate and measured responses and to prevent excessive and potentially damaging release of inflammatory agents.

There are very few instances of neuropeptides found within the CNS which have not been located in immune tissues and it is beyond the scope of this chapter to inventory all neuropeptides in the immune system. Focus will be on critical analysis of functional roles that selected immunoneuropeptides might play in mediating immune responses to chronic stress, emotional or inflammatory, and to explore potential mechanisms for facilitation of these actions. Of particular importance in this respect are the family of corticotropin-releasing factor (CRF) peptides, arginine vasopressin (AVP), proopiomelanocortin (POMC) products, and opioid peptides. Because of the integral role of these peptides within the CNS in mediating the hypothalamo-pituitary-adrenal (HPA) axis and sympathetic nervous system (SNS) responses to stress, acute or chronic, much recent research has focused on potential roles for these peptides within immune

cells in mediating immune responses to stress, both independent of or under control by glucocorticoids and catecholamines. Many inflammatory diseases are associated with elevated HPA axis and SNS activity and can therefore be considered as chronic stress paradigms (Harbuz, 2002). Inflammatory diseases themselves can be influenced by stressful life events, usually (but not always) negatively (Solomon, 1997; Harbuz et al., 2002; Turner-Cobb, 2002; Jessop et al., 2004). Our understanding of the underlying mechanisms by which the neuroendocrine pathways activated by stress can alter immune functions may lead to insight into the roles of the SNS, HPA axis, and immunoneuropeptides in the etiology of inflammatory autoimmune diseases and in the modulation of preexisting disease.

2 CRF Family of Peptides

CRF is a 41-residue peptide first identified in the hypothalamus as an integral transducer in HPA axis and SNS responses to stressors. In addition to its widespread distribution in the CNS, CRF is present in peripheral blood mononuclear cells (PBMC) and neutrophils (Stephanou et al., 1990; Aird et al., 1993), principally in macrophages (Brouxhon et al., 1998; Jessop, 2002) although also found in T cells and B cells. Rodent models of inflammation such as adjuvant arthritis (AA), experimental allergic encephalomyelitis (EAE), and systemic lupus erythematosus (SLE) are also models of chronic inflammatory stress in which the HPA axis and SNS are activated. In these models, splenic and thymic contents of CRF can remain unaltered, increased, or decreased, depending on the disease phase and severity (Jessop et al., 1995a, 2001). CRF bioactivity (Hargreaves et al., 1989), mRNA, and peptide levels (Crofford et al., 1992) are elevated in inflamed rat synovial tissues and in synovium from patients with rheumatoid arthritis (RA) (Crofford et al., 1993). CRF in synovial tissue is probably derived from both infiltrating macrophages and from sympathetic and peripheral afferent nociceptive (PAN) nerves. Dramatic upregulation of CRF-R₁ receptors in mouse splenocytes occurs in response to injection of lipopolysaccharide (Radulovic et al., 1999) and CRF-R₁ receptors are increased within inflamed synovial tissue in arthritic rats (Mousa et al., 1996). There is positive correlation in RA patients between synovial fluid CRF and serum amyloid-A concentrations (Nishioka et al., 1996). This evidence for an early increase of synovial CRF in association with an acute phase protein is consistent with involvement of CRF at the onset of inflammation, a role supported by reports of increased expression of CRF-R₁ (McEvoy et al., 2001) and CRF mRNA (Murphy et al., 2001) in synovial cells in recently diagnosed RA patients.

In contrast to the antiinflammatory effects of hypothalamic CRF and its involvement in activating the HPA axis and stimulating secretion of adrenal glucocorticoids, there is a body of evidence which suggests that immune-derived CRF can act as a proinflammatory peptide (Karalis et al., 1997). CRF has been reported to stimulate lymphocyte proliferation and cytokine release (Singh, 1989; Singh and Leu, 1990). Immunoneutralization of peripheral CRF reduces inflammation associated with carrageenin-induced arthritis in rats (Karalis et al., 1991), and knockdown of CRF mRNA translation by oligonucleotide cDNA probes inhibits rat splenocyte proliferation (Jessop et al., 1997a). In a transgenic mouse model, overexpression of hypothalamic CRF is associated with a reduction in B cell numbers (Stenzel-Poore et al., 1996) and an increase in T lymphocytes (Stenzel-Poore et al., 1996; Boehme et al., 1997), while CRF deficiency in a knockout mouse model is accompanied by increased circulating concentrations of the proinflammatory cytokine interleukin (IL)-6 (Venihaki et al., 2001). It is however difficult to assign a direct role to immune-derived CRF in these models since manipulation of total CRF gene expression inevitably modulates central CRF expression, which will lead to alterations in catecholamine and glucocorticoid release and consequent indirect immunomodulatory effects.

Not all groups have found proinflammatory effects of peripheral CRF and direct antiinflammatory effects have been reported (Thomas et al., 1993). Infusion of CRF from osmotic minipumps at nanomolar concentrations had no effect upon the severity of inflammation in AA (Harbuz et al., 1996), while peripheral injection of CRF prevented inflammation in a rat model of EAE (Poliak et al., 1997). Since CRF was protective in adrenalectomized rats in this study, its antiinflammatory effects may have been exerted through direct influence on the immune system or via pituitary-derived ACTH, rather than through corticosterone release. To reconcile these conflicting observations, it is entirely possible that CRF

can exert pro- or antiinflammatory effects depending on the dose of CRF administered, the type (and severity of) of inflammation, and the timing of administration relative to disease onset.

There is little information about the mechanisms that control immune system CRF responses to stress and inflammation. The CRF gene contains glucocorticoid and estrogen response elements, but unlike the extensive body of knowledge extant on steroid control of hypothalamic CRF expression, few studies have been undertaken on the sensitivity of immune system CRF expression to glucocorticoids and estrogens. While adrenalectomy (ADX) alone had no significant effect on rat spleen or thymic contents of CRF, stimulation of CRF expression in the thymus, but not in the spleen, in response to a central injection of IL-1 β was not observed in ADX rats, evidence that CRF expression in the thymus may be more sensitive to the presence of circulating glucocorticoids than in the spleen (Jessop et al., 1997b). Even less is known about immune system CRF sensitivity to catecholamines. Rat spleen and thymic macrophages positive for CRF-immunoreactivity (ir) exist in proximity to noradrenergic fibers (Brouxhon et al., 1998), but the degree of sympathetic control over immune system CRF expression is unknown. Given the importance of glucocorticoid and catecholamine influence over immune functions during acute and chronic stress, it would be a valuable experimental program to investigate the kinetics of expression of CRF and other stress neuropeptides in the immune system following metyrapone or 6-hydroxydopamine administration.

One attractive hypothesis is that CRF may act in synergy with the proinflammatory peptide substance P (SP) to modulate pathways of inflammation. CRF and its receptors are located in sympathetic efferent neurons and PAN neurons (Udelsman et al., 1986; Elenkov and Chrousos, 1999) in which CRF is colocalized with SP in capsaicin-sensitive fibers (Skofitsch et al., 1984). SP, released from both PAN neurons and immune cells, is an important mediator of the acute inflammatory response through stimulation of histamine secretion from mast cells and cytokines from monocytes and macrophages. These are also target cells for activation by CRF (Elenkov and Chrousos, 1999), and there is potential for SP stimulation of CRF release from macrophages, thereby potentiating release of histamine and cytokines. In addition to mediating inflammation, both SP (Chancellor-Freeland et al., 1995) and CRF (Jessop et al., 1997b) in immune cells are responsive to stressors and may interact to mediate the multiple effects of stress on immune function (Jessop et al., 2001). Establishing a functional relationship between CRF and SP in the onset and severity of inflammation may lead to the development of SP and CRF antagonists as antiinflammatory agents. The challenge will be to design a CRF antagonist which is effective in low doses at the site of inflammation without leaking into the systemic circulation where it has the potential to exert proinflammatory effects by inhibiting HPA axis activity at the anterior pituitary, leading to decreased circulating concentrations of glucocorticoids.

In addition to CRF(1–41), several groups have noted heterogeneous forms of CRF-ir in human immune tissues (Stephanou et al., 1990; Crofford et al., 1993; Ekman et al., 1993; Woods et al., 1996; Baker et al., 2003) but little attempt has been made to characterize these variant forms. A more hydrophobic form of CRF-ir has been reported in resting human PBMC (Baker et al., 2003). This variant is the predominant form of CRF-ir in human PBMC while the form of CRF-ir which coelutes with CRF(1–41) is present in significant amounts only in T cells. Existence of variant forms of CRF-ir in immune tissues may reflect differential degradation of CRF under different experimental conditions into fragments with their own selective bioactivity. Thus products of the CRF gene within the immune system may be multifunctional with specific functions determined by the nature of the peptide isoform expressed within, and released from, subpopulations of immune cells.

Urocortin 1 (Ucn), a 40-residue peptide which shares 44% amino acid homology with CRF, has limited expression in the brain but is widely distributed throughout immune tissues (Bamberger et al., 1998; Kageyama et al., 1999; Baigent et al., 2001). Ucn binds with high affinity to type 1 and 2 CRF receptors, both classes of which are present in the immune system (Baigent, 2001) and are upregulated during inflammation (Radulovic et al., 1999). Ucn 1 mRNA has been detected in rat spleen and thymus by riboprobe hybridization (Kageyama et al., 1999) but not by polymerase chain reaction using Ucn cDNA-specific PCR primers (Park et al., 2000). Upregulation of Ucn 1 mRNA and immunoreactivity in synovial tissues from RA patients has been reported (Kohno et al., 2001; Uzuki et al., 2001) but these studies did not determine whether synovial Ucn 1 is of immune or neural origin. Ucn 1, along with CRF, has been identified in cultured human mast cells (Kempuraj et al., 2004), and both peptides can induce mast cell degranulation (Theoharides et al., 1998; Singh et al., 1999). Unlike CRF, few studies have been published on the

immunological effects of Ucn 1, which has been implicated in dendritic development, with differential effects depending on cell culture conditions (Swinny et al., 2004). Ucn 2 and 3 are recently characterized selective ligands for the CRF-R₂ receptor. These peptides have not so far been reported in immune tissues but do have the potential to mediate immune responses since Ucn 2 can upregulate IL-10 expression (Sashinami et al., 2005).

It is still controversial whether immune cells produce authentic Ucn, since some groups have failed to locate either Ucn I-ir or mRNA in the immune system. One explanation for discrepancies between laboratories may be the observation that the intensity of CRF and Ucn I expression in cultured human mast cells depends on the period of culture and also on the source of the cells (Kempuraj et al., 2004). Chromatographic studies have revealed multiple CRF-like peptides in immune tissues, which may be novel products of the CRF or Ucn genes or even undiscovered genes of the CRF family. Assumptions that all immunomodulatory actions of the CRF family can be ascribed to CRF and Ucn I alone may not be valid. Further characterization of the physico- and biochemical properties of CRF and Ucn isoforms, conditions and timing of their expression, and affinities of these ligands for CRF receptor subgroups is a prerequisite for the development of stable CRF/Ucn analogs as modulators of inflammation.

3 Arginine Vasopressin

AVP is expressed in human thymic epithelial cells (Martens et al., 1996; Vanneste et al., 1997) and has been recently reported to be colocalized with CRF in subsets of PBMC (Baker et al., 2003), which also express AVP receptors (Elands et al., 1990). AVP is located predominantly in rat splenic B lymphocytes (Jessop et al., 1995b) and splenic contents of AVP increase around the time of inflammation in AA (Jessop et al., 1995a). Levels of AVP in spleens and thymuses from female rats are two- to fivefold greater than in males (Chowdrey et al., 1994), and AVP receptor density is significantly higher in female PBMC (Elands et al., 1990). This sex dimorphism may be of relevance with regard to the considerably higher incidence of RA in women, since AVP has been proposed as a proinflammatory agent in RA (Chikanza and Grossman, 1998). This proposal is consistent with the observation that immunoneutralization of AVP attenuated inflammation in Lewis rats (Patchev et al., 1993). However, the role of AVP in modulating inflammation may be complex, since AVP can potentiate CRF-induced ACTH (Reder, 1992; Smith et al., 1986) and β -endorphin (Kavelaars et al., 1989) release from human PBMC, and both ACTH and β -endorphin seem to exert predominantly antiinflammatory effects (see below).

Colocalization of CRF and AVP in human PBMC (Baker et al., 2003) confers potential for cosecretion in an integrated immunomodulatory pathway within inflamed tissues. The highly significant correlation of CRF and AVP observed in macrophages, but not in T cells and B cells, suggests that all macrophages contain both neuropeptides while T cells and B cells may differentially express CRF and AVP. A similar pattern is found in the hypothalamus, where about 50% of parvocellular cells within the paraventricular nucleus coexpress CRF and AVP while the remainder express CRF alone (Whitnall et al., 1985). The percentage of cells coexpressing CRF and AVP in the hypothalamus increases considerably following adrenalectomy or stress (Sawchenko et al., 1992). Therefore it is possible that alterations in coexpression of CRF and AVP in immune cells may occur in activated PBMC during inflammatory diseases, with consequent effects on release of ACTH and β -endorphin.

4 Proopiomelanocortin Peptides

POMC mRNA and POMC peptide products, ACTH and β -endorphin, are found within a wide variety of cells (Blalock 1999) but reports on the characteristics and distribution of these peptides have been controversial, with widely variable amounts and forms of immunoreactivity reported between laboratories. Such discrepancies may be due in part to differing degrees of lymphocyte activation in experimental systems. Only activated T lymphocytes and B lymphocytes contain significant amounts of ACTH, while the expression of ACTH in macrophages is constitutive and independent of cell activation

(Lyons and Blalock, 1995). β -Endorphin contents in a range of lymphocyte subtypes also vary widely throughout the course of inflammation (Rittner et al., 2001). Discrepancies have also arisen from differential POMC precursor processing in immune tissues into forms other than those that exist within the pituitary. Although it has now been convincingly demonstrated that the immune system is capable of producing full-length POMC mRNA and ACTH(1–39) peptide (Lyons and Blalock, 1997), immune tissues also contain a wide range of high-molecular weight forms and small fragments of ACTH and β -endorphin (Lolait et al., 1986; Buzzetti et al., 1989; Jessop et al., 1994). Due to differing methods of measurement and processing prior to assay, levels of total immunoreactivity and chromatographic profiles are not always comparable between laboratories.

Antiinflammatory actions of pituitary ACTH through stimulation of glucocorticoids have been recognized for decades but ACTH and other POMC peptides can be antiinflammatory in their own right. A considerable range of immunomodulatory effects have been reported in studies using synthetic ACTH, β -endorphin, or α -melanocyte-stimulating hormone (α -MSH) (Jessop, 1998). In most of these reports, POMC peptides attenuate immune cell proliferation or block the actions of inflammatory cytokines. The literature however is inconsistent, and reports of biological effects of POMC peptides should be interpreted according to whether the concentration employed is in the range reported within immune tissues, which is frequently not the case for *in vitro* studies. Immune cell status is also critical. The biological effects of ACTH are dependent on the degree of cell activation, since activated lymphocytes in culture display a substantially increased number of high-affinity ACTH binding sites (Clarke and Bost, 1989; Johnson et al., 2001). *In vivo* determination of the degree of POMC expression in immune tissues and cellular responses to synthetic POMC peptides can depend on many factors such as the immunological history of the animal, transportation from breeding facilities, breeding and housing conditions, and prior exposure to stress. Data from *in vitro* cell culture studies can be influenced by the degree of cell activation, the type of antigen employed to activate cells, and the culture milieu, there being a particular requirement for a critical number of CD4⁺ cells (Gaillard et al., 1998). Discrepant reports in the literature on the immunomodulatory effects of β -endorphin may also be explained by immune status. Activation of T cells by cytokines secreted during inflammatory autoimmune diseases and during infection dramatically increases the affinity and number of opioid-binding sites (Roy et al., 1991). The well-recognized biphasic effects of β -endorphin (Heijnen et al., 1987; Williamson et al., 1988; Pasnik et al., 1999) may also lead to empirical confusion, because depending on the concentration and *in vitro* conditions, β -endorphin may inhibit, stimulate, or leave cell proliferation and activity unaffected. Although most reports highlight the antiinflammatory effects of POMC peptides, one study that used oligonucleotide antisense cDNA probes to inhibit total POMC peptide translation in splenocytes *in vitro* resulted in increased mitosis (Fulford et al., 2000), which suggests a net proliferative synergy of POMC peptides in this experimental system.

Despite these caveats, there have been promising reports that POMC peptides may form the basis of antiinflammatory strategies, acting through melanocortin (MC) or opioid receptors. MC-1 and MC-3 receptors have been reported only in macrophages and monocytes and not in resting T lymphocytes and B lymphocytes (Smith et al., 1987; Star et al., 1995; Bhardwaj et al., 1997; Getting et al., 1999). MC-2 receptors are present in some, but not all, T cells, B cells, and macrophages (Johnson et al., 2001). However, it is not possible to be categorical about whether or not a cell subtype expresses a class of receptors, since receptors that are not expressed on resting leukocytes may be upregulated during antigen presentation or inflammation (Johnson et al., 2001). ACTH (4–10) has an antiproliferative effect on mouse macrophages, which can be blocked by an MC-3 receptor antagonist (Getting et al., 1999), and MC-3 agonists can attenuate neutrophil migration and macrophage secretory activity in a mouse model of inflammation (Getting et al., 2001). α -MSH is a potent antagonist of IL-1 β activity (Lipton and Catania, 1998) and can induce expression of the antiinflammatory cytokine IL-10 through α -MSH-specific MC-1 receptors on monocytes (Bhardwaj et al., 1996). Use of MC-1 and MC-3 agonists as antiinflammatory agents has the advantage that the pituitary MC-2 receptor involved in steroidogenesis will not be activated. Therefore systemic application of these compounds in the treatment of chronic inflammatory disorders need not result in hypercortisolaemia and associated problems such as obesity, hypertension, and depression.

μ -Opioid receptors are located in all major leukocyte subtypes, and there are many reports of immunomodulatory effects of opioid peptides (Jessop, 1998). In one study, β -endorphin prolonged skin

allograft survival in mice while naloxone accelerated rejection (Sacerdote et al., 1998) demonstrating that endogenous β -endorphin can tonically inhibit the immune response in tissue transplantation. Although many of the effects of opioid peptides are naloxone reversible (Machelska et al., 1999; Takeba et al., 2001; Menzebach et al., 2003) and are therefore mediated through δ -, κ -, or μ -opioid receptors, there is a body of evidence in support of opioid actions which cannot be explained through classical receptors. Naloxone specifically blocks N-terminal β -endorphin receptor binding, but β -endorphin may also act through C-terminal binding to a naloxone-insensitive class of opioid receptors (McCain et al., 1982; Shahabi et al., 1990; Owen et al., 1998). Morphine has been shown to bind to activated T lymphocytes but no trace of μ -opioid receptor mRNA was detected, suggesting a novel morphine binding site on T cells (Madden et al., 2001). Evidence for novel receptors in immune cells highlights the difficulties in investigating functional mechanisms mediating neuropeptide effects on immune function using pharmacological tools developed for investigation of neuropeptides within the CNS.

Many studies have demonstrated changes in POMC expression in immune tissues during inflammation. POMC mRNA and peptide products are elevated in rat immune tissues in inflammatory autoimmune diseases such as AA, EAE, and SLE (Jessop, 2001). In a rat model of AA, ACTH in the thymus was elevated in response to inflammation whereas ACTH in the spleen was increased well before clinical signs of inflammation (Jessop et al., 1995a), suggesting tissue-specific control of POMC expression. In contrast to the early increase in splenic ACTH, β -endorphin contents in the spleen were only elevated around the time of inflammation, which is consistent with differential POMC processing in the spleen and possible separate functions for β -endorphin and ACTH in modulating the course of AA. Increased POMC expression was observed in inflamed rat paw tissue (Mousa et al., 2004) and β -endorphin immunoreactivity has been observed in inflamed synovial tissue (Przewlocki et al., 1992). While β -endorphin contents are increased in inflamed tissues, lymph node contents of β -endorphin are decreased, which may reflect differential peptide trafficking in response to inflammation (Cabot et al., 1997).

There have been few reports on immune system ACTH expression in human pathologies, but biologically active ACTH is produced by HIV-infected lymphocytes in culture (Hashemi et al., 1998), and β -endorphin levels in mononuclear leucocytes are reduced in patients with chronic fatigue syndrome (Conti et al., 1998). Alterations of β -endorphin levels in peripheral human blood lymphocytes in the predominantly T_H1 -mediated diseases, multiple sclerosis, RA, and Crohn's disease, have led to the hypothesis that β -endorphin may be influential in mediating the switch from the T_H1 to the T_H2 profile of predominantly antiinflammatory cytokines (Panerai and Sacerdote, 1997). Naloxone reduced production of IL-4 and increased secretion of IL-2 and IFN- γ , a cytokine secretory profile that is consistent with an endogenous opioid-induced bias toward inhibition of proinflammatory T_H1 -type cytokines and stimulation of T_H2 -type antiinflammatory cytokines (Sacerdote et al., 2000). β -endorphin inhibited secretion of IL-6 (albeit a T_H2 -type cytokine) from mouse spleen slices (Straub et al., 1998) and IL-8 from synovial fibroblasts taken from RA patients (Raap et al., 2000) demonstrating that β -endorphin can attenuate important immune responses such as antibody production, neutrophil activation, and T cell chemotaxis. Leukocyte production of β -endorphin is stimulated by the T_H1 cytokine IL-1 (Kavelaars et al., 1989) and also by the T_H2 cytokine IL-4 (Kraus et al., 2001) indicating that β -endorphin can respond to changes in the T_H1/T_H2 cytokine balance, a balance that can be altered in favor of T_H2 cytokines by catecholamines and glucocorticoids released in response to stress.

An alternative antiinflammatory mode of action for opioids is the induction of apoptosis in immune cells. Morphine was able to induce DNA fragmentation in mouse thymocytes *in vivo* but not *in vitro* (Fuchs and Pruett, 1993), suggesting an indirect effect, while other *in vitro* studies have shown that β -endorphin enhances spermidine transport (Ientile et al., 1997) and morphine increases Fas expression (Yin et al., 1999). Induction of cell death by apoptosis through the Fas/Fas ligand pathway would be a potent antiinflammatory mechanism for immune system-based opioid peptides, but this mechanism remains controversial since a number of groups have failed to demonstrate any effects of opioids on apoptosis (Jessop et al., 2002).

POMC peptides in the immune system, as in the anterior pituitary, are responsive to acute stressors such as restraint (Jessop et al., 1997b), hyperglycemia (Jessop and Kvetnansky, unpublished observations), or hypoglycemia (Gaillard et al., 1998). ACTH and β -endorphin contents in splenocytes and thymocytes are

increased following central injection of IL-1 β (Jessop et al., 1997b; Panerai et al., 1997), and ACTH and β -endorphin are secreted in response to CRF (Smith et al., 1986). Stress-induced increases in immune system ACTH and β -endorphin may be secondary to local secretion of immune system CRF. Synthetic CRF upregulates POMC gene expression in mouse lymphocytes (Galin et al., 1991) and stimulates the secretion of ACTH and β -endorphin from leukocytes (Smith et al., 1986; Kavelaars et al., 1990). Although some groups have been unable to observe an effect of CRF on immune system POMC expression or ACTH secretion, failure to elicit a POMC response to CRF may be due to differential activation of lymphocytes and consequent variable numbers of CRF receptors. A functional relationship between CRF and POMC expression has been demonstrated in inflammation whereby CRF injected into inflamed tissue produced an analgesic effect which was reversed by antisera to β -endorphin (Cabot et al., 1997; Schafer et al., 1997) or to the adhesion molecule selectin (Machelska et al., 1998), indicating complex interactions between CRF, β -endorphin, and selectin in mediating peripheral pain transmission. There was a strong correlation, implying interdependence, between splenic contents of ACTH, β -endorphin, and CRF in the acute response to central injection of IL-1 β (Jessop et al., 1997b). CRF receptors are present in mouse splenic macrophages and neutrophils and in human lymphocytes (Baigent, 2001). Thus, the components are present for a functional interaction of CRF with POMC expression in the immune system. However, in AA, splenic ACTH contents are elevated many days before the expression of CRF (Jessop et al., 1995a), a time course inconsistent with stimulation of POMC expression by CRF in this disease model.

Immune system POMC expression can be regulated by glucocorticoids as in the anterior pituitary (Smith et al., 1986) but not under all circumstances, since the stimulatory effects of IL-1 β on POMC expression are glucocorticoid-dependent in the thymus but not in the spleen (Jessop et al., 1997b). There is also potential for SNS regulation of immune system POMC, since sympatho-adrenergic neurons abut neuropeptide-positive splenocytes (Felton et al., 1987). Noradrenaline induced secretion of β -endorphin from rat immune cell suspensions (Binder et al., 2004), a propranolol-reversible effect (Mousa et al., 2004), and secretion of β -endorphin from human mononuclear cells was stimulated by the β -adrenergic agonist isoprenaline (Kavelaars et al., 1990). Much however remains to be learned about the mechanisms that control POMC expression in immune cells. Glucocorticoid and catecholaminergic regulation of POMC expression in immune tissues is a potentially fertile area of investigation into mechanisms whereby immune functions can be influenced by CNS responses to acute or chronic stress. Neurotransmitters such as GABA, serotonin, and dopamine can also exert multiple influences on immune functions. These neurotransmitters are responsive to stress and are located in immune cells as well as in peripheral neurons, but little is known about their effects on POMC or expression of any other immunoneuropeptides. Selective peripheral lesioning of these neurotransmitter systems with neurotoxic agents should in future provide valuable information about which neuronal systems exert tonic control over immunopeptide expression under normal conditions or during stress.

5 Non-POMC-Derived Opioid Peptides

The non-POMC-derived opioid peptides, enkephalins, dynorphin, nociceptin/orphanin FQ (OFQ), and endomorphins (EMs) and their respective δ -, κ -, nociceptin, and μ -opioid receptors, have all been located within immune tissues (Peluso et al., 1998; Sharp, 2003). Partial blocking of preproenkephalin mRNA translation using antisense probes resulted in increased human T cell proliferation and cytokine secretion (Kamphuis et al., 1998) and inhibition of mouse thymocyte (Linner et al., 1995) and rat splenocyte (Fulford et al., 2000) proliferation, results which are consistent with immunomodulatory roles for enkephalins in several species and cell types. T cell proliferation was attenuated in a mouse preproenkephalin-deficient model (Hook et al., 2003). Apparent paradoxical pro- or antiinflammatory phenomena associated with disruption of preproenkephalin mRNA translation may be the consequence of opposing effects of the preproenkephalin gene products on immune cell activation under different culture conditions. Preproenkephalin A mRNA and met-enkephalin peptide immunoreactivity are present in activated rodent T cells (Linner et al., 1995; Kamphuis et al., 1997) and are abundant in inflamed tissue surrounding the joints of arthritic rats (Przewlocki et al., 1992). Cells containing opioid immunoreactivity in this study were

identified as T lymphocytes, B lymphocytes, macrophages, and monocytes adjacent to PAN neurons. Both met- and leu-enkephalin, and β -endorphin, inhibited release of TNF- α , IL-1, and matrix metalloproteinases from synovial cells in patients with RA through a naloxone-reversible mechanism (Takeba et al., 2001), implying a complex and possibly integrated series of pathways involving μ and δ receptors. Met-enkephalin and β -endorphin levels are elevated in synovial tissues from RA patients (Stein et al., 1993). These patients reported a lower pain threshold following direct naloxone injection into the inflamed joint compared with intravenous administration. This observation may reveal a tissue-specific mechanism of endogenous opioid action, which may be targeted by localized administration of antiinflammatory opioid analogs in doses sufficient to be efficacious but small enough not to cause cardiovascular or respiratory effects through leakage into the general circulation.

OFQ mRNA has been reported in the porcine immune system (Pampusch et al., 2000) and in human PBMC (Arjomand et al., 2002). OFQ peptide has been observed in neutrophils in synovial fluid from RA patients (Fiset et al., 2003) but it is not yet clear whether OFQ mRNA translation occurs under quiescent noninflamed conditions. OFQ inhibited proliferation of human mononuclear cells (Peluso et al., 2001) and stimulated leukocyte infiltration at sites of inflammation (Serhan et al., 2001). A recent report has revealed differential effects of low doses of OFQ on human T cell proliferation depending on the state of cell activation (Waits et al., 2004). These activities of OFQ are likely to be mediated specifically through OFQ receptors, which have been reported in human lymphocytes (Fiset et al., 2003) and in a wide range of immune cells in other species (Mollereau and Mouledous, 2000; Pampusch et al., 2000).

Dynorphin is found in rat lymphocytes (Hassan et al., 1992) and can enhance IL-2 secretion and lymphocyte proliferation (Ni et al., 1999). At ultralow concentrations, dynorphin inhibits TNF- α secretion from glial cells (Kong et al., 1997). Dynorphin-positive sympathetic nerve terminals have been reported in apposition to T cells in rodent livers, conferring neuroanatomical potential for a functional role of dynorphin in T cell regulation (Kaiser et al., 2003). Dynorphin, along with met-enkephalin, is released in response to CRF stimulation of lymphocytes obtained from inflamed rat hind paw tissue (Cabot et al., 1997). Although there is no doubt that dynorphin and OFQ of neural origin play important roles in mechanisms mediating neurogenic pain, these peptides have not been investigated biochemically or chromatographically in any detail in immune cells, and there is little information about the degree to which dynorphin and OFQ and their receptors respond to cell activation during the onset of inflammation either in animal models or in human autoimmune diseases.

The opioid tetrapeptide EMs, EM-1 and EM-2, which have very high selectivity and affinity for μ -receptors, are located within rat and human immune tissues (Jessop et al., 2000; Seale et al., 2004), and synovial tissue levels of the EMs are increased during inflammation (Jessop et al., 2002). EMs have been reported to exert opposite effects on superoxide anion production by neutrophils depending on the degree of cell activation (Azuma et al., 2000). In a rat model of acute inflammation, EM-1 was effective in attenuating the inflammatory response to SP, an effect reversed by naloxone (Khalil et al., 1999), and EM-1 attenuated the inflammatory effects of kaolin/carogeenan treatment, an effect mediated through μ -receptors (McDougall et al., 2004). However, one careful study found no evidence for EM-1 involvement over a range of immune functions in vivo in normal rats although EM-1 acted as a potent analgesic (Carrigan et al., 2000). This suggests that immune responses to EMs may only be evident during inflammation when opioid receptors are upregulated in activated cells, but that analgesic effects of EM-1 are not dependent on upregulation of receptors on peripheral nerve terminals.

Although pharmacological studies strongly associate EM-1 and EM-2 with μ -opioid receptors through which are mediated the analgesic, respiratory, and tolerance effects of morphine, EMs may exert some immunomodulatory effects through novel receptors. Central injection of EMs induces phenomena that cannot be explained by the existence of a common receptor for EMs and morphine. EM-1 and EM-2 differentially induced analgesia in mice where similar responses were predicted on the basis of action through μ -receptors (Ohsawa et al., 2001; Sakurada et al., 2001). The central effects of morphine on corticosterone release could not be blocked by preadministration of EM-1 or EM-2 (Coventry et al., 2001). This cumulative evidence suggests that EMs can exert effects independent of the morphine receptor. At least two μ -opioid receptor subtypes have been reported through which EM-1 and EM-2 may act, as well as through other novel μ -opioid receptor variants, although evidence for these is controversial. Any

preferential association of EMs with selective μ -opioid receptor subtypes remains to be investigated. EMs, like dynorphin (Kong et al., 1997) and β -endorphin (Williamson et al., 1988), have been reported to modulate immune functions *in vitro* at concentrations lower than 10^{-15} M. Since ligand occupancy of the classical opioid receptors at these doses is negligible, this pharmacology supports the existence of novel opioid receptors in the immune system or heterodimerization of existing receptors in immune cells to form complexes with extremely high affinity for opioid peptides (Jordan and Devi, 1999). Therefore the potential exists for EMs to exert physiological actions at low concentrations through novel opioid receptors or receptor complexes (Jessop, 2006).

Intact, undegraded EM-1 and EM-2 have been detected in human PBMC (Jessop et al., 2002) whereas multiple forms of immunoreactivity have been reported in the spleen, thymus, and plasma (Jessop et al., 2000), suggesting extensive degradation. Thus EMs can be transported encapsulated and protected within lymphocytes in biologically active forms to target inflamed tissue. This is consistent with the mechanism proposed for directing β -endorphin to inflamed tissue through lymphocyte trafficking (Cabot et al., 1997) and selectin-dependent sequestration (Machelska et al., 1998). In this way opioid peptides can be released from circulating lymphocytes directly at sites of inflammation to act as antiinflammatory and analgesic agents through inhibition of cytokine and SP release. Such targeted delivery would enable low concentrations of opioids to exert maximally effective paracrine actions at inflamed sites. An alternative to targeted delivery within peripheral lymphocytes is the possibility that freely circulating peptides, e.g., ACTH and β -endorphin released from the anterior pituitary, might exert immunomodulatory effects. While this may be a plausible scenario in conditions of excessive ACTH secretion such as in Cushing's syndrome, in normal circumstances concentrations are too low and proteolytic degradation too rapid for opioid peptides to exert physiologically relevant systemic effects.

EMs and other opioid peptides are located in neuronal projections innervating respiratory tissues (Groneberg and Fischer, 2001) where they can exert bronchodilatory effects. Although not so far identified in immune cells in this system, circulating opioid-containing lymphocytes might also be sequestered in inflamed bronchial tissues in asthma. The role of opioids in mediating the T_H1/T_H2 cytokine switch in this predominantly T_H2 disease is worthy of closer investigation.

6 Variant Forms of Peptides in Immune Tissues

The field of immunoneuropeptide research has not always been without controversy between laboratories as to the forms and physiological relevance of these compounds, but many disputes have arisen through attempts to compare neuropeptides within the CNS and immune cells in terms of equivalent form and concentration. Such comparisons may be misleading, since there is evidence that immunoneuropeptides may exist as fragments that are biologically active at very low concentrations. While enzymatic processing of mature neuropeptides is traditionally considered to be the principal mechanism by which bioactivity is inactivated in blood and other tissues, there is also mounting evidence for proteolytic conversion into bioactive isoforms which may either bind to the same receptor and modify or inhibit the actions of the parent neuropeptide or bind to a receptor which is not recognized by the parent peptide (Hallberg and Nyberg, 2003). Thus, what has been perceived to be largely a process of peptide deactivation by degradation may actually be quite the opposite and represent a novel regulatory mechanism of neuropeptide actions, one which is of particular importance in the immune system because of the multiplicity of peptide isoforms abound in different immune cell types. One striking feature of the literature on neuropeptides is the number of observations of variant peptides, precursor forms, and fragments in immune tissues in comparison with the CNS, in which neuropeptides generally appear in their precursor and processed bioactive forms only. Complex processing of neuropeptides in immune tissues is no doubt due to the actions of enzymes such as proprotein convertases, carboxypeptidases, endopeptidases, and cathepsins which are present in much greater concentrations in immune tissues than in the CNS.

The phenomenon of peptide processing is even more pronounced in inflamed immune tissues, one example being the wide range of ACTH and β -endorphin immunoreactive compounds which are present in rat spleen extracts compared with noninflamed controls (Jessop et al., 1994). Given the importance of

distinguishing between selective processing of neuropeptides in immune tissues and artifact fragments generated during the extraction procedure, it is critical that tissues are extracted under conditions that irreversibly denature proteases. Immune tissues contain large amounts of acidic proteases such as cathepsins, so extraction of tissues with acidic solvents is ineffective since acidification only induces reversible denaturation. It is necessary to heat extracts to irreversibly denature proteases prior to assay (Jessop et al., 1987).

One example of controlled processing of neuropeptides into variant bioactive forms in the immune system is the generation of C-terminal fragments of β -endorphin, which can exert immunoproliferative effects through a novel subset of naloxone-insensitive receptors (Owen et al., 1998). These actions may be quite distinct from those mediated through N-terminal binding to μ -receptors and would not be affected by acetylation that renders β -endorphin inactive at μ -receptors. There is considerable evidence for biphasic concentration-dependent effects of neuropeptides on immune functions, many of which could be explained by the processing of the mature bioactive peptide within immune cell subtypes under resting or activated conditions into smaller fragments which can act as partial or full antagonists either at the same or different receptors. For example, β -endorphin(1–31) has full analgesic bioactivity through the μ -opioid receptor (and to a lesser extent through the δ and κ receptors) while the (1–27) form antagonizes this activity. The multiplicity of β -endorphin and ACTH isoforms in rat and human immune tissues is well documented (Lolait et al., 1986; Buzzetti et al., 1989; Jessop et al., 1994). Therein lies potential for differential processing of POMC under specific conditions resulting in immunostimulatory or immunosuppressive effects for selective peptide products.

Posttranslational modification of neuropeptides is also a mechanism that can differentially influence immune functions. β -Endorphin is almost entirely lacking in analgesic activity when acetylated. One early study characterized β -endorphin as present in multiple acetylated forms in resting mouse splenocytes but predominantly existing in the bioactive nonacetylated form (Lolait et al., 1986). Little is known about differential acetylation of β -endorphin in activated immune cells or in inflammatory disease in any species. The splenocyte proliferative response to β -endorphin(1–31) could be blocked by the acetylated (1–27) form (Kusnecov et al., 1989). This observation does not reveal whether the antagonistic action was due to the 1–27 form or to the acetylation, but it highlights the potential for differentially modified forms of β -endorphin to alter activity of the native peptide within immune tissues.

7 Conclusion

Almost all neuropeptides found within the CNS are also located in immune cells, but the degree of expression is much more variable than in the CNS, with expression of many immunoneuropeptides depending on immune cell activation. Many neuropeptides are either never expressed or are expressed at very low levels in nonactivated cells. There is some evidence that expression of some immunoneuropeptides is responsive to glucocorticoids and catecholamines and that these neuropeptides can modulate the effects of stress on immune functions. Stress can influence the production of CRF and POMC peptides from leukocytes and also determine T cell differentiation into distinctive T_H1/T_H2 cytokine secretory profiles. Therefore neuropeptides may be involved in mediating the effects of stress on production of specific cytokines with pro- or antiinflammatory activity. It is now accepted that, while most stressors in common activate the HPA axis and the SNS, each type of stressor i.e., acute, repeated, or chronic, physical or psychological, is characterized by subtle and unique differences in the secretory profile of neuropeptides, neurotransmitters, and glucocorticoids, which act as stimulatory or inhibitory agents. Therefore, stress can influence immune functions either directly through catecholamine and glucocorticoid binding to adrenoceptors or glucocorticoid receptors or exert indirect effects through modulating secretion of neuropeptides which can then selectively mediate specific immune responses to different types of stress. This would permit a very finely tuned range of immune responses, mediated through integrated endocrine and paracrine pathways, to maintain homeostasis of cytokine and chemokine balance either in response to acute stressful episodes or to the chronic stress of inflammatory disease.

Most autoimmune diseases are sensitive to stress, yet very little is known of the mechanisms whereby stress can exacerbate, or ameliorate, inflammation. It is possible that neuropeptides in immune cells, like

their counterparts in the HPA axis, may selectively determine stress-induced predisposition to, or onset and severity of, some inflammatory diseases through stimulation of a pro- or antiinflammatory cytokine profile. Identification of these neuropeptides and their receptors, and their specific effects on cytokine secretion and actions, will represent an important step toward our understanding of how stress influences inflammation. Although glucocorticoids remain the most powerful of all antiinflammatory agents, their clinical application in chronic inflammation and autoimmune diseases is limited due to systemic side effects. The challenge now is to develop selective antiinflammatory drugs with efficacy equal or better than glucocorticoids. Immunoneuropeptides have an exciting potential to be developed as a new generation of nonglucocorticoid antiinflammatory drugs. One area of promise is the direct injection into inflamed tissue of small amounts of a long-acting compound or local implants of slow release polymer beads impregnated with an antiinflammatory agent. This would be a useful strategy for the application of low dose/high potency endomorphin analogs to exert antiinflammatory effects without systemic release and consequent nonspecific effects.

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3 Effects of the Immune System on Brain Neurochemistry

A. J. Dunn

1	<i>Introduction</i>	38
2	<i>Neurochemical Responses to Immune Activation</i>	39
2.1	Responses to Bacterial and Viral Infections	39
2.2	Responses to Endotoxin (LPS)	40
3	<i>Neurochemical Responses to Cytokines</i>	41
3.1	Interleukin-1	41
3.1.1	Catecholamines	41
3.1.2	Tryptophan and Serotonin	43
3.1.3	Acetylcholine, Histamine, and the Amino Acids	44
3.1.4	Other Neurochemical Responses	44
3.2	Interleukin-2	45
3.3	Interleukin-6	45
3.4	Tumor Necrosis Factor- α	45
3.5	Interferons	46
3.5.1	Interferon- α	46
3.5.2	Interferon- γ	47
3.6	Other Cytokines	47
4	<i>Effects of Intracerebral Administration of Cytokines</i>	47
5	<i>Mechanisms of the Effects of the Cytokines</i>	48
6	<i>Participation of Cytokines in the Responses to LPS and Infection</i>	49
7	<i>Functional Significance of the Neurochemical Responses to Cytokines</i>	50
7.1	Neurochemical Involvement in the HPA Response to IL-1	50
7.2	Neurochemical Involvement in Behavioral Responses to Cytokines	51
8	<i>Relationships of Cytokine-Induced Effects on Neurotransmission to Depression</i>	51
9	<i>Conclusions</i>	52

Abstract: It has been recognized for some time that the immune system can affect the central nervous system altering behavioral and neuroendocrine activity. The focus of the research has been on cytokines, the hormones of the immune system, because cytokines are known to be secreted by immune cells when they are activated, and administration of cytokines to animals can elicit many effects on the brain, specifically neuroendocrine and behavioral effects. Cytokine administration also affects various neurotransmitter systems, which may underlie the neuroendocrine and behavioral effects. The most well-studied effect is the cytokine activation of the hypothalamo-pituitary-adrenocortical (HPA) axis. Interleukin-1 (IL-1) administered peripherally or centrally is a potent activator of the HPA axis. This property is shared by certain other cytokines, IL-2, IL-6, tumor necrosis factor- α (TNF α), and possibly the interferons (IFNs), although none is as potent or effective as IL-1. Administration of IL-1 also induces norepinephrine (NE) release in the brain, most markedly in the hypothalamus. Small changes in brain dopamine (DA) are sometimes observed, but these effects do not appear to be regionally selective. IL-1 also increases brain concentrations of tryptophan and the metabolism of serotonin (5-HT) throughout the brain in a regionally nonselective manner. IL-2 has similar but more modest effects on DA, NE, and 5-HT. IL-6 also increases tryptophan and 5-HT metabolism, but does not affect NE. TNF α also activates the HPA axis, but affects NE and tryptophan only at high doses. IFN α induces fever and HPA axis activation in man, but such effects have not been confirmed in rodents. The reported effects of IFNs on brain catecholamines and serotonin have been very inconsistent. However, interferon- γ has profound effects on the catabolism of tryptophan, converting it to kynurenine and quinolinic acid, effectively reducing its concentration in plasma. This action may limit brain 5-HT synthesis by decreasing plasma tryptophan.

Endotoxin (lipopolysaccharide, LPS) elicits HPA and neurochemical responses very similar to those of IL-1. Because LPS is known to stimulate the secretion of IL-1, IL-6, and TNF α , it is likely that these cytokines mediate at least some of the responses. Bacterial and viral infections also induce HPA activation, and increase brain tryptophan and brain NE and 5-HT metabolism. These effects are strikingly similar to those of IL-1 (as are the behavioral effects), suggesting that IL-1 secretion, which accompanies many infections, may mediate these responses. Studies with IL-1 antagonists and using IL-1-knockout mice are consistent with this possibility, although in most cases the antagonism is incomplete, suggesting the existence of multiple mechanisms. The neurochemical responses to cytokines are likely to underlie the physiological responses. The NE response to IL-1 appears to be instrumental in the HPA activation, but this is not the only mechanism by which IL-1 activates the HPA axis. Neither the noradrenergic nor the serotonergic systems appear to be involved in the major behavioral responses studied. The significance of the serotonin response is not known.

List of Abbreviations: COX, cyclooxygenase; CRF, corticotropin-releasing factor; CVOs, circumventricular organs; DA, Dopamine; 5,7-DHT, 5,7-dihydroxytryptamine; DNAB, dorsal noradrenergic ascending bundle; DOPAC, 3,4-dihydroxyphenylacetic acid; GM-CSF, Granulocyte/macrophage colony-stimulating factor; 5-HIAA, 5-hydroxyindoleacetic acid; HPA, hypothalamo-pituitary-adrenocortical; 5-HT, 5-hydroxytryptamine; HVA, homovanillic acid; IDO, indoleamine-2,3-dioxygenase; IFNs, Interferons; IL-1, Interleukin-1; IL-1ra, IL-1-receptor antagonist; LPS, Lipopolysaccharide; MHPG, 3-methoxy, 4-hydroxyphenylethyleneglycol; NDV, Newcastle disease virus; NE, Norepinephrine; NOS, nitric oxide synthase; NTS, nucleus tractus solitarius; 6-OHDA, 6-hydroxydopamine; OVLT, organum vasculosum laminae terminalis; PVN, paraventricular nucleus; SRBC, sheep red blood cells; TNF α , tumour necrosis factor- α ; VNAB, ventral noradrenergic ascending bundle

1 Introduction

The first indication of an immune effect on brain neurotransmission appeared over 30 years ago, when Pohorecky et al. (1972) reported that endotoxin (lipopolysaccharide, LPS) increased the turnover of norepinephrine (NE), but not serotonin (5-hydroxytryptamine, 5-HT), in the brain. However, this was not definitive because, although LPS is a powerful stimulator of the immune system, it has many nonimmune effects. Subsequently, Besedovsky et al. (1983) demonstrated that injection of sheep red

blood cells (SRBC) into rats altered the turnover of NE in the hypothalamus (assessed by its depletion following inhibition of synthesis) and that this response correlated with the presence of an immune response. Carlson et al. (1987) then showed that SRBC administration decreased the NE content of the hypothalamic paraventricular nucleus (PVN) and the 5-HT content of the hypothalamic paraventricular and supraoptic nuclei, suggesting activation of both these neurotransmitters. These changes appeared only at the peak of the immune response to SRBC, and no changes were observed in the other hypothalamic or extrahypothalamic regions studied. Because immune activation was known to result in the synthesis and secretion of cytokines, it was postulated that cytokines function as messengers from the immune system to the brain. Subsequent studies have confirmed that peripheral administration of purified cytokines can indeed induce neurochemical changes in the brain, particularly the metabolism of NE and 5-HT.

A second seminal discovery, also came from Besedovsky's laboratory, namely that purified natural and recombinant interleukin-1 (IL-1) administered intraperitoneally (ip) to rats potentially activated the hypothalamo-pituitary-adrenocortical (HPA) axis, elevating plasma concentrations of adrenocorticotropin (ACTH) and corticosterone (the major glucocorticoid hormone in the rat) (Besedovsky et al., 1986). This property of IL-1 was rapidly confirmed by several other groups in several different species (see Dunn, 2000b). Because brain NE has been implicated in the regulation of the HPA axis (Plotsky et al., 1989), the possibility arose that IL-1 was involved in both the noradrenergic response and the HPA activation.

2 Neurochemical Responses to Immune Activation

2.1 Responses to Bacterial and Viral Infections

There have been many indications that plasma concentrations of glucocorticoids are elevated in sick animals. Bacterial infections were shown long ago to increase plasma concentrations of corticosterone, indicating an activation of the HPA axis (Kass and Finland, 1958; Beisel, 1981). More recently, Smith et al. (1982) reported that administration of Newcastle disease virus (NDV) to mice elevated plasma concentrations of corticosterone. More recent work has extended the observation of HPA activation to other viruses (see Silverman et al., 2005). In our own laboratory, it has been a common observation that mice and rats sick from a variety of causes (mostly unknown) exhibit elevated plasma corticosterone. We subsequently showed that NDV administration also elicited neurochemical changes. The NE catabolite, 3-methoxy,4-hydroxyphenylethyleneglycol (MHPG), the dopamine (DA) catabolite, 3,4-dihydroxyphenylacetic acid (DOPAC), and the 5-HT catabolite, 5-hydroxyindoleacetic acid (5-HIAA) were all increased in a number of brain regions (Dunn et al., 1987; Dunn and Vickers, 1994). Free tryptophan was also elevated throughout the brain. These HPA and neurochemical changes lasted for several hours.

NDV does not truly infect mice in the sense of producing live virus, so we studied infection of mice with an active virus, influenza virus. Infusion of the virus into the lungs (the natural site of influenza virus infection) induced a chronic elevation of plasma corticosterone (Dunn et al., 1989), in contrast to the transient elevation seen with most commonly studied stressors. The changes in corticosterone were accompanied by neurochemical ones. MHPG and MHPG:NE ratios were elevated in all brain regions studied, but the magnitude of the response was greater in the hypothalamus than that in the other brain regions studied (Dunn et al., 1989). DOPAC and DOPAC:DA ratios, and homovanillic acid (HVA) and HVA:DA ratios were not significantly altered. Tryptophan concentrations were elevated in all regions studied, as were those of the 5-HT catabolite, 5-HIAA, and 5-HIAA:5-HT ratios. All of these changes appeared around 36 h after influenza virus infection and persisted as long as the animals appeared sick.

Changes in plasma corticosterone have been associated with infection with other bacteria and viruses, such as *Mycoplasma fermentans* (Weidenfeld et al., 1995), Pichinde virus (Guo et al., 1993), herpes virus (Ben Hur et al., 1996), and lymphocytic choriomeningitis virus and murine cytomegalovirus (Miller et al., 1997). It seems likely that infections rather generally cause activation of the HPA axis.

There is a striking similarity between the neurochemical and physiological responses to infections and other stressors commonly studied in the laboratory (e.g., footshock or restraint, see [Table 3-1](#)). In the

■ Table 3-1

Comparison of brain neurochemical and plasma corticosterone responses to viral infection, LPS, and the studied cytokines

Stimulus	Corticosterone	NE	DA	Tryptophan	5-HT
Influenza virus	+	+	0	+	+
LPS	+	+	+	+	+
IL-1 α /IL-1 β	+	+	0	+	+
IL-2	0	+	+	nd	0
IL-6	+	0	0	+	+
TNF α	+	(+)	0	(+)	0
IFN α	0	0	0	0	0

Adapted from Dunn (2001)

+ increased; 0 no change; nd not determined; (+) increased only at high doses

brain, the major stress-related response to footshock occurs in noradrenergic neurons, but responses occur in dopaminergic and serotonergic neurons too (Stone, 1975; Dunn and Kramarcy, 1984). The NE response is widespread and appears to affect both the locus coeruleus (A6) system, which innervates dorsal structures, such as the cortex, hippocampus, and cerebellum via the dorsal noradrenergic ascending bundle (DNAB), as well as the nucleus tractus solitarius (NTS, A1/A2) system innervating ventral structures, such as the hypothalamus, via the ventral noradrenergic ascending bundle (VNAB). The DA response is also widespread, such that all the major neuronal systems (nigrostriatal, mesolimbic, mesocortical) show responses, but the magnitude of the response appears much greater in the prefrontal and cingulate cortices compared with other major dopaminergic regions, such as the striatum. There is also a robust elevation of tryptophan concentrations in all regions of the brain. This increase is quite uniform and does not appear to be related in any obvious way to the extent of the serotonergic innervation of the region (Curzon et al., 1972; Dunn, 1988).

Nevertheless, the responses associated with what have been termed “immune stressors” differ from those associated with physical and psychological stressors. The noradrenergic responses in the hypothalamus (the A1/A2, VNAB system) to infections and illness are much greater than those in the dorsal regions innervated by the DNAB. DA responses are not usually observed during infections. When they occur they are meager and not focused on the prefrontal cortex as they are after footshock and restraint. It is also important that the HPA axis activation is continuous, not transient as it is to electric shock, restraint, and other commonly used laboratory stressors.

2.2 Responses to Endotoxin (LPS)

That LPS has the capability to activate the HPA axis has been known since the 1950s. High doses of LPS significantly decreased the brain content of NE, but did not alter that of 5-HT (Pohorecky et al., 1972). This occurred when the LPS was injected ip or intracerebroventricularly (icv). LPS also accelerated the disappearance of icv-administered [^{3} H]NE. In mice, low ip doses of LPS (e.g., 30 μ g/kg) rapidly induced elevations of plasma ACTH and corticosterone reaching peaks around 2 h (Dunn, 1992a). MHPG concentrations and MHPG:NE ratios were also increased throughout the brain (Masana et al., 1990; Mefford and Heyes, 1990; Dunn, 1992a; Delrue et al., 1994; Lacosta et al., 1999). As was the case with influenza virus infections, the response was greatest in the hypothalamus, suggesting a relatively greater activation of the VNAB compared with the DNAB. LPS also induced small increases of DOPAC in most brain regions, including prefrontal cortex, hypothalamus, and brain stem (Masana et al., 1990; Mefford and Heyes, 1990; Dunn, 1992a; Delrue et al., 1994; Molina-Holgado and Guaza, 1996; Lacosta et al., 1999). The peak responses for both DA and NE occurred around 2 h (Dunn, 1992a). Tryptophan and 5-HIAA were also increased, but in a regionally nonselective manner (Heyes et al., 1989; Mefford and Heyes, 1990;

Dunn, 1992a; Delrue et al., 1994; Molina-Holgado et al., 1996). In addition, these indoleamine responses reached a peak much later at around 6–8 h (Dunn, 1992a). Very similar changes were observed following icv LPS at lower doses (Dunn, 1992a).

These neurochemical changes indicate increased neurotransmitter catabolism, which may or may not reflect their release. However, *in vivo* microdialysis studies indicate increased extracellular concentrations of DA, NE, and 5-HIAA, suggesting increased release of NE, DA, and 5-HT in the medial prefrontal cortex and hypothalamus of rats following ip LPS administration (15 µg/kg; Lavicky and Dunn, 1995). Other studies have indicated increased 5-HT in microdialysates from the hippocampus (Linthorst et al., 1995b, 1996), and of NE, MHPG, 5-HT, and 5-HIAA from the preoptic area (Linthorst et al., 1995a). LPS injection (100 µg ip) was reported to increase DA and 5-HIAA in microdialysates from the nucleus accumbens (Borowski et al., 1998). Thus LPS administration induces a pattern of neuroendocrine and neurochemical responses quite similar to those described earlier for bacterial and viral infections, but with the addition of some changes in cerebral DA that are not regionally selective.

3 Neurochemical Responses to Cytokines

3.1 Interleukin-1

3.1.1 Catecholamines

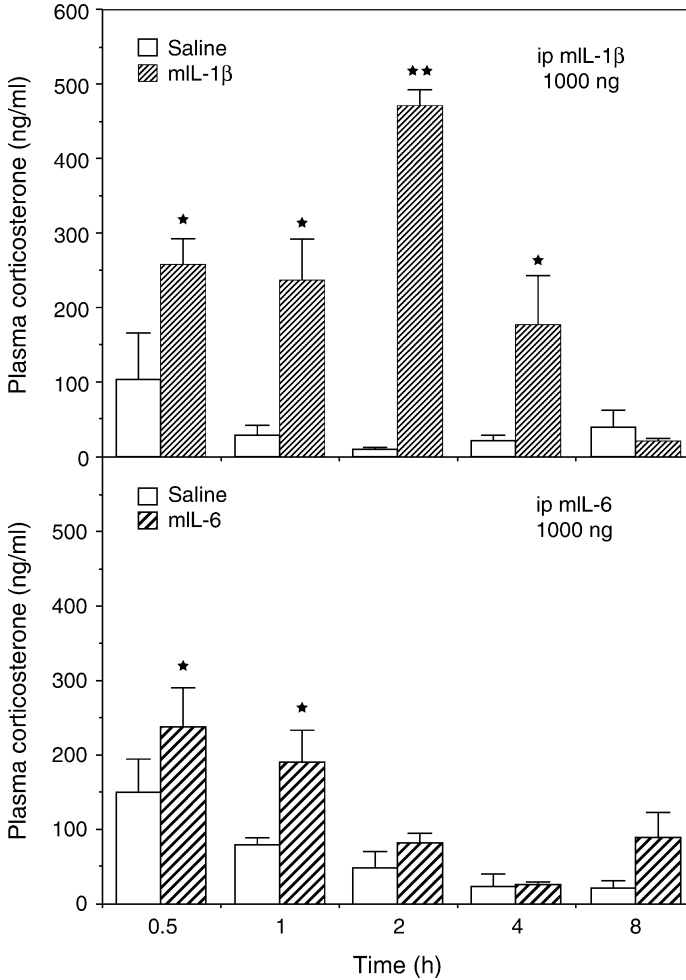
Ip administration of recombinant IL-1 β substantially increased brain concentrations of MHPG and MHPG:NE ratios in mice (▶ [Figure 3-1](#)) (Dunn, 1988; 1992a; Zalcman et al., 1994) and rats (Kabiersch et al., 1988). The responses in the hypothalamus were greater than those in the cortex and other brain regions studied. Within the hypothalamus, the greatest response was found medially (Dunn, 1988). The selectivity for the hypothalamus suggested a preferential activation of the VNAB, although the medial hypothalamus also receives a small noradrenergic input from the locus coeruleus. An IL-1-induced increase in hypothalamic NE turnover was indicated by the accelerated loss of cerebral NE following inhibition of synthesis by metyrosine in rats (Terao et al., 1993). This effect was also greatest in the hypothalamus of the regions studied. IL-1 also decreased the hypothalamic content of NE in rats (Fleshner et al., 1995), suggesting that NE was released faster than it could be replenished by synthesis. The different forms of IL-1 (IL-1 α and IL-1 β) elicited similar responses as did IL-1 from different species (human, rat, mouse) consistent with the known affinities of these forms of IL-1 for the IL-1 type I receptor (Liu et al., 1996). Similar noradrenergic responses were observed in response to intravenous (iv) (Dunn and Chuluyan, 1992) or subcutaneous injections, and to icv administration at considerably lower doses (Dunn, 1992a). These neurochemical effects of IL-1 are not due to contamination with endotoxin because heat-denatured IL-1 was inactive (Dunn, 1988).

DA metabolism is not substantially affected by IL-1 as indicated by changes in DOPAC or HVA (Kabiersch et al., 1988; Dunn, 1988). However, Zalcman et al. (1994) reported increased DOPAC in mouse prefrontal cortex and certain other brain regions. We have occasionally observed statistically significant increases of DOPAC or DOPAC:DA ratios following ip IL-1, but such responses have not been observed consistently. In the few experiments that showed increases in DOPAC, the anatomical pattern was not typical of responses to physical stressors, which show a strong preference for the medial prefrontal cortex compared with other brain regions. Zalcman et al. (1994) reported a similar anatomical pattern. Interestingly, administration of low doses of IL-1 β to rats in the first few days of life was reported to decrease DA permanently in the hypothalamus and in the superior cervical ganglion (Kabiersch et al., 1998).

The effect of IL-1 on NE most likely reflects increased synaptic release, because *in vivo* microdialysis studies have indicated increased extracellular concentrations of NE in the medial hypothalamus (Smagin et al., 1996; Ishizuka et al., 1997; Merali et al., 1997; Wiczorek and Dunn, 2006a, b). One study using push–pull cannulation also suggested increased hypothalamic PVN NE release (MohanKumar and Quadri, 1993). DA was not altered in dialysates from prefrontal cortex, nucleus accumbens, or hippocampus by ip IL-1 β (Merali et al., 1997). IL-1 β has also been reported to increase the activity of the synthetic enzyme, tyrosine hydroxylase, in the median eminence of rats (Abreu et al., 1994).

Figure 3-1

Plasma ACTH and corticosterone responses in mice following administration of mouse IL-1 β and IL-6. Mouse IL-1 β (100 ng/mouse) (*upper figure*) or mouse IL-6 (1 μ g/mouse) (*lower figure*) was injected ip, and samples collected at various subsequent times. Plasma ACTH and corticosterone was determined by radioimmunoassay. The data from the lower figure are derived from Wang and Dunn (1998). *Significantly different from the saline group (* $p < 0.05$, ** $p < 0.01$). Reproduced with permission from Dunn (2001)



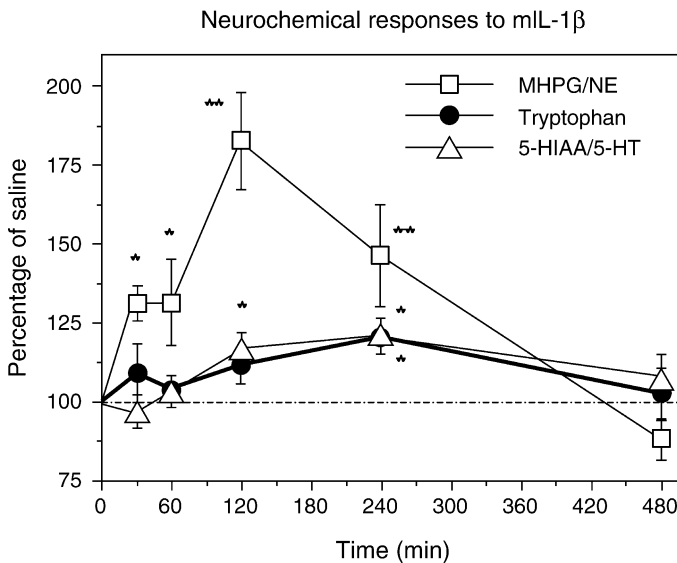
IL-1 also activates the adrenal medulla and the sympathetic nervous system, although this response is weaker than that induced by commonly used laboratory stressors (Berkenbosch et al., 1989; Niiijima et al., 1991; Kannan et al., 1996). Intravenous IL-1 β increases the electrical activity of vagal nerves innervating the thymus (Niiijima et al., 1995). NE turnover in the spleen and pancreas was also increased by ip IL-1 β , but not by IL-6 (Akiyoshi et al., 1990; Terao et al., 1994). NE release was also increased in a splenic dialysis study (Shimizu et al., 1994). In contrast, in vitro studies have shown that IL-1 β decreased NE release from the rat myenteric nerves (Hurst and Collins, 1993), from the perfused spleen (Bognar et al., 1994), and from the heart (Foucart and Abadie, 1996).

3.1.2 Tryptophan and Serotonin

Brain concentrations of the amino acid tryptophan, the precursor to 5-HT, are increased in all regions of mouse brain studied following peripheral IL-1 administration (see [Figure 3-2](#), Dunn, 1988; Mefford and Heyes, 1990). These changes are followed by increases in 5-HIAA, and are most readily observed by increases in 5-HIAA:5-HT ratios (see [Figure 3-2](#)) (Dunn, 1988; Mefford and Heyes, 1990; Zalcman

■ **Figure 3-2**

Catecholamine and indoleamine responses to IL-1 β . Mice were injected with 100 ng mL-1 β in the same experiment as the upper panel of [Figure 3-1](#). Squares: hypothalamic MHPG:NE ratios; filled circles: hypothalamic tryptophan; triangles: hypothalamic 5-HIAA:5-HT ratios. *Significantly different from the saline group (* $p < 0.05$, ** $p < 0.01$)



et al., 1994). Increases in 5-HIAA in push-pull samples from the hypothalamus were detected after intrahypothalamic injection of IL-1 β (Mohankumar et al., 1993). Peripheral administration of IL-1 did not alter microdialysate concentrations of 5-HT from the hippocampus, but 5-HIAA was increased in nucleus accumbens and hippocampus (Merali et al., 1997). Icv IL-1 β increased 5-HT in dialysates from the hippocampus (Linthorst et al., 1995b). Studies with voltammetry found increases in 5-HIAA in the hypothalamus (Gemma et al., 1994) and the dorsal raphe nucleus after peripheral or icv IL-1 β (Clement et al., 1997).

The brain content of tryptophan increases in response to a large variety of stimuli, including several psychotropic drugs, increases in body temperature, and several different stressors (Tagliamonte et al., 1971; Curzon et al., 1972; Dunn, 1988; Dunn and Welch, 1991; Chaouloff, 1993). In our experience, increases in brain tryptophan are a very sensitive index of an animal's state of health. Illness associated with infection, wounds or surgery, or food or water deprivation is almost invariably associated with increases in brain tryptophan. These increases are often accompanied by increases in 5-HIAA, probably driven by the increased tryptophan. Peripheral administration of relatively high doses of tryptophan results in increases in brain tryptophan and 5-HIAA (Lookingland et al., 1986; Dunn, unpublished observations), although the increased 5-HIAA may not reflect increased synaptic release (Joseph and Kennett, 1983; De Simoni et al., 1987).

The increases in tryptophan and 5-HIAA in response to footshock, restraint, IL-1, and LPS appear to depend upon peripheral sympathetic activity, because they can be blocked by pretreatment with the ganglionic blocker, chlorisondamine, and largely prevented by the β -adrenergic receptor antagonist, propranolol, but not by the α -adrenergic receptor antagonist, phentolamine, or the muscarinic receptor antagonist, scopolamine (Dunn and Welch, 1991). Thus, the increases in brain tryptophan appear to reflect sympathetic activation. This is consistent with the ability of β_2 -adrenergic agonists, such as clenbuterol, to increase brain concentrations of tryptophan (Edwards et al., 1989). However, β_2 -adrenergic antagonists do not prevent the IL-1-induced increases in brain tryptophan, although some attenuations have been observed (unpublished observations). Recent studies in our laboratory have shown that both β_2 - and β_3 -adrenergic agonists increase brain tryptophan, and that the effects of β_3 -adrenergic agonists can double the brain concentrations of tryptophan in mice (Lenard et al., 2003). This may explain why the β_2 -adrenergic receptor antagonists were ineffective.

The noradrenergic and indoleamine responses to IL-1 and LPS appear to involve distinct mechanisms. The noradrenergic response to ip IL-1 reaches a peak at around 2 h paralleling the increases in plasma ACTH and corticosterone, whereas the peak responses in tryptophan and 5-HIAA appear later at around 4 h (Figure 3-2, Dunn, 1992a). The peak noradrenergic responses to LPS are similar, around 2 h, but the tryptophan and 5-HIAA responses occurred around 8 h (Dunn, 1992a). Moreover, the anatomic patterns are distinct; the noradrenergic responses are far greater in the hypothalamus than those in the cortex and brain stem, whereas the magnitudes of the increases in tryptophan and 5-HIAA are very similar among all the brain regions studied. The NE and 5-HT responses can be dissociated. Mice of the endotoxin-resistant, C3H/HeJ, strain exhibit very small plasma corticosterone responses to LPS and no elevation of MHPG, but the increases in tryptophan and 5-HIAA are very similar to those in the control strains (Dunn and Chuluyan, 1994). The corticosterone and neurochemical responses to IL-1 are similar in both endotoxin-resistant and normal strains. Conversely, the nitric oxide synthase (NOS) inhibitor, L-NAME, can prevent the tryptophan and 5-HIAA response to both IL-1 and LPS, without obvious alterations in the plasma corticosterone and NE responses (Dunn, 1993). The evidence suggests that the isoform of NOS involved may be iNOS (NOS2) based on the use of selective NOS inhibitors (Dunn, 2002b). Thus the NE and indoleamine responses appear to be activated by distinct mechanisms.

3.1.3 Acetylcholine, Histamine, and the Amino Acids

Ip IL-1 β decreased the secretion of acetylcholine from the hippocampus, as measured by microdialysis in freely moving rats (Rada et al., 1991). This effect occurred only at relatively high doses of 20 or 50 μ g/kg. It is to be noted that iv IL-1 β increased the electrical activity of vagal nerves (Nijima, 1995). Histamine turnover (assessed by accumulation following inhibition of degradation by the monoamine oxidase inhibitor, pargyline) was increased in the rat hypothalamus by 25 ng IL-1 β icv (Kang et al., 1994), and increased release of histamine from the rat hypothalamus was observed following intrahypothalamic injection of IL-1 β in a microdialysis study (Niimi et al., 1994). Ip injection of hIL-1 β (20 μ g/kg, but not 10 μ g/kg) decreased hippocampal concentrations of glutamate, glutamine, and GABA 1 h later (Bianchi et al., 1995).

3.1.4 Other Neurochemical Responses

Peripheral administration of IL-1 increased the expression of Fos protein in a number of brain regions (Chang et al., 1993; Veening et al., 1993; Ericsson et al., 1994). The structure most markedly affected is the hypothalamic PVN, which contains the cell bodies of the neurons that synthesize corticotropin-releasing factor (CRF) involved in the activation of the HPA axis. Increases in Fos are also commonly observed in the central amygdaloid nucleus, the medial preoptic area, the bed nucleus of the stria terminalis, and the NTS. mRNA for Fos (c-fos) is also induced in most of the same structures (Brady et al., 1994; Rivest and Rivier, 1994; Day and Akil, 1996). The Fos response to IL-1 appears to depend on activation of the PVN by

noradrenergic neurons because it is markedly attenuated in mice depleted of NE with 6-hydroxydopamine (6-OHDA) (Swiergiel et al., 1996).

The rate of protein synthesis in the brain was altered by sc injection of hIL-1 β in the rat (Williams et al., 1994). Increases were observed in the subfornical organ, choroid plexus, medial habenula, dentate gyrus, and the anterior and posterior lobes of the pituitary, but decreases were observed in the cingulate cortex and the pineal gland.

3.2 Interleukin-2

IL-2 (200 ng ip) increased MHPG concentrations and MHPG:NE ratios in the hypothalamus of BALB/c mice 2 h after injection (Zalcman et al., 1994). However, there were no changes in plasma corticosterone concentrations. DOPAC:DA ratios were also increased in the prefrontal cortex. Another study found that peripheral IL-2 reversed the decreases in brain NE and 5-HT in olfactory bulbectomized rats as well as the behavioral and immunological deficits (Song and Leonard, 1995). Surprisingly, peripheral IL-2 administration induced decreased DA in microdialysates from the nucleus accumbens (Anisman et al., 1996). Icv IL-2 (500 ng, but not 50 ng) was reported to increase 5-HT and 5-HIAA in hippocampal dialysates (Pauli et al., 1998). Ip injection of human IL-2 (5 μ g/kg) induced small increases in glutamine in the cortex and hippocampus of mice 1 h later (Bianchi et al., 1995).

3.3 Interleukin-6

Administration of IL-6 has frequently been reported to elevate plasma concentrations of ACTH and corticosterone. Human IL-6 (200 ng, ip) injected into mice elevated 5-HIAA in the hippocampus, and 5-HIAA and DOPAC in the prefrontal cortex (Zalcman et al., 1994). Injection of mIL-6 into mice iv or ip elicited modest increases in plasma ACTH and corticosterone; such that maximal concentrations were much lower than those observed with IL-1 (Wang and Dunn, 1998). Corticosterone concentrations reached a peak 30–60 min after injection and returned to baseline by 2 h. Iv or ip mouse IL-6 consistently elevated tryptophan in all brain regions at around 2 h, and 5-HIAA in the brain stem at the same time, but had no effect on MHPG or DOPAC in any brain region (Wang and Dunn, 1998). Icv mIL-6 had similar endocrine and neurochemical effects at lower doses. Our results are consistent with the lack of effect of IL-6 on NE turnover (using metyrosine to block catecholamine synthesis) in rats (Terao et al., 1993). The effect of IL-6 on serotonin was confirmed by a microdialysis study that showed increases in dialysate 5-HT and 5-HIAA from the hypothalamus of rats injected ip with rat IL-6 (2 μ g per rat) (Barkhudaryan and Dunn, 1999). A similar response to rat IL-6 was observed in microdialysates from the striatum (Zhang et al., 2001). This result is consistent with an amperometric study in which it was found that similar doses of IL-6 increased the apparent release of 5-HT from the striatum following stimulation of the dorsal raphe nucleus (Zhang et al., 2001). Thus, these results strongly suggest an effect of IL-6 on cerebral serotonin metabolism. Because IL-1 and LPS administration both stimulate the synthesis and secretion of IL-6, it is possible that IL-6 mediates the indoleamine responses to IL-1 and LPS. IL-6 (0.1–0.5 μ g, ip) did not alter brain concentrations of glutamate, glutamine, aspartate and GABA in mice (Bianchi et al., 1997a).

Changes in Fos protein have also been reported to peripheral administration of IL-6, but the results have varied. The PVN was affected in some studies (Niimi et al., 1997), but not others (Callahan and Piekut, 1997). Other structures involved include the central amygdala, the bed nucleus of stria terminalis, the superoptic nuclei, the NTS, and the superchiasmatic nucleus (Tinsley et al., 2001).

3.4 Tumor Necrosis Factor- α

The human forms of IL-1 and IL-6 are active on the respective cytokine receptors in the mouse and the rat so that the responses to the human and rodent cytokines are similar, but this is not the case for tumor necrosis factor- α (TNF α). Although the amino acid sequence homology between mouse and human TNF α

is 79% (Fransen et al., 1985), mouse TNF α is a glycosylated dimer, whereas hTNF α is not glycosylated (Sherry et al., 1990). Human TNF α does not bind to mouse type 2 TNF receptors (mTNF-R2), whereas mouse TNF α binds to both mTNF-R1 and mTNF-R2 (Lewis et al., 1991). hTNF α essentially lacks some actions of mTNF α in mice (Brouckaert et al., 1992), so that physiologically relevant effects should only be studied using the homologous cytokines. In an early study, we observed no effect of injection of either hTNF α or mTNF α into mice on cerebral catecholamines or indoleamines (Dunn, 1992b). However, mTNF α elevated plasma corticosterone, whereas hTNF α did not. Consistent with this, Terao et al. (1993) found no effect of hTNF α on NE turnover in rats. In a more recent study in mice (Ando and Dunn, 1999), mTNF α increased brain MHPG and tryptophan, but only at relatively high doses (1 μ g/mouse or more). Icv mTNF α (50 or 100 ng) did not alter hippocampal dialysate concentrations of 5-HT or 5-HIAA, but did elevate body temperature and plasma corticosterone (Pauli et al., 1998). A more recent report indicated that hTNF α (4 μ g ip) increased hypothalamic MHPG in mice, and that chronic treatment sensitized this response (Hayley et al., 1999).

By contrast, TNF α inhibited NE release from the median eminence (Elenkov et al., 1992). Similarly, TNF α inhibited NE release from hippocampal slices (Ignatowski and Spengler, 1994). Interestingly, chronic treatment with the antidepressant, desmethylinipramine, reversed this effect, such that TNF α stimulated NE release. In the periphery, TNF α inhibited NE release from the rat myenteric plexus (Hurst and Collins, 1994) and the mouse heart (Foucart and Abadie, 1996).

3.5 Interferons

There are two types of interferons, Type I interferons (IFN α and IFN β), which share a common receptor (the type I receptor, which consists of two subunits: IFNAR-1 and IFNAR-2), and Type II interferons (IFN γ), which act on the Type II receptor (IFNGR).

3.5.1 Interferon- α

The reported effects of interferons on brain catecholamines have been exceedingly varied (see also the review of Schaefer et al., 2003). Shuto et al. (1997) reported that chronic (but not acute) administration of hIFN α (15×10^6 Units ip) to mice induced small decreases in whole brain (minus cerebellum) DA or DOPAC, but there were no changes in the DOPAC:DA ratio, nor in NE. They also reported a decrease in the apparent turnover of DA, assessed by blocking its synthesis with metyrosine. Kumai et al. (2000) found that seven daily treatments of rats with hIFN α (300,000 U/kg sc) increased the DA and NE contents of the cortex, hypothalamus, and medulla, but not of the hippocampus or thalamus. We observed no effects of a single ip injection of mice with mouse IFN α (1,000 or 10,000 U/mouse) on DA, NE, or any of their metabolites 2 h later (Dunn, 2001). The interferon used was the same used in the studies of Crnic et al. (supplied by Dr. Crnic), which had been shown to decrease locomotor activity and feeding (Crnic and Segall, 1992; Segall and Crnic, 1990). In contrast, a single icv injection of IFN α (200 or 2,000 U of hIFN α A/D) in rats was reported to decrease frontal cortical NE (Kamata et al., 2000). However, in another study, 1,000 U hIFN α injected icv into rats increased the DOPAC:DA ratio in the hippocampus (primarily caused by a substantial decrease in the measured hippocampal DA), but no such effect was observed in the prefrontal cortex or striatum (De La Garza and Asnis, 2003). However, these results were based on a very small number of rats. Interestingly, in a subsequent study from the same authors, no effects of acute hIFN α administration (10^5 U/kg ip) on DA or NE were observed, although 14 daily treatments with hIFN α decreased prefrontal cortex DA (De La Garza et al., 2005). In preliminary studies, using hIFN α or homologous IFN α injected at various doses ip or icv into mice and rats, we have not observed consistent changes in DA, NE, or their catabolites.

There are few data on the effects of IFNs on brain 5-HT. A single icv injection of 200 or 2,000 U of hIFN α to rats was reported to decrease the 5-HT content of the frontal cortex, and both 5-HT and 5-HIAA were decreased in the midbrain and the striatum (Kamata et al., 2000). However, we observed no effects of

ip human or mouse IFN α on 5-HT or 5-HIAA in mice at doses (400–16,000 U/mouse) that induced behavioral changes (Dunn, 2001). However, in another study, a single icv injection of IFN α (1,000 U) increased 5-HIAA:5-HT ratios in the prefrontal cortex, but not in the striatum or the hippocampus of rats (De La Garza and Asnis, 2003). The latter effect was prevented by pretreatment with the COX inhibitor, diclofenac. In a subsequent report, no effect of acute hIFN α administration (10^5 U/kg ip) on 5-HT or 5-HIAA was observed, whereas 14 daily treatments with hIFN α decreased 5-HT and 5-HIAA:5-HT ratios in the amygdala (De La Garza et al., 2005). In preliminary studies, using hIFN α or homologous IFN α injected at various doses ip or icv into mice or rats, we have observed no consistent changes in tryptophan, 5-HT, or 5-HIAA.

3.5.2 Interferon- γ

IFN γ has profound effects on tryptophan metabolism and hence may indirectly affect brain 5-HT. IFN γ and to a lesser extent IFN α , induce indoleamine-2,3-dioxygenase (IDO) in circulating immune cells, primarily macrophages. IDO converts tryptophan to kynurenine, which is subsequently converted to quinolinic acid (Moffett and Namboodiri, 2003). Thus, administration of IFN γ , or induction of endogenous IFN γ secretion, decreases plasma concentrations of tryptophan and increases plasma kynurenine. Tryptophan is an essential amino acid, and appears to be the rate-limiting amino acid for protein synthesis (Wunner et al., 1966). Tryptophan is also the essential precursor for 5-HT, and there is good evidence that 5-HT synthesis in the brain depends on available tryptophan (Wurtman and Fernstrom, 1976). Thus, the peripheral catabolism of plasma tryptophan may limit the availability of tryptophan to the brain for 5-HT synthesis. This led Bonaccorso et al. (2002) and Capuron et al. (2003) to speculate that these peripheral effects of IFNs may cause depression by limiting brain 5-HT synthesis. It has also been speculated that this could account for the depression-inducing properties of the interferons observed clinically (Meyers, 1999).

Chronic treatment of mice with IFN γ increased brain concentrations of quinolinic acid and the activity of the enzyme IDO, a key enzyme in indoleamine degradation (Saito et al., 1991). In a voltammetric study, Clement et al. (1997) found that peripheral administration of recombinant IFN γ had no effect on the appearance of 5-HIAA in the dorsal raphe nucleus, although IL-1 β and TNF α increased 5-HIAA. However, icv administration increased 5-HIAA following all three cytokines. In cultured immune cells, IFN γ stimulated the activity of GTP cyclohydrolase, an enzyme critical for the synthesis of tetrahydrobiopterin (Werner et al., 1993). Tetrahydrobiopterin is an essential cofactor for NO synthase, and also for the biosynthesis of catecholamines and serotonin.

3.6 Other Cytokines

Granulocyte/macrophage colony-stimulating factor (GM-CSF) administration to rats (5 and 10 μ g ip) significantly reduced hypothalamic glutamate, glutamine, aspartate, and GABA, as well as NE and 5-HT, but not DA (Bianchi et al., 1997b).

4 Effects of Intracerebral Administration of Cytokines

Many investigators have studied the responses to intracerebral administration of cytokines. Such studies have often been performed to demonstrate direct actions of cytokines on brain tissue. However, the results are difficult to interpret because of the tissue damage inherent in the introduction of the injectors, as well as the microdialysis probes, the push–pull cannulae, or the electrodes used. The lesions induce macrophage and lymphocyte invasion, and microglial and astroglial proliferation. Needless to say; this will result in substantial local production of cytokines and other factors. This presumably elevates the basal concentrations of cytokines and creates the potential for interaction of the injected substances with any of the endogenous factors.

For example, Shintani et al. (1993) reported that anterior hypothalamic infusion of IL-1 β increased microdialysate concentrations of DA, NE, and 5-HT from the anterior hypothalamus. Subsequently, they showed that infusion of the IL-1 receptor antagonist (IL-1ra) into the same region attenuated the increases in plasma ACTH and microdialysate DA, NE, and 5-HT in response to immobilization (Shintani et al., 1995). They concluded that hypothalamic IL-1 plays a role in the HPA and neurochemical responses to the stressor. The problem with this interpretation is that the insertion of microdialysis probes creates a substantial lesion in the brain, and thus a site for the local production of cytokines, such as IL-1 and IL-6, as demonstrated by Woodrooffe et al. (1991). Thus the net effect on plasma ACTH and dialysate amines will reflect the sum of the effects of the stressor and the local cytokine activity. Attenuation of the response to immobilization may indicate only the removal of the contribution of local IL-1 or other mediators, rather than an attenuation of the response to the stressor. Thus, the intracerebral antagonists may simply antagonize the artifacts of the cannulae and probe insertions. We have not observed any attenuation by icv IL-1ra of the neurochemical responses to footshock or restraint in mice (Dunn, 2000a).

5 Mechanisms of the Effects of the Cytokines

The foregoing research has described a variety of effects of peripherally administered cytokines on brain neurochemistry. Cytokines are too large to readily pass the blood–brain barrier. However, cytokines can affect the brain by several mechanisms (see [Table 3-2](#) and Dunn, 2002a). They could act on one or

Table 3-2
The principal known mechanisms for cytokine signaling of the brain

1.	Cytokines can act on the brain at sites where the blood–brain barrier is weak or nonexistent (i.e., the circumventricular organs, CVOs).
2.	Cytokines can be transported into the brain to a limited extent by selective uptake systems (transporters), thus bypassing the blood–brain barrier.
3.	Cytokines may act directly or indirectly on peripheral nerves that can send afferent signals to the brain.
4.	Cytokines can act on peripheral tissues, inducing the secretion of molecules whose ability to penetrate the brain is not limited by the barrier. A major target appears to be endothelial cells, which bear receptors for IL-1 (and endotoxin).
5.	Cytokines can be synthesized by immune cells that infiltrate the brain

Adapted from Dunn (2002a)

other of the brain regions that lack a blood–brain barrier, the circumventricular organs (CVOs), such as the median eminence, organum vasculosum laminae terminalis (OVLT), or the area postrema. Some researchers believe that IL-1, especially when administered iv, may act directly on CRF-containing terminals in the median eminence to initiate HPA axis activation (Rivier, 1995). Local application of IL-1 in the median eminence elevates plasma ACTH and corticosterone in rats, and these responses were prevented by local administration of α - and β -adrenergic receptor antagonists (Matta et al., 1990), or indomethacin (McCoy et al., 1994).

An alternative possibility is that cytokines may cross the blood–brain barrier using specific uptake systems. Banks et al. (1991) have demonstrated specific uptake of many cytokines from the blood to the brain. Uptake has been demonstrated for IL-1 α , IL-1 β , IL-1ra, IL-2, IL-6, and TNF α , as well as the soluble receptors for IL-1 and TNF α . However, the capacity of these uptake systems is quite low, so that it is not clear that such uptake enables active concentrations of the cytokines to be attained in the appropriate brain regions (Banks et al., 1995), although some functional consequences have been demonstrated (Banks et al., 2001).

Yet another important mechanism for cytokine actions on the brain involves actions on nerves that send afferents to the brain, such as the vagus. It was demonstrated that subdiaphragmatic vagotomy prevented the cerebral Fos response to ip LPS (Wan et al., 1994). Subsequently, several groups demonstrated that vagal lesions could prevent the CNS effects of ip IL-1 or LPS on behavior (Watkins et al., 1994; Bret-Dibat et al., 1995). Subdiaphragmatic vagotomy prevented the decrease in hypothalamic NE induced by ip IL-1 β (Fleshner et al., 1995), indicating an involvement of the vagus nerve. Abdominal vagotomy also attenuated the IL-1 β -induced increase in hypothalamic dialysate NE (Ishizuka et al., 1997; for a review see Watkins et al., 1995). It is believed that IL-1 binds to receptors on paraganglion cells associated with the vagus (Goehler et al., 1999). The vagal afferents terminate in the nucleus tractus solitarius (NTS) in the brain stem, which also contains the cell bodies of the A1/A2 noradrenergic projection system, thus providing an obvious pathway for activation of the VNAB and thence the PVN.

In experiments in which we measured hypothalamic NE release in the rat by microdialysis in parallel with measurements of plasma ACTH and corticosterone, we found that subdiaphragmatic vagotomy completely blocked the increase in apparent NE release, while it only attenuated the increases in plasma ACTH and corticosterone (Wieczorek and Dunn, 2006b). However, there may be species differences, because we observed that subdiaphragmatic vagotomy in mice only slightly attenuated the IL-1-induced increases in hypothalamic MHPG (Wieczorek et al., 2005).

Yet another possibility is that cytokines act on peripheral tissues that can synthesize lipophilic messengers that can readily pass the blood-brain barrier. An interesting example is the cerebral blood vessels. Receptors for IL-1 and LPS are known to be present on endothelial cells, including those in the brain. These receptors appear to be coupled to cyclooxygenases (COX) that enable production of prostaglandins, leukotrienes, thromboxanes, and other lipid mediators that can easily pass into and through the brain. Despite the remarkable sensitivity of the brain to IL-1, the brain contains very few receptors for IL-1. Binding sites for IL-1 have not been adequately demonstrated in the rat, and in the mouse they appear only in the hippocampus (Takao et al., 1990; Haour et al., 1992). mRNA for IL-1 receptors has also been difficult to demonstrate in the parenchyma of normal brain. However, IL-1 receptors are common in the capillary endothelium and choroid plexus.

Cytokines can be synthesized by immune cells that can infiltrate the brain. It is well established that peripheral LPS administration initiates a process that results in macrophages invading the brain (appearing as microglia) which appear to migrate through the brain parenchyma (Quan et al., 1999). It is likely that these various mechanisms operate in parallel, so that when cytokines are administered, more than one mechanism may operate.

The cyclooxygenase inhibitor, indomethacin, prevented the increases in MHPG (Dunn and Chuluyan, 1992) and NE turnover (Terao et al., 1993, 1995). Indomethacin also prevented the IL-1-induced increase in microdialysate NE from rat medial hypothalamus (Wieczorek & Dunn, 2006a). The opiate receptor antagonist, naloxone, had no effect on NE turnover, but an antibody to CRF attenuated the response (Terao et al., 1993). However, we have observed normal neurochemical responses to ip mL-1 β in mice lacking the gene for CRF (Dunn, unpublished observations).

6 Participation of Cytokines in the Responses to LPS and Infection

There are remarkable similarities in the neurochemical, behavioral, and HPA responses to IL-1 and those to LPS and to bacterial and viral infections. Thus, an obvious question is to what extent does IL-1 account for the HPA and neurochemical responses. Surprisingly, studies with antibodies to IL-1 and IL-1ra suggest that IL-1 is not critical for the responses to LPS (Swiergiel et al., 1997b; Dunn, 2000a). Although LPS is a potent stimulator of IL-1 production, little IL-1 appears in the first hour, and the peak response in the plasma is around 2 h. Therefore, if IL-1 were the mediator of responses to LPS, the endocrine, neurochemical, and behavioral responses should be delayed more than that which is actually observed (Dunn, 1992a, 2000a). Studies with antibodies to IL-1 and with IL-1ra failed to show blockade of either the increases in plasma ACTH and corticosterone, the behavioral responses, or those in MHPG or tryptophan (Dunn, 1992b, 2000a; Swiergiel et al., 1997b), although there was a trend toward an attenuation of the HPA activation at

the later times (Dunn, 2000a), as might be expected from the time considerations mentioned earlier. These results are consistent with observations on the responses to LPS in IL-1 β -knockout mice (Fantuzzi et al., 1996). Because LPS is a potent stimulator of IL-1 β synthesis and secretion, it is unlikely that IL-1 does not contribute to the HPA, behavioral, and neurochemical responses to LPS (Swiergiel et al., 1997b; Dunn, 2000a). However, IL-1 β is clearly not the only factor involved.

Experiments with antibodies to IL-6 have suggested a role for IL-6 in the HPA responses to both the administration of IL-1 (Wang and Dunn, 1999) and LPS (Perlstein et al., 1993; Wang and Dunn, 1999). In our experiments, the sensitivity to IL-6 antibody was confined to the later phases of the HPA response, consistent with the delay necessary for the production and secretion of IL-6. Pretreatment with a neutralizing monoclonal antibody to mouse IL-6 also attenuated the increases in tryptophan and 5-HIAA following LPS administration to mice, but not that to IL-1. This suggests that IL-6 contributes only to the indoleamine responses to LPS, and is not involved with those to IL-1 (Wang and Dunn, 1999). Treatment with a neutralizing antibody to TNF α also failed to prevent the HPA and neurochemical responses to LPS, even when supplemented with IL-1ra (Dunn, 1992b).

7 Functional Significance of the Neurochemical Responses to Cytokines

A critical question is whether any of the neurochemical responses observed following cytokine administration can be linked to functional responses. The most obvious candidates are the HPA responses and various behavioral responses.

7.1 Neurochemical Involvement in the HPA Response to IL-1

NE and 5-HT have both been implicated in HPA activation (Plotsky et al., 1989), and are thus obvious candidates for mediating the responses to IL-1 (Turnbull and Rivier, 1999). The responses in hypothalamic NE response and those in plasma ACTH and corticosterone are closely linked in time, and in our experiments, have been highly correlated over a large number of experiments involving a large number of different manipulations. This association was confirmed in microdialysis studies in freely moving rats in which a very close temporal relationship between extracellular concentrations of NE in the hypothalamus and plasma concentrations of corticosterone was observed following both iv and ip IL-1 β (Smagin et al., 1996; Wiczorek and Dunn, 2006a, b). This association is supported by some other studies. For example, when the noradrenergic neurotoxin 6-OHDA was injected into the VNAB or the PVN of rats, it resulted in depletions of PVN NE of 75% or more, and the plasma corticosterone response to ip IL-1 was largely prevented (Chuluyan et al., 1992). A similar result was obtained using icv IL-1 in 6-OHDA-treated rats (Weidenfeld et al., 1989). Similarly, 6-OHDA treatment prevented the increase in plasma corticosterone following infection with herpes simplex virus, whereas a lesion of serotonergic systems with 5,7-dihydroxytryptamine (5,7-DHT) did not (Ben Hur et al., 1996). Surprisingly however, when whole brain NE was depleted by around 98% using 6-OHDA in mice, minimal impairment of the plasma corticosterone response to IL-1 β was observed (Swiergiel et al., 1996, and other unpublished observations). In addition, α - and β -adrenergic receptor antagonists had little effect on the response to IL-1 in rats (Rivier, 1995) and mice (Dunn, 2000b), and the α_1 -adrenergic receptor antagonist, prazosin, attenuated the response in mice only at high doses (Dunn, 2000b). Thus it is likely that brain NE is involved in the HPA response to IL-1, but it does not appear to be essential.

The role of CRF in the HPA response is clearer. The original studies on IL-1 action on the HPA axis implicated CRF in this response, because an antibody to CRF largely prevented the increases in plasma ACTH and corticosterone to IL-1 (Berkenbosch et al., 1987; Sapolsky et al., 1987; Uehara et al., 1987) and IL-1 increased secretion of CRF into portal blood (Sapolsky et al., 1987). Studies in CRF knockout mice showed that although several behavioral responses were normal, the plasma corticosterone response to IL-1 was dramatically reduced but not completely absent (Dunn and Swiergiel, 1999). It seems likely that the small response to IL-1 may reflect a direct effect of IL-1 on the pituitary or the adrenal cortex, but this is clearly not the major mechanism involved in the HPA response to IL-1.

7.2 Neurochemical Involvement in Behavioral Responses to Cytokines

Illness is associated with familiar alterations in behavior known as sickness behavior (Dantzer et al., 2001). Sickness behaviors include decreases in activity, in feeding, in exploratory behaviors and sexual activity, and increases in sleep. Similar behaviors are elicited by IL-1 and LPS (Swiergiel et al., 1997a, b; Dantzer et al., 2001). We have studied the drinking of sweetened milk by mice as a simple model of sickness behavior (Swiergiel et al., 1997a). The reductions in ingestive behavior induced by IL-1 are largely prevented by cyclooxygenase inhibitors, such as aspirin, indomethacin, and ibuprofen (Hellerstein et al., 1989; Dunn and Swiergiel, 2000). However, such inhibitors are less effective on LPS-induced behaviors (Swiergiel et al., 1997a; Dunn and Swiergiel, 2000), and have only small effects on the behavioral changes associated with influenza virus infection (Swiergiel et al., 1997a). Some evidence indicates that the responses are largely mediated by the COX2 isozyme, although a role for COX1 is not excluded (Dunn and Swiergiel, 2000; Swiergiel and Dunn, 2001).

We have used the milk drinking model to study the potential involvement of cerebral NE and 5-HT. Both neurotransmitters are excellent candidates because they have previously been implicated in hypophagia (Cooper and Clifton, 1996). NE appeared more likely to be involved because it responds more rapidly to IL-1 than serotonin, and the time course of the hypophagia paralleled its response more closely. Thus, we tested the ability of adrenergic receptor antagonists to reverse the hypophagia. However, neither α_1 -, α_2 -, nor nonselective α - or β -adrenergic receptor antagonists, alone or in combination, induced significant reductions in IL-1- or LPS-induced hypophagia in mice (Swiergiel et al., 1999b). Likewise, pretreatment with 6-OHDA or DSP-4 to deplete cerebral NE failed to alter the hypophagic response to IL-1 or LPS (Swiergiel et al., 1999b).

However, studies of other cytokine-induced behaviors has produced some evidence implicating noradrenergic mechanisms. For example, 6-OHDA pretreatment or prazosin prevented the antinociceptive effect of icv hIL-1 α determined in the hot-plate test (Bianchi and Panerai, 1995).

Surprisingly, we have also failed to find any effect of cerebral 5-HT depletion (with 5,7-DHT), or pretreatment with a variety of 5-HT receptor antagonists (Swiergiel and Dunn, 2000). However, this is consistent with the lack of effect of NOS inhibitors that prevent the increases in tryptophan and 5-HIAA in responses to IL-1 and LPS (Dunn, 1993). It is also consistent with the observation that administration of IL-6 which induces increases in brain tryptophan and 5-HIAA like those to IL-1 (Wang and Dunn, 1998), but it fails to alter milk drinking in mice (Swiergiel et al., 1997b).

8 Relationships of Cytokine-Induced Effects on Neurotransmission to Depression

The potential relationships of the cytokine-induced changes in cerebral neurotransmission to human mood states can only be speculated upon. The strongest links have been suggested between cytokines and depression, although schizophrenia has often been associated with immune abnormalities (Hinze-Selch and Pollmächer, 2001). Both NE and serotonin have been implicated in clinical depression, primarily because the major therapeutic drugs inhibit reuptake of NE or 5-HT, or both. There is also some evidence for a hyperactivity of cerebral noradrenergic systems in depressed patients (e.g., Wong et al., 2000) and substantial evidence that a significant majority of depressed patients hypersecrete cortisol (Kasckow et al., 2001). These facts suggest that IL-1, which activates the HPA axis, as well as cerebral NE and 5-HT metabolism, is a potential mediator of depression.

Several authors have noted the similarities between sickness behavior and depression (Yirmiya, 1996; Yirmiya et al., 1999; Charlton, 2000), so that it has been argued that sickness behavior in animals may be a model for depression. Yirmiya (1996) showed that LPS administration to rats reduced their consumption of saccharin solution and argued that this reflected anhedonia, perhaps resembling the anhedonia, which is a hallmark of depression. In a creative experiment, they showed that chronic treatment with the antidepressant, imipramine, reversed the reduction of saccharin ingestion by LPS, reinforcing the concept that LPS treatment could model depression (Yirmiya, 1996). The ability of antidepressant drugs to prevent the

reduction in pleasurable activities by LPS has been replicated by some (Connor and Leonard, 1998; Castanon et al., 2001; Yirmiya et al., 2001), but not others (Connor and Leonard, 1998; Dunn and Swiergiel, 2001). Yirmiya et al. (1999), subsequently reported that the protective effect of antidepressants was evident only with LPS and not with IL-1. They speculated that the effect of the antidepressants was associated with LPS-induced IL-1 production. However, it is not easy to explain why inhibitors of neurotransmitter reuptake should exert such an effect. A study from another laboratory showed that chronic treatment of rats with the atypical antidepressant, tianeptine, attenuated sickness behavior induced by IL-1 or LPS, but only when these agents were administered peripherally, not icv (Castanon et al., 2001). It has been postulated that the sickness behavior induced by peripheral IL-1 or LPS is mediated by induction of IL-1 centrally because icv IL-1 and LPS can induce some sickness behaviors and IL-1 antagonists can attenuate the effects of peripheral IL-1 and LPS (Dantzer et al., 2001). However, if cerebral IL-1 is responsible for the sickness behaviors, then tianeptine treatment should have attenuated the responses to icv IL-1, unless its site of action is peripheral, which would conflict with most theories of antidepressant action.

The similarities between the responses to IL-1 and depression do not pass close scrutiny. Although depression is frequently associated with decreased activity, anorexia, and loss of libido, it can also be associated with hyperactivity and overeating. Proponents of the cytokine theory have pointed out that both depression and IL-1 administration can be associated with abnormalities in sleep (Dantzer et al., 1999; Yirmiya et al., 1999; Charlton, 2000). However, IL-1 increases the duration of slow-wave sleep (Krueger and Majde, 1995), whereas by far the most common sleep abnormality in depressed patients is insomnia. Administration of cytokines to humans can induce some symptoms of sickness, but this is as true of IL-1 and the interferons as it is of IL-1, yet the interferons have not been shown to induce sickness behavior, or the cerebral noradrenergic activation, although IFN α elevates ACTH in man. Elevated circulating concentrations of IL-1 have been reported in a few studies of depressed patients, but this has not been a universal finding, although IL-6 is consistently elevated (Zorrilla et al., 2001). Thus, cytokine administration and depression may share certain aspects, perhaps because they are both stress-like states. However, the clinical report found increased concentrations of IL-1, but not IL-6, in the CSF of depressed patients (Levine et al., 1999).

9 Conclusions

Administration of certain cytokines to animals can alter CNS neurotransmission. [Table 3-2](#) summarizes the effects of cytokines reported on catecholamines and indoleamines, as well as those on the HPA axis (Dunn, 2001). Cytokine administration can also elicit a number of effects on the brain, including neuroendocrine and behavioral effects. The most well-documented effect is the activation by IL-1 of the HPA axis, which is accompanied by a stimulation of hypothalamic NE metabolism, probably reflecting increased NE release. IL-1 also increases brain concentrations of tryptophan and the metabolism of serotonin. IL-6 induces effects on tryptophan and 5-HT similar to those of IL-1. TNF α has effects on the HPA axis similar to those of IL-6, but affects NE and tryptophan only at high doses. IFN α had little, if any, effect on the parameters studied. The effects of IL-1 are remarkably similar to those observed following administration of endotoxin and infections, such as influenza virus. They also resemble quite closely the responses that are observed to stressors commonly studied in laboratory animals, such as electric shock or restraint ([Table 3-1](#)). The major differences are that the NE response to shock or restraint is very uniform throughout the brain, whereas that to IL-1, LPS, or infection is significantly greater in the hypothalamus. In addition, responses of DA systems are normally observed to shock or restraint, with especially prominent responses in the limbic cortex, whereas DA responses are seldom observed in response to IL-1 and immune stimuli, and when they do occur, the response in the limbic cortex is similar to that in other brain regions. A small DA response occurs throughout the brain to peripheral administration of LPS. The neurochemical responses to cytokines are likely to underlie the endocrine and behavioral responses. The NE response to IL-1 appears to be related to the HPA activation. Neither the noradrenergic nor the serotonergic systems appear to be involved in the hypophagic responses. The significance of the indoleaminergic responses is not known.

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4 **Age-Related Alterations in Autonomic Nervous System Innervation of Lymphoid Tissue**

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1	<i>Introduction</i>	62
2	<i>Bone Marrow</i>	63
2.1	Aging and the Bone Marrow	63
2.2	NA Nerves in Bone Marrow	63
2.3	Aging and Sympathetic Innervation of Bone Marrow	63
2.4	Functional Role of NA Nerves in Bone Marrow	63
3	<i>Thymus</i>	64
3.1	Structure and Function of the Thymus	64
3.2	Age-Related Changes in the Thymus	64
3.3	Sympathetic Innervation of the Thymus	65
3.4	Sympathetic Innervation in the Aging Thymus	66
3.5	Functional Studies and Significance	66
4	<i>Spleen</i>	68
4.1	Structure and Function of the Spleen	68
4.2	Sympathetic Innervation of the Spleen	69
4.3	Sympathetic Innervation in the Aging Spleen	69
4.4	Can Age-Related Changes in NA Innervation Be Reversed?	71
4.5	Age-Related Changes in the Spleen and Immune Function	72
4.6	Functional Studies and Significance	73
5	<i>Summary and Closing Remarks</i>	76

Abstract: Complex bidirectional signaling occurs between the nervous and immune systems that impacts the functions of both systems. In response to stressors, the central nervous system has modulatory effects on the immune system through release of hormones, paracrine signals, and by direct contacts between nerves and immune cells. Conversely, in response to antigens and pathogens, immune system activation results in production of cytokines that induce altered neural activity. Progressive age-related decline in brain and immune functions in the elderly results in impaired cognition, reestablishment of homeostasis at age-adjusted set points, and immune senescence. This chapter reviews our understanding of how normal aging alters the cross talk between the autonomic nervous system and the immune system to alter immune functions with age. Changes in the relationship between noradrenergic (NA) nerves and immune cells with age have been demonstrated, including altered density and compartmentation of NA nerves in lymphoid organs, norepinephrine (NE) turnover, density and coupling of adrenergic receptors, and availability and functional capacity of target lymphoid cells. Strain-dependent changes in how NA nerves age in secondary lymphoid tissue have been reported. These changes alter nerve-to-immune signaling, and subsequently immune functions in old rodents. These findings suggest that impaired sympathetic regulation of immune function occurs in aged individuals, which may contribute to the increase in susceptibility to infectious diseases, autoimmunity, and cancer in the elderly. Age-related changes in sympathetic nervous system (SNS) modulation of immune functions are likely to contribute to the reduced immunocompetence observed in clinical studies with elderly subjects. Understanding the age-related changes that occur in SNS modulation of immune functions could be useful for developing novel strategies for treating diseases, disease prevention, and improving overall health in the elderly.

List of Abbreviations: α MNE, α -methylnorepinephrine; Con A, concanavalin A; CGRP, calcitonin gene-related peptide; CRH, corticotropin-releasing hormone; DN, dual negative; DP, dual positive; GM-CFU, granulocyte/macrophage colony-forming units; IFN- γ , interferon- γ ; MAO-B, monoamine oxidase B; MHC, major histocompatibility complex; NA, noradrenergic; NE, norepinephrine; NGF, nerve growth factor; NPY, neuropeptide Y; PALS, periaarteriolar lymphatic sheath; SCID, severe combined immunodeficiency; 6-OHDA, 6-hydroxydopamine; SNS, sympathetic nervous system; SP, single positive; TH, tyrosine hydroxylase; VIP, vasoactive intestinal polypeptide; SOD, superoxide dismutase

1 Introduction

The sympathetic nervous system (SNS) supplies norepinephrine (NE)-containing nerves to primary and secondary lymphoid organs (reviewed in Bellinger et al., 2001b). Cells of the immune system receive signals from these nerves through the interaction of NE, the primary neurotransmitter of noradrenergic (NA) nerves, and specific subsets of adrenergic receptors expressed on immune cells (reviewed in Sanders et al., 2001). NE-adrenergic receptor interaction signals changes in cellular biochemistry, gene expression, and protein synthesis that result in alterations in immune cell activity and function (reviewed in Lorton et al., 2001; Madden, 2001).

Recently, there has been renewed interest in the role of the parasympathetic nervous system in neural modulation of the immune system (reviewed in Czura and Tracey, 2005), and clearly this arm of the autonomic nervous system plays an important role in neural-immune regulation. Cells of the immune system express cholinergic receptors, and cholinergic drugs alter immune responses; however, cholinergic parasympathetic innervation of primary and secondary lymphoid organs remains controversial, and there is virtually no information regarding age-related changes in parasympathetic modulation of the immune system. Therefore, we focus on aging and SNS-immune system interactions and do not consider parasympathetic innervation in this chapter.

As individuals age, striking changes occur in neuroendocrine, autonomic, and immune functions, which alter the dynamics of brain-immune interactions. The structure, cellularity, and lymphoid composition of primary and secondary lymphoid organs reflect a functional loss of immune competence (Goidl, 1987). There is a remodeling of sympathetic innervation of primary and secondary lymphoid organs and an increase in sympathetic tone concomitant with age-related changes in the local microenvironment and immune senescence. This is presumed to be an adaptive response to maintain appropriate contact between

these nerves and immune target cells (reviewed in Bellinger et al., 2001c). Remodeling of NA nerves in lymphoid tissue is accompanied by altered NA signaling of immune target cells, raising the question of the role of altered NA neuronal-immune target cell signaling in immune senescence. Still, regardless of whether a causal relationship between these age-related changes exists, it is clear age-associated alterations in the SNS and immune functions promote autoimmunity and affect the capacity for host defense against pathogens and cancer. This chapter summarizes the evidence for age-related changes in sympathetic NA innervation of lymphoid organs and the impact on sympathetic modulation of the aging immune system in health and illness.

2 Bone Marrow

2.1 Aging and the Bone Marrow

The morphology of the bone marrow is altered with normal aging. The most prominent changes begin at the onset of puberty, accompanied by thymic involution, which includes an increase in adipose tissue and myeloid cells and a decrease in lymphoid cells in the thymus (Dominguez-Gerpe and Rey-Mendez, 1998). The frequency of thymocyte precursors in the bone marrow of old mice is reduced 40-fold. The immune system is dependent on the migration of these T cell precursors from the bone marrow to the thymus for sustained T-cell production (Donskoy and Goldschneider, 1992). The ability of old bone marrow cells to colonize thymic organ cultures in vitro (Eren et al., 1988, 1990; Globerson et al., 1992; Globerson, 1994) and the thymus in vivo (Tyan, 1977) is reduced.

2.2 NA Nerves in Bone Marrow

NA and peptidergic nerves supply the parenchyma of bone marrow in young adult rodents (Weihe et al., 1989; Muller and Weihe, 1991; Tabarowski et al., 1996; Afan et al., 1997). Coursing in small nerve bundles, they enter the bone through the nutrient foramina along with the vasculature, and extend from these plexuses into the adjacent parenchyma among hematopoietic cells (Felten et al., 1985; Felten and Felten, 1991; Tabarowski et al., 1996). NA nerves in the bone marrow have been demonstrated to contain both NE and neuropeptide Y (NPY).

2.3 Aging and Sympathetic Innervation of Bone Marrow

On the basis of fluorescence histochemistry for localizing catecholamines, the density, distribution, and staining intensity of NA nerves in bone marrow from aged male F344 rats (21-month-old) does not appear altered with advancing age (Bellinger et al., 2001c). However, the effects of aging on neurotransmitter metabolism and turnover, or on sympathetic signaling of bone marrow target cells, have not been investigated.

2.4 Functional Role of NA Nerves in Bone Marrow

Sympathetic innervation of bone marrow is presumed to modulate cell proliferation and differentiation of myeloid and lymphoid cells. Peak concentrations of catecholamines in bone marrow positively correlated with the proportion of bone marrow cells in G2/M (undergoing cell division) phases of the cell cycle (Cosentino et al., 1998; Maestroni et al., 1998), supporting sympathetic regulation of hematopoiesis. Both NE and dopamine concentrations in murine bone marrow have a diurnal rhythm. They peak in concentration at night, which parallel the peak in cellular proliferation rates (Maestroni et al., 1998). This may be a finding that is unique to bone marrow, since rhythmicity of catecholamine concentrations was not detected in either rat thymus or spleen (Kelley et al., 1996).

In normal mice and in mice that have been transplanted with syngeneic bone marrow cells, chemical sympathectomy with 6-hydroxydopamine (6-OHDA) or administration of prazosin, an α_1 -adrenergic receptor blocker, increased myelopoiesis and inhibited lymphopoiesis (Maestroni et al., 1992; Maestroni and Conti, 1994). Further, treatment of bone marrow cultures with either NE or the α_1 -adrenergic agonist, methoxamine, reduced proliferation of granulocyte/macrophage colony-forming units (GM-CFU), consistent with the expression of high affinity α_1 -adrenergic receptors on unfractionated bone marrow cells (Maestroni and Conti, 1994).

Under certain conditions, increased SNS activity may drive migration of progenitor cells into the circulation; chemical sympathectomy in young adult mice increased their numbers in the blood, and reduced bone marrow cellularity (Afan et al., 1997). Sympathectomy transiently increased proliferation of cultured bone marrow cells, particularly progenitor cells and GM-CFU. A similar transient increase in proliferation of bone marrow cells *in vivo* was reported in sympathectomized mice (Madden et al., 1994). Collectively, these findings support an inhibitory role for the SNS in spontaneous cell proliferation in bone marrow.

Contrary to these studies, Benestad and coworkers (1998) were unable to demonstrate effects of either neonatal chemical sympathectomy (which permanently destroys NA nerves) or unilateral surgical denervation of the sciatic nerve on proliferation of hematopoietic cells in the bone marrow. These discordant findings likely result from the differences in the methods employed. Despite studies indicating age-related changes in bone marrow cellularity, proliferation, and cellular migration into the thymus, the influence of the SNS on these processes in aged rodents has not been investigated.

3 Thymus

3.1 Structure and Function of the Thymus

The thymus is composed of incomplete lobules. These lobules are partially separated by septa derived from the connective tissue capsule, which envelops the organ. Each lobule consists of the cortex, a peripheral zone of densely packed lymphocytes, which surrounds the medulla, a lightly staining central zone. The medulla contains Hassall's corpuscles, which are several layers of flattened, concentrically arranged epithelial cells. Hassall's corpuscles are degenerative bodies that increase in size and number throughout life. The thymic stroma consists of dendritic cells, macrophages, and epithelial reticular cells. The epithelial reticular cells have thin cytoplasmic processes that attach to each other by desmosomal contacts.

Differentiation of pre-T cells into mature T cells, which recognize and respond appropriately to foreign antigen, occurs primarily in the thymus. Pre-T cells are stem cells that originate from the bone marrow. These stem cells enter the thymus in the subcapsular region, where they differentiate from a $CD4^-CD8^-$ phenotype (dual negative, DN) to an intermediate $CD3^{lo}CD4^+CD8^+$ (dual positive, DP) phenotype (von Boehmer, 1988). DP thymocytes undergo a selection process in the cortex, which requires T cell receptor (CD3) interactions with major histocompatibility complex (MHC) molecules on the surface of thymic stromal cells (Robey and Fowlkes, 1994). Positively selected thymocytes upregulate their expression of CD3 and downregulate their expression of either CD4 or CD8 to become mature $CD3^{hi}CD4^+CD8^-$ or $CD3^{hi}CD4^-CD8^+$ (single positive, SP) thymocytes. Most thymocytes fail the process of positive selection and undergo apoptosis in the thymus. The surviving mature SP thymocytes reside in the medulla. These cells subsequently undergo negative selection in the corticomedullary junction, then leave the thymus through the vasculature to seed secondary lymphoid organs with mature SP T cells.

3.2 Age-Related Changes in the Thymus

The thymus starts to atrophy after puberty. However, despite this atrophy, the thymus remains a site of T cell selection throughout adulthood. The process of thymic atrophy proceeds as a gradual regression in size, weight, and cellularity (reviewed in Bodey et al., 1997). Adipose tissue displaces and disrupts the

thymic stroma. This is particularly true for the cortical epithelial network that supports early T cell differentiation and selection (George and Ritter, 1996). While the underlying mechanisms of age-related thymic involution are not entirely understood, studies do support that both genetic mechanisms (Aspinall, 1997; Kuro-o et al., 1997; Nabarra et al., 1997; Takeda et al., 1981) and altered hormone levels (Simpson et al., 1975; Bellamy et al., 1976) contribute to thymic atrophy.

Development of bone marrow-derived stem cell precursors into thymocytes diminishes with age. Transplanting young bone marrow into old recipients does not reverse the decrease in T cell generation in the thymus of the old, indicating that the microenvironment of the aged thymus has a decreased capacity to support thymocyte differentiation (Mackall et al., 1998). Thus, there is an increase in the number of $CD4^- CD8^-$ DN immature thymocytes and a reduction in the number of $CD4^+ CD8^+$ DP cells with aging (Thoman, 1995, 1997a, b), which suggests a block in T cell differentiation from the DN to DP phenotype. This leads to a reduction in naive T cell output with age. The decrease in T cell output from the thymus contributes to the age-associated dysregulation of the immune system, including reduced cell-mediated immunity, increased autoantibody production, and increased susceptibility to infectious disease (Thoman and Weigle, 1989). The impairment in T cell development that occurs in the aging thymus has been attributed to defects in stromal cells and to disruption in T cell receptor rearrangement and β -selection efficiency (Aspinall, 1997). Several studies indicate that there is an age-related decrease in bone marrow precursor and thymocyte interactions with stromal cells (Farr and Sidman, 1984; Farr and Anderson, 1985; Globerson et al., 1992). Other thymic tissue components, including nerves, endothelial cells, fibroblasts, and connective tissue may also contribute to the age-related disruption in T cell development. Von Patay and coworkers (1998, 1999) reported that NE stimulated a 14-fold increase in the production of IL-6 by young cultured thymic epithelial cells. Given the role of IL-6 in T cell development (Ma et al., 2000), these findings support an indirect role of the SNS in maintaining the internal milieu.

3.3 Sympathetic Innervation of the Thymus

NA nerves arising from the postganglionic neurons in the upper paravertebral ganglia of the sympathetic chain (primarily the superior cervical and stellate ganglia) innervate the thymus (Bulloch and Pomerantz, 1984; Nance et al., 1987; Tollefson and Bulloch, 1990). These NA nerves enter the thymus with blood vessels as moderately dense plexuses. They course through the capsule and interlobular septa or continue with the vasculature into the subcapsular cortex, a compartment where stem cells from the bone marrow reside. In the septa, NA nerves course along blood vessels, where they often are observed adjacent to mast cells and macrophages. In the capsule and interlobular septa, NA nerves reside adjacent to mast cells (Bellinger et al., 1990; Muller and Weihe, 1991), $ED1^+$ and $ED3^+$ macrophages (Muller and Weihe, 1991; Bellinger et al., 2001b), and corticotropin-releasing hormone (CRH)-immunoreactive cells (Brouxhon et al., 1998). In the thymic cortex, the highest density of NA nerves occurs near the corticomedullary junction, a site of T cell differentiation and maturation. This also is the site where mature T cells exit the thymus to enter the circulation. NA nerves primarily associate with the medullary sinuses in the medulla. Some of the NA nerves that supply the medullary sinuses extend from these plexuses to enter the surrounding parenchyma and reside among mature thymocytes and thymic epithelial cells (reviewed in Bellinger et al., 2001b).

Nerves containing other neurotransmitters such as NPY, tachykinins (substance P, neurokinin A, and neurokinin B, collectively), substance P, calcitonin gene-related peptide (CGRP), CRH, and vasoactive intestinal polypeptide (VIP) have been shown to innervate the thymus in young adult rodents (Bellinger et al., 2001a,b). The location of the ganglia from which these thymic nerves originate is not entirely clear. In the young adult rat thymus, there is, at least to some extent, overlapping distribution of all of these neurotransmitter-specific nerves. In general, the peptidergic innervation is not as robust as that observed for sympathetic NA nerves, with the exception of NPY. Chemical sympathectomy with 6-OHDA destroys NPY^+ nerves in the rat thymus (Kendall and al-Shawaf, 1991), indicating that NPY colocalizes with NE in NA nerves. CGRP- and substance P/neurokinins-containing nerves are presumed to be afferent sensory nerves based on the known presence of these neurotransmitters in sensory neurons; however, Bulloch and coworkers (1991) have suggested that $CGRP^+$ fibers derive from the vagus nerve. Treatment with a

neurotoxin that is selective for small nonmyelinated afferent nerves, capsaicin, depletes substance P in the thymus (Geppetti et al., 1987), providing support for derivation of these thymic nerves from sensory neurons. While substance P and CGRP are largely colocalized in sensory nerves, Geppetti and coworkers (1988) have suggested that these neurotransmitters are not colocalized in the thymus, based on the thymic concentrations of substance P and CGRP. Both CGRP- and substance P-containing thymic nerves do appear to arise from cervical dorsal root ganglia based on anterograde tracing studies (Elfvin et al., 1993). Few studies have examined age-related changes in peptidergic innervation of the thymus. One laboratory has reported that the distribution of CGRP⁺ nerves in the aged thymus remains similar to that observed for young adult rats; however, the density of these fibers are reduced in the aged thymus (Bulloch et al., 1991).

3.4 Sympathetic Innervation in the Aging Thymus

Our laboratory completed a longitudinal study of sympathetic innervation with age in the F344 rat thymus. We observed a dramatic elevation in NE concentration and an increase in the density of NA nerves and NE concentration as the thymus involutes with age (Bellinger et al., 1988). Despite the thymic involution that occurs with increasing age, the total NE content in the thymus remained constant, suggesting that the increase in NE concentration and NA nerve density observed in the aging thymus likely results from an age-related loss in tissue volume. However, Zirbes and Novotny (1992) completed a similar quantitative study and discovered an increase in the density of NA nerves in the aged rat thymus that exceeded the reduction in thymic volume. In contrast to our finding, this study suggests that sprouting of sympathetic nerve fibers occurs in the aged rodent thymus. The reported differences in sympathetic innervation of the aging thymus may be due to differences in the rat strain or housing conditions between these two studies. We also have observed age-related increases in thymic sympathetic innervation in other strains of rats and in several mouse strains. These increases in the density of NA nerves in the thymus in these strains occurred with puberty and involution of the thymus (Madden et al., 1997; Bellinger et al., 2001c). As the thymus involutes with increasing age after puberty, thymocytes reside among a higher density of NA nerve fibers, suggesting that they are exposed to higher concentrations of NE, which has been implicated for the altered thymocyte function that occur in the elderly. Substantiation of this hypothesis awaits measurement of NE release and turnover and studies exploring the role of thymic sympathetic innervation in thymocyte function with age.

3.5 Functional Studies and Significance

Studies using young adult rodents have provided most of our knowledge regarding the role of sympathetic innervation in modulating thymic function. In young adult rodents, research using bioassays, radioligand-binding assays, Northern blot analysis, and signal transduction studies have provided evidence for heterogeneous expression of α - and β -adrenergic receptors on thymocytes (Morgan et al., 1984; Durant, 1986; Kendall and al-Shawaf, 1991; Morale et al., 1992; Marchetti et al., 1994; Cook-Mills et al., 1995) and thymic epithelial cells (β_1 and β_2) (Kurz et al., 1997; von Patay et al., 1998). β -adrenergic receptors are expressed at very low levels (below the level of detection) on unfractionated thymocytes. In contrast, mature T cells residing in the thymus (Madden, 2001) and mature corticosterone-resistant thymocytes express β -adrenergic receptors at levels equivalent to those seen on peripheral T cells (Fuchs et al., 1988; Radojcic et al., 1991). These studies suggest that immature T cells express few to no β -adrenergic receptors and that β -adrenergic receptor expression is induced later in the differentiation process. This interpretation is consistent with findings that β_2 -adrenergic receptors localize primarily to the medullary region of the rat thymus (Marchetti et al., 1994). Despite the lower expression of β -adrenergic receptors in unfractionated thymocytes and equivalent expression in mature thymocytes and T cells in spleen and lymph nodes, the efficiency of β -adrenergic receptor coupling to adenylate cyclase in unfractionated thymocytes and mature thymocytes is comparable to that seen in T cells from the spleen or lymph nodes (Niaudet et al., 1976). Further, unfractionated thymocytes generate an isoproterenol-induced cAMP response that is equivalent to that of

splenocytes, even though spleen cells express a far greater number of β -adrenergic receptors (Madden, 2001). These studies indicate that β -adrenergic receptors expressed on thymocytes are exquisitely sensitive to β -adrenergic receptor signaling.

Evidence that the SNS-to-thymocyte signaling plays a significant role in thymocyte development and functions comes from studies using mice that lack dopamine- β -hydroxylase (*dbh*^{-/-}), an enzyme that converts dopamine to NE. *dbh*^{-/-} knockout mice did possess a similar number and frequency of thymic subpopulations (CD4⁺, CD8⁺, CD4⁻/CD8⁻, CD4⁺/CD8⁺) compared with age- and sex-matched *dbh*^{+/-} controls under normal housing conditions (Alaniz et al., 1999). However, when *dbh*^{-/-} mice were housed in nonspecific pathogen-free conditions, the thymuses showed marked involution and reduced immature CD4⁺/CD8⁺ DP thymocytes. These findings suggest that while *dbh*^{-/-} mice do not have intrinsic developmental defects, under pathogen-free conditions *dbh*^{-/-} mice had higher mortality rates and impaired resistance to primary and secondary infection. These findings support a role for sympathetic innervation in maintenance of the normal microenvironment required for T cell development, differentiation, and responsiveness to environmental antigens in lymphoid organs.

The SNS's role in thymocyte development is further supported by studies using young adult thymocytes, which show that exposure of thymocytes in vitro to β -adrenergic receptor agonists elevates expression of thymocyte Thy-1 and TL antigens, suggesting enhanced thymocyte differentiation after β -adrenergic receptor stimulation (Singh and Owen, 1976). Findings from other studies indicated that thymocyte proliferation is enhanced upon removal of β -adrenergic receptor signaling. When cells obtained from thymic rudiments are implanted into the anterior chamber of mice, sympathectomized by unilateral ablation of the superior cervical ganglia, the number of thymic cells were increased (Singh, 1985). Similarly, removal of NA innervation following chemical sympathectomy with 6-OHDA resulted in an increased cellular proliferation in the thymic cortex as indicated by an elevated bromodeoxyuridine uptake (Kendall and al-Shawaf, 1991).

Other reported effects of chemical sympathectomy in the thymus include an increase in apoptosis, reduced cellularity, and decreased spontaneous thymocyte proliferation ex vitro (Delrue-Perollet et al., 1995; Tsao et al., 1996). The 6-OHDA-induced increase in apoptosis was prevented by pretreatment with desipramine (Tsao et al., 1996), a drug that inhibits 6-OHDA uptake and prevents NA nerve terminal destruction. While these findings suggest that ablation of NA nerves was necessary for this effect, in vitro studies of cultured thymocytes indicate that this interpretation may be incorrect, as incubating thymocytes in 10⁻⁵ M 6-OHDA also induced apoptosis. Further, coincubation of 6-OHDA with desipramine also prevented this effect (Tsao et al., 1996), indicating a direct effect of these treatments on thymocytes and suggesting these cells have a mechanism for catecholamine uptake. In the in vivo study, the extent of sympathetic nerve loss was not determined, an important factor given that NA nerves in the thymus are more resistant to the neurotoxic effects of 6-OHDA compared with the spleen (Kostrzewa and Jacobowitz, 1974; DL Bellingier personal observations). 6-OHDA could also have induced thymocyte apoptosis by inducing the elevated corticosterone levels seen secondary to chemical denervation (Delrue-Perollet et al., 1995; Kruszkowska et al., 1998). Thus, further studies are required to determine the mechanisms of these thymocyte responses. In summary, these findings suggest that sympathetic innervation in the thymus of the young adult may inhibit proliferation and reduce thymocyte apoptosis; however, more detailed and direct studies of catecholaminergic effects on specific T cell subsets are required.

Thymocyte development and proliferation also have been assessed after in vivo chronic β -adrenoceptor blockade using aged mice. Madden and Felten (2001) exposed 2- and 18-month-old male BALB/c mice to the β -adrenergic receptor antagonist, nadolol, by subcutaneous pellet implantation for 4 weeks. In this study, flow cytometric analysis of T cell populations in the thymus and peripheral blood were used to examine thymocyte differentiation and naive T cell output. In aged mice, chronic β -adrenoceptor blockade increased the frequency of immature CD4⁻CD8⁻ thymocytes and reduced the frequency of CD4⁺CD8⁺ thymocytes. An increase in the number of mature CD4⁻CD8⁺ thymocytes was observed in the absence of any change in the frequency of the CD4⁺CD8⁻ population in nadolol-treated aged mice. These findings suggest that β -adrenoceptor stimulation counteracts the age-related changes in thymocyte maturation. Since nadolol treatment did not alter CD3^{hi} expression in the CD4⁺CD8⁺ population, the immediate precursors to the SP thymocytes (Petrie et al., 1990), the observed increase in CD4⁻CD8⁺ cells was unlikely to

be mediated by more CD4⁺ CD8⁺ cells undergoing positive selection. Changes in thymocyte proliferation following nadolol treatment apparently did not contribute to the *in vivo* nadolol-induced effects since chronic β -adrenoceptor blockade *ex vivo* did not alter thymocyte proliferation in either age group. However, a more detailed analysis using additional thymocyte differentiation markers in combination with such functional measures as proliferation and apoptosis will be needed to further characterize the nadolol-induced changes observed in this study.

An additional study was completed to determine whether the nadolol-induced increase in CD8⁺ T cells observed in the thymus of aged mice was due to an inability of these cells to migrate out of the thymus into the circulation. In this study, old mice treated with nadolol the percentage of peripheral blood CD4⁺ and CD8⁺ T cells expressing high and low levels of the adhesion molecule CD44 were examined (MacDonald et al., 1990; Boyd et al., 1993). In old mice, treatment with nadolol elevated the percentage of peripheral blood CD4⁺ and CD8⁺ T cells expressing the naive (CD44^{lo}) phenotype. This occurred without changes in the percentages of total peripheral blood CD4⁺ or CD8⁺ T cells (Madden and Felten, 2001). There are several mechanisms by which β -adrenoceptor blockade could increase peripheral blood naive T cells; one of these mechanisms is by an increased emigration of mature T cells from the thymus. Future studies will use more direct approaches to examine if mature thymocyte emigration into the peripheral blood is increased by chronic blockade of β -adrenoceptors.

These findings indicate that β -adrenoceptor blockade alters thymocyte differentiation in aged mice, and suggest that the age-associated increase in sympathetic innervation of the thymus observed in aged mice modulates thymocyte maturation. That the observed nadolol-mediated effects were restricted to old animals suggest that the SNS has a greater influence on thymocyte maturation in aged than in young adult animals. Finally, observations that experimentally altered sympathetic activity in the aged thymus impacts thymocyte differentiation and immune function has implications for improving immune function in elderly patients, who are immunocompromised. Pharmacological interventions that prevent sympathetic-to-thymocyte signaling via β -adrenoceptors could provide a novel way to improve immune functions by increasing naive T cell output and improving T cell reactivity to novel antigens in the elderly patient.

4 Spleen

4.1 Structure and Function of the Spleen

The young adult spleen is a filter concerned with clearing the blood of particulate matter and worn out or damaged red blood cells, and with immune defense against blood-borne pathogens. It also functions as a reserve for mature erythrocytes that can be recruited to the circulation in response to increased demands. The parenchyma of the spleen consists of white pulp and red pulp. The red pulp consists of blood vessels, which are of large caliber and irregularly shaped (venous sinuses) and of the reticular tissue that occupies the spaces between the sinusoids (cords of Billroth). The spleen has a connective tissue capsule with inward extensions (trabeculae). The capsule and trabeculae are continuous with the reticular network that forms the interior of the organ and holds in its framework the free cells of the spleen. The red color of the red pulp is due to the large numbers of erythrocytes that reside in the lumen of the sinuses and infiltrate the cords of Billroth. Besides reticular cells and erythrocytes, fixed and wandering macrophages, monocytes, lymphocytes, plasma cells, platelets, and granulocytes also reside in the red pulp.

The white pulp contains lymphatic tissue organized in sheaths (periarteriolar lymphatic sheath, PALS) around central arteries and lymphoid nodules appended to the sheaths called follicles. The PALS is composed of lymphoid cells, primarily T lymphocytes, while follicles consist primarily of B lymphocytes. In the PALS, antigen-presenting cells process blood-borne antigens and present them to T cells. Antigen-specific T and B cells interact in the PALS to initiate an immune response. Adjacent to the PALS is a zone consisting of sinuses and loose lymphoid tissue called the marginal zone. In this zone, macrophages showing active phagocytosis are abundant, while lymphocytes in this zone are scant. The marginal zone harbors blood-borne antigens, and thus plays a major role in the immunologic activity of the spleen. Macrophages in the marginal zone produce cytokines that regulate T helper (T_H) cell development toward

either T_H1 or T_H2 cells, a process that pushes the response toward a cellular or humoral-mediated acquired immune response, respectively. The marginal zone also is the site of entry for T and B cells into the spleen before becoming segregated in their specific splenic compartment.

4.2 Sympathetic Innervation of the Spleen

Sympathetic innervation of the rodent spleen has been studied extensively (reviewed in Bellinger et al., 2001b). NA nerves travel along the splenic artery and enter the spleen as a dense vascular plexus at the hilus. These nerves continue to travel along the splenic arterial and venous branches under the capsule and along the trabeculae. From the vascular plexuses in the trabeculae, NA nerves continue to travel with the vasculature as the blood vessels branch to form the central arterioles, which are surrounded by white pulp. In the white pulp, NA nerves branch away from the central arterial plexus and enter into the PALS. Studies using double-label immunocytochemistry for tyrosine hydroxylase (TH) and pan T lymphocyte markers demonstrate there is a close association between NA nerves and T lymphocytes in this compartment. Similarly, NA nerve fibers extend into the marginal zone where they course along blood vessels or are observed as individual fibers adjacent to macrophages, and T and B lymphocytes. Direct contacts have been observed between lymphocytes in the PALS and NA nerves using electron microscopy. The intervening extracellular space between these direct contacts is about 6 nm, a smaller synapse than observed between NA nerves and more traditional targets such as smooth muscle cells within the vasculature. In contrast to the PALS and marginal zones, only an occasional sympathetic nerve fiber is typically seen within the B cell follicles of the white pulp.

In the young adult spleen, nerves containing neurotransmitters other than NE have been demonstrated using immunocytochemistry. For example, NPY has been observed to colocalize with NE as demonstrated by the loss of NPY-containing nerves after chemical sympathectomy with 6-OHDA (Romano et al., 1991). Neuropeptide-containing nerves also supply the rodent spleen. The peptidergic nerves innervating the spleen include substance P, CGRP, met-enkephalin, VIP, cholecystokinin, neurotensin, IL-1, CRH, and arginine-vasopressin (Bellinger et al., 2001b). These neuropeptide-containing nerves do not colocalize with NA nerves based on the difference in density and distribution from that observed for splenic NA nerves.

4.3 Sympathetic Innervation in the Aging Spleen

In contrast to the dramatic age-related increase in the density of sympathetic NA innervation in the thymus, splenic NA innervation is diminished markedly in middle-aged to old (>17-month-old) F344 rats (Bellinger et al., 1987, 1992; Felten et al., 1987b). With increasing age there is a progressive loss in sympathetic innervation in all compartments of the spleen (approximately 75% lower than in young adult rats) and a 50% reduction in splenic NE content by 27 months of age (Bellinger et al., 1987; Felten et al., 1987b). The reduction in NA innervation in the spleen with age is due to degeneration of NA nerves, and ultimately to the loss of neuronal cell bodies in the superior mesenteric/cealic ganglionic complex, the source of sympathetic innervation to the spleen in rodents (Santer et al., 1980; personal observations DL. Bellinger, D. Lorton). The fact that nerve density is reduced by 75%, while NE concentrations in the spleen are reduced by only 50%, reflects an increase in NE turnover in the remaining age-resistant NA nerves in the spleen (Bellinger et al., 2001c). These age-resistant nerves are observed in increased density near the hilus with a striking depletion of nerve fibers with increasing distance from the hilus compared with spleens from young adult rodents. This pattern of nerve loss resembles a peripheral neuropathy, whereby the terminal fields die back and then are capable of sprouting back into the target tissue after damage or upon removal of the injurious environment and is suggestive of a metabolic insult.

Loss of NA nerves visualized by histochemical or immunocytochemical staining may result from an actual loss of these NA nerve fibers or from an inability of NA nerves still present to synthesize enough NE or TH to be detected by these methods. To resolve this issue, α -methylnorepinephrine (α MNE), a compound that is taken up into sympathetic nerve terminals by high-affinity carriers, was administered to aged rats (Bellinger et al., 1993, 2001c). This drug, after being transported into the NA nerve terminal,

persists because it cannot be metabolized by monoamine oxidase. Further, it can be visualized using histochemical methods. In aged rats, α MNE treatment did not reveal additional sympathetic nerve fibers in spleens compared with vehicle-treated aged rats. However, additional studies comparing NE uptake in spleen slices from 3- and 21-month-old rats indicate that [^{3} H]NE uptake remains functional in old animals, and in fact is enhanced (Bellinger et al., 1993, 2001c), suggesting a compensatory hyperactivity in the surviving NA nerve fibers. Together these studies indicate that there is a loss of NA nerves in the aged spleen.

Recent studies from our laboratory indicate that splenic NE turnover rates in aged F344 rats are increased at 10, 12, and 15 months and decreased at 24 months of age compared with 8-month-old rats (D.L. Bellinger, unpublished data). NE turnover time in spleens from F344 rats was slightly reduced between 10 and 15 months of age, but significantly increased in 24-month-old rats compared with 8-month-old rats (D.L. Bellinger, unpublished data). These data support the anatomical data and suggest that during middle age, NA nerves in the spleen compensate for reduced innervation by increasing sympathetic activity in the remaining nerve fibers. Heightened sympathetic activity in the age-resistant nerves in spleens from aged F344 rats is consistent with evidence for an overall elevation in sympathetic tone in humans with advancing age (Ziegler et al., 1976; Lake et al., 1977; Esler et al., 1981; Krall et al., 1981). While uptake of NE from the circulation into splenic NA nerve terminals could help explain the elevated splenic NE concentrations despite the loss of NA nerve terminals during middle age, this does not appear to be the case since we did not observe any age-related differences in plasma NE concentrations between middle age and young rats (D.L. Bellinger unpublished data). These findings suggest that increases in NE utilization in middle age rats are due to changes in sympathetic nerve activity and/or rate of NE metabolism that occur in the splenic NA nerves.

Species and strain differences have been observed with regard to sympathetic NA innervation of the spleen in aged animals (Madden et al., 1997; Bellinger et al., 2001c, 2002). In aged BALB/c and C57BL/6 mice, we observed an increase in the density of splenic NA nerves in the parenchyma of the white pulp, especially near the hilus compared with young adult mice using histochemical methods (Madden et al., 1997; Bellinger et al., 2001c). Sympathetic innervation of the white pulp was variable in areas of the spleen that were more distal to the hilus. Some white pulp regions contained a normal density of NA nerves, while others were depleted. The variable decrease in density of NA nerves in white pulps distal to the hilus, in parallel with increased nerve density at the hilus, suggests there may be some loss of nerves in distal white pulps with compensatory sprouting of NA nerves into the hilus. Splenic NE concentrations were slightly elevated in aged mice, which raises the possibility that NE metabolism is enhanced in these aged mouse strains. Strain differences in sympathetic innervation of the rat spleen with age have also been observed. For example, the density of sympathetic innervation of spleens from aged Brown Norway and Brown Norway $\times$ F344 (F1) rats was equivalent to that of young adults. In contrast, there is a striking age-related loss of NA nerves in spleens from Lewis rats that occur with advancing age (Bellinger et al., 2002). These observations indicate that, unlike the thymus, age-related changes in splenic sympathetic innervation may be dependent on undefined genetic influences. Whether the different patterns of age-related changes in sympathetic innervation of the spleen observed in rodents occur in humans and whether different patterns are indicative of differences in "successful" or "unsuccessful" aging with regard to immunocompetence needs to be investigated.

We have begun to investigate the mechanisms involved in the age-related decrease in sympathetic innervation that occurs in spleens from F344 rats. The integrity of NA nerves in target tissues critically depends on the level of trophic support provided by target and/or stromal cells in the microenvironment and on antioxidative enzyme systems that provide protection from reactive oxygen species. One possible mechanism is that loss of NA innervation in the spleen is related to long-term exposure to antigens, which leads to repeated increases in NE turnover (Besedovsky et al., 1979; Elfvin et al., 1993). During a primary immune response, NE turnover is increased, an effect that results in the production of NE and oxidative metabolites that generate free radicals (Besedovsky et al., 1979; Fuchs et al., 1988). The continuing production of oxidative metabolites following NE release and metabolism over time may lead to subsequent auto-destruction of NA nerve terminals with age (reviewed in Felten et al., 1992). This hypothesis is supported by observations that in young adult rodents treated chronically with NE, there is a striking loss of NA nerves supplying the spleen (Felten et al., 1987a). However, when aging F344 rats were repeatedly immunized with lipopolysaccharide from 12 to 17 months of age, our laboratory did not observe a

significant acceleration in the age-related decline in splenic NA innervation (D.L. Bellinger unpublished data). This finding does not support the hypothesis; however, while lipopolysaccharide has inflammatory and mitogenic actions, it is not T cell dependent antigen challenge. To further explore this hypothesis, similar studies should be completed using antigens that require prolonged T cell activation or repeated exposure to superantigens. Reduced production of neurotrophic factors, such as nerve growth factor (NGF) and neurotrophin-3, and/or diminished capacity of oxidative stress systems in the aged spleen also may contribute to the loss of splenic NA innervation.

The hypothesis that the age-related reduction in splenic NA innervation is the result of an antigen-driven response is being tested further in our laboratory by examining the possibility that cytokines produced during an immune response can locally regulate sympathetic neurotransmission. These studies have revealed that ganglion cells in the superior mesenteric/celiac complex can synthesize IL-2 receptors (Bellinger et al., 2001b). David Snyder has demonstrated that IL-2 can elevate release of NE from synaptosomal preparations (NA nerve terminals) from the spleen, supporting that IL-2 receptors are present on NA nerve terminals and that these receptors can modulate NE release from sympathetic nerve terminals (D.L. Bellinger personal communication). Collectively, these observations support that IL-2 produced locally during an immune response may regulate release of NE from nerve terminals in the spleen. Further studies are needed that explore age-associated changes in IL-2 production and IL-2 receptor expression in vivo, the effects of IL-2 on NE release, and any alterations in IL-2 signaling of NA nerves with age.

4.4 Can Age-Related Changes in NA Innervation Be Reversed?

ThyagaRajan and coworkers (1998a) examined whether the loss of NA innervation in the aged F344 rat spleen was reversible by chronic treatment with the irreversible and selective monoamine oxidase B (MAO-B) inhibitor, L-deprenyl. L-deprenyl was selected as a potential drug to prevent the age-associated loss in sympathetic innervation in the spleen of F344 rats because of its neuroprotective properties for catecholamine neurons. The mechanisms by which L-deprenyl exerts its protective effects on catecholaminergic neurons are not entirely clear. L-deprenyl has been demonstrated to: (1) slow Parkinson's disease progression by improving nigrostriatal dopaminergic neurotransmission (Birkmayer et al., 1985; Tetrad and Langston, 1989); (2) enhance regrowth of transected axons of facial motoneurons at postnatal day 14 (Ansari et al., 1993); (3) improve rat ganglion cell survival after damage to optic nerves (Buys et al., 1995); and (4) increase neurotrophic factor production and increase the activity of superoxide dismutase (SOD) and catalase (Ansari et al., 1993; Seniuk et al., 1994). SOD and catalase are principal scavenging enzymes that protect neurons from damage caused by oxidative stress. Thus, neuroprotective effects of L-deprenyl could occur via multiple mechanisms to maintain sympathetic innervation and promote sympathetic nerve growth into target organs.

Treatment of 21-month-old male F344 rats with L-deprenyl for 9 weeks partially restored NA innervation in the old rat spleen (ThyagaRajan et al., 1998a). The restoration of sympathetic splenic innervation in aged F344 rats induced by L-deprenyl was accompanied by an increase in splenic NE concentration (per mg wet weight) and NE content (per whole spleen). However, the NE levels in treated old rats did not reach the concentration measured in young rat spleens (ThyagaRajan et al., 1998a). That the increase in splenic NA innervation and NE levels in L-deprenyl-treated old rats was of functional significance was confirmed by examining natural killer cell activity and concanavalin A (Con A)-induced IL-2 production to determine if L-deprenyl-treated old rats can reverse age-related decreases in these immune responses. The age-related decline in splenic natural killer cell activity and Con A-induced IL-2 production was partially reversed by deprenyl treatment of aged rats (ThyagaRajan et al., 1998b). These findings indicate that in old animals the NA nerves have retained plasticity responses and support that the age-related reduction in sympathetic innervation is reversible and can be partially restored to improve immune functions with age. They also suggest that manipulation of sympathetic innervation may be a unique way to counteract some parameters of age-associated decline in immune function. This notion is consistent with studies demonstrating that long-term L-deprenyl treatment reduced spontaneous mammary tumor growth in old rats (ThyagaRajan et al., 2000). While L-deprenyl effects on tumor growth are probably mediated via multiple mechanisms,

it is likely that one of these mechanisms is through deprenyl-induced sympathetically mediated changes in tumor immunity. Further research will be needed to confirm this hypothesis.

Double-label immunocytochemistry studies have revealed that the reduced splenic NA innervation observed in old rats is accompanied by changes in the density of specific immune cell populations in the spleens of old F344 rats. We found a gradual reduction in the density of T lymphocytes in the PALS and ED3⁺ macrophages in the marginal zone of the F344 rat spleen with increasing age, which occurred in parallel with the loss of sympathetic nerve fibers (Bellinger et al., 1992). The most striking loss of cells in these compartments occurred in regions distal to the hilus, paralleling the age-related reduced density of NA nerves with increasing distance from the nerve entry points in the hilus. It is interesting to speculate that NA nerve loss is causally related to the reduced cell density of T lymphocytes and macrophages in the spleen; however, this is difficult to prove. Lymphocytes and macrophages in the spleen are sympathetic target cells and, in the case of lymphocytes, synthesize NGF mRNA and protein (Santambrogio et al., 1994; Barouch et al., 2000), while macrophages can sequester and release NGF, in the absence of mRNA (Carlson et al., 1995). NGF and other neurotrophins are essential for sympathetic nerve survival in young animals (Yankner and Shooter, 1982; Levi-Montalcini, 1987), but neurotrophin production, signaling capacity, and functional significance have not been extensively studied in aged rodents. Despite the production of NGF by lymphocytes, some studies do not support a neurotrophic role for lymphocytes in maintenance of sympathetic innervation of the spleen. In the spleen and thymus of severe combined immunodeficiency (SCID) mice, which are deficient in T and B lymphocytes, sympathetic nerve distribution is robust in young adults (Mitchell et al., 1997; S.Y. Stevens, unpublished data). Further, in young adult mice, depletion of lymphocytes after acute treatment with hydrocortisone or cyclophosphamide did not affect the density of splenic NA nerves (Carlson et al., 1987). These findings suggest that lymphocytes may not provide the critical neurotrophic support required for maintenance of sympathetic innervation in the spleen in these mice. However, there is data to support a role for marginal zone macrophages in providing neurotrophic support for NA nerves in the spleen. In transgenic mice that overexpress NGF in the skin and other epithelial structures, macrophages in the splenic marginal zone possess increased NGF intracellular levels. This zone was hyperinnervated in the NGF transgenic mice compared with control mice (Carlson et al., 1995), suggesting that macrophages provide neurotrophic support for NA nerves in the spleen. Further, with increasing age, NA nerves may become more vulnerable to changes in the percentages of macrophage and lymphocyte subsets present in the aged spleen and in the capacity of these cells to provide neurotrophic support.

4.5 Age-Related Changes in the Spleen and Immune Function

With advancing age, cell-mediated immunity is more impaired than humoral-mediated immunity or antibody responses (reviewed in Pawelec et al., 2002). Despite the decreased T cell reactivity toward foreign antigens, there is an age-related increase in responses to autoantigens. Impairment of antigen- and mitogen-induced proliferation, cytotoxic T cell effector function, natural killer cell activity, cytokine production, IL-2 receptor expression, and signal transduction are frequently reported to occur in old T cells (reviewed in Fulop, 1994). The aging process impacts many of the steps required for T cell maturation and activation. In general, IL-2 production is impaired in aging, an effect that dampens clonal expansion of T lymphocytes in response to mitogen and antigen challenge (reviewed in Pahlavani and Richardson, 1996). There is a dysregulation of IL-4 and interferon- γ (IFN- γ) production by T lymphocytes in aging animals and in humans; however, the direction of changes reported is variable (reviewed in Caruso et al., 1996; Rink et al., 1998). The variability in age-related changes in IL-4 and IFN- γ can be attributed to species differences, the stimulating agent used in vitro, variability in the in vitro culture conditions, and the source of lymphocytes employed between laboratories. IL-4 and IFN- γ are critical cytokines for regulation of humoral- and cell-mediated immune responses, respectively. They also are crucial in antimicrobial and antineoplastic defenses in the elderly. With advancing age, there is an increase in cytokine production by T cells in mice (Hobbs et al., 1993, 1994; Caruso et al., 1996) and humans (Castle et al., 1997), such as IL-5 (Hobbs et al., 1993; Caruso et al., 1996) and IL-10, that suggest a possible shift in aging toward humoral-mediated immune responses (reviewed in Hobbs et al., 1993, 1994).

With advancing age, there also is a shift in the immune cell populations present in lymphoid compartments in secondary lymphoid organs and the peripheral blood. For example, there is an increase in the $CD4^+/CD8^+$ ratios in human peripheral blood and in the murine spleen, and a decrease in $CD4^+/CD8^+$ ratios in murine lymph nodes and peripheral blood (reviewed in Mackall and Gress, 1997). The $CD4^+$ and $CD8^+$ populations contribute differently to the host immune defense, and thus any change in their ratio can influence a host's immune response to immune challenge. Changes in the ratio of subsets of $CD8^+$ lymphocytes that can impact immune function also occur with aging. The findings of an increase in $CD8^+ CD28^-$ cells and reduction in $CD8^+ CD28^+$ cells is consistent with the age-associated decrease in proliferative ability in aged humans (Merino et al., 1998) and hyporesponsiveness to costimulation by $CD28$ in T cells from aged mice (Engwerda et al., 1994). The decrease in proliferative responses are particularly prominent in the $CD8^+$ population (Merino et al., 1998). With aging, there is a shift toward T-memory lymphocytes, indicated by an increase in the ratio of T memory to naive T cells that occurs in lymphoid organs and the peripheral blood in humans and animals (Ernst et al., 1990; Utsuyama et al., 1992; Jackola et al., 1994). This shift toward T-memory lymphocytes contributes to the reduction in antigen-induced lymphocyte proliferation and the altered patterns of cytokine production observed with age (Hobbs et al., 1993, 1994).

4.6 Functional Studies and Significance

Many experimental approaches have been used to explore SNS regulation of immune functions in young adult rodents. Use of sympathetic nerve-targeting toxins, such as 6-OHDA, to produce a chemical sympathectomy, and specific β -adrenergic antagonists to block β -adrenergic receptor activation before immunization, reduced the T_H2 -driven antibody response and decreased the delayed-type hypersensitivity reaction to a contact-sensitizing agent (Madden et al., 1989; Kohm and Sanders, 1999). Transgenic mice that lack DBH, *dbh*^{-/-}, have decreased immune reactivity to infectious agents and protein antigen challenge (Alaniz et al., 1999). These studies are in contrast to the generalized immunosuppressive role attributed to the SNS with regard to immune functions. Similarly, Dhabhar and McEwen (1999) demonstrated that in vivo administration of epinephrine prior to antigen sensitization enhanced the delayed-type hypersensitivity response and increased cellularity in the draining lymph nodes. Collectively, these findings indicate that the SNS can have an augmenting effect on immune reactivity.

In contrast to these immune-enhancing effects of the SNS, stressors or agents that elevate sympathetic activity also have been shown to reduce T cell responses, antiviral immune reactivity, and natural killer cell activity. These effects were blocked by pretreatment with β -adrenergic receptor antagonists or ganglionic blockade (Irwin et al., 1988; Cunnick et al., 1990; Dobbs et al., 1993; Fecho et al., 1996). In mice, chemical sympathectomy with 6-OHDA enhanced antigen-induced proliferation and T_H1 and T_H2 cytokine production (Kruszewska et al., 1995, 1998). Sympathetic denervation with 6-OHDA prior to intraperitoneal immunization with a protein antigen also elevated serum antibody levels in rats and in C57Bl/6 mice, but not BALB/c mice (Madden et al., 1995; Kruszewska et al., 1998). A number of important factors contribute to differences in SNS effects on immune reactivity that are reported in the literature. The outcome of sympathetic manipulation on immune function is dependent upon genetic background, site of immunization, and the types of immune cells involved in the immune response (i.e., antigen-presenting cell type, type of T_H cell). Studies using purified cell populations in cell culture models have begun to elucidate some of the mechanisms underlying the sympathetic modulation of immune functions observed in in vivo and ex vivo experiments.

Stimulation of β -adrenergic receptors increased alloreactive T lymphocyte cytotoxicity and antibody-secreting cells in unfractionated effector cells in vitro (Sanders and Munson, 1984; Hatfield et al., 1986). Highly purified lymphocyte populations recently have been used to determine mechanisms of altered T and B cell responses following β -adrenergic receptor stimulation. Mixed cultures containing antigen-specific B cells and T_H cell clones have been used to determine mechanisms by which β -adrenergic receptor agonists alter antigen presentation and synthesis (Sanders et al., 1997). Addition of β -adrenergic agonists to this mixed culture after antigen challenge elevated antibody production (Sanders et al., 1997) by upregulating B cell accessory molecule expression and by enhancing B cell responsiveness to IL-4 (Kasprowicz et al.,

2000). In contrast, addition of β -agonists prior to antigen-induced activation of T_H1 cell clones decreased the number of antibody-forming cells and lowered IFN- γ production (Sanders et al., 1997), suggesting that β -adrenergic receptor stimulation inhibits T_H1 responses. However, T_H cell clones also exhibited differential responsiveness to β -adrenergic receptor stimulation that was dependent upon the activating stimulus and the type of T_H cell cultured (i.e., T_H1 clone versus naive $CD4^+$ cells that were grown in T_H1 -promoting conditions) (Ramer-Quinn et al., 1997; Swanson et al., 2001). Collectively, these studies indicate that the timing relative to immune challenge, the type of immune cells involved, and the immune cell's activational or maturational state contribute to the complexity of sympathetically mediated modulation of immune functions. The complexity likely reflects the important role of the SNS in "fine tuning" immune responses to maintain homeostasis.

Changes in sympathetic innervation of lymphoid organs predict that sympathetic modulation of immune functions are altered in aged F344 rats, and may be an important model to explore the mechanisms by which immune functions are compromised in the elderly population. We have used this model to determine if the reduced sympathetic innervation of lymphoid tissue in the aged spleen is sufficient to maintain functional links with the immune system given the SNS's ability to often compensate for lost innervation of target tissues. We also have explored the possibility that a causal relationship exists between the age-associated reduction in splenic sympathetic innervation and age-associated impairment in T cell proliferation. To explore these possibilities, young (3-month-old) and old (17-month-old) F344 rats were chemically sympathectomized, and the functional impact on ex vivo T cell responses in naive animals and antigen-specific antibody responses in vivo was assessed (Madden et al., 1995, 2000; Bellinger et al., 2001c). Young rats were remarkably resistant to sympathectomy-induced changes in T lymphocyte proliferation and cytokine production. In contrast, splenic T cell activity in aged rats was reduced when sympathetic innervation of the spleen was further depleted. Age-associated impairments in Con A-induced proliferation were augmented in sympathectomized old animals compared with vehicle-treated old animals (Madden et al., 2000). In immune-challenged rats, no differences were observed in serum IgM and IgG anti-KLH antibody responses between untreated 3- and 17-month-old rats. In both young and old rats, sympathectomy increased anti-KLH IgG, IgG1, IgG2b, and IgM antibody responses. In contrast, KLH-specific proliferation of splenocytes was enhanced in old but not young F344 rats (Madden et al., 1995; Bellinger et al., 2001c).

If spleen cells in the aged rats were no longer receiving signals from sympathetic nerve terminals, then removal of remaining NA innervation by sympathectomy should have no impact on immune reactivity. These results indicate that the SNS retains the ability to modulate immune reactivity in aged F344 rats despite the reduction of splenic sympathetic innervation. The reduction in Con A-induced T cell proliferation after sympathectomy suggests that splenic NA innervation in old animals, although diminished, can still exert a positive regulatory role on T lymphocyte functions. The increase in antibody responses after sympathectomy in both young and old rats supports an inhibitory role for splenic NA innervation in modulating humoral immune responses in both young and old F344 rats, despite the reduced sympathetic innervation of splenic lymphoid tissue in the aged rats.

The differences in immune function observed after sympathectomy between young and old rats indicate that old animals are more susceptible to complete loss of sympathetic innervation than young animals. Perhaps, this occurs because compensatory mechanisms become limited in aged animals, which already have lost sympathetic innervation, while young rats rapidly evoke compensatory mechanisms to maintain T lymphocyte activity following NE depletion. The maintenance of sympathetic signaling to splenic immunocytes in the face of an age-associated loss in splenic NE levels suggests there is an upregulation of compensatory mechanisms in intact aged rats. This notion is supported by evidence of an increased sensitivity in synaptic signaling in response to available NE. These compensatory mechanisms include: increased spleen cell β -adrenergic receptor density (Ackerman et al., 1991; Bellinger et al., 2001c), augmented β -adrenergic receptor-induced intracellular cAMP signaling (D.L. Bellinger, unpublished data), and elevated NE reuptake and turnover in the remaining age-resistant NA nerves in the aged F344 rat spleen (Ackerman et al., 1991; Bellinger et al., 2001c). However, the findings presented above suggest that the compensatory capacity of sympathetic signaling is approaching its maximum in the old rat, and further compensation in response to sympathectomy may be limited. In contrast, the young animals, in which splenic sympathetic innervation has not been compromised, compensate more rapidly and efficiently for

the loss of NA nerves induced by 6-OHDA. This may negate or at least limit the impact of sympathetic nerve destruction on immune reactivity.

Kruszewska and coworkers (1995) observed a similar sympathectomy-induced increase in anti-KLH IgG₁ and IgG₂ antibody production in C57Bl/6 mice, a T_H1-predominant strain. However, no significant sympathectomy-induced alterations were observed for serum anti-KLH antibody responses in BALB/c mice, a T_H2-predominant strain. Both murine strains had enhanced antigen-induced T_H1 and T_H2 cytokine production *in vitro* after sympathectomy compared with vehicle-treated mice, providing no support for a T_H1 versus T_H2 dominant cytokine production in these strains (Kruszewska et al., 1995, 1998). We have obtained no evidence of a dominance of one T_H response compared with the other based on IgG isotypes in F344 rats. Unfortunately, splenocytes from KLH-immunized rats, which are immunized in the absence of adjuvant, do not synthesize and release detectable levels of cytokines in response to KLH *in vitro*; thus, we were unable to examine cytokine production in the F344 rats (D.L. Bellinger, unpublished observations). Our findings of elevated serum antibody levels in sympathectomized young and old F344 rats is directionally opposite to the sympathectomy-induced decreases in cell-mediated and antibody responses in young mice reported in other studies (Livnat et al., 1987; Madden et al., 1989). Similarly, elevated serum antibody concentrations in 6-OHDA-treated young and old F344 rats is not consistent with reports of suppressed antibody responses in SCID mice reconstituted with antigen-specific T_H2 cell lines and antigen-specific B cells (Kohm and Sanders, 1999). In the latter study, this effect was partially reversed by treatment with NE.

The inconsistent findings observed in immune responses between sympathectomized F344 rats and mice may be due in part to the fact that, in contrast to the F344 rats, sympathetic innervation is relatively well-maintained in old mice. To investigate this possibility, young and old mice were chemically sympathectomized and immunized with KLH (Bellinger et al., 2001c). In contrast to F344 rats, old mice not treated with 6-OHDA had reduced anti-KLH antibody responses compared with young mice. In young mice, sympathectomy decreased serum anti-KLH antibody titers (IgM, IgG, IgG₁, and IgG_{2A}), KLH-induced proliferation, and increased IL-2, IL-4, and IFN- γ production by KLH-stimulated splenocytes six days after KLH immunization. While the cytokine production findings are similar to the effects of sympathectomy in mice reported by Kruszewska and coworkers (1995), the decrease in serum anti-KLH antibody titers and KLH-induced proliferation was not consistent with their previous reports using C57Bl/6 mice (Kruszewska et al., 1995, 1998). The different findings between these studies may, in part, be due to differences between housing conditions used in the two studies (single versus group housed). The lack of sympathectomy-induced changes in immune reactivity to KLH in aged mice is consistent with preliminary data from our laboratory showing a striking attenuation of cAMP production in splenocytes stimulated with isoproterenol. An altered ability of peripheral blood lymphocytes from elderly subjects to respond to NE has been reported by other investigators (Feldman et al., 1984; O'Hara et al., 1985). The altered responsiveness to NE in these studies was due to a defect in β -adrenergic receptor coupling with adenylate cyclase or the catalytic capacity of adenylate cyclase (Krall et al., 1981; Doyle et al., 1982; Scarpace and Abrass, 1983). These findings support an age-related inability of the SNS to signal immune cells in these strains of aged mice and in elderly humans. It is unclear if this is an adaptive and/or protective compensatory mechanism in response to the age-related increase in sympathetic activity and requires further investigation.

Our findings indicate two patterns of aging for sympathetic innervation and signaling to immunocytes in the spleen. In the spleen of aged F344 rats, NA nerves are markedly reduced in density, but yet the capacity to respond to sympathectomy is intact and is associated with an intact β -adrenergic receptor signaling capacity within immune cells. In the two strains of aged mice, in which splenic NA innervation is largely intact, NE released from NA nerves fails to activate β -adrenergic receptor intracellular signaling pathways in splenocytes from these mice (Madden et al., 1997; Bellinger et al., 2001c). The biological relevance of variations in SNS-immunocyte signaling between aged mice and F344 rats is not known. Further, it is not known whether similar differences exist in aging humans, or whether they contribute to individual or racial differences in the ability to successfully handle immune challenges in the elderly. Such differences in sympathetic modulation of immune reactivity may explain, or be an influential factor in, differences that occur with age in human populations, for example, in response to vaccination (Webster, 2000) and the susceptibility to infectious diseases, cancer, and autoimmune disorders.

5 Summary and Closing Remarks

NA sympathetic innervation is an important component of the microenvironment of lymphoid organs; maintenance of these nerves relies on the secretion of neurotrophins and other neuroprotective mechanisms. The dramatic changes in sympathetic innervation of lymphoid organs with age predict a change in the internal milieu of lymphoid organs occurs with advancing age, which will impact regulation of immune functions. The SNS's role in immune modulation is complex, affecting numerous immune functions, including cytokine production, effector functions, cellular trafficking, immunocyte activation, and clonal expansion. SNS signaling via NE release and interaction with adrenergic receptors on immunocytes leads to a variety of complicated responses. As a modulatory neurotransmitter, it differentially influences multiple cell types at multiple stages during the immune response depending on the timing relative to immune challenge, the activation state of the target immune cells, the site of infection, and the type of immune challenge. Determining the nature and timing of complex neural-immune interactions is one of the major challenges in this field of study.

Findings from our laboratory suggest that the SNS in some strains of rodents may exert a greater influence on immune responses when the host is immunocompromised, as in aging, perhaps because of dampened or ineffective feedback mechanisms and/or readjusted homeostatic set points in aging. Since the SNS is critical for host immune competence, the altered sympathetic activity in lymphoid organs that occurs with aging may put the elderly at increased risk for diseases associated with reducing immune competence, including infectious diseases, autoimmunity, and cancer. This may be especially true under stressful life events in which the elderly have a reduced ability to cope. Investigating the temporal relationship between age-related changes in immune function and NA innervation of lymphoid organs may provide some insight into these issues. Such research may lead us to ways to reduce the risk of developing cancer and autoimmune disorders, improve the effectiveness of vaccines, and improve the ability of the elderly to handle infectious diseases. Future research in this area also may lead to the development of behavior-based therapies that either reduce stress or improve coping strategies in order to prevent disease or improve disease outcome.

The association between sympathetic dysfunction and immune senescence in aging suggests that adrenergic agents alone and in combination with other treatments could be useful for improving immune responsiveness to vaccines and be effective in preventing or treating some age-related illnesses. This would have the advantage of reducing or eliminating treatment side effects, to which the elderly are more sensitive. However, before such strategies can be developed further, research is necessary to better understand the SNS's role in immune modulation in the elderly. It will be important in these studies to examine strain and species differences, as these may lead to a predisposition of certain strains to particular types of illnesses whose frequency increases with advancing age. Clearly, aging in humans is highly variable. Understanding the factors that contribute to "successful" and "unsuccessful" aging will provide insight to reduce the risk of developing diseases that are associated with aging and perhaps improve treatments for these disorders.

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5 **Drugs that Target Sympathetic–Immune Pathways for Treatment of Autoimmune Diseases**

D. Lorton · C. Lubahn · D. Bellinger

1	<i>Introduction</i>	85
2	<i>General Concepts of Neural–Immune Interactions: SNS, Stress, and the Stress Response</i>	86
3	<i>SNS–Immune Cell Signaling</i>	87
3.1	Sites of Sympathetic Nerve–Immune Cell Signaling	87
3.2	Effects of the SNS on Immune Responses	87
3.2.1	Inhibition of Lymphocyte Responsiveness	89
3.2.2	Enhancement of Lymphocyte Responses	89
3.2.3	Macrophage and Natural Killer Cell Functions	90
3.2.4	Modulation of Trafficking of Immune Cells	90
3.3	SNS and Immune Cell Cytokine and Monokine Production	91
3.3.1	Catecholamine Modulation of Cytokine and Monokine Production	91
3.3.2	Anti-Inflammatory Effects of Catecholamines	93
3.3.3	Pro-Inflammatory Effects of Catecholamines	94
3.4	In Vivo Effects of the SNS on Immune Functions	94
4	<i>Dysregulation of the SNS in RA and Animal Models of RA</i>	95
4.1	Inflammatory Cytokines Induce Changes in SNS Activity	96
4.2	SNS Innervation and Immune Cell Reorganization in Lymphoid Organs of AA	98
4.3	Parallel Redistribution of Sympathetic Nerves and Immunocytes in Lymphoid Organs	99
4.4	Potential Mechanisms of Sympathetic Nerve and Immunocyte Reorganization	100
5	<i>SNS Intervention and Disease Outcome in Autoimmune Disease</i>	101
5.1	Sympathectomy in Arthritis Models—The Dual Role of the SNS Based on its Target	101
5.2	SNS Manipulation in Arthritis Models—Time-Dependent Effects	103
5.3	β -Antagonist Treatment and Disease Severity	104
5.4	Role of α -AR in Disease Development	104
6	<i>Changes in AR Expression with Disease Development</i>	104
7	<i>Is There a Relationship Between Immune Dysregulation and SNS Dysregulation?</i>	105
8	<i>Can Adrenergic Agents Restore Immune System Homeostasis in RA?</i>	107
8.1	Cytokine Imbalance in RA and Current Treatment Strategies	107

8.2	Anti-TNF- α Therapies	108
8.3	Potential Benefits of Sympathetic Drugs in Treating RA over Anti-TNF- α Drugs	109
8.3.1	The SNS Regulates TNF- α Production in AA	109
8.3.2	Potential Advantages of SNS Regulation of Immune Functions	110
9	Conclusion	111

Abstract: Autoimmune disorders often share two common characteristics, dysregulation of the immune system and the nervous system stress pathways, the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis. Dysregulation of these systems are likely to be functionally related, as there is bidirectional communication between the immune system and the central nervous system (CNS). The CNS modulates immune function by signaling target cells of the immune system through autonomic and neuroendocrine pathways. These immune cells relay information back to autonomic, limbic, and cortical areas of the CNS to affect neural activity and consequently modify behavior, hormone release, and autonomic function (Besedovsky and del Rey, 1996; Maier and Watkins, 1998). In this manner, immune cells function as a sense organ, informing the CNS of peripheral events relating to infection and injury. Equally important, homeostatic mechanisms are needed at all levels to turn off the immune response when the pathogen and injurious condition are eliminated and the repair process is completed. In individuals with rheumatoid arthritis (RA) and other autoimmune diseases, there is a failure of the homeostatic regulation leading to long-term immune activation that has serious health consequences.

This chapter summarizes changes that occur in SNS to immune signaling in autoimmune diseases using RA as a specific example and presenting similarities of other autoimmune disease. Evidence that the SNS can enhance or suppress inflammation and immune function, that SNS dysregulation is a critical component of the immune system dysregulation which drives RA pathology, and that the SNS may be targeted in RA to restore immune system homeostasis and prevent disease pathology, will be presented. The potential for using drugs that target the SNS to treat RA and other autoimmune diseases also will be explored.

List of Abbreviations: AA, adjuvant-induced arthritis; ACTH, adrenocorticotrophic hormone; ANS, autonomic nervous system; AR, adrenergic receptor; CFA, complete Freund's adjuvant; CIA, collagen-induced arthritis; CNS, central nervous system; Con A, concanavalin; CREAE, experimental allergic encephalomyelitis; CRH, corticotrophin releasing hormone; DTH, delayed-type hypersensitivity; EAE, experimental allergic encephalomyelitis; EAMG, experimental autoimmune myasthenia gravis; EPI, epinephrine; HPA, hypothalamic-pituitary-adrenal; IFN, Interferon; Ig, immunoglobulin; IL, Interleukin; KLH, keyhole limpet hemocyanin; LPS, lipopolysaccharide; MHPG, metabolite, 3-methoxy-4-hydroxy-phenylglycol; NA, noradrenergic; NE, norepinephrine; NGF, nerve growth factor; NK, natural killer; NPY, neuropeptide Y; PBMC, peripheral blood mononuclear leukocytes; PFC, plaque-forming cell; PNS, parasympathetic nervous system; PVN, paraventricular nucleus; RA, rheumatoid arthritis; RBC, red blood cells; SEB, staphylococcal enterotoxin; 6-OHDA, 6-hydroxydopamine; SNS, sympathetic Nervous System; SWC, streptococcal cell wall-induced arthritis; SympX, sympathectomy; TH, tyrosine hydroxylase; TNF, tumor necrosis factor

1 Introduction

The sympathetic nervous system (SNS) regulates immune system responses via the release of norepinephrine (NE) and epinephrine (EPI). These neurotransmitters bind to specific receptors on cells of the immune system, which in turn alter intracellular second messenger pathways in these cells. Functional studies indicate that the SNS, through release of NE and EPI, can exert significant effects on antigen- and mitogen-induced proliferation, antibody and cytokine production, natural killer (NK) cell activity and delayed-type hypersensitivity (DTH). The SNS also can either inhibit or promote inflammatory responses depending upon the adrenergic receptor (AR) subtypes expressed by specific immune target cells. This dual role of the SNS in modulating inflammation is dependent on the involved organs and tissue compartments, timing of distinct effector mechanisms, availability of respective ARs on target cells, and an intricate shift from β - to α -AR signaling during the inflammatory process. With chronic inflammation that occurs in rheumatoid arthritis (RA), SNS functions that modulate inflammation are altered due to changes in innervation patterns within immune organs and the affected joints (nerve fiber loss and/or sprouting). Further, SNS regulation of inflammation in RA is also altered due to changes in immunocyte AR expression, reorganization of immunocytes within target immune organs, and the altered activational state of large populations of immunocytes.

Altered sympathetic regulation of immune functions can significantly alter disease severity in animal models of inflammatory and autoimmune diseases, including adjuvant-induced arthritis (AA), a model of RA, and encephalitis allergic myelitis, a model of multiple sclerosis. In the case of AA, the direction of disease severity changes after manipulations of the SNS is opposite depending upon the disease stage in which the SNS was manipulated. In very early stages of inflammatory joint disease, the SNS plays a predominant proinflammatory role in the joint, largely through interaction with α -AR. However, in secondary lymphoid tissues at this disease stage, the SNS modulates development of the immune response that secondarily exerts an antiinflammatory effect in the chronic disease stage, largely through interaction with β_2 -AR. In contrast, in late stages of inflammatory joint disease, the SNS plays a predominant antiinflammatory role largely via β_2 -AR on cells of the immune system and a proinflammatory role via α -AR predominantly on activated macrophages and target tissues in the joint. Similarly, changes in sympathetic-to-immune signaling occur in other autoimmune diseases with inflammatory components such type 2 diabetes, lupus, and multiple sclerosis. Since patients with RA most often enter the clinic in the chronic phase of the disease, antiinflammatory (β_2 -AR-mediated) and inhibitory proinflammatory (α -AR-mediated) adrenergic drugs that target sympathetic pathways could be promising therapeutic options in treating this chronic inflammatory joint disease, as well as in other autoimmune diseases.

The objectives of this chapter are to review literature supporting neural-immune dysfunction in autoimmunity in general and RA specifically; to develop a unifying hypothesis to explain the mechanism through which the SNS regulates immune responses to antigens that initiate AA in a rat model based on data from our laboratories and from basic and clinical research; and to explore whether there is a great, and thus, far untapped potential of adrenergic therapies in the treatment of RA and perhaps other autoimmune diseases.

2 General Concepts of Neural-Immune Interactions: SNS, Stress, and the Stress Response

The SNS and the parasympathetic nervous system (PNS) make up the autonomic nervous system (ANS). In general, the SNS and PNS regulate the activities of structures that are not under voluntary control and which function below the level of consciousness. They control respiration, circulation, digestion, body temperature, metabolism, sweating, and secretions of some endocrine glands. Their functions are to regulate the constancy of the internal environment of the organism, referred to as homeostasis. The SNS is tonically active at all times. The degree of sympathetic activity varies over time and from organ to organ so that body functions are finely controlled and kept within the normal functioning range in a constantly changing environment. While the SNS and its associated adrenal medulla are not essential to normal life in the confines of a laboratory setting, under conditions of stress, the lack of sympathetic functions can become life threatening.

The activity of the SNS becomes elevated and can discharge as a unit under conditions of rage and fear. When this occurs, sympathetic nerves innervating structures over the entire body are simultaneously affected. SNS activation produces a characteristic set of physiological responses that include an accelerated heart rate, rise in blood pressure, increase in blood glucose levels, a shift in circulation away from skin and splanchnic bed to the skeletal muscles, and dilation of bronchioles and pupils. This prepares the organism for a “flight or fight” response and is referred to as the stress response (for review, see Chrousos and Gold, 1992).

Stressors activate the SNS resulting in an increase in outflow of noradrenergic (NA) neurons in the periphery. Activation of this pathway occurs through the paraventricular nucleus (PVN) of the hypothalamus. This hypothalamic nucleus is connected to NA areas of the brain stem, such as the locus coeruleus and A2 and C2 regions by several neuronal pathways (Sawchenko et al., 1996). A wide range of physical, psychological, or immune/inflammatory stimuli can activate the SNS.

From the 1980s onward, homeostasis of inflammatory and antigen-specific immune reactions has been added to the long list of body functions regulated by the SNS. Cells of the immune system are exposed to NE released from sympathetic nerves that reside within lymphoid organs (Micalizzi and Pals, 1979; Bellinger et al., 2001) or that are present in target tissue with immune cell infiltration. Under highly

stressful conditions, EPI and NE also are released from the adrenal medulla to elevate plasma catecholamines, which can interact with AR-expressing immune cells throughout the body. Additionally, NE released from nerves in target organs innervated by sympathetic nerves can spillover into the plasma if sympathetic activity is elevated in response to highly stressful stimuli.

Catecholamines released from sympathetic nerves modulate a variety of immune cell functions. These include cellular proliferation, cytokine and antibody production, lytic activity, and leukocyte homing and migration. Thus, the SNS is an important efferent immune modulator following exposure to stressors, often acting in concert with hypothalamic-pituitary-adrenal (HPA)-axis activation (Elenkov et al., 1996; Sheridan et al., 1998; Elenkov and Chrousos, 2002). As our knowledge of the underlying immunological mechanisms at work in autoimmune disorders has improved, more attention has been directed to understanding the links between the immune system and the SNS and HPA-axis pathways.

3 SNS-Immune Cell Signaling

3.1 Sites of Sympathetic Nerve-Immune Cell Signaling

Sympathetic nerve-to-immune cell signaling occurs in secondary lymphoid organs and at sites of inflammation. Even though sympathetic nerve communication with immune cells in the inflamed arthritic joints is important, sympathetic nerve-to-immune cell interactions that occur within the secondary lymphoid organs is also of equal significance. The role of different immune cell populations in RA pathology is initiated in the secondary lymphoid organs where antigens are processed, immune cells differentiate and become activated, and their homing patterns are established. Clearly, sympathetic nerves signal-specific immune cell populations in the secondary lymphoid organs that modulate immune functions to impact disease pathology just as sympathetic signaling to specific immune cell populations in the arthritic joints affect disease processes.

Early studies showing that the development of AA is dependent upon secondary lymphoid organs illustrate the importance of secondary lymphoid organs in the disease process. Development of AA can be prevented if lymph nodes that drain the site of complete Freund's adjuvant (CFA) challenge are removed within 5 days of the challenge (Newbould, 1964a, b). AA can be elicited by direct CFA injection into the inguinal lymph nodes (Newbould, 1964a, b; Azuma et al., 1972). Injection of rhodamine-labeled CFA at the base of the tail results in widespread localization of CFA (Vernon-Roberts et al., 1976), including the draining and peripheral lymph nodes, peritoneum, spleen, and lungs. Both lymph node and spleen cells obtained from AA rats can be used to transfer the disease to normal recipients (after concanavalin (Con)-A expansion) and to thymectomized, irradiated, bone marrow cell-reconstituted recipients (Taurog et al., 1983). These findings suggest that antigen processing of CFA occurs in the draining lymph nodes and other secondary lymphoid tissues. Further, chronic synovial infiltration with lymphocytes is dependent on the generation and release of activated precursor cells from peripheral lymph nodes, spleen, and gut-associated lymphoid tissue (Kelly and Harvey, 1978; Issekutz and Issekutz, 1991; Mikecz and Glant, 1994; Spargo et al., 1996; Holm et al., 2002). Similarly, ED3⁺ and ED1⁺ macrophages that originate from secondary lymphoid organs and bone marrow-derived peripheral blood monocytes outnumber the mature resident ED2⁺ macrophages in the inflamed joints (Dijkstra et al., 1985; Verschure et al., 1989; Carol et al., 2000). Thus, immune cells that drive chronic inflammation and joint destruction are activated and expanded within secondary lymphoid organs prior to migration into arthritic joints. Immunological and neural signals that these cells receive while residing in the lymphoid organs are critically important in determining their functions once they home into the joints. In this manner, sympathetic-to-immune cell signaling within secondary lymphoid organs play an important role in initiating and perpetuating the disease pathology.

3.2 Effects of the SNS on Immune Responses

Recently a linkage between the corticotropin-releasing hormone (CRH) gene, or a closely linked one, and the genetic etiology of RA was reported (Fife et al., 2000). Since CRH in the hypothalamus regulates both

glucocorticoid production and SNS activity, this study supports a role for a defective HPA axis and SNS in RA. While studies have focused on CRH in altered regulation of adrenocorticotrophic hormone (ACTH) and glucocorticoids in RA, the role of altered CRH in the dysregulation of the SNS in RA has been largely ignored. CRH responses in the PVN influence and regulate sympathetic tone (Sundar et al., 1990; Irwin et al., 1992; Friedman and Irwin, 1995). This would be expected to affect innate, humoral, and cellular immunity in response to the arthritogenic antigen and contribute to the development and progression of RA and AA. This section will focus on the modulatory role of the SNS on the immune system and explore how altered sympathetic activity before onset or with disease development affects disease pathology in RA.

Increases in local or systemic cytokine levels that occur following an immune system challenge or with disease pathology affects the activity of central nervous system (CNS) sites that regulate SNS outflow to peripheral tissues, including lymphoid organs. SNS outflow to target tissues from central autonomic pathways consists of a two-neuron chain. Preganglionic cholinergic neurons in the intermediolateral cell column of the spinal cord innervate postganglionic neurons. Primary and secondary lymphoid organs are innervated by postganglionic NA neurons. Within lymphoid organs NA innervation is regional and specific (reviewed in Bellinger et al., 2001). NA nerves supply the vasculature and continue into the parenchyma. In the parenchyma, NA nerves supply specific lymphoid compartments to interact with specific subsets of immune cells. NE is released from NA nerve terminals following activation of sympathetic nerves and is available to interact with immune cells either by direct contact (6 nm extracellular spaces) with lymphocytes and macrophages or by diffusion (paracrine effects) with immunocytes that are located short distances from the terminal.

SNS activation also results in release of EPI from the adrenal gland (reviewed in Bellinger et al., 2001). Preganglionic neurons arising from the intermediolateral cell column of the spinal cord also innervate adrenal medullary cells. These cells are embryologically analogous to postganglionic sympathetic neurons. EPI is a methyl derivative of NE and like NE can bind ARs to modulate cellular responses, albeit with differing affinities for each AR subtype.

Activation of the immune system by mitogens or antigens results in an increase in plasma catecholamine concentrations (Jones and Romano, 1989; Qi et al., 1991; Zhou and Jones, 1993). This triggers the activation of cerebral and peripheral catecholamine metabolism (Besedovsky et al., 1985; Berkenbosch et al., 1989; Shanks et al., 1994; Dunn, 2000). Following an immune challenge, the release of catecholamines is accompanied by the release and metabolism of adenosine and ATP (Barajas-Lopez and Huizinga, 1993; Hasko, 2001). Neuropeptide Y (NPY), colocalized within sympathetic terminals, is also released along with NE after an immune challenge (Romano et al., 1991; Zukowska et al., 2003; Bedoui et al., 2004).

SNS activation after an antigen challenge is mediated by proinflammatory cytokines that are produced by antigen-presenting cells in response to the antigen. These proinflammatory cytokines feedback to CRH-positive neurons in the PVN of the hypothalamus. Activation of these neurons signals an increase in SNS activity that induces NE release from sympathetic nerves in primary and secondary lymphoid organs and EPI release from the adrenal medulla (Sundar et al., 1990; Irwin et al., 1992; Friedman and Irwin, 1995). Interleukin (IL)-1, tumornecrosis factor(TNF)- α , IL-6, and IL-2 mediate the activation of PVN neurons resulting in the increased SNS outflow that follows an antigen challenge (Berkenbosch et al., 1989; Hurst and Collins, 1994; Zalcman et al., 1994; Foucart and Abadie, 1996). The catecholamines released from sympathetic terminals after SNS activation bind to β - and α -ARs (primarily β_2 -ARs) expressed on immunocytes. It is through this interaction that NE and EPI exert their regulatory effects on immunocytes (Madden, 2001; Sanders et al., 2001).

ARs are seven-transmembrane domain cell-surface receptors, which are divided into three major groups based on ligand affinity: β -, α_1 - and α_2 -AR. NE has a higher affinity for α -AR, but at high physiologic concentrations will bind to β -AR. Similarly, EPI preferentially binds to β -AR, but also can bind to α -ARs at high physiologic concentrations. These ARs are further divided into the following subtypes: α_{1a} -, α_{1b} -, α_{1d} -, α_{2a} -, α_{2b} -, α_{2c} -, β_1 -, β_2 -, and β_3 -AR. All of the AR subtypes are G-protein-coupled (reviewed in Lorton et al., 2001). The α_2 -ARs are coupled to G α_i/o , which when activated inhibits cAMP production and activates ERK/MAPK pathways. α_1 -ARs are coupled to G $\alpha_q/11$. Activation of α_1 -ARs turns on the PLC/PKC/ERK1/

2 MAPK pathway. β_1 - and β_2 -ARs are coupled to $G_{\alpha s}$, which when activated increases cAMP through the PKA/cAMP pathway.

A variety of immune cells express ARs; these include T and B lymphocytes, and macrophages (reviewed in Madden, 2001 and Sanders et al., 2001). NE or EPI binding to AR on immune cells activates the receptors to alter immune responses (Lorton et al., 2001; Madden, 2001; Sanders et al., 2001). The most common AR expressed by immune cells is β_2 -AR (reviewed in Madden, 2001). With the exception of bone marrow cells, T and B cells do not normally express α -AR (reviewed in Sanders et al., 2001). Macrophages express β_2 -AR and α_2 -AR (Spengler et al., 1990). Under normal conditions, radioligand binding studies indicate that α_1 -ARs are not normally expressed on resting murine lymphocyte or on human blood cells (Casale and Kaliner, 1984; Fuchs et al., 1991). In lipopolysaccharide (LPS) stimulated human whole blood cultures, treatment with an α_1 -AR agonist did not affect TNF- α or IL-6 production, but did make a small contribution to the enhanced IL-10 production (van der Poll et al., 1994, 1996). While evidence does not support a significant role for α_1 -AR in regulating immune functions under normal conditions, their expression by macrophages and monocytes can be induced in vitro by glucocorticoids and β_2 -AR agonists (Roupe van der Voort et al., 1999). Further, monocyte expression of α_1 -AR has been detected in some disease conditions, including RA (Heijnen et al., 1996).

3.2.1 Inhibition of Lymphocyte Responsiveness

In early in vitro studies, treatment of human lymphocytes with catecholamines or isoproterenol, a β -AR agonist, inhibited mitogen-induced proliferation (Hadden et al., 1970), an effect that is blocked by propranolol, demonstrating a β -AR-mediated mechanism. In the presence of hydrocortisone, NE increased mitogen proliferation, an effect that is blocked by phentolamine, an α -AR antagonist, supporting an α -AR-mediated mechanism. EPI has no effect on mitogen-induced lymphocyte proliferation at submicromolar concentrations. However, when mitogen-stimulated lymphocytes are treated with EPI in the presence of a β -AR antagonist or an α -AR antagonist, EPI enhances and inhibits, respectively, mitogen-induced proliferation. These findings suggest that β - and α -AR stimulation have opposite effects.

Stimulation of β -AR expressed on T lymphocytes and B lymphocytes inhibits mitogen-induced proliferation (Johnson et al., 1981), cytotoxicity by cytotoxic T lymphocytes (Strom et al., 1973), IL-2-stimulated proliferation responses (Beckner and Farrar, 1988), IL-2 synthesis (Didier et al., 1987), chemotaxis, and antibody production (Watson et al., 1973; Watson, 1975) by increasing intracellular cAMP. On the basis of the inhibitory effects observed with β -AR stimulation in these early studies, the view emerged that increases in SNS activity suppress lymphocyte proliferation and effector functions, and by extension, immune responsiveness. This view has been challenged due to more recent studies exploring the effects of cAMP and adrenergic agonists on immune reactivity, which indicates a more intricate and complex role for catecholamines in modulation of immune functions. The effects of catecholamines on immune responses are critically dependent upon the time relative to the immune challenge or stressor, the dose, cell type(s), lymphoid organ, and the subtype of AR stimulated.

3.2.2 Enhancement of Lymphocyte Responses

In vivo, NE enhances plaque-forming cell (PFC) responses. This response is mimicked by treatment with a β_2 -AR agonist, when the agonist is added at the start of the culture and blocked by propranolol, a β -AR antagonist if administered within 6 h of the NE or the β_2 -AR agonist (Sanders and Munson, 1984a, b, 1985a, b). The NE or β_2 -AR enhanced-PFC response is not inhibited by addition of an α -AR antagonist. β -AR blockade in the presence of NE unmasked NE-mediated enhancement of the PFC response on the fourth day of culture, which is mimicked by an α_1 -AR agonist and inhibited by an α -AR antagonist, indicating that the response is mediated via α_1 -ARs. Thus, these findings suggest that NE enhanced antibody responses by interaction with β_2 -AR stimulation.

3.2.3 Macrophage and Natural Killer Cell Functions

The killing of virus-infected and tumor cells by interferon (IFN) γ -activated macrophages is inhibited by treatment with NE and EPI (Koff and Dunegan, 1985, 1986). NE- and EPI-induced suppression of infected- and tumor cell killing by activated macrophages is mediated by an increase in macrophage intracellular cAMP levels. Treatment of human monocytes with NE, EPI, or phenylephrine (an α -AR agonist) also elevates the synthesis of complement components (Lappin and Whaley, 1982). Addition of phentolamine or prazosin, an α_1 -AR antagonist, but not propranolol, blocks the catecholamine-induced increases in complement component synthesis.

NK cell lytic activity is inhibited after NE, EPI, or isoproterenol (β -AR agonist) treatment in vivo when the adrenergic stimulation occurs during the incubation of NK cells with the target cells (Hellstrand et al., 1985; Whalen and Bankhurst, 1990; Takamoto et al., 1991). In contrast, pretreatment of NK cells with EPI, followed by removal of EPI prior to addition of the target cells, increases cytolytic activity (Hellstrand et al., 1985; Takamoto et al., 1991). The effects of EPI pretreatment on NK cell lytic activity are mediated by β_2 -AR (Maisel et al., 1990). Thus, stimulation of β_2 -AR expressed on NK cells can enhance or suppress the lytic activity of NK cells depending upon the timing of β -AR stimulation relative to the NK cell and target cell interactions.

In animal studies, administration of NE, EPI, or a β -AR agonist increases the number of NK cells in the circulation (Van Tits et al., 1990; Schedlowski et al., 1996; Shakhar and Ben-Eliyahu, 1998). These findings are consistent with other reports that acute stress or exhaustive exercise, which increase the circulating catecholamine levels, increase circulating NK cells, an effect that is mediated via β_2 -AR (Murray et al., 1992; Benschop et al., 1994). It is unclear whether the NK cells released into the circulation are able to localize to the site of infection or tumors, given in vitro studies showing β_2 -AR agonists suppress NK cell adherence to cultured endothelial cells (Benschop et al., 1994, 1997). Treating rats with a β -AR agonist also decreases NK cell activity ex vivo and the promotion of tumor growth and metastasis in vivo (Shakhar and Ben-Eliyahu, 1998).

3.2.4 Modulation of Trafficking of Immune Cells

Lymphocyte and monocyte circulation through the body are critical for surveillance and recognition of foreign entities, as well as for appropriate effector functions (Westermann and Pabst, 1990; Shakhar and Ben-Eliyahu, 1998). The SNS releases catecholamines that regulate lymphocyte and monocyte trafficking. A rapid and transient increase in circulating NK cells, lymphocytes, and monocytes in the blood is observed with mental and physical stressors and with physical exercise (Benschop et al., 1996; Schedlowski et al., 1996; Mills et al., 1997). The effect of these stressors and exercise are blocked by β -AR antagonists and mimicked by EPI or isoproterenol treatment (Gader, 1974; Crary et al., 1983; Benschop et al., 1996; Schedlowski et al., 1996; Mills et al., 1997). In guinea pigs treated with NE or isoproterenol an increase in lymphocyte and granulocyte release from the spleen is observed (Ernström and Sandberg, 1973). Phentolamine and propranolol blocks the NE- or isoproterenol-mediated effects on lymphocyte and granulocyte release from the spleen, respectively. The observed effects are not due to altered vascular smooth muscle contractility of the blood vessels, because treatment with NE or isoproterenol does not alter blood flow. Similarly, a dramatic increase in the release of PFCs and the duration of release of PFCs from the spleen is observed in guinea pigs treated with EPI (Ernström and Söder, 1975). No changes in splenic blood flow or smooth muscle contraction are observed, suggesting a direct effect of EPI on lymphocyte trafficking. Pretreatment of lymphocytes with isoproterenol in vitro prior to adoptive transfer is demonstrated to increase the homing of these lymphocytes to the spleen and lymph nodes (Carlson et al., 1997). Further, administration of 6-hydroxydopamine (6-OHDA), a neurotoxin that selectively destroys NA nerves, alters lymphocyte trafficking (Madden et al., 1994b). Lymphocytes obtained from untreated mice and adoptively transferred to sympathectomized recipients migrated in larger numbers to Peyer's patches, mesenteric, inguinal, and axillary lymph nodes. However, lymphocytes from sympathectomized mice that were transferred to untreated recipients have reduced migration to these lymph nodes. These findings demonstrate that depletion of sympathetic innervation alters lymphocyte migration into secondary lymphoid organs.

Mechanisms for catecholamine-induced changes in lymphocyte and monocyte trafficking have not been extensively studied and thus are not entirely clear. They do not seem to result from catecholamine-mediated changes in adhesion molecule expression by immune cells or the endothelium (Carlson et al., 1996; Benschop et al., 1997). While β_2 -AR agonists decrease T cell and NK cell adhesion to endothelial cells in culture (Carlson et al., 1996), treatment with NE or EPI does not change immune or endothelial cell expression of cell adhesion molecules tested in vitro or in vivo (Carlson et al., 1996; Schedlowski et al., 1996; Benschop et al., 1997). These data indicate that immune cells are less likely to attach firmly to blood vessel endothelium in the presence of catecholamines. There is some evidence to support that under conditions of stress, catecholamines do modulate adhesion molecule expression. Exercise to exhaustion elevates plasma levels of ICAM-1 by a β -AR-dependent mechanism (Rehman et al., 1997). Alternatively sympathetic innervation of the spleen regulates leukocyte egress by altering flow resistance and by a flow resistance-independent β -AR-mediated pathway (Rogauch et al., 1999). Further research is required to gain an understanding of the mechanisms and significance of catecholamine-induced changes in immune cell trafficking during local immune and inflammatory responses in health and disease.

3.3 SNS and Immune Cell Cytokine and Monokine Production

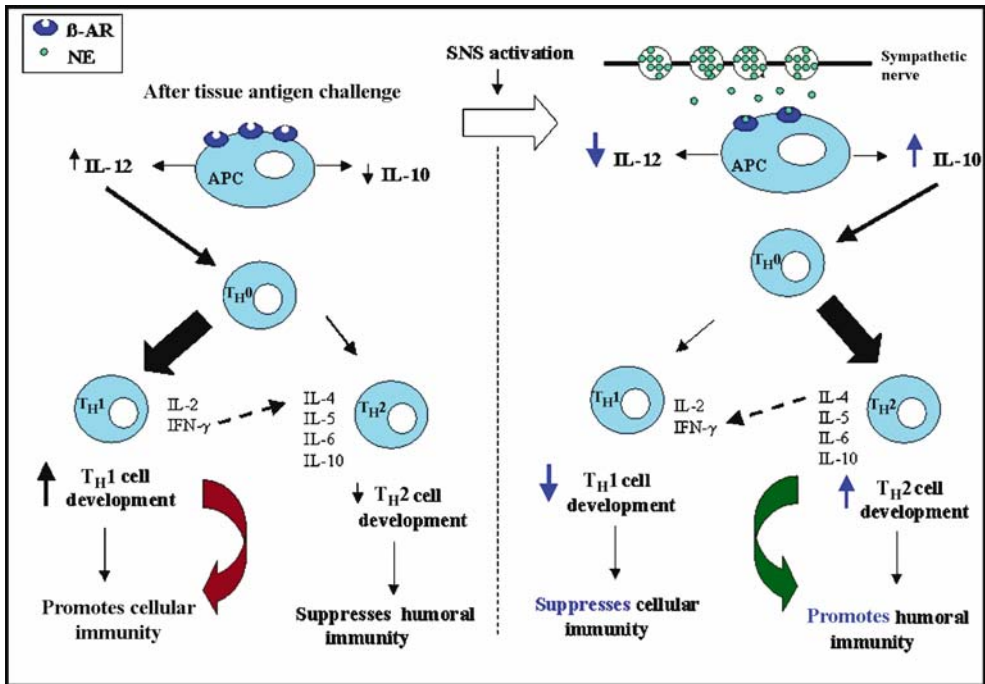
3.3.1 Catecholamine Modulation of Cytokine and Monokine Production

Catecholamines regulate cytokine production and are intimately involved in determining the balance of T_H lymphocyte subsets and in initiating and sustaining inflammation (Webster et al., 1998). The subsets of T_H lymphocytes, T_{H1} and T_{H2} cells, determine the cellular or humoral dominance of an immune response by virtue of the cytokine pattern they produce (Arai et al., 1990; Kroemer et al., 1993; Paul and Seder, 1994) (🔗 [Figure 5-1](#)). Whether an immune reaction to challenge is dominated by a cellular- or humoral-mediated response is dependent upon the nature and concentration of the antigen, the degree of antigen-induced activation of $CD4^+$ T_H cell subsets, and the relative production of different T_{H1} and T_{H2} cell cytokines produced. T_{H1} and T_{H2} cells result from the differentiation of T_{H0} cells that primarily produce IL-2. T_{H1} cells produce IL-2 and IFN- γ . In contrast, T_{H2} lymphocytes synthesize IL-4, IL-5, IL-6, and IL-10. The different cytokine patterns lead to different functions of the two T_H cell subtypes. T_{H1} cells play critical roles in the generation of delayed-type hypersensitivity and cytotoxic responses (Kupfer and Singer, 1989). In contrast, T_{H2} cells regulate B cell growth, differentiation, antibody production, and immunoglobulin (Ig) isotype switching (Snapper and Paul, 1987; Stevens et al., 1988; Mosmann and Coffman, 1989a, b). The balance of cytokines produced by T_{H1} and T_{H2} lymphocytes plays an important role in the development and progression of autoimmune diseases. Altering the balance of T_{H1} to T_{H2} cell cytokines has been shown to have therapeutic value for ameliorating some autoimmune diseases (Elliot et al., 1994).

The cytokines produced by one T_H cell subtype regulate the synthesis of cytokines produced by the other subset. For example, IFN- γ produced by antigen-activated T_{H1} lymphocytes can inhibit T_{H2} lymphocyte expansion (Scott, 1991; Sher et al., 1992; Aguilera et al., 1997). Further, the IFN- γ -induced suppression of T_{H2} lymphocyte expansion alters the secretion of IgG2a. Similarly, the IL-4 and IL-10 produced by T_{H2} cells inhibits T_{H1} cell growth and their production of T_{H1} -type cytokines (Kroemer et al., 1993; Scott, 1993; Paul and Seder, 1994). In this manner, DTH and antibody responses are regulated directly and indirectly by cytokines produced by both T_{H1} and T_{H2} lymphocytes. Macrophages also produce cytokines that regulate cellular and humoral immune responsiveness (Unanue and Allen, 1987) (🔗 [Figure 5-1](#)). In responses to an antigen challenge, macrophages produce IL-12. IL-12 and IFN- γ stimulate the development of T_{H1} lymphocytes that promote cell-mediated immunity. Cytokines produced by the developing T_{H1} lymphocytes then inhibit the development of T_{H2} cells and thus dampen humoral immunity. In contrast, IL-10, which is largely produced by monocytes and macrophages, and IL-4 stimulate T_{H2} cell development and thus promote humoral-mediated immunity. Cytokines produced by the developing T_{H2} lymphocytes then suppress the development of T_{H1} lymphocytes and, in this manner, dampen cell-mediated immunity. In addition to regulating cellular- and humoral-mediated immunity,

■ Figure 5-1

Immune responses are regulated by antigen-presenting cells (APC) and by T helper (T_H) lymphocyte subclasses T_H1 and T_H2 , components of innate and acquired (adaptive) immunity, respectively. T_H1 cells secrete $IFN-\gamma$ and IL-2, which promote cellular immunity. T_H2 cells produce IL-4, IL-5, IL-6, and IL-10 that promote humoral immunity. T_H0 cells (naïve $CD4^+$ T lymphocytes) are bipotential and serve as precursors of T_H1 and T_H2 cells. Differentiation of T_H0 cells toward T_H1 or T_H2 is influenced by cytokines, particularly IL-12, TNF- α , and IL-10 produced by APC. Cellular-mediated immune responses provide defense against intracellular bacteria, protozoa, fungi, and some viruses. In contrast, humoral-mediated immune responses provide protection against multicellular parasites, extracellular bacteria, some viruses, soluble toxins, and allergens. The SNS regulates T_H0 cell development by controlling APC release of IL-10 and IL-12 and by reducing $IFN-\gamma$ production by T_H1 cells. Solid line arrows represent stimulation. Dashed lines represent inhibition. Ag, antigen; APC, antigen-presenting cell; T, T lymphocyte; T_H , T helper cell; IL, interleukin; TNF, tumor necrosis factor; IFN , interferon. Activation of the SNS stimulates release of NE and EPI from sympathetic nerves and the adrenal glands, respectively, which simulate production of IL-10 and inhibit release of IL-12 by APC. NE and EPI also inhibit $IFN-\gamma$ and IL-2 production by T_H1 cells. Thus, NE and EPI shift the production of cytokines by APC and T_H1 cells in a manner that promotes development of humoral immunity and suppresses development of cell-mediated immunity. APC, antigen-presenting cell; IL, interleukin; IFN , interferon; T_H , T helper; NE, norepinephrine; EPI, epinephrine



macrophages also produce cytokines that play roles in antigen processing and presentation and in inflammatory processes.

The synthesis of numerous cytokines by lymphocytes and macrophages is regulated by catecholamines. By regulating the production of T_H cell cytokines, catecholamines dampen T_H1 lymphocyte development and promote development of T_H2 lymphocytes (reviewed in Webster et al., 1998). NE and EPI binding to lymphocyte β_2 -AR elevates intracellular cAMP. This results in a potent inhibitory effect on T_H1 cell IL-2 and $IFN-\gamma$ production, and thus, promotes T_H2 cell development and humoral immunity (reviewed in Monastera and Secchi, 1993; van der Poll et al., 1994; Szabó et al., 1997; Haskó et al., 1998; Webster et al., 1998).

Adrenergic agonist-induced cAMP elevation is a more potent inhibitor of T_H1 cell cytokine production than the IL-4 produced by T_H2 cells. In addition, NE and EPI inhibit LPS-induced IL-12 production by human peripheral blood mononuclear cells (PBMC) ex vivo (Elenkov et al., 1996). Since IL-12 production promotes T_H1 lymphocyte development, the NE-mediated inhibition of IL-12 production favors T_H2 lymphocyte development. NE and EPI also stimulate mononuclear leukocytes to produce IL-10 (Elenkov et al., 1996; van der Poll et al., 1996). NE-induced production of IL-10 promotes humoral immunity by stimulating $CD4^+$ T_H0 cells to differentiate to T_H2 cells. Thus, NE and EPI release from sympathetic nerves and adrenal chromaffin cells indirectly enhance development of T_H2 lymphocytes, and by doing so, promote humoral-mediated immunity.

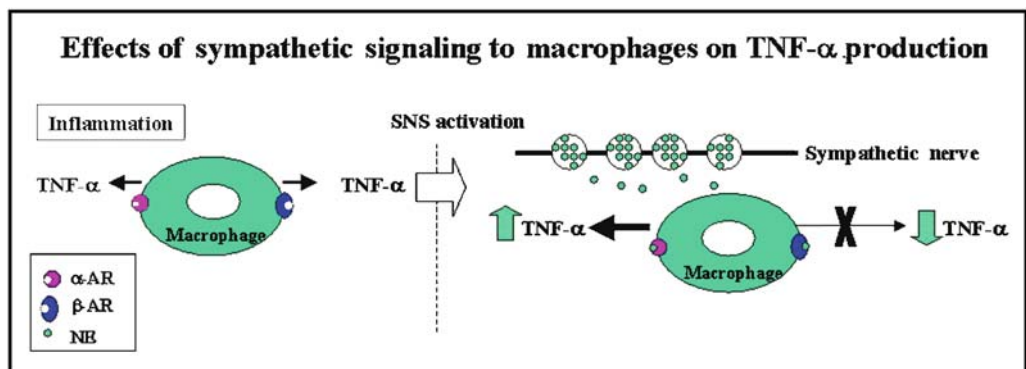
Sanders and coworkers (Ramer-Quinn et al., 1997; Sanders et al., 1997) have shown that β_2 -AR are expressed on T_H1 lymphocyte clones and absent on T_H2 lymphocyte clones. Consistent with these findings, in vivo functional studies have revealed that T_H1 and T_H2 lymphocytes are differentially regulated by β -AR agonists. Treatment of T_H1 cell clones with terbutaline, a β_2 -AR agonist, prior to activation with enriched populations of antigen-specific B lymphocytes or anti-CD3 antibodies, inhibits T_H1 cell clone production of IL-12 (Wacholtz et al., 1991; Bartik et al., 1993; Chen and Rothenberg, 1994; Sanders et al., 1997) and IFN- γ (Sanders et al., 1997). The production of IL-4 and IL-10 by T_H2 cell clones is not affected by terbutaline treatment (Ramer-Quinn et al., 1997; Sanders et al., 1997). In addition, IL-2-dependent T cell proliferation is inhibited by terbutaline treatment. This finding is consistent with observations of reduced IL-2 production. These findings support the differential regulation of T_H cell subsets by catecholamines and provide a mechanism by which sympathetic nerves can modulate clonal expansion of activated T_H cell subsets.

3.3.2 Anti-Inflammatory Effects of Catecholamines

The SNS modulates the proinflammatory cytokine production of activated macrophages, including TNF- α , which plays a major role in initiating and sustaining inflammatory responses (Figure 5-2). Elenkov and coworkers (1995) have demonstrated that in LPS-treated mice sympathetic activation inhibits TNF- α production. Pretreatment of LPS-treated mice with propranolol (β -antagonist) increases plasma

■ Figure 5-2

Norepinephrine (NE) regulates inflammation by altering macrophage production of pro- and antiinflammatory cytokines through interaction of NE with β_2 - or α -adrenergic receptors (AR) expressed on macrophages. Selective activation of these two receptor populations on macrophages/monocytes elicits opposite effector functions with β -AR stimulation being inhibitory and α -AR stimulation being excitatory to tumor necrosis factor (TNF)- α and IL-12 production. Thus, shifts in expression of β - and α -AR subtypes on monocytes/macrophages in RA patients is likely to contribute significantly to disease pathology



TNF- α . Pretreatment with an α_2 -AR antagonist, which would be expected to either decrease the release and availability of NE or to target macrophage α_2 -AR, also reduces plasma TNF- α levels. Treatment with chlorisondamine, a ganglionic blocker, prior to LPS administration inhibits the α_2 -AR antagonist effects, supporting an effect of the α_2 -AR antagonist on presynaptic NA terminals to alter NE release. In contrast, pretreatment with the β -agonist, isoproterenol, in LPS-treated mice inhibits plasma TNF- α . Thus, effects of sympathetic activation on TNF- α production in LPS-treated mice are mediated via β -AR.

Other studies also have reported inhibitory effects of catecholamines on TNF- α production after LPS treatment. Administration of EPI or isoproterenol to LPS-treated animals and humans reduces plasma TNF- α levels (Monastra and Secchi, 1993; van der Poll et al., 1994, 1996; Szabó et al., 1997; Haskó et al., 1998). Similarly, in vitro studies show that β -AR agonists inhibit macrophage TNF- α production (Severn et al., 1992; Spengler et al., 1994; Ignatowski and Spengler, 1995; Chou et al., 1996a; Haskó, 2001).

Catecholamines also increase activated macrophage production of the antiinflammatory cytokine, IL-10. IL-10 dampens inflammatory responses by reducing TNF- α production (Gerrard et al., 1980, 1993; van der Poll et al., 1996; Szabó et al., 1997; Haskó et al., 1998). Thus, effects of sympathetic activation on plasma TNF- α levels could be indirectly mediated by increasing IL-10 production. This is not the case in LPS-treated mice, since LPS-treated IL-10-deficient mice treated with a β -agonist also is observed to have decreased plasma TNF- α levels compared to LPS-treated wild-type mice (Haskó et al., 1998). These data support a direct effect of sympathetic activation on macrophage TNF- α production, which is mediated via β -AR activation.

3.3.3 Pro-Inflammatory Effects of Catecholamines

Under normal conditions, α_1 -AR do not appear to play a significant role in regulating immune functions. This AR subtype is not normally expressed on peripheral blood monocytes/macrophages (PBMC). However their expression can be induced on these cells in some diseases. For example, PBMC obtained from patients suffering from juvenile RA express functional α_1 -AR, while PBMC from healthy donors do not (Heijnen et al., 1996). The expression of α_1 -AR on macrophages is likely to contribute to the disease process by increasing the production of proinflammatory cytokines (Heijnen et al., 1996). Expression of α_1 -AR can be induced in human monocytes by treatment with dexamethasone or terbutaline suggesting that glucocorticoids and stimulation of β_2 -AR regulate α_1 -AR subtype expression by human monocytes. The induction of α_1 -AR on cells of monocytic origin has not been explored in animal models of RA. Future studies are needed to investigate the significance of monocyte α_1 -AR expression in the inflammatory and disease processes of RA and to determine, (monocyte α_1 -AR expression occurs in other chromo inflammatory diseases.

3.4 In Vivo Effects of the SNS on Immune Functions

The effects of surgical and chemical sympathectomy (SympX) on cytokine and antibody production provide support for SNS modulation of immune functions. Antibody production is increased following surgical denervation of the submaxillary lymph nodes compared with sham-denervated lymph nodes (Esquifino and Cardinali, 1994). Chemical SympX inhibits DTH responses in mice (Madden et al., 1989). IL-2 production and cytotoxic T lymphocyte generation in the draining lymph nodes is decreased in these SympX mice, providing a potential mechanism for the inhibitory effects of SympX on DTH responses.

SNS effects on immune responses are to some extent dependent upon the CD4⁺ T_H predominance of the animal strains examined (Kruszewska et al., 1995, 1998). The effect of 6-OHDA-induced SympX on cytokine and antibody production was explored in BALB/cJ mice and C57BL/6 mice that have a predominant T_H2 and T_H1 cytokine profile, respectively (Kruszewska et al., 1995). Primary antibody responses to keyhole limpet hemocyanin (KLH) in the T_H1-predominant C57BL/6 mice is increased after SympX. In this strain, SympX increases IgM and IgG (IgG1 (T_H2-associated) and IgG2a (T_H1-associated)) anti-KLH antibody titers. In the T_H2-predominant BALB/cJ mice, anti-KLH IgM and IgG responses are not altered after SympX. In both mouse strains, SympX enhances splenocyte KLH-induced proliferation in vitro. IL-2 and

IL-4 production are elevated after SympX in both mouse strains; however, the T_H1 -dominant strain synthesizes much more IL-4 in response to SympX than the T_H2 -dominant strain (Kruszewska et al., 1998). IFN- γ production in response to KLH challenge is increased after SympX in the T_H1 -predominant strain but not in the T_H2 -dominant strain. These findings are consistent with a tonic inhibitory mechanism of immune regulation by the SNS. They support a differential responsiveness by T_H1 - and T_H2 -dominant strains of mice to changes in SNS activity indicating that T_H predominance impacts the effect of SympX on the immune response to an antigen challenge.

Changes in immune functions that occur after SympX differ depending upon the lymphoid organ that is examined. In SympX adult mice, mitogen-induced B cell proliferation is dramatically increased in the lymph nodes; however, it is reduced in the spleen (Madden et al., 1994a). In the spleens of SympX mice, Con-A-induced T cell proliferation is reduced as are IL-2 and IFN- γ production. In the lymph nodes of these SympX mice, production of IFN- γ is dramatically increased, but IL-2 is not altered. SympX-induced changes in lymphocyte homing may explain some of the differences observed in the spleen and lymph nodes after SympX (Madden et al., 1994a). Our laboratory also has observed differences in cytokine responses in lymph nodes and spleen following CFA challenge to induce arthritis in rats (unpublished observations D. Lorton and C. Lubahn). While in these studies the arthritic rats were not SympX, the sympathetic innervation of lymphoid organs undergoes an injury and sprouting response remodeling similar to SympX (Lorton et al., 2005), which is accompanied by a reorganization of the immune cell populations (Bellinger et al., 2001).

In summary, the SNS regulates all aspects of innate and acquired immune responses to challenge in vivo, including proliferation, cytokine production, antibody production, cytotoxic responses, and lymphocyte migration. The direction of the sympathetically-mediated response can differ based upon the AR stimulated, the lymphoid organ examined, the age of the individual, genetic factors (e.g., T_H cell predominance), and the nature of the immune-activating signal received by immune cells. Given that catecholamines regulate development of cellular and humoral immunity and pro- and antiinflammatory cytokine production, there is a potential for pharmacological manipulation of sympathetic pathways that can be used in treating autoimmune and chronic inflammatory diseases. Targeting sympathetic pathways could be therapeutically beneficial in treating diseases where there is dysregulation of immune functions and/or a significant inflammatory component. In autoimmune disease such as RA, the dysregulation of the immune system results in autoantibody production, robust cell-mediated immune responses, chronic inflammation, and the generation of autoaggressive immune cells within affected joints. Given that the primary function of the SNS is maintenance of homeostasis of most body functions, including immunologic homeostasis, it is difficult to believe that the SNS is not involved in the striking dysregulation of immune functions that occurs in autoimmune diseases. Pathology driven alterations in the bidirectional communication between the SNS and the immune system are likely to profoundly contribute to the disease processes of autoimmune diseases in general and RA specifically. In fact there is convincing evidence that the SNS plays a significant role in the pathophysiology of RA and that SNS to immune signaling can be beneficially targeted to retard the disease processes.

4 Dysregulation of the SNS in RA and Animal Models of RA

Immune system dysfunction is central to the pathophysiology of RA. Dramatic changes in SNS communication with the immune system are evident in RA patients and animal models. This altered communication is likely to hamper the ability of the SNS to maintain immune system homeostasis in RA and be critically involved in the immune system dysfunction that drives joint inflammation and cell-mediated immunity in RA. Numerous studies have reported that the onset of RA and juvenile RA and subsequent flares often occur following stressful events in the patient's life. These studies support a role for stress-induced increases in SNS activity in the pathology of inflammatory arthritis (Walker et al., 1999; Hermann et al., 2000). Reports of autonomic dysfunction in RA and juvenile RA patients further support this notion. In children with RA, Kuis et al. (1996) observed increased resting heart rates and high levels of the NE metabolite 3-methoxy-4-hydroxy-phenylglycol (MHPG) in the urine. Similarly, abnormal cardiovascular tests during orthostatic

stress (Leden et al., 1983), Valsalva maneuver (Leden et al., 1983), deep breathing (Perry et al., 1989), pupil size (Perry et al., 1989), and perspiration responses (Kalliomaki et al., 1963) indicate sympathetic dysfunction occurs in RA patients. The directionality of sympathetic changes in RA patients is indicative of increased sympathetic tone with disease development and progression.

Cardiac sympathetic activity is elevated in RA patients who were recently diagnosed and had active disease (Remick, 2003; Dekkers et al., 2004; Evrengul et al., 2004). In contrast, cardiac parasympathetic activity remains at normal levels. Similarly, using heart rate variability to detect changes in the balance between SNS and PNS balance, RA patients have an increase in sympathetic activity (Remick, 2003; Evrengul et al., 2004). Changes in sympathetic control could be involved in ventricular tachyarrhythmias and in the higher incidence of sudden death from cardiovascular complications in this disorder. Beneficial use of functional indicators of sympathetic activity for early diagnosis, for early initiation of RA therapy, and to identify risks of cardiovascular complications have recently been proposed (Remick, 2003; Evrengul et al., 2004; Harle et al., 2005a). Treatment of RA patients with adrenergic-targeting drugs has demonstrated some success in reducing disease severity. In clinical studies, RA patients treated with propranolol, a β -antagonist, or regional sympathetic blockade with guanethidine decreases the symptoms associated with the disease (Levine et al., 1986b, 1988), supporting a SNS role in RA pathology.

Altered sympathetic activity also has been reported in an animal model of RA. Plasma levels of NE are significantly elevated on days 4 and 7 after CFA challenge to induce AA in Lewis rats, the time required for antigen processing. Plasma NE levels peak again on day 21 after CFA challenge, a time when inflammatory responses peak in this model (Tanaka et al., 1996). Considering that NE in the plasma results from spillover into the blood from sympathetic nerves that innervate peripheral target organs (Micalizzi and Pals, 1979), these findings indicate that an increase in SNS activity occurs during specific time periods in the disease process.

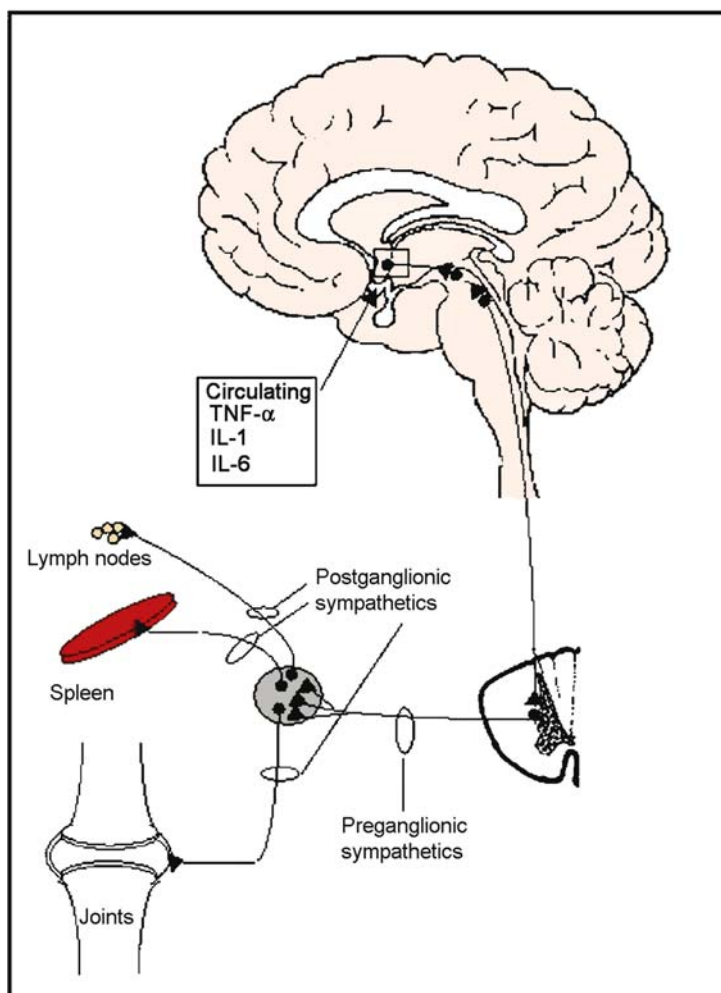
4.1 Inflammatory Cytokines Induce Changes in SNS Activity

Enhanced SNS activity observed in RA patients is presumably in response to immunological events that occur in the disease (🔗 [Figure 5-3](#)). This assumption is based on reports that immunization with bacterial antigens such as LPS increases NE release and turnover in secondary lymphoid organs (Pardini et al., 1983; Jones et al., 1986; Tang et al., 1999). Splenic NE levels are reduced after antigen challenge with sheep red blood cells (RBC); however, it is unclear if the changes in NE levels are due to altered NE release, production, and/or reuptake by the nerve terminals. Reduced NE concentrations in secondary lymphoid organs can also result from an increase in organ weight from antigen-induced proliferation. Recently, Kohm and coworkers (2000) have shown that activation of antigen-specific T_H2 cells and B cells in vivo by a soluble protein antigen increased the rate of NE release and turnover in secondary lymphoid organs. While the mechanisms involved in the increase in NE turnover in secondary lymphoid organs after antigen challenge are not well understood, there is some evidence to support that cytokines, like IL-1, IL-2, TNF- α , and IL-6, produced by activated immunocytes signal the nervous system to induce an increase in sympathetic activity. These cytokines can act locally at the site of production to inhibit presynaptic release of NE or indirectly through activation of CNS pathways to induce increased sympathetic activity and NE turnover (Barbany et al., 1991; Saito et al., 1991; Soliven and Albert, 1992; Hurst and Collins, 1993, 1994; Shimizu et al., 1994; Zalzman et al., 1994). Increased sympathetic activation increases the NE available to interact with AR, which likely modulates the ensuing immune response to the antigen challenge as a negative feedback mechanism.

Effects of immune challenge with arthritis-inducing superantigens on sympathetic activity have not been extensively examined, but are of interest given the robust immune response that they elicit and difficulties in their clearance. Increases in splenic NE concentrations occur 2 h after challenge with the staphylococcal enterotoxin (SEB) superantigen (Del Rey et al., 2002). Splenic NE levels return to baseline values within 2 days of the challenge. These findings indicate that immunization with superantigens can increase sympathetic activity. Tanaka and coworkers (1996) also have reported increases in sympathetic activity after CFA challenge to induce AA. After adjuvant challenge, NE plasma levels are elevated on

Figure 5-3

Activation of central nervous system (CNS) pathways by circulating cytokines, including $\text{TNF-}\alpha$, IL-1, and IL-6, that increase sympathetic nervous system (SNS) activity to peripheral target tissues. Inflammatory cytokines act at the level of the hypothalamus to increase the activity of CRH-positive neurons in the paraventricular nucleus (PVN). These neurons project to neurons in the rostroventrolateral medulla (RVLM). Neurons in the RVLM tonically controls the discharge of neurons in the intermediolateral (IML) cell column of the thoracic spinal cord. Neurons in the IML cell column project their axons via preganglionic nerves to neurons in sympathetic ganglia. Neurons within sympathetic ganglia project their axons to the peripheral target tissue. In rheumatoid arthritis (RA), the SNS regulates immune functions within secondary lymphoid tissues, such as the lymph nodes and the spleen to influence disease pathology. The SNS also regulates blood flow, and functions of synovial fibroblasts and macrophages, as well as, immune cells that infiltrate into the joint in rheumatic joints. Osteoblasts and osteoclast also express adrenergic receptors (ARs) and represent additional target cells within the joints



days 4 and 7, times for antigen processing, and on day 21, a time of acute inflammation. Whether NE turnover is altered after CFA challenge has not been examined. The differences in the timing of increased sympathetic activity and the single and biphasic responses between the SEB and CFA challenge in these two

studies could be due to the adjuvant vehicle and the development of joint inflammation that occurs in CFA-challenged rats. No studies have explored how the high levels of proinflammatory cytokines and activated immunocytes in the circulation and arthritic joints with active or chronic inflammation affects NE turnover in lymphoid organs, the primary source of immune cells that migrate into the affected joints.

The immune and nervous systems communicate by numerous routes. The expression of, and susceptibility to, inflammatory and autoimmune diseases are differentially affected by blocking this communication at a variety of signaling sites in the stress pathways (Harbuz et al., 1994; Baerwald et al., 2000; Straub and Cutolo, 2001). Inflammatory mediators are produced with peripheral inflammation in RA. These mediators signal the brain (Banks and Kastin, 1997; Banks et al., 2002) and afferent peripheral nerves (Bluthe et al., 1994; Watkins et al., 1995) using a variety of second messenger systems (Karanth et al., 1993). The chronically high proinflammatory cytokine production in the synovium, which also enters the circulation, plays a key role in the disease pathology (Brennan et al., 1995; Feldmann et al., 1996; Feldmann and Maini, 2001), partly by activating neuroendocrine and sympathetic pathways (Baerwald et al., 1997; Straub and Cutolo, 2001). We have proposed that the chronic inflammation and/or the inability to effectively process and clear the arthritis-inducing antigen in RA and AA results in prolonged neuroendocrine and sympathetic activation. This may eventually lead to the dysregulation of neuroendocrine and sympathetic systems, observed in RA patients and in RA animal models. Dysregulation of the neural signaling to the immune system reduces the effectiveness of these negative feedback pathways to damp inflammation and immune responses that perpetuate the disease.

4.2 SNS Innervation and Immune Cell Reorganization in Lymphoid Organs of AA

The occurrence of SNS dysfunction with a dysregulation of immune and inflammatory responses in RA is supported by findings of altered sympathetic innervation patterns and a reorganization of immune cells in lymphoid organs from AA rats. Our laboratory has explored whether changes in sympathetic innervation occur in AA rats 28 days after CFA challenge. In AA rats there is a significant decrease in NE concentration (pmol NE/gram wet weight) in the spleen and lymph nodes (inguinal and popliteal lymph nodes) compared with non-AA rats (Lorton et al., 1997). However, when the total NE content (pmol NE/whole organ weight) is determined, the amount of NE per whole organ is not altered in these organs with the exception that NE content is elevated in the popliteal lymph nodes. The reduced NE concentration without changes in the total NE content in the organ was originally interpreted to result from an increase in organ volume. These findings indicate that cells in the spleen and inguinal lymph nodes are exposed to a lower NE concentration in their microenvironment as AA develops. On the other hand, in the popliteal lymph nodes, the site that receives the highest antigen load, the increase in total NE content indicates a change in NE metabolism or an increase in sympathetic outflow occurs in AA rats. Subsequent studies have revealed that this interpretation is overly simplistic.

Examination of splenic sympathetic innervation in AA rats with severe disease revealed a striking reorganization of sympathetic nerves (Lorton et al., 2005) (reviewed in Lorton and Lubahn, 2004). At entry points into the spleen there is a dramatic increase in the density of sympathetic nerves innervating the lymphoid compartments (white pulp, sites of antigen processing) in arthritic rats compared with nonarthritic rats. The volume density of sympathetic nerves innervating the central arterioles and white pulp compartments at nerve entry points in AA rats is more than double that observed for non-AA rats (Lorton et al., 2005). In contrast, at regions distant to nerve entry points, the density of sympathetic nerves supplying the central arteriole and white pulp areas of spleens is reduced by 50% in arthritic rats compared with nonarthritic rats. These findings are indicative of a dying back of sympathetic nerves in regions distant to the nerve entry regions as the disease progresses and a subsequent attempt to replace, reorganize, and repair lost innervation with a sprouting response at nerve entry points. Interestingly, in arthritic rats, sympathetic nerves appear to sprout into the red pulp compartment of the spleens of AA rats. Thus, the density of sympathetic nerves is dramatically increased in the red pulp of arthritic rats compared with nonarthritic rats (164% and 198% of control for nerve entry and distal regions, respectively). This is a

compartment that normally is sparsely innervated and is populated by effector immunocytes that express AR (e.g., activated macrophages, plasma cells, cytotoxic T cells and NK cells).

A marked reduction of sympathetic nerves that innervate the synovium in inflamed joints of RA patients also has been reported (reviewed in Miller et al., 2000; Straub and Härle, 2005). Loss of synovial sympathetic nerves is associated with an increase in semaphorin 3C (Miller et al., 2004), a sympathetic nerve repellent, and an increase in the density of brain-derived neurotrophic factor-positive cells in the synovium (Weidler et al., 2005). Similarly, a loss of sympathetic innervation occurs in the synovium of antigen- and hydrogen peroxide-induced arthritis models (Mapp et al., 1994). Thus, not only are the sympathetic innervation of the lymphoid organs altered, but the inflamed arthritic joints are also affected. Given the autonomic dysfunction that has been reported for a wide variety of sympathetic target organs in RA patients, sympathetic peripheral neuropathy could be more wide spread and should be examined. In preliminary studies, we have found a 20% decrease in the density of sympathetic nerves that supply the heart in arthritic rats compared with nonarthritic rats (unpublished observations D. Lorton). These studies suggest that there is global SNS dysfunction in experimental animal models of RA and in RA patients. Further, they provide evidence that the SNS's ability to modulate immune functions is compromised in this disease.

4.3 Parallel Redistribution of Sympathetic Nerves and Immunocytes in Lymphoid Organs

Changes in the relationship between immune target cell populations and sympathetic innervation has been examined using double-label immunohistochemistry for immune cell markers and tyrosine hydroxylase (TH), the rate-limiting enzyme for NE synthesis (Bellinger et al., 2001). The altered pattern of splenic sympathetic innervation observed in arthritic rats is paralleled by changes in the distribution of specific immune subsets of target cells. We have observed a substantial decrease in the density of ED3⁺ macrophages in the marginal and parafollicular zones of spleens from arthritic rats compared with nonarthritic rats. The loss of ED3⁺ macrophages in these zones in arthritic rats is particularly striking in regions most distal to NA nerve entry points into the spleen and paralleled the loss of TH⁺ nerves in these regions. We have observed similar decreases in ED3⁺ macrophages in these splenic compartments after loss of NA nerves with 6-OHDA treatment in nonarthritic rats, supporting a relationship between the loss of NA nerves and the reduced numbers of ED3⁺ macrophages. This relationship is further supported in arthritic rats by the observed increase in ED3⁺ macrophages present in the red pulp which corresponds to the increase in TH⁺ sympathetic nerves present in this compartment in AA rats compared with non-AA rats. Perhaps the NA nerves that sprout back into the spleen after injury are following ED3⁺ macrophages into the red pulp as they migrate into this compartment after activation with CFA.

In arthritic rats, we also have observed a dramatic decrease in the density of IgM⁺ B lymphocytes within the follicles (Bellinger et al., 2001). The follicles in AA rats are largely devoid of IgM⁺ B lymphocytes. This decrease in IgM⁺ B lymphocytes was observed at entry points of NA nerves and in regions distant to nerve entry points. Thus, changes in the density of IgM⁺ B lymphocytes do not parallel the changes in sympathetic innervation observed in the spleens of AA rats. Consistent with this finding, we have not found differences in the density of IgM⁺ B lymphocytes in the spleen after loss and sprouting of sympathetic nerves with 6-OHDA treatment. Whether the decrease in density of IgM⁺ B lymphocytes is due to a depletion of these cells or whether these cells undergo isotype switching after the adjuvant challenge is unclear.

We observe no differences in the distribution or density of CD8⁺ T lymphocytes in arthritic compared to nonarthritic rats (Bellinger et al., 2001). However, the density of CD4⁺ T lymphocytes is reduced in the white pulp, particularly in regions near entry points of sympathetic nerves compared with nonarthritic rats (Bellinger et al., 2001). Further, CD4⁺ T lymphocytes are much more prevalent in the red pulp of arthritic compared with nonarthritic rats. Thus, as for ED3⁺ macrophages, the redistribution of CD4⁺ T lymphocytes parallels the loss of sympathetic innervation of white pulp regions close to sympathetic nerve entry points and increases within the red pulp of arthritic rats. A similar decrease in CD4⁺ T lymphocytes has

been observed in 6-OHDA-treated nonarthritic rats in regions of sympathetic nerve loss. These findings support a relationship between changes in sympathetic nerve density and the density and distribution of CD4⁺ T cells in arthritic rats. Altered sympathetic innervation of lymphoid organs also occurs in other autoimmune disease animal models, including New Zealand mouse strains used as models for hemolytic anemia, lupus-like syndrome (Bellinger et al., 1989), and experimental allergic encephalomyelitis (EAE) (Breneman et al., 1993).

In summary, our findings indicate that with disease development there is a loss of sympathetic innervation of white pulp compartments, a normally innervated region of the spleen involved in modulation of antigen presentation and processing. In AA rats with severe disease, there is also an increase in the sympathetic innervation of the red pulp, a region that normally receives only a sparse innervation of NA nerves and where activated immunocytes reside and a site of immune cell exiting from the spleen. Changes in ED3⁺ macrophages and CD4⁺ T cells, target cells of sympathetic nerves, paralleled the altered sympathetic innervation, while changes in IgM⁺ B cells do not, in AA rats. These findings are indicative of a disease-associated pathology of sympathetic nerves in secondary lymphoid organs, which may occur in response to changes in the activation and distribution of immune target cells within the white pulp compartments in response to the adjuvant challenge that induces arthritis.

Changes in sympathetic innervation in white pulp would be expected to alter the SNS's ability to modulate antigen processing of future antigen challenges. Given that sympathetic nerves, through release of NE and binding to β -AR, promote development of T_H2 cells and impede development of T_H1 cells, dying back of nerve fibers in the splenic white pulp may support aggressive cell-mediated immune responses. The abnormal innervation of the red pulp allows for SNS signaling to effector cells (e.g., activated macrophages and T cells) that reside in this compartment prior to homing to arthritic joints. Signals these effector cells receive in the red pulp may prime them in a manner that promotes joint inflammation once they arrive in the arthritic joints (e.g., altered cytokine production).

4.4 Potential Mechanisms of Sympathetic Nerve and Immunocyte Reorganization

The mechanisms involved in the reorganization of sympathetic nerves within the spleen in arthritic rats have not been extensively examined. Preliminary findings indicate that altered neurotrophic support from T cells and macrophages contributes to the changes in splenic sympathetic innervation. We have observed a dramatic increase in nerve growth factor (NGF) levels (50%) in spleen samples taken from nerve entry regions and a significant decrease (20%) in NGF concentrations in distal regions in arthritic rats compared with nonarthritic rats (Lorton et al., 2003). In spleens from arthritic rats, we have found a redistribution of NGF⁺ macrophage-like cells, with NGF⁺ cells being largely depleted from the white pulp and more abundant in the red pulp at nerve entry points and in regions distant to nerve entry points. At least some of the NGF⁺ cells within the red pulp are ED3⁺ macrophages (unpublished observations D. Lorton and C. Lubahn). Thus, these findings indicate that changes in NGF levels and distribution of NGF⁺ cells within the spleens of arthritic rats parallel the observed loss of sympathetic nerves in white pulp and increase in red pulp observed in severe disease. The higher NGF levels located at sympathetic nerve entry regions of the spleen would be expected to provide trophic support and entice sympathetic nerves to sprout into this region. This could provide an explanation for the hyperinnervation observed in this region in spleens from arthritic rats. Conversely, the reduced NGF levels found in regions distant to sympathetic nerve entry regions could explain the loss of sympathetic innervation in white pulps distant to the points of nerve entry. The redistribution of NGF⁺ cells from white pulp to red pulp is consistent with sprouting sympathetic nerves following NGF-producing target cells into the red pulp rather than returning to the white pulp.

An alternative mechanism that should be investigated to explain the loss of sympathetic nerves in the white pulp distant to nerve entry is the cumulative damage from reactive oxidative metabolites of NE. This potential mechanism is supported by reports of increased sympathetic tone in RA and RA animal models (reviewed in Lorton and Lubahn, 2004) that would lead to an increase in NE release or turnover during

earlier disease stages. An age-related loss of nigrostriatal dopaminergic and locus coeruleus NA neurons in the CNS has been demonstrated to occur by this mechanism (Finch, 1973; McGeer et al., 1977; Sladek and Blanchard, 1981). Addition of pergolide, a powerful antioxidant that reduces dopamine metabolism, in the diet for 2 years protects dopamine neurons in the substantia nigra (Felten et al., 1992). Demonstrations that reducing superoxide dismutase results in apoptotic death of sympathetic neurons and PC12 cells, an adrenal chromaffin cell line (Troy and Shelanski, 1994; Troy et al., 1997; Park et al., 1998), further support the sensitivity of catecholamine neurons to oxidative stress. In addition, chronically high NE levels infused via osmotic minipumps damages sympathetic nerves in peripheral target tissues, an effect blocked by treatment with desipramine, an NE uptake blocker, or superoxide dismutase, an antioxidative enzyme. Similarly, in Fischer 344 rats, there is an age-associated decline in splenic sympathetic innervation that can be partially reversed by treatment with L-deprenyl, a monoamine oxidase-B inhibitor (Park et al., 1998; Thyagarajan et al., 1998). These studies support the notion that an increase in sympathetic tone accompanied by elevated release of NE and an increase in oxidative metabolites resulting from NE degradation may be involved in the lost white pulp innervation in spleens of arthritic rats. Thus, examining the effects of oxidative stress on sympathetic nerve loss within the white pulps of AA rats is a reasonable mechanism to explore.

5 SNS Intervention and Disease Outcome in Autoimmune Disease

In Lewis rat models of EAE and experimental autoimmune myasthenia gravis (EAMG), chemical SympX following immunization to induce the autoimmune disease increases disease severity (Agius et al., 1987). Rats antigen challenged to induce EAE or chronic/relapsing experimental allergic encephalomyelitis (CREAE) and treated with the β -agonist isoproterenol or the β_2 -agonist terbutaline protect against development of EAE and CREAE (Chelmicka-Schorr et al., 1989; Wiegmann et al., 1995). Further, in EAMG, and in experimental allergic neuritis, an induced inflammation of peripheral nerves (similar to Guillian-Barré disease), treatment with terbutaline after disease onset reduces disease severity (Chelmicka-Schorr et al., 1993; Kim et al., 1994). These findings suggest a protective role for the SNS in autoimmune diseases.

AA is an exception to the observations that β -AR stimulation plays a protective role in autoimmune diseases. Systemic depletion of NE by treatment with guanethidine or reserpine attenuates the inflammation and joint destruction in AA rats (Colpaert et al., 1983; Levine et al., 1985, 1986a, b, 1991). We have confirmed that chemical SympX reduces disease severity in AA rats (Lorton et al., 1999). The seemingly disease-promoting effects of the SNS in the AA model is consistent with clinical studies in RA patients demonstrating that administration of propranolol, a β -AR antagonist, or regional sympathetic blockade with guanethidine reduces symptoms associated with this disease (Levine et al., 1986b). We believe the reported discrepancy in disease outcome in RA and animal models of RA and other autoimmune diseases following SNS manipulation can be explained, in part, by dissecting out the SNS's differing functional roles in lymphoid organs and the inflamed joints. Additionally, the differences in disease outcome between AA and other animal models of autoimmune disease may be due to differences in the timing of SNS manipulation (e.g., during the initiation stage, disease onset, or acute and chronic disease stages).

5.1 Sympathectomy in Arthritis Models—The Dual Role of the SNS Based on its Target

Animal studies investigating the role of the SNS in arthritis have used the AA Lewis rat model. Most of the experiments that have explored the SNS's role in RA pathology have administered drugs that continuously manipulate the SNS from the time of CFA challenge through severe disease development. Results from these studies clearly demonstrate that overall peripheral sympathectomy or blockade of AR (particularly via β -AR) prior to or during injection of adjuvant diminishes the severity of joint inflammation during the entire observation period of approximately 30 days (Levine et al., 1988; Lorton et al., 1999). In contrast,

similar treatment strategies in other autoimmune models indicate a protective role for the SNS. Drugs used to induce SympX target all of the peripheral sympathetic nerves, resulting in nearly total body depletion of sympathetic nerves. In models of autoimmune disease where the SNS is protective, loss of sympathetic nerves is thought to alter the function(s) of immunocytes relevant to the disease process. In AA rats, these methods of denervation remove sympathetic nerves in both the lymphoid organs and the arthritic joints. The SNS's roles in modulating immune functions in the lymphoid organs, the sites of antigen processing, the inflamed joints, and sites of the effector phase of the immune response are likely to differ as the target cells for sympathetic nerves in these sites differ. Normal targets in the lymphoid organs are vascular smooth muscle, naive macrophages, naive T lymphocytes and B lymphocytes, and dendritic cells. In contrast, cellular targets in the affected joints include activated macrophages, differentiated T lymphocytes and B lymphocytes, synoviocytes, osteoblasts, osteoclasts, mast cells, vascular smooth muscle, and primary sensory afferents. Given the difference in target cell populations between these sites, our laboratory has proposed that in AA the SNS plays a dual role in modulating immune functions. The SNS would be expected to regulate antigen processing within lymphoid organs and to impact immune functions involved in joint inflammation and destruction in the effector phase of the immune response (● [Figure 5-3](#)).

To explore this possibility, we have developed a method involving the injection of 6-OHDA into the fat pads surrounding inguinal and popliteal lymph nodes to destroy sympathetic nerves in lymphoid organs with sparing of the sympathetic innervation of the hind limbs (Lorton et al., 1996). Sympathetic denervation of the lymphoid organs with sparing of the hind limb innervation is observed to increase disease severity in AA rats (Lorton et al., 1996), a finding that is consistent with the protective role of the SNS demonstrated in other autoimmune disease models. Target-specific effects on different organ and tissue compartments likely explain these differences in disease outcome with whole body SympX compared with local 6-OHDA application into the lymph nodes. Specifically, sympathetic nerves that target sites other than the lymphoid organs are responsible for the attenuated affects, most likely sympathetic nerves supplying the skin (the site of challenge) or the affected joints. Collectively, these studies indicate a dual role for the SNS dependent on nerve target tissue-specific regulation. Since opposite effects on arthritis are seen with systemic sympathectomy, which destroys sympathetic innervation of secondary lymphoid tissues too, our data suggests that inhibiting sympathetically mediated events in the periphery (such as neurogenic inflammation) abrogates the detrimental effects of targeted immune organ sympathectomy on arthritis. Alternatively the significant but modest attenuation of arthritis with systemic sympathectomy may reflect the summing of both positive and negative effects of the SNS on multiple targets over the course of the disease.

The mechanisms responsible for our finding that targeted sympathectomy of secondary lymphoid organs prior to adjuvant injection exacerbates AA are not clear. Selective denervation may (1) alter antigen presentation and processing, (2) drive an aggressive cell-mediated effector immune response, (3) stimulate proinflammatory cytokine production by activated macrophages and recruit nonspecific T cell activation, and (4) impair downregulation of several regulatory elements (Lorton et al., 1999). In support of sympathetic effects being mediated via interaction with antigen-presenting cells (APC), several studies have shown that the SNS can affect their migration and ability to prime T_H1 lymphocytes (Panina-Bordignon et al., 1997; Haskó and Szabó, 1998; Maestroni and Mazzola, 2003).

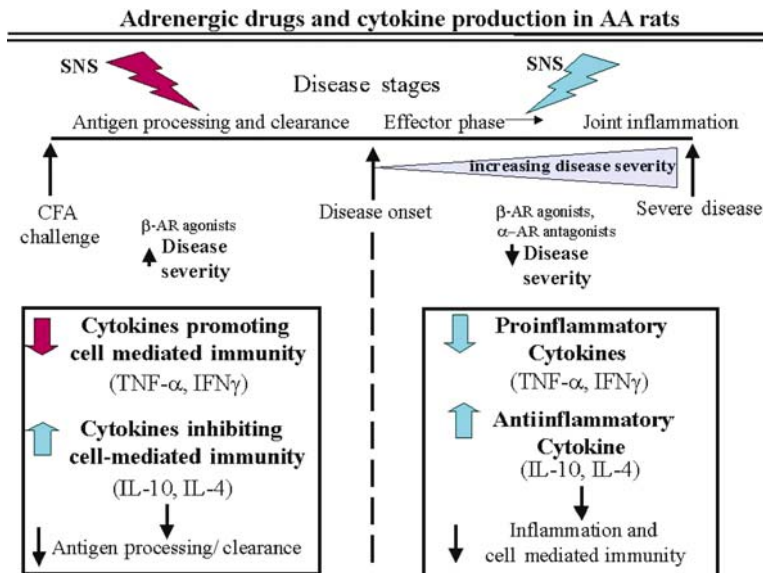
Similarly, the mechanisms by which NE exerts a negative effect on inflammation in the inflamed joints are not entirely understood. Sympathetic nerves in the synovium travel along the local vasculature of the joint capsule (Halata and Groth, 1976; Loren et al., 1976; Langford and Schmidt, 1983; Levine et al., 1986a). The SNS could alter joint inflammation by inducing changes in blood flow and promoting edema, and by reducing the clearance of destructive components from the joint capsule. The SNS also could target activated macrophages, lymphocytes, and polymorphonuclear leukocytes to promote inflammation through interaction of NE with AR expressed by these cells. Additionally synoviocytes, osteoblasts, and osteoclasts express AR and are potential targets of the sympathetic innervation of the joint. NE released from sympathetic nerves could signal these cell types to alter tissue metalloprotease production by synoviocytes and differentiation of osteoblasts to osteoclasts to promote cartilage and bone destruction.

5.2 SNS Manipulation in Arthritis Models—Time-Dependent Effects

There is conflicting data in the literature regarding the effects of β_2 -agonists on disease severity. Coderre et al. (1991) have reported that continuous infusion of EPI or salbutamol, a β_2 -agonist, starting 2 days before CFA challenge and continuing through day 28 after adjuvant immunization increased disease severity. In contrast, Malfait and coworkers (1999) have observed that treatment with salbutamol beginning at disease onset and continuing every other day for 10 days dramatically reduced disease severity of established collagen-induced arthritis (CIA) in mice. We have proposed that this discrepancy is due to the timing of adrenergic treatment relative to the disease stage, and that systemically blocking sympathetic neurotransmission prior to and after disease onset has directionally opposite effects on disease severity (► Figure 5-4). Indeed, we have found that administration of a β_2 -agonist, terbutaline, or an α -antagonist,

■ Figure 5-4

Different functions of the sympathetic nervous system (SNS) in regulating immune cell functions during different disease stages. Sympathetic activation during disease initiation stages modulates antigen processing and clearance by targeting functions of the innate and early events in acquired immunity. Increasing sympathetic activity during this stage would be expected to inhibit antigen processing and clearance by targeting naive immune cells involved in these processes (antigen-presenting cells, T_H0 cells). Sympathetic signaling to these cells drives cell-mediated immune responses in the face of a tissue antigen, and thus, increases disease severity due to inadequate antigen clearance of relevant tissue antigens. In contrast, after disease onset, increasing sympathetic activity targets activated macrophages to reduce production of proinflammatory cytokines and elevated production of antiinflammatory cytokines. Additionally, during this disease stage, sympathetic activation drives humoral-mediated immunity and dampens cell-mediated immunity at a time when cellular immunity results in tissue damage. It is through these actions after disease onset that the SNS functions to reduce disease severity



phentolamine, increases or decreases disease severity when given from the time of adjuvant injection or from disease onset, respectively (Lubahn et al., 2004). Similarly, sympathectomy prior to injection of collagen II to induce CIA markedly decreases arthritis severity whereas sympathectomy at later stages of the chronic disease, at day 55, markedly increases severity (Harle et al., 2005b). Collectively, these studies

support different functional roles for the SNS in inflammatory arthritis from the time between adjuvant immunization and disease onset (during antigen processing) and the time between disease onset and the chronic disease stage.

5.3 β -Antagonist Treatment and Disease Severity

Treatment of AA rats with nonspecific β - or specific β_2 -AR antagonists from disease onset through severe disease have yielded consistent decreases in disease severity (Levine et al., 1988; Felten et al., 2001). The mechanisms for the inhibitory effects of β -antagonists on disease severity are likely to differ during the initiation and effector phases of the disease. A decrease in disease severity with β -antagonist treatment during the initiation phase is consistent with effects of sympathectomy and the opposite effect of administering β -agonists on disease severity during this same time period. During the initiation phase, we are proposing that β -antagonists promote $CD4^+$ T_H0 cell development to T_H1 cells by blocking the inhibitory effects of NE released from sympathetic nerves during antigen challenge on T_H1 cell development. Antagonizing β -AR promotes a shifting of the immune response to the arthritogenic antigen toward a more robust cell-mediated response. This would be expected to lead to a more efficient processing and clearance of the tissue arthritogenic antigen. The beneficial effects on disease severity of administering a β -antagonist after disease onset is less easily explained given that stimulating β -AR after disease onset ameliorates disease severity. The beneficial effects observed after treatment with the β -antagonists may simply be due to the disease-ameliorating effects on antigen processing during the disease initiation stage, with the summing of negative effects after disease onset.

5.4 Role of α -AR in Disease Development

Although the role of α -AR in development of AA has not been well studied, there is some convincing evidence that this class of receptors plays a profound role in the development of AA. As for the role of β -AR, the effect of α -AR treatments on disease severity is dependent upon the disease stage when the treatments are administered. Treatment of AA rats with α - or α_2 -antagonists starting from adjuvant challenge and continued through development of severe disease are found to increase disease severity (Levine et al., 1988; Coderre et al., 1990, 1991; Lubahn et al., 2004). In contrast, if treated with phentolamine, a nonspecific α -AR antagonist, at disease onset, soft tissue swelling and joint destruction are significantly reduced (Lubahn et al., 2004). Consistent with these findings, yohimbine, a selective α_2 -antagonist, or prazosin, a selective α_1 -antagonist, administered to AA rats at disease onset and continued through severe disease also attenuates soft tissue swelling and joint destruction (Coderre et al., 1991; D. Lorton and C. Lubahn, unpublished observations). These data suggest that the effect of an α -AR antagonist is also dependent upon the disease stage in which the antagonist is administered.

The mechanisms for the α_2 - and α_1 -antagonist-mediated changes in disease severity are not clear. During the initiation and effector disease stages, α_2 -AR may target immune cells that express α_2 -AR to alter antigen processing within the secondary lymphoid organs and inflammation in the arthritic joints, respectively. Alternatively, the effects of α_2 -AR on disease outcome could be indirectly mediated through presynaptic α_2 -AR that would alter the availability of NE released from sympathetic nerves, which is then available for binding to β_2 -AR expressed by immune target cells. Both α_1 - and α_2 -antagonist may also affect immune cell migration from secondary lymphoid organs, homing of immune cells into the arthritic joints, and plasma extravasation and edema by targeting endothelial cells and vascular smooth muscle, which could impact disease severity.

6 Changes in AR Expression with Disease Development

Increases and decreases in sympathetic activity and catecholamine availability can up- and downregulate target cell expression of β -ARs. In RA patients and in experimental animal models of RA, data supports that

at least during some disease stages, sympathetic tone is elevated. The increase in sympathetic tone during these disease stages would be expected to downregulate β_2 -AR expression by target immunocytes. Similarly, the observed changes in sympathetic nerve density in white and red pulp compartments in the spleen in severe disease stages should alter the availability of NE for interaction with target cells and subsequently result in a downregulation of immune target cell β_2 -AR expression. Reduced β -AR expression and functional responses to catecholamines has been reported for PBMC collected from RA patients (Baerwald et al., 1992, 1997). Other studies have reported that the density of β -AR on PBMC are unchanged. However, in these studies they observe a decrease in the expression and activity of the G-protein-coupled kinase necessary for β_2 -AR-induced responses in PBMC of RA patients (Lombardi et al., 1999) and in splenocytes and mesenteric lymph node cells obtained from AA rats (Lombardi et al., 2001). Similarly, in PBMC from children with juvenile RA, stimulation of β_2 -AR with β_2 -agonists fails to induce an increase in cAMP (Lombardi et al., 1999), while PBMC obtained from age-matched children respond with an increase in intracellular cAMP. Interestingly, Xu and coworkers (2005) recently have reported that β_2 -AR gene single-nucleotide polymorphisms are observed in RA patients and are associated with disease activity and higher levels of rheumatoid factor. These studies indicate that there is a reduced capacity of the SNS to regulate immune functions of lymphocytes and monocytes via interaction of NE with β_2 -AR.

While α_1 -ARs are not expressed by circulating immunocytes under normal conditions, Heijnen and coworkers (1996) have observed α_1 -AR expression on peripheral blood monocytes obtained from juvenile RA patients. α_1 -ARs are not expressed on peripheral blood monocytes from healthy donors in this study. Treatment of the mononuclear cells with an α_1 -agonist increases production of IL-6 indicating that α_1 -ARs are functional and could contribute to chronic inflammation by promoting proinflammatory cytokine production (Heijnen et al., 1996). Monocyte α_1 -AR expression can be induced by chronic stimulation of β_2 -AR in vitro (Roupe van der Voort et al., 1999). If this occurs in vivo in RA and juvenile RA patients, the induction of α_1 -AR expression and the decrease in β_2 -AR expression on PBMCs in RA patients would be consistent with an increase in sympathetic activity in these patients. A sympathetically-mediated β_2 -AR to α_1 -AR expression shift on monocytes, and by extension this to macrophages, could have a profound effect on the production of proinflammatory cytokine production in RA and juvenile RA given that stimulation of β_2 -AR generally inhibits and α_1 -AR generally promotes production of proinflammatory cytokines by monocytes and macrophages. Studies that examine immunocyte AR subtype expression in RA and animal models of RA are required to explore this possibility.

7 Is There a Relationship Between Immune Dysregulation and SNS Dysregulation?

The imbalance between pro and antiinflammatory cytokine production and the robust cell-mediated immunity, which is characteristic of RA, drives the disease pathology (reviewed in Feldmann and Maini, 1999, 2001; Arend, 2001; Van Roon et al., 2002). The SNS is activated with increases in production of proinflammatory cytokines and robust cell-mediated immune responses. Normally the increase in sympathetic activity results in sympathetic signaling to immune cells. This sympathetic-to-immune cell signaling functions as a negative feedback loop to dampen proinflammatory cytokine production by macrophages and inhibit T_H1 cell development as the antigen is being cleared. The inhibitory effects of the SNS on T_H1 cell development are largely mediated through NE binding to β -AR expressed on immune cells (🔗 [Figure 5-1](#)). In contrast, sympathetic modulation of pro- and antiinflammatory cytokine production by macrophages occurs through NE interaction with either β_2 - or α -ARs or with both (Abrass et al., 1985; Spengler et al., 1990) (🔗 [Figure 5-2](#)). Selective activation of these AR populations elicits opposite effector functions, with β -AR stimulation being largely inhibitory and α -AR stimulation being largely excitatory. Numerous studies have demonstrated that β_2 -AR stimulation inhibits TNF- α and IL-12 and increases IL-10 production. In contrast, α_2 -AR stimulation increases TNF- α production (Severn et al., 1992; Monastra and Secchi, 1993; Spengler et al., 1994; van der Poll et al., 1994, 1996; Ignatowski and Spengler, 1995; Panina-Bordignon et al., 1997). The sympathetically-mediated negative feedback system under normal conditions is responsible for maintaining immune system homeostasis.

Proper functioning of this feedback system may be critical for returning immune system function to normal levels following an antigen exposure.

If the SNS is responsible for maintaining immune system homeostasis, why does the SNS fail to restore homeostasis of immune functions in RA? While this question cannot be completely answered at present, some conclusions can be ascertained from existing research. On the basis of studies which indicate that sympathetic tone is elevated in RA patients, it appears that the high levels of circulating proinflammatory cytokines present during active disease periods do signal appropriate CNS nuclei to activate this negative feedback loop. These studies suggest that the immune-to-SNS signaling arm of the feedback loop is functional. In contrast, studies demonstrating that sympathetic innervation of arthritic joints (Miller et al., 2000) and lymphoid compartments of secondary lymphoid organs (Lorton et al., 2005) is lost or reorganized in chronic disease stages suggests that sympathetic-to-immune target cell signalling is impaired in RA. In RA, the increase in sympathetic activity associated with production of proinflammatory cytokines would be expected to increase NE turnover, with an increase in NE released, which is then available to interact with immune cells. A chronic increase in sympathetic activity is likely to alter immune cell AR expression, since β -AR expression is normally down- and upregulated in the presence of high and low levels of NE exposure, respectively. An increase in turnover also may be responsible for destruction of sympathetic terminals within the white pulp of the spleen due to an increase in oxidative stress with increased NE production and degradation. The sprouting of NA nerves into the red pulp of the spleen would increase the availability of NE in this compartment to interact with activated immune cells within this compartment and thus, β -AR expression as well. The downregulation of β -AR expression on PBMC in RA patients has been demonstrated, but whether the decreased β -AR is due to altered NE availability has not been explored. The changes in the sympathetic innervation and β -ARs expressed on immunocytes suggest a reduced capacity of the SNS to regulate immune functions of lymphocytes and monocytes/macrophages through NE interaction with β_2 -ARs in RA patients. This reduced capacity of the SNS to regulate T_H cell development and monocyte/macrophage functions is likely to contribute to the robust cell-mediated immune response within the arthritic joints and to the imbalance between cellular- and humoral-mediated immunity that characterize this disease.

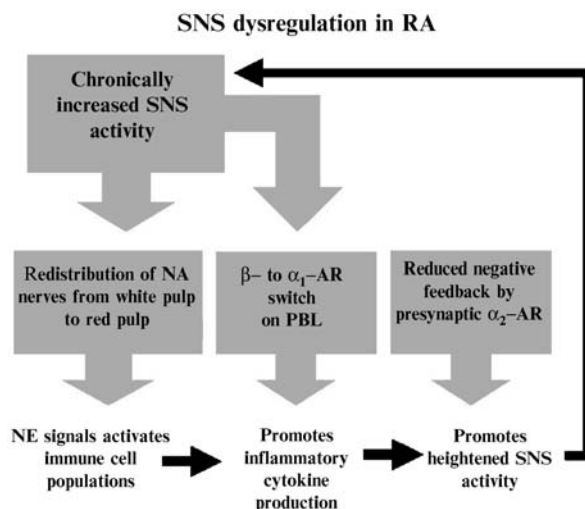
Chronic stimulation of β_2 -AR expressed on monocytes or macrophages would also be expected to contribute to disease pathology. Initially, stimulation of β_2 -AR would be expected to reduce production of proinflammatory cytokines, such as TNF- α and IL-12, which are important in development of an appropriate cell-mediated immune response in the face of a tissue antigen challenge. If this occurs during the induction stage of the disease this would be expected to reduce the capacity of the cell-mediated immune response to adjuvant challenge to clear an antigen that is already difficult to clear. This may be why treatment of AA rats with β -agonists during the induction stage of the disease results in increased disease severity. This hypothesis needs to be tested.

Chronic stimulation of β_2 -AR would be expected to also occur after disease onset and through severe disease development since proinflammatory cytokines are produced in high levels in the inflamed joints. The chronic stimulation of β_2 -AR during these disease stages would be expected to initially inhibit production of proinflammatory cytokines that drive the disease pathology. This may explain why treatment with β_2 -agonist starting from disease onset and continued through the development of severe disease reduces disease severity (Lubahn et al., 2004). However, chronic stimulation of β_2 -AR could also negatively impact disease pathology by inducing the expression of macrophage α -AR, especially α_1 -ARs, which are abnormally induced on circulating monocytes in RA patients (Heijen et al., 1996). Repeated stimulation of α -AR with the increase in sympathetic activity being driven by the presence of proinflammatory cytokines from the joints would be expected to further promote proinflammatory cytokine production, perhaps setting up a feed-forward rather than the normal negative feedback role of the SNS (Figure 5-5). This may explain why treatment with α -AR antagonists has a beneficial effect when administered from disease onset through severe disease development. While there is some limited evidence to support this hypothesis at present, more research is necessary to prove whether these hypotheses are correct.

While sympathetic denervation and β -agonist treatments significantly alter disease severity as described above, the changes are not dramatic given the critical role the SNS plays in regulating immune functions. The dual positive and negative effects of β_2 -AR stimulation during the disease expression period (decreasing

■ Figure 5-5

Flow diagram of the proposed consequences of chronically increased SNS activity for sympathetic nerve integrity and distribution and immunocyte expression of adrenergic receptors (ARs) in lymphoid targets, which promotes proinflammatory cytokine production in RA patients. Proinflammatory cytokines then promote heightened SNS activity to set up a feed forward loop



proinflammatory cytokine production and inducing macrophage α -AR expression, respectively) could explain why sympathetic denervation and β -AR agonist treatments do not have dramatic effects. After considering reported changes in AR density (Baerwald et al., 1992, 1997, 2000), shifts in immune cell AR subtype expression (Heijnen et al., 1996; Rouppe van der Voort et al., 1999), and effects of AR drugs during different disease stages (Coderre et al., 1991; Malfait et al., 1999; Lubahn et al., 2004), we have been able to develop an adrenergic treatment that dramatically reduces disease severity (Lorton et al., 2003; Lubahn et al., 2004). Lewis rats were administered a β_2 -AR agonist and an α -AR antagonist (SH1293) at disease onset (day 12) and continued through 28 days postadjuvant challenge to induce AA. SH1293 treatment significantly decreases paw swelling in arthritic rats by 50% compared with vehicle-treated arthritic rats (▶ Figure 5-6). SH1293 treatment results in a 60–70% reduction in X-ray scores in arthritic rats compared with vehicle-treated arthritic rats. These data indicate the importance of the SNS in the disease pathology and the need for a better understanding of the dynamic SNS changes occurring at different disease stages. Our findings using SH1293 indicate both β_2 - and α -ARs mediate SNS effects in inflammatory arthritis.

8 Can Adrenergic Agents Restore Immune System Homeostasis in RA?

8.1 Cytokine Imbalance in RA and Current Treatment Strategies

In RA, the balance of pro- to antiinflammatory mediators is shifted, such that the proinflammatory cytokines produced by macrophages are predominant (▶ Table 5-1). Cytokines that promote cell-mediated immune responses also are produced to a greater extent than those that promote humoral immune responses. Studies show that shifting the cytokine profiles of T_H0 cells toward T_H2 cells (promoting humoral immunity) or neutralizing TNF- α or IL-1 production after disease onset significantly reduce disease severity in experimental animal models (Feldmann and Maini, 1999, 2001; Hossain et al., 2001;

■ Figure 5-6

Radiographs of hind limbs taken 28 days postimmunization from adjuvant-induced arthritic rats treated with twice-daily injections of vehicle, terbutaline, phentolamine, or SH1293 initiated at disease onset. A radiograph representative of the ankle joint from a nonarthritic rat (Non-AA) is included for comparison. Destructive joint damage as indicated by reduced soft-tissue shadows, decreased osteoporosis (increases in bone luminescence), erosions (increased bone integrity), cartilage loss joint space narrowing), and decreased periosteal bone formation (bone formation in the surrounding soft tissue) compared with vehicle-treated arthritic animals



Salomon et al., 2002; Van Eden et al., 2002; Van Roon et al., 2002; Mauri et al., 2003). However, therapies that have targeted T cells have provided disappointing results clinically. At present, the most promising treatments for RA patients in the clinic are TNF- α neutralizing antibody treatments or therapies that inhibit TNF- α or IL-1 binding to their specific receptors (reviewed in Feldmann and Maini, 2001). In order to explore the potential use of AR drugs that regulate immune functions for treating RA patients, it is first necessary to provide a brief summary of current RA treatments that are based on the role of proinflammatory cytokines, such as TNF- α in RA. An assessment of the potential benefits of AR drugs that may restore the SNS's regulatory role in immune system homeostasis will follow this summary.

8.2 Anti-TNF- α Therapies

The rationale for using anti-TNF- α therapies in RA patients is based on studies demonstrating that TNF- α blockade reduced IL-1 production in RA synovial cell cultures (Brennan et al., 1989) and on the presence of TNF- α in the RA synovial membrane and cartilage-pannus junction (Chu et al., 1991, 1992). Subsequently, studies have shown that anti-TNF- α antibodies given systemically reduced clinical and histopathological signs of both CIA and AA in mice and rats (Piguet et al., 1992; Thorbecke et al., 1992; Williams et al., 1992; Wooley et al., 1993). Transgenic mice that overexpress human TNF- α were found to spontaneously develop a severe erosive arthritis that is prevented by treatment with antihuman TNF- α monoclonal antibodies, lending further support for TNF- α based therapies (Keffer et al., 1991). Anti-TNF- α antibody therapy also reduces IL-1 and IL-6 production in an animal model of sepsis (Fong et al., 1989), which supports the notion that blocking TNF- α alters the expression of other proinflammatory cytokines. Thus, a clear benefit of TNF- α inhibition is demonstrated to reduce proinflammatory cytokine production and decrease disease severity in RA animal models.

Presently, drugs in clinical trials or practice to block TNF- α are biological, protein-based drugs, either monoclonal anti-TNF- α antibodies or soluble TNF-receptor (TNF-R) Fc fusion proteins (e.g., infliximab (Remicade), Centocor; and etanercept (Enbrel), Amgen). Randomized, placebo-controlled, multicenter clinical trials of human TNF- α inhibitors have demonstrated their consistent efficacy in controlling inflammation and joint damage in approximately two-thirds of patients tested for up to 2 years (reviewed in Feldmann and Maini, 2001). Clinical results are notable, with patients reporting alleviation of pain, stiffness, tiredness, and lethargy within hours, decreased numbers of swollen and tender joints within 2 to 4 weeks,

■ Table 5-1

Cytokine production by synovial macrophages and T cells in joints of RA patients illustrating that macrophages are the largest producers of cytokines within arthritic joints. Cell source and levels of cytokines produced in synovium in rheumatoid arthritis

*Macrophages/^Fibroblasts		T lymphocytes	
Cytokine	Production in RA synovium	Cytokine	Production in synovium
TNF- α	$\uparrow\uparrow^{a,b}$	IL-2	$\downarrow\downarrow^c$
IL-1	$\uparrow\uparrow^{a,b}$	IL-3	$\downarrow\downarrow^c$
IL-1ra	$\uparrow\uparrow^d$	IL-4	$\downarrow\downarrow^{c,e}$
IL-6	$\uparrow\uparrow^f$	IL-6	$\downarrow\downarrow^f$
IL-10	$\uparrow\uparrow^{g,h}$	Interferon- γ	$\downarrow\downarrow^f$
M-CSF	$\uparrow\uparrow^c$	GM-CSF	$\downarrow\downarrow^f$
GM-CSF	$\uparrow\uparrow^a$	TNF- α	$\downarrow\downarrow^c$
Interferon- α	$\uparrow\uparrow/\downarrow\downarrow^i$	TNF- β	$\downarrow\downarrow^e$
TGF- β	$\uparrow\uparrow^a$		
Fibroblast growth factor	$\uparrow\uparrow^j$		
Chemokines (IL-8, MCP-1, etc)	$\uparrow\uparrow^{k,l}$		

*Macrophages refer to tissue macrophages or type A synoviocytes

^Type B synoviocytes or tissue fibroblasts

$\uparrow\uparrow$ Present

$\downarrow\downarrow$ Absent or present in very low concentrations

Abbreviations: TNF, tumor necrosis factor; TGF, transforming growth factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; M-CSF, macrophage colony-stimulating factor; mcp, monocyte chemoattractant protein

^aChu et al. (1992)

^bFirestein et al. (1990)

^cFirestein et al. (1988)

^dSeitz et al. (1994)

^eMiossec et al. (1990)

^fChen et al. (1993)

^gCush and Lipsky (1988)

^hKatsikis et al. (1994)

ⁱFrucht et al. (2001)

^jMelnyk et al. (1990)

^kKoch et al. (1992)

^lLoetscher et al. (1994)

and long-term protection against progressive joint destruction (Fong et al., 1989; Maini et al., 1998, 1999; Weinblatt et al., 1999; Kavanaugh et al., 2000; Lipsky et al., 2000). Thus, the concept that TNF- α is an effective therapeutic target in RA has been validated and other therapeutics that target TNF- α are under development.

8.3 Potential Benefits of Sympathetic Drugs in Treating RA over Anti-TNF- α Drugs

8.3.1 The SNS Regulates TNF- α Production in AA

The role of ARs in regulating macrophage TNF- α production in streptococcal cell wall (SCW)-induced arthritis has been explored (Chou et al., 1996b, 1998). Treatment of SCW-induced arthritis with the β -agonist, isoproterenol, reduces LPS-induced TNF- α release from peritoneal macrophages obtained from

arthritic rats (Chou et al., 1996b). Treatment with the β -antagonist, propranolol, facilitates LPS-stimulated TNF- α production in arthritic and nonarthritic rats; however, peritoneal macrophages from arthritic rats are much more sensitive to the β -AR antagonist-induced increases in TNF- α production (Chou et al., 1998). Macrophage treatment with the α_2 -antagonist, idazoxan, inhibits LPS-induced TNF- α production in arthritic and nonarthritic rats; however, inhibition of TNF- α production is much greater in macrophages from arthritic rats (Chou et al., 1998). Salbutamol, a β_2 -agonist, also is found to reduce TNF- α release from peritoneal macrophages obtained from CIA mice (Malfait et al., 1999). These findings provide support that TNF- α production by activated macrophages is regulated by β - and α -ARs.

To examine SNS effects on balancing the production of pro- and antiinflammatory cytokines in our laboratory, CFA-challenged rats are treated with terbutaline, a β_2 -agonist, phentolamine, an α -antagonist, or SH1293. Treatment is started at disease onset and continues through severe disease (day 28 post-CFA challenge). On day 28, spleen and draining lymph node cells are harvested and cultured without further mitogen or antigen stimulation to evaluate pro- and antiinflammatory cytokines production. Terbutaline, phentolamine, or SH1293 treatment started at disease onset, which reduces disease severity, shifts the production of pro- and antiinflammatory cytokines toward an antiinflammatory profile in spleen (▶ [Table 5-1](#)) and draining lymph nodes (data not shown) as indicated by differences in the ratios of IL-10 to TNF- α production. These AR drug treatments also shift T_H cytokine production toward a T_H2 profile as indicated by the ratios of IL-4 to IFN γ production. The observed shift in cytokine profiles support the fact that these AR drugs, if given after disease onset, promote a balancing of pro- to antiinflammatory cytokine production and of cell- to humoral-mediated immunity in secondary lymphoid organs. These shifts in immune functions would be expected to decrease disease severity and support the potential benefits in using adrenergic drugs for treatment of RA. In contrast to these AR drugs, current anti-TNF- α treatments result in low-level production of antiinflammatory cytokines in RA patients, suggesting sympathetic drugs that target β - and α -AR may have benefits over the anti-TNF- α therapies.

8.3.2 Potential Advantages of SNS Regulation of Immune Functions

Targeting multiple pro- and antiinflammatory cytokines that are involved in disease pathology may prove more beneficial than TNF- α -based therapies that reduce TNF- α but also decrease production of antiinflammatory cytokines. Support for treatments that target pro- and antiinflammatory cytokines is provided by studies demonstrating that combining anti-TNF- α therapies with IL-10 or IL-1Ra in murine CIA and rat AA models, respectively, reduce joint pathology to a greater extent than anti-TNF- α therapy alone (Walmsley et al., 1996; Feige et al., 1999, 2000). In SCW-induced arthritis, combined IL-10/IL-4 treatment reduces TNF- α and IL-1 β levels in the synovium (Joosten et al., 1997; Lubberts et al., 1998). Further, the balance between IL-10 and IL-12 for regulation of T_H1 and T_H2 responses seems to be critical for disease development, with IL-10 suppressing and IL-12 increasing TNF- α and IFN γ production in the synovium (Yin et al., 1997). The administration of cytokines that downregulate macrophage function, e.g., IL-4 (van Roon et al., 1995; Horsfall et al., 1997; Joosten et al., 1997, 1999; Kim et al., 2001) or IL-13 (Bessis et al., 1998, 1999; Woods et al., 2000, 2002), reduces joint inflammation and destruction. If the pathogenesis of RA is due to an imbalance in pro- and antiinflammatory cytokine production and a dysregulation in cellular and humoral immunity as animal and human studies indicate, it suggests that there may be an inability of the SNS to maintain homeostasis of the immune system in this disease. Under normal conditions, the SNS critically regulates the balance between cellular- and humoral-mediated immunity and macrophage pro- and antiinflammatory cytokine production for maintenance of homeostasis. SNS dysfunction may account for the SNS's inability to reestablish immune system homeostasis in RA patients. It is possible that AR treatments could be developed to correct this SNS dysfunction, and thereby restore immune system homeostasis in RA patients.

Anti-TNF- α treatment is not effective in all RA patients. One-third of the patients treated with anti-TNF- α do not respond to this treatment, supporting the need for other alternative treatments. Another disadvantage of anti-TNF- α therapy is that these treatments have to be repeatedly injected or infused to sustain their beneficial effects (Tugwell, 2000). The reason why repeated long-term treatment with

anti-TNF- α therapies is necessary to sustain beneficial effects in RA patients is that infliximab and etanercept only remove the TNF- α that is produced, but do not inhibit the synthesis or release of TNF- α from immune cells. Thus, when the treatments are stopped, synovial and circulating TNF- α again increases and the disease progression starts again. Additionally, potent antiinflammatory immune mediators, serum IL-1Ra, soluble TNF-R, and IL-10, are diminished after infliximab treatment (Charles et al., 1999). Thus, the increase in TNF- α production, after treatment is stopped, is accompanied by reduced production of antiinflammatory mediators necessary to reduce inflammation and restore a balance of pro- to antiinflammatory cytokine production. In contrast, β_2 -agonists and α -antagonists inhibit macrophage production of TNF- α and increase antiinflammatory cytokine production (e.g., IL-10, IL-4), resulting in a balancing of the pro- and antiinflammatory cytokine levels. It is likely that with AR drug treatments that restore homeostasis, long-term therapy may not be necessary for continued prevention of joint destruction. If a short course of AR drugs produces long-term decreases in TNF- α synthesis through direct inhibition of TNF- α production and indirectly by balancing pro- to antiinflammatory cytokine production, short-term AR therapy may be all that is needed to balance cytokine production over a considerable period of time. Collectively, these findings support that targeting the SNS to treat RA would have the benefits of altering the production of multiple pro- and antiinflammatory cytokines and cytokines that dampen cell-mediated immunity while promoting humoral-mediated immunity.

9 Conclusion

The SNS can enhance or inhibit immune reactivity through release of its primary neurotransmitter, NE, and through NE interaction with ARs expressed on immune cells. Manipulation of the SNS may be useful in modulating production of cytokines critically involved in RA, to restore “cytokine balance” necessary to prevent joint pathology. While sympathetic denervation and treatment with β - and α -AR agonists and antagonists significantly alter disease severity as described above, the resulting changes have not been dramatic considering the critical role of the SNS in modulating immune function. This is likely to be because of the lack of understanding of the dynamic changes in sympathetic activity, in the AR subtypes expressed on target cells, and in AR and second messenger coupling or activation that occur during different disease stages, and the role of cotransmitters in sympathetic nerve terminals. Altered sympathetic innervation of spleen and lymph nodes and striking changes in disease outcome after treatment with adrenergic drugs that target both β - and α -AR in AA rats support an important role for the SNS in RA, particularly in regulating bone and cartilage destruction in affected joints. Once an understanding of the temporal and spatial SNS-to-immune cell signaling changes that occur in arthritic joints and lymphoid organs in RA over the disease course is gained, rational development of adrenergic drug treatments to reduce joint destruction in RA patients is a possibility. Clearly, additional data are required to devise logical strategies for clinical interventions that will restore immune functions in RA patients. These types of studies are essential for designing therapeutic approaches for clinical interventions aimed at ameliorating joint destruction and restoring the balance between innate-, humoral-, and cellular-mediated immune functions in RA patients. An added benefit of using adrenergic-based treatments for RA is that α -antagonists that do not cross the blood-brain barrier can be used that also inhibit nociception. This chapter underscores the importance of increasing our understanding of SNS modulation of immunity in order to develop new strategies for the treatment of RA.

While this chapter has focused on the SNS, glucocorticoids also modulate immune functions in a similar manner as the SNS. Dysregulation of HPA-axis pathways involved in control of glucocorticoid pathways occurs in RA patients and is likely to contribute to the dysregulation of immune functions observed in RA patients. Further, synergistic effects of catecholamines and glucocorticoids on immune functions have been reported. Thus, while treatments that target one stress pathway may provide relief from inflammatory arthritis, targeting both pathways may prove more beneficial. This approach is already being used in the treatment of asthma with favorable results. Finally, while this review has focused on RA, other autoimmune diseases exhibit strikingly similar changes in sympathetic and immune dysfunction as RA. This suggests that there may be similar changes in the ability of the SNS to modulate immune functions in

other autoimmune diseases that promote disease pathology and that the SNS may be a target for therapeutic intervention in other autoimmune diseases.

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6 Neuro-Immune Associative Learning

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1	<i>Introduction</i>	124
1.1	Cross Talk between Central Nervous System and Immune System	124
1.2	Classical Conditioning	125
1.3	Neuroimmune Associative Learning	125
1.4	Historical Overview	126
2	<i>Evoking Neuroimmune Associative Learning Responses</i>	127
3	<i>Conceptual Framework for Neuroimmune Associative Learning</i>	129
3.1	Association Phase	129
3.2	Recall Phase	129
4	<i>Principles of Neuroimmune Associative Learning</i>	131
4.1	General Learning Rules of Neuroimmune Associative Learning	131
4.2	Features of Neuroimmune Associative Learning	132
5	<i>Mechanisms of Neuroimmune Associative Learning</i>	133
5.1	Neurobiology of Association and Recall	133
5.2	Peripheral Mediation of the Conditioned Effects on Immunity	137
6	<i>Clinical Implications</i>	139
6.1	Animal Models	139
6.2	Human Studies	139
7	<i>Methodological Considerations for Neuroimmune Associative Learning Experiments</i>	141
8	<i>Closing Remarks</i>	142

Abstract: Neuroimmune associative learning constitutes the concomitant presentation of a neutral stimulus (conditioned stimulus, CS) and an immunomodulating agent (unconditioned stimulus, US). Representation of the CS alone is able to activate the centrally stored engram often resulting in changes in immune parameters that mimic those formed by the US (conditioned immune response). This experimental model enables to study and dissect the afferent (at association) as well as efferent (at recall) pathway of brain-immune communication. In this chapter, we will introduce this behavioral conditioning paradigm, review the available literature focusing on features of the conditioned and unconditioned stimuli, and the conditioned responses, the so far known mechanisms steering the conditioned response, and the underlying learning principles. Moreover, a theoretical framework modeling the pathways as well as the guidelines how to design neuroimmune associative learning experiments will be provided. Finally, the clinical feasibility of this behavioral approach will be discussed as a supplement to standard therapies with the aim of optimizing individual healing conditions.

List of Abbreviations: CRs, conditioned responses; CTA, conditioned taste avoidance; SRBCs, sheep red blood cells; 6-OHDA, 6-hydroxydopamine

1 Introduction

Clinical observations and experimental evidence demonstrate the intensive bidirectional communication between the central nervous system (CNS) and the immune system (for review, see Elmquist et al., 1997; Straub and Schedlowski, 2002; Tracey, 2002; Dantzer, 2004; Glaser and Kiecolt-Glaser, 2005). We termed the process of sensing and encoding immune inputs to the CNS, and further associating them with the memory traces of exteroceptive clues as *neuroimmune associative learning*. Retrieving such engrams may result in a complex repertory of physiological responses affecting neurobehavioral, endocrine, as well as immune parameters. Furthermore, neuroimmune associative learning paradigms can be employed to experimentally study the principles by which the immune and the nervous system exchange information.

1.1 Cross Talk between Central Nervous System and Immune System

One put a lymphocyte into a culture dish, added an antigen, and out came an antibody. So who needed a nervous system? (Spector, 1996).

The paradigm of total independence of the immune system has been dismissed by experimental evidence accumulating mainly during the last three decades. Here are only some relevant issues related to the present topic enlisted.

A whole series of neuropeptides, neurotransmitters, and neuroendocrine hormones are endogenously produced by immune cells (for review, see Blalock, 1994; Tayebati et al., 2002; Warthan et al., 2002) and many cytokines are found to be produced and have significant biological activity in the central and peripheral nervous system (Blalock, 2005).

1. Stimulation or silencing of distinct brain areas affects immune functioning by different mechanism (for a detailed review, see Meisel et al., 2005; Wrona, 2006; Ziemssen and Kern, 2007).
2. There is evidence for rich neural connections with lymphoid tissue (Steinman, 2004; Straub, 2004), and receptors for neurotransmitters are also present on lymphocytes.

Immune-to-brain pathway: On the afferent pathway, peripheral immunological changes are signaled to the CNS, and different ascending pathways (neural and humoral) have been identified. The vagus nerve provides the major neural pathway identified to date. The initial chemosensory transduction events occur in immune cells, which respond to specific chemical components expressed by dangerous microorganisms. These immune chemosensory cells release mediators, such as cytokines, to activate neural elements, including primary afferent neurons of the vagal sensory ganglia. Primary afferent activation initiates local reflexes (e.g., cardiovascular and gastrointestinal) that support host defense (Goehler et al., 2000).

This neural afferent pathway is complemented by a humoral afferent pathway that involves cytokines or other immunotransmitters transported in the blood or produced at the level of the circumventricular organs. It is possible that such immunotransmitters cross the blood–brain barrier or originate a second wave of cytokines produced in the brain parenchyma (Banks, 2006). Depending on their source, these locally produced cytokines can either activate neurons that project to specific brain areas or diffuse by volume transmission into the brain parenchyma to reach their targets. Activation of neurons by cytokines can be direct or indirect. The way the neural pathway of transmission interacts with the humoral pathway remains to be elucidated; however, it has been proposed that each pathway may engage in different conditions (e.g., localized vs. systemic infections), and thus codify different information (Dantzer et al., 2000).

Brain-to-immune pathway: There are three main pathways: the hypothalamo–pituitary–adrenal (HPA) axis (humoral), the sympathetic–adrenal–medullary axis and the parasympathetic nervous system including the vagus nerve (both neural). Activation of the HPA axis results in the production of glucocorticoid hormones and catecholamines (Meisel et al., 2005), regulating cytokine balance (for review, see Ziemssen and Kern, 2007) and vice versa. The sympathetic nervous system regulates immunity by innervation of lymphoid organs and the release of noradrenaline, and a hormonal component that regulates immunity systemically through the release of adrenaline from the medulla of the adrenal glands (Sternberg, 2006). Coupling of the sympathetic nervous system and the HPA axis leads in the spleen to stronger effects through activation of β -adrenoceptors and glucocorticoid receptors (Straub, 2004).

In summary, the CNS and immune system intensively and extensively interact, sharing pathways, messengers, and their receptors.

1.2 Classical Conditioning

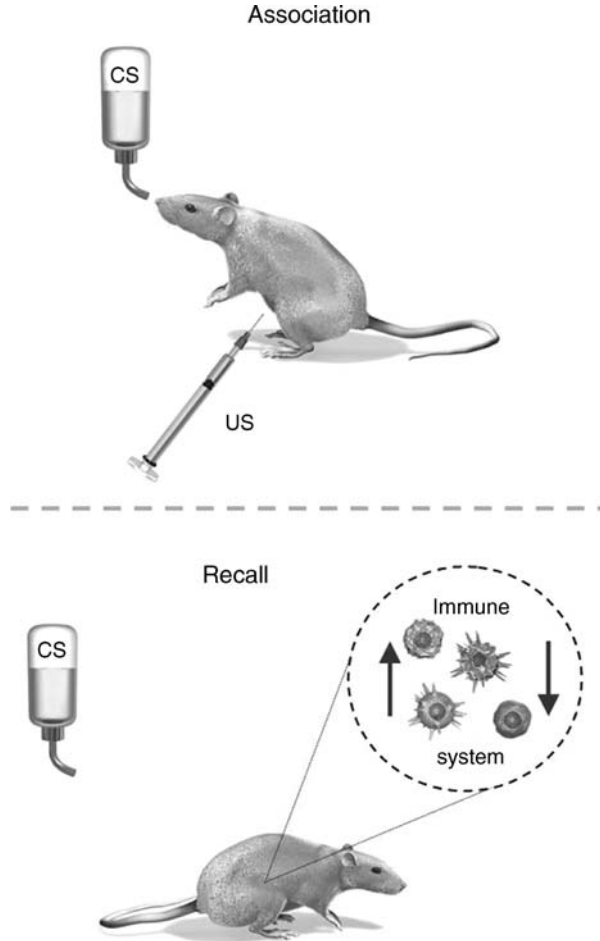
Since the CNS has the capability to alter the activity of the immune system as well as of many other organs/systems, it can be hypothesized that behavioral approaches such as classical conditioning may be able to modulate immune functions. Any paradigm of associative learning or classical conditioning comprises two basic phases: *association* and *recall*. During association phase, a neutral stimulus (to become a conditioned stimulus: CS) is paired with an unconditioned stimulus (US), which elicits vigorous responding (unconditioned response, UR) (Domjan, 2005). Contingent pairing(s) of the CS and the US leads to the establishment of a temporal/causal association of both stimuli, stored within the CNS. This learned association can then be identified during recall by the emergence of new responses to the CS, which can be now presented in the absence of the US. These new responses are termed conditioned responses (CRs) and they usually mimic the URs.

1.3 Neuroimmune Associative Learning

During an association trial, a gustatory/olfactory stimulus as a CS (e.g., saccharin solution; taste, chocolate milk; flavor, or camphor; odor) precedes the administration of an immunomodulating agent, that is, US (e.g., cyclosporine A, cyclophosphamide, polyinosinic: polycytidylic acid or antigens). Paired administration of these stimuli leads to a CS–US association. During recall, the CS is able to produce some effects, formerly ascribed just to the immunomodulating compound (US) (Figure 6-1). This phenomenon implies that the total effects of a drug are composed of pharmacological effects per se plus potential CRs (Pacheco-López et al., 2006). Neuroimmune associative learning has been reported employing visual and/or auditory stimuli such as a light or a tone as CS (MacQueen et al., 1989; Palermo-Neto and Guimaraes, 2000; Irie et al., 2002; Costa-Pinto et al., 2005), or touch stimuli such as scratching or heating of the skin as CS (Metal'nikov and Chorine, 1926; Nicolau and Antinescu-Dimitriu, 1929). However, the naturalistic relation of postprandial immunotoxicological consequences facilitates neuroimmune associative learning that employs gustatory/olfactory clues as CS. It contributes to the feasibility, duration, and magnitude of the association. Therefore, it is not surprising that stimuli causing gastrointestinal irritation get more easily

■ Figure 6-1

Neuroimmune associative learning. During association, animals are exposed to a conditioned stimulus (CS) paired with an immunomodulating unconditioned stimulus (US). During recall, the CS is presented without the US, evoking the formerly learned association. This is often accompanied by avoidance behavior and a complex physiological response that may also affect peripheral immune functions



associated with the postprandial taste/odor memories (e.g., after saccharin intake) than with an acoustic or visual stimulus, since under natural circumstances the former are more likely to become associated (Domjan et al., 2004).

1.4 Historical Overview

The first documented cases of behavioral conditioned immune effects are of anecdotal character and date back to the late nineteenth century. Mackenzie (1886) reported a patient who was suffering from a severe coryza at the sight of an artificial rose, which the presence of natural roses invariably produced in her case. Others reported similar findings in that environmental stimuli that have been associated with an allergen in the past provoke allergic symptoms in sensitive patients by a picture of a hay field (Hill, 1930, cited in Ader and Cohen, 1992).

The first scientific reports of classically conditioned immune functions arose from Russian scientific contemporaries of I. P. Pavlov. Lukyanenko (1961) cites an observation of Makukahin (1911) and a report by Voronov and Riskin (1925) as possibly the first ones to demonstrate “*conditioned leukocytic reactions*.” However, according to Luk’yanenko they could not be interpreted properly and were not carried forward. In the 1920s, Metal’nikov and his colleagues at the Pasteur Institute in Paris, France systematically documented that an immune response could be elicited without the presence of an antigen but just by evoking a previous experience, that is, CS exposure. After scratching or heating the skin of guinea pigs with a warmed metallic plate as CS, the animals received intraperitoneal (i.p.) injections of various bacteria-derived compounds such as small doses of either *Bacillus anthrax*, a *Staphylococcus* filtrate, tapioca emulsion, or *Vibrio cholera* as US. After association phase and a delay to allow the return to baseline levels was completed, the CS alone yielded significant and rapid influx of polymorphonuclear leukocytes into the peritoneal cavity (Metal’nikov and Chorine, 1926, 1928). As a proof of the biological relevance of the elicited conditioned response on the immune system, it was reported that conditioned animals survived to lethal doses of *V. cholera* or *Streptococcus* if they were previously behaviorally evoked. Follow-up experiments replicated and extended these findings (Nicolau and Antinescu-Dimitriu, 1929; Ostrovskaya, 1929). Additional evidence occurred largely provided by A. O. Dolin in the Soviet Union and his fellows Krylov, Flerov, and Luk’yanenko. Both specific and nonspecific immune reactions and both immunosuppression and immunoenhancement due to conditioning were achieved in mice, rats, guinea pigs, rabbits, dogs, oxen, monkeys and also in humans (Dolin and Krylov, 1952; Doroshkevich, 1954; Vygodchikov, 1955). At the same time, but less recognized, reports from Romania showed a conditioned increase in phagocytic activity of blood polymorphonuclear cells in dogs (Benetato, 1955; Baciú et al., 1965).

In addition, a series of reports published in Switzerland documented conditioned asthma-like response by using an auditory CS in guinea pigs (Noelpp and Noelpp-Eschenhagen, 1951a–c, 1952a–c). Such findings were replicated by Ottenberg et al. (1958), and extended to humans (Dekker et al., 1957; Turnbull, 1962).

In 1975, Ader and Cohen published their seminal work in which they constructed the term behaviorally conditioned immunosuppression, which retrospectively set the stage for the field of psychoneuroimmunology.

During that time, R. Ader was working on extinction of conditioned taste aversion employing saccharin taste as CS paired with a drug that induced significant visceral malaise. He realized that some rats of the conditioned group died during the course of a series of extinctions trials (i.e., CS representation). Moreover, those animals had received the largest of three different amounts of saccharin and displayed the most pronounced conditioned taste avoidance (CTA) behavior (Ader, 1974). Knowing the additional immunosuppressive properties of the drug employed as US, cyclophosphamide: CY, Ader and his colleague, immunologist N. Cohen, tested the hypothesis that the increased mortality on the conditioned animals during extinction phase was related to a compromised immune status resulted from a conditioned immunosuppressive response. This hypothesis was systematically assessed and confirmed showing that rats receiving paired administration of saccharin and CY not only displayed a strong CTA but also a reduced production of antibody titers on challenge with sheep red blood cells (SRBC) (Ader and Cohen, 1975). This initial finding was verified by independent laboratories basically under the same conditions (i.e., Rogers et al., 1976; Wayner et al., 1978), and further extended and elaborated (Ader et al., 1982).

2 Evoking Neuroimmune Associative Learning Responses

As a synopsis, [Table 6-1](#) is provided summarizing several neuroimmune associative learning protocols in which immune functions are evaluated after recall phase.

Different antigens have been applied as US eliciting conditioned immunoenhancement effects, for example, T-dependent antigens such as ovalbumin (Chen et al., 2004; Huang et al., 2004), keyhole limpet hemocyanin (Ader et al., 1993), T-independent antigens such as lipopolysaccharides (Bull et al., 1991), or superantigens such as staphylococcal enterotoxin (Pacheco-Lopez et al., 2004), as well as viral synthetic patterns such as poly I:C (Solvason et al., 1993; Coussons-Read et al., 1994; Demissie et al., 1995; Hsueh et al., 1995, 1999, 2002; Kuo et al., 2001; Chao et al., 2005). On the other hand, immunosuppressive drugs

Table 6-1
Overview of conditioning protocols affecting immunity

Conditioned response	Unconditioned stimulus	Conditioned stimulus	Species	References
NK-cell activity ↑	Poly I:C	Camphor odor	Mouse	Hsueh et al. (1995, 1999, 2002); Kuo et al. (2001)
NK-cell activity ↑	Interferon-β	Camphor odor	Mouse	Solvason et al. (1993)
NK-cell activity ↑	Arecoline	Camphor odor	Mouse	Demissie et al. (1995)
Neutrophil activity ↑	Poly I:C	Camphor odor	Mouse	Chao et al. (2005)
Antibody response ↓	Cyclophosphamide	SAC	Rat	Ader et al. (1975, 1979); Cohen et al. (1979)
Antibody response of IgM and IgG isotypes ↑	Hen egg-white lysozyme	SAC	Rat	Alvarez-Borda et al. (1995); Madden et al. (2001)
Antibody response and T-lymphocyte proliferation ↓	Cyclophosphamide	SAC	Mouse	Neveu et al. (1986)
Anti-SRBC antibody titers ↑	Sheep red blood cells	SAC/LiCl	Rat	Jenkins et al. (1983)
Anti-OVA antibody titers ↑	Ovalbumin	SAC	Rat	Chen et al. (2004)
Anti-OVA antibody titers ↑	Ovalbumin	Electro-stimulation (2 ms/2 Hz/2 or 4 V)	Rat	Huang et al. (2004)
Anti-OVA antibody titers ↑	Ovalbumin	electroacupuncture	Rat	Huang et al. (2004)
Antibody response ↓	Keyhole limpet hyacinine	Chocolate milk	Mouse	Ader et al. (1993)
Secretion of mast-cell protease II ↑	Egg albumin antigen	Flashing light and noise by ventilation fans	Rat	MacQueen et al. (1989)
Primary mixed lymphocyte response ↓	Antilymphocyte serum	SAC	Rat	Kusnecov et al. (1983)
Splenocyte proliferation, IL-2 and IFN-γ production, and mRNA expression and synthesis ↓	Cyclosporine A	SAC	Rat	Exton et al. (1998, 2000); Pacheco-Lopez et al. (2005)
T helper:T suppressor subset ratio ↑	Levamisole	SAC	Rat	Husband et al. (1987)
NK-cell activity, IL-2, lymphocyte proliferation ↓	Morphine sulfate	Distinctive environment	Rat	Coussons-Read et al. (1994)
IL-2, IFN-γ, and corticosterone plasma levels ↑	Staphylococcal enterotoxin B	SAC	Rat	Pacheco-Lopez et al. (2005)
Nitric oxide ↓	Heroin	Distinctive environment	Rat	Szczytkowski and Lysle (2007)
Corticosterone production ↑	Interleukin-1 β	SAC/LiCl or peppermint odor	Mouse	Dyck et al. (1990)

like cyclophosphamide (CY) and cyclosporine A (CsA) have been successfully employed as US, resulting in conditioned immunosuppressive effects when associated with an appropriate CS such as taste/flavor (Ader and Cohen, 1975; Exton et al., 2001).

Morphine sulfate as US paired with a distinctive environment as CS served to achieve a conditioned suppression of NK-cell activity, IL-2 production, and lymphocyte proliferation (Coussons-Read et al., 1994).

3 Conceptual Framework for Neuroimmune Associative Learning

The following section aims to provide a framework which takes basic assumptions about stimuli and sites of action within neuroimmune associative learning into account. Within classical conditioning theory, a prerequisite for its occurrence is that the CNS must sense both the CS (changes in the external environment) and the US (changes in the internal environment), where the signals are then processed, associated, consolidated and recalled at evocation time (Eikelboom and Stewart, 1982; Bovbjerg, 2003; Pacheco-López et al., 2006, 2007b). Accordingly, the CNS must initiate both the UR and the CR. Translated to neuroimmune associative learning, this implies that only immune changes that are detected by the brain can serve as US, in turn, only immune changes that are executed by the brain should then be called CR. However, the nature of the US and the CR in most of the neuroimmune associative learning protocols so far reported is not evident (🔗 [Figure 6-2](#)).

3.1 Association Phase

During association phase, there are two possible USs that may be detected by the brain. A *directly perceived US*, which is defined as a stimulus that itself is recognized by the CNS. The second kind is an *indirectly perceived US*, which is defined to be signaled by intermediary molecules that are then indeed detected by the CNS. In that case those molecules are the genuine US and the applied drug is a sham US, or indirectly perceived US. This has important implications, for example, pairing of a CS with a compound that unconditionally decreases body temperature as US could result in a conditioned hypothermic response; however, conditioned hyperthermia is also possible, that is, “paradoxically conditioned effects” or “counterconditioning effects” (e.g., Bull et al., 1991). The proposed framework predicts such possibilities also for conditioned immune responses and provides a tentative explanation. As Eikelboom and Stewart (1982) proposed, it is necessary to correctly determine the nature of the US and UR to predict the direction of the CR.

There are two possible afferent pathways for any US, regardless whether directly or indirectly perceived – a humoral and a neural pathway (see 🔗 [Section 1.1](#)). Within the neural pathway, the US information, on detection, may be translated into neural activity. This sensing process requires immunoceptive capacities of the CNS (Goehler et al., 2000; Blalock, 2005).

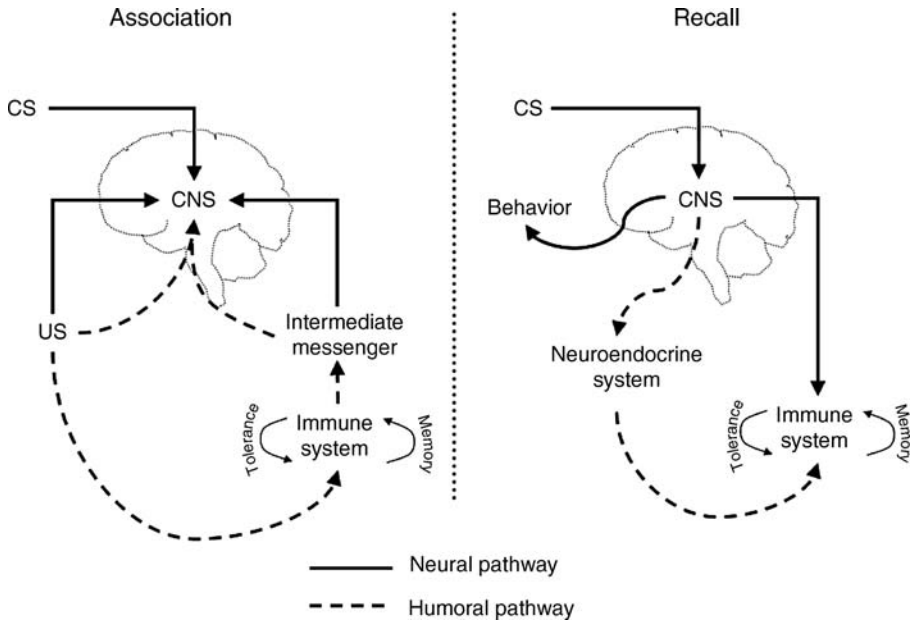
The *humoral afferent pathway* is required more often for indirectly perceived USs that induce molecules that reach the brain via the blood stream as well as for USs that are not detected locally by the immune system. In addition, if an indirectly perceived US affects several cell types, all of the involved molecules become candidates for serving as a genuine US to be detected by the CNS. This implies a more complex, longer, and therefore maybe slower signaling process for indirectly perceived US compared with directly perceived US. Therefore, it can be assumed that the CNS may take longer to respond to an indirectly perceived US than to a directly perceived US.

3.2 Recall Phase

The CR represents the ultimate proof that an association has formerly taken place. There are two possible pathways by which immune functions can be modulated by the CNS: the humoral efferent pathway and the neural efferent pathway (see 🔗 [Section 1.1](#)). During recall phase, humoral efferent pathway may affect

■ Figure 6-2

Theoretical framework for neuroimmune associative learning. At association, the conditioned stimulus (CS) can potentially be associated with two possible unconditioned stimuli (US). The US directly detected by the CNS is defined as a directly perceived US. The stimulus that needs one or more intermediary molecules released by another system to be detected by the CNS is termed indirectly perceived US. Any US, indirectly or directly perceived, has two possible afferent pathways to the CNS: a neural afferent pathway and a humoral efferent pathway. At recall, the CNS can modulate immune function via these two pathways. The humoral efferent pathway may imply changes in neuroendocrine mediators that directly or indirectly modify the immune response. The neural efferent pathway is supported by direct innervations of primary and secondary lymphoid organs



neurohormones that in turn affect immune responses. These peripheral effects are diffuse and long-lasting as any neuroendocrine responses. Direct innervation of primary and secondary lymphoid organs (Elenkov et al., 2000; Mignini et al., 2003; Tracey, 2007) may be part of the neural efferent pathway. Since several immune parameters such as T-cell differentiation (Sanders and Kohm, 2002a; Sanders and Straub, 2002b), hematopoiesis (Miyan et al., 1998; Artico et al., 2002), T- and B-cell activity (Downing and Miyan, 2000), NK-cell activity (Katafuchi et al., 1993; Hori et al., 1995), and inflammatory responses (Czura and Tracey, 2005; Pavlov and Tracey, 2005) are affected by neural activity, and they may also be subjected to be affected by neuroimmune associative learning protocols. It may also be the case that besides the analyzed and reported parameters there are still others not yet identified that are controlled or initiated by the conditioning procedure. The picture becomes more complex considering that the immune system undertakes sensitization (memory) and habituation (tolerance) processes, basically independent from the CNS. In addition, it is known that several immune functions underlie circadian rhythms (Buijs et al., 2006). These conditions complicate the prediction of the final nature and magnitude of the CR, often requesting experimental trials. In this regard, some immune parameters modulated at recall may be the bizarre reflection of neural activity that cannot be explained by orthodox learning and memory rules. For instance, one such scenario would be that the delay between two recall trials is not long enough for certain immune functions to return to baseline levels. Such successive recall trials may yield an additive effect in these and related immune parameters rather than an extinction, which may occur in the neural correlates that elicited these conditioned immune effects. In summary, immune responses can be affected recalling neuroimmune engrams, but this does not necessarily imply that such immune responses were behaviorally conditioned.

4 Principles of Neuroimmune Associative Learning

The following section introduces findings of neuroimmune associative learning experiments and discusses them with regard to rules of orthodox learning and memory theory that are commonly accepted to apply when nonimmune USs are employed. This is insofar of interest as the conditioned taste aversion paradigm, which is employed in the majority of neuroimmune associative learning experiments, has certain unique features. It has been reported that odor–immune association can be established under long interstimulus intervals (e.g., up to 24 h) (Hsueh et al., 1992). Taste–visceral associations are weak with interstimulus delays longer than 4 h (Hiramoto et al., 1992; Solvason et al., 1992). Taste/odor–immune association could occur after only a single CS–US association, whereas visual/auditory/touch–immune association requires further reinforcement trials (Domjan, 2005). Therefore, given the CNS–immune communication complexity, it seems worth to take a closer look at rules applying to a given neuroimmune associative learning.

4.1 General Learning Rules of Neuroimmune Associative Learning

Intensive *training* by increasing the number of CS–US trials during association strengthens the conditioned response. This is a commonly agreed principle in diverse conditioning paradigms and was first described by Pavlov (1927) and also applying to neuroimmune associative learning (Espinosa et al., 2004). Although one learning trial conditioning (e.g., pairing antigenic challenge with saccharin, Alvarez-Borda et al., 1995; Madden et al., 2001) has been reported many times, it is not a phenomenon general to all USs employed in neuroimmune associative learning protocols. Therefore, a paradigm including several association trials may produce more reliable CRs (Espinosa et al., 2004). In this regard, it has been reported that the magnitude of the conditioned effects on the immunity is larger after intensive learning (Niemi et al., 2007).

Extinction is defined as the reduction in magnitude and duration of the conditioned response as a consequence of unreinforced trials (i.e., CS alone) (Szczytkowski and Lysle, 2007). The extinction rate was directly related to the volume of CS presented on the association trial (Ader, 1974). This principle has been proven to apply to neuroimmune associative learning in many other studies (e.g., Bovbjerg et al., 1984; Lysle et al., 1988). In contrast, a recent finding indicates that evoking a consolidated taste–CsA engram several times resulted in a stronger conditioned immunosuppression (Niemi et al., 2007). Similar heterodox findings have been reported on an analog taste–immunosuppression engram resulting from pairing saccharin and cyclophosphamide. Here, the immunosuppression was also more pronounced after several CS unreinforced exposures (Ader and Cohen, 1975; Rogers et al., 1976; Wayner et al., 1978). Possible explanations for such peculiar results are due to an insufficient delay between recall trials (too-near trials), in that immune functions do not have enough time to return to baseline levels, an additive effect may result in a cumulative immunosuppression. An alternative hypothesis is that the conditioned immunosuppressive state occurring at first recall is sensed by the CNS and acts as a reinforcement to induce reconsolidation (Berman and Dudai, 2001; Eisenberg et al., 2003; Dudai, 2006) of the taste–immunosuppression engram and hereby working as an additional association trial.

Regarding the phenomenon of *passive forgetting* (the retention rate after a delay between association and recall phases), Markovic et al. (1988) found excellent retention of CS–US association 8 weeks after association. Here, rats were sensitized with ovalbumin injections; afterward, conditioning comprised saccharin as CS and ovalbumin as US. No extinction occurred during a 6-day test period in terms of the CTA behavior.

Contingency is defined as the occurrence of CS and US and is important for achieving associative learning. Ader and Cohen (1982) demonstrated this in showing that a partial reinforcement protocol significantly reduced the conditioned immunosuppressive effect. Apparently, contingency in neuroimmune associative learning adheres also to common learning principles.

Backward association would be the case if the US precedes the occurrence of the CS. Odor–poly I:C backward conditioning resulted in conditioned increased NK-cell activity (Solvason et al., 1992). This should not be possible for directly (fast) perceived US (see ▶ [Section 3](#)), whereas an indirectly perceived US, which takes longer to be sensed by the CNS, is more likely to occur in parallel with sensory and

encoding steps of the CS, and therefore may become associated. In this case, a backward conditioned group may help to delineate the nature of a given US or to discover the genuine US, respectively. An alternative approach would be to systematically vary the CS–US interstimulus interval. In addition, if the UR kinetic is known, the determination of the time point of the most pronounced CR enables extrapolation to the genuine US that mediates the relevant information.

Latent inhibition, the CS preexposure may retard or diminish CS–US engram, thus reducing the strength of the CR at recall. Such learning interference phenomena have been documented also in neuroimmune associative learning (e.g., saccharin–ovalbumin Chen et al., 2004). However, the naturalistic relation among stimuli should be considered to estimate the relevance of such a phenomenon in a given conditioning protocol.

4.2 Features of Neuroimmune Associative Learning

Neuroimmune associative learning across life span: The strength of neuroimmune associative learning seems to be age-dependent. For instance Spector et al. (1994) report that old mice (24 months old) were able to display a conditioned enhancement of NK activity in an odor–poly I:C conditioning protocol, but young (3 months old) mice showed a stronger conditioned response. This reflects very much the unconditioned response, which is detectable, although weaker in aged mice. This assumption is in line with reports demonstrating a diminished conditioned immunosuppression in aged animals (Gorczynski, 1987b, 1991). Two main factors could be the basis of aging effects on neuroimmune associative learning. Learning and memory deficits are a common characteristic of CNS senescence (Nomura and Hori, 1996). However it should also be considered that innervations to the immune organs change across life span, reducing significantly during elderly stages. As has been reviewed (🔗 [Section 3.2](#)), such neuroimmune efferent neural pathways may be essential to induce the conditioned effect on immunity. Thus, it cannot be assumed that a given conditioning protocol could be extrapolated to any stage of life.

Gender effects: Few systematic attempts have been undertaken to elucidate the role of gender or estruous cycle on neuroimmune associative learning. Spector et al. (1994) reported conditioned increases in NK-cell activity in both male and female mice. However, it should be taken into account that a significant effect of gonadal hormones on the neural (Kritzer et al., 2007) as well as on immune processes (Cutolo et al., 2006) has been documented. Thus, it may not be surprising to find gender effects in a given neuroimmune associative learning protocol.

Compensatory or paradoxical conditioned effects: These effects on immune responses have been reported after evocation of a given neuroimmune associative learning. For example, Gorczynski and Kennedy (1984) varied the time of day at which the initial association trial began and found that conditioned suppression was developed by taste cues paired with CY during the light portion of the diurnal cycle, whereas association that began during the dark portion of the diurnal cycle resulted in either no CR or a conditioned immunoenhancement. The authors hypothesized that the background level of neuroendocrine hormones is critical to the direction of the CR to the taste cue paired with CY. However, Siegel et al. (1987) posited an alternative hypothesis, indicating that the observation that most conditioning studies of regulatory responses use stimuli other than taste cues suggest that the immunosuppression results in evoking a taste–CY association may be unique to the use of taste cues. Other cues, inadvertently present in the association regimen may control CRs different than those CRs elicited by taste cues (e.g., cue to consequence specificity, Garcia and Koelling, 1967b).

Consciousness: An interesting finding is that association and recall phases can occur in anesthetized subjects. Hsueh et al. (1992) reported that mice associate CS (camphor odor) and US (poly I:C) under anesthesia. Second, if conditioned consciously, they could recall under anesthesia, even when CS/US interstimulus interval was separated by 1–2 days. Since this unusual long interval differed from conscious learning where the organism seeks information using a logical perceptual relation among events, the authors reasoned that CS/US learning must be taken place unconsciously by different rules.

Immune history: Different immune histories among subjects may result in a divergent response to the same immune stimulus. This yields a different immune reaction signaled to the CNS that may become

associated with a CS. Therefore, at recall phase, the conditioned response may vary in magnitude or even in direction depending on the initial immune reaction. Evidence supporting this hypothesis comes from a study showing that in an anaphylaxis model, physiological responses to first and second antigen exposure differs drastically: CTA response (Djuric et al., 1988; Markovic et al., 1988), immunological, neural, and behavioral responses (Costa-Pinto et al., 2005). For instance, a recent study documented a highly significant difference in lipopolysaccharide-induced CTA behavior depending on the individual immune history (i.e., tolerant vs. naïve) (Pacheco-López et al., 2007a). Drug tolerance is another phenomenon that may affect neuroimmune associative learning. Repeated treatment with a drug may reduce its specific effect on the organism, a phenomenon termed drug tolerance. Therefore, if used as US with several CS–US pairings, the signaling of this drug may alter with each administration. If so, this yields to different information possibly becoming associated with the CS (e.g., Dyck et al., 1987). The first pairing may involve a different US signal than the second, where it may be less pronounced.

Another key finding comes from experiments of odor–poly I:C conditioning in which several immune responses (e.g., NK cell, neutrophil, and cytotoxic T cell) are affected on recall. However, a systematic analysis led to the conclusion that the enhanced immune responses after recall depended on the immunological history of the particular subject (Demissie et al., 1997, 2000).

Species differences: Differences among species in a given conditioning paradigm do also exist. Although conditioned immunosuppression in a saccharin–CSa conditioning paradigm is apparent in all three species, it is reported that mice did not develop CTA behavior (Niemi et al., 2006), but rats do whereas humans have reported a reduced palatability of the conditioned taste (Goebel et al., 2002). Dissociation of CTA behavior and conditioned immunosuppression was found here in mice (e.g., Bovbjerg et al., 1984).

CS/US administration route: The delivery route of CS and US may affect the conditioned response. For instance, it was found that the mode of administration of saccharin (CS) and ovalbumin (US) significantly affects the conditioned behavioral response. The most effective mode to induce a conditioned taste aversion behavior was CS periorally and US intraperitoneally. Strikingly, this mode yielded the mildest symptoms of anaphylactic shock (UR) compared with CS intravenously/US intravenously mode and CS periorally/US intravenously (weakest CTA response) (Markovic et al., 1988). Such results are in concordance with the naturalistic relation of postprandial immunotoxicological consequences. Although according to established associative learning rules, the extent of the UR determines the extent of the CR (Domjan, 2005); the magnitude of the CR is often smaller than the UR. However, in the earlier case changing the route of administration led to violation of this law.

Neuroimmune engram specificity: Solvason et al. (1991) demonstrated that a given CS creates specific and independent neuroimmune engrams. Two odors (camphor and citronella oil) were paired with poly I:C as a US. Mice were able to discriminate between the two CS when reexposed to either one of them in the sense that they showed a CR to that CS that had been paired with the US during association phase, but not to the other one.

Different CS served successfully in different conditioning protocols. Among the reported data are environments (Coussons-Read et al., 1994; Szczytkowski and Lysle, 2007); visual (MacQueen et al., 1989) and auditory (Harris and Fitzgerald, 1989; MacQueen et al., 1989); stimuli or such complex procedures like surgical sham skin grafts (Gorczyński et al., 1982) (see also [Table 6-1](#) and [Table 6-2](#)). In general, it can be assumed that novelty, intensity, distinctiveness, and uniqueness are relevant characteristics of an effective CS.

5 Mechanisms of Neuroimmune Associative Learning

5.1 Neurobiology of Association and Recall

The neural network involved in taste–visceral associative learning includes mainly sensory and hedonic pathways (Sewards and Sewards, 2002; Sewards, 2004). Among the involved brain structures are consistently the nucleus tractus solitarius, parabrachial nucleus, medial thalamus, amygdala, and insular cortex (Yamamoto et al., 1994). In particular, the insular cortex subserves the association, retrieval, retention, and extinction of taste–visceral memories (Nerad et al., 1996; Bermudez-Rattoni et al., 1997, 2004; Pacheco-López et al., 2007b),

■ Table 6-2

Conditioned immune responses on animal disease models

Disease model	Conditioned response	Unconditioned stimulus	Conditioned stimulus	Species	References
Allergy	Delayed-type hypersensitivity response ↓	Cyclophosphamide	SAC	Mouse	Roudebush and Bryant (1991)
	Delayed-type hypersensitivity response ↑	Cyclophosphamide	SAC	Mouse	Bovbjerg et al. (1987)
Tumor model	Survival time of tumor-bearing animals ↑	Poly I:C	Camphor odor	Mouse	Ghanta et al. (1987, 1988)
	Tumor growth ↓	Allogenic DBA/2 spleen cells	Camphor odor	Mouse	Ghanta et al. (1990)
	Myeloma growth ↓	Poly I:C	Camphor odor	Mouse	Ghanta et al. (1995)
	cytotoxic T-lymphocyte response to YC8 tumor ↑				
	Increased and decreased development of DMBA-induced tumors ↑↓	Cyclophosphamide	SAC	Mouse	Gorczyński et al. (1985)
Autoimmune disease	Proteinuria and mortality ↓	Cyclophosphamide	SAC	Mouse	Ader and Cohen (1982)
Grafting	Cytotoxic T-lymphocyte precursor cells ↑	C57BL/6 lymphoid cells inoculated i.p.	Sham skin grafts	Mouse	Gorczyński et al. (1982)
	Heart graft survival ↑	Cyclosporine A	SAC	Rat	Grochowicz et al. (1991)
Arthritis	Arthritic inflammation		SAC-vanilla drink	Rat	Klosterhalfen and Klosterhalfen (1983)
Asthma	Histamine release ↑	Bovine serum albumin (with prior sensitization)	Methylsulfide and triethylamine odor	Guinea pig	Russell et al. (1984); Dark et al. (1987); Peeke et al. (1987)
	Histamine release ↑	Ovalbumin (with prior sensitization)	Dimethylsulfide odor	Guinea pig	Irie et al. (2001, 2002, 2004)

and it has been postulated that the insular cortex may integrate gustatory and visceral stimuli (Sewards and Sewards, 2001). More recently, using the neuronal activity marker c-Fos, it was possible to confirm the preponderant role of the insular cortex in conditioned increase of antibody production (taste–ovalbumin) (Chen et al., 2004). In particular, reexposure to the CS yielded significant increase in c-Fos expression in all insular areas (granular, dysgranular, and agranular) 120 min postrecall, confirming previous observations employing an excitotoxic lesioning approach (Ramírez-Amaya and Bermúdez-Rattoni, 1999).

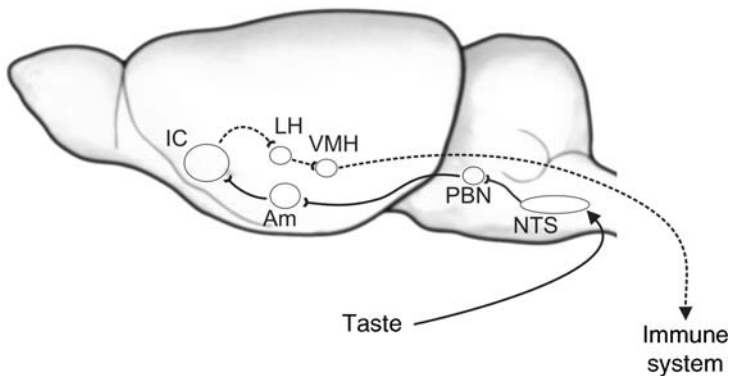
Regarding other forebrain structures, the amygdala seems to play an important role during the formation of aversive ingestive associations (Reilly and Bornova, 2005), and is also relevant for

limbic–autonomic interaction (Swanson and Petrovich, 1998). A series of reports has indicated that the insular cortex and the amygdala are key structures in conditioned immunosuppression after evoking taste–CY and odor–CY associations (Ramírez-Amaya et al., 1996, 1998).

It has also been proposed that the ventromedial hypothalamic nucleus, widely recognized as a satiety center (Vettor et al., 2002), is intimately associated with sympathetic facilitation in peripheral tissues (Saito et al., 1989), including modulation of peripheral immune reactivity (Okamoto et al., 1996). In agreement with previous reports employing a *taste–CY* engram (Ramírez-Amaya et al., 1996, 1998, 1999), the neural substrates involved in the immunosuppression resulting in evoking a taste–CsA association in rats have been identified (Pacheco-Lopez et al., 2005). The conditioned effect on the immune system that reduced splenocyte responsiveness and cytokine production (IL-2 and IFN- γ) was affected by brain excitotoxic lesions. These data show that the insular cortex is essential for acquiring and evoking this conditioned response. In contrast, the amygdala seems to mediate the input of visceral information necessary at association time, whereas the ventromedial hypothalamic nucleus appears to participate in the output pathway to the immune system, needed to evoke the behaviorally conditioned immune response (► Figure 21-3).

■ Figure 6-3

At evocation time, conditioned animals are reexposed to the conditioned stimulus (CS). This gustatory information is centrally processed through brain stem relays (nucleus tractus solitarius: NTS, parabrachial nucleus: PBN), reaching the insular cortex (IC). This neocortex, together with the amygdala (Am), is indispensable during the association phase, and is also necessary in evoking conditioned ingestive behavior (aversion/avoidance). The ventromedial nucleus of the hypothalamus (VMH) is essential for evoking the immunosuppressive conditioned response in the periphery, also recruiting other hypothalamic nuclei such as the lateral hypothalamus (LH)



Several attempts have been undertaken to elucidate central processing in conditioning protocols applying antigen as US. Brain lesions of specific brain areas revealed that, again, the insular cortex and the amygdala are indispensable for associating saccharin with an immune response induced by a protein antigen (Ramírez-Amaya and Bermúdez-Rattoni, 1999). Although the hippocampal plasticity is modulated by neuroimmune interactions (Avital et al., 2003; Ikegaya et al., 2003; Balschun et al., 2004), this limbic structure seems not to be involved in the taste–immune association process (Ramírez-Amaya and Bermúdez-Rattoni, 1999). In the same report, the authors successfully conditioned an almond odor with hen egg lysozyme antigen, and insular cortex lesion blocked this association. Interestingly, this neocortex is not essential for odor-conditioned aversive behavior (Kiefer et al., 1982).

In a modified classical passive avoidance paradigm, ovalbumin-immunized mice avoid a context that has been paired with the allergen (Costa-Pinto et al., 2005). Ovalbumin aerosol was employed as an aversive

US, the CS was a usually preferred dark compartment of a box. After association phase, animals avoided this compartment and spent more time in the bright (usually aversive) compartment of the box. The expression of c-Fos expression was tracked after airway ovalbumin challenge and it was found to be enhanced in the hypothalamic paraventricular nucleus and the central nucleus of the amygdala in sensitized mice. Therefore, it is likely that the immediate hypersensitivity reaction in allergic asthma, which is characterized by IgE-mediated mast-cell deregulation with concomitant histamine release, plays a major role in the reported brain activity (Costa-Pinto et al., 2005). In addition, these structures are commonly linked to emotional behavioral patterns that are also involved in conditioned taste aversion (Bermudez-Rattoni, 2004).

Regarding neural mechanisms behind the odor–poly I:C conditioning paradigm, it has been reported that IFN- β is the major input to the CNS during association phase. It can replace poly I:C as US to acquire a conditioned increase in NK-cell activity; interestingly, this is not the case for IFN- α (Solvason et al., 1988). This association can be achieved by intravenous (i.v.) administration of IFN- β at a dose of 10,000 IU, but not at 1,000 IU. Further, if IFN- β was directly delivered into the CNS via the cisterna magna, a smaller dose (100 IU) sufficed to be associated with the CS. In turn, the CR was blocked by injecting anti-IFN- β (100 neutralizing units) into the cisterna magna 24 h before association (Solvason et al., 1993). The afferent pathway for association yielding conditioned increased NK-cell activity seems to be a different one to that of the conditioned fever in the same associative learning conditioning protocol (Rogers et al., 1992). The i.p. injection of sodium carbonate blocked CS–US association for enhancement of NK-cell activity, but left conditioned fever response unaffected. Conversely, indomethacin treatment which is known to prevent prostaglandin synthesis blocked the conditioned fever response but not the conditioned NK-cell response. Using a pharmacological approach, the neurochemical features of the conditioned effect enhancing NK-cell activity in rodents (odor–poly I:C association) have been described in detail by one group. Administering lidocaine centrally blocked both association and recall of the CR (Rogers et al., 1994a) and the CNS sensory processes of the CS, but not of the US. Similarly, peripheral and central treatment with monosodium glutamate (Ghanta et al., 1994) or sodium carbonate (Rogers et al., 1994b) apparently blocks association, but not CS perception. Moreover, the hypothalamic arcuate nucleus is necessary for association, but not for recall of the CR (Ghanta et al., 1994). Recall in this paradigm seems to be mediated by central opioid pathways; peripheral injection of the opioid receptor antagonist quaternary naltrexone (not penetrating the CNS) does not affect recall of the CR. Naltrexone given before association did not prevent the conditioned effect (Solvason et al., 1989), indicating independency of central opioid pathways.

Central catecholamines seem to be essential, and glutamate – but not GABA – is also required at recall stage (Hsueh et al., 1999; Kuo et al., 2001). In particular, reserpine treatment before recall, which unspecifically depletes central and peripheral catecholamine contents, blocked the CR (Hiramoto et al., 1990). More recently, it has been shown that α - and β -adrenoceptor antagonists or dopamine (DA)-1 and DA-2 receptor antagonists given shortly before recall also blocked the CR (Hsueh et al., 1999).

Furthermore, it has been demonstrated that cholinergic, as well as serotonergic, central systems are required in triggering conditioned NK-cell response (Hsueh et al., 2002). At association, acetylcholine is believed to act through nicotinic, M_2 -, and M_3 -muscarinic receptors, whereas at recall M_1 -, M_2 - and M_3 -muscarinic receptors have been identified to be crucially involved. In both association and recall phase, serotonin acts through 5-HT $_1$ and 5-HT $_2$ receptors to affect the CR.

Neurotransmitter contents in certain brain areas during recall stage have been analyzed, revealing a significantly higher norepinephrine content in the cerebellum and dopamine content in the striatum and hippocampus in conditioned animals compared with controls (Hsueh et al., 1999). Interestingly, glutamate contents at recall in this paradigm in the same brain areas did not differ between groups (Kuo et al., 2001).

In an identical conditioning protocol (camphor odor, poly I:C) with enhanced neutrophil activity as CR instead of NK-cell activity (Chao et al., 2005) measured levels of tyrosine hydroxylase in several brain areas 24 h after recall to localize action sites of catecholamines. Conditioned animals displayed significantly more tyrosine hydroxylase expressing neurons in the hypothalamus, cortex, and locus coeruleus compared with control animals. But this is unlikely to be related to a neural memory process since a 24 h memory trace is rather unusual.

In addition to classical neurotransmitters, cytokines have been demonstrated to play an important role within the CNS, modulating neuronal and glial function in nonpathological settings such as learning and memory processes (Balschun et al., 2004; Dantzer, 2004; Tonelli et al., 2005). Specifically, proinflammatory cytokines, such as IL-1, IL-6, and TNF- α , have been shown to modulate spatial learning tasks, as well as long-term potentiation phenomena (Gibertini, 1996; Schneider et al., 1998; Fiore et al., 2000; Banks et al., 2001; Matsumoto et al., 2001, 2002; Rachal Pugh et al., 2001; Lynch, 2002). In this sense, it can be assumed that cytokines may be a significant factor in the associative processes occurring during behavioral conditioning of immune functions. Apart from these neuromodulatory properties, proinflammatory cytokines seem to play an important part in the afferent pathway between the immune system and the CNS (Besedovsky and del Rey, 1996; Turnbull and Rivier, 1999; Dantzer, 2004). Therefore, it can be hypothesized that central cytokines may act as mediators in the brain during an “immune-sensing” phase in the association phase. These hypotheses are supported by observations that (1) receptors for these proinflammatory cytokines are expressed in the CNS (Szelényi, 2001; Sredni-Kenigsbuch, 2002), (2) peripheral immune changes affect central cytokine production and cytokine receptor expression in the brain (Pitossi et al., 1997; Del Rey et al., 2000), and (3) cytokines can act as unconditioned stimuli to induce conditioned taste aversion/avoidance (Tazi et al., 1988; Dyck et al., 1990; Janz et al., 1991; Hiramoto et al., 1993).

The underlying mechanisms when using SEB as US are completely unknown. However, systemic IL-2 administration has been found to modify central monoamine activity (Lacosta et al., 2000). Similarly, striatum catecholamine concentrations followed a dose–response curve in reaction to increased peripheral SEB immunization (Pacheco-Lopez et al., 2004). Therefore, association may involve T-cell-derived cytokines like IL-2 signaling to the CNS.

5.2 Peripheral Mediation of the Conditioned Effects on Immunity

The available data suggest that the effects of conditioning could be mediated by a preferential effect on T cells. Conditioned suppression of lymphoproliferative responses in rats and mice, for example, has been observed in response to T-cell mitogens but not (or less reliable) in response to B-cell mitogens (Neveu et al., 1986; Kusnecov et al., 1988; Lysle et al., 1990, 1991). Immune adoptive transfer experiments also suggest that conditioning may be mediated by T-cell changes (Gorczyński, 1987a). Splenocytes from conditioned or experimentally naïve animals were transferred into irradiated naïve or conditioned animals that were or were not subsequently reexposed to the CS. The observed increases or decreases in the antibody-forming cell response to SRBC depended on the donor cells and the conditioning treatment experienced by the recipient. The separate transfer of enriched T and B cells into naïve or conditioned animals suggested that conditioning effects were attributable to the adoptively transferred T cells (Gorczyński, 1991). However, the specificity of whether conditioning can modulate the antibody response to different types of antigens has not been resolved.

With respect to adrenocortical influences, it is reasonable to hypothesize that conditioned alterations in immunologic reactivity could be mediated by conditioned neuroendocrine changes. In experiments in which humoral immune responses were assessed in conditioned animals, the recall of a taste–LiCl association did not affect antibody responses to SRBC, in contrast to the immunosuppression that was observed after evoking the taste–CY engram (Ader et al., 1979). This finding indicates that an immunosuppressive drug was needed to cause immunosuppression during association phase, thus supporting a taste–immunosuppression engram interpretation.

In follow-up experiments, circulating levels of adrenocortical steroids were artificially elevated by exogenous administration of corticosterone at the time of antigen injection to mimic the stress response occurring in animals reexposed to the taste paired with sickness (Ader et al., 1979). Elevated glucocorticoid levels did not significantly lower antibody titers. These data would appear, *a priori*, to exclude the possibility of a stress-mediated phenomenon in the genesis of conditioned immunosuppression.

However, experimental data, in which the delayed-type hypersensitivity (DTH) response was used as an index of T-cell function, support the hypothesis that changes in the immune function of animals subjected

to conditioned taste aversion might be better viewed as a secondary consequence of the psychological conflict affecting thirsty animals that are exposed to a taste solution previously associated with sickness (Kelley and Dantzer, 1988). According to this hypothesis, the immunosuppressive status after evocation phase is a consequence of the conflict of the strong motivation to drink in fluid-deprived animals against the aversive memories associated to the CS. Accordingly, it should be possible to induce immunosuppression in conditioned animals even when no immunosuppressive drug is used during association phase (as US). After a *taste-LiCl* association, conditioned animals showed a strong conditioned taste aversion, but also an immunosuppressive status in the T-cell function only when a forced choice test was implemented, but not in a two-bottle preference procedure which eliminates the psychological conflict of thirst versus aversive memories (Kelley et al., 1984, 1985). In contrast to this stress hypothesis, a serum corticosterone time-course study performed to examine the possible involvement of glucocorticoids in conditioned immunosuppression of the DTH response has been published (Roudebush and Bryant, 1991). Animals were sacrificed 30, 60, 90, 120 min and 24 h after evoking a taste-CY engram. No significant differences in serum corticosterone levels were detected between nonconditioned controls and the conditioned groups at any time point. Supporting this line of thinking, several other research groups have consistently reported that the suppression of splenic T-cell proliferation was independent of the stressor-induced increase in adrenocortical activity (Mormede et al., 1988; Lysle et al., 1990; Exton et al., 1998).

Importantly, both conditioned and stressor-induced alterations in immune and nonspecific defense responses have been attributed to the action of central and peripheral catecholamines. For instance, it has been reported that both chlorpromazine and amitriptyline, both centrally acting, abolish the immunosuppressive status elicited by recalling a taste-CY engram (Gorczyński and Holmes, 1989). Furthermore, it has also been reported that the β -adrenergic antagonist, propranolol, blocked the immunosuppressive effects of a conditioning stress paradigm (Lysle et al., 1992). Supporting the involvement of peripheral catecholamines, nadolol (α/β -adrenergic antagonist that does not cross the blood-brain barrier) blocked the electric shock-induced suppression of splenic, but not peripheral, blood lymphocyte proliferation following mitogenic stimulation *ex vivo* (Cunnick et al., 1990). In this regard, we have previously revealed that the immunosuppressed status after evoking a taste-CsA engram is not related to the activation of the HPA axis and is merely mediated by the neural innervation of the spleen, via noradrenaline- β -adrenoceptors-dependent mechanisms (Exton et al., 1999, 2002; Xie et al., 2002). It has been demonstrated that splenocyte reactivity is modulated in part by tonic inhibition from the splenic nerve (Okamoto et al., 1996) and sympathetic splenic innervation seems to be under the central control of the ventromedial hypothalamus (Katafuchi et al., 1993, 1994). While electrical stimulation of the ventromedial hypothalamus has been found to arouse sympathetic activity (Saito et al., 1989), the lateral hypothalamus seemed to do the opposite (Bernardis and Bellinger, 1993). Thus, the lateral hypothalamus may exert immunoenhancing properties (Wrona and Trojnar, 2003) in part by antagonizing the ventromedial hypothalamus, and thereby reducing sympathetic tone inhibition. Importantly, such hypothalamic regulation of sympathetic activity seems to be modulated by the insular cortex (Allen et al., 1991; Cechetto and Chen, 1992; Oppenheimer et al., 1992; Butcher and Cechetto, 1998).

Concerning the conditioned enhancement of NK-cell activity, several mechanisms have already been elucidated. On the neural efferent pathway, sympathetic innervation of the spleen seems not to be responsible for mediating the conditioned response. Despite peripheral sympathectomy using 6-hydroxydopamine (6-OHDA) between association and recall, the CR still occurred (Hiramoto et al., 1990). However, splenic denervation should give final evidence. Interestingly, applying the same conditioning protocol, but measuring neutrophil activity as UR and CR, respectively, peripheral sympathectomy before recall completely abrogated the CR (Chao et al., 2005). On the humoral efferent pathway, elevated plasma adrenocorticotrophic hormone (ACTH) levels and splenic IFN- α expression were measured in conditioned animals at recall time (Hsueh et al., 1994b); no effect was found for β -endorphin concentrations. Peripheral administration of the synthetic glucocorticoid dexamethasone blocked recall, but not association of the CR, assumingly by negative feedback inhibition of HPA axis activity (Hsueh et al., 1994a). Dexamethasone treatment before recall resulted in elevated neutrophil activity in conditioned animals compared with controls, indicating that this specific CR is independent of HPA activity (Chao et al., 2005).

6 Clinical Implications

6.1 Animal Models

► **Table 6-2** summarizes conditioned effects affecting critical immune responses in animal disease models with potential clinical application.

The DTH response refers to an overreaction produced by the immune system given a presensitized immune state of the host. A conditioned decrease (Roudebush and Bryant, 1991) and increase (Bovbjerg et al., 1987) of this response has been observed by pairing saccharin with CY. Similarly, Exton et al. (2000) reported a conditioned suppression of the contact hypersensitivity reaction by pairing saccharin with CsA.

Conditioning protocols attested a significant impact on tumor-related models. For instance, tumor growth was demonstrated to be enhanced as well as delayed by applying different US (CY and cimetidine, a histamine type II receptor antagonist) (Gorczynski et al., 1985). Moreover, the survival time of tumor-bearing mice has been prolonged in conditioned animals (Ghanta et al., 1988).

Evoking a taste–CY engram, Ader and Cohen (1982) demonstrated a conditioned retardation in proteinuria and mortality in New Zealand hybrid mice which are prone to develop an autoimmune disease resembling human lupus erythematosus. Most interestingly, in a follow-up study they could show that the lupus-prone MRL-lpr/lpr mice displayed a weaker CTA compared with congenic controls. The authors interpreted that the lupus-prone animals seek to regain homeostasis by consuming the tasting solution to achieve the therapeutic immunosuppressive status elicited by evoking the taste–CY engram (Grota et al., 1987).

Another example of conditioning effects on immunity is documented by grafting experiments. Gorczynski et al. (1982) performed skin grafting as CS in combination with i.p. injected lymphoid cells of another mouse strain as US. Reexposing the conditioned animals to the sham-grafting procedure, they showed an increase in cytotoxic T-lymphocyte precursor cells specific for alloantigens on the grafted tissue. In addition, Grochowicz et al. (1991) reported a conditioned prolongation of the survival time of heart allografts in rats evoking a saccharin–CsA engram. In follow-up experiments, Exton and colleagues (1999) demonstrated a significant prolongation of the survival of transplanted hearts, including long-term survival (>100 days) of transplants in 20% of the animals that were conditioned and additionally subtherapeutically treated with CsA. In addition, employing taste–CsA association Klosterhalfen and Klosterhalfen (1983, 1990) extended the conditioned immunosuppression to an arthritis model. Before induction of adjuvant arthritis, rats were dosed with cyclophosphamide (US) after presenting a distinctive saccharin/vanilla solution (CS). Conditioned rats showed no external signs of a proliferation of inflammation, whereas approximately half of the animals in the control groups developed small lesions.

A series of studies dealt with conditioning of asthma-like symptoms, anaphylactic shock (Noelpp and Noelpp-Eschenhagen, 1951c; Djuric et al., 1988; Palermo-Neto and Guimaraes, 2000), or histamine release, respectively (Dark et al., 1987; Irie et al., 2001, 2002, 2004) that have been already reviewed (► **Sections 1.3, 1.4, and 4.2**).

6.2 Human Studies

This section summarizes the few, but promising research reports of conditioned effects modulating immune responses in human subjects, indicating potential therapeutic outcomes of this behavioral approach.

A Japanese study from the early 1960s reported a conditioned dermatitis response in adult male subjects elicited by evoking a specific association (CS: blue solution topically applied also contains 2% raw extract *Rhus vernicifera*: US) (Ikemi et al., 1962). A case report of asthmatic patients suffering from skin sensitivities to house-dust extract and grass pollen shows a conditioned effect. The subjects were exposed to these allergens by inhalation (Dekker et al., 1957). After a series of conditioning trials, they experienced allergic attacks after inhalation of the neutral solvent used to deliver the allergens. This work showed not only fast conditioning of the asthmatic attack (CR), but also tenacious retention, that is, lack of extinction. Similarly,

presenting a novel-tasting and novel-appearing drink as CS with house-dust mite allergen as US to patients with allergic rhinitis yielded an associative learning (Gauci et al., 1994). In addition, pairing an olfactory cue as CS with nasal challenge with seasonal grass allergens to investigate conditioning of the immediate hypersensitivity reaction in hay fever (seasonal allergic rhinitis) produced conditioned increase of histamine release and decrease nasal airflow at recall (Barrett et al., 2000). Furthermore, there was evidence of extinction and a follow-up experiment showed a more pronounced conditioned effect after three association trials compared with one, indicating a reinforcement effect. An elevated mast-cell tryptase in mucosa was observed when an intranasal saline application was given simultaneously with the CS at recall. Another type of allergic reaction, the DTH response, was tested in healthy volunteers who received five monthly tuberculin skin tests (Smith and McDaniel, 1983). In this conditioning protocol, both tuberculin (US) and saline were injected; the latter was taken from a green vial (CS-) and the former was drawn from a red vial (CS+). On the test day, the color labeling of the substances was reversed. Although the saline injections did not induce a skin reaction (erythema and induration), the severity of the symptoms was significantly blunted in all the subjects tested when the tuberculin was drawn from the green vial (i.e., conditioned compensatory effect), where subjects expected their reactions to be negative. However, a similar protocol using various allergens (e.g., mite dust, fur) taken from colored vials did not result in conditioned modulation of skin reactions in the subjects tested (Booth et al., 1995).

Another series of experiments investigated the role of conditioning in the context of cancer treatment and chemotherapy (Bovbjerg, 2003). Some chemotherapeutic agents such as CY have immunosuppressive effects. For the patient, often the treatment visits to the hospital become aversive and act as CS-US association trials pairing whereby a variety of features such as white coat, distinct smell when entering the building, the clinic itself, its smell, the clinician's voice, and so on may act as distinctive salient stimuli (CS) that are contingently paired with the chemotherapy (US) (e.g., CY). Immune function was assessed in cancer patients in the hospital before chemotherapy and compared with assessments conducted at home. Proliferative responses to T-cell mitogens were lower for cells isolated from blood samples taken in the hospital (i.e., after recall) than for home samples (Bovbjerg et al., 1990). These results were replicated in ovarian cancer patients (Lekander et al., 1995) and pediatric cancer patients receiving chemotherapy (Stockhorst et al., 2000). In addition, chemotherapy patients often develop conditioned/anticipatory nausea (Andrykowski, 1988; Bovbjerg et al., 1990; Morrow et al., 1991; Matteson et al., 2002), anxiety (Jacobsen et al., 1993; DiLorenzo et al., 1995), and fatigue (Bovbjerg et al., 2005) responses to reminders of chemotherapy. To resolve such severe consequence of chemotherapy treatment, one may use some of the learning principles that have been shown to apply to neuroimmune associative learning (Section 4), for example, latent inhibition to prevent such undesirable conditioning effects. Several visits to the clinic where the chemotherapy will take place should be recommended before starting the chemotherapy. Another possible behavioral prophylactic therapy may be accomplished before chemotherapy, a specific novel stimulus that could be administered under the physician's control, for example, a novel and distinctive tasting/flavored/colored/sparkling solution. Such artificial stimulus will be categorized and associated to negative chemotherapy hedonic values; however, it may prevent the development of possible associations to other high-protein/caloric food input (Scalera, 2002).

Regarding conditioning of cellular immune parameters, one group assessed the conditionability of augmentation of NK-cell numbers and their lytic activity in healthy subjects. Although a conditioned response was evoked after pairing a given taste with subcutaneous administered adrenaline (Buske-Kirschbaum et al., 1992, 1994), these effects could not be replicated (Kirschbaum et al., 1992). In multiple sclerosis patients, four monthly CY infusions (US) were paired with an anise-flavored syrup (CS) (Giang et al., 1996). Long-term treatment with CY decreases blood leukocyte numbers, which often leads to leukopenia. After a long period such as 6 months of administering the placebo infusion paired with the drink, 8 out of 10 patients showed a conditioned reduction in peripheral leukocytes numbers. In addition, by pairing subcutaneously interferon- γ injections (US) with a distinctively flavored drink (CS), it was possible to induce an elevation of neopterin and quinolinic acid serum levels after evoking such an association in healthy volunteers (Longo et al., 1999). However, it has been hypothesized that more than a single associative learning trial pairing a distinctive taste (CS) with interferon- β injections (US) is

necessary to produce immune-conditioned effects (Goebel et al., 2005). This view is supported by experimental data for healthy male volunteers where the immunosuppressive drug cyclosporine A (US) was paired four times with a distinctively flavored/colored solution (CS) (Goebel et al., 2002), inducing taste-immune associative learning. After association, the drink (CS) alone induced conditioned inhibition of ex vivo cytokine (IL-2 and IFN- γ), mRNA expression, and cytokine release, as well as of the proliferative responsiveness of human peripheral blood lymphocytes, similar to the CsA effect. In addition, a study with patients suffering from allergic house-dust mite rhinitis received pairing of a novel-tasting drink (CS) and the H1-receptor antagonist, desloratadine (US) on five consecutive days. On reexposure, conditioned patients displayed a decreased basophil activation, assessed in a skin prick test, and subjective symptom score to a degree that was similar to drug treated control group (Goebel et al., 2007).

In summary, the reported results imply that introduction of behavioral conditioning to supplement standard pharmacotherapeutic treatment may enable the physician to maintain some desired physiological state or diminish pathological states. The outlook will be of reducing undesired side effects and maximizing the effects of pharmacological therapies with a lower cumulative amount of active drug than is currently used (Ader and Cohen, 1985).

7 Methodological Considerations for Neuroimmune Associative Learning Experiments

The following methodological considerations may improve neuroimmune associative learning establishment in new settings as well as its further experimental development and clinical translation.

Ader (2003) suggested the following experimental groups to be included in conditioning protocols, testing its impact on immune parameters:

Conditioned group: It constitutes the primary experimental group. After CS-US pairing during association, this group is reexposed to the CS during recall.

Conditioned not reexposed group: This group is identically treated as conditioned group during association phase, and it is not reexposed to the CS during recall phase. It serves as a control for any direct or indirect immunomodulating effects of the association procedure per se, as well as for possible residual effects of the US at time of recall.

Unconditioned response group: This group is also identically treated to conditioned group during association, and receives additionally US reexposure during recall without the CS. This is the pharmacological control that defines the magnitude and direction of the UR, and also controls for immunological and other assays being performed to gather the data.

Latent inhibition group: This group is preexposed to the CS before submitted to the entire conditioning procedure identical to conditioned group. Here, a reduced CR is expected since preexposure should weaken the strength of association.

Noncontingent conditioned group: This group receives the same number and amount of stimuli as conditioned group, but CS and US are not timely paired. During recall, this group is also reexposed to the CS. It controls for nonassociative factors, and certifies the neutrality of the CS in terms of immunological effects.

Placebo group: This group is exposed to the CS at times as conditioned group is, but never receives the US; instead, an immunologically neutral stimulus such as saline is administered as placebo. It controls for residual CS effects and possible procedural artifacts like handling, injections, and so on.

The difficulty for the investigator lies not so much in inducing conditioned immune alterations, but in employing the proper controls, both immunological and psychological, to demonstrate the predictability and reliability of such effects, and consequently, the controllability. Therefore, the more control groups one employs, the better the data are to interpret. Although it is a matter of costs and in the case of human studies, in particular such involving patients, an ethical issue.

Some considerations about the nature of both the US and CS are provided in the following paragraphs, complementing previous elaborations (Spector, 1987).

The *unconditioned stimulus* should be known in detail in terms of immunologic sequel, and the sequel must be reproducible. This means that applying a drug with an attenuating effect with subsequent administration is useless for more than one association trial, since different immunological statuses would be paired with the CS. In addition, the dose level must be reproducible. For ethical reasons, the drug should have minimal side effects when experimenting with humans.

The *conditioned stimulus* should be easily perceived by each subject. This means that it should be novel and distinct. This is not trivial, since the experimenter needs to find a stimulus that is new to a diverse population that encountered numerous tastes, odors, colors, and so on in daily routine. Note also that for instance about 30% of the human population may be anosmic to camphor odor (Spector, 1987). The CS must also be continuously perceived by each subject without attenuation on repetition, similarly to the US. It must be immunologically neutral and harmless. The conditioning procedure must be carried out in a constant environment, since its cues have been proven to be part of the CS. The conditioning procedures often inadvertently manipulate additional stimulus components that may not be identified explicitly (e.g., the deodorant or voice of the experimenter, the smell of the room, the sound of a church bell nearby, etc.). A lack of attention to environmental components of the CS may make conditioned effects in the immune system appear to be small, although they may just be masked by the presence of environmental cues. In addition, it should be taken into account that the development of the CR depends on the modality of the CS; aversions produced by association with drug-induced visceral illness are often specific to gustatory or olfactory components of the CS (Garcia and Koelling, 1967a). The procedure should be carried out at the same time of the day, since most immunological parameters underlie circadian rhythm.

Finally, some issues should be solved to standardize a given conditioning protocol with a clinical perspective. For instance, it is unknown how long the conditioned response lasts. Is reconditioning possible (to maintain the conditioned response in a longer perspective, extinction must be avoided)? When does forgetting set in? Are side effects of the US also conditioned? How controllable is the conditioned response in a population that in contrast to a laboratory animal comprises differing immune and psychological history/status? In addition, other factors such as age, gender, and cultural background must be taken into consideration by health practitioners implementing behavioral conditioning as a supplement therapy.

8 Closing Remarks

Neuroimmune associative learning is one of the most fascinating examples which uncover the multiple interactions among the nervous, endocrine, and immune systems. Contributions to this line of research are coming from various fields of expertise, from the molecular to the behavioral level. This behavioral model is unique in elucidating the CNS-immune interactions, because it unites the afferent (during association phase) and efferent (during evocation phase) pathway between these systems in one model, but with the experimental possibility to dissect such interactions. The potential clinical application to use CRs affecting immunity as supplementary treatment in a therapeutic setting is a further argument to promote the understanding of the principles behind neuroimmune associative learning.

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7 The Brain Immune System: Chemistry and Biology of the Signal Molecules

A. Galoyan

1	<i>Introduction</i>	155
2	<i>The Functional System of Neurosecretory Hypothalamus–Endocrine Heart</i>	156
3	<i>The Brain Neuroendocrine Immune System</i>	157
4	<i>The Cytokine System</i>	159
4.1	Inducible Character of Cytokine Formation and Reception	159
4.2	Locality of Cytokine Action	160
4.3	Superfluity of Cytokine System	160
4.4	Interrelationship and Interdependency in the Cytokine System	163
4.5	Cytokines and Their Brain Receptors	164
4.6	Brain Born Cytokines	164
5	<i>Chemokine System</i>	164
5.1	CXC-Chemokines	166
5.2	CC-, C- and CX3C-Chemokines	166
5.3	Chemokine Receptors	166
5.4	Chemokines in the Neurons	167
6	<i>Antibody Production in the Brain</i>	168
7	<i>The Discovery of the New Brain Immunomodulators</i>	168
7.1	Thymosin β_4 (1–39)	168
7.2	Isolation of a Fragment of MBP from Bovine Hypothalamus	169
7.3	Hypothalamic Immunophilin-Receptor of FK-506 Immunosuppressor: FK-506 Binding Protein (FKBP-12)	171
7.3.1	Iphs as Cytokines	171
7.3.2	Targets of the Iph–Immunosuppressant Complexes	172
7.3.3	Iphs in the Nervous System	172
7.4	Thymosin β_1 (Ubiquitin) Isolated from the Neurosecretory Granules of Neurohypophysis is a Calmodulin-Binding Endogenous Protein, a Calmodulin Antagonist	173
7.5	Isoforms of Macrophage Migration Inhibitory Factor in Bovine Brain	173
8	<i>Discovery of the Neuroendocrine Immune System of Brain</i>	175
8.1	Neurosecretion of Interleukins by Magnocellular Cells of Hypothalamus: Bioassay Methods of Interleukin Identification	175
8.2	Discovery of New Brain Neurosecretory Cytokines	176
9	<i>Biological Properties of PRP-1</i>	178

9.1	The Neuronal Activity of PRP-1	178
9.2	Antibacterial Activity of PRP-1	178
9.3	PRP Regulation of the Thymocyte Differentiation in Neonatal and Fetal Thymus	179
9.4	PRP is a Regulator of Myelopoiesis	180
9.5	PRP-1 is a Stimulator of Bone Marrow Stem Cells	180
9.6	Neuroprotective (Antineurodegenerative) Properties of PRPs	181
9.6.1	Anti-Snake Venom Effect of PRP-1 and PRP-3, Their Action in Spinal Cord Injury and After Nerve Transsection	181
9.6.2	The Crush Syndrome	182
9.6.3	Effect of PRP on Aluminum Neurotoxicosis	183
9.6.4	The Influence of PRP on the Model of Alzheimer's Disease Induced by Intracerebroventricular Injection of β -Amyloid Peptide (25–35)	183
9.6.5	Neurotrophin-Like Properties of PRP-1	183
10	Discussion	183
11	Conclusion	186

Abstract: Understanding the brain immune system is impossible without the apprehension of the complicated mechanisms of innate and adaptive immunity as a whole. Meanwhile, discovery of a great number of new immunomodulators of brain produced by neurosecretory cells of hypothalamus, such as interleukins, new cytokines – proline rich peptides, immunophilin isoforms (receptors of immunosuppressors – FK-506, rapamycin, cyclosporine), tyrosine β_1 (ubiquitin), etc. – is the basis for new image on brain immune system. Biosynthesis of new cytokines in the neurosecretory cells also testify the existence of neuroendocrine immune system of brain, which has close neurohumoral interrelations with central immune organs, particularly bone marrow. The focus of this chapter therefore, is on the main principles of studying the brain immune system, as well as the main literary and data obtained on cytokines, types of cytokine receptors, and the presence of common ways of signal transduction from receptors into cell nucleus, interrelationship and interdependency in the cytokine system, cytokines and their brain receptors. Understanding the role of cytokines in the complicated mechanisms of the immune response is of great importance. There are a number of chemoattractant-cytokines known as chemokines and their receptors. It is also known as the role of the chemokines in the neuronal function. It is supposed to establish the integrative role of brain neuroendocrine immune system signal molecules in the regulation of the brain immune defense and “peripheral” immune system. The proline-rich peptides (PRPs) take part in the endocrine protective mechanisms for the immune response at various pathologic states of the organism. Though at present the morphological, neurochemical, neuroimmunological mechanisms of the brain immune system are not yet outlined, new data appear on signal molecules of the brain immune system and on their role in adaptation mechanisms of the brain immune defense and periphery. Based on huge body of novel data reviewed in this submission, the conventional conception concerning the neurosecretory function of hypothalamus and the hypothalamic mechanisms of adaptation (taking into account also the role of corticotrophin releasing factor in the HPA-axis) have to be reconsidered.

List of Abbreviations: BDNF, brain-derived neurotrophic factor; Cyp, cyclophilin; G-CSF, granulocyte-colony-stimulating factor; GM-CSF, granulocyte–macrophage-colony-stimulating factor; INF, interferon; Iph, immunophilin; MBP, myelin basic protein; MLCK, myosin light chain kinase; NF-AT, nuclear factor of activated T cells; NK, natural killer; NMDA, *N*-methyl-D-aspartate; NSO, *N. supraopticus*; NPV, *N. paraventricularis*; NVAG, neurophysin–vasopressin-associated glycoprotein; PDE, phosphodiesterase; PRPs, proline-rich peptides; PPI, peptidyl-prolyl-cis–trans-isomerase; RANTES, regulated and normal T-cell expressed and secreted; SDF-1, stromal cell derived growth factor; SIAM, soluble immune activator marker; SLC, secondary lymphoid tissue chemokine; T β_4 , thymosin β_4 ; TNF, tumor necrosis factor; Trk ATrk B, and Trk C, the family of tyrosine kinases; P.S. trk-a; TRPV1, caspacin receptor protooncogene encoding a transmembrane glycoprotein the product is a tyrosine kinase receptor

1 Introduction

Nowadays it is impossible to understand the brain immune system without understanding complicated mechanisms of innate and adaptive immunity as a whole. At present, significant data on the presence of immunity components in the brain are available. In this chapter, it is attempted to find specific and nonspecific components of the brain immune system. More and more data become available that the brain immune system components are common with those of “peripheral” immune system. Cytokines, chemokines, and other known factors of intercellular communication are generated not only in blood cells (lymphocytes, macrophages, neutrophils, and monocytes) but also in neurons and glia along with the synthesis classical neurotransmitters. Some of them are expressed in neurons in lesions or in neurons of special functional state. For example, “brain-born” cytokines (Besedovsky et al., 2001) are expressed in hippocampus during the long-term potentiation (LTP). Electrically active neurons actively suppress MHC inducibility of surrounding glia cells and neurons. After incubation of neurons with tetrodotoxin, MHC inducibility is restored (Neuman et al., 1995, 1996; Neuman and Wekerle, 1998). Thus, electrically active neurons modulate immune reactivity of CNS by downregulating MHC expression. “The functional significance of the MHC expression was still subject to debate. The conventional wisdom that immune

recognition molecules are not present in the CNS has now been challenged by the study of Shatz and colleagues (Corriveau et al., 1998) whose data suggest the widespread use of a network of MHC I receptor-ligand systems in electrically active neurons at time when they are undergoing remodeling and synaptic plasticity. . . . The study of MHC I expression in neurons has been planned by large numbers of conflicting reports. . . . Moreover, the study provides new direction to the search for molecular mechanisms underlying complexity in the brain by suggesting the need to reevaluate whether the mechanisms by which T-cell clones generate diversity are also utilized by individual neurons" (Darnell, 1998).

Taking into account the large body of experimental evidence concerning mainly the chemistry and biology of the newly discovered cytokines and other signal molecules of immune system (immunophilin, etc.) that are produced by neurosecretory magnocellular nuclei of hypothalamus and by being very sensitive to the different neurotransmitters and other chemicals neurosecretory cells of NSO (*N. supraopticus*) and NPV (*N. paraventricularis*), I wrote: "It becomes evident that in addition to the known intimate structural neurohumoral interaction between the nervous and immune systems, some neuronal cells of the brain seem to fulfill the T- and B-lymphocyte functions" (Galoyan, 1997, p. 169). Studying molecular-genetic mechanisms of immune response by neuronal cells, elucidation of all components of the brain immune system and mechanisms of their participation in the immune response is the main goal of molecular neuroimmunology.

It is necessary to gain more information about immunocompetent neural cells as well as about immune molecules of brain neurons. It is clear that the main components of antigen presentation to T-lymphocytes are inducible as shown by Syken and Shatz (2003): "...their documentation of $\beta 2$ microglobulin expression in neurons is consistent with the presence of functioning MHC I signaling system". Although not all components at the MHC I signaling system have been yet examined in the neurons, those that have been identified seem well suited for providing specific signaling pathways. The clearest example from the paper by Syken and Shatz (2003) is in the visual system. Presynaptic lateral geniculate nucleus (LGN) neurons express the TCR component CD3 ζ , and postsynaptic layer IV neurons in the visual cortex express MHC I in dendritic processes. These observations led Shatz and colleagues to suggest two related neuronal activities for MHC I molecules in the brain (Syken and Shatz, 2003). The authors showed that the regulation of parenchymal class II MHC after local injection of IFN- γ is site-specific. "To activate endogenous T-cells, adult CDF rats were immunized with a normal antigen (myelin Basic protein, MBP). Two weeks later, the proinflammatory cytokine IFN- γ (100–10,000 U/site) was administered into two neurochemically and anatomically distinct sites: the hippocampus (area CA1) and brainstem (nucleus of the solitary tract). Monoclonal antibody R73 was used to detect T cells on cryostat sections. The greatest difference was seen 48 h after the injection of 300 U of IFN- γ at each site, when several times more parenchymal T cells were present in the brainstem than in the hippocampus. Most parenchymal T cells were CD4⁺/class II restricted. It can be suggested that the local regulatory environment contributes to site-specific immune regulation (Phillips and Lampson, 1999).

The neurosecretory hypothalamic cells of NSO and NPV are the gold mine for brain immune system and particularly brain neuroendocrine immune system. The discovery of neurosecretion by NPV and NSO of hypothalamus (Scharrer and Scharrer, 1954) and the biosynthesis of vasopressin and oxytocin in these nuclei as well as that of releasing factors of adenohipophysial hormones (Guillemin, 1978; Schally, 1978) can be considered as the foundation of modern molecular neuroendocrinology. During the last 43 years we added a new dimension to this area discovering novel functional systems. The phenomenon of hypothalamic neurosecretion of a number of organotropic and imminomodulatory molecules will be reviewed below. These systems are closely related to each other, and they are single links in difficult adaptation of organism to different pathogenic influences.

2 The Functional System of Neurosecretory Hypothalamus–Endocrine Heart

The neurosecretion of organotropic (cardioactive) neurohormones and their protein carriers by NSO and NPV has been demonstrated in our works. The isolation of cardioactive (coronary-dilatory) peptide neurohormones from hypothalamus, their specific carriers and precursors can be considered as an

important event in this field. Coronary dilatory activities of mammalian hypothalamic extracts were first reported in 1962 (Galoyan, 1962), and tentatively designated as neurohormones “C” and “K”. In 1964 first specific proteins of bovine hypothalamus possessing coronary dilatory properties were discovered. These proteins were precursors and carriers of substances NC and NK (Galoyan, 1964, 1965). Their coronary dilatory activity was shown to be involved in lymph flow regulation (Srapionian et al., 1997). Later the NC and NK substances produced by NPV and NSO were isolated from the neurosecretory granules of hypothalamus neurohypophyseal system (Galoyan and Sahakian, 1971). Multiple forms of NC were revealed in studies of its physical, chemical, and biochemical properties (Galoyan et al., 1986). The cardioactive protein–hormonal complexes belong to neurochemical systems involved in the regulation of coronary circulation (Galoyan and Srapionian, 1983) and in blood clotting mechanisms (Coggin et al., 2002). Neurosecretory peptides of hypothalamus possess both cardiotropic and immune activities. It was shown that cardioactive neurohormones and protein hormonal complexes are produced also by the neurosecretory cells of hypothalamus. They are localized mainly in synaptosomes of hypothalamus as well as in neurosecretory granules of hypothalamus–neurohypophyseal system. In 1967, we demonstrated the phenomenon of neuropeptide neurosecretion by atrial ganglionic cells (Galoyan and Rostomian, 1967). These observations were made prior to reports of Dr. De Bold and associates regarding the synthesis and storage of atrial natriuretic peptides within the atrium (see reviews of Chobanian, 2000; Guillemin, 1999). Later on we isolated bovine atrial coronary active peptides as well as novel cardioactive protein hormonal complexes. It was discovered that atrial ganglionic cells and incretory cells of pancreas produce hormones (noninsulin, such as glucagons, etc.), which are stimulators (liberins) of hypothalamus coronary dilatory hormone release into general circulation (Galoyan, 1965; Galoyan et al., 1971). Now it is time to consider the neuroendocrine heart and neurosecretory hypothalamus as the functional system playing an exclusively important role in the adaptation of the organism in general (Galoyan and Sahakian, 1971; Galoyan, 1986, 1992a). Organotropic (cardioactive) atrial peptides and signal molecules of brain neuroendocrine immune system form a coupled regulation system.

3 The Brain Neuroendocrine Immune System

The discovery of cytokine secretion by hypothalamus magnocellular nuclei (NPV and NSO) as well as of biosynthesis of a new class cytokines (proline-rich peptides, PRPs) illustrates the capability of the brain neuroendocrine cells to synthesize cytokines along with vasopressin, oxytocin, and cardioactive neurohormones. The discovery of strong antibacterial (in vivo) and antiviral (in vitro) properties of hypothalamic PRPs as well as strong antineurodegenerative and immunotropic properties of PRP family cytokines provides evidence for the existence of brain protective mechanisms against infection (Aprikyan and Galoyan, 1999). In other words, brain immune system specificity is the capability to produce in neurosecretory cells the protective neuropeptides, which oppose different infections in vivo. To get some idea about brain immune system specificity it is necessary to identify immunocompetent cells and immune molecules and to determine their role in the immune defense of the brain and the organism as a whole. In the present chapter, an attempt is made to compare the components of peripheral and brain immune systems and to characterize the specificity of the last.

We concentrated our research on signal molecules of brain immune system produced by neurosecretory cells of NPV and NSO and on the role of these substances in providing (in cooperation with glial cells) the protection of the brain during various infections and degenerative disorders and in the regulation of immunocompetent cells in the organism (Galoyan, 1997, 2004). The accumulated literature data on CNS interaction with immune system, expression of some cytokines and their receptors in human neurons in culture, in astrocytes and microglia, provide evidence about the existence of brain immune system. The present chapter provides a general introduction to the latest data concerning chemistry and biology of newly discovered cytokines and other signal molecules of the immune system that are produced by magnocellular nuclei (NPV, NSO) of hypothalamus and, in our opinion, participate in the generation of brain immune response (like T- and B-lymphocytes and macrophages). The experimental results obtained so far give substantial grounds to claim that cytokines arising from NPV and NSO neurons can participate in brain

immune response in cases of viral invasions (including retrograde ones via n. vagus and without break-up of brain–blood barrier), autoimmune and neurodegenerative disorders, traumas, etc., along with glial cells and brain MHC I and II complexes (Brightman et al., 1995; Fleshner et al., 1995; Laye et al., 1995; Kreutzberg, 1996). The understanding of molecular mechanisms of central regulation of adaptation to various factors is impossible without the knowledge of fine genetic, morphological, biochemical, neuroendocrine, and neurophysiological processes in functioning of hypothalamic nuclei, particularly of NPV and NSO. Considering the immunomodulators of the brain immune system as an integral part of the adaptation mechanism, we concentrated on the search of signal molecules of immune system in the brain itself, mainly in NPV and NSO, and on the role of these substances in providing, in cooperation with glial cells, the protection of brain in various infections and degenerative disorders. The existence in the brain, mainly in the hypothalamus, of the multiple immune molecules (immunomodulators) playing a pivotal role in the brain immune system is of great importance. The aim of this chapter is not to show neuroendocrine, neurophysiologic, and neurochemical mechanisms of the immune system regulation of the organism by the brain (there is a great deal of literature about it), but to solve the problem of whether the brain itself is an immune organ, and also to define cellular, neurochemical, and immunological properties of the brain that serve for its immune defense when the blood–brain barrier is not damaged, in spite of the penetration of the infection into brain.

Is the brain an organ of the immune system? To better understand the problem, at least four aspects should be taken into consideration:

- Interrelations of the CNS with the immune system. The CNS is immunologically distinct owing to the presence of the blood–brain barrier (BBB). In certain cases (patients with multiple sclerosis, etc.) the existence of a number of immune cells and humoral factors in the brain is a result of infiltration of T-lymphocytes into the CNS.
- Does the expression of a large quantity of cytokines and their receptors in human neurons, astrocytes, and microglia (mainly in the culture of these cells) provide evidence that the antibodies are formed by the brain cells themselves? It is known that microglia is considered as the resident macrophages of the CNS. It was shown that viruses can be transported into the brain parenchyma through the axons (Brightman et al., 1995).
- Are neurons principally inducible regarding the major histocompatibility complex (MHC) expression? It is not known why the healthy CNS tissue is devoid of MHC antigens. “Electrically active neurons actively suppress MHC inducibility of surrounding glia cells. After incubation of the neurons, e.g., by tetrodotoxin, MHC inducibility is restored” (Neuman et al., 1996; Neuman and Wekerle, 1998). Moreover, similar data are obtained by the authors referring to the neurons of the hippocampus. Probably, for the expression of MHC in neurons, special demands for nerve tissue are required.
- Self–nonself discrimination by the immunocompetent cells of the brain in the adaptive immune system.

“Phylogenetically the immune system of mammals is divided into two branches, innate immunity and adaptive immunity. There are important differences between these systems with regard to the problem of self–nonself discrimination. In the more ancient innate system immunity is evoked by biochemical differences that distinguish the pathogen from the host. The antigen receptors that recognize these biochemical differences are encoded in the germ line of the host so that self–nonself discrimination is genetically determined. However, in the more recently evolved adaptive immune system, which is found only in vertebrates, discrimination can be made between host’s own proteins and those of a closely related species, even when the differences involved consist of only a few amino-acid residues. The number of B-cell and T-cell clonotypes in a mammal greatly exceeds the number of genes in the genome, so that the antigen receptors on these cells are generated by somatic recombination of alternative segments of DNA that encode them. Because the selection of these alternatives is an essentially random process, it follows that the antigen receptors so generated do not exclude that set which recognizes self-antigens. Consequently, self–nonself discrimination in the adaptive immune system is an acquired characteristic, not an inborn one. How is this self-tolerance acquired? Self–nonself discrimination among T cells is mediated principally in two ways: by deleting the majority of auto-reactive T cells in the thymus and by active suppression of auto-reactive T cells

in the periphery by a specialized set of T cells termed 'Treg.' I believe that neurosecretory cells of hypothalamus can be very sensitive to self- and nonself antigens, taking into account their high sensitivity to different chemical agents (Galoyan, 1965). However, the issue of self–nonself discrimination by brain immunocompetent cells needs further investigation. It is obvious that mechanism of development of all neurodegenerative diseases of brain (up to the cell death) includes a stage of autoantibody formation like it is in the case of tumor cells. A number of disorders belong to this group, including encephalomyelitis, spongy-like encephalomyelitis, paraneoplastic encephalomyelitis, autoimmune neurological disorders, autoimmune diseases induced by intracellular vesicle-associated proteins, multiple sclerosis, several neurodegenerative diseases. They all are subjects for studying the role of the brain immune system.

It is noteworthy that the multidisciplinary approach should be applied to the unraveling of molecular mechanisms of action of the brain immune system. At present we still do not have a complete knowledge about brain immune system components. Taking into account the specificity of immunological properties of brain cells, it makes sense to examine first the immune system signal molecules of the brain and those of the peripheral nervous system as well. Let us survey the defence arsenal of the immune system as a whole and that of the brain immune system in particular.

4 The Cytokine System

“Cytokines are small proteins (approximately 25 kDa) that are released by various cells in the body, usually in response to an activating stimulus, and they induce responses through binding to specific receptors. They can act in an autocrine manner, affecting the behavior of the cell that releases the cytokine, in paracrine manner, affecting the behavior of adjacent cells, and some cytokines are stable enough to act in an endocrine manner, affecting the behavior of distant cells, although this depends on their ability to enter the circulation and on their half-life in the blood. There are two major structural families of cytokines: the hematopoietin family, which includes growth hormones and also many interleukins with roles in both adaptive and innate immunity, and the tumor necrosis factor (TNF) family, which again functions in both innate and adoptive immunity and includes many members that are membrane-bound.” (See Janeway et al., 2005).

Cytokines traditionally are divided into several groups: interleukins (ILs), factors of interaction between leukocytes; TNFs; interferons (IFNs), cytokines with antiviral activity; colony stimulating factors (CSFs), hematopoietic cytokines; chemokines, chemotactic cytokines; growth factors. Cytokines produced in response to certain factors form a unique structural–functional system, the cytokine network, within which biological actions of single cytokines are regulated. The most important principles of cytokine system activity are inducibility, functioning locality, superfluity, interrelation, and interdependency.

4.1 Inducible Character of Cytokine Formation and Reception

The production of cytokines in response to stimulating effects is determined by the level of gene induction. At the same time, it is known that certain cytokines (IL-12 and IL-15) are produced in small quantities spontaneously. Cytokines, according to their origin, are formally divided into monokines (produced by monocytes and macrophages; stromal and epithelial cell products are similar) and lymphokines (produced by lymphocytes). Monokines induce inflammation, and lymphokines induce antigen-specific immune response. Monokine production in comparison with that of lymphokines is characterized by a higher rate of release. Retardation of certain lymphokine production is determined by time necessary for lymphocyte activation. The presence of high-affinity receptors on the cell surface in sufficient quantities is a necessary condition for the development of cellular response to cytokines; their role is to transduce cytokine signals into cells. Usually, a small quantity of cytokine receptors is found on the surface of quiescent cells. Often they lack components (subunits) determining receptor affinity to cytokines. As a

result, quiescent target cells usually do not respond to physiological concentrations of exogenous cytokines. Only very high doses of cytokines can affect inactive target cells. Generally, under the influence of inducers the quantity of receptors increases up to a necessary level; sometimes additional polypeptide chains appear in their structure (Yarilin, 1997).

4.2 Locality of Cytokine Action

Invariance of conditions of onset of cytokine secretion and amplification of expression of cytokine receptors on target cells are the main prerequisites for providing local character of cytokine action. Different pathogens (bacteria, viruses, parasites, etc.) are localized in the organism, as a rule, in a certain micro-volume where producer cells and target cells of cytokine action are activated. Locality of cytokine action also depends on the exact ratio between time and expression period of cytokine genes and their receptors. Thus, there are different mechanisms of providing local character of cytokine action as well as preventing cytokine release from the sites of their production. In the case of cytokine release into the bloodstream, their quick removal takes place.

4.3 Superfluity of Cytokine System

The superfluity of cytokine production means that each type of immune system cells is able to produce several cytokines, and each single cytokine can be secreted by different cells (Table 7-1). Besides, cytokines are characterized by polyfunctionality and overlapping of final effects. A number of cytokines induces T-cell proliferation or participates in the process (IL-2, 4, 7, 9, 13, 15, and TNF- α). Even greater number of cytokines participate in proliferation of B cells (IL-2, 7, 15, 1, 12, TNF- α , and INF γ). The reliability of such redundancy is enhanced by the fact that the production of cytokines with a similar spectrum of activity is carried out by different types of cells. For example, IL-2 and IL-15 are produced by dendritic cells and macrophages, respectively (Hsieh et al., 1993; Simbirtsev, 1998).

The overlapping of cytokine effects occurs not only on the cellular but also on the receptor level. There are five main classes of cytokine receptor types (Table 7-2) (Yarilin, 1997; Hirano, 1999; Hibi and Hirano, 2000). Within each family, the homology of receptor structures can be very high for different cytokines, the circumstance determining the similarity of these cytokine effects. Besides, the receptor complexes of different cytokines may have common polypeptide chains. For example, receptors for IL-2, -4, -7, -9, -13, and -15 have common γ -chain; receptors for IL-3 and IL-5 have common β -chain. According to the presence of common polypeptide chains, the cytokine receptors are classified into different families (Fukada et al., 1999).

Next level of cytokine overlapping effect realization is related to the presence of common ways of signal transduction from receptors into cell nucleus. The interaction of proximal cytoplasmic domains of tyrosine kinase receptor Jak (Janus tyrosine kinase) plays a key role in signal transduction. Four representatives of this family, which participate in signal transduction from cytokine receptors type I and type II are known (Jak1, Jak2, Jak3, Tyk2). The binding with cytokine receptor leads to transphosphorylation between tyrosines of two Jak molecules, as a consequence a complex of two Jak subunits acquires strong kinase activity. In its turn, Jak activates another tyrosine kinase localized on the cytoplasmic domain of cytokine receptor. The subsequent activation of tyrosine kinases results in the switching on signal transduction pathways, so that the signal from Jak kinase is transmitted in several directions. One of them is connected with the activation of Grb2/SHC/Sos complex with subsequent involving of p21ras and activation of serine/threonine kinase cascade, which kinases participate in mitogenic enzyme activation. This pathway is induced as by the action of majority of IL as well as by receptor-antigen interactions. Thus, a limited amount of tyrosine kinases and a discrete cascade of signaling pathways provide even greater overlapping of cytokine effects on this level (Simbirtsev, 1999; Hirano, 1999). The transcription factors of the STAT family (signal transducer and activator of transcription) participate in the realization of cytokine action on the gene level.

■ Table 7-1

Producer cells, target cells, and biological effects of cytokines

Cytokines	Main producer cells	Main target cells	Biological effect
IL-1	Monocytes, endothelial tissue, macrophages, fibroblasts, glial cells	T-, B-, NK-cells, macrophages, monocytes, endothelial cells, granulocytes, eosinophils	T-, B-, NK cells activation. Induction of cytokines and adhesion factors (IL-1, 2, 3, 4, 6, 8, TNF, G-CSF, M-CSF, RANTES, E-selectin, ICAM, INCAM) synthesis. Stimulation of bone marrow stem cells, glial cells, B-lymphocytes, T-lymphocytes, keratinocytes, fibroblasts, endotheliocytes proliferation. Activation of a number of acute phase proteins, ACTH, corticosteroids
IL-2	T-cells	T-, B-, NK-cells monocytes	Enhancement of T-, B-, NK-cells growth
IL-4	T-cells	Macrophages, T-, B-, NK-cells	Enhancement of T (Th2) and B-cells growth. Stimulation of SIAM, G-CSF, GM-CSF production. Inhibition of IL-1, TNF, IL-6, IL-8 production
IL-5	T-cells	B-cells	Enhancement of eosinophils, B-cells differentiation
IL-6	Monocytes, macrophages, and other cells	T-, NK-cells, activated B-cells, monocytes	Enhancement of apoptosis, phagocytosis, SIAM, TNF, and ACTH receptors production. Inhibition of T-cells proliferation. Suppression of IL-1 and TNF- α production
IL-10	Th2-cells, B-cells, mastocytes	T-, B-, NK-cells macrophages	Activation of T-helpers (Th2), SIAM production, inhibition of T-helpers (Th1), TNF, TGF, M-CSF, G-CSF production, H ₂ O, O ₂ ⁻ release, cytotoxicity
IL-12	Monocytes, macrophages, dendritic cells, neutrophils	NK-cells, CD4 ⁺ and CD8 ⁺ T-cells B-cells (B1)	Inducement of IFN- γ , TNF- α , TNF- β production by NK-cells. Activation of NK, providing T-helpers (Th1) development. Enhancement of surface antigens and receptors expression (CD56, CD2, ICAM-1, β and γ -chains of IL-2-R)
IL-15	Astrocytes microglia cells	T-, B-, NK-cells monocytes	Activation of T-lymphocytes proliferation. Promoting CTL differentiation. Activation of NK
TNF- α	Monocytes, macrophages, T-cells	Macrophages, Monocytes, granulocytes, eosinophils, T-, B-cells, endothelial cells	Activation of lymphocytes, neutrophils, eosinophils, fibroblasts, osteoclasts, neural cells. Induction of IL-1, IL-6, IL-10, IFN- γ production, and GM-CSF, G-CSF synthesis by endotheliocytes. Increasing CTL activity and proliferation. Enhancement of phagocytosis, degranulation, superoxide production, and adhesion molecules synthesis in polymorphonuclear leukocytes (E-selectin, ICAM-1, INCAM)
IFN- α	Leukocytes	Macrophages, eosinophils, B-, T-, NK-cells	Activation of NK, B cells, and other cells. Possess antiviral activity

■ Table 7-1 (continued)

Cytokines	Main producer cells	Main target cells	Biological effect
IFN-β	Many cells infected by virus	Macrophages, granulocytes, B-, NK-cells	Activation of NK. Possess antiviral activity
IFN-γ	T-cells	Macrophages, granulocytes, T-, B-, NK-cells, endothelial cells	Activation of monocytes, macrophages, fibroblasts, T-suppressors, B-cells. Stimulation of MHC I and II expression and antigen presentation. Possess antiviral activity
GM-CSF	T-cells, macrophages, fibroblasts, endothelial cells	T-cells, macrophages, granulocytes, eosinophiles, endothelial cells	Stimulation of granulocytes, macrophages, eosinophiles colonies of megakaryocytopoiesis, phagocytosis, oxygen metabolism

■ Table 7-2
Main types of cytokine receptors

Receptors' family	Cytokine	Interferon	TGF-R/TNF-R	Immunoglobulin-like	Tyrosine kinase
Ligands	IL-2, 3, 4, 5, 6, 7, 9, 12, GM-, G-CSF, EPO, prolactin, growth hormone	IFN-α, -β, -γ	TNF-α (I and II), LT-α and β, and Fas, CD30L, CD40L	IL-1 (I and II)	M-CSF (c-fms), stem cell factor (c-kit)
Structural-functional features	4 Cis residues in N-terminal. 2–4 extracellular domains	A couple of Cys residues surrounded by 1–2 extracellular domains in N- and C-terminals	2–4 extracellular domains, in each 3–4 Cys residues	Immunoglobulin-like structure	Cytoplasmic domain has tyrosine kinase activity

They bind gene regulatory sequences involved in the accomplishment of IL and IFN function, for example, acute phase protein genes, and promote their expression. Phosphorylation of STAT proteins leads to their homo- and heterodimerization: STAT dimers can then translocate into the nucleus, where they activate various genes. The proteins encoded by these genes contribute to the growth and differentiation of particular subsets of lymphocytes (Kisseleva et al., 2002; Aaronson and Horvath, 2002). Four representatives of STAT family affecting the cytokines in different combinations are known (▶ Table 7-3) (Simbirtsev, 1999; Hirano, 1999). As a result, the same genes can be activated by different cytokines.

The activation of different pathways of signal transduction originating from cytokine receptors in its turn influences the cytokine functional activity. It is supposed that cellular reaction in response to signal passes may be described by two possible models, by the model of “receptor conversion” and by “orchestral” model.

According to the first model, the effect of a definite signal depends on the structural–functional state of ligand. For example, upon the action of IL-6 on a cell (target cell A) a specific receptor complex is expressed. At the same time, IL-6 in complex with IL-6α (soluble form of IL-6 receptor) induces gp130 expression on another cell surface (target cell B); in other words, it promotes the formation of high affinity IL-6 receptor.

■ Table 7-3

Transcription factors of STAT family and corresponding signal inducers

STAT1	STAT3	STAT5	STAT6
Activated upon phosphorylation of tyrosine at position 440 of IFN- γ -R α -chain. The signal inducer is IFN- γ	Activated upon phosphorylation of tyrosine at positions 767, 824, 905, 915 of gp130. The signal inducer is IL-6	Activated upon phosphorylation of tyrosine at positions 332, 510 of IL-2-R α -chain. The signal inducer is IL-2	Activated upon phosphorylation of tyrosine at position 575, 603, 631 of IL-4-R α -chain. The signal inducer is IL-4

In this case, the cellular response to this signal will be different from that of IL-6 separate action. Similar example is IL-12, which is a heterodimer of two proteins: 35 kDa (p35, a cytokine) and 40 kDa (p40, soluble form of IL-12 receptor). Only p75 complex is biologically active (Freidlin, 1999).

The “orchestral” model is based on the idea of cytokine pleiotropic effect. Each cytokine possesses its individual structural–functional characteristics and is able to manifest unique biological activity, inducing certain cellular response. On the other side, the same cytokine can influence pleiotropically on different cells. One of the reasons of such pleiotropy can be the diversity of signal transduction pathways; as a result, the signal from one cytokine is transmitted inside different cells in different directions, causing different final effects. Moreover, in different cell populations the syntheses and regulation of activity of molecules participating in the signal transduction are different as well as are the DNA sites responsible for the transcription of signal transduction factors. The important reason of cytokine pleiotropic activity is interrelationship and interaction of transduction pathways. However, the cytokines induce the effect of multiple and varying signals in the cell. The specific response on a given signal depends mainly on the equilibration of these signals. Because of so-called conductor mechanisms, different directions of signal transduction are regulated (orchestrated), the inconsistency between incoming signals is eliminated, and the specific cellular response on the stimulus is formed. Different factors (signal duration and strength, interplay of transduction pathways, different factors of transduction, etc.) can play the role of the “conductors” (Hirano, 1999).

4.4 Interrelationship and Interdependency in the Cytokine System

The cytokines have a strong regulatory influence on each others’ production. Cytokines secreted by one type of Th-cells significantly influence another subpopulation of leukocytes, suppressing their differentiation and effector functions. For example, IFN- γ inhibits the proliferation of Th2-cells, and IL-10 inhibits the cytokine synthesis by Th1-cells. Cytokine functional interdependency can be increased through enhancing one cytokine action by another or by complementarity of their effects. For example, IL-1, IFN- α , and IL-6, can enhance mitogenic activity of IL-2, IL-4, and IL-7 without being independent growth factors for thymocytes and mature T-cells. Negative interrelationships among cytokines are observed at the cell level, e.g. mutual inhibition of action of IL-2 and IFN- α on the proliferation and differentiation of T-lymphocytes and other cells. It is also observed at the level of their receptors (e.g. inhibitor soluble immune activator marker (SIAM) binds IL-1 receptors and blocks them). Thus, cytokines are the components of the common functional field, and they are formed and act in definite combinations. This coherence is enhanced by interactions of cytokines at the level of their production and their effector properties. The basis of high reliability of cytokine chain functioning is the considerable level of overlapping and doubling of cytokine effects. At the same time, the cytokine system is well structured, and there is a strict principle of cytokine synthesis in consequence of induction. Data here present the evidence about complex structure of cytokine chains.

An influence on any part of this system should affect the chain functioning as a whole as well as the functioning of its parts. Thus, a single cytokine is dynamically connected with many other cytokines.

4.5 Cytokines and Their Brain Receptors

A great body of evidence for the presence of cytokines and cytokine receptors in the brain exists in the literature (🔗 [Tables 7-4-7-6](#)).

We can see from the tables here that many cytokines and some of their receptors are present in neurons, astrocytes, oligodendrocytes, and microglia cells. However, the neuronal origin of these cytokines is not proved. There are data to show that normal human brain expresses immune-reactive IL-1 β , especially in hypothalamus neurons (Breder et al., 1988). Rat hypothalamus and hippocampus express mRNAs for chemokine CXC and for IL-8 (Licini and Wang, 1992). A large number of studies have documented the production of TNF, IL-1, or IL-6 by endothelial, glial, and astrocyte cells in the CNS. Reverse transcription-polymerase chain reaction (RT-PCR) was used to study the expression of various cytokines and cytokine receptors in purified populations of human neurons, astrocytes, and microglia (obtained from human fetal brain at 12–18 weeks of gestation). Human astrocytes produce IL-1 β , IL-6, IL-8, IL-9, IL-11, IL-15, TNF- α , and granulocyte-macrophage-colony-stimulating factor (GM-CSF), but they do not produce IL-2, IL-3, IL-4, IL-7, IL-10, IL-12, and IL-13. Human microglia cells express IL-6, IL-12, IL-15, and TNF- α , but do not express IL-2, IL-3, IL-4, IL-5, IL-7, IL-8, IL-9, IL-10, IL-11, IL-13, and GM-CSF. The RT-PCR analysis with purified populations of human neurons demonstrated the expression of IL-1RI, IL-1RII, IL-6R, IL-8R, IL-9R, IL-11R, IL-15R, and GM-CSFR. These results suggest that cytokines secreted by astrocytes and microglia (IL-1 β , IL-6, IL-8, IL-9, IL-11, IL-15, and GM-CSF) should have functional roles in survival, differentiation, and regeneration of CNS neurons (Lee and Kim, 1997).

4.6 Brain Born Cytokines

Cytokine expression in the brain may take place at its different functional states. A clear increase in IL-1 β gene expression, triggered by glutamatergic neurons through *N*-methyl-D-aspartate (NMDA) receptors was observed in hippocampal slices and in freely-moving rats during the course of LTP (Schneider et al., 1998). Besedovsky and coworkers showed that the IL-6 gene is also overexpressed during *in vivo* and *in vitro* LTP (Pitossi et al., 1997). These data present the first evidence that cytokine gene expression in the brain can be triggered by presynaptically induced activity of a discrete population of neurons (Besedovsky et al., 2001). Does the expression of a large quantity of cytokines and their receptors in human neurons, astrocytes, and microglia (mainly in the culture of these cells) suggest the formation of antibodies or antibody-like compounds by the brain cells themselves? It is known that cells of microglia are considered as the resident macrophages of the CNS.

5 Chemokine System

Among the cytokines released by tissues in the earliest phase infection, there are members of the family of chemoattractant cytokines known as chemokines. They were initially named interleukins: interleukin-8 (now known as CXCL8) was the first chemokine to be cloned and characterized. All chemokines are related in amino acid sequence, and all their receptors are integral membrane proteins containing seven membrane-spanning helices. The chemokine receptors transduce signals through G-proteins. Chemokines are proteins of low molecular weight, regulating activation of cells and its migration toward the inflammation site. Their molecular weights range from 8 to 12 kDa, and amino acid sequence homologies, from 20 to 80%. The presence of disulfide bonds between cysteine residues is a characteristic feature of chemokines. These bonds contribute to a unique spatial conformation of chemokines necessary for the manifestation of biological activity through the interaction with specific receptors. In accordance with the position of cysteine residues in polypeptide chains all chemokines are divided into four groups: CXC (α -chemokines), CC (β -chemokines), C (γ -chemokines), and CX₃C (δ -chemokines). In α -chemokines any other amino acid residue (X) is between two first cysteines, whereas in β -chemokines two first cysteine residues are side by side. In γ -chemokines there are only two cysteine residues instead of four, and they are distant from each other.

■ Table 7-4

Cytokines and cytokine receptors in the brain (Sternberg et al., 1989)

Cytokine	Neuron	Astrocytes	Oligodendrocytes	Microglia
IL-1	C/R	C/R	C/R	C
IL-2	C/R		R	R
IL-3	C/R	C/R	R	R
IL-4		R	R	R
IL-5		C		C/R
IL-6	C/R	C/R		C/R
IL-7		R	C/R	R
IL-8	R	C/R		R
IL-9		R		
IL-10		C/R		C/R
IL-11		C		
IL-12				C
IL-15		C		C
TNF	R	C/R	R	C/R
IFN- γ		C/R		R
TGF- β		C/R	C/R	C/R
GM-CSF	R	C/R	R	R
M-CSF		C	R	C/R

C, cytokine; R, receptor

■ Table 7-5

Central effects of cytokines (Breder et al., 1988)

Fever	(IL-1, TNF, IL-6, IL-8, MIP-1, CNTF)
Sleepiness	(IL-1, TNF)
Anorexia	(IL-1, TNF, IL-6)
HPAA activation	(IL-1, TNF, IL-6, and other gp 130-user cytokines)

Hypothalamus–pituitary–adrenal axis

■ Table 7-6

Cytokines in CNS diseases (Licini and Wang, 1992)

Meningitis (bacterial) ^a	IL-1, TNF
Cerebral malaria ^a	TNF
Multiple sclerosis/EAE ^{a,b}	TNF, IL-1, IL-12, IL-18
Alzheimer's disease	IL-1, TNF, IL-6
Cerebral ischemia ^a	IL-1, TNF
AIDS dementia	TNF
Myasthenia gravis ^a	IL-12
Stroke ^a	TNF

^aDiseases where inhibition of cytokines is protective in animal models^bEAE, experimental autoimmune encephalomyelitis

In δ -chemokines cysteines are separated by three other residues. The chemokine production is characterized by inducibility (chemokines involved in inflammatory processes—eotaxin, MIP, MCP, regulated and normal T-cell expressed and secreted (RANTES)) and by constitutive expression (chemokines involved in homing processes—stromal cell derived growth factor (SDF-1), BCA-1, secondary lymphoid tissue chemokine (SLC), MIP-3). Chemokine action on target cells is characterized by selectivity. CXC-chemokines, including IL-8, affect generally neutrophilic granulocytes and some types of mononuclear cells (except of monocytes), whereas CC-chemokines are chemoattractant mainly for monocytes, being also active toward other mononuclear cells. Molecules of chemokine family affect practically all leukocyte types, but each chemokine has its individual biological activity.

5.1 CXC-Chemokines

Regarding the ability for endothelial cell activation and angiogenesis regulation, CXC-chemokines are classified into two types: CXC-chemokines that have a common amino acid sequence Glu-Leu-Arg before first cysteine bond, have the common receptor CXCR2, are capable of activating neutrophils, and are angiogenic, and CXC-chemokines that do not have this amino acid residue sequence and that are angiostatics. The best known representative of CXC-chemokine subclass is IL-8. First, cell spectrum affected by the chemokine is the broadest in comparison with those of other members of this subclass. Second, IL-8 production is induced by activating the cells by different biologically active substances, including components of bacterial cell walls, viruses, a number of cytokines, lectins, etc. IL-8 is produced by monocytes or macrophages and endotheliocytes. However, minor production of IL-8 is observed in many other cells: lymphocytes, neutrophilic granulocytes, epithelial cells, fibroblasts, hepatocytes, etc. The strongest inducers of IL-8 synthesis are bacterial LPS and anti-inflammatory cytokines IL-1 and TNF. IL-8 synthesis starts in response to different exogenous and endogenous stimulants when their levels increase at the inflammation site during developing local defense reaction against pathogen invasion.

But, unlike other anti-inflammatory cytokines regulating the development of local inflammatory reactions, IL-8 synthesis can be induced by intravascular blood coagulation in sites of damaged tissues. Perhaps, it is connected with the activation of producer cells by the release of different mediators during blood coagulation. The induction of IL-8 synthesis by cells at the site of inflammation is accomplished in three main ways: (1) direct activation of the synthesis by bacterial cell wall components and viral proteins; (2) activation by cytokines and other biologically active substances appearing at the inflammation site; (3) stimulation of the synthesis upon intravascular blood coagulation. This multifactor system of IL-8 synthesis activation points at an important role of the cytokine in the regulation of inflammation (Simbirtsev, 1999).

5.2 CC-, C- and CX₃C-Chemokines

The most numerous are chemokines of CC subclass. Their main function is to activate monocytes or macrophages, lymphocytes, basophiles, and eosinophils, but not neutrophilic leukocytes. Lymphotactin is the only representative of C-chemokines possessing chemoattractant properties regarding T-lymphocytes. This polypeptide is synthesized by T-lymphocytes, NK-cells, mastocytes, and is unique because it displays the selectivity only to the lymphocytes. Among CX₃C-chemokines the most studied is fractalkine (neurotactin). It is the only membrane-bound polypeptide among proteins of chemokine family, which is synthesized by endothelial cells and expressed on cell surface. Fractalkine action is to attract monocytes and T-lymphocytes to the inflammation site. Both membrane-bound and soluble forms of fractalkine are biologically active (Simbirtsev, 1999).

5.3 Chemokine Receptors

Nowadays more than 20 different chemokine receptors possessing different affinities to one or more ligands of chemokine family are known. There are two types of high affinity receptors for IL-8, type I and type II receptors with 77% amino acid sequence homology. IL-8 receptors belong to the rhodopsin receptor

superfamily. Both receptors have seven transmembrane domains and transmit the signal through the interaction with G-proteins. A big chemokine group binds also IL-8 receptor, which corresponds to their common function—neutrophil activation (Ketlinski and Kalinina, 1995). Another receptor for IL-8, which binds the ligand with less affinity, is found on erythrocyte surface. This receptor is less specific, because it binds chemokines CXC and CC. This is DARC (Duffy-antigen receptor for chemokines) expressed on epithelial cells and brain glial cells. DARC can bind IL-8 and “present” it to circulating neutrophils when expressed on the postcapillary venular endothelium. It is important to note that using this molecule malaria plasmodium enters the erythrocyte. HIV uses receptors CXCR4 for CXC-chemokines and CCR5 for CC-chemokines as cofactors for penetrating the cells of human immune system (Redwine et al., 1999). Receptors for CC-chemokines are found on different cells: CCR1, on premonocytes; CCR-2, on monocytes; CCR3, on eosinophiles; CCR4, on basophiles and T-cells; and CCR5, on macrophages. It is significant that different chemokine receptors have different levels of specificity. For example, from CC receptors CCR6 and CCR8 bind only one ligand, and CCR7 is less specific and can bind two ligands. CCR9 has the lowest specificity. It binds all CC-chemokines and is expressed on all cell types. Most of the chemokines can bind to many receptors. For example, RANTES binds to CCR1, CCR3, CCR4, CCR5, and DARC receptors; MIP-1 α binds to CCR1, CCR4, and CCR5. However, at low concentrations chemokines bind only one receptor. Chemokine derivatives with minor mutations in amino acid sequence can play the role of chemokine receptor blockers. For example, mutant IL-8 derivative, which has a changed sequence upstream of arginine binds the corresponding receptor but does not induce chemotaxis. RANTES derivatives act in the same way: Met-RANTES (with redundant methionine residue in the amino acid sequence) and AOP-RANTES (with alkyl residue bound to the N-terminal serine). Met-RANTES or AOP-RANTES binding the corresponding receptor stimulates Ca^{2+} mobilization. However, in contrast to RANTES, both the derivatives do not induce chemotaxis and also inhibit RANTES-induced chemotaxis of eosinophils (Wells et al., 1999). It is important that T-helpers (Th1 and Th2) express different chemokine receptors on their surface. The main receptors expressed on Th1-cells are CCR5 and CXCR3, and to lesser extent these cells express CCR1, CCR2, CCR3, CCR7, and CXCR4. Th2-cells mainly express CCR4 and CCR8, and less of CCR1, CCR2, CCR3, CCR5, CCR7, CXCR4, and CXCR3. This selectivity of chemokine receptor expression on Th1 and Th2 cells determines a strict control over the cell migration to the inflammation site. Thus, the protective immune response (cellular or humoral) to a given pathogen is regulated (Wells et al., 1998, 1999). Now a large quantity of chemokines (more than 50) is known, and a lesser quantity of chemokine receptors.

The ability of the organism to develop defense reactions is completely affected upon chemokine gene destruction. Such serious dysfunctions are observed already during the embryonic development (e.g., upon CXC-chemokine gene lesion). There is an evidence of a significant role of chemokines in the immune response and during ontogenesis. Perhaps, a limited quantity of chemokine receptors and a great number of chemokines ensure the optimal regulation of cell migration (Simbirtsev, 1999).

5.4 Chemokines in the Neurons

Recently, chemokines and their receptors have been detected on the cells of both central and peripheral nervous systems (Meucci et al., 1998; Oh et al., 2001; Tran and Miller, 2003). The expression of chemokine receptors is not limited to microglial cells, and their function is indicative of neuron-dependent effects. Neuronal cells from both the hippocampus and dorsal root ganglia respond to chemokines with a transient calcium flux, suggesting the expression of various chemokine receptors on neuronal cells (Zou et al., 1998; Oh et al., 2001; Abbadie et al., 2003). Disruption of CXCR4 causes many proliferating granule cells to invade the cerebellar anlage, indicating a critical role of this chemokine receptor during brain development (Zou et al., 1998; Oh et al., 2001; Abbadie et al., 2003). The chemokine receptors play a role in modulating the sense of pain (Abbadie et al., 2003). These studies suggest that chemokines are involved in the development of the nervous system as well as in the CNS sensory processes (Szabo et al., 2002; Zhang et al., 2004). Data obtained by Zhang et al. (2004) indicate that proinflammatory chemokines are capable of desensitizing μ -opioid receptors on peripheral sensory neurons, providing a novel potential mechanism for peripheral

inflammation hyperalgesia. Immunohistochemical staining showed that CCR1 and μ -opioid receptors were co-expressed on small to medium diameter neurons in rat dorsal root ganglion. The introduction of either CXCL12 or CCL5 (RANTES) into rat cerebellar periaqueductal gray matter impaired DAMCO-induced analgesic effect, suggesting that MORS could be inhibited by chemokines. It was shown that CCL2, CCL5, and CXCL8 are capable of inducing transient calcium flux in a subpopulation of DRG (dorsal root ganglia) neurons. Recent studies have suggested that chemokines, the pivotal mediators of innate and adaptive immunity, also participate in inflammation-induced hyperalgesia. RT-PCR analysis showed that a spectrum of chemokine and cytokine receptors is expressed by the cells of rat root ganglia. Immunohistochemical staining of DRG showed that CCR1 is co-expressed with vanilloid receptor 1 (caspacin receptor proto-oncogene encoding a transmembrane glycoprotein the prodalt is a tyrosine kinase receptor, TRPV1) in more than 85% of small-diameter neurons. CCR1 on neuronal cells is functional as was demonstrated by CCL3-induced calcium flux and PKC activation (Zhang et al., 2004). CCR1, one of the proinflammatory chemokine receptors, is expressed on activated T-cells, monocytes, and neutrophils.

Chemokines can interact with TRPV1 in sensory neurons. Upon binding to their receptors on nociceptors, chemokines stimulate these neurons directly. The chemokine-mediated recruitment of macrophages and microglia and their activation in skin and nerve tissues might contribute to both inflammatory and neuropathic pain states. It has also been shown that astrocytes, microglia cells, and some neurons can produce cytokines (Fontana et al., 1982; Breder et al., 1988; Fabry et al., 1994; Pitossi et al., 1997; Schneider et al., 1998).

6 Antibody Production in the Brain

A number of diseases are known as slow human infections: kuru, Creutzfeldt-Jacob disease, subacute sclerosing panencephalitis (SSPE), progressive multifocal leucoencephalopathy, multiple sclerosis, Parkinsonism, Alzheimer's disease, Huntington's chorea, Schilder's disease, metachromatic leucodystrophy, and myoclonic epilepsy. Agents associated with the slow encephalomyelitides elicit antibodies, and standard immunological techniques can be used to detect them (Connolly et al., 1971). The IgG concentration is greatly increased in the CNS of SSPE patients. The major part of this IgG derives from a nonvascular source and is most likely synthesized within CNS (Culter et al., 1967; Koler et al., 1972; Touatellotte, 1976). Homogenous IgG bands were also observed in brain extracts and in blood sera of some patients. IgG accumulation was demonstrated in neurons and glia, in plasma cells, and in lymphocytes of perivascular infiltrations. A specific increase in γ -globulins has been found in the cerebrospinal fluid of patients having a variety of neurological disorders, most conspicuously in patients with multiple sclerosis.

7 The Discovery of the New Brain Immunomodulators

7.1 Thymosin β_4 (1–39)

The term "thymosin" was introduced by Goldstein et al. (1970, 1972) and Hooper et al. (1975) and was used for a biologically active fraction isolated from bovine thymus tissue (Rebar et al., 1981). Among the members of T β -thymosin (T β) family, T β_4 is the major peptide in different cells of human, calf, rat, or mouse. Thymosin β_4 (T β_4) has been reported to induce terminal deoxynucleotidyl transferase activity in vivo and in vitro, inhibit the migration of guinea pig peritoneal macrophages, stimulate the hypothalamic secretion of luteinizing hormone-releasing hormone (Rebar et al., 1981), or induce phenotypic changes in the Molt-4 leukemic cell line (Azakawa et al., 1994). Although several biological activities of T β_4 have been reported in the literature, its physiological role and biochemical mechanisms of action on the brain and immune cells still remain unknown. We discovered T β_4 (1–39) in the hypothalamus and elucidated its primary structure and fundamental biochemical mechanisms of action (Galoyan et al., 1992b). T β_4 (1–39) is the primary activator of calcium-calmodulin-dependent enzymes (cAMP PDE, myosin light chain kinase (MLCK), etc.) in concentrations of 10^{-9} – 10^{12} M without participation of Ca^{2+} and CaM. This polypeptide

is a Ca^{2+} -CaM-replacing molecule for the regulation of Ca^{2+} -CaM-dependent enzymes in the hypothalamus. Thus, a new level of Ca^{2+} -CaM-activated enzyme regulation was discovered. We determined the epitopes in the structure of $\text{T}\beta_4$ (1–39), which are responsible for the activity. $\text{T}\beta_4$ (1–39) as well as fragments 25–31, 11–19, 16–38 are strong activators of cAMP PDE, MLCK, cAMP-dependent protein kinase, etc. However, $\text{T}\beta_4$ -derived peptides Ac-SDKP and Ac-ADKP are inhibitors of cAMP PDE (Voelter et al., 1995).

The primary structure of $\text{T}\beta_4$ (1–39)



The peptides caused incomplete competitive inhibition of phosphodiesterase (PDE) activation by calmodulin. A 20-fold increase in the constant for PDE activation by calmodulin was accompanied by an insignificant elevation of the maximum rate of cAMP hydrolysis. In this case, the value of the inhibition constant (K_i) was about 600 nM. In the absence of calmodulin, the saturation concentration of these peptides reduced the enzyme activity nearly 2–3-fold. The effect of the peptides on PDE was noncompetitive with respect to cAMP ($K_i \sim 100$ nM). The data are characteristic of noncompetitive binding of both peptides to the enzyme with respect to changes in the enzyme properties during its interaction with the peptides. All the reactions of the enzyme mentioned here were reversible. Activating effect of these fragments can be explained by the presence of 5, 2, and 6 lysine residues in each peptide, respectively (Galoyan, 1997). Experiments by Gurvits and Sharova demonstrated that cyclic AMP PDE and 5'-N (5'-nucleotidase) make a coupled system (Galoyan et al., 1989; Galoyan, 1997). This system promotes quick conversion cAMP–5'-AMP–adenosine. The latter is of extreme importance for the regulation of vascular smooth muscle tone and for the formation of new cAMP in the brain through adenylate cyclase activation. We found that 5'-nucleotidase isolated from hypothalamus had the PDE activity (Galoyan et al., 1989). The existence of functional interrelation between PDE and 5'-nucleotides (5'-N) was demonstrated. The first-order rate constants were determined for adenosine formation using the two substrates, during 2-min incubation. It was found in kinetic studies of 5'-N that the first-order rate constant for 5'-AMP formed from cAMP under the action of PDE exceeded the first-order rate constant for exogenous 5'-AMP hydrolysis by a factor of 100. This can be a result of directed transfer of intermediate (5'-AMP in this case) from one active center of the enzyme to another if they are conjugated. It was found that the first-order rate constant for cAMP is 2.0 min^{-1} , and that for 5'-AMP is 0.02 min^{-1} . These data show that adenosine is formed mainly from cAMP. Moreover, neither cAMP (3–60 μM) nor N^{6-} , O^{2-} dibutyl cAMP (3–60 μM) can activate the hydrolysis of [^{14}C] 5'-AMP if the latter is the substrate. These data indicate the existence of functional interrelations between cAMP, PDE, and 5'-N. The Lineweaver-Burk plots show that both cAMP PDE and 5'-N are characterized by two K_m and $V_{\max}$ values, the fact indicating the existence of two catalytic sites on both enzymes, with high and low affinities for the substrates. For cAMP PDE the values of K_{m1} and K_{m2} are 2.5×10^{-6} and 3.7×10^{-5} M, respectively, and those of $V_{\max1}$ and $V_{\max2}$ are 6.3×10^{-6} and $3.7 \times 10^{-5} \text{ mol min}^{-1} \text{ mg}^{-1}$, respectively. If 5'-AMP is the substrate, the values of K_{m1} and K_{m2} for 5'-N are 5×10^{-6} and 1×10^{-4} M, respectively, and those of $V_{\max1}$ and $V_{\max2}$ are 5×10^{-6} and $7 \times 10^{-9} \text{ mol min}^{-1} \text{ mg}^{-1}$, respectively. cGMP PDE and 5'-GMP 5'-N are characterized by the same K_m values (Galoyan et al., 1989). We showed that cAMP PDE isolated from hypothalamus and also 5'-N are Ca^{2+} , CaM-activated enzymes. It was demonstrated that cAMP PDE and 5'-N activities are the properties of the same protein. The fact that the two enzymatic activities are associated with the monomer isolated from bovine hypothalamus provides evidence that the same molecule possesses cAMP PDE and 5'-N activities. $\text{T}\beta_4$ (1–39) (1 $\mu\text{g/ml}$) stimulated both cAMP PDE and 5'-N activities.

7.2 Isolation of a Fragment of MBP from Bovine Hypothalamus

We isolated from bovine hypothalamus several biologically active compounds, which proved to be stimulators of the basal activity of calmodulin-dependent cyclic nucleotide PDE. One of these stimulants has been purified to homogeneity by reverse phase HPLC. Amino acid sequence analysis showed that the

sample was indeed a peptide containing 14 amino acid residues corresponding to residues 72–85 of MBP with one exception: Glu was found in place of Gln (72) (Gurvits et al., 1992). The following amino acid sequence was obtained for this peptide: Glu-Lys-Ala-Gln-Gly-His-Arg-Pro-Gln-Asp-Glu-Asn-Pro-Val. Full 169 amino acid residue sequence of bovine spinal cord myelin MBP is shown here (Eylar et al., 1971):

AAQKRPSQRS KYLASASTMD HARHGFLPRH RDTGILDSLQ
 RFFGSDRGAP KRGSGKDGHH AARTHIGSL **PQKAQGHRPQ**
 DENPVVHFFK NIVTRPTPPP SQGKGRGLS SRSFWGAEGQ
 KPGFGYGGRA SDYKSAHKGL KGHDAQGTLS KIFKLGGGRDS
 RSGSPMARR

The fragment (72–85) of the protein (in bold letters) is similar to the sequence of the isolated peptide, with the exception of glutamine in position 72, where, according to our data, glutamic acid occurs. This substitution could take place because of in vitro glutamine desamidation, since our protocol included the homogenization of fresh tissue in an acidic medium (the solution of 0.25–0.5% acetic acid). It should be pointed out that in the peptide we obtained two other glutamines, which, despite this treatment, retained their amidated form; they correspond to glutamines 75 and 80 in the MBP sequence.

It was shown in previous studies that glutamine and asparagine residues neighboring basic amino acids (for example, lysine or arginine) are subjected to desamidation. In our case, glutamine-72 is followed just by lysine-73. MBP is one of the major myelin structural proteins. Properties laid in MBP structure can determine its functions as a phospholipid acceptor protein. Basic amino acids are distributed randomly in primary structure of the protein, without evident periodicity; they can interact with phosphate groups of phospholipids. Among 14 amino acid residues of the sequenced peptide, lysine, histidine, and arginine are present, which can participate in such interactions. MBP is also methylated by enzymes of the brain and other tissues. It has been shown previously that only arginine-106 is methylated. In our peptide, arginine is present in a position corresponding to arginine-79 in MBP, and it is not yet known whether it can be methylated. Besides, it was shown that threonine-98 of MBP is glycosylated as a result of *N*-acetylgalactosamine transferase action. A question arises if MBP glycosylation and deglycosylation take place during its functioning as a component of the myelin membrane. There are no threonine residues in our peptide (72–85). It should be noted that the bovine MBP structure (1–169) is highly homologous to the structure of rabbit and guinea pig MBPs, though some differences exist. In rabbit and guinea pig MBP the amino acid residues glutamine-75 and histidine-77 are absent, and serine is substituted for alanine-74 and proline-79, respectively. These substitutions are of importance because MBP is known to be one of the best substrates for cAMP-dependent protein kinases and Ca^{2+} -phospholipid-dependent protein kinases. The important discovery was that MBP participates in the induction of experimental encephalomyelitis. The studies of immunopathological role of MBP revealed that peptide fragments of MBP are also immunogenic (Hashim and Eylar, 1969; Eylar et al., 1971; Eylar, 1972). It was shown that guinea pig MBP fragment (114–123), which includes tryptophan residue, namely, Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys-Pro, is the most effective of all peptides studied as inducers of the disease. In the case of rabbit MBP, the fragments 114–123 and 44–89 possess the same property. The amino acid sequence 44–89 includes the sequence 68–74, which is structurally similar to the fragment (114–123). It was also found that even such amino acids of MBP as tryptophan, glutamine, and lysine are important for encephalogenic activity. It is worth noting that the peptide we studied was enriched enough in glutamine and glutamic acid and contained lysine. Such amino acid composition is favorable for the peptide immunogenicity. It is quite probable that under certain conditions various MBP peptide fragments can be formed by brain proteases, including the peptide under consideration, which possesses the encephalogenic activity. Studying functional properties of such peptides can help us to better understand pathogenesis of the disease and to suggest some methods of its treatment. MBP is a major component of myelin of all mammals. Much is known about the encephalotogenic epitopes of MBP (Alvord, 1984). The portion of the molecule (residues 116–124) that causes EAE in strain 13 guinea pigs is conserved among various species. On the other hand, the portion of the molecule (residues 74–87) that produces EAE in Lewis rats varies among different species. I believe that residues 72–85 also can produce EAE in different species.

7.3 Hypothalamic Immunophilin-Receptor of FK-506 Immunosuppressor: FK-506 Binding Protein (FKBP-12)

In 1992, we discovered immunophilin (Iph) in bovine hypothalamus peptidyl-prolyl cis-trans isomerase; its primary structure was determined by mass-spectral analysis and Edman degradation (Galoyan and Gurvits, 1992; Gurvits and Galoyan, 1995). The molecular weight of FKBP isolated from hypothalamus and that of FKBP earlier isolated from bovine thymus was 11,778 Da. This protein takes part in brain biochemical processes by changing the conformation of various enzymes and synaptic proteins; it participates thus in the formation of regulatory mechanisms of the immune system of the brain and of the organism as a whole. All proteins of this family (FKBPs of 12, 12.6, 14, 33, 38, 52, and 51 kDa) contain at least one domain that is homologous to FKBP12 (the FKBP-domain) (Callebaut and Mornon, 1995). FKBPs have distinct structures, cellular localization, functions, and other properties. The 12 kDa FKBP has been initially isolated from human erythrocyte and lymphocyte membranes (Cunningham, 1995) and from bovine hypothalamus (Gurvits and Galoyan, 1995; Galoyan, 1997); it effectively binds inositol-1,4,5-triphosphate ($K_d \sim 96$ nM) and inositol-1,3,4,5-tetraphosphate ($K_d \sim 14$ nM), and the inositolpolyphosphate binding inhibits the peptidyl-prolyl cis-trans isomerase activity of this FKBP (Gunningham, 1995). FKBP (107 amino acid residues) exhibits 85% identity with the amino acid sequence of FKBP12; in mammalian tissues it is associated with one ryanodine receptor isoform (Sewell et al., 1994).

The primary structure of hypothalamic immunophilin

GVQVETISPGDGRTPFKRGQTCVVHYTGMLEDGKKFDSSRDNRNPKFKFMLGKQEVIRGWEEGV
AQMSVGORAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKE

Taking into account the existence of Iph in the neurosecretory cells of the hypothalamus, one can suggest that multiple forms of peptidyl-prolyl-cis-trans-isomerase (PPIase), and particularly of Iph, are synthesized by the hypothalamic neurosecretory nuclei and play a role in protein folding; they also can take part in the regulation of biosynthesis of interleukins in the same nuclei as well as in the metabolism of secondary messengers (Ca^{2+} , cyclic nucleotides) in the brain. There are endogenous activators and inhibitors of Iph, among which are $T\beta_4$ (1–39) and $T\beta_1$. Iph can take part in the mechanisms of signal transduction in neurosecretory neurons of hypothalamus. The effect of Iph and of its fragments (1–9, 1–15) on the brain and immunocompetent cells was investigated. By an immunohistochemical method (avidin-biotin peroxidase complex), using antisera to 1–15 fragments of Iph, we observed Iph immunoreactivity (Iph-IR) in the NSO of frog and rat hypothalamus (Abrahamyan et al., 2001a, b). The immunoreactivity of Iph fragment was determined also in NPV varicosities. Iph-IR was revealed in several groups of cells concentrated particularly in the medulla oblongata where the cross- and the longitudinal sections of myelinated single nerve fibers and bundles were also found. They extended for a long distance through the Iph-IR neurons mainly in the paramedian gigant- and parvicellular reticular nuclei. Calcineurin is a target for FK-506-FKBP complexes in cells. Precisely by that mechanism the transcription of interleukin-2 gene is inhibited in lymphocytes, suppressing the immune response. It can be suggested that a similar complex could be formed by Iph and its endogenous ligands; this complex could regulate the level of formation of interleukins in neurosecretory cells of hypothalamus.

Cardenas et al. (1994) showed the possibility of complex formation between FKBP12 and calcineurin (with catalytic subunits), both in the presence and absence of immunosuppressor FK-506. Three Iph families, FK506/rapamycin-binding proteins (FKBP), cyclosporin A-binding proteins (cyclophilins, CyP), and parvulins, are recognized based on their structure and sensitivity to inhibitors.

7.3.1 Iphs as Cytokines

Some Iphs are secretory proteins that may potentially be involved in distant cell communication. During bacterial lipopolysaccharide-induced stimulation, macrophages secrete CyPA (Sherry et al., 1992). Exogenous CyPA exerts a chemotactic effect on eosinophils and neutrophils. The maximal chemotactic effect was observed at 10 nM CyPA, and cyclosporin A blocked this effect. CyPB is also a secretory protein, which has

been found in blood and milk. Its secretion is stimulated by fibroblast growth factor (FGF) and by epidermal growth factor (EGF) (Allain et al., 1996). The plasma membrane of human lymphocytes and the Jurkat cells contain CyPB receptor-binding sites, $\sim 35,000$ per cell, $K_d \sim 12$ nM. The N-terminal 24-amino acid fragment of CyPB interacts with this receptor (Mariller et al., 1996). After binding, the ligand–receptor complex internalizes, and then CyP dissociates and undergoes proteolysis. Cyclosporin does not influence CyPB binding to its receptor and its translocation into the cell. Stimulation of mast cells with anti-IgE causes FKBP12 secretion (Bang et al., 1995). Extracellular FKBP12 exerts a chemotactic effect on neutrophils, and this FKBP12-induced activation is blocked by FK506 and rapamycin. In spite of structural and functional heterogeneity, Iphs form a group of enzymes that share one common property: they all possess peptidyl-prolyl *cis*–*trans* isomerase (rotamase) activity. The involvement of PPI rotamase activity in protein folding is now well recognized. However, the nature of functional coupling between Iph native proteins, which form joint structural–functional complexes, is still unclear. As stated earlier, reversible *cis*–*trans* isomerization of imide bonds in oligopeptides and native proteins may have a regulatory role. It is logical to suggest that the regulatory effect of Iphs as components of various protein complexes is due to its rotamase activity. However, there is not enough experimental evidence that could confirm the involvement of Iph rotamase activity in such regulatory reactions.

7.3.2 Targets of the Iph–Immunosuppressant Complexes

In the course of search of target proteins for Iph-immunosuppressor complexes, which are responsible for immunosuppression, the calcium and calmodulin-dependent protein phosphatase known as calcineurin (CaN) has been identified (Liu et al., 1991; Friedman and Weissman, 1991). FK506 and cyclosporin A, when bound to Iph, inhibit CaN activity, and CaN inhibition might mediate immunosuppression: IL-2 gene transcription is regulated by the nuclear factor of activated T cells (NF-AT). The cytoplasmic form of NF-AT is phosphorylated, and it must be dephosphorylated in order to be translocated into the nucleus and activate the IL-2 gene transcription. Thus, FK506 and cyclosporin A appear to regulate immunosuppression by increasing the phosphorylation level of transcription factors required for IL-2 formation.

Rapamycin acts via a different mechanism: It influences a later, calcium independent stage in the T-cell cycle. Rapamycin inhibits the ability of IL-2 to stimulate the transition of T cells from G1 to S phase by blocking several steps in growth factor action on translational regulators. The proteins referred to as TOR1 and TOR2 (targets of rapamycin), RAFTs or FRAPs (rapamycin and FKBP targets) were found to be involved in the phosphorylation of cytoplasmic protein kinases (Kunz et al., 1993; Brown et al., 1994).

7.3.3 Iphs in the Nervous System

After the discovery of Iph, the majority of researchers were studying their activity in tissues and cells of the immune system. However, a substantial level of ubiquitously distributed Iphs was also revealed in the nervous system. The finding that Iphs are much more abundant in the brain than in lymphocytes suggested important roles for the Iphs in neural functions. A few selected examples of the evidence for the neurotropic Iph functions are presented below. First, FKBP and CyP protein and mRNA localizations are quite similar to those of CaN that suggest a physiologic link of Iphs to CaN. The limbic system is enriched in the Iphs and CaN, with high levels in the hippocampus. High levels of FKBP and CaN, but not CyP, were revealed in the caudate nucleus; the brain stem displays CyP, but neither CaN nor FKBP (Steiner et al., 1992). The protein whose phosphorylation level is enhanced by the treatment with immunosuppressors is nitric oxide synthase (NOS). NOS is a calcium–calmodulin-dependent enzyme, and the activation of NMDA receptors stimulates NOS activity because these receptors possess a calcium channel activity and allow calcium to enter the cell upon glutamate action (Dawson et al., 1993). Being a physiologic neurotransmitter, glutamate elicits neurotoxicity via NMDA receptors when released in excess following cerebral vascular stroke. By enhancing

NOS phosphorylation, the immunosuppressors might inhibit NO formation and block glutamate/NMDA neurotoxicity in the cerebral cortex and other brain regions.

In the nervous system, another protein that binds to Iph is the type I receptor for TGF β . TGF β induces the long-term synaptic facilitation, protects hippocampal neurons from ischemic damage and amyloid peptide-induced neurotoxicity, and promotes the axonal regeneration (Ren and Flander, 2004; Zhang et al., 1997). Iph is a releasing factor for serotonin from mast cells. FK506 inhibits this release (Hultsch et al., 1991). Potent effects of FK506 are inhibiting spontaneous and depolarization of induced release of dopamine and acetylcholine from PC12 cells (Hirsch et al., 1993). The effects of FK506 on retinal ganglion cells after optic nerve crush were also reported (Freeman and Grosskreutz, 2000). The purpose of the study was to determine the physiologic consequence of Iph (FKBP12) presence in rat retina, particularly in the retinal ganglion cells. It was demonstrated that FK506 confers neuroprotection on cells due to its ability to interfere with apoptotic mechanisms after optic nerve crush.

7.4 Thymosin β_1 (Ubiquitin) Isolated from the Neurosecretory Granules of Neurohypophysis is a Calmodulin-Binding Endogenous Protein, a Calmodulin Antagonist

By the use of HPLC chromatography, a new protein has been isolated from peptide–protein fraction of hypothalamus, and also from neurosecretory granules, which was eluted together with Iph, superoxide dismutase, and parvalbumin group (acetonitrile gradient, 40–44%). Two peptides registered at the wavelength of 210 nm were eluted in 41 and 42.1% of acetonitrile at a flow rate of 1.0 ml/min (Galoyan et al., 1992c; Galoyan, 1997; Gurvits and Galoyan, 2001). Employing mass–spectrometry analysis and micro-sequencing we succeeded in establishing their primary structures (Galoyan et al., 1992a). We have identified 30 N-terminal amino acid residues of the Thymosin β_1 /ubiquitin. The peptides contained 74 and 76 amino acid residues. The amino acid sequences of the obtained proteins were completely identical to those of T β 1 (1–74) and (1–76). Data obtained indicate that ubiquitin (T β 1) plays an important role in the proteolytic breakdown of interleukin precursors produced by hypothalamic neurosecretory nuclei.

The primary structure of thymosin β_1 (ubiquitin)

MQIFVKTLTGKTITLEVEPSDTIEDVKAKIQDKEGIPPDQQLIFAGKQLEDGRTLSDYNIQKESTL
HLVLRRLR₇₄–GG₇₆

It was shown that ubiquitin forms rather stable complexes with CaM in the presence of calcium ions (0.2 mM). Thus, one of the fundamental functional properties of T β 1 (ubiquitin) has been established on the basis of the fact that at high calcium ion concentrations (resulting in Ca²⁺-dependent conformational changes) it binds to CaM not permitting the latter to activate the enzymes depending on it, including cAMP PDE and MLCK. Under different pathological conditions of the organism when calcium level in the tissues considerably increases, T β 1 acts in the brain probably as a CaM antagonist preventing proteolytic protein degradation. T β 1 inhibits the phosphorylation of myosin light chain and other substrates. Thus, we isolated from bovine hypothalamus proteins, which in the presence of high calcium concentrations (0.2 mM) act as CaM antagonists and change the direction of metabolic shifts. Data obtained indicate that the immunocompetent cells and the pool of immunomodulators are located mainly in the hypothalamic neurosecretory neurons of NSO and NPV.

7.5 Isoforms of Macrophage Migration Inhibitory Factor in Bovine Brain

In the course of study of primary structures and molecular mechanisms of action of immunologically active compounds of the nervous system we have isolated two thermostable proteins from the soluble fraction of total bovine brain. The purification procedure was mainly based on DEAE-servacel ion-exchange chromatography and reverse-phase HPLC. The proteins were identified by the N-terminal Edman degradation and

database search as macrophage migration inhibitory factor (MIF). The N-terminal sequences for MIF1 and MIF2 were found to be identical. Molecular masses for MIF1 and MIF2 were determined as 12,369.21 and 12,299.7 Da by mass-spectrometry analysis. In addition, we have also isolated a third peptide with the same N-terminal sequence and M_r of 9,496.2 Da, probably, a proteolytic fragment of MIF. Using *p*-hydroxyphenylpyruvate as a substrate, we have not revealed tautomerase activity of either MIF1 or MIF2. A comparatively simple purification procedure was worked out, which may widely be used for simultaneous isolation in one run of the MIF isoforms (Gurvits et al., 2000). The protein known as macrophage migration inhibitory factor (MIF) was originally described as one of the first cytokines to be secreted from activated lymphocytes and inhibited the random migration of macrophages *in vitro* (Bloom and Bennet, 1966; David, 1966). More recently MIF was characterized as an immunological mediator that counter-regulates glucocorticoid action (Calandra et al., 1995). A multifunctional nature of MIF was suggested in further studies that demonstrated its occurrence in a wide variety of tissues and cell types. However, despite the fact that MIF has been widely studied in relation to the immune system, its physiological functions in the immune response are far from being clear. In relation to the nervous system, the role of MIF seems even much more obscure. MIF was previously rediscovered as being an anterior pituitary gland hormone; its presence was demonstrated in other regions of the nervous tissue (Galat et al., 1993, 1994; Nishino et al., 1995; Bucala, 1996). However, the biological role of MIF in terms of "where and when" its activity is expressed remains to be elucidated. Moreover, although MIF also was found to exhibit a number of catalytic functions (*D*-dopachrome- and phenylpyruvate tautomerase activities), the physiological significance of MIF enzyme activity is unclear (Bendrak et al., 1997; Rosengren et al., 1997).

The primary structure of the brain MIF



By searching the database, the primary structure of the N-terminus of each of the isolated proteins was found to be the exact match for that of the known MIF from calf brain cytosol, which has 114 amino acid residues (Galat et al., 1993, 1994). Comparison of the MIF isolated from bovine brain with other MIFs (human, rat, murine, chicken, etc.) demonstrates a high degree of similarity in amino acid sequences with many homology motifs (Swope et al., 1998). As mentioned before, the molecular masses of isolated proteins of fractions 6 and 7 were estimated as 12,369.2 (MIF1) and 12,299.7 (MIF2) Da, respectively. Mass-spectrometry analysis showed another peak of $M_r = 29,568.9$ in both fractions. For example, fraction 6 showed a protein identical to bovine MIF revealed in two peaks (m/z of 12,369.2 and 6,184.6) and an additional single peak having m/z of 29,568.9. As Edman microsequence analysis of the MIF proteins demonstrated no additional meaningful N-terminal amino acid sequence, we concluded that the fractions contain a protein that either is acetylated at the N-terminus or has the N-terminal amino acid sequence identical to that of MIF. The latter suggestion is less likely, because this protein was not revealed by Western-blot analysis. In addition, it should be noted that the existence of two MIF isoforms characterized by *pI* values equal to 9.5 and 9.4 has been reported previously (Galat et al., 1993). Two MIF isoforms have also been demonstrated with the use of RP-HPLC system. Sequence analysis and Western blotting revealed that one isoform was identical to bovine MIF and the other was an N-terminally modified form of MIF (Nishino et al., 1995). Based on the evidence presented earlier, we also concluded that there existed at least two MIF isoforms in the bovine brain and that the two proteins have identical N-terminal amino acid sequences. As both immunologic and enzymatic activities were reported to be expressed by the oligomeric structure of MIF, we suggested that our study might give additional information on MIF structure. Using *p*-hydroxyphenylpyruvate as a substrate, we have not revealed tautomerase activity of MIFs.

It still remains to be revealed whether the difference between MIF1 and MIF2 subunits found by RP-HPLC is of importance for MIF oligomeric structure. As MIF was found to be multifunctional, the results presented could contribute to further understanding of structural-functional relationships of MIF isoforms involved in the regulation of a variety of fundamental neuroimmunological processes. The precise physiological functions of MIF1 and MIF2 in relation to the immune response in the central nervous system remain to be elucidated.

8 Discovery of the Neuroendocrine Immune System of Brain

8.1 Neurosecretion of Interleukins by Magnocellular Cells of Hypothalamus: Bioassay Methods of Interleukin Identification

Secretory granules were isolated from bovine hypothalamus and neurohypophysis and dissected out immediately after the animals were slaughtered. Homogenization of the tissues and the procedures of granule preparation, purification, and lysis were carried out in the medium containing protease inhibitors (phenylmethylsulphonyl fluoride, benzamidine, and leupeptin) (Galoyan, 1997; Markossian et al., 1999). Experiments directed at neuronal cytokine-like activity determination were performed with lysates of the neurosecretory granules. Protein concentration in the granule lysates was determined according to Ohnishi and Barr using BSA as a standard (Ohnishi and Barr, 1978).

The presence of IL-1 α and β activities in the granule lysates was assayed by their ability to maintain the proliferation of the T-helper cell clone D10.G4.1 (Kaye et al., 1983, 1984; Kurt-Jones et al., 1985). The cells (2×10^4) in 200 μ l of a culture medium containing 2.5 μ g of concanavalin A (ConA) were incubated for 72 h at 37 °C in the presence of 7% CO₂ with various concentrations of the granule lysates. During the last 6 h of incubation 0.5 μ Ci of [³H]-thymidine was present in the medium. A quantity of IL-1, which induced 50% of maximal proliferation of cells, was accepted as one unit of IL-1 activity. The inhibition of IL-1 α and β activities was achieved by the addition of rabbit antisera to these ILs. The presence of IL-2 activity in the granule lysates was assayed by their ability to maintain the proliferation of the IL-2-dependent line of the cytotoxic T-lymphocytes (CTLL-2) (Simon et al., 1985; Seratte et al., 1987). The cells (2×10^4) in 100 μ l of the culture medium were incubated for 24 h at 37°C with various concentrations of the granule lysates, individual neuropeptides, or IL-2 standard.

Six hours before the end of the incubation, 0.5 μ Ci of [³H]-thymidine was added. Neutralization of IL-4, which can also maintain the proliferation of the CTLL-2 cells, was achieved by the addition of a monoclonal antibody to IL-4 (11B11). The results were evaluated by comparison with the activity of IL-2 standard (100 U/ml).

IL-6 activity in the granule lysates was assayed by their ability to maintain the growth of the IL-6-dependent mouse B-cell hybridoma (line B9) (Aarden et al., 1987). The cells (2.5×10^3) in 200 ml of the culture medium were incubated for 72 h at 37°C with various concentrations of the lysates of neurosecretory granules of neurohypophysis (NGN). During the last 6 h, 0.5 μ Ci of [³H]-thymidine was present in the medium. A quantity of IL-6, which induced 50% of maximal cell proliferation, was accepted as one unit of IL-6 activity. Cytotoxic activity of TNF- α in the granule lysates was assayed by its ability to lyse TNF- α -sensitive fibroblast cell line L₉₂₉ (Sugarman et al., 1985; Hoffman et al., 1989). The TNF- α neutralization was conducted by the use of rabbit antiserum to mouse TNF- α . The protein quantity that induced 50% lysis of fibroblasts was accepted as one cytotoxic unit. It was of great interest to detect TNF- α in the neurosecretory granules, taking into account that TNF- α produced in the brain (Turnbull et al., 1997) can block the stimulation of the HPA axis that occurs during neuronal inflammation. The ability of NGH (neurosecretory granules of hypothalamus) and NGN (neurosecretory granules of neurohypophysis) lysates to affect the expression of inflammatory cytokines IL-1 α and β , IL-2, IL-6, and the secretion of TNF- α was studied on astrocytes obtained from a primary glial cell culture from BALB/c mice (Aloisi et al., 1997). Astrocytes (2×10^4) were kept in a culture medium for 8 h, then the neurosecretory granule extracts, LPS, and polypeptides were added, and the incubation continued for 24 h. The interleukins were determined in the supernatants of the LPS-stimulated glial cells by the earlier-mentioned methods. Antigen presentation was assessed by measuring the ConA-specific T helper cell clone D10.G4.1 proliferation in response to ConA-pulsed astrocytes or peritoneal macrophages (Virgin et al., 1985).

For the differentiation of cytokines, antibodies against IL-1 α , IL-1 β , TNF- α , and IL-4 were used. The cytokines of the neurosecretory granules produced by magnocellular nuclei of hypothalamus play an important role in the immune system of brain. We were able to isolate cytokines from the neurosecretory granules of hypothalamus (NGH) and neurohypophysis (NGN). The effects of the NGH and NGN lysates on the IL-1 (α and β)-dependent clone D10.G4.1. proliferation was demonstrated. The results revealed that in the presence of both types of the granule lysates the IL-1 (α and β) activities were enhanced.

Hence, the lysates of NGH and NGN themselves possessed the IL-1-like activities. The activities in both cases were dependent on protein concentrations in the granule lysates. Thus, the NGH lysate at a dilution of 1:10 possessed the highest activity. Upon addition of rabbit anti-IL-1 α serum to the cell culture the IL activities decreased by 75%. Both IL activities were removed entirely by simultaneous administrations to the same culture of antisera to IL-1 α and IL-1 β . The contents of IL-1 (α and β) in NGH lysates were 75 and 24%, respectively. In the case of NGN, the highest activity of the NGN lysate was at a dilution of 1:20. The simultaneous additions of antisera to IL-1 (α and β) to the culture removed the effects of the NGN lysate completely. The experiments presented an evidence that the activities of both IL-1 (α and β) in NGN were higher. If the total activity of IL-1 (α and β) in control experiments was 15.6 U/ml, then on the addition of the NGH lysate (1:10) their combined activity grew to 65.5 U/ml, and on the addition of the NGN lysate (1:20) it grew to 84.3 U/ml. Taking into consideration that the initial protein concentration in the NGH lysate was 37.5 mg/ml, and in the NGN lysate, 19.4 mg/ml, it was possible to conclude that the IL concentration in the neurohypophysis was twice as high in comparison with that in the hypothalamus.

The IL-2 activity was also revealed in both NGH and NGN lysates by their ability to stimulate the proliferation of the IL-2-dependent line of cytotoxic T lymphocytes. We used an IL-2 standard (100 U/ml) to maintain the proliferation of these cells as a control. After the addition of monoclonal antibodies to IL-4 to the cell culture the activity of IL-2 remained unchanged. The IL-2 activity was higher in the NGN lysate in comparison with that in the NGH lysate (82.3 and 63.4 U/ml, respectively) upon additions of the 100- μ l aliquots of the granule lysates into the culture medium. Bearing in mind the fact that the protein concentrations per ml of the NGN and NGH lysates were 19.4 and 37.5 mg/ml, respectively, it becomes obvious that the concentration of IL-2 in the neurohypophysis was two times higher than that in the hypothalamus.

IL-6 is produced by a variety of cells, including macrophages, T cells, and B cells. We succeeded to observe the existence of IL-6 activity in the NGN lysates. The experiments performed with IL-6-dependent mouse B-cell hybridoma showed that the activity of IL-6 upon the addition of 100 μ l of lysate was lower than at 50 μ l. The level of activity was nondependent on the time of culture incubation (72 and 96 h).

The TNF- α activity was determined in both types of neurosecretory granules by the ability of TNF- α to lyse fibroblasts. The existence of TNF- α activity in the NGH lysate was shown. The results allowed us to conclude that TNF- α content in NGN was 3–4 times higher than that in NGH (Galoyan et al., 1998).

The biological properties of interleukins, particularly of “brain-born” cytokines, are given consideration in the chapter by Besedovsky and del Rey (1996), “Brain cytokines as integrators of the immune-neuro-endocrine network”, and also in some other papers: (Besedovsky et al., 1983, 1999; Berkenbosch et al., 1987; del Rey and Besedovsky, 1987; Rothwell and Hopkins, 1995; Rothwell, 1997, 1999; Dinarello, 1998; McCann et al., 2000; Besedovsky et al., 2001; Lehtimäki et al., 2003; Dunn, 2004).

8.2 Discovery of New Brain Neurosecretory Cytokines

More than 50 years has passed since the discovery of the primary chemical structures of vasopressin and oxytocin (produced by neurosecretory cells of hypothalamus) and their syntheses by Nobel Prize winner Vincent Du Vigneaud (1954). However, except for vasopressin and oxytocin, which are synthesized in neurosecretory cells of NSO and NPV of hypothalamus and were isolated from the neurosecretory granules of neurohypophysis, nothing more was known before our investigations. We have succeeded in the discovery and isolation of a family of organotropic (cardioactive) neurohormones and their protein carriers (Galoyan, 1965, 1997) as well as a series of novel cytokines from neurosecretory granules of bovine hypothalamus and neurohypophysis, and have also succeeded in the determination of their primary structure. For the first time we showed the neurosecretion of interleukins as well as new immunomodulators produced by the neurosecretory cells of hypothalamus. Neurosecretory granules were isolated from bovine neurohypophysis by a modified method described elsewhere (LaBella, 1968; Galoyan and Sahakian, 1971; Markossian et al., 1999). The neurohypophyses were dissected out directly after the animals were slaughtered, and then they were homogenized in 0.25 M sucrose (tissue/buffer ratio 1:10, w/v) in the

presence of the protease inhibitors (5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, and 10 μ M leupeptin). The procedures of granule preparation and purification were carried out in a medium containing the earlier-mentioned protease inhibitors at 4°C. From the granule lysate we were able to isolate and identify PRPs by HPLC system and microsequence analysis. Using the RP300 C18 column we separated six major peptides from neurohypophysis secretory granule acid extract fractionated preliminarily by ultramembranes Centriprep 50 and Microcon 3. Elution was accomplished using aqueous 0.1% TFA (buffer A) and 0.08% TFA in acetonitrile (buffer B). The peptides were eluted in the region from 17 to 27% of buffer B, at its concentrations of ~17.3 (I), 18.1 (II), 21.5 (III), 23.1 (IV), 24.1 (V), and 26.9% (VI). All of them were subjected to Edman microsequence analysis. Samples III and V were subjected to rechromatography on the Vydac C18 column before sequencing. Analysis of amino acid sequences of the peptides (▶ [Table 7-7](#)) showed that the peptides corresponding to peaks V and VI were vasopressin and oxytocin, respectively.

■ **Table 7-7**

The primary structure of neuropeptides isolated from neurosecretory granules of bovine neurohypophysis

Ala-Gly-Ala-Pro-Glu-Pro-Ala-Glu-Pro-Ala-Gln-Pro-Gly-Val-Tyr	(25–39)	PRP-1
Ala-Gly-Ala-Pro-Glu-Pro-Ala-Glu-Pro-Ala-Gln-Pro-Gly-Val	(25–38)	PRP-2
Ala-Gly-Ala-Pro-Glu-Pro-Ala-Glu-Pro-Ala-Gln-Pro-Gly	(25–37)	PRP-3
Ala-Pro-Glu-Pro-Ala-Glu-Pro-Ala-Gln-Pro	(27–36)	PRP-4
-H--Pro-Lys-Gly-NH ₂	Vasopressin	
H--Pro-Leu-Gly-NH ₂	Oxytocin	

The peptides corresponding to other peaks represented C-terminal fragments 27–36, 25–37, 25–38, and 25–39 of the neurophysin–vasopressin-associated glycoprotein (NVAG).

These peptides were designated PRPs: PRP-1, PRP-2, PRP-3, and PRP-4, respectively. PRP-1 peptide consists of 15 amino acid residues and has an apparent molecular mass of 1,475.26 Da. It was very interesting to study the physiological properties of human PRP, which also consists of 15 amino acid residues. According to mass–spectral analysis, the molecular mass of human PRP-5 is 1,560.5 Da. This polypeptide differs from bovine PRP in three amino acid residues. In the structure of bovine 15-amino acid PRP the Ala is in a position against Phe-31 in the human peptide. In place of Gly-Val in bovine PRP, the Asp-Ala is in human PRP (37–38). As it is shown below, the positions of proline residues in human and bovine PRPs are the same:

Bovine PRP-1: Ala-Gly-Ala-Pro-Glu-Pro-Ala-Glu-Pro-Ala-Gln-Pro-Gly-Val-Tyr

Human PRP-5: Ala-Gly-Ala-Pro-Glu-Pro-Phe-Glu-Pro-Ala-Gln-Pro-Asp-Ala-Tyr

In 1985, Richter (1985) by the method of gene engineering determined that the hypothalamic NVAG is a continuous molecule. NVAG is formed in NPV. The nucleotide and amino acid sequences of NVAG as a precursor form of rat and calf vasopressin were elucidated, and possible mechanisms of its fragmentation have been discovered. In ▶ [Figure 7-1](#) the complete amino acid sequence of bovine NVAG is shown.

Thus, bovine vasopressin precursor (preprovasopressin) consists of 168 amino acid residues, and its molecular mass is 17,310 Da. Rat NVAG also consists of 168 amino acid residues and has molecular mass of 17,826 Da (▶ [Figure 7-1](#)). The glycosylation site Asn-Ala-Thr and leucine-rich central part are well conserved in all species studied so far. The consecutive leucine residues may represent native processing signals for converting the glycoprotein into subfractions (Richter, 1985). Although glycoprotein NVAG is localized in the vasopressin-producing magnocellular neurons, the biological role of this glycoprotein remains to be clarified in detail.

The effect of PRP-1 is much wider: This cytokine is a unique regulator of several functions of the organism (as well as mediator of hypothalamus–neurohypophysis–bone marrow axis) (Aprikyan and Galoyan, 1999, 2000, 2001, 2002; Galoyan et al., 2000a, b, c; Galoyan et al., 2001; Galoyan and Aprikyan, 2002; Galoyan, 2004, 2005).

Amino acid sequence of bovine neurophysin-vasopressin-associated glycoprotein (Richter, 1985)

9.1 The Neuronal Activity of PRP-1

9.2 Antibacterial Activity of PRP-1

In our recent publications immunological methods of investigations of antibacterial properties of PRPs were described (Galoyan, 1997; Aprikyan and Galoyan, 1999, 2000) as well as some fundamental biochemical mechanisms of PRPs' antibacterial action (Galoyan, 2004). Data obtained indicate that PRP-1 is a strong antibacterial agent. Antibacterial properties of PRP-1 were tested with the following species of bacteria: *Salmonella typhimurium*, *Salmonella cholerae* ssp, *Salmonella typhi*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella flexneri*, *Shigella sonnei*, *Staphylococcus aureus*, *Streptococcus pneumoniae*. All these

highly virulent strains of bacteria were received from I. Mechnikov Institute for Vaccines and Sera (Moscow, Russia) and displayed typical cultural, biochemical, and morphological properties. Before use, the microorganisms were cultured in 100 ml of 2.5% nutrient broth (Difco Lab., Detroit, MI) for about 18 h at 37 °C. Then the bacteria were collected by centrifugation for 10 min at 1,500g, washed twice with PBS, and suspended in gelatin-Hanks-balanced salt solution (HBSS) at a concentration of $1\text{--}2 \times 10^8$ bacteria/ml. Five hundred microliters of each sample dilution was injected i.p. into mice. Aliquots of suitable dilutions were also seeded onto agar dishes for precise counting of bacterial cells. Determination of CFU (colony-forming units) of microorganisms was performed by agglutination with type-specific standard sera. Female pathogen-free lines of mice, i.e. BALB/c, C57BL/6J, C3H/HeJ, (CBAXC57BL/6J), F₁, at the age of 6–8 weeks (Laboratory of Animals “Stolbowaya” of the RAMS, Moscow), were used in the experiments.

Determination of lethal doses (LD) of bacteria for mice. Bacteria of each strain were injected i.p. into 10 mice in doses ranging widely in CFU. The surviving mice were counted daily for 21 days, and the 50% and 100% LDs (LD₅₀ and LD₁₀₀) were calculated, counting 21-day survivors, using Probit analysis.

Growth of bacteria in the internal organs of infected mice. At different time points after bacterial challenge mice were killed by cervical dislocation, and their peripheral blood, liver, spleen, intestine duodenum, mesenteric lymphatic nodes, intestine jejunum, and intestine crassum were obtained aseptically. Hundred microliters of each tissue homogenate dilution was spread over nutrient agar broth. Colonies of bacteria were counted after 18 h of incubation at 37°C.

Determination of antibacterial antibody formation in blood of infected mice. In blood sera of mice infected with *S. typhimurium* and *S. cholerae suis* the titers of anti-O-antibodies to *Salmonella* O-antigens were estimated by passive hemagglutination test with erythrocyte ultrasound-O-diagnostic system.

Determination of bactericidal activity of macrophages (MF). The method of direct measurement of the bactericidal activity of peritoneal macrophages (PMF) was used with minor modifications. Briefly, 1.0 ml of a suspension of viable *S. typhimurium* in sublethal dose in 0.1% gelatin-Hanks solution with 10% newborn calf serum was injected i.p. into mice. After 3 min the mice were killed, and peritoneal cells were collected. To remove the extracellular bacteria, the cell suspension was washed 3 times with ice-cold gelatin-Hanks solution and centrifuged for 4 min at 110g; the MF concentration was then adjusted to 6×10^6 cells/ml. MF containing bacteria were reincubated at 37°C, and at various time points the number of viable intracellular bacteria was determined by a microbiological assay. PRP-1 was administered 1 h before the challenge of mice with bacteria. The direct action of PRP-1 on the growth of bacteria in *in vitro* cultures was estimated microbiologically. It was shown that PRP-1 had no etiotropic effect. PRP-1 did not change quantitative and qualitative parameters of bacterial growth.

Our studies demonstrated that PRP-1 possesses strong antibacterial properties. It increased the survival index of mice infected with lethal doses of bacteria 1.64–3.8 times ($p < 0.001$) and had a pronounced effect on the infection development, and enhanced the production of specific antibacterial antibodies in infected mice 1.48–2.2 times ($p < 0.001$). PRP-1 also stimulated bactericidal activity of peritoneal macrophages 1.45–2.5 times by means of stimulation of intracellular killing of bacteria after phagocytosis ($p < 0.001$) and their clearance from the circulation. PRP-1 increased IL-1 synthesis by peritoneal macrophages (PMF) in mice infected with *S. typhimurium* 3.6 times ($p < 0.001$) (Galoyan, 2004). It was shown that administration of PRP-1 did not affect the phagocytic function of human granulocytes and monocytes but dramatically enhanced spontaneous as well as chemotaxis- and protein kinase C-dependent oxidative burst of cells (Davtyan et al., 2005a, b).

9.3 PRP Regulation of the Thymocyte Differentiation in Neonatal and Fetal Thymus

We studied the influence of PRP-1 on T-cell development during fetal life and the early neonatal period using differentiation of mouse thymocytes in FTOC (fetal thymus organ culture) and NTOC (neonatal thymus organ culture). Initially, we investigated the quantitative changes in thymocytes obtained from fresh fetal thymus lobes (*in vivo*) or from FTOC (*in vitro*). PRP-1 was injected *in vivo* or *in vitro* on the 13th day of gestation. It was shown that PRP-1 enhanced the number of cells by a factor of 1.23–3.1 ($p < 0.001$)

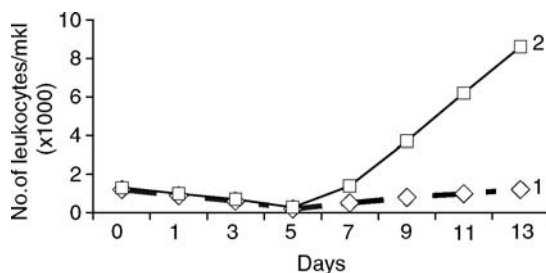
in vivo and 1.34–3.5 ($p < 0.001$) in vitro in fetal thymus lobes on the 13–19 days of gestation (Aprikyan and Galoyan, 2001). Of special interest are two fundamental findings concerning PRP-1 action. First, simultaneously with a decrease in the level of immature DP thymocytes (after the 17th day of gestation), an increase was observed in the generation of mature SP thymocytes. Second, the degree of generation of $CD4^{+}$ -thymocytes was higher than that of $CD8^{+}$ -thymocytes. It may result in subsequent reinforcement of T-lymphocyte functions.

9.4 PRP is a Regulator of Myelopoiesis

At present a number of well-characterized biomolecules are known to stimulate myelopoiesis. Nevertheless, a search for remedies capable of regulating myelopoiesis remains a challenge. We investigated the regulation of myelopoiesis by PRP-1 as well as the role of the peptide in the prevention of infection by *P. aeruginosa* in mice with leukopenia. After cyclophosphamide (CPA) administration a profound leukopenia was induced in both control and PRP-1-treated mice, and the nadir was reached on day 5 of experiments. However, the number of leukocytes in PRP-1-treated mice rapidly recovered after day 5, and starting from day 7 it was 2.8–7.2 times higher ($p < 0.001$) than that in control mice, in which leukopenia lasted for a longer period (Galoyan and Aprikyan, 2002) (Figure 7-2).

■ Figure 7-2

Effect of PRP-1 on peripheral blood leukocytes of CPA-treated mice. 1, control mice; 2, PRP-1-treated mice



Starting from day 7, the leukocyte population in PRP-1-treated mice consisted mainly of neutrophils with segmented nuclei and monocytes. At the same time, the incremental ratios of neutrophils were higher than those of monocytes (Table 7-8).

9.5 PRP-1 is a Stimulator of Bone Marrow Stem Cells

Taking into consideration a huge body of experimental evidence concerning the PRP action on the hematopoiesis during lymphocytopenia induced by cyclophosphamide (see Galoyan, 2004), we have performed recently in cooperation with the laboratory of Prof. L. Korochkin (Institute of Gene Biology of the Russian Academy of Sciences) a study on the effect of human and bovine synthetic PRPs (10–15 amino acid residues) on human stromal stem cells in culture (Galoyan et al., 2005, unpublished). Mesenchymal stem cells of bone marrow were isolated from tubular bones of abortion material on the 19–21 weeks of gestation. The mononuclear cell fraction was obtained by treating bone marrow by classic methods (Calter et al., 2000). We have received clonogenic culture of mesenchymal stem cells (MSC) from human fetal bone marrow and primary culture of bone-marrow stroma from patients. To study the differentiation of hematopoietic stem cells the following CD-markers were used: CD3, CD10, CD14,

■ Table 7-8

Effect of PRP-1 on the number of peripheral blood leucocytes in CPA-treated mice

Day	Treatment	Number of cells/ μ l				
		Neutrophils		Eosinophils	Monocytes	Lymphocytes
		Segmented	Banded			
0	None	210 $\pm$ 22	41 $\pm$ 7	12 $\pm$ 0.7	61 $\pm$ 4	1,063 $\pm$ 51
1	Control	4 $\pm$ 0.3	1 $\pm$ 0.1	1 $\pm$ 0.1	3 $\pm$ 0.1	596 $\pm$ 25
	PRP	10 $\pm$ 0.7	2 $\pm$ 0.1	2 $\pm$ 0.1	6 $\pm$ 0.3	521 $\pm$ 22
3	Control	1 $\pm$ 0.1	1 $\pm$ 0.1	0 $\pm$ 0	5 $\pm$ 0.3	186 $\pm$ 8
	PRP	1 $\pm$ 0.1	0 $\pm$ 0	0 $\pm$ 0	8 $\pm$ 0.4	347 $\pm$ 14
5	Control	2 $\pm$ 0.3	2 $\pm$ 0.3	0 $\pm$ 0	1 $\pm$ 0.1	192 $\pm$ 8
	PRP	1 $\pm$ 0.1	0 $\pm$ 0	0 $\pm$ 0	2 $\pm$ 0.1	116 $\pm$ 6
7	Control	11 $\pm$ 2	3 $\pm$ 0.3	0 $\pm$ 0	9 $\pm$ 0.6	411 $\pm$ 18
	PRP	1,013 $\pm$ 83	9 $\pm$ 0.8	15 $\pm$ 0.8	94 $\pm$ 6	436 $\pm$ 18
9	Control	57 $\pm$ 4	11 $\pm$ 0.9	3 $\pm$ 0.2	21 $\pm$ 1.8	684 $\pm$ 24
	PRP	5,108 $\pm$ 112	111 $\pm$ 12	83 $\pm$ 6	613 $\pm$ 22	523 $\pm$ 21
11	Control	61 $\pm$ 4	16 $\pm$ 1.1	5 $\pm$ 0.3	26 $\pm$ 1.8	861 $\pm$ 36
	PRP	6,421 $\pm$ 168	131 $\pm$ 12	94 $\pm$ 8	681 $\pm$ 28	783 $\pm$ 31
13	Control	73 $\pm$ 7	22 $\pm$ 1.3	6 $\pm$ 0.4	31 $\pm$ 2.2	936 $\pm$ 44
	PRP	7,621 $\pm$ 213	133 $\pm$ 11	96 $\pm$ 8	744 $\pm$ 31	917 $\pm$ 42

CD15, CD16, and CD34, and the antibodies against CD14, CD15, CD10, CD11a, CD11b, CD3, CD4, and CD16. Using the markers of hematopoietic cells, such as CD15 (neutrophils), CD14 (monocytes), CD16 (NK-killers), and CD34, we showed that only CD14 cells manifested differences in a PRP-containing medium. We observed a contradiction between data on the PRP-1 effect in vivo (in lymphocytopenia) and the data on direct effect of the PRP-1 on the stromal cells. Data obtained indicated that PRP-1 is able to stimulate colony formation (CFU) by hematopoietic stem cells both in vivo and in vitro. Notably, in vitro PRP enhanced the number of CFU, whereas in PRP-treated rat bone marrow the CFU number was decreased. To understand the regulatory effect of neurosecretory cytokines produced by hypothalamus NSO and NPV on bone marrow under normal and pathological conditions, it was of great importance to determine the existence of PRP-1 in human and animal bone marrow and to study its localization. The localization of PRP-1 was studied in the bone marrow of an adult healthy human individual died from trauma and bone marrow of 3-month human embryos resulted from miscarriages. We were able to demonstrate by immunohistochemical methods the localization of PRP-1 in bone marrow granulocytes and their progenitor cells (Galoyan et al., 2004c; Ter-Tadevosyan et al., 2006). Thus, bone marrow is a target of PRP-1; there exists a humoral axis between neurosecretory hypothalamic nuclei (NSO and NPV) and the bone-marrow.

9.6 Neuroprotective (Antineurodegenerative) Properties of PRPs

9.6.1 Anti-Snake Venom Effect of PRP-1 and PRP-3, Their Action in Spinal Cord Injury and After Nerve Transsection

In our previous works we discovered that PRP-1 and PRP-3 display a powerful antineurodegenerative effect upon snake venom action (*Vipera Raddei Boettger* 1898, cobra venom) (Galoyan et al., 2000b, 2001). Of some interest is a pronounced effect of a small dose (35 μ g) of PRP-1 even under conditions of paroxysmal bursting of neuronal activity upon preliminary application of a maximal dose of the venom. In this case, a

considerable (8-fold) BA decrease and gradual disappearance of bursts is observed. Thus, again the adaptive effect of PRP-1 takes place, even in its minimal dose, in comparison with the preceding case. In this case high efficiency of PRP-1 small doses is observed. The absence of effect of systemic venom injections is revealed after preliminary injection of PRP-1. The application of the venom revealed only 2-fold decrease in BA frequencies without a pronounced initial excitatory effect, the result confirming protective properties of PRP-1.

Of special interest is the effect of PRP-1 on injured nerve cells under conditions of preliminary SC hemisection. As a rule, in this case, on injury side, under the section, a multiple decrease in the level of BA frequency takes place (Sarkissian et al., 2001). After 10-min action of PRP-1, the rehabilitation of activity to the normal level and even to a level 2 times higher takes place, which makes evident the great perspective of PRP-1 use for clinical practice. In other experiments the PRP-1 promoted nerve regeneration 3 and 4 weeks following rat nervus ischiadicus transection. There were no effects upon stimulation of the injured nerve distal stump in the control (without PRP-1 treatment) because of the absence of fusion between transected nerve stumps. In contrast, in the experiments with PRP-1 treatment, the complete coalescence of transected fibers together with restoration of the motor activity of the affected limb was shown (Sulkhanyan et al., 2003, Galoyan et al., 2005b).

9.6.2 The Crush Syndrome

During the crush, profound neurodegenerative changes take place in the brain. This is one of the reasons for studying the effect of neuroprotector PRP-1 on morphological and biochemical changes during crush syndrome. There are numerous literature data showing that in crush syndrome the intoxication of the organism occurs mostly during decompression when toxic metabolites are released into the blood from damaged tissues, reaching the brain because of the damage of blood-brain barrier. Available clinical data show that the mortality is most frequently caused by the intoxication of the organism starting from decompression. The general intoxication of the organism in crush syndrome is accompanied with a sharp depression of immunity. In our experiments on rats, during 2-h compression the protein synthesis decreased in cytosol by 37.7%. In 2-h decompression, the protein synthesis decreased in cytosol by 53.7%; in 2-h compression the synthesis decreased by 68.8% in comparison to that in the intact group. Under the influence of PRP-1, the protein synthesis in brain cytosol increased 5-fold in comparison with that of experimental group of animals without PRP-1, and by 54 and 128%, as compared with intact and control groups, respectively. In crush, no reliable changes were observed in brain ER, whereas in 2-h decompression the synthesis of membrane proteins decreased by 51.9%. Under the action of PRP-1, [^{14}C]-glucose utilization was stimulated by 66% in slices of large hemispheres of intact animals. After 2 h compression glucose oxidation was inhibited by 36%. Glucose utilization rate in decompression was inhibited in 2 and 24 h by 81 and 40%, respectively, in comparison with intact animals, and was equal to the level in intact animals after 48 h. In 2-h decompression 10-fold stimulation of glucose utilization under PRP-1 action was registered in 24 and 48 h after decompression; it was significantly higher than that in the control group (2-h compression). Our electron microscopy studies indicated that 2-h compression results in increasing the sizes of cell organelles (in comparison with those of intact animals). We observed the round-shaped mitochondria (M) and lightened matrix. In most of M large spots of focal lysis were seen. The outer mitochondrial membrane was maintained, though sometimes it looked destroyed. The cristae lost their typical parallel arrangement and adopted a honeycomb-like configuration. Certain M were transparent, and besides, normal cristae honeycombs-like structures were observed. Some M were irreversibly damaged. An increase in the distances between the cristae and respiratory ensembles located on the cristae occurred, which was accompanied by the uncoupling of oxidative phosphorylation reactions. In the period of 2-h decompression followed by 2-h compression the M swelled. Their sizes increased strongly, and they had round shape. Outer membrane of some mitochondria was damaged. Washing out the mitochondrial matrix took place. Often mitochondrial configuration was of honeycomb type, but they were more swollen, and the decay of their structure occurred. The introduction of PRP-1 in the case of 2-h decompression, which followed 2-h compression, was accompanied by transformation of brain cell morphology from damaged to intact type. The size of M decreased, the matrix maintained the majority of the organelles, and

its electron density was higher, but in some cases the matrix still was washed out. The outer membrane was maintained, but in rare cases it was still destroyed. The restoration parallel arrangement of cristae, disappearance of swollen M, and normalization of the disposition of respiratory ensembles on the cristae was characteristic of this series of experiments (Kevorkian et al., 2001; Galoyan, 2004).

9.6.3 Effect of PRP on Aluminum Neurotoxicosis

During past years the evidence accumulated in the literature that aluminum can present in brain neurons and induce the formation of amyloid proteins causing Alzheimer-like disease. Our data proved that PRP-1 can prevent aluminum neurotoxicity by removing aluminum from its place of accumulation in the nervous system. Thus, hopefully PRP-1 can be used as a remedy for prophylaxis and treatment of Alzheimer-like disease induced by aluminum (Shakhlamov et al., 2002; Galoyan et al., 2004b).

9.6.4 The Influence of PRP on the Model of Alzheimer's Disease Induced by Intracerebroventricular Injection of β -Amyloid Peptide (25–35)

The pathomorphological analysis of brain sections of animals that received $A\beta(25-35)$ injections showed significant cellular neurodegeneration of almost all regions of hippocampal complex and almost full destruction of dental fascia. A single PRP-1 administration before the $A\beta(25-35)$ injection did not influence the process of neurodegeneration. However, in rats that regularly received PRP-1 every other day during 4 weeks after $A\beta(25-35)$ injections, full restoration of all hippocampal gyrus and dental fascia and regeneration of cells were observed. Electrophysiological analysis demonstrated changes of the hippocampal single neuron spike activity under the stimulation of entorhinal cortex of the cerebral hemisphere. In the hippocampal neurons of intact animals, upon tetanic stimulation both the tetanic and posttetanic potentiations were observed, in some cases being accompanied by posttetanic depression of different levels and duration. The PRP-1 administration every other day during one month resulted in the prevention of $A\beta(25-35)$ intoxication and in restoration of hippocampal neurons to the normal state that suggests therapeutic perspectives for PRP-1 (Galoyan et al., 2004a).

9.6.5 Neurotrophin-Like Properties of PRP-1

The effect of neurotrophin on the biosynthesis of GFAP was studied on the embryonic astrocyte culture 14 days after adequate quantities of BDNF, GDNF, or PRP-1 were added to the medium. All the neurotrophins under investigation were capable of producing a stimulating effect on the biosynthesis of GFAP by embryonic astrocytes (Chekhonin et al., 2001). A conclusion was made that BDNF, GDNF, and PRP-1 neurotrophic factors are capable of producing a stimulating effect on the biosynthesis of gliofibrillar protein by astrocytes.

10 Discussion

The biosyntheses of immune system signal molecules in the neurosecretory nuclei of hypothalamus (NPV, NSO) indicate that parvo- and magnocellular cells of these nuclei are immunocompetent cells. All the experimental data reviewed here confirms to a certain extent the existence of the neuroendocrine immune system of the brain, signal molecules of which participate in molecular mechanisms of immune response and neuronal survival. The most interesting recent finding is the broad spectrum of action of PRP-1—this cytokine is a unique mediator of the hypothalamus–neurohypophysis–bone marrow–thymus axis. PRP-1 may participate in hematopoietic stem cell differentiation. Our data demonstrated that PRP-1 is a stimulator of myeloid as well as lymphoid precursors. It regulates myeloid and lymphoid lineages differentiation.

With Marks and coworkers we studied the effect of PRP-1 on caspase activities in neuroblastoma N2A cells. PRP-1 causes definite inhibition of caspases 3 and 9 and activates of caspases 2 and 6 in neuroblastoma N2A cells. As is known, these caspases cause degradation of some cellular proteins, including precursors of amyloid peptides. There are literature data on positive role of caspase-2S in amyloid peptide formation. In vitro effect of PRP-1 on the activity of the earlier-mentioned 4 caspases of neuroblastoma N2A cells indicates that in vivo PRP-1 probably prevents the development of neurodegenerative processes. Our data show that PRP-1 alters the cytosolic caspases of differentiated but not of nondifferentiated N2A cells providing another example of biological activity of PRP-1 (Galoyan et al., 2000c). PRP-1 can be considered a stimulator of immune cells, myelopoiesis, T-cell differentiation, and hematopoiesis.

Also, PRP-1 is a unique regulator of protein, carbohydrate, and lipid metabolism, with antioxidant action in different pathological conditions. PRP-1 can protect erythrocyte membrane during hemolysis preventing release of troponin C from cardiomyocytes into blood during experimental infarction (Danelyan et al., unpublished data). PRP-1 also inhibits membrane phospholipase A2 activity in rabbit heart tissue, erythrocyte membrane, blood lymphocytes, and cardiomyocytes during cardiopulmonary insufficiencies. It prevents H_2O_2 accumulation and inhibits the formation of oxygen active radicals (O_2^- , HO^- , etc.) in various toxicoses, thus maintaining the cell membrane integrity (Ghazaryan et al., 2001). PRP-1 also maintains the level of glycerophosphate dehydrogenase and glycerol kinase activities, which are the key enzymes in glycolipid biosynthesis. In other words, PRP-1 takes an active part in phosphatidogenesis, utilization of glucose, glycolytic pathways, etc. In [Table 7-9](#) biological activities of PRP-1 are summarized.

A broad spectrum of PRP-1 biological effects on both immune and nervous systems is accompanied by noticeable stimulation of carbohydrate, protein, and lipid metabolism. In the brain, both in normal state and in crush syndrome, a powerful PRP-1 stimulates glucose utilization. Thus, after 2-h decompression glucose utilization increases ~ 10 times under PRP-1 action. In other organs, such as heart, liver, and kidneys, glucose utilization is stimulated to a lesser extent. High level of glucose utilization in the brain proves the PRP's role in carbohydrate metabolism, especially in that of glucose. Simultaneous 3- to 4-fold increase in protein biosynthesis in subcellular compartments of the brain and noticeable regeneration of nervous tissue were shown. PRP-1 is a potent antibacterial (in vivo) and antiviral (in vitro) agent. This property is realized through an increase in macrophage and T-cell activity, stimulation of antigen presentation function of macrophages, antibody formation, etc. In mice thymus development, PRP-1 affects T-lymphocyte differentiation in both embryonic and postnatal periods and exerts powerful regulatory effect on myelopoiesis in vivo as well.

Our experiments showed that during lymphocytopenia caused in mice by cyclophosphamide and during *P. aeruginosa*-caused toxicosis, the action of PRP-1 not only abolished lymphocytopenia, but also augmented the content of neutrophils and other cells of myeloid lineage. PRP-1-induced differentiation of TCD4^+ and TCD8^+ lymphocytes in the thymus during postnatal and embryonic development was shown. It is possible that progenitors of lymphoid and myeloid lines are also involved in the process. Data obtained indicate that PRP-1 probably affects stem cells of myeloid and lymphoid origin. The bone marrow contains two well-characterized types of stem cells: hematopoietic and mesenchymal stem cells.

Despite a large body of evidence obtained during our work, there is still not enough information for complete understanding of broad spectrum of PRP molecular mechanism of action; therefore, it was important to assume the existence of PRP-binding domains in many proteins. In other words, it is possible to accept the suggestion that PRP-1 can produce an effect on many biochemical and immunological systems by direct interaction with PRP-1-binding domains (SH2, SH3, and WW domains) (Heldin et al., 1998; Schlessinger, 2000; Pawson et al., 2002). SH2 (Src homology 2) domains recognize Y-P motif in peptide structure, and SH3 (Src homology 3) domains as well as WW domains can recognize PRPs. Functionally, WW domain contains elements of SH3 and SH2 domains as it recognizes proline-rich ligands; in some cases it is regulated by phosphorylation. The SH3 domain is the most widespread protein recognition module in the proteome, and more than 1,500 different SH3 domains can be identified in protein databases (Ren et al., 1993; Yu et al., 1994). SH2 domains are found in a large number of proteins, which have distinct biochemical activities. 111 SH2 domains are found in proteins of diverse functions, including those participating in the regulation of protein and lipid phosphorylation, phospholipid metabolism, transcrip-

■ Table 7-9

Biological properties of PRP-1

Metabolic activity under pathological conditions	
↓ phospholipase A ₂ ; Normalizes PL metabolism; ↓ lipid peroxidation; ↓ activity of caspase-3 and caspase-9; ↑ utilization of glucose; ↑ protein biosynthesis; ↑ creatine kinase activity; ↑ activity of caspase-2 and caspase-6; ↑ glycerol-3-phosphate kinase	
Antibacterial and antiviral effects	
↑ bactericidal activity of macrophages (phagocytosis, intracellular killing of bacteria, IL-1 synthesis, antigen-presenting function, accumulation and viability); ↑ antibody production; ↑ survival of mice in several infections; ↓ growth of bacteria in internal organs and enhancement of their elimination from the body; ↓ in vitro replication of EMV in Hep-2 cells; ↑ interferon- γ level in mitogen-stimulated blood mononuclears.	
Myelogenesis	Lymphopoiesis (T-cell differentiation)
Recovery myelopoiesis in profound leukopenia induced by CPA in mice infected with <i>P.aeruginosa</i>	↑ growth and differentiation of mice fetal and neonatal thymocytes in vivo and in vitro
Immunomodulatory action	
↑ immunocyte activation (T-cell proliferation; NK-cell, macrophage, monocyte, and neutrophil functions; astrocytes proliferation and expression of proinflammatory cytokines IL-1, IL-6); ↓ proliferation of Jurkat cells (human lymphoma cell culture) in vitro; ↑ IL-2-dependent proliferation of T-lymphocytes, antibody-dependent T-cell and LAK-cell cytotoxicity; ↑ cytokine production (IL-1 α , IL-1 β , IL-2, IL-6, TNF- α).	
Neuroprotective properties	
Restoration of background and evoked neuronal activity of spinal cord inhibited by <i>Vipera Raddei</i> and Cobra venoms; ↑ regeneration after spinal cord hemisection and nerve transection	
Neurohormonal properties	
↑ prolactin release into general circulation; ↑ expression of human GH in mice fibroblasts (clone BALB/c-GH).	
Neurotrophin-like properties	
↓ stimulation of biosynthesis of glial fibrillar acid protein (GFAP) in cell culture of astrocytes	

tion regulation, cytoskeletal organization, and control of Ras-like GTP-ases (Pawson et al., 2002). Many functional proteins contain SH2 domains: adaptors, scaffold proteins, kinases, phosphatases, transcription factors, small GTP-ase signaling molecules, signaling proteins, etc. (Pawson et al., 2002). SH2 domains interact with phosphotyrosine-binding domains (PTB) originally characterized by their ability to recognize phosphorylated Asn-Pro-X-Tyr motifs, such as those found in the RTKs for nerve growth factor (Pawson et al., 2002). Proteins containing SH2 domains can inhibit Jak kinases. PRP-1 metabolic, neuroprotective, and anti-infectious effects can explain its anti-oxidative, anti-free-radical, neurotrophic, antineurodegenerative, immunotropic, antibacterial, and antiviral properties.

11 Conclusion

For the first time neuroimmunology becomes a scientific part of the Handbook of Neurochemistry and Molecular Neurobiology, which is the evidence that during last 20–25 years in the worldwide bibliography the incontestable data appeared on the role of immunocompetent cells and organs in molecular mechanisms of brain and peripheral nervous system neurons. All the cellular elements of the blood, including the cells of the immune system arise from pluripotent hematopoietic stem cells of the bone marrow.

Specialized classes of lymphocytes recognize target pathogenic microorganisms or cells infected with them. Both innate immunity and adaptive immune response depend on the activities of white blood cells. In many cases, an adaptive immune response confers lifelong protective immunity to reinfection with the same pathogen. Activated macrophages secrete cytokines and chemokines. The building of “brain immune system” just started, and there are a lot of unsolved problems. It differs from immune response mechanisms that are formed in the periphery from brain immunocompetent cells.

The mechanisms of antibody formation in the brain, self–nonself discrimination, the role of single cytokines, chemokines, and their receptors in the brain are to be understood. Our discovery of neurosecretion by immunocompetent cells and cytokines answers the question about brain immune system availability. The cells of hippocampus (and maybe of other brain regions) express cytokines in different functional states of the central nervous system (Besedovsky et al., 2001). The microglia cells function as macrophage and dendrite cell. The microglia cells, like macrophages, phagocytize microorganisms and apoptotic products of neural and other cells, as well as apparently have antigen-presenting function, presenting molecules to MHC I.

Many questions are addressed to molecular neuroimmunology, and their solution can lead to the elucidation of the role of MHC and macrophage-like (neuroglia), immunocompetent neural cells, and mediators like PRPs. Old data on the possibility of immunoglobulin biosynthesis in the central nervous system do not exclude their role in immune response formation (Culter et al., 1967; Koeler et al., 1972; Touatellotte, 1976). As our data have shown, PRP-1 stimulates the formation of antibodies against a number of microorganisms, as well as the antigen-presenting function of macrophages. The antibody formation in the brain in autoimmune diseases was shown. Taking into consideration that all neurodegenerative brain diseases are accompanied to some extent by the development of autoimmune diseases, I can conclude that the mediators of the brain neuroendocrine immune system (PRP-1 and others) can prevent the development of brain autoimmune pathologies such as encephalomyelitis, spongiform encephalopathy, autoimmune neurological disorders, multiple sclerosis, and Alzheimer’s disease. Cytokine expression in the brain during autoimmune diseases is not excluded and might serve as one of the defense mechanisms. According to our data, signal molecules of brain neuroendocrine immune system (PRP-1 and PRP-3) prevent the morpho-functional neurodegenerative processes in the brain and spinal cord (which were induced by transection of spinal cord, snake venoms, aluminum neurotoxicosis, upon crash syndrome, and also upon intoxication of animals by amyloid peptides) by immune defense mechanism (25–35 и 1–40) (Galoyan, 2004; Galoyan et al., 2005b). The distinguishing feature of signal molecules of the brain neuroendocrine immune system is that entering general circulation they exert influence on immune organs and immunocompetent cells of the organism, including bone marrow cells. These molecules regulate the differentiation of lymphatic and myeloid cell lines during embryonic as well as during postnatal development.

The effect of PRP-1 on the differentiation of T-lymphocytes in fetal and neonatal thymus cell cultures, as well as on the regulation of myelopoiesis, is similar to that of transcription factor PU-1, which plays a critical role in the generation of both lymphoid and myeloid lineages (Walsh et al., 2002). Mice lacking PU-1 function die 1–3 days before their expected birth (Scott et al., 1994). I believe that PRP is a unique transcription factor for the production of progenitor cells of lympho- and myelopoiesis or a factor regulating the expression of transcription factors (GM-CSF, PU-1, GATA-3, IKAROS, etc.). The transcription factor PU-1, a hematopoiesis-specific member of the ets family (Moreau-Gachelin et al., 1990; Klemsz et al., 1990) is expressed in monocytes or macrophages and in B-lymphocytes, but also in erythrocytes and granulocytes. Thus, a factor replacing PU-1 (PRP-1) is present in the neurosecretory granules of hypothalamus. The

participation of PRP-1 in the transdifferentiation of brain stem nervous cells into hematopoietic cells cannot be excluded (Bjornson et al., 1999). We can assume that PRP-1 stimulates the formation of TH-1 and TH-2 (taking into account the formation of TCD4⁺ and TCD8⁺ lymphocytes), which define the type of immune defense and humoral and cellular immunity. As was shown, PRP-1 is a powerful neuroprotector in neurodegenerative pathologies of the brain and spinal cord. We can affirm that brain endocrine immune system and the immune system in general are conjugated regulation systems supplementing functions of each other.

Iph regulates the secretion of neurotransmitters (Steiner et al., 1996). It was shown that VDJ recombination required the expression of RAG-1 and RAG-2 proteins. RAG-1 is expressed in the brain (Chun et al., 1991). Moran and Graeber (2004) showed the inducibility of «immune» genes in the CNS tissues under various pathological conditions, during microbial infection and neuronal degeneration. MHC class I or Ib antigens are required to regulate the synaptic pruning on neuronal bodies that undergo retrograde degeneration after axonal transection (Oliveira et al., 2004). It was shown that during LTP of brain hippocampal cells the cytokine expression (IL-1 β , etc.) takes place (Besedovsky et al., 2001). MHC expression in the nervous system is actively regulated by neurons (Neuman et al., 1995, 1996; Curriveau et al., 1998; Neuman and Wekerle, 1998; Syken and Shatz, 2003; Wekerle, 2005). Recent work showed that MHC class I genes and CD3 ξ , an obligate component of T-cell receptors, are expressed in neurons, are regulated by neuronal activity, and function in neuronal development and plasticity (Syken and Shatz, 2003). CD ξ is a critical transmembrane signaling component of T-cell receptor complexes, which binds MHC1 ligands (Howe and Weiss, 1995). Both Immunoglobulin gene segment rearrangement and T-cell receptor gene segment rearrangement depend on the proteins RAG-1 and RAG-2. The RAG-1 and RAG-2 heterodimer is a component of V(D)J recombinase, which is active on very early stages of lymphoid development. RAG proteins are inactive during the burst of cell proliferation that follows these first successful rearrangements. Thus, the biosynthesis of neurotransmitters, biologically active peptides of type I and II MHC, RAG-1, and of a number of proteins—immunomodulators and cytokines—takes place in neurons (Galoyan, 2004).

Of current interest is to find functional–biochemical and genetic mechanisms of interconnection among neurotransmitter peptides and immune molecules (MPCI and II) to understand neuronal and neuro-immune functions of brain immunocompetent cells.

For brain immune system conception we should take into consideration the penetration of blood immunocompetent cells in the brain parenchyma (Brightman et al., 1995; Phillips and Lampson, 1999). We should find out the interrelationship of hematopoietic cells and brain immunocompetent cells and their contribution to the mechanisms of immune response of brain neurons. The discovery in the hypothalamus neurosecretory nuclei (NPV and NSO) of FK-506 immunosuppressor receptors (Iphs) possessing peptidyl-prolyl cis–trans isomerase activity brings us closer to the understanding of fundamental theoretical questions of the contemporary immunology theory of brain tissue transplantation. We take into consideration that some brain peptides (Neurohormone C, T β ₄ (1–39), etc.) can bind Iph, which is an important event for the regulation of biosynthesis of interleukins (IL-2) in the hypothalamic neurosecretory nuclei. There are connections between Iph–peptide–neurohormonal complexes and brain calcineurin. The understanding of Iph role in both biochemistry and physiology of the neuronal membrane will contribute to solving the problem of brain tissue transplantation. It is known that FK-506 and cyclosporin A, when bound to Iph and CyP, inhibit calcineurin activity, which results in the inhibition of IL-2 biosynthesis in T-lymphocytes. IL-2 gene transcription is regulated by the NF-AT. Cytoplasmic form of NF-AT is phosphorylated by CaN and must be dephosphorylated in order to be translocated into the nucleus and to activate the IL-2 gene transcription. Basu and Srivastava (2005) showed that dendritic cell (DC)-like sensory neurons express vanilloid receptor 1 (VR1), and that VR1 ligand capsaicin (CP), which has been shown previously to engage VR1 and transmit the perception of pain, similarly engages VR1 on the DCs and transmits the immunological inflammatory response. It was also shown by the authors that the receptors for SEMA4D, Slit2, Eph, Semaphorin 3A, and others, are expressed on DC as well as on neurons.

The discovery of the brain neuro-endocrine immune system (biosynthesis of signal molecules in NPV and NSO immunocompetent cells, elucidation of their primary structure, and their chemical synthesis)

turns a new page in our understanding of molecular mechanisms of the immune system regulation by neurosecretory cytokines and ensuring neuronal survival. The effect of recently discovered PRP-1 is much broader—this cytokine is a unique mediator of the hypothalamus–neurohypophysis–bone marrow–thymus axis. PRP-1 may participate in hematopoietic stem cell differentiation.

A huge work is ahead on elucidating the roles of all brain immune system signal molecules (Iph (1–39), TP_1 , MIF, MBP (72–85), studying their interactions, as well as the formation of new cytokines in the brain and peripheral immune system in normal and pathological states.

The data listed in the article prompt us to revise the role of hypothalamic neurosecretion in the regulation of hypothalamic–pituitary–cortical axis, as well as in the brain immune system and adaptation in general. Basing on a huge body of novel data reviewed here, the conventional conceptions concerning the neurosecretory function of hypothalamus and the hypothalamic mechanisms of adaptation have to be reconsidered.

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8 The Dialect of Immune System in the CNS: The Nervous Tissue as an Immune Compartment for T Cells and Dendritic Cells

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1	<i>Introduction</i>	198
2	<i>Physiological and Anatomical Features Contributing to Immune Privilege in the CNS and the Role of the BBB</i>	199
2.1	Physiological Features Contributing to Immune Privilege in the CNS	199
2.1.1	A Lack of Conventional Lymphatic Drainage from the CNS to Peripheral Immune Tissues ..	199
2.2	The BBB in Health and Inflammatory Disease of the CNS	200
2.2.1	A. Components of the BBB: The Neurovascular Unit	200
2.2.2	The BBB in Inflammation	203
3	<i>Soluble Mediators and Cell Surface Molecules that Promote Immune Privilege</i>	205
3.1	Neuropeptides as Soluble Regulators of Immune Privilege in the CNS	205
3.2	Anti-inflammatory Cytokines Produced by CNS-Resident Cells: TGF- β , IL-10	206
3.3	Cell Surface Molecules Expressed on CNS-Resident Cells that Regulate Immune Privilege: PD-1–PD-L1, FasL–Fas Interactions	206
4	<i>The Role of APCs in the Brain Tissue that Promote the Initiation of T-Cell-Mediated Immunity against CNS Antigens</i>	207
4.1	Naïve and Effector T-Cell Activation	207
4.2	CNS-Resident APCs	208
4.2.1	APCs in the Perivascular Space	208
4.2.2	APCs in the Brain Parenchyma	208
4.3	DCs Amplify Immune Responses in the CNS	209
5	<i>Conclusion</i>	211

Abstract: There is a growing body of evidence shedding light on the mechanism of immune regulation in the central nervous system (CNS). There are multiple elements that contribute to the special anti-inflammatory environment in this tissue. Unique physiological and anatomic features, soluble mediators and cell surface molecules and immune cells promote the immune privilege status of the CNS. These multiple mechanisms together shape immune responses in the inflamed CNS. A deeper understanding the mechanism of immune privilege in the CNS will provide the basic of successful therapeutic interventions for immune-mediated CNS diseases, such as multiple sclerosis (MS).

List of Abbreviations: α -MSH, α -melanocyte-stimulating hormone; AP, area postrema; APCs, antigen-presenting cells; BBB, blood–brain barrier; CNS, central nervous system; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; ECM, extracellular matrix; GM-CSF, macrophage colony-stimulating factor; HUVEC, human umbilical vein endothelial cell; ICAM-1, intercellular adhesion molecule-1; IFN- γ , interferon- γ ; IL, Interleukin; JAM, junction associated molecule; MIP, macrophage inflammatory protein; MOG, myelin oligodendrocyte glycoprotein; MRP, multidrug resistance-associated proteins; MRP, multidrug resistance-associated proteins; MS, multiple sclerosis; NVU, neurovascular unit; NVU, neurovascular unit; pDCs, plasmacytoid DCs; PD-L1, programmed death ligand 1; PECAM-1, platelet–endothelial cell adhesion molecule-1; PLP, proteolipid protein; SST, Somatostatin; TEER, transendothelial electrical resistance; TEER, transendothelial electrical resistance; TJs, tight junctions; TNF- α , tumor necrosis factor- α ; VCAM-1, vascular cell adhesion molecule-1; VIP, vasoactive intestinal peptide; ZO, zonula occludens

1 Introduction

Immune responses are greatly reduced in certain organs, including the central nervous system (CNS), eye, fetus, testes, and hair follicles (reviewed in Niederkorn, 2006). This phenomenon, called “immune privilege” (Billingham and Boswell, 1953) has been linked to the existence of several anatomical and physiological protective mechanisms in these tissues. To some degree, immune responses are regulated in every organ in a different manner, they are controlled in organs with limited capacity for regeneration, such as the CNS (🔗 [Figure 8-1](#)). In the CNS, immune-mediated inflammation could lead to devastating consequences. The idea of “immune privilege” in the CNS was originally described based on the relative hospitality of the brain towards transplanted allografts (Shirai, 1921; Billingham and Boswell, 1953; Barker and Billingham, 1977). Recently, the immune-privileged status of the CNS has been redefined. Additionally, it has been pointed out that privilege is not absolute; it varies with age and brain region and is mainly due to the lack of professional antigen-presenting cells (APCs), conventional lymphatic vessels, and the presence of blood–brain barrier (BBB) (Bechmann et al., 2007). In the last few years, our view regarding immunity in the CNS became more complex as we came to understand that (1) immune surveillance that involves the migration of immune cells into the CNS might be critical for protection of the brain and spinal cord (reviewed in Hickey, 2001) and (2) that there is bidirectional communication between the immune and the nervous systems that is further augmented under pathological conditions (Carson et al., 1999). Nevertheless, it is well appreciated that there is a unique microenvironment in the CNS that profoundly influences immune cell functions and the dialect of immune system in the nervous tissue. This microenvironment in the CNS is sustained by (1) existing physiological and anatomical features, such as the lack of lymphatic drainage and the presence of BBB, (2) anti-inflammatory suppressive factors and cell surface molecules expressed by CNS-resident cells (Carson et al., 1999), and (3) the lack of molecules in CNS-resident cells to stimulate immune cells (🔗 [Figure 8-1](#)).

In the inflamed CNS, this anti-inflammatory microenvironment shifts to a more pro-inflammatory condition as activated immune or CNS cells release proinflammatory mediators. This affects immune privilege in the CNS and promote immune responses (Wekerle, 2006). In this chapter we will discuss the current view of immunological privilege in the CNS and focus on the regulatory mechanisms that influence T lymphocyte and dendritic cell (DC) accumulation and function in the brain. We will take a careful look at the role of (1) physiological and anatomic features contributing to immune privilege with a special focus on the BBB; (2) soluble mediators and cell surface molecules that promote immune privilege; and (3) the role

■ Figure 8-1

Some of the critical physiological, anatomical features and molecules that contribute to the regulation of immune responses in tissues. VIP (vasoactive intestinal peptide), α -MSH (α -melanocyte stimulating hormone), SST (somatostatin); GALT (gut-associated lymphoid tissue); MALT (mucosa-associated lymphoid tissue), BALT (bronchial-associated lymphoid tissue); PDL-1 (programmed death ligand 1); PD-1 (programmed death molecule 1)

Physiological features:

Lymphatic drainage

Anatomical features:

Tight junctions between endothelial or epithelial cells

Immune suppressive soluble factors:

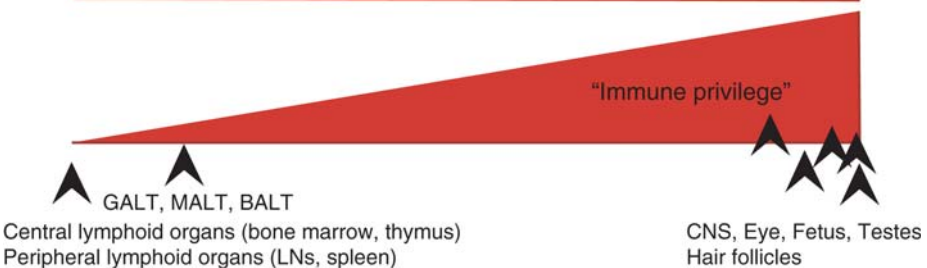
TGF- β , IL-10, VIP, α -MSH, SST, sFasL

Immune stimulatory molecules:

Major histocompatibility antigen Class II, costimulatory molecules

Immune suppressive molecules:

PD1-PDL1, FasL-FAS



of APCs in the brain tissue that promote the initiation of T-cell-mediated immunity against CNS antigens, with special interest in DCs. The consensus is that these multiple mechanisms together shape immune responses in the inflamed CNS.

2 Physiological and Anatomical Features Contributing to Immune Privilege in the CNS and the Role of the BBB

2.1 Physiological Features Contributing to Immune Privilege in the CNS

2.1.1 A Lack of Conventional Lymphatic Drainage from the CNS to Peripheral Immune Tissues

Lymphoid drainage plays a critical role in the delivery of antigens and antigen-bearing cells from tissues to lymph nodes where immune responses will be initiated. Lymph, an extracellular fluid that is produced by

continuous filtration from blood, is collected into an extensive system of vessels that returns it to the blood. Afferent lymphatic vessels drain fluid from tissues and carry the lymph with cells and antigens to lymph nodes. Lymph will be filtered through these draining lymph nodes and exit through the efferent lymphatic vessels that will return it to the bloodstream. Although conventional lymphatic drainage is absent in the CNS, pathways for the lymphatic drainage of interstitial fluid and proteins from the brain have been shown (Weller et al., 1992; Kida et al., 1993, 1995). There have been multiple potential pathways proposed for cerebral extracellular fluid drainage from the brain. It was suggested that extracellular fluid drain from the brain to blood across the arachnoid villi and to lymph along certain cranial nerves (primarily olfactory) and spinal nerve root ganglia (Cserr et al., 1992). It was shown that the clearance of ^{125}I -HSA from the cerebrospinal fluid (CSF) is almost equally distributed between lymphatic and arachnoid villi pathways (Boulton et al., 1996). Following intraventricular administration of radiolabelled serum albumin (^{125}I -HAS) in conscious sheep, the 6-h recoveries of ^{125}I -HSA in the lymph (sum of cervical and thoracic duct) and blood were $8.2\% \pm 3.0\%$ and $12.5\% \pm 4.5\%$, respectively and at 22 h, $25.1\% \pm 6.9\%$ and $20.8\% \pm 4.1\%$, respectively (Boulton et al., 1996). Using radiolabelled albumin as a marker of flow, it was described in rabbits, cats, and sheep that a minimum of 14–47% of protein injected into different regions of brain or CSF passes through lymph (Cserr et al., 1992). Intracerebrally injected protein antigens (ovalbumin or myelin basic protein), in the presence of intact BBB, elicited humoral immune responses, with antibody production in cervical lymph nodes and spleen (Harling-Berg et al., 1989, 1991; Cserr et al., 1992; Gordon et al., 1992), and also affected cell-mediated immunity (Harling-Berg et al., 1999). When inflammatory conditions were induced by the small trauma at the intracerebral injection, protein antigens were collected in the cervical lymph nodes and induced T-cell activation in the periphery (Qing et al., 2000). Furthermore, it was also shown that antigen may be more immunogenic when administered into the CNS than into the periphery (Gordon et al., 1992; Qing et al., 2000). These data indicate that the afferent arm of the immune response to antigens, within the CNS, is intact.

2.2 The BBB in Health and Inflammatory Disease of the CNS

2.2.1 A. Components of the BBB: The Neurovascular Unit

The concept of a barrier between the brain and the rest of the body was formed based on several experiments. In 1885, Paul Ehrlich discovered that the intravenous injection of an acidic dye (coerulein-s) caused staining of various organs, but left the brain uncolored (Ehrlich, 1885, 1906). Roux and Borrel (1898), Biedl and Kraus (1898) and Lewandowsky (1900) observed, respectively, that tetanus toxin, bile, and sodium ferricyanide, induced cerebral symptoms when injected into the cerebral fluid, but did not cause any cerebral symptoms when injected into the periphery (intravenously). Edwin Goldman (Goldman, 1913) injected water-soluble dye (trypan blue) intravenously and observed that the dye was selectively excluded from the brain tissue. Goldman concluded that there is a highly regulated and restrictive structure between the rest of the body and the CNS, but placed the site of this barrier in the choroids plexuses. Using horseradish peroxidase as a tracer, Reese and Karnovsky (1967) demonstrated the fine structure of the BBB and indicated that it is located at the level of the vascular endothelial cells. They proposed that the existence of tight interendothelial junctions and the absence of micropinocytosis in the brain endothelial cells are the two major components that contribute to the restricted cellular migration and molecular diffusion across the BBB. It was later shown that tight junctions (TJs) formed between neighboring endothelial cells restrict the paracellular flux and the lack of any significant number of endocytotic vesicles limits the transcellular flux across the BBB (Rubin and Staddon, 1999). To support the nutritional requirement of the brain, selective intracellular transport systems regulate the passage of nutrients such as glucose and amino acids and some transcytosed larger molecules such as transferrin across the BBB (Albrecht et al., 1990; Maher et al., 1994; Simpson et al., 1994; Joo, 1996; Staddon and Rubin, 1996). As part of this restricted transport system, cerebral endothelial cells express multidrug-resistance protein *mdr-1 α* (also called P-glycoprotein)

that actively transports low molecular weight toxic molecules out of the brain to the circulation (Schinkel et al., 1994). Besides *mdr-1 α* , additional multiple transport systems are expressed at the BBB, and by regulating efflux and influx of endogenous or exogenous substances, the BBB, and to a lesser extent, the blood–cerebrospinal barrier in the ventricles, represent the main interface between the CNS and the rest of the body (Girardin, 2006).

There are variations in the level of BBB restriction in different regions of the CNS. Brain microvessel permeability in areas of the CNS involved in neuroendocrine feedback is much less restricted. One of these areas is the capillaries of the hypothalamus tuber cinereum, where rich and close connections between fenestrated endothelial cells and neurons result in a relatively free diffusion of releasing and inhibitory hormones into the circulation. This zone, expressing high capillary permeability together with extensive blood–tissue surface area, and wide Virchow–Robin spaces (perivascular areas that are formed by the invagination of the subarachnoid space, presumably “dragged” into the depth of the brain during brain vascularization in early stages of embryonic development), is a gateway for flooding other tuberal compartments with blood-borne factors (Shaver et al., 1992). It is likely that the proximal arcuate parenchyma is exposed to ever changing gradients of neuroactive substances on a moment-to-moment basis. There is an absence of BBB at the area postrema (AP) that allows the diffusion of materials from the blood to the brain tissue, providing an important area of the brain for neuroendocrine feedback mechanisms. It was demonstrated that these areas, also termed circumventricular organs, not only lack a BBB but are also deficient in expression of BBB markers (Albrecht et al., 1990, #78). These areas were also proposed to contribute to immune cell infiltration into the CNS during inflammatory conditions of the CNS, such as in multiple sclerosis (MS) or in its animal model, experimental autoimmune encephalomyelitis (EAE) (Schulz and Engelhardt, 2005). It was shown that in EAE, immune cells migrate from the blood stream into the CNS parenchyma and into the CSF through the circumventricular organs, such as the AP, the subfornical organ (SFO), the organum vasculosum of the lamina terminalis (OVLT), and the median eminence (ME) (Schulz and Engelhardt, 2005). However, in most areas of the brain, under normal conditions the BBB is a stringent gatekeeper for hematopoietic cells. The most prominent characteristic of sustaining the integrity of the BBB is the capacity of cerebral endothelial cells to form TJs.

There are several molecules that contribute to the formation of TJs (reviewed by Stevenson and Keon, 1998; Nusrat et al., 2000). These include transmembrane molecules occludin, claudins, and junction associated molecule (JAM) that provide homo-dimeric association and contribute to the formation of a tight barrier. Homodimeric interactions between endothelial cells and leukocytes are also important for the migration of T cells across the BBB. One of these molecules, platelet–endothelial cell adhesion molecule-1 (PECAM-1, CD31) was shown to contribute to leukocyte migrations across the BBB and to BBB integrity. It was described that in vivo, PECAM-deficient mice develop early onset of EAE, due to increased vascular permeability of the BBB (Graesser et al., 2002). It was also demonstrated that an injected chimeric soluble sPECAM-1 fused to human IgG-Fc impairs leukocyte entry through the BBB and reduces CNS autoimmunity (Reinke et al., 2007). sPECAM-Fc impaired migration of lymphocytes across brain endothelial monolayers and diminished the severity of EAE when administered at the onset of symptoms. However, in mice transgenic for sPECAM-Fc, the chronically elevated levels of sPECAM-Fc hastened onset of EAE disease (Reinke et al., 2007). Transmembrane molecules on cerebral endothelial cells associate with several intracellular components to further strengthen TJ structures and provide signaling components and attachment to the actin cytoskeleton. These intracellular components include the zonula occludens (ZO), cingulin and AF-6 and 7H6 (Tsukita and Furuse, 1999; Kniesel and Wolburg, 2000; Wolburg et al., 2003; Hawkins and Davis, 2005).

Out of these intracellular molecules, ZO proteins were proposed to be critical for TJ formation. ZO proteins are intracellular molecules of the membrane associated guanylate kinase homologs (MAGUKs) family. There are at least three members of this ZO family, ZO-1, ZO-2, and ZO-3, although ZO-3 has not been associated with the formation of endothelial TJs (Inoko et al., 2003). ZO-1 is a key structure molecule of TJs and interacts with many TJ-associated proteins including ZO-2, occludin, claudin, cingulin, and JAMs (Bazzoni and Dejana, 2004). ZO-1 is largely responsible for bridging transmembrane molecules with the cytoskeleton binding F-actin in an ATP-dependent interaction (Fanning et al., 1998). ZO-1 expression is

regulated under inflammatory conditions and immunohistochemical staining of ZO-1 is disrupted in MS, both in areas of ongoing damage and in noninflamed white matter (Plumb et al., 2002; Kirk et al., 2003). However, the regulation and role of this molecule in inflammatory diseases is currently confusing because supernatant from both pro- and anti-inflammatory helper T cells are equally capable of dysregulating ZO-1 expression, and disrupting ZO-1 and ZO-2 integrity of brain endothelial cell monolayer in vitro (Biernacki et al., 2001).

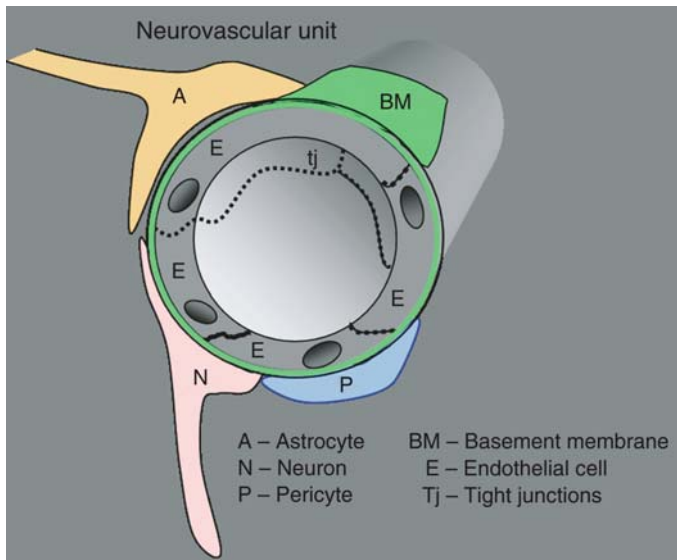
These data show that the TJ is a complex structure that is made up of transmembrane junctional spanning molecules involved both in maintaining barrier integrity and in facilitating cellular migration. Intracellular proteins provide connections between membrane proteins and the cytoskeleton as well as responding to intracellular signals to adjust TJ structure and behavior. The critical role of TJ disruption in CNS inflammatory diseases led to the development of numerous therapeutically targets.

Cerebral microvascular endothelial cells together with neighboring astrocytic, pericytic, and neuronal cells, as well as the extracellular matrix (ECM) between them, constitute a “neurovascular unit” (NVU) (Hawkins and Davis, 2005) (► [Figure 8-2](#)).

The NVU is critical for maintaining the immune-privileged nature of the CNS and regulating cellular transmigration (Fabry et al., 1994; Glabinski et al., 2003). As a result, brain microvascular endothelial cells depend on constant support from surrounding astrocytes, neurons, and pericytes in order to maintain their BBB ability. This is supported by studies showing that the exposition of cerebral endothelial cells to glia cells promote the expression of P-glycoprotein (Bauer and Bauer, 2000) and of γ -glutamyl-transpeptidase (el Hafny et al., 1996), both considered as marker proteins of the BBB. The contribution of pericytes to

■ Figure 8-2

Cerebral microvascular endothelium (E) together with astrocytes (A), pericytes (P), neurons (N), and the basement membrane (BM), constitute a “neurovascular unit” that is essential for the health and function of the CNS. Tight junctions (TJ) between endothelial cells of the BBB restrict diffusion of water-soluble substances from blood to brain and the migration of blood cells. This picture shows the unit at the level of capillaries, while pre- and postcapillary levels are strikingly different in that they have and additional basement membrane and perivascular spaces. The two basement membranes differ in their laminin content



elevated endothelial occludin and multidrug resistance-associated proteins (MRP) MRP-6 mRNA expression was also demonstrated (Berezowski et al., 2004; Hori et al., 2004). Astrocytes and pericytes can also influence the transendothelial electrical resistance (TEER) as a direct measure of BBB integrity in vitro with endothelial cells (Giese et al., 1995; Hayashi et al., 2004). The nature of the underlying mechanism of the NVU regulation is still under controversy. One possible pathway is assigned to soluble molecules such as pericyte-secreted transforming growth factor- β (TGF- β) (Dohgu et al., 2004) or astrocyte-secreted basic fibroblast growth factor (bFGF) (Sobue et al., 1999). Although the use of conditioned media obtained from cultured rat brain astrocytes or C6 glioma cells reveal some BBB-supporting capacity (Rubin et al., 1991; Dehouck et al., 1994; Hurst and Fritz, 1996; Rist et al., 1997; Igarashi et al., 1999), the induction of BBB features is generally more effective in cocultures, where glial processes are allowed to contact basal surface of the endothelium (Abbott, 2002). Additionally, by measuring the TEER of cerebral endothelial cell cultures on filters covered with different purified ECM proteins, it was suggested that the basement membrane placed between endothelium and surrounding astrocytes and pericytes may be involved in the differentiation of the BBB phenotype (Tilling et al., 1998). The molecular mechanisms through which both ECM and glial cells affect the BBB are still poorly understood. However, an impact of endogenously derived extracellular matrices from both astrocytes and pericytes was recently demonstrated to tighten cerebral endothelial cells remarkably inducing TEER values in vitro (Hartmann et al., 2007). In summary, it is obvious that a NVU represents a complex interplay of a variety of components including glial cells, neurons, and ECM which are orchestrated in a way that leads to a strong BBB phenotype.

2.2.2 The BBB in Inflammation

Inflammation overall induces a shift in the expression level of adhesion molecules, in the structure of TJ proteins and in the production of mediators that control the entry of lymphocytes and the “leakiness” of the BBB. There are multiple inflammatory molecules that contribute to this shift.

For example, it was shown that interleukin (IL)-1 and IL-6 are expressed in response to inflammation of the CNS and modify the integrity of the BBB. These cytokines could be produced either by resident brain cells or infiltrating white blood cells. In vitro treatment of brain endothelial cells with IL-1 or lipopolysaccharide (LPS) leads to the secretion of IL-6 by endothelial cells that may amplify the inflammatory status of the BBB (Fabry et al., 1993; Reyes et al., 1999). This is compensated by an orchestrated response of the NVU, in which, as a response to IL-6 production by endothelial cells, astrocytes upregulate integrin molecules (β -4 and α -5 integrin subunits) in an attempt to increase BBB integrity, supporting the idea that the NVU is a functionally unified structure (Milner and Campbell, 2006). IL-1 also increases leukocyte adhesion to, and migration across, monolayers of mouse brain endothelial cells and decreases BBB integrity as measured by decrease in TEER across an in vitro BBB model comprised of rat endothelial cells and astrocytes (Waldschmidt et al., 1991; Fabry et al., 1995; de Vries et al., 1996). To support the importance of studying brain endothelial cells in the context of the NVU, it was shown that IL-6 treatment of brain endothelial cells alone leads to decreased TEER measurements (de Vries et al., 1996). In accord with this, IL-6-deficient mice are resistant to EAE. However, the mechanism of this resistance remains controversial and a deficiency in the development of proper autoreactive T-cell or Th2 responses in these mice cannot be excluded (Okuda et al., 1998; Samoilova et al., 1998b). Others have suggested that the resistance of IL-6-deficient mice to EAE might be mainly due to enhancement of Th2 responses (Eugster et al., 1998; Okuda et al., 2000). It was also shown that there is a striking difference between myelin oligodendrocyte glycoprotein (MOG)-immunized wild-type and IL-6-deficient mice in the expression of endothelial vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) molecules. These molecules are dramatically upregulated in the CNS in wild type but not in IL-6-deficient mice, suggesting that the absence of VCAM-1 on endothelial cells of the BBB in IL-6-deficient mice is responsible for their resistance to EAE (Eugster et al., 1998). IL-6 binds to target cells via the ligand-binding protein IL-6 receptor (IL-6R) and the affinity-converting and signal-transducing glycoprotein 130 (gp130). Interestingly, soluble IL-6R (sIL-6R) that has an agonistic role in inflammatory events was shown in the CSF of patients with

neuroinflammatory diseases (Michalopoulou et al., 2004). This indicates a unique regulatory mechanism mediated by the IL-6–IL-6R pathways in diseases such as MS.

When discussing proinflammatory cytokine production by cerebral endothelial cells, we also must take into consideration that brain endothelial cells have a unique feature of polarization, when their luminal (blood-facing) and abluminal (brain-facing) cell membranes differ in their lipid, receptor, and transporter compositions. Interestingly, it was proposed that constitutive LPS-stimulated secretion of cytokines is polarized in favor of luminal secretion (Verma et al., 2006). It would be interesting to analyze polarized secretion of inflammatory cytokines by cerebral endothelial cells in the context of the NVU.

Additional proinflammatory cytokines in the inflamed brain, such as interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α), can also influence the integrity of the BBB. These proinflammatory cytokines are produced in Th1 cell-mediated immune responses (Mustafa et al., 1991; Schmidt et al., 2003). CNS-resident cells, such as astrocytes, can also secrete TNF- α (Chung et al., 1991). Both TNF- α and IFN- γ cytokines can increase the adhesion of leukocytes to brain endothelial cells (Brankin et al., 1995), and in the presence of IL-1, can increase the number of leukocytes migrating across an *in vitro* BBB (Waldschmidt et al., 1991). The effect of IFN- γ on brain endothelial cells is well documented. IFN- γ was shown to decrease TEER, increase permeability, and decrease occludin expression in human umbilical vein endothelial cell (HUVEC) layers (Oshima et al., 2001). In parallel with these changes, human brain endothelium was shown to increase major histocompatibility complex (MHC) class II in the presence of IFN- γ indicating the potential for antigen presentation functions of these cells (Huynh and Dorovini-Zis, 1993). It was shown that a surge of IFN- γ expression preludes the onset of MS and that IFN- β significantly attenuates the IFN- γ -induced decrease in occludin and VE-cadherin expression and BBB deterioration (Minagar et al., 2003). The additional important role of IFN- γ in regulating IL-17 producing T helper cell migration across the BBB was also suggested (reviewed in Steinman, 2007). While IL-17 cytokine was shown (1) to promote angiogenesis and elicit neovascularization in rat cornea (Numasaki et al., 2003), (2) to antagonize bFGF-induced signaling in human umbilical endothelial cells (Yang et al., 2003), and (3) to induce the production of fractalkine chemokine by ocular microvessel endothelial cells (Silverman et al., 2003), the direct effect of IL-17 on the BBB has not been documented. For the migration of IL-17 producing helper T cells into the brain (a novel group of helper T cells that had been suggested to play a critical role in maintaining inflammation and causing tissue damage in the CNS) additional inflammatory cytokines, such as IL-6, TNF- α , and IFN- γ are required to upregulate adhesion molecules on the BBB (Steinman, 2007).

Besides IFN- γ , IL-1, and IL-6 other proinflammatory cytokines were shown to modify the integrity of the NVU. Most notably, multiple members of the TNF superfamily (TNFSF), a group of cytokines that comprises 19 ligands and 28 receptors, were shown to affect the BBB. Ligand-mediated activation of TNFSF receptors plays a role in the pathogenesis of multiple CNS diseases such as MS and cerebral ischemia. Treatment with TNF increases the permeability of brain endothelial cells to fluorescently labeled dextran (Mark and Miller, 1999; Mayhan, 2002). One member of the TNFSF, TNF-like weak inducer of apoptosis (TWEAK) that binds a small cell surface receptor known as fibroblast growth factor-inducible 14 (Fn14), has been shown to regulate the permeability of the NVU and development of an inflammatory response in the CNS under physiological and pathological conditions (reviewed in Yepes, 2007; Zhang et al., 2007).

In summary, multiple proinflammatory mediators influence the integrity of the BBB. The NVU is crucial in CNS homeostasis and is an important factor in maintaining immune privilege in the CNS, and it is clear that a more complex circuit will be devised by future studies. It is, however, noteworthy that the diffusion of solutes is a function of capillaries, while recruitment of immune cells takes place at the level of postcapillary venules which are separated from the neuropil by perivascular (Virchow–Robin) spaces. In fact, leukocytes passing the vascular wall do not necessarily enter the brain parenchyma, but are put on hold in these perivascular spaces (Tran et al., 1998). Perivascular restimulation by antigen presenting (dendritic) cells appears to be required for progression across the glia limitans (Greter et al., 2005; Becher et al., 2006). Thus, the extension of meaning of the term BBB from a barrier for solutes to a barrier for cells is often misleading and two differentially regulated steps (1) passage of the vascular wall and (2) progression across the glial limitans are required for immune cells to enter the brain (Bechmann et al., 2007).

3 Soluble Mediators and Cell Surface Molecules that Promote Immune Privilege

In the following paragraphs we will discuss the major anti-inflammatory mechanisms that contribute to the unique nature and dialect of immune responses in the CNS. We will focus on anti-inflammatory neuropeptides, cytokines, and immune regulatory molecules that control immunity in the microenvironment of nervous tissues.

3.1 Neuropeptides as Soluble Regulators of Immune Privilege in the CNS

Regulation of immune responses in the CNS is a complex interaction of soluble and cell surface molecules and mediators that include immunosuppressive neuropeptides. These neuropeptides can target immune cells and pathways of adaptive and innate immunity. Several neuropeptides prevent activation of helper T cells and promote the development of regulatory T cells (Tregs), thus participating in the maintenance of immunological privilege. In the next paragraph, we selected for discussion four neuropeptides – vasoactive intestinal peptide (VIP), α -melanocyte-stimulating hormone (α -MSH), neuropeptide Y (NPY), and somatostatin (SST) – as they are well known for their role in controlling inflammation and autoimmunity in the CNS. These will serve as examples to show the complexity of neuropeptide mode of action in immune responses.

VIP neuropeptide, a 28 amino acid peptide, that has been found in multiple organs, including the gut, pancreas, and hypothalamus, has an important role in vasodilation. (Pozo and Delgado, 2004; Delgado et al., 2004b; Gonzalez-Rey and Delgado, 2005). VIP has multiple functions throughout the body, but also has a strong anti-inflammatory effect on microglia and macrophage, as well as on T cells. VIP has been shown to prevent experimental MS by downregulating both inflammatory and autoimmune components of this disease (Fernandez-Martin et al., 2006.) Additionally, it was shown that VIP treatment blocks bacterial LPS-stimulation-induced TNF- α , IL-1 β , IL-6, and nitric oxide (NO) production by primary microglia cells (Ganea and Delgado, 2002; Delgado, 2002a, b; Delgado et al., 2002a, b, 2003). VIP also inhibited LPS and IFN- γ -induced production of macrophage inflammatory protein (MIP)-2, MIP-1 α , keratinocyte derived chemokine (KC), regulated upon activation of normal T cell-expressed and - secreted (RANTES) chemokines by microglia (Delgado and Ganea, 2001a, b; Delgado et al., 2002a), and the production of γ -interferon inducible protein (IP-10) (Delgado, 2003). More importantly, VIP induces macrophage and immature DC stimulation of Th2 cells, indicating that this neuropeptide is critical in the regulation of the autoimmune helper cell profile in the CNS (Delgado et al., 2004a). Besides regulating Th2 immune responses, VIP was shown to induce the development of Tregs expressing CD25⁺ forkhead-box transcription factor p3 (Foxp3)⁺ and glucocorticoid-induced TNF receptor (GITR)⁺ molecules (Chorny et al., 2006; Gonzalez-Rey et al., 2006; Delgado et al., 2006a, b) VIP-induced development of Th2 and Treg responses contribute to the regulatory and anti-inflammatory responses in the CNS.

The MSHs are produced by cells in the intermediate lobe of the pituitary gland and in the hypothalamus and they induce the production of melanin. One of these hormones, α -MSH is a 13 amino acid peptide that is cleaved from the pro-opiomelanocortin hormone. α -MSH inhibits the production of TNF- α and iNOS by in vitro cultures of LPS-stimulated astrocytes or amyloid- β protein-stimulated microglia (Wong et al., 1997; Galimberti et al., 1999). More importantly, when α -MSH is microinjected into the brain, it can counteract a systemic LPS response by decreasing TNF- α , IL-1b, IFN- γ , MCP, and IL-8 secretion (Catania et al., 1999). Additionally, α -MSH was shown to inhibit fever responses to TNF- α or IL-1 (Robertson et al., 1988). Based on these data, α -MSH is likely to play a critical role in regulating immune responses in the CNS and to contribute to the maintenance of CNS immunological privilege.

NPY is a 36 amino acid peptide that was shown to be produced by astrocytes (Barnea et al., 1998). Macrophage respond to NPY by reducing IL-6 release (Straub et al., 2000) and enhancing phagocytosis, chemotaxis, and adhesion (De la Fuente et al., 1993; Dureus et al., 1993). Alone, NPY leads to paradoxical cytokine responses in Th cell lines, inducing IL-4 from Th1 cells and IFN- γ from Th2 cells, but combined

with antigen or anti-CD3, NPY induces IL-4 secretion from both splenocytes and Th2 cell lines (Kawamura et al., 1998; Levite, 1998, 2001; Levite and Chowers, 2001). A comprehensive understanding of the NPY anti-inflammatory characteristics in neural-immune regulation will require further intense study.

SST, present as 28 and 14 amino acid peptide isoforms, contributes to immune functions and hormone control (Krantic, 2000). SST can be produced by astrocytes (Schwartz et al., 1994; Mercure et al., 1996) and will suppress basal and stimulated IL-6 secretion in astrocytes. In vitro, microglia have depressed proliferative responses to mitogenic factors when exposed to SST (Feindt et al., 1998). Similarly, application of SST induces macrophage apoptosis while IL-10, IFN- γ , TNF- α , and LPS actually induce SST secretion by macrophage (Sandor et al., 2003). Likewise, IFN- γ also induces SST secretion by T cells while SST suppresses T-cell IFN- γ production (Blum et al., 1993, 1998, 1999). However, Th2 cell lines have been shown to respond to SST with an increase of IL-4, but also an increase of IFN- γ , IL-2, and IL-10 (Levite, 1998, 2001; Levite and Chowers, 2001). Additionally, SST and antigen treatment of Th1 cell lines resulted in an uncharacteristic production of IL-4 (Levite, 1998, 2001; Levite and Chowers, 2001). This indicates that cytokine secretion may be regulated by SST.

3.2 Anti-inflammatory Cytokines Produced by CNS-Resident Cells: TGF- β , IL-10

In general, inflammatory responses are controlled by the release of antagonistic anti-inflammatory cytokines. TGF- β is one example for these regulatory anti-inflammatory cytokines. TGF- β cytokine is secreted in the CNS by neurons and glia cells, as well as by macrophages (De Groot et al., 1999). TGF- β treatment reduces the IL-1, IFN- γ , or TNF- α -induced migration of lymphocytes across an in vitro BBB. Additionally, intracerebral or systemic injections of TGF- β into mice lessened the number of lymphocytes found in the CNS in both actively and passively induced EAE (Fabry et al., 1995). Besides TGF- β , another anti-inflammatory cytokine that has been shown to play an important role in regulating T-cell migration across the BBB and influencing anti-nervous tissue immunity is IL-10. This cytokine is produced by astrocytes and microglia during inflammation (Ledeboer et al., 2000), and by activated Tregs (Zhang et al., 2004). IL-10 mitigates the effects of IFN- γ on HUVECs, maintaining near normal TEER and occludin expression levels and minimizing the increase in permeability (Oshima et al., 2001). Mice deficient in IL-10 have an early and severe form of EAE as compared to wild-type (Bettelli et al., 1998; Samoilova et al., 1998a), while mice that overexpress IL-10 in activated T cells are completely resistant to disease induction (Bettelli et al., 1998). TGF- β and IL-10 work to control inflammation and antagonize the effects of proinflammatory cytokines such as IL-1, IFN- γ , and TNF- α . The balance of these and probably other pro- and anti-inflammatory cytokines in CNS is crucial in maintaining the immune privilege nature of the nervous tissue.

3.3 Cell Surface Molecules Expressed on CNS-Resident Cells that Regulate Immune Privilege: PD-1–PD-L1, FasL–Fas Interactions

Recently the induction of the programmed death ligand 1 (PD-L1 or B7-H1) has been identified as a mechanism of peripheral immune tolerance. This cell surface glycoprotein belongs to the Ig superfamily. Its expression has been shown in the heart, skeletal muscle, placenta, and lung (Dong et al., 1999), but brain cells were also found to transcribe this gene (Salama et al., 2003; Magnus et al., 2005). Mice deficient in programmed death molecule 1 (PD-1) develop lupus-like autoimmune diseases in organs, where its ligand is present under normal conditions suggesting a crucial role for PD-1 in the maintenance of immune tolerance (Nishimura et al., 1998, 2001). Moreover, PD-L1–PD-1 signaling was found to be critical in type I diabetes, fetomaternal tolerance, and tumor-tolerance (Okazaki et al., 2001, 2002, 2003; Okazaki and Wang, 2005). In the course of EAE, blockading studies demonstrated a central role for PD-L1–PD-1 signaling in the termination of neuroinflammation (Liang et al., 2003; Salama et al., 2003; Zhu et al., 2006; Carter et al., 2007; Cheng et al., 2007). Microglia were identified as a cellular source of PD-L1, further emphasizing their tolerogenic capacity.

FasL (CD95L) and its receptor Fas (CD95) are crucial for activation-induced cell death of lymphocytes (Dhein et al., 1995a, b), but were also found to maintain immune privilege in the anterior chamber of the eye (Griffith et al., 1995, 1996; Ferguson, 1997; Ferguson and Griffith, 1997a, b, 2006) and the testes (Bellgrau and Duke, 1999). The first evidence that T-cell apoptosis in the brain is dependent on the expression of FasL was derived from studies showing that, in EAE, a remarkably lower number of apoptotic cells are found in mutant mice deficient in Fas (B6-*lpr*) and its ligand (FasL) (B6-*gld*) compared with normal animals (C57BL/6J), even though the total number of infiltrating cells appears to be similar (Waldner et al., 1997; Sabelko-Downes et al., 1999). Using adoptive transfer of lymphocytes lacking FasL into normal animals or normal T cells into mice lacking Fas, Sabelko-Downes et al. (1999) demonstrated a dual role for Fas–FasL signaling in EAE: they found that both tissue damage and the elimination of T cells and thus, termination of inflammation, crucially depend on FasL-induced cell death in EAE. This function of FasL as a “double-edged sword” has been confirmed in a variety of models for brain pathologies (Choi et al., 2003, 2004; Choi and Benveniste, 2004).

Interestingly, FasL is located at the glia limitans, the first layer of the brain parenchyma proper reached by infiltrating T cells (Bechmann et al., 1999). In vitro, astrocytes were shown to kill activated T cells in a FasL-dependent way (Bechmann et al., 2000, 2002). Thus, the constitutive expression of this death ligand beyond the BBB appears to provide an immunological brain barrier capable of eliminating infiltrating cells. In the presence of highly proinflammatory signals, astrocytes themselves become susceptible to FasL-induced apoptosis (Becher et al., 1998). Thus, the immune privilege of the brain may depend on the number of incoming lymphocytes, whether they apoptose upon entry into the CNS, or whether the inflammatory response is sufficient to kill the resident astrocytes (Bechmann et al., 2007).

4 The Role of APCs in the Brain Tissue that Promote the Initiation of T-Cell-Mediated Immunity against CNS Antigens

In the following paragraphs we will consider the role of CNS-resident cells in regulating T-cell activation and the initiation of CNS immune responses. We will focus on the proposed antigen presentation properties of resident cells within the CNS parenchyma (glial cells: microglia, astroglia) as well as infiltrating DCs.

4.1 Naïve and Effector T-Cell Activation

T cells recognize antigen using their unique T-cell receptor (TCR) when fragments of antigen bind to either class I or class II MHC molecules on the surface of an APC. Class I MHC-restricted T cells are almost exclusively CD8+ (cytotoxic/suppressor) phenotypes, whereas class II-restricted T cells are CD4+ T helper phenotypes (Unanue, 1984; Kourilsky and Claverie, 1989; Townsend and Bodmer, 1989; Brodsky and Guagliardi, 1991). In addition to antigen presentation in the context of MHC molecules, secondary signals are also required for the successful activation of peripheral T cells. These secondary signals are provided by costimulatory molecules (Tivol et al., 1996; Schweitzer and Sharpe, 1998; Sharpe and Freeman, 2002; Greenwald et al., 2005; Keir and Sharpe, 2005). It is generally accepted that autoimmune T cells are first stimulated in the peripheral lymphoid organs, then enter the CNS and will encounter APCs at the target site for a secondary restimulation. Their fate in the CNS is dependent on this interaction. Absence of antigen or APCs could lead to the elimination of these T cells from the brain parenchyma and thus termination of the immune response. However, their secondary restimulation could result in further activation and survival of these cells. Activated autoimmune T cells in the CNS could induce the recruitment of mononuclear cells into the brain parenchyma, inducing a broad inflammatory reaction and destruction of the myelin sheet. Throughout this process, autoimmune T cells will first encounter residential cells of the BBB and later, interact with resident APCs in the perivascular space and in the brain parenchyma. In the next paragraphs we will outline the role of antigen presentation at these two compartments and their role in initiation of CNS autoimmunity.

4.2 CNS-Resident APCs

4.2.1 APCs in the Perivascular Space

Recent data indicate that the perivascular space that surrounds the tightly closed endothelial wall in the CNS, provides a fertile environment for the activation of T cells. As we discussed above, during CNS inflammation, blood-borne immune cells access the CNS via the BBB (Hickey et al., 1991) and brain microvessel endothelial cells together with astrocytes, pericytes, and neurons, form the NVU (Hawkins and Davis, 2005). During inflammation, the BBB becomes compromised and allows cellular traffic into the perivascular space. In the perivascular space, antigen-presenting cell can facilitate inflammatory reaction in the CNS. Two major cell types were proposed to regulate CNS immunity in this compartment.

First, it was proposed that perivascular microglia and macrophage are capable of phagocytosis and expression of high levels of class II antigen (Bo et al., 1994; Ulvestad et al., 1994b). Furthermore, using irradiated bone marrow chimeras in CD45-congenic rats, highly purified populations of microglia and nonmicroglial but CNS-associated macrophages (CD45^{high} CD11b/c⁺) have been isolated from the adult CNS. It was demonstrated that the minority CD45^{high} CD11b/c⁺ transitional macrophage population, and not the resident microglia, are the effective APCs for EAE, activating CD4(+) myelin basic protein (MBP)-reactive T cells (Matyszak et al., 1997). The depletion of systemic macrophage results in the depletion of the perivascular microglia-macrophage population also and leads to the inhibition of the onset of EAE (Cuadros and Navascues, 1998). Using CD11b-HSVTK transgenic mice, which express herpes simplex thymidine kinase in macrophages and microglia, Heppner et al. (2005) demonstrated that ganciclovir blocked microglial activation repressed the development of EAE (Heppner et al., 2005). They concluded that microglial paralysis inhibits the development and maintenance of inflammatory CNS lesions.

Second, DCs were proposed to accumulate at the perivascular space. DC localization in the proximity of inflamed microvessels in MS lesions (Serafini et al., 2006), and the production of astrocyte-derived chemokines that promote DC recruitment into the CNS (Ambrosini et al., 2005) strongly suggest that brain microvessel endothelial cells regulate DC recruitment into the CNS.

Perivascular cells are probably continuously, slowly, recirculating between the blood and the CNS, carrying CNS- or blood-originated antigens to the perivascular space (Hickey and Kimura, 1988). In different neuropathological diseases, infections, or during posttraumatic healing and inflammatory processes, a high number of macrophages can “join” these cells and readily enter the CNS.

4.2.2 APCs in the Brain Parenchyma

The most frequently proposed cell type for antigen presentation in the CNS parenchyma is the microglia cell. These cells are of mesodermal origin and they function as the resident macrophages in the CNS. Developing microglial cells enter the CNS from the blood, through the ventricular space or the meninges (Cuadros and Navascues, 1998). Following their migration into the developing CNS, microglial cells are distributed more or less homogeneously through the entire nervous parenchyma. Microglial cells moving through the nervous parenchyma are ameboid microglia, which apparently differentiate into ramified microglia after reaching their definitive location. Microglial cells within the developing CNS are involved in clearing cellular debris and withdrawing misdirected or transitory axons. Serial analysis of gene expression (SAGE) in a microglial cell line indicated that these cells produce multiple cytokines such as endothelial monocyte-activating polypeptide I (EMAP I), adhesion molecules such as CD9, CD53, CD107a, CD147, CD162, and mast cell high affinity IgE receptor (Inoue et al., 1999). Microglia in vitro can express the essential molecules necessary for competent antigen presentation, such as class II MHC antigens (Tomimoto et al., 1993; al-Sabbagh et al., 1994; Wucherpfennig, 1994; Xu and Ling, 1994; Jiang et al., 1995; Walker et al., 1995; Aloisi et al., 1998; Perry, 1998). Additionally, in vivo infusion and intracerebral injection of IFN- γ also upregulates class II MHC on microglia cells (Wong et al., 1984; Steiniger et al., 1988; Vass and Lassmann, 1990). Class II expression on microglia cells is tightly regulated. It was demonstrated that IFN- γ -induced surface expression of class II MHC molecules on a microglial cell line can be inhibited by

the cytokines TGF- β 1, IL-4, and IL-10, but not IL-13 (O'Keefe et al., 1999). In several nervous tissue diseases, such as EAE, MS, AIDS-mediated dementia complex, Alzheimer's and Parkinson's diseases, class II upregulation on microglial cells has been demonstrated (Hofman et al., 1986; Achim et al., 1991; Achim and Wiley, 1992; Dickson et al., 1993; McGeer et al., 1993). Microglia cells also express costimulatory molecules, such as B7, LFA-3, and ICAM-1 (Ulvestad et al., 1994a; Benveniste et al., 1995; De Simone et al., 1995). Several laboratories have also demonstrated that microglia can present antigens *in vitro*. Interestingly, when microglial cells were compared to other CNS macrophages in antigen presentation assays, it was demonstrated that these macrophages are superior in their APC properties compared to microglia (Ford et al., 1995). In summary, microglia cells are critical elements in the initiation of CNS immune responses owing to their ability to present antigens and produce several inflammatory mediators in the brain parenchyma.

Astrocytes have also been shown to modulate the activity of pathogenic T cells by presenting myelin antigens in combination with pro- or anti-inflammatory signals. These cells are star-shaped and belong to the glial cell family. Astrocytes were shown to upregulate class II MHC molecules upon exposure to IFN- γ and IFN- γ , with TNF- α (Hirsch et al., 1983; Fontana et al., 1984; Wong et al., 1984; Vidovic et al., 1990). In addition, astrocytes can also be activated by other proinflammatory stimuli, such as IL-1 β , LPS, or viral proteins, to express adhesion molecules, such as ICAM-1, VCAM-1, and CD62E (E-selectin) (Frohman et al., 1989; Aloisi et al., 1992; Hurwitz et al., 1992; Ballesta and Benveniste, 1995; Benveniste et al., 1995; Rosenman et al., 1995). It was recently demonstrated that IFN- γ -activated HLA-DR2 and HLA-DR4 expressing astrocytes efficiently present immunodominant and subdominant MOG peptides to T cells (Kort et al., 2006). The hierarchy of the presented MOG epitopes was comparable to that of professional APCs, including DCs and microglia (Kort et al., 2006). Importantly, astrocytes were poor in processing and presenting native MOG protein and induced a mixed Th1/Th2 cytokine response in MOG-specific T cells, whereas DCs induced a predominantly Th1 cell response. Astrocytes are also playing an important role in regulating immune responses in the CNS by producing a plethora of proinflammatory cytokines, such as TNF- α , IL-1, IL-6, macrophage-CSF, and granulocyte-macrophage-CSF (for more details, see (Benveniste, 1988, 1992, 1994, 1998; Benveniste et al., 1990; Bethea et al., 1990, 1992; Baldwin et al., 1993; Norris and Benveniste, 1993; Merrill and Benveniste, 1996; Boulton et al., 1999; Van Wagoner and Benveniste, 1999). Collectively, the results suggest that astrocytes should also be considered for modulation of anti-MOG T-cell responses in the CNS.

4.3 DCs Amplify Immune Responses in the CNS

DCs are highly efficient APCs and largely considered to be the only cell type responsible for the initiation of primary immune responses through activation of naïve T cells (Steinman, 1991). DCs can also induce immune tolerance. Since the first discovery of DCs in the CNS (Matyszak and Perry, 1996), DCs have emerged as pivotal players in the development and maintenance of CNS autoimmunity and inflammation.

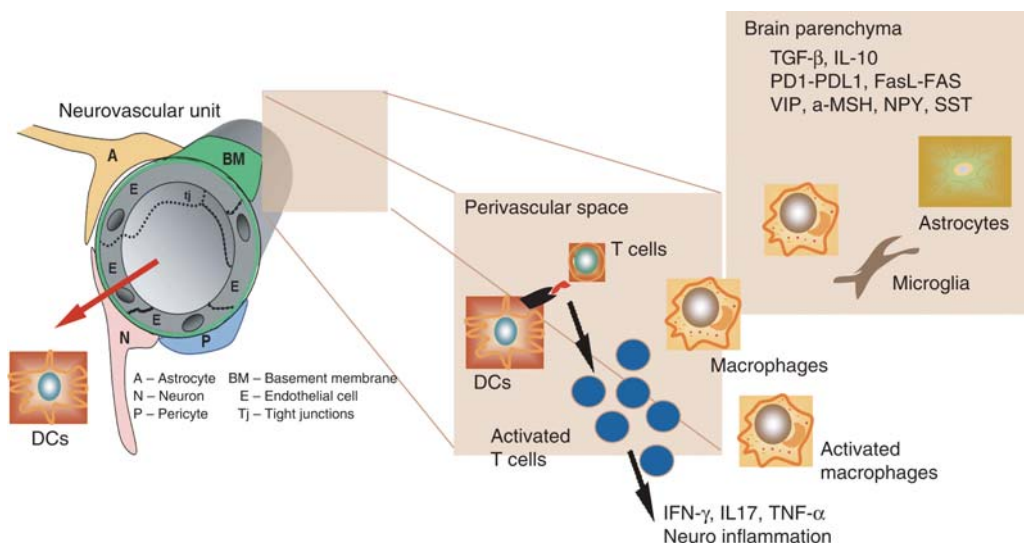
DCs are rarely detected in the healthy CNS. However, when they are present, they localize to vascular-rich tissues including the meninges and choroid plexus (Matyszak and Perry, 1996; Hanly and Petito, 1998; McMenamin, 1999; Serot et al., 2000; Greenwood et al., 2003). Numerous studies have demonstrated a substantial accumulation of DCs in the brain and spinal cord in response to local inflammation induced by autoimmunity, infection, or trauma (Hanly and Petito, 1998; McMenamin, 1999; Suter et al., 2003; McMahon et al., 2005; Newman et al., 2005; Bailey et al., 2007). The mechanism(s) by which DCs accumulate in the CNS under inflammatory conditions are not well understood. DCs are present under normal conditions in the choroid plexus (Matyszak and Perry, 1996; McMenamin, 1999; Serot et al., 2000) and in the healthy CSF (Pashenkov et al., 2001, 2002a, c). In addition, DCs in the CNS could arise through differentiation of resident microglia cells. *In vitro*, microglia cells can differentiate into DCs in the presence of the growth factor granulocyte-macrophage colony-stimulating factor (GM-CSF) (Fischer and Bielinsky, 1999; Fischer et al., 2000; Fischer and Reichmann, 2001; Santambrogio et al., 2001). Several cell types in the CNS, including endothelial cells, can produce GM-CSF (Hart et al., 1992). Interestingly, GM-CSF-deficient mice fail to develop EAE following immunization with encephalitogenic peptides (McQualter et al., 2001), raising the possibility that GM-CSF-induced differentiation of DCs may play a role in CNS autoimmune

disease. Additional cytokines known to induce DC differentiation have also been detected in the CNS, including TNF- α , IL-1, and IL-6 (Becher et al., 2001; Willenborg and Staykova, 2003). As discussed above, FMS-like tyrosine kinase 3 ligand (Flt-3L) can induce DC proliferation and differentiation *in vivo* (Maraskovsky et al., 1996; Pulendran et al., 1997; Shurin et al., 1997), and has been shown to specifically recruit DCs to the brain parenchyma (Curtin et al., 2006). When Flt-3L expression was increased locally in the brain, recruitment and activation of plasmacytoid DCs (pDCs) were detected, with no effect on myeloid DCs or other immune cells in the brain parenchyma (Curtin et al., 2006). Plasmacytoid DCs (CD11b⁻CD11c⁺ B220⁺ CD8a⁺PDCA⁺) were also detected in the CNS of mice with a relapsing type of EAE, although pDCs were relatively inefficient in stimulating CD4⁺ T cells to produce IL-17 (Bailey et al., 2007). It was also proposed that myeloid DCs (mDCs) uniquely biased Th17 and not Th1 cell differentiation that correlates with their expression of TGF- β , IL-6, and IL-23 (Bailey et al., 2007). As we discussed above, another possible mechanism of DC accumulation in the CNS is via recruitment of DCs from the periphery. It is well accepted that DCs express a dynamic and broad profile of chemokine receptors that enable trafficking between peripheral tissues and secondary lymphoid organs during an immune response (reviewed in McColl, 2002). Importantly, two of these receptors, CCR2 and CCR5, have specifically been implicated in the regulation of CNS inflammation. Deficiency in either CCR2 or its ligands was shown to confer resistance to EAE induction in mice (Izickson et al., 2000; Huang et al., 2001). In the CCR2-deficient mice, EAE resistance was accompanied by a lack of mononuclear cell infiltration into the CNS (Huang et al., 2001). In addition, CNS-resident cells in CCR2-deficient mice fail to upregulate additional chemokines and chemokine receptors. Further supporting a role for CCR2 in CNS inflammation, MCP-1, a ligand for CCR2, was significantly upregulated in response to traumatic brain injury (Pashenkov et al., 2002b). Interestingly, DCs are recruited to the ischemic CNS in high numbers (Fischer and Reichmann, 2001), although the role of these cells is unknown. CCR5 is another example of a possible mediator of DC recruitment to the CNS because it is present on infiltrating leukocytes in both the brain parenchyma and the CSF (Trebst et al., 2001; Pashenkov et al., 2002b). Interestingly, in MS patients, the nonfunctional allele of CCR5 is associated with a prolonged remission interval and decreased recruitment of mononuclear cells to the CSF (Sellebjerg et al., 2000).

Once they are in the CNS, DCs can inhibit T-cell responses (Suter et al., 2003) leading to protection from EAE (Kleindienst et al., 2005). However, other data suggest that DCs can contribute to EAE induction (Dittel et al., 1999; Weir et al., 2002). Supporting a positive role for DCs in CNS immune responses, DCs were recently shown to be the only CNS APC population capable of inducing memory cytotoxic T-cell responses in lymphocytic choriomeningitis virus (LCMV) infection (Lauterbach et al., 2006). Furthermore, CNS-resident F4/80⁻CD11c⁺ CD45^{high} DC cells isolated from brains of animals experiencing relapsing EAE (R-EAE) or Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD) can efficiently present endogenous myelin proteolipid protein (PLP) antigen and activate naïve PLP₁₃₉₋₁₅₁-specific T cells *in vitro* (McMahon et al., 2005). These studies suggest that local CNS APCs, likely DCs, can activate infiltrating naïve T cells in inflamed brain tissue. Additional support for this comes from data showing that in the complete absence of a peripheral lymphoreticular system (i.e., lymph nodes, Peyer's patches, and spleen), T cells entering the CNS can recognize target antigens and induce inflammation (Greter et al., 2005). In addition, DC-SIGN (CD209), a molecule believed to be expressed by DCs (Geijtenbeek et al., 2000a, b, c), can be detected on cells in proximity to invading T cells in acute and chronic active human MS lesions (Greter et al., 2005). Furthermore, boosting DC numbers in the brain by systemic injection of Flt-3L leads to a substantial increase in the severity of EAE clinical symptoms (Greter et al., 2005). Conversely, inhibition of Flt-3 signaling ameliorates EAE, providing further evidence that DC numbers in the brain correlate with autoimmune responses (Whartenby et al., 2005). In addition to presenting antigen locally in the CNS, DCs can deliver antigens from the CNS to peripheral lymph nodes, activate autoreactive naïve T cells, and induce homing of these cells to the nervous tissue (Karman et al., 2004). We demonstrated that DCs presenting CNS-derived antigens mediate either cooperative or competitive interactions between T-cell populations with different antigen specificities (Karman et al., 2006). In addition, we verified an MMP-dependent migration of DC across brain microvascular endothelial cells *in vitro* (Zozulya et al., 2007). These data have significant clinical implications in therapeutic strategies aimed at blocking a specific antigen-mediated T-cell responses in the CNS. In addition, these data suggest that

■ Figure 8-3

The dialect of immune system in the CNS



DC-mediated enhancement of T-cell responses to CNS-derived antigens may occur much earlier than previously thought.

5 Conclusion

Every tissue has a different gradient of immunoregulatory mediators, lymphocyte, macrophage, DC traffic, and costimulatory environment. These conditions are more fully understood in tissues like the skin or mucosa. In recent literature there is a growing body of evidence shedding light on the mechanism of immune regulation in the CNS. In this chapter we discussed some of the critical elements in the CNS that contribute to the special anti-inflammatory environment in this tissue (Figure 8-3). We concluded that (1) immune responses are regulated in every tissue by unique tissue elements, (2) immune privilege is dynamic, and (3) there is a highly regulated communication between the immune and the nervous systems and that this bidirectional communication is further augmented under pathological conditions. A better understanding of the mechanism of immune privilege in the CNS will provide the basis of better therapeutic interventions for immune-mediated CNS diseases, such as MS.

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9 Immunoproteasome Activity in the Nervous System

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1	<i>Introduction</i>	224
2	<i>Intracellular Systems Involved in Protein Degradation</i>	224
3	<i>The Proteasomes</i>	225
3.1	The 26S Proteasome, 20S Proteasome, 11S Reg-20S Proteasome	225
3.2	The Immunoproteasome	226
3.3	The Proteasome and the Immunoproteasome in the Nervous System	228
3.4	The Immunoproteasome and Neurological Diseases	229
3.4.1	Parkinson's Disease	230
3.4.2	Alzheimer's Disease	230
3.4.3	Huntington's Disease	231
3.4.4	Familial Amyotrophic Lateral Sclerosis	231
3.4.5	Glioblastoma Multiforme	231
4	<i>Conclusion</i>	232

Abstract: Intracellular protein degradation is prevalently carried out by the nonlysosomal ubiquitin proteolytic system; to enter this pathway, the protein has to be tagged by oligomers of ubiquitin, because in this form it becomes a selective target of the proteasome, the central protease of the pathway. The repertoire of proteins degraded by the proteasome is continually growing, and it has been elucidated that the proteasome activity is essential in a wide range of vital cellular processes extending from cell division and differentiation, DNA repair, transcription factor and regulatory protein processing, and membrane receptor internalization to inflammatory response and antigen presentation. However, from the beginning the proteasome has emerged as an important determinant in the defense against oxidative stress by providing the degradation of oxidized, damaged, misfolded, or unfolded proteins, thereby preventing their accumulation. Furthermore, the considerable progress made in the proteasome characterization in various cell and tissue types has uncovered that the proteasome has great structural and functional plasticity, which constitutes a prerequisite for its selective and efficient targeting by different challenges. However, the characterization of proteasome expression, activity, function, and structural properties in the central nervous tissue is not entirely defined and is very poor with regard to neurodegenerative diseases. This chapter focuses on what is known about the proteasome properties in the central nervous system in health and degenerative conditions.

List of Abbreviations: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; APP, amyloid precursor protein; A β , β -amyloid protein; CNS, central nervous system; fALS, familial ALS; GBM, glioblastoma multiforme; HD, Huntington's disease; IFN, interferon; LBs, Lewy bodies; MCP, multicatalytic peptidase; NF- κ B, nuclear factor kappa B; NFTs, neurofibrillary tangles; NGF, nerve growth factor; PD, Parkinson's disease; PGPH, peptidyl glutamyl peptide hydrolyzing; PHFs, paired helical filaments; PSs, presenilins; RUP, regulated ubiquitin/proteasome-dependent processing; SCA1, spinocerebellar ataxia type 1; SCAs, spinocerebellar ataxias; SNc, substantia nigra pars compacta; SOD, superoxide dismutase; TNF, tumor necrosis factor; Ub, ubiquitin; UPS, ubiquitin proteolytic system

1 Introduction

Proteins, besides having a scaffolding role in cell architecture, play a crucial part in all cellular functions; for these reasons their turnover has to be under strict control to guarantee cell homeostasis and vitality. Since protein synthesis does not give rise to storage, this process is regulated by specific needs, which in turn are dictated by developmental situations and environmental conditions. This presumes that each protein is subjected to destruction as soon as its presence is no longer indispensable, thus concurring to explain why each protein has a well-defined half-life. Protein synthesis is heavily regulated by gene transcription and mRNA translation; by contrast, the mechanisms underlying protein degradation are still not entirely understood.

2 Intracellular Systems Involved in Protein Degradation

Protein degradation takes place: (1) at acidic pH in the lysosomes, resulting in the resolution of the protein into the amino acids concurring to its backbone; (2) at neutral pH, in different cellular compartments, including the cytoplasm, nucleus, mitochondria, and the cellular membrane. This second modality not necessarily results in the full destruction of the protein molecule, but often leads to its partial degradation, an event defined protein processing, which frequently concerns proteins capable of developing catalytic activity and/or involved in signal generation. Since protein degradation and processing are both irreversible events, it emerges that these events are harmful for the cell homeostasis if they proceed in a deregulated manner. A step forward to a better understanding on the processes at the core of protein degradation and their implication in cellular physiology and pathology was given by the discovery of the ubiquitin (Ub) proteolytic system (UPS). A growing number of studies have shown that this pathway plays a crucial role in a variety of basic cellular functions, including regulation of cell-cycle progression, cell growth and

differentiation, processing of transcriptional factors such as the nuclear factor kappa-B usually referred as NF- κ B, morphogenesis, apoptosis, aging, cancerogenesis, inflammatory response, and immune surveillance (Coux et al., 1996; Hershko and Ciechanover, 1998; Tanaka and Kasahara, 1998; Bochtler et al., 1999; Rock and Goldberg, 1999; Noda et al., 2000; Hallermalm et al., 2001; Husom et al., 2004), as well as degeneration of neuronal networks and long-term synaptic plasticity (Naujokat and Hoffmann, 2002; Ciechanover, 2006). However, at the beginning, this pathway was considered to be prevalently devoted to the selective removal of misfolded or unfolded proteins because of mutations, and of proteins that underwent an incorrect postsynthetic processing or damage by external agents (Glickman and Ciechanover, 2002; Kisselev and Goldberg, 2001); consequently, this pathway is also involved in the protein quality control, which maintains the health of the cell. Therefore, UPS provides a clue for the understanding of the molecular mechanisms underlying various neurodegenerative diseases (Tanaka et al., 2004).

UPS is at work in the cytoplasmic and nuclear compartments and is characterized by unique features including: (1) tagging of the protein substrate selected for destruction or processing with oligomers of ubiquitin, a low-molecular mass protein largely conserved along the phylogenesis; (2) targeting of the marked protein to the central protease of the pathway, the 26S proteasome, which, in a Ca^{2+} -independent manner, cuts it into small peptides (comprising 8–12 amino acids) which in turn are cleaved by various aspecific aminopeptidases further. These two steps are both dependent on energy supply relying on the cleavage of the phosphoanhydride bonds of ATP.

3 The Proteasomes

3.1 The 26S Proteasome, 20S Proteasome, 11S Reg-20S Proteasome

The 26S proteasome is a high-molecular mass polymeric protein comprising a core particle, identified as the 20S proteasome or multicatalytic peptidase (MCP) harboring the catalytic activity sites, which is capped at each extreme by two particles with the role of regulatory components; the latter are classified as the PA700 or 19S complex or 19S regulator (19S Reg) and the 11S regulator (11S Reg) or PA28.

The 20S proteasome, which in the cell is also present as an independent unit, is a barrel-shaped complex of approximately 700 kDa, built up of four stacked rings, each constituted by seven subunits. The two external rings are formed by highly homologous α subunits, the two inner ones by highly homologous β subunits. The former play a scaffolding role, control the access to the catalytic chamber, and interact with regulatory factors and protein complexes; the latter concur to define the inner cavity of the barrel and are responsible for the catalytic function, which in eukaryotic cells is restricted to the β 1, β 2, and β 5 subunits. These subunits have distinct peptidase activities that are selective for the cleavage of peptide bonds on the carboxyl side of acidic (β 1), basic (β 2), and hydrophobic (β 5) amino acid residues. These activities, identified with the use of synthetic tetra- or penta-fluorogenic peptides, are defined peptidyl glutamyl peptide hydrolyzing (PGPH) or caspase-like, trypsin-like, and chymotrypsin-like activities because they mimic the activities of known proteolytic enzymes and are carried by the β 1, β 2, and β 5 subunits, respectively (Coux et al., 1996; Baumeister et al., 1998). A characteristic proteasome feature is the catalytic center including the N-terminal threonine, the hydroxyl group of which behaves as a catalytic nucleophile and primary proton acceptor in the cleavage of the peptide bond (Seemuller et al., 1995). Further evidence supporting this peculiarity came from the discovery that the antibiotics lactacystin and epoxomicin selectively react with the N-terminal threonine of the β 5 subunit, and less so with that of the β 1 and β 2 subunits, resulting in the irreversible inactivation of the corresponding peptidase activities (Fenteany et al., 1995; Meng et al., 1999). Since each of the active subunits is present in two copies, the central cavity harbors six active sites.

The 19S Reg, about 700 kDa in size, plays the role of recognizing the ubiquitinated proteins and converting them into a form suitable for degradation by the catalytic core, the 20S proteasome. These two steps, which imply unfolding of the native protein and the association of the regulator with the 20S proteasome that commutes into the 26S proteasome, run parallel with the hydrolysis of ATP, and thus they are energy consuming. Furthermore, in capping the core particle, the regulator enables it to selectively degrade Ub-tagged proteins, enhances its peptidase activity, and finally makes peptidase and proteolytic

activities dependent on ATP. In degrading the protein, the active sites of the core particle continue to act on the protein substrate until it is cleaved in fragments that are small enough to exit the catalytic chamber through its narrow ports. Therefore, and surprisingly, the 26S proteasome does not inevitably lead to full degradation of the targeted protein and in some cases it only degrades some well-defined protein segments leaving other parts of the protein intact, thereby yielding a protein with different biological activity with respect to its precursor. This process is referred as regulated ubiquitin/proteasome-dependent processing (RUP) (Rape and Jentsch, 2004) and is essential for the function of certain transcription factors and crucial for their regulation. The best characterized proteins subjected to this proteasome-mediated processing belong to the mammalian NF- κ B family, which is central to inflammatory and immune responses. Protein digestion by proteasomes is processive and the majority of the resulting peptides have a length of 4/14 amino acids (Rock and Goldberg, 1999). Genetic studies have established a hierarchy of the proteasomal active sites according to their implication in the progression of the proteolytic process and their importance for cell growth; in this regard, the chymotrypsin-like activity is the most involved, followed by the trypsin-like one, the caspase activity being the least important (Arendt and Hochstrasser, 1997). The importance of the chymotrypsin-like activity for the proteasome function was exploited in a large array of studies in mammalian cells, and cell-permeable inhibitors of this activity were instrumental in the disclosure of proteasomes and their implication in numerous vital processes (Wolf and Hilt, 2004).

Structurally, the 19S Reg is made up of 17–18 subunits, which aggregate into two different entities defined as the base and the lid, the latter representing the upper part of the regulator. The base is constituted by six different subunits behaving as ATPases and belonging to the AAA family (ATPases associated with a variety of cellular activities), and two additional non-ATPase subunits. It binds to the outer α rings of the 20S proteasome and is responsible for the ATP-dependent opening of the proteasome central gate; in so doing, it confers to the core particle proteolytic activity on ubiquitinated substrates, as well as the ability of reverse chaperone-like unfolding of the protein substrates. The lid is composed of at least ten non-ATPase subunits, which carry the binding sites for ubiquitinated and nonubiquitinated substrates, and also for enzymes of the breakdown and recycling of the Ub chains. These subunits are thought to play a role in the recognition of polyubiquitinated proteins and are essentially required for their degradation; thus, the lid is believed to provide the specificity to proteolysis (Ferrell et al., 2000).

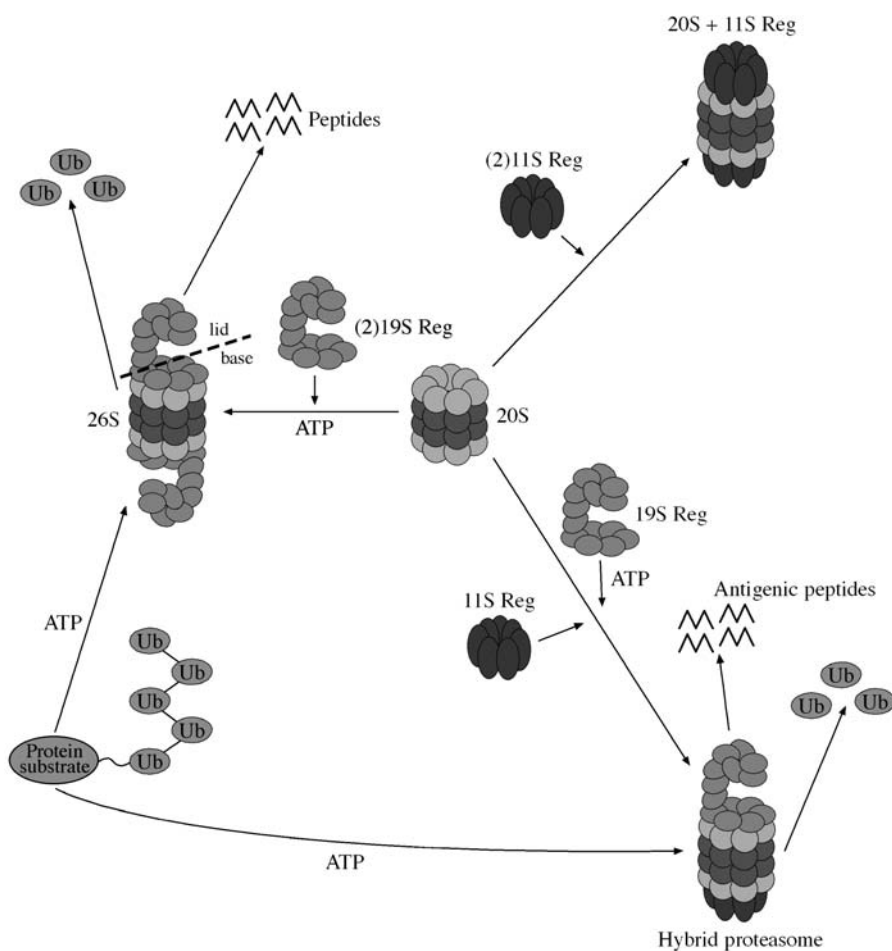
The 11S regulator (11S Reg), 280 kDa in size, is constituted by two types of subunits, α and β , distributed to form two heptameric rings at each extreme of the 20S proteasome, each ring comprising 3 α and 4 β subunits. The interaction of the two particles is not dependent on energy supply and does not enable the core particle to degrade folded proteins even if tagged with Ub tails; contrastingly, its role consists in enhancing the core peptidase activity, particularly the chymotrypsin-like activity (Rechsteiner et al., 2000). The regulator is expressed only in organisms with an adaptive immune system (Bose et al., 2004); in mouse it is highly distributed in the spleen, thymus, and lung, moderately abundant in the liver, and almost absent in the brain (Rechsteiner et al., 2000), as opposed to human brain where its presence is undoubted (Piccinini et al., 2005). Evidence has been presented that the 19S Reg and 11S Reg can simultaneously bind the 20S proteasome, thus giving origin to the so-called hybrid proteasome (Hendil et al., 1998) (● [Figure 9-1](#)).

3.2 The Immunoproteasome

The 20S proteasome shows great plasticity with regard to structural and functional properties. In fact, in cultured cells stimulated by the proinflammatory cytokines interferon (IFN)- γ and tumor necrosis factor (TNF)- α , three subunits harboring the peptidase activity, identified as LMP2, Mecl-1, and LMP7 and defined as inducible subunits, were generated. They were homologous to the three active-site bearing subunits β 1, β 2, and β 5, respectively, and because of increased expression, they were incorporated into the newly assembled proteasome, substituting the corresponding constitutive subunits upon proteasome synthesis. This replacement also occurs during an immune response to pathogens, following release of the two cytokines, in activated dendritic cells; whereas professional antigen-presenting cells, such as those present in the thymus, spleen, and lymph nodes, express the inducible subunits constitutively (Kloetzel, 2004). As a consequence of these substitutions, new 20S core complexes are formed with different

■ Figure 9-1

Proteasomes and degradation of ubiquitin-tagged proteins. 20S, 20S proteasome; 26S, 26S proteasome; 19S Reg, 19S regulator; 11S Reg, 11S regulator; Ub, ubiquitin; (2)19S and (2)11S, two molecules of the regulator, one at the top and the other at the bottom of the 20S proteasome. Ubiquitinated proteins are taken up by the 26S proteasome or the hybrid proteasome, resolved, in an ATP-dependent manner, inside the 20S proteasome into short peptides; the latter are released together with free ubiquitin molecules that are recycled. Peptides generated by the hybrid proteasome are selectively transported to MHC class I molecules for presentation to cytotoxic T lymphocytes and for this reason they are defined antigenic peptides



proteolytic properties leading to the generation of short peptides with hydrophobic and basic amino acidic residues at their C terminus. In fact, LMP2, as opposed to its constitutive counterpart $\beta 1$, is void of PGPH activity and instead is selective, similar to LMP7, for carrying chymotrypsin-like activity; in addition, with respect to $\beta 2$, MECL-1 has enhanced trypsin-like activity. For this specificity, the proteasome becomes the main provider of peptide ligands for MHC class I proteins that serve as T-cell epitopes. The first indication that proteasomes may be implicated in the activation of the immune response was the finding that LMP2 and LMP7 are encoded in the MHC class II region in the direct neighborhood to genes coding for TAP, the transporter associated with antigen presentation. Furthermore, stimulation of cells with IFN- γ also triggers the synthesis of the α and β subunits of the 11S Reg; and of note, the expression profile of these two types of

subunits largely correlates with that of the proteasome-inducible subunits. In concert with the 19S Reg, these five IFN- γ -inducible components of the proteasome system form the immunoproteasome, a definition that well exemplifies the connection between the hybrid proteasome and the cellular immune response. (Fruh et al., 1994; Tanaka and Kasahara, 1998; Bochtler et al., 1999; Noda et al., 2000; Klotzel, 2004) (► [Figure 9-1](#)). It emerges that the constitutive proteasome and the immunoproteasome coexist in the cell, but their balance varies in relation to cell and tissue types and environmental conditions (Noda et al., 2000; Ding and Keller, 2001; Carrard et al., 2003; Piccinini et al., 2005).

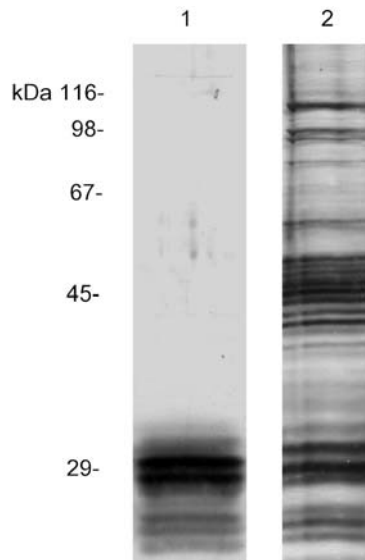
3.3 The Proteasome and the Immunoproteasome in the Nervous System

Proteasomes are widely documented to be distributed in the cytoplasm, nuclei, dendrites, axons, and synaptic buttons of a variety of cells in the central nervous system (CNS) including pyramidal cells, granular cells in the hippocampus, Purkinje cells, and glial cells (Azaryan et al., 1989; Okada et al., 1991; Lucas et al., 1992; Akaishi et al., 1996; Piccinini et al., 2000, 2003, 2005; Keller et al., 2002; Wojcik and Di Napoli, 2004; Mishto et al., 2006a, b). In the CNS, with the inclusion of synaptic terminals, the proteasome is expressed ubiquitously although not homogeneously, as revealed by immunohistochemical analysis; and throughout the CNS it is present both in neuronal and glial populations (Ding and Keller, 2001). Increased proteasome activity is associated with neuronal differentiation, whereas activity and expression of proteasomes decrease in neurodegenerative disorders and with age, thereby concurring to the elevation of protein oxidation, protein aggregation, and neurodegeneration evident in the aging CNS (Keller et al., 2000, 2002).

Brain proteasomes have been purified and extensively characterized with regard to their structure and function (Piccinini et al., 2000, 2003, 2005). Homogeneous 20S and 26S proteasomes from bovine brain and 20S proteasome from human brain display the same structural and functional properties characterizing standard proteasomes (► [Figure 9-2](#)). In this context, it is surprising that, although the

■ Figure 9-2

Electrophoretic pattern of the subunits concurring to the constitution of the 20S and 26S proteasomes isolated from bovine brain. Resolved subunits by denaturing gel electrophoresis are visualized by silver staining. Sizes of the subunits range from 29 to 21 kDa for the 20S proteasome (lane 1) and from 120 to 21 kDa for the 26S proteasome (lane 2)



brain is an immune-privileged organ (Neumann et al., 1995; Xiao and Link, 1998), proteasomes from the human brain cortical region incorporate the three inducible subunits, and these subunits structurally do not differ with respect to their counterparts from tissues involved in the immune response (Piccinini et al., 2003). However, in comparison with the proteasome from the latter tissues, the inducible subunits are expressed in a reduced amount in the human brain proteasome, and this proteasome has a significantly higher PGPH activity and lower chymotrypsin-like and trypsin-like activities (Piccinini et al., 2003). In the normal brain, a polymorphism at codon 60 of LMP2 subunit is described, which is void of any effect on LMP2 expression in terms of mRNA or mature protein although capable of modifying the proteasome peptidase activity (Mishto et al., 2006a, b). Furthermore, induction of immunoproteasome is associated with neuronal differentiation (Wojcik and Di Napoli, 2004) and progression in aging, an observation that indirectly confirms a role of immunoproteasomes in immunosenescence and agrees with the theory of chronic neuroinflammation in the aged brain (Mishto et al., 2006b). Lastly, in the human brain the 11S Reg is expressed (Piccinini et al., 2005), but not in the mouse brain (Noda et al., 2000). In primary microglia cultures treated for a prolonged time with IFN- γ , the expression of the subunit LMP7 is enhanced as is that of MECL-1; the latter event runs parallel with the disappearance of the constitutive counterpart β 2 (Stohwasser et al., 2000). Therefore, the presence of the inducible subunits in brain proteasome supports the view that the brain, particularly the human brain, is potentially capable of triggering an immune response at least with regard to the generation of antigenic peptides. In fact, neurons and resting microglia are cells with poor and absent immunoreactivity, particularly the former; however, in brain, under appropriate stimuli, microglia cells become the major antigen-presenting cells and respond to pathologic events (Neumann et al., 1997; Mavria et al., 1998; Xiao and Link, 1998). Immunostaining reveals that immunoproteasomes are distributed in human brain areas such as hippocampus and cerebellum, and localized in neurons, astrocytes, and endothelial cells (Mishto et al., 2006a, b).

3.4 The Immunoproteasome and Neurological Diseases

Primary dysfunctions of UPS and impaired activity of proteasomes in various neurological disorders, including Alzheimer's disease (AD) (Lopez et al., 2000; Keller et al., 2002; Lindsten and Dantuma, 2003; Mishto et al., 2006b), Parkinson's disease (PD) (Leroy et al., 1998; McNaught and Olanow, 2003), and Huntington's disease (HD) (Jana et al., 2001; Diaz-Hernandez et al., 2003; Zhou et al., 2003), are widely documented and recently reviewed (Ciechanover, 2006). Impaired proteasome-mediated protein degradation contributes to the accumulation of misfolded or modified proteins in neurodegenerative disease, such as paired helical filaments in AD (Keller et al., 2000, 2002; Keck et al., 2003) and aggregation of α -synuclein in PD (Takeda et al., 1998; Snyder et al., 2003; Ciechanover, 2006). Actually, protein aggregates are described to underlie different diseases and their causing toxicity in some diseases is referred to as proteasome function inhibition (Bence et al., 2001). In addition, several studies using pharmacological cell-permeable proteasome inhibitors illustrate that inhibition of proteasome activity determines mitochondria-mediated apoptotic neuronal death in a variety of primary neuronal culture models (Qiu et al., 2000; Piccioli et al., 2001). By contrast, inhibition of proteasome activity has been referred to prevent apoptosis in some neuronal death models, such as cultured sympathetic neurons deprived of nerve growth factor (NGF) (Sadoul et al., 1996), cerebellar granule neurons exposed to low potassium dosages (Canu et al., 2000), and cortical neurons exposed to β -amyloid fragments (Favitt et al., 2000). This discrepancy is closely related to the extent of proteasome inhibition, with widespread neuronal apoptosis relying on partial proteasome activity inhibition, and reduced progression toward apoptosis relying on near-complete blockade of proteasome activity (Suh et al., 2005). A characteristic trait of a wide number of neurodegenerative diseases—AD, PD, Lewy body (LB) dementia, amyotrophic lateral sclerosis (ALS), CAG expansion (poly Q) diseases, such as HD, spinocerebellar ataxias (SCAs), and spinobulbar muscular atrophy (Kennedy's syndrome)—is the accumulation of Ub conjugates and/or inclusion bodies consisting of ubiquitinated material, proteasome, and defined disease-specific proteins surrounded by disorganized filaments. This accumulation is a feature of neurofibrillary tangles in AD, brainstem LBs in PD, LBs in LB dementia, Bunina bodies in ALS, and nuclear inclusions in CAG

diseases (Ciechanover, 2006). Some of these neuropathological aspects also affect, although to a lesser extent, the normal aging of the brain (Selkoe, 1994; Ciechanover, 2006).

3.4.1 Parkinson's Disease

An important player in the pathogenesis of PD that supports the involvement of UPS, and in particular of proteasome proteolytic activity, in the disease is α -synuclein, a small 140-amino acid residue protein thought to intervene in dopamine neurotransmission and release via effects on vesicular storage; this role is impaired in familial forms of PD owing to protein mutations (Ciechanover, 2006). α -Synuclein is the major component of LBs, and its association with LBs is concomitant with its aggregation, an event that is strengthened when it is present as a mutant protein. Accumulation of aggregated α -synuclein is considered to contribute to the pathophysiology of PD; the underlying mechanism concerns the ability of the protein to inhibit the 26S proteolytic activity. This effect relies on α -synuclein-mediated interaction with the 19S Reg resulting from its binding with the S6' subunit (also defined tat-binding protein 1 or Rtp5), the docking protein for ubiquitin-tagged proteins and essential for interaction of these proteins with the 26S proteasome (Lam et al., 2002; Snyder et al., 2003). However, mutations in the α -synuclein gene have not been detected in sporadic cases, which represent the vast majority of patients with PD. Nonetheless, a dysfunction in protein handling in the substantia nigra pars compacta (SNc) in patients affected by sporadic PD is disclosed by an increase in the level of oxidatively damaged proteins, a diffuse increase in protein aggregation, and the accumulation of a wide range of poorly degraded proteins within LBs. Of interest, in these patients the three major peptidase activities of the 20S proteasome are downregulated in the SNc; furthermore, in dopaminergic neurons of the SNc, but not in other brain regions, a selective loss of the proteasome α subunits is described, which is in turn responsible for a destabilization of the proteasome catalytic core along with impaired proteasome assembly (McNaught and Jenner, 2001; McNaught and Olanow, 2003).

3.4.2 Alzheimer's Disease

A pathological hallmark of the disease is the intracellular accumulation of neurofibrillary tangles (NFTs) resulting from the association of Ub-reactive material with the tau-based paired helical filaments (PHFs) and senile plaques including the β -amyloid precursor protein (APP) and its proteolytic product β -amyloid protein ($A\beta$). Additional and probably distinct players in the development of the disease are presenilin-1 and -2 (PS) implicated in the processing of APP carried out by the γ -secretase proteolytic complex (Ciechanover and Schwartz, 2004). This accumulation has been related to dysfunctions of proteasome catalytic activity (Keller et al., 2000; Lopez Salon et al., 2000). $A\beta$ is described to interfere with the proteolytic step of UPS by affecting the 26S proteasome proteolytic activity through a direct interaction with the catalytic core, the 20S proteasome, and in particular the $\beta 5$ subunit, resulting in a downregulation of the chymotrypsin-like activity (Gregori et al., 1995). Furthermore, PHFs have been uncovered as strong proteasome inhibitors. The underlying mechanism does not concern the proteasome amount, but rather the interaction between PHFs and the proteasome, in which the 19S complex constitutes the docking element, resulting in a depression of the proteasome chymotrypsin-like activity. This impairment clearly affects the hippocampus straight gyrus, although appreciable in other regions, and its severity correlates with the amount of PHFs and the accumulation of oxidatively damaged proteins (Keck et al., 2003). Immunoproteasomes are distributed in different regions of the brain in AD patients with a significant increase of LMP2 in the hippocampus, not related to LMP2 polymorphism, but surprisingly associated with unaltered chymotrypsin-like and PGPH activities, as opposed to what was reported above (Keck et al., 2003), and significant decrease of trypsin-like activity (Mishto et al., 2006b). Lastly, both PS-1 and -2 are highly controlled by multiple proteolytic activities, which for PS-2 can be identified in that of the proteasome, since in vivo PS-2 is subjected to ubiquitination and its degradation is blocked by proteasome inhibitors (Kim et al., 1997; Steiner et al., 1998). Therefore, accumulation of PS-2 due to impaired proteasome activity in AD can be associated with accumulation of $A\beta$, this in turn hindering the proteasome function.

3.4.3 Huntington's Disease

HD is an autosomal, dominant, neuropathic disorder relying on a CAG triplet-repeat coding a polyglutamine (polyQ) motif in the N-terminal region of the protein huntingtin (htt), a trait shared also by other (at least eight) neurological diseases. In HD and spinocerebellar ataxia type 1 (SCA1) brains, as well as in mouse and cell models of most triplet-repeat disorders, these aggregates are immunopositive for Ub and proteasome subunits. Sequestration of ubiquitinated proteins and proteasome subunits into polyQ-containing aggregates raised the suggestion that the efficiency of UPS is impaired in CAG triplet-repeat disorders, and thus this impairment is at the heart of the neurological dysfunction associated with these disorders, because of altered turnover of crucial proteins caused by downregulated proteasome function (reviewed in Diaz-Hernandez et al., 2003). Unexpectedly, the three major proteasome peptidase activities tested in brain extracts from a mouse model of HD are not depressed, but intriguingly, the chymotrypsin-like and trypsin-like activities in extracts from affected and aggregate-containing regions, such as cortex and striatum, are even upregulated. In parallel LMP2 and LMP7 immunodecoration reveals an accumulation of the two proteins along with unvaried total proteasome amount. The above findings in the HD animal model are reproduced in the brain and in particular in the striatum and cortex of HD patients. Although the latter observations throw additional light on the role of the immunoproteasome in HD pathogenesis, they offer information only with regard to the efficiency of the proteasome catalytic core not subjected to the influence of the 19S Reg and its inability to degrade Ub-tagged proteins in an *in vitro* context, quite dissimilar to the *in vivo* one. By contrast, the increased expression of the immunoproteasome, which is likely to reflect an *in vivo* situation, could be taken as a marker of the mechanisms underlying the neurodegeneration characterizing the disease, accounting for a neuroinflammatory stress.

3.4.4 Familial Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative fatal disease of unknown etiology characterized by progressive loss of spinal cord, cortical motor neurons, and brainstem. Usually, ALS has a sporadic trait but familial in 5–10 of cases, and in 10%–20% of the latter it relies on mutations in the gene encoding Zn/Cu ion-dependent superoxide dismutase (SOD1). Neuropathological traits of the familial (fALS) and sporadic forms are inclusion bodies comprising aggregated and ubiquitinated proteins surrounded by a network of poorly organized filaments; SOD1 is a component of these inclusion bodies in fALS associated with SOD mutations. Intracellular inclusions enriched in SOD1, as well as in ubiquitinated materials, are also detectable in motor neurons and astrocytes of an animal model of fALS related to SOD1 mutations, constituted by transgenic mice overexpressing human mutant SOD1, in late stages of the disease. In the spinal cord of symptomatic transgenic mice, accumulation of soluble mutant SOD1 relies not on increased transcription but rather on impaired degradation. This anomaly runs parallel with decreased amounts of the constitutive proteasome and increased levels of the immunoproteasome; furthermore, a remarkable quantitative decrease of the constitutive proteasome also occurs in motor neurons of presymptomatic transgenic mice, more evident with progression of the severity of the disease (reviewed in Cheroni et al., 2005). Therefore, the suggestion that, as concerns fALS associated with SOD mutations, toxicity of the disease at least part is related to impaired function of proteasomes, and in particular of immunoproteasomes, is reliable.

3.4.5 Glioblastoma Multiforme

The most aggressive tumor affecting the brain is glioblastoma multiforme (GBM), which specifically targets astrocytes. GBM largely relies on genetic alterations associated with necrosis, angiogenesis, and clonal selection. Upregulation of UPS and of the transcription nuclear factor NF- κ B are traits of GBM (Hayashi et al., 2001; Nagai et al., 2002). In the 20S proteasome isolated from fresh specimens of the human GBM, the inducible subunits LMP2, MECL-1, and LMP7 are much more incorporated than in the control

proteasome; but surprisingly, the chymotrypsin-like activity is strongly depressed with unvaried trypsin-like activity (Piccinini et al., 2005). Reasons for this discrepancy that concerns 70% of the specimens examined are still unclear; however, it can be taken as a marker of the functional dysfunction affecting this tumor tissue and in particular of an altered immunological attitude.

4 Conclusion

Collectively, impaired proteasome proteolytic function appears to have a considerable part in acquired and inherited neurological diseases, thus strengthening the view that proteasomal protein degradation, and more in general UPS, is a critical process for neuronal health. There is evidence that oxidative events are at the origin of dysfunctions of the proteasome activity in aging and that these events occur during the course of PD. Furthermore, inclusion bodies enriched in disorganized proteins including ubiquitinated materials are a frequent feature of neurological diseases associated with aging; accumulation of these materials is related to impaired proteasome proteolytic activity, possibly owing to proteasome sequestration inside the inclusions as well as to an inhibitory action of proteins characterizing the inclusions. Searching for the origin of the oxidative events in these diseases and for a relation among these events—inclusion bodies formation and impaired proteasome function—will be helpful for the identification of a dysfunction unifying all these diseases, thus providing a novel impetus to design new therapeutic molecules.

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10 Regulation of the Inflammatory Response in Brain

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1	<i>Basic Components of the Neuroinflammatory Response</i>	237
1.1	Innate Immunity in the CNS	237
1.2	Antigen Presentation—Formation of the Immunological Synapse Between T Cells and the MHC-Expressing Cell	237
1.3	Microglia—Regulation of APC Function	242
1.4	Macrophages—Specialized Monocytes in the CNS	248
1.5	Astrocytes and the Immune Response	249
1.6	T Lymphocytes	251
1.7	B Lymphocytes	252
1.8	The Blood–Brain Barrier	253
2	<i>Concluding Remarks on the Regulation of the Inflammatory Response</i>	255

Abstract: Neuroinflammation is a pathological condition of the central nervous system (CNS) that happens in response to invasion of a multitude of infectious pathogens, and also in autoimmune diseases. The infectious pathogens each have special properties as to the invasion modes, the natural reservoir, the host reservoir, the immune response that they elicit under normal and under immunocompromised conditions, the target tissue, and the “target region” within the CNS. In addition, there is great variation in susceptibility to specific infectious agents in humans (and mice), reflecting genetic predispositions. In autoimmune diseases such as multiple sclerosis (MS), paraneoplastic encephalitis, etc., there is also considerable variance in the development, progression, and outcome of the disease in different patients. The CNS is a highly vulnerable tissue, which is physically protected against external forces by a rigid osseous shell. In addition, for protection against potentially destructive endogenous inflammatory responses it possesses properties of an immune-privileged or specialized organ. As a consequence, the inflammatory response within the CNS and its connective tissue coverings differs considerably from the response that would be elicited in other bodily parenchymal organs by the same pathogen. Although most of the components, such as cells (granulocytes, lymphocytes, macrophages/microglia) and mediators (cytokines, chemokines, enzymes, adhesion molecules, activation factors etc.), of the immune response that are involved are the same in the CNS as in the periphery, they are differentially regulated according to CNS requirements. The CNS does not depend on resident tissue leukocytes or infiltrating professional macrophages or neutrophils to recognize and clear pathogens. Instead, the CNS has established its own immune system, based upon activation of resident innate immune cells such as microglia and astrocytes. Evolution has established a system that recognizes “nonself” such as microbial products, and “altered self” such as apoptotic cells, amyloid fibrils, and prions by pattern recognition receptors like those of the Toll-like receptor (TLR) family. Glial cells may rapidly respond to pathogens by producing chemokines and cytokines. Only in a second step, and with delay, leukocytes from the periphery can enter the CNS following chemokine gradients. The entry of leukocytes from the periphery into the CNS is one of the crucial steps in neuroinflammation and is considered an important regulative element. Another hallmark in this scenario is a process considered as “activation.” Not only can the different subpopulations of leukocytes exist in resting/quiescent forms and in highly activated states but also that endothelial cells vary from basal to activation states—processes that obviously require complex regulation. These regulatory processes will decide which inflammatory leukocytes will be permitted to enter at which site of the CNS. (1) The exceptional immunologic situation of the CNS is the consequence of at least five well-defined causes. The healthy CNS parenchyma is patrolled by only very few lymphocytes, and leukocytes/macrophages reside within specialized loci such as the plexus and the meninges. (2) There are no identifiable lymphatics within the CNS tissue. (3) The major histocompatibility complex (MHC) molecules I and II are barely detectable in the healthy state and only become upregulated in pathological conditions. (4) Allogeneic tissue grafts survive within the CNS but are readily rejected after implantation elsewhere in the body, which is the basic observation wherefrom the idea of “immune privilege” was derived. (5) Additionally, the functional properties as well as the morphology of the blood–brain barrier (BBB) within the nervous system are unique. All of these features contribute to avoid inflammation of the CNS. On the other hand, some observations have added fuel to the fiery discussion on a putative exceptional role of the CNS in immunological terms. After CNS parenchymal damage, antigens are readily transported into the cervical lymph nodes, where they may encounter professional antigen-presenting cells (APC), and efficient T-cell or B-cell responses will be triggered. These cells may recirculate into the CNS across the BBB, where they encounter their specific antigen, and the whole cascade of defense proceeds. The invasion of leukocytes across the BBB can be highly efficient. It involves the tight regulation of expression of adhesion molecules and their receptors on both the invaders (leukocytes) and the frontier (BBB). Furthermore, the appropriate secretion of chemoattractants contributes to leukocyte invasion of the brain via the BBB. Several severe systemic inflammatory conditions have been shown to induce activation of microglia, since these cells function as sensors of inflammation. But even relatively minor deviations from bodily homeostasis may be sufficient to alert microglia and to put them into a state that results in release of proinflammatory or regulatory mediators according to the activating stimulus, thereby contributing to a special “immune milieu” within the CNS.

The regulatory elements of diverse neuroinflammatory processes and the network of variables that contribute to the process of inflammation, leading to a final net outcome in healing or defective healing of the CNS, are discussed herein.

List of Abbreviations: ACTH, adrenocorticotrophic hormone; APC, antigen-presenting cells; BBB, blood–brain barrier; BDNF, brain-derived neurotrophic factor; CNS, central nervous system; c-SMAC, supramolecular activating complex; CVE, cerebrovascular endothelial cells; GA, glatiramer acetate; GFAP, glial fibrillary acidic protein; GM-CSF, granulocyte/macrophage colony-stimulating factor; HIV, human immunodeficiency virus; IP-10, IFN- γ -induced protein-10; MBP, myelin basic protein; MCP-1, monocyte chemotactic protein-1; M-CSF, macrophage colony-stimulating factor; MHC, major histocompatibility complex; MHV, murine hepatitis virus; MNGC, multinucleated giant cells; MOG, myelin oligodendroglial glycoprotein; MS, multiple sclerosis; NGF, nerve growth factor; NT-3, neurotrophin-3; PAMP, pathogen-associated molecular patterns; PRR, pattern recognition receptors; TLR, Toll-like receptor; VIP, vasoactive intestinal peptide

1 Basic Components of the Neuroinflammatory Response

1.1 Innate Immunity in the CNS

The innate immune system is considered the first line defense in recognition of microbial pathogens, and triggers the consecutive host defense response. Key players in the scenario are so-called pattern recognition receptors (PRR), which communicate the attack of a pathogen to the host. PRR bind to a conserved set of molecular patterns confined to a group of microbes termed pathogen-associated molecular patterns (PAMP). Toll-like receptors (TLR) are important PRR in the mammalian universe, and most mammals have between 10 and 13 TLR, which bind to PAMP. While some of the TLR are specialized in the recognition of bacterial surface structures (e.g., lipopolysaccharide), others recognize nucleic acids (CpG-containing DNA) to detect motifs of viral DNA or RNA intracellularly in endosomal compartments in which the host's own nucleic acids ("self") are normally not accessible—a mechanism that may be involved in development of autoimmunity (Leadbetter et al., 2002). Activation of the innate immune system by TLR/PAMP recognition stimulates the antimicrobial response of the host by inducing inflammation through production of proinflammatory cytokines. This will be followed by the adaptive immune response with production of chemokines, recruitment of leukocytes, and their activation. Factors regulating the individual antiinflammatory response of the host after microbial sensing through TLR in the CNS in order to terminate inflammation are currently under investigation. During the CNS inflammatory response, the bactericidal and cytotoxic nitric oxide (NO) produced by the enzyme iNOS, as well as proinflammatory cytokines such as tumor necrosis factor (TNF), interferon (IFN)- γ , interleukin (IL)-1, IL-6, and IL-12 play important roles. These factors are not different from those involved in innate immune responses in peripheral organs. Cellular components involved in innate responses within the CNS are macrophages/microglia, astrocytes, cerebrovascular endothelial cells (CVE), natural killer cells (NK cells), and granulocytes. In different infectious contexts, the link between the initial "innate" TLR signaling and the ensuing adaptive immunity is influenced by a multitude of external factors and triggers, which remain to be analyzed in detail. One key mechanism of this linkage is the upregulation of costimulatory molecules such as B-7.1 (CD80) and B-7.2 (CD86) on the surface of innate immune cells (e.g., macrophages) to activate T cells. The molecular and cellular innate immunity pathways leading to protective immunity or pathological consequences of immune reactions (e.g., excessive inflammation or autoimmunity) remain to be fully established (Beutler, 2004).

1.2 Antigen Presentation—Formation of the Immunological Synapse Between T Cells and the MHC-Expressing Cell

We first discuss some general features of antigen presentation before going into details on antigen presentation in the CNS.

Antigen presentation is the central event that is mandatory for a T-cell response against infectious pathogens, and of unintentional autoreactive T-cell responses against "self" in autoimmune diseases. It is a

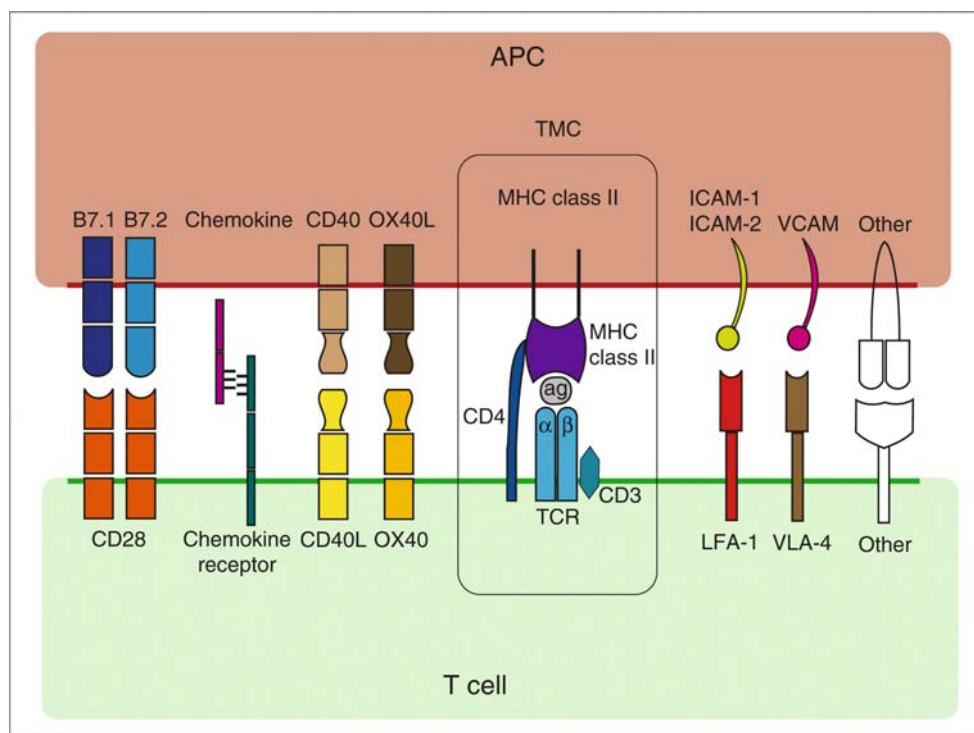
process that involves different specialized cells termed antigen-presenting cells (APC), which take up, process, and present antigen or antigen fragments on their cell surface. These fragments, which are generally peptides, are bound to MHC molecules, and therefore recognizable for T cells. MHC historically stands for major histocompatibility complex, a genetic locus containing indispensable genes that determine the success or failure of graft rejection of two individual organisms. Highly polymorphic allelic variants of MHC loci lead to a high number of different heterodimeric combinations that determine the individual response to a given inflammatory challenge. Human MHC molecules, also referred to as HLA (human leucocyte antigen), consist of the class I (A, B, and C) and the class II (DP, DQ, and DR) loci. While MHC class I molecules are found on all nucleated cells, MHC class II molecules are only expressed on macrophages, dendritic cells, thymic epithelium, and B cells. During the ontogenetic development of the immune system, an enormous number of antigen-specific T cells with differing specificities develop. An individual T cell recognizes only one complex of antigenic peptide and bound MHC molecule, while other T cells recognize other combinations. Even though one peptide may be bound to several MHC molecules, the ensuing T-cell response will only occur if the peptide binds MHC on the APC and the TCR on the T cell simultaneously, an important process that is called MHC restriction of T-cell recognition. Unless a peptide is not bound to a self MHC molecule, it will not be recognized by the TCR, and even expanded and activated T cells cannot recognize proteins in the absence of APC. A major feature of the normal appropriate antigen presentation is the regulated MHC class II expression on APC. Inappropriate MHC class II expression has been found in various inflammatory and autoimmune diseases, whereas MHC class II deficiency leads to dramatic immunodeficiency during infections of mice and humans (Grusby and Glimcher, 1995). Therefore, tight regulation of the MHC class II expression is a prerequisite for APC and T-cell interaction during antigen presentation and functional T lymphocyte development. Regulation of MHC class II expression occurs at the transcriptional level with three *cis*-acting sequences, known as W, X, and Y boxes. Additionally, the essential class II transactivator (CIITA) has been identified as a key player of antigen presentation (Harton and Ting, 2000). Subsequent formation of the “intrinsic immunological synapse” involves a complex machinery of events on both the T cell and APC side (● [Figure 10-1](#)). Tightly regulated interactions between adhesion molecules and costimulatory molecules and their counterreceptors are required. At the interface, a regulated sequence of molecular events occurs: binding of ligands such as LFA-1 (CD11a)/ICAM (CD54) as well as other costimulatory molecules (CD40, B7.1 (CD80), B7.2 (CD86)), TCR arrangement in clusters, consecutive centralization and coalescence of TCR, finally leading to the so-called central supramolecular activating complex (c-SMAC) (Krummel and Davis, 2002). These synapses may form according to local and systemic requirements and will resolve after signal transduction is achieved.

Besides the MHC-restricted binding of the CD4⁺ T cell to the MHC class II molecule of the APC, a multitude of additional interactions between the above mentioned receptors and ligands of specific systems must take place to guarantee an efficient binding. These interactions involve members of the B7 family on the APC, which bind to CD28 on T cells. The B7/CD28 interaction leads to firm and prolonged binding. Moreover, this interaction enables T cells to respond to B7-expressing infected cells but not to B7-negative normal cells in the vicinity. In addition to the B7/CD28 interaction, other interacting molecules comprise the integrin family with ICAM-1 and ICAM-2 on APC and LFA-1 on T cells, or the VCAM–VLA-4 interaction, as well as members of the TNF family (e.g., CD40–CD40L or OX40–OX40).

It has to be mentioned that in the case of severe infections due to some pathogens, there is an important exception to the normal MHC-restricted T-cell response described here. Enterotoxins from *Staphylococcus aureus* for example may stimulate a huge number of T cells at once—much more than if the stimulation was confined to a single antigen-specific T cell. Staphylococcal enterotoxins have been shown to bind to other parts of the MHC molecule and the TCR in a manner that one “superantigen” can stimulate many T cells, leading to a massive release of proinflammatory cytokines. This is an interesting example of an unbalanced immune response although one may speculate that the stimulation of many T cells could also be beneficial in acute cases of severe infections where a response by a few antigen specific T cells would be too “slow.” A further exception to the described classical antigen presentation is the involvement of another small subgroup of T cells, the so-called $\gamma\delta$ T cells, which account for about 5% of the circulating T cells. These cells recognize phospholipids antigens, using their TCR but not the MHC molecules. Again, this means that such an interaction is not MHC dependent. The activation of $\gamma\delta$ T cells upon microbial stimulation results

■ Figure 10-1

Cell adhesion between a $CD4^+$ T cell and an antigen-presenting cell (APC). The antigenic peptide (ag), which is recognized by the T-cell receptor (TCR), is presented by the MHC class II molecule. The invariant CD3 molecule mediates consecutive signal transduction. A multitude of additional costimulatory molecules and adhesion molecules and their respective receptors stabilize the contact between the two cells by forming concentric rings around the centrally located trimolecular complex (TMC) consisting of MHC molecule, TCR, and antigenic peptide (B7.1 = CD80 and B7.2 = CD86 on the APC interact with CD28 on the T cell. Chemokines bind to their receptors both on T cells and APCs, members of the TNF receptor superfamily like CD40 on the APC binds to CD40L (CD154) on the T cell, and OX40 (DD134) on the activated $CD4^+$ T cell binds to OX40L on the APC. The adhesion molecules ICAM-1, and -2 (intercellular adhesion molecule 1 and 2) as well as VCAM (vascular cell adhesion molecule) on the APC bind to LFA-1 (lymphocyte function-associated antigen 1) and VLA-4 (very late antigen-4) on the T cell, respectively



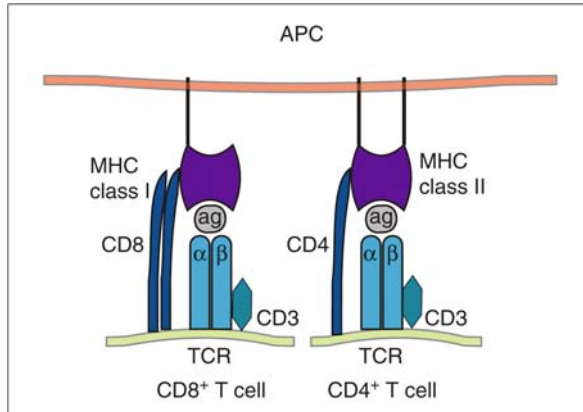
in production of $IFN-\gamma$ and IL-10. Recent studies have shown that $\gamma\delta$ T cells play an active role in the regulation and resolution of pathogen-induced immune responses (Skeen et al., 2004).

Since T cells communicate with APC through formation of a synapse, a short introduction in T-cell immunology is given at this point. T lymphocytes can be subdivided into $CD4^+$ and $CD8^+$ cells, which both express the T-cell antigen receptor (TCR α/β). $CD4^+$ cells carry also the CD4 coreceptor, which binds to a conserved region of MHC-II. $CD8^+$ cells are defined by the CD8 coreceptor, which binds to a conserved region of MHC-I (► Figure 10-2).

T cells expressing the CD8 molecule are named cytotoxic T cells (T_C), mainly designed for killing, especially killing of cells infected with intracellular pathogens such as viruses. They secrete perforins, granzymes, and proinflammatory cytokines such as TNF and $IFN-\gamma$. T cells with the surface marker CD4 are called T helper cells (T_H), and can again be subdivided into T_{H1} and T_{H2} cells. They will only bind to cells carrying the MHC class II molecule, e.g., macrophages, B cells, dendritic cells, and microglial cells within the CNS. These T_H cells can be distinguished by the profile of secreted cytokines. T_{H1} cells activate

■ Figure 10-2

Antigen recognition by $CD4^+$ and $CD8^+$ T cells and binding to the appropriate MHC molecules. The MHC molecules on the APC present bound antigenic peptide (ag) to the T-cell receptor. The coreceptors CD4 and CD8 facilitate and stabilize the binding. MHC class I consists of one transmembrane heavy chain (α chain), which is built up by three domains, α_1 , α_2 , and α_3 noncovalently bound to the β_2 m protein. The MHC class II molecule is formed by two transmembrane protein chains α and β , each consisting of two domains α_1 and α_2 as well as β_1 and β_2 .



macrophages and $CD8^+$ T-cell function by secreting mainly $IFN-\gamma$, IL-2, and TNF. Further they mediate B-cell differentiation toward Ig isotypes that fix complement. They have been extensively characterized in the context of EAE and MS (Owens et al., 2001; Owens and Babcock, 2002). Additionally, they are able to mediate resistance against certain intracellular bacteria, fungi, and protozoans. T_H2 cells induce humoral responses with proliferation, differentiation, and antibody secretion of B cells, by secreting cytokines such as IL-4, IL-13, IL-10, and IL-5. T_H2 cells also inhibit numerous macrophage inflammatory features. They were found to mediate resistance to certain intestinal helminths. Additional T_H cell subsets are included in the group of suppressive $CD4^+$ $CD25^+$ regulatory T cells, which comprise the so-called nT_{reg} (naturally occurring regulatory T cells) and the aT_{reg} (adaptive regulatory T cells), comprising Tr1 cells producing high levels of IL-10 and low levels of transforming growth factor- β (TGF- β) and IL-5, and T_H3 cells producing preferentially TGF- β and varying amounts of IL-4 and IL-10 (Bluestone and Abbas, 2003). Most important is the modulating influence of one T_H subset on other subsets by their cytokine profile. The transcriptional repressor Foxp3 has been identified as being crucial for regulatory T-cell development, since conventional $CD4^+$ T cells and T_{reg} originate in the thymus from a common T-cell precursor, and exit to the periphery as Foxp3 $^+$ fully functional T_{reg} , or as Foxp3 $^-$ naive T_H0 cells that will differentiate into T_H1 or T_H2 effector cells or into a T_{reg} (Wing et al., 2006).

Professional APC are only dendritic cells (DC), B cells, and macrophages. Among these only DC can induce the differentiation of naive T lymphocytes into antigen-specific T lymphocytes. They have the important and complicated task of taking up the pathogen and subsequently degrading its large proteinaceous three-dimensional structure into small linear peptides of 9–15 amino acids, couple the degraded peptide to the MHC binding groove, transport the MHC–peptide complex to the cell surface, and present it to T cells. These intracellular processes are pathogen dependent, and involve the phagolysosome, Golgi apparatus, as well as the endoplasmic reticulum (ER). In addition, as described above APC are able to express costimulatory molecules complementary for counter receptors on T cells that are important for the full activation of unstimulated T cells, since it is not sufficient to present the mere MHC–peptide complex on the cell surface. However, costimulation is not antigenspecific. CD4 and CD8 are cell-surface molecules, mentioned above, which ensure the stable binding of T lymphocytes to MHC class II and class I.

In summary, antigen presentation in general is tightly dependent on (1) the antigen itself and its properties within an individual host organism, (2) the MHC molecule repertoire, and (3) the T-cell receptor repertoire available for recognition of the MHC-peptide complex, leading to a hierarchic assembly of immunodominant antigens versus anergic antigens on the one hand and on the other to the involvement of a large and complex expression of costimulatory molecules, adhesion molecules, and soluble mediators.

In the CNS, antigen presentation has some crucial properties that are different from other compartments of the body. MHC molecules are not constitutively expressed within the healthy CNS, but their expression can be induced effectively during CNS inflammation (Neumann and Wekerle, 1998). Nevertheless, in the healthy CNS, all glial cells and neurons express MHC class I at low levels (Huh et al., 2000; Neumann, 2001), and microglial cells even express MHC class II and present antigen to specific T cells, e.g., after IFN- γ stimulation (Aloisi, 2001). In the CNS, MHC class I and class II expression can be induced or upregulated under many pathological conditions including infection which leads to neuroinflammation. Thus, invading cytotoxic CD8⁺ T cells do not come across MHC⁺ APC within the CNS parenchyma unless tissue damage has occurred. The diverse cell populations within the brain parenchyma have different abilities to induce MHC upregulation. Since microglial cells show high and acute induction of MHC class II upregulation in inflammatory CNS diseases, they are believed to be intimately involved in the immune response. They are certainly the most important and the most potent APC in vitro (Becher and Antel, 1996; Aloisi et al., 1998; Matyszak et al., 1999; Juedes and Ruddle, 2001). During the effector phase of EAE, activation of microglial cells is an important issue concerning the leukocyte invasion of the CNS (Heppner et al., 2005). Astrocytes are far less effective APC owing to their lack of costimulatory molecules. Astrocytic myelin-antigen presentation to CD4⁺ T cells through MHC class II molecules has only been shown in vitro (Mossner et al., 1990; Aloisi, 2001), and their contribution to the in vivo situation remains speculative (Prat et al., 2000; Becher et al., 2000b; Stuve et al., 2002). Oligodendrocytes in active multiple sclerosis lesions do not express MHC class II molecules (Lee and Raine, 1989), and therefore they cannot serve as competent APC. These issues have brought up a fervid international debate about antigen presentation in EAE and MS research. EAE is the currently most accepted model disease for multiple sclerosis, and can be induced by active immunization with myelin antigen (e.g., myelin oligodendroglial glycoprotein (MOG), myelin basic protein (MBP), etc.), leading to the invasion of the CNS with autoaggressive myelin-reactive T cells, which recognize their target and lead to a whole cascade of inflammatory immune-mediated events. Adoptive transfer of myelin-reactive CD4⁺ T_H cells can also induce EAE. These T cells depend on the presentation of antigen by APC in the context of MHC class II molecules in order to recognize their cognate antigen within the CNS. The questions remains: where the crucial antigen-presenting events happen and what the responsible cells are. In the rat EAE model using MBP as an antigen, it has been shown that autoreactive T cells circulate into secondary lymphoid organs where they acquire activation markers and diverse chemokine receptors before entering the CNS (Flugel et al., 2001), but this migration pattern is not essential for disease development. Furthermore, Becher and coworkers have shown recently that although microglial cells are important for EAE development, they are not necessarily required for antigen presentation to autoaggressive T cells in vivo (Greter et al., 2005), postulating DC in perivascular localization as well as plexus-associated regions and meningeal DC as the major mediators of antigen presentation in EAE and MS. This is a very important finding, since in the CNS, unlike in other parenchymal organs, APC such as DC and macrophages are present only in special locations and do not have access easily to all parts of the organ (Hart and Fabre, 1981; McMenamin, 1999). They have the unique ability to take up antigen in the periphery and transport it to lymphoid organs, where the consecutive immune response is initiated (Banchereau and Steinman, 1998). Further, as already stated above, their antigen-presenting capacity is much stronger than that of macrophages, since they can directly activate naive T cells, while macrophages cannot (Iwasaki and Medzhitov, 2004). Also, tissue-resident macrophages of the CNS, with their special localization residing in the meninges, the choroid plexus, and in perivascular spaces, are known to contribute to the organ-specific T-cell activation (see below). DC in the CNS can recognize pathogens that will invade the brain parenchyma through the blood-CSF barrier, and transport them to cervical lymph nodes or to the spleen. Moreover, it has been postulated that DC may accumulate within perivascular spaces and even infiltrate the brain parenchyma during infectious diseases of the CNS and during

autoimmune diseases such as EAE and MS (Fischer et al., 2000; Fischer and Reichmann, 2001; Hohlfeld and Wekerle, 2004; McMahon et al., 2006). Further, they express a pattern of cytokines that is thought to be mainly immunosuppressive (Serot et al., 2000; Suter et al., 2003). These properties of CNS DC are reminiscent of immature tolerogenic DC in the periphery, which are known to induce T-cell tolerance. However, there is an interesting ongoing debate on their role and specific contribution in CNS inflammation: While their number increases drastically in infectious conditions, they may change their surface markers or phenotype, although full characterization as well as the exact profile of proinflammatory mediators secreted by DC is still lacking (McMahon et al., 2006).

The antigen-presenting ability of APC within the CNS is tightly controlled by a multitude of factors from the neuronal microenvironment such as cytokines and growth factors (▶ [Figure 10-3](#)). In the quiescent state, neurons produce immuno-downregulatory nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3), while astrocytes produce TGF- β leading to secretion of IL-4 and IL-10. Contrarily, under inflammatory conditions neurons, glial cells, and also endothelial cells produce chemokines such as CCL2, CCL5, CCL19, CCL21, CXCL10, CXCL12, CXCL19, and cytokines such as IL-1 β , and TNF. All of these factors promote attraction, activation, and transmigration of inflammatory leukocytes (▶ [Figure 10-4a, b](#)) (Neumann et al., 1996; Neumann et al., 1997; Neumann and Wekerle, 1998; Neumann et al., 2002b).

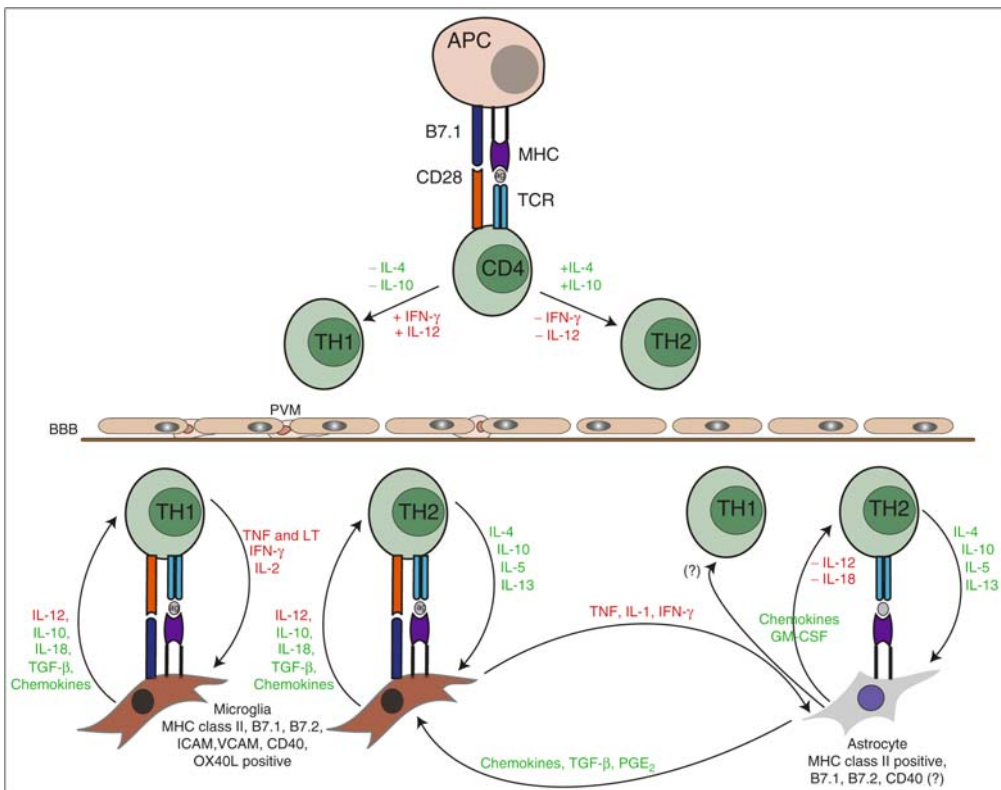
From the mechanisms of antigen presentation described above, it becomes clear that cell–cell interactions are of critical importance for the control of the immune response in general and in infectious diseases of the CNS. There is a well-defined interaction between TCR on T cells with processed peptide fragments in MHC molecules on APC (▶ [Figure 10-1](#) and ▶ [Figure 10-2](#)). This antigen-specific recognition step is critical since specific pathogen detection is required for an effective clearance and termination of the infection, while inappropriate responses to APC presenting “self-antigen” can lead to autoimmune reactions such as MS. Taken together, in MS it is still a matter of debate what the primordial APC are that communicate with T cells, where this initial response happens, and what mechanisms and peculiarities are the ones required for propagation of neuroinflammation thereafter.

1.3 Microglia—Regulation of APC Function

CNS-derived glial cells may be subclassified as microglia and macroglia, the latter comprise oligodendrocytes, astrocytes, and also ependymal cells and their derivatives. Initially, glial cells as a whole were thought to provide only structural and functional support for neurons. Nowadays, their complex physiological roles and their impact in different pathological states especially in inflammatory and immune diseases are widely recognized glial cells are defined herein already. Microglial cells were first described by Nissl in 1899 as “Stäbchenzellen,” i.e., rod cells (Nissl, 1899), and recognized as a distinct glial cell form by del Rio-Hortega and Penfield (1927). Microglial cells are immune cells of the brain with phagocytic activity, building up a relatively stable and resident population within the CNS under physiological and even under pathological conditions. They represent about 10% of the total adult brain cell population, and originate from microglial/macrophage precursors in the yolk sac and the bone marrow. They are detectable from the very early embryonic state. Most of the microglial cells, however, appear during early postnatal development, arising from distinct initial *in situ* proliferation (Alliot et al., 1991; Dalmau et al., 1998; Alliot et al., 1999; Pessac et al., 2001). Besides microglial cells, the CNS contains further monocyte/macrophage populations, which include the plexus choroideus macrophages, meningeal macrophages, and perivascular macrophages (see next paragraph). The latter are, similar to microglial cells, also resident monocytes that are found around parenchymal blood vessels. All of these cells with their specialized localization have been described as being closely involved in the immune surveillance of the neuronal parenchyma (Owens et al., 1998; Tran et al., 1998; Aloisi et al., 2000a, b). Interestingly, microglial cells can adopt immunological, morphological, and functional phenotypes indistinguishable from macrophages and dendritic cells (Hepp-

■ Figure 10-3

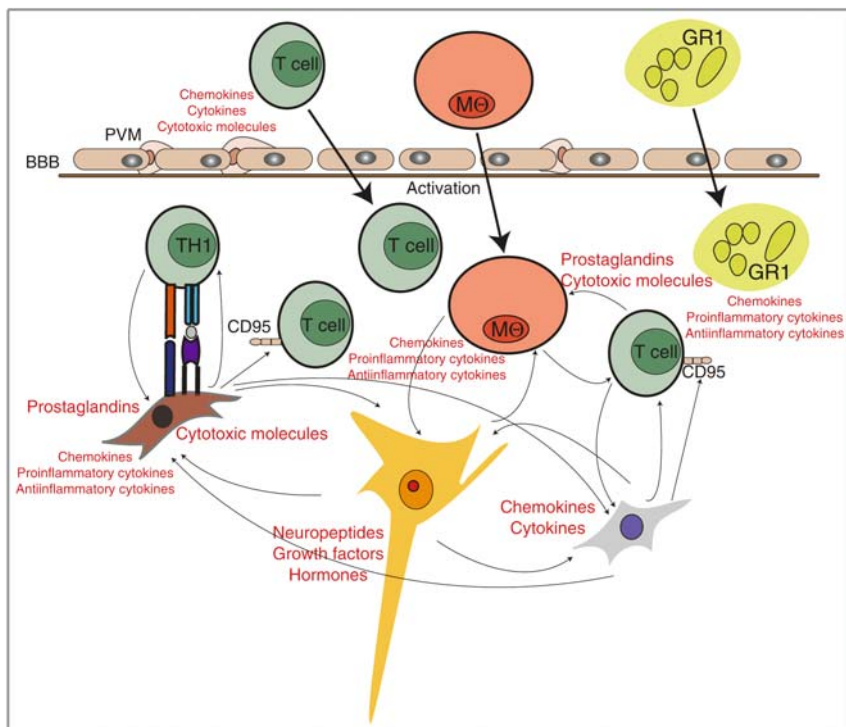
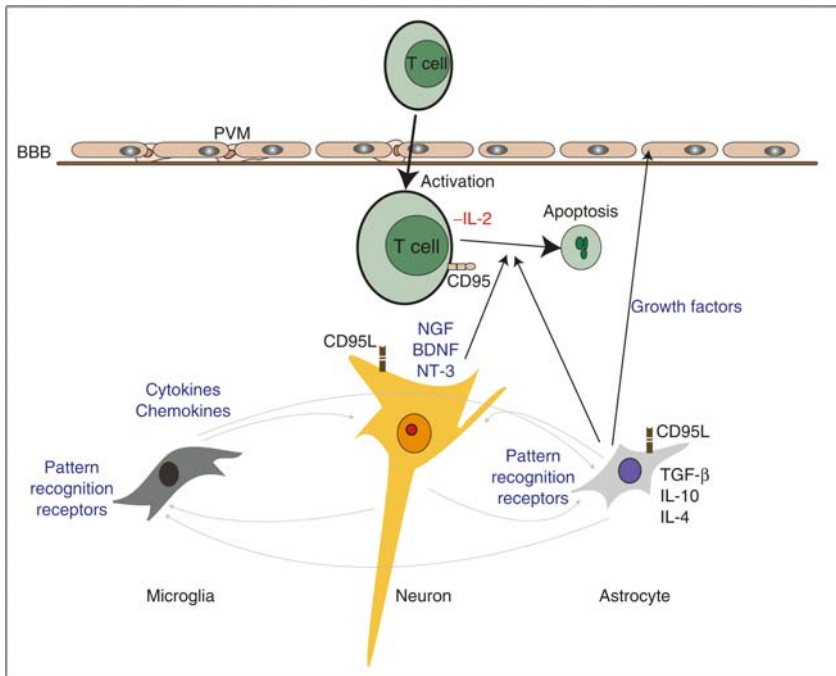
Regulation of antigen presentation within the CNS. After priming in the peripheral lymphoid organs, $CD4^+$ T cells cross the BBB and produce T_H1 - or T_H2 -type cytokines upon recognition of the target antigen on CNS APCs. To accomplish this, T cells migrate through the perivascular space of cerebral blood vessels and get restimulated by MHC class II⁺ perivascular macrophages. In the CNS parenchyma, T cells interact with activated microglia expressing MHC class II and adhesion/costimulatory molecules like B7.1 (CD 80). Microglia efficiently restimulate both T_H1 and T_H2 cells and release proinflammatory mediators (green) and regulatory mediators (red) (including chemokines, IL-10, IL-12, IL-18, TGF- β), which regulate T helper phenotype, recruitment, and activation. Conversely T_H1 cells produce TNF, LT, IFN- γ , and IL-2 (red), and activate stimulated microglial cells, whereas T_H2 cells produce IL-4, IL10, IL-5, and IL-13 (green). Astrocytes also produce immunoregulatory mediators (such as chemokines, colony-stimulating factors, and pro- as well as antiinflammatory cytokines) but show poor MHC class II and adhesion molecule expression (in vitro); their APC function may be limited to T_H2 restimulation in the presence of antigenic peptides. Astrocytes can antagonize IL-12 and IL-18 production by microglia (red). Abbreviations: APC, antigen-presenting cell; ICAM-1, intercellular adhesion molecule 1; VCAM, vascular cell adhesion molecule; IFN- γ , interferon- γ ; IL-1, interleukin 1; IL-2, interleukin 2; IL-4, interleukin 4; IL-10, interleukin 10; IL-5, interleukin 5; IL-13, interleukin 13; IL-12, interleukin 12; IL-18, interleukin 18; IL-10, interleukin 10; LFA-1, leukocyte function-associated antigen 1; LT, lymphotoxin; MHC, major histocompatibility complex; PVM, perivascular macrophages; TCR, T-cell receptor; TGF- β , transforming growth factor- β ; T_H1 , T helper cell 1; TNF, tumor necrosis factor



ner et al., 1998; Graf, 2002). In the resting state, they display a special phenotype that is generally considered less differentiated than other monocytes/macrophages, since they have a ramified morphology, an extremely slow turnover, no phagocytic activity, and functionally they are CD45^{low}, expressing low levels of MHC class II. This downregulated resting phenotype is thought to be the consequence of an immunosuppressive neuronal microenvironment, with release of immunosuppressive factors like TGF- β , IL-1, neurotrophins, etc., which inhibit MHC class II expression on microglia (Neumann and Wekerle, 1998). In addition, neuropeptides and other mediators such as vasoactive intestinal peptide (VIP), pituitary adenylyl cyclase-activating polypeptide, and adrenocorticotrophic hormone (ACTH) produced by neurons in vitro can ensure a quiescent unactivated state of microglial cells (Hetier et al., 1991; Kim et al., 2000). In this context, the CD200 (expressed on neurons) and CD200R (expressed on microglial cells within the CNS) family have given interesting insight into neuronal–microglial cross talk. OX2 (CD200)-deficient mice have shown spontaneously activated microglia with clinically more severe EAE (Hoek et al., 2000). As an early sign of activation in inflammatory diseases, they upregulate MHC class II and adhesion molecules such as LFA-1, ICAM-1, and VCAM, and they also express the costimulatory molecules CD40, B7.1, and B7.2 complementing classical MHC-restricted T-cell responses. Further, they acquire phagocytic properties and express receptors necessary for active endocytosis such as mannose receptors and Fc receptors (Aloisi et al., 2000a). After stimulation with macrophage colony-stimulating factor (M-CSF) in vitro, microglial cells turn into a macrophage morphology with ameboid phagocytic properties, while granulocyte/macrophage colony-stimulating factor (GM-CSF) induces a dendritic cell-like phenotype (Carson et al., 1998; Fischer and Reichmann, 2001; Mitrasinovic et al., 2001; Santambrogio et al., 2001). It has been elegantly shown in vitro, that after IFN- γ stimulation or LPS priming, microglia upregulate MHC class II, express costimulatory and adhesion molecules, process antigen, and re-stimulate T cells, but their relative capacity in priming T cells is lower than that of DC (Aloisi et al., 1998; Aloisi et al., 1999b; Aloisi et al., 2000a). Isolated CD45^{low} microglia from normal rodent CNS behave as poor APC (Carson et al., 1998). Virtually all kinds of inflammatory diseases of the CNS lead to microglial activation with strong upregulation of cell membrane markers such as MHC class II, adhesion molecules (ICAM, VCAM, and PCAM), F4/80, MAC-1 etc., in mice and humans (Hailer et al., 1997; Bechmann et al., 2001; Stenzel et al., 2004). More importantly, the type and combination of expressed costimulatory molecules seems specific for different maladies, since for example CD80 (B-7.1) is only expressed on strongly activated APC in the EAE model (Racke et al., 1995) and in MS (Kuchroo et al., 1995). Interestingly, MHC class II molecules, CD40, and B7 can be inhibited by cytokines such as TGF- β suggesting that their gene expression, which is regulated on the transcriptional level, can be inhibited by the same mechanism (O’Keefe et al., 2002).

■ Figure 10-4

The glioneuronal micromilieu regulates immunological mechanisms within the brain. *The upper panel illustrates the physiologic situation (i.e., without an inflammatory stimulus), which is characterized by secretion of immunosuppressive factors/mediators in order to maintain an immunologically downregulated state. Activated T cells may cross the blood–brain barrier (BBB), but regularly undergo apoptosis under normal conditions, because they do not recognize antigen. Astrocytes produce immunosuppressive TGF- β , IL-4, and IL-10. Neurons produce neurotrophins such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and neurotrophin-3 (NT-3), which have been shown to prevent expression of proinflammatory cytokines on astrocytes and microglial cells. Neurons and astrocytes express fas-ligand (CD95L). Both astrocytes and microglial cells constitutively express PRR. Perivascular macrophages are not activated and secrete low or no inflammatory mediators. The lower panel shows inflammation which activates neurons, astrocytes, and microglial cells as well as endothelial cells and perivascular macrophages to produce chemokines, cytokines, growth factors, and cytotoxic mediators as well as prostaglandins. A chemokine gradient is thought to attract inflammatory leukocytes (T cells), granulocytes (GRA), macrophages($\mu\phi$), and microglial cells to the lesion site. Specific antigen recognition stimulates T cells locally and leads to enhanced production of cytotoxic mediators and proinflammatory cytokines. Antigen presentation to invading T cells is assured by activated microglial cells, which have upregulated their MHC production*



A paradigm of effective immune responses in the CNS is the balanced regulation of the T_H1 and T_H2 response. Microglial cells have been shown to respond strongly to T_H1 cytokines (e.g., IFN- γ and TNF) with prompt activation, but they also downregulate their activity upon exposure to T_H2 cytokines. Polarization of T_H1 versus T_H2 cells, their homing capacity to infectious foci, and effector function is regulated by a distinct spectrum of chemokines, the acquisition of chemokine receptors as well as loss of lymph node receptors, and proliferative capacity (Sallusto et al., 2000a, b). Also, within the CNS during T-cell reactivation, the type of APC and the spectrum of secreted cytokines may affect T_H -cell phenotype generation (Aloisi, 2001). In this scenario, IL-12 seems to be a major player in skewing the response toward a T_H1 type, and microglia are the major source of IL-12 within the CNS in vitro and in MS and EAE (Aloisi et al., 1997; Stalder et al., 1997; Pagenstecher et al., 2000). On the other hand, T_H1 cells are the major producers of antiinflammatory mediators such as PGE₂, thereby also contributing to a downregulatory immune response that may represent an intrinsic negative feedback mechanism (Aloisi et al., 1999a). Microglia exposed to TGF- β and microglia-derived TGF- β may induce an antiinflammatory milieu (Ma and Streilein, 1999). It is likely that microglial cells are able to amplify as well as downregulate the immune response depending on the inflammatory cytokine milieu. Thus, TNF induces a positive feedback loop, whereas B7-H1 expression downregulates T-cell activation in vitro (Kuno et al., 2005; Magnus et al., 2005). There seems to be substantial microglia/T-cell interactions in both directions. Skewing the T-cell response toward the T_H2 phenotype, which is the presumed therapeutic mechanism of glatiramer acetate (GA), has been linked to favorable outcomes in MS (Farina et al., 2002). GA-reactive T_H2 -biased cells were found to modulate the cytokine profile of microglia producing high levels of IL-10 and low levels of IL-12 (Kim et al., 2004). Consistent with these findings, Becher and coworkers (2000a) have defined monocytic APC subtypes according to the IL-10/IL-12 secretion profile, defining the subsequent T_H1/T_H2 response, with APC1 producing low amounts of IL-10 and high amounts of IL-12, and APC2 vice versa. In this context, it could be shown in vitro that soluble molecules such as TNF, IL-6, or IP-10 released by T_H1 and not T_H2 cells that infiltrate the CNS can stimulate resident microglia (Seguin et al., 2003), arguing for an environmental impact exerted by the CNS on the T cell–APC cross talk. Further, Aktas and coworkers (2003) have elegantly shown that soluble products derived from T_H1 cell lines generated in response to self antigen (MBP) upregulate expression of MHC class II, B7.1, B7.2, CD40, and ICAM-1 on microglia, and that co-culture of unstimulated T cells with these macrophages and microglial cells results in a sustained production of more T_H1 -type soluble products.

Microglia produce and secrete a large panel of cytokines and chemokines particularly in order to regulate recruitment of leukocytes into the inflamed CNS (Hanisch, 2002; Lee et al., 2002). Under basal conditions, human microglia express mRNA transcripts for cytokines, chemokines, and their receptors such as IL-1 β , IL-6, IL-8, IL-10, IL-12, IL-15, MIP-1 α , MIP-1 β , MCP, IL-1RI, IL-1RII, IL-5R, IL-6R, IL-10R etc., but mRNA transcripts for some cytokine receptors such as IL-2R, IL-3R, IL-4R, and IL-7R were not found. Chemokine production by microglial cells, promoting leukocyte infiltration in consequence, represents an important early innate defense mechanism and plays a key role in the adaptive response as well (Babcock et al., 2003; Owens et al., 2005). The TLR ligands lipopolysaccharide (LPS) or peptidoglycan (PGN), incubation with the gram-positive bacterium *Staphylococcus aureus*, and also IFN- γ , TNF, and IL-1 β can stimulate the production of MCP-1, MIP-1 α , MIP-1 β , RANTES, and IP-10 by microglia in vitro (Kielian et al., 2002; Olson and Miller, 2004). Microglial proliferation and activation can be induced by proinflammatory cytokines such as IL-1 β , IL-4, and IFN- γ and more effectively, by colony-stimulating factors like M-CSF or GM-CSF in vitro (Lee et al., 1994). In the murine entorhinal dentate axotomy model, chemokines induce macrophage and T-cell infiltration with expression of MCP-1/CCL-2 as well as their receptor CCR2, which are of critical importance, while RANTES and CCL5 were found less important. MCP-1/CCL-2 is produced by microglia, macrophages, endothelial cells, neurons, and astrocytes, but microglia followed by astrocytes are the main producers. MCP-1, MIP-1 α , MIP-1 β , RANTES, and IP-10 were detected in the rat EAE model (Miyagishi et al., 1997). In the humans MCP-1, MIP-1 α , MIP-1 β , and RANTES produced by microglia/macrophages were found in MS plaques (Simpson et al., 1998). Interestingly, blocking antibodies to chemokines like RANTES and IP-10 reduces or inhibits development of virally induced EAE by inhibiting the infiltration of inflammatory cells into the CNS (Lane et al., 2000; Fife et al., 2001), but contrarily the use of RANTES- or IP-10 deficient mice has shown an increased susceptibility to EAE, which

has been attributed to compensatory upregulation of other chemokines from the network (Klein et al., 2004). Recently, it has been shown that microglia and astrocytes by producing IL-10 inhibit the *Neisseria meningitidis*- and *Borrelia burgdorferi*-induced immune responses, thereby contributing to an immunosuppressive milieu in chronic infections (Rasley et al., 2006).

Microglial cells express a large panel of TLR in normal human cultured CNS tissue, and in a variety of diseases including MS, and TLR expression was most abundant in active MS lesions in perivascular localization (Bsibsi et al., 2002; Jack et al., 2005a, b). In vitro, neonatal mouse microglia express TLR1–TLR9 (Bsibsi et al., 2002; Olson and Miller, 2004), and human CNS tissue has been shown to express TLR2, TLR3, and TLR4 in vivo, with a more widespread and higher activation as compared with astrocytes (Bsibsi et al., 2002). TLR ligation on APC is followed by immediate upregulation of costimulatory molecules, thus promoting their fully active status, an important connection between innate and adaptive immunity in general. Microglial cells are a major source of proinflammatory cytokines in the inflamed CNS. They produce a wide range of cytokines in response to IFN- γ , viral, parasitic, and bacterial stimuli (Zhao et al., 2001; Olson and Miller, 2004; Jung et al., 2005). Upon TLR linkage, e.g., by stimulation with LPS, human microglia was shown to express pro- and antiinflammatory cytokines (Becher and Antel, 1996) and chemokines (e.g., IP-10) (Jack et al., 2005a). The expanding array of “danger signals” such as heat-shock proteins, hyaluran, fibrinogen etc., which are able to stimulate TLR signaling, contribute to the induction of potentially competent APC. In CNS inflammation, the TLR-mediated activation of microglial cells leads to an inflammatory cascade, which largely depends on a functional microglia–T cell–macrophage cross talk.

Finally, microglia is probably involved in the termination of the inflammatory response, which is mainly mediated by apoptosis of CNS leukocytes. Excessive stimulation of microglia leads to an expression of Fas (CD95) and Fas-ligand (L) (CD95L), which may be involved in apoptotic pathways in EAE (Kohji et al., 1998; Takeuchi et al., 2006). Macrophages and microglia undergo apoptosis via activation of caspase-3 in experimental brain abscess (Stenzel et al., 2005a, b). Very interestingly, a constitutive upregulation of APC-related molecules on human microglia as compared with rodents was described, which raises the question as to whether this reflects differences in the environment, and it has been speculated that this basal expression may predispose humans to develop diseases such as MS (Jack et al., 2005a). High levels of TLR4 stimulation can mediate microglial apoptosis (Jung et al., 2005), another mechanism possibly involved in the termination of CNS inflammation (Jones et al., 1997).

NO production and consecutive free-radical chain reactions by inflammatory leukocytes, microglial cells, and macrophages can be useful and harmful at the same time, and therefore have to be regulated tightly. Primarily, NO and reactive oxygen species are produced to destroy pathogens, thereby protecting the vulnerable CNS tissue. Conversely, the overproduction of NO in pathological states, may be detrimental, causing the secretion of proinflammatory cytokines such as TNF and IL-12, as well as edema (Stenzel et al., 2005a). In MS lesions, iNOS production was found strongly upregulated (Hill et al., 2004), and macrophages as well as microglia are major producers of the enzyme (De Groot et al., 1997; Fabrizi et al., 2001). NO and toxic free radicals may cause strong damage especially to oligodendrocytes and neurons in autoimmune diseases of the CNS (e.g., MS and EAE), or Rasmussen's encephalitis and paraneoplastic disorders. Importantly, there is some debate as to the source of NO in humans. Unlike in the murine situation, human microglial cells may not represent the major producers of NO, whereas astrocytes are good producers of NO (Jana et al., 2005). Bacterial products such as LPS and bacterial DNA can induce iNOS in rat and mice astrocytes. However, human astrocytes do not upregulate iNOS in response to LPS. In addition to whole viruses themselves, isolated viral products, like viral nucleic acids or proteins, are strong inducers of iNOS in astrocytes. Cytokines, like IL-1 β and IFN- γ , alone can induce iNOS in rodent astrocytes. Other cytokines, like TNF- α , usually induce iNOS in conjunction with IL-1 β or IFN- γ . However, human astrocytes behave differently with respect to cytokine-mediated expression of iNOS. For example, IFN- γ , which is a potent inducer of iNOS in rat or mouse astrocytes, fails to induce it in human astrocytes.

In summary, microglial cells as APC of the CNS are potentially able to perform a variety of duties in the inflammatory response. They recognize the pathogen, initiate a primary immune response, by producing mediators of inflammation, regulate the T-cell response, and participate in tissue homeostasis and antiinflammatory regulatory responses. In addition, they can phagocytose debris. In the case of sustained inflammation, activated microglia may contribute to uncontrolled tissue damage through prolonged

production of proinflammatory mediators and enzymes such as iNOS. These properties are modulated by infiltrating immune cells, the mediators they produce, and particularly the neuronal milieu such as damaged oligodendrocytes and neurons. Microglial cells and macrophages develop special properties in different inflammatory contexts, involving important and fine-tuned mechanisms of cell-surface expression of activation markers, and an intense cross talk to resident and invading cells of the CNS. Whether microglial cells and their phenotypic action in special conditions are beneficial or, by contrast, destructive, seems to depend on regulatory signals that are not completely identified. Although these properties have become clearer during the past decades, important questions remain unanswered and are still a matter of debate and investigative efforts. Importantly, it has become clear that “microglial activation” does not follow stereotyped schemes, that activation is highly heterogeneous, even with opposing resultant activity, and that the micromilieu within the CNS plays an important role (Schwartz et al., 2006).

1.4 Macrophages—Specialized Monocytes in the CNS

Monocytes/macrophages are key players in nearly every inflammatory and autoimmune disease of the CNS, and also play important roles in ischemia and neurodegenerative diseases. They consist of a highly heterogeneous group of cells that can be distinguished by their morphology, the surface antigens they express, the turnover, and the anatomical site they reside at. Studies on rat bone marrow chimeras have revealed that these include (1) macrophages from the meninges, (2) macrophages from the Virchow–Robin space, (3) perivascular macrophages (a resident CNS monocyte, found around parenchymal microvessels), (4) macrophages of the choroid plexus, (5) microglia (resident macrophages of the CNS), and (6) multinucleated giant cells (MNGC) found in some forms of encephalitis or specific infections of the CNS (Hickey et al., 1992; Lassmann and Hickey, 1993; Lassmann et al., 1993).

After lethal irradiation and allogenic bone marrow scavenging, meningeal macrophages emerge relatively quickly from the bone marrow. In 2–3 months, over half of these cells are “donor type,” and represent cells with the quickest turnover. These meningeal macrophages express low levels of MHC class I and II (and are CD4⁺ in rats and humans). They also carry adhesion molecules such as ICAM-1. They become activated and increase in number during meningitis caused by bacteria and fungi, but the regulating factors to which they respond remain unknown. Next, macrophages that reside in the Virchow–Robin space come from the bone marrow more slowly, with 50% being replaced after 2–3 months after irradiation. Morphologically indistinguishable from meningeal macrophages, they however neither express MHC class II nor CD4 in the healthy state. Further, perivascular cells are even slower than the former and only 30% arrive from the bone marrow in 2–3 months. Functionally interesting, they have long branching processes, and establish a close and intense contact to cerebral blood vessels. Immunologically, they express MHC class I and II, and CD4 (in rats and humans) and a variety of adhesion molecules upon stimulation. Systemic LPS injection leads to a production of enzymes involved in eicosanoid metabolism by perivascular macrophages, serving as a sentinel alert. Importantly, perivascular cells may serve as Trojan horses, since they are readily able to take up and transport soluble material into the CNS parenchyma. Their antigen-presenting capacity is further reflected by the ability to sense the CSF for “stranger signals,” take them up, process them, and present them to T cells. Choroid plexus macrophages have a similar turnover and are also competent APC (Nathanson and Chun, 1989). Finally, microglia have the slowest turnover. As mentioned previously, microglial cells are immunologically quiescent in the healthy state with no detectable MHC molecules, no CD4 expression (in case of human microglial cells), and very low adhesion molecule expression. However, upon activation by an infectious pathogen, they quickly upregulate the above mentioned molecules. They can be distinguished from the perivascular cells and other macrophages by their expression level of CD45. Microglial cells are CD45 low as compared with other macrophages and have a poorer antigen-presenting capacity than conventional macrophages (Sedgwick et al., 1991b). Microglial phenotypes may be diverse, and it has been postulated that the response is mainly dependent on the preconditions of a given destructive stimulus, and they can change their phenotype accordingly (Schwartz et al., 2006). However, the majority of macrophages in the injured CNS (ischemic infarcts or necrosis in infections or demyelinating lesions) are not derived from microglia but

enter the brain from the periphery (Lassmann et al., 1993). Further, once having transformed into a macrophage, they do not become microglia again later in the disease healing process, and perivascular macrophages transform neither into microglia nor into parenchymal macrophages. Regulators such as IL-4, insulin-like growth-factor, IFN- γ , and TNF of microglial function (i.e., their commitment to a specialized sensor of defined damage) are only beginning to be identified (Butovsky et al., 2006).

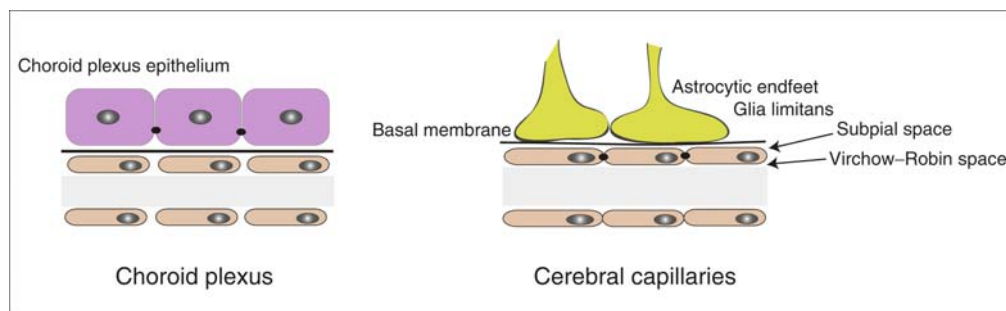
Taken together, while all of these monocytes originate from the bone marrow, it is still unclear how these cells differentiate within the CNS. The immunosuppressive influence of the neural environment, which is in different reach anatomically for these cells, probably acts as an immunological primer, governing the desired and the required response. All of these cells have been much less intensely studied than microglia or astrocytes.

1.5 Astrocytes and the Immune Response

Astrocytes are the main population of the macroglial cell family in the CNS, which also comprise oligodendrocytes and ependymal cells, and they fulfill a multitude of physiological and pathological duties. They play a role in the maintenance of normal neuronal functioning, and in the physiological composition of the BBB by apposition of their endfeet onto the abluminal site of capillary endothelial cells (Figure 10-5). Astrocytes contribute to homeostasis of the brain regulating energy household, composition of the extracellular fluid, and uptake of substances through the BBB (Kettenmann and Ransom, 2004). However, they are not mere supporters of the complicated network of neuronal functioning. Also, they are not a stable, stationary population, but undergo proliferation and activation both in vitro and in vivo. Activated astrocytes strongly upregulate glial fibrillary acidic protein (GFAP), an important intermediate filament protein of the cytoplasm, contributing to stability and motility of the cells. GFAP is the sole marker of astrocyte activation in vivo to date. However, GFAP expression is not yet correlated with specific gene transcription, and thus multiple activation states are assumed (Eddleston and Mucke, 1993; Falsig et al., 2004a, b). In response to various harmful conditions such as inflammation, stroke, intracerebral hemorrhage, brain tumors, and neurodegenerative diseases, activated GFAP-positive astrocytes serve a bordering function (Stenzel et al., 2004) and support neuronal growth, by release of neurotrophic factors, and play their part in forming pseudocystic cavities or glial scars after resorption of necrotic CNS tissue (e.g., after

■ Figure 10-5

(Left) Characteristics of the blood–brain barrier (BBB) at the blood–CSF level and (right) the BBB at the parenchymal level. Complex tight junctions are formed between epithelial cells of cerebral capillaries and epithelial cells of the plexus, whereas the capillaries of the choroid plexus are fenestrated and do not form tight junctions. The endfeet of astrocytes of the glia limitans are covered by a basal membrane building the superficial border of the brain parenchyma, separating it from the subpial space (lamina limitans gliae superficialis) and the perivascular space (Virchow–Robin space; lamina limitans gliae perivascularis). Any cell entering the CNS from the blood must cross the glia limitans



ischemic stroke through an unspecific mechanism called astrogliosis) (Pekny and Nilsson, 2005). Upon activation (i.e., during infections), which is characterized by upregulation of GFAP, astrocytes can exert a variety of other immunological functions as detailed below. GFAP-deficient mice have been shown to develop more severe EAE with less well-defined lesions and decreased astrogliosis (Liedtke et al., 1998). Moreover, it has been demonstrated in a model of acute bacterial infection (e.g., *S. aureus*-induced brain abscess) and in a model of chronic parasitic infection (e.g., *Toxoplasma* encephalitis) that GFAP^{-/-} mice develop less well-defined lesions, a higher pathogen load, and higher leukocyte numbers with increased production of proinflammatory cytokines (Stenzel et al., 2004).

Astrocytes express high levels of TLR3 and low levels of TLR1, TLR4, TLR5, and TLR9, leading to proinflammatory responses characterized by production of TNF, IL-6, IL-12, IFN- β , IL-10, and CXCL-10 (Bsibsi et al., 2002; Jack et al., 2005b). Additionally, astrocytes produce high levels of iNOS upon TLR stimulation in vitro (Esen et al., 2004), and iNOS is mainly produced by astrocytes in CNS infections such as Theiler's murine hepatitis virus (MHV) or human immunodeficiency virus (HIV) (Oleszak et al., 1997; Zhao et al., 2001), as well as in EAE and MS (Tran et al., 1997; Liu et al., 2001).

Astrocytes can play a role in immunological responses by producing mediators and factors normally found in microglial/macrophage activation (Aloisi, 2001; Aloisi, 2003; Ambrosini and Aloisi, 2004; Ambrosini et al., 2005). They may function as APC, both expressing MHC class II and other costimulatory molecules (Fontana et al., 1984; Neumann et al., 2002a). Upon IFN- γ stimulation, MHC class II molecules were upregulated in vitro, and intrathecal injection of IFN- γ induced an increase in the number of class I and class II MHC-expressing astrocytes within the rat nervous system in vivo, leading to a progressive appearance of these cells. Also, after intrathecal stimulation with IFN- γ , astrocytes express MHC antigens inconsistently in a low density and patchy distribution in comparison to microglial cells or macrophages (Vass and Lassmann, 1990; Lassmann et al., 1991). Further, TNF enhances IFN- γ -induced MHC class II expression on astrocytes but has no effect by itself. To date it is believed that only very strong inflammatory stimuli are sufficient for MHC class II expression and competent antigen presentation by astrocytes in vivo. Astrocytes can process and present antigen to T cells in vitro (Fontana et al., 1984), however, as mentioned, it is still a matter of debate whether astrocytes have a strong APC potential in vivo, since it has been shown that they do not regulate the costimulatory molecules CD80 (B7.1) and CD86 (B7.2), which may preclude priming of naive T cells (Aloisi, 2001; Falsig et al., 2006). Conflicting results on B7.1 and B7.2 expression in vitro and in vivo have been reported. While B7.2 expression was documented on microglia, macrophages, and astrocytes in the EAE model (Issazadeh et al., 1998), others did not find any B7.1 or B7.2 expression on astrocytes (Cross and Ku, 2000). Further in MS, B7.1 and B7.2 expression was found on microglia and not on astrocytes by some authors (Williams et al., 1994; Windhagen et al., 1995; Zeinstra et al., 2003), while others demonstrated the expression of these molecules on astrocytes (Zeinstra et al., 2003). The lack of costimulatory molecules could play a role in maintaining the immune privilege of the CNS, and it has been speculated that astrocytes downregulate T-cell responses in the CNS. In this scenario, during EAE and MS, antiinflammatory mediators such as TGF- β or IL-10 are produced by astrocytes (Aloisi et al., 2000a, b). Data on CD40 expression on astrocytes are also conflicting. While some authors actually did find astrocytic CD40 expression in vitro, especially after cytokine stimulation (Tan et al., 1998), others were unable to detect any CD40 (Aloisi et al., 1998; Nguyen and Benveniste, 2000). In vivo, astrocytic CD40 expression was not observed (Togo et al., 2000). On the other hand CD40-ligand (-L), a key inducer of costimulatory molecules was found to be upregulated on astrocytes both in vitro and in vivo, and the endoplasmic reticulum chaperone gp86 that binds MHC class I peptide, and an inducer of CD80 (B7.1), CD86 (B7.2), and IL-12, was found to be upregulated on activated astrocytes. The chaperone gp86 can be taken up by cross-presentation of the antigen via the scavenger receptor A (CD91) after cell damage. Additionally it has been shown that astrocytes produce chemokines involved in macrophage and T-cell recruitment such as monocyte chemoattractant protein-1 (MCP-1) and IFN- γ -induced protein-10 (IP-10). This could lead to the hypothesis that astrocytes, besides presenting antigen to CD4⁺ T cells, may also activate microglia to upregulate costimulatory molecules, promote monocyte invasion into the CNS (Fontana et al., 1984; Owens and Babcock, 2002; Babcock et al., 2003), and induce a leakage of the BBB (Proescholdt et al., 1999). As mentioned before, studies conducted by Tan and coworkers (1998) clearly show that astrocytes have the ability to express the costimulatory molecules B7.1 and B7.2 and the MHC class II molecule

in vitro, indicating that they may efficiently process and present self antigen and activate naive and memory T cells. On the other hand, transgenic targeting of MHC class II expression on astrocytes did not support the issue of target recognition by activated T cells in vivo (Stuve et al., 2002). If astrocytes were not fully competent APC, they may promote T-cell apoptosis or T-cell anergy (Gold et al., 1996) (● [Figure 10-4a](#)). Other studies suggest that astrocytes can only activate T cells after priming by microglial cells (Sedgwick et al., 1991a), and bias the putative T-cell response toward a T_H2 type (Aloisi et al., 1999b; Becher et al., 2000b) (● [Figure 10-3](#) and [Figure 10-4b](#)).

Astrocytes have consistently been shown to produce a wide and important range of cytokines and chemokines in vitro and in vivo, as well as in inflammatory diseases, and therefore it is unquestionable, despite the ongoing discussion about their APC capacity, that they play a major role in CNS immune and inflammatory diseases. They were however shown to inhibit IL-12 secretion by in vitro-activated microglia, a mechanism that is thought to contribute to limiting destruction in CNS inflammation (Aloisi et al., 1997).

1.6 T Lymphocytes

The presence of T cells in the CNS as well as the process of entry into the CNS is a hallmark of many neuroinflammatory conditions. Their primary goal is to patrol the body in search of foreign or altered self-antigens. In autoimmune diseases (e.g., MS), paraneoplastic diseases, Rasmussen's encephalitis, and other chronic inflammatory conditions, T cells are thought to initiate the immune response with a whole cascade of consecutive reactions. A key process in many neuroinflammatory diseases is the entry of T cells into the CNS, and the mechanisms involved may constitute targets for therapeutic options.

While the basic principles of T-cell immunology in general have been outlined above, we now focus on the T-cell traffic into the CNS. Naive/resting T cells are excluded from penetrating the normal noninfected brain parenchyma, only activated T cells of both the $CD4^+$ and the $CD8^+$ phenotype are able to enter the CNS in an antigen- and MHC-independent manner (Hickey, 1991; Hickey et al., 1991). Interestingly, the antigen-specific part of the T-cell journey starts after the mere entry, since persistence of T cells within the CNS tissue is dependent on the recognition of the antigen coupled to the MHC complex. T cells disappear quickly after an equally rapid and effective entry into the CNS, if they do not encounter their antigen coupled to the appropriate MHC complex. The antigen recognition phase is followed by the recruitment of large numbers of leukocytes such as macrophages, more T cells, and NK cells. This part of the inflammatory process is clearly dependent on the properties and changes endothelial cells are undergoing, because attraction of these leukocytes requires expression of a large and specific panel of adhesion molecules (e.g., VLA-4, ICAM-3, LFA-1, and others) and chemokines (e.g., CCL1, CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL19, CCL20, CXCL1, CXCL8, CXCL10, CXCL12, etc.) and the corresponding chemokine-receptors (Engelhardt et al., 1998; Ambrosini and Aloisi, 2004).

Conversely, termination of the inflammatory process triggered by encephalitogenic T cells must be contained, because it has become increasingly clear that much of the damage done to the CNS comes from secondary events such as edema and vascular damage and not from the invading leukocytes themselves (Stenzel et al., 2005a). An important feature of limiting the inflammatory process, be it with or without treatment, is the accurate and timely disappearance of leukocytes from the CNS, where the prolonged production and leverage of proinflammatory cytokines and other mediators such as NO may be harmful to the vulnerable tissue (Stenzel et al., 2005b). Leukocytes to be terminated undergo apoptosis and are phagocytosed by microglia and macrophages. This holds for T cells in EAE (Pender, 1999) as well as for granulocytes and macrophages in experimental brain abscess (Stenzel et al., 2005a, b). Interestingly, the phagocytic process influences the immune response, by downregulating the production of proinflammatory cytokines (Magnus et al., 2001). During the last decade, T-cell apoptosis has become an important field of investigation in various inflammatory situations of the CNS. In the EAE model, myelin antigen-specific T cells appear to be targeted to apoptotic death (Tabi et al., 1995), and this seems to be due to their inability to produce IL-2 (Ford et al., 1996), thereby providing an explanation why the antigen-specific inflammatory T cells die and T cells that do not recognize antigen survive. Although neurons,

astrocytes, microglia, and T cells express Fas or FasL under inflammatory conditions, only neurons and astrocytes constitutively express FasL and are able to induce apoptosis in Fas-positive T cells, another mechanism contributing to immunosuppression in the CNS (Bechmann et al., 1999; Flugel et al., 2000) (► [Figure 10-4a](#)).

CD4⁺ T cells (i.e., T_H cells) are believed to play a central role in the initiation and perpetuation of human multiple sclerosis and its murine model, EAE. Previously IFN- γ producing T_H cells (i.e., T_H1 cells) induced by IL-12 were considered to be essential in the pathogenesis of EAE. With the discovery of another heterodimeric IL-12 family member, IL-23, the role of the IL-12/IFN- γ axis in development of EAE was reconsidered. IL-23 shares the p40 and the IL-12R β 1 subunit with IL-12 (Brombacher et al., 2003). Previous studies using anti-p40 antibody treatment or p40- or β 1-gene-deficient mice could not distinguish effects mediated by IL-12 from those by IL-23. Using IL-23p19^{-/-} mice, which lack only IL-23 but not IL-12, revealed that IL-23 is the critical cytokine for autoimmune inflammation of the brain (Cua et al., 2003). In a model of chronic cerebral cryptococcosis, it has been demonstrated that the absence of IL-23 is associated with an impaired inflammatory response, which was attributed to a reduced recruitment and activation of mononuclear cells (Kleinschek et al., 2006). Initially, direct actions of IL-23 on inflammatory macrophages were characterized to be the underlying disease-promoting mechanism. This initial view was substantially broadened by the observation that IL-23 is able to induce the proinflammatory cytokine IL-17 in a subset of activated CD4⁺ T cells (Aggarwal et al., 2003). Thus, a so-called IL-23/IL-17 axis was established in addition to the previously described IL-12/IFN- axis. To verify the functional significance of the IL-23/IL-17 pathway in EAE, passive transfer studies revealed that IL-23-dependent CD4⁺ T cells, producing IL-17A, IL-17F, IL-6, and TNF, are essential for induction of EAE (Langrish et al., 2005). In addition, even therapeutic treatment during active EAE with either anti-IL-23p19 or anti-IL-17A antibodies prevented disease relapse (Chen et al., 2006). IL-17-producing T_H cells were very recently found to constitute a novel unique T_H subset termed T_H17 cells (Harrington et al., 2005; Park et al., 2005). A series of papers published within the past several months has demonstrated that IL-6 and TGF- β are the crucial external triggers for the differentiation of naive T_H cells into these so-called T_H17 cells. Moreover, IL-23 is required for the expansion and survival of T_H17 cells (Mangan et al., 2006; Veldhoen et al., 2006). The very recent description of the central role of T_H17 cells in EAE has already enabled preclinical experimentation for therapeutic intervention during EAE (Chen et al., 2006). Presently, a distinct T_H17 lineage has not yet been characterized in humans although IL-17-producing T-cell clones have been isolated from rheumatoid arthritis patients (Aarvak et al., 1999). On the basis of the novel concept of the pathogenic role of T_H17 cells in EAE, it is clear that results from studies looking at T_H17 cells in humans with MS should appear soon. Potentially, this will point to novel therapeutic strategies in the clinic.

1.7 B Lymphocytes

B lymphocytes as major constituents of the so-called “humoral response” produce soluble antibodies called immunoglobulins, which bind antigen leading to its elimination by involvement of either the complement cascade or the activation of phagocytes. In general, B cells present antigen, aiming to activate T_H cells, which in turn stimulate the specific antibody response of the B cell itself. They express MHC class I and class II antigens on their surface. By virtue of their specific membrane-bound antigen receptor, they bind proteinaceous antigens, which are engulfed and processed subsequently. Their profile of chemokine and adhesion molecule expression on the cell surface, however, differs from other APC, which leads to a preferential T_H2 response of antigen-recognizing CD4⁺ T cells with secretion of IL-4, IL-5, and IL-10 (Abbas et al., 1996). B cells are much less intensely investigated in inflammatory diseases and models for CNS infections than T cells, the other antigen-specific population of the adaptive immune system. Little is known about the molecules that may be important in regulating the entry of B cells into the inflamed CNS tissue. In an elegant model using single microinjections of antigen into the CNS of rats, Cserr and colleagues have shown that leakage of this antigen from the CNS will activate the specific memory B cells (Cserr et al., 1992; Cserr and Knopf, 1992). This will lead to active traffic into the CNS where the reactivated B cells can differentiate

into plasma cells. B cells—similarly as T cells—are able to enter the CNS, seek their antigen, and develop their specific effector functions when they encounter their antigen (Knopf et al., 1998). Not only in acute pathogen-triggered infectious diseases of the CNS (Pfister et al., 1994) but also in multiple sclerosis, B cells seem to play an important role in the immunopathogenesis. Several lines of evidence argue for a participation of B cells in the immune response. Intrathecal synthesis of IgG and evidence of oligoclonal IgG bands in the CSF have been known for more than six decades to occur regularly in MS patients (Kabat et al., 1942). Lassmann and coworkers have established four different neuropathological lesion types, with type II involving also antibody-mediated mechanisms (Lucchinetti et al., 2000). Ongoing studies are aiming to identify the target of the B-cell response (Colombo et al., 2003; Owens et al., 2003; Cepok et al., 2005b), and to precisely characterize the pattern of the B-cell response (Baranzini et al., 1999; Cepok et al., 2005a). Recently, follicular B-cell structures within the meninges have been identified in the EAE model, suggesting generation of germinal centers and local B-cell production within the CNS (Magliozzi et al., 2004). The role of B cells is only beginning to be investigated in different immunopathologies, the precise interaction of the specific B-cell-dependent humoral immunity with the CNS innate immune system (e.g., microglial and astrocyte activity) or with the T-cell response and their regulatory mechanisms have not yet been identified.

1.8 The Blood–Brain Barrier

Homeostasis of the internal environment within the CNS is tightly regulated to maintain a stable milieu of nutritional substances, neurotransmitters, ions, hormones, and other mediators, which is the basis for a normal functioning of the neuronal network. There are two mechanical barriers as parts of the innate immune system, consisting of (1) the blood–brain barrier (BBB), separating the blood within cerebral capillaries from the extracellular neuronal and glial CNS tissue, and (2) the blood–cerebrospinal fluid barrier, separating the blood within the plexus choroideus from the CSF in the ventricles. Both barriers are important structures; only the CNS parenchyma is a compartment, which is in consequence an immune-privileged compartment, whereas the subarachnoid space containing the CSF is not (► [Figure 10-5](#)). Therefore, the CSF is more readily accessible by pathogens and inflammatory cells/leukocytes, and consequently may contain higher numbers of cytokines, chemokines, antibodies, and other proinflammatory mediators than the parenchyma, *sensu stricto*. This is the case especially under pathological conditions i.e., the bacteremia/sepsis/meningitis-sequence. The BBB consists of astrocytes of the glia limitans, whose endfeet sheeted by a basal membrane build up the outer border of the CNS parenchyma, separating it from the subpial space and the Virchow–Robin perivascular space. Therefore, the “glia limitans” is the critical tissue barrier for all cells and molecules that have a tropism for the CNS from the blood. Tight junctions between the CVE further contribute to the innate barrier of the cerebral epithelium, while the choroid plexus epithelium is widely fenestrated (► [Figure 10-5](#) and ► [Table 10-1](#)).

Disruption of the BBB is the hallmark of many inflammatory diseases and facilitates the entry of inflammatory leukocytes. In the case of a hematogenous route of infection, the pathogen that gains access to the CNS must disrupt the complex and unique structures of the BBB. Two main types of passages are described: one is the transcellular–vesicular or receptor-mediated pathway, the other is the paracellular migration mode. The invasion of a host is a multistep process that starts at the site of infection with adhesion of the pathogen to the epithelium, colonization of a mucous membrane (e.g., the nasopharyngeal epithelium), and penetration of the endothelium into the blood stream with establishment of bacteremia (viremia or parasitemia). Within the vascular system, pathogens have to overcome innate and adaptive immune mechanisms depending on their nature. The final step is the invasion into the CNS or the transcytosis through the BBB. Some pathogens such as viruses and *Haemophilus influenzae* have the possibility to directly infect and destroy the cerebrovascular endothelium (Soilu-Hanninen et al., 1994; Wiley et al., 1986). *Cryptococcus neoformans* cells invade the CNS by transcellular crossing of the endothelium of the BBB without affecting the monolayer integrity by triggering the formation of microvillus-like membrane protrusions (Chang et al., 2004). In the case of bacterial

■ Table 10-1

Characteristics of the cerebral vascular endothelium as compared with other vascular beds of most peripheral parenchymal organs

Feature	BBB	Other vascular beds
Tight junctions	Abundant	Rare
Fenestration and intercellular gaps	Rare	Abundant
Perivascular space	Small	Large
Pinocytosis	Rare	Abundant
Glucose transporters	Present	Absent
Electrical resistance	High	Low
Mitochondrial content	High	Low
Paracellular diffusion of hydrophilic molecules	Restricted	High
Transcellular flux	Low	High
Adhesion molecules	Few	Many

meningitis, *H. influenzae*, *Streptococcus pneumoniae*, *Escherichia coli*, and *Listeria monocytogenes* are all able to invade the epithelium by a transcellular or a paracellular way, and gain entry to the CSF either at the site of the choroid plexus or at cerebral microvessels. Some pathogens such as the obligate intracellular bacterium *L. monocytogenes* may further use leukocytes as carriers. A curious entry into the brain parenchyma is used by malarial parasites, which alter the erythrocyte surface to produce “knobs” that are highly adhesive for endothelial cells and are recognized by ICAM, VCAM, and E-selectin. The lining up within the cerebral capillaries causes microvascular obstruction, and consecutive cerebral infarction, resulting in an entry of the parasite without a formal invasion through a barrier (Miller et al., 1994). Pathogens may also use the natural polarized carrier function of macromolecules from the vascular compartment (luminal) to the CNS tissue (abluminal), or receptor-mediated endocytosis such as the platelet-activating factor (PAF) receptor (Cundell et al., 1995). Other routes of bacterial entry into the CNS include mechanical spread from a contiguous source of infection such as sinusitis and mastoiditis. *S. pneumoniae* has been shown to enter the CNS through a nonhematogenous route in experimental animals after intranasal infection and otitis media (Marra and Brigham, 2001). For a comprehensive review on the topic of microbial invasion through the BBB, the reader is referred to a very recent review (Kim, 2006).

Astrocytes play a central role in the building up and maintenance of the BBB (🔗 [Figure 10-5](#)). It has been shown in vitro that co-culture of astrocytes with CVE leads to an increase of tight junctions, decreased permeability, and increased electrical resistance, while absence of astrocytes leads to loss of these special features (Hayashi et al., 1997). Moreover, astrocytes can induce BBB properties on peripheral vascular beds (Janzer and Raff, 1987), and several growth factors such as TGF- β , basic fibroblast growth factor (bFGF), and glial-derived neurotrophic factor (GDNF) mediating these BBB functions have been isolated (Abbott, 2002). Recently, astrocyte-derived Src-suppressed protein kinase C substrate (SSeCKS) was found to regulate angiogenesis and tight junction formation in the BBB by decreasing VEGF and increasing Ang-1 secretion (Lee et al., 2003). The latter molecule signals through Tie-2 receptors on cerebral endothelial cells, a process that is intimately involved in tight junction formation (Rieckmann and Engelhardt, 2003). Proinflammatory cytokines may alter and disrupt the BBB and facilitate microbial entrance into the adjacent tissue. In this scenario also chemokines as key molecules have been found to play an important role in regulating leukocyte traffic to the infected CNS. Endothelial cells, microglia, and astrocytes within the CNS all produce chemokines and chemokine receptors under inflammatory conditions (Andjelkovic et al., 1999a, b; Hesselgesser and Horuk, 1999). They play important roles in MS, EAE, and experimental brain abscess as well as in pneumococcal meningitis and neuroborreliosis (Gerard and Rollins, 2001; Kastenbauer et al., 2005; Rupprecht et al., 2005; Stenzel et al., 2005a; Klein et al., 2006). Chemokines are small secreted proteins that have been classified into four main subgroups based on the relative position of

the first two cystein residues. Chemokines act on distinct leukocyte populations, and each subpopulation has its partly redundant chemokine receptors (Murphy, 2002; Ambrosini and Aloisi, 2004; Owens et al., 2005). Chemokines are transported through endothelial cells from the abluminal to the vascular/luminal site and presented to leukocytes, thereby stimulating their entry into the CNS (Middleton et al., 2002). These tissue-infiltrating leukocytes have been shown to establish a chemokine milieu and a gradient, which actively regulates leukocyte movement within the brain (Sedgwick et al., 2000). Moreover, astrocytes have the possibility to produce significant amounts of pro- and antiinflammatory cytokines and chemokines involved in leukocyte recruitment, a mechanism that actively contributes to the establishment of an immunological micromilieu that distinguishes the intravascular and the parenchymal compartment. As mentioned above, monocytes that have crossed the glia limitans may adopt microglial cell-like morphology and function under the influence of immunoregulatory TGF- β secreted by astrocytes within the CNS (Bechmann et al., 1999).

Leukocyte movement across the BBB during inflammation of the CNS is a complex process, described in detail elsewhere (Engelhardt and Ransohoff, 2005). It is dependent on a close interaction between both the leukocyte and the endothelial cell. The process of diapedesis does not destroy or breach the endothelial layer, and paracellular as well as transcellular migration is possible (Engelhardt and Wolburg, 2004). Involvement of the cell junction appears to be regulated by transendothelial migration itself through a number of molecules such as PECAM-1, the JAM family, or CD99. Classically, in the inflamed brain, leukocyte migration is considered a complex multistep process involving (1) tethering and rolling, (2) activation, (3) adhesion strengthening, and (4) final transmigration of the activated T cell. Alternatively, in the noninflamed spinal cord white matter, Engelhardt and coworkers have provided *in vivo* evidence that encephalitogenic T cells interact with the cerebral microvasculature without rolling and that $\alpha 4$ -integrin mediates the G protein-independent capture, and subsequently the G protein-dependent adhesion strengthening of T cells to microvascular VCAM-1. LFA-1 was found to mediate neither the G protein-independent capture nor the G protein-dependent initial adhesion strengthening of encephalitogenic T cells but was rather involved in T-cell extravasation across the vascular wall into the parenchyma. This process is thought to be involved in immunosurveillance of the CNS by T cells, postulating different trafficking mechanisms during immunosurveillance and disease (Engelhardt and Wolburg, 2004; Engelhardt and Ransohoff, 2005).

2 Concluding Remarks on the Regulation of the Inflammatory Response

The constituents of the proinflammatory immune response in inflammatory CNS injury are a constantly growing family with a broad and intense spectrum of interaction. First, the specialized immune status of the CNS has long been recognized and relies mainly on the BBB physiology and the expression of multiple TLR on resident cells of the CNS such as neurons, astrocytes, and microglia. The pathways that are taken after invasion of a pathogen, or, in the case of autoimmune-mediated diseases, may vary enormously, which in general is constrained by the nature and the context of the stimulus. This is, among many factors, dependent on the type of pathogen, the route of infection, the site where the pathogen enters the CNS, the immune status of the human organism at the beginning of the disease, genetic factors affecting the quality and intensity of the immune response (e.g., complement system), as well as factors influencing the persistence or “time-to-eradication” within the CNS. Consequently, the intracellular signal transduction pathways activated and the genetic regulation of downstream mechanisms are the subcellular and molecular targets of the inflammatory response. A further important issue is the possibility of different compounds of the immune response to communicate and cooperate with one another, which may in turn be impaired or enhanced under pathological conditions. As a general example of the concept of balanced defense against infection and damage elicited by the inflammatory response, an efficient T_H1 response produces proinflammatory cytokines and NO in order to combat the pathogen, mediators that in turn may be cytotoxic and harmful for the vulnerable CNS tissue.

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11 Evolutionary Origins of the Brain's Immune Privilege. Implications for Novel Therapeutic Approaches: Gene Therapy

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1	<i>Introduction</i>	264
2	<i>The Treatment of Neurological Diseases Using Gene Therapy</i>	265
3	<i>Gene Therapy for Pain</i>	266
4	<i>Adenovirus as a Gene Transfer Vector</i>	267
5	<i>Immune Responses to Adenoviral Vectors</i>	268
5.1	Innate Immune Response	269
5.2	Adaptive Immune Response	271
5.2.1	Cell-Mediated Immune Response	271
5.2.2	Humoral Immune Response	274
6	<i>Future Directions</i>	275

Abstract: Researchers have conducted numerous pre-clinical and clinical gene transfer studies using recombinant viral vectors modified from a wide range of pathogenic viruses such as adenovirus, adeno-associated virus, herpes simplex 1 virus, and lentivirus. Herein, we examine the utility of each vector system to treat disorders of the nervous system as well as offer a summary of various strategies and clinical outcomes of gene therapy approaches to treat neurological disorders such as Parkinson's disease, Alzheimer's disease, and glioma. As viral vectors are derived from pathogenic viruses, they have an inherent ability to induce a vector specific immune response. The role of the immune response against the viral vector gene delivery vehicle has been implicated in the inconsistent and unpredictable translation of pre-clinical success into therapeutic efficacy in human clinical trials using gene therapy to treat neurological disorders. The effects of the innate and adaptive immune responses on therapeutic gene expression mediated by viral vectors are discussed. Furthermore, the immune responses against gene therapy vectors and the resulting loss of therapeutic gene expression are examined in the context of the architecture and neuroanatomy of the brain immune system.

List of Abbreviations: AAV, adeno-associated virus; APCs, antigen-presenting cells; BBB, blood–brain barrier; BDNF, brain-derived neurotrophic factor; CAR, coxsackievirus–adenovirus receptor; CNTF, ciliary neurotrophic factor; CTLs, cytotoxic T cells; DC, dendritic cells; DIC, disseminated intravascular coagulation; GBM, glioblastoma multiforme; GDNF, glial-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; HD, Huntington disease; HPRT, hypoxanthine–guanine phosphoribosyltransferase; HSV, herpes simplex virus; IRFs, interferon regulatory factors; ITR, inverted terminal repeats; LNS, Lesch–Nyhan syndrome; MAPK, mitogen-activated protein kinase; MS, multiple sclerosis; NGF, neural growth factor; NK, natural killer; NKT, natural killer T; PI-3K, phosphoinositide-3-OH kinase

1 Introduction

Gene therapy has the potential to become the next generation of biopharmaceuticals. By utilizing therapeutic genes and cDNAs delivered directly to infected organs by employing novel gene transfer strategies, gene therapy offers hope to a wide variety of major unmet medical needs from diseases ranging from macular degeneration (Saishin et al., 2005) to brain cancer (Ali et al., 2005). Over the course of the last decade, astounding advances have been made in the techniques and reagents permitting the transfer of therapeutic genes into cells. Numerous preclinical and clinical gene transfer studies have been carried out using viral vectors modified from pathogenic viruses or artificial nonviral liposome-based approaches. Nonviral methods have made significant advances in the last few years; however, its efficiency remains below that achieved by viral vectors. Viral-derived gene transfer vectors are currently considered the gold standard as tools for gene transfer. Unfortunately, viral capsid proteins, as well as low level production of other viral proteins in situ, can elicit an immune response that curtails or eliminates expression of the therapeutic gene (Thomas et al., 2001). In the most extreme cases, the human body's immune system has been shown to elicit serious adverse events in human clinical trials that have resulted in the death of one patient (Raper et al., 2003). Understanding, modulating, and overcoming the complex relationship between gene therapy viral vectors and the human body's immune system are of critical importance if gene therapy is to succeed in human clinical trials.

Retrovirus, lentivirus, adenovirus, adeno-associated virus (AAV), and herpes simplex virus (HSV)-derived vectors are modified from pathogenic viruses, both of human origin as well as other species (Kay et al., 2001). Each vector system has its own inherent tropism and expression levels for particular target tissues. These characteristics are adapted to optimize each vector to each type of therapeutic need. For transduction of continually dividing bone marrow cells, an integrating retroviral or lentiviral vector ought to be used, while for the transduction of terminally differentiated muscle or brain cells, adenoviral, HSV-1, and AAV-derived vectors are ideal. Lentivirus-derived vectors are especially "flexible" since they transduce both dividing and nondividing cells, thus making this system a versatile one for the transduction of most organ systems. However, each vector system also has drawbacks. For example, a drawback to lentiviral vectors include the derivation of these vectors from pathogenic viruses such as HIV, the increased

probability of insertional activation of oncogenes following integration, and the inability to produce vector preparations of high titer that challenge its clinical applicability in human patients. Disadvantages for AAV vectors include a small cloning capacity limiting the size of their expression cassettes and inability to produce high titer preparations. Adenoviral vectors also have their own inherent disadvantages such as semitransient expression due to its episomal nature, potential cytotoxicity at high doses, and most importantly, its high immunogenicity.

Thus, in the absence of one single ideal vector, the field of gene therapy has evolved to its current strategy in which each vector system has its particular target niche. The viral vector origin, the size of transgenic constructs that can be inserted into different vectors, their intrinsic ease or difficulty of production, their capacity to infect and express in dividing or nondividing target cells, and their ability to evade detection or elimination by the immune system all contribute to the choice of vector.

Adenoviral vectors have been shown to provide reliable, efficient, persistent, targeted high levels of expression, following gene transfer into various tissues and organs. Adenoviruses do not cause major lethal diseases but are effectively neutralized by the humoral and cellular host immune system. Thus, the immune system raises one of the most important challenges to the clinical success of adenoviral vectors. This chapter focuses on the immune response to adenoviral vectors used in the context of gene therapy for the treatment of brain diseases and its implications for pursuing human clinical trials using promising gene transfer vehicles.

2 The Treatment of Neurological Diseases Using Gene Therapy

Gene therapy is currently being explored as an investigational therapy for many neurological diseases in both preclinical and human clinical trials. Lesch–Nyhan syndrome (LNS) is a rare genetic disorder characterized by growth retardation, compulsive self-injurious behavior usually manifesting through biting of lips and/or buccal mucosa, and eventually progressing to biting of fingers and hands, head banging, dysarthria, and cognitive moderate retardation among other movement disorders. LNS is caused by the absence of hypoxanthine–guanine phosphoribosyltransferase (HPRT) activity. This deficiency is due to a genetic alteration in the HPRT gene. The HPRT deficiency is an X-linked recessive disease with the gene localized to the long arm of the X chromosome, locus Xq26-q27 (Seegmiller, 1971), making LNS a potential target disease for a gene therapy approach (Southgate et al., 1999). Therapeutic approaches concern the replacement of HPRT within the brain of affected children.

Huntington's disease (HD) is an autosomal dominant genetic disorder characterized by motor alterations such as chorea, abnormal gait, motor and cognitive impairment, dementia, and depression. The HD alterations are caused by a mutated gene found on chromosome arm 4p16.3 located in the gene "interesting transcript 15" (*IT15*) (The Huntington's Disease Collaborative Research Group, 1993; Albin and Tagle, 1995). The *IT15* gene contains CAG trinucleotide repeats that encode the amino acid glutamine. Normal individuals show 10–34 CAG repeats but the HD patients can present more than 40 CAG repeats. The genetic basis of the disease is not completely known, and it is not possible to treat the disease in its starting point. However, gene therapy is now being developed in order to counteract striatal degeneration. Many studies are underway investigating the therapeutic effects of ciliary neurotrophic factor (CNTF) in HD models. CNTF promotes the survival and differentiation of a number of neuronal populations, such as hippocampal neurons, stimulates the growth of neurites, and plays a crucial role in the induction of reactive gliosis (Winter et al., 1995; Hudgins and Levison, 1998), stimulating the expression of glial fibrillary acidic protein (GFAP) and neural growth factor (NGF) in brain astrocytes (Clatterbuck et al., 1996; Levison et al., 1998; Semkova et al., 2002). CNTF also has an antiinflammatory function (Meazza et al., 1997). It has been demonstrated that CNTF can inhibit the production of tumor necrosis factor (TNF) (Benigni et al., 1995) and protect oligodendrocytes from its deleterious effect (D'Souza et al., 1996). Other growth factors such as nerve growth factor (NGF) (Kordower et al., 1999), glial-derived neurotrophic factor (GDNF) (McBride et al., 2003; McBride et al., 2006), and brain-derived neurotrophic factor (BDNF) (Bemelmans et al., 1999) have also been explored as potential therapeutic genes in the development of gene therapy treatments for HD.

Multiple sclerosis (MS) is a chronic neurological, autoimmune disease characterized by demyelinated lesions in the central nervous system (CNS) that can occur along the extent of the neuroaxis. Common symptoms of MS are incoordination, sensory disturbances, visual impairment, and paralysis, which usually worsen as the disease progresses. The disease usually starts suddenly with an acute attack, which lasts for a few days to weeks, followed by stabilization of the disease or remission. Treatment strategies, many of which can be implemented using gene therapy vectors, have been proposed and have been investigated in preclinical and clinical trials with the aim of developing gene therapy to treat MS. These strategies include (1) inhibiting leukocyte migration into the CNS by targeting the blood–brain barrier (BBB), (2) reducing the production of antimyelin antibodies, (3) expressing immune-modulating molecules within the affected CNS, (4) targeting differentiation factors to oligodendrocytes precursors to repair damaged myelin, (5) abrogation of the myelin-specific T-cell tolerance in the thymus and in the periphery, and (6) using stem cells to replace affected oligodendrocytes (Larocque et al., 2006; Martino and Pluchino, 2006).

Parkinson's disease is a chronic neurodegenerative disorder in which patients experience strong tremors, suffer very serious impairments in their capacity to move, and in some cases display strong uncontrollable movements called dyskinesias. The disease usually starts in middle age (40–50 years of age) and can progress slowly for 10–40 years of the patient's life. The disease is due to the progressive death of cells in a region of the midbrain known as the substantia nigra, which controls fine aspects of motor behavior. These cells, referred to as dopaminergic nigrostriatal neurons, normally provide a chemical transmitter called dopamine to another part of the brain, called the striatum. The physical effects of Parkinson's disease are a result of lack of dopamine in the striatum. Currently, several gene therapy strategies using variety of neural growth factors, various kinds of dopamine replacement techniques, and modifications of basal ganglia neurotransmission are being evaluated for their effectiveness in treating Parkinson's disease by stopping the degradation of dopaminergic nigrostriatal neurons, or modifying the affected neural circuitry (Bohn, 2000; Hurtado-Lorenzo et al., 2004; Chen and Le, 2006; Forsayeth et al., 2006).

Glioblastoma multiforme (GBM) is the most common type of brain tumor in adults and accounts for 25% of all brain tumors (Counsell and Grant, 1998; Pobereskin and Chadduck, 2000). GBM is a malignant cancer, with a mean survival of ~6–12 months postdiagnosis. Aggressive surgical resection is usually combined with chemotherapy or radiotherapy to reduce tumor burden; however, the tumor invariably recurs within 6–12 months. Gene therapy strategies have been explored in preclinical and human clinical trials as alternatives to treat glioblastoma by using conditional and direct cytotoxicity and immune-stimulatory approaches in a wide variety of viral vector systems (Castro et al., 2003; Curtin et al., 2005; King et al., 2005).

3 Gene Therapy for Pain

Pain is very complex symptom that can be caused by various types of pathological stimuli. Neural pain pathways have been well mapped, with the peripheral components involving the dorsal root ganglion neurons, while more psychological aspects of pain perception involve the cerebral cortex. Theoretically, pain perception could be modified by interfering with neural pathways anywhere along the pain circuits, but most emphasis has been given to modifying the function and signaling capacity of dorsal root ganglion neurons.

Of the various types of vectors in current use, herpes simplex type 1 vectors are ideally suited for the treatment of pain at the level of DRG neurons. HSV-1 establishes latency within DRG neurons, and increased understanding of the HSV1 promoters active during latency has allowed the production of vectors that express therapeutic transgenes during latency. Adenoviral vectors also have potential as gene delivery vehicles for treatment of chronic pain as they are capable of infecting both dividing and nondividing cells, but their potential immunogenicity would likely negatively impact therapeutic gene expression with repeat systemic administration, as would be required in patients receiving treatment for chronic pain (Cope and Lariviere, 2006). Furthermore, pain signaling pathways could potentially be inadvertently stimulated due to the proinflammatory effects of adenoviral vectors (Tsai et al., 2000; Castro et al., 2001). Nevertheless, direct delivery into sensory ganglia may reduce any inflammatory effects.

Preclinical studies on chronic pain using various gene therapy vectors have been performed with mixed results (Cope and Lariviere, 2006). Adenoviral, AAV, and HSV vectors as well as naked DNA and naked RNA delivery systems using a variety of therapeutic genes from β -endorphin (Finegold et al., 1999) to proopiomelanocortin (Lu et al., 2002) have been tested in a rodent model of baseline thermal pain and mechanical insensitivity with no positive effect on sensitivity to thermal pain. Only an adenoviral vector expressing IL-2 had an analgesic effect in the thermal pain model in rodents (Yao et al., 2003). On the contrary, a multitude of gene therapy strategies elicited an analgesic effect in a rodent model of inflammatory pain sensitivity, including HSV-1-mediated delivery of proenkephalin A to treat polyarthritic thermal hyperalgesia (Braz et al., 2001) and adenoviral delivery of IL-10 to treat inflammatory mechanical allodynia (Milligan et al., 2005a,b). Gene therapy has also proven itself efficacious in neuropathic pain models such as treatment of a lumbar spinal nerve ligation, or mechanical allodynia with an HSV-1 vector expressing GDNF (Hao et al., 2003). Also, treatment of streptozotocin diabetic peripheral neuropathy or an acrylamide intoxication model of neuropathic pain using an adenoviral vector expressing neurotrophin-3 has had positive outcomes (Pradat et al., 2001).

Currently, gene therapy approaches for chronic pain have yet to be utilized in a human clinical trial; yet several research groups are in the early implementation stages. Chronic pain associated with terminal cancer is a practical choice for gene therapy as patients undergoing therapy would have more limited life expectancy, and thus be suitable candidates for such an experimental treatment. Currently, the research group of Fink, Glorioso, and Mata are in discussions with the FDA and the recombinant DNA advisory committee of the NIH to obtain approval for a clinical trial using an HSV vector to treat intractable pain associated with metastatic cancer (Mata et al., 2006).

4 Adenovirus as a Gene Transfer Vector

The main advantages of adenoviral vectors to treat neurological diseases are their ability to transduce both dividing and nondividing cells, their strong episomal expression without integration into the host genome, the ease of genetic manipulation by a variety of novel vector production systems, the feasibility to target specific populations of cells of tissue types, the ability to produce high titers of vectors, the production of high level, regulated transgene expression in many cell types, and their capacity to encode up to 30 kb of transgene sequences in the newer generation of high-capacity adenoviral vectors.

Adenoviruses are icosahedral nonenveloped viruses that contain a linear 36-kb double-stranded DNA genome. Although roughly 50 different serotypes of adenovirus have been identified based on various factors such as hemagglutination groups, oncogenic potential, and percentage of G-C DNA content, serotypes 2 and 5 have been most extensively studied for use in gene therapy applications. The adenoviral capsid structure consists primarily of 240 hexon proteins and 12 penton proteins onto which 12 fiber structures are positioned. The wild-type adenoviral genome contains short inverted terminal repeats (ITR) at both the 5'- and 3'-ends, which are required for viral DNA replication and packaging of viral DNA into the capsid structure.

Adenovirus binding to the host cell surface and cell entry has been examined in detail (Russell, 2000). Attachment to the cell occurs via interactions between the adenoviral fiber knob proteins and the cellular coxsackievirus-adenovirus receptor (CAR) (Bergelson et al., 1997). Internalization is then mediated by an interaction between an arginine-glycine-aspartic acid (RGD) motif on adenoviral penton base capsid proteins and α_v integrins on host cells' membrane. It has been shown that ablation of the native RGD motif and fiber-binding domains eliminates the native tropism of adenoviral vectors (Einfeld et al., 2001), thus allowing engineering of novel ligands to target the adenovirus to specific tissue types (Wickham, 2003). Following entry, the virus is internalized into the cell via endocytotic pathways mediated by clathrin-coated vesicles (Wickham et al., 1993; Li et al., 2001); there are also less well-understood interactions between fiber proteins and heparan sulphate proteoglycans present on cellular membranes. The interaction of the adenovirus with the cell membrane also activates various cell-type specific signaling pathways. Examples include the phosphoinositide-3-OH kinase (PI-3K) and Raf/mitogen-activated protein kinase (MAPK) pathways (Russell, 2000). Following binding, vectors are internalized into endosomes, from which

adenoviral vectors escape to the cytoplasm through a pH-dependent change in adenoviral penton base and an interaction with the $\alpha_v\beta_5$ integrins (Wickham et al., 1994; Greber et al., 1997; Wang et al., 2000). Adenovirus particles then travel to the nucleus via microtubules where the viral genome enters the nucleus through the nuclear pore (Greber et al., 1997). Transcription of the adenoviral early proteins (E1–E4) is initiated, which modulates host cellular functions, and in turn, influences the transcription of late (L1–L3) genes of the viral genome coding for structural proteins involved in the formation and assembly of the capsid structure.

To transform a wild-type adenovirus into an adenoviral vector as well as enhancing their safety profile, essential viral genes (i.e., E1a) are removed from the viral virus rendering them replication deficient. The deleted essential viral genes are replaced by an expression cassette containing a therapeutic gene under the control of a promoter that can be constitutively expressed in many tissues (i.e., murine CMV) (Gerdes et al., 2000), a tissue or cell-type specific promoter (i.e., GFAP for expression in astrocytes) (Smith-Arica et al., 2000), or a regulatable promoter whose expression depends on the presence of an inducer (i.e., TetOn) (Xiong et al., 2006).

Current replication-defective adenoviral vectors can be classified into several categories, each with their own advantages and disadvantages. Briefly, first generation vectors are deleted in the E1 region, rendering them unable to replicate in host cells in the absence of the native E1a protein. These vectors usually also have deletions in the E3 region to increase cloning capacity. Second and third generation adenoviral vectors contain additional deletions in the E2 or E4 regions to increase safety, reduce toxicity, and expand cloning capacity (Brough et al., 1996; Lusky et al., 1998) as well as modifications in their fiber or penton structures to allow targeting of specific cells of tissues (Einfeld and Roelvink, 2002). While the advances in adenoviral vectorology have improved the safety profile of the vector, they still have not addressed its high immunogenicity. The latest generation of high-capacity, helper-dependent (HC-Adv) or “gutless” adenoviral vectors have the entire viral coding region deleted, only retaining the inverted ITRs (necessary for replication) and the packaging signal (Morsy and Caskey, 1999). These new helper-dependent adenovirus vectors have increased efficiency of transduction, reduced immunogenicity and vector toxicity, and extend the length of transgene expression in various target tissues, such as the liver and brain. Most importantly, they allow long-term regulated expression even in the presence of a pre-existing antiadenovirus immune response.

5 Immune Responses to Adenoviral Vectors

Adenoviral vectors can be administered systemically or directly to the diseased tissues or tumors. If injected systemically into the bloodstream, they will immediately encounter immune cells and antibodies, triggering an early inflammatory response. At high doses, interaction with platelets can lead to disseminated intravascular coagulation (DIC), the cause of death of Jesse Gelsinger (Raper et al., 2003).

To avoid the initial interaction with circulating antibodies and monocytes, direct injections into particular tissues or tumors may be implemented as well as pegylation of vector capsids to reduce capsid protein availability to the immune system. Entry of gene therapy vectors into target cells is the first step in viral infection toward in vivo expression of therapeutic genes. It can also trigger a variety of specific and nonspecific cellular host inflammatory and immune responses, which depend on the cell type infected.

Classical adenovirology studies determined that in the absence of the adenoviral E1a protein, the vector genome would fail to replicate using low multiplicity infections. To achieve high levels of in vivo therapeutic gene expression with gene therapy vectors, however, higher vector loads are used, between 100 to 10,000 vector particles per cell. In these circumstances, cumulative basal expression is detected from the remaining viral transcriptional cassettes present in all vector systems, apart from the HC-Adv. Thus, although not predicted by classical experiments, the use of high MOI causes adenoviral expression of the remaining native proteins encoded within the recombinant vector genome that can be toxic, inflammatory, or provide antigenic epitopes to prime the immune system.

5.1 Innate Immune Response

One of the many challenges that face the successful use of adenoviral vectors in clinical gene therapy is the inflammation and immune responses associated with administration of adenoviral vectors. As stated, viral infection with wild-type virus is a complex process that requires virus entry and the subsequent replication of genetic material, production of viral gene products, and the assembly and release of new viral particles. As a result of concomitant evolution and natural selection, the host immune system is thus tuned to recognize and eliminate potentially infectious pathogens at each stage in the viral replication cycle. For gene therapy applications, the inflammatory and immune response will vary depending on the virus dose, site of administration, surgical technique and proficiency, and type of vector used. In addition, there is also the possibility of the transgene itself inducing a specific immune response.

The innate immune response is the earliest line of defense generated by the host to protect against the constant barrage of pathogens. Although the pathways to trigger innate immune responses are specific to each particular pathogen, once innate immune response has been initiated it results in a standard set of consequences designed to prevent the entry of microorganisms into tissues or, once they have gained entry, eliminate them prior to the occurrence of disease. Following injection of recombinant adenoviral vectors carrying therapeutic transgenes, the innate immune response is activated very rapidly (i.e., within minutes to hours), representing the first line of defense against viral infection.

Other important components of the innate immune system include granulocytes, neutrophils, natural killer (NK) cells, natural killer T (NKT) cells, in addition to the macrophages and dendritic cells (DC). All these lymphocytes are rapidly recruited to the site of viral infection to participate in antiviral responses directly, by killing infected cells and producing antiviral cytokines, as well as indirectly by the production of chemokines that act to recruit other immune cells into infected tissues. Later, these cytokines and chemokines can also be involved in the activation of the adaptive immune response (Guidotti and Chisari, 2001).

Interactions between host cell-surface receptors and viral vectors stimulate various intracellular signaling pathways including activation of NF- κ B (Alexopoulou et al., 2001), AT-2/c-Jun (Paludan et al., 2001), interferon regulatory factors (IRFs) (Sato et al., 2000), and MAP kinases (Dong et al., 2002). The final destination of these pathways is the production of inflammatory cytokines, such as IFN- α/β , IL-6, and IL-12 (Schnell et al., 2001; Higginbotham et al., 2002; Malmgaard et al., 2002), as well as the production of a variety of chemokines, including CCL5, CXCL10, and MIP and MCP families of molecules (Muruve et al., 1999; Borgland et al., 2000; Bowen et al., 2002; Tibbles et al., 2002). The major defense mechanism for the body against viral infection, i.e., the production of antiviral cytokines and chemokines, provides many functions and can act to decrease transcription of transgenes contained in vectors used for gene therapy primarily through cytotoxic and noncytotoxic mechanisms (Qin et al., 1997; Sung et al., 2001).

It has been shown that several of the innate immune mechanisms mentioned above play a significant role in the very early clearance of the adenoviral vectors. Studies that utilized systemic administration of vector to organs such as the liver and lung demonstrated that inflammation mediated by macrophages, activation of NK cells, and production of cytokines including TNF- α resulted in elimination of transgene expression (Worgall et al., 1997; Peng et al., 1999; Worgall et al., 1999). Adenovirus-induced activation of NF- κ B also stimulates production and secretion of CCL5 and CXCL10, key regulators of the innate antiviral immune response (Lieber et al., 1998; Bowen et al., 2002).

The immune response to the careful injection of adenoviral vectors into the CNS differs greatly from the response seen following injection to peripheral organ systems. Several factors, including the presence of a BBB, lack of lymphatic capillaries, absence of dendritic cells in the naive noninflamed brain, and low level constitutive expression of major histocompatibility complex (MHC) expression, have caused the brain to be considered a relatively immune-privileged organ. Thus, comparison of immune responses in the brain versus those seen in peripheral organs is difficult and has generally been misunderstood. Systemically, immune responses completely eliminate both vector and transgene expression in 2–3 weeks following systemic injection of adenovirus (Elkon et al., 1997). On the contrary, expression of transgene following direct injection into the CNS can be sustained for substantially longer periods of time. Studies performed by Byrnes, Wood, and Charlton (Byrnes et al., 1995; 1996a, b; Wood et al., 1996a; Kajiwarra et al., 1997;

Kajiwar et al., 2000). Zermansky and colleagues (2001) and Thomas and colleagues (2000a) have demonstrated that expression of transgene from first generation adenoviral vectors can be detected for up to 13 months in the absence of systemic immunization against adenovirus. It is important to note that the dose of vector injected dictates the duration of long-term expression in the CNS. Injection of first generation adenoviral vectors at doses higher than 1×10^8 IU causes a massive activation of the innate immune response in the brain, completely abolishing transgene expression and produce a brain lesion, even in the absence of the priming of the systemic immune response (Thomas et al., 2001). However, lower doses of vector (i.e., $<1 \times 10^8$ IU) elicit only transient, mild inflammation when delivered intracranially, which appears to be dependent on the quality of the vector preparation (Zirger et al., 2006).

Although the amount of immune cells that are able to gain access to the brain is limited, astrocytes and microglia themselves initiate an innate immune response. Injection of first-generation adenovirus vectors and HC-Advs into the brain results in the stimulation of a rapid local inflammatory reaction. This early response is characterized by recruitment of macrophages and nonantigen-specific lymphocytes, increased expression of MHC class I antigens, and activation of local microglia and astrocytes (Byrnes et al., 1995). This response can first be detected at vector doses above 1×10^6 IU. The existence of a threshold for adenoviral vector-mediated gene therapy in the murine CNS has been established at 1×10^8 IU. Injections below this threshold result in cytokine and cellular-mediated inflammation, which is acute, transient and attenuated within 30 days, and most importantly does not result in a reduction of transgene expression in the brain. Injection of vectors above this 1×10^8 IU threshold results in increased cytotoxicity, irreversible inflammation, elimination of transgene expression, and loss of neuronal and glial cells (Lowenstein, 2003). Recent studies performed in our laboratory have shown that an intracranial injection of 1×10^8 IU elicits an upregulation of innate proinflammatory mRNAs, i.e., those coding for the interferon (IFN)-regulated protein interferon regulatory factor 1, 2', 5'-oligoadenylate synthetase (OAS) and double-stranded RNA-dependent protein kinase in addition to those coding for the chemokines RANTES, IFN- γ -inducible protein 10, and monocyte chemoattractant protein 1. However, when lower doses are administered (i.e., 1×10^7 IU) no statistical differences were observed in the levels of interferon regulatory factor OAS, T-cell-specific guanine nucleotide triphosphate-binding protein, or double-stranded RNA-dependent protein kinase at 3 and 7 days when compared with saline injections (Zirger et al., 2006).

Various experiments have been conducted to determine whether gene expression from the viral vectors is necessary to generate an immune response against adenovirus. To study this phenomenon, adenoviral vectors that could not express genes from their genomes, either because of psoralen/UV inactivation or because capsids were unable to package vector genomes, were used. Even though all these vectors cause similar inflammation when injected into the brain (Thomas et al., 2000a), in mice systemic injection of psoralen/UV-inactivated vectors did prime a systemic immune response, which resulted in the generation of specific cytotoxic T cells (CTLs) (Kafri et al., 1998), while injection of similar vectors in rats did not prime a systemic immune response that could abolish transgene expression from the brain, suggesting that in rats psoralen/UV-inactivated vectors did not prime a systemic immune response (Thomas et al., 2001). Thus, whether genome expression is needed to prime systemic antiadenoviral immune responses and whether it is species specific remain to be determined. Using gene expression microarrays, it was demonstrated that the number of genes with altered expression following infection of target cells is substantially reduced when infection proceeds with adenovirus virions devoid of viral genomes, compared with capsids containing vector genomes (Stilwell et al., 2003; Stilwell and Samulski, 2004).

Antigen-presenting cells (APCs) are involved in the initial stages of recognition of foreign infectious agents. Examples of APCs include conventional dendritic cells and macrophages, which are located in many different tissues throughout the body. These cells demonstrate a high level of phagocytic activity facilitating the capture of foreign antigens for further processing (Banchereau and Steinman, 1998). Although the recognition process is nonspecific, the ability of the APCs to recognize foreign antigens is the first event in a defined sequence of events that leads to the eventual activation of effector T and B cells (Jooss and Chirmule, 2003). Importantly, dendritic cells are the primary cells responsible for transporting the pathogen's antigenic epitopes to the lymph nodes where these antigens will be presented to naive T lymphocytes to prime the adaptive immune response. Thus, dendritic cells bridge the translation from the nonspecific

innate inflammatory responses to the adaptive antigen-specific immune responses, based on recombination and selection of antigen-specific effector T cells.

5.2 Adaptive Immune Response

The adaptive immune response is the long-term, specific, immune response that is tailored to the invading pathogen. The kinetics of this response is slower than those of the innate immune system, necessitating days to weeks to reach its maximum level, rather than hours in the case of the innate immune response as described above. The delay in achieving the peak of the adaptive immune response is explained by the need of specific T cells expressing the correct TCR to expand following their encounter with the corresponding antigenic epitopes displayed on dendritic cells. The adaptive immune response is classified into cell-mediated or humoral responses.

5.2.1 Cell-Mediated Immune Response

In response to the antigen, cellular effector T cells provide a specific, long-term memory compared with the shorter-lived response achieved by the innate immune system. Foreign antigenic epitopes displayed on MHC molecules on the surface of infected cells are recognized by activated T-lymphocytes. T cells, upon interactions with their specific antigens, secrete cytokines such as IFN- γ or TNF- α , or kill target cells through mechanisms that utilize the protein perforin and/or Fas-ligand.

Adenoviral vector administration induces adaptive immune responses in hosts, occurring within 5–7 days after virus administration (Yang et al., 1994a). Administration of adenoviral vectors on their own (i.e., in the absence of any adjuvants) is sufficient to stimulate adaptive responses, as they induce both the innate as well as the adaptive immune responses. Antigens that do not stimulate innate immune responses rely on other agents such as CpG sequences present on plasmids, LPS, or adjuvants such as Freund's to activate the innate response and are necessary to activate the adaptive immune response. The adaptive immune response is defective in the absence of stimulating the early inflammatory innate immune response.

In peripheral organs, such as the liver and lung, as well as in the CNS, it has been determined by immune-cell deletion studies that both CD8⁺ and CD4⁺ T cells play an important role in the antiadenoviral vector response (Yang et al., 1994b; 1995b). CD8⁺ CTLs are one of the major effector cells involved in the eventual clearance of virus from the host (Yang et al., 1994b). Following infection with wild-type adenovirus, the immunodominant epitopes recognized by the immune system reside in the native E1a and E1b proteins. Given that E1-deleted adenoviral vectors do not express these proteins, it is unclear which immunodominant peptides are the culprit targets resulting in the elimination of transgene expression.

It has now been determined that peptides embedded within the viral capsid, e.g., within the fiber protein, contain antigenic peptides recognized by the immune system following infection with adenoviral vectors. While adenoviral vectors cannot replicate within noncomplementing cells including human cells, owing to the high MOI used to transduce cells, transcriptional cassettes from the wild-type genome still are expressed. Following infection with an early generation vector, these antigens will be expressed and produced in transduced cells, resulting from the cumulative basal level of expression of the multiple copies of vector genomes transduced into the target cell; thus they will be available for presentation by DC to stimulate a specific adaptive immune response. However, following infection with an HC-Adv vector devoid of any viral coding sequences, the only antigenic epitopes present will be those of the vector capsid proteins; interestingly although present, they will only be available to the immune system while the vector is uncoating. However, following uncoating, there will not be any antigenic epitopes produced from an HC-Adv genome, as the viral protein-encoding sequences including the immunodominant sequences with the fiber protein are absent. This makes HC-Ad so resistant to immune responses, even when administered in subjects with a preexisting antiadenoviral immune response. Potential immune responses to the transgene, however, could continue to be recognized, even when expressed from an HC-Ad; this is explained, of course, by the continued expression of the transgene.

Adenovirus-specific CTL recognition does not necessarily require *de novo* gene expression, and adenoviral capsid proteins themselves can be processed and complexed with MHC class I molecules to serve as targets for CTL recognition and induction of antiviral CTL responses (Byrnes et al., 1995; Kafri et al., 1998; Molinier-Frenkel et al., 2000). In addition, cytotoxic ^{51}Cr release assays have also shown that CTLs can be generated against the therapeutic transgene contained in viral vectors in mice immunized with a lacZ-expressing adenovirus (Yang et al., 1996). Immune responses against transgenes will of course be more difficult to address; however, in the treatment of diabetes and hemophilia, a percentage of patients are known to develop blocking immune responses while continuing to respond to higher doses of therapeutic proteins. The interactions between the immune responses and effective gene therapies during clinical trials will have to be addressed individually and will likely be specific to each vector and transgene employed. Methods to induce tolerance both in the treatment of diabetes and hemophilia as well as for the treatment of organ transplantation have been developed and could be adapted to gene therapy. While the field of gene therapy has been reluctant to utilize short-term, transient immune-suppression regimes at the start of the therapies as is the case in organ transplants, their potential in making clinical trials safer is starting to be appreciated. The immune-suppression paradigm has direct applicability to the clinical use of HC-Ad vectors. When administering an HC-Ad vector, an immune-suppression regime could be applied during vector administration and the first few days thereafter to suppress immune function during the few days needed for vector uncoating and clearance of viral capsid proteins, as these epitopes represent the only antigenic particles within an HC-Ad vector besides the transgene. Once HC-Ad vectors have uncoated and their genomes become localized to the nuclei, no other antigenic proteins will be expressed from these genomes. Thus, at this stage, the only potentially immunogenic proteins produced will be the transgene.

The central role of the adaptive immune response in eliminating transgene expression has been elucidated in a variety of studies using immunodeficient mice (Yang et al., 1994a, b; 1995a, b; Barr et al., 1995; Dai et al., 1995; Yang and Wilson, 1995; Zsengeller et al., 1995). Yang and colleagues (1994a) first postulated direct killing of transduced hepatocytes by CTLs, potentially through apoptotic mechanisms. However, direct *in situ* demonstration of the killing of target cells by CD8^+ *in vivo* has been elusive. Thus, recent work by Guidotti and Chisari (2001) on the clearing of hepatitis B virus from the liver in a transgenic model has provided strong evidence that CD8^+ CTLs inhibit viral genome replication through the secretion of $\text{IFN-}\gamma$ with only limited toxicity to liver cells. Therefore, the precise cellular and immune mechanism for inhibition of transgene expression in the brain remains to be determined.

Despite low basal MHC class I and II expression in the brain, inflammation increases their expression, a necessary step to allow immune-cell infiltration of the brain. Studies in the brain by Byrnes and colleagues (1996b) have shown that the early innate inflammation can be reduced following depletion of CD4^+ and CD8^+ T cells, and that following injection of adenoviral vectors in the brain, lymphocytes are mainly CD8^+ T cells in sensitized animals, whereas in unprimed animals CD4^+ T cells dominate (Ohmoto et al., 1999). However, the role of this early inflammation is unclear, especially since it appears not to abolish long-term transgene expression. An interesting hypothesis however is that it may "mark" the brain for infiltration by antigen-specific T cells. This hypothesis remains to be proven.

In contrast to what is seen in other organs, however, transgene expression levels in the brain remain largely unaltered in the presence of these presumably antigen-nonspecific T lymphocytes that enter the brain during the early innate inflammatory responses (Wood et al., 1996a). This suggests that the mechanism underlying persistent transgene expression in the brain is due to an ineffective priming of a T-cell-mediated response, resulting in the absence of a systemic immune response that is able to clear vector or transgene product from the naive brain (Byrnes et al., 1996a; Wood et al., 1996b; Kajiwarra et al., 1997; Lowenstein, 2002). This phenomenon is thought to be due to the lack of a functional DC system present in the naive brain (Lowenstein, 2002), although once brain inflammation occurs, various populations of DCs have been shown to infiltrate the brain parenchyma (Hart and Fabre, 1981; Lowe et al., 1989; Matyszak and Perry, 1996; Carson et al., 1999; Fischer and Bielinsky, 1999; McMenamin 1999; Fischer et al., 2000; Fischer and Reichmann, 2001; Santambrogio et al., 2001; Lowenstein, 2002; Stevenson et al., 2002; Greter et al., 2005; McMahon et al., 2005; Ponomarev et al., 2005). Thus, the naive immune status of the brain can be manipulated experimentally.

It is of paramount importance to determine whether adenoviral vectors used as gene transfer systems are able to produce stable and long-term expression of transgene in the presence of a systemic immune challenge because a large portion of the human population has been naturally exposed to adenovirus during childhood. For successful, safe, and efficacious outcomes of human clinical trials using adenoviral vectors, this clinical paradigm must be addressed and investigated in preclinical models. Upon injection of first generation adenoviral vector into brain parenchyma only, a local inflammatory response is elicited. This localized inflammation is a nonspecific response characterized by influx of T cells, macrophages, and other cells of the innate immune system, and only found to be present for one month after vector administration. Importantly, this inflammatory response does not result in elimination of transgene expression (Byrnes et al., 1995; 1996a; Kajiwaru et al., 1997). As previously discussed, the inability to mount a response that eliminates transgene expression may be the result of the failure of the brain to produce an effective antigen-specific T-cell response against the injected adenoviral vector (Byrnes et al., 1995; 1996a; Kajiwaru et al., 1997).

Activation of an antiadenoviral immune response causes antigen-specific T cells to infiltrate the area of brain parenchyma previously infected with adenoviral vectors. While CD8⁺ T cells infiltrate the brain parenchyma proper and target infected cells, CD4⁺ T cells remain in the perivascular compartment. T-cell infiltration also leads to activation of further brain inflammatory cells, such as microglia, and also causes activation of brain astrocytes. Glial activation is limited to the area of T-cell infiltration. The result of T-cell infiltration and brain inflammation is the elimination of adenoviral-mediated transgene expression if first generation vectors are injected (Byrnes et al., 1996a; Thomas et al., 2000b). Recent experiments from our laboratory (Zirger et al., 2005) have demonstrated that the systemic immune response-induced loss of transgene expression (i.e., infected cells expressing the transgene) is dependent on both CD4⁺ and CD8⁺ T cells. Further experiments with knockout mice have shown that IFN- γ , perforin, and TNF- α are mediators of the immune system in our models. The cellular consequences of these interactions, however, are complex, and their details are still being elucidated. While we have strong evidence that immune-mediated cell death of both astrocytes and neurons occurs, it is likely that functional inhibition of transgene expression also occurs. Thus, both cytotoxic and noncytotoxic mechanisms contribute to immune-mediated transgene expression loss. These experiments highlight one of the major limitations for the use of first generation E1-deleted vectors for gene therapy, and one of the major limitations for the use of viral vectors since immune responses against virions and transgenes have been detected when utilizing AAV and lentiviral vectors (Peden et al., 2004; Abordo-Adesida et al., 2005). However, newer generations of viral vectors that express less amounts of proteins encoded by the viral genome, e.g., HC-Ad, and a better understanding of the kinetics of viral vector uncoating and the immunogenicity of viral capsid proteins as well as detailed kinetic analysis of how T cells recognize cells infected by various different types of viral vectors will indicate the power and limitations of the various viral vector systems vis-a-vis the immune system.

Further, we have recently described the microanatomical substrate that underlies communication between T cells and infected brain cells. Immunological synapses are the anatomical basis of intercellular communication in the immune system. We have recently demonstrated their existence *in vivo* in the brain during the clearing of astrocytes transduced by adenoviral vectors from the brain. We hypothesize that the formation of immunological synapses between CD8⁺ T cells and infected astrocytes is necessary for the elimination of adenovirally transduced cells. Thus, understanding further the detailed molecular events involved in the formation, function, and consequences of immunological synapse formation may aid in developing better ways to avoid elimination of transgene-expressing cells from the brain.

As stated earlier, the development of HC-Ad or "gutless" adenoviral vectors, which have the entire viral genome deleted, has generated newer gene transfer systems that are less immunogenic, but more importantly do not express those proteins that provide the antigenic epitopes targeted by the immune system. Recent studies have shown that unlike the effects seen with first generation vectors, the HC-Ad vectors are able to achieve long-term expression of transgene in the brain following the induction of a systemic antiadenoviral immune response (Thomas et al., 2000a). These results have shown for the first time that stable transduction and gene expression from adenoviral vectors is possible in the brain even after peripheral immune challenge that results in the generation of a systemic antiadenoviral immune response.

This experimental paradigm closely mimics the situation that will most likely be encountered in human clinical trials using adenoviral gene therapy vectors.

Before gene therapy is widely accepted as a safe and efficacious biopharmaceutical and implemented as standard practice to treat neurological diseases, the potential immunogenicity of therapeutic transgenes encoded by adenoviral vectors used for gene therapy must be thoroughly characterized. Although most of the work on the immune response to adenoviral vectors is focused on the viral capsid or the viral proteins associated with the viral vector, studies have shown that the transgene encoded by viral vectors may play a role in priming an immune response against virally transduced cells and the resulting elimination of transgene expression. Viral vector delivery of human and murine variants of the same therapeutic transgene into mouse muscle results in immune responses directed against only the foreign (human) transgene-encoded proteins but not the endogenous (self) protein (Chenuaud et al., 2004). Furthermore, it was shown that long-term expression can be attained following a single injection of the vector containing the gene for the self protein (Tripathy et al., 1996). It has also been determined that while CTL responses directed against viral antigens are primarily responsible for transgene elimination, CTL responses can also be generated against the β -galactosidase transgene. In preparation for clinical trials, the immunogenicity of both the vector and the transgene will need to be considered. While the immunological consequences may not impose limitations on clinical trials needing short-term transgene expression, immune responses against the transgene where long-term expression is needed could be decisive. In this case, application of various immune-suppressive, or even regimes that induce tolerance, as have been developed for the treatment of patients suffering from diabetes or hemophilia and who developed immune responses against the therapeutic protein, will need to be considered.

5.2.2 Humoral Immune Response

Although data suggest that the chief mechanism resulting in the clearance of virus-infected cells and elimination of transgene expression from adenoviral vectors is cellular immunity, including the recruitment of macrophages and T lymphocytes into the brain, recent evidence has also shown that the humoral arm of the adaptive immune response also plays a role. Circulating antibodies are responsible for blocking infectivity and transduction after vector delivery. This is especially important in subjects preexposed to the vector or vector readministration. Upon injection of first generation E1-deleted adenovirus vectors into the brain, it has been shown that circulating antibodies against both the adenovirus vector and the transgene product can be detected in serum. These studies detected antiadenovirus antibodies in the serum in as little as two days after injection with a peak at day 30 as well as the presence of CD4⁺ T cells and B cells in the brain (Kajiwar et al., 2000). The kinetics of the humoral response also differs greatly from those seen in the cellular immune response.

It is of extreme importance in the context of gene therapy to understand whether transgene expression is able to elicit an humoral immune response. Previous studies have detected transgene-specific neutralizing antibodies in serum when adenoviral vectors were injected intravenously (Juillard et al., 1995). Similarly, infusion of lacZ-expressing adenovirus leads to the production of β -galactosidase-specific antibodies (Yang et al., 1996). Results from Kajiwar and colleagues (2000) have also shown antibody production against β -galactosidase transgene at 30 days postadenoviral injection in the brain. When injected into the brain parenchyma, generation of antibodies to adenovirus occurs much slower or not at all than when injected peripherally in the ventricles, where antibodies can be detected between 7 and 15 days.

Some gene therapy applications will most likely require readministration of adenoviral vectors as a treatment paradigm. Repeat administration of adenoviral vectors as gene transfer systems in the periphery results in the loss of transgene expression due to the presence of neutralizing antibodies. However, the presence of neutralizing antibodies was not detected in serum after injection into the brain (Kajiwar et al., 2000). Because it is the neutralizing antibodies that act to sequester virus and prevent subsequent adenoviral infection, their absence in these studies provides preliminary evidence that repeated administration of adenoviral vectors as gene transfer vehicles may be possible. Moreover, the use of HC-Adv in combination

with the regulatable Tet-On system (Wei) will eliminate the need for repeat vector administration for diseases such as Parkinson's, which will probably require repeat dosing. The long-term persistence of expression and vector genomes and the ability to "turn on" and "turn off" transgene expression suggests that a single administration of a regulatable HC-Ad vector could provide controlled therapeutic benefits for over 1 year.

To achieve repeat administration if necessary, the vector serotype could be exchanged for the follow-up injections, a chimeric fiber system could be used, the viral capsid could be PEG coated, or transient immune-suppression during the first week postinfection could be considered. These methodologies can work experimentally and in patients, and these are currently being optimized.

6 Future Directions

Viral vectors currently comprise the best available vehicles for efficient gene transfer. Considerable gains in the field of gene therapy have been made in the last decade; however, the immunogenic properties of these vectors persists as a major drawback to the use of first generation adenoviral vectors in clinical procedures. Currently, the use of adenoviral vectors for gene therapy applications could lead to inflammation, toxicity at high doses, and transgene elimination and local damage in cases where subjects may have been previously exposed to adenovirus or could be infected in the future. This inflammation would occur not only at the site of virus injection but also in synaptically linked brain regions (Wood et al., 1996a).

Despite these potential problems, the field of vectorology has made great strides forward in the use of adenoviral vectors as gene transfer vectors. Using current vector technology, the field of gene therapy has also seen promising results in human clinical trials for solid tumors and potentially for esophageal and pancreatic cancers using an adenoviral vector expressing TNF- α under the control of a radiation-inducible promoter (McLoughlin et al., 2005), for age-related macular degeneration using an adenoviral vector expressing pigment epithelium-derived factor (Campochiaro et al., 2006), and for glioma using a conditionally cytotoxic adenoviral vector expressing HSV1-TK in the presence of ganciclovir (Immonen et al., 2004). The development of newer adenoviral gene transfer systems and the production of gene-deleted and "gutless" vectors that are less immunogenic will have a great impact on the field. The field of gene therapy is anxiously awaiting the initiation of the first human clinical trial using the novel HC-Ad vector technology.

Immunosuppression has been shown to enhance the expression of transgenes from adenoviral vectors in the periphery (Kass-Eisler et al., 1994), and development of new immunosuppressive therapies could lead to a controllable modulation of the inflammatory response. Co-administration of several E1/E3a-deleted adenoviral vectors encoding YTS 191 (anti-CD4), YTS 169 (anti-CD8), and TIB 213 (anti-CD11a) have also proven to be very efficient in blocking both cellular and humoral immune reactions (Guerette et al., 1996). Another option that is currently being explored is the use of alternate "stealth" adenoviral serotypes that may evade recognition by the immune system (Morral et al., 1999; Parks et al., 1999).

Initial excitement surrounding adenoviral gene therapy that blossomed in the early 1990s has been curtailed with the realization that the host immune system can mount an effective response after delivery of the viral vectors. In fact clinical trials using adenoviral gene therapy were temporarily put on hold by the FDA following the death of Jesse Gelsinger while the field reevaluated the immunological consequences to adenoviral vectors. Much of the current work in the field of adenoviral-mediated gene therapy is focused on the development of increasingly less immunogenic and more effective vector systems. Although it is important that future work using viral vectors as gene transfer systems produce vectors that are able to target specific populations of cells and express high levels of therapeutic transgene from the smallest dose possible, one can not bypass the importance that the host immune system and the role it plays in gene therapy. Therefore, we must strive to uncover the molecular nature of the innate, the adaptive, and the humoral immune responses to adenoviral vectors as we move forward into human clinical trials. Elucidation of all aspects of the immune response to the delivery of gene therapy treatments and understanding of its mechanisms are of critical importance before the application of gene therapy can become a clinical success.

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12 Adenosine: An Endogenous Regulator of the Brain Immune System

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1	<i>Adenosine in Regulating Brain Function in Health and Disease</i>	284
2	<i>Metabolism of Adenosine in the Central Nervous System</i>	285
3	<i>Modulation of Astrocyte Function by Adenosine</i>	286
4	<i>Adenosine Regulates the Function of Resident Microglia</i>	287
5	<i>Regulation of Inflammation in the Brain by Adenosine Receptors on Infiltrating Immune Cells</i>	287
6	<i>Conclusions</i>	288

Abstract: Adenosine receptors are major targets of caffeine, the most commonly consumed drug in the world. There is growing evidence that they could also be promising therapeutic targets in a wide variety of conditions including Parkinson's disease, stroke, and Alzheimer's disease. The immune system of the brain that comprises astrocytes, microglia, and infiltrating immune cells is normally quiescent, but it becomes activated following pathophysiological events, such as ischemia, trauma, inflammation, or infection. Adenosine is an endogenous purine nucleoside that is liberated into the extracellular milieu at sites that encounter these "stressful" stimuli. There are four known subtypes of adenosine receptors (A_1 , A_{2A} , A_{2B} , and A_3) and these receptors are expressed on cells of the brain immune system. Adenosine receptor activation on immune-competent cells in the brain has both protective actions and injurious consequences. A more thorough understanding of adenosine receptor function in the brain immune system will bring the goal of therapeutic application of adenosine receptor modulators considerably closer.

List of Abbreviations: CCL2, C-C motif ligand 2; C/EBP β , CCAAT/enhancer binding protein β ; GFAP, glial fibrillary acidic protein; IL, interleukin; MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor- κ B; NTPase, nucleoside triphosphate diphosphorylase; PLC, phospholipase C; PTG, protein targeting to glycogen; TNF- α , tumor necrosis factor- α

1 Adenosine in Regulating Brain Function in Health and Disease

Extracellular adenosine is a local modulator with a generally cytoprotective function in the body. One of the most widely recognized effects of adenosine is its ability to control, both under physiological and pathophysiological conditions, central nervous system functions. Adenosine binds to one or more of four cell-surface receptors, which are the adenosine A_1 , A_{2A} , A_{2B} , and A_3 receptors (Ralevic and Burnstock, 1998; Fredholm et al., 2001). These receptors are members of the large family of G protein-coupled receptors. Classically, adenosine receptor signaling is thought to result from inhibition or stimulation of adenylyl cyclase (also known as adenylate cyclase), although it is now clear that other pathways, such as phospholipase C (PLC), Ca^{2+} and mitogen-activated protein kinases (MAPKs), are also relevant. Adenosine is present in the extracellular space at low levels, and metabolically stressful conditions increase dramatically its extracellular concentrations. Under physiological situations adenosine has important roles in the modulation of a wide variety of brain functions, which include regulation of sleep and arousal, locomotion, anxiety, cognition, and memory (Ribeiro et al., 2002). For example, although several mechanisms have been offered to explain the stimulant effects of caffeine, adenosine receptor blockade most likely accounts for the primary mode of action (Huang et al., 2005). In contrast to the low adenosine levels observed in physiological situation, metabolic stress associated with hypoxia, ischemia, trauma, and excessive neuronal activity triggers large increases in the levels of extracellular adenosine. These high levels of released adenosine resulting from pathophysiological stimuli have an important role in governing subsequent tissue damage. Although in general the actions of extracellular adenosine are beneficial, in certain scenarios adenosine receptor activation exacerbates tissue injury (Picano and Abbracchio, 2000; Cunha, 2005).

The protective and regenerative functions of adenosine after acute injury fall into several categories, which include increasing the ratio of oxygen supply to demand; protecting against ischemic damage by cell conditioning; triggering antiinflammatory responses; and promoting angiogenesis (Picano and Abbracchio, 2000; Cunha, 2005). Early after the onset of harmful stimuli, adenosine, via activating A_1 receptors, exerts a potent presynaptic feedback inhibitory effect on the release of injurious excitatory neurotransmitters, mainly glutamate. Concurrently, A_1 receptor stimulation induces hyperpolarization of the postsynaptic membrane, decreases NMDA receptor signaling, and limits calcium influx, preventing both the generation and propagation of excitatory action potentials. Subsequently, adenosine receptor activation contributes to the isolation of damaged tissue by aiding formation of an astrocytic scar and facilitation of astrocytic support of neurons (de Mendonca et al., 2000). Finally, in the long term, adenosine has a role in facilitating removal of dead cells and debris by inducing microglial proliferation and phagocytosis. In addition, there is indirect evidence that adenosine might contribute to tissue remodeling late after the insult

by inducing angiogenesis, and thus promoting the replacement of dysfunctional blood vessels (Fischer et al., 1995). Although the predominant theory has been that adenosine is protective, there is emerging evidence that in the adult brain, adenosine has injury-enhancing effects following ischemic insults. These injurious effects relate to late activation of A_{2A} receptors on immune cells (Cunha, 2005).

The modulatory actions of adenosine receptor activation in response to brain tissue injury have been proposed to involve mainly neuronal cells and have been summarized extensively in a number of excellent reviews (Vizi, 2000; Cunha, 2001; Dunwiddie and Masino, 2001; Stone, 2002). In this chapter, we overview the central nervous system effects of adenosine, both protective and harmful, that are mediated by modulating immune events in the wake of harmful insults, including metabolic, traumatic, and acute or chronic inflammatory conditions. First, we discuss the mechanisms by which extracellular adenosine concentrations are regulated during metabolic stress in the central nervous system. Second, since cells of the immune system in the central nervous system, which include astrocytes, microglia, and infiltrating immune cells, are key players in the modulatory effects of adenosine subsequent to tissue injury, it will be important to delineate how adenosine modulates the function of these cell types.

2 Metabolism of Adenosine in the Central Nervous System

Extracellular adenosine levels are quite variable, depending on the tissue and the degree of stress facing the tissue, and so the basal levels of stimulation of the four adenosine receptor subtypes by the endogenous agonist vary enormously. Processes affecting its generation, intra and extracellular metabolism, release, and cellular uptake determine the local concentrations of adenosine that are available for binding to its receptors (Ralevic and Burnstock, 1998; Fredholm et al., 2001). The sources of extracellular adenosine are either release of adenosine from the intracellular space via specialized transporters or conversion of released adenine nucleotides (ATP, ADP, and AMP) by a cascade of ectonucleotidases, which include CD39 (nucleoside triphosphate diphosphorylase) (NTPDase) and CD73 (5'-ectonucleotidase) (Zimmermann, 2000; Linden, 2001; Sperlagh et al., 2006). All cell types that are present in the central nervous system can serve as a source of extracellular adenosine. The cellular sources of adenosine, however, differ depending on the type of the stimulus triggering the release of adenosine. For example, during high-frequency neuronal firing and seizure, neurons release large amounts of ATP (Mitchell et al., 1993; Cunha et al., 1996), which is then metabolized to adenosine via CD39 and CD73. In contrast, glial elements could also be a significant source of extracellular adenosine, especially after episodes of ischemia and hypoxia (Parkinson and Xiong, 2004; Parkinson et al., 2005). Ischemia leads to intracellular adenosine accumulation via dephosphorylation of ATP to adenosine by the metabolic enzyme 5'-nucleotidase and by inhibiting the activity of the salvage enzyme adenosine kinase, thereby preventing the rephosphorylation of adenosine (Lynch et al., 1998). When adenosine accumulates intracellularly, it is released into the extracellular space through bidirectional, equilibrative nucleoside transporters (Jennings et al., 2001; Parkinson and Xiong, 2004; Parkinson et al., 2005). It was recently demonstrated that high extracellular levels of both ATP and adenosine are achieved by treatment of hippocampal slices with the proinflammatory cytokine interleukin (IL)-1 β (Sperlagh et al., 2004). This release of ATP and adenosine in response to IL-1 β was secondary to the activity of both glutamate receptors and tetrodotoxin-sensitive sodium channels. Nevertheless, the exact cellular source and mode of ATP and adenosine release remain undetermined. Ischemia, head trauma, seizure activity, and inflammation induce a rapid increase in extracellular adenosine level exceeding the resting concentration by 30- to 100-fold (von Lubitz, 1999). While baseline adenosine concentrations in the brain are in the range of 30–300 nM in the extracellular space (Rudolph and Schubert, 1997), extracellular adenosine levels following 15 min of ischemia can increase to 10–50 μ M (Hagberg et al., 1987). Adenosine is degraded to inosine by adenosine deaminase. Conventional thinking held that inosine had no biological efficacy; however, recent studies have revealed that inosine is a potent neuroprotective and antiinflammatory substance (Hasko et al., 2000b, 2004). Inosine is finally converted to the stable end product uric acid, a metabolite which possesses antiinflammatory properties and as such is a potential candidate agent for the therapy of multiple sclerosis (Spitsin et al., 2001).

3 Modulation of Astrocyte Function by Adenosine

Astrocytes are the most abundant type of glial cell in the brain. They regulate the external chemical environment of neurons by removing excess ions, notably potassium, and recycling neurotransmitters released during synaptic transmission. In response to injurious stimuli to the central nervous system, astrocytes proliferate, display morphological changes (hypertrophy of cell bodies, thickening and elongation of astrocytic processes), and exhibit enhancement of glial fibrillary acidic protein (GFAP) expression (Liberto et al., 2004). This process is termed astrogliosis and it is associated with augmented secretion of growth factors and neurotrophins that provide support for neuronal growth. Astrogliosis, however, may also lead to the creation of neuronal scars (Liberto et al., 2004). Activated astrocytes also become immunocompetent; they produce a plethora of inflammatory cytokines, increase expression of major histocompatibility complex II on the cell surface, and secrete free radicals (Dong and Benveniste, 2001).

Astrocytes express all four adenosine receptor subtypes, the stimulation of which can radically alter various aspects of astrocyte function. One of the best-studied features of this regulatory function of adenosine is its effect on astrocyte proliferation, survival, and cell death. Adenosine inhibits astrocyte proliferation acting at the high-affinity A_1 adenosine receptors (Rathbone et al., 1991), which indicates that under physiological conditions in which A_1 receptor expression is high, there may be a tonic inhibition of astrocyte proliferation. On the other hand, the activation of A_{2A} receptors increases astrocyte proliferation and activation (Hindley et al., 1994; Brambilla et al., 2003). Because the expression of A_{2A} receptors is upregulated following hypoxia, trauma, or inflammation (Cunha, 2005), we propose that adenosine may be a key factor in inducing astrogliosis following ischemic or traumatic events. Stimulation of the low-affinity A_{2B} receptor augments reactive astrogliosis in tumor necrosis factor- α (TNF- α) pretreated but not naive cells (Trincavelli et al., 2004). Interestingly, A_3 receptor stimulation induces the apoptotic cascade in astrocytes (Abbraccio et al., 1997; Appel et al., 2001; Di Iorio et al., 2002). Von Lubitz (1999) proposed the concept that this apoptotic process induced by A_3 receptors during severe metabolic stress may have a role in isolating the most damaged tissue by eliminating cells from regions of irreversible injury. This process might help to reallocate energetic resources to tissue regions that are less injured (the penumbra) providing a better chance for survival of the penumbra zone.

Besides regulating the proliferation and survival of astrocytes, adenosine can substantially influence the secretory functions of these cells. A_1 receptor activation facilitates the release of nerve growth factor (Ciccarelli et al., 1999), which may indirectly promote neuronal survival and growth. Stimulating A_{2A} receptors inhibits both the expression of inducible nitric oxide synthase and nitric oxide production by astroglial cells following stimulation with the combination of lipopolysaccharide and interferon- γ or coadministration of TNF- α and IL-1 β (Brodie et al., 1998). Since nitric oxide production can contribute to the pathophysiology of many diseases that involve the central nervous system (Licinio et al., 1999), the inhibition of nitric oxide production by adenosine may be an important protective mechanism during inflammatory conditions in the brain.

A_{2B} receptor activation has been documented to trigger the release of IL-6 from astrocytes (Fiebich et al., 1996a, b; Schwaninger et al., 1997), which involves a transcriptional mechanism relying on the transcription factors nuclear factor (NF)- κ B and NF-IL6 or CCAAT/enhancer binding protein (C/EBP) β (Schwaninger et al., 1997). Because IL-6 possesses neuroprotective effects against hypoxia and glutamate neurotoxicity (Maeda et al., 1994), the stimulation of A_{2B} receptors represents a means of damage control during central nervous system injury. Also, A_{2B} receptor-mediated NF-IL6 activation contributes to the induction of the gene of protein targeting to glycogen (PTG) (Allaman et al., 2003), which is one of the protein phosphatase-1 glycogen-targeting subunits that has been implicated in the control of glycogen level in a number of tissues. Stimulation of the transcription of PTG by A_{2B} receptor activation leads to a protracted synthesis of glycogen, which may cause repletion of the early loss in glycogen levels following ischemia (Allaman et al., 2003). Finally, A_3 receptor activation elicits the synthesis of the neuroprotective chemokine, chemokine (C-C motif) ligand 2 (CCL2) (formerly known as monocyte chemoattractant protein-1 or MCP-1), by astrocytes (Wittendorp et al., 2004). These findings indicate that although adenosine might exacerbate tissue injury by way of inducing excessive astrogliosis, in the majority of conditions adenosine induces a neuroprotective astrocyte phenotype.

4 Adenosine Regulates the Function of Resident Microglia

Microglia comprise between 5% and 15% of the resident cell population of the central nervous system (Minagar et al., 2002). Microglia are the tissue macrophages of the central nervous system and are found in all regions of the brain and spinal cord (Stoll and Jander, 1999). Even though microglia and infiltrating macrophages express many of the same molecules, several recent studies clearly demonstrate that microglia and infiltrating macrophages are phenotypically different cell types and have different functions. Most notably, microglia and peripheral macrophage populations appear to differ in the rate that they are replaced by bone marrow stem cells: parenchymal microglia are only slowly or rarely repopulated by bone marrow stem cells, while all other macrophage populations are replaced 100% within days to months (Melchior et al., 2006). Microglia rapidly react to different kinds of injury and these responses are relatively homogeneous regardless of the noxious agent. The responses include morphological alterations, proliferation, upregulation of cell surface molecules, and production of soluble mediators. Most disorders affecting the central nervous system involve activation and possibly dysregulation of microglia.

Microglia have been documented to express A₁, A_{2A}, and A₃, but not A_{2B} receptors. Adenosine has been demonstrated to stimulate proliferation of naive microglial cells, which requires simultaneous stimulation of A₁ and A₂ receptors (Gebicke-Haerter et al., 1996). In contrast, adenosine can inhibit the proliferation of activated microglial cells, because phorbol 12-myristate 13-acetate-stimulated microglial proliferation was reduced following treatment with an A₁ receptor agonist (Si et al., 1996). Similar to findings with astrocytes, adenosine receptor activation can lead to apoptosis of microglial cells (Ogata and Schubert, 1996). Because the nonselective adenosine receptor agonist 2-chloro-adenosine, but not selective A₁, A₂, and A₃ agonists, could evoke apoptosis, the authors proposed that this effect was mediated by an atypical adenosine receptor. On aggregate, these observations indicate that adenosine receptor activation can distinctly impact microglial proliferation/apoptosis, where the outcome of the response depends on many factors, such as the receptor subtypes expressed and the environment in which these functions are explored.

Although the proliferation and/or apoptosis of microglia can be influenced by different adenosine receptors, the secretory function of microglia is predominantly regulated by A_{2A} receptors. For instance, A_{2A} receptor activation produces an upregulation of cyclooxygenase-2 and the release of prostaglandin E₂ (Fiebich et al., 1996b). Because prostaglandin E₂ has many proinflammatory effects, it can be proposed that A_{2A} receptors have proinflammatory functions. Alternatively, on the basis of recent evidence that other cyclooxygenase-2 products, such as prostaglandin D₂ and 15-deoxy-prostaglandin J₂, are instrumental in the resolution of inflammation (Gilroy et al., 2004), the induction of cyclooxygenase-2 activity by adenosine in microglial cells might also have beneficial, tissue restitutory functions. In support of such a beneficial role, it is also notable that A_{2A} receptor activation induces the synthesis and release of nerve growth factor (Heese et al., 1997). A recent study demonstrated that A₃ receptor activation on microglia suppresses lipopolysaccharide-induced TNF- α production by microglial cells through a phosphatidylinositol-3-kinase/Akt-dependent but extracellular signal-regulated kinase (ERK)1/2-independent mechanism (Lee et al., 2006). In another study microglia were shown to express A₃ receptors, the stimulation of which resulted in an increased ERK1/2 phosphorylation (Hammarberg et al., 2003). Collectively, it is difficult to predict how adenosine affects microglial functions; it appears that adenosine has both pro- and antiinflammatory effects.

5 Regulation of Inflammation in the Brain by Adenosine Receptors on Infiltrating Immune Cells

The role of infiltrating immune cells in the central nervous system is controversial and depends on the underlying disease. While the infiltrating hemopoietic cells protect the brain from invading microorganisms and eliminate debris at sites of tissue injury they can also contribute to tissue damage, as their uncontrolled activation can impart further damage on the affected tissues. These cells secrete a large number of potentially neurotoxic mediators including proinflammatory cytokines, various injurious free radicals, and proinflammatory lipid products. Inflammation has been implicated as a secondary injury

mechanism following ischemia and stroke. Ischemia in the central nervous system is followed by infiltration of various inflammatory/immune cell types, including neutrophils, monocytes and macrophages, and lymphocytes (Stoll et al., 1998). Bacterial meningitis is associated with pleocytosis of neutrophils into the cerebrospinal fluid (Kim, 2003). Immigration of monocytes and macrophages into the central nervous system is also an early feature of HIV-1 infection, and subsequent recruitment of macrophages could be a key factor in the development of HIV-1-associated dementia (Rappaport et al., 1999). All four adenosine receptor types have been found on infiltrating hemopoietic cells, and the adenosine receptors regulate brain immune events in a cell type-specific and stimulus-dependent manner.

A₁ receptor-knockout mice demonstrate more severe symptoms and a worsened course of disease in experimental allergic encephalomyelitis, which is an animal model of multiple sclerosis. The heightened demyelination and clinical course observed in A₁-knockout mice was associated with an augmented activation of macrophages in the brain parenchyma (Tsutsui et al., 2004). Macrophages obtained from A₁ receptor-knockout mice had increased expression of the proinflammatory molecules IL-1 β and matrix metalloproteinase-12 compared with macrophages from wild-type controls, incriminating A₁ receptors on macrophages as initiators of crucial antiinflammatory processes (Tsutsui et al., 2004). In agreement with these observations in mice, a diminished expression of A₁ receptors was found in brain macrophages of patients with multiple sclerosis, which was proposed as a potential contributing factor to the excessive inflammatory response seen in affected patients (Johnston et al., 2001).

A_{2A} receptors on bone marrow-derived cells were recently incriminated as pivotal contributors to brain injury following ischemia. Selective inactivation of A_{2A} receptors on bone marrow cells in chimeric mice protects against ischemic brain injury following middle cerebral arterial occlusion (Yu et al., 2004). This protection is associated with decreased expression of macrophage-derived proinflammatory mediators, such as IL-1, IL-6, and IL-12 in the brain, demonstrating an injurious proinflammatory effect of A_{2A} receptor stimulation in this model. Interestingly the protective, antiinflammatory effect of A_{2A} receptor deficiency of bone marrow-derived cells appears to be specific for the ischemic brain, because selective inactivation of A_{2A} receptors on bone marrow cells aggravates ischemic liver injury. Similar to this antiinflammatory role of A_{2A} receptor activation in the liver, A_{2A} receptor activation has antiinflammatory properties in a rat model of endotoxin-induced meningitis, where A_{2A} receptor stimulation prevents pleocytosis and breakdown of the blood–brain barrier (Sullivan et al., 1999). Furthermore, A_{2A} receptor stimulation was recently observed to attenuate HIV-1 Tat-induced production of TNF- α by macrophages (Fotheringham et al., 2004). These latter observations underline the prevailing notion that A_{2A} receptor stimulation is antiinflammatory by deactivating macrophages and neutrophils (Hasko et al., 1996, 2000a; Hasko and Cronstein, 2004). Alternatively, the observation that selective blockade of A_{2A} receptors on bone marrow-derived cells prevents injury after middle cerebral arterial occlusion (Yu et al., 2004) indicates that the mechanisms leading to and protecting from injury might be distinct in the ischemic brain when compared with other tissues.

6 Conclusions

There are several lines of evidence supporting the notion that adenosine receptors might be valid targets for drug development to treat several disease states afflicting the central nervous system (Ralevic and Burnstock, 1998; de Mendonca et al., 2000; Picano and Abbracchio, 2000; Fredholm et al., 2001; Ribeiro et al., 2002; Cunha, 2005). Selective agonists are well advanced in clinical trials for the treatment of pain and neuropathy. Selective antagonists have entered clinical trials for the treatment of Parkinson's disease (Schwarzschild et al., 2003). On the basis of our increasing knowledge of adenosine receptor function in the brain immune system, there is optimism that the list of adenosinergic therapies in the brain will grow longer in the near future.

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13 Neuroimmune Cross Talk

G. Juhász

1	<i>Introduction to Neuroimmunology</i>	294
2	<i>Immune System of the Brain; Activation by Inflammation</i>	295
2.1	Microglia and Other Brain Macrophages	296
3	<i>Mediator and Modulator Molecules of Neuroimmune Cross Talk</i>	298
3.1	Neuropeptides as Neuroimmune Modulators	298
3.2	Hormones in Neuroimmune Communication	299
3.3	Brain Cytokines	300
4	<i>Synaptic Innervation of Immune System by Autonomic Nervous System</i>	302
4.1	Cholinergic Anti-Inflammatory Pathway	302
4.2	Nonneuronal Cholinergic System and Immune Modulation	303
5	<i>Synaptic Communication is not the Privilege of Neurons</i>	303

Abstract: Immune system and the nervous system share the job of keeping the homeostatic control and responding to changes in external and internal environment. Similarity of their functions and common use of receptors, ligands and other cell-to-cell communication molecules by cells of nervous and immune system origin supports the idea that the two systems function in close cooperation. This chapter intends to provide an overview of the field of neuroimmune cross talk including innervation of immune system, innate immune system of the brain, commonly used mediators and transmitters, and the similarity of neuronal and immune synapse. We did not focus on autoimmune diseases of the brain and only barely touch the medical neuroimmunology because we aimed to give a survey of basic facts and principles of neuroimmune cross talk, providing a firm background for understanding medical consequences of interaction between the two systems.

List of Abbreviations: ACTH, adrenocorticotrophic hormone; APC, antigen-presenting cell; BDNF, brain-derived nerve growth factor; CGRP, Calcitonin gene-related peptide; CRF, Corticotrophin-releasing factor; CRH, corticotropin-releasing hormone; ICAM, integrin-mediated cell adhesion molecules; LPS, lipopolysaccharides; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein; NCAM, Neuronal cell adhesion molecules; NOS, nitric oxide synthase; NGE, nerve growth factor; PVN, paraventricular nucleus; RAMP, receptor activity-modulating protein; SMAC, supramolecular activation cluster; TLR, toll-like receptor; VIP, vasoactive intestinal polypeptide

1 Introduction to Neuroimmunology

The nervous and immune systems provide a mutual function, preserving integrity and homeostasis of the organism. These two systems are composed of networks of cells, monitoring, processing, and responding to changes in external and internal environment including extracellular space of tissues. Similarity of their functions and common use of receptors, ligands and other cell-to-cell communication molecules by cells of nervous and immune system origin supports the idea that the two systems function in close cooperation. Indeed, the amount of experimental evidences for neuronal control of immune system and impact of immune response on neuronal functions is growing exponentially. The main frontier of neuroimmune cross talk is at the barrier regions such as skin, lung, and gut, where the interaction of mast cells and eosinophils with neurons is confirmed. Behavioral changes under inflammatory reactions modifying mood and motor activity as well as sleep are common experiences in infectious diseases. The severe influence of depression and other psychological disorders on immune reaction has also been extensively reported. Thus, evidently the bilateral interaction of nervous and immune systems is a rapidly growing field of molecular and cellular neuroscience (Blalock, 2005).

History of comparative studies of immune and nervous system goes back to the Neuroscience Study Program books edited by F. O. Smith and particularly to the scientific efforts of G. Edelman. So the idea is old that immune cells and their molecular compartments have several similarities to the nerve cells. The main outcome of genome era that the number of genes is much lower than expected led us to a conclusion that same proteins can play different roles in different cell phenotypes. In fact, there are receptors, proteins in the signalosomes, and synapses which are in use in nerve cells and immune system cells, such as caveolines, cholinergic receptors, and so on. Here, we give a review of common structural elements with and without functional similarities.

The efferent part of neuroimmune cross talk is the action of neurotransmitters and modulators on immune cells. This chapter provides an insight to mechanisms of immune cell activation or depletion by neuropeptides such as calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP), substance P. The cholinergic anti-inflammatory pathway and adrenergic control of immune cells are direct efferent actions of CNS on immune system. Nicotinic-type acetylcholine receptor is present on microglia and on some other immune system cells. As of the other side of the neuroimmune cross talk system is concerned, specific behavioral effects can be achieved by inflammatory cytokines. There are immune system elements in the brain such as microglia, the resident macrophage of the brain. Microglia is a typical immune system cell activated by surrounding neuronal and glial events. Complement system elements

expressed on astrocytes are also possible targets of peripheral immune reaction. The open areas of the blood–brain barrier (BBB) compose an interface between blood and brain. Peripheral immune response induced proinflammatory cytokines and other small blood-derived peptides can enter the brain and induce changes in nervous function by activation of endogenous cytokines and neurotransmitters.

Our aim is to call attention of neuroscientists for investigation and importance of neuroimmune cross talk and spread out the idea that neurosciences can learn novel scope and new targets of research from immunology. At the present stage of knowledge, we try to give a general scope to neuroimmune cross talk principles but we do not give a detailed, complete review of its literature; thus, we skipped several features of the topic and aimed to make a short but informative survey of neuroimmunology.

2 Immune System of the Brain; Activation by Inflammation

Brain is isolated from the blood by BBB. BBB does not allow the free penetration of blood immune cells into the brain and limits the spread of large molecules from the blood to the brain. BBB is built from epithelial cells covering the brain surface and endothelial walls of capillary blood vessels and end feet of astrocytes are also involved. Permeability of BBB depends on cell adhesion molecules, connecting the epithelial cells such as CD40, vascular adhesion molecule-1, intercellular adhesion molecule-1, E-selectin. There are open areas of BBB under physiological conditions, allowing direct communication between blood and brain tissue and penetration of large molecules even cells. Circumventricular organs are such open places locating at strategic position near to the middle ventricular system. Because of anatomical vicinity, the blood-derived molecules easily reach pineal gland, subcommissural organ, subfornical organ, and particularly reach organum vasculosum, median eminence, and hypophysis. All these structures are close to the autonomic nervous system centers in the brain. Area postrema is very close to the medullar autonomic nuclei including solitary tract (Rubin and Staddon, 1999).

Endothelial cells of BBB could release neurotransmitters and modulators as a response to cytokine signals from the blood. In addition, activation of BBB epithelial cells induces leakage of BBB; in turn, neurotoxic and inflammatory mediators can penetrate the brain. Penetrability of BBB is controlled by inflammatory cytokines, metal ions such as Al, organic solvents, and many other compounds. During inflammation, opening of BBB is a major factor of brain symptoms. Inflammation activates genes of cyclooxygenase (COX2) and enhances transcription via nuclear factor of kappa B (NFκB) pathway. That was disclosed by using lipopolysaccharide (LPS) for induction of inflammation. LPS also activates mitogen-activated protein kinase (MAPK) pathway through soluble CD14, induces release of IL1β and TNFα from epithelial cells, and also activates nitric oxide synthase (NOS) (Wong et al., 2000). These mediators could regulate BBB so that permeability increase allows entrance of blood cytokines into the brain and enhances infiltration of macrophages (see later in this chapter). Such an effect on BBB can be attenuated by glial cells, dexamethasone, and NOS inhibitors. Cerebrovascular dysfunctions can also be observed during inflammation but changes in cerebral blood flow data are controversial (Hedley and Pippa, 2002).

For neuroimmune communication, brain should detect inflammatory mediators as well. Components of innate and adaptive immune systems are expressed in the brain in experimental endotoxin shock and expression of immune system molecules spread from the circumventricular organs to the deeper structures of the nervous system to areas controlling autonomic nervous system and neuroendocrine regulatory system. It is nicely shown by LPS receptor CD14 expression. Recently, Toll-like receptor (TLR) has been demonstrated in resting and LPS-activated brain; expression of TLR2, TLR4, and TLR9 was convincingly established on microglia, oligodendroglia, and astrocytes. TLR4 receptor mRNA has been found in the circumventricular organ, hypothalamus and medulla, but in contrast to CD14, TLR4 mRNA is downregulated in LPS infection (Opal and Huber, 2002). Data on TLR4 protein are contradictory because of the poor selectivity of TLR4 antibodies available. TLR2 and TLR9 mRNA was found in microglia. TLR and CD14 both activate NFκB pathway and enhance transcription of proinflammatory cytokines. LPS also activates NOS in the brain and increases the release of proinflammatory and anti-inflammatory cytokines synchronously suggesting a delicate immune counterimmune regulation in the brain. Prostaglandin E2 is also activated in the brain

under inflammation and its role in fever genesis is extensively studied (Blatteis et al., 2004). Finally, it has to be noted that free radicals and superoxides are generated in the brain during inflammation.

The outcome of inflammatory reaction in the brain is complex. Cytokines, NO, and prostaglandins modulate neurotransmission particularly the β -adrenergic system and production of corticotrophin-releasing factor (CRF), ACTH, and vasopressin. Autonomic nervous system is also controlled by inflammation as we will describe later in detail. Intracellular changes in brain cells under inflammatory influence are both destructive as apoptotic cell death induced by NO or TLR4 receptor activation and protective as heat shock protein expression (Rotwell, 1999). Apoptotic cell death can be induced by LPS via IL1 β , TNF α . Some data support that epileptic activity in the brain is enhanced by IL1 β and reduced by endogenous antagonist of it (ILR1a) (Vezzani and Granata, 2005). Investigation of inflammatory reaction and its molecular consequences in the brain is a promising field for research to understand how severe brain diseases are as neurodegenerative disease and psychiatric diseases can develop in subjects involved sustained inflammatory diseases (Kronfol and Pemick, 2000). Statistical epidemiology data support a correlation between frequency of inflammation and expectance of neurodegenerative disease and depression. On the other hand, manipulation of immune reaction and inflammatory processes via neuronal influence on the peripheral immune system is also an old experience in Chinese and other Oriental medicines.

Brain cells such as astrocytes, microglia, and oligodendroglia express complement system proteins. The classical and the alternative complement activation pathway is present in the brain and plays an important role when BBB is broken and immunoglobulins or various LPS can enter the brain under infections (Guillemin and Brew, 2004). The classical pathway, activated by IgG or IgM molecules via C1 converted to C1b by C1-convertase, is activated in neurodegenerative disorders such as Alzheimer's disease. Amyloid- β can bind the C1 receptor directly (Yasojima et al., 1999). The alternative pathway activated by membrane lipids and liposaccharides and mediated by C5 converted to C5b by C5-convertase is involved in viral and bacterial infections of the brain. The brain complement system and its endogenous inhibitors have particular therapeutic importance in medication of neurodegenerative disorders. The glial localization of complement system supports the particular importance of glial cells in pathomechanisms of neurodegeneration based on protein aggregation (Yasojima et al., 1999).

As it has been demonstrated, several functional elements of immune system such as tissue resident macrophages, cytokine-releasing cells, and the complement system are present in the brain. It is interesting to note that only few components of immune system proteins are expressed in neurons but all of them are found in different glia cells. The microglia is equipped with all immune system protein components because microglia cells are the resident macrophages of the brain as we are to describe in the next chapter. Actually, it is clear that brain and immune system communicate under inflammation and early and late immune response. The immune system proteins containing glia cells mediate the immune system actions to neurons in great extent but there are several examples of direct action of immune system on neurons. The molecular substrate of neuroimmune cooperation is the immune system of the brain on one hand and the neurotransmitter or neuromodulator receptors on the immune cells particularly on macrophages and lymphocytes on the other.

2.1 Microglia and Other Brain Macrophages

Microglia cells are resident macrophages in the nervous system. The name "microglia" was introduced by del Rio Hortega in 1919. Recently, the name of microglia remained in use but it turned out that it is more real to classify the microglia cells into the group of central nervous system macrophages. CNS contains pericytes, perivascular macrophages, and the real microglia; thus, they all form the group of resident macrophage cells. The controversial nomenclature derives from the controversial origin and function of different CNS macrophages. The most common hypothesis of the cellular origin of CNS macrophages is that they are derived from monocytes (Guillemin and Brew, 2004). In developing brain, however, the pial macrophages can be an additional source of brain macrophages. In embryogenesis, macrophages can migrate from any direction into the developing CNS but in developed brain the source of migration is restricted to the blood vessels. Differentiation of primary embryonic macrophages is governed by microenvironmental factors and

finally they differentiate into four different types of brain macrophages listed earlier. There are evidences suggesting that different brain macrophage types can be transformed to each other in certain conditions and such transformations are influenced by diseases (Guillemin and Brew, 2004).

The characteristic features of different brain macrophages are as follows: *Pericytes* are of mesodermal origin and migrate from the blood vessels at the end of vascularization. *Perivascular macrophages* are very similar to the blood-derived macrophages and they are a subpopulation of migratory macrophages. Because of their similarity to pericytes, the differentiation of them can be done only by marker proteins (Guillemin and Brew, 2004). *Infiltrating macrophages* are blood macrophages actually infiltrating through the BBB around the blood vessels. They have all markers of blood macrophages generally described, so they are not differentiated brain macrophages. *Microglia cells* are tissue-resident macrophages in CNS and their origin and functional roles are not disclosed properly. They are definitely not glia cells because of their macrophage origin. Microglia can be differentiated by characteristic marker proteins using immunostaining.

In normal brain under physiological conditions, there are resting microglia cells in all brain areas as well as perivascular macrophages and pericytes. A few blood-derived macrophages also “patrolling” and a spontaneous infiltration of macrophages through the BBB can be observed. In pathological conditions, when either a large number of cells die or inflammation of the nervous system occurs, the CNS macrophages are activating. They can proliferate and migrate in some extent and if the BBB is damaged, the number of infiltrating macrophages can increase significantly (Sharshar et al., 2005). The antigen-presenting cell (APC) of CNS is the pericyte, which can enhance constriction of blood vessels and they can convert to smooth muscle cells. Pericytes are essential elements of BBB and involved in pathomechanisms of hypoxia, hypertension, diabetic retinopathy, trauma, Alzheimer’s disease, multiple sclerosis, and brain tumors. The time sequence of macrophage activation in the case of an injury is that pericytes and perivascular macrophages activate first, than blood monocytes and lymphocytes cross the BBB. The migration of macrophages is controlled by chemokines particularly the astrocyte-released monocyte chemoattractant protein-1 (MCP-1). Simultaneously, microglia cells are activated and they became capable for phagocytosis (Guillemin and Brew, 2004). That process is one of the novel hypotheses of Alzheimer’s disease (Kaltschmidt et al., 1997). It has to be noted that recently several new aspects of microglia activation have been determined such as neuroprotective role of microglia activation. So the pathophysiological role of CNS inflammatory reaction is in focus of research interest.

Activated microglia cells have specific markers allowing identification of activation by immunostaining. Because it is a considerably long list of proteins available in several review papers (Graeber et al., 1999), we give only a brief survey of categories of marker proteins. One-third or even more of the microglia activation marker list contains cell adhesion molecules and membrane proteins such as integrins, CAMs, immune receptors, and so on. The others are miscellaneous proteins of cellular metabolism and transport procedures. The long list of activation marker proteins suggests that the activation process is a complex and fundamental change in cellular phenotype of macrophages. The phenotypes of activated, maturing, infiltrating macrophages are different. CNS macrophages, as other macrophages, have several phenotypes controlled by activation and microenvironmental changes (Yasojima et al., 1999). Cell culture conditions as well as other microenvironmental conditions significantly change the macrophage phenotype, so it has to be taken into account in planning experimental protocols. On the other hand, one of the major challenges in CNS immunology is to understand the phenotype–function relations of CNS macrophages. Novel methodology of laser capture microdissection and systems biology approaches including gene arrays and proteomics are promising. In addition, we should emphasize that BBB particularly the endothelial wall of blood vessels is an important interface between immune and nervous systems. The BBB epithelial cells and the participating astrocytes, macrophages are in focus of research interest for understanding the early mechanisms of neurodegenerative and autoimmune diseases of the brain. To give even a brief summary of the actual stage of BBB research, it requires a complete chapter and it has been done in excellent reviews elsewhere, so here we just attract the attention to the importance of that issue.

3 Mediator and Modulator Molecules of Neuroimmune Cross Talk

Neuroimmune mediators are small diffusible compounds released and detected by both, CNS cells and immune cells. They are the information mediator molecules and substrates of the common biochemical language of CNS and immune system. Neuroimmune modulators are hormones, cytokines, neuropeptides, and chemokines.

3.1 Neuropeptides as Neuroimmune Modulators

Neuropeptides are involved in communication between immune system and CNS. Participation of VIP, substance P, and CGRP in neuroimmune communication has been properly established but some role of other neuropeptides like neuropeptide-Y, galanin, somatostatin, and neurokinin A has also been reported. Immune cells express neuropeptide receptors and they also express neuropeptides. The main hot spots of neuroimmune communication are the open areas of the BBB, where immune cells can be in direct contact with neurons and glia cells and communicate to each other via releasing small molecules such as neuropeptides (Rubin and Staddon, 1999).

VIP is present in the immune system. It was observed in lymphocytes, mast cells, and other leukocytes. Expression of VIP gene has been described in CD4 and CD8 thymocytes and in spleen lymphocytes. Lymphocytes do only not express VIP mRNA and accumulate VIP in the cell body, but they release VIP on immune stimulation (Delgado et al., 2004). There are controversial studies on macrophages expressing VIP. Expression and secretion of VIP depends on immune stimulation of the organism by LPS or cytokines (IL1 β , IL6, or TNF). Any process increasing cAMP levels or activating PKC increases VIP expression and secretion (Delgado et al., 2004). VIP and pituitary adenylate cyclase-activating peptide (PACAP) are bound to a receptor of B1 receptor subfamily expressed in immune cells and CNS cells. In CNS, the microglia is the resident macrophage and responds to VIP and expresses VIP. Microglia activation is a hallmark of Alzheimer's disease and also activated by cytokines released under CNS inflammation. Microglial activation and also the VIP secretion from microglia cells can be modulated by VIP. VIP release and expression is modulated by small dose of LPS. It has been disclosed that VIP is neuroprotective in CNS inflammatory diseases.

CGRP is a 37 amino acid neuropeptide binding to its G-protein-coupled receptor. Receptor activation requires a receptor activity-modulating protein (RAMP). CGRP has neurotrophic and neurotropic effects in CNS and also regulates antigen presentation of immune system cells. On the other hand, CGRP is the most potent endogenous vasodilator. The epidermal Langerhans cells belonging to the dendritic cell family of the immune system are controlled by CGRP-containing axons and CGRP downregulates antigen presentation by Langerhans cells (Tsatsaris et al., 2002). Thus, CGRP is a typical communication molecule having roles both in CNS and immune system.

Neurotrophins are also involved in modulation of immune system. Neurotrophins are a group of polypeptide growth factors that control development and maintain functions of the nervous system. Particularly, nerve growth factor (NGF), neurotrophins, namely NT-3, NT-4, NT-5, NT-6, NT-7, and brain-derived nerve growth factor (BDNF) are involved in immune system modulation. Neurotrophin receptors (tyrosine kinase receptor, Trk A, B, C forms) are expressed on all cells of immune system and in several components of the lymphoid system. The number of receptors increases after activation of immune cells and neurotrophins enhance cytokine release. Neurotrophins are candidate molecules for regulating communication in the immune system and also could control neuroimmune cross talk. NGF is involved in the pathomechanism of autoimmune inflammatory diseases (Vega et al., 2003).

Substance P is also involved in immune system modulation. Substance P is expressed in lymphocytes and it is a key transmitter in pain sensation. The sickness response, which is the response of brain to peripherally released cytokines, is transmitted by substance P in great extent. The sickness-induced hyperalgesia develops when central IL and TNF release is increased from astrocytes expressing substance P receptors (Hartung and Toyka, 1989). The origin of hyperalgesia is the spinal cord glutamate and substance P containing neuron population. Astrocytes expressing glutamate and substance P receptors

can be activated by substance P and in turn they release brain cytokines. Microglia is able to activate the same astrocyte population as substance P and the released substances from astrocytes further activate the microglia forming a positive feedback loop. It suggests that substance P is a key element of the molecular mechanism controlling inflammatory processes in CNS (Wiedermann et al., 1987).

3.2 Hormones in Neuroimmune Communication

Interaction of stress response with immune system has been widely studied in the past twenty years. Stress is defined as a state of disharmony in homeostasis provoked by psychological or/and environmental influences called stressors. In stress response, the neuronal and the immune systems interact forming a network of regulation of the internal and external homeostasis. Cytokines released by activated immune cells influence CNS and CNS acts on the immune system. Using hypothalamic–pituitary–adrenal axis, CNS controls the immune system by downregulating the immune response. That control is responsible for many different pathological modulations in immune reaction during and after psychiatric disorders. Interestingly, molecular mechanisms involved in immune system control of stress-induced psychiatric disorders like anxiety or depression are amazingly similar (Black, 1994).

Evidences for CNS influence on immune system derive from electrolytic lesion experiments destroying different areas of the hypothalamus. Immune suppression and facilitation were evoked by lesions depending on the hypothalamic area destroyed (Khansari et al., 1990). Hypophysectomy prevented the effects of hypothalamic lesions indicating that pituitary gland is necessary for CNS action on immune system. Molecular substrates of CNS–immune system cross talk in stress are the neurotransmitter and hormone receptors expressed in lymphocytes and macrophages as well as the cytokine receptors in hormonal system and in CNS (Black, 1994).

CRF is the main coordinator molecule of stress response and CRF containing neurons are scattered all over the brain but the highest density of CRF neurons is in the paraventricular nucleus (PVN) of the hypothalamus. PVN receives pathways from many different brain structures including limbic system and forebrain. Somatic and viscerosensory systems also send axons to the PVN, so it is a highly integrative center in the hypothalamus. Monoamine neurotransmitters such as serotonin, noradrenaline, as well as acetylcholine, glutamate transmit the information toward PVN. CRF is the key component of the stress mechanism and initiate many different processes. Some of them reach the immune system as modulator or regulator. Immune system sends a feedback to CRF releasing PVN neurons via cytokines (Licinio and Wong, 1997). Blood cytokines enter the brain and induce release of brain cytokines, initiating behavioral and sensory processing changes. Cytokine receptors are all over the CNS, so an increase in brain cytokines could result in a complex change in neuroendocrine modulation. Since most of the known hormones have some influence on the immune system, we will focus on only those which have been investigated in detail. They are as follows: corticosteroids, catecholamines, endogenous opiates, growth hormone, prolactin. It has to be noted, however, that somatostatin, cholecystokinin, oxytocin, melatonin, sex steroids, thyroxine, VIP, and arginine–vasopressin have also influences on the immune system but at the present stage of knowledge the mechanism of their action is not clear.

Corticosteroids have profound immune suppressive effect when the hormone levels increase in stress (Roy and Loh, 1996). Besides the corticosteroid action on lymphoreticular system, marked antiallergic and anti-inflammatory effects of them can be observed. Adrenalectomy or depletion of adrenaline-induced corticosteroid elevation on any other way depletes the immune-suppressive effect of stress but not entirely because some other molecular mechanisms of stress-induced immune suppression remain intact. It is supported by the fact that in aged stressed rats the tumor implantation is more successful than in young stressed rats due to the elevated corticosteroid levels in aged rats' brain.

Catecholamines, such as norepinephrine and epinephrine, also accompany molecular mechanisms of stress. They change lymphocyte, monocyte, and leukocyte activation and plasma epinephrine level is a good indicator of stress and inversely related to specific immune system functions. β -adrenergic receptors are expressed on lymphocytes and macrophages, in turn β -adrenergic agonists and antagonists down- or upregulate immune functions, respectively. β -agonists applied before initiating immune response can

increase antigen production. The late immune response, however, is suppressed by β -agonists as it is indicated by decreased antibody production and response to cytokines. Interestingly, ablation of sympathetic nervous system enhances autoimmune reaction, augmenting experimental allergic encephalitis, experimental autoimmune multiple sclerosis. It suggests that sympathetic nervous system activity generally downregulates immunity and the absence of sympathetic influence enhances autoimmunity (Pavlov and Tracey, 2004).

Endogenous opiates are a large group of peptides found in the brain and pituitary gland, which have antinociceptive effect. Endogenous opiates have three groups. One is the endorphin family, the second is the enkephalin family, and the third is the dynorphin. Opiates bind to their specific mu, delta, and kappa opiate receptors. The opiate level increases in stress in conjunction with catecholamine release. The opiate effect on immune system is clearly established but the results are conflicting. Opiates are mostly immunosuppressive, as it is supported by application of opioid receptor blockers like naltrexone (Roy and Loh, 1996). Natural killer cells were depressed and interferon release from lymphocytes was also decreased by opiates and mitogenesis of lymphocytes was depleted. However, some early studies reported that opiates can be immunostimulatory. Contradictions in experimental data on opiate action on immune system are mainly derived from differences in experimentation protocols. Because of complexity of CNS control on immune system, the age and sex in *in vivo* or *in vitro* conditions can controversially influence the results.

Growth hormones and prolactin are immunoenhancing agents of pituitary gland. Immune-suppressive effect of hypophysectomy can be antagonized by growth hormone and prolactin. Growth hormone and prolactin receptors are expressed on lymphocytes and macrophages. Growth hormone receptors can influence T lymphocytes, monocytes, stem cells, whereas prolactin controls T lymphocytes and macrophages. Both hormones are essential for thymus cell differentiation via receptors of thymus epithelial cells. Prolactin and growth hormone levels elevate in early phase of stress response and it is proposed that prolactin buffers the immune-suppressive effect of stress. It is unlikely because the prolactin level decreases in late phase of the stress response to the prestress value and the immune suppression could develop. Growth hormone and prolactin release is controlled by many other hormones and neurotransmitters like serotonin, CRF, oxytocin, and so on; so this complex regulatory network of prolactin release makes investigation of endocrine influence on immune system very difficult (Williams and Frohman, 1986).

Compounds mediating CNS–endocrine–immune system cross talk are neurotransmitters, neuropeptides, and hormones. Immune system cells like lymphocytes, macrophages, killer cells, and stem cells express their receptors and several neuropeptides and neurotransmitters are synthesized and released by immune cells not only by neurons (Feldt, 1991). Neurotransmitters and modulators from the effectors of CNS mediate the controlling messages from CNS structures, mainly from hypothalamic regions to the immune cells. In spite of the immense data, many important questions of neuroimmune communication remain unsolved. We do not know how the neurotransmitters and modulators reach the targeted immune cells; there are many inconsistent data on hormonal and neuropeptide actions on immune cells. At the actual state of knowledge, however, it can be suggested that molecular mechanisms of neuronal and hormonal control mechanisms of immune system could be important targets of development of novel methods for medication of autoimmune and neurodegenerative disorders.

3.3 Brain Cytokines

Brain cytokines are the main communication molecules in neuroimmune cross talk. Cytokines are soluble protein molecules that allow for communication between cells and external environment. Cytokines are expressed in the brain and modulate neuronal survival and cell death. To date, all cytokines were found in CNS cells including neurons. In addition to CNS cytokine effects, cytokines released by immune cells during inflammation can modulate physiological, neuroendocrine, and behavioral response to inflammation (Larson, 2002). Cytokines can modulate body temperature inducing fever, enhance sleep and modulate the hypothalamo–pituitary–adrenal gland axis, and initiate sickness behavior (Dantzer et al., 1998). In addition, cytokines effect depression and anxiety and modulate cell survival in stroke and hypoxia; so the scale of cytokine action on brain is fairly wide. Cytokines bind their specific receptors expressed on neurons,

astrocytes, oligodendrocytes, and microglia cells. Neurons express receptors for IL1, IL2, IL3, IL6, IL8, TNF α , and GM-CSF. In addition, neurons express the protein of IL1, IL2, IL3, and IL6. Astrocytes express receptors for IL1, IL3, IL4, IL6, IL7, IL8, IL9, IL10, TNF α , TGF β , and GM-CSF. Expression of cytokine proteins has been found in astrocytes including protein of IL1, IL3, IL5, IL6, IL10, IL11, IL15, TNF α , IFN, TGF β , GM-CSF, and M-CSF. Oligodendrocytes express cytokine receptors in great extent but only a few cytokine proteins are expressed by oligodendrocytes. Oligodendrocytes have receptors for IL1, IL2, IL3, IL4, IL7, IL15, TGF β , GM-CSF, and M-CSF. However, only IL1 and TGF β proteins are expressed in oligodendrocytes. Microglia cells, one of the tissue resident macrophages in the brain, express receptors of IL2, IL3, IL4, IL5, IL6, IL7, IL8, IL10, TNF α , IFN, TGF β , GM-CSF, and M-CSF. Protein of IL1, IL5, IL6, IL10, IL12, IL15, TNF α , TGF β , and M-CSF has also been found in microglia cells.

One of the main questions of cytokine action in the brain is that how and why such large molecules as cytokines (15–20 kDa) penetrate the BBB. Peripherally released cytokines act like hormones stimulating the hypothalamo–pituitary–adrenal axis and regulate neuroendocrine functions, sympathetic nervous system responses, behavioral events like fever, sickness, and sleep (Larson, 2002). Cytokines not only simply penetrate the BBB but also cross it at penetrable spots of the barrier like the periventricular organs. Cytokines also bind their receptors on cerebral blood vessels and signal the brain via NO and prostaglandins (De Simoni and Imeri, 1998). A rapid action of peripheral cytokines can reach the brain on vagal nerve (Floto and Smith, 2003). After peripheral cytokine stimulation on CNS, the central mechanisms of stress response are activated and corticotropin-releasing hormone (CRH) and adrenocorticotrophic hormone (ACTH) are released from the hypothalamus and from the pituitary gland, respectively. In turn, adrenal gland releases glucocorticoids and as a final step cortisol in humans and corticosterone in rodents are released. As a feedback regulation, glucocorticoids suppress the release of some cytokines, not all of them. IL12, TNF α , and IL1 are the most sensitive cytokines for glucocorticoid suppression (Blalock, 2005). IL6 is relatively resistant to glucocorticoids. Summarizing the data of glucocorticoid effect on cytokines, it can be stated that glucocorticoids suppress proinflammatory cytokines and stimulate anti-inflammatory cytokines such as IL4 and IL10. Thus, we can conclude that glucocorticoids play a role in physiological adjustment of immune response. The molecular mechanism of glucocorticoid action on cytokines is unknown.

In addition to the neurohumoral routes of modulation of immune system by CNS, there are some more direct routes because autonomic and peripheral nervous systems innervate the immune system. Dense innervations can be observed in spleen, thymus, bone marrow, and lymph nodes (Mignini et al., 2003). In these innervations, the immune cells and the nerve cells are in close apposition, so they could form synapse like communication contacts (see more details in the following chapters). In such a close contact neuropeptides, neurotransmitters could exert effects on immune system. Molecular mechanisms of that borderline of immune and nervous systems are unknown and we are to give brief summary on the immunological and neuronal synapses later to shed some light to the relevance of ideas on neuroimmune synaptic communication.

There are evidences of interferences between neuropeptidergic communication and cytokine release. For example, VIP can enhance release of IL3, GM-CSF, IL6, TNF α , IFN, IL1 β , and IL1 α from astrocytes (Delgado et al., 2002). So the given variety of neuropeptides and cytokines in a small microenvironment containing neuronal endings and immune cells raises the possibility of complex interactions to be disclosed in near future. As we are just getting understand the importance and basic principles of neuroimmune cooperation in maintenance of homeostasis of the body, it is an open area of investigation. We have already understood that when the hypothalamus–pituitary–adrenal axis is damaged or altered in function, the subjects are enormously sensitive for inflammatory diseases while overstimulation of endocrine system can lead to either impaired immune function or increased susceptibility for infectious diseases. Depression of hormonal stress reaction was associated with autoimmune diseases like allergy, inflammatory diseases like rheumatoid arthritis, posttraumatic stress disorder, chronic fatigue, and atypical depression (Lechin et al., 2002).

Recently, clinical psychiatry was attracted by neuroimmune cross-talk principle because of the effects of cytokines on brain cells changes in psychiatric disorders like major depression, Alzheimer's disease, and schizophrenia. From early 1980s, several authors published observations on stress and cytokine receptor mRNA changes and changes in concentrations of different cytokines in humans. The stress induction and subjects were very different from examination stress to negative emotions and from volunteered students to

multiple sclerosis patients. The cytokines changes varied by experimental conditions and also by subject groups. The conclusion of these studies is that negative emotional stress and any kind of stress-inducing anxiety fair suppress immune response and decrease cytokine secretion (Lechin et al., 2002). Positive emotions and positive stress like examination stress of students could enhance immune response and cytokine release. Some cytokines have been tested in clinical practice and some of them induced psychiatric adverse effects. Interferons induced fatigue, depression, cognitive impairments, psychosis, and suicidal ideation (Gutterman, 1994). IL1 induced somnolence, confusion, and delusions. TNF α induced anorexia and fatigue. In schizophrenia, IL2 receptor concentration and IL6 secretion increase in the blood (Kronfol and Pemick, 2000). So it is an accepted idea that some types of schizophrenia are related to viral or bacterial infections.

One of the novel ideas of Alzheimer's disease pathophysiology is that cytokines play a critical role in its development and progression. Cells associated with amyloid plaques can produce cytokines; amyloid- β by itself can stimulate microglia to secrete proinflammatory cytokines. IL1, IL6, TGF β , and TNF α have been associated with Alzheimer's disease progression. TNF α and TGF β are present in senile plaques and contribute to the lesions around the plaques. IL1 level is elevated in the brain tissue of Alzheimer's disease patients. IL1 induces increased release of neurite extension factor S100 β from activated astrocytes and in turn, the enhanced neurite growth promotes neuritic plaque formation. IL1 also regulates heparin sulfate proteoglycan synthesis, which is an important promoter factor of amyloid- β aggregation (Tarkowski et al., 2003).

4 Synaptic Innervation of Immune System by Autonomic Nervous System

Autonomic nervous system innervates all organs in the body including immune system. Accumulating evidences support that immune system is modulated by stress induced by the environment or by the pathological change in the internal environment under different diseases. Autonomic nervous system has two distinct parts: the sympathetic and the parasympathetic system. In physiological conditions, the balance of the two subsystems maintains the homeostasis, stabilizes blood pressure, heart rate, and so on. Sympathetic nervous system uses noradrenaline for neurotransmission and parasympathetic nerves are cholinergic. The involvement of autonomic nervous system in modulation of inflammatory and immune response is an exciting new field of research.

4.1 Cholinergic Anti-Inflammatory Pathway

The cholinergic anti-inflammatory pathway was discovered recently when the anti-inflammatory role of vagal nerve was demonstrated in endotoxemia and shock (Pavlov et al., 2003). It is a previously unrecognized cholinergic pathway for neuronal inhibition of inflammatory reaction and a novel interface between brain and immune system. The immune-to-brain communication can use two distinct mechanisms: one is neuronal and the other is humoral. Vagal nerve afferent fibers can be activated directly by cytokines released by dendritic cells, macrophage-associated immune cells, or indirectly via chemosensitive cells (Steven et al., 1998). The vagal afferents project to the solitary tract nucleus (NTS) and the connections of NTS are able to activate the PVN, in turn that activation reaches one of the main regulatory centers of hormonal system. Physiological evidences support that vagal nerve fails to suppress high-dose endotoxin-induced inflammation but it is efficient in suppression of reaction to mild or moderate doses. So the vagal cholinergic pathway has a particular importance in certain types of moderate inflammatory reactions (Pavlov et al., 2003).

The humoral mechanism of NTS activation by immune system is mediated by blood cytokines crossing the BBB entering the cerebrospinal fluid at circumventricular organs. Area postrema, one of the penetration sites, is very close to NTS and so the entering cytokines could activate NTS neurons. Cytokines can also bind to the capillary endothelial cell cytokine receptors and enhance release of different neurotransmitters as NO (Bianchi et al. 1995).

Brain-to-immune communication is the efferent part of cholinergic anti-inflammatory pathway. The humoral pathway is the hypophysis–adrenal gland axis, which reduces inflammation via releasing

glucocorticoids and catecholamines. Cholinergic fibers participate in PVN–pituitary communication. Parasympathetic fibers also innervate immune system and vagal nerve fibers reach the thymus. This direct effect of vagal efferents on immune cells has not been properly established yet. Vagal efferents are able to suppress the release of inflammatory cytokines such as IL1 β , IL6, IL18, and TNF α (Steven et al., 1998).

Acetylcholine has also a direct influence on immune cells. It turned out that immune cells express nicotinic acetylcholine receptor proteins (Hunyady et al., 1997). Lymphocytes not only express the receptor but also synthesize acetylcholine as well. Acetylcholine is immune-suppressive *in vitro*, decreasing levels of ILs and TNF. Vagotomy results in increased immune reaction, suggesting that vagal tone is important for immune homeostasis. Vagal stimulation has anti-inflammatory effect in local inflammation models such as carrageenan-induced inflammation in rodents (Tracey, 2002). It is a fast immunomodulatory effect, much faster than humoral regulation of immune system. Using pharmacological agents, anti-inflammatory effect of CNI-1493 requires vagal nerve; bilateral vagotomy eliminates all effects of it. Nicotinic acetylcholine receptor- $\alpha 7$ subunit is the essential component of the receptor necessary for cholinergic anti-inflammatory reaction (Pavlov et al., 2003). Clinical implications of cholinergic anti-inflammatory pathway include the application of implanted vagal nerve stimulator for suppression of immune response in autoimmune diseases, application of cholinergic agonists in endotoxemia and sepsis, and new methods for the treatment of autoimmune diseases.

4.2 Nonneuronal Cholinergic System and Immune Modulation

A nonneuronal cholinergic system is a network of nonneuronal cells producing acetylcholine. Such cells are widely expressed in the upper airway. Epithelial cells lymphocytes, macrophages, and mast cells could produce acetylcholine and they also express cholinergic receptors (see Wessler and Kirpatrick, 2001). The cholinergic gene encodes expression of vesicular acetylcholine transporter and choline–acetyl transferase protein and contains the regulatory sequences for controlling their expression. When a cell becomes cholinergic, the concentration of choline and acetyl CoA is about 50 and 5 μ M, respectively. The acetyl group is derived from pyruvate. Interestingly, almost all mammalian cells have the components of acetylcholine synthesis but the concentrations of the enzymes are not sufficient for high quantity acetylcholine synthesis. The main functional role of the nonneuronal cholinergic system is that it could enhance the neuronal cholinergic influence locally.

5 Synaptic Communication is not the Privilege of Neurons

Cells all over the body communicate through the extracellular space by specific communication molecules having receptors on the surface of communicating cells. The extracellular space is not a space in the common sense, but it is filled up by special protein molecules forming the extracellular matrix. At particular spots of the membrane, the communication is more intensive than on the rest of the cell surface. The communication “hot spots” were named as synapses in the nervous system. In the last decade, it turned out that synapses are adhesive junctions transmitting information by directed secretion. As it has been revealed, synapses have a complex but amazingly uniform molecular construction. Comparison of immune system with nervous system disclosed that both systems use specific molecular recognition events between discrete cells, cell–cell adhesion, positional stability, and directed secretion for communication necessary for their responsive functions. Both systems have sophisticated molecular mechanisms for information storage. The neuronal synapse concept is >100 years old. The immune system synapse has been determined only recently. The function of the synapse in the immune system is to make the freely spreading small compounds direction specific; they coordinate cell migration and antigen recognition during immune response. The two synapses were approached so differently that it seems very promising to compare them to understand the functions of the synaptic communication.

The critical difference between immune and nervous synapses is in their function and in the wiring of the cellular networks behind. CNS is hard-wired, keeping the main wiring intact throughout the life. The

immune system is more flexibly wired. In CNS, the cells are anchored to each other after the early ontogenesis preventing cell migration. The axons and dendrites are long; in turn, most of the CNS synapses act at a large distance from the cell body and nucleus. CNS synapses can be translocated and diminished but the dendrites and axons remain in place. One of the important consequences of that is the absence of direct translational control on CNS synapse and the synaptic transmission feedback to the transcription is slow and late. CNS synapses can alter their efficacy by clustering and remodeling their receptor sets (Dustin and Colman, 2002).

In contrast the immune system operates by rapidly migrating T cells and by their partner cells, the dendritic cell (DC). Each T cell expresses a different antibody but the spot where the antigen enters the body to associate with the DC cell cannot be predicted. Therefore, it is essential for T cells to make as many random contacts with DC cells as possible to find the matching DC cell and from a synapse (Dustin and Colman, 2002). Migrating T cells are similar to the growth cone of the neurons in early ontogenesis but much faster. By other words, T cells and DC cells cover greater distances than any neuron, but when they form a synapse they remain attached and proximal to the gene transcriptional machinery of the nucleus. DC cell is an APC destructing the antigen to peptides binding to the major histocompatibility gene complex molecule (MHC) and 300 MHC-p complexes on an APC can activate T cell via T-cell receptors and induce T-cell proliferation. One of the daughter cells of the T cell that became a memory cell can be activated by 50 MHC-p molecules. T-cell synapse is the best studied immunological synapse and it can be compared with the neuronal synapse in many aspects (Shaw and Allen, 2001). For prototypic synapse we have to create criteria differentiating the synapse from other cell-cell contacts.

Criterion 1. It is that cells remain individuals. It means that synapse is a contact but there is no continuity between cells. The synapse is formed by two opposing membranes and a fluid in the synaptic cleft in between.

Criterion 2. It is the adhesion. Cells at the early stage of synapse formation recognize each other and they make an adhesion contact. The pre- and postsynaptic membrane regions are tightly locked together.

Criterion 3. It is the stability. Cell adhesion molecules clamp the pre- and postsynaptic membrane together. This clamp is very stable in time but some adhesion molecules can change conformation like N-cadherin during functioning of the synapse; but in spite of that, the synaptic structure remains considerably stable.

Criterion 4. It is the directed secretion. At the presynaptic side, a specific secretory apparatus is assembled and activated by signaling events. On the postsynaptic side, specific receptors are clustered and a molecular machinery transforming the secretory signal to a relevant intracellular signal. A special zone of the synaptic microdomain surrounds the communicating central zone of the synapse limiting the lateral spread of secreted molecules. That microdomain could change in response to secretory activity.

In CNS synapse, the pre- and postsynaptic membrane is connected to the synaptic scaffold. It is the two apposed parallel plates of the membranes attached by filamentous material spanning the synaptic cleft. The pre- and postsynaptic thickenings are also attached to a cytomatrix and that complex molecular scaffold recovers in synaptosome preparations after treatment with detergents (Loscher et al., 1985).

The CNS synapse has neurotransmitter machinery integrated with the scaffold and its interaction with the scaffold is not understood. We do not concern with the synaptic mechanism of neurotransmitter release in this chapter since we did it earlier. Several groups of recognition and adhesion molecules are in the scaffold. Cadherins are the major adhesion molecules of CNS synapse, but some others such as integrins are also important in synaptic adhesion. It is suggested that cadherins are important factors in recognition process in the recognition phase of synapse formation and later they clamp together the synaptic membranes. Neuronal cell adhesion molecules (NCAM) are also important elements of the synaptic scaffold. Presynaptic density proteins are a protein family involved in the synapse formation (Dustin and Colman, 2002).

The immune synapse construction is different from the CNS synapse but there are several features of CNS synapses like receptor clustering, or quantal release of transmitters, which can be interesting aspects for immunologists and oppositely, some lines of investigations on the immune synapse can be very promising in CNS synapse research. After the discovery of T cells, APCs, and lymphocyte adhesion molecules in the early 1980s, it became clear that T cell–APC cell communication passes all criteria of synaptic communication. When T cell and APC cell interact, they remain as independent cells. Cell

adhesion molecules clamp them together and MHCp–T cell interaction is a stop signal stabilizing the connection for long term. Secretion of immune cells is vectorial, so the immune synapse has all principal properties of a CNS synapse but it works differently to some extent and there are surprising similarities as well (Dustin and Colman, 2002).

The immune synapse is at the micron scale and it is a supramolecular activation cluster (SMAC) of particular proteins. The interaction zone between T cell and APC cell has a central SMAC surrounded by a ring of integrin-mediated cell adhesion molecules (ICAM) and microtubules are radially connected to the SMAC (Dustin and Colman, 2002). When the immune synapse is immature, the antigen presentation for T cell is at the peripheral zone of the synapse and the center is dominated by adhesive protein interactions. The first stage of immune synapse is dominated by adhesion molecule–receptor interactions and each molecule pair has their own distance determining the synaptic gap (Dustin and Colman, 2002). The SMAC formation is less known but it is hypothesized that an actin–myosin-dependent capping process forms the SMAC. There are no direct evidences for that.

The function of immune synapse is complex. One role is the induction of T-cell proliferation via integration of signals. Synapse can sustain signal patterns from APC cells in the early stage of the synapse. Later, the synapse became active and the SMAC zone is involved in secretion of small molecules such as cytokines and cytotoxic agents. The small and soluble molecules are held by SMAC, so they cannot diffuse freely away from the synaptic area. Comparing the immune synapse with CNS synapse in immune synapse integrins forms the gasket of the synapse while cadherins have the same role in the CNS synapse. Immune synapse changes functions within minutes and it is clearly demonstrated that in the early stage of synapse formation the molecules of the synaptic region play a different role dominantly in cell recognition and adhesion, later the secretion is the main function of the synapse (Shaw and Allen, 2001). As the synapse formation is faster in the immune system than in CNS and the size of the immune synapse is big, immune synapse is an ideal model for studying the early phase of synapse formation.

Recently, immunologists verify more and more CNS synapse proteins in the immune synapse. Agrin has a crucial role in neuromuscular synapse as clustering protein keeping the receptor molecules at the middle of the synapse. It has been identified in the immune synapse between T cells and APC cells (Mossman et al., 2005). That finding stimulates synaptologists and suggests that CNS and immune system synapses are variations of the same theme and organized by the same principles. The linkage of receptors to the synaptic scaffold by rapsyn, the importance of supramolecular structure of the synapse in general, the functional significance of central or peripheral location of a certain receptor molecules, and so on are common principles in both synapses. The scaffolding proteins are the same in the two synaptic structures. Postsynaptic density protein 95 (PSD95), gephyrin, GRIP, homer, Pick1, GABARAP are scaffolding proteins identified in CNS synapse first than in immune synapse (Mossman et al., 2005). Although the actual state of knowledge about CNS synapse is considerably more detailed compared with what it is known about immune synapse but immunologists found some important new aspects of synapse formation. One is that synapses can be formed rapidly and their lifetime can be as short as some minutes. It was disclosed because immunologists focused on synapse formation. In addition, the importance of protein charge and size in structure of membrane was studied by the immunologists in detail. Immune synapse develops quickly, so immunologists disclosed many novel mechanisms of molecular arrangement in cell contacts which could help in understanding neuronal development of CNS synapses. In conclusion, immunologists and neurobiologists can learn from each other about the synaptic communication and this is a promising new interdisciplinary research area for the future.

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14 Role of Glia in CNS Inflammation

S. Pawate · N. R. Bhat

1	<i>Introduction</i>	311
1.1	Microglia	312
1.2	Astrocytes	313
1.3	Glial Cells in Innate and Adaptive Immunity	313
2	<i>Molecules and Mechanisms of Inflammatory Signal Transduction in Glial Cells</i>	313
2.1	Microbial Products and Toll-Like Receptors	313
2.1.1	Toll-like Receptor Signaling	314
2.2	Cytokines and Cytokine Signaling	314
2.2.1	Interleukin-1 β	315
2.2.2	Interferon- γ	315
2.2.3	TNF α	315
2.2.4	IL-6 Family of Cytokines	315
2.2.5	CD40–CD40 Ligand	315
2.3	Chemokines	316
2.4	Disease-Specific Abnormal Proteins	316
2.5	Downstream Signaling: NF κ B	316
2.6	Downstream Signaling: MAP Kinases	316
2.6.1	p38 MAP Kinase	317
2.6.2	JNK	317
2.6.3	ERK	317
3	<i>Glial Mediators of Inflammation</i>	318
3.1	Cytokines, Chemokines	318
3.2	Reactive Oxygen Species and NADPH Oxidase	318
3.3	Nitric Oxide	318
3.4	Proteases	319
3.5	Mediators of Excitotoxicity	319
3.6	Antiinflammatory Mediators	319
4	<i>Role of Glial Cells in Neuroinflammatory and Neurodegenerative Diseases</i>	319
4.1	Glia in Alzheimer Disease	319
4.1.1	Introduction	319
4.1.2	Pathology and Pathogenesis	320
4.1.3	Role of Glial Cells	320
4.2	Glia in Parkinson's Disease	321
4.2.1	Introduction	321
4.2.2	Pathology	321
4.2.3	Oxidative Stress	322
4.2.4	α -Synuclein and Neuromelanin	322
4.2.5	Role of Glial Cells in PD	322

4.3 Glia in Amyotrophic Lateral Sclerosis 323

4.3.1 Introduction 323

4.3.2 Pathology and Pathogenesis 323

4.3.3 Role of Glia in ALS 323

4.4 Glia in Multiple Sclerosis 324

4.4.1 Introduction 324

4.4.2 Pathology 324

4.4.3 Role of Glial Cells 324

4.5 Glia in Other Neurological Diseases 325

4.5.1 HIV Encephalitis 325

4.5.2 Prion Diseases 325

4.5.3 Traumatic Brain Injury and Stroke 325

5 *Concluding Remarks* 326

Abstract: Inflammation is being increasingly recognized to play pathogenic roles in diverse neurological conditions and diseases including not only primary inflammatory diseases such as multiple sclerosis and HIV dementia but also primary degenerative disorders such as Alzheimer's and Parkinson's, as well as in brain injuries resulting from stroke and trauma. In the CNS, microglia, the resident macrophages, and astrocytes, the major endogenous glial cell type, together represent the main immunocompetent cell system that responds to any number of brain injuries, infections, and disease conditions, thereby mediating the local inflammatory response, i.e., "neuroinflammation." The stimuli that activate these cells come in the form of microbial products, cytokines, products released by injured neurons, and disease-specific abnormal molecules. Activated glial cells in turn release several potentially neurotoxic mediators including cytokines, chemokines, reactive oxygen species, and nitric oxide, which target bystander neurons and oligodendrocytes. Although in acute settings, glial activation may be beneficial by clearing out debris and allowing repair to occur via released trophic factors, situations of their chronic response would entail deleterious neurodegenerative outcome. A better understanding of these processes would help devise appropriate neuroinflammation-targeted interventions against neurological diseases.

In this chapter, we review the mechanisms of glial cell activation by different stimuli, associated inflammatory signaling pathways, and the induced release of inflammatory mediators. This is followed by a review of the current literature highlighting the roles of glial cells in some of the common neurodegenerative and inflammatory diseases including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, prion disease, multiple sclerosis, and HIV encephalitis.

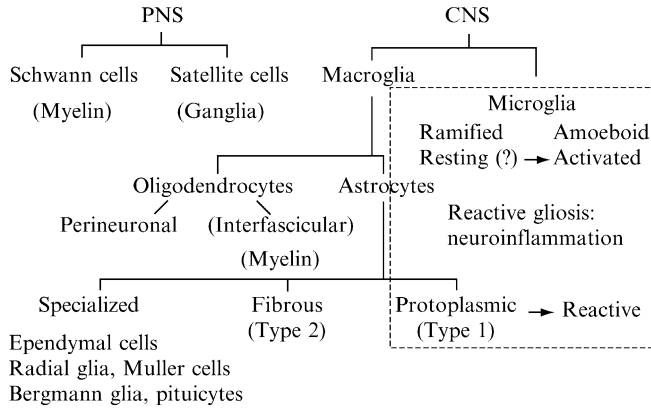
List of Abbreviations: ALS, amyotrophic lateral sclerosis; APCs, antigen-presenting cells; APP, amyloid precursor protein; BBB, blood brain barrier; BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt–Jakob disease; CNTF, ciliary neurotrophic factor; CSAIDs, cytokine-suppressive anti-inflammatory drugs; DOPAC, dihydroxyphenylacetic acid; ERK, extracellular signal regulated kinase; FFI, fatal familial insomnia; GAS, gamma-activated sequence; GSS, Gerstmann–Sträussler–Scheinker syndrome; HAART, highly active antiretroviral therapy; HAD, HIV-associated dementia; HERV, human endogenous retroviruses; ICAM-1, intercellular adhesion molecule-1; IKK, I κ B kinase; iNOS, inducible NOS; IRAK, IL-1R-associated kinase; IRF, IFN-regulatory factor; JNK, c-Jun N-terminal kinase; LDL, low density lipoprotein; LIF, leukemia inhibitory factor; LPS, lipopolysaccharide; LRP, lipoprotein (LDL)-like receptor related protein; LTA, lipoteichoic acid; MAP, mitogen-activated protein; MAPKAPK2, MAP kinase-activated protein kinase-2; MAPKK, MAPK kinase; MAPKKK, MAPK kinase kinase; MMPs, Matrix metalloproteases; MSKs, mitogen- and stress-activated kinases; NCAM, neural cell adhesion molecule; NF κ B, nuclear factor κ B; NOSs, nitric oxide synthases; ODN, oligodeoxynucleotide; OSM, oncostatin M; PAMPs, pathogen-associated molecular pattern; PET, positron emission tomography; RAGE, receptor for advanced glycation end products; ROS, reactive oxygen species; SNpc, substantia nigra pars compacta; SOD1, superoxide dismutase-1; STAT, signal transducer and activator of transcription; TIR, Toll/IL-1R; TLRs, Toll-like receptors; VMAT2, vesicular monoamine transporter 2

1 Introduction

Glial cells constitute the major part of the cellular composition of the CNS outnumbering the neurons by greater than 2:1. Among the glia are astrocytes, which were long thought, rather incorrectly, to have simply a supportive role for the neurons (glia=glue that supports neuronal networks) and oligodendrocytes, which myelinate the CNS axons (🔗 [Figure 14-1](#)). Besides these "macroglia," interspersed throughout the brain, are microglia that represent resident macrophage-like cells and function as the prime immune regulators of the CNS. Under normal conditions, the "resting" or ramified microglia perform homeostatic and surveillance roles. However, they become readily activated in response to injury (i.e., stroke), infection, and under a variety of disease settings including but not limited to primary neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) as

■ Figure 14-1

Glial cell classification. Glial cell types mediating neuroinflammation are bracketed with broken lines



well as primary inflammatory brain diseases such as multiple sclerosis (MS) and HIV-associated dementia (HAD). Activated microglia mediate and modulate local immune and inflammatory processes, as part of a neuroinflammatory response common to the previous conditions. These specialized roles of microglia are shared by astrocytes, which act as a second immune effector cell type upon undergoing a process called “reactive astrogliosis.” The description of glial (microglia and astrocyte) response to conditions of injury has evolved over the years starting from “reactive gliosis” to the recent terminology, “neuroinflammation.” According to a recent definition of the term, “neuroinflammation encapsulates the idea that microglial and astrocytic responses and actions in the CNS have a fundamentally inflammation-like character, and that these responses are central to the pathogenesis and progression of a wide variety of neurological disorders” (Mrak and Griffin, 2004). It follows, therefore, that suppression of neuroinflammation forms an important and a versatile therapeutic strategy to treat these conditions.

The immune functions of microglia and astrocytes were reviewed by Aloisi (2001) and Dong and Benveniste (2001), respectively. In this chapter, we will review the role of these glia, in neuroinflammation, with a focus on the work done since the publication of the reviews mentioned earlier.

1.1 Microglia

The prevailing notion is that microglia are of a monocytic origin and that they would have invested the brain during development before the establishment of the blood brain barrier (BBB) and become resident macrophages of the CNS. Microglia comprise about 10% of the total glial population (Vilhardt, 2005). In the resting state, they occur throughout the brain and possess a dendritic-ramified morphology. Although the functions of microglia in this state are yet undefined, they may provide trophic support for neurons and glia. There is new evidence that the “resting” microglia are, in fact, highly active, constantly surveying the surroundings. Thus, Nimmerjahn et al. (2005) performed a two-photon imaging of microglia in vivo, and found that the resting microglia continuously extend and retract their processes, monitoring their micro-environment. Focal injury or disruption of the BBB caused their immediate activation, resulting in their process extension toward the injured sites to shield them off from the healthy tissue.

Microglial activation follows a graded response and change in their morphology with the contraction of ramified cellular processes and conversion into an amoeboid macrophage-like state. The activated microglia proliferate; express increased levels of myeloid markers, receptors, and adhesion molecules; and display diverse functions including migration, phagocytosis, role in innate and adaptive immunity, and release of various inflammatory mediators, as will be discussed in the following sections.

1.2 Astrocytes

Astrocytes are the predominant glial cell type in the CNS. Although they can be broadly classified into protoplasmic (found predominantly in the gray matter) and fibrous (white matter-enriched) varieties, they do display further heterogeneity with respect to their function, pharmacology, and localization. Among astrocyte subtypes are ependymal cells that line the ventricles, radial glia that, in the developing brain, provide a scaffold for migrating cortical neurons, Bergman glia of the cerebellum, and Muller glia of the retina. In addition to providing structural and nutritional support to neurons, astrocytes regulate ionic and neurotransmitter balance in the neuronal microenvironment thereby regulating synaptic transmission, maintain BBB integrity by interacting with capillary endothelium, and even participate in the formation, maturation, and maintenance of synapses (Nedergaard et al., 2003; Newman, 2003; Ransom et al., 2003; Slezak and Pfrieger, 2003).

Relevant to the subject of this chapter, they also contribute significantly to immune and inflammatory processes in the CNS in cross talk with microglia.

1.3 Glial Cells in Innate and Adaptive Immunity

The primary purpose of the immune system is defense against infections (Chaplin, 2006). Innate immunity, an evolutionarily ancient mechanism of host defense common to plants and animals, is the initial, antigen-nonspecific response, accomplished by recognition of diverse microbial products, which are collectively known as pathogen-associated molecular patterns (PAMPs). Adaptive immunity is more evolved and represents the antigen-specific response of the vertebrate species to deal with the pathogenic challenge unmet by the innate immunity. It is mediated by T and B lymphocytes involving great variability and rearrangement of receptor gene segments, which enable specific recognition of foreign antigens and immunological memory of infection. Unfortunately, such immune responses to nonpathogen antigens can lead to allergy and autoimmunity. Adaptive immune mechanism involves recognition by helper T (Th) cells of an antigen only when it is presented by antigen-presenting cells (APCs) such as dendritic cells, macrophages, and B cells. APCs first internalize the antigen and then display it on their cell surface bound to a class II MHC molecule to be recognized by Th cells. An additional costimulatory molecule is then produced by the APC, resulting in Th cell activation.

As noted earlier, microglia are the major innate immune cells of the CNS and respond to injury and infection by releasing reactive oxygen species (ROS), nitric oxide (NO), and a large number of inflammatory mediators. While there are conflicting views on the role of astrocytes as APCs, activated microglia are known to upregulate their expression of MHC class II as well as costimulatory molecules (i.e., B7-1, B7-2, CD40, and ICAM-1), and hence, function as primary APCs (Olson and Miller, 2004). Microglia, therefore, are central players in both innate and adaptive immune responses in the CNS.

2 Molecules and Mechanisms of Inflammatory Signal Transduction in Glial Cells

A wide variety of substances have been shown in in vitro models to activate microglia. These range from microbial products, of which bacterial lipopolysaccharide (LPS) is the best studied, to cytokines (such as TNF α , IL-1 β , and IFN γ), chemokines, and to endogenous regulators such as heat shock proteins and an increasing number of identified disease-specific abnormal proteins. The major activators, and their signaling pathways, will be briefly reviewed below.

2.1 Microbial Products and Toll-Like Receptors

Toll-like receptors (TLRs) (Akira and Takeda, 2004; Pasare and Medzhitov, 2005; Sandor and Buc, 2005; Kawai and Akira, 2006) are the primary recognition systems that detect microbial products.

There are 12 different TLRs, which can be grouped into five subsets: (1) TLR4, which recognizes LPS and is perhaps the best characterized TLR, (2) TLR3, which recognizes double-stranded RNA, (3) TLR5, which recognizes bacterial flagellin, (4) TLR9 subfamily, which consists of TLR7, TLR8, and TLR9, which recognize viral and bacterial nucleic acids, with CpG DNA (microbial DNA containing large number of unmethylated cytosine–guanine nucleotides) recognition by TLR9 being the best characterized, and (5) TLR2 subfamily, composed of TLR1, TLR2, TLR6, and TLR10, which recognize bacterial lipid-based structures such as mycobacterial lipoteichoic acid (LTA), lipoproteins, and lipopeptides.

Using quantitative real-time PCR, Jack et al. (2005) found that microglia expressed TLRs 1–9, whereas astrocytes expressed TLR3, low levels of TLR 1, 4, 5, and 9, while the remaining TLRs were rare to undetectable. Bsibsi et al. (2002) used RT-PCR as well as immunohistochemistry on brain and spinal cords of control and MS patients, and showed a broad expression of TLRs in microglia and a more restricted expression in astrocytes.

A recent study by Ebert et al. (2005) compared microglial activation (as determined by NO production) in response to several TLR agonists including LPS, pneumolysin (TLR4 agonists), CpG oligodesoxynucleotide (ODN, a TLR9 agonist) and tripalmitoyl-S-glycerol-cysteine, and heat-killed *Acholeplasma laidlawii* (HKAL) (TLR-2 agonists), and found that TLRs 2, 4, and 9 all elicited comparable responses. They further showed that low concentrations of different agonists had additive or superadditive effects, suggesting that different microbial products together can activate microglia even at very low concentrations.

2.1.1 Toll-like Receptor Signaling

TLRs and interleukin-1 receptor (IL-1R) share a conserved cytoplasmic domain, which is known as the Toll/IL-1R (TIR) domain. There are three highly homologous regions known as boxes 1, 2, and 3 within the TIR domain. However in the extracellular domain, the TLRs have leucine-rich domains (Bell et al. (2003)) whereas IL-1Rs have immunoglobulin-like domains.

In the MyD88-dependent pathway of TLR signaling, ligand binding to TLRs leads to dimerization of the TLRs and recruits myeloid differentiation primary-response protein 88 (MyD88). MyD88 has a TIR domain in its C terminus, through which it binds to the TIR domain of TLR/IL-1R. The N-terminal domain of MyD88 has a death domain (DD), which allows it to recruit DD-containing IL-1R-associated kinase (IRAK) 4. IRAK4 then associates and phosphorylates IRAK1. This is followed by the recruitment of TNF receptor-associated factor 6 (TRAF6). Phosphorylated IRAK1/TRAF6 then dissociates from the receptor and associates with TAK1, TAB1, and TAB2, leading to phosphorylation of TAK1. IRAK1 is then degraded at the membrane and the TRAF6–TAK1–TAB1–TAB2 complex translocates to the cytosol, where TRAF6 is ubiquitinated and TAK1 is activated. TAK1 leads to activation of downstream signaling molecules such as mitogen-activated protein (MAP) kinases and nuclear factor κ B (NF κ B), which are discussed further later.

MyD88-independent signaling through TLRs is also being unraveled. This pathway activates IFN-regulatory factor (IRF) 3 and involves the late phase of NF κ B activation, both of which lead to the production of IFN β and the expression of IFN-inducible genes.

2.2 Cytokines and Cytokine Signaling

Cytokines, a class of glycoproteins and polypeptides, which include interleukins (IL), TNF α , and interferons, are involved in virtually every aspect of immunity and inflammation (reviewed by Gosain and Gamelli, 2005). Normally, they are present in the CNS at extremely low to undetectable levels, but accumulate rapidly in response to pathophysiological events. Cytokines may be proinflammatory (IL-1 β , IL-6, TNF α , IFN γ) or antiinflammatory (IL-4, IL-10, TGF β). Growth factors such as NGF and GDNF also belong to the cytokine family.

2.2.1 Interleukin-1 β

IL-1 α , IL-1 β , and IL-1 receptor antagonist (IL-1Ra) are members of the IL-1 family (Stylianou and Saklatvala, 1998), the former two capable of initiating signal transduction upon binding to the IL-1 receptor. IL-1Ra, as the name implies, binds to the IL-1 receptor but does not result in receptor activation. IL-1 β is induced by LPS, cytokines, and other stimuli and is thought to play a major role in neuroinflammation in response to stress, injuries, and infections. Binding of IL-1 β to its receptor results in the initiation of signal transduction similar to the one described earlier for TLR.

2.2.2 Interferon- γ

IFN γ is a prominent cytokine released by Th1 CD4+ cells and NK cells. There is some evidence for endogenous production of this cytokine by glial cells. IFN γ activates microglia and astrocytes by stimulating the receptor-associated tyrosine kinases, JAK1 and JAK2 and their targets, that is, signal transducer and activator of transcription (STAT) proteins. Upon phosphorylation, STATs translocate to the nucleus and bind to the γ -activated sequence (GAS) element of IFN γ -responsive genes (Schindler and Brutsaert, 1999; Ganster et al., 2001; O'Shea et al., 2002; Pestka et al., 2004). There is evidence that, in addition to the JAK-STAT pathway, IFN γ also signals through MAP kinase pathways (referenced in Pawate et al., 2004).

2.2.3 TNF α

TNF α is produced not only by macrophages and microglia but also by T cells, B cells, and astrocytes. Signaling by TNF α occurs through two TNF receptors, TNFR1 (p55) and TNFR2 (p75) (reviewed in MacEwan, 2002), leading to the activation of several intracellular pathways including NF κ B and MAP kinase pathways. In addition, TNFR1 activates a cascade of death protease signaling while TNFR2 tends to be antiapoptotic.

2.2.4 IL-6 Family of Cytokines

IL-6 belongs to a family of ten or so cytokines including IL-6 itself, IL-27, leukemia inhibitory factor (LIF), oncostatin M (OSM), and ciliary neurotrophic factor (CNTF), which commonly use a receptor complex containing the subunit, gp130, and signal through JAK/STAT pathway. In contrast to IFN γ , which prefers JAK/STAT1, the IL-6 family utilizes JAK/STAT3 module. IL-6 cytokines elicit pleiotropic responses in addition to being inflammatory mediators, and in the CNS, primarily signal astrocyte activation in response to injurious signals as well as astroglialogenesis during normal development.

2.2.5 CD40-CD40 Ligand

CD40 is a transmembrane glycoprotein that belongs to the TNF receptor superfamily (van Kooten and Banchereau, 2000), expressed in various cell types including macrophages, endothelial cells, vascular smooth muscle cells, and B cells, where it was originally discovered (Armitage et al. 1992; Kehry, 1996). Both astrocytes and microglia express CD40, which acts as a costimulatory molecule and by heterologous interaction with its ligand, serves to amplify local immune responses. Its ligand, CD40L, is a member of the TNF family and is present as a trimer, expressed by activated CD4+ T cells as well as monocytes/macrophages. Binding of CD40L to CD40 results in the initiation of signal transduction by protein tyrosine kinases, leading to downstream activation of MAP kinases.

2.3 Chemokines

Chemokines are small, biologically active compounds that regulate leukocyte trafficking during inflammatory responses (Murphy et al., 2000). Growing evidence indicates a crucial role for these chemoattractants in neuro–glia–vascular interactions. The two major types are CC chemokines, typified by the presence of two adjacent cysteines near the N terminus; and CXC chemokines in which the corresponding cysteine residues are separated by one amino acid. The other two are C and CX3C types.

Specific chemokines, expressed rather disproportionately among neurons and all three glial cell types, are monocyte chemoattractant protein-1/CCL2, stromal cell-derived factor 1/CXCL12, fractalkine/CX3CL1, IFN γ -inducible protein 10/CXCL10, macrophage inflammatory protein 1 α /CCL3, and CCL5 (Gebicke-Haerter et al., 2001). In addition to playing a role in immune response, the chemokine system (chemokines plus their cognate receptors) contributes to normal brain development and function. Chemokine receptors signal through the G-protein-coupled receptors and activate intracellular signaling pathways including MAP kinase cascades, NF κ B, and JAK/STAT (Cartier et al., 2005).

2.4 Disease-Specific Abnormal Proteins

Several disease-specific abnormal proteins are thought to activate microglia, including amyloid- β (A β) in Alzheimer disease, α -synuclein in Parkinson disease, prion protein in prion diseases, HIV-gp41, gp120, and HIV-tat. These will be discussed further in the context of specific diseases, later in this chapter.

The mechanisms by which several of the disease-specific factors interact with and activate microglia seem to involve members of the scavenger receptor (SR) family. Scavenger receptors (Husemann et al., 2002) are a family of cell surface receptors, which include SR class A (which is expressed in microglia but not in astrocytes or neurons); SR class B1 (which is expressed in astrocytes and vascular smooth muscle cells but not in microglia (Husemann and Silverstein, 2001)); CD36, receptor for advanced glycation end products (RAGE); low density lipoprotein (LDL)-like receptor related protein (LRP); and others. Scavenger receptors recognize several ligands including LDL, fibrillary β -amyloid, and myelin (Coraci et al., 2002) and mediate oxidative stress in the form of ROS generation as has been shown in the case of CD36 interacting with A β (Ricciarelli et al., 2004) and RAGE with AGE products (Dukic-Stefanovic et al., 2003). Another molecule that may act as a receptor for A β thereby inducing microglial activation is formyl peptide receptor 2 (Iribarren et al., 2005).

2.5 Downstream Signaling: NF κ B

Intracellular signaling via the NF κ B represents the major mechanism of proinflammatory signaling in glial cells as in several other immune cells. NF κ B is a dimeric complex of various subunits that belong to the Rel family (p105/50, p100/52, p65 [RelA], RelB, and c-Rel), which share a N-terminal 300 amino acid Rel homology domain allowing DNA binding, dimerization, and nuclear localization (Delhalle et al., 2004; Kaltschmidt et al., 2005; Memet, 2006). Homo- or heterodimers of these subunits are bound to an inhibitory unit called I κ B. Phosphorylation of I κ B on two serine residues by I κ B kinase (IKK) leads to its ubiquitination and degradation by the 26S proteasome, exposing the NF κ B, which translocates to the nucleus and activates DNA transcription. In addition to LPS, cytokines (TNF α and IL-1 β), chemokines, virus, injury, and oxidative stress are among the more than 150 agents shown to activate NF κ B (Pahl, 1999). The downstream targets of NF κ B include several inflammatory mediators, including cytokines, chemokines, adhesion molecules, COX-2, and more than 150 other products.

2.6 Downstream Signaling: MAP Kinases

MAP kinases are evolutionarily conserved proline-directed serine–threonine kinases (i.e., they phosphorylate serines or threonines that neighbor proline residues), which play important roles in cell proliferation, inflammation, and apoptosis (Kyosseva, 2004; Qi and Elion, 2005). The three major mammalian MAP

kinases are p38 (Zarubin and Han, 2005), c-Jun N-terminal kinase (JNK) (Vlahopoulos and Zoumpourlis, 2004), and extracellular signal-regulated kinase (ERK) (Rubinfeld and Seger, 2005). The MAP kinase module consists of three sequential kinases: MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK or MKK), and MAPK. MAPK activation occurs when they are phosphorylated by MAPKKs on both Thr and Tyr residues. Targets of MAPKs include downstream kinases, specific cellular substrates, and transcription factors that mediate cytoplasmic and nuclear signal transduction leading to metabolic and transcriptional responses.

Because of their prominent roles in glial inflammatory signal transduction (Koistinaho and Koistinaho, 2002; Kim et al., 2004), MAP kinases have emerged as attractive targets in the treatment of neuroinflammatory and neurodegenerative diseases. In contrast to NF κ B, which is also involved in normal physiology so that a global inhibition might result in serious side effects, specific inhibition of either p38 or JNK may be a viable option as a potential treatment of these diseases.

2.6.1 p38 MAP Kinase

p38 MAP kinase exists in four isoforms. p38 α and β are expressed ubiquitously including in microglia and astrocytes, whereas γ and δ are more restricted. The α -isoform appears to predominantly mediate proinflammatory signal transduction in a variety of immune cells including glia. The kinase is activated in response to stress (i.e., ultraviolet light, osmotic and heat shock), LPS, cytokines (IL-1 β , TNF α), and other stimuli. The upstream kinases involved in p38 activation are MKK3 and MKK6, which are, in turn, activated by multiple upstream activators and signaling complexes targeted by cytokine and other innate immune receptors. Downstream targets of p38 include kinases such as MAP kinase-activated protein kinase-2 (MAPKAPK2) and mitogen- and stress-activated kinases (MSKs), and specific transcription factors such as ATF2, C/EBP, and SP1, which are involved in the induction of a number of proinflammatory mediators.

It is becoming apparent that p38 MAP kinase represents a key proinflammatory signaling pathway in a variety of immune cells including glia and a potential target for antiinflammatory intervention. In fact, the kinase was originally identified as a target of a class of potent antiinflammatory agents termed cytokine-suppressive antiinflammatory drugs (CSAIDs) in LPS-activated macrophages (Lee et al., 1994). Using these drugs as a tool, it was early on discovered that the kinase pathway also plays an essential proinflammatory role in glial cells (Bhat et al., 1998). It is of interest that minocycline, a tetracycline derivative, which has shown remarkable neuroprotective activities in a number of neurodegenerative/inflammatory disease models (thereby prompting its clinical trials), is thought to mainly act as an antiinflammatory agent by targeting microglial p38 MAP kinase (Stirling et al., 2005).

2.6.2 JNK

The three main subtypes of JNK (JNK1, JNK2, and JNK3) are encoded by three different genes (Vlahopoulos and Zoumpourlis, 2004). Alternative splicing results in a total of about ten isoforms. JNKs 1 and 2 are expressed ubiquitously, whereas JNK3 is expressed mainly in the brain, heart, and testis. JNK is potently activated in glial cells in response to different stimuli (referenced in Pawate et al., 2006) including LPS, proinflammatory cytokines (TNF α , IL-1 β), and other physical and chemical stress. The upstream activators of JNK are MKK4 and MKK7, which are in turn activated by a number of kinases. Downstream targets of JNK that mediate nuclear responses including the expression of proinflammatory mediators are: c-Jun, ATF-2, Elk-1, and AP-1, an important transcription factor consisting of c-Jun and c-Fos, or dimerized c-Jun.

2.6.3 ERK

ERK1 (p44) and ERK2 (p42) are expressed ubiquitously and primarily mediate cell growth and proliferation responses. ERK is commonly activated in response to mitogens such as EGF, bFGF, and PDGF

involving the upstream kinase, Raf, and its targets MEK1 and 2, which are the immediately upstream activators of ERK. A number of transcription factors including Elk1, c-Myc, c-Jun, and CREB act as the nuclear targets of activated ERK and mediate nuclear signaling and transcription. Although the main function of the ERK pathway is to signal growth and differentiation, it also cooperates with the previous stress kinases (p38 and JNK) in its action as a mediator of proinflammatory and stress signals, especially in immune cells including glia (Koistinaho and Koistinaho, 2002).

3 Glial Mediators of Inflammation

Activated glia, in particular, microglia, release several inflammatory mediators that are potentially toxic to neurons and oligodendrocytes. These include, but are not limited to, cytokines, chemokines, reactive oxygen and nitrogen species, eicosanoids, excitotoxins, and proteases.

3.1 Cytokines, Chemokines

The two major cytokines released by activated microglia are TNF α and IL-1 β . In addition, activated glia produce several other proinflammatory cytokines including IL-6, IL-12, IL-15, and chemokines such as IL-8, MIP-1, SDF-1 α , IP-10, and MCP-1 (Kim and de Vellis, 2005) as well as certain antiinflammatory cytokines including IL-4 and IL-10, thus setting up an autocrine feedback loop.

TNF α and IL-1 β play major roles in neuroinflammation by inducing the expression of adhesion molecules and chemokines, thereby attracting leukocytes to the region through compromised microvasculature. TNF α also plays a role in cell/tissue destruction.

3.2 Reactive Oxygen Species and NADPH Oxidase

ROS, which include superoxide anion, hydrogen peroxide, and hydroxyl radical, are released by activated microglia. These ROS have been shown to be toxic to neurons and oligodendrocytes. NADPH oxidase (Babior, 2002) is an enzyme complex consisting of two membrane-bound subunits (gp91phox and p22phox) and several cytosolic components (p47phox, p67phox, p40phox, and the small GTPases Rac1 and Rac2). On activation, the cytosolic components translocate to the membrane resulting in the functional enzyme complex. While the superoxide generated exerts potent microbicidal and adverse tissue destructive effects, the dismutation product of superoxide, H₂O₂, has important roles in inflammatory cellular signaling in both microglia and astrocytes (Pawate et al., 2004).

As a prime mediator of oxidative stress, NADPH oxidase is implicated in a number of neurodegenerative diseases/conditions including Alzheimer disease, Parkinson disease, and stroke.

3.3 Nitric Oxide

While NO is produced by several nitric oxide synthases (NOSs), the production of large amounts of NO by immune cells as a result of an induced expression of inducible NOS (iNOS) represents an important event in neuroinflammation (Bhat and Feinstein, 2006). A number of studies with rat and mouse species have shown that both microglia and astrocytes express iNOS in response to inflammatory stimuli. However, human astrocytes, but not microglia, seem to have the capacity to express significant NO (Kim and de Vellis, 2005). Of relevance to pathophysiological roles of NO, it reacts with superoxide radical to form peroxynitrite (ONOO⁻), a potent neurotoxin believed to be responsible for neuronal apoptosis and degeneration (Estevez and Jordan, 2002).

3.4 Proteases

Matrix metalloproteases (MMPs) (Nakanishi, 2003) are released by activated microglia and are thought to play major roles in inflammation and tissue destruction (Rosenberg, 2002, 2005; Jian Liu and Rosenberg, 2005). MMPs are involved in the disruption of BBB in response to ischemia and result in vasogenic edema and hemorrhage. MMPs are also thought to be important in the development of contrast-enhancing lesions seen in multiple sclerosis.

3.5 Mediators of Excitotoxicity

Activated glial cells release NMDA receptor agonists such as glutamate (Matute et al., 2006; Takeuchi et al., 2006), D-serine (Hayashi et al., 2006), and quinolinic acid (Obrenovitch, 2001), which may play roles in excitotoxic damage to neurons.

3.6 Antiinflammatory Mediators

The plasticity of glial response allowing a beneficial, protective outcome involves the release of several counterregulatory molecules. These antiinflammatory mediators include IL-1Ra, Th2 cytokines (TGF β , IL-4, and IL-10), and the product of hemoxygenase-1 (HO-1) activity, that is, carbon monoxide (CO). The Th2 cytokines antagonize and suppress Th1-type immune responses. Recent attempts at therapeutic vaccination to treat neurodegenerative diseases seem to make use of this potential immune deviation leading to the production of Th2 products including microglia-derived neurotrophic molecules (Schwartz et al., 2006). It should be emphasized, however, that several of the so-called antiinflammatory molecules (e.g., TGF β) can display multifunctionalities, and hence, their simple classification becomes tentative, and largely, context-dependent.

4 Role of Glial Cells in Neuroinflammatory and Neurodegenerative Diseases

As noted in [Section 16.1](#), neuroinflammation is a common and most often, a pathogenic feature of a large number of brain injuries and diseases ([Figure 14-2](#)). A distinction can be made differentiating primary inflammatory and secondary neurodegenerative (as in MS and HIV dementia) versus primary neurodegenerative and secondary inflammatory (as in AD and PD) diseases. The former are characterized by infiltration of leukocytes from the blood and the presence of cellular and humoral immune responses characteristic of adaptive immunity. On the other hand, leukocyte infiltration is not significant in neurodegenerative diseases such as AD and PD. Even in cases where it is considered a secondary event, neuroinflammation characterized by an innate immune response certainly contributes to the progression of the disease, and hence, represents an important treatment target for limiting the chronicity of the ensuing neuropathology.

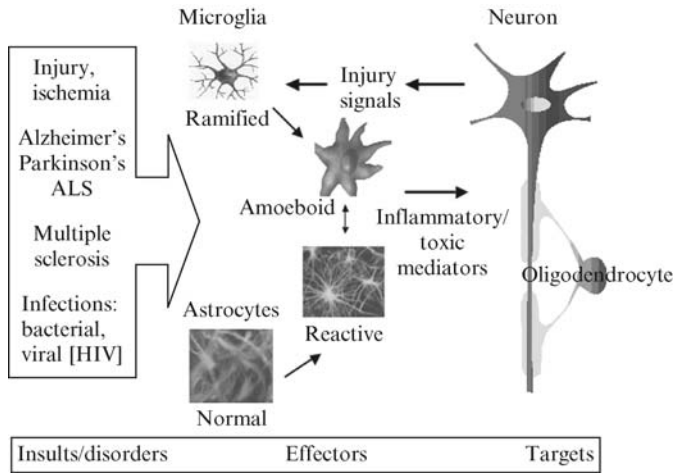
4.1 Glia in Alzheimer Disease

4.1.1 Introduction

Alzheimer's disease is the most common cause of dementia in the older population and is the commonest neurodegenerative disease, which results in progressive cognitive and behavioral impairment, ultimately resulting in death (Kawas, 2003; Cummings, 2004; Blennow et al., 2006). It affects less than 1% of the

■ Figure 14-2

Glia-mediated neuroinflammation in response to brain injuries, infections, and neurodegenerative diseases



population under the age of 65, but increases exponentially thereafter, so that by age 85, it has a prevalence of 25–33%. In the USA, there were 4.5 million people affected by it in 2000, a number expected to increase to 13.2 million by 2050, resulting in staggering economic burden.

4.1.2 Pathology and Pathogenesis

The pathological hallmarks of Alzheimer disease are the β -amyloid deposition in extraneuronal plaques (Wang et al., 2006) and the presence of intraneuronal neurofibrillary tangles composed of hyperphosphorylated Tau (Avila, 2006). The precise pathogenic role of either of these entities is still not clarified.

A β is a protein fragment of either 40 or 42 amino acids, generated from the cleavage of its precursor protein, the amyloid precursor protein (APP). APP is a transmembrane glycoprotein, which may be processed by a nonamyloidogenic pathway by α -secretase, or in an amyloidogenic pathway by β -secretase. α -Secretase cleaves the APP within the A β peptide residue 17, yielding the peptide products that do not aggregate. In the amyloidogenic pathway, the β -secretase cleaves APP at the beginning of A β sequence to release an extracellular fragment called sAPP- β and an intracellular fragment called C99. A third enzyme, γ -secretase, then cleaves C99 to release the amyloidogenic peptide A β 40 or A β 42. Normally, brain A β is degraded by the peptidases such as insulin-degrading enzyme, neprilysin, and by endothelin-converting enzyme. When the degradation is inefficient, the insoluble A β accumulates and is deposited in plaques.

Tau is a normal axonal protein that functions in promoting microtubule assembly and stability. Its phosphorylation status is regulated by multiple kinases and phosphatases. Hyperphosphorylated Tau tends to aggregate and form insoluble fibers found in the neurofibrillary tangles, thus impairing neuronal function.

4.1.3 Role of Glial Cells

Neuroinflammation is thought to play an important role in AD pathogenesis, with both microglia (Streit, 2004, 2006) and astrocytes (Tuppo and Arias, 2005) playing important roles. Evidence for the role of neuroinflammation is derived from several sources. In epidemiological studies, prolonged use of nonsteroidal antiinflammatory drugs has been shown to have a protective effect against AD (Akiyama et al., 2000)

although clinical trials have suggested that NSAIDs lose their effectiveness before the onset of clinical symptoms (Breitner, 2003). Pathologically, reactive microglia and astrocytes are found around the fibrillary amyloid plaques. Recently, Cagnin et al. (2006) used positron emission tomography (PET) and [^{11}C](R)-PK11195, a specific ligand of the peripheral benzodiazepine-binding site, to systematically study microglial activation *in vivo*. Significant microglial activation was found to be present in the brains of patients with neurodegenerative dementia even at early and possibly preclinical stages of the disease. *In vitro* evidence continues to support the idea that A β 1–40 acts as a potent activator of microglia (Garcao et al., 2006) leading to neuronal death in microglia–neuron cocultures. The neuronal death was reduced by IL-6 antibody, suggesting that IL-6 contributes to neuronal death. In another study, Floden et al. (2005) found that NMDA receptor antagonists memantine and 2-amino-5-phosphopentanoic acid as well as soluble TNFR protect neurons from microglial-conditioned media-dependent death after the microglia had been stimulated with A β . It is to be pointed out that neurodegeneration *in vivo* may or may not involve actual neuronal loss and that AD-associated dementia most likely ensues from synaptotoxicity. Again, glia-derived toxic products potentially participate in this type of neuronal damage.

Recently, microglia have been proposed to link the two hallmarks of Alzheimer disease, i.e., the amyloid plaque and the neurofibrillary tangle (Kitazawa et al., 2005). Thus, activation of microglia by secreted APP or a prototype inflammogen (LPS) results in the elevation of cytokines (i.e., IL-1 α , IL-1 β , and TNF α) and subsequent tau hyperphosphorylation and neuronal loss (Li et al., 2003). The cytokine (i.e., IL-1)-induced tau hyperphosphorylation seems to be mediated by the activation of p38MAPK in neurons.

It is also becoming clear that glia-released inflammatory mediators may act as “primary” effectors of neurodegeneration, especially in sporadic cases of AD and in aging. Cytokines have been shown to induce APP expression and processing resulting in an increased generation of A β . Cytokines and oxidative stress also induce the expression/activity of β - and γ -secretases, thereby contributing to increased APP processing and A β generation, and hence, a vicious cycle of A β -mediated glial activation and chronic inflammation.

Streit (2006) has advanced an alternative hypothesis of microglial role in AD, which states that microglia are, in fact, neuroprotective. However, microglial senescence in aged brains impedes this neuroprotective activity, leading to neuronal loss/degeneration seen in AD.

4.2 Glia in Parkinson's Disease

4.2.1 Introduction

Parkinson's disease (Feany, 2004; Samii et al., 2004; Nutt and Wooten, 2005) is the second most prevalent neurodegenerative disease after Alzheimer's disease, affecting 1% of the population over the age of 60. PD results from the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). This results in the cardinal clinical features of tremor, rigidity, bradykinesia, and postural instability. Dementia and autonomic instability may also occur, especially later in the course of the disease. It is estimated that clinical features arise when the loss of dopamine is in excess of 80%, suggesting a long preclinical course.

4.2.2 Pathology

Pathologically, PD is characterized by the loss of dopaminergic neurons in the SNpc, as noted earlier. Histologically, intracellular Lewy bodies are a pathological hallmark of PD. Lewy bodies are found not only in substantia nigra, but are widespread throughout the brain and in autonomic ganglia as well. There are also other conditions, such as dementia with Lewy bodies and multiple system atrophy, where these abnormal accumulations can be found.

4.2.3 Oxidative Stress

Oxidative stress is thought to play a central role in PD. Dopaminergic neurons are especially susceptible to oxidative damage because of the presence of dopamine metabolites. Immediately after synthesis, dopamine is taken up into synaptic vesicles by the vesicular monoamine transporter 2 (VMAT2). In synaptic storage vesicles, low pH and absence of MAO hinder dopamine breakdown (Lotharius and Brundin, 2002). However in the cytoplasm, dopamine metabolism by MAO-A leads to the production of dihydroxyphenylacetic acid (DOPAC) and hydrogen peroxide, while intracellular autooxidation of dopamine generates superoxide, H_2O_2 , and dopamine-quinine (Hald and Lotharius, 2005). In the presence of ferrous ions, the H_2O_2 can be converted into hydroxyl radical by the Fenton reaction. Superoxide is toxic to cells if not scavenged rapidly, as it can oxidatively damage cellular components including DNA, lipids, and proteins. Iron levels are higher in the substantia nigra than in other areas of the brain and are further elevated in PD patients, thus potentially pointing to oxidative damage as a mechanism for cell death. Increasing evidence indicates that, in addition to neuronally generated ROS, that produced by activated glia substantially contribute toward oxidative stress in PD (see later).

4.2.4 α -Synuclein and Neuromelanin

Aggregated α -synuclein protein is the major constituent of the Lewy bodies. Its normal function is not well known. It has been suggested that it plays a role in the recycling of vesicles that have released their neurotransmitter content into the synaptic cleft (Lotharius and Brundin, 2002). Normally, the ubiquitin proteasomal system and the 20S proteasome system are involved in the degradation of unwanted proteins. Dopamine-dependent oxidation of intracellular targets such as α -synuclein may render them less soluble and likely to aggregate, and the aggregates are thought to be cytotoxic. While increased levels of α -synuclein characterize sporadic forms of PD, its mutations lead to familial cases of the disease. There is a recent report indicating the possibility that aggregated α -synuclein may trigger an activation of microglia resulting in NADPH oxidase-mediated ROS production and the release of neurotoxic inflammatory mediators (Zhang et al., 2005). The occurrence in vivo and the associated mechanisms of α -synuclein release from neurons, however, are not certain.

Neuromelanin (NM), a pigment derived from cytosolic (nonvesicle associated) catecholamines, present in the SN and the locus ceruleus, has received attention as a significant player in PD (Zecca et al., 2006). NM binds avidly to metals. As mentioned earlier, there is an increase in iron in the SN of PD patients. Therefore, NM acts as a neuroprotector in two ways: by trapping cytosolic catecholamines and scavenging metal ions (Zecca et al., 2006). However, excess NM on a prolonged scale has an inhibitory effect on proteasomal function (Shamoto-Nagai et al., 2006) leading to neuronal death. It has also been found that the extracellular NM is a potent activator of microglia (Wilms et al., 2003) leading to an upregulation of cytokines such as $TNF\alpha$ and IL-6 and NO involving signaling via $NF\kappa B$ and p38 MAPK pathways.

4.2.5 Role of Glial Cells in PD

Microglia are increasingly thought to play an important role in the pathogenesis of Parkinson's disease (reviewed in Trisman et al., 2003; Kim and Joh, 2006). Activated microglia are found in abundance around the surviving neurons in the substantia nigra of Parkinson disease patients (McGeer and McGeer, 1998; Knott et al., 2000; Mirza et al., 2000) as well as in animal models of MPTP-induced parkinsonism (Kohutnicka et al., 1998; Czlonkowska et al., 2000). In autopsies of patients who had MPTP-induced Parkinsonism, marked glial activation was found (Langston et al., 1998). In fact, several of the environmental neurotoxins that are being linked to PD also cause glial activation in animal models. Although activation of glia may represent either a protective or a toxic (or both) response, given that activated microglia release several inflammatory mediators that are potentially toxic to neurons, it is likely that the

outcome is more detrimental in nature. To directly test if glial activation per se leads to dopaminergic neurodegeneration, studies have used the strategy of LPS administration. Thus, its direct brain infusion (Gao et al., 2002), systemic administration (Qin et al., 2007), or even maternal exposure to low-level LPS (Carvey et al., 2003) have all been shown to cause progressive and cumulative loss of dopaminergic neurons over time involving a neuroinflammatory response.

In terms of effector molecules, reactive nitrogen and oxygen species have received much attention as toxic mediators. Thus, in glial cells in the SNpc of PD patients there is a marked activation of iNOS (Hunot et al., 1996). Wu et al. (2003) showed that the NADPH oxidase gp91 subunit is upregulated in MPTP-treated mice and that the inactivation of NADPH oxidase attenuates MPTP toxicity. Inhibition of cyclooxygenase 2, another enzyme capable of producing ROS (in addition to potentially toxic prostaglandin products), also reduces MPTP toxicity (Teismann et al., 2003). In the LPS model, there occurs a sustained elevated brain level of the cytokine, TNF α potentially playing a neurotoxic role (Carvey et al., 2003; Qin et al., 2007), as has also been indicated by studies with knockout models (Kim and Joh, 2006).

4.3 Glia in Amyotrophic Lateral Sclerosis

4.3.1 Introduction

ALS (Rowland and Schneider, 2001; Krivickas, 2003; Boillee et al., 2006a) is much less common than the two neurodegenerative diseases discussed earlier, with an incidence of 1 per 100,000 and a prevalence of 5 per 100,000. It is characterized by selective degeneration of upper and lower motor neurons, leading to progressive paralysis of extremity, respiratory and bulbar muscles, leading to death over 2–5 years.

4.3.2 Pathology and Pathogenesis

There is a prominent loss of lower motor neurons in the anterior horns of the spinal cord and brainstem motor nuclei, with gliosis and microglial proliferation. There is also loss of the upper motor neurons in the motor cortex.

4.3.3 Role of Glia in ALS

Neuroinflammation is a prominent feature of ALS (Mcgeer and Mcgeer, 2002) and microglia play an important role (Sargsyan et al., 2005; Weydt and Moller, 2005). Evidence for glial involvement comes from several observations. Thus, immunohistochemical analysis of autopsy specimens shows the presence of microglia in ALS motor cortex (Troost et al., 1993). There have been reports of increased COX-2, ROS, NO, TNF α , and other cytokines in mouse models of ALS (Vukosavic et al., 1999; Nguyen et al., 2001; Almer et al., 2002). In human patients, Turner et al. (2004) used PK11195, a ligand for the “peripheral benzodiazepine-binding site” expressed by activated microglia, and performed PET scans to confirm widespread activation of microglia.

The precise signals that activate microglia in ALS are not clear. It is presumed that injured neurons release agents that activate microglia, with the CX3C chemokines receiving some attention, as have neural cell adhesion molecule (NCAM), purines, and pyrimidines (Sargsyan et al., 2005).

In approximately 10% of cases, ALS is familial, and in 20% of these cases, mutations in superoxide dismutase-1 (SOD1) have been noted (Rosen et al., 1993). SOD1 transgenic mice have served well as a model for ALS with which to address mechanisms of disease pathogenesis and treatment options. With respect to the occurrence of inflammation, Alexianu et al. (2001) performed immunohistochemistry for CD11b, IgG, Fc γ RI, intercellular adhesion molecule-1 (ICAM-1), CD3, and glial fibrillary acidic protein in spinal cords of these mice at 40, 80, and 120 days of age. They found activation of microglia at 80 days and

astrocytes and T cells at 120 days indicating a progressive course of glial activation and leukocyte infiltration. By far, the most convincing evidence for the role of microglia in ALS disease progression has come from the use of chimeric mice differentially expressing mutant SOD1 in neurons and glia. Thus, Boillee et al. (2006b) used a Cre recombinase technique to selectively express mutant SOD1 in motor neurons or microglia and found that mutant SOD1 expressed in microglia renders neighboring motor neurons apoptotic, which otherwise are relatively more protected (by an interaction with normal neighbors) even in the presence of the mutant SOD1 expressed in them. It is still not clear whether the microglia with mutant SOD1 cause neuronal death by release of toxic mediators or by failing to exert their usual neuroprotective functions. In this regard, a recent study (Urushitani et al., 2006) suggests an interesting scenario involving chromogranin-mediated neuronal release of mutant SOD1 into the extracellular space where it can then potentially trigger microglial activation.

Dysfunctional astrocytes also contribute to the progression of ALS because of the loss of their glutamate transporter, EAAT2, which would otherwise be engaged in removing excessive amount of extracellular glutamate, an excitotoxin (Boillee et al., 2006a).

4.4 Glia in Multiple Sclerosis

4.4.1 Introduction

Multiple sclerosis (MS) is the most common inflammatory demyelinating disorder of the CNS (Noseworthy et al., 2000; Frohman et al., 2006; Hauser and Oksenberg, 2006), affecting mostly young adults aged 20–40 years. The pathogenesis of MS continues to be only partially understood (see Chapter 21 of this book for more details).

4.4.2 Pathology

The hallmark of MS is the inflammatory demyelinating plaque (Frohman et al., 2006) (see Chapter 21 of this book for more details), consisting of a well-demarcated hypocellular area characterized by the loss of myelin. Following an extensive pathological investigation, Lucchinetti et al. (2000) classified MS lesions into four subtypes. Types I and II showed prominent inflammation, with IgG and complement deposition in type II lesions. Types III and IV were suggestive of a primary oligodendroglialopathy.

Contrary to the long-held belief that the axons are relatively spared, a reexamination of this issue has confirmed significant axonal pathology/loss in MS specimen (reviewed in Hauser and Oksenberg, 2006). Besides an ongoing neuroinflammation, potential occurrence of the so-called “virtual hypoxia” and associated excitotoxicity has been evoked as a mechanism of this pathology.

4.4.3 Role of Glial Cells

Microglia may play important roles in the genesis of the lesions. Deng and Sriram (2005) proposed three models of microglia-associated demyelination, based on the pathology noted earlier. In model 1, T-cell infiltration leads to microglial activation, damaging the myelin through a combination of cytokine, complement, and antibodies. In model 2, microglial activation is the central event, occurring presumably by exposure to infectious agents, as in HTLV-associated myelopathy, leading to TLR-mediated signaling and toxicity to oligodendrocytes and the myelin sheath. In model 3, the primary oligodendroglialopathy leads to activation of microglia as a secondary response. Perhaps, related to model 3, a recent study has found elevated levels of syncytin, a glycoprotein encoded by human endogenous retroviruses (HERV), in glia within the demyelinating lesions of MS and that astrocytic expression *in vitro* of this protein causes toxicity toward cocultured oligodendrocytes (Antony et al., 2004).

4.5 Glia in Other Neurological Diseases

4.5.1 HIV Encephalitis

HIV dementia occurred in about 20% of HIV-infected patients before the availability of highly active antiretroviral therapy (HAART) and caused a rapidly progressive subcortical dementia. In treated patients, the disease appears to be milder and the incidence is lower, although Anthony et al. (2005) found evidence of ongoing neuroinflammation even in HAART-treated patients. The virus does not infect the neurons directly. Neuronal death in HIV infection occurs because of the infection of macrophages/microglia and astrocytes (Mattson et al., 2005), leading to the production of TNF α , IL-6, and IL-1 β , and chemokines such as MCP-1 and SDF-1, as well as the release of neurotoxic HIV proteins gp120, gp41, Tat, Nef, Vpr, and Rev. The released proteins can also induce the activation of microglia, further stimulating mediator expression. The gp120 was shown to act through the p53 pathway in neurons (Garden et al., 2004). Several of the HIV-associated proteins may signal through chemokine receptors, in particular, CXCR4.

4.5.2 Prion Diseases

Prion diseases are caused by proteinaceous infectious particles that lack nucleic acids and include scrapie, a disease of sheep, bovine spongiform encephalopathy (BSE), and Creutzfeldt–Jakob disease (iatrogenic, variant, and familial forms), Kuru, Gerstmann–Sträussler–Scheinker syndrome (GSS), and fatal familial insomnia (FFI) in human beings. Creutzfeldt–Jakob disease (CJD) is the most common of the prion diseases, but still rare, with an incidence of 1 in 1,000,000 (Knight, 2006). However, interest in this group of diseases was sparked by the outbreak of variant CJD or “mad cow disease.” CJD results from abnormal posttranslational modifications of the normal prion protein, which change it from predominantly α -helical to β -sheet structure, leading to protein accumulation.

Pathologically, CJD is characterized by spongiform changes in the brain, gliosis, and the presence of activated microglia. However, there is little evidence of inflammatory infiltrate and no antibody response against prion protein. The activated microglia produce not the usual proinflammatory cytokines (TNF α , IL-1 β , IL-6), but transforming growth factor- β 1 and prostaglandin E2 (Perry et al., 2002), resulting in the term “atypical inflammation” being used to describe the pathogenesis of CJD. Neuronal loss in prion diseases has been shown to be predominantly due to apoptosis both in mice infected with scrapie and in CJD (Giese et al., 1995; Gray et al., 1999), potentially involving microglial nitric oxide release. With regard to astrocytes in prion disease, a recent study with rodent brain cultures has demonstrated a potent gliotrophic activity of a synthetic peptide homologous to GSS amyloid protein, in parallel to its neurotoxic effect, both effects being dependent on the presence of endogenous prion protein (Fioriti et al., 2007).

4.5.3 Traumatic Brain Injury and Stroke

Traumatic brain injury (Nortje and Menon, 2004) results in (1) direct mechanical injury to brain tissue and (2) delayed secondary injury (Zhang et al., 2006). Microglia/macrophages are thought to play a major role in the delayed injury. Microglial accumulation consisting of distinct subpopulations at the site of injury is an early event (Ladeby et al., 2005).

Stroke results from the sudden cessation of blood flow to a part of the brain. The tissue directly affected by the loss of oxygen dies, but there is a surrounding area known as the “ischemic penumbra” that is potentially viable. There is prominent microglial activation in this area. The role of microglia in ischemic brain has been reviewed recently (Lai and Todd, 2006). There is evidence that microglial activation occurs in response to the ischemic death of neurons, but there is also evidence that glial activation occurs very early after the onset of hypoxia and plays a role in neuronal death.

5 Concluding Remarks

While both acquired and innate mechanisms of immunity play roles in classical “inflammatory” CNS diseases, the latter plays a predominant role in a number of neurodegenerative diseases. Intracerebral immune response to exogenous (injury, infection, autoimmune) and endogenously derived triggers (disease products) is mediated by microglia and astrocytes as an innate immune response with both beneficial and detrimental outcome. Although transiently activated microglia are most likely beneficial for reparative processes including phagocytosis and clearance of abnormal products such as A β , when chronically activated, they secrete a number of inflammatory neurotoxic mediators including cytokines such as TNF- α and IL-1 β , chemokines, prostaglandins (products of cyclooxygenase 2, COX-2), complement proteins, and reactive oxygen and nitrogen species. Due to the autocrine nature of the cytokines produced and due to interactions between microglia and astrocytes, a self-propagating “cytokine cycle” may ensue – often leading to a chronic inflammatory cascade and hence, neuronal cell damage. Suppression of neuroinflammation is increasingly viewed as an important and general therapeutic approach to treat neurodegenerative diseases. However, the development of specific strategies for immunosuppression relies on a clear understanding of the molecular mechanisms and mediators involved in CNS immune response elicited by glial cells.

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15 Autoimmune Processes in the Central Nervous System

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1	<i>Immunological Reactivity within the Central Nervous System</i>	335
2	<i>Factors that Contribute to the Immunologically Privileged Status of the CNS</i>	335
3	<i>Loss of Tolerance as a Mechanism of Autoimmunity in the CNS</i>	336
4	<i>Infectious Agents and Autoimmunity within the CNS</i>	336
4.1	Molecular Mimicry as a Possible Cause of Autoimmunity within the CNS	337
4.2	Autism as an Autoimmune Disease	337
4.3	Lyme Disease	338
4.4	Acute Disseminated Encephalomyelitis	338
4.5	Sydenham's Chorea	338
4.6	Protective Effects of Bacterial and Helminth Infections in CNS Disease	338
5	<i>Autoimmunity in Human Neurological Diseases</i>	339
5.1	Neuromyelitis Optica	339
5.2	Neuromyotonia	339
5.3	Schizophrenia	339
5.4	Lethargic Encephalitis	340
5.5	Stiff-Man Syndrome	340
6	<i>Autoimmunity Caused by CNS Insult</i>	341
7	<i>Autoimmunity as a Mediator of CNS Repair</i>	341
7.1	Antibody-Mediated Remyelination	341
7.2	MBP-Specific T Cells Aid Recovery from Spinal Cord Damage	342
8	<i>Multiple Sclerosis</i>	342
9	<i>Experimental Autoimmune Encephalomyelitis</i>	343
10	<i>A Viral Etiology for Multiple Sclerosis</i>	343
11	<i>Theiler's Murine Encephalomyelitis Virus as a Model for MS</i>	344
11.1	Age-Related Susceptibility to TVID	344
11.2	The Early Disease Induced by Theiler's Virus	344
11.3	The Late Demyelinating Phase of Theiler's Virus Infection	344
11.4	Genetic Control of Susceptibility to TVID	345
11.5	Similarities between TVID and EAE	345
11.6	Aberrant MHC Class II Expression	345

12	<i>The Role of the BBB in TVID and MS</i>	346
12.1	Demyelinating Viruses and the BBB	346
13	<i>Mast Cells in Demyelination</i>	347

Abstract: In this chapter we discuss the factors that contribute to the unique immunological environment of the central nervous system and the mechanisms that may account for the development of autoimmunity within the CNS, including infectious agents as inducers of autoimmune disease. Consideration is given to a variety of human neurological diseases of autoimmune or presumed autoimmune etiology: autism, neuromyelitis optica, neuromyotonia, schizophrenia, lethargic encephalitis and stiff-man syndrome. Also, we discuss autoimmunity as a possible mediator of CNS repair and examples of the protective effects of bacterial and helminth infections on CNS disease. Multiple sclerosis and models of multiple sclerosis are discussed with special attention given to the Theiler's virus-induced demyelination model.

List of Abbreviations: BBB, blood–brain barrier; CNS, The central nervous system; CTL, cytotoxic T cell; CVE, cerebrovascular endothelial cells; DTH, delayed type hypersensitivity; EAE, experimental autoimmune encephalomyelitis; EL, encephalitis lethargica; GAD, glutamic acid decarboxylase; ICAM-1, intracellular adhesion molecule-1; INS, insulin; LFA-1, lymphocyte functional antigen-1; MHC, major histocompatibility complex; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; NMO, Neuromyelitis optica; NMT, neuromyotonia; OCB, Oligoclonal bands; PGE₂, Prostaglandin E₂; SC, Sydenham's chorea; SCI, Spinal cord injury; TGF- β , transforming growth factor- β ; TVID, Theiler's virus-induced demyelination; VAA, vasoactive amine

1 Immunological Reactivity within the Central Nervous System

The central nervous system (CNS) has been considered to be an immunologically privileged site since the early transplantation work of Peter Medawar (Medawar, 1948). Medawar noted that skin transplants survived for longer periods of time in the brain than in other peripheral sites. Antigens injected directly into the CNS parenchyma remain invisible to the immune system but a vigorous delayed-type hypersensitivity (DTH) response may be induced following peripheral antigen administration (Matyszak and Perry, 1995). In contrast, allografts injected into the ventricles are rapidly rejected (Mason et al., 1986). Thus, the immune response in the CNS parenchyma differs considerably from the immune response in other compartments. The reason for diminished immune activity within the CNS is thought to be to avoid immunologically mediated tissue damage that would have devastating consequences on the neurological functioning of the organism. However, the disadvantage of this phenomenon is that the CNS becomes an ideal environment for persistent viral infections.

2 Factors that Contribute to the Immunologically Privileged Status of the CNS

In order to develop an immune response, an antigen is taken up by an antigen-presenting cell, processed, and presented in the context of major histocompatibility complex (MHC) class II to a T cell. The interaction between the costimulatory molecule B7 on the antigen-presenting cell and CD28 on the T cell is also a requirement for T-cell activation. One of the most important factors that contribute to the immunological inactivity of the CNS is the lack of expression of MHC within the CNS.

Other characteristics that were originally thought to be involved in the immunologically privileged status of the CNS are the lack of lymphatic drainage and the paucity of professional antigen-presenting cells within the CNS (Cserr and Knopf, 1992). Additionally, the blood–brain barrier (BBB) was thought to protect the CNS from immunological damage. However, research has now demonstrated that these concepts are incorrect. There is a connection between the brain and cervical lymph nodes and spleen (reviewed in Bradbury and Cserr, 1985). Both microglial cells (Matsumoto et al., 1992) and astrocytes have been shown to function as antigen-presenting cells (Fontana et al., 1984; Borrow and Nash, 1992) and dendritic cells have been detected in the CNS (Greter et al., 2005). In addition, the BBB does not in fact prevent immune cells from entering the CNS and activated T cells continually pass through into the CNS in

surveillance mode (Wekerle et al., 1986; Hickey et al., 1991). Although DTH responses in the brain are suppressed (Harling-Berg et al., 1991; Streilein, 1995), humoral immunity appears to be enhanced (Harling-Berg et al., 1989). Interestingly, the isotype of antibody produced in the CNS appears to be biased toward noncomplement-fixing IgG subclasses. There are also low levels of complement in the CNS that reduces the inflammatory reactions mediated by complement-fixing antibody.

In addition, there appears to be active immunosuppression in the CNS (Brent, 1990), which may be mediated by transforming growth factor- β (TGF- β) (Streilein and Wilbanks, 1992). Constitutive FasL expression within the CNS is also thought to play a role in protecting the CNS from immunological damage. Both astrocytes (Fontana et al., 1982) and microglia (Keane, 1997) release prostaglandin E₂ (PGE₂) following stimulation with LPS. PGE₂ inhibits the proliferation of T cells and also decreases MHC class II expression on macrophages (Keane, 1997). Lipocortins or annexins are calcium- and phospholipid-binding proteins that are induced by glucocorticoids and mediate anti-inflammatory effects (Goulding et al., 1990). Lipocortins 1, 2, 4, and 5 are detected within the CNS (Elderfield et al., 1992) and may also be involved in the anti-inflammatory milieu of the CNS.

3 Loss of Tolerance as a Mechanism of Autoimmunity in the CNS

The thymus plays a key role in the education of T cells and the deletion of T cells that recognize self-proteins. The thymic epithelium has been shown to translate a diverse array of organ-specific antigens, which represent all of the tissues in the body (Derbinski et al., 2005). T cells that recognize these self-determinants are deleted. However, in autoimmune diseases self-reactive T cells clearly escape this purging process and may become pathogenic. Research in the diabetes field has led to a better understanding of the process of tolerance. Members of the insulin (INS) gene family of self-proteins are expressed in the thymic stroma in precise hierarchy and location. IGF2 is expressed at the highest levels in thymic epithelial cells followed by IGF1 in thymic macrophages and INS at the lowest levels in thymic medullary epithelial cells and/or dendritic cells (Geenen and Brilot, 2003). Thus IGF2 is better tolerated than INS. In the biobreeding (BB) rat, an animal model of diabetes, there is a defect in IGF2 expression which is thought to explain the absence of tolerance to INS-secreting β cells in these animals. Investigations of loss of tolerance to myelin in mice have shown that the thymus contains significant amounts DM-20, a proteolipid protein (PLP) isoform in which 35 residues (including the encephalitogenic sequence PLP139–151) have been removed by alternative splicing (Anderson et al., 2000; Klein et al., 2000). Thus failure to delete potentially autoaggressive T cells with specificity for PLP139–151 probably arises through the lack of thymic expression of this self-peptide. However, this mechanism does not apply in the case of myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE). Despite the expression of MOG in the thymus, T cells reactive to MOG fail to be eliminated (Fazilleau et al., 2006). One reason for this may be the fact that MOG is only expressed in the medullary thymic epithelial cells, whereas both myelin basic protein (MBP) and PLP are expressed in this site and also in cortical thymic epithelium (Gotter et al., 2004).

4 Infectious Agents and Autoimmunity within the CNS

Autoimmunity can result following infections with either viruses, bacteria, or parasites. In this section, we review some of these infectious agents.

Autoimmune reactions have been reported in acute and chronic viral infections both with RNA and DNA viruses in animals and in man (Notkins et al., 1984). Autoantibodies produced during these viral infections are usually of low titer and disappear when the viral infections are cleared by the host immune system, and thus are probably not involved in the disease process. Cell-mediated immune reactions against autoantigens (CMAI) however persist longer, but their pathogenic role is largely unknown. Such CMAI has been well documented in disseminated postinfectious encephalomyelitis, in measles encephalitis, or

following rabies vaccination (Hemachudha et al., 1987). Both clinical disease and neuropathological changes may be mediated by autoantigen-specific immune reactions similar to what is observed following the adoptive transfer of MBP-specific T cells and subsequent induction of EAE in rats and mice. Indeed, measles virus infection of Lewis rats leads to both subacute and acute disease processes of the CNS. Animals developing such subacute measles encephalitis contain T cells primed for MBP, MBP-specific CD4⁺ cells, which when adoptively transferred to naïve syngeneic recipients cause EAE (Liebert et al., 1988). Similar findings have been reported in JHM coronavirus-induced subacute demyelinating encephalitis (Watanabe et al., 1983). The finding that viral infections may enhance the susceptibility to EAE has been made in another set of studies showing that a preceding infection with either measles virus or Semliki Forest virus potentiates both the development and severity of EAE. To explain these later findings, several hypotheses have been proposed. First, the virus-induced damage to CNS tissue facilitates the subsequent priming of clonal expansion of preexisting myelin-reactive T cells. Secondly, in measles virus infection, it is possible that cell-surface alterations occur in infected cells resulting in the exposure of cellular components together with the viral envelope proteins (Notkins et al., 1984). Such exposure of cellular antigens in infected brain cells could potentially lead to the development of immunity to fragments of MBP that do not normally elicit immunogenic responses. Thirdly, as a result of viral infection there are changes in the integrity of the BBB, which can allow the entry of antigen-specific CD4⁺ T cells into the CNS.

4.1 Molecular Mimicry as a Possible Cause of Autoimmunity within the CNS

The term “molecular mimicry” was originally formulated by Damian in 1964 to describe the phenomenon of shared antigens between host and parasite (Damian, 1987). If an infectious agent possesses an antigenic determinant that is similar to a host molecule, the determinant may not evoke an immune response or may be recognized as foreign and an immune response elicited that also attacks the host antigen. The degeneracy of the T-cell repertoire may account for the occurrence of molecular mimicry at the T-cell level. There are several instances of molecular mimicry in autoimmune diseases of the CNS. In herpes-induced keratoconjunctivitis, T cells with specificity for the viral protein UL6 cross-react with a corneal antigen (Zhao et al., 1998). In patients with Guillian–Barre syndrome following gastrointestinal infection, antibodies raised against *Campylobacter jejuni* cross-react with human gangliosides (Yuki, 1999). T cells, isolated from multiple sclerosis (MS) patients and shown to recognize MBP, also react with peptides derived from Epstein Barr virus, influenza type A, and human papillomavirus (Wucherpfennig and Stominger, 1995).

4.2 Autism as an Autoimmune Disease

Since autoimmune diseases are sometimes suspected of being triggered by viruses, recently investigators have been interested in virus serology in autism. Very little is known concerning the etiology or pathogenesis of this disorder that affects over half a million Americans alone. Current theories include genetic factors, immune factors, environmental factors, and neural factors. It has been found that many children with autism have elevated levels of antibodies to measles virus, but not to human herpes virus-6, cytomegalovirus, or rubella virus (Singh et al., 1998; Singh, 2001). The elevated levels of measles antibodies were associated with brain autoantibodies, which led the authors to postulate a pathogenic association of measles virus to autoimmunity in autism (Singh et al., 1998; Singh, 2001). Additionally these same authors found that several children with autism had unusual measles–mumps–rubella (MMR) antibodies, which showed a temporal association with MBP autoantibodies that were used as a marker of CNS autoimmunity in autism (Singh et al., 2001). Although the association between autism and autoantibodies is controversial, these findings are reported here since it is important to explore the possibilities of viral autoantibodies and human diseases. Indeed, it may be relevant in future research to characterize the molecular basis of cellular and humoral immunity to viral antigens in children with autism.

4.3 Lyme Disease

Lyme disease is caused by the spirochete *Borrelia burgdorferi*, and is primarily transmitted to humans by ticks. The disease is presented by a wide variety of symptoms including fever, a skin “bull’s-eye” and has rheumatological as well as neurological consequences. The neurological problems are due to inflammation of the central and peripheral nervous systems. Oligoclonal bands (OCB) of immunoglobulin with restricted heterogeneity are often observed in cerebrospinal fluid (CSF) samples. Phage lambda gtl expression libraries from *B. burgdorferi* and human brains were screened with CSF antibody probes from patients with Lyme disease. It was found that patients produced antibodies against *B. burgdorferi* as well as CNS proteins. It is possible that this autoimmune response may be essential for the development of demyelinating disease in neurological Lyme borreliosis (Schluesener et al., 1989).

4.4 Acute Disseminated Encephalomyelitis

Acute disseminated encephalomyelitis (ADEM) is a demyelinating disorder of the CNS sharing many similarities with MS. Demyelination in MS is considered to be an autoimmune process mediated by autoreactive T cells against myelin epitopes. It has been postulated that in ADEM there is in vivo activation of autoreactive T cells by superantigens of *Streptococcus pyogenes* resulting in a dramatic demyelination. In vitro analysis of monocytes and T-cell clones indicated that: first, the T-cell receptor (TCR) repertoire was compatible with in vivo expansion induced by *S. pyogenes* exotoxins; secondly, MBP-reactive T cells showed cross-reactivity to *S. pyogenes* supernatant and exotoxins; thirdly, cytokine mRNA expression revealed a Th2-biased cytokine profile (Jorens et al., 2000). Thus, it was concluded that *S. pyogenes* may have induced activation of pathogenic myelin-reactive T cells contributing to the dramatic inflammatory demyelination.

4.5 Sydenham’s Chorea

Some infectious agents directly invade the CNS, whereas others cause an immune-mediated disorder of the CNS without direct CNS invasion by the microorganism. The classic postinfectious disorder of the CNS is Sydenham’s chorea (SC) (Sydenham, 1848), a psychiatric disorder occurring after infection with Group A streptococcus (GAS). In post-streptococcal CNS disease, several different immune mechanisms could cause dysfunction of the CNS including toxin-, T-cell-, B-cell-, antibody-, cytokine-, or superantigen-mediated disease. Elevated levels of antineuronal antibodies have been reported in 46% of patients with acute SC (Husby et al., 1976), and only 1.8% to 4% are controls. Further studies have supported antibody reactivity in acute SC (Morshed et al., 2001). However, the presence of autoantibodies in serum, and CSF, is only one inclusion criterion for an autoimmune-mediated disorder. Several studies support the presence of antineuronal antibodies and their pathogenicity in post-streptococcal disease in the CNS (Dale, 2005). The role of cellular immunity in SC has so far not been examined, but could be of critical importance. It is possible that several different immune mechanisms could result in the clinical symptoms of SC, complicated further by genetic, environmental, or neurochemical factors. However, despite these complications post-streptococcal disorders of the CNS remain an intriguing model of neuropsychiatric disease.

4.6 Protective Effects of Bacterial and Helminth Infections in CNS Disease

Interestingly, certain bacterial or helminth infections can result in a reduction in autoimmunity in the CNS. For example, it has been shown that during experimental infection with BCG (the vaccine strain of *Mycobacterium bovis*) overexpansion of activated CD4+ T cells is prevented by the interferon- γ (IFN- γ)-dependent promotion of apoptosis within this population (Dalton et al., 2000). Controlled elimination of Th1 cells is necessary to avoid the pathologies related to overaccumulation of these cells. The protective effect of BCG infection has been analyzed in an EAE model of human MS. In this model, the fate of genetically marked encephalitogenic CD4+ T cells was determined after transfer to BCG-infected and -uninfected recipients (O’Connor et al., 2005). The results support the hypothesis that the protection

conferred by mycobacterial infection results, in part, from the generation of an internal environment that is hostile to the survival of activated CD4+ T cells. Other bacteria showing a protective effect in reducing autoimmunity in the CNS include *Bordetella pertusis*, *Mycobacterium tuberculosis*, and *M. bovis* BCG. These findings have implications for autoimmune-, atopic-, vaccine-, and pathogen-induced immune responses during chronic mycobacterial infections.

In contrast to most bacterial infections, helminth infections induce a Th2-type immunity (Pearlman et al., 1993). The correlation between helminth infections and a lower incidence of autoimmune diseases has been suggested by several studies, most notably in MS. For example, MS occurs rarely in areas endemic with shistosome infections. Thus, it is possible that “natural Th2 preconditioning” could influence the development of Th1-modulated autoimmunity in the CNS. Indeed in experimental mice where a Th2 environment was induced, by intraperitoneal and subcutaneous *Schistosoma mansoni* ova immunization, there was a significant protection from EAE (Sewell et al., 2002). Since some intestinal helminthic infections produce little pathology, infection or treatment with helminth components has potential therapeutic applications for CNS autoimmunity, including MS. Other parasites that have been shown to have protective effects in autoimmune disease include *S. mansoni* live infection or ova, *Trypanosoma brucei brucei*, Malaria, and *Trichuris trichuria*.

5 Autoimmunity in Human Neurological Diseases

Several human neurological diseases, apart from MS, have been identified as probably involving autoimmune mechanisms. Such diseases include neuromyelitis optica (NMO), encephalitis lethargic syndrome, neuromyotonia (NMT), and possible schizophrenia. The most intensively studied disease in this group is “stiff-man syndrome” (SMS).

5.1 Neuromyelitis Optica

NMO, also known as Devic’s disease, is an idiopathic demyelinating disease of the CNS, and is characterized by attacks of optic neuritis and myelitis. Although the causes of NMO are unknown, several lines of evidence suggest the involvement of B-cell autoimmunity. In human cases, the lesions are seen in the spinal cord and optic nerves. Analysis of these lesions indicated that demyelination was present across multiple spinal cord levels, associated with cavitation, necrosis, and acute axonal pathology (spheroids) in both gray and white matter (Lucchinetti et al., 2002). The inflammatory infiltrates in the lesions are characterized by macrophages associated with large numbers of perivascular granulocytes and eosinophils, together with rare CD3(+) and CD8(+) T cells. Active lesions show a marked perivascular deposition of immunoglobulins and complement C9 neoantigen. Additionally, there is serum autoantibody NMO-IgG in active patients (Wingerchuk, 2006). These findings taken together support a role for humoral immunity in the pathogenesis of NMO.

5.2 Neuromyotonia

There is increasing evidence that autoimmunity is implicated in the pathogenesis of peripheral nerve hyperexcitability, neuromyotonia (NMT). In NMT, patient’s plasma or IgG can transfer the electrophysiological features to mice, and can reduce voltage-gated potassium channel currents in vitro. Indeed antibodies to voltage-gated potassium channels can be detected in the serum of many patients, who have peripheral nerve hyperexcitability. Thus, NMT can occur as antibody-mediated autoimmune ion channelopathies like myasthenia gravis and the Lambert–Eaton myasthenic syndrome (Newsom-Davis, 2004). These findings offer alternative approaches to the treatment of NMT.

5.3 Schizophrenia

Research on schizophrenia has focused on studies of structural and functional brain abnormalities, but recently has changed direction with the emphasis on possible etiological factors. One hypothesis is that schizophrenia is caused by an infection or is the result of an autoimmune reaction against the CNS. Several

studies attempted to identify a specific infection agent or an antibody directed against CNS tissue have not produced a consistently replicable finding (Kirch, 1993). However, schizophrenia is more likely to be a heterogeneous disorder resulting from multiple factors including genetic, environmental factors, as well as autoimmune mechanisms.

5.4 Lethargic Encephalitis

Lethargic encephalitis has been known for centuries and the most recent epidemic ravaged the world between 1916 and 1927, and was named by von Economo as encephalitis lethargica (EL) (Von Economo, 1931). Since EL was epidemic during the same period as the 1918 influenza pandemic, it was originally proposed that EL was caused by influenza virus. However, recent reports have consistently failed to demonstrate evidence of neurotropic viral particles. The finding of OCB in the CSF (Williams et al., 1979), and the successful treatment of some cases with steroids, has led to the hypothesis that this phenotype may be immune-mediated. Recently, cases of an EL-like syndrome have been reported, often following pharyngeal infections. These EL-like patients have been examined for the presence of autoantibodies, particularly against basal ganglia (Dale et al., 2004). It was found that either intrathecal OCB or a mirrored pattern of OCB was seen in 69% of the patients. By contrast, all CSF PCR studies were negative suggesting that a neurotropic viral encephalitis is unlikely. Histopathological findings in EL show perivascular lymphocytic cuffing of the basal ganglia. Cellular infiltration consists of both T and mature B lymphocytes. Additionally, there were secondary reactive astrocytes and macrophage activation but no other striking pathological features. The hypothesis that the EL phenotype could be etiologically similar to Sydenham's chorea is appealing. In EL, it is possible to demonstrate autoantibodies reactive against discrete basal ganglia autoantigens in 95% of the patients (Dale et al., 2004). CSF examination confirmed that the autoantibodies are present in the CNS, although at present it is unknown whether these antibodies are produced either intrathecally or peripherally. Future proteomic studies should be able to identify the auto antigens involved. Dale has proposed that this EL phenotype may occur secondary to postinfectious autoimmunity with vulnerability of deep gray matter neurons (Dale et al., 2004).

5.5 Stiff-Man Syndrome

SMS is a rare human CNS disease, characterized by chronic rigidity of the body musculature with superimposed painful spasms (Moersch and Woltman, 1956). Although the etiology of SMS is unknown, an autoimmune mechanism has been postulated based on the presence of autoantibodies against γ -aminobutyric acid (GABA)-converting enzyme glutamic acid decarboxylase (GAD) in up to 60% of SMS patients (Solimena et al., 1990). The pathogenic significance of these autoantibodies is uncertain. The frequent finding of pancreatic autoreactivity in SMS patients and the higher than predicted association of SMS with INS-dependent diabetes mellitus (IDDM) suggests that these two diseases might have an autoimmune pathogenesis involving shared autoantigens predominantly expressed in neuron-endocrine tissues such as the brain and pancreas (Solimena and De Camilli, 1991). To further refine the role of autoimmunity in SMS, the autoimmune recognition of a second IDDM-associate autoantigen, pancreatic 37/40-kDa IDDM autoantigen (coded for by the gene called ICA), has been investigated (Martino et al., 1996). Human ICA 105 is restricted to the pancreas and brain and its distribution within the CNS is similar to GAD. Anti-ICA 105 and anti-GAD antibodies have been detected in 75% of patients with SMS. Thus ICA 105 represents another putative neuroendocrine autoantigen in SMS.

In a subset of cases, SMS has an autoimmune paraneoplastic origin. The presence of high-titer autoantibodies directed against gephyrin has been reported in a patient with clinical features of SMS and mediastinal cancer (Butler et al., 2000). Gephyrin is a cytosolic protein selectively concentrated at the postsynaptic membrane of inhibiting synapses, where it is associated with GABA and glycine receptors. It has been suggested that these mixed GABA/glycine synapses are the primary targets of autoimmunity in SMS. The question remains as to how these autoantibodies arise? It is possible that T cells or other

associated antibodies directed against surface antigens may mediate the disease. These autoantibodies could be generated by the spread of an autoimmune response against macromolecular complexes, including both surface antigens and associated intracellular proteins.

6 Autoimmunity Caused by CNS Insult

Injury to nerves, such as transection or crush injury, can result in autoimmune reactions. Examples of such injury are best shown in studies with rats involving facial nerve transection and optic nerve crush injury.

Nervous tissue expression of immunological signaling molecules and lymphoid tissue immune responses has been studied in male rats of the Lewis and Brown Norway (BN) strains following facial nerve transection (Olsson et al., 1992). Within 4 days of nerve transection, in both strains of rat, IFN- γ -like immunoreactivity was detected in the cytoplasm of axotomized motor neurons. In addition, there was a similar induction of MHC class I and class II and CD4 molecules on surrounding glial cells. T-lymphocyte infiltration was also observed in the facial nuclei ipsilateral to the axotomy in all rats. Autoreactive T cells, to myelin or peptides of MBP, secreting IFN- γ , increased markedly in the superficial cervical lymph nodes. Some, but not all, of the axotomized Lewis rats developed widespread perivascular infiltration of mononuclear cells in the CNS. This later finding, reminiscent of EAE, may have immunological consequences.

Spinal cord injury (SCI) initiates many destructive processes mediating tissue injury at the site, and in close proximity, of primary trauma. These processes are collectively referred to as secondary injury. Several lines of evidence implicate immunologic activation in promoting progressive tissue pathology and/or inhibiting neural regeneration after traumatic injury to the CNS. The significance of immune cells within the spinal cord lesions remains somewhat controversial. Lymphocytic infiltration is also observed after CNS injury. Since myelin-reactive antibodies are elevated following CNS injury, both B and T cells are likely to be activated (Palladini et al., 1987). These findings, coupled with pathophysiologic data, show a striking resemblance between SCI and inflammatory demyelinating diseases such as EAE (Popovich et al., 1996). Witebsky postulated that three criteria need to be fulfilled to establish the autoimmune nature of a disease (Rose and Bona, 1993). First, disease induction in normal individuals must be effected by the transfer of autoreactive antibodies or autoreactive T cells. Secondly, evidence must be obtained by reproducing the disease in animals. Thirdly, autoantibodies and/or autoreactive T lymphocytes must be isolated from the diseased organ. In SCI, data show that the second criteria is satisfied and experimental results fulfill the third condition, namely, CNS-reactive antibodies have been isolated from both animals and patients with SCI (Palladini et al., 1987). Additionally, low-level T-cell proliferation to MBP has been measured after SCI, as well as the generation of encephalitogenic T cells (Popovich et al., 1996). However, in SCI immunopathogenic responses are rare. Autoimmune reactions occurring after SCI could be due to T-cell recognition of myelin, which contains proteins that are normally sequestered behind the BBB in adults. It is highly likely, as in other autoimmune diseases, that both genetic and environmental factors play a role in the outcome of SCI.

7 Autoimmunity as a Mediator of CNS Repair

Rather than being purely deleterious, it is possible to use autoimmunity as a tool to mediate CNS repair.

7.1 Antibody-Mediated Remyelination

It has clearly been demonstrated that autoreactive antibodies can enhance endogenous myelin repair in the Theiler's virus model of MS (Bieber et al., 2001). Intracerebral inoculation of Theiler's murine encephalomyelitis virus (TMEV) into susceptible SJL mice results in acute encephalitis that is resolved in 14–21 days, which is followed by chronic viral persistence. This persistent TMEV infection leads to chronic demyelination and loss of motor function, and is used as an animal model of human MS. In the SJL strain, demyelination is

evident within 30 days after infection, and paralysis eventually occurs by 6–9 months. Spontaneous remyelination is very limited in SJL mice, where <10% of the total demyelinated lesion is repaired. Thus, SJL mice make an excellent model to study strategies promoting remyelination. In this model, it was found that transfer of antiserum or purified immunoglobulin (Rodriguez and Lennon, 1990) from uninfected animals immunized with spinal cord homogenate (SCH) enhanced remyelination, demonstrating a beneficial role for the humoral immune response against SCH in promoting myelin repair. Hybridomas were subsequently generated from SJL mice following SCH immunization. Two mouse monoclonal antibodies (mAbs) were identified that enhance this remyelination and are polyreactive IgM antibodies. These mAbs bind to antigens expressed on the surface of oligodendrocytes, suggesting that the remyelinating promoting activity of these mAbs involve the direct stimulation of myelin-producing cells (Asakura and Rodriguez, 1998).

7.2 MBP-Specific T Cells Aid Recovery from Spinal Cord Damage

Since the CNS is termed an “immune-privileged site,” immune responses with the CNS are restricted (Streilein, 1995). In the CNS, under normal conditions, activated T cells can cross the BBB and enter the CNS parenchyma. However, only T cells capable of reacting with a CNS antigen persist there (Hickey et al., 1991). Following axotomy in the CNS, there is a significantly greater accumulation of endogenous T cells in the injured PNS axons than in the injured CNS axons. In addition, the CNS shows a high potential for elimination of T cells via apoptosis (Smith et al., 1996), whereas such elimination is less effective in the PNS and other tissues. These findings taken together have led investigators to conclude that the T-cell response to CNS injury is limited, and has raised the question of whether boosting the T-cell response at the site of CNS injury can affect the outcome of secondary degeneration. Studies with Lewis rats, subjected to spinal cord contusion at T7 or T9 and given systemic injection of anti-MBP T cells at the time of contusion, or 1 week later, results in significantly better recovery than that observed in control rats treated with T cells directed against ovalbumin (Hauben et al., 2000). Not only passive T-cell transfer but also active immunization with MBP promotes recovery from SCI. The earlier results raise various questions concerning the mechanism of this neuroprotective effect. Results with thymectomized rats and nude mice demonstrate that the observed neuroprotection is T-cell-dependent (Yoles et al., 2001), although the precise type of T cell involved is as yet unclear. The beneficial T cells might operate by producing cytokines and neurotrophic factors, whose primary targets are probably astrocytes, microglia, and even neurons. Regardless of the mechanism of the T-cell-mediated neuroprotective effect, the experimental evidence clearly shows that active or passive immunization with CNS myelin-associated antigens can be beneficial for neuronal recovery (Schwartz and Cohen, 2000). The nature of the regulatory mechanism controlling beneficial autoimmunity has yet to be elucidated. A number of endogenous mechanisms have been proposed (Schwartz, 2001), and the role of such mechanisms in neuronal protection should be examined.

8 Multiple Sclerosis

MS is the most common demyelinating disease of the CNS occurring at a prevalence of 250,000–350,000 in the USA (Anderson et al., 1992) and the national annual costs of this disease were estimated to be \$6.8 billion (Whetten-Goldstein et al., 1998). MS usually affects people between the ages of 15 and 50, although there is an increasing number of juvenile MS patients who develop disease. Eighty percent of MS patients have a relapsing–remitting disease, which eventually progresses to a chronic progressive disorder. The MS lesion is characterized by plaques throughout the white matter of the brain and spinal cord. Demyelination is accompanied by inflammatory cell infiltrates consisting of plasma cells, macrophages/microglia, T and B lymphocytes. In common with other autoimmune diseases, relapsing–remitting MS is more common in women than men, with a ratio of 2:1. Autoimmune responses to myelin components MBP, PLP, and MOG have been detected in MS patients, suggesting an autoimmune etiology for MS (Stinissen et al., 1997).

MS is a complex disease which follows a markedly varying course in different patients and also patients' responses to therapy vary considerably. Recently, MS lesions have been classified into four separate

categories, which may account for the heterogeneity in disease (Lucchinetti et al., 2000). Type I MS lesions were similar to T-cell-mediated demyelination and type II lesions resembled T-cell plus antibody-mediated autoimmune encephalomyelitis. Type III and IV lesions involved primary oligodendrocytes dystrophy (virus- or toxin-induced).

9 Experimental Autoimmune Encephalomyelitis

EAE is the most widely used animal model of MS. EAE was first developed in 1933 (Rivers et al., 1933). Originally, the disease was induced by injections of whole brain or spinal cord homogenates with adjuvants (Kabat et al., 1947). However, more recently encephalitogenic CNS proteins or peptides have been used to induce EAE. This disease is mediated by CD4⁺ T cells that recognize certain myelin epitopes and infiltrate the CNS causing inflammatory demyelination.

In order for a T-cell clone to be pathogenic and induce EAE, it must home to the target organ, recognize antigen within target tissue, and produce a combination of cytokines to initiate tissue injury. The first critical step in this process is the interaction of lymphocytes with the cerebrovascular endothelial cells (CVE). The interaction between CVE and T lymphocytes is mediated by a number of different adhesion molecules: lymphocyte functional antigen-1 (LFA-1) binding to intracellular adhesion molecule-1 (ICAM-1) and ICAM-2, VLA-4 to VCAM-1 and fibronectin. Experiments with encephalitogenic T-cell clones to MBP (Kuchroo et al., 1993) and PLP (Barten and Ruddle, 1994) have shown increased expression of adhesion molecules (in particular VLA-4) and tumor necrosis factor (TNF) contribute to the pathogenicity of the T cells.

10 A Viral Etiology for Multiple Sclerosis

The etiology of MS is unknown, although epidemiological studies have implicated an infective agent as a probable initiating factor (Acheson, 1977; Gilden, 2001). An epidemiological survey reported that the increased risk of developing MS was associated with mumps, measles, and Epstein-Barr virus infections at an older age (Miguel et al., 2001). In addition, exacerbations of MS are frequently preceded by viral infections (Sibley et al., 1985). It is also intriguing that the antiviral agent IFN- β has been reported to have a beneficial effect on relapsing–remitting MS (IFN- β Multiple Sclerosis Study Group, 1993). IFN- β also inhibits the progression of relapsing–remitting EAE in mice (Yu et al., 1996). The exact mechanism of IFN action in these conditions is not completely understood. IFN- β has many diverse biological roles ranging from antiviral to immunomodulatory effects (Belardelli and Gresser, 1996). Since MS is thought to be initiated by a viral infection, the effectiveness of IFN may be related to its role in viral inhibition. However, the autoimmune aspects of MS may be downregulated through a number of different mechanisms because type I IFNs have been shown to inhibit DTH responses (also important in Theiler's virus-induced demyelination (TVID) and EAE); alter the homing and trapping of lymphocytes; enhance NK and T-cell activity and cytotoxicity; enhance Fc γ receptors effects (Belardelli and Gresser, 1996); inhibit T-cell proliferation; and decrease the production of IFN- γ (Noronha et al., 1993). IFN- β has also been shown to decrease the migration of T lymphocytes in vitro by interfering with the production of T-cell matrix metalloproteinases, which mediate T-cell migration through matrices. Both NK cells, CD8 and CD4 T cells, were also affected by this treatment. The authors demonstrated that IFN- β decreased the matrix metalloproteinase levels which in turn prevented the cleavage of fibronectin (Stuve et al., 1996).

A number of different viral agents have been isolated from the brains of MS patients, including measles, mumps, parainfluenza type I (Allen and Brankin, 1993), and human herpes simplex type 6 (HHSV6) (Challoner et al., 1995). In common with other autoimmune diseases, stressful life events may precipitate the onset and clinical relapses in MS patients (Whitacre et al., 1994). One mechanism of stress-induced exacerbation might be via increased glucocorticoid levels resulting in immunosuppression and reactivation of latent viruses such as herpes virus.

Viruses are also known to cause demyelination in animals: measles virus in rats; JHM mouse hepatitis virus, Semliki Forest virus, and Theiler's virus in mice; visna in sheep; herpes simplex in rabbits

(Dal Canto and Rabinowitz, 1982). Therefore, in order to understand the pathogenesis of MS, animal models of virus-induced demyelination such as Theiler's virus infection represent a highly relevant approach. Theiler's virus infection in mice is not only an excellent model for the study of the pathogenesis of MS, but also a model system for studying disease susceptibility factors, mechanisms of viral persistence within the CNS, and virus-induced autoimmune disease.

11 Theiler's Murine Encephalomyelitis Virus as a Model for MS

Max Theiler first described TMEV in 1934 after isolating this virus from the brain of a paralyzed mouse (Theiler, 1934). Theiler noted that the incubation period was 7–30 days and the mice remained infected up to 150 days after injection. The mice developed acute necrosis of the ganglion cells in the anterior horn of the spinal cord. TMEV was originally classified as an enterovirus in the Picornaviridae family, as it was biologically similar to poliovirus. However, after the sequencing of TMEV, it was reclassified as a cardiovirus as it showed marked similarity to mengovirus.

11.1 Age-Related Susceptibility to TVID

Theiler's virus infection in mice became a model for MS in the 1970s, after Lipton demonstrated that the virus induced inflammatory demyelination (Lipton, 1975). The virus is administered intracranially to mice that are 4–6 weeks of age because younger animals develop fatal encephalitis and older animals become resistant (Steiner et al., 1984). This window of opportunity is a common theme in neurotropic viral infections that induce demyelination. It is also interesting to note that exposure to the putative causative agent of MS occurs before or during adolescence. The susceptibility age phenomenon is probably not due to immune system development because mice and humans have mature immune systems during adolescence. A potential explanation is that there are a crucial number of oligodendrocytes at a susceptible time of development available for infection. Evidence to support this hypothesis is that mice are still actively myelinating at this stage and humans continue myelination until about 20 years of age. In addition, mature oligodendrocytes appear to be relatively resistant to TMEV infection *in vitro* whereas immature oligodendrocytes are susceptible to the lytic effects of TMEV (O'Shea et al., 1997).

11.2 The Early Disease Induced by Theiler's Virus

During early Theiler's virus infection, neurons are infected and, in resistant strains of mice, this early gray matter disease is the only manifestation of infection. However, in susceptible mice the virus also infects and persists in oligodendrocytes, astrocytes, and macrophages/microglial cells (Aubert et al., 1987; Blakemore et al., 1988). Theiler's virus must establish a persistent infection in the CNS to cause later demyelinating disease (Aubert et al., 1987). Strains of mice that are resistant to develop TVID are able to clear the early viral infection effectively from the CNS. Susceptible strains of mice fail to clear the CNS infection, in part due to inadequate natural killer cell (NK), cytotoxic T-cell (CTL) and CD4⁺ T cell responses (Borrow et al., 1992, 1993). During the early infection, virus replicates to high levels in the brain and spinal cord (Welsh et al., 1989). At ~4 weeks postinfection, the viral titers decrease and this coincides with the development of high-neutralizing antibody titers. In this early phase of the disease, the virus infects neurons and mice may develop polio-like disease, that is, flaccid hind limb paralysis.

11.3 The Late Demyelinating Phase of Theiler's Virus Infection

The mechanisms of demyelination in the TVID model are complex. Demyelination is partly mediated by direct viral lysis of oligodendrocytes (Roos and Wollmann, 1984). CTL reactivity has also been implicated in demyelination (Rodriguez and Sriram, 1988). Bystander demyelination mediated by virus-specific DTH

T cells recruiting macrophages to the CNS is a further mechanism (Clatch et al., 1987; Gerety et al., 1991). Finally, autoimmunity, at both the B- and T-cell level, has been detected in TVID (Welsh et al., 1987, 1989, 1990; Miller et al., 1997; Borrow et al., 1998). The autoimmune reactivity seen in TVID may result from viral damage to oligodendrocytes, uptake and processing of myelin antigens by macrophage/microglial cells, and subsequent activation of autoreactive T cells. The autoreactive T cells have been shown to be pathogenic and are able to demyelinate in vitro (Dal Canto et al., 2000). Epitope spreading has also been noted in TVID whereby SJL mice first develop T-cell responses to viral epitopes, followed by T cells to PLP139–151 (the dominant encephalitogenic peptide in this strain of mice). Intramolecular epitope spreading to PLP178–191 and followed by PLP56–70 is then observed and finally intermolecular spreading to MOG92–106 (Miller et al., 1997).

11.4 Genetic Control of Susceptibility to TVID

A number of studies have reported that viral persistence and demyelination in susceptible strains of mice are under multigenic control. Overall there is good correlation between viral load, pathology, and clinical symptoms within different strains of mice infected with TMEV which suggests that the host's ability to control the viral replication during the persistent infection is related to disease status (Brahic et al., 2005). The most important genes implicated in susceptibility to demyelination are the MHC class I and the T-cell receptor genes (Melvold et al., 1987). Another gene locus on chromosome 6 not linked to the T-cell receptor locus has also been implicated in demyelination (Bureau et al., 1992). Two additional loci, one close to IFN- γ on chromosome 10 and one near MBP on chromosome 18, have been associated with viral persistence in some strains of mice (Bureau et al., 1993). Immune recognition of Theiler's virus is clearly an important element in susceptibility to demyelination, as indicated by the genetic association with MHC and the T-cell receptor, although other undefined factors are also involved.

11.5 Similarities between TVID and EAE

The lesions induced by Theiler's virus not only resemble those seen in EAE, but also occur in the same locations within the spinal cord (Blakemore et al., 1988). Similar strains of mice are susceptible to EAE and Theiler's virus; SJL mice being the most susceptible to both diseases (Linthicum and Frelinger, 1982; Rodriguez et al., 1986). Treatments of both diseases with immunosuppressive regimens and monoclonal antibodies to MHC class II (Steinman et al., 1981; Freidmann et al., 1987) and CD4 (Waldor et al., 1984; Welsh et al., 1987) have proved successful. Interestingly, local x-irradiation of the spinal cord in EAE and Theiler's virus infection resulted in the exacerbation of the lesions probably through the increased permeability of the BBB (Love et al., 1987). Taken together, these facts indicate that there may be similar mechanisms of demyelination operating in both conditions. Furthermore, autoimmune phenomena occur in Theiler's virus-infected mice and autoreactive T cells have been shown to mediate demyelination in vivo (Dal Canto et al., 2000). Just as EAE has been transferred by the intravenous injection of T-cell clones against an encephalitogenic determinant (Ben-Nun et al., 1981), autoreactive T cells in TVID may also be an important part of the disease pathogenesis.

11.6 Aberrant MHC Class II Expression

The ability of antigen-presenting cells to express MHC class II antigens has been shown to be an important initiating event in the evolution of an immune response (Schwartz et al., 1978).

Aberrant class II expression occurs on both target cells and endothelial cells in a number of autoimmune diseases and is thought to be important either in the initiation of the inflammatory process or in the subsequent perpetuation of the disease process (Bottazzo et al., 1983).

The induction of class II expression on astrocytes has been found to correlate with susceptibility to demyelination induced by JHM virus and in EAE in SJL mice (Massa et al., 1987). The relevance of aberrant class II expression on astrocytes is unclear because the predominant antigen-presenting system in CNS is the microglia (Matsumoto et al., 1988). Furthermore, the class II-expressing astrocytes are able to present both MBP (Fontana et al., 1984) and viral antigens to T cells (Borrow and Nash, 1992). Astrocytes also express the ICAM-1 in response to a number of lymphokines (Frohen et al., 1989). This intergrin molecule is the ligand for LFA-1 and their association is intimately involved in antigen presentation.

The effectiveness of monoclonal antibodies directed at MHC class II antigens in the treatment of both EAE (Steinman et al., 1981) and TVID (Friedmann et al., 1987) also provides evidence of the importance of aberrant MHC class II expression and subsequent antigen presentation in the evolution of the inflammatory process. Presumably, the monoclonal antibodies are acting either at the BBB preventing MHC class II expression on the endothelial cells or in the lymphoid tissue interfering with T-cell activation.

Differential virus-induced MHC expression on cells within the CNS may also account for defective viral clearance from the white matter in TVID-susceptible mice and may explain why virus is confined to the gray matter in resistant strains of mice. MHC expression in the CNS is induced during TVID (Lindsley et al., 1992). MHC class I expression has been shown to be mediated by type I IFN, since IFN- α/β knock out mice fail to express MHC class I following infection with TMEV (Njenga et al., 1997). TMEV infection of SJL/J and CBA CVE results in the upregulation of expression of MHC class I on CVE (Sapatino et al., 1995; Welsh et al., 1995) via IFN- β production (unpublished data). In addition, IFN- γ upregulates the expression of MHC class II on CVE (Welsh et al., 1993) and astrocytes (Borrow and Nash, 1992) derived from strains of mice that are susceptible to TVID. Other researchers have demonstrated that the IFN- γ -inducible MHC class II expression in SJL CVE is confined to endothelial cells derived from the CNS (Jemison et al., 1993). Increased MHC class II expression on cells within the CNS may lead to increased antigen presentation and inflammation. The fact that there is a correlation between susceptibility to TVID and increased MHC expression in the CNS suggests that the CNS of autoimmune susceptible mice is a more inflammatory environment than that of TVID-resistant mice.

12 The Role of the BBB in TVID and MS

The BBB is composed of CVE, astroglia, pericytes, perivascular macrophages, and basal lamina. In inflammatory conditions of the CNS such as MS, and two animal models of MS: EAE and TVID, T cells enter the CNS and mediate tissue damage. Lymphocyte cuffing around postcapillary venules is a characteristic of all these conditions and suggests that the main route of cellular migration (and viral migration in the case of TVID) may occur across the BBB. Heterotropic brain transplants from EAE-susceptible (SJL/J) to resistant mouse strains suggest that regulation of susceptibility to disease resides at the level of the cerebral endothelium and not within the CNS parenchyma or lymphoid/bone marrow-derived compartment (Goldowitz et al., 1987). Therefore, the BBB is critical in the development of inflammatory CNS disease.

The role of CVE in MS is likely to be more important than originally suspected. In progressive MS, there is rapid trafficking of T cells from the vasculature across the BBB and into the CNS (Hafler and Weiner, 1987). Furthermore, antibodies to CVE were described in MS patients (Tanka et al., 1987) and rhesus monkeys injected with CVE were shown to develop EAE (Tsukada et al., 1988). In patients with MS, therapy with IFN- γ caused exacerbations which may have resulted from increased MHC class II expression on CVE (Panitch et al., 1987) and subsequently, antigen presentation which would allow increased access of T cells into the CNS. Interestingly, cytotoxic activity against CVE has also been described in MS patients (Tsukada et al., 1993).

12.1 Demyelinating Viruses and the BBB

A number of viruses that have been implicated in MS have been shown to infect CVE. CVE possess many specialized receptors (transferrin receptor, γ -GTP, acetylated LDL) that may also function as specific viral receptors. Infection of CVE may allow the virus to gain access to the CNS or may result in the upregulation

of various molecules involved in lymphocyte adhesion. Herpes simplex virus infection of CVE causes an upregulation in the expression of ICAM-1 and increased adhesion of naive syngenic splenocytes (Brankin et al., 1995). Alteration in the function of the BBB may also result from systemic infection with virus resulting in increased cytokine production. It is interesting to note that MS patients often suffer from disease relapses following viral infections (Sibley et al., 1985).

Interactions at the BBB in Theiler's virus infection have been shown to be important in the development of demyelinating disease. Hyperexpression of ICAM-1 on vascular endothelial cells was observed in the spinal cord of TMEV-infected mice and suppression of TVID was observed following treatment with monoclonal antibody to ICAM-1 or LFA-1 (Inoue et al., 1997a). In addition, fibrin deposition within the spinal cord correlated with the degree of demyelinating disease seen in SJL mice infected with TMEV, indicating that increased permeability of the BBB is an important component in TVID neuropathogenesis (Inoue et al., 1997b).

13 Mast Cells in Demyelination

Susceptibility to both TVID and EAE is controlled by a number of genes including those for MHC and T-cell receptors. In addition, genes for vasoactive amine (VAA) sensitization have been implicated in susceptibility to EAE (Linthicum and Frelinger, 1982) and the administration of cyproheptadine (a serotonin and histamine blocker) has been shown to reduce the edema and inflammation in this condition (Waxman, et al., 1984). The strains of mice that are susceptible to EAE and TVID are also sensitive to VAA and therefore VAA may also play a role in the development of TVID. One possible source of VAAs in the CNS is mast cells which have been demonstrated in the thalamus, hypothalamus, meninges, and in close proximity to the dorsal root ganglia in association with blood vessels (Orr, 1988). It is precisely at these sites that demyelination occurs in both EAE and TVID (Blakemore et al., 1988). Regional and temporal increases in the histamine content of the brain and spinal cord occur concomitantly with the appearance of EAE in the rat (Orr and Pace, 1984). Furthermore, mast cells have been shown to be essential for the early onset and severity of disease in EAE using mast cell-deficient mice (Secor et al., 2000). Mast cells are regularly present in the border zone of MS plaques in close association with small vessels but are absent from normal brain tissue (Kruger et al., 1990). Recently, BBB abnormalities were detected not only in focal lesions but also in diffusely abnormal white matter as detected by postmortem MRI (Vos et al., 2005). Interestingly, hydroxysine, a histamine-1 (H1) receptor antagonist, has been shown to be effective in EAE and a recent pilot clinical trial in MS has shown promising results (Logothetis et al., 2005).

The role of VAAs in CNS inflammation is hypothesized to be related to their ability to increase the permeability of the BBB allowing the influx of elevated numbers of T cells into the CNS. TNF- α -secreting CD4+ T cells specific for MBP have been shown to cause EAE (Ben-Nun and Cohen, 1971; Powell et al., 1990) and similar Th1 virus-specific T cells have been implicated in the pathogenesis of TVID (Clatch et al., 1987). Thus, the passage of these cells into the CNS is an important initiating event in the pathogenesis of both conditions. Mast cells secrete and release TNF- α that has been shown to cause myelin loss by direct toxic effect on oligodendrocytes (Selmaj and Raine, 1987). In addition, activated mast cells have been shown to secrete proteases that also destroy myelin (Theoharides, 1990). Furthermore, the mast cell proteases release an encephalitogenic peptide from MBP (Johnson et al., 1998) that can be presented to T cells by either astrocytes or endothelial cells and cause T-cell proliferation.

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16 Experimental Autoimmune Encephalomyelitis

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1	<i>Introduction</i>	356
1.1	History	357
2	<i>Models of Experimental Autoimmune Encephalomyelitis</i>	358
2.1	EAE in Rodents	359
2.2	EAE in Nonhuman Primates	360
2.3	Transgenic Models Including Humanized Models	360
3	<i>Immunopathology of EAE</i>	361
3.1	EAE Lesions in Different Animal Models	361
3.1.1	Lesions in the Guinea Pig Model	361
3.1.2	Lesions in Mouse Models	361
3.1.3	Lesions in the Lewis Rat Model	362
3.1.4	Lesions in the DA Rat Model	363
3.1.5	Lesions in Nonhuman Primate Models – The Marmoset Model	363
3.2	Inflammation	364
3.2.1	T Cells	364
3.2.2	T Helper 1 (Th1) Cells	364
3.2.3	T Helper 17 (Th17)	366
3.2.4	Regulatory T Cells (Tregs)	367
3.2.5	Epitope Spreading	368
3.2.6	Antigen-Presenting Cells	368
3.2.7	B Cells	368
3.3	The Role of the Innate Immune System in EAE	370
3.3.1	Toll-Like Receptors in EAE	370
4	<i>The Blood–Brain Barrier in EAE</i>	371
4.1	Structural Components of the BBB	372
4.2	Functions of the Structural Components of the BBB	372
4.3	Role of the Blood–Brain Barrier in EAE	372
5	<i>Conclusion and Future Outlook</i>	373

Abstract: Experimental autoimmune encephalomyelitis (EAE) is a well-characterized animal model of multiple sclerosis (MS), a common inflammatory demyelinating disease of the central nervous system (CNS). EAE is induced in susceptible animal species by immunization with myelin proteins or their immunodominant peptides in adjuvant substances, which facilitate the initiation of immune responses. Antigen-presenting cells (APCs) prime autoreactive CD4⁺ T cells to react against myelin components and migrate from the lymph nodes draining the immunization site, to the blood stream, and to the CNS through the blood–brain barrier. In the CNS, myelin-reactive T cells are reactivated by local APC, such as microglia, which present myelin peptides to them. Antigen recognition in the CNS leads to the expansion of myelin-reactive T cells, which produce inflammatory molecules such as cytokines and chemokines and initiate an inflammatory cascade. Other inflammatory cells, such as macrophages and B cells, are recruited to the CNS and contribute to damage to central myelin by direct attack and myelin “stripping” (by macrophages), the production of inflammatory cytokines, reactive oxygen species and nitric oxide, the production of antimyelin antibodies, and the deposition of complement. Immune mechanisms of EAE support the concept that MS is an immune-mediated disease initiated by autoreactive T cells. Adoptive-transfer EAE, in which immune cells from mice with EAE are transferred to healthy recipients, directly demonstrated for the first time the role of myelin-reactive CD4⁺ T cells in autoimmune demyelination. Other evidence for the involvement of immune mechanisms in the pathogenesis of MS include the presence of perivenular T and B lymphocytes in MS lesions, the responsiveness of MS patients to immunomodulatory and immunosuppressive treatments, and the contribution of genes of the immune response to disease susceptibility.

The possibility to manipulate experimental variables such as animal species and strains, myelin proteins and other components of the immunization protocols, and the use of genetically modified animals has rendered EAE a valuable source of information on potential disease mechanisms in MS, in which ethical considerations obviously limit mechanistic interventions. In addition, the administration of drugs and biologicals during different phases of EAE has enabled researchers to use the disease for preclinical testing of experimental treatments. Because of the phylogenetic distance between rodents and humans, treatment efficacy in EAE does not necessarily translate into efficacy in MS. In certain cases, the use of primate models of EAE may help bridge the gap between rodent and human immuno- and neurobiology. In addition, humanized animal models are being developed, in which human immune molecules are transgenically expressed in mice to improve the relevance of experimental findings to MS.

List of Abbreviations: ADEAE, Antibody-augmented demyelinating EAE; APCs, antigen-presenting cells; BBB, Blood–brain barrier; CFA, complete Freund’s adjuvant; CNPase, 2’,3’-cyclic nucleotide 3’-phosphodiesterase; EAE, Experimental autoimmune encephalomyelitis; EAL, Early active lesions; HLA, human leukocyte antigen; IFA, incomplete Freud’s adjuvant; IFN, Interferon; MAG, myelin-associated glycoprotein; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; MTb, *Mycobacterium tuberculosis*; PAMPs, pathogen-associated molecular patterns; PGN, peptidoglycan; PLP, proteolipid protein; PRRs, pattern recognition receptors; rMOG, recombinant MOG; TCR, T-cell receptor; TLRs, Toll-like receptors; TNF, tumor necrosis factor; TRAF6, tumor-necrosis factor-receptor-associated factor 6

1 Introduction

Experimental autoimmune encephalomyelitis (EAE) is a well-characterized animal autoimmune disease. It has been used extensively as a model system to study basic immune function and is the most informative animal model used for the study of multiple sclerosis (MS). Like multiple sclerosis, EAE is an inflammatory and demyelinating autoimmune disease, the development of which varies depending on several genetic and environmental determinants. It was previously thought that CD4⁺ T helper type 1 (Th1) cells producing interferon- γ (IFN- γ) mediated EAE. However, recent data have shown that it is interleukin-17 (IL-17) producing effector CD4⁺ T cells (Th17 cells) that are primarily involved in disease pathogenesis. Though

Th17 cells are now thought to be the main cells involved in disease progression, this is not to the exclusion of other types of immune cells. Th1 cells are still thought to play a role along with Th2, CD8⁺ T cells, macrophages, and B cells.

1.1 History

The origin of EAE arose from vaccination with rabies-infected rabbit spinal cord by Louis Pasteur from 1885. “Neuroparalytic incidents” which caused paralysis or death were observed in around 1 in 1,000 vaccines. It was the topic of intense debate as to the immune mechanisms that caused these neuroparalytic incidents. It was later proved that “contamination” by spinal cord components in the inoculum caused this demyelinating disorder. In the 1920s, Koritschoner and Schwinburg observed that spinal cords of rabbits that were inoculated with human spinal cord caused inflammation (Koritschoner and Schwinburg, 1925). In the 1930s, Thomas Rivers, at Rockefeller University, reproduced the encephalitic complications associated with rabies vaccination by repetitive immunization of rhesus monkeys with central nervous system (CNS) tissue. He along with colleagues, D.H. Sprunt and G.P. Berry, submitted the seminal article entitled, “Observations on Attempts to Produce Disseminated Encephalomyelitis in Monkeys” which was published in the *Journal of Experimental Medicine* on February 21, 1933 (Rivers et al., 1933).

EAE has since become the most extensively studied experimental model not just in neuroimmunology, but in the whole field of research in autoimmune disease. Numerous developments in the field have occurred in the last seven decades. In 1949, Olitsky and Yager demonstrated the principle of strain specificity that has been taken to indicate that genetic factors underlie disease susceptibility in specific animal strains (Olitsky and Yager, 1949). The demonstration that EAE could be adoptively transferred (Lipton and Freund, 1953) by cells (Paterson, 1960) but not serum (Chase, 1959) has been the most influential lesson that EAE has taught us so far on disease mechanisms in MS. The importance of T lymphocytes was directly documented in EAE by the demonstration that these cells are effective and sufficient to adoptively transfer disease from affected animals to healthy recipients (Paterson, 1960; McFarlin and Pettinelli, 1981). The histological and ultrastructural features of EAE have been advantageously compared with those of MS (Lampert and Carpenter, 1965; Raine, 1997). Even the evolution of the name of the experimental disease (from “experimental allergic encephalomyelitis” to “experimental autoimmune encephalomyelitis”) has reflected our dynamic understanding of the immunopathogenesis of the disease (Pedotti et al., 2003; Steinman, 2006).

Advancements also occurred in the methods for induction of disease. The use of Freund’s adjuvant allowed for induction of disease only after a single injection (Kabat et al., 1951) compared with the need for multiple injections (up to 80 per animal) over 1-year period previously. In addition in the 1950s, the addition of heat-inactivated *Mycobacterium tuberculosis* (MTb) to the adjuvant – complete Freund’s adjuvant (CFA) was found to enhance the response to sensitization with CNS tissue. Since then EAE has been induced in many different species including mice, rats, guinea pigs, rabbits, macaques, rhesus monkeys, and marmosets. However, for various reasons including the number of immunological tools, the availability, life span, and fecundity of the animals and the resemblance of the induced disease to MS, mice and rats are the most commonly used species. For many years, it was believed that EAE was mediated by a single immune-cell population (T helper 1, Th1 cells) principally directed against a single antigen, myelin basic protein (MBP). Although MBP was the major component in the CNS extract that caused disease, other components of myelin, such as proteolipid protein (PLP) were later shown to induce disease. It has since become clear that EAE can be induced by different T-lymphocyte populations, including those producing IFN- γ and tumor necrosis factor- α (TNF- α) (Th1), but also those producing IL-17 and other cytokines (Th17). In addition, EAE has been induced in immunosuppressed mice by IL-4-producing T cells (Th2) which are otherwise considered anti-inflammatory. Over the years, the study of myelin antigens in EAE (and MS) has revealed several other targets of autoimmune inflammation, including 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), myelin-associated glycoprotein (MAG), and, perhaps most importantly, myelin oligodendrocyte glycoprotein (MOG). In addition, recent observations have clearly shown that EAE severity can be modulated by the presence of antibodies directed against several myelin antigens, indicating that T- and B-cell responses can cooperate and act in synergy in disease induction (Cross et al., 2001; Oliver et al., 2003).

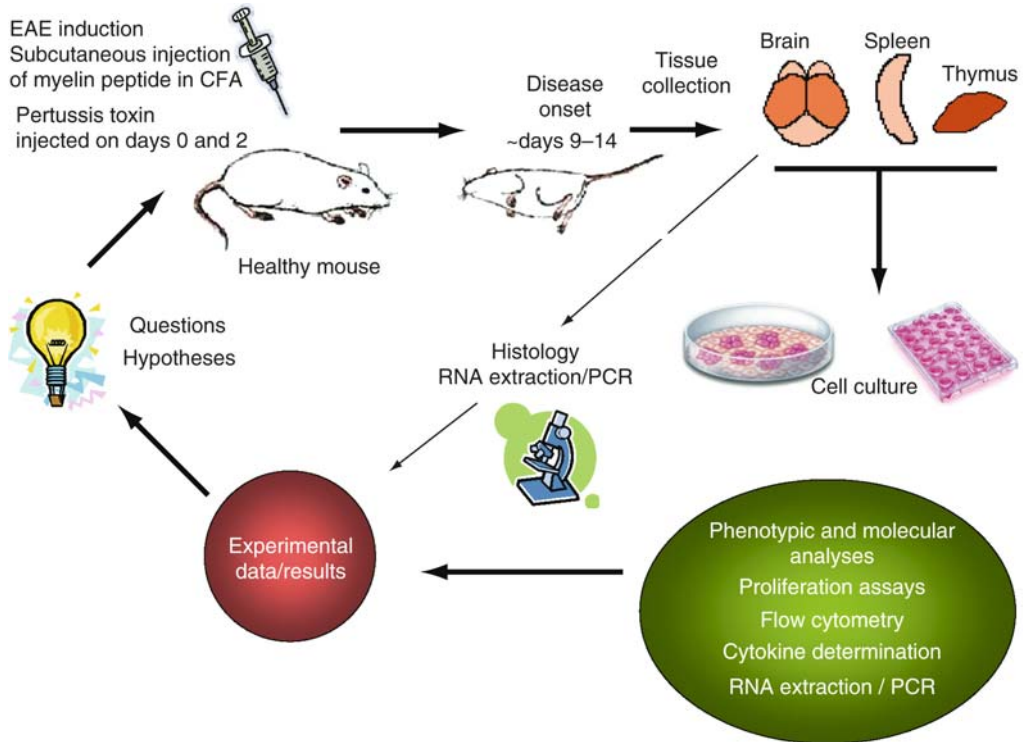
2 Models of Experimental Autoimmune Encephalomyelitis

Many variations of EAE have been developed including acute monophasic, relapsing–remitting, and chronic progressive models. In addition, models for optic neuritis (often the first sign of disease in MS) have also been developed. Each of these EAE models can be used as a useful tool to address specific aspects of MS pathogenesis (Mendel et al., 1995; Kornek et al., 2000; Pomeroy et al., 2005; Gold et al., 2006).

Active immunization with CNS tissue or myelin peptides, such as MBP and PLP in CFA, results in high incidence of disease in susceptible animal strains (🔗 [Figure 16-1](#)). The first clinical signs of EAE typically

■ **Figure 16-1**

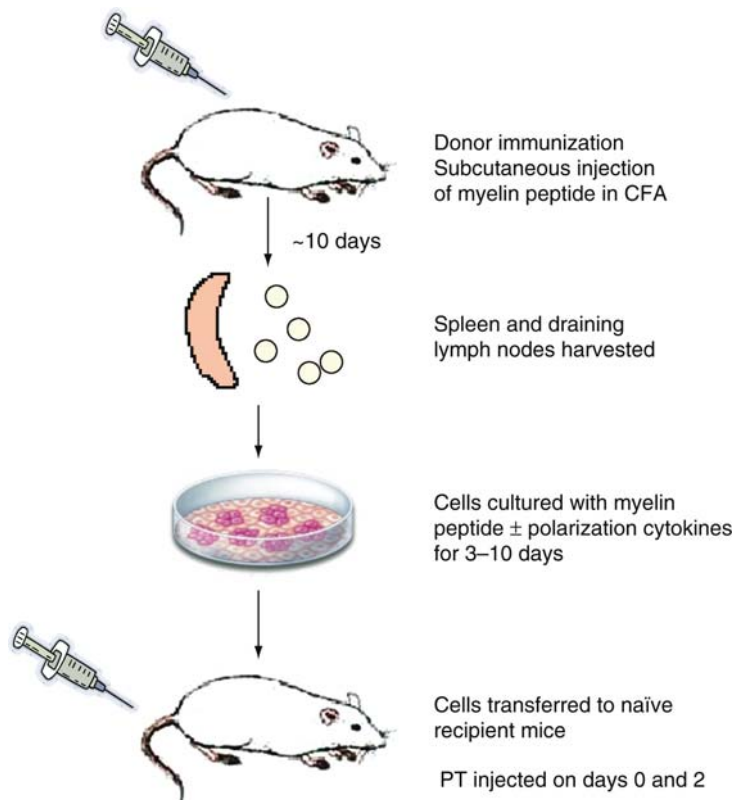
Use of EAE in neuroimmunology research. Healthy mice are immunized with a subcutaneous injection of strain-specific, immunogenic myelin peptide in complete Freud's adjuvant (CFA) and pertussis toxin is injected on days 0 and 2. Disease onset, evident as progressive neurological disability, occurs between days 9 and 14. Tissues are collected and are either directly analyzed histologically and by PCR or are cultured and later harvested for phenotypic and molecular analysis. Results are analyzed, interpreted, and used to evaluate the original hypothesis of the experiment and plan further studies



develop after 9–12 days, with some variations dependent on animal strains and immunization regimens. After this “induction phase,” disease course is even more variable, depending on the aforementioned variables. For example, MBP induces an acute self-limiting disease in guinea pigs, while immunization with CNS tissue homogenates results in chronic relapsing–remitting or progressive disease in the same species (Raine et al., 1977; Alvord et al., 1985). Adoptive-transfer EAE (AT-EAE), in which pathogenic, myelin-specific CD4⁺ T cells are transferred from affected mice to healthy recipients, is also used to induce disease and has confirmed the importance of T cells in disease induction (🔗 [Figure 16-2](#)). AT-EAE has

Figure 16-2

Adoptive-transfer EAE. Healthy mice are immunized with a subcutaneous injection of strain-specific, immunogenic myelin peptide in complete Freud's adjuvant (CFA). The spleen and lymph nodes are harvested and are cultured with myelin peptide $\pm$ polarization cytokines for 3–10 days. Cells are transferred intravenously into naïve recipients and pertussis toxin (PT) is administered on days 0 and 2



become a major experimental tool for studying the function of myelin-reactive, “effector” T cells. These autoreactive effector cells are induced and expanded in lymph nodes draining the immunization sites and in other peripheral lymphoid organs such as the spleen during the “induction phase” of disease. AT-EAE studies enable researchers to “bypass” the induction phase and focus on the “effector phase” of disease by analyzing the events that follow the injection of myelin-reactive T cells into naïve recipients. Of note, effector T cells can also be stimulated *in vitro* by various biological agents before adoptive transfer, which provides an opportunity to directly manipulate the pathogenic population. In addition, the model can be established with either donor or recipient mice lacking specific genes to address the role of various molecules in different aspects of disease development and regulation.

2.1 EAE in Rodents

The identification of MOG as a myelin autoantigen has improved the use of EAE as a model for MS. Similar to other myelin proteins that have been used to induce EAE, MOG induces an encephalitogenic T-cell response; however, it also induces a demyelinating autoantibody response (Lebar et al., 1986). Extensive demyelination in T-cell-mediated brain inflammation and increased disease severity was observed due

to demyelinating anti-MOG antibodies in animals immunized with MOG. Such combination of T- and B-cell-mediated effector mechanisms more closely mimics the complexity of MS.

The relevance of both genetic and environmental factors in disease susceptibility became apparent when other rat strains were investigated using MOG-induced EAE (Becanovic et al., 2003). It was also revealed that MOG could induce EAE in strains that were previously considered resistant. This was the case with the Brown Norway rat where a possible involvement of T helper cell 2 became apparent as disease was hyperacute and the demyelinating lesions were associated with eosinophilic infiltrate (Steffler et al., 1999).

For quite some time, the use of certain rodent models in EAE was limited by insufficient severity of the disease course. However, this problem was overcome by using pertussis toxin to increase disease severity. In addition, more susceptible mouse strains were developed. There are many standard mouse models that are now used widely. Relapsing models are created using SJL mice induced with PLP139–151, PL/J mice induced with MBP and Biozzi ABH mice immunized with CNS tissue homogenates or MOG. Chronic progressive models are created using C57/BL6 mice induced with MOG protein or MOG35–55 peptide.

2.2 EAE in Nonhuman Primates

Nonhuman primates are phylogenetically closer to humans than rodents and have therefore been used to establish EAE models. The marmoset, a small outbred monkey originating from South America, was found to be susceptible to EAE induced by immunization with CNS tissue homogenates or recombinant MOG (rMOG). Similar to MOG-induced EAE in rodents, both an encephalitogenic T-cell response and a demyelinating autoantibody response are observed in the marmoset and contribute to disease pathogenesis (von Büdingen et al., 2004). However, there are some limitations with the use of marmosets as opposed to rodent models. Firstly, it is more difficult to genetically manipulate the components of the immune and nervous systems that are involved in the pathogenesis of chronic inflammation and tissue damage. There are also other technical difficulties including the limited availability of reagents that have been developed to work with the marmoset compared with rodents. The use of an outbred species in comparison with inbred rodent strains leads to higher variability in the incidence and clinical course and in the pathological phenotype of the disease. This makes systematic studies more difficult but, importantly, enables closer modeling of MS which occurs in outbred human subjects.

2.3 Transgenic Models Including Humanized Models

Transgenic mouse models have been developed by modifying T-cell receptor (TCR) gene expression such that a large proportion of the T-cell population express receptors specific for myelin epitopes. These cells are not deleted during thymic selection and are functionally autoreactive. In 1993, Goverman et al. developed an MBP transgenic mouse model which developed spontaneous EAE when housed under nonsterile conditions (Goverman et al., 1993). Lafaille et al. (1994) developed a similar MBP-TCR transgenic mouse model but with a higher proportion of autoreactive T cells by crossing with RAG-1-deficient mice. Within a year, all these mice develop spontaneous EAE. Bettelli et al. (2003) established a MOG-TCR transgenic mouse model in which over 30% of animals develop spontaneous optic neuritis, which is the most common presenting clinical episode of human MS. The predisposition for inflammation in the optic nerve independent of EAE in these mice likely reflects the higher proportion of MOG in myelin of the optic nerve relative to the spinal cord. More recent developments in the field of myelin-reactive transgenic mice have resulted in modification of the MOG-TCR model to include myelin-specific B cells also (30% of total B-cell population), which results in enhanced B-cell MOG antigen presentation to MOG-TCR T cells and subsequent severe EAE. The distinct clinicopathological profile of this model closely reflects that of the human condition, Devic's disease (Bettelli et al., 2006a; Krishnamoorthy et al., 2006).

Additional models have been developed by inserting transgenes such as human leukocyte antigen (HLA) DR2, which is associated with susceptibility to MS, into mice (Friese et al., 2006). The creation of these models enable us to address the importance of genetic variation in the complex disease, MS (Gregersen et al., 2004). Double transgenic mice have also been created with incorporate human HLA molecules and myelin-reactive human TCRs. Models that incorporate multiple susceptibility factors allow

for the clinical heterogeneity of MS to be more clearly demonstrated and therefore will hopefully enable therapeutic advancement. Other models have been created in marmosets that reflect the crucial aspects of pathology associated with MS. Many knockout mouse models have been created where important immune system genes have been knocked out by homologous recombination, leading to major advancements in understanding the complexity of inflammatory demyelination.

The use of stem cell technology will also allow for humanization of animal models. For example the use of human hematopoietic stem cells to reconstitute a depleted mouse immune system (Gimeno et al., 2004; Traggiai et al., 2004; Shultz et al., 2005) may advance models of multiple sclerosis and aid in the development of therapeutic strategies. However, due to ethical concerns, this technology is currently limited.

3 Immunopathology of EAE

Immunopathology aims in understanding the structural characteristics associated with immune responses to diseases. Pathology studies on several EAE models have contributed to our understanding of EAE pathophysiology and are briefly reviewed here.

3.1 EAE Lesions in Different Animal Models

3.1.1 Lesions in the Guinea Pig Model

In the strain 13 guinea pig model, EAE lesions are most apparent in brain and spinal cord white matter. A classic chronic EAE lesion in this model often displays signs of inflammation around its periphery where parenchymal and perivascular mononuclear cells containing myelin debris suggest ongoing myelin breakdown. Plasma cells are also often observed at the margins of lesions, whereas the presence of small lymphocytes is unusual. The center of the lesions contains an increased number of blood vessels along with relatively undamaged naked axons. Enlarged perivascular Virchow–Robin spaces which contain fibroblasts, macrophages, and collagen deposits are also observed in the center of the lesions. Extensive gliosis and oligodendroglial sparing are two other manifestations which suggest the chronicity of the lesions. The meninges (i.e., the three membranes; pia mater, arachnoid mater, and dura mater that surround the brain and spinal cord) which lie above these chronic lesions are characteristically adherent, extremely fibrotic, and are attached to the pial surface by glial bridges.

Traits such as a central fibrous astrogliotic center and a peripheral myelin breakdown are typical of chronic lesions, as compared with an expanding lesion often observed in relapsing animals. Expanding lesions contain closely packed and recently demyelinated fibers at their periphery, as well as recent perivascular infiltrates which give lesions a more “fleshy texture” (Raine, 1997).

3.1.2 Lesions in Mouse Models

The mouse model of chronic relapsing EAE shows lesions which are densely gliotic. The depletion of axons is more extensive, whereas the degree of demyelination is relatively low. The active sensitization of mice by injection of CNS emulsions induces lesions which are more damaging, less demyelinating and more confined than lesions formed in adoptively transferred models.

The ultrastructure of a chronic lesion in the actively induced murine model shows a fibrous astrogliotic center with scattered demyelinated axons, whereas lesions in the adoptively transferred model distinctly exhibit a higher degree of axonal sparing, as well as a higher tendency for old lesions to show remyelination. Other characteristics of lesions in the actively induced model include hemorrhage, vascular damage, and nerve fiber depletion – traits which are less noticeable in the adoptively transferred murine model.

Several murine models exhibit an uncommon heterotopic regeneration of peripheral nervous system (PNS) fibers into the subarachnoid space (SAS) above the spinal cord. It has been suggested that the development of these fibers could originate from regenerating sprouts from PNS fibers, which have been cut off at the root entry zones. This occurrence is very rare in the guinea pig model (Raine, 1997).

In the C57BL/6 mouse, monophasic or a chronic, sustained form of EAE is induced by immunization with MOG35–55 in CFA. The former is characterized by multifocal, confluent areas of mononuclear inflammatory infiltration in the peripheral white matter of the spinal cord with associated demyelination (Day, 2005). Inflammatory infiltrates include both macrophages and CD4⁺ T cells. In the brain, there is meningitis and perivascular inflammatory cuffing in the cerebellum and hind brain white matter. The sustained form of EAE, induced with a “booster” injection of MOG35–55 at day 7 postimmunization, or with higher doses of peptide at the first immunization, shows similar pathology but a reduced tendency for inflammation and demyelination to resolve after the peak of disease. This model lends itself to studies of the chronic phase of disease (Bannerman et al., 2005).

In the SJL/J mouse, a relapsing model of EAE is induced by immunization with PLP139–151 and is followed by spreading of myelin reactivity to other immunological determinants of PLP and MBP during disease relapses (Vanderlugt and Miller, 2002). Disease can also be induced by MBP84–104 or by whole spinal cord homogenate, all in CFA, and is characterized by lesions in the optic nerve, brainstem, spinal cord, cerebellum, and cerebral cortex. Initially the inflammatory infiltrate includes lymphocytes and neutrophils in the meninges and the white matter with a perivascular distribution. As the infiltrate begins to resolve, there is more evident progression of white matter damage and gliosis, demyelinated axons, and macrophages containing myelin debris. At the time of clinical relapse, there are chronic lesions with low degree of inflammation but with gliosis and some initial remyelination, and smaller, acute lesions with more prominent inflammatory infiltrate (Day, 2005). The clinical course provides a useful model for the study of spontaneous relapses, a feature that is typically observed in the most frequent clinical course of MS.

Occasionally, neurological symptoms develop in mice which do not fit the usual description of clinical EAE. Such symptoms include head tilt and spinning, poor coordination and ataxia, often in the absence of “classical” ascending flaccid paralysis. This “atypical” or “nonclassical” EAE has been reported in a low proportion of animals within experimental groups, particularly in knockout mice and also in alternate mouse strains immunized with myelin peptide that has been shown to induce classical EAE in other strains as was observed in PL mice immunized with MOG35–55 (Muller et al., 2005).

3.1.3 Lesions in the Lewis Rat Model

The immunopathology of EAE has also been investigated in other rodent models such as the Lewis rat and the Dark Agouti (DA) rat. The susceptibility of the Lewis rat to EAE is useful as the disease can be both actively induced or passively transferred in this strain. EAE resulting either from the active immunization with MBP or from the passive transfer of activated MBP-specific T lymphocytes primarily manifests itself by severe inflammation of the CNS, while primary demyelination is almost absent. This makes the MBP-immunized Lewis rat a good model to study the effect of CNS acute inflammation. The lesions show some perivascular inflammation which causes a few venules in the cerebellum and brainstem of the animals to form a surrounding cufflike border. Similar inflammatory cuffs are observed in the white matter of the spinal cord, while demyelination is almost undetectable in any area of the CNS. In cases where demyelination does occur, the phenomenon is limited to the dorsal root entry and ventral root exit areas of the spinal cord (Meeson et al., 1994).

In studies carried out by Matsumoto and Fujiwara (1987) to investigate the immunopathology of EAE at the preclinical stage, spleen cells from rats previously immunized with guinea pig MBP in CFA were used to adoptively transfer EAE in Lewis rats. Several observations on the development of lesions were recorded, including a higher number of inflammatory OX19⁺ and OX18⁺ T cells and macrophages in the SAS on day 3 posttransfer (PT). The inflammatory cells spread through Virchow–Robin spaces by day 4 PT, while, at this point, perivascular lymphocytic infiltration was almost undetectable in the gray matter. The rats exhibited acute partial paralysis of the lower limbs (paraparesis) by day 5 PT. In addition, detected by day 5 PT was an increased number of inflammatory foci in the white and gray matter, as well as scattered T-lymphocyte infiltration in the parenchyma. The inflammation subsided from day 6 PT, and by day 8 PT, all the previously inflamed lesions showed only a few inflammatory cells in the parenchyma and SAS, and the rats recovered from EAE.

Even though the Lewis rat model does not show primary demyelination, the introduction of demyelinating antibodies can induce the formation of demyelinating lesions which closely resemble the MS plaque. Antibody-augmented demyelinating EAE (ADEAE) can be induced in the Lewis rat by injecting the animal with doses of antibodies to surface components such as MOG. Such treatment results in lesions exhibiting increased demyelination as well as increased perivascular inflammation and clinical symptoms (Di Bello et al., 1999). However, once the clinical symptoms have subsided, remyelination occurs.

Proinflammatory cytokines are capable of provoking axonal damage by forming reactive oxygen intermediates such as NO, which favors the degeneration of the myelin membrane. High concentrations of NO have been shown to be the cause of axonal degeneration and neuronal death (Tamatani et al., 1998; Ahmed et al., 2001). Studies have shown that axonal loss in the Lewis rat is observed only during the third relapse in the interleukin-12 model of relapsing EAE (Ahmed et al., 2002).

3.1.4 Lesions in the DA Rat Model

The Dark Agouti (DA) rat model is a relapsing EAE model, which exhibits the disease following immunization with syngeneic spinal cord tissue (obtained from genetically identical members of the same species). This model is characterized by demyelination in a chronic relapsing disease course. EAE in the DA rat is manifested by lesions in the spinal cord, where inflammatory cell infiltration is apparent in the perivascular spaces and subpial region. Such inflammatory lesions occur during the peak stage of acute EAE and during the first and second attacks of chronic relapsing (CR) EAE. Demyelinating lesions are neither observed in acute EAE, nor at the first attack of CR EAE. However, at the second attack of CR EAE, the dorsal column of the spinal cord shows distinct demyelination.

Immunohistochemical studies to assess the phenotype of infiltrating cells show that most of the infiltrating cells present in acute EAE are TCR $\alpha\beta$ -positive T cells and ED-1-positive macrophages (Tanuma et al., 2000). In CR EAE, the majority of infiltrating cells are ED-1-positive macrophages and few T cells.

3.1.5 Lesions in Nonhuman Primate Models – The Marmoset Model

The common marmoset monkey (*Callithrix jacchus*) shares a close phylogeny with humans, thus making the animal an interesting candidate as a nonhuman primate EAE model. EAE can be induced in the marmoset by immunization with rMOG. Immunization results in a relatively long asymptomatic period followed by a primary progressive, chronic, or relapsing–remitting disease course. Similar to human disease, a substantial lesion load can often be observed in the brain of marmosets with EAE even in the absence of neurological dysfunction (Smith et al., 2005).

Studies carried out by Merkler et al. (2006) on MOG-induced EAE in the marmoset revealed demyelinated lesions which were clearly distinguished in the subcortical white matter. Macrophages in those lesions were found to contain MBP-positive myelin degradation products, which suggested ongoing active demyelination. Furthermore, cortical demyelination resulted in three main types of lesions: leukocortical lesions which involved the white matter and neocortex; intracortical lesions; and subpial lesions which stretched from the pial surface to the neocortex. The cortical lesions showed a higher axonal preservation than white matter lesions, suggesting a lower degree of damage.

Histopathological studies were also carried out on marmosets where EAE was induced by intracutaneous injections of an emulsion consisting of human brain white matter dispersed in demineralized water and emulsified in CFA (Mancardi et al., 2001). The lesions formed showed demyelination in the white matter of the cerebral hemispheres and were primarily localized around the wall of lateral ventricles, as well as in the corpus callosum, optic nerves, and optic tracts. The demyelinated areas contained perivascular cuffs of mononuclear cells which confirm inflammation, but no granulocytes. The lesions formed in the spinal cord were characterized by demyelination in the ventral, lateral, and dorsal columns, particularly in the outer areas of the spinal tracts.

Immunohistochemical tests showed that the inflammatory infiltrates were made up of macrophages, T lymphocytes and rare B cells (Mancardi et al., 2001). Luxol fast blue (LFB)- or Periodic acid-Schiff (PAS)-positive myelin degradation products were detected in the cytoplasm of macrophages. Early active lesions (EAL) and late active lesions were both observed, inactive lesions were almost nonexistent.

A study by Mancardi et al. (2001) also showed some evidence of axonal damage in the EAE-induced marmoset. Demyelinated areas, stained with both anti-amyloid precursor protein (APP) and anti-nonphosphorylated neurofilaments (SMI-32) monoclonal antibodies, resulted in a different immunoreactivity pattern between APP⁺ and SMI-32⁺ axons. SMI-32 staining was relatively more uniform along the whole axon length, whereas APP staining was only evident in short sections of the axon which exhibited constrictions or swellings, as well as a “beaded-like” appearance suggestive of axonal damage or degeneration.

3.2 Inflammation

EAE is mediated by CD4⁺ T cells. However, although CD4⁺ T cells dominate the perivascular regions of the inflammatory focus in EAE induced by MBP and PLP, MS lesions contain more CD8⁺ than CD4⁺ T cells. CD4⁺ T cells in MS lesions have been shown to have either pathogenic or neuroprotective functions dependent on the cytokines that are produced. Babbe et al. (2000) demonstrated that active MS lesions have a predominance of CD8⁺ T cells. On further investigation, it was found that expansion of the CD8⁺ T-cell repertoire was more antigen-driven than the CD4⁺ T-cell repertoire. Overrepresentation of CD8⁺ T cells was seen in cerebrospinal fluid of MS patients and T cells were found to be stable over several months. TCR V gene expression pattern was involved in the expansion of CD8⁺ T cells in some patients which indicates clonal expansion (Jacobsen et al., 2002).

3.2.1 T Cells

A significant advance in the field of autoimmunity arose from the understanding of the crucial role of autoreactive CD4⁺ T cells. Autoreactive T cells are generally deleted by thymic selection and even autoreactive T cells for tissue antigens such as myelin antigen are also deleted as these antigens are expressed in the thymus. However, for various reasons not all autoreactive T cells are deleted. Usually, the autoreactive T cells that do not undergo negative selection are located in the periphery and peripheral tolerance ensures that they do not become pathogenic. These autoreactive T cells cannot become activated unless they come in contact with major histocompatibility complex (MHC) molecules presenting relevant antigens together with the corresponding costimulatory molecules. In EAE once these autoreactive T cells become activated, they can interact with endothelial cells and cross the blood–brain barrier (BBB) to initiate disease. Once in the CNS, these autoreactive T cells have to be activated by local antigen-presenting cells (APCs) along with the corresponding costimulatory molecules before they can cause inflammation and tissue injury.

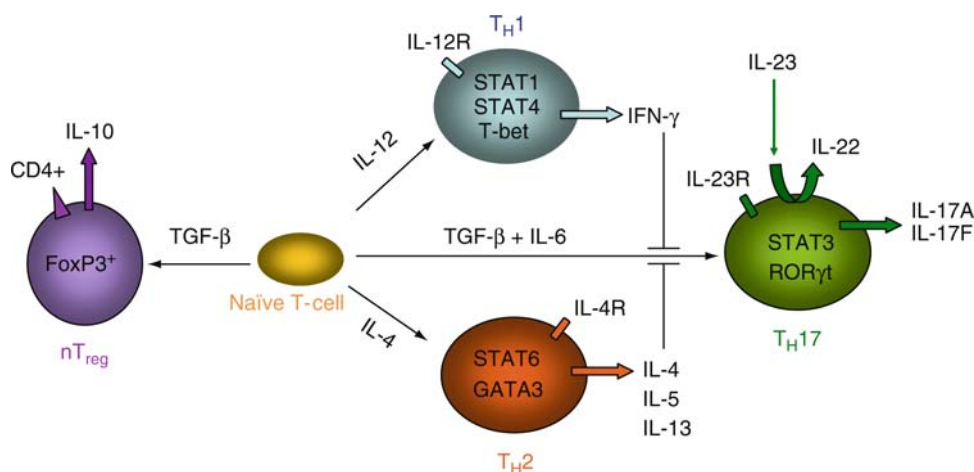
3.2.2 T Helper 1 (Th1) Cells

In the 1980s the finding that EAE could be adoptively transferred by a single transfer of MBP-sensitized lymph node cells or T cells (later to be named CD4⁺ T cells) in mice paved the way for many further studies about the role of T cells in EAE (Pettinelli and McFarlin, 1981; Mokhtarian et al., 1984). In 1986, Mosmann et al. provided definitive evidence that murine CD4⁺ T-cell clones, otherwise indistinguishable from one another in terms of surface antigen expression, could nevertheless be assigned to two different subsets on the basis of distinct, nonoverlapping cytokine secretion patterns in response to stimulation with specific antigens or polyclonal mitogens (Mosmann et al., 1986). The T cells were designated T helper type 1 cells (Th1), characterized by the secretion of interleukin (IL)-2 and IFN- γ but not IL-4 and IL-5, and T helper type 2 cells (Th2), characterized by the secretion of IL-4 and IL-5 but not IL-2 or IFN- γ . Th1 T cells can

mediate proinflammatory or cell-mediated immune responses, whereas Th2 T cells promote humoral immunity (► [Figure 16-3](#)). As more and more cytokines have been described and characterized, the Th1/Th2 hypothesis has evolved to include other cytokines that are not necessarily secreted by CD4⁺ T cells but that promote the development of either Th1 or Th2 cells. Thus cytokines such as IL-12, although not secreted by T cells, have been assigned to the Th1-associated group of cytokines, whereas cytokines such

■ **Figure 16-3**

T-cell differentiation. Naïve T cells differentiate into four different subsets of cells depending on the cytokines present during initial activation. (i) IL-12 induces a Th1 differentiation program via activation of the transcription factors, STAT1, STAT4, and T-bet, resulting in IFN- γ production. (ii) IL-4 induces Th2 differentiation via the transcription factors, STAT6 and GATA₃, resulting in the production of IL-4, IL-5, and IL-13 by Th2 effector cells. (iii) In the presence of IL-6, TGF- β drives Th17 differentiation via activation of the transcription factors, ROR γ t and STAT3. IL-23 also supports the Th17 lineage of effector T cells which produce IL-17A and IL-17F. (iv) However, when naïve T cells are exposed to TGF- β only (without IL-6) the transcription factor FoxP3⁺ is activated resulting in the differentiation of regulatory T cells which produce IL-10



as IL-10 have been assigned to the Th2-associated group of cytokines. In the 1990s, IL-12 was implicated in exacerbating EAE by inducing IFN- γ and promoting Th1 cell development (Leonard et al., 1995). The Th1/Th2 paradigm was applied to EAE as changes in cytokine profile were observed. IFN- γ and IL-12 were mainly produced at disease onset, whereas production of IL-4 and IL-10 was associated with disease recovery. In 1994, Racke et al. showed that retinoids can modulate an immune response dominated by Th1 T cells to one in which the protective cytokines Th2 cells predominate, a concept known as immune deviation (Racke et al., 1995).

IL-12 was thought to be the main cytokine in regulating effector function in EAE (Leonard et al., 1995). Exogenous IL-12 was found to induce relapses in an otherwise typically monophasic form of EAE in Lewis rats (Smith et al., 1997). It was later found that relapses of EAE were prevented with anti-IL-12 Ab (Constantinescu et al., 1998). The disease promoting effects of IL-12 were found to be antagonized by IL-10 produced by an antigen nonspecific CD4⁺ T cell which itself is regulated by IL-12. Thus a regulatory IL-10/IL-12 immunoregulatory circuit was thought to control susceptibility to EAE as well as other autoimmune diseases (Segal et al., 1998).

Some paradoxical observations began to challenge the view that the Th1 cells are essential for EAE pathogenesis and that Th2 cells are invariably protective. An important observation was that mice deficient in IFN- γ , a “signature” Th1 cytokine, developed more severe EAE than wild-type mice (Ferber et al., 1996). TNF- α , another Th1 cytokine, was also found to be not required for EAE susceptibility (Frei et al., 1997). Furthermore, immune deviation to a Th2 phenotype was found to exacerbate disease in

certain circumstances by increasing concentrations of pathogenic autoantibodies (Genain et al., 1996). Th2 cells were also found to be potentially encephalitogenic, at least in immunodeficient mice (Lafaille et al., 1997). Because of these discrepancies, the accuracy of the Th1/Th2 paradigm in EAE came under question.

IL-12 is a heterodimeric cytokine formed by a large (p40) subunit and a small (p35) subunit. It was shown that IL-12 p40^{−/−} mice were completely resistant to disease (Segal et al., 1998). However a novel protein, IL-23, was discovered to share the p40 subunit with IL-12 (Oppmann et al., 2000). This discovery raised many questions which led to a re-evaluation of the original findings pertaining to IL-12. Key experimental data that linked EAE to Th1 autoimmunity were based on the protection from disease associated with inhibition of IL-12 p40. Therefore, once it was uncovered that IL-23 also contained this subunit it was unclear if inhibition was due to lack of IL-12 or IL-23, or both. The deletion of IL-12 p40 previously eliminated the expression of IL-12 (p40p35) but also IL-23 (p40p19). Becher et al. (2002) and Gran et al. (2002) then determined that it was in fact the p35 subunit, specific to IL-12, that was required for disease susceptibility. This indicated that IL-12 was not strictly required for the induction of EAE and a role of IL-23 was suggested. In 2003, Cua et al. directly showed that IL-23 rather than IL-12 is crucial for autoimmune inflammation by using IL-23 knockout mice. The p19 subunit of IL-23 (that is not shared with IL-12) was knocked out and these mice were resistant to EAE. They also showed that IL-23 acts more broadly as an end-stage effector cytokine by direct actions on macrophages compared with IL-12 (Cua et al., 2003). Further evidence strengthened the finding that IL-23 is more crucial in EAE than IL-12. The IL-12Rβ1, which is a subunit of the receptors for both IL-23 and IL-12, was found to be the receptor crucial in development of disease (Zhang et al., 2003). In addition, IL-12 was found to suppress EAE when administered to mice during the early phase of EAE induction. Such suppressive effect was mediated by the induction of IFN-γ (Gran et al., 2004). IL-23 was found to cause production of the proinflammatory cytokine IL-17 from CD4⁺ T cells of both effector and memory phenotype, whereas IL-12 had only marginal effects on IL-17 production (Aggarwal et al., 2003). IL-17 was found to be associated with several chronic inflammatory diseases including rheumatoid arthritis synovium (Kotake et al., 1999), psoriasis (Teunissen et al., 1998) and also multiple sclerosis (Kurasawa et al., 2000). However, IL-17 was not found to be associated with Th1 or Th2 phenotypes. Therefore, a new lineage of T helper cells became evident. An association between IL-17-producing T-cell effectors (Th17 cells) and immune pathogenesis was proven when it was shown that PLP peptide-primed CD4⁺ T cells induced to produce IL-17 in vitro by culture in the presence of IL-23-induced severed EAE in recipient mice after passive transfer. By contrast, Th1 cells cultured in vitro in the presence of IL-12 did not (Langrish et al., 2005). Genes associated with cytotoxicity (IFN-γ, FasL, and granzymes) are expressed in IL-12-polarized Th1 cells, whereas genes associated with inflammation (IL-17, IL-17E, IL-6, TNF-α, and proinflammatory chemokines) are expressed in IL-23-polarized Th17 cells. This suggests that Th17 cells are crucial in the immunopathogenesis of EAE.

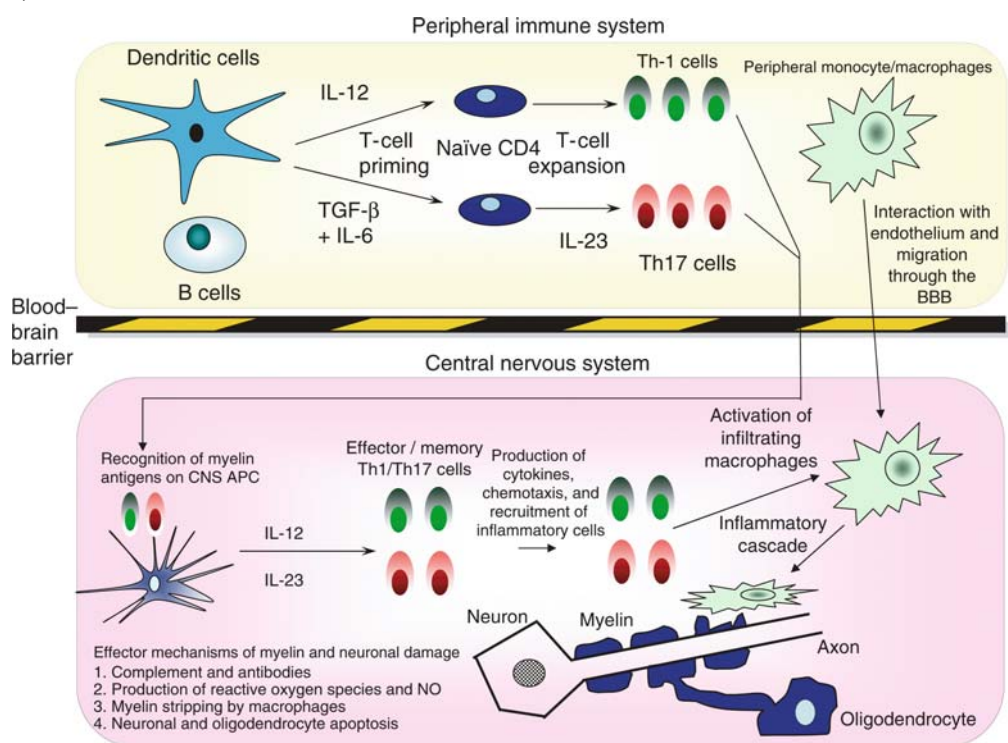
3.2.3 T Helper 17 (Th17)

While the distinction of the IL-23-driven IL-17-producing T helper cell population was clearly established by Langrish et al. (2005) in AT-EAE, the definitive report of the Th17 lineage was made in models of EAE and collagen-induced arthritis (also a murine model of autoimmune disease). Both these models had long been associated with Th1 responses. From the initial characterization of Th17 cells in late 2005, a plethora of papers in rapid succession have taught us much about the regulation of this population. Most striking was the surprising role of transforming growth factor-β (TGF-β) in the presence of IL-6 in the de novo differentiation of Th17 cells (🔗 [Figure 16-3](#)). IL-1β and TNF have also been shown to support Th17 polarization, whereas IL-27 and IL-2 are potent negative regulators of Th17 immune responses. The transcription factor that drives Th17 development was identified as RORγt. The specific role of IL-23 in supporting Th17 immunity is somewhat controversial in that IL-23 may drive de novo differentiation of Th17 cells when IFN-γ and IL-4 are neutralized but appears to play a central role in the activity of differentiated and memory T cells. Consistent with this, it has been proposed that the site of action of IL-23 in EAE is the CNS; however, recent findings by Thakker et al. (2007) report to the contrary that in fact

IL-23 functions in the induction phase of the disease which correlates to peripheral immune responses. Studies by Li et al. (2007) have shown enhanced IL-23 expression in CNS lesions of MS patients; however, functional human studies are still required to elucidate the precise role of IL-23 and downstream Th17 regulation in human MS. A schematic view of the current understanding of EAE pathogenesis is presented in [Figure 16-4](#).

■ Figure 16-4

Pathogenesis of EAE. In the peripheral immune system, antigen-presenting cells (mainly DCs but also B cells and macrophages) produce IL-12 and IL-23 which prime naïve $CD4^+$ T cells during activation by interaction of the T-cell receptor with cognate antigen presented by MHC class II complexes. Activated Th1 and Th17 cells cross the blood-brain barrier into the CNS. Resident antigen-presenting cells (APCs) interact with activated myelin-specific Th1 and Th17 cells which become reactivated. Inflammatory cytokines and chemokines are produced and an inflammatory cascade is initiated. Infiltrating macrophages are activated and attack the myelin sheath on neurons



3.2.4 Regulatory T Cells (Tregs)

$CD4^+CD25^+Foxp3^+$ regulatory T cells (Treg) represent a naturally occurring and inducible immunoregulatory population of T cells. In contrast to effector T cells, Tregs have been shown to protect against tissue damage and inhibit autoimmune disease (Sakaguchi, 2004). Therefore autoimmune responses appear to be regulated by a balance between effector cells and regulatory T cells (Tregs). $CD4^+CD25^+Foxp3^+$ regulatory T cells occur naturally in the thymus and control effector T-cell responses in the periphery. TGF- β can act on naïve T cells to induce Foxp3 and generate induced Treg cells that suppress effector T-cell immune

responses (● [Figure 16-3](#)). However, in the presence of TGF- β and IL-6, there is a predominant generation of Th17 cells (Bettelli et al., 2006b). This suggests that not only is there an antagonist relationship between Th17 and Treg cells but there is also a dichotomy in their generation. During a steady state in the immune system when there is no inflammation present, TGF- β induces the differentiation of Foxp3⁺ Treg cells. However once inflammation is initiated, IL-6 is produced by the activated innate immune system and this suppresses the generation of TGF- β -induced Treg cells and generates Th17 cells (Bettelli et al., 2007). There is a Treg population present in the CNS during EAE. However, these Tregs are functional in suppressing MOG-specific responses of naïve T cells but they fail to inhibit antigen-specific effector T cells, which have been isolated from the target organ at the acute disease phase (Korn et al., 2007).

3.2.5 Epitope Spreading

Epitope spreading is the de novo activation of autoreactive T cells by autoepitopes released secondary to inflammatory tissue damage that occurs during disease progression. The description of epitope spreading in autoimmune disease was uncovered in EAE (Lehmann et al., 1992). A hierarchical order of epitope spreading has been demonstrated in EAE and also that there is a pathological role of epitope spreading in chronic disease progression. In relapsing EAE studies in SJL mice, PLP139–151 has been found to be the dominant encephalitogenic epitope, PLP178–191 is the secondary and MBP84–104 is weakly encephalitogenic. In relapsing EAE induced by PLP139–151 in SJL mice, expansion of PLP178–191-specific T cells is observed between recovery from the acute clinical episode and the first relapse, that is, intramolecular spreading (spreading from one epitope to another on the same molecule). During the second relapse, expansion of MBP84–104-specific T cells is observed, that is, intermolecular spreading (spreading from an epitope on one molecule to an epitope on another molecule) (Vanderlugt et al., 2002). The development of this T-cell reactivity to these endogenous myelin epitopes correlates with the amount of myelin destruction observed during the acute disease phase. In addition, T cells that have been isolated from the CNS of mice with advanced disease proliferate to the spread epitopes PLP178–191 and MBP84–104 and these cells can initiate disease in naïve recipients. Even more significant is the fact that peptide-specific tolerance is induced to relapse-associated epitopes during remission from acute disease that blocks disease progression. It should, however, be noted that epitope spreading is not an absolute requirement for EAE relapses (Vanderlugt et al., 2002; Miller et al., 2007).

3.2.6 Antigen-Presenting Cells

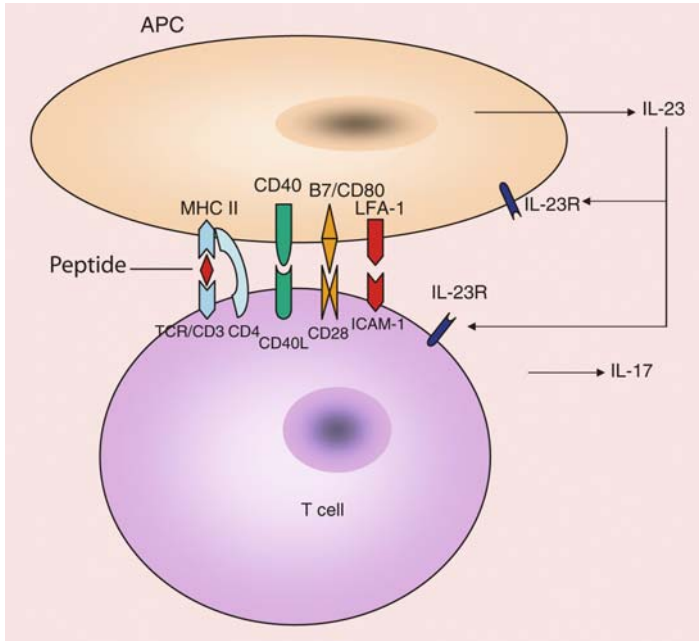
Epitope spreading is clearly significant in EAE but to initiate disease, re-presentation of myelin epitopes by CNS APCs is required (Bailey et al., 2007). Though microglia and macrophages can act as APCs, it has been found that dendritic cells (DCs) are the most efficient APCs in presenting myelin epitopes and driving the activation of naïve myelin-specific CD4⁺ T cells (● [Figure 16-5](#)). Consistent with this fact, DCs have been shown to be present in the CNS of mice with EAE and also in humans with MS (they are sparse in the healthy CNS). CD11c⁺ DCs are found in clusters and also isolated pairs with CD4⁺ T cells in the parenchyma and around blood vessels in relapsing EAE (Miller et al., 2007). Three types of DC are present in the CNS of mice during relapsing EAE, namely CD11b⁺ myeloid DCs (mDCs), plasmacytoid dendritic cells (pDCs), and CD8⁺ DCs. The most efficient among these was found to be the mDCs – these cells drive TCR transgenic T-cell population expansion and the production of pathogenic cytokines IL-17 and IFN- γ . The expression profiles of mDC and CD8⁺ DCs are similar; however, there was a distinct difference in the proliferation, differentiation of naïve CD4⁺ T cells, and their production of IL-17 (Bailey et al., 2007).

3.2.7 B Cells

B lymphocytes develop in the bone marrow and are a major cell type of the adaptive immune system. B cells are responsible for antigen-specific humoral immune responses by producing and secreting immunoglobulins (antibodies) in response to antigens. Autoimmune diseases result from the breakdown of the

■ Figure 16-5

Antigen presentation to autoreactive T cells. Antigen-presenting cells endocytose, process, and present self-antigens via MHC class II on the cell surface. Autoreactive T cells expressing cognate T-cell receptors for the specific antigen interact with the MHC/antigen complex and the T cell becomes activated. Costimulatory signals are delivered via interaction of additional cell surface receptors on the APC and T cell including CD40/CD40 ligand, CD80/CD28, and LFA-1/ICAM-1. T-cell-polarizing cytokines are secreted by the APC to direct T-cell differentiation in a lineage-specific manner. Depicted is the production of IL-23 by APCs to support Th17 development. See [Figure 16-3](#) for further detail of T-cell-polarizing cytokines



mechanisms responsible for self-tolerance, which in turn triggers an immune response against the body's self-antigens. Studies on the pathogenesis of EAE have confirmed that the disease can be passively transferred by myelin-reactive T cells, but at the same time, there has been increasing evidence which suggests that B cells also play important roles during the course of the disease.

The overall role of B cells in EAE is unclear. Studies have shown that B cells are involved in the priming of autoreactive naïve T cells in models of MBP-induced EAE (Fillatreau et al., 2002). Studies by White et al. (2000) show that myelin-reactive B cells accumulate in the CNS of Lewis rats with acute MBP-induced EAE. These B cells become apoptotic just before spontaneous clinical recovery takes place, and although these cells have not been shown to have a pathogenic function by themselves, it has been suggested that they may contribute to the pathogenesis of the disease by acting as APCs. Studies by Ziemssen and Ziemssen (2005) confirmed that B cells act as APCs for CD4⁺ T cells, and that they also provide costimulatory support to T-cell activation in the immune periphery and the CNS.

Contrasting studies have shown that B cells can be beneficial in EAE. Mice that lacked B cells failed to recover from MOG-induced EAE. In addition, B cells from normal mice B6, which were stimulated with the autoantigen and anti-CD40, synthesized the cytokine IL-10, and subsequently, animals recovered from EAE, whereas mice with B cells that could not be activated through CD40 did not produce any IL-10, and did not show any signs of recovery.

Research investigating the role of B cells in EAE has been tackled from several angles, and has resulted in a number of possible mechanistic roles to be proposed for this cell type, as well as its products, in the pathogenesis of CNS inflammatory demyelination (Cross et al., 2001). These include: (i) B cells acting as

critical APCs, whereas the antibody acts in the uptake and processing of the antigen (Tamatani et al., 1998), (ii) the costimulation of T cells, and (iii) demyelination brought about by myelin opsonization, where antimyelin antibodies contribute to opsonization and increase the level of myelin phagocytosis.

Conversely, it has also been suggested that B cells may downregulate the disease progression by bringing a balance in cytokine production, promoting remyelination as well as inducing T-cell anergy (Cross et al., 2001).

3.3 The Role of the Innate Immune System in EAE

3.3.1 Toll-Like Receptors in EAE

We cannot limit our study of EAE and MS to the adaptive immune system as the cross-recognition of viral and myelin antigens by the adaptive immune system may not be frequent enough to account for autoimmunity (Sospedra and Martin, 2005). The innate arm of the immune system, however, has the capacity to quickly and efficiently recognize structures that are far less specific to individual viruses and other pathogens. Toll-like receptors (TLRs) are mammalian innate immunity receptors that play a key role in the control of pathogens (Akira et al., 2001; Bsibsi et al., 2002). They are a class of membrane-bound pattern recognition receptors (PRRs) that recognize conserved pathogen-associated molecular patterns (PAMPs). TLRs on APCs are important for the recognition of microbial pathogens and subsequently the initiation of inflammatory immune responses (Barton and Medzhitov, 2003; Pasare and Medzhitov, 2003a; Takeda et al., 2003).

Endogenous TLR ligands have recently begun to be identified and are of particular relevance to autoimmunity. TLR ligation by endogenous ligands presents a self-trigger of innate immunity and sterile inflammation, which can be pathological in itself as well as activate the adaptive immune response and autoreactive T cells. The relevance of this phenomenon to EAE and MS remains to be investigated.

All TLRs except TLR3 signal through the MyD88-dependent pathway (Akira and Takeda, 2004; Takeda and Akira, 2005). MyD88 is an adaptor protein that contains a TIR domain in the C-terminal region and a death domain in the N-terminal region (Barton and Medzhitov, 2003; Takeda et al., 2003; Takeda and Akira, 2004). On TLR activation, MyD88 is recruited through its TIR domain to the receptor and then in turn, interacts with the TIR domain of the TLR. MyD88 then recruits IL-1 receptor-associated kinase-4 (IRAK4) – a death domain containing serine/threonine kinase (Takeda and Akira, 2005). IRAK4 becomes activated and phosphorylates IRAK1 (Takeda and Akira, 2004). IRAK1 then associates with tumor-necrosis factor-receptor-associated factor-6 (TRAF6). This activation of TRAF6 leads to the activation of two distinct signaling pathways, JNK and NF- κ B (Muzio et al., 1997; Wesche et al., 1997). The activation of the NF- κ B pathway controls the expression of the genes required for inflammatory and adaptive immune responses. Inflammatory cytokines produced in response to TLRs, including IL-6, IL-1 β , IL-12 p40, and TNF- α , are controlled by this pathway (Takeda et al., 2003; Kawai and Akira, 2006).

The MyD88-independent pathway is used by TLR3 and TLR4 and leads to the activation of the transcription factor IFN-regulatory factor-3 (IRF-3) and also late phase NF- κ B and leads to the production of IFN- β and costimulatory molecules (Kawai et al., 1999, 2001; Yamamoto et al., 2003). This pathway signals through the adaptor molecules TIRAP/Mal and TIR-domain-containing adaptor protein inducing IFN- β (TRIF, also known as TIR-domain-containing molecule 1 or TICAM-1) (Yamamoto et al., 2002; Oshiumi et al., 2003). A fourth TIR domain containing adaptor, TRIF-related adaptor molecules (TRAM/TICAM-2) was found to be involved in TLR4- but not TLR3-mediated activation of IRF-3 and induction of IFN- β and IFN-inducible genes (Yamamoto et al., 2003). In TRIF- and TRAM-deficient mice TLR4 ligand-induced inflammatory cytokine production was not observed indicating that MyD88-dependent and MyD88-independent/TRIF-dependent pathways are required for TLR4-induced inflammatory cytokine production (Takeda and Akira, 2005). Two noncanonical IKKs, TBK1 and IKKi/IKKe, are critical regulators that mediate IRF-3 activation in this MyD88-independent pathway. They phosphorylate IRF-3, which then translocates to the nucleus and activates type 1 interferon promoters in particular IFN- β (Sharma et al., 2003).

Dendritic cells (DCs) are unique APCs as they can initiate primary immune responses (Banchereau et al., 2000). Immature DCs are highly endocytotic and they reside in tissues. TLRs induce the maturation of (DCs) and therefore also control the activation of the adaptive immune system (Akira et al., 2001). DCs migrate to the lymph nodes when a foreign pathogen is recognized by TLRs and endocytosed by the DC. The upregulation of MHC class II and costimulatory molecules CD80/CD86 on DCs provide the signals necessary for T-cell activation and the maturation of DCs. TLR engagement on DCs inhibits the immunosuppressive effects of CD4⁺CD25⁺ regulatory T cells on effector T cells via IL-6 expression (Pasare and Medzhitov, 2003b).

There is a plethora of evidence suggesting that the MyD8-dependent pathway has a role in proinflammatory diseases and autoimmunity. When EAE is induced by active immunization, CFA is used which contains inactivated MTb. This has been found to activate the Toll/IL-1 pathway by activating TLR1, and this pathway is crucial for the expansion of autoreactive T cells and the development of EAE (Hansen et al., 2006). Injections of *Staphylococcus aureus* peptidoglycan (PGN) (which stimulates TLR2) with the encephalitogenic peptide MOG35–55 emulsified in incomplete Freud's adjuvant (IFA) into female C57BL/6 mice (8–12 weeks old) at the axillary and inguinal regions has been found to induce EAE (Visser et al., 2005). Research by this same group also found that most MS CNS lesions contain an increased number of APCs that are rich in PGN. Therefore, it is highly likely that PGN activates proinflammatory mechanisms by stimulating TLR2 on DCs.

Other TLR agonists have also been used to induce EAE as well as other autoimmune diseases such as autoimmune arthritis and autoimmune myocarditis. The role of various TLR agonists on the activation of autoreactive encephalitogenic immune cells (T cells) and their role in EAE induction have been studied. It was found that the MyD88-dependent TLR agonists like PGN and lipoproteins, combined with IFA are potent inducers of EAE (Hansen et al., 2006). However the MyD88-independent agonist (TLR3) polyI:C combined with IFA does not induce EAE. It is still unsure of the exact mechanisms by which TLR agonists induce autoimmunity but it is hypothesized that under normal circumstances, an autoreactive T cell will not become activated by a DC that has endocytosed a self-antigen and is expressing a self-peptide on its surface because there is no costimulatory molecule present. However once an infection is present, the DC becomes activated and presents a self-peptide to an autoreactive T cell which expresses costimulatory molecules and cytokines for instance IL-12, and the autoreactive T cell becomes activated.

TLR expression is strongly increased within the CNS during EAE (Prinz et al., 2006). This group used mutant MyD88-deficient (MydD88^{-/-}) mice to show that the MyD88-dependent pathway is proinflammatory. These mice were resistant to EAE on stimulation with different TLR agonists. The basis for this was that mice were unable to produce an encephalitogenic immune response due to the absence of NFκB-induced cytokines and chemokines. Therefore the DCs could not prime naïve T cells to become autoreactive toward the CNS (Prinz et al., 2006).

The MyD88-independent pathway has been shown to be anti-inflammatory. When this pathway is induced by stimulation of TLR3, which is the only TLR that is completely MyD88-independent, increased levels of IFN-β were detected. This study involved injecting the TLR3 agonist polyI:C into 6–8-week-old SJL/J mice with relapsing EAE. Clinical scores and histological analysis of inflammatory lesion clearly demonstrated that the endogenous IFN-β that was produced suppresses the murine model of EAE (Touil et al., 2006). The use of neutralizing anti-IFN-β antibodies reinforced this concept. The suppression of EAE was found to be associated with increased levels of CCL2 (MCP-1) in the peripheral compartment of the immune system. This was found to be IFN-β-dependent (Touil et al., 2006).

4 The Blood–Brain Barrier in EAE

The blood-brain barrier (BBB) is a physical barrier between the blood vessels in the CNS and the CNS itself. The concept of the BBB was first introduced by Paul Ehrlich in the late 1800s, when he discovered that the intravenous injection of dyes into the bloodstream stained all the tissues in most organs except the brain. Further work by scientists showed that this selectively permeable barrier is a specialized system of epithelial-like high-resistance tight junctions that cement together the capillary endothelial cells which make up the

capillaries of the brain and spinal cord (Pardridge, 2007). The BBB protects the CNS from harmful substances in the blood stream, while at the same time acting as a system of cellular transport mechanisms. The BBB is a major contributor to the maintenance of the CNS homeostasis by regulating soluble factor and cellular exchange between the CNS and the blood.

4.1 Structural Components of the BBB

The three cell types of the brain microvasculature which make up the BBB are the capillary endothelium, the capillary pericyte, and the astrocyte foot process (Ballabh et al., 2004).

The endothelial cells of the BBB are distributed along the length of the vessel and completely around the lumen. Cerebral capillary endothelial cells contain tight junctions, which seal cell-to-cell contacts between adjacent endothelial cells forming a continuous blood vessel. A thin basement membrane known as the basal lamina supports the abluminal surface of the endothelium.

It has been suggested that pericytes in the BBB could be derived from microglia. Pericytes in the periphery of the blood vessels are flat, undifferentiated, contractile connective tissue cells, which develop around capillary walls.

Astrocytes are glial cells which envelop >99% of the BBB endothelium on the abluminal side. Intercellular adhesion between astrocytes in the BBB has been observed in the form of gap junctions and adherens junctions.

4.2 Functions of the Structural Components of the BBB

The endothelial cell is the principal barrier cell which controls cerebral microvascular permeability. The capillary endothelial cells inhibit the passage of molecules across the barrier by an extremely low pinocytotic activity. The lack of fenestrations in the endothelial cells, as well as the presence of tight junctions (TJ), limits the diffusion of molecules across membranes. At the same time, the cells have developed specific transport systems which carry nutrients into the CNS.

4.3 Role of the Blood–Brain Barrier in EAE

The BBB makes the CNS an immunologically privileged region (Engelhardt, 2006). In the healthy CNS, lymphocyte entry is maintained at a low level, and activated T lymphocytes can freely cross the BBB to conduct immunological surveillance in the CNS (Muller et al., 2005). If these T cells do not encounter their antigen, they exit the CNS, but if they do, an inflammatory reaction can be triggered, which could damage the BBB.

The loss of the BBB integrity is a pathological feature that has been associated with neurological diseases such as MS and its animal model EAE (Fabis et al., 2007). The network of blood vessels in the CNS in a healthy individual closely regulates the passage of solutes and cells of the immune system into the cerebral compartment. During the disease progress in EAE, the BBB becomes more permeable, thus allowing immunocompetent cells and plasma elements to easily access the CNS. This disrupts the existing homeostatic neuronal environment, and results in the formation of inflammatory lesions (Paul and Bolton, 2002). In vitro-activated CD4⁺ T cells have been shown to migrate across the BBB into the CNS, and their migration was followed by an onset of molecular events that resulted in inflammation, loss of barrier properties, and demyelination (Engelhardt, 2006). These events did not occur in experiments which employed resting T cells with the same antigen specificity.

Studies carried out on EAE models have demonstrated that the onset of clinical signs of EAE is generally accompanied by changes in the BBB permeability (Fabis et al., 2007). MOG-immunized KO and congenic wild-type mice were examined for changes in BBB permeability by measuring the uptake of the fluid-phase marker sodium fluorescein (NaF). All the mice which developed clinical signs of EAE also displayed a significant increase of BBB permeability toward NaF.

Studies have also shown that the immunization of Lewis rats with guinea pig MBP results in the detection of increased levels of gadolinium into the CNS of the spinal cord, the brainstem, and the cerebellum early after the onset of clinical signs of EAE (Floris et al., 2004). However, later studies have shown that the cerebellar BBB of SJL/J mice immunized with peptide 139–151 of myelin PLP (PLP139–151) become more permeable to rabbit IgG before the clinical signs of EAE are observed (Muller et al., 2005). It can thus be suggested that the permeability of the BBB increases in different regions of the CNS at different stages of the disease progression of T-cell-induced EAE.

Even though the precise mechanisms which take place during BBB dysfunction are relatively unknown, several inflammatory, endothelial, and neuronal cell sources believed to contribute to its occurrence have been identified. They include causal mediators of neurovascular dysfunction during EAE such as the cytokines TNF- α and interleukin-1, arachidonic acid metabolites, and free radicals among others. Further research studies on the loss of function and integrity of the BBB in EAE have to be carried out to investigate whether this occurrence is a primary manifestation in the disease development, or a secondary response to immune cell activation.

5 Conclusion and Future Outlook

EAE remains the best characterized animal model of autoimmunity and a very important tool for studies of pathogenesis and experimental treatments in MS. The availability of different animal species and strains, immunization protocols (including the use of distinct myelin antigens and adjuvants, and the use of active or AT-EAE), and routes of drug administration (e.g., intraperitoneal, intravenous, and intraventricular) at different stages of disease (preinduction, induction, clinical onset, peak, remission, and relapse) enables the researcher to choose optimal tools to address specific questions. For example, the use of PLP-induced disease in the SJL mouse allows for excellent insight into mechanisms of spontaneous relapses. In addition, murine models are often optimal in terms of availability of biological reagents. However, a rat model with similar clinical features (e.g., MOG-induced relapsing disease in the DA rat) will offer the advantage of a larger CNS when imaging studies are needed. To bridge biological gaps between rodent and human immune nervous systems, particularly for the purpose of preclinical studies, the use of nonhuman primates such as the marmoset will be valuable and informative.

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17 Immunological Aspects of Central Nervous System Demyelination

S. Pawate · S. Sriram

1	<i>Introduction</i>	380
2	<i>Hypothesized Mechanisms of CNS Demyelination</i>	381
2.1	Autoimmune Attack on Myelin and Oligodendrocyte	381
2.1.1	Introduction	381
2.1.2	CNS Autoantigens Capable of Causing EAE and Implicated in MS	383
2.1.3	Effector Cells in CNS Demyelination	384
2.1.4	Mediators of Inflammation	386
2.1.5	Mechanisms for Autoimmunity	387
2.2	Viral Models of CNS Demyelination	388
2.3	Primary Oligodendrogliopathy	388
2.4	Primary Axonal Damage with Secondary Inflammation	389
3	<i>Conclusions</i>	389

Abstract: The mechanism of the pathogenesis of lesions found in multiple sclerosis (MS) is a focus of intense research. The prevailing view has been that MS is an inflammatory demyelinating disease mediated by autoreactive CD4+ T lymphocytes, a hypothesis based on experimental autoimmune encephalitis, a mouse model of autoimmune demyelination induced by inoculation with myelin proteins. Axonal loss in MS has been thought to represent a secondary phenomenon. However, more recently it was reported that the earliest pathological change in MS lesions was a widespread oligodendrocyte apoptosis in the virtual absence of infiltrating lymphocytes or macrophages. Axonal injury as an early event, independent of inflammatory demyelination, is also gaining appreciation. In this chapter, the current literature on the autoimmune model will be reviewed in detail. Virally mediated demyelination, based on Theiler's virus infection model; Barnett-Prineas model of oligodendrocyte apoptosis; and the "inside-out model" positing a primary role for axonal injury, will also be reviewed. EAE appears to be a model of ADEM rather than MS. This, in combination with the new findings of oligodendrocyte apoptosis and axonal injury, and the fact that most of the disability that accumulates in patients with MS correlates with axonal loss, suggests that MS may need to be thought of more as a neurodegenerative disease and that neuroprotective treatments are most likely to limit its effects.

List of Abbreviations: CRF, corticotropin-releasing factor; CVOs, circumventricular organs; DA, Dopamine; DNAB, dorsal noradrenergic ascending bundle; DOPAC, 3,4-dihydroxyphenylacetic acid; GM-CSF, Granulocyte/macrophage colony-stimulating factor; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine; 5,7-DHT, 5,7-dihydroxytryptamine; HPA, hypothalamo-pituitary-adrenocortical; HVA, homovanillic acid; IDO, indoleamine-2,3-dioxygenase; IFNs, Interferons; IL-1, Interleukin-1; IL-1ra, IL-1-receptor antagonist; LPS, Lipopolysaccharide; MHPG, 3-methoxy,4-hydroxyphenylethyleneglycol; NDV, Newcastle disease virus; NE, Norepinephrine; NOS, nitric oxide synthase; 6-OHDA, 6-hydroxydopamine; OVLT, organum vasculosum laminae terminalis; PVN, paraventricular nucleus; SRBC, sheep red blood cells; TNF α , tumour necrosis factor- α ; VNAB, ventral noradrenergic ascending bundle

1 Introduction

The myelin sheath that surrounds axons is a lipid-rich membrane that contains several proteins that are quite specific to it, and facilitates the saltatory conduction of nerve impulses. Destruction of myelin (demyelination) can occur by a variety of mechanisms, including ischemic (Kelley, 2006), Toxic [for example, cyclosporine (Munoz et al., 2006)], and Metabolic [as occurs in vitamin B12 deficiency (Scalabrino, 2001) and copper deficiency (Kumar et al., 2004)]. Inflammatory demyelination underlies the pathogenesis of several central nervous system (CNS) diseases such as MS and acute disseminated encephalomyelitis (ADEM) (Menge et al., 2005) as well as peripheral nervous system (PNS) diseases such as Guillain-Barre syndrome (Hughes and Cornblath, 2005) and chronic inflammatory demyelinating polyradiculoneuropathy (Lewis, 2005). In this chapter we will discuss the mechanisms of demyelination in the CNS, using MS as the prototypical disease.

Our review will outline the current understanding of the immunological basis of MS, which is one of the most common demyelinating diseases of the CNS (Noseworthy et al., 2000). MS affects young adults with preponderance in women. At least 350,000 individuals are affected with MS in the USA. There is indirect evidence that the incidence of the disease is increasing in the developing world (Orton et al., 2006). Clinically, MS presents as three major clinical phenotypes, (1) relapsing remitting disease, (2) secondary progressive disease, and (3) primary progressive disease. There is no one "test" for MS and the diagnosis rests on the composite of clinical presentation, magnetic resonance imaging studies, and the presence of immune abnormalities present in the cerebrospinal fluid. Irrespective of the clinical presentation, the one constant feature of all the different clinical subtypes involves loss of myelin (demyelination) and damage to the underlying axons. The central histological feature of MS is the MS plaque (Frohman et al., 2006), which is described as acute, (prominent inflammatory cells and activated microglial cells), subacute, and chronic (paucity of immune cells and prominent gliosis). The lesions are typically located in the optic nerves, periventricular areas, cerebellum, floor of the fourth ventricle, and in cervical spinal cord. Although less prominent, demyelinating lesions are seen within the gray matter as well (Bo et al., 2006; Kutzelnigg and

Lassmann, 2006). Within the demyelinating plaque there is both loss of myelin and oligodendrocytes. The destruction of axons, although not as prominent as loss of myelin, is nonetheless an important component of the pathology of MS (Kornek and Lassmann, 2003). It is now clear that axonal loss is an important cause of the physical disability of MS (Peterson and Trapp, 2005).

It is not entirely clear whether the target involved in MS is the myelin sheath, the oligodendrocyte, or both. Since the core feature in the pathology of MS is demyelination (Frohman et al., 2006), there has been great interest in understanding the factors that lead to loss of myelin and impaired integrity of axons.

The oligodendrocyte myelinates 30–50 axons of the CNS. The fusion of the cell membranes as it wraps around the axon forms the multilamellated myelin structure consisting of major dense and intraperiod lines. The formation of myelin membranes allows for the rapid saltatory conduction of nerve impulses to proceed from one node of Ranvier to the next, because the sodium channels are confined to the nodes of Ranvier, while the potassium channels are located along the axolemma. Hence, loss of myelin or the myelin producing cells leads to disruption of normal conduction and the attendant neurological deficits. A comprehensive review of oligodendrocyte and myelin biology can be found in Baumann and Pham-Dinh (2001).

2 Hypothesized Mechanisms of CNS Demyelination

The following hypotheses have been put forward to explain the process of demyelination in MS (🔗 [Figure 17-1](#)):

1. Autoimmune attack of myelin membranes and/or oligodendrocytes
2. Infection of oligodendrocytes (virally mediated demyelination)
3. Apoptosis of oligodendrocytes with secondary inflammation (Barnett Prineas model)
4. Primary axonopathy and secondary demyelination and inflammation

2.1 Autoimmune Attack on Myelin and Oligodendrocyte

2.1.1 Introduction

Current views strongly endorse an autoimmune basis as a cause of MS. In the autoimmune model, MS is considered to be a T-cell-mediated autoimmune disease, mediated by autoreactive T cells that recognize antigens present on myelin or oligodendrocytes. For these reasons we will explore the underlying immune processes that are implicated in MS (Weiner, 2004). Most of the evidence for this hypothesis comes from the experimental autoimmune encephalomyelitis (EAE) model (reviewed in Rao and Segal, 2004).

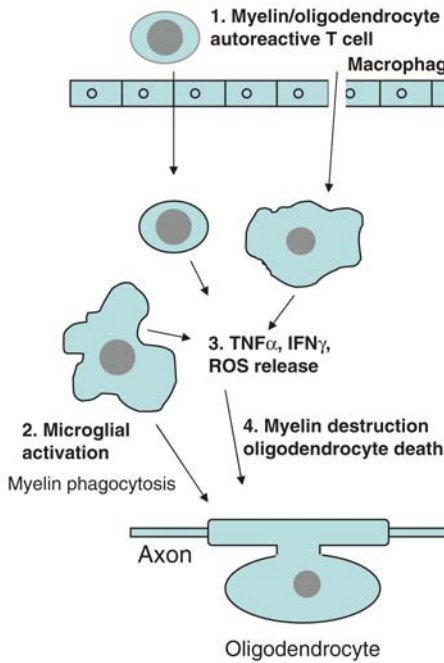
EAE is in most animal models a CD4+, T-cell-mediated, inflammatory, autoimmune demyelinating disease of the CNS (Owens and Sriram, 1995). The disease can be induced in a number of experimental laboratory animals, including primates, by the injection of whole brain homogenate, or a purified preparation of myelin antigens. The major myelin antigens that have well established murine disease models are myelin basic protein (MBP), proteolipid protein (PLP), or myelin associated oligodendrocyte protein (MOG) in adjuvant. Additional myelin antigens (minor antigens) have been shown capable of causing inflammatory changes in the CNS. However, their roles in MS, which remain unclear, are described below and have been recently reviewed exhaustively by Sospedra and Martin (2005).

The role of T cells was shown by the development of paralytic signs in mice that were injected exclusively with T cells reactive to whole myelin antigens. Subsequent studies have shown that peptide fragments of myelin proteins are sufficient to induce EAE. In most animal models, EAE (unlike MS) is a monophasic disease and resembles acute demyelinating encephalomyelitis (ADEM). However, altering the immunization protocols results in a chronic form of EAE, referred to as chronic relapsing EAE (CR-EAE) (Lublin et al., 1981). In the chronic relapsing model, animals go through multiple episodes of relapses and remissions, a clinical picture that closely mimics MS. In addition, the ability to alter the number and severity of relapses after the disease is established offers means to test new therapies in a clinically relevant

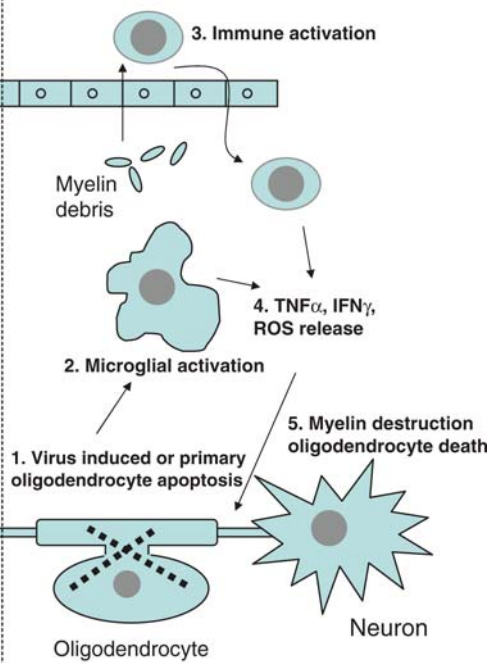
■ Figure 17-1

Models of demyelination in multiple sclerosis. a. Primary autoimmune attack. Autoreactive Th lymphocytes initiate the autoimmune attack when they gain entry across the blood–brain barrier. This leads to recruitment of microglia and macrophages and the release of inflammatory mediators, leading to the destruction of myelin, death of oligodendrocytes, and phagocytic clearance of the debris. b. Primary oligodendroglipathy. Oligodendrocyte apoptosis is the primary event, leading to activation of microglia, which are overwhelmed by the amount of myelin debris. Circulating autoreactive T lymphocytes are then activated by the myelin debris and initiate the immune attack on myelin. c. Axonal injury. Axonal injury is the primary event, leading into microglial, and in turn, T-cell activation. The stereotypical autoimmune destruction of myelin ensues as a secondary event

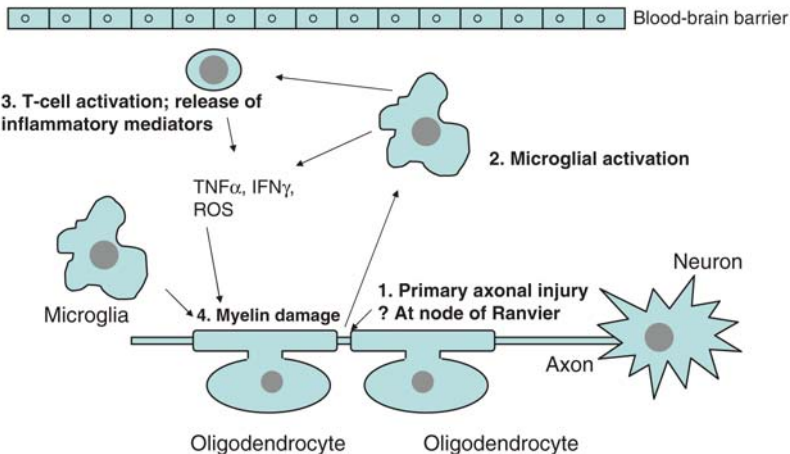
(a) T cell-mediated autoimmunity



(b) Primary oligodendroglipathy



(c) “Inside-out” model (primary axonal damage)



situation. MS shows close similarities in clinical, pathologic, and immunologic features to chronic relapsing experimental allergic encephalitis (EAE), and autoimmune response to myelin antigens is seen in patients with MS (Zamvil and Steinman, 1990; Martin et al., 1992).

2.1.2 CNS Autoantigens Capable of Causing EAE and Implicated in MS

2.1.2.1 Myelin Basic Protein (MBP) MBP (Boggs, 2006) is the second most common protein in the myelin sheath, constituting 30–40% of the CNS myelin proteins, and located at the inner surface of myelin membranes. MBP, along with lipids that line the myelin membrane, plays an important role in the maintenance of myelin sheath structure and function. The noncovalent interactions between MBP and lipids help to maintain its structure. MBP has a net positive charge, and this helps it to maintain compaction of myelin sheath via interactions with acidic lipid moieties.

There are five MBP isoforms with 14.0–21.5 kDa molecular weights (Campagnoni and Macklin, 1988). The most abundant 18.5 kDa isoform has been the most commonly used in immunological studies. The peptide fragments of MBP that are capable of causing EAE differ between strains. In SJL/J mice the encephalitogenic peptides reside between residues 89–103, while in PL/J mice the predominant epitope is at the N terminus (residues 1–14). MBP shows several posttranslational modifications such as deimination, phosphorylation, deamidation, methylation, and N-terminal acylation (Musse et al., 2006), resulting in charge variants C1 to C8 (least cationic). The loss of cationicity has been correlated with severity of demyelination (Beniac et al., 1999) with extensive loss of cationicity occurring in Marburg variant of MS.

The ability of MBP to cause an MS-like disease in humans has forged more evidence for its role in human disease. However, even after three decades of research, clear evidence for a role for MBP in MS remains inconclusive. While an earlier report suggested that the presence of anti-MBP and anti-MOG (see below) antibodies predicted relapses after the first clinical event in MS (Berger et al., 2003), a more recent report found no relation between the presence of anti-MBP antibodies or anti-MOG antibodies, and progression to clinically definite MS (Kuhle et al., 2007).

2.1.2.2 Proteolipid Protein (PLP) PLP is the most abundant myelin protein (50%), and highly hydrophobic. In mice, there are two main transcripts, the full-length 276 amino acid wild-type protein and the DM-20, an isoform that lacks 35 amino acids. The latter is mainly expressed in brain and spinal cord prior to myelination but is also expressed in peripheral lymphoid organs (Endoh et al., 1986). The major encephalitogenic and immunodominant PLP (139–154) peptide is the sequence that is contained in full-length PLP, but not in DM-20 (thus not subjected to thymic negative selection). PLP (139–151) and PLP (178–191) are main targets of high-avidity T cells and there is an increase in the frequency of these cells in a subset of MS patients. EAE is readily induced in susceptible strains of mice following injection of peptides that span residues 139–151. The clinical and histological disease closely resembles that induced by MBP.

2.1.2.3 Minor Encephalitogenic Proteins These proteins form less than 1% of the myelin antigens of the oligodendrocyte-myelin unit.

Myelin-Associated Glycoprotein (MAG). MAG is a large (approximately 100 kDa) myelin glycoprotein (Schachner and Bartsch) located at the inner surface of the myelin sheath opposing the axon surface. Loss of MAG is an early event in CNS demyelination in a subset of MS patients (Aboul-Enein et al., 2003). However, the presence of an immune response to MAG in MS patients is not evident. Thus while anti-MAG neuropathy (Van den Berg et al., 1996) is a well-defined polyneuropathy characterized by anti-MAG antibodies and a monoclonal immunoglobulin (Ig) M paraproteinemia, the role of MAG in MS remains unclear.

Myelin Oligodendrocyte Glycoprotein (MOG). MOG is a 218 amino acid transmembrane glycoprotein of the Ig superfamily and, unlike MBP and PLP, is present mainly on oligodendrocytes (Bernard et al., 1997). MOG constitutes only 0.01–0.05% of total myelin proteins, but is located strategically on the outer surface of the oligodendrocyte membrane thus serving as a target for putative autoantibodies. MOG and peptide fragments of MOG are capable of inducing EAE (Wekerle and Linington, 2006). Unlike other myelin antigens, MOG antigens appear to be a target for cellular and humoral immune responses in MS (Bernard et al., 1997).

Unlike EAE induced by either MBP or PLP, antibodies to MOG markedly potentiate EAE. In addition the lesions produce demyelinating lesions (type II lesions) in the periventricular region in rodents and primates that resemble closely the immunopathology of MS (Genain and Hauser, 2001; Iglesias et al., 2001). In addition, the T cell response to MOG is not restricted to CD4⁺ T cell population. Bettelli et al. (2003) suggested that the higher level of expression of MOG in optic nerves may explain ON involvement in MS. Like other myelin antigens, clear evidence for the role of MOG antigens and the role of anti MOG antibodies in MS remains an area of investigation.

Additional myelin proteins that are implicated as potential autoantigens in MS include, 2', 3'-Cyclic Nucleotide 3' Phosphodiesterase (CNPase), Myelin-Associated Oligodendrocytic Basic Protein (MOBP), Oligodendrocytic-Specific Glycoprotein (OSP), and Transaldolase-H (Tal-H). CNPase is located in oligodendrocytes, mainly around the nucleus and in the paranodal loops. CNPase is immunogenic both in rodents and in humans, and studies of the reactivity to either recombinant or native CNPase and to overlapping CNPase peptides have located a number of areas with promiscuous binding to several HLA-DR alleles, including the MS-associated DR15 molecules (Rosener et al., 1997; Muraro et al., 2002). A C-terminal area residues (343-373) is one of the immunodominant epitopes that is recognized preferentially by high-avidity myelin-specific T cells of MS patients (Bielekova et al., 2004).

MOBP (Kaye et al., 2000) is exclusively expressed in oligodendrocytes and whose function is not clear and no clear animal model is currently available. Indirect evidence of reactivity to MOBP has been reported. (Bielekova et al., 2004; Montague et al., 2006). Arbour et al. (2003) used MOBP and showed a positive correlation between antigen-specific T cell proliferation and interferon- γ production with clinical relapses and MRI lesion activity.

OSP/Claudin-11 is a protein similar to PLP (Chow et al., 2005) that has essential functions in maintaining compact myelin. Several OSP peptides induce EAE in SJL/J mice, and OSP-specific antibodies are found in the CSF of RR-MS patients (Bronstein et al., 1999; Vu et al., 2001).

Transaldolase, a key component of the pentose phosphate pathway, is expressed in oligodendrocytes, Schwann cells, and lymphoid tissues. High-affinity antibodies against Tal-H have been found in the serum and CSF of MS patients (Banki et al., 1994), and Tal-H also stimulates proliferation of MS peripheral blood mononuclear cells (Colombo et al., 1997).

2.1.3 Effector Cells in CNS Demyelination

2.1.3.1 CD4⁺ T Cells The ability of CD4⁺ T cells to induce a paralytic demyelinating disease has been the framework to implicate a similar process in MS. Indeed, CD4⁺ cells are seen in the CNS of patients with MS. Although EAE is more reflective of acute disseminated encephalomyelitis (ADEM) in humans, rather than MS (Stuerzebecher and Martin, 2000), there is speculation that MS might represent the opposite spectrum of ADEM, having similar immunological substrates.

Although the autoantigen in MS remains unclear, the associations of MS with certain alleles of the MHC class II antigens have suggested that MS is likely to represent a disorder mediated by CD4⁺ effector cells. It is believed that activation of CD4 T cells that react to CNS antigens leads to its amplification in the periphery and its subsequent entry into the CNS. In addition, mice that are transgenic for both human HLA DR alleles and the TCR gene product that recognizes MBP peptide in the context of human HLA DR molecules have been capable of inducing an autoimmune demyelinating disease. One of the major stumbling blocks in proving a causal role for MBP-reactive CD4⁺ cells has been the presence of MBP reactive T cells in healthy volunteers as well as MS patients. This has led to a number of studies that have examined not only the frequency of CD4⁺ cells that recognize myelin antigens in peripheral blood but also the pattern of activation in normal individuals and MS patients. There is some evidence that the phenotypes of myelin reactive cells are different in healthy controls, in whom they are likely to be Th2 phenotype, and MS patients, in whom they are likely to be Th1 phenotype (Crawford et al., 2004). A number of studies have provided with sometimes contradictory evidence for the role of CD4⁺ cells since the frequency of autoreactive cells varied between laboratories and between MS patients and controls.

The most persuasive argument for the role of CD4+ MBP reactive T cells was the serendipitous observation that some of the MS patients being treated with altered peptide ligands of MBP, led to a marked increase in the frequency of MBP reactive T cells along with increase in the number of enhancing lesions in the brain (Martin et al., 2000). It is clear that the role for CD4 T cells while suggestive in MS is yet unproven conclusively. Most importantly, *in vivo* therapy with anti CD4 antibodies did not lead to a decrease in the number of active lesions in MS patients receiving therapy (van Oosten et al., 1997).

Current viewpoints suggest that expansion of circulating myelin autoantigen reactive CD4+ T cells could lead to its entry into the CNS leading to its recognition of its cognate antigen in the CNS (Bielekova et al., 2004). Activation and amplification of autoreactive T cells leads to the development of an inflammatory milieu within the CNS. At present, it is not clear what set or pattern of chemicals (cytokines or chemokines) are necessary and sufficient for producing the typical pathological picture of MS. While Th1 cells have traditionally thought to mediate MS pathogenesis, recently there has been evidence that the IL-23/IL-17 axis (Touil et al., 2006) may be more important than the Th1 cells in MS.

2.1.3.2 CD8+ T Cells Although the focus to a large extent has been on CD4 T cells there is recent re-examination of the role of CD8 cells. A number of pathological studies have shown that CD8+ T cells predominate the inflammatory infiltrate in MS, even though they represent less than 30% of cells in the peripheral blood (Cabarrocas et al., 2003). Laser capture amplification of CD8+ cells have shown that those obtained from MS brains show a limited pattern of expression of their T cell receptor. Prominent oligoclonal expansions of CD8+ memory T cells have been found in the CSF (Jacobsen et al., 2002) and in MS brain tissue and a persistence of CD8+ TCC in CSF and blood (Skulina et al., 2004). Furthermore, CD8+ T cells that react to MOG from wild-type C3H mice are encephalitogenic and induce a disease phenotype that resembles MS more closely with respect to the presence of ataxia and spasticity than some of the CD4+ T cell-mediated EAE models (Huseby et al., 2001). Since MHC class I antigens are expressed on OC it is conceivable that activation of CD8+ autoreactive cells may be a crucial factor in the development of MS. While CD8+ cells being effectors in CNS demyelination is attractive there is very little evidence for a direct role of myelin antigen specific CD8 positive cells in MS.

2.1.3.3 $\gamma\delta$ T Cells $\gamma\delta$ T cells (Born et al., 2006) are an important subtype of T-cells that constitute only a small fraction of circulating lymphocytes, but play roles in a variety of immune responses. $\gamma\delta$ T cells are seen in MS lesions, predominating in chronic lesions (Selmaj et al., 1991). Since $\gamma\delta$ T cells have the potential to kill targets that do not require MHC class I or II antigen expression, and $\gamma\delta$ T cells are known to be cytotoxic to oligodendrocytes *in vitro*, they may play important roles in the pathogenesis of demyelination (Zeine et al., 1998).

2.1.3.4 B Cells and Immunoglobulins The role of immunoglobulins in MS stems from immune characteristics of the spinal fluid in MS. One hallmark of MS is the presence of increased levels of IgG and oligoclonal bands in the cerebrospinal fluid (CSF). Oligoclonal bands (OCBs) are seen in the CSF of approximately 90% of MS patients, but are also found in the CSF of patients with a wide variety of CNS infections. The presence of OCBs and elevated IgG are thought to represent an intrathecal immune response to an infectious agent and the OCBs are antibodies synthesized within the CNS to a putative infectious agent or to as yet undefined autoantigens. Although MS is thought to be a T-cell-mediated autoimmune disease, there is evidence that even in prototypic T-cell-mediated diseases, there is a role for B cells and possibly autoantibodies (Lucchinetti et al., 2000; Cross et al., 2001; Berger et al., 2003; reviewed in Cross and Stark, 2005). EAE cannot be induced in animals depleted of B-cells. But if at the same time they were given serum from MBP immunized mice they develop EAE. Abs can opsonize myelin, leading to membrane attack complex (MAC) deposition and complement-mediated cytolysis and phagocytosis by macrophages. They may target cells such as mast cells to the site of inflammation. B cells can serve as APCs for autoreactive T cells. Thus it was found that epitope specificity of the antibodies generated during EAE, the encephalitogenic T cell epitopes, and the immunodominant T and B cell epitopes in humans often overlap (Wang and Fujinami, 1997; Wucherpfennig et al., 1997) While it may be reasonable to conclude that oligoclonal bands seen in the CSF are pathogenic, these antibodies do not show reactivity to myelin antigens.

2.1.3.5 Dendritic Cells and Microglial Cells Although controversial there is evidence for the presence and function of dendritic cells in the CNS (Pashenkov et al., 2001). Dendritic cells (DC) are professional antigen presenting cells (APC) that play a major role in regulating both innate and adaptive immunity (for a recent review, see Adams et al., 2005). There is recent evidence to suggest that as antigen presenting cells, DCs are more potent than microglia. Both microglia and DCs act as effector cells in two overlapping ways. First, they are potent inducers and secretors of proinflammatory cytokines that activate and expand T cells that are presumably autoreactive (Adams et al., 2005). They also can induce ROS and chemical mediators that are detrimental to the myelin-oligodendrocyte unit. There is also evidence that DCs and microglial cells can alter the pattern of cytokine response so as to attenuate the immune response or shift them from a Th1 to Th2 (Zhang et al., 2002). Finally, microglia and DCs act as the final effectors in demyelination and cause the stripping and phagocytosis of myelin membranes resulting in pathological picture of the demyelinating plaque.

2.1.3.6 Immunoregulatory Cells *T reg cells.* T reg cells represent CD4+ population of antigen specific cells that presumably regulate the potency and duration of the immune response. They express CD25 and down regulate expansion of T cells and decrease the expansion of proinflammatory cytokines. Depletion of T reg cells leads to organ-specific autoimmune diseases in mice (Sakaguchi et al., 1985). The role of T reg cells in MS is actively being pursued but their effect in MS is not clear. Viglietta et al. (2004) showed that in MS patients, there was loss of function of T reg cells. Venken et al. (2006) showed a normal T reg function in SPMS but not in RRMS.

NK cells. NK cells (Johansson et al., 2006) constitute about 10% of the population of circulating lymphocytes. They are involved in innate immunity and recognize ligands on a number of somatic cells that lead to its activation which in turn leads to lysis of the target cells. In contrary to expectations, NK cell depletion leads to exacerbation of EAE (Xu et al., 2005) and impaired NK cell activity is seen in MS patients undergoing relapse (Hirsch and Johnson, 1985). These studies suggest that NK cells may play a role in down regulating the autoimmune response.

2.1.4 Mediators of Inflammation

Inflammatory molecules that are responsible for the destruction of the oligodendrocyte myelin unit can act in the following ways: (1) they can disrupt the integrity of myelin sheath or the oligodendrocyte or activate apoptotic process, (2) they can attract inflammatory cells to the site of the lesion.

2.1.4.1 Direct Attack on the Oligodendrocyte/Myelin Sheath Agents that actively participate in the destruction of either oligodendrocytes and/or myelin sheath include tumor necrosis factor alpha, interferon- γ , NO, and ROS.

Tumor necrosis factor (TNF) alpha. TNF α is well known to be toxic to oligodendrocytes in vitro. TNF α is made by T cells and microglial cells, and astrocytes in the CNS. TNF receptors are present on oligodendrocytes and it is possible that activation of the death receptors on the TNF receptor pathway promotes apoptosis of oligodendrocytes (Selmaj and Raine, 1988). In mouse models of EAE, anti TNF antibody is protective and transgenic mice that are induced to express TNF in astrocytes show demyelination in vivo (Probert et al., 1995). Contrary to expectation, MS patients treated with anti TNF antibodies showed worsening of the neurological signs, which would suggest that the role of TNF in MS is not straightforward (Magnano et al., 2004).

Interferon (IFN) γ . IFN γ , which is produced mainly by T cells have been suggested as mediator of demyelination. IFN γ treatment of MS patients resulted in a transient worsening of their clinical symptoms attacks (Panitch et al., 1987). However, there is no direct evidence that it plays a role in MS. Also to complicate issues further, mice that do not express IFN γ receptors show worsening of EAE (Chu et al., 2000).

Reactive Oxygen Species (ROS). ROS are primarily generated by activated macrophages and ROS, which include superoxide radical O $_2^{\bullet-}$, hydrogen peroxide (H $_2$ O $_2$), and hydroxyl radical (HO $^{\bullet}$) can damage lipids, proteins, and nucleic acids of cells, resulting in disturbed mitochondrial function which may induce cell death (Bolanos et al., 1997; Merrill and Scolding, 1999). ROS also play an important role in inflammatory

signaling by microglia and astrocytes in response to agents such as LPS and IFN- γ , resulting in the induction of inducible nitric oxide (iNOS) and the release of nitric oxide (Pawate et al., 2004).

ROS are implicated as mediators of demyelination and axonal damage in both MS and experimental allergic encephalomyelitis (EAE) (Cross et al., 1997; van der Goes et al., 1998; Liu et al., 2001; Gilgun-Sherki et al., (2004). Peroxynitrite, formed by the interaction of NO with superoxide, has been shown to be toxic to myelin and axons (Liu et al., 2001; Garthwaite et al., 2002). Both ROS and iNOS act to disrupt the integrity of the myelin membranes thereby promoting release of cytochrome C and the activation of the apoptosis pathway.

2.1.4.2 Mediators of Cell Expansion and Recruitment Since T cells are presumed to be the main mediators of CNS demyelination, it is not surprising that factors that induce their activation and expansion are thought to play a role in MS. Since neither OC nor myelin express sufficient quantities of MHC Class I or Class II antigens, it is unlikely that T cells directly target the OC or myelin membranes. More than likely, the autoimmune response is likely to induce an inflammatory milieu that directly affects the function of OC and myelin leading ultimately to its loss and destruction.

2.1.5 Mechanisms for Autoimmunity

2.1.5.1 Molecular Mimicry Factors that induce the development of MS include those that pertain to the genetic make up and the presence of appropriate environmental event. Development of EAE is closely associated with genes that regulate immune response however; the presence of these genes alone does not guarantee the development of EAE. In fact, animals that are bred in germ-free environment have a reduced incidence of EAE (Goverman et al., 1993). The most accepted mechanism by which autoimmunity is induced in the host, who possesses the genetic risk factors, is through a process called molecular mimicry. This phenomenon represents the presence of a shared immunologic epitopes between a self antigen and a microbe (Fujinami et al., 1983). In MS it is believed that shared antigenic determinants between putative infectious pathogens and myelin antigens in a genetically susceptible individual as a likely explanation for development of autoreactivity and ultimately autoimmune disease. This process although not proven has been proposed in other systemic autoimmune disorders (Benoist and Mathis, 1999).

Molecular mimicry in MS has been hypothesized (Fujinami and Oldstone, 1985; Sospedra and Martin, 2006). Markovic-Plese et al. (2005) studied the extent of cross-reactivity of a CD4⁺ T-cell clone (TCC) specific for the immunodominant influenza virus hemagglutinin (Flu-HA) peptide derived from a patient with MS. They showed cross-reactivity against 14 Flu-HA variants, 11 viral, 15 human, and 3 myelin-derived peptides (Olson et al., 2005). Lee et al. (2005) showed that monoclonal antibodies to HTLV-1-tax cross-reacted with hnRNP A1, indicating a role for molecular mimicry in HTLV-1 induced tropical spastic paraplegia.

While molecular mimicry remains a possible idea, there have been few concrete examples of mimicry playing a role in the pathogenesis of chronic inflammatory disease. Molecular mimicry is much better established in the case of Guillain-Barre syndrome (GBS), a demyelinating disease of the peripheral nervous system. GBS is usually preceded by a bacterial or viral infection. Antibodies to GM1 ganglioside have been detected in patients with axonal GBS, which follows a campylobacter jejuni infection. The *C. jejuni* strains isolated from these patients have a lipooligosaccharide with a structure similar to GM1 (Yuki, 2001). Yuki et al., (2004) further proved molecular mimicry by inducing GBS in rabbits by repeatedly immunizing them with lipooligosaccharide.

2.1.5.2 Bystander Activation According to this model, microbial infections lead to significant activation of APCs such as dendritic cells. These activated APCs could potentially activate preprimed autoreactive T cells, which can then initiate autoimmune disease (bystander activation of autoreactive immune T cells) (Fujinami et al., 2006). Bystander activation can occur because of superantigen exposure (Brocke et al., 1993) through TLR activation by microbial products (Waldner et al., 2004). Specific immune responses to microbial antigens could thus result in immune attacks on local antigens in the CNS.

2.1.5.3 Epitope Spreading Epitope spreading has been shown as a possible mechanism in the development of chronic relapsing EAE (Miller et al., 1995). Epitope spreading can occur after initiation of the T cell response by either molecular mimicry or bystander activation, and involves the diversification of epitope specificity from the initial focused, dominant epitope-specific response to other epitopes on the same protein or on other proteins (Vanderlugt and Miller, 2002).

2.2 Viral Models of CNS Demyelination

Besides the autoimmune hypothesis described above, the other major hypothesis promoted to explain the pathogenesis of MS is infection. An infectious agent can persist in the CNS, driving inflammatory responses that result in damage to the oligodendrocyte and myelin. Although several infectious agents have been proposed as causative in MS (Gilden, 2005), none has stood up to closer scrutiny. Direct evidence of pathogen mediated demyelination has not been proven since ultrastructural studies have failed to show the presence of viral particles in oligodendrocytes. Direct viral infection mediated demyelinating syndromes are well recognized (for example, progressive multifocal leukodystrophy), but none of these produce a clinical and pathological picture that resembles MS. In the second mechanism, a viral infection is followed by an autoimmune demyelination, as occurs in acute disseminated encephalomyelitis.

PML is usually seen in immunosuppressed individuals although 80% to 90% of the population is infected. Around 5% of patients with AIDS develop PML, accounting for 85% of cases (Power et al., 2000), and recently, three cases of PML occurred in MS patients receiving a combination of beta-interferon and Natalizumab. The neuropathology of PML is characterized by demyelination in the relative absence of inflammatory reaction, and enlarged oligodendrocytes that contain intranuclear viral inclusion bodies. Thus demyelination is thought to occur as a result of oligodendrocyte destruction.

Thieler's virus, a single-stranded RNA virus and a member of the Picornaviridae family, causes neurological disease in susceptible mice strains. Inoculation of TMEV directly into the brain initially causes an acute disease resembling polioencephalomyelitis, and this is later followed in susceptible strains by a chronic demyelinating disease in the spinal cord. The development of demyelinating disease is dependant upon the viral strain used and the strain of mice used to induce viral infection. Extensive demyelination occurs, with infiltration by CD4+ and CD8+ T cells, some monocytes/macrophages, and few B cells and plasma cells. (Oleszak et al., 1988).

TMEV has been proposed as a model of virus-induced demyelination (Oleszak et al., 2004) that has many similarities with MS. For example, susceptibility to both TMEV in mice and MS in humans are MHC dependent, and the neuropathology of TMEV lesions and MS lesions show similarities.

2.3 Primary Oligodendrogliopathy

MS shows marked heterogeneity in clinical course and response to therapy (Noseworthy et al., 2000). This variability suggests that diverse pathogenic mechanisms underlie the disease. Consistent with this idea, the examination of a large group of biopsies and autopsies has facilitated the classification of active demyelinating lesions in MS into two large groups (Lucchinetti et al., 2000). In one group, the lesions are similar to those found in EAE, the most widely used animal model of MS; in the other, the lesions have features suggestive of primary loss of oligodendrocytes rather than autoimmunity.

The study by Barnett and Prineas describes pathological findings from patients who died during or shortly after the onset of a fatal relapse (Barnett and Prineas, 2004). The earliest structural change shared by all newly forming lesions was profuse oligodendrocyte apoptosis in tissue areas with early microglial activation, in the virtual absence of infiltrating lymphocytes or myelin phagocytes, whereas "older" lesions in the same patients contained these cells. These observations contrast with the commonly held view that inflammation is a primary event in MS and suggest instead that, at least in a subset of MS, autoimmunity might well be a secondary amplifying response to massive oligodendrocyte apoptosis. In addition, Barnett and Prineas propose that this could reflect distinct lesion stages rather than a dichotomy (of apoptosis

versus autoimmunity) in the mechanisms originating focal damage in MS. Further evidence that primary oligodendroglialopathy may in fact be a principal player in MS, is the observations that demyelinating lesions in the gray matter show paucity of inflammatory cellular response in spite of having large areas of demyelination that span sometimes the entire cortical mantle (► [Figure 17-1](#)).

Implicit in this alternate view of CNS demyelination is the idea that the inflammatory response is a reaction to the activation of microglial cells that have phagocytosed large amounts of myelin. Myelin debris can activate macrophages and lead to the recruitment and infiltration of the CNS by lymphocytes.

Crucial to the new observations is the nature of the signals that initiate oligodendrocyte apoptosis and the ensuing lesion. Candidate triggers include viruses that can directly kill oligodendrocytes *in vitro* by apoptosis. Also likely candidates are bacterial products, such as LPS that can induce apoptosis and oligodendrocytes are known to express receptors that recognize these receptors (Lehnardt et al., 2002).

Oligodendrocyte apoptosis, and so white matter damage, can also be caused by increased extracellular levels of glutamate generated during an inflammatory response. Elevated glutamate levels lead to oxidative stress and excitotoxicity in oligodendrocytes, and this response has been associated with MS (Matute et al., 2006; Vallejo-Illarramendi et al., 2006). Also, endogenous retroviral products including syncytin (Antony et al., 2004), which generate reactive oxygen radicals can impair the functioning of glutamate transporters and thus contribute to the elevation of extracellular glutamate concentration.

2.4 Primary Axonal Damage with Secondary Inflammation

While we have focused thus far on inflammatory demyelination, another feature of MS is the loss of axons. Axonal injury and loss correlates with the accumulation of disability in MS. Axonal injury has traditionally been thought to be a late event. However it is becoming evident that axonal injury may be a very early event. In a mouse model of mouse hepatitis virus (MHV) induced demyelinating disease, Dandekar et al. (2001) showed axonal injury in areas devoid of demyelination. Trapp et al. (Trapp et al., 1998) were able to use immunohistochemistry and confocal microscopy to show evidence of axonal transection in lesions from MS brains in autopsy specimens. Evangelou et al. (2000) demonstrated axonal injury even in normal appearing white matter. Bitsch et al. (2000) showed that axonal injury is independent of demyelination, and association with macrophages and CD8 cells but not CD4 cells. Thus there is an emerging “inside-out” model (Tsunoda and Fujinami, 2002) that posits that injury to axons may be the primary event and inflammatory demyelination may be a consequence of it. Rammohan (2003) proposed a model of primary axonal injury wherein the most logical location of involvement would be the node of Ranvier, the location that generates the nerve action potential. An antibody- and complement-mediated, or alternately CD8 T cell-mediated attack on the node of Ranvier may occur, because the axolemma at the node of Ranvier has the potential to express MHC class I molecules. The idea that axonal injury is independent of demyelination was further supported by DeLuca et al. (2006) who found little correlation between lesion load and axonal loss (► [Figure 17-1](#)).

3 Conclusions

Multiple sclerosis has been traditionally thought of as an inflammatory demyelinating disease of the central nervous system, with loss of axons being a secondary phenomenon. The focus of research in MS for the last several decades has therefore been on demyelination, and as described in this chapter, a great deal of knowledge has accumulated on the mechanisms of inflammatory demyelination, including the role of various cells and chemical mediators. The currently available treatments of MS, that is, beta-interferons, glatiramer acetate, and mitoxantrone, are modulators of the immune process that results in demyelination.

Several findings raise questions about the centrality of autoimmune demyelination in MS. While in the early relapsing-remitting stage of MS, contrast-enhancing lesions, indicative of a disruption of blood–brain barrier, are well correlated with clinical relapses, there is poor correlation between the number of lesions seen on MRIs and the extent of progression. Brain atrophy, indicative of progressive axonal loss, correlates

better with the accumulation of disability in the later, progressive phase of the disease. Axonal loss has been shown to occur very early in MS, even in the normal-appearing white matter (Bjartmar et al., 2001).

Further support for the notion that axonal loss and demyelination may be independent of each other comes from autopsy studies of brains of patients who had received autologous bone marrow transplants (Bruck, 2005). In these specimens, there was no presence of inflammatory markers such as T cells, but significant staining for amyloid precursor protein (APP). This suggests that even in the absence of inflammation, neurodegeneration was proceeding in these patients. Thus, MS may need to be considered a pan-cerebral neurodegenerative disease. Although an immune inflammatory process cannot be excluded, there is a need to address the chronic neurodegenerative aspects that may be immune independent. It follows that neuroprotective treatments aimed at halting the loss of axons may offer the best hope of delaying or preventing the disability in progressive MS.

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18 The Inflammatory Component of Neurodegenerative Diseases

C. C. Ferrari · F. J. Pitossi

1	<i>Inflammatory Response in the Central Nervous System</i>	396
2	<i>Parkinson's Disease</i>	398
2.1	Introduction	398
2.2	The Inflammatory Reaction in PD	398
3	<i>Prion Disease</i>	400
3.1	Introduction	400
3.2	The Inflammatory Reaction in Prion Disease	401
4	<i>Alzheimer's Disease</i>	402
4.1	Introduction	402
4.2	The Inflammatory Reaction in Alzheimer's Disease	402
5	<i>Concluding Remarks</i>	402

Abstract: Inflammation is a key component in the immunological defense of the organism against health-threatening pathogens. On the other hand, a dysregulated inflammatory response can lead to tissue damage and disease. In the central nervous system (CNS), the inflammatory response obeys different rules than the periphery. The presence of a selective blood–brain barrier, the lack of dendritic cells, the chronic downregulation of costimulatory molecules, and a bias towards an immunosuppressive environment are mainly responsible for the characteristic features of the inflammatory response in the CNS. Until recently, with the exception of multiple sclerosis, the inflammatory component present in major chronic neurodegenerative diseases has been regarded as a logical consequence of cell death with no major functional relevance but to remove cellular debris. However, inflammation is now known to have toxic and protective effects in chronic neurodegenerative diseases such as Parkinson's, Alzheimer's, and prion diseases. Microglial activation appears as a common feature to all these pathologies. However, microglial cells do not seem to be activated to an end-stage, proinflammatory phenotype during chronic neurodegeneration. Instead, they appear to be in a “primed” state where major proinflammatory cytokine production is tightly controlled. These observations are in agreement with the noninflammatory nature of apoptotic cell death. In addition, evidence is accumulating to favour the hypothesis that these “primed” microglial cells will produce an exacerbated amount of proinflammatory cytokines if a proinflammatory stimulus hits them in this state. The resulting exacerbated inflammatory response can lead to a toxic effect on the degenerating cells, exacerbating disease and aggravating symptoms.

These observations are seminal to understand the pathophysiology of neurodegenerative diseases and prevent putative disease exacerbation by inflammation.

List of Abbreviations: APP, amyloid precursor protein; CJD, Creutzfeld-Jacob diseases; CNS, central nervous system; GSS, Gertsman-Straussler-Scheinker syndrome; IFN- γ , interferon- γ ; IL-1 α , interleukin 1 α ; IL-1 β , interleukin 1 β ; IL-4, interleukin-4; IL-6, interleukin-6; IL-8, interleukin-8; IL-10, interleukin-10; LPS, bacterial lipopolysaccharide; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PS1, presenilin 1; PS2, presenilin 2; 6-OHDA, 6-hydroxydopamine; SN, substantia nigra; TNF α , tumor necrosis factor- α ; TSEs, transmissible spongiform encephalopathies

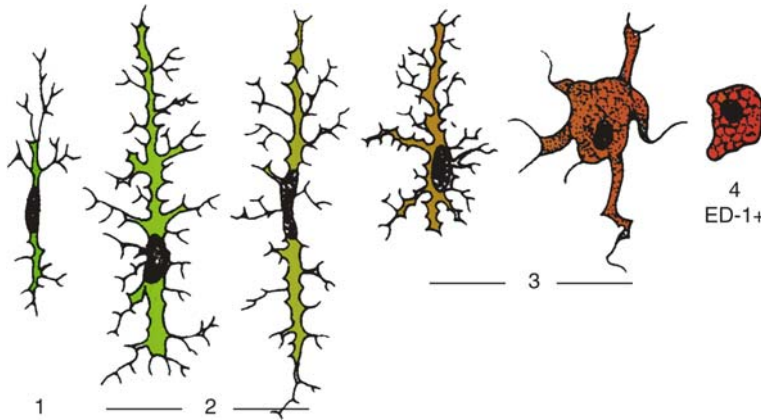
1 Inflammatory Response in the Central Nervous System

Inflammation is pivotal in the defense of the body against infection and injury. On the other hand, its exacerbation or deregulation can lead to deleterious effects and disease. Inflammation in the central nervous system (CNS) has distinct characteristics from the one elicited in the periphery (Perry et al., 1995). The presence of a selective blood–brain barrier, the lack of dendritic cells, the chronic downregulation of costimulatory molecules and a bias toward an immunosuppressive environment are mainly responsible for the unique features of the inflammatory response in the CNS. In addition, the inflammatory response in the CNS varies according to the different CNS compartments studied. For example, the ventricles and meninges behave more similarly to the periphery in terms of immunoreactivity than the cerebral parenchyma (Perry et al., 1995).

In the CNS, a main component of the inflammatory response is played by the macrophage resident system, the microglial cells. Microglia cells represent about 10% of the nervous system cell population (Perry and Gordon, 1988) and are evenly distributed throughout the brain, but the substantia nigra (SN), a main brain region affected in Parkinson's disease (PD), exhibits the highest concentration of microglia in the brain (Lawson et al., 1990). While in the intact brain microglia exhibits a resting phenotype, they can rapidly change their morphology and physiology in response to brain insults, becoming activated. The morphological changes associated with activation can be divided into at least four stages (Kreutzberg, 1996) ([▶ Figure 18-1](#)). *Stage 1* includes microglia in a resting state. Their morphology consists of rod-shaped soma with fine and ramified processes. They express low or undetectable cell-surface antigens such as CD45 and major histocompatibility complex (MHC) class I and II molecules. *Stage 2* defines activated ramified microglia. At this state of activation, the cells present elongated-shaped cell body with long and thicker

■ Figure 18-1

Morphological changes during microglial activation. (Adapted from Keutzberg et al., 1996). At least four different groups of microglial morphology can be distinguished according to their degree of activation. Microglial cells express the ED-1 marker only at *Stage 4* of activation (see text)



processes. The appearance of amoeboid microglia defines *Stage 3* of activation. They present round-shaped body with short, thick and stout processes. At *Stage 4* of activation, microglia is called phagocyte cells. They are round-shaped cells with vacuolated cytoplasm and no processes, resembling a macrophage at the light microscopy level. This end-stage microglial activation can also be defined by the detection of the ED-1 intracellular glycoprotein with specific antibodies.

When activated, microglia can start expressing membrane proteins such as MHC class II and can secrete a plethora of molecules, including cytokines that will determine the functional outcome of this activation. Although microglial morphology characteristic of an activated state have always been associated with the production of proinflammatory molecules, this vision has been recently revisited, mainly due to the results obtained in models of chronic neurodegeneration (see later) (Perry, 2004; Perry et al., 2007). In fact, it has been realized that a confounding feature is based on the observation that microglial cells with a similar morphological stage of activation (i.e., *Stage 3*) could have different profile of secreting molecules depending on the stimulus that triggered the activation and the milieu in which they reside (Perry et al., 2007). We have found only one main correlation: *Stage 4*, ED-1-positive, end-stage-activated microglia has been consistently found to correlate with a proinflammatory profile (Pott Godoy, unpublished).

Microglial activation is a feature of many neurodegenerative diseases (Vila et al., 2001). It is clear that microglia plays a crucial role in tissue homeostasis in neurodegenerative diseases by removing dead cells. Until recently, this has been microglia's main function. However, it has been shown that microglial cells become activated during chronic neurodegeneration and can also exhibit neurodegenerative or neuroprotective functional roles, depending on the products they secrete and the composition of the milieu at that moment. Microglial activation can contribute to neurodegeneration through the release of cytokines, nitric oxide, and/or free radicals such as superoxide radicals (Czlonkowska et al., 2002; Iravani et al., 2002; Ajmone-Cat et al., 2003; Arimoto and Bing, 2003). Alternatively, it can promote neuroprotection by secreting neurotrophic factors and also cytokines (Vila et al., 2001). Thus, cytokines can have both, neurodegenerative and neuroprotective effects on neuronal demise in the SN (Vila et al., 2001; Cho et al., 2006).

In this chapter, we will focus on the current knowledge of the characteristics of the inflammatory reaction and the functional relevance of inflammation in Parkinson's, Alzheimer's, and prion diseases, three chronic neurodegenerative diseases not previously regarded as inflammatory ones.

2 Parkinson's Disease

2.1 Introduction

PD is a neurodegenerative disorder characterized by the progressive loss of dopaminergic neurons in the SN (Lang and Lozano, 1998a). The loss of dopamine in the nigrostriatal system causes most clinical symptoms of this disease such as: tremor at rest, bradykinesia (slowness of movement), rigidity and difficulty with balance. Lewy bodies and dystrophic neurites accompany neurodegeneration. The aetiology of idiopathic PD, the major form of PD, remains unknown. Specific gene mutations have been linked to familial PD. Genetic Parkinsonism could account for not more than 10% of all PD cases (reviewed in Dawson and Dawson, 2003). These gene mutations have been found in genes such as parkin, α -synuclein, and DJ-1, among others (reviewed in Dawson and Dawson, 2003). Current therapeutic treatments deal with the replacement of dopamine content or with surgical interventions to reduce the hyperactivity of specific regions in the basal ganglia (Lang and Lozano, 1998b). However, no treatment is available that could reduce the progression of the neurodegenerative process. Important features of the pathology of the disease have been elucidated, but still a clear comprehension of the pathological process in PD is lacking.

2.2 The Inflammatory Reaction in PD

A main pathological feature of PD is microglial activation. Robust microglial activation was consistently found in animal models and PD patients (McGeer et al., 1988; Hunot et al., 1999; Langston et al., 1999; Liberatore et al., 1999; Mogi and Nagatsu, 1999; Mirza et al., 2000; He et al., 2001; Vila et al., 2001; Cicchetti et al., 2002; Orr et al., 2002; Depino et al., 2003; Hirsch et al., 2003; Sugama et al., 2003; Hald and Lotharius, 2005). Microglial activation is present for long periods of time in the SN. Indeed, in PD patients, microglial activation was observed as long as 16 years after self-administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston et al., 1999). The functional role of microglial activation in PD is still not clear.

Contrary to microgliosis, astrogliosis is not as pronounced and has not been consistently found in PD patients or animal models (Mirza et al., 2000). Although reactive astrocytes have been associated with brain trauma, increasing evidence indicates that astrogliosis would also be involved in beneficial effects, especially those related to the expression of neurotrophic factor and reuptake of glutamate (Boka et al., 1994; Chen et al., 2005; Nagatsu and Sawada, 2005). Several studies described the astrocyte reaction in several models of PD, such as MPTP and 6-hydroxydopamine (6-OHDA) and PD cases, usually found in the later stages of the disease (Forno, 1992; Sheng et al., 1993; Langston et al., 1999; Mirza et al., 2000; Rodrigues et al., 2001; Depino et al., 2003; Hirsch et al., 2003).

Cytokines are major mediators of microglial function. Several proinflammatory cytokines such as interleukin 1 β (IL-1 β), tumor necrosis factor- α (TNF- α), interferon- γ (IFN- γ), and interleukin-6 (IL-6) have been shown to be increased in the ventricular cerebrospinal fluid and in postmortem striata of PD patients, compared with control patients (Mogi et al., 1994; Mogi and Nagatsu, 1999). In addition, bacterial lipopolysaccharide (LPS), a potent inflammogen and cytokine inducer, has been shown to be toxic for dopaminergic neurons in vitro and in vivo (Castano et al., 1998; Kim et al., 2000; Iravani et al., 2002). The effect of LPS cannot be mimicked by the acute intranigral injection of TNF- α or IL-1 β alone (Castano et al., 2002; Depino et al., 2005). One crucial parameter on the final effect of a given cytokine on neuronal vitality is the duration of its expression. For example, the acute injection of IL-1 β in the SN was not toxic for dopaminergic neurons in vivo, either when cytokine was injected alone (10 ng or 1000 Units) or in combination with 1000 U of TNF- α and 100 U of IFN- γ in the SN (Castano et al., 2002; Depino et al., 2005). On the contrary, the chronic expression of IL-1 β in the SN elicited most of the characteristics of PD, including progressive neurodegeneration, akinesia, and glial activation. These symptoms were evident after 21 days of continuous expression (Ferrari et al., 2006). These data suggest that IL-1 β per se is able to mediate inflammatory-mediated toxic effects in the SN if its expression is sustained for at least 21 days in the SN. Furthermore, the prenatal administration of LPS during the second week of pregnancy reduces the

amount of dopaminergic cells in the SN and renders them more susceptible to the toxic effects of other stimulus such as 6-OHDA (Carvey et al., 2003; Ling et al., 2004). Only one study has shown that the previous administration of IL-1 β ameliorate the neurotoxic effect in the SN of a subsequent intrastriatal injection of 6-OHDA (Saura et al., 2003). Anti-inflammatory treatments have shown to be neuroprotective in mice models of PD (He et al., 2001; Wu et al., 2002; Sanchez-Pernaute et al., 2004) suggesting that inflammatory processes and microglial activation might contribute to the degeneration of the SN neurons. In addition, several anti-inflammatory agents such as minocycline and naloxone, have been shown to ameliorate neurodegeneration in PD models (Liu et al., 2000a; Liu et al., 2000b; Wu et al., 2002; Sanchez-Pernaute et al., 2004; Hald and Lotharius, 2005), leading to envisage a deleterious role of inflammation on neurodegeneration.

Thus, taken the presence of microglial activation on postmortem tissue and the toxic effects of proinflammatory cytokines on dopaminergic cells, it has been suggested that microglial activation and subsequent proinflammatory cytokines secretion were detrimental for neuronal viability in the SN. However, different features of microglial activation and cytokine profile were detected during neurodegeneration in the SN when they were studied in an animal model of PD that allowed for a temporal and detailed analysis of the pathological process (Depino et al., 2003). In addition, it should be emphasized that microglial activation does not always lead to proinflammatory secretion (Perry et al., 2007).

We have studied microglial activation at the morphological and molecular levels in a progressive model of neurodegeneration of the SN by the intrastriatal injection of 6-OHDA (Depino et al., 2003). 6-OHDA is taken by the synaptic terminals of the SN in the striatum. The incorporation of the 6-OHDA neurotoxin into the neurons of the SN produces a progressive neurodegeneration in that region that is accompanied by motor disabilities (Sauer and Oertel, 1994). Triggered by the neurodegeneration in the SN, morphological microglial activation was evident from 6–30 days postlesion (Depino et al., 2003). Microglial cells become activated up to *Stage 3* of activation, but will not proceed to *Stage 4*, ED-1-positive, end-stage activation in that region. The observation that microglia did not become fully activated during neurodegeneration suggested a tight control of microglial activation in this model.

This microglial activation in the SN was accompanied by an atypical cytokine production: Interleukin-1 (IL-1) α and β mRNAs were found to be elevated 30 days post 6-OHDA injection (2- and 16-folds, respectively), but no induction for IL-1 α or β at the protein level was detected by ELISA (Depino et al., 2003). As a control, a classical proinflammatory stimulus, namely LPS, was capable of driving microglial cells to end-stage activation (*Stage 4*) inducing those proinflammatory cytokines at the mRNA but also at the protein level. In addition, TNF- α mRNA was hardly or not detected in the SN at any time point studied. Our data point out to a tight control of key proinflammatory cytokine production in our model of PD where microglial cells are “primed” by increased untranslated proinflammatory cytokine transcripts.

Central inflammation could transform the “primed” state of microglia into an end-stage, proinflammatory state, which could exacerbate neurodegeneration. It is known that a systemic inflammatory response which circulates in the blood can communicate with the brain (Pitossi et al., 1997; Combrinck et al., 2002; Perry, 2004). There are different routes of communication between brain and periphery: circumventricular organs, blood brain barrier and the sensory afferents of the vagus nerve (Maier et al., 1998; Palin et al., 2004; Dantzer and Kelley, 2007). Thus, not only central, but also peripheral infections might have an impact on the neurodegeneration of the SN and thus, be considered a risk factor in PD (Perry et al., 2003, 2007).

These observations are in agreement with the biology of the chronic neurodegenerative model used: neuronal cell death in the SN is supposed to be apoptotic (He et al., 2001), which by definition does not cause inflammation. Similarly, activated macrophages in the periphery do not express proinflammatory cytokines during phagocytosis of an apoptotic cell (Fadok et al., 1998). The brain preserves its anti-inflammatory milieu by different means such as increasing anti-inflammatory cytokine production and restricting the entry of plasma proteins and immune cells from the periphery by the blood–brain barrier (Perry et al., 1993; Aloisi, 2001). The control of IL-1 α and β and TNF- α expression at different levels suggests additional mechanisms of preserving this anti-inflammatory milieu during chronic neurodegeneration in the SN. This lack of TNF- α mRNA induction, and the absence of IL-1 α and β induction at the protein level, does not seem to correlate to what was found in postmortem samples from PD patients

(Mogi and Nagatsu, 1999; Nagatsu and Sawada, 2005). However, the time of analysis of both approaches is different: postmortem samples reflect the final episodes of PD, whereas the study on an animal model of PD allows studying its progression. In addition, animals are kept in a pathogen-free environment and patients are not. Moreover, cytokines from the periphery can induce cytokines in the brain (Pitossi et al., 1997), opening the possibility that cytokines present in postmortem samples could also be a consequence of peripheral infections. In fact, most PD patients die from respiratory tract infections (Ebmeier et al., 1990; Nakashima et al., 1997; Beyer et al., 2001).

In summary, during chronic neuronal death in the SN, microglia seems to be in a “primed” but not end-stage activation state, with no production of proinflammatory cytokines. It has been proposed that a second, proinflammatory stimulus is required to shift microglial activation to end-stage (*Stage 4*) leading to the secreting of proinflammatory cytokines in the SN, exacerbating the neurodegeneration. This “second hit” hypothesis should account for the observation of end-stage microglial activation in postmortem tissues from PD patients. Thus, these studies do not exclude that proinflammatory cytokines such as IL-1 α and β and TNF- α could be functionally involved in the last stages of the human disease.

Importantly, this “atypical” microglial activation without proinflammatory cytokine production has been previously tested in models of other neurodegenerative diseases such as prion and Alzheimer’s diseases (ADs) as described later (reviewed in Perry et al., 2007). Even more relevant, the “second hit” hypothesis has been already demonstrated in prion disease and AD models (Sly et al., 2001; Perry et al., 2002).

3 Prion Disease

3.1 Introduction

Prion diseases are fatal and transmissible neurodegenerative diseases that include Creutzfeldt–Jacob diseases (CJD), fatal familial insomnia, Gertsmann–Straussler–Scheinker syndrome (GSS) and kuru in humans, and transmissible spongiform encephalopathies (TSEs) in both domestic (including the “mad cow disease” in cattle and scrapie in sheep) and wild animals. No effective therapies are available for any of these diseases nowadays.

Sporadic CJD affects equally men and women and the symptoms include fatigue, sleep disorders, behavioral or cognitive changes, visual loss, cerebellar ataxia, and motor deficits (Johnson, 2005). At present, the incidence of the sickness in the United Kingdom appears stabilized with approximately 160 new cases per year, but still an unknown number of undiagnosed cases remain undiscovered, that exhibit this illness subclinically. No clinical symptoms were available to define the CJD; indeed, the definitive diagnosis is the postmortem analysis of the patient’s brain. Variants of this sickness in humans are the iatrogenic and the familial CJD. Iatrogenic CJD is produced when the infection has occurred as a consequence of a medical procedure (corneal transplant, dural grafts, hormonal treatments from human pituitary glands, and contaminated neurosurgical material) (Johnson, 2005). Familial CJD are transmitted as autosomal dominant traits with mutations in *Prnp*, the gene that encodes the prion protein (Hsiao et al., 1989).

The main cause of these diseases is a proteinaceous infection from a particle called prion. Prusiner (1982) discovered that the infectious protein is a misfolded isoform of the prion protein (PrP^C), expressed in neurons and glia in a pathogenic form (PrP^{Sc}) (scrapie). PrP^{Sc} is characterized by increased β -sheet content, proteases resistance and detergent insolubility (Sly et al., 2001) reviewed in Abid and Soto (2006).

The damage produced by prions is evidenced in the CNS as deposit of PrP^{Sc} viewed as amyloid plaques, vacuolisation of neurons, gliosis and neuronal apoptosis (de Armond et al., 1980; Gray et al., 1999; Lewicki et al., 2003; Johnson, 2005; Aguzzi and Heikenwalder, 2006).

As we previously said, the main target of the prion protein is the brain, but it also has been described in extra cerebral localizations, including the lymphoid organs and sites of inflammation (reviewed in Aguzzi and Heikenwalder, 2006; Isaacs et al., 2006). In addition, peripheral myeloid cells can carry the prion infectious agents for weeks in vitro (Manuelidis et al., 2000; Aucouturier et al., 2001) The immune

system also contributes to the pathogenesis by amplifying the prion protein in the lymphoid organs (Aguzzi, 2003).

A better understanding of the protein conversion, the characterization of early symptoms and its effects in the periphery would probably help discover new therapeutic strategies against prion diseases.

3.2 The Inflammatory Reaction in Prion Disease

Prion diseases are characterized by deposits of insoluble protein plaques, neurodegeneration and activation of microglia and astroglia. Inflammatory components, such as increased number of astrocytes and microglia have been described in postmortem brains together with an increased expression of proinflammatory cytokines, IL-1 β , interleukin-8 (IL-8), and anti-inflammatory cytokines, interleukin-4 (IL-4) and interleukin-10 (IL-10) in the cerebrospinal fluid of CJD patients (Lusky et al., 1998; Van Everbroeck et al., 2002). Microglial activation and astrogliosis have also been extensively studied in several prion diseases and animals models (Williams et al., 1994; Peyrin et al., 1999; Russelakis-Carneiro et al., 1999; Vidal and Legrain, 1999; Lucking et al., 2000; Eikelenboom et al., 2002; Van Everbroeck et al., 2002; de Almeida et al., 2005; Unterberger et al., 2005; Veerhuis et al., 2005; Boche et al., 2006). The kinetics and magnitude of the response varies according to the prion disease in question (Manuelidis et al., 2000; Lu et al., 2004). In addition, transcripts of myeloid cell recruitment factors such as MIP-1 α , MIP-1 β , and MCP-1 were upregulated before the symptoms of the disease (Lu et al., 2004). Moreover, microglia can show prion infectivity and exerts changes in morphology and cytokine expression (Baker et al., 2002). Therefore, microglia can act as an agent to distribute the prion protein throughout the brain parenchyma. On the other hand, *in vitro* studies demonstrated that activated microglia assist in the removal of neurodegenerative neurons in prion damage areas (Bate et al., 2001; de Almeida et al., 2005). Microglia even in the later stage of CJD did not exhibit end-stage activation with macrophage morphology and only rarely are cells positive for phagocytic activity markers such as ED-1 (Manuelidis et al., 1997). The PrP^{Sc} protein can induce microglial activation together with the expression of proinflammatory cytokines, such as IL-1 β and IL-6, with no increased TNF- α and anti-inflammatory cytokines such as IL-10 and TGF- β (Williams et al., 1994; Peyrin et al., 1999; Baker et al., 2002; Greenwood et al., 2003). Several studies have demonstrated, however, the expression of TGF- β in animal models of prion diseases (Cunningham et al., 2002; Perry et al., 2002). These studies described atypical microglial activation in an ME7 model of murine scrapie characterized by the lack of IL-1 β , IL-6, and TNF- α expression but dominated by high expression of TGF- β as the mayor cytokine involved. This suggests a central role for TGF- β in minimizing brain inflammation, promoting the evolution of atypical inflammation and involving vascular extracellular matrix components (Cunningham et al., 2002; Perry et al., 2002). In summary, as described in previous paragraphs for PD, neurodegeneration in the prion model also leads to an “atypical”, not proinflammatory microglial activation. Indeed the findings of, “atypical” microglial activation and the “second hit” hypothesis of the exacerbation of neurodegenerative diseases were first postulated by Hugh Perry and colleagues from results coming from this experimental prion disease model (Perry et al., 2002).

As stated earlier, this “atypical” inflammatory state of the microglia can be driven to an inflammatory state with either central or peripheral proinflammatory challenge. The central injection of LPS in a model of murine prion disease induced a dramatic increase of IL-1 β expression, neutrophil infiltrate, and iNOS expression in the brain of prion-infected animals compared with the same challenge in non-infected animals (Cunningham et al., 2005). In addition, Perry and colleagues have also demonstrated that a systemic inflammatory challenge in an animal with chronic neurodegeneration exacerbated the inflammatory response in the brain (Combrinck et al., 2002; Perry, 2004; Cunningham et al., 2005; Perry et al., 2007). In particular, systemic inflammation induced by LPS induced the expression of proinflammatory cytokines, iNOS transcription, microglial expression of IL-1 β and an increment in apoptosis in the brains of mice with prion disease (Cunningham et al., 2005). In addition, the inhibition of an anti-inflammatory cytokine such as TGF- β by expressing decorin induced iNOS expression in microglial cells, inducing the exacerbation of brain inflammation and neurodegeneration in a model of murine prion disease (Boche et al., 2006). Similarly,

prion disease was exacerbated in the absence of IL-10 (Thackray et al., 2004). In summary, the atypical inflammatory response can be exacerbated by either inducing central or inflammatory challenges or inhibiting anti-inflammatory molecules. This shift in the inflammatory profile toward inflammation can in turn, increment brain damage and neurodegeneration.

4 Alzheimer's Disease

4.1 Introduction

AD was originally recognized by Alois Alzheimer in 1907 as a particular form of dementia. Nowadays, AD affects more than 20 million people worldwide (Blennow et al., 2006). In addition, as for other neurodegenerative diseases, the prevalence of AD is expected to increase due to the increments in life expectancy of the world population (Blennow et al., 2006). AD causes a progressive and permanent decline in memory and cognitive abilities, leading to dementia. The majority of AD cases (approximately 90%) occurs sporadically and is late in onset, usually occurring after 65 years of age (Pratico and Delanty, 2000; Blennow et al., 2006). Familial AD, only accounts for approximately 10% of cases and symptoms and usually has an earlier onset (Blennow et al., 2006). Mainly, three genes have been implicated in AD pathogenesis: amyloid precursor protein (APP), presenilin 1 (PS1), and presenilin 2 (PS2) (Bird, 2005). The AD brain shows a consistent pathology amongst patients. Two main histological features of amyloid plaques and neurofibrillary tangles have been described mainly in the temporal neocortex and hippocampal regions of the AD brain (Selkoe, 1991; Taguchi et al., 2005).

4.2 The Inflammatory Reaction in Alzheimer's Disease

Activated microglia has been found in postmortem brains from AD patients, specially surrounding amyloid- $\beta_{1-40/42}$ plaques (reviewed in Akiyama et al., 2000). It is not clear whether the activating stimulus is the amyloid itself and/or the degenerating processes of neurons. This type of activation has also been defined as "atypical", with similar general features as found in prion and PD models (reviewed in Perry, 2004). Since amyloid plaques persist over time, it has been proposed that this chronic stimulus down-regulates the microglial response, similarly to the unresponsiveness observed in microglia after repeated LPS challenges (Ajmone-Cat et al., 2003).

Most if not, all inflammatory mediators have been described in postmortem brains from AD patients (Akiyama et al., 2000). However, even the results on the quantitation of a single cytokine such as IL-1 β are lacking consistency among the different reports (Perry et al., 2007). In general, proinflammatory cytokine expression is tightly controlled and only modest or subtle levels of proinflammatory cytokines such as IL-1 β or TNF- α are found in animal models of AD. For example, mRNA levels of IL-1 α , IL-1 β and TNF- α were found 2–3-fold increased in the cortex of transgenic mice expressing the Swedish double mutation under the control of the prion protein promoter compared with wild-type littermates (Sly et al., 2001). In transgenic models of AD, microglial cells were found to be "primed" and responded with increased inflammation, exacerbated transcription of cytokines, increased TAU hyperphosphorylation and/or altered amyloid beta processing when challenged with LPS (Sly et al., 2001; Kitazawa et al., 2005). Importantly, systemic infections have been shown to be associated with an exacerbated rate of cognitive decline in AD patients, highlighting the functional relevance of the exacerbation of inflammation in this disease (Holmes et al., 2003).

5 Concluding Remarks

Taken together, the inflammatory profile observed in chronic neurodegenerative diseases such as PD, prion, and AD differs greatly from the one elicited by other stimuli such as infections or trauma. This particular microglial activation includes morphological activation till *Stage 3* (not to end-stage activation) and a tight

control of proinflammatory cytokine production, especially at the translational level, with increased untranslated mRNAs for key proinflammatory cytokines. Once called “atypical”, these features of microglial activation are started to be regarded as typical for chronic neurodegenerative diseases. It has also been proven for prion and AD that microglial cells are “primed” during neurodegeneration and respond with an exacerbated proinflammatory response if a second proinflammatory stimulus hits the neurodegenerative brain area. This “second hit” could shift the microglial secretory profile toward inflammation, which in turn exacerbates neurodegeneration and disease progression. Since brain inflammation can be exacerbated from the periphery, data are accumulating to start thinking on peripheral infections or inflammatory events as risk factors for health decline in these diseases.

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19 Mechanisms of Inflammation in HIV-associated Dementia

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1	<i>HIV Life Cycle</i>	408
2	<i>HIV Infection of CNS Inflammatory Cells</i>	408
3	<i>HIV-1 Replication by Cell Type in CNS</i>	409
3.1	Microglial HIV-1 Replication	409
3.2	Astrocyte HIV-1 Replication	410
3.3	Neuronal HIV-1 Replication	410
4	<i>HIV gp120 Protein</i>	411
5	<i>Overview of Cytokines and Chemokines in HAD Critical Mediators of Inflammation</i>	411
5.1	Interleukin-1	413
5.2	Tumor Necrosis Factor- α	415
5.3	Interleukin-6	415
5.4	Interferon- γ	416
5.5	Interleukin-4	417
6	<i>Granulocyte Macrophage Colony-Stimulating Factor</i>	417
7	<i>Nitric Oxide and Inducible Nitric Oxide Synthase</i>	417
8	<i>Cyclooxygenase-2</i>	418
9	<i>Quinolinic Acid (QUIN)</i>	419
10	<i>Platelet-Activating Factor</i>	419
11	<i>Prostaglandin E₂</i>	419
12	<i>Summary and Future Directions</i>	419

Abstract: Human immunodeficiency virus type-1 (HIV-1) causes neuropsychiatric impairment in ~60% of HIV-infected patients (Fischer-Smith and Rappaport, 2005, *Expert Rev Mol Med* 7(27): 1–26). HIV-1 is the most common viral cause of dementia and HIV-associated dementia (HAD) is the major form of dementia in the USA in the population <60 years old (Janssen, 1992; McArthur JC, Sacktor N, Selnes O, 1999, *Semin Neurol* 19(2): 129–150). HIV-1 enters the central nervous system (CNS) during the early stage of infection (Kramer-Hammerle S, Hahn A, Brack-Werner R, Werner T, 2005, *Gene* 358: 31–38) and then typically remains latent there for decades. Many factors can contribute to the neuropathology HAD, particularly the inflammatory action of glial cells (Corasaniti MT, Bagetta G, Rotiroli D, Nistico G, 1998, *Biochem Pharmacol* 56(2): 153–156; Kaul R, Dong T, Plummer FA, Kimani J, Rostron T, et al., 2001, *J Clin Invest* 107(10): 1303–1310; Kaul R, Garden GA, Lipton SA, 2001, *Nature* 410(6831): 988–994). If opportunistic infections have been ruled out as the etiology for neuropsychiatric impairment in HIV-positive patients, major clinical symptoms include deficits in short-term memory and concentration, lower extremity weakness, as well as slowness of hand movement, and depression (Janssen RS, Nwanyanwu OC, Selik RM, Stehr-Green JK, 1992, *Neurology* 42(8): 1472–1476; Reger M, Welsh R, Razani J, Martin DJ, Boone KB, 2002, *J Int Neuropsychol Soc* 8(3): 410–424). The terms AIDS dementia complex (ADC), and HAD, are synonymous in describing these symptoms caused by HIV in CNS infection.

List of Abbreviations: AZT, azidothymidine; BBB, blood–brain barrier; COX-2, cyclooxygenase-2; CSF, cerebral spinal fluid; EGCG, epigallocatechin-3-gallate; 5-LOX, 5-lipoxygenase; GM-CSF, granulocyte macrophage colony-stimulating factor; HAART, highly active antiretroviral therapy; HAD, HIV-associated dementia; HIV-1, Human immunodeficiency virus type-1; α -MSH, α -melanocyte-stimulating hormone; IFN- γ , Interferon- γ ; iNOS, inducible nitric oxide synthase; JAKs, Janus-associated kinases; MAPK, mitogen-activated protein kinase; MGCs, multinucleated giant cells; MIP, macrophage inflammatory proteins; MRI, magnetic resonance imaging; OND, other neurological diseases; PAF, platelet-activating factor; PBMCs, peripheral blood mononuclear cells; PCR, polymerase chain reaction; PGE₂, Prostaglandin E₂; PHS, prostaglandin H synthase; PKC, protein kinase C; RT-PCR, reverse transcriptase-PCR; SCID, severe combined immunodeficient; STAT, signal transducer and activator of transcription; TGF, Tumor growth factor; TNF-Rs, tumor necrosis factor receptor; TNF- α , tumor necrosis factor- α

1 HIV Life Cycle

Because of the variability of human immunodeficiency virus type-1 (HIV-1) phenotypes, strains are defined by their coreceptor usage of either CCR5 or CXCR4, and termed R5- or X4-viruses, respectively (Wu and Marsh, 2001). Among the human chemokine receptors, CXCR4 appears most important for HIV-1 entry into lymphocytes, whereas CCR5 is more crucial for monocytes, macrophages, and microglia (Moore et al., 2004). After cell entry, reverse transcription of the HIV RNA genome occurs, generating a double-stranded DNA, a preintegration complex of viral DNA with integrase, and other viral protein such as Vpr. This preintegration complex facilitates integration of the HIV DNA into the host cell chromatin, generating the provirus and eventually mature HIV-1. High levels of viral DNA remain nonintegrated within the nucleus, directing expression of further viral transcripts as well (Wu and Marsh, 2001, 2003; Kilzer et al., 2003). Production of infectious virus particles is facilitated by the generation of viral transcripts, proteins such as Nef, Tat, and Rev, viral assembly, release, and maturation. RNA genomes are produced in a later phase. During the assembly phase, Gag and Gag-Pol polyproteins, envelope proteins, and viral RNA are assembled into immature virus particles at the cell membrane where they are released. Finally, mature virus results after the cleavage of Gag and Gag-Pol polyproteins by HIV-1 protease (Bukrinskaya, 2004; Seelamgari et al., 2004; Nielsen et al., 2005).

2 HIV Infection of CNS Inflammatory Cells

HIV-1, through various techniques, has been shown to be present in cells of the spinal cord, brain, and peripheral nerves in vitro and in vivo (Merrill et al., 1991; Dunfee et al., 2006). Neurons are not productively infected by HIV (Corasaniti et al., 1998). The virus does however productively infect T cells, macrophages,

microglial cells, and astrocytes (Corasaniti et al., 1998; Kaul et al., 2001a, b). Tissue macrophages and brain microglia, which express lower levels of CD4 and CCR5 than CD4⁺ T cells in periphery, serve as the initial major targets for HIV-1 infection (Levy et al., 1998; Dunfee et al., 2006). Indeed, HIV-associated dementia (HAD) is believed to be sustained by viral replication in these cell types (Persidsky et al., 1997; Merrill et al., 1991; 33–35).

HIV-1 invades the central nervous system (CNS) by infecting T-cells and monocytes (Gonzalez-Scarano and Martin-Garcia, 2005), which express the HIV-1 receptors, CD4 and CD8, and several chemokine receptors that function as coreceptors; most prominently CXCR4. These receptors aid in docking of the virus to the cell and membrane fusion, resulting in viral entry into the cell (Zaitseva et al., 2003; Moore et al., 2004). The well-characterized dichotomy between the distribution and number of HIV-infected cells and brain parenchymal damage point to an indirect mechanism for CNS damage (Michaels et al., 1988; Price et al., 1988; Geleziunas et al., 1992; Merrill and Chen, 1992; Epstein, 1993). Thus it is believed that neuron cell damage occurs due to the action of soluble factors including viral proteins, cytokines, and chemokines released by HIV-1-infected macrophages, astrocytes, and microglial cells. These soluble factors activate uninfected macrophages, microglia, and astrocytes and perpetuate CNS damage (Kaul et al., 2001a, b; Okamoto et al., 2001).

3 HIV-1 Replication by Cell Type in CNS

HIV-1-infected cells are classified as “productive” or “restricted” producers of viruses and both types occur in the CNS. Perivascular macrophages and microglia are the resident brain immunocompetent cells and considered “productive” (Kaul et al., 2001a, b; Anderson et al., 2002). In vitro works have suggested that long-lived human brain-derived mixed microglial cultures infected with R5 HIV-1 maintain replication competent HIV-1 for up to 2.5 months (Jordan et al., 1991; Vallat et al., 1998; Albright et al., 1999). Viral production in glial cells as a whole can be modulated by the level of inflammatory signaling within these cells (Trkola et al., 2004) or the addition of activating compounds such as cytokines (Albright et al., 2004). For example, an inflammatory cell environment characterized by high levels of tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) can reactivate viral production (Gorry et al., 1998; Kramer-Hammerle et al., 2005) in both restricted and productively infected cells (Gorry et al., 1998; Sabri et al., 1999; Wang et al., 2004).

3.1 Microglial HIV-1 Replication

The pathoneumonic signature of HIV infection in the brain is productive viral replication in brain macrophages, microglia, and multinucleated giant cells (MGCs; Gabuzda et al., 1986; Koenig et al., 1986; Stoler et al., 1986; Wiley et al., 1986). These immune cells secrete viral- and cell-expressed neurotoxins that cause neuronal injury and death, further leading to inflammatory cell proliferation and myelin pallor during the later stages of HAD (Giulian et al., 1990, 1993; Pulliam et al., 1991; Genis et al., 1992). Microglia-like macrophages express the CD4/CCR5 receptors and coreceptors used by HIV-1 (Jordan et al., 1991; Vallat et al., 1998; Albright et al., 1999) and are productive producers of virus (Kaul et al., 2001a, b; Anderson et al., 2002). These “productive” immune cells play a critical role in the transmission of the infection and rapid evolution of viral genome and eventually die. However, peripheral macrophage populations are replenished quickly, which allows for migration of more infected monocytes into the CNS where they can then take up residency and differentiate into macrophages. In vitro studies demonstrated that HIV-1 replication takes place in primary microglia grown from adult (Watkins et al., 1990; Albright et al., 2000), infant (Ioannidis et al., 1995), and fetal brain (Sundar et al., 1995; McCarthy et al., 1998). Many reports have demonstrated HIV-1 in macrophages and microglial cells by double-labeling immunohistochemical and/or *in situ* hybridization techniques in brain and spinal cord (Koenig et al., 1986; Koyanagi et al., 1987; Gendelman et al., 1989; Kure et al., 1990). Investigations of the progression of HIV-1 infection in purified primary human microglial cultures have demonstrated that productive infection was more efficient by R5-tropic strains of HIV-1 compared with X4-tropic strains (Ioannidis et al., 1995). Several reports have shown that this macrophage/microglia tropism relates to specific sequences of the

HTLV envelope glycoprotein (*env*) gene, implicating viral entry processes specific for macrophages (O'Brien et al., 1990; Shioda et al., 1991). It has been suggested that the genetic heterogeneity of HIV-1 in patients results in strains of HIV-1 that are selected for replication in macrophages. Then macrophages spread HIV-1 to the tissues, including the CNS where HIV-1 can have its neurocognitive effects.

3.2 Astrocyte HIV-1 Replication

Astrocytes are restricted producers, resisting efficient viral production (Kaul et al., 2001a, b; Anderson et al., 2002). A large proportion of astrocytes are actively or latently infected with HIV-1, likely resulting in neuronal dysfunction *via* loss of supporting growth factors, excitotoxicity due to dysregulation of neurotransmitter reuptake, and decreased integrity of the blood–brain barrier (BBB) permitting further penetration of HIV-1 into the brain. Infection of astrocytes is characterized by near-exclusive synthesis of early regulatory gene products of HIV-1 (Blumberg et al., 1996). These glial cells lack CD4 receptor, although they do express CXCR4 and possibly other HIV-1 coreceptors including CCR5 (Gorry et al., 2003). Immunopositivity of astrocytes for HIV-1 structural proteins has also been reported (Brack-Werner, 1999). *In situ* hybridization or *in situ* polymerase chain reaction (PCR) also showed the presence of HIV-1-specific nucleic acids in astrocytes (Nuovo et al., 1994; Bagasra et al., 1996; Takahashi et al., 1996). Other studies reported the presence of HIV-1 DNA and Nef protein in astrocytes as well (Trillo-Pazos et al., 2003). These cells typically continue to survive as a viral reservoir (Gorry et al., 2003). Restricted infection is thought to result from inhibition of HIV-1 replication at various stages from entry to maturation of progeny virions. Of particular importance for HAD, studies in astrocytes indicated a cell-specific block in nucleocytoplasmic Rev shuttling which in turn causes nuclear retention and subsequent degradation of Rev-dependent HIV-1 mRNA (Brack-Werner, 1999; Ludwig et al., 1999; Neumann et al., 2001; Gorry et al., 2003). Although only 1% of astrocytes may be potentially infected, the total number of infected astrocytes contributing to neuropathology has been suggested to be substantial in multiple studies (Takahashi et al., 1996; Rutka et al., 1987).

3.3 Neuronal HIV-1 Replication

Although HIV does not productively infect neurons (Nuovo et al., 1994; Bagasra et al., 1996), neural damage/death and brain atrophy are readily observed in the HAD brain and have been correlated with severity of cognitive symptoms (Everall et al., 1991, 1993a–c; Masliah et al., 1992; Petit and Roberts, 1995). Productive infection of neurons themselves has yet to be observed in the brains of HAD patients. Investigations have largely demonstrated an absence of any *in vivo* infection of neurons, although some works have reported the presence of HIV-1 DNA and proteins in neuronal cells (Nuovo et al., 1994; Bagasra et al., 1996). *In vitro* studies indicated restricted infection of primary neurons (Ensoli et al., 1995) and neuronal cell lines by X5 and R4 strain HIV-1 (Mizrahi et al., 1994; Obregon et al., 1999). Neural loss in the neocortex has been estimated between 18% and 38% (Ketzler et al., 1990; Everall et al., 1991, 1993a–c). It has been suggested that mild dendritic injury may lead to behavioral alterations in HIV-associated minor cognitive/motor disorder (MCMO), and that this injury precedes the loss of neural loss typically associated with HAD (Thompson et al., 2005). Clinical correlations using magnetic resonance imaging (MRI) confirm that HIV infection is associated with progressive cortical atrophy of the gray and white matter in the brain, particularly in the later stage of the disease (Dal Pan et al., 1992; Aylward et al., 1993; Hall et al., 1996; Stout et al., 1998). Several works demonstrated a correlation between the deterioration of neurocognitive function and reduction in volume of certain brain structures including the basal ganglia and caudate nucleus. Volumetric MRI scans have indicated that cortical atrophy associated with HIV infection may be secondary to neuronal loss and demyelination. Both cross-sectional and longitudinal cohorts have indicated the degree of atrophy to be correlated with the degree of cognitive and motor dysfunction (Dal Pan et al., 1992; Aylward et al., 1993; Hall et al., 1996; Stout et al., 1998). Further, neuropsychiatric impairment has been related specifically to white matter damage in HAD (Grassi et al., 2002). Specifically,

a variable degree of white matter neuropathology with myelin damage ranging from pallor to widespread breakdown and loss leading to accumulation of lipid macrophages and axonal damage has been correlated with HIV-1 in the cerebrospinal fluid (CSF) in multiple works (Santosh et al., 1995; Lawrence and Major, 2002; Gonzalez-Scarano et al., 2005; Banks et al., 2006). Neuron cell damage and death may occur ultimately as a direct result of the release of cytokines and other neurotoxins from infected microglial cells (Everall et al., 1991).

4 HIV gp120 Protein

It is important to understand the role of HIV-1 coat protein gp120 because of its interaction and synergism (Giunta et al., 2004, 2006) with certain inflammatory cytokines (Corasaniti et al., 1998). This protein has been proposed as a likely etiologic agent of the neuronal loss in HAD and neuropsychiatric symptoms (Pugh et al., 2000) because it causes the death of neurons in culture (Giunta et al., 2006). It has also been shown that brain cortical cell death caused in rats and mice by intracerebroventricular (i.c.v.) injection of gp120 occurs *via* apoptosis (Corasaniti et al., 1998; Giunta et al., 2006). The glycoprotein has been revealed in the CSF of AIDS patients (Buzy et al., 1992) and its presence has been demonstrated in the brain of patients with HAD (Jones et al., 2000). The neurotoxic potential of gp120 has been shown in several *in vitro* studies, in which different types of neurons maintained in culture, such as rodent cortical (Dawson et al., 1993), and hippocampal (Dreyer et al., 1990) neurons, mouse cortical neurons (Giunta et al., 2006), and retinal ganglion cells (Dreyer et al., 1990; Lipton et al., 1991) die following exposure to the viral protein. Neuronal cell death triggered by gp120 *in vitro* seems to be related to the gp120-induced secretion of neurotoxic factors (such as cytokines, eicosanoids, excitotoxins, and free radicals) by macrophages/microglial cells present in the mixed cell culture preparations (Kaul et al., 2001a, b; Giunta et al., 2004) as the blockade of inflammatory cytokine signaling, such as interferon- γ (IFN- γ), in neurons confers a neuroprotective effect (Giunta et al., 2006). Several *in vivo* studies support the neurotoxic potential of the coat viral protein. Expression of gp120 in astrocytes of transgenic mice causes neuropathological changes similar to those reported in the HAD brain (Toggas et al., 1994). Binding of gp120 to chemokine receptors, CXCR4 or CCR5, and CD4 molecule allows HIV-1 entry into cells expressing the appropriate receptors including T cells and macrophages, and microglia (Clapham and Weiss, 1997). In brain, chemokine receptors have been identified in macrophages/microglia, astrocytes, and neurons (Lavi et al., 1998). In the latter cell type, activation of CXCR4 and CCR5 receptors affects neuronal Ca^{2+} signaling (Meucci et al., 1998). Interestingly, *in vitro* evidence suggests that interaction of these receptors with the HIV-1 coat protein gp120 is responsible for neuronal cell death (Hesselgesser et al., 1998) and this can be reduced by natural ligands of CXCR4 and CCR5 receptors, such as the α -chemokine SDF-1 α , and β -chemokines MIP-1 α , MIP-1 β , and regulated upon activation, normal T-cell expressed and secreted (RANTES) in rat hippocampal neurons (Meucci et al., 1998) and in human neuroblastoma cells (Catani et al., 2000). Subchronic i.c.v. injection of recombinant gp120 in adult rats causes biochemical (Bagetta et al., 1995, 1996) and ultrastructural (Bagetta et al., 1996) alterations typical of apoptosis in the brain neocortex, where cells undergoing apoptosis are neurons but not astrocytes (Corasaniti et al., 2001c). The most salient soluble factors, chemokines and cytokines, involved in the neuropathogenesis of HAD will be discussed in the following section as they are the central mediators of inflammation in this disease.

5 Overview of Cytokines and Chemokines in HAD Critical Mediators of Inflammation

Inflammatory cytokines are considered to play an important role in the pathogenesis of HAD (Pulliam et al., 1994; Nath et al., 1999; Okamoto et al., 2001). These substances are produced predominantly by HIV-1-infected or -activated macrophages and microglia in the brain and induce neural cell death (Okamoto et al., 2001). Indeed, the pathological correlates of HAD are glial cell activation and cytokine dysregulation. These findings

occur in the presence of a small number of productively infected cells within the brain (Nath et al., 1996). Thus with the lack of productive infection of neurons, neuron cell damage has been widely suggested to critically require: (1) HIV-bearing monocyte/macrophage infiltration into the CNS (Koenig et al., 1986; Nottet, 1999), (2) inflammatory activation of resident glia (Hall et al., 1996; Kaul et al., 2000; Ghafouri et al., 2006), and (3) production of HIV proteins including gp120 and Tat (Nath et al., 1996; Li et al., 2005). Cytokines are generally thought to function as major mediators of inflammation in HIV-1 infection based on a large amount of data obtained *in vitro* in animal models as well as in human post-mortem brain tissue. The cells of the immune system and glial cells of the CNS use many of the same cytokines as communication signals (Beneviste et al., 1994). Brain macrophages and microglia express neurotoxins by secretion of viral proteins (e.g., gp120) (Merrill et al., 1989; Lipton et al., 1990; Dawson et al., 1991; Lipton, 1996), while the virus itself immunologically activates macrophages to produce high levels of neurotoxic cytokines, quinolate (QUIN), and nitric oxide (NO). Chronic immune stimulation mediated by IFN- γ production both outside and within the CNS continues the process of macrophage activation leading to further neural injury. The activation of infected macrophages results in production of cellular factors, which then cause hyperresponsive activation in uninfected macrophages, a self-perpetuating cycle. Thus, HIV-infected macrophages are initiators as well as augmenters for neuron cell damage (Gendelman et al., 1994). It is because of this, the study of microglial and macrophage inflammatory factors are essential for elucidating new therapies for this neurodegenerative disorder (Corasaniti et al., 1998). There is a complex circuitry of interactions mediated by cytokines, during the BBB damage characteristic of HAD, which involves lymphoid/mononuclear cell infiltration into the brain (Beneviste, 1994). Activated macrophages, which successfully penetrate the BBB, produce cytokines such as IL-1, TNF- α , and IL-6, which cause other glial cells to express their own cytokines (Beneviste, 1994). Activated astrocytes and microglia that secrete proinflammatory cytokines and granulocyte macrophage colony-stimulating factor (GM-CSF), may contribute to the propagation of brain inflammatory responses initiated by immune cells, as well as augmentation of HIV-1 expression in the CNS.

Whether immune and inflammatory responses within the CNS are propagated or suppressed depends on several variables, including: (1) activation status of microglia and astrocytes, (2) cytokine receptor expression levels on these cells, (3) the presence of cytokines with dual immune-enhancing and immune-suppressing effects including IFN- γ , IL-1, TNF- α , IL-6, tumor growth factor (TGF- β), (4) the concentration and neuroanatomical location of these cytokines in the CNS, and (5) the timing in which each cell type is exposed to various cytokines. The interaction of these factors determines the ultimate outcome of immunologic and inflammatory events in the CNS, as well as HIV replication (Beneviste, 1994).

Chemokines and their receptors are critically involved in the inflammation characteristic of HAD as well. Upregulation of specific chemokines in individual brain regions contributes to the neuropsychiatric deficits of the disease. Importantly, chemokines and their receptors represent the portal of entrance of HIV into the CNS (Dou et al., 2004; Li et al., 2005). All CXCR chemokine receptor variants are expressed in the brains of HAD patients (Brandimarti et al., 2004). Immunohistochemical analysis of HIV-infected brains revealed expression of four HIV-1 coreceptors, CCR2, CCR3, CCR5, and CXCR4. Importantly, hippocampal neurons were CCR2-, CCR3-, and CXCR4-positive (Vander Meer et al., 2000). Furthermore within other brain regions, neurons and glial cells were positive for CCR2, CCR3, and CXCR4, whereas only primary microglia were CCR5-positive. The highest areas of expression were observed in subcortical regions including the limbic system and are in accord with the symptoms of HAD in light of this subcortical region's critical role in memory, effect, and emotion. The presence of chemokines and their receptors is especially elevated in areas of neuron-glial cell interaction. This phenomenon is likely involved in the recruitment of inflammatory cells from the periphery and formation of microglial nodules and MGCs. This is supported by detection of high levels of expression of CCR1, CCR3, CCR5, and CXCR4 in the microglial nodules (Sanders et al., 1998; Vallat et al., 1998). In addition, CCR3 and CXCR4 are expressed at high levels in the hippocampal pyramidal neurons as well as in the entorhinal cortex for CCR3. Moreover, in comparison with non-neurocognitively impaired AIDS patients, the brain tissue of patients with HAD demonstrated overexpression of the CX₃C chemokine, fractalkine/CX3CL1 (Tong et al., 2000; Pereira et al., 2001). The overexpression of fractalkine/CX3CL1 was also discovered in brain neurons of pediatric HAD patients (Tong et al., 2000), while the same phenomenon was found to be true in astrocytes in adult patients (Pereira et al., 2001).

CSF chemokine levels in HIV-infected patients with and without HAD have been investigated in various works. There is a consensus that CSF chemokine concentration of MCP-1/CCL2, MIP-1 α /CCL3, MIP-1 β /CCL4, RANTES/CCL5, IL-8/CXCL8, and fractalkine/CX3CL1 is positively correlated with the severity of neurocognitive deficits and viral load, pointing to HIV-induced brain damage. The importance of neuronal-, microglial-, and astrocyte-expressed CCR5 in the brain is under debate in the neuropathogenesis of HAD. In vitro studies suggested that activation of CCR5 by RANTES or MIP-1 α / β confers neuroprotection against HIV-1 gp120-induced apoptosis (Meucci et al., 1998; Kaul and Lipton, 1999). Concurrently, other in vitro investigations have suggested that neurovirulent strains of HIV are largely M-tropic with increased CCR5affinity (Power et al., 1998). Additionally, it has been demonstrated that CCR5 activation via its specific ligand promotes apoptosis in neuroblastoma cell lines (Cartier et al., 2005). Thus, CCR5 seems to operate as a neuronal death receptor in HIV-1-induced brain damage.

5.1 Interleukin-1

Infiltrating activated macrophages, microglia, and astrocytes produce IL-1 (Beneviste et al., 1994). Elevated IL-1 β levels have been reported in the CNS of individuals affected by HAD (Perrella et al., 1992; Tyor et al., 1992) as well as other diseases. This cytokine likely stimulates reactive astrocytosis (Vitkovic et al., 1995). Indeed, CSF IL-1 α has been shown to be more frequently detectable in HAD patients than in other neurological disorders (Perrella et al., 1992). High concentrations of IL-1 have been demonstrated in azidothymidine (AZT)-treated, HIV-1-infected children with progressive encephalopathy. Fifty percent of these patients showed elevated IL-1 β , whereas low levels were also detected in some asymptomatic children (13%; Laverda et al., 1994). In an investigation of the distribution of cytokine receptors in the human brain using fluorescent-labeled cytokines combined with confocal laser microscopy imaging, (Masliah et al. 1994) demonstrated phycoerythrin-conjugated IL-1 β -labeled dendritic processes of neurons in the neocortex. Further in cases with moderate HAD, an average 35% increase in intensity of labeling was observed compared with cases without HAD or with severe HAD (Masliah et al., 1994). Its ability to induce damage in the CNS is modulated by the type of cell producing IL-1 as well as the chronicity of exposure. The chronic effects of IL-1 in the CNS have been consistently correlated with activation of glial cells. Whether glial cells, neurons, or both are involved in the CNS responses to HIV is uncertain (Tringali et al., 1998). Using a human neuronal cell line, IL-1 β caused no significant cell death (Westmoreland et al., 1996).

However, many other reports indicate this cytokine plays a major role in the neuropathogenesis of HAD (Zhao et al., 2001) *via* indirect mechanisms. For example, in a study of cerebral white matter obtained at autopsy from AIDS patients, double-label immunohistochemistry revealed increased expression of IL-1 β , which is known to upregulate the TNF receptors (TNF-Rs). This resultant upregulation of TNF-Rs in AIDS further points to the action of macrophages and microglia in amplifying the TNF- α response (Sippy et al., 1995). Evidence for the role of IL-1 and transforming growth factor- β 1 (TGF- β 1) indicates that these cytokines likely control HIV-1 brain expression and astrocytosis, two pathopneumonic signatures of HAD (Vitkovic, 1997). For example, in vitro investigation demonstrated that astrocyte SDF-1 production was directly dependent on IL-1 β , from macrophage-conditioned media following both HIV and immune activation (Peng et al., 2006). Further, production of SDF-1 was prevented by IL-1 β receptor antagonist (IL-1ra) and IL-1 β siRNA treatment of human macrophage-conditioned media. These observations were further confirmed in severe combined immunodeficient (SCID) mouse model of HAD where reactive astrocytes showed a significant increase in SDF-1 expression, as observed by immunocytochemical staining. These observations provide direct evidence that IL-1 β , produced from HIV-1-infected and/or immune competent macrophage, positively modulate SDF-1 astrocyte expression, and contribute to chronic SDF-1-mediated CNS regulation during HAD. Moreover IL-1 β increases the permeability of the BBB, allowing for further HIV-infected monocytes to enter the brain parenchyma (Brabers and Nottet, 2006).

Neuronal damage in the neocortex by gp120 is preceded by microglial activation enhancement by IL-1 β (Bagetta et al., 1999). IL-1 also induces overstimulation of the NMDA receptor *via* several pathways, resulting in neuronal apoptosis secondary to increased intracellular Ca²⁺ (Brabers and Nottet, 2006).

HIV gp120 activates brain microglial cells and astrocytes, and *in vivo* activation of microglia and astrocytes leads to the release of IL-1 β (Pugh et al., 2000). The importance of gp120-mediated IL-1 β release has been suggested to be related to the memory impairments associated with HAD as central IL-1 β negatively affects cognitive function (Pugh et al., 2000). Intracerebral injection of gp120 in adult rats causes enhanced expression of IL-1 β (Corasaniti et al., 2001c), similar to what is seen in HAD (Tyor et al., 1992; Petito and Roberts, 1995). Other investigations, by immunohistochemical analysis of brain tissue from gp120-treated rats, revealed that upregulation of IL-1 β expression occurred at 24 h from *i.c.v.* injection of a single dose of gp120 with more augmented expression after 1 week of treatment (Bagetta et al., 1999). Pharmacological modulations have demonstrated that overexpression of IL-1 β is critical for the expression of neuronal cell death secondary to gp120 in the neocortex of rat as well. That is, neuronal cell loss was minimized by Ac-YVAD-CMK (Bagetta et al., 1999), a specific inhibitor of caspase 1, the enzyme which cleaves pro-IL-1 β yielding the mature, 17-kDa active form of the cytokine (Thornberry et al., 1992). Moreover, a neuroprotective effect was conferred by inhibition of IL-1 β by the endogenous IL-1ra, an antagonist of the IL-1 receptor (Bagetta et al., 1999). Importantly, this neuroprotection by IL-1ra further strengthens the role of IL-1 β (Bagetta et al., 1999) in gp120-mediated neuronal death.

The mechanisms leading to gp120-mediated upregulation of IL-1 β are not fully understood. It has been proposed that gp120-enhanced neocortical expression of IL-1 β originates from *in situ* cleavage of mitochondrial pro-IL-1 β as supported by investigations using immunoelectron microscopy analysis and measurement by ELISA of cytokine levels in subcellular fractions of rat brain neocortical homogenates after treatment with gp120. In this scenario it was found, after 1 week, that IL-1 β immunoreactivity was localized in the mitochondria and cytosol (Corasaniti et al., 2001d). Specifically, elevation of mitochondrial IL-1 β levels was paralleled by a decrease of pro-IL-1 β in conjunction with enhanced expression of the p20 component of activated caspase-1 (Corasaniti et al., 2001d). Work by this same group demonstrated that SDF-1 α , the ligand for CXCR4 chemokine receptors, was efficacious in preventing early enhancement of IL-1 β expression as well as death of neocortical neurons caused by gp120 in rats suggesting that CXCR4 chemokine receptors act as the transmembrane molecules recruited by gp120 to induce IL-1 β expression (Corasaniti et al., 2001c). Investigations with CHP100 neuroblastoma cultures have further confirmed that gp120 enhances IL-1 β levels and secretion (Corasaniti et al., 2001a). It has also been demonstrated that *i.c.v.* administration of gp120 induced the expression of IL-1 β mRNA in the hypothalamus as early as 3 h postinjection. IL-1ra pretreatment of rats significantly attenuated gp120-induced anorexia and loss in body weight; however, it had no effect on gp120-induced reduction in locomotor activity. Interestingly, pretreatment with either IL-1ra and pentoxifylline simultaneously or with IL-10 produced effects that were similar to that of IL-1ra alone (Barak et al., 2002). Zhao et al. (2001) analyzed expression of IL-1 with respect to HIV-1 infection and glial activation. In HAD, IL-1 was expressed at abnormally high levels in areas of HIV-1-infected microglial nodules with HIV-1 p24 immunoreactivity as well as in regions of diffuse white matter gliosis. Cell-type-specific expression of IL-1 and caspase-1 was observed in macrophages and microglia. Double immunocytochemistry showed a regional colocalization of microglial IL-1 and caspase-1, further supporting the paradigm that autocrine and paracrine interactions between HIV-1-infected macrophages and microglia lead to expression of proinflammatory and neurotoxic molecules (Zhao et al., 2001). Behavioral testing revealed that *i.c.v.* injection of gp120 produced memory impairments on hippocampally dependent contextual fear conditioning, but not hippocampally independent auditory-cue fear conditioning following postconditioning gp120 administration. Central administration of gp120 also leads to elevated IL-1 β expression in the hippocampus and frontal cortex but not in the hypothalamus (Pugh et al., 2000). These memory impairments were blocked by α -melanocyte-stimulating hormone (α -MSH), IL-1ra, as well as heat denaturation of suggesting it impairs contextual fear conditioning by binding to its receptors on glial cells (Pugh et al., 2000). There is also evidence that HIV-1 Tat protein induces IL-1 β expression in monocytic cells, whereas astrocytic cells showed an increase in mRNA for IL-1 β with a lack of translation of IL-1 β protein (Nath et al., 1999). An exposure to Tat for a few minutes was sufficient for a several hours of release of IL-1 β . This prolonged IL-1 β production was thought to be maintained by a positive feedback loop of Tat-induced nuclear factor- κ B activation and independent of extracellular calcium, further suggesting a transient exposure of HIV protein; in this case, Tat may be sufficient to initiate a cascade resulting in cerebral dysfunction (Nath et al., 1999). Moreover, like gp120,

HIV-1 Tat's neurotoxicity is augmented by IFN- γ (Giunta et al., 2006). IL-1 is known to stimulate production of IL-6, GM-CSF, and TNF- α in cultured astrocytes and astrocytoma cell lines (Vitkovic et al., 1995); thus these factors will be reviewed in the following sections.

5.2 Tumor Necrosis Factor- α

Concentrations of TNF- α are increased in the CNS and correlate with the severity of HAD (Munno et al., 1992; Perrella et al., 1992; Achim et al., 1993; Tyor et al., 1995; Vitkovic et al., 1995). TNF- α has been consistently demonstrated to be elevated in the brains of AIDS patients and functions as a central mediator of cerebral damage (Sippy et al., 1995; An et al., 1996). Thus, CSF levels of this cytokine provide a good marker of infection of the CNS in the HIV-1 infection (Calvo Manuel et al., 1995). In an investigation to determine the cellular localization and degree of protein and mRNA expression of the specific TNF receptors (TNF-Rs) in brain tissues from AIDS patients, cerebral white matter obtained at autopsy revealed a dramatic upregulation of TNF-Rs on activated macrophages and microglia in all AIDS patients while no upregulation was detected on astrocytes. Staining was particularly observed in individuals with opportunistic brain infection as well those with HAD. TNF- α , known to upregulate the TNF-Rs, was also increased. Importantly, asymptomatic HIV-positive patient without AIDS showed no increase in TNF-Rs, suggesting macrophages and microglial cells play a central role in the augmentation of the TNF- α response (Sippy et al., 1995). Interestingly, unlike IL-1 β , TNF- α elevations have been shown not to be elevated in children treated with AZT (Laverda et al., 1994). Wesselingh et al. (1994) evaluated TNF- α in the brain, spinal cord, and peripheral nerves of AIDS patients with HAD, vacuolar myelopathy, and peripheral sensory neuropathy compared with AIDS patients without neurological disease and seronegative controls. Reverse transcriptase-PCR (RT-PCR) revealed a consistent profile of increased TNF- α in all the three syndromes compared with AIDS patients without neurological disease. In another clinical study of TNF- α in autopsied brain specimens of HAD patients, it was found that mild cases showed elevated levels of TNF- α in deep gray matter, whereas in more severe cases elevated TNF- α levels were also found within cortical white and cortical gray matter.

Although HIV infection itself has been found to correlate with serum TNF- α levels (Munno et al., 1992), neither of the previous study found any serum levels of TNF- α that would correlate with HAD severity (Perrella et al., 1992; Achim et al., 1993). This phenomenon was further confirmed by Pulliam et al. (1996), who isolated peripheral blood mononuclear cells (PBMCs) from 16 HAD patients and 13 healthy HIV-seropositive individuals, revealing peripheral production of TNF- α was not a significant predictor of HAD (Pulliam et al., 1996). Further confirmation comes from a study of peripherally infected macrophages from 11 patients with AIDS dementia. Here the percentage of HIV macrophage infectivity did not correlate with the severity of dementia as well (Pulliam et al., 1993). These results are also consistent with an animal investigation. Here, central administration of HIV gp160 to male rats dramatically increased mRNA expression TNF- α in the hypothalamus, hippocampus, brainstem, and cerebellum, peaking 2 h after administration. In line with human studies, multiple cytokine mRNAs were detected in peripheral tissues, but their expression was not altered by central administration of gp160 (Gemma et al., 2000).

5.3 Interleukin-6

Infiltrating activated macrophages, microglia, and astrocytes produce IL-6 (Beneviste et al., 1994). In vitro studies have shown that exposure to HIV-1 Tat protein dose dependently results in an increase in IL-6 protein and mRNA productions in astrocytic cells. Exposure to Tat for a several minutes was enough to provoke a sustained release of this cytokine for several hours, which was hypothesized to result from a positive feedback loop of Tat-induced nuclear factor- κ B activation and IL-6, independent of extracellular calcium (Nath et al., 1999). In vitro examination by Fiebich et al. (2001) revealed that prostaglandin E₂ (PGE₂)-stimulated IL-6 release in U373 MG human astrogloma cells and primary rat astrocytes was

inhibited by specific inhibitors of p38 mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) intracellular signaling (Fiebich et al., 2001). An et al. (1996) determined, from 36 asymptomatic HIV-1-positive, 8 AIDS patients either affected by HAD or exempt from any neuropathological changes ($n = 10$), that HIV proviral DNA was present in 17 out of the 36 brains from HIV-positive pre-AIDS cases. Most (15 of 17) of PCR-positive brains showed minimal to severe expression of MHC class II antigen and IL-6 could be detected predominantly within white matter even at this early stage, suggesting the presence of cytokines such as IL-6 may trigger the cascade of events leading to brain damage (An et al., 1996).

In accord with this study, it was also found that brain tissue from HAD patients displayed increased expression of IL-6, which like IL-1 and TNF- α , upregulates TNF-Rs. Asymptomatic HIV-infected brains displayed no such upregulation (Sippy et al., 1995). An in vitro study revealed that, like TNF- α , IL-6 production can be triggered in human primary astrocytes and astrocytoma cell lines by IL-1 in serum-free medium. As with IL- β , high concentrations of IL-6 were found in the CSF children with HAD (50% and 37%, respectively), whereas minimal levels were detected in <15% of asymptomatic children (Laverda et al., 1994). In contrast, an earlier clinical investigation found, using RT-PCR, that no significant differences were evident in the amount of IL-6 mRNAs in the brains from 24 HIV-infected patients compared with subjects without dementia and HIV-uninfected control subjects (Wesselingh et al., 1993). In an examination of CSF and serum concentrations of this same cytokine in 30 patients with HAD, and in 20 HIV-seronegative subjects without neurological disease, IL-6 was more frequently detectable in former patients than in the latter. IL-6 was also detectable in CSF of HAD patients with minimal symptoms. However, the majority of both HAD and non-neurologically impaired patients did not contain detectable serum levels of IL-6 (Perrella et al., 1992). Torre and colleagues (1992) found a moderate increase of IL-6 levels in the CSF of HAD patients compared with control subjects.

In vitro studies indicated gp120-activated human brain endothelial cells derived from children in upregulating IL-6 expression (Stins et al., 2001). Pulliam et al. (1996) found levels of PBMCs IL-6 were significantly higher in HAD patients without opportunistic infections compared with those with opportunistic infection. Interestingly, this group also found trend toward worse cognitive and motor performance in patients suffering from HAD, but who had no opportunistic infections as well. Further, supernatant from PBMC of HAD patients was significantly more neurotoxic than those from healthy HIV-seropositive individuals. This same group also demonstrated, in vitro via examination of HIV-infected macrophages from 11 patients with HAD, that IL-6 production did not correlate with HIV macrophage infectivity suggesting that cytokine is strain-specific (Pulliam et al., 1994).

5.4 Interferon- γ

Chronic immune stimulation mediated by IFN- γ production both outside and within the CNS continues the process of macrophage activation leading to further neural injury. The activation of infected macrophages results in production of cellular factors, which then cause hyperresponsive activation in uninfected macrophages, a self-perpetuating cycle. Thus HIV-infected macrophages are initiators as well as augmenters for neuron cell damage (Gendelman et al., 1994). IFN- γ has been implicated in numerous reports publications as having a role in HAD (Brenneman et al., 1988; Dreyer et al., 1990; Merrill et al., 1992; Gelbard et al., 1993; Giunta et al., 2006). In human primary astrocytes, CD23 protein membrane expression was dose-dependently upregulated by IFN- γ (Dugas et al., 2001). IFN- γ is implicated in the neurotoxicity of HAD and is elevated in the HAD brain (Shapshak et al., 2004). Indeed, IFN- γ induces apoptosis in many cells including neurons (Lee et al., 1999; Balabanov et al., 2006; Simon et al., 2006) *via* phosphorylation of intracellular activation of signal transducer and activator of transcription (STAT) proteins. STATs remain latent in the cytoplasm until activated by Janus-associated kinases (JAKs). IFN- γ receptor ligation causes JAKs to tyrosine phosphorylate the intracytoplasmic side of the IFN- γ receptor leading to STAT1 activation (migration to the nucleus). Although STATs are not exclusively activated by JAKs, one of the best studied pathways for STAT activation is through the JAKs, a system termed as the JAK/STAT pathway. STAT1 is known to be phosphorylated by JAK1 in response to IFN- γ (Ramana et al., 2002) in vitro and in vivo in neurons (Takagi et al., 2002; Giunta et al., 2006). Chronic HIV-1 infection among individuals with

progressive disease has been correlated with a six- to tenfold increase in levels of activated STATs in PBMCs after exposure to virions (Kohler et al., 2003). In normal cells, STAT1 activation is transient (minutes to hours; Horvath, 2004). Hyperactive cytokine-induced STAT signaling could potentially lead to neural apoptosis, as it is known to bind apoptosis-inducing mediators. For example, STAT1 modulates caspase transcriptional activation in thymocytes (Simon et al., 2006), cardiomyocytes (Stephanou et al., 2000), and fibroblasts (Kumar et al., 1997). In further confirmation, STAT1-deficient fibroblasts were resistant to TNF- α -induced apoptosis, an important known mediator of neuron death in HAD (Kumar et al., 1997). In addition, in mice STAT1 deficiency conferred neural resistance to ischemic injury and death (Takagi et al., 2002), as well as resistance the neurotoxicity of HIV-1 gp120 and Tat in vitro and in vivo (Giunta et al., 2006).

5.5 Interleukin-4

Unlike the previous cytokines discussed, IL-4 is an inhibitor of macrophage activation and has been reported to protect against HIV-1-related disease progression in white adults (Singh et al., 2004). Because of this, the massive increase in macrophage activation later in HIV infection may be secondary to the diminution in production by Th2, CD4-positive cells which produce IL-4 (Tyor et al., 1995). This is further supported by observations of decreased IL-4 in HAD compared with AIDS patients without neurological disease (Wesselingh et al., 1994).

6 Granulocyte Macrophage Colony-Stimulating Factor

Infiltrating activated macrophages, microglia, and astrocytes produce GM-CSF. (Beneviste et al., 1994). IL-1 stimulates GM-CSF in cultured astrocytes and astrocytoma cell lines (Vitkovic, 1995). Perrella et al. (1992) detected GM-CSF by ELISA in the CSF and serum of 30 HAD patients, and 20 subjects with other neurological diseases (OND). A higher incidence of detectable GM-CSF in the CSF of HAD patients compared with OND patients was observed, although it did not reach statistical significance (Perrella et al., 1992). Stimulation with GM-CSF induced macrophage inflammatory proteins (MIP-1 α and MIP-1 β) and enhances RANTES expression after HIV-1 infection of microglia, while blockade of GM-CSF-induced microglial proliferation had no effect on β -chemokine expression. The functional significance of the GM-CSF-induced β -chemokines was suggested by the finding that this soluble factor induced a loss of exogenous β -chemokines anti-HIV-1 effects (Si et al., 2002). In human primary astrocytes and astrocytoma cell lines U251 and 253 exposed to IL-1, GM-CSF-positive/GFAP-positive cells were detected in three of seven cases containing GM-CSF immunoreactivity. Therefore, discordance may exist between human astrocytic cytokine expression in vitro and in tissue (Vitkovic, 1995).

7 Nitric Oxide and Inducible Nitric Oxide Synthase

As with inflammatory cytokines, the inducible nitric oxide synthase (iNOS) enzyme plays an important role in the pathogenesis of HAD (Zhao et al., 2001). The excitotoxic, glutamate-mediated cell death triggered by gp120 in neuroblastoma cultures proceeds through the recruitment of Ca²⁺-dependent iNOS (Corasaniti et al., 1995) along with membrane lipid peroxidation (Corasaniti et al., 1998). CD23 ligation is an alternative pathway in the induction of astroglial inflammatory product synthesis and participates in CNS inflammation. CD23 antigen was colocalized with iNOS and nitrotyrosine on brain tissue from patients with HAD, suggesting that CD23 participates in iNOS activation of astrocytes in vivo (Dugas et al., 2001). Cell surface stimulation of CD23 expressed by astrocytes induced NO production, implicating this receptor in the astrocyte inflammatory reaction (Dugas et al., 2001). In HAD brain autopsies, iNOS was found to be expressed at high levels in areas of HIV-1 infection characterized by microglial nodules with HIV-1 p24 immunoreactivity as well as in areas of diffuse white matter gliosis. Expression of iNOS was cell-type-specific in activated astrocytes. Further this same group found, by double immunocytochemistry,

a regional colocalization of astrocyte iNOS and microglial IL-1 and caspase-1 suggesting that autocrine and paracrine interactions between HIV-1-infected macrophages and microglia, activated microglia, and astrocytes lead to expression of proinflammatory and neurotoxic molecules (Zhao et al., 2001).

8 Cyclooxygenase-2

Immunohistochemical analysis has demonstrated that subchronic i.c.v. treatment with gp120 augments expression of cyclooxygenase-2 (COX-2) in the neocortex of rat (Bagetta et al., 1998). A role for IL-1 β in mediating gp120-enhanced COX-2 expression is suggested by the hypothesis that IL-1 β may stimulate COX-2 production (Feng et al., 1993). HIV-1-infected monocytes overexpress inflammatory molecules that facilitate BBB penetration. Prostanoids are an example of such molecules and are lipid mediators of inflammation that result from COX-2 activity (Pereira et al., 2000). Using in vitro cocultures of HIV-infected macrophages and brain endothelium, an upregulation of COX-2 expression was observed by both cell types. This upregulation occurs through an IL-1 β -dependent mechanism in macrophages, whereas in endothelial cells it occurs *via* an IL-1 β -independent mechanism. Thus, interactions between HIV-infected monocytes and brain endothelium result in COX-2 expression and, as such, might contribute to the neuropathogenesis of HIV infection by increasing BBB permeability (Pereira et al., 2000). Further, in vivo models have indicated that i.c.v. injection of gp120 causes an early enhancement of both IL-1 β (Bagetta et al., 1999; Corasaniti et al., 2001c) and COX-2 (Corasaniti et al., 2000) expression in the rat neocortex. Moreover in CHP100 neuroblastoma cells, upregulation of COX-2 expression occurs on gp120 exposure (Maccarrone et al., 1998) and are sensitive to IL-1 β -mediated gp120-induced cytotoxicity (Corasaniti et al., 2001a). Caspase-1 inhibitors (Corasaniti et al., 2001a), administered at concentrations known to prevent enhancement of IL-1 β levels and death of CHP100 cells caused by gp120 (Corasaniti et al., 2001a), are able to block augmentation of COX-2 expression otherwise induced by gp120. The observation that inhibition of caspase-1 reduces gp120-mediated death of CHP100 cells through reduction of secreted IL-1 β combined with the ability of caspase-1 inhibitors to block expression of COX-2 enhanced by gp120 indicate IL-1 β as the signal through which the gp120 enhances COX-2 production. In addition, Western blot analysis demonstrated induction of COX-2 by gp120 as early as 6 h following a single dose of the viral coat protein (Corasaniti et al., 2000). The induced COX-2 by gp120 in the rat brain (Bagetta et al., 1998; Corasaniti et al., 2000) is functional as supported by the evidence that gp120 increases PGE₂ production in the rat brain neocortex (Corasaniti et al., 2000; Maccarrone et al., 2000). This may account for the significant increase in body temperature previously reported in rat as well (Bagetta et al., 1999). A central role for augmented COX-2 production in neuron cell death mediated by gp120 is also substantiated by data obtained in vitro. Human CHP100 neuroblastoma cells, intracellular concentrations of PGE₂, and leukotriene B₄ are induced by gp120 increases via upregulation of the arachidonate-metabolizing enzymes, prostaglandin H synthase (PHS) and 5-lipoxygenase (5-LOX) (Maccarrone et al., 1998). Consistent with a role of the arachidonate cascade in gp120-mediated cell demise, inhibitors of COX activity of PHS, such as indomethacin, and inhibitors of 5-LOX, such as caffeic acid, provide protection against neuroblastoma cell death mediated by gp120 (Corasaniti et al., 1995; Maccarrone et al., 1998).

It is generally accepted that under physiologic conditions, the level of expression of COX-2 appears to positively correlate with the state of activation of excitatory, glutamate-mediated, synaptic transmission (Yamagata et al., 1993). In vitro and in vivo data suggest that gp120 enhances glutamate transmission through release of excitotoxins from astrocytes acting at the NMDA, but not non-NMDA, receptors in the brain (Corasaniti et al., 2001b). Taken together, these data support the notion that enhanced expression of COX-2 and accumulation of PGE₂ observed here may be the consequence of abnormal activation of glutamate neurotransmission in the gp120-treated rat neocortex. In contrast, one in vitro study found, through pretreatment with MK801; a selective antagonist of the NMDA receptor complex, which failed to counteract gp120-enhanced COX-2 expression observed 6 h posttreatment (Corasaniti et al., 2000). However, systemic pretreatment with competitive and noncompetitive NMDA receptor antagonists or free-radical scavengers of the 21-aminosteroid family reduced gp120-induced apoptosis in the rat neocortex (Corasaniti et al., 2000), supporting an excitotoxic, glutamate-mediated cell death (Corasaniti et al., 2001b).

It has also been demonstrated that products of the arachidonic acid cascade stimulate the Ca^{2+} -dependent release of glutamate from astrocytes, leading to the hypothesis that this mechanism may have physiological as well as neuropathological consequences in the brain (Bezzi et al., 1998). Thus to rationalize the observed lack of MK801 effect on COX-2 production with the neuroprotection conferred by the NMDA receptor antagonists and by the 21-aminosteroid, U-74389G, it can be inferred that IL-1 β is central to the gp120-evoked fast induction of COX-2 which may elevate synaptic glutamate which would then trigger a self-potentiating cycle, leading to cell death *via* oxidative stress and subsequent apoptotic signaling (Corasaniti et al., 2001b).

9 Quinolinic Acid (QUIN)

Activated macrophages produce (QUIN), a neurotoxin, in HAD (Smith et al., 2001). The level of the kynurenine pathway metabolite, QUIN, is increased in the brain and CSF of HIV-1-infected patients, and is correlated with quantitative measures of neurological impairment (Saito, 1995). It has been demonstrated that, as early as 72 h, there is significant increases in QUIN production in macrophages stimulated by platelet-activating factor (PAF) and HIV-Nef protein with less production by Tat stimulation. In contrast, IL-1 β , IL-6, TGF- β 2, and gp41 stimulation produced no significant increase in QUIN production, suggesting that some of the neurotoxicity of PAF, nef, and Tat may be mediated by QUIN (Smith et al., 2001). Conversion of L-tryptophan to QUIN has also been shown *in vitro* in brain tissue following macrophage infiltration, and in macrophages stimulated by IFN- γ or HIV infection. Macrophages *in vitro* have a high capacity for QUIN production following exposure to IFN- γ , TNF- α , IL-1 β , and IL-6 compared with other cell types (Saito, 1995). Within the brains of 10 AIDS postmortem specimens, homogenization and extraction of cortical gray/white and deep gray matter showed that CSF QUIN concentrations were substantially increased compared with control values. However in this study, QUIN concentrations were not correlated with the severity of HAD (Achim et al., 1993).

10 Platelet-Activating Factor

In the human neuronal cell line, NT2N, PAF was demonstrated to be highly neurotoxic. Interestingly, pentoxifylline, which inhibits the effects of TNF- α , had a significant protective effect (Westmoreland et al., 1996). This is in accord with results from a study using the SCID mouse model of HAD. Here, an antagonist and a matrix metalloproteinase inhibitor, which also inhibits TNF- α release, were administered to the animals in conjunction with a basal ganglia-targeted inoculation of human HIV-infected monocyte medium. The drugs administered in combination dramatically reduced brain inflammation, microglia activation, and astrogliosis (Persidsky et al., 2001).

11 Prostaglandin E₂

Increased levels of PGE₂ have been found in CSF of HAD patients (Griffin et al., 1994). Like TNF- α , a possible role for PGE₂ in HAD is suggested by the observation that increase of PGE₂ levels in the CSF are associated with severity of dementia in AIDS patients (Griffin et al., 1994). *In vitro* examination revealed PGE₂-stimulated IL-6 release in U373 MG human astrogloma cells and primary rat astrocytes *via* an EP4-like receptor independently of cAMP (Fiebich et al., 2001).

12 Summary and Future Directions

Taken together, HAD is a devastating effect of HIV infection which critically requires the interaction of the glial cell inflammatory cascade to confer its neuropsychiatric effects. HAD is poorly controlled by the current standard of care for highly active antiretroviral therapy (HAART). Paradoxically in the setting of

HAART, HIV-1 infection has become chronic and recent works demonstrate a rise in the prevalence of HAD (Wang et al., 2006). This may be due to the fact that HAART does not target the inflammatory cascade which is critical to HAD symptom development (Janssen et al., 1992; McArthur et al., 1999). Thus the study of inflammation in HAD may shed light on new therapeutic options.

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20 **Role of Poly(ADP-ribose) Polymerase in Brain Inflammation and Neuroinjury**

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1	<i>Introduction</i>	428
1.1	Poly(ADP-ribose) Polymerase and Its Activation	428
2	<i>PARP Inhibition in Stroke</i>	433
2.1	General Considerations	433
2.2	Effect of PARP Deficiency and PARP Inhibition in Stroke	435
3	<i>PARP Inhibition and Neurotrauma</i>	441
4	<i>PARP and Neuroinflammation</i>	443
4.1	Role of PARP in Meningitis	443
4.2	Role of PARP in Multiple Sclerosis and Experimental Allergic Encephalomyelitis	444
5	<i>PARP and Neurodegeneration</i>	448
6	<i>Conclusions and Future Directions</i>	450

Abstract: Poly(ADP-ribose) polymerase-1 (PARP-1) is a DNA-binding protein, which is primarily activated by nicks in the DNA molecule. It regulates the activity of various enzymes, including itself, and those involved in the control of DNA metabolism. Upon binding to DNA breaks, activated PARP cleaves NAD^+ into nicotinamide and ADP-ribose and polymerizes the latter on nuclear acceptor proteins including histones, transcription factors, and PARP itself. Poly(ADP-ribosylation) contributes to DNA repair and to the maintenance of genomic stability. Evidence obtained with pharmacological PARP inhibitors of various structural classes, as well as animals lacking the PARP-1 enzyme indicate that PARP plays an important role in cerebral ischemia/reperfusion, stroke, neurotrauma, neuroinjury, and neurodegeneration. Overactivation of PARP consumes NAD^+ and ATP culminating in cell dysfunction and necrosis. PARP activation can also act as a signal that initiates cell death programs, for instance through apoptosis-inducing factor (AIF) translocation. PARP has also been shown to associate with and regulate the function of several transcription factors. Of special interest is the enhancement by PARP of nuclear factor (NF)- κB -mediated transcription, which plays a central role in the expression of inflammatory cytokines, chemokines, adhesion molecules, and inflammatory mediators. Via this mechanism, PARP is involved in the upregulation of numerous proinflammatory genes that play a pathogenetic role in the later stage of central nervous system (CNS) diseases. Here, we review the roles of PARP in DNA damage signaling and cell death and summarize the pathogenetic role of PARP in neuroinflammation and neuroinjury.

List of Abbreviations: AIF, apoptosis-inducing factor; APP, amyloid precursor protein; CNS, central nervous system; EAE, experimental allergic encephalomyelitis; ICAM-1, intercellular adhesion molecule-1; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; MCAO, middle cerebral artery occlusion; MS, multiple sclerosis; NOS, nitric oxide synthase; PARG, poly(ADP-ribose) glycohydrolase; PARP, poly(ADP-ribose) polymerase; $\text{PARP}^{-/-}$ cells/mice, cells/mice homozygous for disrupted poly(ADP-ribose) polymerase genes; ROS, reactive oxygen species; SCI, spinal cord injury; TBI, traumatic brain injury; $\text{TNF-}\alpha$, tumor necrosis factor- α .

1 Introduction

1.1 Poly(ADP-ribose) Polymerase and Its Activation

Poly(ADP-ribose) polymerases (PARP) represent an expanding family of enzymes with complex regulatory functions (Hiromatsu et al., 1992a; De Murcia et al., 1994; Menissier-de Murcia et al., 1997; Szabó et al., 1997a; Szabó and Dawson, 1998; LeRhun et al., 1998; Rudat et al., 1998; De Murcia and Shall, 2000; Szabó, 2000; Davidovic et al., 2001; Virág and Szabó, 2002). Poly(ADP-ribose) polymerase-1 (PARP-1; EC 2.4.2.30) (also known as poly(ADP-ribose) synthetase (PARS)), the major PARP isoform, is the major isoform present in the nucleus, which is responsible for most of the poly(ADP-ribose) formation in health and disease. Because the role of the minor PARP isoforms is poorly understood, in this review we alternate the use “PARP-1” and “PARP”.

Several overviews and monographs appeared in recent years about the structure and functions of PARP (Szabó and Dawson, 1998; Szabó, 2000; Virág and Szabó, 2002; Zhang, 2002). PARP is a DNA damage sensor and signaling molecule and binds to both single- and double-stranded DNA breaks, becomes activated, forms homodimers and initiates an energy-consuming cycle by transferring ADP ribose units from NAD^+ onto nuclear acceptor proteins such as histones, transcriptional factors, DNA replication factors, and signaling molecules (AP-1, DNA-PK, NF- κB , Oct-1, p53) with PARP itself being the major acceptor. Auto-poly(ADP-ribosylation) represents a major regulatory mechanism for PARP, resulting in the downregulation of the enzyme activity. Poly(ADP-ribosylation) alters the activity of the other acceptor proteins also, due to the high negative charge of the attached ADP-ribose. In case of the histones, this process leads to electrostatic repulsion between DNA and loosening of the chromatin structure thereby making certain genes more accessible for gene transcription (Oikawa et al., 1980; Poirier et al., 1982; Park et al., 1983; Satoh and Lindahl, 1992; Lautier et al., 1993; Herceg et al., 2001). The effect of PARP on the function of these proteins is carried out by noncovalent protein–protein interactions and by covalent poly

(ADP-ribosylation) (for review see Virág and Szabó (2002)). Poly(ADP-ribosylation) is a dynamic process as the polymer is rapidly degraded by two enzymes: poly(ADP-ribose) glycohydrolase (PARG) and ADP-ribosyl protein lyase. These enzymes are involved in the catabolism of poly(ADP-ribose) with PARG cleaving ribose–ribose bonds of both linear and branched portions of poly(ADP-ribose) and the lyase removing the protein proximal ADP-ribose monomer (Davidovic et al., 2001).

The biological role of PARP is complex. One of the most important functions is related to the fact that mild genotoxic noxious stimuli cause PARP activation that facilitates DNA repair and cell survival. Under pathophysiological conditions, reactive species (such as hydrogen peroxide, hydroxyl radical, and peroxynitrite) trigger severe DNA damage and overactivation of PARP (Szabó, 1996; Szabó et al., 1996, 1998). Peroxynitrite is considered a key trigger of DNA strand breakage because it can travel significant distances and readily crosses cell membranes. When activated by DNA breaks, PARP initiates an energy-consuming cycle resulting in the depletion of NAD^+ and ATP, slowing the rate of glycolysis and mitochondrial respiration, eventually leading to cellular dysfunction and necrotic cell death. Necrosis is the least desirable form of cell death as the cellular content is released into the tissue exposing neighboring cells to proteases and various proinflammatory factors, thereby triggering positive feedback pathways of inflammatory tissue injury (Virág and Szabó, 2002). PARP-overactivation-induced necrosis is also related to intracellular acidification (Herceg et al., 2001; Affar et al., 2002).

Another important function of the PARP enzyme is the regulation of the production of inflammatory mediators such as inducible nitric oxide synthase (iNOS), intercellular adhesion molecule-1 (ICAM-1), and major histocompatibility complex (MHC) class II (Hiromatsu et al., 1992a; Ehrlich et al., 1995; Szabó et al., 1997b; Zingarelli et al., 1998; Simbulan-Rosenthal et al., 2000). NF- κ B is a key transcription factor in the regulation of this set of proteins and PARP has been shown to act as a coactivator in the NF- κ B-mediated transcription. Similarly, PARP works with AP-1-mediated transcription in a cooperative fashion.

Other roles of PARP include its involvement in the regulation of replication and differentiation. It has been shown that poly(ADP-ribose) polymer can also serve as an emergency source of energy used by the base excision machinery to synthesize ATP (Oei et al., 2000). Furthermore, poly(ADP-ribose) may also serve as a signal for protein degradation in oxidatively injured cells (Ullrich et al., 2000).

It must be stressed that PARP functions a double-edged sword: on one hand, moderate activation of PARP can be of physiological importance via enhancement of DNA repair. On the other hand, overactivation of PARP represents an important mechanism of tissue damage in various pathological conditions associated with oxidative and nitrosative stress, including stroke and neurotrauma (see below). Although it is beyond the subject of this chapter, we would like to mention that PARP activation plays a significant pathogenetic role in a variety of diseases, including myocardial reperfusion injury (Zingarelli et al., 1997, 1998), heart transplantation (Szabó et al., 2002a), heart failure (Pacher et al., 2002b, c), circulatory shock (Szabó et al., 1997a; Oliver et al., 1999; Jagtap et al., 2002; Pacher et al., 2002a; Soriano et al., 2002; Shimoda et al., 2003), and autoimmune β -cell destruction associated with diabetes mellitus (Burkart et al., 1999; Pieper et al., 1999). Activation of PARP and beneficial effects of various PARP inhibitors have been demonstrated in various forms of endothelial dysfunction such as the one associated with circulatory shock, hypertension, atherosclerosis, preeclampsia, and aging (Szabó et al., 1997a; Hung et al., 2002; Martinet et al., 2002; Pacher et al., 2002e, f). Furthermore, recent data suggest that activation of PARP importantly contributes to the development of endothelial dysfunction in various animal models of diabetes and in humans (Garcia Soriano et al., 2001; Soriano et al., 2001; Pacher et al., 2002d; Szabó et al., 2002b). In addition, it has recently been demonstrated that PARP activation plays a pathogenetic role in diabetic nephropathy, neuropathy, and retinopathy (Szabó et al., 2001; Minchenko et al., 2003; Li et al., 2004).

Our knowledge with respect to the pathogenetic role of PARP in stroke and neurotrauma was generated mainly from studies using pharmacological inhibitors of PARP and from studies using genetically modified animals lacking PARP-1, the major PARP isoform. Pharmacological inhibitors of PARP (Tentori et al., 2002; Southan and Szabó, 2003) and genetic deletion of PARP (Shall and DeMurcia, 2000; Beneke et al., 2004) have been the subjects of separate recent reviews. In ▶ [Table 20-1](#), we overview some of the protective effects of PARP inhibitors and PARP deficiency in neuronal lines *in vitro*. The vast majority of published studies demonstrate that PARP inhibition or PARP-1 deficiency is protective (as judged by inhibition of cell

Table 20-1
Protection against various forms of neuronal injury by pharmacological inhibition or genetic inactivation of PARP in vitro

Experimental model	Inducer of injury	Mode of PARP inhibition	Effect of PARP inhibition	References
Rat hippocampal neurons	Nitric oxide	3-AB, Nicam	Improved neuronal conductance	Wallis et al. (1993)
Rat cortical cultures	NMDA, SNAP	Benzamide, 3-AB, AmNAP, ISQ	Reduced cell death	Zhang et al. (1994)
Cerebellar granule cells	Glutamate	Benzamide, 3-AB, 3-APH	Reduced cell death	Cosi et al. (1994)
Rat and mouse cortical cultures	NMDA, SNAP, SIN-1	PARP ^{-/-} phenotype, Benzamide, DHQ	Reduced cell death	Eliasson et al. (1997); Yu et al. (2002)
NSC39 spinal cord line	Peroxytrite, H ₂ O ₂	Benzamide, nicam, 3-AB, PHT	Reduced cell death	Cookson et al. (1998)
Cerebellar granule neurons	3-Acetylpyridine	3-AB	Protection against cell death	Wullner et al. (1997)
Rat cortex	H ₂ O ₂	Benzamide, nicam	Reduced cell death	Hlivert et al. (1998)
C6 astrocytoma cells	Peroxytrite	INH2BP	Reduced cell death	Endres et al. (1998b)
Rat motoneurons	OGD	3-methoxybenzamide	Reduction of ATP depletion	Tasker et al. (1998)
Rat hippocampal neurons	Homocysteine	3-AB	Reduction of NAD depletion and cell necrosis	Kruman et al. (2000)
Rat hippocampal neurons	SIN-1, NOC-9	Nicam	Reduction of cell necrosis and apoptosis	Lin et al. (2000)
Mouse neurons and astrocytes	H ₂ O ₂ , NMDA, MNNG	Benzamide, PARG inhibitors	Reduction of cell necrosis	Ying and Swanson (2000); Ying et al. (2001)
Mixed rat cortical cells	OGD	gallotannin and nobotanin B	Reduction of cell necrosis	Moroni et al. (2001)
Mixed rat cortical cells	OGD	Benzamide, PHT, DPQ	Reduction of cell necrosis	Abdelkarim et al. (2001)
Hippocampal slices	OGD	PJ34	No effect on apoptotic death	Moroni et al. (2001)
Hippocampal slices	Fluid percussion injury	Benzamide, PHT, DPB-ISQ	Improved neuronal conductance	Wallis et al. (1996)
Rat cortical slices	H/R	3-AB	Reduced LDH leakage	Oka et al. (2000)
Human A72 glioma	H ₂ O ₂	Benzamide, 3-AB, ISQ	Reduced cell death, maintained ATP	Lee et al. (2001)
Mouse BV-2 microglia mouse hippocampal cultures	NMDA	PARP ^{-/-} phenotype	Amelioration of glial migration and of secondary neuronal injury	Ullrich et al. (2001)
HcN2 cells	Tertiary butylhydroperoxide	Nicam	Inhibition of cell necrosis, preservation of cell morphology and differentiation	Sonee et al. (2002)

PC12 cells	H ₂ O ₂	Aminobenzamide	Reduction in neuronal cell death, preservation of pyridine nucleotide levels	Cole et al. (2002)
Primary cortical neurons	Peroxyinitrite	PARP ^{-/-} phenotype, INH2BP	Reduction in cell death, inhibition of the mitochondrial release of AIF	Du et al. (2003a)
Cultured mouse cortical cells	OGD	TiQ-A	Reduction of cell necrosis	Chiarugi et al. (2003)
Rat PC12 cells	H ₂ O ₂	FR217304	Reduction of NAD ⁺ depletion	Iwashita et al. (2004a)
Cortical neuronal cultures	Glucose depletion	ISQ	Reduction of cell necrosis	Suh et al. (2003)
Cultured rat neurons	H ₂ O ₂	DPQ	Elevation of postinsult ATP synthesis, reduction of cell necrosis, cell morphology shifts toward apoptosis	Aito et al. (2004)
Mixed cortical cell culture	NMDA	DPQ	Inhibition of the high dose NMDA-induced neuronal necrosis (but not the low dose NMDA-induced apoptosis)	Meli et al. (2004)
Primary cortical neuronal culture	H ₂ O ₂ , in combination with oligomycin	3-AB	Protection against the cell necrosis elicited by hydrogen peroxide and oligomycin	Huang et al. (2004)
Cultured SK-N-MC human neuronal cell line	Peroxyinitrite, H ₂ O ₂	INO-1001	Increased cell viability	Komjati et al. (2004)
Primary spinal neuron cultures	Peroxyinitrite	Nicam, 3-AB, INH2BP	Improved cell viability	Scott et al. (2004)

Abbreviations: 3-AB, 3-aminobenzamide; AIF, apoptosis-inducing factor; 3-APH, 3-aminophthalhydrazide; AmNAP, 4-amino-1,8-naphthalimide; ATP, adenosine triphosphate; CCl₄, chronic constriction injury of the sciatic nerve; CNS, central nervous system; dLGN, dorsal lateral geniculate nucleus; DPQ, 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]1(2H)-isoquinoline; FR247304, 5-chloro-2-[3-(4-phenyl-3,6-dihydro-1(2H)-pyridinyl)propyl]-4(3H)quinazolinone; H/R, hypoxia/reoxygenation; icv, intracerebroventricular; INH2BP, 5-iodo-6-amino-1,2-benzopyrone; ISQ, 1,5 dihydroxyisoquinoline; LDH, lactate dehydrogenase; MNNG, *N*-methyl-*N*-nitrosoguanidine; Nicam, nicotinamide; NMDA, *N*-methyl-*D*-aspartate; NOC-9, 6-(2-hydroxy-1-methyl-2-nitrosodiazirino)-*N*-methyl-1-hexamine MAHMA NONOate; OGD, oxygen-glucose deprivation; PARP, poly(ADP-ribose) polymerase; PC12 cells, pheochromocytoma cells; PHT, 6(5h)-phenanthridinone; PJ34, *N*-(*tert*-oxo-5,6-dihydro-phenanthridin-2-yl)-*N,N*-dimethylacetamide; SIN-1, 3-morpholino-sydnimine; SNAP, the NO donor *S*-nitroso *N*-acetyl penicillamine; t-BuOOH, *t*-butyl-hydroperoxide; TiQ-A, thieno[2,3-*c*] isoquinolin-5-one

necrosis and preservation of cellular NAD levels) against various forms of neuronal damage (induced by, for instance, reactive oxygen and nitrogen species, *N*-methyl-D-aspartate (NMDA) receptor activation, or oxygen–glucose deprivation). The protective effect of the PARP inhibitors is dose-dependent, consistent with the potency and the cell uptake of the PARP inhibitor tested, and in many cases incomplete (does not provide full normalization of cell functions or cell viability).

Reactive oxygen and nitrogen species are important triggers of neuronal damage in stroke and neurotrauma. The involvement of superoxide and the protective effect of superoxide neutralizing strategies (Lafon-Cazal et al., 1993; Fagni et al., 1994), as well as the involvement of NO and the protective effect of nitric oxide synthase (NOS) inhibition (Tewari et al., 1995) have been well established in various forms of central nervous system (CNS) injury, including reperfusion of the ischemic brain (stroke). Infarct volume following vascular stroke is markedly diminished in animals treated with NOS inhibitors and in mice with neuronal isoform of NOS (bNOS) gene disruption (Tewari et al., 1995). On the basis of chemical considerations, it was proposed over a decade ago that peroxynitrite, and not NO or superoxide separately, is the major cytotoxic mediator in neuronal injury during stroke (Gu et al., 1995). Reperfusion injury in the CNS is associated with activation of NMDA receptors, which are now believed to play a key role in the pathogenesis of stroke and which trigger the simultaneous production of superoxide and NO. There is now indirect evidence, which suggests that activation of the NMDA receptor is associated with a marked increase in a hydroxyl radical-like reactivity in the brain which may be blocked by inhibition of NOS (Hammer et al., 1993), and which is presumably due to peroxynitrite generation.

Ischemia/reperfusion injury of the brain is frequently modeled in the laboratory by exposing primary neuronal cultures to glutamate or its agonists, or to various reactive oxygen species (ROS), NO donors, peroxynitrite, or by combined oxygen–glucose deprivation (● [Table 20-1](#)). In cerebellar granule cells, glutamate induces a rapid increase in poly(ADP-ribose) immunoreactivity (Cosi et al., 1994). PARP inhibitors have been shown to offer protection in these models of brain injury—both in models where injury was induced by glutamate and in response to chemical compounds, which generate NO. The rank order of potency of different classes of PARP inhibitors correlates with the degree of protection (Zhang et al., 1994). Moreover, protection by PARP inhibition is not associated with changes in calcium influx induced by glutamate. In addition, primary cortical cultures from PARP^{-/-} mice (mice homozygous for disrupted poly(ADP-ribose) polymerase genes) were found resistant to toxicity induced by NMDA, by compounds which generate NO, and by combined oxygen–glucose deprivation (Wallis et al., 1993, 1996; Cosi et al., 1994; Zhang et al., 1994, 1995; Tewari et al., 1995; Didier et al., 1996; Snyder, 1996; Eliasson et al., 1997). Delaying the treatment of PARP inhibition relative to the stimulus of neuroinjury produced a significant therapeutic window of opportunity in vitro, as demonstrated in a model system consisting of primary rat hippocampal neurons exposed to the NO donor NOC-9 and the peroxynitrite generator compound SIN-1 (3-morpholino-sydnominine), with nicotinamide being used to block the activity of PARP (Lin et al., 2000). As opposed to NMDA-mediated neuronal injury, which is PARP-dependent, non-NMDA receptor-mediated form of neuroinjury, e.g., the one induced by the excitatory amino acid AMPA does not involve the PARP pathway (Mandir et al., 2000). Chiarugi et al. (2003) reported that a novel PARP inhibitor, thieno [2,3-*c*]isoquinolin-5-one (TIQ-A), exerts a significant neuroprotective effect on cultured mouse cortical cells stimulated by oxygen- and glucose deprivation. TIQ-A is potent PARP inhibitor with an IC₅₀ value of ~0.45 μM (Chiarugi et al., 2003). Treatment with 5-chloro-2-[3-(4-phenyl-3-6-dihydro-1 (2H)-pyridinyl)propyl]-4(3H)quinazolinone (FR247304) (10⁻⁸ M to 10⁻⁵ M) has also been shown to protect against ROS-induced PC12 cell injury in vitro (Iwashita et al., 2004a). Incubation of SK-NMC cultured neuronal cells with peroxynitrite for 2 h reduced cell viability by 57%, an effect which was dose-dependent, but not fully reversed by INO-1001, an isoindolinone-based PARP inhibitor (Komjati et al., 2004). Similarly, INO-1001 dose dependently protected against hydrogen peroxide-induced PARP activation and restored the viability of the hydrogen peroxide-treated cells. The concentration for half-maximal inhibition of PARP and restoration of cell viability ranged between 0.1 and 1 nM for PARP inhibition and was approximately 300 nM for restoration of cellular viability (Komjati et al., 2004).

In cells exposed to various oxidants, overactivation of PARP has been considered to be the key trigger of ATP depletion and necrosis. Oxidants may trigger delayed cell death also by mechanisms involving loss of mitochondrial membrane potential, release of cytochrome *c* to activate caspases (Yang J.C. et al., 1998), and

translocation of apoptosis-inducing factor (AIF) into the nucleus (Yu S.W. et al., 2002). The role of cellular energy status and PARP in these processes has not been fully clarified. In Aito's cortical culture model, consisting predominantly neurons, PARP inhibition by DPQ during a moderate to severe acute insult reduced the extent of acute ATP depletion and had a distinct protective effect on cell survival and morphology. A smaller but still significant effect was seen when DPQ was applied posttreatment (Aito et al., 2004). Since PARP is activated by DNA damage, its nuclear location has usually been emphasized. However, rapid cellular depletion of NAD^+ and ATP upon oxidant exposure would be difficult to explain by nuclear effects alone. Recently, significant PARP activity was shown also in mitochondria, and its selective inhibition preserved NAD^+ content, prevented AIF translocation, and reduced cell death due to oxidants (Du et al., 2003a). Aito and colleagues' data on decreased ATP resynthesis after oxidant exposure are consistent with compromised mitochondrial function (Aito et al., 2004). Since no significant improvement by DPQ could be shown, mechanisms other than transient energy depletion are probably involved, e.g., direct inhibition of the respiratory chain oxidants (Almeida and Bolanos, 2001).

Many additional *in vitro* studies utilizing PARP inhibitors or PARP-1 deficiency in primary neurons or neuronal cell lines, not discussed here in detail, are reviewed in [Table 20-1](#). At this point, we have to note that many pharmacological PARP inhibitors possess additional pharmacological activities, including oxidant and free radical scavenging effects (Southan and Szabó, 2003; Szabó, 2003a; Czapski et al., 2004; Zhang and Rosenberg, 2004), which necessitates some caution in the interpretation of the results.

Additional approaches to prevent PARP activation include oxidant and free radical scavenging, which prevents the generation of DNA strand breaks and hence the activation of PARP (Szabó, 2003a; Cuzzocrea et al., 2004).

In addition, recent studies indicate that metabolic interventions with tricarboxylic acid cycle substrates or NAD repletion can also counteract PARP-mediated cell necrosis (Ying et al., 2002, 2003). Paradoxically, inhibition of PARP has been shown to exert neuroprotective effects *in vitro* and *in vivo* (Ying et al., 2001; Lu et al., 2003), but again, nonspecific effects of the compounds used (e.g., gallotannin) make the interpretation of the results difficult, or perhaps impossible (Falsig et al., 2004).

2 PARP Inhibition in Stroke

2.1 General Considerations

In the 1990s, there was very little compelling evidence that pharmacological intervention could radically alter outcome after cerebral ischemia, even in experimental animals. By 1996, the pace of advance was such that a large number of drugs targeted at neurotransmitter receptors, and toward related mechanisms involved in ischemic damage, had advanced to clinical trials for the treatment of stroke and head injury (Dyker and Lees, 1998). This transformation of the pharmacology of cerebral ischemia was achieved due to two major reasons: first, the elucidation of neurochemical cascades initiated by ischemia, which revealed potential targets for intervention, and second, the systemic assessment of drug efficacy using robust endpoints (i.e., quantitative histopathology) in pertinent animal models (McCulloch et al., 2002). Animal models of focal cerebral ischemia are generally recognized as the most pertinent to human stroke. The pattern of ischemia produced after middle cerebral artery occlusion (MCAO) results in a sharp boundary between viable and damaged tissue on histological examination and lends itself to volumetric assessment of the lesion in gray matter (Osborne et al., 1987). While the volume of ischemic lesion is generally well defined when putative antiischemic drugs are evaluated, the histological definition of the lesion is often rudimentary. At best, it involves mapping the distribution of eosinophilic neuronal perikarya, at worst, it means simply mapping the area of tissue pallor using triphenyltetrazolium (TTC) staining. This limited histological definition of ischemic pathology in many pharmacological investigations has contributed to a neglect of ischemic white matter pathology, where the assessment of ischemic damage is difficult (McCulloch et al., 2002).

Since elucidation of the excitotoxic cascade, numerous other pathological mechanisms have been identified by which neuroprotection can be achieved in ischemia. The ischemic cascade involves many

events, occurring in parallel, that can independently lead to brain cell injury and death. These events, which are largely initiated by energy depletion resulting from loss of blood flow, include membrane depolarization, release of massive amounts of neurotransmitters, a huge increase in intracellular calcium, metabolic acidosis, enzyme activation, induction of an inflammatory response, and apoptosis. On the basis of their proven ability to reduce gray matter damage in animal models, a large number of drugs targeted at neurotransmitter receptors and related mechanisms involved in ischemic damage have advanced to clinical trials in stroke and head injury (Dyker and Lees, 1998). The neuroprotectants tested to date comprise a wide range of compounds from over ten classes, including calcium channel blockers, antioxidants, NMDA and glutamate antagonists, sodium channel blockers, potassium channel openers, growth factors, and leukocyte adhesion inhibitors (Maynard, 2002). At present, the outcome of clinical trials of neuroprotective drugs has been uniformly disappointing (Grotta, 1994; De Keyser et al., 1999; STAIR, 1999; Fisher, 2001; STAIR II, 2001; Lees, 2002; Maynard, 2002). The notable exception in clinical trials for stroke is the benefit demonstrated with tissue plasminogen activator (tPA) (NINDS tPA stroke study group, 1995), where the putative mechanism of action is restoration of blood flow and not neuroprotection (McCulloch et al., 2002). The failure to translate the insight gained in animal models into clinical therapy may be due to a combination of reasons.

First, it was suggested that the inability of the first generation of neuroprotective drugs to improve function after human stroke is related to their inability to protect white matter (Dewar et al., 1999). Many drugs (e.g., NMDA antagonists) are targeted at receptors that are not present to any extent in axons or oligodendrocytes. The protection of myelinated fiber tracts by drugs has been largely neglected in preclinical investigations of drug action. However, in humans, it is obvious that improved functional outcome after drug treatment depends not only on protection of cortical gray matter but also the simultaneous protection of associated white matter (McCulloch et al., 2002).

Second, although the incidence of stroke patients over age 75 is equal in men and women, mortality is almost double in women in this age group. This suggests that stroke and stroke-related mortality may be influenced by gender. These facts should direct stroke studies to testing in older animals, including females (STAIR, 1999; Fisher, 2001; STAIR II, 2001; Maynard, 2002). (In this respect, the recently reported gender difference in the protection against stroke by PARP deficiency is worth mentioning (see below) (Hagberg et al., 2004).

Third, since most stroke patients arrive with an occluded blood vessel to the hospital, the testing of protective drugs should be done in posttreatment models of both permanent and transient stroke.

Recent reports have shown that drug pharmacokinetics may change when an agent is administered to rodents with stroke compared with normal animals. Consequently, drug pharmacokinetic studies need to be performed on animals poststroke.

Many drugs shown to protect the brain after stroke in animal models have exhibited U-shaped dose-response curves. This means that the optimally protective dose is not necessarily the maximally tolerable dose, an assumption typically made in designing clinical trials. (STAIR, 1999; Fisher, 2001; STAIR II, 2001; Maynard, 2002). Similarly, additional experiments need to be determined if a bolus-plus-infusion dosing paradigm is better than a single dose of drug. The latter schedule is often used in preclinical studies, but the former is common in clinical practice.

Furthermore, preclinical studies have indicated that different doses may be protective under different co-morbid conditions. The protective dose for stroke patients with diabetes may not be the same as for patients with hypertension. It is also unlikely that a drug will have the same therapeutic window in different co-morbid conditions.

Some studies have indicated that some protective agents are able to achieve functional brain protection while not affecting the lesion size. This is not surprising given our understanding of brain plasticity following injury. Thus, the use of both infarct volume and functional or behavioral assessments as endpoints is necessary to judge the efficacy of any compound.

Finally, a word about the number of patients enrolled in a clinical trial (2,000–20,000 persons). While conducting simple, large clinical trials with broad entry criteria may lead to generalized treatment for more patients, smaller trials could show efficacy in specific patient populations, such as the aged, female patients, or diabetic patients (STAIR, 1999; Fisher, 2001; STAIR II, 2001; Maynard, 2002).

The general principles stated above, based on numerous ischemic studies of the past should be kept in mind while interpreting the preclinical data obtained so far with PARP inhibitors and PARP deficiency, and should be considered when designing stroke studies in the future, including the ones where PARP inhibitors would be tested in patients suffering from stroke or neurotrauma.

2.2 Effect of PARP Deficiency and PARP Inhibition in Stroke

The in vitro systems discussed in the previous section only model a component of the complex chain of events initiated in vivo following an ischemic insult or stroke (► [Tables 20-1](#) and ► [20-2](#)). Nevertheless, the pathophysiological relevance of these observations is supported by the observation that increased poly(ADP-ribosylation) has been demonstrated in the reperfused brain (Endres et al., 1998a). In PARP-1^{-/-} mice, a markedly reduced infarct volume is observed after transient MCAO (Eliasson et al., 1997; Endres et al., 1997). The reduction in infarct volume was observed in PARP-1^{-/-} mice that had either a genetic background identical to the wild-type strain (Endres et al., 1997) or a mixed 129/C57B6 genetic background (Eliasson et al., 1997). Thus, the reduction in infarct volume was due to the absence of the PARP gene product and not to other genetic variables. PARP activation was examined, following focal ischemia in the ipsilateral hemisphere, by evaluation of (ADP-ribose) polymer formation or levels of NAD⁺. This observation also demonstrates that among the multiple isoforms of PARP, PARP-1 appears to play the main role in the enhanced poly(ADP-ribosylation) in the brain during stroke. ADP-ribose formation was increased and NAD⁺ was decreased following focal ischemia in wild-type tissue, while no (ADP-ribose) formation was observed in PARP^{-/-} tissue (Eliasson et al., 1997) and NAD⁺ levels were spared (Endres et al., 1997). PARP activation is mainly related to NO production by the neuronal isoform of NOS, because in mice deficient in this enzyme, when subjected to MCAO/reperfusion, PARP activation was found to be markedly diminished (Endres et al., 1998a). The protection observed in the PARP^{-/-} mice exceeds the degree of protection reported for any other transgenic model, including the bNOS^{-/-} mice. This observation suggests a common role for PARP activation by other excitotoxic mechanisms, in addition to the production of free radicals and NO. With respect to the mechanism of the neuroprotection seen in PARP-deficient mice (physical absence of PARP vs. lack of its catalytic activity), a recent study provided a definitive answer: in PARP^{-/-} mice that were also treated with viral transfection of wild-type PARP-1, the protection from MCA occlusion was lost with restoration of the gene product (Goto et al., 2002). Thus, we can conclude that it is the catalytic activity of the enzyme (and not its physical association or other function) that leads to neuronal necrosis in stroke and neuroinjury.

Additional studies, comparing reductions in infarct volume in response to various application regimens and PARP inhibitors of various structural classes yielded the following findings.

PJ34 (*N*-(6-oxo-5,6-dihydrophenanthridin-2-yl)-2-(*N,N*-dimethylamino)acetamide.HCl) administration (10 mg/kg i.v. bolus for 3 min) before MCAO or 10 min before reperfusion partially protected rats (70%) against ischemic brain damage (Abdelkarim et al., 2001). Significant reduction in infarct volume was also seen in a mouse model of stroke (Abdelkarim et al., 2001). The neuroprotective effect of PJ34 in stroke has subsequently been confirmed by two independent studies, where PJ34 was used as a reference compound (Iwashita et al., 2004a; Park et al., 2004).

A novel potent PARP-1 inhibitor, FR247304, produced 47% cortical infarct size reduction and 14% decrease in the striatal infarct size in transient MCAO in rats. The intraperitoneal (ip) application of the compound at 32 mg/kg occurred twice: 10 min preocclusion and then 10 min prior to recirculation (Iwashita et al., 2004a).

On the basis of the results of the biological evaluation of imidazobenzodiazepines, Ferraris et al. (2003) reported a very potent neuroprotective effect of the “drug 5g” in both the transient and permanent model of cerebral ischemia in rats (MCAO). The administration of “drug 5g” (20 mg/kg i.p.) occurred twice: 30 min preocclusion and then 30 min after ischemia. This treatment regimen resulted in 59% reduction of infarct volume in the transient MCAO model and 39% reduction of infarct volume in the permanent MCAO model (Ferraris et al., 2003).

Table 20-2
Protection against various forms of neuronal injury by pharmacological inhibition or genetic inactivation of PARP in vivo

Experimental model	Inducer of injury	Mode of PARP inhibition	Effect of PARP inhibition	References
Mouse	MCAO I/R	PARP ^{-/-} phenotype, 3-AB	Reduced infarct size, maintained NAD, improved neurological function both acutely and after several days	Endres et al. (1997); Eliasson et al. (1997); Goto et al. (2002)
Rat	MCAO, BCO, I/R	DPQ	Reduced infarct size, improved neurological status at high doses, DPQ loses protective effect	Takahashi et al. (1997, 1999)
Rat	MCAO I/R	3-AB	Reduced infarct size, attenuation in glutamate and PEA release	Lo et al. (1998)
Rat	MCAO permanent	3-AB	Reduced infarct size	Tokime et al. (1998)
Mouse	MCAO, I/R	INH2BP	Reduced infarct size, improved neurological status	Endres et al. (1998b)
Rat	MCAO permanent	GPI-6150	Reduces infarct size in transient and permanent cerebral ischemia	Williams et al. (1999)
Neonatal rat	MCAO I/R	3-AB	Reduced infarct size, improved neurological status both short (2 days) and long term (17 days), reduced neutrophil infiltration, reduced nitrosative stress	Ducrocq (2000)
Rat	MCAO I/R	3-AB, Nicam	Reduced infarct size, improved neurological status, at high doses nicam becomes detrimental	Sun and Cheng, 1998
Mouse, rat	MCAO I/R	PJ34	Reduced infarct size, improved neurological status	Abdelkarim et al. (2001); Park et al. (2004)
Rat, mouse	MCAO permanent	PJ34	Reduced infarct at 4 h permanent MCAO	Abdelkarim et al. (2001)
Rat	Global brain ischemia	3-AB	Reduced infarct size, improved cortical NAD and phosphocreatine levels, no change in ATP	Plaschke et al. (2000a)
Rat	MCAO I/R	3-AB	Long-term (28 day) improvement in neurological status and reduced infarct volume	Ding et al. (2001)
Rat	MCAO permanent	Nicam	Reduced infarct size, improved neurological status both with acute (1 day) and delayed (7 days) determinations, protective effect diminishes at higher dose of the compound	Ayoub et al. (1999); Sakakibara et al. (2000); Mokudai et al. (2000); Ayoub et al. (2002)
Rat	MCAO permanent	DPQ	Reduced infarct size but not DNA damage	Giovanelli et al. (2002)
		MK801	Reduced both infarct size and DNA damage	

Rat	MCAO I/R	Nicam	Reduced infarct size, improved neurological status	Yang et al. (2002)
Rat	MCAO I/R MCAO permanent	"5g"	Reduced infarct volume	Ferraris et al. (2003)
Mouse Rat	MCAO I/R MCAO I/R	3-AB 3-AB	Reduced infarct volume and neutrophil infiltration Infarct volume and water content decreased, number of TUNEL-positive cells decreased, immunoreactivity of PAR, CD11B, ICAM-1, COX2 decreased	Couturier et al. (2003) Koh et al. (2004)
Rat	MCAO I/R	Nicam or 3-AB in combination with melatonin	Combination of PARP inhibitor and antioxidant causes enhanced neuroprotection	Gupta et al. (2004)
Rat	MCAO I/R	FR247304 3-AB	Reduced infarct volume	Iwashita et al. (2004a)
Rat	MCAO I/R and permanent	PJ-34 INO-1001	Reduced infarct volume, improved neurological outcome in short-term (24 h) and long-term (1 week) follow-up,	Komjati et al. (2004)
Mouse	Hypoxia/ ischemia	PARP ^{-/-} phenotype	immunoreactivity of PAR, NT, and APP was reduced, immunoreactivity changes of AIF restored	Hagberg et al. (2004)
SHR rats, diabetic and nondiabetic rats	MCAO	Nicam	Gender difference in brain protection	Skakakibara et al. (2002)
Rat	MCAO I/R and permanent	DR2313	Neuroprotection with dose difference between SHR, diabetic, and nondiabetic rats	Nakajima et al. (2004)
Rat	MCAO I/R	4-ANI	Reduced infarct volume even in 2 h posttreatment regimen	Kabra et al. (2004)
Mouse Rat Mouse	Cortical trauma Cortical trauma Intraatrial NMDA	PARP ^{-/-} phenotype GPI-6150 PARP ^{-/-} phenotype	Neuroprotective effect is attributed to the reduction of NAD ⁺ depletion and ATP fragmentation Improved neurological function Reduced lesion size, protection from cell death Protection from cell death	Whalen et al. (1999) LaPlaca et al. (2001) Mandir et al. (2000)
Mouse	Intraatrial AMPA	PARP ^{-/-} phenotype	No protection from cell death	Mandir et al. (2000)

Table 20-2 (continued)

Experimental model	Inducer of injury	Mode of PARP inhibition	Effect of PARP inhibition	References
Mouse	t-BuOOH (icv)	Nicam	Maintenance of NAD, NADP, and NADPH levels in many brain regions	Klaidman et al. (1996, 2001)
Gerbil	Short BCO	Benzamide, PHT, DPB-ISQ	No effect on hippocampal apoptotic death	Moroni et al. (2001)
Rat	Short global ischemia and hypothermia	3-AB	Enhancement of CA1 hippocampal apoptotic death	Nagayama et al. (2000)
Mouse	Global ischemia reperfusion	Nicam	Partially restored brain NAD ⁺ and ATP levels, partially restored mitochondrial respiration	Klaidman et al. (2003)
Mouse	Monocular deprivation	PARP ^{-/-} phenotype	Protection from cell death in dLGN	Nucci et al. (2001)
Rat	CCI	Benzamide	Reduced neuronal death, reduced hyperalgesia and mechanical allodynia	Mao et al. (1997)
Mouse	Nigral grafting	PARP ^{-/-} phenotype	No improvements in implanted cell survival or graft recipient status	Kaminski et al. (1999)
Guinea pig	I/R of the cochlea	3-AB	Improved neuronal conductance	Tabuchi et al. (2001)
Rat	I/R of the retina	3-AB	Reduced cell death	Lam (1997); Chiang and Lam (2000)
Rabbit	SAH	3-AB	Attenuated vasospasm	Satoh et al. (2001)

Abbreviations: 3-AB, 3-aminobenzamide; AIF, apoptosis-inducing factor; APP, amyloid precursor protein; ATP, adenosine triphosphate; AmNAP, 4-amino-1,8-naphthalimide; BCO, bilateral carotid occlusion; CCI, chronic constriction injury of the sciatic nerve; CNS, central nervous system; COX-2, cyclooxygenase 2; dLGN, dorsal lateral geniculate nucleus; DPQ, 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]1(2H)-isquinoline; FR247304, 5-chloro-2-[3-(4-phenyl-3,6-dihydro-1(2H)-pyridinyl)propyl]-4(3H)quinazolinone; GPI-6150, 1,11b-dihydro-[2H] benzopyrano[4,3,2-de]isquinolin-3-one; H/R, hypoxia/reoxygenation; icv, intracerebroventricular; ICAM-1, intercellular adhesion molecule-1; I/R, ischemia/reperfusion; INH2BP, 5-iodo-6-amino-1,2-benzopyrone; ISQ, 1,5 dihydroxyisquinoline; MCAO, middle cerebral artery occlusion; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; MPP(+), 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NAD⁺, nicotinamide adenine dinucleotide; Nicam, nicotinamide; NMDA, N-methyl-D-aspartate; NT, nitrotyrosine; PAR, poly(ADP-ribose); PARG, poly(ADP-ribose) glycohydrolase; PARP, poly(ADP-ribose) polymerase; PHT, 6(5h)-phenanthridinone; PJ34, N-(oxo-5,6-dihydro-phenanthridin-2-yl)-N,N-dimethylacetamide; SAH, subarachnoid hemorrhage induced by icv injection of blood; SIN-1, 3-morpholino-sydnominine; SNAP, the NO donor 5-nitroso N-acetyl penicillamine; STZ, streptozotocin; t-BuOOH, t-butyl-hydroperoxide; TNF- α , tumor necrosis factor α ; TUNEL, terminal deoxynucleotidyltransferase (TdT)-mediated dUTP-biotin nick end labeling; "5g" imidazobenzodiazepine; DR2313, 2-methyl-3,5,7,8-tetrahydrothiopyrano[4,3-d]pyrimidine-4-one

The isoindolinone-based PARP-1 inhibitor, INO-1001, significantly reduced infarct size following both transient and permanent focal cerebral ischemia (MCAO) in rats. INO-1001 at 3 mg/kg/24 h total dose (administered i.p. over 24 h time period) provided 86% reduction in total hemispheric infarct size, when its administration began 2 h after occlusion in transient MCAO, and remained effective at the 4 h and 6 h postocclusion treatment regimen in transient MCAO. In the permanent MCAO model, INO-1001 produced 62% reduction in total hemispheric infarct size when drug administration followed immediately the onset of permanent occlusion, and it remained effective even after 2 h in the postocclusion treatment regimen (Komjati et al., 2004).

As mentioned earlier, since most stroke patients arrive with an occluded blood vessel to the hospital, the testing of protective drugs is preferred in posttreatment models, both in permanent and transient stroke experiments. We must emphasize that the therapeutic window of intervention is rather large (up to 4–6 h after the onset of ischemia in the middle cerebral artery ischemia–reperfusion models), as demonstrated using both nicotinamide (Ayoub and Maynard, 2002) and a phenanthridinone derivative PJ-34 (Abdelkarim et al., 2001) and at the isoindolinone-based INO-1001 (Komjati et al., 2004).

Several studies have indicated that some protective agents are able to achieve functional brain protection in stroke (and also in neurotrauma; see below), while not affecting the lesion size. This is not surprising given our understanding of brain plasticity following injury. Thus, the use of both infarct volume and functional or behavioral assessments as endpoints is necessary to judge the efficacy of any compound. It is important to emphasize that the protective effect of PARP inhibition on neurological function is a lasting one, remaining significant even after 5–21 days of ischemia–reperfusion (Ding et al., 2001). Goto and his coworkers reassured the observation that PARP-1 deficiency provides both early and prolonged protection from experimental focal stroke (Goto et al., 2002). Marked improvements in the neurological status were observed after PARP inhibition with INO-1001 in either transient- or permanent MCA occluded rats (Komjati et al., 2004). In the 1-week follow-up studies in rats with permanent MCAO, the protective effect of PARP inhibition was maintained until the end of the observation period (Komjati et al., 2004). Many drugs (e.g., NMDA antagonists) are targeted at receptors that are not present to any extent in axons or oligodendrocytes. The protection of myelinated fiber tracts by drugs has been largely neglected in preclinical investigations of drug action. However, in humans, it is obvious that improved functional outcome after drug treatment depends not only on protection of cortical gray matter but also the simultaneous protection of associated white matter (McCulloch et al., 2002). The treatment with the isoindolinone-based PARP-1 inhibitor INO-1001 not only reduced the infarct volume, but also the accumulation of amyloid precursor protein (APP), a marker of axonal damage which was diminished by treatment with this compound (Komjati et al., 2004).

Interestingly, in many studies where more potent PARP inhibitors were used and full dose–response curves were obtained, the protection provided by PARP inhibitors diminishes when the dose of the agent is increased, i.e., a bell-shaped dose response is observed (e.g., Takahashi et al., 1997). This observation may be related to intrinsic neurotoxic effects of the particular compounds used or may suggest that the nonselective and complete inhibition of all PARP isoforms may not be a more desirable future therapeutic approach.

Although the incidence of stroke patients over age 75 is equal in men and women, mortality is almost double in women in this age group. This suggests that stroke and stroke related mortality may be influenced by gender and age. Recent results published by Hagberg et al. (2004) indicate that hypoxia–ischemia activates PARP-1 in neonatal brains, and that the sex of the animal strongly influences its role in the pathogenesis of brain injury. Seven-day-old wild-type and PARP-1 gene-deficient mice were used to test the effect of hypoxia/ischemia (Hagberg et al., 2004). PARP-1 genetic deficiency produced significant protection in the total group of animals, but analysis by sex revealed that males were strongly protected in contrast to females. Separate experiments showed that PARP-1 was activated over 1–24 h in both gender, but the decrease of brain NAD^+ level during early ischemia was seen only in males (Hagberg et al., 2004). The genes of AIF, as well for several proteins involved in perinatal hypoxia ischemia that may be related to PARP-1, are localized on the X chromosome and may, in addition to NAD^+ , be differentially expressed in males and females. In adult rodents, females sustain less injury than males after experimental ischemia (Hurn and Macrae, 2000). This resistance acquired after puberty (Payan and Conrad, 1977) depends on the estrous cycle and is lost after menopause in accordance with the putative effect of sex steroids, especially estrogen

(Hurn and Macrae, 2000; Stein, 2001). The exact cellular and molecular mechanisms of this gender difference need to be clarified, but these findings must be kept in mind when designing future preclinical and clinical investigations involving PARP inhibitors.

Another interesting recent and hitherto unexplained finding concerns the possible interaction of PARP and iNOS in the pathogenesis of stroke. Park and coworkers reported in a murine model of stroke that the PARP inhibitor PJ34 reduced infarct volume and attenuated postischemic iNOS mRNA upregulation by 72%. Curiously, coadministration of PARP and iNOS inhibitors or treatment of iNOS-null mice with PARP inhibitors, abrogated the protective effect afforded by iNOS or PARP inhibition alone. The loss of neuroprotection was associated with upregulation of the inflammatory genes iNOS, ICAM-1, and gp91 (phox). These findings unveil a previously unrecognized deleterious interaction between iNOS and PARP that may be relevant to the development of combination therapies for ischemic stroke. This example points out the importance of detailed characterization of various interactions of experimental stroke therapies prior to testing in the clinical arena.

► **Table 20-2** overviews the protection afforded by benzamides, isoquinolinones, phenanthridinones, and other classes of PARP inhibitors and compares their effects with that of genetic PARP deficiency. Taken together, there are multiple lines of clear evidence suggesting the importance of PARP pathway in the pathogenesis of stroke injury. Although the correlation between cerebrocortical NAD⁺ and ATP levels and neuronal injury in stroke is far from being understood (Paschen et al., 2000; Plaschke et al., 2000b), it is likely that the energetic/cell necrosis pathway is the main but not exclusive mode of PARP inhibitors' acute neuroprotective actions in stroke. Findings such as the suppression of NMDA-induced glutamate efflux and overall neurotransmitter dysregulation by PARP inhibitors (Lo et al., 1998) may be a direct consequence of an overall maintenance of cellular energetic status and reduction of cell necrosis.

Nevertheless, it is important to note here that there may also be some mild levels of injury (e.g., in a model of 15 min ischemia in the rat), where a mild degree of PARP activation, without NAD depletion may even be beneficial, and its inhibition may not be desirable (Nagayama et al., 2000). Also, while the oxygen-glucose deprivation-induced cell necrosis can be largely prevented by PARP inhibitors (as demonstrated in mixed cortical cell cultures), the same approach is unable to reduce primarily apoptotic type cell death (such as the CA1 pyramidal cell loss in organotypic hippocampal slices) (Moroni et al., 2001). The notion that PARP inhibition in stroke should be reserved for the most severe forms of the disease—and ones that are associated with predominantly necrotic type cell death—should certainly be considered and further explored in future studies. Studies demonstrating an important role for PARP-1 in the regulation of gene transcription have further increased the intricacy of poly(ADP-ribosylation) in the control of cell homeostasis and challenge the notion that energy collapse is the sole mechanism by which PAR formation contributes to cell death. Furthermore, oxidants may trigger delayed cell death also by mechanisms involving loss of mitochondrial membrane potential, release of cytochrome *c* to activate caspases (Yang et al., 1998), and translocation of AIF into the nucleus (Yu et al., 2002). A recent study, conducted in a transient MCAO model in rats demonstrates that AIF staining strongly diminished in the necrotic core of the striatum, while it is enhanced at the borderline ischemic territories of the white matter (capsula externa). Inhibition of PARP reshifted the location of the apoptotic marker to the axons in the ipsilateral striatum (Komjati et al., 2004). This observation proves that AIF translocation occurs in stroke in vivo, and this process can be suppressed by pharmacological inhibition of PARP.

In addition to the acute neuroprotective effects it is also conceivable that modulation by PARP of inflammatory molecule expression may also contribute to the protection. Excitotoxic brain lesions initially result in a primary destruction of brain parenchyma and, subsequently, in a secondary damage of neighboring neurons hours after insult. This secondary damage of initially surviving neurons accounts for most of the volume of the infarcted area and loss of brain function after stroke. One major component of secondary neuronal damage is the migration of macrophages and microglial cells toward the site of injury, where they produce large quantities of toxic cytokines and oxygen radicals. As discussed above, PARP inhibitors suppress the expression of proinflammatory cytokines and chemokines, thereby interrupting positive feedback cycles of mononuclear cell migration (Skaper, 2003). An additional mechanism—specifically relevant for the pathogenesis of CNS injury—may involve the regulation of the expression of integrin C11a by PARP, with subsequent suppression of microglial migration (Ullrich et al., 2001).

We must not forget, that in human beings (as opposed to experimental animal models), stroke develops on the basis of underlying vascular disease (hypertension, diabetes, hyperlipidemia, etc). One of the major underlying mechanisms of injury relates to endothelial dysfunction, vasospasm, and atherosclerosis. Emerging data indicate the potential role of PARP in the development of endothelial dysfunction associated with a variety of vascular diseases including diabetes, hypertension, aging, and hypercholesterolemia/early stage of atherosclerosis (see for more details: Garcia Soriano et al., 2001; Soriano et al., 2001; Pacher et al., 2002d, e, f, 2004; Du et al., 2003b; Benko et al., 2004; Piconi et al., 2004; Szabó et al., 2004; Zhang et al., 2004). The mechanisms whereby PARP activation leads to endothelial dysfunction are diverse, but they generally include oxidant-mediated DNA damage and PARP activation. The specific mechanisms include mitochondrial reactive species generation, poly(ADP-ribosylation) of GAPDH and depletion of NADPH, a cofactor of endothelial NO synthase (as in hyperglycemia/diabetic complication), NADPH oxidase activation, reactive nitrogen species generation, and DNA strand breakage (as in angiotensin II-induced endothelial dysfunction). The recent prevention trials demonstrating the reduced incidence of stroke in patients treated with angiotensin-converting enzyme inhibitors or statins demonstrate the powerful effect of this approach in the clinical practice. Similarly, reduction of oxidative/nitrosative stress in the vasculature and inhibition of endothelial PARP activation may also be useful in the future in preventing vascular (endothelial) dysfunction, and thereby reducing the degree of the contribution of preexisting vascular factors that are crucial in the development of stroke.

3 PARP Inhibition and Neurotrauma

Similar to the situation in stroke, at the beginning of the deleterious cascade, traumatic brain injury (TBI) and spinal cord injury (SCI) induce the activation of NOS leading to the production of NO (Wu et al., 1994; Yamanaka et al., 1995; Hamada et al., 1996; Sakamoto et al., 1997; Muralikrishna Rao et al., 1998; Cherian et al., 2000; Liu et al., 2000, 2002; Xu et al., 2001; Chatzipanteli et al., 2002; Diaz-Ruiz et al., 2002; Nakahara et al., 2002; Yune et al., 2003; Lee et al., 2004; Vaziri et al., 2004). Moreover, free radical production is triggered by TBI (Goss et al., 1997; Nishio et al., 1997; Fabian et al., 1998; Shohami et al., 1999; Tyurin et al., 2000; Pratico et al., 2002) and SCI (Azbill et al., 1997; Baldwin et al., 1998; Liu et al., 1999; Lee et al., 2004; Vaziri et al., 2004). The involvement of oxidative stress and NO have been well established both in brain (Hall et al., 1988; Hamm et al., 1996; Mésenge et al., 1996, 1998a, b; Mikawa et al., 1996; Petty et al., 1996; Lewen et al., 2001; Marklund et al., 2001; Pineda et al., 2001; Aoyama et al., 2002; Flentjar et al., 2002) and spinal cord trauma (Hamada et al., 1996; Sharma et al., 1996, 2003; Zhang et al., 1997; Chikawa et al., 2001; Farooque et al., 2001; Kamencic et al., 2001; Leski et al., 2001; Suzuki et al., 2001; Chatzipanteli et al., 2002; Sugawara et al., 2002; Pearse et al., 2003; Takahashi et al., 2003; Yune et al., 2003; Luo and Shi, 2004). As superoxide anions are produced after TBI (Fabian et al., 1998) and SCI (Liu et al., 1998), the cytotoxic oxidant peroxynitrite is formed. Indeed, it has been demonstrated in the traumatic spinal cord (Scott et al., 1999; Liu et al., 2000; Xu et al., 2001; Lee et al., 2004; Vaziri et al., 2004) and brain tissue (Mésenge et al., 1998a; Whalen et al., 1999; Besson et al., 2003; Satchell et al., 2003). Additionally, peroxynitrite is involved in neuronal cell death and neurological deficits following TBI (Hall et al., 1999; Lacza et al., 2003) and SCI (Bao and Liu, 2002). Treatment with L-NAME (*N*^ω-nitro-L-arginine-methylester), a NOS inhibitor, promoted neurological recovery and reduced nitrotyrosine formation and the number of nitrotyrosine-positive neurons after closed head injury in mice (Mésenge et al., 1998a). Moreover, 3-bromo-7-nitroindazole, a neuronal NOS inhibitor, significantly decreases the production of poly(ADP-ribose) in damaged cerebral cortex after cryogenic lesion demonstrating that cold lesion-induced PARP activation depends, at least in part, on prior activation of neuronal NO synthase isoform (nNOS) (Hortobagyi et al., 2003).

Taken together, the above listed data indicate that following brain and spinal cord trauma, NO leads to peroxynitrite formation (via combination with superoxide anion), which in turn induces DNA strand breaks rendering PARP active. Massive DNA breakage has been reported after TBI (Rink et al., 1995; Colicos and Dash, 1996; LaPlaca et al., 1999; Satchell et al., 2003), and peroxynitrite production and poly(ADP-ribosylation) colocalize in areas of necrosis in injured spinal cord (Scott et al., 1999) and traumatic brain tissues (Besson et al., 2003). PARP is markedly activated as early as 30 min after TBI and its activation

persists for 3 days after TBI (LaPlaca et al., 1999; Besson et al., 2003). Overactivation of PARP contributes to energy failure (NAD and consequently ATP depletion), and thus leads to cell death. Cleavage of PARP by caspase-3, occurring only 7 days after TBI (LaPlaca et al., 1999), is one of the mechanism to inactivate PARP, thus preserving cell energy stores required during apoptosis. More recently, Satchell and coworkers (2003) studied protein nitration, as a marker of peroxynitrite production, and poly(ADP-ribosylation) for 21 days after controlled cortical impact in mice. Both are found to be persistently increased compared to normal brains, with relative peaks seen at 8 and 72 h (Satchell et al., 2003). As PARG rapidly degrades polymers of ADP-ribose (Davidovic et al., 2001), it suggests that ADP-ribosylation, i.e., PARP activation, is a prolonged phenomenon. This pattern of PARP activation is likely related to the continuing presence of peroxynitrite in the lesioned tissue. It is also conceivable that a massive early DNA single-strand breakage, which remains unrepaired for prolonged periods of time, is responsible for the prolonged pattern of PARP activation. Furthermore, levels of ATP in rat spinal cord tissues are decreased following SCI (Colak et al., 2003). It is therefore possible, if not likely, that the activation of PARP in spinal cord neurons following trauma causes neurotoxicity through energy depletion.

Like cerebral ischemia, TBI and SCI share many pathophysiological events, and it is not unexpected that PARP may be involved in the consequences of acute brain and spinal cord trauma. Indeed, PARP inhibition protects hippocampal slices against percussion-induced loss of CA1 pyramidal cells-evoked response in vitro (Wallis et al., 1996). Whalen and coworkers (1999) showed that motor and cognitive deficits of mice submitted to TBI are less severe when the PARP-1 gene is inactivated. The prototypical PARP inhibitor, 3-aminobenzamide (3-AB), and other benzamide derivatives have been found to be neuroprotective of neurological deficits and brain lesions after closed head injury in mice (Mésenge et al., 2005) and after TBI induced by fluid percussion (Besson et al., 2003). Delaying the treatment of PARP inhibition relative to the TBI produces a therapeutic window of opportunity of 2 h, with 3-AB being used to block the activity of PARP (Verrecchia et al., 2000). GPI-6150 (1,11b-dihydro-[2H]benzopyrano[4,3,2-de]isoquinolin-3-one), another PARP inhibitor, reduces the injured area in the very early phase (24 h) after TBI (LaPlaca et al., 2001). Moreover, partial inhibition of poly(ADP-ribosylation) by the PARP inhibitor INH2BP (5-iodo-6-amino-1,2-benzopyrone) preserves brain NAD levels and improves long-term functional outcome after TBI (Satchell et al., 2003). Recently, PJ34 and INO-1001, two novel water-soluble PARP inhibitors, were found to be neuroprotective in a model of TBI caused by fluid percussion in rat. Indeed, repeated treatment with PJ34 and INO-1001 decrease the neurological deficit 3 days after injury and these protective effects still persist at 7 days postinjury (Besson et al., 2005). It is important to emphasize that the protective effect of PARP inhibition on neurological function is a lasting one remaining significant even 7 (Besson et al., 2003; unpublished data) or 21 days (Satchell et al., 2003) after TBI. These data strengthen the hypothesis that the activation of PARP has a deleterious effect on the neurological consequences in the early and late phase after TBI.

Pharmacological inhibition of PARP activation may also be a viable approach for improving the outcome of stem cell transplantation. This can be achieved by two ways. First, it may make the host tissue environment more receptive for a graft. Second, it may directly protect the transplanted cells from necrosis induced by peroxynitrite. Indeed, treatment with the PARP inhibitor PJ34 improves the neurological score after cryogenic lesion, and increases it even further following stem cell transplantation. High peroxynitrite production coincides with the loss of the majority of the grafted cells, and inhibition of the PARP activation cascade increases the number of surviving cells (Lacza et al., 2003). This PARP inhibition approach may ultimately lead to an optimized grafting strategy.

In cerebral ischemia studies in which different PARP inhibitors were used and full dose-response curves were obtained, the protection provided by PARP inhibitors diminishes when the dose of agent is increased, i.e., a bell-shaped dose response is observed (Takahashi et al., 1997). Consistent with this observation, similar data have been noticed with 3-AB in a model of closed head injury (Mésenge et al., 1999). The beneficial effects of PJ34 and INO-1001, two potent PARP inhibitors, on the neurological score have been seen despite no significant benefit on brain lesion volume suggesting that the protective effect by PARP inhibition could not be attributed to salvaging significant amounts of lesioned tissue (Besson et al., 2005). This observation has been also noticed in both pharmacological inhibition and genetic intervention in models of TBI induced by controlled cortical impact (Whalen et al., 1999; Satchell et al., 2003) and cold

injury (Lacza et al., 2003) indicating that functional improvement does not always correlate with the extent of brain damage. In addition, more complete inhibition of PARP-1 with INH2BP impairs spatial memory acquisition independent of injury and is associated with ribosylation of 14-3-3 γ , a protein implicated in learning and memory (Satchell et al., 2003). These observations suggest that complete and nonselective inhibition of all PARP isoforms may produce some potential adverse effects, limiting its potential as a future therapeutic approach for TBI.

The role of PARP in SCI is less understood. The phenomenon of PARP activation in spinal cord sections after spinal cord trauma has been reported in 1999 (Scott et al., 1999). In spinal cord, a recent *in vitro* study shows that peroxynitrite is responsible for DNA strand breakage and PARP activation in primary spinal cord neurons (Scott et al., 2004). In addition, PARP activity contributes to peroxynitrite neurotoxicity *in vitro*, as spinal cord neurons treated with PARP inhibitors showed a decreased peroxynitrite-induced cell death, suggesting PARP inhibition may provide a useful therapeutic approach to reduce secondary neuronal injury following spinal cord trauma. At present, there is only one *in vivo* study available on the outcome of spinal cord trauma in animals, where cell death induced by peroxynitrite is inhibited in the presence of PARP inhibitors (Scott et al., 2004). These data suggest in which PARP is inhibited (and no studies have been performed with mice in which PARP is genetically inactivated). In this one recent study, Genovese and coworkers (2004) demonstrate that in a spinal cord trauma model induced by the application of vascular clips to the dura via a four-level T5–T8 laminectomy, treatment of the mice with the PARP inhibitors, 3-AB or 5-aminoisoquinolinone (5-AIQ) significantly reduced the degree of (1) spinal cord inflammation and tissue injury (histological score), (2) PAR formation, (3) neutrophil infiltration, and (4) TUNEL positivity. Treatment with these PARP inhibitors also reduced DNA binding of NF- κ B and of I κ B- α degradation indicating that PARP regulates some proinflammatory pathways during spinal cord trauma (Genovese et al., 2004). In a separate set of experiments, we have also demonstrated that PARP inhibitors significantly ameliorated the recovery of limb function (evaluated by motor recovery score) (Genovese et al., 2004). These encouraging initial studies need to be repeated and confirmed with PARP inhibitors of other structural classes.

PARP activation has been demonstrated in human neurotrauma studies. For instance, a recent study shows that PARP activity is present in neurons of pericontusional tissue of patients suffering from severe TBI (Ang et al., 2003). Future interventional studies with clinically useful PARP inhibitors are needed, however, to define the role of PARP in the pathogenesis of neurotrauma in humans.

4 PARP and Neuroinflammation

4.1 Role of PARP in Meningitis

ROS along with reactive nitrogen intermediates are known to be mediators of brain damage in bacterial meningitis (Koedel and Pfister, 1999). More recently peroxynitrite has also been proposed to play a principal role in the development of bacterial meningitis. Several studies demonstrate that interfering with peroxynitrite protects against the pathological changes associated with bacterial meningitis (Kastenbauer et al., 1999, 2001, 2002; Irazuzta et al., 2000). In bacterial meningitis, the disease signs mainly result from CNS complications such as cerebrovascular alterations, brain edema, hydrocephalus, and increased intracranial pressure (Pfister et al., 1993). All these factors contribute to brain injury and an unfavorable clinical outcome. While there is substantial evidence that peroxynitrite is a central mediator of meningitis-associated CNS complications, the mechanisms underlying peroxynitrite-induced brain injury in the disease have yet to be defined. Peroxynitrite is able to cause cell death through a variety of different mechanisms including tyrosine nitration, lipid peroxidation, and inhibition of mitochondrial respiration (Szabó, 2003b). Peroxynitrite may also trigger cell death by inducing DNA strand breakage, resulting in the activation of the nuclear enzyme PARP (Szabó, 2003b).

Over the last few years it has been suggested that PARP activation plays a role in the development of meningitis-associated CNS complications. PARP was activated in the CNS during experimental pneumococcal meningitis in rats and mice (Koedel et al., 2002). More importantly, mice with a targeted disruption

in the gene for PARP-1 had an improved clinical outcome following infection with *Streptococcus pneumoniae* (Koedel et al., 2002). These PARP-1 knockout mice were protected from meningitis-associated CNS complications, including blood–brain barrier (BBB) breakdown and increased intracranial pressure (Koedel et al., 2002). The suppression of pathophysiological alterations in PARP-1-deficient animals was associated with a marked reduction in meningeal inflammation, evidenced by lower numbers of leukocytes detected in cerebrospinal fluid, as well as a reduction in inflammatory cytokine levels in the brain (Koedel et al., 2002). Similar protective effects were observed in a rat model of pneumococcal meningitis following pharmacological inhibition of PARP using 3-AB (Koedel et al., 2002).

A number of studies have suggested that a loss of neurovascular integrity is a fundamental event in the development of CNS complication in meningitis. PARP activity is thought to contribute to BBB breakdown in bacterial meningitis, as a significant reduction in disease-associated BBB permeability was observed in both PARP-1 knockout mice and 3-AB-treated rats. In addition, cerebrovascular reactivity to hypertension and hypercapnia were maintained indicating that endothelial function was preserved in the absence of PARP activity (Koedel et al., 2002). Further support for the involvement of PARP in endothelial dysfunction in meningitis is provided by work demonstrating PARP activation and NAD depletion in brain endothelial cells exposed to *S. pneumoniae* (Koedel et al., 2002). Moreover, pneumococci-induced brain endothelial cell death was prevented by the presence of 3-AB (Koedel et al., 2002).

CNS complications during meningitis are also thought to occur as a consequence of an exaggerated inflammatory response to bacterial products. Therefore, PARP activity may be involved in the disease process by promoting inflammatory cell infiltration into the CNS and facilitating the production of proinflammatory mediators. Accordingly, in pneumococcal meningitis CNS infiltration of leukocytes as well as the brain levels of interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α were reduced following PARP-1 deletion or pharmacological inhibition of PARP (Koedel et al., 2002). PARP activity may assist inflammatory cell migration by upregulating the expression of adhesion molecules on brain endothelial cells (Zingarelli et al., 1998). PARP has been shown to be functionally associated with NF- κ B, a key regulatory molecule involved in the transcription of inflammatory mediators (Hassa and Hottiger, 1999; Oliver et al., 1999). Conceivably, PARP may modulate the production of proinflammatory cytokines in pneumococcal meningitis through the actions of NF- κ B. Noteworthy in this regard, it has previously been established that NF- κ B activation plays a fundamental role in the development of the host inflammatory response to bacterial meningitis (Koedel et al., 2000).

4.2 Role of PARP in Multiple Sclerosis and Experimental Allergic Encephalomyelitis

Experimental allergic encephalomyelitis (EAE) is an autoimmune disease of the CNS that is widely employed as an animal model for the human demyelinating disorder multiple sclerosis (MS). While the etiology of EAE is reasonably well characterized, with the disease generally believed to occur as a consequence of CD4⁺ T cell autoreactivity, the cause of MS remains largely undefined. However, several recent reports have proposed that peroxynitrite, a reactive molecule that is rapidly formed when nitric oxide (NO) and superoxide combine, is involved in the pathogenesis of both disorders (Hooper et al., 1997; Van der Veen et al., 1997; Cross et al., 1998; Hooper et al., 1998b; Hooper et al., 2000; Scott et al., 2001). Infiltrating immune cells, together with resident CNS cells, are capable of producing peroxynitrite and a number of studies have demonstrated the presence of nitrotyrosine residues, a marker of peroxynitrite production, in CNS tissues from MS patients (Hooper et al., 1997; Cross et al., 1998) and animals with EAE (Cross et al., 1997; Van der Veen et al., 1997; Hooper et al., 2000; Scott et al., 2001). Furthermore, uric acid, a selective inhibitor of certain peroxynitrite-mediated reactions not only exerts therapeutic effects in a PLS/JL mouse model of EAE (Hooper et al., 1997, 1998a, b, 2000) but promotes recovery from preexisting disease signs (Hooper et al., 1998a, b, 2000). Peroxynitrite scavengers such as mercaptoethylguanidine and desferrioxamine, along with a peroxynitrite decomposition catalyst that isomerizes peroxynitrite to produce inactive nitrate, have also been shown to influence clinical signs of EAE (Pedchenko and LeVine, 1998; Cross et al., 2000). As peroxynitrite is known to be a major trigger of PARP activation in pathophysiological

conditions (Szabó, 1996), the formation of peroxynitrite and subsequent activation of PARP may be important events in the etiology of EAE.

Emerging data directly implicates PARP in the pathogenesis of EAE. The increased presence of poly (ADP-ribose) residues in CNS tissues from animals with EAE indicates that PARP activity may be involved in this disease process (Scott et al., 2001). Consistent with this observation, several PARP inhibitors have been shown to have beneficial effects in different models of EAE (Scott et al., 1998, 2001, 2004; Chiarugi, 2002). In the Lewis rat model of EAE, 3-AB, benzamide, INH2BP, and 6(5H)-phenanthridinone prevented disease progression (Scott et al., 1998, 2001; Chiarugi, 2002). Inhibiting PARP using 3-AB and INH2BP resulted in a delay in the disease onset as well as a reduction in the incidence and severity of disease signs (Scott et al., 1998, 2001). Similarly, benzamide and 6(5H)-phenanthridinone prolonged the time of disease development and lessened the duration and severity of EAE (Chiarugi, 2002). Pharmacological inhibition of PARP is also protective in murine models of the disease as INH2BP abrogated disease signs in PLSJL mice with EAE (Scott et al., 2001). Likewise, PJ34, a novel and potent PARP inhibitor, markedly reduced neurological signs and improved survival in a PLSJL mouse model of EAE (Scott et al., 2004).

The main features of MS and EAE include invasion of inflammatory cells into the CNS, breakdown of the BBB, local inflammation, and demyelination. In the case of EAE, inflammatory processes trigger changes in BBB function, which are important for the infiltration of cells into CNS tissues and, ultimately, for the pathogenesis of the disease (Hooper et al., 2000; Kean et al., 2000). While PARP activity is thought to be involved in disease development, the specific pathways through which PARP exerts its effects in EAE have not been well defined. In the past, PARP was thought to contribute to disease pathogenesis primarily by inducing cell death (Szabó, 2000). However, more recently it has been recognized that PARP activity modulates the transcription and translation of genes involved in inflammation (Szabó, 2000; Kraus and Lis, 2003). Consequently, PARP is likely to mediate the pathogenic effects in EAE through multiple mechanisms.

Particular emphasis has been placed on the ability of PARP to induce cell death, and PARP inhibitors have been shown to prevent free radical-induced toxicity in a variety of different cell types (reviewed in Virág and Szabó (2002)). Both EAE and MS are characterized by oligodendrocyte destruction and demyelination (Sobel, 1995), with damage occurring to mature oligodendrocytes as well as to remyelinating cells (Prineas, 1975). Several reports have demonstrated that NO induces oligodendrocyte death *in vitro* (Merrill et al., 1993; Mitrovic et al., 1994a, b, 1995, 1996). Many of the cytotoxic actions previously attributed to NO are now known to be mediated through peroxynitrite (Hausladen and Fridovich, 1994; Zingarelli et al., 1996). Therefore, oligodendrocyte damage in certain CNS conditions may result from peroxynitrite rather than from NO, and we recently established that oligodendrocytes undergo cell death *in vitro* following exposure to peroxynitrite (Scott et al., 2003). Notably, peroxynitrite-mediated oligodendrocyte toxicity was associated with DNA strand breakage and activation of PARP indicating that this enzyme may play an active role in the cell death process (Scott et al., 2003). However, when oligodendrocytes were treated with the PARP inhibitors, 3-AB and INH2BP, this failed to protect the cells from cell death (Scott et al., 2003). In addition, primary oligodendrocytes isolated from PARP-1-deficient mice were also highly susceptible to peroxynitrite-induced cytotoxicity (Scott et al., 2003). Taken together these results suggest that PARP activity does not directly mediate oligodendrocyte destruction during EAE. Instead PARP may be activated in oligodendrocytes as a protective mechanism since PARP has been shown to promote cell survival (reviewed in Bürkle, 2001). Specifically, PARP has been implicated in the maintenance of genomic stability (Masutani et al., 2000), as well as in the regulation of gene transcription and proteosomal function (Ullrich et al., 1999; Kameoka et al., 2000). Moreover, Vispé et al. (2000) have recently identified a novel cellular defense pathway, mediated by PARP, which regulates transcription in response to DNA damage.

In addition to myelin degradation and loss of oligodendrocytes, axonal degeneration is a key feature of MS. Importantly, axonal injury is thought to be the main cause of neurological deficits and disability in MS patients (Neumann, 2003). At present it is not clear whether PARP activity directly contributes to axonal destruction during the disease. However, as PARP activation has previously been implicated in neurotoxicity it is possible that enzyme activity contributes to disease development by directly damaging axons. Moreover, the direct injection of peroxynitrite into rat brain has been shown to cause prominent injury to both myelin and axons (Touil et al., 2001), an effect that may be mediated through PARP.

It is also possible that the therapeutic effects of inhibiting PARP activity during EAE are mediated by preventing the death of astrocytes. We have previously shown that peroxynitrite induces astrocyte death *in vitro* via the activation of PARP (Endres et al., 1998a, b). Astrocytes have been shown to protect CNS cells from the toxic effects of free radicals (Drukrach et al., 1998; Tanaka et al., 1999), and they are also required for maintaining the integrity of the BBB (Andjelkovic and Pachter, 1998). Moreover, a loss of astrocyte function has been associated with neurodegenerative disorders such as Parkinson's and Alzheimer's diseases (Schipper, 1996). Therefore, it is possible that PARP inhibitors suppress disease development by preserving the functional activity of astrocytes.

As well as directly causing energetic changes and inducing cell death, activation of PARP may exert other effects that are pertinent to the development of EAE. Another potential mechanism through which PARP activation may contribute to the pathogenesis of EAE is through effects on neurovascular integrity. Under normal circumstances the BBB regulates CNS homeostasis by limiting soluble factor and cellular exchange between the CNS and the blood (Andjelkovic and Pachter, 1998). BBB breakdown is a characteristic feature of EAE and is coupled with an influx of immune cells, fluids, and proteins into the CNS (Andjelkovic and Pachter, 1998). The entry of substances that are normally excluded from the CNS is thought to contribute directly to the disease pathology. Therefore, a loss of neurovascular integrity has been proposed to be a prerequisite for disease development, and BBB breakdown is a fundamental event in the pathogenesis of MS and EAE. Although the precise mechanisms leading to loss of neurovascular integrity are presently unknown, peroxynitrite has been proposed to be involved in this process (Kastenbauer et al., 1999; Hooper et al., 2000, 2001; Kean et al., 2000; Nag et al., 2001). Consequently, activation of PARP may also play a role in inducing BBB breakdown during EAE. It is noteworthy in this regard that PARP activity has previously been implicated in mediating endothelial dysfunction in models of pneumococcal meningitis (Koedel et al., 2002). In this case, BBB disruption was shown to be reduced in either pneumococcal-infected mice with a targeted mutation in the gene for PARP-1 or in rats administered the PARP inhibitor, 3-AB (Koedel et al., 2002). We have also demonstrated that neurovascular integrity is maintained in EAE by inhibiting PARP activity (Scott et al., 2004), confirming that in neurological conditions PARP activity contributes to a breakdown in the BBB. One of the ways through which PARP activity has been suggested to modulate neurovascular integrity is by inducing endothelial cell cytotoxicity, as brain endothelial cells exposed to pneumococci *in vitro* were protected from cell death by the presence of 3-AB (Koedel et al., 2002). Moreover, neuroendothelial cells are dependent on elevated intracellular levels of ATP to maintain their normal function (Oldendorf et al., 1977). They may be sensitive to toxicity resulting from overactivation of PARP. However, this is unlikely to be the case in EAE because cell invasion into the CNS tissues is highly selective rather than nonspecific. We therefore consider that the effects of PARP inhibitors on BBB function in EAE are more likely to be an indirect consequence of the modulation of the inflammatory response.

Recent attention has focused on the role of PARP in inducing inflammation in other models (reviewed in Virág and Szabó, 2002). Importantly, EAE is considered to be a neuroinflammatory disorder, and there is accumulating evidence that the CNS infiltration of inflammatory cells is pivotal to disease development. Therefore, it is feasible that PARP activity may regulate immune cell migration during EAE. Accordingly, PARP has been shown to modulate inflammatory cell trafficking outside the CNS (Virág and Szabó, 2002). For example, the accumulation of inflammatory cells in various tissues after either hemorrhagic shock or myocardial ischemia reperfusion injury was attenuated in mice deficient in PARP-1 (Zingarelli et al., 1998; Liaudet et al., 2000). Additionally, pharmacological inhibition of PARP reduced leukocyte recruitment in carrageenan- and zymosan-induced models of inflammation as well as in arthritis and peritonitis (Szabó and Dawson, 1998; Szabó et al., 1997b, 1998; Cuzzocrea et al., 1998; Mabley et al., 2001). The therapeutic effects of PARP inhibitors in EAE were accompanied by a decrease in inflammatory cell infiltration (Chiarugi, 2002; Scott et al., 2004). In particular, PJ34 treatment appeared to have a greater influence on T cell migration during the disease, an observation that is particularly noteworthy since EAE is widely believed to be an autoimmune condition mediated by CD4⁺ T cells (Scott et al., 2004).

Inflammatory cell infiltration may be reduced in EAE-immunized animals dosed with PARP inhibitors merely as a consequence of maintained neurovascular integrity. However, PARP has been proposed to alter leukocyte migration in other models through modifying the expression of adhesion molecules

(reviewed in Virág and Szabó (2002)). Several *in vitro* studies have demonstrated that PARP regulates the expression of the adhesion molecules ICAM-1, E-selectin, and P-selectin on endothelial cells (Hiromatsu et al., 1992b; Zingarelli et al., 1998; Sharp et al., 2001). Furthermore, in experimental models of colitis and myocardial ischemia reperfusion injury, the expression of both ICAM-1 and P-selectin were downregulated in PARP-1-deficient mice (Zingarelli et al., 1998, 1999). We have provided evidence that PJ34 suppresses the expression of ICAM-1 in the CNS of MBP-immunized mice (Scott et al., 2004). Therefore, PARP activity may contribute to EAE pathogenesis by facilitating inflammatory cell migration through the upregulation of ICAM-1 expression.

PARP may also influence inflammatory cell infiltration during EAE by modulating the production of chemokines. Chemokines play a critical role in regulating cell trafficking in a variety of situations. Moreover, a recent report has revealed that in endotoxic shock inhibiting PARP activity, either through pharmacological means or by using gene knockout animals, reduces the production of the chemokines MIP-1 and MIP-2 (Hasko et al., 2002). Since chemokines, along with adhesion molecules, are thought to be responsible for the transmigration of cells through a vascular endothelium, it is possible that PARP inhibitors prevent CNS inflammation in EAE via effects on chemokines. Indeed, an important role for chemokines in EAE pathogenesis has already been established as not only have encephalitogenic T cells been shown to produce high levels of chemokines but also the expression of both chemokines and chemokine receptors is known to be upregulated in the CNS during the disease (reviewed in Babcock and Owen (2003)). Additionally, administration of antichemokine antibodies ameliorates clinical signs of EAE (Karpus et al., 2003). Correspondingly, the beneficial effects of PARP inhibitors in EAE may occur as a result of a reduction in chemokine levels.

During the last several years, greater emphasis has been placed on the ability of PARP to regulate the expression of inflammatory mediators. Accordingly, PARP inhibitors have been shown to act as antiinflammatory agents in a broad range of conditions (reviewed in Virág and Szabó (2002)). One of the main ways through which PARP inhibitors exert antiinflammatory effects is thought to be by interfering with the expression of proinflammatory cytokines such as TNF- α and interferon (IFN)- γ (reviewed in Virág and Szabó (2002)). In EAE, IFN- γ and TNF- α expression appears to be downregulated in the CNS following the administration of PJ34, which may help account for the decreased CNS inflammation observed in these animals (Scott et al., 2004). The expression of iNOS, another proinflammatory molecule, was also reduced in CNS tissues from drug-treated mice (Scott et al., 2004). Modifying iNOS expression may well be another indirect antiinflammatory action of inhibiting PARP activity in EAE. iNOS has previously been proposed to play a principal role in the development of EAE, perhaps through the formation of NO and the subsequent generation of peroxynitrite (Giovannoni et al., 1998). Therefore, PARP inhibitors may prevent peroxynitrite production and so interfere with the various reactions that have been attributed to this molecule. Notably, our laboratory has recently demonstrated that peroxynitrite is involved in mediating the BBB breakdown and inflammatory cell infiltration associated with EAE (Hooper et al., 2000; Kean et al., 2000). While these effects may occur in response to peroxynitrite-induced activation of PARP, as we have suggested above, other peroxynitrite-mediated reactions may be responsible for the loss of neurovascular integrity and CNS inflammation observed in EAE. In this case, PARP inhibitors would not only modulate these peroxynitrite-mediated reactions but would also inhibit PARP activation, almost like a negative feedback loop.

Many of the proinflammatory actions of PARP are thought to be due to its effects on gene transcription. Moreover, a variety of transcription factors, including NF- κ B and AP-1, have been shown to be downregulated in the absence of PARP or following pharmacological inhibition of the enzyme (Chiarugi, 2002; Chiarugi and Moskowitz, 2003; Zingarelli et al., 2003, 2004). As both NF- κ B and AP-1 play fundamental roles in mediating immune and inflammatory responses, interfering with the proper functioning of these molecules may have wide ranging consequences. For instance, the capacity of PARP inhibitors to prevent iNOS expression is related to their effects on NF- κ B activation (Le Page et al., 1998). Likewise, the ability of PJ34 to regulate chemokine production has been shown to be associated with suppressed activation of NF- κ B (Hasko et al., 2002). Therefore, the downregulation of IFN- γ , TNF- α , iNOS, and ICAM-1, observed in spinal tissues from EAE-immunized mice successfully treated with PARP inhibitors, may be a result of impaired NF- κ B activation (Chiarugi, 2002; Scott et al., 2004).

In addition to the effects of PARP on inflammatory processes, there is evidence that PARP activity may modulate immune cell function (Broomhead and Hudson, 1985; King et al., 1989; McNerney et al., 1989; Weltin et al., 1995; Chiarugi, 2002). As EAE is thought to be triggered by autoreactive T cells, the ability of PARP inhibitors to protect against disease development may be related to direct effects of the PARP inhibitor on T cell function. However, there is some controversy as to whether or not PARP inhibitors directly interfere with T cell reactivity, which may be explained by differences in the experimental approaches and PARP inhibitors utilized (Broomhead and Hudson, 1985; McNerney et al., 1989; Weltin et al., 1995; Chiarugi, 2002). For example, several widely used pharmacological inhibitors of PARP have also been found to scavenge free radicals in addition to preventing enzyme activity (Szabó et al., 1998). We failed to detect any effect on antigen-specific proliferative responses of T cells from EAE-immunized mice when PJ34 was added to the culture medium. However, the *in vitro* proliferative response of cells from EAE-sensitized mice treated with PJ34 was significantly reduced by comparison with those from control animals (Scott et al., 2004). The results of cell-mixing experiments suggest that, in this case, the major effect of PJ34 treatment *in vivo* may be manifested at the level of T cell function (Scott et al., 2004). Lymphocyte proliferation may be reduced in animals administered PJ34 due to the accumulation of DNA strand breaks in these cells (Greer and Kaplan, 1986). Alternatively, PJ34 treatment may prevent T cell proliferation by nonselectively inhibiting CD38, a newly identified member of the PARP family of enzymes, which has been shown to be involved in lymphocyte signaling (Lund et al., 1998). Further insight into the mechanisms through which PARP may regulate T cell activity has been provided by a study illustrating that PARP inhibitors reduce IL-2 transcription in lymphocytes *in vitro* (Chiarugi, 2002). This investigation also demonstrated that IL-2 expression was decreased in spinal cord tissues from EAE-immunized rats treated with two structurally unrelated PARP inhibitors (Chiarugi, 2002). Since IL-2 is one of the main regulators of T cell proliferation and activation, it is possible that PARP modulates T cell function in EAE by modifying the production of this cytokine. In view of the fact that T cells play a pivotal role in the pathogenesis of EAE, it is quite possible that the therapeutic effects of PARP inhibitors, may, in fact, be due to modulation of T cell activity.

Although given that in some cases drug treatment was not started until 7 days following immunization, it is more likely that PARP inhibitors modify, rather than suppress, the immune response. For example, a bias from a largely T_H1 inflammatory response to a less inflammatory T_H2 response would be consistent with a reduction in PARP-mediated proinflammatory processes as well as the effects of PARP inhibitors on the clinical course of EAE. T_H2 cells produce cytokines with antiinflammatory properties and their activity has been linked to disease remission in EAE (Bettelli and Nicholson, 2000). Furthermore, altering the immune response in EAE toward a T_H2 phenotype has been shown to change disease progression (Bettelli and Nicholson, 2000). Since the T_H bias of an immune response is reflected by the antibody isotypes that are elicited, the switch from predominantly IgG2a to IgG1 and IgG2b antibody isotype production observed in PJ34-treated mice confirms that the antigen-specific T cell response changes from T_H1 to T_H2 . PARP activity may promote the T_H1 response through its known effects on the expression of proinflammatory genes (Chiarugi, 2002). Therefore, PARP inhibition may primarily be therapeutic in EAE by selectively interfering with the T_H1 inflammatory response that is pathogenic in this autoimmune disorder. As a result PARP inhibition in EAE manifests in a reduction in most aspects of CNS inflammation, including the loss of BBB integrity. However, it is important to remember that PARP activation has also been implicated in neurotoxicity (Virag and Szabó, 2002; Skaper, 2003). Consequently, inhibiting PARP may also be beneficial in EAE by maintaining cell viability, thus preventing functional deficits as well as reducing the release of new autostimulatory antigens.

5 PARP and Neurodegeneration

It has been suggested that PARP activation contributes to the pathogenesis of other forms of brain injury and neurodegenerative disorders. For instance, PARP activation has been implicated in the pathogenesis of Parkinson's disease, a chronic progressive neurologic disorder, related to the degeneration of the neurons of the substantia nigra that contain melanin—another disease where activation of NMDA receptors plays a

crucial role in its pathogenesis. The synthetic heroin analog, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), can selectively damage neurons in the nigrostriatal dopaminergic pathway and produce Parkinsonism in experimental animals (Przedborski and Jackson-Lewis, 1998; Blum et al., 2001). There is evidence for both the production of reactive oxygen intermediates (Cassarino et al., 1997; Hung and Lee, 1998) and NO-derived radicals/oxidants (Schulz et al., 1995a, b, c; Ara et al., 1998; Beal, 1998; Ferrante et al., 1999; Liberatore et al., 1999) in the pathogenesis of MPTP neurotoxicity. In brain injury induced by MPTP, the neuronal NO synthase is the source of cytotoxic NO and peroxynitrite. Accordingly, protection is provided by the neuronal NO synthase inhibitors 7-nitro-indazole or S-methylthiocitrulline (Schulz et al., 1995c; Przedborski et al., 1996; Matthews et al., 1997b; Ferrante et al., 1999). Furthermore, genetically engineered mice, which lack the bNOS gene, are resistant to toxicity induced by MPTP as compared to wild-type littermates (Matthews et al., 1997a). Direct evidence for the involvement of PARP in the pathogenesis of toxicity induced by MPTP comes from a mouse model of Parkinson's disease. MPTP treatment reduces striatal dopamine and cortical norepinephrine levels by more than 50% in these animals, while simultaneous treatment with each of five different inhibitors of PARP ameliorates the catecholamine depletion induced by MPTP (Cosi et al., 1996). The protective potency of benzamide and its derivatives parallels their efficacy as enzyme inhibitors (Cosi et al., 1996). Furthermore, recent studies have demonstrated that mice lacking functional PARP are also resistant against MPTP neurotoxicity (Mandir et al., 1999). Recent work, using "classical" PARP inhibitors, such as nicotinamide (Yang et al., 2004) as well as a variety of novel potent PARP inhibitors of various structural classes (Iwashita et al., 2004b), further implicates the PARP pathway in MPTP-induced neurodegeneration. Similar to the situation in stroke, there is evidence for nuclear/mitochondrial cross talk and PARP activation in MPTP neurotoxicity (Wang et al., 2003). Signal transduction pathways may also be affected. Mandir and coworkers (2002) demonstrated that p53 is heavily poly(ADP-ribosylated) by PARP-1 following MPTP intoxication and suggested that this posttranslational modification may serve to stabilize p53 and alter its transactivation of downstream genes. These influences of PARP-1 on mitochondrial and nuclear factors (including AIF, cytochrome c, NF- κ B, p53, etc.) may underlie the mechanisms of MPTP-induced Parkinsonism and other models of neuronal death.

There is also evidence for the role of the PARP pathway in other experimental models of neurodegeneration, including a model of methamphetamine-induced (nigrostriatal dopaminergic) neurotoxicity (Iwashita et al., 2004c).

NF- κ B is a transcription factor with key roles in the response to injury and inflammation. Various stimuli including inflammatory cytokines and ischemia converge on NF- κ B activation. The close relationship between NF- κ B and PARP-1 is demonstrated (Le Page et al., 1998; Hassa et al., 1999; Oliver et al., 1999) and discussed in the previous section. The activation of NF- κ B and p53 has been demonstrated in neurons exposed to cytotoxic concentrations of glutamate or β -amyloid, suggesting an active role of these transcription factors in acute and chronic neurodegeneration. (Chiarugi, 2002). Given that the cross talk between NF- κ B and p53 is central to the transcriptional decision making mechanisms that regulate cell fate (Chiarugi A, 2002), this transcriptional hypothesis might be pertinent to mechanisms underlying the initial inflammatory response associated with PARP-1-dependent gene activation not involving massive genome rupture, ATP depletion, overactivation of PARP1, and cell death (Chiarugi A, 2002). Another marker of the activated microglia is the phosphorylation of p38^{MAPK} signal transduction pathway, which seems to occur in most chronic neurodegenerative diseases such as Alzheimer's disease, MS, and HIV-associated dementia (Bhat et al., 1998; Kaul et al., 2001; Koistinaho and Koistinaho, 2002). Microglia from APP transgenic mice also show p38^{MAPK} activation (Koistinaho et al., 2002). Ha (2004) found a defective nuclear signaling of p38^{MAPK} in PARP-1^{-/-} glial cells in response to inflammatory stimuli. The study demonstrates in PARP-1^{-/-} glial cells a loss of several stress-activated transcription factors, as well as decreased expression of genes for cytokines and cellular adhesion molecules (Ha et al., 2002). These findings indicate that PARP-1 is an essential host factor among factors that actively mediate excessive production of proinflammatory molecules in glial cells, which may in turn contribute to the initiation of neuronal injuries. The role of PARP in HIV-associated neurodegeneration or in Alzheimer's disease is much less characterized than in models of Parkinson's disease (presumably because relevant preclinical models are scarce). Nevertheless, there appears to be some indication for the evidence of PARP in these diseases in

human brain samples. For instance, in autopsies of AIDS patients there appears to be an upregulation of PARP-1 (the enzyme itself, and not the product of the enzyme) (Wiley et al., 2000). It remains to be determined whether this upregulation is associated with increased catalytic activity of the enzyme, and whether PARP inhibition would influence the course of this disease. Similar to HIV-associated neurodegeneration, there is only circumstantial evidence for the role of PARP in amyotrophic lateral sclerosis (ALS)-mediated neuropathologies. In transgenic mice expressing a human Cu/Zn SOD mutation, i.e., SOD1(G93A), used as an *in vivo* model of ALS, immunohistochemical studies investigating the changes of PARP expression show an increase in glial cells (Chung et al., 2004). There is also an apparent overexpression of PARP in human ALS samples, at least in the astrocytes, while the PARP expression in motoneurons appears to be suppressed (Kim et al., 2003, 2004). As PARP is a constitutive enzyme, which is generally regulated on the level of its enzymatic activity (rather than its level of expression), studies of the type quoted above are not very helpful in pointing toward the functional role of this enzyme in the disease. Although there are murine models of ALS, interventional studies using potent PARP inhibitors or crossing of ALS animals with PARP-1-deficient animals has not yet been performed. In one study, the compound INH2BP, which has relatively modest potency, failed to demonstrate effects on the progression of the disease (Andreassen et al., 2001).

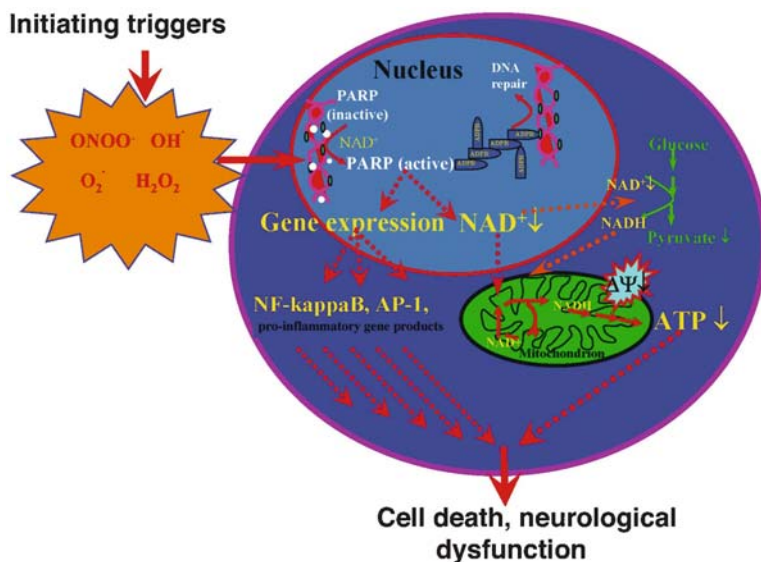
6 Conclusions and Future Directions

Evidence in support of the protective actions of PARP inhibitors in *in vivo* models for human acute and chronic ischemic, neuroinflammatory and neurodegenerative diseases is rapidly growing. Prototypical benzamide and quinoline compounds as well as more novel PARP inhibitors (e.g., GPI-6150, PJ34 or INO1001) (Abdelkarim, 2001; Mazzon, 2001; Komjati, 2004) have been shown to have protective effects in animal models relevant to human diseases related to inflammation and ischemia, including stroke. The mechanism by which these inhibitors of PARP provide protection might not entirely be related to the preservation of cellular energy stores, but might also include other PARP-mediated mechanisms (e.g., controls of transcription factors and related gene expression and modulation of mitochondrial release of cell death factors) (▶ [Figure 20-1](#)). The regulation by PARP of these factors may be even more important in its pathogenesis in neuroinflammation and neurodegeneration. Overall, PARP inhibitors block several common pathway(s) of tissue injury, such as NF- κ B activation or oxidative/nitrosative stress-induced cytotoxicity, and the relative contribution of direct cytoprotective effects, effects on signal transduction and inflammatory pathways, and effects on mitochondrial functions and proteins may be different from experimental model to experimental model, as well as from disease to disease. It is clear that PARP is not the only factor involved in the pathogenesis of cell and organ injury in response to oxidant or nitrosative stress in neuroinjury. The relative importance of PARP in mediating oxidant injury is also dependent on cell type. Furthermore, the protection is lost when cells are challenged with extremely high concentrations of oxidants which trigger cytotoxic effects independent of PARP, such as direct inhibitory effects on the mitochondrial respiratory chain or inhibitory effects on other intracellular energetic or redox processes. Finally, direct interactions of the oxidants with proteins, lipids, arachidonic acid, and other molecules may also play a significant role in the development of cellular injury. It is likely that there are important synergistic interactions between PARP activation and these other cellular processes of cytotoxicity. In fact, recent work demonstrates additive or synergistic neuroprotective actions of peroxynitrite decomposition catalyst and PARP inhibitor compounds in stroke (Sharma et al., 2004). From the evidence presented here, it seems that inhibition of PARP alone can “tip the end of the balance” and significantly influence the outcome of acute and chronic CNS diseases.

An exciting development in PARP research has been the discovery of new poly(ADP-ribosylating) enzymes. It is not known at present, how PARP inhibition therapy affects the function of these minor isoforms and whether or not inhibition of the minor PARP isoforms contributes to the well-established *in vivo* effects of PARP inhibitors. Bearing in mind that results of most pharmacological studies could be reproduced by using PARP-deficient animals and cells, we conclude that PARP-1 is the major target of PARP inhibitors in stroke and neuroinjury. Development of isoform-selective PARP inhibitors and generation of

■ Figure 20-1

PARP-dependent cytotoxic pathways involving nitric oxide (NO^*), hydroxyl radical (OH^*), superoxide (O_2^-), and peroxynitrite (ONOO^-) in the pathogenesis of CNS diseases. Ischemia, reperfusion, release of excitatory amino acids, followed by NMDA receptor activation triggers the production of reactive oxygen species (ROS) from a variety of cellular sources, and may also induce the activation of the neuronal isoform of NOS (bNOS). During 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity, a key source of the ROS is the mitochondrion (as opposed to NMDA receptor activation) (not shown in the current scheme). In neuroinflammation and other forms of neurodegeneration, reactive oxygen and nitrogen species can be derived from a variety of cellular and extracellular sources, including inducible nitric oxide synthase (iNOS). NO , in turn, combines with superoxide to yield peroxynitrite. OH^* (produced from O_2^- via the iron-catalyzed Haber–Weiss reaction) and peroxynitrite induce the development of DNA single-strand breakage, with consequent activation of poly(ADP-ribose) polymerase (PARP) (Dawson, 1995). There may be an additional, calcium-dependent cellular activation of PARP as well (Virag et al., 1999; Homburg et al., 2000). Depletion of the cellular NAD^+ leads to inhibition of cellular ATP-generating pathways leading to cellular dysfunction. NO alone does not induce DNA single-strand breakage, but may combine with (O_2^-) (produced from the mitochondrial chain or from other cellular sources) to yield peroxynitrite. PARP activation, via a not yet fully understood fashion, promotes the activation of nuclear factor (NF) κB , AP-1, MAP kinases, and the expression of proinflammatory mediators, adhesion molecules such as ICAM-1, endothelin, various cytokines, and chemokines of iNOS. By promoting proinflammatory mediator production, neutrophil recruitment, and oxidant generation, positive feedback cycles are triggered, which may play important roles in the pathogenesis of neuroinflammation, but also in the delayed stage of stroke and neurotrauma. PARP activation and mitochondrial oxidant production form another positive feedback cycle. Not shown on this scheme are the pathways whereby PARP regulates the mitochondrial release of apoptosis-inducing factor (AIF) (and possibly other death signals), initiating additional positive feedback cycles of injury (Hong et al., 2004). Ultimately, PARP activation leads to cell dysfunction, cell death (via the necrotic, rather than apoptotic route), and to the upregulation of a variety of inflammatory pathways. The relative contribution of each of the above-mentioned pathways depends on the type and stage of the particular neuroinflammatory or neurodegenerative disease



knockout mice deficient in the novel PARP enzymes will clarify the biological roles of the additional PARP inhibitors. It must be emphasized at this point that data obtained from knockout studies cannot always be extrapolated to situations where PARP is present but is inhibited by pharmacological agents (Simbulan-Rosenthal et al., 2000, 2001).

Table 20-3
The role of PARP in neuronal injury during neuroinflammation and neurodegeneration

Experimental model	Inducer of injury	Mode of pharmacological or genetic intervention	Role of PARP	References
Newborn piglet	<i>Escherichia coli</i> meningitis	3-AB	Decrease in lactate production, neutrophil infiltration, TNF- α production, peroxidation, and ATP depletion	Park et al. (2001)
Mice, rats	<i>Streptococcus pneumoniae</i> meningitis	3-AB, PARP ^{-/-} phenotype	Improved clinical outcome (less BBB breakdown, less increase in ICP, lower leukocyte number, and inflammatory cytokine level in CSF)	Koedel et al. (2002)
Mice	EAE	INH2BP	PARP activation	Scott et al. (2001)
Rat	EAE	3-AB, Benzamide, INH2BP	Prevents disease progression	Scott et al. (1998, 2001)
Rat	EAE	PHT	Prevents disease progression, downregulates NF κ B expression, reduced IL-2 expression in spinal cord tissue	Chiarugi et al. (2002)
Mice	EAE	INH2BP	Abrogated disease signs	Scott et al. (2001)
Mice	EAE	PJ34	Reduced neurological signs, improved survival, maintained neurovascular integrity, suppressed expression of ICAM-1 in the CNS, IFN- γ and TNF α and iNOS expression downregulated	Scott et al. (2004)
Rat	Peroxyntirite	-	Axonal injury and myelin damage induced through PARP activation	Touil et al. (2001)
Mouse	MPTP	PARP ^{-/-} phenotype	Reduced cell death, improved function, protection against dopamine loss	Mandir et al. (1999)
Mouse	MPTP	Benzamide, ISQ, nicotinamide	Maintained striatal NAD ⁺ and ATP protection against dopamine loss	Cosi et al. (1994, 1996); Yang et al. (2004)
Rat	MPP(+)	3-AB	No protection against the impairment of neuronal dopamine transporter activity	Barc et al. (2001)
Mouse	MPTP	FR255595	Improved neurological outcome and protection against neuronal loss	Iwashita et al. (2004b)
Mouse	Methamphetamine	FR261529	Attenuated the damage to dopaminergic neurons without changes in dopamine metabolism	Iwashita et al. (2004b)

Abbreviations: 3-AB, 3-aminobenzamide; ATP, adenosine triphosphate; BBB, blood-brain barrier; CNS, central nervous system; CSF, cerebrospinal fluid; DPQ, 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]1(2H)-isoquinolinone; EAE, experimental allergic encephalomyelitis; ICAM-1, intercellular adhesion molecule-1; ICP, intracranial pressure; INH2BP, 5-iodo-6-amino-1,2-benzopyrone; ISQ, 1,5 dihydroxyisoquinoline; LPS, bacterial lipopolysaccharide (endotoxin); MPP(+), 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine;

The marked beneficial effect of PARP inhibitors in many animal models suggests that PARP inhibitors can be exploited to treat human diseases. However, before potent PARP inhibitors could be used in humans, crucial safety issues must be addressed. It is important to point out that some degree of PARP-1 or compensatory poly(ADP-ribosyltransferase) activity may be essential for DNA repair (Burkle et al., 2001) and for many global cellular functions (Burkle et al., 2001) as well as memory formation (Satchell et al., 2003). Thus, partial PARP-1 inhibition may be a more desirable approach to the treatment of brain injury than complete inhibition. The various potential problematic issues surrounding the clinical development and testing of PARP inhibitors including the risk/benefit ratio has been discussed recently (Southan and Szabó, 2003). Briefly, it appears that the risk of interfering with DNA repair and genetic processes is relatively small, when the PARP inhibitor is given in short courses of administration (i.e., several days only). Also, when PARP inhibitors are used to treat severe life threatening or serious debilitating diseases, the regulatory agencies tend to be more lenient with respect to side effect profiles.

Overall, the weight of evidence points toward the importance of PARP in the pathogenesis of stroke, neurotrauma, and neuroinflammation. There is direct evidence for the involvement of PARP in Parkinson's disease and indirect evidence for a variety of other chronic neurodegenerative diseases. The body of literature overviewed in the current chapter (see also [Tables 20-1–20-3](#)) should encourage the advanced preclinical and eventual clinical testing of potent, CNS-permeable PARP inhibitors for acute and chronic neuroprotection.

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21 Schizophrenia and Immune Responses

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- 1 *Background* 468

- 2 *Does the Immune System Contribute to the Etiology of Schizophrenia?* 469
 - 2.1 Similarity Between Schizophrenia and Some Autoimmune Disease 469
 - 2.2 Autoimmune Diseases and the Genetics of Schizophrenia 469
 - 2.3 HLA Antigens in Schizophrenia 470
 - 2.4 Materno-Fetal Incompatibility 471
 - 2.5 Maternal Immune Response to Viral Infections 471
 - 2.6 Viral Antibodies in Patients with Schizophrenia 473
 - 2.7 Autoantibodies in Schizophrenia 473
 - 2.8 Antineuronal Antibodies 474

- 3 *Immune Changes Associated with Schizophrenia: Coincidence, Neuromodulators, or Consequence?* 474
 - 3.1 The Immune System as Neuromodulator 474
 - 3.2 Interleukin 1 and Interleukin 1 Receptor Antagonist 475
 - 3.3 Interleukin 2 475
 - 3.4 Interleukin 6 476
 - 3.5 Interferons 476
 - 3.6 Other Cytokines 477
 - 3.7 Hypothalamic–Pituitary Axis 477
 - 3.8 The Acute Phase Response in Schizophrenia 478
 - 3.9 Immunoglobulin Isotypes in Schizophrenia 478
 - 3.10 T Lymphocytes and B Lymphocytes in Schizophrenia 479
 - 3.11 Vaccine Response 479

- 4 *Effects of Antipsychotic Medication on Immune Markers* 480

- 5 *Treatment of Schizophrenia with Immunosuppressants* 481

- 6 *Summary* 481

Abstract: The etiology of schizophrenia is, as yet, unclear. The multiple-hit theory suggests that environmental insults combine with genetic susceptibility to increase the risk of developing clinical disease. In schizophrenia, alterations in immune responses have been described for many decades but their significance is uncertain. Immunological responses are at least partially genetically determined, but largely stimulated by environmental factors. In the presence of a genetic vulnerability of schizophrenia, immune responses may be relevant in a number of different ways: They may result in the neurodevelopment abnormalities seen in the condition; may modulate the ongoing course of illness; may be a direct result of the illness or its treatment or may merely be linked to an associated condition. Here we explore the published clinical, epidemiological and laboratory data on the immune system as it relates to schizophrenia, and look at the opportunities for future work in the field.

List of Abbreviations: ANA, antinuclear antibodies; BBB, blood–brain barrier; BDV, borna disease virus; BMI, body mass index; DST, dexamethasone suppression tests; EBV, Epstein-Barr Virus; GAD, glutamic acid decarboxylase; HHV, human herpesvirus; HPA, hypothalamic–pituitary axis; HSP, heat-shock proteins; HSV, herpes simplex virus; IAA, insulin autoantibodies; ICA, islet cell antibodies; IDDM, insulin dependent diabetes mellitus; IFN, interferon; IL, interleukin; LIF-R, leukemia inhibitory factor receptor; LPS, lipopolysaccharide; MHC, major histocompatibility complex; PBMs, peripheral blood mononuclear cells; PPI, prepulse inhibition; PUFAs, polyunsaturated fatty acids; RBC, red blood cell; SLE, systemic lupus erythematosus; TNF, tumor necrosis factor

1 Background

Schizophrenia is a psychotic illness affecting almost 1% of the worldwide population. Onset is commonly in late adolescence or young adulthood. Diagnosis of schizophrenia is made by recognition of categorized clinical features. Psychotic features such as delusions or hallucinations are classified as positive symptoms whereas poor motivation, social withdrawal, day–night reversal, poor self-care, and poverty of thought are considered negative symptoms. Other common features include disorganization in thought and speech. Disturbance in attention, executive function, and working memory are also observed and greatly contribute to the functional disability associated with the disorder. Schizophrenia is a chronic illness, thus management is expensive and lifelong both in terms of human resources and intervention.

The etiology of schizophrenia is, as yet, unclear. It is possible that schizophrenia, as we define it, is more than one disease and it is almost certain there is more than one cause. There is undoubtedly a significant genetic component, with heritability in the region of 80%, although this is likely to be related to a combination of many genes rather than a single “schizophrenia” gene. A multiple-hit theory has been proposed. This theory suggests that environmental insults combine with genes conveying a susceptibility to schizophrenia increasing the chance of developing the clinical phenotype.

Environmental factors linked to schizophrenia have been considered throughout gestation and natural life span. First trimester famine (Susser and Lin, 1992; St Clair et al., 2005) and maternal exposure to an influenza epidemic (O’Callaghan et al., 1991a; Cooper, 1992) have been implicated, progressing to obstetric complications (Cannon et al., 2002) and perinatal viral infections (Koponen et al., 2004). Later adult risk factors include head injury (Abdel Malik et al., 2003), regular cannabis use (Henquet et al., 2005), psychosocial disadvantage, and isolation (Kohn, 1968). For example, schizophrenia is more common in cities and occurs more frequently in ethnic minority populations (Fearon et al., 2006).

By the time a person presents with schizophrenia, brain changes are often evident on MRI (Nopoulos, 1995). The most consistent abnormalities are enlargement of the ventricles and dilatation of the cortical sulci, along with decreased cerebral volume of the cortices and hippocampus. These enlargements are present from the first episode of illness. Harrison (1999) reviewed the neuropathology of schizophrenia and found good evidence for preferential involvement of the temporal lobe and moderate evidence for an alteration in normal cerebral asymmetries. Neuropathologically, reduced neuropil and neuronal size, rather than number, account for the volume changes, suggesting alterations in synaptic, dendritic, and axonal organization. A relative absence of gliosis, or scar tissue, suggests that these structural changes may date

from before the third trimester of intrauterine development (Waddington, 1993). Minor physical and dermatoglyphic (fingerprint) anomalies in schizophrenic patients of both sexes and the notably high incidence of obstetric complications in male patients, provide further evidence of intrauterine insult (O'Callaghan et al., 1991b, 1992; Bracha et al., 1992). The theory that the neuropathology predates the onset of symptoms is reinforced by studies suggesting that those who go on to develop schizophrenia are more likely to have shown abnormalities of behavior in childhood (Jones et al., 1994).

There is good evidence to show that the monoamine neurotransmitters, dopamine and serotonin, are important in the pathogenesis of schizophrenia. More recently, glutamatergic deficiency (Carlsson and Carlsson, 1990) and reduced GABAergic function have been suggested as causative factors (Deutsch et al., 2001). Dopamine agonists, such as L-dopa and amphetamine, along with glutamatergic antagonists such as ketamine can produce acute psychosis. All the drugs used for the treatment of schizophrenia over the last four decades have in common an antagonism of dopamine. More modern "atypical" neuroleptics often exhibit both dopamine and serotonin receptor blockade.

Immune abnormalities in schizophrenia have been described for many decades predating the invention of antipsychotic medication (Lehmann-Facius, 1939). There are a number of possible roles that the immune system may play in the causation of schizophrenia. First, the early neurodevelopmental changes described above may be the result of immunological insults to the developing brain, resulting in the later development of the schizophrenia phenotype. Second, the immune system may modulate the ongoing clinical course of the illness. Third, the immunological findings associated with schizophrenia could be the result, rather than the cause of the illness, or linked to other immune conditions occurring coincidentally. Last, although some findings date from the 1930s, the effect of neuroleptics on the immune system has to be explored and accounted for in the interpretation of most modern studies.

2 Does the Immune System Contribute to the Etiology of Schizophrenia?

2.1 Similarity Between Schizophrenia and Some Autoimmune Disease

Various similarities exist between schizophrenia and autoimmune disease. For example, the remitting/relapsing course of disease is common to both, as is a variable age of onset: Both systemic lupus erythematosus (SLE) and thyrotoxicosis display a correlation in age of onset within families and this is also seen in families with multiple members diagnosed with schizophrenia. Both schizophrenia and autoimmune disease can be precipitated by drugs, physical injury, or infection, and both have similar monozygotic twin concordance rates. Patients with insulin-dependent diabetes mellitus (IDDM), like in schizophrenia, cluster toward winter births. (Knight et al., 1992).

Schizophrenia-like symptoms can be directly induced by autoimmune illnesses, introducing the possibility of misdiagnosis. Such illnesses may be chronic, as in CNS-SLE, or acute, such as Sydenham's chorea. SLE and antiphospholipid syndrome, a disorder that sometimes coexists with SLE, can mimic symptoms of schizophrenia (Brey and Escalante, 1998; Khan et al., 2000). More acutely, Sydenham's chorea can cause a psychosis that is associated with obsessional thoughts and abnormal involuntary movements, which wax and wane in line with changes in systemic antibrain antibody titers (Swedo et al., 1993).

2.2 Autoimmune Diseases and the Genetics of Schizophrenia

Epidemiological associations between schizophrenia and various autoimmune diseases have been well described and are both positive and negative. Positive associations are those where a higher incidence of a condition occurs in the schizophrenic population, and negative associations are where the two conditions are less likely to co-occur. As well as the increased risk of autoimmune conditions in general (Ganguli et al., 1987), there are particular associations between schizophrenia and disorders such as coeliac disease (Eaton et al., 2004), supported by reports of gluten-free diets improving symptoms (Dohan and Grasberger, 1973). There are negative associations with conditions such as rheumatoid arthritis (Eaton et al., 1992) and

possibly with IDDM (Finney, 1989). Mothers of patients with schizophrenia also have lower rates of rheumatoid arthritis than controls, mirroring the findings in the patients themselves (Baldwin, 1979; Gilvarry et al., 1996). Although autoimmune disorders tend to occur together, negative associations can result from genes having opposing effects on the risk of developing different disorders.

The schizophrenia–rheumatoid arthritis connection is particularly interesting and has been explored by Torrey and Yolken (2001). Both disorders have a prevalence of approximately 1% in North America and Europe, have monozygotic twin concordance rates of approximately 30%, and are more common in urban areas. Significantly, both diseases have been associated with similar class II HLA antigens and have been suspected of having infectious etiology. It is possible that the inverse correlation between these two diseases derives from a mutually exclusive response to a common infectious and/or immune etiology and that once an individual develops either disease they are relatively immune to the other.

The propensity of autoimmune conditions linked with schizophrenia is not limited to just the sufferers but also extends to the families. Therefore, the inheritance of schizophrenia may be related to that of autoimmune disorders. People with a schizophrenic first-degree relative are more likely to have a parent or sibling with an autoimmune disease (Wright et al., 1996b). Some examples of this relationship include the following. Autoimmune thyroid diseases, both thyrotoxicosis and myxedema, are more common in first-degree relatives of psychotic patients. An excess of IDDM is found in families of patients with schizophrenia compared with controls (odds ratio (OR) = 9.65) (Wright et al., 1996). Furthermore, mothers of patients with schizophrenia are more likely to have diabetes mellitus during pregnancy than controls, (OR = 7.76) (Cannon et al., 2002) although here the type of diabetes was unspecified.

However, the most convincing evidence comes from a recent, large epidemiological study using national registers in Denmark. There, a past history of any autoimmune disease was associated with an increase in risk for schizophrenia in the order of 45%. Furthermore, in this study, schizophrenia was associated with a larger range of autoimmune diseases than previously thought. Nine different autoimmune disorders had higher prevalence rates among patients with schizophrenia than among comparison subjects, whereas 12 autoimmune diseases had higher prevalence rates among parents of schizophrenia patients than among parents of comparison subjects. Thyrotoxicosis, coeliac disease, acquired haemolytic anaemia, interstitial cystitis, and Sjögren's syndrome had higher prevalence rates among both patients with schizophrenia and families of such patients than in their respective control groups (Eaton et al., 2006).

2.3 HLA Antigens in Schizophrenia

Reviews of early HLA studies in schizophrenia were considered limited by their use of unstandardized diagnostic criteria (Wright et al., 2001). Nevertheless, several groups have reported associations of HLA: A9, A28, A10, DRB1*01, and DRw6 with schizophrenia, although the cumulative evidence thus far remains weak.

Wright and coworkers (1996a) reported a negative association between schizophrenia and IDDM in patients with HLA genotype DQB1*0602 suggesting this allele may be protective against IDDM. Another HLA type examined, DRB1*04, is a genotype known to be associated with rheumatoid arthritis with a gene locus on chromosome 6p21.3. This chromosome was examined in 94 patients with schizophrenia and 92 unrelated mothers of schizophrenic patients. Decreased HLA DRB1 *04 gene frequency was found in both the schizophrenics and the unrelated mothers, suggesting this allele may have a protective role against schizophrenia as well as a positive association with rheumatoid arthritis, and therefore, supportive of the previously described negative association between rheumatoid arthritis and schizophrenia.

Thus, we have some evidence that HLA alleles at DQB1*0602 and DRB1*04 (or linked loci) may protect against schizophrenia. Wright suggests possible mechanisms for this. First, some property of the HLA molecules encoded by these alleles may directly protect against schizophrenia. Second, a negative genetic association with HLA DQB1*0602 and DRB1*04 may be secondary to a positive association at a linked locus. As a third possibility, he suggests that pedigrees with a schizophrenic member may have a propensity

for autoimmune diseases and the presence or absence of various HLA alleles may influence whether IDDM, rheumatoid arthritis, or schizophrenia develops.

Genetic linkage studies have found evidence of a susceptibility locus for schizophrenia on the short arm on chromosome 6 near the HLA region at 6p21.3. Although there have been some negative studies, linkage has been found for loci D6S274, D6S296, and D6S291, which map close to the HLA region (Wright et al., 1996a). A German linkage and association study likewise was not in favor of a direct involvement of the HLA system in schizophrenia, but was compatible with the existence of a susceptibility gene in the major histocompatibility complex (MHC) region at chromosome 6p21.31 (Schwab et al., 2002).

In addition to direct effects on the occurrence of schizophrenia, it appears that HLA type may be relevant in response to treatment. There is an increased frequency of HLA-A1 in patients with schizophrenia who do not respond to conventional drugs, but do respond to clozapine. HLA-A1 also predicts a low risk of agranulocytosis on clozapine treatment (Lahdelma et al., 2001).

2.4 Materno-Fetal Incompatibility

Exposure to maternal antibodies against paternally derived antigens in the developing fetus is one possible risk factor for schizophrenia. An example of materno-fetal incompatibility is rhesus (Rh) incompatibility, where a materno-fetal genotype interaction induces an adverse intrauterine environment, which increases later life susceptibility to schizophrenia. In a meta-analysis, Rh incompatibility was associated with a twofold increase in the incidence of schizophrenia (Cannon et al., 2002).

However, Rh is not the only antigen that can spark a maternal immune response. For example, maternal antibodies against paternally derived HLA inherited by the fetus are detectable in the circulation of ~20% of primigravidae and ~40% of multigravidae (Payne and Rolfs, 1958; van Rood et al., 1958).

Given the complex inheritance of schizophrenia, it is useful to explore patterns consistent with materno-fetal incompatibility of unknown antigens as a possible etiologic factor. One such pattern is birth order. Increased illness liability with advancing birth order is consistent with progressively increasing materno-fetal immune reactions. Studies of rates of schizophrenia in relation to birth order have proved inconsistent, with some noting higher rates in later born children, whereas others finding no such effect, or even the reverse (Sham et al., 1993; Westergaard et al., 2001).

However, if a substantial proportion of cases of schizophrenia result from materno-fetal incompatibility, then later born children in multiply affected families might be expected to exhibit a more severe form of the illness than their older siblings. Consistent with this, later born children in families multiply affected by schizophrenia have a reduced likelihood of regaining their premorbid level of functioning after an acute episode of illness, and also present with the condition at an earlier age than their older affected siblings (Gaughran et al., in press). Additionally, patients with many older siblings are less likely to recover than those with few (Farina et al., 1963). Materno-fetal incompatibility is therefore an etiologic theory that merits further investigation.

2.5 Maternal Immune Response to Viral Infections

It has been suggested that some cases of schizophrenia may have a viral origin and that the effects of the virus on the developing brain may be immunologically mediated. Influenza epidemics have been followed by an increase in births of people who later go on to develop schizophrenia (Cooper, 1992). Schizophrenia has also been associated with maternal exposure to rubella and elevated first trimester maternal influenza antibodies (Brown et al., 2001, 2004a). The offspring of mothers with elevated levels of total IgG and IgM immunoglobulins and antibodies to herpes simplex virus type 2 are also at increased risk for the development of schizophrenia and other psychotic illnesses (Buka et al., 2001). Postnatal Coxsackie B5 infection likewise results in a higher risk of schizophrenia (Rantakallio et al., 1997).

It has been proposed that virus induced maternal antibodies may cross the placenta and the immature blood–brain barrier to cross-react with fetal brain tissues. These antibodies may interfere with neurodevelopment, resulting in schizophrenia in later life (Wright et al., 1993). Rabbits inoculated with influenza A virus produce an antibody that cross-reacts with a protein in the human hippocampus, cortex, and cerebellum leading to suggestions that certain mothers, perhaps those with enhanced antibody resistance to viral infections, are immunologically predisposed to such a reaction (Laing et al., 1996). Interestingly, relatives of patients with schizophrenia go to the doctor with fewer viral infections than do controls although they present with similar rates of bacterial infections (Carter and Watts, 1971).

Respiratory infection with the human influenza virus in pregnant mice yields offspring that display highly abnormal behavioral responses as adults. As in schizophrenia and autism, these offspring display deficits in prepulse inhibition (PPI) in the acoustic startle response. Moreover, these mice are deficient in exploratory behavior in both open-field and novel-object tests, and they are deficient in social interaction. The abnormal behavior in the infected mice responds to antipsychotic drugs. Therefore, maternal viral infection has a profound effect on the behavior of adult offspring, possibly via an effect of the maternal immune response on the fetus (Shi et al., 2003).

Schizophrenia is therefore associated with both maternal exposure to infection and obstetric complications (O'Callaghan et al., 1991a, 1992). Cytokines, including interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α , are altered in amniotic fluid or neonatal cord blood in pregnancies complicated by infection (Gillmore and Jarskog, 1997). Given that maternal cytokines can cross the placenta, birth trauma, associated with a disturbed blood–brain barrier (BBB), could facilitate invasion of immune activating agents into the developing CNS (Muller and Achenheil, 1998). In a well-designed epidemiological study, levels of IL-8, a cytokine elevated in viral infections, were significantly higher during the second trimester in mothers of offspring with schizophrenia spectrum disorders than in comparison mothers (Brown et al., 2004b). This could be another mechanism accounting for the neurodevelopmental changes that ultimately present as schizophrenia.

Evidence that the neurodevelopmental insult derives from the maternal immune response, rather than from the viruses themselves, is provided by studies that exposed pregnant rats to the synthetic cytokine releaser polyriboinosinic–polyribocytidylic acid (poly I: C). Their offspring exhibited latent inhibition disruption in adulthood, which was reversed by antipsychotics, a clinical feature of schizophrenia in the human. In addition, prenatal immune activation led to morphological alterations in the hippocampus and the entorhinal cortex in the adult offspring, consistent with the well-documented mesolimbic dopaminergic and temporolimbic pathology in schizophrenia (Zuckerman et al., 2003). Therefore, prenatal poly I: C administration may provide a neurodevelopmental model of schizophrenia that reproduces a putative inducing factor, mimics the temporal course as well as some central abnormalities of the disorder, and predicts responsiveness to antipsychotic drugs.

Another substance capable of inducing an immune response similar to that resulting from infection is bacterial endotoxin lipopolysaccharide (LPS). The effect of peripheral administration of LPS to pregnant rats upon PPI and immune function in adult offspring was measured. Impaired ability to “gate out” sensory and cognitive information is considered to be a central feature of schizophrenia and is manifested, among others, in disrupted PPI of the acoustic startle reflex. Prenatal LPS treatment disrupted PPI and this was reversed by antipsychotics. Serum levels of IL-2 and IL-6 were also increased (Borrell et al., 2002).

Cytokines affect neurodevelopment if given to the neonate. Sublethal doses of four proinflammatory cytokines, IL-1 α , IL-2, IL-6, and interferon- γ (IFN- γ), were administered to rat pups. These animals displayed alterations in physical development; but behavioral abnormalities also emerged at different developmental stages, depending on the type of cytokine administered (Tohmi et al., 2004).

Gillmore and coworkers (2004) studied the effect of cytokines generated in response to infection, namely interleukin-1 β (IL-1 β), TNF- α , and interleukin-6 (IL-6)-on the dendritic development of cortical neurons. At higher doses, each cytokine reduced the number of primary dendrites, nodes, and total dendritic length, while neuron survival was reduced by 14%–21%. Inflammatory cytokines can thus reduce dendrite development and the complexity of developing cortical neurons, reminiscent of the neuropathology observed in schizophrenia. These findings also support the hypothesis that cytokines play a key mechanistic role in the link between prenatal exposure to infection and risk for schizophrenia.

2.6 Viral Antibodies in Patients with Schizophrenia

Viral infections may play a role in the etiopathogenesis of some cases of schizophrenia. Increased serum and CSF IgG antibody to cytomegalovirus and *Toxoplasma gondii* are found in untreated schizophrenia. This group also had reduced serum antibody to human herpesvirus (HHV) type 6 and varicella-zoster virus (Leweke et al., 2004). Antibodies to herpes simplex virus (HSV) type 1 and 2, and Epstein Barr virus (EBV) were similar in cases and controls. Other infections include borna disease virus (BDV), which has been especially linked to negative symptoms of schizophrenia (Terayama et al., 2003).

Although the antibody levels to these viruses are increased, the question of whether schizophrenia is precipitated by an acute exacerbation of a viral infection has yet to be addressed. Antibodies to 5 herpesviruses (HSV type 1, CMV, EBV, varicella-zoster virus, and HHV type 6), as well as 6 other viruses (measles, rubella, mumps, influenza A and B, and Japanese encephalitis viruses) were recorded over time in eight patients with acute onset or exacerbation of schizophrenia (Fukuda et al., 1999). No change in antibody levels or new antibody was observed. It was therefore felt unlikely that an active infection or reactivation of these viruses had a direct causal relationship to schizophrenia. Moreover, in a longitudinal general population sample in the UK, the number of common childhood viral illnesses per individual did not relate either to the number of soft neurological signs, or to any particular adult outcome. Schizophrenia, affective psychosis, and epilepsy were not associated with common childhood illness although they were associated with an increased, but small, frequency of previous meningitis and tuberculosis (Leask et al., 2002).

2.7 Autoantibodies in Schizophrenia

Is it possible that antibody damage to the brain could result in the syndrome we know as schizophrenia?

Antibodies to brain and nonbrain tissue in schizophrenia have been widely reported. Antibodies to non-CNS specific antigens reported in schizophrenia are many, and include antihistone antibodies, anti-ganglioside antibodies, rheumatoid factor, anticardiolipin antibodies, lupus anticoagulant, and antibodies to nicotinic acetylcholine receptors (Chengappa et al., 1991, 1992b; Yannitsi et al., 1991; Stevens and Weller, 1992; Mukherjee, 1994). Platelet autoantibodies inhibiting dopamine uptake have also been described, which is interesting in the context of the dopamine hypothesis for schizophrenia (Kessler and Shinitzky, 1993). Symptom-dependent correlations have been established between platelet-associated antibody levels and degree of psychosis as measured by brief psychiatric rating scale scores (Sinyakov et al., 2003).

Not only unmedicated patients with schizophrenia, but also their healthy relatives have high levels of anticardiolipin antibodies, (Chengappa et al., 1991; Firer et al., 1994) implying a possible association with antiphospholipid syndrome, a disorder characterized by anticardiolipin antibodies and lupus anticoagulant, resulting in "sticky blood."

Antinuclear antibodies (ANA), as associated with SLE, have long been linked to antipsychotic medication, but high levels are also found in drug-free patients, and so it is reasonable to conclude that the elevation cannot be entirely explained by medication. Increased ANA, anti-double-strand DNA, and anti-single-strand DNA titres have been reported in patients with schizophrenia and in their healthy first-degree relatives with no significant difference in autoantibody systems between the patients and their relatives (Sirota et al., 1991). This is noteworthy as SLE can present with a schizophrenia-like picture.

Non-right-handed, neuroleptic-naïve men with schizophrenia have high autoantibody levels (Chengappa et al., 1992a). Non-right-handedness is especially prevalent in schizophrenia (Gur, 1977). Chengappa and coworkers (1992a) suggested that if a fetus was subjected to a dominant hemisphere insult, this could expose the immune system to released intracellular antigens, precipitating an antibody response, while simultaneously resulting in non-right-handedness. The association of non-CNS antibodies with a past history of obstetric complications in schizophrenia (Chengappa et al., 1995) may also be explained in this way.

The high co-occurrence and familial aggregation of IDDM and schizophrenia suggests a shared etiology. IDDM is well established as an immunological disorder, associated with glutamic acid decarboxylase

(GAD) antibodies, islet cell antibodies (ICA), and insulin autoantibodies (IAA). IDDM, like schizophrenia, is associated with exposure to Coxsackie B virus, which shares sequence similarity with GAD (Horwitz et al., 1998).

GAD is a GABA-synthesizing enzyme, located in pancreatic islet cells and nervous tissue. As discussed, reduced GABAergic function may be a causative factor for schizophrenia (Deutsch et al., 2001). Reduced brain expression of GAD67 has been described in schizophrenia (Guidotti et al., 2000). Gaughran and coworkers (2005) therefore tested the hypothesis that exposure to maternal GAD antibodies was an etiologic factor in schizophrenia, but found no elevation of GAD antibody levels in mothers of people with the disorder. These mothers however, were not sampled during pregnancy, but rather after their adult children were diagnosed with schizophrenia. Therefore, although diabetes-related exposure to maternal GAD antibodies is unlikely, as these antibodies tend to persist over time, further studies are needed to examine the possibility of a transient maternal GAD antibody response to coxsackie virus infection affecting a genetically vulnerable fetus.

2.8 Antineuronal Antibodies

Lehmann-Facius first recorded antibrain antibodies in schizophrenia as far back as 1939. High titres of antibrain antibodies have been found in the serum and CSF, not just of patients but also from their relatives (Pandey et al., 1981). These antibrain antibodies are more frequent in those with a family history of schizophrenia.

Antibodies to specific brain regions, including the hippocampus, septum, cingulate gyrus, amygdala, and frontal cortex have been described, along with antibodies to neurones, glia, and blood vessels (Kuznetsova and Semenov, 1961; Kelly et al., 1987). Antibodies to the septal region of the brain, in particular, have been described in a number of studies. (Heath et al., 1989; Henneberg et al., 1994). This is especially interesting in the light of work showing higher frequency of enlarged cavum septi pellucidi in patients with childhood-onset schizophrenia (Nopoulos et al., 1998).

Antibodies to the 60 and 70 kDa human heat-shock proteins (HSP) have been described. Especially high anti-HSP70 titers are seen in never-medicated patients, whereas high anti-HSP60 titers are mainly found in patients treated with neuroleptics. Since HSPs are involved in diverse neuroprotective mechanisms, antibodies against them may inhibit neuroprotective processes, thus leaving the brain vulnerable to schizophrenia (Schwarz et al., 1999).

3 Immune Changes Associated with Schizophrenia: Coincidence, Neuromodulators, or Consequence?

3.1 The Immune System as Neuromodulator

Schizophrenic patients may exhibit abnormal EEG recordings (Heath et al., 1967). Abnormal EEGs can be induced in similar sites in monkey brains by injection of IgG isolated from the blood of acutely ill schizophrenic patients into the monkeys' lateral ventricles. A later study injected rhesus monkeys with prepared IgG fractions from acutely ill patients with schizophrenia and nonschizophrenic controls. Positive EEG recordings in the monkeys were found in more than 1 in 4 tested in the schizophrenia group, compared with 1 in 13 positive EEGs from control serum fractions. This suggests a substance in the serum in acute schizophrenia that is able to affect brain function (Bergen et al., 1980).

More recently, evidence has come to light of close links between the CNS, the endocrine system, and the immune system, much of which are mediated by cytokines. Besides the developmental impact already discussed, cytokines in the CNS are involved in various regulatory mechanisms including initiation of inflammatory immune responses in the CNS; regulation of the blood-brain barrier; CNS repair after injury; regulation of the hypothalamic-pituitary axis (HPA); modulation of dopaminergic, serotonergic, noradrenergic, and cholinergic neurotransmission.

Communication between the brain and the immune system is a two way process. Activated microglia and astrocytes produce and release cytokines. (Muller and Achenheil, 1998). Conversely, cytokines activate T cells, which trigger proliferation of oligodendrocytes, and affect the differentiation of brain cells. Cytokines influence the release of glutamate, dopamine, and serotonin and therefore may have a role in the pathogenesis of schizophrenia. The subtle neuropathological abnormalities in schizophrenia, such as alterations in neuronal number and density, are consistent with the actions of cytokines on neuronal survival and programmed cell death.

Recent studies have suggested that dysfunctions of both pro- (IL-1 β , IL-6, and TNF- α) and anti-inflammatory (IL-1Ra and IL-10) cytokines could be involved in the pathophysiology of schizophrenia. Is there a relationship between a number of specific cytokines and schizophrenia?

3.2 Interleukin 1 and Interleukin 1 Receptor Antagonist

IL-1 is implicated in a variety of central activities, including fever, sleep, ischemic injury, and neuroimmune and neuroendocrine interactions. IL-1 mediates psychological stress responses by regulating monoamine metabolism and secretion of corticotropin-releasing factor, and has therefore been investigated in various psychiatric diseases.

Administration of IL-1 β causes a profound decrease of glutamate transmission in hippocampal CA1 pyramidal neurons (Luk et al., 1999). IL-1 also enhances dopaminergic sprouting and regulates astroglia-derived dopaminergic neurotrophic factors, such as acidic and basic fibroblast growth factor or glial cell line-derived neurotrophic factor. Dopaminergic neurons themselves express IL-1 receptors (Ho and Blum, 1998). IL-1 β , synergistically with IL-6, modulates the serotonin response in rat hypothalamus (Wu et al., 1999).

IL-1 β serum levels are raised in acute schizophrenia (Katila et al., 1994; Theodoropoulou et al., 2001) as are levels of IL-1 receptor antagonist (IL-1Ra) (Maes et al., 1996; Toyooka et al., 2003). In contrast, IL-1Ra and its mRNA levels are decreased in the prefrontal cortex of schizophrenic patients. There appears to be a genetic basis for this, in that the frequencies of IL-1 β and IL-1Ra allele 1 are significantly higher in schizophrenic patients compared with controls, with a protective effect of the IL-1Ra allele 2 against schizophrenia (Zanardini et al., 2003; Kim et al., 2004). Additionally, bifrontal-temporal gray matter volume deficits and generalized white matter tissue deficits were found in IL-1 β allele 2 carriers with schizophrenia, suggesting that genetically determined differences in IL-1 β may influence brain morphology in schizophrenia (Meisenzahl et al., 2001).

3.3 Interleukin 2

IL-2 influences dopamine release in the striatum (Lapchak, 1992) and potentiates its release in mesencephalic cell cultures (Alonso et al., 1993). Peripheral application of IL-2 causes increased catecholaminergic neurotransmission in the hippocampus and frontal cortex (Zalcman et al., 1994) and it is a potent modulator of hippocampal acetylcholine release (Hanisch et al., 1993).

Recombinant IL-2 (rIL-2) is used with some success in cancer immunotherapy. Its administration, however, has been reported to induce symptoms similar to the positive and negative features of schizophrenia, such as visual and auditory hallucinations, paranoia, delusions, agitation, irritability, cognitive impairment, and fatigue. (Denicoff et al., 1987).

In a Scottish series, compared with patients given chemotherapy alone, patients receiving rIL-2 immunotherapy showed significant cognitive impairment on the trail making test B and the digit symbol substitution test. One patient developed repeated transient psychotic episodes associated with rIL-2 infusions and another regularly became confused (Walker et al., 1997).

Interestingly, in one study, older mice treated with rIL-2 showed impairment of acquisition of a passive-avoidance task, along with degenerative changes maximal in the cA3 region of the hippocampus (Nemni et al., 1992). In the older treated mice, there was a loss of neurones, without accompanying reactive astrocytosis or increase in numbers of oligodendroglia, in the cA1, cA3, and cA4 hippocampal regions. These psychological and neuropathological changes are remarkably similar to those described in schizophrenia.

The findings with respect to peripheral IL-2 levels in schizophrenia are contradictory. Gattaz and coworkers (1992) found no change, while Theodoropoulou and coworkers (2001) found lower IL-2 serum levels in patients than controls, with more cells expressing IL-2 receptors in medicated chronic schizophrenic patients compared with drug-naïve patients. This finding was recently replicated in Chinese patients (Mahendran et al., 2004). However, in other studies serum IL-2 levels were higher in schizophrenia compared with controls (Ebrinc et al., 2002). Licinio and coworkers (1991) found elevated CSF IL-2 in drug-free patients. High CSF IL-2 has been thought to predict schizophrenic relapse (McAllister et al., 1995).

The findings regarding serum soluble IL-2 receptor (sIL-2R α) levels, a marker of immune activation, are somewhat more consistent. The majority of laboratories have found serum sIL-2R α to be increased in schizophrenia, although not all concur (Barak et al., 1995; Gaughran et al., 1998). Barak and coworkers (1995) reported low sIL-2R α CSF levels. Rapaport (1993) found increased sIL-2R α in discordant monozygotic twins while unaffected siblings of patients with schizophrenia also have higher serum sIL-2R α levels than controls (Gaughran et al., 2002).

Increased serum sIL-2R α is present before neuroleptic treatment and is higher in patients with tardive dyskinesia, negative symptoms, and soft neurological signs (Rapaport and Lohr, 1994; Hornberg et al., 1995). Patients with increased sIL-2R α and decreased IL-2 production seem to follow a worse clinical course (Hornberg et al., 1995). Muller and coworkers (1997) observed that sIL-2R α levels increased in patients after clinical improvement, although this has not been a uniform finding (Gaughran et al., 2001).

Muller attributed the symptoms of schizophrenia to excess intracranial IL-2 and suggested that the symptoms decrease because of neutralization with treatment, mediated by sIL-2R α . Plasma sIL-2R α binds IL-2, thereby competing for the binding of free IL-2 to cellular IL-2R on the responding cells. Increased sIL-2R α therefore mediates an immunosuppressive effect due to its capacity to reduce IL-2 availability.

A reduction in IL-2 production in an *in vitro* assay from stimulated peripheral blood mononuclear cells (PBMs), a standard test of cellular immunocompetence, has been repeatedly reported in schizophrenia, although, again, there have been dissenters (Kim et al., 1998; Cazzullo et al., 2001). The reduced IL-2 production reported in schizophrenia is also seen in certain autoimmune diseases (Caruso et al., 1993) and has been explained as *in vivo* overproduction of IL-2 causing T-cell exhaustion, with consequent *in vitro* hypo-responsiveness of stimulated peripheral blood mononuclear cells.

3.4 Interleukin 6

IL-6 modulates the serotonin response in the rat hypothalamus (Wu et al., 1999) and reduces interstitial dopamine levels (Song et al., 1999). Elevated serum IL-6 and serum IL-6R (sIL-6 and sIL-6R respectively) have been reported in schizophrenia and associated with treatment resistance (Lin et al., 1998). Levels of IL-6 are highest in acute episodes of schizophrenia (Frommberger et al., 1997) and are associated with longer duration of illness (Ganguli, 1994). High CSF sIL-6R levels are found in patients with marked positive symptoms of schizophrenia (Muller et al., 1997).

A significant and inverse correlation exists between CSF IL-6 and red blood cell (RBC) membrane polyunsaturated fatty acids (PUFAs) levels in patients with schizophrenia. Decreased membrane PUFAs may thus be related to increased phospholipase A2 activity mediated through the proinflammatory cytokines (Yao et al., 2003). Treatment of schizophrenia with fish oils is currently being evaluated.

3.5 Interferons

Once again, the published evidence is somewhat contradictory. Becker and coworkers (1990) found no alterations in serum interferon during the first psychotic attacks but decreased production of interferon (INF)- α and INF- γ has been reported (Moises et al., 1985; Arolt et al., 2000), and Cazzullo and coworkers (2001) described higher production of INF- γ in drug-free and drug-naïve patients.

It is possible that there may be a variation based on symptoms, as Inglot and coworkers (1994) found that patients with a high interferon response had predominately positive symptoms, whereas those with a low interferon response had mainly negative symptoms. In Preble and Torrey's 1985 study, high titers of interferon were found in serum from 24.4% of patients with psychosis and from 3.1% of controls. Interferon-positive patients were more likely to have had a recent onset or exacerbation of their illness and to be on low dose or no medication. No interferon was detected in the CSF of 65 patients or 20 control subjects. Decreased INF- γ production indicates a worse clinical course (Hornberg et al., 1995).

3.6 Other Cytokines

TNF- α levels and IL-3 like activity are high in schizophrenia (Sirota et al., 1995; Theodoropoulou et al., 2001); likewise, serum IL-18, a T_H1 proinflammatory cytokine produced by macrophage-like cells (Tanaka et al., 2000).

Cazzullo found no difference in IL-4 or IL-10 production in drug-free patients (2001). However, Kaminska and coworkers (2001) observed decreased serum IL-10 levels in schizophrenia, whereas Maes and coworkers (2002) found higher serum IL-8 and IL-10 levels. Differences in both allelic and genotypic frequencies of the IL-10 gene in the Chinese population have been studied and suggest IL-10 can confer susceptibility to the development of schizophrenia (Yu et al., 2004).

In chronic schizophrenia, there is a positive correlation between IL-8 and negative symptoms with a negative relationship between IL-2 and IL-8 (Kaminska et al., 2001; Zhang et al., 2002). Patients with low concentrations of serum IL-2 or IL-8 at baseline show greater improvement after treatment (Zhang et al., 2004). This suggests a correlation between baseline IL-2 and IL-8 levels and therapeutic outcome.

A word of caution is appropriate in the interpretation of cytokine studies. The inconsistent cytokine abnormalities reported in schizophrenia have led some observers to be sceptical as to their significance in the pathogenesis of the disorder. Cytokine levels are affected by age, body mass index (BMI), gender, smoking habits, ongoing or recent infectious diseases, and prior medication; so it is difficult to compare like with like (Haack et al., 1999). For example, people with psychotic illnesses such as schizophrenia smoke more than the general population and this may affect findings unless controls are carefully chosen.

3.7 Hypothalamic-Pituitary Axis

Given the close relationship between glucocorticoid status and immune activity, it is important to include the HPA in our review. However, the precise relationship of the hypothalamic-pituitary axis (HPA) axis to schizophrenia is uncertain. Early studies focussed on basal cortisol secretion, cortisol or ACTH responses to stressors, dexamethasone suppression tests (DST), and cortisol responses to pharmacological probes (Marx and Lieberman, 1998). Other work has concentrated on diurnal variation in cortisol secretion.

Basal cortisol level studies in patients with schizophrenia have been inconclusive, with individual studies reporting both high and low basal cortisol secretion. Following neuroleptic treatment, plasma cortisol levels decrease, perhaps due to reduced noradrenergic activity (Wik, 1995).

Higher salivary cortisol levels are linked to poorer performance on memory and frontal tasks and are associated with positive, disorganized symptoms and overall severity (Walder et al., 2000). The HPA axis may influence the behavioral expression of vulnerability to schizophrenia. Salivary cortisol levels, along with minor physical anomalies, and dermatoglyphic asymmetries are higher in adolescents with schizotypal personality disorder than those with other personality disorders and controls (Weinstein et al., 1999).

No differences in basal serum or CSF levels of ACTH secretion have been reported in schizophrenia. However, reduced cortisol and ACTH responses have been observed in relation to physical stressors such as lumbar puncture, suggesting a disturbance of the flexibility of the HPA axis (Breier et al., 1998; Kathol et al., 1992). Conversely, the ACTH response to acute metabolic stress induced by 2-deoxy-D-glucose is exaggerated (Elman et al., 1998). Schizophrenic patients show blunted salivary cortisol responses to

psychosocial stressors, but not to physical stressors, in spite of similar increases in heart rate to controls (Jansen et al., 2000).

The rates of DST nonsuppression cited in schizophrenia vary between 5% and 44%, with nonsuppression linked to negative symptoms and cognitive impairment (Newcomer et al., 1991). The DST abnormalities in schizophrenia do not seem to be a result of comorbid depression, as depressed patients with schizophrenia do not have higher rates of DST nonsuppression than nondepressed patients (Garyfallos et al., 1993).

Approximately two-thirds of patients with chronic schizophrenia exhibit abnormal diurnal variation of saliva cortisol. This group has more negative symptoms than those with normal diurnal variation (Kaneko et al., 1992). Functional studies describe reduced ACTH and cortisol responses to apomorphine in schizophrenia, implying dysfunction in the dopaminergic systems (Mokrani et al., 1995).

There is some evidence to suggest a cytokine role in the regulation of the HPA axis in schizophrenia. HPA axis activation may be mediated by the proinflammatory cytokines such as IL-1, IL-6, and TNF- α , known to be increased in schizophrenia (Altamura et al., 1999).

3.8 The Acute Phase Response in Schizophrenia

The acute phase response is an innate body defense seen during acute illnesses and involves the increased production of certain blood proteins termed acute phase proteins. Acute schizophrenia is accompanied by increased serum and/or plasma concentrations of acute phase proteins. α 1-antitrypsin, α 2-macroglobulin, haptoglobin, ceruloplasmin, and thyroxine-binding globulin, fibrinogen, complement component 3, C4, α 1-acid-glycoprotein, and hemopexin are all increased in the serum of patients with schizophrenia.

Albumin (a negative acute phase protein), transferrin, and retinol-binding protein levels are reduced. Hemopexin levels are only increased in acutely ill patients while complement C3 and complement hemolytic activity is decreased in chronically ill patients (Spivak et al., 1993; Wong et al., 1996; Maes et al., 1997).

Maes and coworkers (1997) found that differences in acute phase reactants between normal volunteers and schizophrenic, manic, and depressed patients disappeared following chronic treatment with psychotropic drugs. Their results suggested that these disorders are accompanied by an acute phase response, which may be normalized by treatment with psychotropic medication. To see this acute phase response, classically associated with physical illness, in schizophrenia, is remarkable when one thinks of the older term for schizophrenia as a “functional” disorder.

IL-1 synergizes strongly with IL-6 in generating the acute phase response and, as discussed earlier, both cytokines are found in increased quantities in schizophrenia (el-Mallakh et al., 1993; Ganguli et al., 1994).

The increased levels of fibrinogen (a proinflammatory protein linked to metabolic syndrome) are interesting in the light of the work showing features of the metabolic syndrome in people with schizophrenia, even before they first receive antipsychotic treatment (Thakore, 2004).

One acute phase marker, haptoglobin, has been subject to genetic study in schizophrenia. Haptoglobin (Hp) is characterized by a molecular variation with three known phenotypes on chromosome 16, Hp1-1, Hp2-1, and Hp2-2. The allele frequency of the Hp phenotypes in schizophrenia was significantly different from that in a local (Italian) population. The frequency of the Hp-2 gene was significantly higher in schizophrenic patients (71.7%) as compared with controls (62.5%). The altered distribution of the Hp phenotypes and genotypes in schizophrenia suggested that genetic variation on chromosome 16 might be associated with the disorder (Maes et al., 2001).

3.9 Immunoglobulin Isotypes in Schizophrenia

Many studies have examined differential immunoglobulin isotype levels in schizophrenia but the results have been inconsistent. Overall, no consistent direction of change in the representation of serum immunoglobulin isotype levels has been clearly demonstrated in schizophrenia.

Centrally, however, it appears that CSF IgG is positively correlated with negative symptoms in schizophrenia and furthermore, patients with these CSF alterations may be at a higher risk of developing negative symptoms (Muller and Achenheil, 1995).

Increased specific IgA antibodies in schizophrenia have been described (Reichelt and Landmark, 1995). More patients than controls showed serum IgA antibody levels above the upper normal limit to gliadin, β -lactoglobulin, and casein. Interestingly, earlier discharge from hospital has been reported after a milk-free, gluten-free diet, implying some association with mucosal antigen challenge and the nature of the immune response thus engendered. This would also suggest removal of an antigen could modulate the course of schizophrenia.

3.10 T Lymphocytes and B Lymphocytes in Schizophrenia

Morphologically atypical lymphocytes, termed "P cells," similar to those found in mononucleosis and other viral diseases, have been reported in patients with schizophrenia over the past four decades and seen in patients who have never taken antipsychotic medication (Lahdelma et al., 1995). Many laboratories have since examined the distribution of peripheral blood lymphocytes in schizophrenia. A number of abnormalities have been reported, although the direction of change is inconsistent.

In a recent study of lymphocyte distribution, morphology and activity were examined not only by light microscopy but also by flow cytometry. In contrast to the previously published data, there were no differences in cell distribution (lymphocytes, polymorphonuclear cells, eosinophil and basophil granulocytes, monocytes), lymphocyte morphology ("atypical lymphocytes" versus "normal lymphocytes"), distribution of lymphocyte subtypes (T cells (CD3⁺), T_H cells (CD3⁺/CD4⁺), T_C cells (CD3⁺/CD8⁺), B cells (CD19⁺), NK cells (CD3⁻/CD56⁺), and state of T-lymphocyte activity (CD25⁺) or HLA-DR⁺ cells) between schizophrenic patients and healthy controls. The authors suggested that the possible immunological alterations in schizophrenia do not correlate with morphological characteristics of lymphocytes or an altered state activity of T lymphocytes (Rudolf et al., 2004).

However, we should not be too quick to dismiss T-cells in schizophrenia as, interestingly, mice deprived of mature T-cells manifest cognitive deficits and behavioral abnormalities, which are remediable by T-cell restoration. T-cell-based vaccination can overcome behavioral and cognitive abnormalities that accompany neurotransmitter imbalance induced by amphetamine. The suggestion that a peripheral T-cell deficit can lead to cognitive and behavioral impairment highlights the importance of properly functioning adaptive immunity in the maintenance of mental activity and in coping with conditions leading to cognitive deficits (Kipnis et al., 2004).

3.11 Vaccine Response

There is impaired responsiveness to routine vaccination in schizophrenia. Before the introduction of neuroleptics, Molholm (1942) described decreased delayed hypersensitivity to guinea pig serum in schizophrenia and, in the same year, Vaughan and coworkers (1949) recorded decreased responsivity to pertussis vaccine. Subsequently, lower antibody production after vaccination against salmonella in unmedicated patients with schizophrenia was observed (Ozek et al., 1971), while psychiatric patients in general have a reduced antibody response to hepatitis B vaccine (Russo et al., 1994).

Given the rise in proportion of elderly patients with schizophrenia and dementia in residential care, Edgar and coworkers (2000) examined the effectiveness of current influenza vaccination strategies in older people with schizophrenia and dementia in residential care. The antibody response rate to influenza vaccination in 74 elderly individuals with a range of psychiatric diseases was extremely low, with only 5 (6.76%) patients responding to all three components of the subunit vaccine. The response to influenza vaccination in institutionalized elderly patients with schizophrenia and dementia is therefore significantly less than recommended levels.

4 Effects of Antipsychotic Medication on Immune Markers

Antipsychotic medications have long been associated with the production of autoantibodies, including antinuclear antibodies, rheumatoid factor, antihistone antibodies, and anticardiolipin antibodies (Gallien et al., 1977; Canoso et al., 1990; Chengappa et al., 1991, 1992b), thus mimicking to some extent the autoantibody profiles characteristic of SLE and antiphospholipid syndrome. Significantly, as mentioned earlier, these antibodies are also seen in unmedicated patients with schizophrenia.

Some authors have described immunosuppressive effects of neuroleptics (Saunders and Muchmore, 1964; Baker et al., 1977), whereas *in vitro* investigations have demonstrated antipsychotics to have an immune-activating function (Zarrabi et al., 1979). These contradictory results suggest that both *in vitro* and *in vivo* effects, as well as short- and long-term effects must be considered when interpreting studies.

There does appear to be, following short-term treatment at least, a difference in effects on the immune system between “typical” and “atypical” antipsychotics. Taking IL-2 as an example, haloperidol, a typical neuroleptic, does not alter sIL-2R α levels (Pollmacher et al., 1997), although clozapine does (Pollmacher et al., 1995). The findings of increased sIL-2R α levels with atypical neuroleptics may be related to their mixed dopamine 2 (D₂) and 5HT_{2a} receptor blockade or their ability to modulate the HPA axis. No difference in sIL-2R α levels was found, however, between patients on typical and atypical neuroleptics after chronic treatment (Ganguli et al., 1995), whereas both haloperidol and clozapine increase production of IL-2 and IFN- γ (Rudolf et al., 2002).

Clozapine, a drug used for treatment-resistant schizophrenia, is widely accepted to have immunomodulatory properties. It increases plasma levels of TNF- α , soluble TNF receptors p55 and p75, and sIL-2r. Increased TNF- α and sIL-2r levels are more pronounced in patients with clozapine-induced fever who also have increased plasma IL-6 levels and granulocyte counts (Pollmacher et al., 1996). The serum concentrations of sCD8 (an activated T cell) are increased 2 months, but not 4 months, after starting treatment with clozapine, suggesting that short-term treatment with clozapine may induce signs of immune activation, which disappear upon prolonged treatment (Maes et al., 2002).

Olanzapine also seems to affect immune cells. In the third month of olanzapine treatment, CD8 is increased and the CD4/CD8 ratio decreased relative to before treatment, suggesting that immune impairment may occur during olanzapine treatment in patients with schizophrenia (Bilici et al., 2003).

Serum leukemia inhibitory factor receptor (LIF-R) concentrations are significantly increased 2 and 4 months after starting treatment with atypical antipsychotics, implying that prolonged treatment with atypical antipsychotics may increase the antiinflammatory capacity of the serum in schizophrenia (Maes et al., 2002).

Plasma IL-12 levels increased significantly after 4 weeks of risperidone treatment, although IL-1 β , IL-2, IL-6, and INF- γ levels were not significantly different (Kim et al., 2001). The use of risperidone in another study was associated with augmented IL-10 (a suppressor of type I cytokines) and decreased INF- γ production (Cazzullo et al., 2002). Zhang and coworkers (2004) found both risperidone and haloperidol reduce elevated serum IL-2 concentrations in schizophrenia although neither influence the higher serum IL-6 or IL-8 concentrations.

In 1989, Heath and coworkers evaluated reactivity of an IgG fraction from the serum of schizophrenic patients and controls against homogenates of tissues of brain septal region, hippocampus, vermal cerebellum, frontal cortex, and the liver of rhesus monkeys. When IgG fractions of unmedicated schizophrenic patients and schizophrenic patients who had received neuroleptic medication for less than 24 h were tested against the septal region homogenate, a precipitin arc was identified, indicating a positive result, with more than 95% of the fractions. In contrast, IgG fractions of schizophrenic patients who had received neuroleptic medication for more than 24 h were rarely positive. Fractions of all control subjects tested negatively. Potentially this is also consistent with an immunomodulatory effect of neuroleptic medication.

Overall, Muller and Achenheil (1998) concluded that the signs of immune activation seen in schizophrenia and depression normalize after 1–2 months of neuroleptic, mood stabilizer, or antidepressant therapy. It is difficult to know how much of this is related to normalization of the acute phase response and other signs of immune activation in acute schizophrenia, and how much is an independent effect of the various medications.

5 Treatment of Schizophrenia with Immunosuppressants

Given the immunomodulatory effects of antipsychotics, some researchers have taken the next logical step and evaluated immunosuppressive therapies in the management of schizophrenia. A pilot study treated 12 chronic schizophrenic patients with glucocorticoids together with neuroleptic drugs. Two became worse and discontinued. However, seven had a greater than 50% reduction in first-rank symptoms. These patients tended to be younger, with a shorter history of illness, and a positive family history of schizophrenia. Immunological investigations, including α 1-antitrypsin levels, haptoglobins, orosomucoid, and serum IgA, IgM, and IgG levels, failed to differentiate between glucocorticoid responders and nonresponders (Smidt et al., 1985).

On the other hand, treatment with the immunosuppressant azathioprine in patients with schizophrenia who had high antiplatelet antibodies titers had only a limited effect. Only two of 11 patients had a reduction in psychiatric symptomatology although the treatment was generally well tolerated (Levine et al., 1997).

Later, the antiinflammatory COX-2 inhibitor, celecoxib, was evaluated as an add-on treatment in schizophrenia, with reasonable results (Muller et al., 2004). Unfortunately, these medications may not be as safe as was originally supposed, but the principle is encouraging.

Interestingly, improvement in negative symptoms of schizophrenia was described following treatment with antibodies to TNF- α and INF- γ in a patient with high TNF- α levels but no elevation in interferon antibodies (Skurkovich et al., 2003). TNF- α antagonists are now a recognized treatment for rheumatoid arthritis.

6 Summary

The research into the relationship between the immune system and schizophrenia has produced varied and often contradictory results. In trying to interpret the literature, one must bear in mind that, in using the clinical definition of schizophrenia, we may well be dealing with a number of different conditions exhibiting the same phenotype. Undoubtedly, there is no one single cause. Cannon (2003) encourages us to think of the neurodevelopmental etiology of schizophrenia in terms of early and late influences on a product of genes, environment, and their interactions. Here we have described how cytokines, antibodies, exposure to infection, HLA, and autoimmune associations could at least participate in the cascade of biological events that results in schizophrenia. This may be as pre- or perinatal factors, combining with neural system genetic vulnerabilities to produce an "at risk" individual or, at a later stage, perhaps through the immune response to adverse psychosocial environmental factors or to altered hormone regulation. The lack of consistency in the findings is to be expected in a dynamic immune system, subject to multiple influences, sampled at differing points in the course of schizophrenia. However, this research provides the building blocks for future expansion of knowledge of the causes and possible treatments for this condition with worldwide impact.

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Index

- Acetylcholine, 44
- Acute disseminated encephalomyelitis (ADEM), 338
- Acute phase response in Schizophrenia
 - haptoglobins, 478
- Adaptive immune response
 - cell-mediated immune response, 271–274
 - humoral immune response, 274, 275
- Adenoviral capsid proteins, 272
- Adenoviral vectors and immune responses, 268
- Adenoviruses, 267
- Adjuvant-induced arthritis, 85
- Adrenergic receptor(s), 62, 64, 66, 67, 73, 76
 - alpha-adrenergic receptor, 93
 - beta-adrenergic receptor, 93
 - cytokine production, 85, 91, 93, 95, 98, 100, 102, 105–111
 - immune cell trafficking, 91
 - immune function in vivo, 85–87, 89
 - inflammation, 104, 105
 - lymphocyte responses, 89
 - monokine production, 91–94
 - natural killer cell activity, 90
- α -Adrenergic receptor, 64, 66, 93
- β -Adrenergic receptor, 66, 67, 73–75, 93
- β -Adrenergic receptor density, 74
- Adrenocorticotropin (ACTH), 39, 40, 42, 44, 45, 48–50, 52
- Age, 132, 142
- Allergic house-dust mite rhinitis, 141
- Alpha synuclein, 322
- Aluminum neurotoxicosis, effect of PRP on, 183
- Alzheimer's disease (AD), 311, 319, 321
 - β -amyloid proteolytic product (A β), 230
 - astroglisis, 402
 - atypical microglial activation, 402
 - cytokines, 402
 - model of, effect PRP on, 183
 - presenilin 2 (PS2), 230
 - proteasome inhibition, 230
 - treatment, 402
- Amyloid beta (A β), 316
- Amyloid plaque, 321
- Amyloid precursor protein, 320
- Amyotrophic lateral sclerosis, 323
- Anaphylactic shock, 133, 139
- Angiotensin II, 441
- Animal models, 139
- Antibody(ies), 383–387
 - production in the brain of, 168
- Antibody-mediated remyelination, 341, 342
- Antigen presentation, 313
- Antigen presenting cell (APCs), 270, 313, 335
- Antigen-presenting cells in CNS
 - antigens
 - astrocytes, 208, 209
 - microglia, 206–209
 - perivascular space, 207, 208
 - T cell activation, 207
- Anti-inflammatory cytokines
 - interleukin 10 (IL-10), 206
 - transforming growth factor β (TGF- β), 206
- Anti-KLH antibody responses, 74, 75
- Antineuronal antibodies, 338
- Apoptosis, 64, 67, 68, 434
- Apoptosis inducing factor, 428, 431, 433, 438, 451
- Arthritis, 134, 139
 - adjuvant-induced arthritis, 85, 86
 - rheumatoid arthritis, 85, 97, 109
- Aspartate, 45, 47
- Asthma-like symptoms, 139
- Asthmatic patients, 139
- Astrocytes, 236, 237, 241–244, 246, 247, 249–255, 313–318, 430, 446, 450
- ATP
 - intracellular levels of, 446
- Atrophy, brain, 389
- Autoantibodies in schizophrenia, 473, 474
- Autoimmune, 381–388
- Autoimmune disease(s), 337, 342
 - lupus, 20, 21
 - RA, 20–23, 25, 27
 - stress and, 85–87, 90, 91, 95, 96, 98, 101, 106, 111
 - sympathetic regulation of, 86
- Autoimmune etiology, 342
- Autoimmune illnesses
 - antiphospholipid syndrome, 469
 - coeliac disease, 469
 - increased risk of schizophrenia with, 471
 - insulin dependent diabetes mellitus (IDDM), 469
 - rheumatoid arthritis, 469–471
 - Sydenham's chorea, 469
 - systemic lupus erythematosus (SLE), 469, 471, 473
 - thyrotoxicosis, 470
- Autoimmunity, 237
- Autonomic nervous system
 - cholinergic anti-inflammatory pathway, 302
 - immune system modulation by stress, 298, 299
 - non-neuronal cholinergic system, 303
 - sympathetic nervous system, 85, 97, 103
 - vagal nerve, 301–303
- Autoreactive antibodies, 341
- AVP
 - co-localisation of CRF with, 23
 - inflammatory effects of, 23
 - receptors, 23
- Axonal injury, 389
- B cells, 238–240, 252, 253
- B Lymphocytes, 68, 69, 72
- Backward association, 131
- Bacteria, 38, 39, 41, 49

- BDNF. *See* Brain-derived neurotrophic factor
- Behavior, 38, 45, 47, 49–52
- Biobreeding (BB), 336
- Blood-Brain barrier (BBB), 335, 346, 472, 474
- circumventricular organs, 201
 - components of, 372
 - functions of, 372
 - in EAE, 371, 372
 - loss of integrity, 373
 - neurotransmitter release, 303
 - neurovascular unit, 200–203
 - open areas, 295, 298
 - permeability in inflammation, 295
 - platelet-endothelial cell adhesion molecule1, PECAM–1, CD31, 201
 - pro-inflammatory mediators: IL-1, IL-6, IFN- γ , TNF- α , 198
 - tight junctions, 200
 - transendothelial electrical resistance (TEER), 203–204
 - virchow-robin spaces, 201, 204
 - zonula occludens molecules, 201
- Bone marrow, 63–65
- Bordetella pertussis, 339
- Borrelia burgdorferi, 338
- Brain
- antibody production in, 168
 - neuroendocrine immune system of, 175–178, 183
- Brain cytokines
- alzheimer's disease, 302
 - changes in psychiatric disorders, 301
 - enhance sleep, 300
 - expressed in brain, 300
 - glucocorticoid suppression, 301
 - modulate body temperature, 300
 - penetrate blood brain barrier, 301
- Brain-derived neurotrophic factor, 265
- Brain function
- cytokines administration of, 6, 7
 - effect of, 7
- Brain-to-immune pathway, 125
- Brown Norway (BN) strains, 341
- Bystander activation, 387
- Catalase, 71
- Catecholamines, 93. *See also* norepinephrine and epinephrine regulation of tumor necrosis factor alpha
- dopamine, 41
 - MHPG, 41
 - norepinephrine, 41, 42
- CD3, 64, 67
- CD4, 64, 65, 67, 68, 73, 74
- CD40 ligand, 315, 316
- CD8, 64, 65, 67, 68, 73
- Cell-mediated immune reactions against autoantigens (CMAI), 336
- Cellular infiltration, 340
- Central nervous system (CNS)
- immunological reactivity in, 335
 - immunosuppression in, 336
 - infectious agents in, 336
 - mechanism of autoimmunity in, 336
 - virus-induced damage to, 337
- Cerebrospinal fluid (CSF), 338
- Cerebrovascular endothelial cells (CVE), 343
- Chemical sympathectomy, 64, 65, 67, 69, 73
- Chemokines, 316, 318, 325, 326, 440, 447, 451
- classification of, 164–166
 - fractalkine, 412, 413
 - interleukin-8 (IL-8), 413
 - MCP-1, 413
 - MIP-1 α , 411, 413, 417
 - MIP-1 β , 411, 413, 417
 - RANTES, 411, 417
 - receptors of, 166, 167
 - role in neurons of, 167, 168
- Chemotherapy, 140
- Chronic β -adrenergic receptor blockade, 67, 68
- Chronic inflammatory demyelinating neuropathy, 380
- Ciliary neurotrophic factor (CNTF), 265
- Circumventricular organs, 125
- Classical conditioning, 125, 129
- CNS-immune communication, 131
- Compensatory conditioned effects, 132
- Complement system
- glial localization, 296
 - neurodegeneration, 296
 - viral and bacterial infections, 296
- Concanavalin A (Con A), 62, 71, 74
- Conditioned not re-exposed, 137, 141
- Consciousness, 132
- Contact hypersensitivity reaction, 139
- Contingency, 131
- Cortical thymic epithelium, 336
- Corticosterone, 39, 40, 42, 44–46, 48–50
- Corticotropin releasing factor (CRF), 38, 44, 48–50
- Corticotropin releasing hormone, 87
- Coxsackievirus-adenovirus receptor (CAR), 267
- Creutzfeldt-Jakob disease, 325
- CRF family of peptides
- AA, 21
 - CRF (1–41), 21–23
 - EAE, 21
 - heterogeneity in human T cells of, 21, 22
 - human PBMC, 21, 22
 - inflammatory effects of, 21
 - R1 and R2 receptors, 21, 23
 - rat splenocyte contents of, 21, 22
 - substance P and, 22
 - urocortins 1, 2 and 3, 22, 23
- CS / US administration route, 133
- Cyclic adenosine monophosphate (cAMP), 66, 74, 75
- 2', 3'-Cyclic Nucleotide 3' Phosphodiesterase (CNase), 384
- Cyclooxygenase, 38, 49, 51
- Cytokine mRNA expression, 10
- Cytokine receptors and production in brain, 6
- Cytokines, 88, 91–94, 96–98, 103, 105–111
- action locality of, 160
 - antipsychotic effects on, 480
 - brain born, 155, 164, 176
 - brain receptors of, 164
 - cytokine releaser polyribonucleoside-polyribocytidilic acid (poly I: C), 472
 - cytokine system, superfluity of, 160–163
 - cytokines in neuromodulation, 474
 - granulocyte-macrophage colony-stimulating factor (GM-CSF), 38, 47
 - induction of, 159, 161, 163
 - interferon- α , 476
 - interferon (IFN)- γ , 315, 384, 386, 411, 412, 415, 416, 419, 472
 - interleukin-1 (IL-1), 38, 39, 41, 42, 315, 380, 413
 - interleukin-1 β , 472
 - interleukin-1RA, 475
 - interleukin-2, 38, 45, 472, 476
 - interleukin-4, 417
 - interleukin-6, 38, 45, 472, 476
 - interleukin-8, 472, 477, 480
 - interleukin-10, 475, 479, 480
 - interrelationship and interdependency of, 163
 - neurosecretion of, 176
 - recombinant interleukin-2, 475, 476

- tumour necrosis factor – α , 38, 315, 318, 323, 386, 409, 412, 415–417, 419, 472, 475, 477, 480, 481
- Cytokines antiinflammatory, 6
- Cytotoxic T cells (CTLs), 270, 344
- Delayed-type hypersensitivity (DTH), 134, 137, 335
- Demyelination, 380–389
- Dendritic cells (DCs), 386
 - CCR2 and CCR5, 210
 - DC-SIGN (CD209), 210
 - FMS-like tyrosine kinase 3 ligand (Flt-3L), 210
 - granulocyte-macrophage colony-stimulating factor (GM-CSF), 209
 - interleukin 17 (IL 17), 210
 - plasmacytoid DCs, 210
 - tissue localization, 209
- Depression, 47, 51, 52
- Dermatitis, 139
- Devic's disease, 339
- Differential peptide processing
 - CRF, 21–23
 - POMC, 23, 24
 - post-translational modification, 29
- Differentiation of pre-T cells, 64
- Disseminated intravascular coagulation (DIC), 268
- Dopamine, 321, 322
- Dopamine- β -hydroxylase, 67
- Dual (Double) negative, 62, 64
- Dual (Double) positive, 62, 64
- Encephalitis lethargica (EL), 340
- Endogenous pyrogens, 7, 8
- β -Endorphin therapeutic genes, 267
- Endothelial cells, 444, 446, 447
- Endotoxin (Lipopolysaccharide, LPS), 38
- Engram specificity, 133
- Enzymes
 - cyclooxygenase-2 (COX-2), 418
 - granulocyte macrophage colony-stimulating factor (GM-CSF), 417
 - inducible nitric oxide synthase (iNOS), 417
 - nitric oxide (NO), 417
 - platelet activating factor (PAF), 419
 - prostaglandin E₂ (PGE₂), 419
 - quinolinic acid (QUIN), 149
- Epinephrine, 85, 92
- Epitope spreading, 388
- Epstein-Barr virus infections, 343
- Excitotoxicity, 319
- Experimental autoimmune encephalomyelitis (EAE), 336, 343, 381
 - history, 357
 - immunopathology of, 361–370
 - innate immune system in, 370, 371
 - in non-human primates, 360
 - models of, 358–361
 - pathology of EAE
 - in guinea pig model, 361
 - in Lewis rat model, 362
 - in mouse model, 361
 - in rodents, 359, 360
 - toll like receptors in, 370
- Experimental groups, 141
- Extinction, 127, 130, 131, 133, 139, 142
- Familial amyotrophic lateral sclerosis (ALS)
 - impaired immunoproteasome function, 231
 - intracellular inclusions, 231
 - SOD 1 mutations, 231
- Fenton reaction, 322
- Fractalkine, 316
- Free radicals (ROS), 70
- Gamma-aminobutyric acid (GABA), 44, 45, 47, 340
- GDNF. *See* Glial-derived neurotrophic factor
- Gender, 132, 142
- Gene therapy for pain, 266, 267
- Gene therapy vectors, 267
- GFAP. *See* Glial fibrillary acidic protein
- Glia, 309–312, 321–323
- Glial-derived neurotrophic factor, 265
- Glial fibrillary acidic protein, 265
- Glioblastoma multiforme (GBM), 266
 - INF- γ inducible subunit accumulation, 231, 232
 - programmed cell death, 231, 232
 - proteasome peptidase activity downregulation, 231, 232
- Glucocorticoids, 6, 88, 89, 94, 111
- Glucose homeostasis, 8
- Glutamate, 44, 45, 47, 319, 324
- Glutamic acid decarboxylase (GAD), 340
- Group A streptococcus (GAS), 338
- Guillain Barre Syndrome, 337, 380, 387
- Heart
 - neurosecretory hypothalamus-endocrine heart system, 156, 157
- Heart allografts, 139
- Heat shock proteins, 313
- Herpes simplex virus infection, 347
- HIV
 - infection of astrocytes, 410
 - infection of microglia, 411
 - infection of neurons, 410
 - life cycle, 408
- HIV associated dementia, 408–419
- HIV encephalitis, 325
- HLA antigens
 - genetic linkage, 471
 - HLA-A1 as predictor of granulocytosis on clozapine treatment, 471
 - maternal antibody to fetal HLA, 471
 - response to treatment, 471
- Hormones
 - immune suppression and facilitation, 299
 - interactions of stress response with immune system, 299
- HPA axis, 38–40, 44, 48, 50–52, 125, 138
- Human studies, 139–141
- Humoral CNS-immune communication, 131, 142
- Huntington's disease (HD), 265
 - CAG triplet expansion (huntingtin), 231
 - immunoproteasome expression increase, 231
 - proteasome peptidase activity modulation, 231
- 6-Hydroxydopamine, 62, 64, 85
- Hyperphosphorylated tau, 320
- Hypothalamo-pituitary-adrenocortical (HPA) axis. *See* HPA axis
- Hypothalamus
 - magnocellular nuclei of, 156, 157
 - neurosecretory hypothalamus-endocrine heart system, 156, 157
- Hypoxanthine-guanine phosphoribosyltransferase (HPRT) activity, 265
- IL1 β
 - epileptic activity reduced by IL1 α , 296
 - epilepsy enhanced by IL1 β , 296
- Immune cytokines
 - brain-controlled homeostatic mechanisms and, 7–9
 - induction and relevance of
 - neuronal signals and, 10
 - peripheral immune signals, 9, 10
 - integrative role of, 13
 - and thermoregulation, 7
- Immune history, 132, 133

- Immune organs, sympathetic innervation in experimental arthritis, 99
- Immune privilege
- definition, 198
 - lymphatic drainage, 199, 200
- Immune senescence, 62, 63, 76
- Immune synapse
- communication hot spots, 303
 - criteria, 304
 - integrins, 305
 - prototypic synapse, 304
 - scaffold, 304, 305
 - supramolecular activation cluster, 305
 - T cells and DC cells, 304
- Immune system, neuroendocrine, of brain, 157–159
- Immune-neuroendocrine network, 4, 5
- Immune-to-brain pathway, 124
- Immunity
- adaptive, 312, 313
 - innate, 313, 317, 319, 326
- Immunological signaling molecules, 341
- Immunological status, factors contribution, 335
- Immunological synapse, 4
- Immunology of EAE
- antigen presenting cells, 367, 368
 - B-cells, 368–370
 - CD 8⁺, 364, 368
 - chemokines, 356, 366, 367, 371
 - cytokines, 356, 364, 365, 367, 370
 - IL-12, 365–367
 - IL-17, 356, 357, 366, 368
 - IL-23, 365–367
 - IL-27, 366
 - epitope spreading, 368
 - macrophages, 361–364
 - Th-2, 357, 365
 - Th-17, 357, 366
- Immunomodulators
- discovery in brain of, 168
 - myelin basic protein fragments, 169, 170
 - thymosin β_4 , 168, 173
- Immunophilins
- cytokine function of, 171, 172
 - role in nervous system of, 172–174
- Immunosuppressors, FKBP-12, 171–173
- Indoleamines, serotonin, 47
- Infection, 38–41, 43, 49–52
- Inflammation in central nervous system, characteristics and basic features, 396
- Inflammation, 85, 87, 91, 93, 95, 97, 98, 100–102, 104, 105, 108, 110, 111, 311–322
- Inflammatory mediators, 311–313, 316, 318, 321, 322
- Innate and adaptive immunity, 247
- Innate immune response, 269–271
- Innate immunity, macrophages, 86, 88
- Innervation of lymphoid organs, 125
- Innervation of lymphoid tissue, 102
- INS-dependent diabetes mellitus (IDDM), 340
- Inside out model, 389
- Interesting transcript 15 (IT15), 265
- Interferon- α , 46, 47
- Interferon- γ , 38, 46, 47
- Interleukins, 85, 88, 92, 109
- Interstimulus delays, 131
- Interstimulus intervals, 131
- Intracellular adhesion molecule-1 (ICAM-1), 343
- Intracellular protein degradation
- in cytoplasm and nucleus, 224
 - in lysosomes, 224
 - pathway involved, 224, 225
 - ubiquitin/proteasome/ATP dependent, 225
 - ubiquitin tagged proteins, 224, 225
- Intracerebral inoculation, 341
- Iron, 322
- KLH-specific proliferation, 74
- Lambert–Eaton myasthenic syndrome, 339
- Latent inhibition, 132, 140, 141
- L-Deprenyl, 71
- Lesch–Nyhan syndrome, 265
- Lethargic encephalitis, 340
- Leukocyte, 316, 319, 324
- Lewy bodies, 321, 322
- Life-span, 132
- Lipopolysaccharide (LPS)
- administration, 7, 70, 71, 311, 313
- LNS. *See* Lesch–Nyhan syndrome
- Lupus erythematosus, 139
- Lyme borreliosis, 338
- Lymphocyte functional antigen-1 (LFA-1), 343
- Lymphocytes
- B lymphocytes, 313
 - infiltration, 341
 - T lymphocytes, 313
- Lymphoid organs, 62–64, 67, 73, 74, 76
- Lymphoid tissue, innervation of, 102
- Macrophage migration inhibitory factor, 173, 174
- Macrophage(s), 86–94, 97, 99, 100, 102, 103, 105–107, 109–111, 236–244, 246–252, 311–313, 315–317, 325
- Magnetic resonance imaging, 380
- Magnocellular nuclei, 156, 157, 175
- Major histocompatibility complex (MHC), 335
- MAP kinase
- ERK, 317
 - c-Jun N-terminal kinase (JNK), 317
 - p38, 317
- Marginal zone, 68, 69, 72
- Mast cells, 347
- Matrix metalloproteases (MMP), 319
- Measles–mumps–rubella (MMR) antibodies, 337
- Mechanisms
- neural network, 133
 - peripheral pathways, 124, 125
- Meningitis
- effect of PARP deficiency, 435–441
 - effect of PARP inhibitors, 443–448
- α -Methylnorepinephrine, 62, 69
- 3-Methoxy- 4-hydroxyphenylethylene glycol (MHPG)/NA ratio, 6
- MHC class I and II expression in brain, 272
- Microglia, 236, 237, 242–244, 246–252, 254, 255, 312–317, 380, 382, 386, 389
- Microglia cells, 335
- activated microglia, 297
 - basic features, 396
 - CNS macrophages, 296, 297
 - in Alzheimer's disease, 402
 - in Parkinson's disease, 398–400
 - in prion's disease, 400–402
 - resident macrophages, 296, 301
- Molecular mimicry, 387
- Monoamine oxidase B (MAO-B) inhibitor, 71
- Mouse monoclonal antibodies (mAbs), 342
- MPTP, 449, 451, 452
- Multiple sclerosis (MS), 140, 266, 312, 324, 342, 343
- as relates to EAE, 356
 - effect of PARP inhibitors, 444–448
 - immunomodulatory treatments of, 356
 - primary progressive, 380
 - relapsing remitting, 380
 - secondary progressive, 380

- Mycobacterium tuberculosis, 339
 Myelin, 381–389
 - autoimmune demyelination, 356
 - peptides, 356, 358
 - proteins, 356, 359
 Myelin basic protein (MBP), 336, 383
 Myelin oligodendrocyte basic protein (MOBP), 384
 Myelin oligodendrocyte glycoprotein (MOG), 336, 383
 Myelin-producing cells, stimulation of, 342
 NAD, intracellular levels of, 432
 NADPH oxidase, 318, 322
 Natural killer cell activity, 71–73
 NE turnover, 62, 69, 70
 Necrosis, 428–432, 435, 440, 442
 Nerve growth factor (NGF), 62, 71, 72
 - in brain astrocytes, 265
 Neural CNS-immune communication, 124, 125
 Neurodegeneration
 - effect of PARP deficiency, 449
 - effect of PARP inhibitors, 448–450
 Neurodegenerative disease, 317, 319–321, 326
 Neuroendocrine autoantigen, 340
 Neuroendocrine immune system of brain, discovery of, 175–178
 Neurofibrillary tangles, 320
 Neurohormones, 156, 157, 176
 Neurohypophysis, neurosecretory granules of, 173
 Neuro-immune associative learning
 - auditory, 125, 127, 131, 133
 - conditioned, CS, 127
 - gustatory, 125, 134, 135, 142
 - olfactory, 125, 140, 142
 - recall
 - behaviorally conditioned immune effects, 130
 - conditioned, CR, 129, 130
 - response, 129, 130
 - unconditioned, UR, 141
 – touch, 125, 131
 – unconditioned stimulus, US
 - genuine / directly perceived, 129, 132
 - sham / indirectly perceived, 129
 - visual, 125, 126, 131, 133
 Neuro-inflammation, 236, 241, 242, 312, 318, 320, 323–326
 Neurological diseases treatment by gene therapy, 265, 266
 Neuromelanin, 424
 Neuromyelitis optica (NMO), 339
 Neuromyotonia (NMT), 339
 Neuronal-glial interactions, 7
 Neurons, effect of PRP (proline rich peptides) on, 181–182
 Neuropeptides
 - alpha-melanocyte-stimulating-hormone (α -MSH), 205
 - BDNF, 298
 - CGRP, 294, 298
 - communication between immune system and CNS, 298
 - neuropeptide γ (NPY), 205
 - somatostatin (SST), 205
 - substance P, 298, 299
 - VIP, 205, 298, 299, 301
 Neuropsychiatric disease, 338
 Neurosecretory hypothalamus-endocrine heart system, 156, 157
 Neurotransmitters, 39, 51
 - acetylcholine, 303
 - glutamate, 298
 - noradrenaline, 299, 302
 - serotonin, 299, 300
 Neurotrauma
 - effect of PARP deficiency, 429
 - effect of PARP inhibitors, 441–443
 Nitric oxide (NO), 318
 - as an activator of PARP, 451
 - in CNS, 432
 NMDA receptors, 319, 321
 Node of Ranvier, 381, 389
 Non-contingent conditioned, 141
 Non-POMC-derived opioid peptides
 - dynorphin, 26–28
 - endomorphins 1 and 2, 26
 - enkephalins, 26
 - involvement in inflammation, 27, 28
 - lymphocyte trafficking of, 28
 - mediation of neurogenic pain by, 27
 - nociceptin/orphanin FQ, 26, 27
 - selective delivery to inflamed tissues of, 28
 Noradrenergic innervation, 62
 Norepinephrine (NE), 62–67, 69–71, 74–76, 85, 92, 93
 Nuclear factor kappa B, 314
 1, 2', 5'-Oligoadenylate synthetase (OAS), 270
 Oligoclonal bands (OCB), 338
 Oligodendrocyte(s), 318, 324, 381, 383, 386, 388, 389, 434, 439, 445
 Oligodendrocytic-specific glycoprotein (OSP), 384
 One learning trial conditioning, 131
 Oxidative stress, 322
 Parkinson's disease (PD), 266, 321–323
 - astrogliosis, 398
 - atypical microglial activation, 400
 - cytokines, 398–400
 - etiology, 398
 - proteasome proteolytic activity, 230
 - 19S reg interaction, 230
 - α -synuclein aggregates, 230
 - treatment, 399
 Partial reinforcement, 131
 Passive forgetting, 131
 Pathogen-associated molecular patterns (PAMPs), 313
 Peptidergic nerves, 63, 69
 Periaarteriolar lymphatic sheath, 62, 68
 Pericyte
 - CNS antigen presenting cell, 297, 305
 - essential elements of BBB, 297
 - pathomechanisms of hypoxia, hypertension etc., 297
 Phospholipid-binding proteins, 336
 Placebo, 140, 141
 Plaque, 380, 381, 386
 Poly (ADP-ribose) glycohydrolase, 429, 431, 438, 442
 Poly (ADP-ribose) polymerases
 - and cell death, 429, 432, 442
 - and DNA repair, 429, 453
 - and neurotrauma, 435
 - and NMDA receptor activation, 432, 451
 - and stroke, 443–441
 - interactions with nuclear proteins, 451
 - isoforms, 428, 439, 443, 450
 - regulation of genes by, 428
 POMC family of peptides
 - ACTH, 23–26
 - biphasic effects of, 24
 - β -endorphin, 23–26
 - increases in response to inflammation of, 25
 - interactions with cytokines, 24, 25
 - MC receptors, 24
 - naloxone-sensitive and insensitive actions of, 25
 - opioid receptors, 24, 25
 - regulation by glucocorticoids, 26
 - Th1/Th2 cytokine balance, 25, 28
 Prion's disease, 325
 - astrogliosis, 401
 - atypical microglial activation, 401

- cytokines, 401
- treatment, 400
- Proenkephalin A, 267
- Progressive multifocal leukoencephalopathy, 388
- Proinflammatory cytokines, 88, 94, 98, 103, 105–108
- Proline rich peptides (PRP), 155, 157, 177–188
- Prostaglandin E2 (PGE2), 336
- Prostaglandins, 326
- Proteasomes, 316, 322
 - antigenic peptide generation, 227, 229
 - brain constitutive and immunoproteasomes, 226, 228, 229
 - constitutive and immunoproteasomes, 228, 229
 - hybrid proteasome, 226
 - immunoproteasome, 226–228
 - INF- γ inducible subunits, 226–228
 - peptidase activity, 225, 226
 - proteasome inhibitors, 229
 - proteolytic activity, 226
 - 20S proteasome, 225, 226
 - 26S proteasome, 225, 226
 - 11SReg–20S proteasome, 225, 226
 - 11S regulator (11S reg), 225–227
 - 19S regulator (19S reg), 225–227
- Proteasomes and neurodegenerative diseases
 - insoluble protein aggregates, 229
 - proteasome proteolytic activity depression, 230
- Proteins, gp120, 411–414
- Proteolipid protein, 383
- Proteolipid protein (PLP) isoform, 336
- PRP (proline rich peptides)
 - action after nerve transection, 181, 182
 - action in spinal cord injury, 181, 182
 - antibacterial activity of, 178, 179
 - anti-snake venom effect of, 181, 182
 - effect on aluminum neurotoxicosis of, 183
 - effect on neurons of, 183
 - effect on thymocyte differentiation of, 179, 180
 - neuroprotective (antineurodegenerative) properties of, 181–183
 - PRP-1 (proline rich peptide 1), 181
 - PRP-3 (proline rich peptide 3), 181
 - regulation of myelopoiesis by, 180
 - stimulation of bone marrow stem cells by, 180, 181
- Raf/mitogen-activated protein kinase (MAPK) pathways, 267
- Rapid eye movement, 10
- Reactive oxygen species (ROS), 318, 322, 386, 387
- Reconditioning, 142
- Red pulp, 68
- Regulatory cell surface molecules
 - FasL–FAS interactions, 206, 207
 - PD1–PDL1, 206
- Reinforcement trials, 131
- REM. *See* Rapid eye movement
- Retention, 131, 133, 139
- Rheumatoid arthritis
 - autoimmunity, 86
 - cytokines, 96–98
 - macrophages, 105–107
 - Th1/Th2 lymphocytes, 91
 - treatment of, 96
- Schistosoma mansoni, 339
- Schizophrenia, 339, 340
 - anatomical brain changes in, 469, 471
 - birth order, 471
 - clinical features and symptoms of, 468
 - etiology, 469
 - immunological changes in, 469
 - neurotransmitters in pathogenesis, 469
 - relationship to autoimmune illnesses, 469
 - rhesus incompatibility, 471
 - treatment of, 481
- Seasonal allergic rhinitis, 140
- Serum antibody levels, 75
- Severe combined immunodeficiency (SCID) mice, 72, 75
- Signal transduction, 155, 160, 162, 163, 171
- Skin grafting, 139
- Spinal cord homogenate (SCH), 342
- Spinal cord injury (SCI), 341
- Spleen, 63, 66–75
- Spleen and rheumatoid arthritis, 85, 97, 109
- Spongiform encephalopathy, 325
- Spontaneous mammary tumor growth, 71
- Stem cells, stimulation by PRP (proline rich peptides) of, 180, 181
- Stiff-man syndrome (SMS), 339–341
- Stimulus, 125, 126, 128–130, 134, 135, 140–142
- Streptococcus pyogenes, 338
- Stress, 40, 52
- Stroke
 - and vascular dysfunction, 432
 - design of preclinical studies, 434
 - effect of PARP deficiency, 435–441
 - effect of PARP inhibitors, 435–441
- Stroke, 325
- Substantia nigra, 321, 322
- Superoxide dismutase (SOD), 62, 71, 323
- Sydenham's chorea (SC), 338
- Sympathetic modulation, 73–75
- Sympathetic nerves, 65–67, 69–75
- Sympathetic nervous system, 62, 125
 - cytokines and activity of, 96–98
 - dysregulation in animal models, 95–101
 - dysregulation in rheumatoid arthritis, 95–101
 - intervention in autoimmune disease, 102
 - lymphoid tissue and innervation of, 97–99
- T-cell activation, requirement for, 335
- T-cell infiltration, 273
- T-cell receptor (TCR), 338
- T cells, 237–244, 246, 248, 250–253, 255
- T lymphocyte, 68, 69, 72
 - CD4 and CD8, and spleen, 91, 94, 99, 100, 104
 - CD4+ T lymphocytes, 380
 - CD8+ T lymphocytes, 385, 388
 - gamma delta T cells, 385
 - natural killer (NK) cells, 386
 - regulatory T lymphocytes (T reg cells), 386
 - rheumatoid arthritis, 109
 - Th1 lymphocytes, 385
 - Th1/Th2 balance and pattern, 91–93
 - Th2 lymphocytes, 384, 386
- Tau, 320, 321
- Th1 cytokine, 73, 75
- Th2 cytokine, 73, 75
- Theiler's murine encephalomyelitis virus (TMEV), 341
- Theiler's virus, 344, 345, 388
- Theiler's virus induced demyelination (TVID), 343, 344, 347
- Thymic epithelial cells, 336
- Thymic involution, 63, 65, 66
- Thymic nerves, 65, 66
- Thymocyte differentiation, 65, 67, 68

- Thymocyte proliferation, 67, 68
Thymosin β_1
 - calmodulin antagonist function of, 173
 - isolation from neurosecretory granules of neurohypophysis of, 173, 176TLR4 receptor
 - expression on glia cells, neurons, 295
 - LPS activated, 295TNP-hemocyanin, 4
Tolerance, 130, 133
Toll-like receptors (TLR), 313, 314
Transaldolase-H (Tal-H), 384
Traumatic brain injury, 325
Trichuris trichuria, 339
Trypanosoma brucei brucei, 339
Tryptophan, 38–40, 43–47, 49–52
Tumor growth, 134, 139
Tumor necrosis factor (TNF), 343
Tumor necrosis factor-alpha (TNF- α)
 - bioactivity, 6
 - catecholamine regulation of, 94, 95Tyrosine hydroxylase, 62, 69
Ubiquitin, 322
Unconditioned response, 125, 132, 141
Unreinforced trials, 131
Uric acid, antioxidant effects of, 444
Vagus nerve, 49
Vasoactive amine (VAA)
 - sensitization, 347Viral infection
 - Coxsackie B5, 471, 474
 - effect of virus-induced maternal response on fetus, 471–472
 - herpes simplex, 471, 473
 - influenza, 471–473, 479
 - rubella, 471, 473
 - viral antibodies in schizophrenia, 473Viruses, 39
Vitamin B12, 380
White pulp, 68–70