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ENTERIC NERVOUS SYSTEM**

Payam Roshan

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Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements for the degree of

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in

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ABSTRACT

There is limited knowledge of immunocyte-myenteric neuronal interaction and the role of iNOS in myenteric neuronal injury. This research sought to examine the role of macrophages, NO, and iNOS inhibitors in myenteric neurodegeneration.

Increased NO synthesis in macrophages and its effects on myenteric neurons were investigated in cell cultures. Using rodent models of inflammation, we further examined NO-dependent neurotoxicity.

In the presence of activated macrophages neuronal injury and degeneration occurred; however the myenteric neurons showed greater resistance to oxidative challenge than cortical neurons. Pretreatment with iNOS inhibitors significantly reduced these inflammatory effects. Myenteric neuronal injury was also evident in experimental colitis, and iNOS selective inhibitor protected the myenteric neurons from inflammation and degeneration.

In conclusion, these results show that activated macrophage-derived NO is important in inflammation-dependent myenteric neurodegeneration, and iNOS inhibitors can protect myenteric neurons from degeneration. Two potential strategies for neuroprotection in gut inflammation are defined.

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LIST OF ABBREVIATIONS

1400W	N-(3-(aminomethyl)benzyl) acetamidine
5-HT	5-hydroxytryptamine
Ach	Acetylcholine
ADC	Acquired immunodeficiency syndrome dementia complex
ANS	Autonomic nervous system
AP-1	Activator protein-1
ATP	Adenosine triphosphate
Bax	Bcl-2 -associated x protein
Bcl-2	B cell leukemia/lymphoma 2
Bcl-X _L	B cell leukemia-x long
BH4	Tetrahydrobiopterin
BN	Bombesin a.k.a. GRP
Casp-3	Caspase 3
Casp-6	Caspase 6
Casp-7	Caspase 7
Casp-8	Caspase 8
Casp-9	Caspase 9
CCK	Cholecystokinin
CD	Crohn's disease
CD4+	Cluster of differentiation antigen 4
cGMP	Cyclic guanidine mono phosphate
CGRP	Calcitonin gene-related peptide
ChAT	Choline acetyl transferase
CNS	Central nervous system
Cyt-c	Cytochrome c

DMEM	Dulbeco's modified Eagle medium
DNBS	2,4-dinitrobenzenesulfonic acid
dUTP	Deoxyuridine triphosphate
ENK	Enkephalin
ENS	Enteric nervous system
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal regulated kinase
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GABA	Gamma amino butyric acid
GAF	Gamma-activated factor
GAL	Galanin
GC	Guanidine cyclase
GFAP	Glial fibrillary acidic protein
GI	Gastrointestinal
GRP	Gastrin releasing peptide
GTP	Guanidine triphosphate
H&E	Hematoxylin and eosin
HIV	Human immunodeficiency virus
IBD	Inflammatory bowel disease
ICC	Immunocytochemistry
IFN	Interferon
IFNR	Interferon gamma receptor
IFN- γ	Interferon gamma
IHC	Immunohistochemistry
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-10	Interleukin-10

IL-12	Interleukin-12
IL-13	Interleukin-13
iNOS	Inducible nitric oxide synthase
IRAK	IL-1 receptor-associated kinase
I κ B α	Inhibitory kappa B alpha
I κ K	Inhibitory kappa B protein kinase
JAK1	Janus kinase 1
JAK2	Janus kinase 2
JNK	C-Jun N-terminal kinase
LBP	LPS-binding protein
L-NAME	N ^ω -nitro-L-arginine methyl ester
L-NIL	L-N6-(1-iminoethyl)-lysine
L-NMMA	L-nitro-monomethyl-arginine
L-NNA	L-nitro-n-arginine
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MPO	Myeloperoxidase
NADP	Nicotinamide-adenine dinucleotide phosphate
NADPH	Reduced nicotinamide-adenine dinucleotide phosphate
NADPH-d	Reduced nicotinamide-adenine dinucleotide phosphate - diaphorase
NCBI	National center for biotechnology information
NF-IL6	Nuclear factor interleukin-6
NFP	Neurofilament protein
NF κ B	Nuclear factor kappa B
NIK	NF κ B inducing kinase
NK	Neurokinin
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NPY	Neuropeptide Y

PACAP	Pituitary adenylyl cyclase activating peptide
PARP	Poly (ADPribose) polymerase
PBS	Phosphate buffer saline
pChAT	Peripheral choline acetyl transferase
PGP9.5	Protein gene product 9.5
PHI	Peptide histidine isoleucine
PTP	Permeability transition pore
RIP	Receptor-interacting protein
RT-PCR	Reverse transcriptase-polymerase chain reaction
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyl sulfate
SOM	Somatostatin
SP	Substance P
STAT1	Signal transducer and activator of transcription 1
TBS	Tris buffer saline
TGF- β	Transforming growth factor beta
Th1	T helper 1
Th2	T helper 2
TK	Tachykinin
TLR4	Toll like receptor 4
TNBS	2,4,6-trinitrobenzenesulfonic acid
TNF	Tumor necrosis factor
TNF- α	Tumor necrosis factor alpha
TNFR1	Tumor necrosis factor receptor 1
TNFR2	Tumor necrosis factor receptor 2
TRADD	TNF receptor-1 associated death domain
TRAF	TNF-receptor 1 associated protein
TUNEL	Transferase dUTP nick end labeling
VIP	Vasoactive intestinal peptide
WB	Western blotting

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CHAPTER 1:

INTRODUCTION

1.1. Innervation of the GI tract

The digestive functions of the gastrointestinal (GI) tract are controlled by intrinsic neural networks comprising the enteric Nervous System (ENS), which itself can be modulated by the autonomic and central nervous systems (Figure 1). Together, these systems comprise four different levels of control and modulation. At Level One are the intramural neurons which constitute the enteric nervous system. Level Two is comprised of the prevertebral ganglia of the sympathetic nervous system. Level Three consists of pre-ganglionic sympathetic and parasympathetic neurons of the autonomic nervous systems (ANS); and the brain (i.e. hypothalamus), Level Four, is positioned at the highest stage in this hierarchy (Wood *et al*, 1999). The extrinsic autonomic neurons connect the ENS to the brain, and form the brain – gut axis (Furness and Costa, 1987). Cell somas of extrinsic neurons are located in sacral part of the spinal cord, sympathetic ganglia, pelvic ganglia (both sympathetic and parasympathetic), and caudal region of the brain stem. The neuronal structures mentioned above are directly or indirectly involved in shaping the patterns of the different gut behaviors occurring throughout the gut wall necessary for optimal digestive function.

Figure 1. Hierarchy in the Neural Control of the Gut.

Four basic levels of enteric neural control: the intrinsic nerve networks of the ENS represent the first level of communication. The prevertebral ganglia of the sympathetic nervous system form the second level. Level three consists of the pre-ganglionic component of the sympathetic and parasympathetic systems, and the brain (specifically hypothalamus) is positioned in the highest level of this hierarchy, level 4. Adapted from Wood *et al*, 1999.

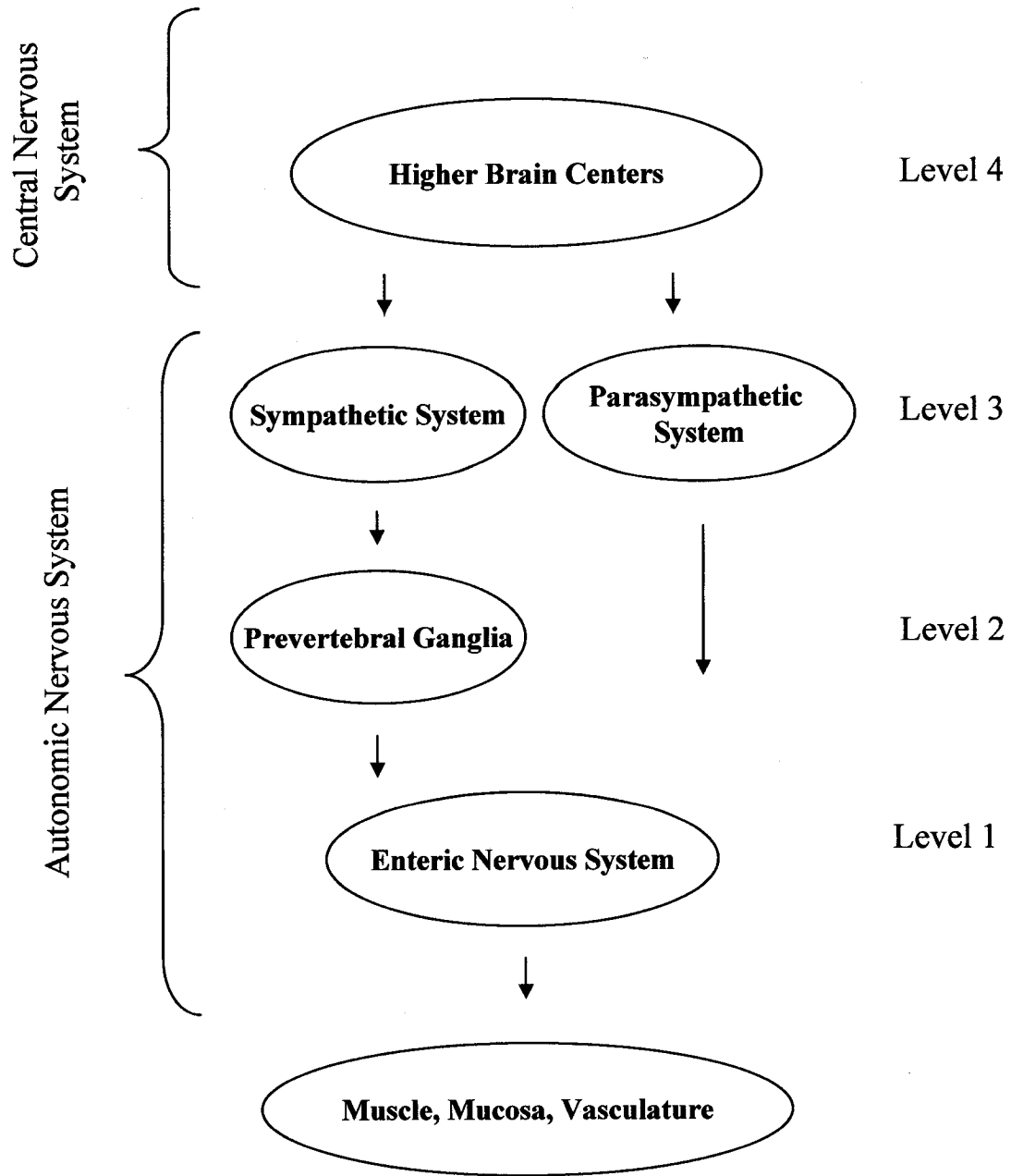


Figure 1

1.1.1. Parasympathetic Innervation

The parasympathetic neurons originate from the sacral part of the spinal cord and the medulla of the brainstem (cranial), and innervation of the GI tract has roots in both cranial and sacral divisions of the system. Parasympathetic innervation is responsible for increased activities in the GI tract; it increases motility, tone and secretion and induces sphincter relaxation (Sharkey and Pittman, 1996). Pre-ganglionic axons of parasympathetic efferent neurons originate from dorsal motor nucleus of the vagus (10th cranial nerve) and supply the postganglionic neurons (networked within the ENS) of the upper abdominal viscera (stomach, small intestine, ascending colon, gallbladder, pancreas, and liver), whilst the lower abdominal viscera (descending colon, rectum, urinary bladder, and genitalia) receive parasympathetic inputs from pelvic nerve. The pelvic nerve contains sacral parasympathetic fibers originating from the second, third and fourth sacral segments of the spinal cord that proceed to form pelvic nerve (Sharkey and Pittman, 1996) (Figure 2). The afferent parasympathetic fibers run with the efferent fibers. Vagal afferent fibers have their cell bodies in the cranial vagal ganglia, and pelvic afferents in the dorsal root ganglia. Visceral afferents have several functions including the detection of environmental changes (stimuli), conveying them into a signal and transmitting the signal (Sharkey and Pittman, 1996).

1.1.2. Sympathetic Innervation

The pre-ganglionic cell somata of the efferent sympathetic division are situated in the intermediolateral grey matter of the thoracic and upper lumbar segments of the spinal cord. After leaving the spinal cord, the fibers traverse the paravertebral ganglia to the

outlying prevertebral ganglia. Prevertebral ganglia consist of the celiac, superior mesenteric, inferior mesenteric and pelvic ganglia. The celiac ganglia innervate the GI tract at the level of the ascending colon; superior mesenteric ganglia innervates the small intestine and ascending colon; inferior mesenteric ganglia supply fibers to the transverse and descending colon, and pelvic ganglia innervate the descending colon and rectum (Luckensmeyer and Keast, 1994) (Figure 2). These sympathetic innervations serve to modulate gut motility and mucosal transport via prejunctional inhibition of acetylcholine (ACh) release from enteric excitatory cholinergic motor neurons (Furness and Costa, 1987). The afferent sympathetic division contains spinal visceral afferents which have their cell bodies in the dorsal root ganglia. The function of these visceral afferents has been discussed in section 1.1.1.

1.1.3. Intrinsic Enteric Innervation

The ENS is the intrinsic neuronal network of the gut (Hansen, 2000; Sharkey and Pittman, 1996). It ultimately regulates all functions of the GI tract: motility, absorption, secretion, and vascular functions are based upon pre-programmed patterns generated by the intrinsic circuitry of the ENS (Sharkey and Pittman, 1996). The basis of this intrinsic nervous system is the interconnected ganglionated neural networks that contain the sensory, interneuron and motor neuronal cell soma. These neuronal cells project their processes within the ganglia, between ganglia and also onto effector cells via the interconnecting primary, secondary and tertiary plexuses. Sensory neurons detect mechanical stimulations or changes in temperature and chemistry. Interneurons process sensory information and control motor neurons. Motor neurons initiate, sustain or

Figure 2. Sympathetic and Parasympathetic Innervation of the Gut.

Sympathetic and parasympathetic innervation of the gut is shown in red and blue respectively. Solid lines represent pre-ganglionic fibers; broken line, post-ganglionic fibers. Adapted from Carpenter, 1976.

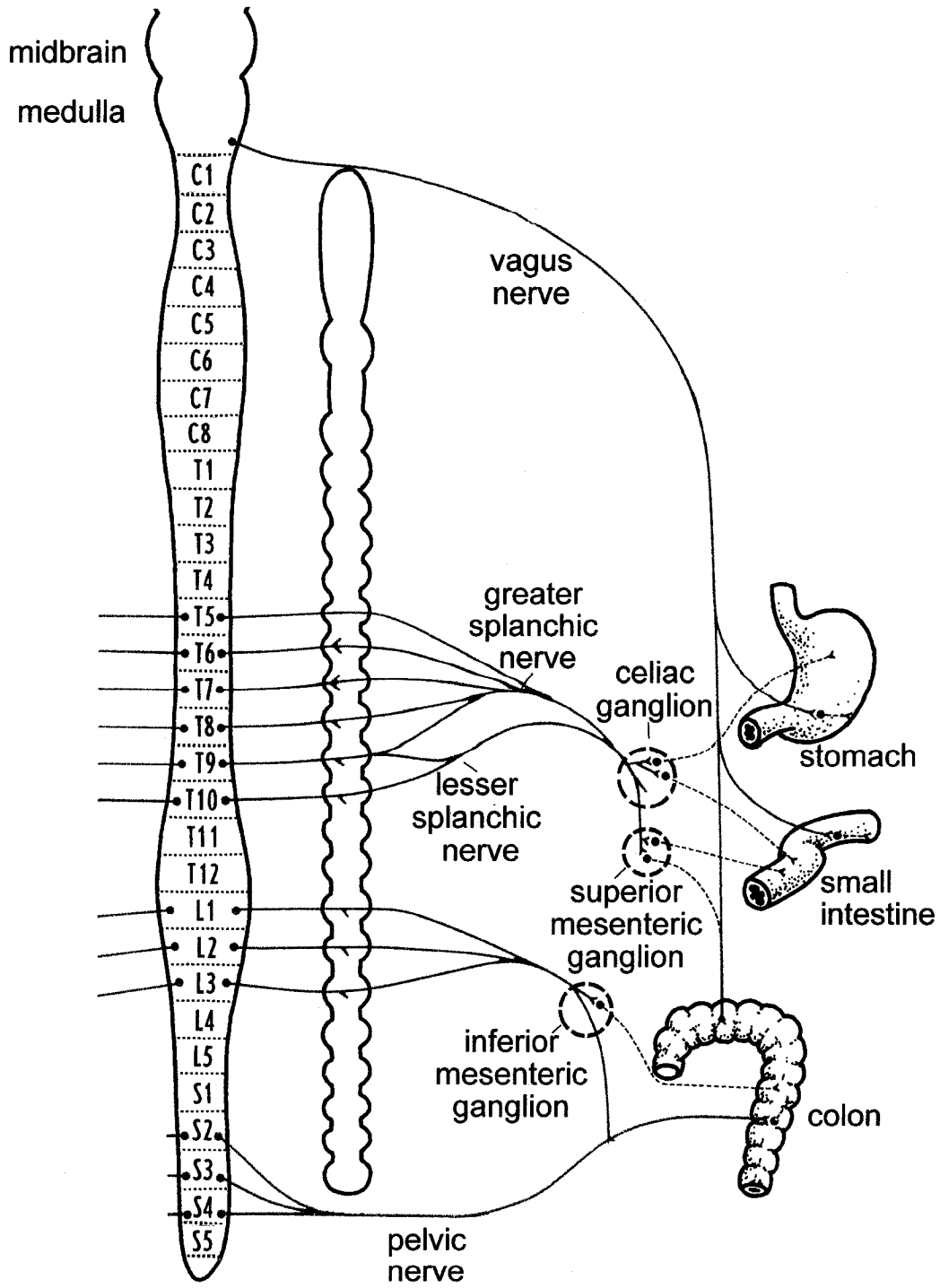


Figure 2

suppress the effector's behavior in the gut (Wood *et al*, 1999; Hansen, 2003; Wood, 2000). The average ratio of the sensory, interneuron and motor neurons is 2:1:1 (Hansen, 2003; Costa *et al*, 2000).

The ENS is different from the other constituents of the ANS and more comparable to brain and spinal cord, and enteric neuronal cell structure, neurochemistry and physiology/pharmacology resemble that of the CNS; on this basis it is sometimes referred to as the second brain, or the brain-in-the-gut (Wood *et al*, 1999). The number of enteric neurons is much larger than the number of pre-ganglionic motor fibers entering the gut by way of the vagus nerves: the number of neurons within the gut is estimated in the range of millions, whilst the efferent vagal fibers are in the thousands (Gershon *et al*, 1994). This suggests that many of the enteric neurons are not under direct individual control by the CNS, rather the enteric neuronal network acts as a whole but under the modulatory control by higher levels of the nervous system.

a. Enteric Plexuses:

Enteric neurons are found in interconnected plexuses located between the outer longitudinal and inner circular muscle layers (myenteric plexus), and in the submucosa between the circular muscle layer and the mucosa (submucosal plexus) (Sharkey and Pittman, 1996; Hansen, 2003). These neural networks (plexuses) contain aggregates (ganglia) of neuronal cell soma, and occur along the alimentary canal from the esophagus to the rectum, in all mammalian species (Furness and Costa, 1987). The location of these plexuses is shown diagrammatically in Figure 3. The myenteric and submucosal plexuses

Figure 3. Enteric Innervation of the Human Intestine.

Note the myenteric nerve plexus located between the circular and longitudinal muscle layers and the submucosal networks (plexuses) located between the mucosa and the circular muscle layer. Adapted from Hansen, 2003.

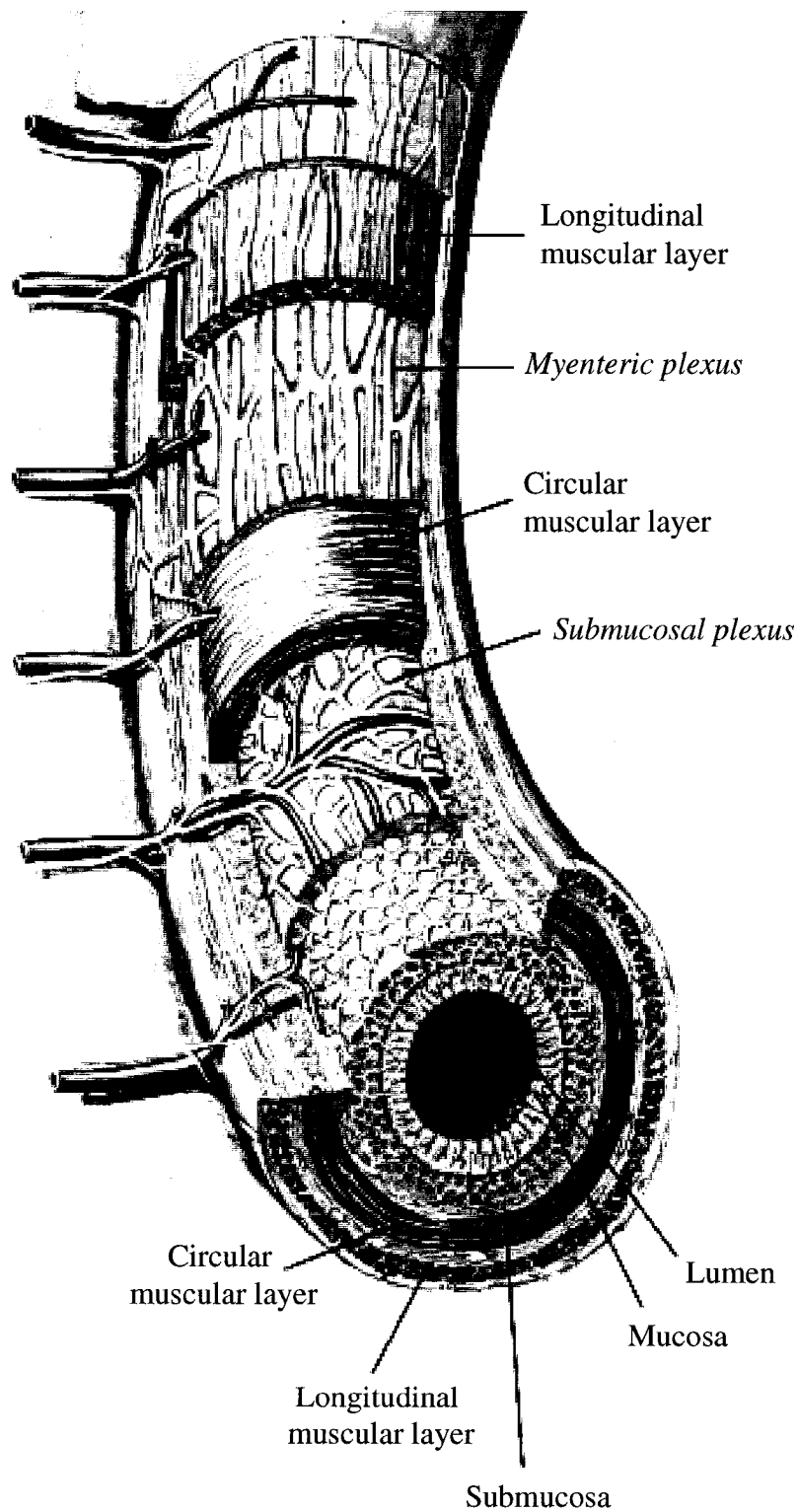


Figure 3

are specialized in controlling gastrointestinal motility and mucosal secretion respectively (Costa *et al*, 1996); however they are interconnected and are ultimately functionally integrated through the myenteric plexus, which is considered to be the primary neuronal network of the gut (Furness and Costa, 1987).

b. Enteric Ganglia:

The enteric ganglia contain neuronal cells and glial cells exclusively; these ganglia do not contain blood vessels, connective tissue, or collagen fibrils; and their metabolic needs rely on simple diffusion of nutrients and oxygen from the proximal vascular network of adjacent tissue (Gershon *et al*, 1994).

Enteric Glia:

The enteric glial cells are the morphological and functional equivalents of CNS astrocytes (Cabarrocas *et al*, 2003; Cornet *et al*, 2001). Glial cells possess an integrated structure of intermediate filaments, and are rich in glial fibrillary acidic protein (GFAP) (Cabarrocas *et al*, 2003). These cells have a protective role and support the enteric neurons; they envelop the enteric neuronal cell somas and axon fibers as well as intestinal blood vessels (Cornet *et al*, 2001; Furness and Costa, 1987). During the course of inflammation the glial cells become activated and capable of synthesizing inflammatory mediators (Tjwa *et al*, 2003). Furthermore, enteric glia may play an essential role in maintaining the integrity of the bowel; since it has been shown that ablation of enteric glia in adult transgenic mice has degenerative effects on myenteric neuronal survival. Bush *et al* (1998) propose that their loss or dysfunction may contribute to the cellular mechanisms of inflammatory bowel disease (IBD).

Enteric Neurons:

The greatest amount of study related to enteric neurons has been undertaken using guinea pig (see Furness and Costa, 1987). Extrapolating the data from lower mammalian species, in man there would be a total of about 80-100 million enteric neurons (Furness and Costa, 1987). Enteric neurons have been classified based on their morphology, projections, electrophysiological properties, and neurotransmitters (Anlauf *et al*, 2003; Costa *et al*, 1996). The ENS in primates and other mammalian species are comparable; however there are minor differences (Anlauf *et al*, 2003).

Morphology: Based on cytoarchitecture, Dogiel (1899) was the first to classify enteric neurons. Dogiel identified three types based mainly upon cell soma, length and shape as well as the number and pattern of cell processes (Brehmer *et al*, 1999). Flattened cells with stellate or angular shapes, 4-20 or more dendrites and a single axon form the Dogiel type I cells. He described type II cells as angular, star or spindle shaped with 3-10 dendrites and one axon. Type III cells were described as having 2-10 thin branching dendrites as they were followed from the cell body (Furness and Costa, 1987). Nevertheless, there are some cells whose shapes do not easily fit Dogiel's classification.

Neurochemistry: Heterogeneity exists in the neurochemistry of the enteric neurons: there are at least 30 different classes of chemicals represented in the ENS. In fact almost all of the neurotransmitter types used at synapses in the brain are represented in the gut (Gershon *et al*, 1994). Neurons in the gut commonly co-localize different neurotransmitters (table 1) (Lomax *et al*, 2000; Costa *et al*, 1996; Furness, 2000), and there is convincing evidence that "plurichemical transmission", the processes ensuing when multiple transmitters are released, occurs in the ENS (Sharkey and Pittman, 1996).

The permutations and combinations of transmitter molecules have been used to define enteric neuronal subpopulations in the submucosal and myenteric plexuses (Gershon *et al*, 1994). For instance, in guinea pig distal colon 25% and 18% of myenteric neurons show nitric oxide synthase (NOS) / vasoactive intestinal peptide(VIP) and choline acetyl transferase (ChAT) / calretinin immunoreactivity respectively (Lomax *et al*, 2000), and VIP / peptide histidine isoleucine (PHI) / neuropeptide Y (NPY) neurons comprise 38% of neuronal population in the submucosal plexus of the rat (Gershon *et al*, 1994). In Table 1 enteric neuronal subtypes in the submucosa and myenteric plexuses of the guinea pig ileum are reviewed.

ACh, glutamate, and tachykinins (TK) are considered to be the three major excitatory neurotransmitters in the ENS; however ACh is commonly recognized as the primary enteric excitatory transmitter. Adenosine triphosphate (ATP), nitric oxide (NO), and VIP are the common enteric inhibitory transmitters. In contrast to the CNS, gamma amino butyric acid (GABA) is considered to be either excitatory or inhibitory neurotransmitter, based on the receptor that is activated post-synaptically (Krantis *et al*, 2000).

The ENS is a target of inflammation and subject to damage and degeneration. In the next sections the general inflammatory process, inflammatory bowel disease in particular, and the effects of inflammation on neurons and glial cells in the gut will be reviewed.

Table 1. Types of Neurons in the ENS.

This table lists the known enteric neurons based upon neurochemical classification derived primarily from data obtained in the guinea pig small intestine. Myenteric neurons represent greater than 97%, and submucosal neurons represent less than 3% of all enteric neurons present within the guinea pig ileum (adapted from Furness, 2000, and Costa *et al*, 1996). Neurons are also classified based on their short / long processes. There are functional classes of myenteric and submucosal neurons (excitatory and inhibitory motor neurons, interneurons and sensory neurons); however the proportions and chemical codings of these categories are not known. **Abbreviations:** BN: bombesin a.k.a. GRP, CCK: cholecystokinin, ChAT: choline acetyltransferase, CGRP: calcitonin gene related peptide, ENK: enkephalin, GABA: gamma amino butyric acid, GAL: galanin, GRP: gastrin releasing peptide, 5-HT: 5-hydroxytryptamine, NFP: neurofilament protein, NK: neurokinin, NOS: nitric oxide synthase, NPY: neuropeptide Y, PACAP: pituitary adenyl cyclase activating peptide, SOM: somatostatin, TK: tachykinin, VIP: vasoactive intestinal peptide.

A. Myenteric Neurons

Note: percentages represent that of all myenteric neurons.

Proportion	Functional Class	Neurotransmitter/Characteristic Protein
12%	Excitatory circular muscle motor neurons	Short: ChAT/TK/ENK/ GABA Long : ChAT/TK/ENK/NFP
16%	Inhibitory circular muscle motor neurons	Short : NOS/VIP/PACAP/ENK/NPY/GABA Long : NOS/VIP/PACAP/Dynorphin/BN/NFP
25%	Excitatory longitudinal muscle motor neurons	ChAT/ Calretinin /TK
~2%	Inhibitory longitudinal muscle motor neurons	NOS/VIP/GABA
5%	Ascending interneurons (local reflex)	ChAT/ Calretinin /TK
5%	Descending interneurons (local reflex)	ChAT/NOS/VIP±BN±NPY
2%	Descending interneurons (secretomotor reflex)	ChAT/5-HT
4%	Descending interneurons (migrating myoelectric complex)	ChAT/SOM
26%	Myenteric intrinsic primary afferent (primary sensory) neurons	ChAT/ Calbindin /TK/ NK ₃ receptor
<1%	Intestinofugal neurons	ChAT/BN/VIP/CCK/ENK

B. Submucosal Neurons

Note: percentages represent that of all submucosal neurons.

Proportion	Functional Class	Neurotransmitter/Characteristic Protein
45%	Non-cholinergic secretomotor vasodilator neurons	VIP/GAL
15%	Cholinergic secretomotor vasodilator neurons	ChAT/ Calretinin / Dynorphin
29%	Cholinergic secretomotor (non-vasodilator) neurons	ChAT/NPY/CCK/SOM/CGRP/Dynorphin
11%	Submucosal intrinsic primary afferent (primary sensory) neurons	ChAT/TK/calbindin

Table 1

1.2. The Inflammatory Cascade: A Review

1.2.1. Definition

In the aftermath of injury caused by physical damage or pathogenic infection, a complex series of events ensue which represent the physiological response of the immune system to the injury. This response is a cumulative homeostatic process, termed inflammation. The ultimate result of inflammation is restoration of homeostasis in the organ (Baumann and Gauldie, 1994; Vaday and Lider, 2000). Amongst the cells involved in the inflammatory process, leukocytes are considered to be the most important.

1.2.2. Leukocytes in Inflammation

The two types of blood circulating leukocytes, mononuclear and polymorphonuclear cells, contribute in different ways to the response to damage or infection. Mononuclear cells consist of two categories: monocytes / macrophages and lymphocytes. In circulating blood, monocytes are relatively immature cells. They may leave the blood stream, enter the tissue, and differentiate into macrophages which are enlarged cells with increased number of mitochondria and lysosomal enzymes and long life span, i.e. months to years (Trowbridge and Emling, 1997). Macrophages mount generalized responses to stimuli: they consume microorganisms and malignant cells by phagocytosis and secrete cytotoxic compounds; moreover they collaborate with lymphocytes as they ingest and process antigens and present them to T cells (Trowbridge and Emling, 1997).

Lymphocytes are cells of the lymphoid system which are specialized to attack specific injurious stimuli: B-lymphocytes produce antibodies that bind to foreign organisms and facilitate their destruction, and T-lymphocytes act through a cell-to-cell contact manner, they become effector cells and produce cytokines and directly kill other cells (Kuby, 1997). Polymorphonuclear leukocytes (granulocytes) comprising neutrophils, basophils, and eosinophils, release cytotoxic compounds from their intracellular granules into the local environment during inflammation. If this event occurs too rapidly or else the response is too strong, this event may also injure the host tissue.

1.2.3. Acute Inflammation versus Chronic Inflammation

A short and relatively severe course of inflammation is referred to as 'acute', whilst that type of inflammation which persists over a long period of time is termed 'chronic'. The cell most commonly associated with the inflammatory acute phase response is the tissue macrophage or blood monocyte. Bacterial products can activate these cells; however regardless of the initiative event of inflammation, monocytes / macrophages are the forerunners of the inflammatory cascade. Activated macrophages release a broad spectrum of inflammatory mediators, of which tumor necrosis factor (TNF) and interleukin-1 (IL-1) families have a crucial role in initiating the next phases in inflammatory response (Baumann and Gauldie, 1994). The released cytokines act on stromal and endothelial cells which in turn produce the second wave of cytokines; these cytokines are highly chemotactic for neutrophils and macrophages and increase the migration of immune cells to the site of affected tissue.

Vascular dilation, a significant event in the process of inflammation, takes place predominantly in the post-capillary venules. The alteration of capillary tone is likely the result of the release of low molecular weight mediators at the site of inflammation, including molecules such as nitric oxide, products of arachidonic acid, and reactive oxygen species (Baumann and Gauldie, 1994). The upregulation and release of these inflammatory mediators are largely related to the migration and activation of mononuclear cells monocytes / macrophages and platelets. Thus activation of these cells results in increased vascular permeability leading to recruitment of even more circulating leukocytes to the site of inflammation. The acute phase lasts for 24-48 hrs, and within a few days the organism returns to its normal function; however if the stimulus persists or the control mechanism is disrupted, the acute phase may convert to a chronic one (Baumann and Gauldie, 1994).

In chronic inflammation the dominant immune cells are macrophages and lymphocytes replacing neutrophil granulocytes (Movat, 1985). Major immune functions in chronic inflammation are elicited through the macrophage phagocytic and cytotoxic actions, lymphocyte cytotoxicity, and natural killer cell activity. In the chronic phase pro-inflammatory cytokines, e.g. IL-1, interleukin 6 (IL-6), or tumor necrosis factor alpha (TNF- α), augment cell mediated immune activities, while regulatory cytokines interleukin 4 (IL-4), interleukin 10 (IL-10) or transforming growth factor beta (TGF- β) suppress the inflammatory response. Imbalance between pro-inflammatory and regulatory cytokines is implicated in diseases like ulcerative colitis (UC) and Crohn's disease (CD) contributing to the perpetuation and relapsing nature of these diseases.

In some inflammatory states, e.g. Crohn's disease, granulomatous inflammation is a hallmark of the chronic stage. It is distinguished by aggregation of activated macrophages sometimes joined together forming giant cells, encompassed by lymphocytes (Lucero *et al*, 2001; Freeman *et al*, 2000; Bogenrieder *et al*, 2003; Boros, 2003).

1.2.4. Th1 versus Th2 Response

Immune reactions are categorized into Th1 and Th2 immune responses. In the Th1 response, macrophages, T cytotoxic cells and CD4+ T helper cells are involved (Trowbridge and Emling, 1997; Kuby, 1997; Strober *et al*, 2002), and CD4+ cells produce interferon (IFN), interleukin 2 (IL-2), interleukin 12 (IL-12) and TNF which mediate macrophage activation and delayed type hypersensitivity reaction (Romagnani, 1999; Luster and MacLean, 2001). The Th1 mediated response is marked by intramural infiltration of immune cells and is sometimes associated with granulomata. For instance Crohn's disease is considered to be a Th1 mediated inflammation.

Th2 mediated response is characterized by the synthesis and secretion of IL-4 and interleukin 5 (IL-5). B-lymphocytes and a subset of CD4+ T helper cells are involved in the process of Th2 mediated response (Trowbridge and Emling, 1997; Kuby, 1997). In Th2 response CD4+ cells produce IL-4, IL-5, IL-6, IL-10 and IL-13, which act as growth or differentiation factors for B cells (Romagnani, 1999; Luster and MacLean, 2001).

The equilibrium between the two types of responses is regulated by the balance in synthesis of IL-2 and IL-4, as IL-2 initiates the production of Th1 cells and IL-4 swings the balance in favor of Th2 immune cells. Th2 mediated inflammation is associated with superficial infiltration of immunocytes, disruption of epithelial and mucosal layers, and in

some cases massive polymorphonuclear cell invasion. Ulcerative colitis has not been clearly shown to be a Th2 mediated inflammation, as it does not meet the respective established criteria. For example the level of pro-inflammatory cytokine IL-4, the promoter of Th2 inflammation, is quite normal in UC patients. Obviously if a Th2 type of response underlies inflammation in UC, it is a highly atypical Th2 inflammation (Strober *et al*, 2002).

1.3. Inflammation and the Enteric Nervous System

Within the CNS, the brain is located within a bony cavity, hence protected against physical trauma. Exogenous proteins are also incapable of permeating the parenchyma of the brain due to the protective blood-brain barrier, except in circumventricular areas. In this system, the endothelial cells form a continuous band of tight junctions that prevent the free diffusion of most substances between the cerebrospinal fluid and blood (Brightman and Reese, 1969).

Within sympathetic and parasympathetic nerves, there are tight junctions between capillary endothelial cells and between perineural fibroblasts surrounding the neurons. These barriers protect neurons from inflammation (Haines, 2002).

Enteric neurons are subject to the same inflammatory influences as other cells within the gut wall. The ENS, however, is not privileged to protective mechanisms associated with the CNS and the ANS.

The ENS lacks a perineurium and the neurons in its avascular ganglia and tracts are exposed to extracellular fluid formed by permeable vessels in adjacent tissues of the gut

(Kiernan, 1996). Although the enteroglial cells form a marginally protective pre-ganglionic sheath around the enteric neurons, it is neither equivalent to the perineurium barrier present in the ANS, nor to the highly impermeable blood-brain barrier in the CNS. All the extracellular spaces of the peripheral nervous system are accessible to plasma proteins, and diffusion occurs more quickly into the ganglia than into the endoneurium. For instance, Allen and Kiernan (1994) have shown that albumin could easily penetrate into the enteric ganglia whereas it does not penetrate the highly impermeable blood-brain barrier.

There are three major target cells in the intestine affected by the presence of inflammatory mediators: smooth muscle cells, glial cells and neurons. Both intrinsic enteric neurons and terminals of extrinsic afferent and efferent nerves are structures targeted by inflammation (Bueno, 2000). Inflammation induces phenotypic changes in enteric neurons and affects the release of neurotransmitters and other mediators in the ENS.

1.3.1. Inflammation and Enteric Neurons

The smooth muscle of the GI tract has an intrinsic rhythmicity. Motor function of the gut wall is superimposed upon this intrinsic tonic activity coordinated by the intrinsic neurons and modulated by the extrinsic neural innervation. Destruction of the enteric nerve plexuses impairs this control and the remaining muscular activity (tonic activity) is insufficient for propulsion of bowel contents through the intestinal tract. The ENS damage also inhibits and impairs intestinal blood circulation, gastrointestinal secretion, and absorption (Giaroni *et al*, 1999).

With damage to inhibitory enteric motor neurons, hypermotility, spasticity, and achalasia are expected, since loss of inhibitory control allows the self-excitabile smooth muscle to contract continuously and obstruct the alimentary canal. For instance the neurogenic forms of chronic intestinal pseudo-obstruction, i.e. paraneoplastic syndrome, Chagas' disease, and idiopathic degenerative disease, are linked with degeneration of the neurons of the ENS (Wood, 2000), and the symptoms of the irritable bowel syndrome may be classified as an expression of early stage of enteric neurodegeneration (Wood *et al*, 1999). In Hirschsprung's disease, a functional disorder of the gut in which there is a loss of enteric neuromuscular inhibition, a congenital absence of ganglion cells in the myenteric and submucosal plexuses of the distal bowel is observed (Cuffari *et al*, 1993). Furthermore, enteric neurodegeneration may arise secondarily in intestinal or extraintestinal diseases. It is found associated with a variety of conditions like diabetes, Parkinson's disease, scleroderma, and myotonic dystrophy (Giaroni *et al*, 1999).

In some diseases the enteric neuronal inflammation and / or degeneration is also associated with a typical aspect of inflammation: immunocyte infiltration. For example both chronic intestinal pseudo-obstruction and IBD are characterized by extensive neuronal cell death accompanied by migration of immune cells to the neuromuscular structures (Schuffler, 1981).

1.3.2. Glial Cells and Inflammation

Astrocytes of the CNS react to inflammation in a vigorous manner which is characterized by morphological changes and cell proliferation, termed as "reactive astrogliosis" (Eddleson and Mucke, 1993; Sharkey and Kroese, 2001). The transition of

astrocytes from the resting to the activated state is associated with the expression of proteins not normally detectable in quiescent astroglia as well as with the upregulation of factors that are found at low levels in resting astrocytes (Eddleson and Mucke, 1993).

Proliferation of enteroglia occurs in normal conditions and is increased in response to inflammation (Bradley *et al*, 1997), and although factors responsible for enteroglia hyperplasia have yet to be definitively determined, the anti-inflammatory cytokine IL-10 enhances glial cell proliferation *in vitro* (Rühl *et al*, 1999). Enteroglia cells are also a potential source of pro-inflammatory cytokine IL-6 in the myenteric plexus when stimulated with other cytokines such as IL-1 β (Rühl *et al*, 2001); IL-1 β itself is upregulated during inflammation in the gut. Moreover, in response to inflammation enteroglia cells express substance P (SP) that triggers the pro-inflammatory activity of macrophage cells (Cabarrocas *et al*, 2003).

1.4. Inflammatory Bowel Disease (IBD)

The IBD consists of two chronic idiopathic inflammatory diseases of the GI tract: ulcerative colitis and Crohn's disease (Yang *et al*, 2001, Pavlick *et al*, 2002). These two diseases are considered together because of their overlapping clinical, epidemiologic and pathogenetic features. They also share complications and some therapies. Men are more likely than women to develop UC and women are at a 20-30% greater risk to develop CD (Tremaine and Dozois, 1997). The highest proportion of IBD patients displays onset of disease between the ages of 15-40 years; however it may occur for the first time anytime between infancy and old age (Peppercorn, 1989). IBD has a yearly incidence of 20,000

cases in the United States or 7.8 cases per 100,000 population (Kubes and McCafferty; 2000; Pavlick *et al*, 2002). Canada has one of the highest rates of incidence of IBD in the world; for example province of Manitoba during the years 1989-1993 had an incidence rate of 29.0 IBD cases per 100,000 population (Kirsner, 2000). It is an uncommon disease in Asia, Africa, and South America.

1.4.1. Symptoms

Massive inflammatory infiltration and extensive mucosal and/or transmural injury including edema, loss of goblet cells, decreased mucous production, crypt cell hyperplasia, erosions, and ulcerations are classical features of IBD (Pavlick, 2002). Rectal bleeding, severe diarrhea, abdominal pain, fever, and weight loss are clinical manifestations commonly associated with these diseases, which significantly reduce the quality of life (Kubes, 2000; Pavlick, 2002).

1.4.2. Ulcerative Colitis or Crohn's Disease?

The mucosa and submucosa of the colon are the layers of the GI tract wall displaying pathophysiology in UC. Disruption of colonic mucosal structure diminishes the ability to absorb water and sodium, leading to diarrhea. The disease may be limited to rectum, involve the colon, or extend to the caecum in a continuous fashion. The major features of UC are diffuse inflammation, crypt abscesses, and chronic mucosal changes (Peppercorn, 1989).

In CD any portion of the GI tract may be involved, it has a discontinuing focal pattern and spreads transmurally. In one third of Crohn's cases only the small intestine is

involved, and most of these cases occurring in the distal ileum (Ileitis); 20% of patients with Crohn's complication have colonic involvement (Crohn's colitis) (Peppercorn, 1989).

1.4.3. Etiology

To date the mechanisms underlying these inflammatory conditions are poorly understood. Experimental and clinical studies suggest that the initiation and pathogenesis of these diseases are multifactorial, and interactions amongst genetic, environmental, and immune factors play a critical role (Fiocchi, 1998; Pavlick *et al*, 2002). IBD appears to be an excessive response to a normal stimulus or a normal response to a chronic abnormal stimulus (Tremaine and Dozois, 1997). A recent conceptual advance in our understanding of the etiology of IBD has arisen from experimental studies suggesting that chronic gut inflammation results from a dysregulated immune response to components of the normal gut flora, as examining the biopsies taken from IBD patients reveals the presence of immune cells such as macrophages, lymphocytes and polymorphonuclear cells (specifically neutrophils) in the inflamed areas of the gut (Pavlick *et al*, 2002; Dijkstra *et al*, 2002; Podolsky and Fiocchi, 2000; Oehmichen and Refferscheid, 1977). Dysregulation of host immune reactions and pathological activation of the immune system are factors acknowledged to be etiological or at least contributory to the perpetuated inflammatory response. Several pathways normally utilized in immune defense converge to promote intestinal inflammation. These contributing factors include pro-inflammatory cytokines, nitric oxide, reactive oxygen species, eicosanoids, bacterial endotoxin, antibodies and autoantibodies (Fiocchi, 1998).

1.5. The Enteric Nervous System in IBD

Lesions of the enteric nervous system have been described frequently in chronic inflammatory bowel diseases. These lesions include hyperplasia, hypertrophy and degeneration of enteric cells.

1.5.1. Ulcerative Colitis

Dysfunction of the gut control of motility and absorption is characteristic of UC, and yet inflammatory tissue disruption does not extend to the neuromuscular layers. Therefore the ENS may be affected by inflammatory cells and mediators diffusing to the deeper layer of the intestine. Neunlist *et al* (2003) found changes in subpopulations of enteric neurons in UC: they observed marked reduction in the proportion of ChAT positive neurons in inflamed and non-inflamed areas of the gut in UC patients. On the contrary, the proportions of neurons containing ChAT / SP and SP were significantly higher in inflamed and non-inflamed areas in comparison with controls; therefore there is a shift from mainly cholinergic to more SP positive innervation in the gut during colitis. Furthermore, glial cells may be affected by intramural infiltration of the inflammatory components (Jacobson *et al*, 1995), and acute colitis is associated with marked increase in Fos expression in these cells (Miampamba and Sharkey, 1999).

1.5.2. Crohn's Disease

Neuropathy in the sympathetic and parasympathetic nervous systems is a well established feature of Crohn's disease; for instance almost half of the CD patients in the study of Lindgren *et al* (1990) have shown disturbances in these parts of the ANS; however pathophysiologic changes in the enteric nervous system in CD need further studies. Major histocompatibility complex (MHC) molecules class II antigens are expressed on axons and dendrites of the neurons and enteroglial elements in the submucosal and myenteric plexuses of CD patients (Geboes *et al*, 1992). MHC type II is involved in the education of thymocytes during intrathymic development and acts as a restriction element in the presentation of antigen to systemic CD4+ cells.

Siemers *et al* (1974) have reported axon dilation, increased number of neurofilaments, and penetration of nerve fiber bundles by plasma and mast cells in CD patients. Moreover axonal necrosis is observed in both inflamed and grossly normal areas of the ileum and colon in Crohn's patients (Brewer *et al*, 1990, Dvorak *et al*, 1993). Steinhoff *et al* have shown that intramural axonal degeneration ensues in CD cases exclusively (1988). However on the contrary, Oehmichen and Reifferscheid (1977) observed extended nerve fiber filaments, sometimes exhibiting a tumor-like distension. Another intramural observation concerning the changes in the enteric neurons is the presence of small axons in the neuronal plexuses (Brewer *et al*, 1990).

According to the study of Oehmichen and Reifferscheid (1977) intramural ganglion cell degeneration occurs in CD as they found various stages of cell deterioration frequently next to each other: deformity in the cells with nuclei positioned close to the cell margin, vacuolar degeneration in the cytoplasm, central chromatolysis and disappeared nuclei, and atrophy.

In the past decades a variety of IBD-like models of inflammation have been used to study the depth of the phenomenon of inflammation and influence and subsequent effects of it on the intestine and its shaping components including the ENS.

1.6. Experimental Models of IBD

An ideal animal model of a disease should have the same causal factors, the same pathology and pathophysiology, and the same clinical manifestations as the disease itself. A variety of animal species and inflammatory inducing factors have been applied to generate an appropriate model of IBD; nevertheless the ideal experimental model as well as treatment has yet to be found due to the unknown etiology of the disease. Animal models of IBD have been used for more than half a century to investigate the nature of these diseases and test therapeutical approaches (Kim and Berstad, 1992, Strober *et al*, 2002, Strober, 1985). These models have been of particular help in characterizing the immunologic basis of this category of disease.

Animal models of IBD could be classified under two general categories: naturally occurring (exposure to infectious agents, genetic defect) and experimentally induced inflammation (exposure to chemicals / toxic dietary substances, pharmacologic agents, or environmental materials; exposure to materials derived from patients; or manipulation of the animal's immune system) (Strober, 1985).

Natural models of inflammatory bowel disease are generally rare and relatively expensive. For instance Madara *et al* (1985) carried out their experiments and studies in Cotton-top tamarins (*Saguinas oedipus*), showing the presence of different symptoms of

the disease in animal and its positive response to sulfasalazine treatment. Unfortunately this endangered species is not widely available, thus limiting its usefulness.

Chemically induced inflammation in rodents has become the most widely used model to study the disease and evaluate novel treatments (Strober *et al*, 1998, Morris *et al*, 1989, Yamada *et al*, 1992). Amongst the chemically induced models of IBD, acetic acid, 2,4,6-trinitrobenzenesulfonic acid (TNBS) / 2,4-dinitrobenzenesulfonic acid (DNBS) – ethanol, formalin, indomethacin, cyclosporine, mitomycin C, and peroxy nitrite are the substances most commonly applied to induce inflammation in the gut (Kirsner, 2000).

In order to induce an inflammation similar to what is observed in IBD the mucosal barrier should be broken and the immune system subsequently activated; the hapten-ethanol model is designed based on this approach. This model was introduced by Morris *et al* (1989) and has been widely used in the past years to analyze the relation of the response to a specific antigen to the overall mucosal immune response leading to colonic inflammation. Ethanol breaks the mucosal barrier, allowing hapten molecules to penetrate the inner layers of the tissue where they bind to host proteins, rendering them immunogenic. The compounds frequently used to elicit immunologic responses are TNBS and DNBS (Morris *et al*, 1989). These hapten molecules are unable to induce an immune response alone; however coupling of the nitrophenyl groups of the hapten to a substance of high molecular weight (e.g. tissue proteins) initiates immunologic activation (Figure 4). These haptens also modify host cell protein lysine residues, rendering these cells prone to recognition and lysis by immune cells. Formation of sulfite radical, a precursor of cytotoxic sulfate and sulfite peroxy radicals, and reduction of TNBS and DNBS to nitro anion radicals with concomitant formation of superoxide radical are other

possible mechanisms for their inflammation-inducing potential (Cavani *et al*, 1995; Chamulitrat, 1997). The other possible mechanism of induction of colitis in this model is that ethanol disruption of the mucosal barrier in the colon represents an initial tissue insult which subsequently leads to increased exposure of the mucosal immune system to mucosal microflora. Antigenic determinants present in the mucosal microflora contribute to the immune response driving this disease (Strober *et al*, 2002). Morris *et al* (1989) showed that ethanol causes widespread acute mucosal damage and the combined administration of ethanol and TNBS results in the development of severe, transmural, granulomatous inflammation of the distal colon which persists for 8 weeks in Sprague-Dawley rats. These effects can not be induced with either ethanol or TNBS alone. The advantages of this experimental approach are: ethanol-hapten combination can induce an IBD-like pathophysiology in a localized and controlled way with little discomfort for the animal and minor effects on its behavior, it is fairly inexpensive, it is easily reproducible, lesions are found in 90 to 100% of the hapten injected animals (Kirsner, 2000), it does not need any kind of pretreatment and sensitization, the inflammation is long lasting enough to examine therapeutic treatments on the animal: the hapten itself is detectable for up to 5 weeks in the tissue (Morris *et al*, 1989) and its inflammatory effects last for even a longer period of time (8 weeks, Kirsner, 2000), it is even possible to generate a chronic inflammation by intrarectal injection of a higher dose of the hapten, and finally the model is more relevant to IBD than other chemically induced inflammations as it involves the use of an immunologic hapten and because the mucosal injury produced by the barrier breaker, ethanol, resolves quickly and is followed by a more enduring phase of inflammation (Yamada *et al*, 1992).

Figure 4. Mechanism of Ethanol and TNBS/DNBS – Induction of Inflammation.

In one of the most commonly used experimental models of colitis, TNBS/DNBS binds to endogenous protein molecules and makes them immunogenic, thus initiating the inflammatory response. *Abbreviations:* H: Hapten, P: protein, T: T-cell, M: macrophage. Diagram adapted from Trowbridge and Emling, 1997.

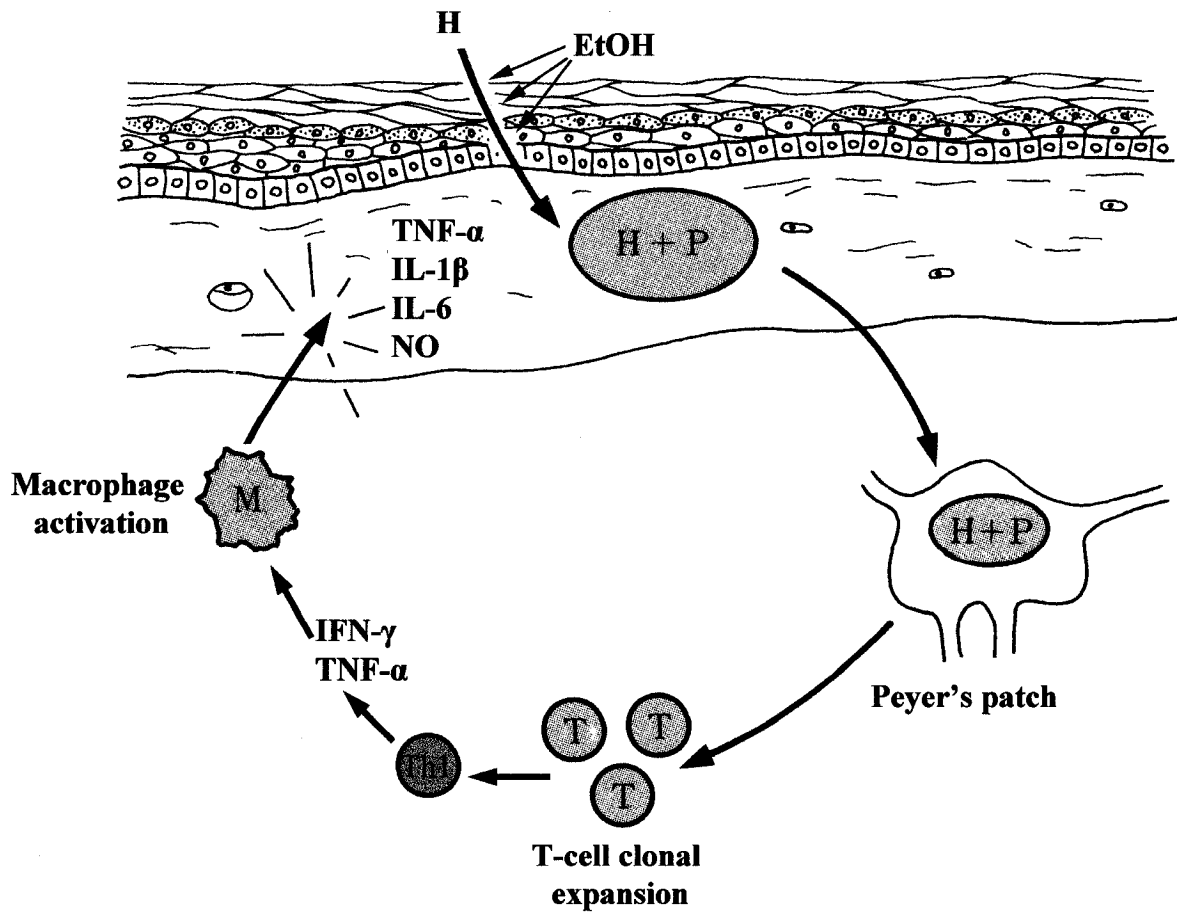


Figure 4

Mucosal inflammations in this “induced” murine model of IBD are IL-12 driven processes which result in the appearance of Th1 cells producing pro-inflammatory cytokines such as IFN- γ and TNF- α (Neurath *et al*, 1995; Davidson *et al*, 1996). In hapten-induced inflammation thickening of the bowel wall, and leukocyte (polymorphonuclear cells, macrophages, and lymphocytes), mast cell and fibroblast infiltration of the full thickness of the gut wall are observed (Morris *et al*, 1989).

Studying experimental murine models of colitis have shown that the induced inflammation is limited to the colon (Neurath *et al*, 1995). This inflammation is TNF- α dependent as it could not be induced in TNF- α deficient mice and is far more severe in mice over-expressing this pro-inflammatory cytokine (Neurath *et al*, 1997). Thus TNF- α is essential in initiating and persistence of the Th1 response. A major insight derived from the study of hapten-induced colitis is that the anti-inflammatory mechanisms inherent in the mucosal immune response can prevent the development of colitis: Elson *et al* (1996) and Fuss *et al* (2002) have demonstrated that oral administration of the hapten prior to its intrarectal injection results in unresponsiveness to this agent and protects mice from development of colitis. This preventive effect was due to the induction of regulatory cells synthesizing TGF- β . Thus TGF- β appears to be an essential regulatory cytokine in colitis; however IL-10 is necessary to the maintenance and effectiveness of the TGF- β response (Fuss *et al*, 2002). Hapten-induced colitis ensues only in certain murine strains, suggesting that genetic factors are involved to determine whether TNBS/DNBS will elicit an inflammatory response (Strober *et al*, 1998).

Neurath *et al* (1995) have shown that T cells extracted from lamina propria of the animals with TNBS-induced colitis transfer the disease to the naïve animals in the

absence of the hapten. This observation suggests that inflammation ensuing in TNBS-injected animals is at least partly due to the reaction of immune cells and/or their inflammatory products with the normal flora components.

In this research a murine model of chemically-induced colitis by DNBS intrarectal injection was used to study the effect of inflammation on survival of the enteric neurons and its dependence on nitric oxide synthesis during the course of inflammation.

1.7. The ENS Response to Inflammation in Experimental IBD

Enteric cells are subject to injury, alteration, and degeneration in IBD. These features are also detectable in experimental models of IBD. Experimental intestinal inflammation in rats is accompanied with a reversible suppression of ACh release from the myenteric plexus in the jejunum (Collins *et al*, 1989), and is associated with significant reduction in choline uptake, and ACh release (Poli *et al*, 2001). In experimental colitis, the release of noradrenaline from enteric neurons in rat transverse and distal colon and terminal ileum is suppressed (Jacobson *et al*, 1995, 1997). Interestingly, this suppression was observed in both inflamed and non-inflamed areas of the gut, suggesting that pro-inflammatory cytokines reaching the normal regions of the gut are sufficient to impact on function of enteric neurons without attracting inflammatory cells. In a rabbit model of colitis, inflammation was associated with a defect in the modulation of secretomotor neurons by ACh and prostaglandin E₂ (Goldhill *et al*, 1993). The pattern of SP immunoreactivity in the submucosa of the guinea pig ileum is also substantially changed by TNBS-induced inflammation (Miller *et al*, 1993).

In the ileum, SP is present in primary afferent and intrinsic enteric nerves. There was an initial reduction in SP immunoreactivity in enteric neurons 24 hrs after the induction of colitis. Similar results have been obtained for SP and calcitonin gene-related peptide (CGRP) in acetic acid-induced ileitis in guinea pig over a shorter time course (Sharkey and Kroese, 2001) and in the parasitized ferret (Palmer and Greenwood, 1993). The enteric neurons also showed reduced or absent expression of protein gene product 9.5 (PGP9.5) during inflammation (Poli *et al*, 2001). This suggests that tissue inflammation influences the nerve cell functions. This effect was reversible as followed by a gradual increase in the intensity and density of immunoreactive nerves.

In TNBS-induced colitis glial cells proliferate (Bradley *et al*, 1997); however it is not clear that glial hyperplasia actually occurs, as it is possible that there is a balance between degeneration and regeneration of these cells during inflammation.

Inflammation in the gut can also result in neuronal degeneration. Sanovic *et al* (1999) detected a significant myenteric and submucosal neuronal cell loss in the inflamed regions of the gut in hapten-induced colitis rats. Neurodegeneration was observed as early as 24 hrs after induction of colitis with only about half of neurons remaining by day four and thereafter, when inflammation had subsided. The number of ganglia was also significantly reduced in submucosal plexus of TNBS injected animals.

1.8. Cell Death Mechanisms

1.8.1. Necrosis

Cell death occurs via one of two mechanisms: necrosis and apoptosis. Necrosis is a cell death mechanism ensuing due to toxicity, massive ischemia or high dose of radiation (Honig and Rosenberg, 2000). In necrosis the cell membrane is disrupted resulting in the loss of control of ion influx and changes in the osmotic pressure gradient that allows the cells to take on more water. Subsequently the cells swell, the chromatin inside the cell takes a more flocculated pattern, and ATP activity in mitochondria is abolished. Eventually the loss of plasma and organelle membrane integrity leads to cell death (Leach, 1998, Hashimoto, 1996). DNA degradation of necrotic cells occurs later by proteases and nucleases destroying histones and nucleic acids (Leach, 1998). A number of pathways have been implicated in necrosis, including generation of reactive oxygen species, phospholipase activation, perturbation of Ca^{2+} homeostasis, ATP depletion, and unspecific DNA and protein damage (see the review by Beyaert and Fiers, 1994).

1.8.2. Apoptosis

Apoptosis or 'programmed active cell death' (Honig and Rosenberg, 2000) is an active process which needs energy in the form of ATP to maintain cellular integrity (Leach, 1998). The cell consumes the energy to synthesize RNA and proteins necessary for the successful completion of the process. Apoptosis occurs in developmental or programmed degenerative changes, growth factor deprivation, mild ischemia, or radiation (Honig and Rosenberg, 2000). It is involved in many diseases; its suppression is a hallmark of cancer, whilst abnormal upregulation of apoptotic pathways contributes to neurological disorders (e.g. Alzheimer, encephalomyelitis, Down's syndrome) (Hashimoto, 1996; Allen *et al*, 1998; Shi, 2002).

Oxidative stress, loss of neurotrophic factor support, endogenous or exogenous toxic factors, or excessive release of excitatory neurotransmitters (excitotoxicity) are assumed to initiate apoptosis in a variety of cells in the nervous system and other systems (Honig and Rosenberg, 2000). Nitric oxide can induce apoptosis in cells. Elevated levels of nitric oxide can also react with superoxide to form the highly cytotoxic peroxynitrite radical, which can induce cell death via several pathways (see section 1.10.3) (Bonfoco *et al*, 1995; Brune *et al*, 1998; Brune *et al*, 1997; Messmer and Brune, 1996; Uchiyama *et al*, 2002; Chung *et al*, 2001).

The apoptotic cell loses its water and ions resulting in condensation of cytoplasm and nucleus. Nuclear membrane invagination progresses to nuclear segmentation (Leach, 1998). The shrinkage of the cell proceeds to the formation of apoptotic bodies which are cellular fragments constituted of cytoplasmic proteins, nuclear material and intact intercellular organelles. The adjacent cells recognize these bodies and eliminate them. The whole process of apoptosis ensues quite quickly in a time frame of about four hrs without provoking inflammatory response.

Regulation of Apoptosis at the Gene Expression Level: A few of the apoptosis affiliated genes have been identified, and amongst them families of caspases, B cell leukemia/lymphoma 2 (Bcl-2), and Fas are well studied. Caspases, a family of at least fourteen members, play a critical role in initiating apoptosis. An extracellular signaling may result in aggregation of caspases through an association of cytoplasmic domain of ligand receptors, e.g. Fas-ligand or TNF- α receptor, and adapter molecules. The alternative pathway for this activation involves alteration in mitochondrial permeability transition pores (PTP), cytochrome c release, association of this protein with apoptosis

aggregating factor (APAF-1) and activation of caspase-9 (Honig and Rosenberg, 2000). Another important group of apoptotic regulatory proteins is Bcl-2 family. It consists of apoptotic suppressing members such as Bcl-2 and B cell leukemia-x long (Bcl-X_L), and apoptotic initiating proteins including Bcl-2-associated x protein (Bax) and Bak (Allen et al, 1998). Fas gene is also involved in triggering apoptosis. Fas gene product Apo-1 is a lymphocyte cell surface receptor and essential for cellular death in this cell type (leach, 1998).

In this study by applying a variety of assays and necessary measurements the occurrence of apoptosis and necrosis in cell culture and in vivo experiments were investigated.

1.9. Inflammatory Cells Involved in IBD

As early as 1977, Oehmichen and Refferscheid reported that macrophage infiltration in inflammatory bowel diseases is often accompanied by ganglion cell loss. They also observed that inflammatory lesions consisted of lymphocyte, neutrophil and in some cases eosinophil infiltration of the bowel wall and reported of a slight inflammatory infiltration in the region of the intramural plexus.

1.9.1. Mast Cells

Mast cells are hemopoietic cells involved in inflammatory and allergic reactions. They store multifunctional mediators and vasoactive substances and release their products on specific cell activation (Valent, 1995). Mast cells are present in the mucosa

in normal conditions. Up to 78% of mast cells are apposed to neurons and adjacent to nerve fibers, where membrane-to-membrane contact between axon-like processes and mast cells exists (Stead *et al*, 1989). The importance of this arrangement is underscored by observations in colonic biopsies from patients with Crohn's disease, where SP enhances mast cell degranulation and histamine release in intestinal tissues with active inflammation (Raithel *et al*, 1999). In an ultrastructural study in patients with IBD, Dvorak *et al* (1992) demonstrated that nerve–mast cell associations were significantly increased in UC (the role of mast cells in intestinal inflammation is reviewed by Sharkey and Mawe, 2002, and Sharkey and Kroese, 2001).

1.9.2. Monocytes / Macrophages

The production of monocytes in the bone marrow, their absolute number in blood circulation, and the percentage of inducible nitric oxide synthase (iNOS) positive circulating monocytes are markedly increased in both UC and CD patients (Dijkstra *et al*, 2002; Podolsky and Fiocchi, 2000). In these patients a broad zone of densely packed macrophages is detectable at the bottom of the ulcers and the fissures of the gut, and the remaining part of the swollen edematous submucosa contains a large number of macrophages (four to five times as much as in healthy colon) (Seldenrijk *et al*, 1989). The distinctive feature of IBD is the almost complete replacement of colonic mucosal macrophages with mature tissue macrophages that have the ability to synthesize inflammatory mediators. These cells enter the lamina propria along the chemotactic gradients of inflammatory mediators and respond to inflammatory stimuli by upregulating the transcription factor nuclear factor kappa B (NF κ B), which in turn

activates cytokine gene transcription (Elson, 2000). This feature is quite unique to inflammatory bowel diseases UC and CD, and is not observed in ischaemic colitis, infective colitis and solitary ulcers of the colon (Allison and Poulter, 1991). Furthermore, random macrophage motility, chemokinesis, is significantly increased in IBD patients (Elson, 2000).

Infiltration of mucosa and muscular layers of the gut with macrophages has also been reported in experimentally induced colitis (Bradley *et al*, 1997; Yamada *et al*, 1993; Poli *et al*, 2001; Morris *et al*, 1989).

1.9.3. Lymphocytes

The gut is a rich source of lymphocytes which play a vital role in defense against foreign antigens (Sharkey and Kroese, 2001). In affected mucosa of IBD patients 4 to 6 fold increase of perforin or granzyme-A mRNA positive intraepithelial lymphocytes are observed. Perforin and granzyme-A are pan-markers widely used to assess cytotoxic activity (Kappeler and Mueller, 2000). Furthermore, there are data showing an interaction between neuronal cells and lymphocytes. For instance SP can affect lymphocyte functions and increase its trafficking through peripheral lymph nodes (O'dorisio, 1988).

Infiltration of lymphocytes and marked increase of the total number of these cells in the site of inflammation have also been observed in murine experimental models of colitis (Yamada *et al*, 1993; Poli *et al*, 2001; Morris *et al*, 1989; Bradley *et al*, 1997).

1.9.4. Neutrophils

Although eosinophils are normally present in the gut, neutrophils are not, and infiltration of the intestine by these cells is a classical feature of active inflammation in the gut as first observed by Oehmichen and Reifferscheid (1977) In IBD patients, and by Jacobson *et al* (1995), Yamada *et al* (1992, 1993), Poli *et al* (2001), Sanovic *et al* (1999) and Morris *et al* (1989) in experimental IBD models. Inhibition of neutrophil function is used as therapeutic approach to treat IBD patients (Elson, 2000).

1.10. Inflammatory Initiators and Mediators in IBD

Bacterial products and inflammatory components, i.e. pro-inflammatory cytokines, reactive oxygen species and nitric oxide, synthesized by infiltrated immunocytes are assumed to play an important role in induction and maintenance of IBD. In the present study we have investigated the possible roles of bacterial endotoxin, pro-inflammatory cytokines TNF- α and IFN- γ , and nitric oxide in the process of enteric neuronal injury.

1.10.1. Enteric Bacterial Endotoxins

Bacterial products play a critical role in initiation and maintenance of experimental IBD, as in germ-free or even pathogen-free conditions most animals undergoing experimental induction of IBD either remain healthy or develop attenuated disease (Ehrhardt *et al*, 1997; Kühn *et al*, 1993; Sadlack *et al*, 1993; Davidson *et al*, 1996). In experimental IBD as well as in CD and UC several pathogenic bacteria including *Vibrio cholera*, *Escherichia coli*, *Helicobacter*, *Campylobacter*, *Salmonella*, *Yersinia*, *Listeria monocytogenes*, *Mycobacteria*, and *Streptococcus pneumoniae* are detected in the

intestine (Caradonna *et al*, 2000; Liu *et al*, 1995). Inflammation in the gut in CD shares some similarities with those observed in the inflammation induced by Shigella and Salmonella infection, and CD patients clinically improve when there is a reduction in bacterial flora in the gut (Sutherland *et al*, 1991).

In terms of immuno-stimulation and inflammatory capacity, the most important component of the bacteria is lipopolycaccharide (LPS) from the cell membrane; also referred to as bacterial endotoxin. LPS is the main outer membrane component of bacteria involved in infection, sepsis and shock. Endotoxins are molecules consisting of a hydrophilic polysaccharide chain and a hydrophobic lipid-A part. Large amounts of LPS are present in blood stream during bacterial infection and an abnormal microflora and/or an increased permeability of the intestinal mucosa have been invoked as cofactors responsible for endotoxemia (Heine *et al*, 2001; Caradonna *et al*, 2000).

Mechanisms of Eliciting Inflammatory Response: Most of the cells responsive to LPS stimulation are from the cellular immune system, and the majority of them respond to small amounts of endotoxin. Among these cells monocytes / macrophages are the most prominent in response to endotoxin stimulation and they synthesize a variety of pro-inflammatory cytokines including IL-1, IL-6, IL-8 and in particular TNF- α , and reactive oxygen species superoxide anion, hydrogen peroxide, and nitric oxide (Heine *et al*, 2001). In many studies LPS stimulation is applied to induce macrophage activation *in vivo* or in cell culture; the works of Boisse *et al* (2003), Drapier and Hibbs (1988), Nau *et al* (2002), and Eskandari *et al* (1999) are some instances of use of this approach. The LPS signal transduction cascade starts with binding of endotoxin to LPS binding protein (LBP), which then associates with cell surface receptor CD14. LBP is present in plasma

at the concentration of 3-10 $\mu\text{g} / \text{ml}$ and its level rises in acute phase of immune response (Guha and Mackman, 2001). LPS then associates with the Toll like receptor (TLR), a transmembrane signaling factor (Figure 5) (Chow *et al*, 1999; Yang *et al*, 1998; Aderem and Ulevitch, 2000), and following it several intracellular signaling pathways are activated, eventually leading to the activation of MAPK pathways (extracellular signal-regulated kinases (ERK) 1 and 2, c-Jun N-terminal kinase (JNK) and p38) and translocation of different transcription factors including NF κ B, activator protein-1 (AP-1) and nuclear factor IL-6 (NF-IL6), ending up in the gene expression of cytokines and other inflammatory mediators such as TNF- α , IL-1, IL-6, IL-8, inhibitory kappa B alpha ($\text{I}\kappa\text{B}\alpha$), and iNOS (Figure 5) (Heine *et al*, 2001; Guha and Mackman, 2001; Medzhitov, 2001).

Since LPS is a strong initiator of macrophage activation and subsequent increased NO production, we applied LPS to co-cultures of macrophages and neurons to study the phenomenon of macrophage activation and its degenerative effects on myenteric cells *in vitro*.

1.10.2. Cytokines

Cytokines are polypeptides with diverse actions on many cell types. These components are produced by lymphocytes, macrophages, granulocytes, endothelial cells, epithelial cells, and fibroblasts (Rogler and Andus, 1998). Cytokines are classically categorized under four different families: interleukins, tumor necrosis factors, growth factors, and interferons. Many cytokines show overlapping or synergistic activities and in some cases share the same related receptors (Rothwell and Strijbos, 1995). Some of the cytokines have pro-inflammatory functions, e.g. IL-1, TNF, IL-2, IL-6, IL-8, or IL-12.

Figure 5. LPS Signal Transduction Pathways in Monocytes / Macrophages.

LPS binding to its receptor on cell surface of monocytes / macrophages leads to stimulation of expression of pro-inflammatory cytokine, iNOS, and other inflammatory mediator genes. Solid-line arrows represent direct relationship between the two components of the pathway; broken-line arrows imply indirect cause-effect relations.

Abbreviations: AP-1: activator protein-1, ERK: extracellular signal-regulated kinases, I κ B: inhibitory kappa B, IRAK: IL-1 receptor-associated kinase, JNK: c-Jun N-terminal kinase, LBP: LPS-binding protein, MAPK: mytogen - activated protein kinase, NF-IL6: nuclear factor-interleukin-6, NF κ B: nuclear factor kappa B, TLR4: Toll like receptor 4, TRAF6: TNF receptor associated factor 6.

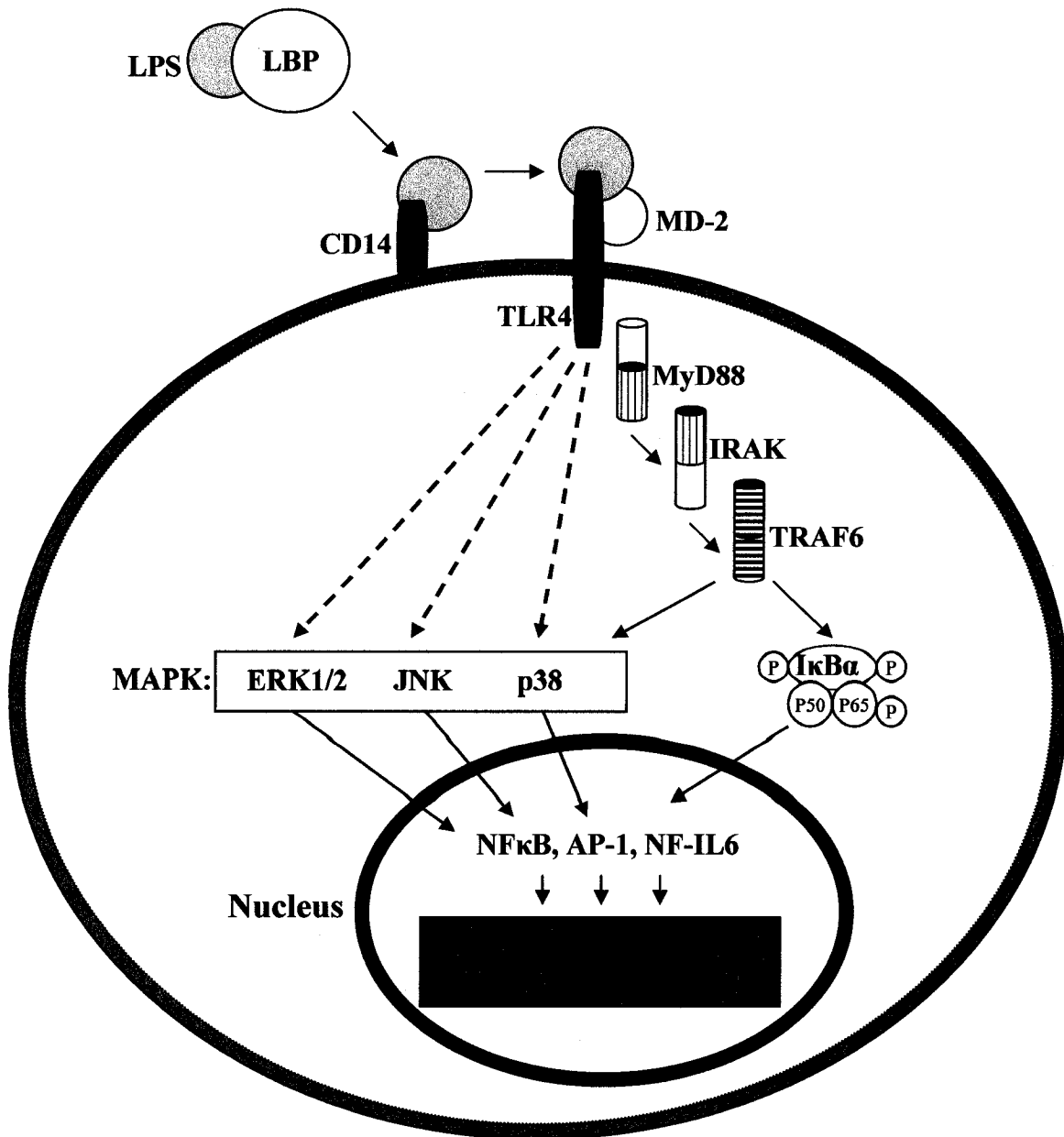


Figure 5

Pro-inflammatory cytokines play a critical role in the modulation of the immune system. Another class of cytokines, i.e. IL-3, IL-4, IL-10, and TGF- β , plays an anti-inflammatory role. Increased levels of the pro-inflammatory cytokines and disturbed balance between the pro-inflammatory and anti-inflammatory cytokines are observed in IBD (Rogler and Andus, 1998).

a. Tumor Necrosis Factor alpha (TNF- α):

Tumor necrosis factor was first described as an endotoxin-induced and macrophage-secreted factor which causes necrosis of tumor cells (Carsewell *et al*, 1975; Smyth and Johnstone, 2000). Over the past two decades the mechanism of TNF- α synthesis (Shurety *et al*, 2001) has come to be better appreciated; TNF- α is known to be produced by several other cell types than macrophages, including lymphocytes, fibroblasts, and hepatocytes (Natoli *et al*, 1998). TNF- α is involved in regulation and modulation of many inflammatory processes including cachexia, antibacterial host defense, contact hypersensitivity reactions, LPS – induced endotoxin shock, and induction of fever (Neurath *et al*, 1997).

TNF- α plays a central role in induction and maintenance of IBD. It is of major importance in the pathogenesis of CD; treatment with anti TNF- α antibody is useful in patients with CD that is unresponsive to steroid treatment (van Dullemen *et al*, 1995), and intravenous infusion of infliximab, a genetically engineered monoclonal antibody against TNF- α in CD patients achieved a long term clinical response (Targan *et al*, 1997, Nahar *et al*, 2003). This antibody is now available for clinical use in the treatment of CD (D'Haens, 2003). The pathologic role of TNF- α within inflammatory damage of the GI

tract makes this cytokine a likely candidate to act as a neurodegenerative agent in the ENS. It is shown that in experimental IBD in rats, TNF- α mRNA and protein expressions are increased in the myenteric plexus (Khan and Collins, 1994), and in TNF- α transgenic mice a striking colonic inflammation is induced after TNBS treatment (Neurath *et al*, 1997).

Receptors and signal Transduction: There are about 100 to 10,000 TNF- α surface receptors per cell, depending on the type of the cell (Larrick and Wright, 1990), and two distinct receptor types: p55 receptor and p75 receptor (Lewis *et al*, 1991; Brockhaus *et al*, 1990). Most cells express these two receptors. The 75kD TNF receptor (TNFR2) has been implicated in lymphocyte proliferation, whilst the 55kD receptor (TNFR1) is involved in TNF-mediated cytotoxicity, apoptosis and NF κ B activation (Figure 6) (Pfeffer *et al*, 1993; Rothe *et al*, 1993; Declercq *et al*, 1995; Perez *et al*, 1990; Lewis *et al*, 1991; Neurath *et al*, 1997). The functional differences of the two distinct TNF- α receptors, however, may not be absolute and the effects produced by them depend on the cell type (Pimentel-Muinos and Seed, 1999).

In all cases TNF-binding of p55 receptor recruits TNF-receptor 1 associated death domain containing protein (TRADD), which acts as an adapter to recruit downstream transducers such as TNF-receptor 1 associated protein 2 (TRAF2) (Tartaglia *et al*, 1993). TRAF2-activated pathway induces NF κ B inducing kinase (NIK) activation (Mailinin *et al*, 1997), which in turn activates the transcription factor NF κ B (Hsu *et al*, 1996). Receptor-interacting protein (RIP), bound to TRADD, can also activate NF κ B. These transcription factor pathways regulate the expression of genes involved in inflammatory processes including iNOS gene in macrophages (Figure 6) (Sanders *et al*, 2001). On the

other hand, a recent study showed that selective iNOS enzyme inhibition suppresses TNF- α expression and release in patients with active UC, suggesting a reciprocal mechanism stimulating TNF- α production by this inducible protein (Kankuri *et al*, 2003).

Soluble forms of both of these receptors, known as TNF-binding proteins (TNF-BP) also exist. The expression of these receptors is associated with a variety of diseases (van Zee *et al*, 1992). These soluble receptors have dual functions: they either compete with TNF membrane-bound receptors and inhibit TNF activity, or prolong and enhance TNF activity by stabilizing the trimeric structure of this cytokine (Bemelmans *et al*, 1993, Aderka *et al*, 1992).

In this study, TNF- α has been used in both *in vivo* and *in vitro* experiments, and its effect on macrophage cells either alone or in combination with IFN- γ and their capability to induce nitric oxide synthesis were studied. Furthermore, the consequences of single TNF- α injection in naïve and DNBS treated mice were investigated.

b. Interferon-gamma (IFN- γ):

IFN- γ , also referred to as IFN type II or immune IFN, is primarily synthesized by Th1 lymphocytes. IFN- γ regulates the occurrence of mucosal inflammation (Strober *et al*, 1997), and in CD its expression is elevated in the intestinal mucosa (Fais *et al*, 1991; Breese *et al*, 1993).

IFN- γ is one of the most potent activators of macrophages, it also increases MHC class I and II expression, and activates neutrophils. IFN- γ in combination with a second signal such as TNF- α or LPS activates NF κ B, which in turn transcriptionally activates

Figure 6. TNF- α and IFN- γ Signal Transduction Pathways.

Binding of pro-inflammatory cytokines TNF- α and IFN- γ induce inflammatory responses including upregulation of inflammatory gene expression. **Abbreviations:** AP-1: activator protein-1, GAF: gamma-activated factor, I κ K: inhibitory kappa B protein kinase, I κ B α : inhibitory kappa B alpha, IFNR: interferon gamma receptor, JAK: Janus kinase, JNK: C-Jun N-terminal kinase, NF κ B: nuclear factor kappa B, NIK: NF κ B inducing kinase, RIP: receptor-interacting protein, STAT: signal transducer and activator of transcription, TNFR: tumor necrosis factor receptor, TRADD: TNF receptor-1 associated death domain, TRAF: TNF-receptor 1 associated protein.

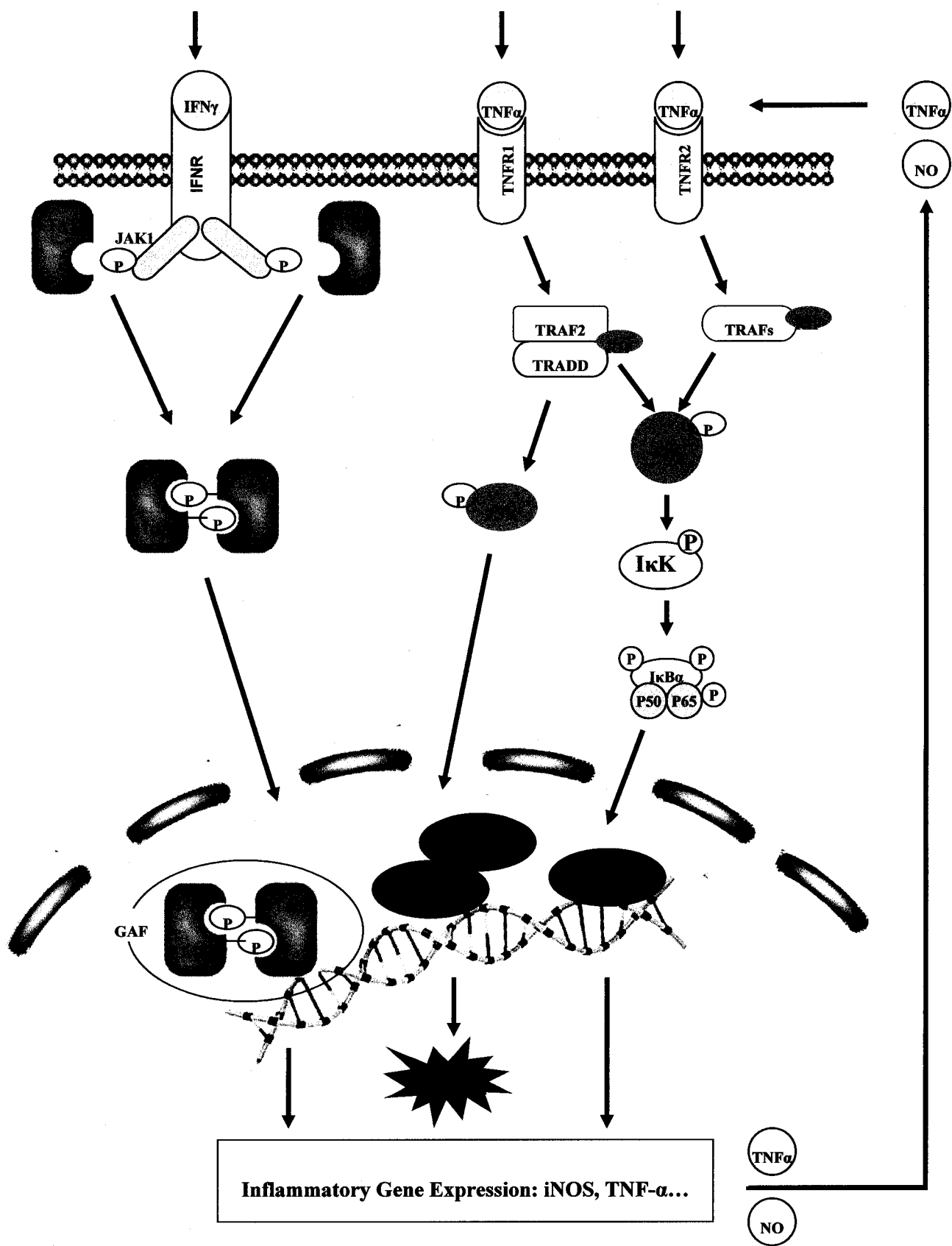


Figure 6

iNOS gene, resulting in NO production by activated macrophages (Schreiber and Schreiber, 2003). High concentrations of IFN- γ in synergy with TNF- α lyse epithelial cells in vitro (Sartor, 1994). Cell culture studies have shown that TNF- α alone can not stimulate and induce macrophage tumoricidal activity whilst combination of this pro-inflammatory cytokine with IFN- γ results in the synergistic induction of tumoricidal activity (Hori *et al*, 1987). TNF- α and IFN- γ also synergize to induce nitrite and nitrate synthesis from L-arginine and cause inhibition of the iron dependent enzyme aconitase in macrophages (Drapier *et al*, 1988), which suggest that the tumoricidal ability of the macrophages was at least partly due to the induction of nitric oxide and its metabolites.

Receptors and Signal Transduction: IFN- γ interacts with a high affinity receptor comprised of two subunits, interferon gamma receptor (IFNGR) -1 and -2, expressed virtually by all cells except erythrocytes. IFNGR1 is required for ligand binding and signaling while IFNGR2 is primarily required for signaling and plays a minor role in binding (Bach *et al*, 1997).

The two IFN- γ receptor subunits are constitutively associated with inactive forms of Janus kinase 1 (Jak1) and Janus kinase 2 (Jak2) (Figure 6). IFN- γ binding to its respective receptor induces activation of Jak1 and Jak2 via phosphorylation. Activated kinases form the phosphorylated sequence on a receptor that is recognized by the SH₂ domain of signal transducer and activator of transcription 1 (Stat1) resulting in phosphorylation and translocation of Stat1 across the nuclear pore (Sekimoto *et al*, 1996). Once inside the nucleus, activated Stat1 also referred to as gamma-activated factor (GAF), affects the transcription of IFN- γ induced genes that encode proteins which

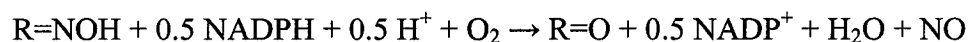
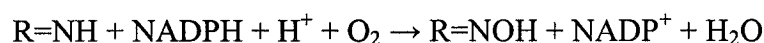
participate in inflammatory and immune responses (Darnell *et al*, 1994; Bach *et al*, 1997).

1.10.3. Nitric Oxide (NO)

NO is a small gaseous molecule involved in different physiologic and pathophysiologic processes, and considered one of the major cytotoxic factors released from activated macrophages and other cells following immunologic activation.

NO Synthesis: NO is synthesized by oxidation of the terminal guanidine nitrogen atom of L-arginine. Its synthesis is carried out by an enzyme, nitric oxide synthase (NOS), of which three different isoforms, based on the cell origin and constitutive or inducible nature of the enzyme, have been characterized. The constitutive types are responsible for NO release in physiological conditions and the inducible one act as part of the immunologic response. NOS isoforms are encoded by three different genes, thereby exhibiting distinctive pathways in expression and regulation that determine special patterns of NO production (Forstermann and Kleinert, 1995). The two Ca^{2+} / calmodulin-dependent constitutive enzymes were identified in neurons and endothelial cells, hence the name neuronal NOS (nNOS) and endothelial NOS (eNOS) respectively. The third type of NOS protein, referred to as inducible NOS (iNOS), is a Ca^{2+} -independent enzyme, the low basal expression of which increases in response to cytokines and LPS challenge of inflammatory cells. It has a calmodulin tightly bound to the enzyme at all times which makes it almost completely insensitive to calcium concentrations and maintains it in a tonically activated state (Calatayud *et al*, 2001).

iNOS gene in human is located in 17cen-q11.2 of chromosome 17 and its protein in predominant form consists of 1153 amino acids with 131 kD molecular weight (Alderton *et al*, 2001). iNOS catalyzes a reaction in which L-arginine, nicotinamide-adenine dinucleotide phosphate reduced (NADPH), and O₂ are the substrates and nicotinamide-adenine dinucleotide phosphate (NADP), citrulline, and nitric oxide are the products:



(R=NH, arginine; R=NOH, N⁰-hydroxy-L-arginine; R=O, citrulline)

Cytotoxic Mechanisms of NO: The initial observation of NO-mediated apoptosis was reported by two independent groups in 1993 (Albina *et al*, 1993, and Sarih *et al*, 1993). Through direct or indirect targeting of mitochondria NO activates the apoptotic pathway. NO interacts with mitochondrial generated superoxide to form peroxynitrite, inducing alteration in PTP of the mitochondrial membrane resulting in release of cytochrome c, and activation of caspase cascades thus apoptosis (Figure 7) (Poderoso *et al*, 1996; Lin *et al*, 1995).

Indirect mitochondria-based mechanisms of NO-initiated programmed cell death include JNK and stress-activated protein kinase (SAPK) activation (Jun *et al*, 1999), and increased ceramide level and Bcl-2 downregulation (Di Nardo *et al*, 2000). NO targets the naked DNA and induces oxidative DNA damage. The damaged DNA elicits a stress response in the cell. This phenomenon involves attachment of poly (ADP ribose) polymerase (PARP) to the strand breaks and synthesis of polymers by the bound enzyme

(Brüne *et al*, 1998, Brüne *et al*, 1997). DNA damage and PARP activation induce the expression of p53 (Messmer and Brüne, 1996), an initiator of apoptosis through increasing the ratio of Bax / Bcl-xL, and induction of PTP dysfunction, cytochrome c release, and caspase family activation, resulting in execution of apoptosis in DNA damaged cells (Chung *et al*, 2001) (Figure 7).

NO and its metabolites can also regulate non-enzymatic lipid oxidation. NO is a hydrophobic gas. Therefore, lipid membrane is a potential sink for it. In this hydrophobic environment, reactive nitrogen species will exhibit different chemistry than in aqueous environments due to higher local concentrations and the lack of hydrolysis reactions. The peroxy nitrite anion (ONO_2^-) can freely pass through lipid membranes and react with lipids within the intramembrane hydrophobic environment. The reactions observed by these nitrogen metabolites in a hydrophobic milieu include oxidation, nitration and chain-breaking reactions (Goss *et al*, 1999; Bloodsworth *et al*, 2000).

NO Involvement in IBD: In 1986 Roediger *et al* published the initial finding of augmented level of NO in patients with active UC. iNOS expression is upregulated in neutrophils and macrophages at the base of the ulcers in the active stage of colitis but virtually absent in the healthy colon and the inactive stage of UC (Ikeda *et al*, 1997; Dijkstra *et al*, 1998; Zhang *et al*, 1998; Dijkstra *et al*, 2002). Based on the work of Kimura *et al* (1997) there is a linear correlation among plasma NO_x concentrations, iNOS activities and endoscopic and histologic grading of inflammation in patients with active UC and CD. Furthermore, gas samples aspirated from IBD patients showed greatly increased NO concentration (Lundberg *et al*, 1994; Reynolds *et al*, 1997).

Figure 7. Main Signaling Components of NO-Initiated Cell Death.

NO and its metabolite peroxynitrite act on cell mitochondria, the nucleus, and the membrane lipids to initiate apoptotic cell death. *Abbreviations:* ATP: adenosine triphosphate, Bax: Bcl-2-associated x protein, Bcl-2: B cell leukemia/lymphoma 2, Casp: caspase, Cyt-c: Cytochrome c, cGMP: cyclic guanine mono phosphate, GC: guanine cyclase, GTP: guanine triphosphate, JNK: C-Jun N-terminal kinase, MAPK: mitogen-activated protein kinase, SAPK: stress-activated protein kinase.

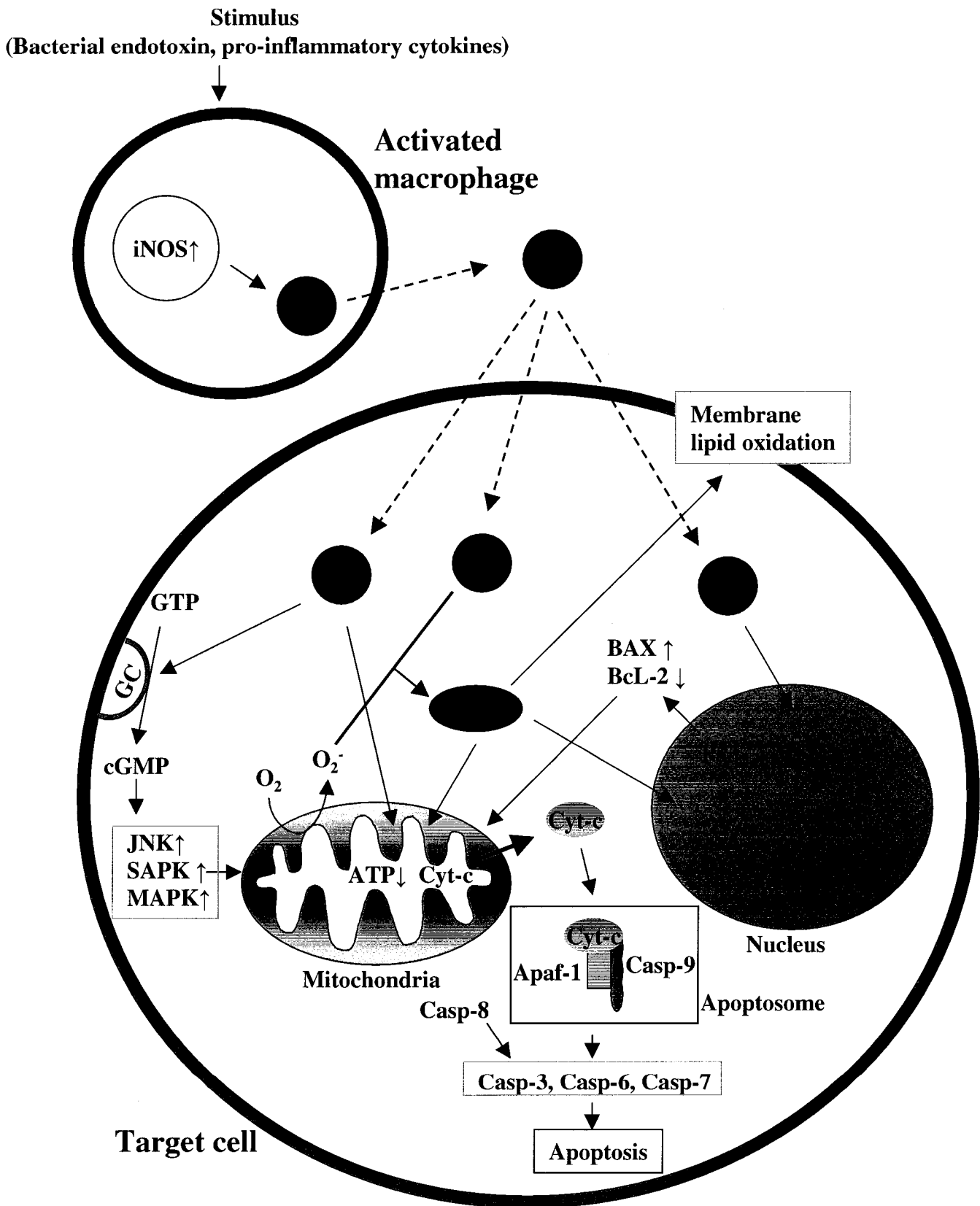


Figure 7

The enhanced production of NO by iNOS has been extensively studied in experimental models of IBD. iNOS immunoreactivity in hapten-induced colitis was detected in rat colon (Zhang *et al*, 1998; Kankuri *et al*, 1999; Blanchard *et al*, 2001) and in guinea pig ileum (Zhang *et al*, 1998). iNOS is involved in initiating and maintenance of inflammation, as in its absence oxidative damage was markedly reduced (Zingarelli *et al*, 1999), and a significant reduction in the onset and severity of colitis was observed in iNOS deficient mice (Kriegelstein *et al*, 2001). Furthermore, Yue *et al* (2001) correlated the colon epithelial cell death in colitic rats with the expression of iNOS. However the findings of McCafferty *et al* (1999) suggest that iNOS ameliorates only the early phase but does not impact on the chronic phase of TNBS-induced colitis. It has been shown that iNOS is produced by different inflammatory cells including macrophages in the inflamed site of the gut in rats (Miampamba and Sharkey, 1999) and neutrophils and macrophages in mice (McCafferty *et al*, 1999).

NO Synthase Inhibition: A large number of NOS inhibitors work through competitive binding to different sites of the NOS enzymes. They are distinguishable based on their mechanism of inhibition, and time- and/or substrate-dependence. Some NOS inhibitors act on the bipterin, haem, flavoprotein or calmodulin binding sites of the enzyme (Alderton *et al*, 2001); however most of the inhibitors identified so far, such as L-nitro-N-arginine (L-NNA), L-nitro-monomethyl-arginine (L-NMMA) and its methyl ester prodrug N^ω-nitro-L-arginine methyl ester (L-NAME), L-N6-(1-iminoethyl)-lysine (L-NIL), and N-(3-(aminomethyl)benzyl) acetamidine (1400W) (Figure 8), are competitive with substrate L-arginine. The selectivity of these inhibitors for enzyme isoforms differ, reflecting the fact that mechanisms involved in their effects on NOSs are

beyond simple binding in competition with L-arginine (Alderton *et al*, 2001). These inhibitors require active enzyme and NADPH substrate to allow inhibition to proceed from the initial weak binding to potent inhibition.

1400W is a highly selective iNOS inhibitor; it has 5000 and 200 times more affinity for human iNOS than eNOS and nNOS respectively (Garvey *et al*, 1997). It has the advantage of penetrating into cells and tissue, and also suppresses the late iNOS-driven phase of endotoxin-provoked leakage with no exacerbation of the early phase.

NO Synthase Inhibition as a Therapeutic Approach: NO synthesis blockade through NOS inhibition is already an experimental approach to ameliorate gut inflammation. In a model of ileitis administration of L-NAME hydrochloride reduced the inflammatory response (Miller *et al*, 1993), and in hapten-induced colitis it reduced myeloperoxidase activity, intestinal muscle hyperplasia, and colonic nitric oxide synthase expression and prevented macrophage infiltration into the muscle (Hogaboam *et al*, 1995). The effect of L-NAME hydrochloride is mediated through its ability to inhibit NO production as shown by Di Lorenzo and Krantis (2002). In experimental colitis, colonic epithelium develops hyporesponsiveness. A relatively selective iNOS inhibitor, L-NIL, normalizes the secretory response and sensitivity (Asfaha *et al*, 1999; MacNaughton *et al*, 1998).

The highly selective iNOS inhibitor, 1400W dihydrochloride, has been applied as a pharmacological tool in a variety of animal models; it has been applied to stop the growth of solid tumors expressing iNOS (Thomsen *et al*, 1997), attenuate the circulatory failure caused by endotoxin (Wray *et al*, 1998), reduce ischemic lesion volume and attenuate neurological dysfunction in cerebral ischemia in rats (Parmentier *et al*, 1999), and abolish

Figure 8. Chemical Structures of L-arginine, L-citrulline and iNOS Inhibitors.

L-arginine and L-citrulline are substrate and product of the NO synthesis reaction respectively. iNOS inhibitors L-NNA, L-NAME, L-NIL, and 1400W act competitively with L-arginine thereby blocking NO synthesis. **Abbreviations:** L-NNA: L-nitro-n-arginine, L-NAME: N^ω-nitro-L-arginine methyl ester, L-NIL: L-N6-(1-iminoethyl)-lysine, 1400W: N-(3- (aminomethyl) benzul) acetamidine.

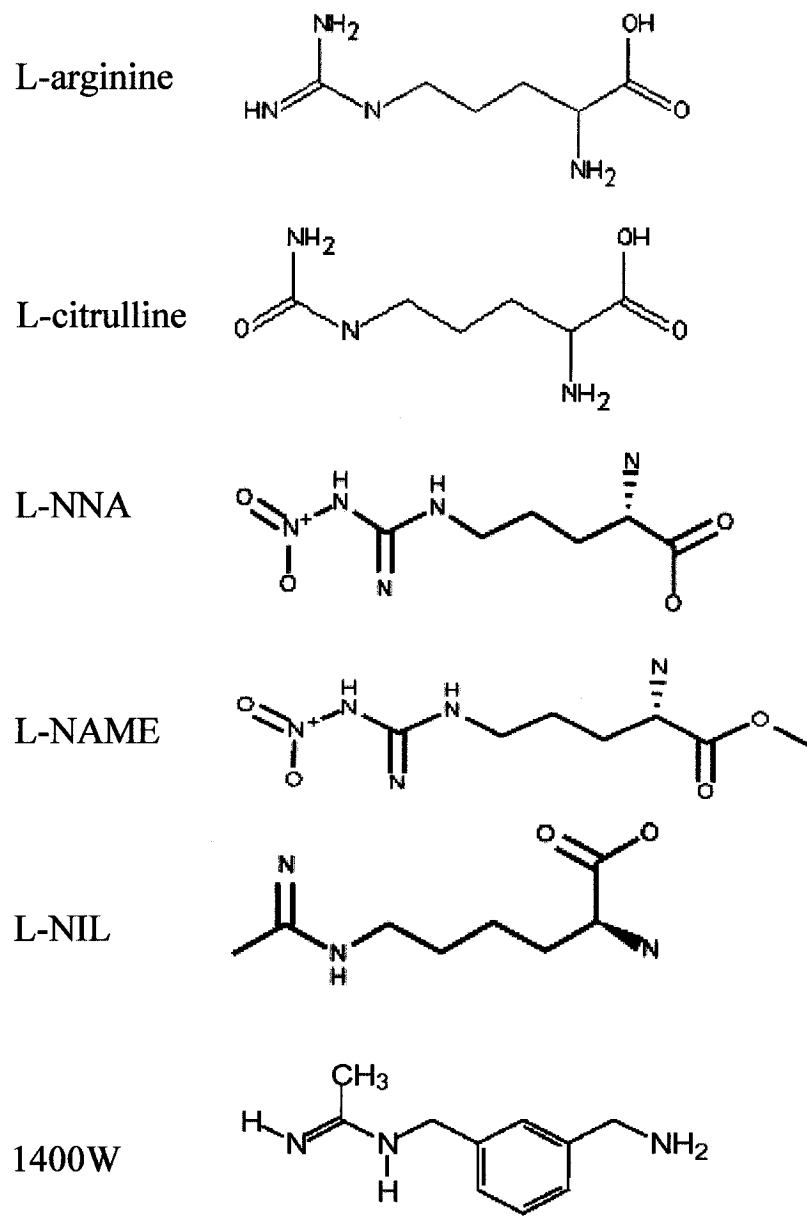


Figure 8

hyperresponsiveness and eosinophil accumulation in ovalbumin sensitized mice (Koarai *et al*, 2000). More recently 1400W dihydrochloride has been applied to some experimental models of colitis. It reduced neutrophil infiltration, macroscopic damage, formation of edema, and histological changes in colon induced by inflammation (Menchén *et al*, 2001; Krieglstein *et al*, 2001; Kankuri *et al*, 2001).

The focus of the research described in this thesis is the effects of NO produced by macrophage cells in cell culture and *in vivo*, and the effectiveness of NOS inhibitors on neuronal survival under conditions of physiological stress in cell culture, and in animal models of colitis.

1.11. Neurodegeneration in the CNS and Involvement of Macrophages and Microglia

There are two major subgroups of glial cells in the CNS: macroglia (consisting of astrocytes, oligodendrocytes, and ependymal cells), and microglia. Microglia are considered to be the resident macrophages of the brain and contribute to 10-20% of the total glial cell population in the CNS (Benveniste, 1997). They share many phenotypic markers and effector molecules with macrophages and rapidly respond to CNS injuries with changes in their morphology, proliferation and upregulation of surface molecules. Together, these constitute microglial 'activation'.

Although the CNS is an immune privileged organ, inflammation can cause a breakdown in the blood-brain barrier, which allows inflammatory cells to infiltrate from the blood circulation into the highly protected regions of the CNS (Schwartz, 2003).

Studies of degenerative diseases such as Parkinson's disease, Alzheimer's dementia, multiple sclerosis, and HIV encephalitis, and experimental models of inflammation in the CNS, suggest a pathogenic role for the endogenous microglial cells and infiltrated macrophages. Parkinson's disease and Alzheimer's disease are both characterized by selective loss of neurons in distinct regions of the brain. In Alzheimer's disease microglia are detectable in the center of senile neuritic and β -amyloid plaques (Uchihara *et al*, 1997) and there is a correlation between the presence of phagocytic microglia and the development of the plaques (Sheng *et al*, 1997). Microglia are consistently activated in substantia nigra of Parkinson's disease patients, distinguished by upregulation in nitric oxide synthase, cyclo-oxygenase-1, and cyclo-oxygenase-2 (Mirza, 2000; Knott *et al*, 2000).

Macrophages play a major role in human immunodeficiency virus (HIV)-induced CNS injury, and in its most frequent manifestation, acquired immunodeficiency syndrome dementia complex (ADC) (Achim and Wiley, 1996). ADC is attributed to direct HIV infection of the brain (HIV encephalitis), and autopsy from ADC patients shows subcortical damage and macrophage infiltration (Navia *et al*, 1986). Most researchers accept the "Trojan horse" hypothesis suggesting that HIV is carried into the brain hiding within the infected macrophages (Peluso *et al*, 1985; Kure *et al*, 1990). Nonviral and viral macrophage products have been implicated in the pathophysiology of ADC (Giulian *et al*, 1993; Adamson *et al*, 1996).

In multiple sclerosis patients, the lesions in the brain contain mononuclear infiltrates and foamy (myelin-laden) macrophages. iNOS protein and synthesized

nitrotyrosine were also co-distributed in a widespread pattern in macrophages in the acute phase of the disease (Oleszak *et al*, 1998; Bagasra *et al*, 1995).

Moreover in different animal models of CNS complications, the presence and role of the inflammatory cells macrophages and microglia have been investigated. In a model of Parkinson's disease, widespread microglial activation was noted in the striatum and the substantia nigra. Blockade of microglial activation was neuroprotective in another model of Parkinson's disease (Wüllner and Klockgether, 2003). In experimental autoimmune encephalomyelitis which mimics multiple sclerosis disease, a characteristic feature is massive infiltration of T cells and macrophages in the CNS and imbalance between pro- and anti-inflammatory cytokines (Stoll and Jander, 1999). In rat brain inflammation, iNOS immunoreactive macrophages and activated microglia were detected in choroid plexus and ependymal cells in the brain (Garcion *et al*, 1998). The work of Vela *et al* (2002) on an experimental injury in the cortex showed that proliferation of microglia and macrophages ensues within 12 hrs post-trauma, and monocyte infiltration starts from the second post-lesion day in the cortex.

The mechanisms of macrophage / microglial cytotoxicity in the CNS have recently received great interest. In one of the more direct approaches a co-culture system of microglia and cerebellar neurons was developed to demonstrate that immuno-stimulation of microglia induces neuronal cell death (Boje and Arora, 1992). This effect was prevented by applying NOS inhibitor L-NNA or NO inactivator oxyhemoglobin, and augmented by NO stabilizer superoxide dismutase, which inactivates superoxide anion, all suggesting that microglial-produced NO plays a neurotoxic role in neurodegenerative disease states.

This observation was confirmed by Chao *et al* (1992), using co-culture of microglia and neuronal cells from the brain. They observed a marked reduction in neuronal cell survival after IFN- γ and LPS treatment, in a correlative pattern with microglial density, concentration of IFN- γ and LPS, and co-culturing duration. NOS inhibitor L-NMMA blocked these effects, suggesting that the neurodegenerative process is associated with neurotoxic free radical NO production. Furthermore, McMillian *et al* (1995) reported that LPS activated microglia induced marked reduction in survival of a specific subtype of neuronal cells, ChAT neurons. This effect was also prevented by pretreating the co-cultures with NOS inhibitor L-NAME hydrochloride, consistent with the theory of NO being at least one of the neurotoxins responsible for the loss of cholinergic neurons after LPS treatment.

It has also been observed in HIV studies that exposure of a co-culture of primary neuronal and microglial cells to gp41, an HIV-1 coat protein, induces iNOS upregulation and NO formation followed by neuronal cell death which was prevented by applying NOS inhibitor L-NAME hydrochloride (Adamson *et al*, 1996).

Taken together, this represents the underlying rationale for the research embodied in this thesis. This research focused on the damaging effect of activated macrophages on enteric versus cortical neuronal cells, comparing their susceptibilities to damage, and the protective role of iNOS inhibitors on neuronal survival.

CHAPTER 2:

MATERIALS AND METHODS

2.1. General Methodology

2.1.1. Tissue Fixation and Sectioning

Tissues and cultures were fixed in modified Zamboni's fixative (consisting of 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 6.9) for 120 min (60 min for cell cultures), rinsed in 10 mM phosphate buffer solution containing 10% sucrose and 0.01% sodium azide several times. Gut segments (5mm) were frozen in CO₂, and 10µm thick cryostat sections were cut and mounted onto subbed slides.

2.1.2. Immunohistochemistry

Tissue sections were encircled with hydrophobic pencil to create a well for antibody incubation (100µl volume). Similarly, coated cover-slips containing cultured cells from 24 well plates were attached to slides and encircled with hydrophobic enamel to create a well for the antibody (50µl volume). Immunohistochemistry on cells grown and fixed in 96 well plates were carried out in the wells in a volume of 50 µl. Primary antibodies (Table 2) were diluted in 10mM phosphate buffer saline (PBS) with 0.3% Triton-X 100 and incubations were carried out overnight at 4°C. After rinsing in 10mM

PBS, secondary antibody (Table 2) was applied for 35 min at 37°C (in 10 mM PBS with 0.3% Triton-X 100). Sections and cell cultures (except for the cultures in 96 well plates) were then coverslipped with fade retardant mounting medium and viewed on a Zeiss Axioplan microscope. Cells in plates were rinsed in 10 mM PBS and examined for fluorescent light microscopy using an inverted microscope (Zeiss; Axiovert 25). Digital images were captured and analyzed using “Northern Eclipse Image Analysis” software.

2.1.3. Western Blotting

Tissue for Western blot analysis was frozen in liquid nitrogen and stored at -80°C. Tissue samples of 100mg weight were thawed, homogenized in 500 µl of 1% sodium dodecyl sulfate (SDS, Invitrogen) for 15 seconds, centrifuged (12,000 g, 10 min, 4°C), and the supernatant was collected. Extracted protein was measured using the Bradford assay (Bio-Rad). SDS gel-loading buffer was added to each protein sample, and the samples were heated for 2 min at 100°C. SDS-PAGE gels were prepared according to the method of Sambrook and Russel (2001), and protein samples were run until they reached the bottom of the gel. Using a semi-dry electro-cell, proteins were transferred from the gel to a nitrocellulose membrane (0.45 µ thickness) at 10 V (350 milliamp) for 1 hr. The nitrocellulose membrane was then soaked in blocking buffer (5% non-fat dry milk in Tris buffer saline, TBS) for 1.5 hrs and incubated with primary antibody for overnight at 4°C (details in Table 2). The membrane was washed in TBS and incubated in alkaline phosphatase conjugated secondary antibody (details in table 2) for 1 hr at room temperature with gentle shaking. The membrane was then washed and incubated in alkaline phosphatase color development buffer (0.1 M Tris, 0.5 mM MgCl₂, pH 9.5),

containing of nitro blue tetrazolium chloride (75 mg/ml; Invitrogen), and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (50 mg/ml; Invitrogen). Once the band developed, the membrane was washed with water and allowed to air dry.

2.1.4. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RNA isolation: 500mg of tissue (frozen in liquid nitrogen, preserved in -80°C) was homogenized in 1 ml Trizol reagent (Gibco BRL). 200 μl chloroform was added to the sample, and it was spun (12,000 rpm, 15 min, 4°C). The aqueous phase was incubated with 500 μl of isopropyl alcohol for 10 min at room temperature, and then spun (12,000 rpm, 10 min, 4°C) to precipitate RNA. The pellet was vortexed with 1 ml of 75% ethanol and spun again (9,000 rpm, 5 min, 4°C). The RNA was air dried, heated at 62°C for 10 min and transferred to -80°C freezer for short-term storage.

cDNA Synthesis and Amplification: Using Superscript One-Step RT-PCR with a Platinum Taq kit (Invitrogen), RNA was reverse-transcribed to cDNA and amplified. cDNA synthesis and pre-denaturation was performed by applying one heating cycle of 50°C for 30 min, followed by a cycle of 94°C for 2 min (Eppendorf Mastercycler Gradient). 40 cycles of denaturation, annealing, and extension were performed to amplify cDNA. At the end of the procedure, 1 cycle of 72°C temperature for 10 min was added to maximize the amplification.

Gel Electrophoresis and Photography: Agarose gel electrophoresis was carried out following the method of Sambrook and Russel (2001). cDNA bands were visualized under ultra-violet light and imaged digitally using the Alpha Imager program.

2.1.5. DNA Binding Fluoro-chrome (Hoechst 33258) Assay

Hoechst 33258 (1:10,000; Sigma) was used to label the cell nuclei. It was applied for 15 min at room temperature after all the other staining reactions were performed.

2.1.6. Transferase Deoxyuridine Triphosphate (dUTP) Nick End Labeling (TUNEL) Assay

A fluorescein – dUTP labeling kit (Roche Applied Science) was used to detect DNA breaks and fragmentations. The cells were permeabilized with 0.1% Triton X-100, 0.1% sodium citrate in PBS (1 min, room temperature), rinsed with PBS and incubated with 50 µl of TUNEL reaction mixture for 60 min at 37°C in dark. The samples were rinsed with PBS, and TUNEL positive cells were detected by fluorescence microscopy.

2.1.7. Fluoro-Jade Histo-fluorescent Labeling

Fluoro-Jade is a marker for identifying degenerative neurons undergoing necrosis (Schmued *et al*, 1997). Fluoro-Jade (Histo-Chem Inc.) staining was carried out according to the method of Schmued *et al* (1997). In brief, air dried slide mounted sections were immersed in 100% ethanol, 70% ethanol, distilled water, 0.06% potassium permanganate (KMNO₄), and again in distilled water, 1 min in each solution. Fluoro-Jade (0.001% dissolved in water) was applied to the sections for 30 min, at room temperature, with gently shaking and protected from light. The sections were rinsed three times in distilled water followed by immersing in xylene, and cover-slipped under distrene plasticizer in xylene for microscopy. Sections were examined with a fluorescence microscope using the same filter set as that used to visualize fluorescein.

2.1.8. Nicotinamide Adenine Dinucleotide Phosphate Hydrogen-diaphorase (NADPH-d) Assay

NADPHd histochemical staining for NOS activity within cultures was performed according to published methods (Nichols *et al*, 1994). In brief, cell culture preparations were placed in a dark, moist chamber for 1 hr at 37°C and exposed to the reaction medium for NOS activity, which consisted of 1 mM NADPH (Sigma), 0.5 mM nitroblue tetrazolium (Sigma), and 0.3% Triton X-100 in 10 mM PB (pH 8). Cultures were given an extensive final wash with 0.1 M PB (pH 8), and NADPH-d staining was examined under light field microscopy.

2.1.9. Myeloperoxidase (MPO) Assay

MPO activity was measured following the method of Stelzner *et al* (2001) and Mullane *et al* (1985). In brief, using a polytron (Kinematica GmbH) 50 mg of tissue was homogenized in 500 µl buffer, consisting of 5% hexadecyltrimethylammonium bromide (Sigma) dissolved in 10 mM PBS, pH 7.0. The tissue was incubated for 10 min at room temperature, followed by three cycles of freezing in liquid nitrogen (5 min) and thawing (15 min, with brief vortexing). The homogenate was spun (20,000 g, 30 min, 4°C) and the supernatant taken. 20 µl of supernatant was added to 180 µl of H₂O₂ – tetramethyl benzidine (Sigma) reagent in a 96-well plate, and incubated for 2 min at room temperature. The reaction was stopped by adding 50 µl of 2 M sulfuric acid. Optical density of wells was measured spectrophotometrically (Versa Max) at 450 nm. A standard curve of peroxidase activity was prepared using horseradish peroxidase (Bio-

Rad) in serial dilution, and MPO activity was expressed as an equivalent of the activity of the relative standard nanograms of horseradish peroxidase converting the same amount of H₂O₂. Data were expressed in nanograms and normalized per gram weight of the tissue.

2.1.10. Statistical Analysis

Data were analyzed using Sigma Stat program (version 3.0). Unless otherwise noted, significance of differences between multiple cell groups was determined using one-way ANOVA followed by All Pair-wise Multiple Comparison Analysis (Tukey test). Significance was considered at p values of less than 0.05.

2.2. Macrophage Activation and NO Synthesis

Mouse macrophage cell line (RAW 264.7) derived from the Abelson murine leukemia virus-induced tumor (organism: *Mus musculus*) was obtained from American Type Cell Culture, and plated in 96 well culture plates at a density of 20,000 cells per well in medium consisting of 89.5% Dulbecco's modified Eagle medium (DMEM; 4mM L-glutamine, 4.5 g/L glucose and 1.5 g/L sodium bicarbonate; Gibco BRL), 10% fetal bovine serum (Gibco BRL) , and 0.5% penicillin streptomycin (Invitrogen). After 24 hrs cells were treated with either 1 µg/ml LPS (from *Escherichia coli*, Sigma), 50 ng/ml recombinant human TNF-α (R&D Systems), 10 ng/ml recombinant rat IFN-γ (Sigma), or a combination of TNF-α (50 ng/ml) and IFN-γ (10 ng/ml). Non-treated cells served as controls. At intervals of 1, 6, 12, or 24 hrs post exposure, cells were fixed as explained in general methodology and stored in PBS at 4°C until processed for immunofluorescence.

Immunocytochemistry and Cytochemistry: Phalloidin-tetramethyl rhodamine isothiocyanate (Sigma) staining was performed to visualize F-actin (Hou *et al*, 2000). Phalloidin binds relatively specifically to actin filaments and is widely used as its indicator (Pastore *et al*, 2003; Maddala *et al*, 2003). Increased cell size and processes were used to distinguish phenotypically activated macrophages from non-activated ones. iNOS immunostaining was carried out to detect upregulation of this enzyme in macrophage cells. Hoechst 33258 was used to label the cell nuclei. The stained nuclei were counted and the resulting value was considered to reflect the total number of cells per well. The numbers of activated cells and iNOS immunoreactive ones were counted and normalized to the total number of macrophage cells. The resulting data were analyzed as described in general methodology.

Measurement of NO Synthesis: RAW 264.7 macrophage cells were plated in 24-well culture plate at the density of 1,000,000 cells/well. The cells received the same treatments described above. In some experiments, macrophage cells were pretreated with NOS inhibitors, either 10 mM N-nitro-L-arginine methyl ester hydrochloride (L-NAME hydrochloride; Sigma) or 10 μ M 1400W dihydrochloride (Tocris) 1 hr prior to exposure to LPS or pro-inflammatory cytokines. The media was collected and stored at 4°C. Nitrate (NO_3^-) and nitrite (NO_2^-), final metabolized molecules from nitric oxide (NO), were measured as an indirect approach to quantify NO synthesis (Palmer *et al*, 1988). In order to measure the total amount of produced $\text{NO}_3^- / \text{NO}_2^-$, a two step quantitative colorimetric system was used (Kamiya biomedical company). Significant differences between different treated groups and respective controls were determined using two-tailed Student's *t* tests.

Table 2. Antibodies Used in the Experiments.

Primary and secondary antibodies applied in the experiments are listed. Information regarding type, host, species and specificity reactions is based on data provided by suppliers, while application and dilution refer to usage of antibodies in our experiments.

N/A: data not available. *Abbreviations:* ICC: immunocytochemistry, IHC: immunohistochemistry, WB: western blotting.

Primary Antibodies

Antibody	Supplier	Type	Host	Species Reaction	Application	Dilution
β -tubulin Type III (TUJ1)	Dr. D. Brown's lab, U of Ottawa, Canada	Monoclonal	Mouse hybridoma cells	Mammalian	ICC	1:10
Caspase-3	Signaling Technology	Polyclonal	Rabbit	Rat, mouse, human	ICC, IHC	1:50
ED-1	Serotec	Monoclonal	Mouse	Rat	IHC	1:50
ED-1	Cymbus	Monoclonal	Mouse	Rat	IHC	1:50
Glial Fibrillary Acidic Protein	Chemicon	Monoclonal	Mouse	Rat, human, porcine, chicken	ICC	1:50
Glial Fibrillary Acidic Protein	Dako	Polyclonal	Rabbit	Bovine, guinea pig, rat, mouse,	IHC	1:400
iNOS	Becton-Dickinson	Polyclonal	Rabbit	Rat, mouse, human	ICC, IHC WB	1:1000 1:1000
Lymphocyte	Accurate Chemicals	Polyclonal	Rabbit	Rat	IHC	1:50
Monocyte / Macrophage	BMA	Monoclonal	Rat	Mouse	IHC	1:50
nNOS	Santa Cruz	Polyclonal	Rabbit	Mouse, rat, human	ICC	1:400
Peripheral Choline Acetyltransferase	Dr. H. Kimura's lab U of Shiga, Japan	Polyclonal	Rabbit	N/A	ICC, IHC	1:1000

Table 2

Primary Antibodies (Continued)

Antibody	Supplier	Type	Host	Species Reaction	Application	Dilution
Protein Gene Product 9.5	UltraClone	Monoclonal	Mouse	Human, rat, guinea pig	ICC, IHC	1:100
Protein Gene Product 9.5	Biogenesis	Polyclonal	Rabbit	Most lower species	ICC, IHC	1:1600
Synapsin-1	Sigma	Polyclonal	Rabbit	Rat, mouse, bovine, human	ICC	1:50
Tumor Necrosis Factor - Alpha	Cedarlane	Polyclonal	Rabbit	Rat	IHC	2.5 ng/ μ l

Secondary Antibodies

Conjugated Dye	Supplier	Host	Specificity Reaction	Application	Dilution
Alexa	Molecular Probes	Goat	Rabbit IgG	IHC	1:200
Cyanine Cy3	Jackson Immuno-research	Donkey	Rabbit IgG	IHC, ICC	1:400
Fluorescein	Amersham Biosciences	Donkey	Rabbit IgG	IHC ICC	1:20 1:50
Cyanine Cy3	Jackson Immuno-research	Goat	Mouse IgG	IHC, ICC	1:400
Fluorescein	Sigma	Goat	Rat IgG	IHC	1:80
Alkaline Phosphatase	Bio-Rad	Goat	Rabbit IgG	WB	1:3000

Table 2 (Continued)

2.3. Pure Myenteric Cell Cultures

Rat ileal myenteric cells (from male Sprague-Dawley, 130-150g, Charles River) were dissociated (Donat *et al*, 1999) and cryopreserved using a proprietary protocol (QBM Cell Science). Cells were stored in liquid nitrogen up to the time of plating. 96-well cell culture plates were coated with Matrigel matrix (1:10, VWR Canlab), and the myenteric cells were plated at a density of 10,000 cells/well. In order to study the neurons and glia in a pure culture of myenteric cells, cultures were fixed on day 7 and cytochemical assays and immunofluorescence were performed.

Immunocytochemistry and Cytochemistry: PGP9.5 is a member of the ubiquitin carboxyl-terminal hydrolase family (Day *et al*, 1990; Wilkinson *et al*, 1989). PGP9.5 is a neuron – specific protein, and polyclonal anti-PGP9.5 was used as a pan-neuronal marker to identify myenteric neurons. Anti-peripheral choline acetyltransferase (pChAT) antibody labeled cholinergic neurons, and anti-neuronal NOS (nNOS) immunostaining and NADPH-d assay were applied to examine the other major subset of myenteric neurons, i.e. nitrenergic neurons. Glial cells were detected by performing anti-GFAP immunostaining.

2.4. Co-Culturing of Myenteric Cells and Macrophages

5 days after plating the myenteric neurons, 10,000 RAW 264.7 macrophage cells were added to each well. In each single experiment one control well was left without macrophages. 7 days following initial plating, the cells either received no treatment, or were exposed to 1 μ g/ml LPS, or a combination of 50 ng/ml TNF- α and 10 ng/ml IFN- γ . One group of cells were pretreated with 10 mM L-NAME hydrochloride and another group received 10 μ M 1400W dihydrochloride, 1 hr prior to, and 8 and 16 hrs after endotoxin / cytokine exposure. Cultures were fixed 6, 12, 18, 24, 48, or 96 hrs following endotoxin / cytokine challenge (60 min in modified Zamboni's fixative), rinsed three times in 10 mM PBS, and stored in 4°C until analyzed by immunocytochemistry.

Immunocytochemistry and Cytochemistry: PGP9.5 immunopositive neurons were counted and the number of neurons in test wells was normalized to the number in control wells (non-treated single cultures of myenteric neurons). In addition micrographs were taken and images were analyzed (Northern Eclipse; Empix) for the intensity of PGP9.5 immunofluorescence, expressed as grey scale value in a range of 0-255 (black - white respectively). The significance of differences in intensity was determined as explained in general methodology. PGP9.5 immunofluorescence was combined with either polyclonal anti-caspase 3 (casp-3), a marker of apoptotic cells, or polyclonal anti-synapsin-1, to enable evaluation of neurons undergoing early stage of apoptosis and neurite degeneration (loss of synapse). Later stage of apoptosis was evaluated using TUNEL Assay and Hoechst 33258 staining; following the protocol described in general methodology. Class III isoforms of β -tubulin (TUJ1) also labels neurons in the gut (Alexander *et al*, 1991). The monoclonal anti- β -tubulin was used as a correlative marker

to evaluate neuronal degeneration. Polyclonal anti-monocyte / macrophage antibody was applied to identify macrophage cells in co-cultures.

2.5. Macrophage - Cortical Neuron Co-Culture

To evaluate differences between the toxic effect of macrophage activation on gut neurons and neurons of the CNS the co-culturing experiment was repeated with rat cortical neurons instead of myenteric cells. Cryopreserved neocortical cells (e18-19, QBM Cell science) were thawed and plated in medium consisting of 77% MEM, 10% Horse serum, 10% fetal bovine serum, 1% L-glutamine (200 mM), 35.6 g/l glucose, and 1% penicillin streptomycin, at the density of 80,000 cells/well, in wells coated with Poly-D-Lysine (Fisher). On day 5 of cortical cell cultivation, macrophage cells (32,000) were added to assigned wells. This ratio of 2 macrophages : 5 cortical cells was determined optimum through empirical approach. On day 7 the co-cultures were treated with either 1 $\mu\text{g}/\text{mL}$ LPS or a combination of 50 ng/mL TNF- α and 10 ng/mL IFN- γ for 48 hrs. Some of the wells were pretreated with 10 mM L-NAME hydrochloride or 10 μM 1400W dihydrochloride, 1 hr prior to endotoxin / cytokine exposure, and subsequently 8 and 16 hrs post treatment. The cells were fixed (modified Zamboni's fixative, 60 min), rinsed in 10 mM PBS, and kept at 4°C.

Immunocytochemistry and Cytochemistry: Polyclonal and monoclonal PGP9.5 antibodies were used to label neurons (see section 2.3). Neurons in selected identical areas in all wells were counted and the number of cells was normalized to that of single cortical neuron culture (baseline control). Significant difference was determined as

explained in general methodology. Polyclonal anti-mouse macrophage antibody was applied to label macrophages. Monoclonal GFAP was used to detect glial cells in the culture. Hoechst 33258 cytochemical staining was carried out to identify cell nuclei and visualize them under fluorescent light.

2.6. iNOS Inhibition in Experimental Model of Colitis in Rats

Sprague-Dawley rats (180-200 g, Charles River) were maintained on a 12 hr light/dark cycle, and rat chow (Ralston Purina) and water *ad libitum*. Animals were stabilized in our facility for 5 days before initiation of any experimental procedure. All experiments were approved by the Animal Care Committee of the University of Ottawa and met the guidelines set out by the Canadian Council on Animal Care. The animals were divided into 4 different groups, and each group had 4 subgroups assigned for different time-points. An unmanipulated group (group 1) was used as baseline control. Group 2 received a single DNBS (ICN Biomedicals) injection. The third group received 1400W dihydrochloride triple injections plus DNBS administration and the fourth group was injected with 1400W dihydrochloride three times. Animals in each group were evaluated at 4 different time-points: 12, 24, 48, and 96 hrs post DNBS (or initial 1400W dihydrochloride) injection.

The animals were fasted 24 hrs prior to any experimental manipulation. Subcutaneous injection of 1400W dihydrochloride (10 mg/kg in 100 μ l saline) was carried out immediately before DNBS administration. Subsequent 1400W dihydrochloride injections were performed at 8 and 16 hrs following initial injection.

DNBS (25 mg in 250 μ l of 50% Ethanol + 50% saline) was administered intrarectally, under light halothane anesthesia. The rats received analgesic subcutaneous injections (buprenorphine hydrochloride; 0.02 mg/kg; Schering-Plough) every 12 hrs and the wellness of the animals was assessed twice a day up to the time of sacrifice. At the end-point the blood was collected through cardiac puncture under anesthesia, and rats were then sacrificed via cervical dislocation. The large intestine (proximal and distal colon and rectum) was excised, opened, and pinned to a silicon elastomer plate, photographs were taken and gross morphologic damage was assessed and scored from 0 (no damage) to 5 (major sites of inflammation and ulceration) (Morris *et al.*, 1989) and the normal and inflamed segments of gut were weighed and their lengths measured. A sample of the tissue was frozen in liquid nitrogen for RT-PCR and western blotting, and another was kept in saline for histochemical studies.

Immunohistochemistry and Histochemistry: In order to observe microscopic changes in tissue architecture, sections were stained with hematoxylin and eosin (H & E) according to standard procedures. Polyclonal anti-PGP9.5, anti-synapsin-1, anti-iNOS, anti-casp-3, and monoclonal anti-GFAP immunostainings were carried out as described above (section 2.1.2.). Polyclonal anti-rat-TNF- α immunofluorescence was used to detect TNF- α upregulation. In order to immunolabel macrophages in different layers of the gut two different monoclonal antibodies against rat macrophage ED-1 receptor were used. Polyclonal anti-rat lymphocyte antibody was also used to detect lymphocyte infiltration. MPO activity was measured following the protocol described in general methodology (section 2.1.9), and treated groups were statistically compared to their respective controls.

Fluoro-Jade histofluorescent labeling and TUNEL assay were performed as explained previously (general methodology sections 2.1.6 and 2.1.7).

Western blotting: Western blotting was performed following the protocol described in general methodology (section 2.1.3). Polyclonal anti-iNOS and alkaline phosphatase conjugated goat anti-rabbit IgG were applied to the membrane as primary and secondary antibodies respectively.

RT-PCR: iNOS rat primers were based on the works of Kankuri *et al* (2001) and Eskandari *et al* (1999) and the sequence of rat nitric oxide synthase (D12520) was extracted from the National Center for Biotechnology Information (NCBI) database. The primers, 5' (sense) Primer: 5'-TTGGGTCTTGTTAGCCTAGTC-3', and 3' (anti-sense) Primer: 5'-TGTGCAGTCCCAGTGAGGAAC-3' correspond to bases 114-134 (5') and 355-375 (3') of the rat iNOS cDNA and amplify a 262 base-pair of the respective cDNA. The method described in general methodology (part 2.1.4) was followed, and 40 cycles of denaturation (94°C, 1 min), annealing (59°C, 2 min), and extension (72°C, 3 min) were performed to amplify iNOS cDNA.

2.7. The Effect(s) of a Combination of DNBS and TNF- α in Mice

To evaluate TNF- α effects we used a smaller rodent model due to the great expense of *in vivo* TNF- α administration. CD-1 mice (40 g) were obtained from Charles River, housed in chambers of 12 hr light/dark cycle, and permitted an *ad libitum* diet of mouse chow (Ralston Purina) and water. All the experimental procedures had the approval of the

Animal Care Committee of the University of Ottawa, and met the guidelines set out by the Canadian Council on Animal Care. The animals were divided into 5 different groups. Group 1, sham operated animals, was used as a control. The second group received a single DNBS injection. The third group received single TNF- α injection. The fourth group received both DNBS and TNF- α , and the fifth group was pretreated with 1400W dihydrochloride before DNBS and TNF- α challenge. The animals were fasted 24 hrs prior to conducting the tests. 4 mg of DNBS (in 100 μ l of 50% saline + 50% ethanol) was administered intrarectally using a 5 cm cannula, under light halothane anesthesia. 24 hrs later some mice received intraperitoneal TNF- α (4 μ g TNF- α in 100 μ l saline) (Sturiale *et al*, 1999). 1400W dihydrochloride (10 mg/kg in 100 μ l saline) was injected subcutaneously, immediately prior to, and 12 and 24 hrs following DNBS administration. Sham operated animals received an intrarectal injection of 100 μ l saline. The wellness of the mice was assessed and recorded twice a day until they were sacrificed. The mice were anesthetized and sacrificed via cervical dislocation 48 hrs after the initial DNBS/TNF- α injection. The large intestine was excised and the segment from the caecum to the anus was sampled. Weight, length, and severity of inflammation of normal and inflamed intestines were measured and assessed. Tissue was then fixed for immunohistochemistry.

Immunohistochemistry and Histochemistry: PGP9.5 staining was performed as described before (section 2.3). Micrographs were taken, and PGP 9.5 intensity, in terms of grey scale value (0-255) in myenteric neurons was measured. In a number of ganglia chosen randomly neurons were counted and the number of myenteric ganglia per unit length of the gut was also counted. Macrophage distribution was assessed using rat anti-

mouse monocyte/macrophage antibody. This antibody was also applied in double-staining with polyclonal anti-PGP9.5 and anti-iNOS (see table 2) antibodies.

In order to label glial cells in the myenteric ganglia, immunostaining was carried out using polyclonal anti-GFAP antibody; micrographs were taken, and the area covered by GFAP immunoreactive cells and the total size of the ganglion were measured. The percentage of GFAP covered portion to the total area was calculated.

TUNEL Assay was carried out as described in general methodology part 2.1.6.

CHAPTER 3:

RESULTS

3.1. RAW264.7 Cell Cultures: Macrophage Activation

RAW264.7 macrophage cells exposed to bacterial endotoxin or pro-inflammatory cytokines displayed marked morphological differences from control cells. Control macrophages showed a generally rounded appearance (Figure 9.A), whilst treated cells, exhibited pronounced elongation and increased cell size (Figure 9.B), properties associated with macrophage activation (Albina *et al*, 1989). This phenomenon was distinguishable 6 hrs post treatment; 56.3±6.1% of LPS and 40.4±6.4% of TNF- α + IFN- γ treated cells showed morphological differentiation related to cellular activation. These changes were long lasting and visible up to 24 hrs post treatment; 52.5±1.6% of LPS and 26.8±2.7% of TNF- α + IFN- γ treated cells showed the distinguished phenotype of activated macrophages at that time-point (Figure 10). Furthermore phalloidin staining revealed multiple pseudopodia on the activated cells, which were not detectable in non-treated cells (Figure 9.A&B) (n \geq 5).

Figure 9. LPS Treatment, Morphological Changes, and iNOS Upregulation in RAW264.7 Cells.

A. Control macrophages show a cylindrical shape; in some cells small processes are visible. **B.** LPS treated macrophages (24 hrs) show a dramatic change in morphology: increased cell size and long pseudopodia enabling the cells to adhere, move and migrate. These are typical features of activated macrophages. iNOS enzyme expression was not detectable in control (**C**) and 1 hr LPS treated cells (**D**); however 12 (**E**) and 24 hrs (**F**) after LPS treatment a significant number of cells showed iNOS immunoreactivity. Bar represents 100 microns.

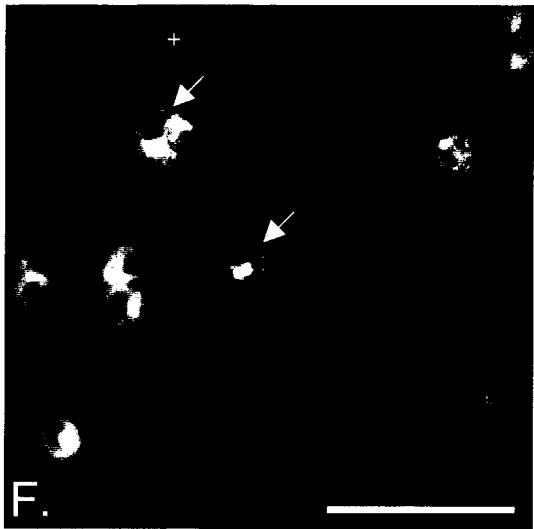
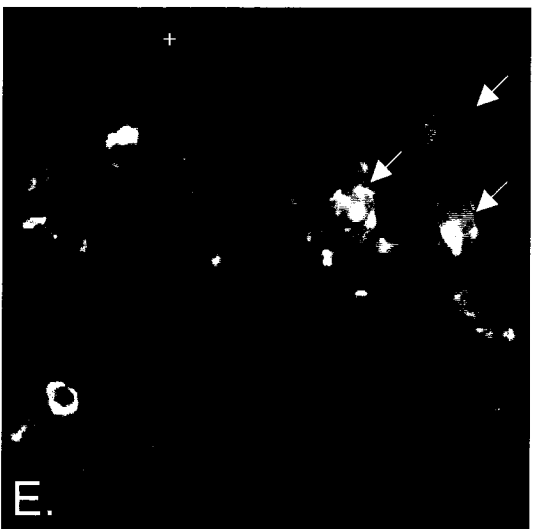
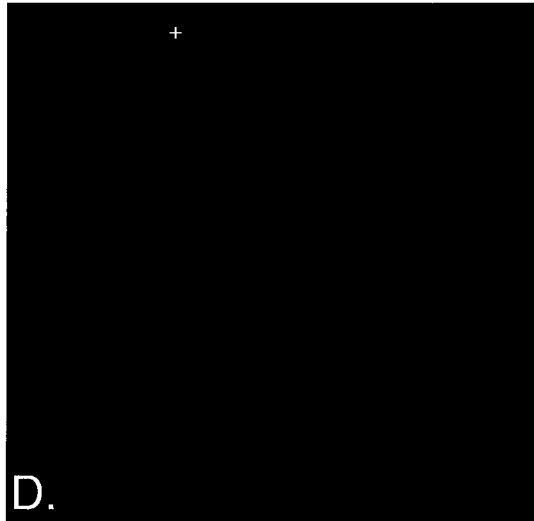
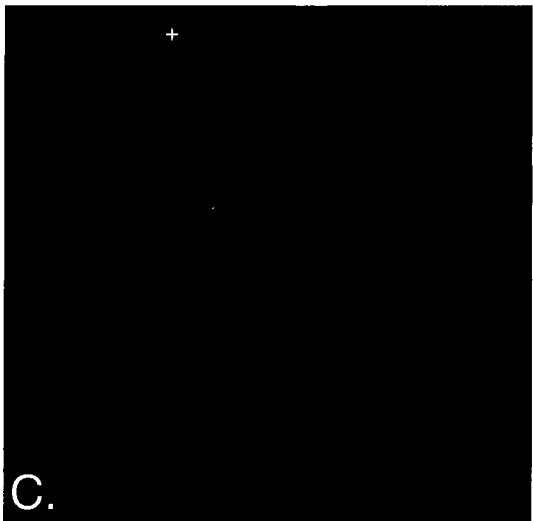
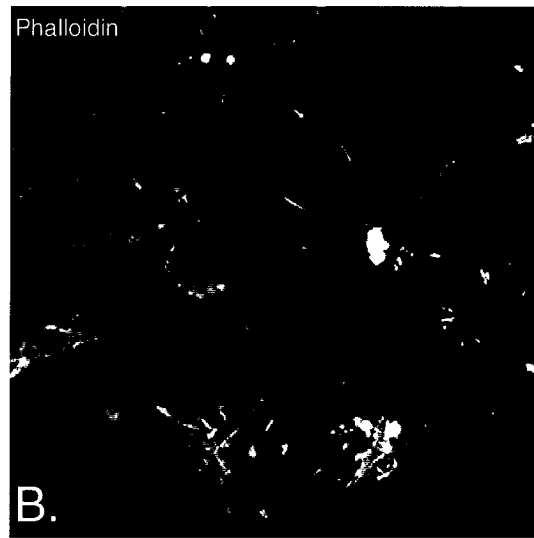
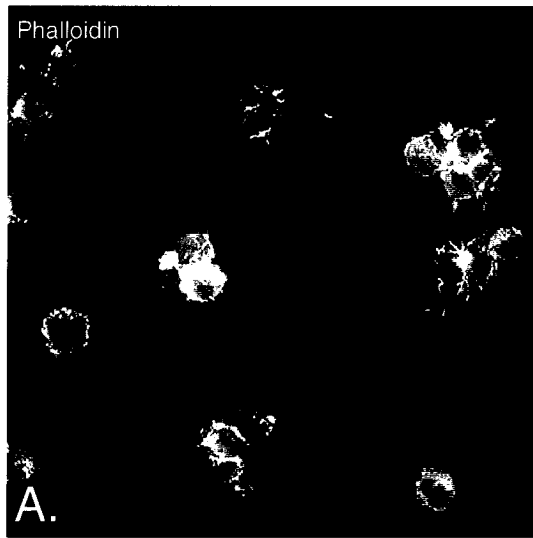


Figure 9

3.2. RAW264.7 Cell Cultures: iNOS Upregulation and Increase in NO Synthesis

In RAW264.7 control and 1 hr treated cell cultures no sign of iNOS immunoreactivity was observed (Figures 9.C-D). 6 hrs after exposure to bacterial endotoxin or pro-inflammatory cytokines, iNOS positive cells were detectable in treated wells (20.8±6.3% of LPS and 8.5±3.2% of TNF- α + IFN- γ treated cells) (n \geq 5). This time-point corresponded with significant increase in macrophage morpho-activation (Figure 10).

Nitrite and nitrate synthesis levels were measured using the Griess reagent assay (Ding *et al*, 1988; Green *et al*, 1982). The results showed that 24 hrs exposure of the cells to LPS increased NO end-metabolites synthesis (28.1±0.6 μ M NO₂, 31.4±0.9 μ M NO₃) compared to controls (1.6±0.1 μ M NO₂, 5.7±1.1 μ M NO₃) (Figure 11.A). Single TNF- α (1.9±0.1 μ M NO₂, 3.9±0.6 μ M NO₃) or IFN- γ (2.5±0.2 μ M NO₂, 4.0±0.4 μ M NO₃) treatment did not induce significant increase in NO metabolite synthesis; whilst a combination of TNF- α and IFN- γ did so (14.5±1.2 μ M NO₂, 10.2±0.9 μ M NO₃) (Figure 11.B). Pretreatment with L-NAME hydrochloride or 1400W dihydrochloride significantly reduced the level of nitrogen metabolite synthesis in either LPS or pro-inflammatory cytokine treated wells (n = 6) (Figures 11.A&B).

3.3. Myenteric Neurons and Glial Cells Showed Normal Appearances in Cell Culture

The neurons of the ENS exhibit variety of phenotypes and neurotransmitters, and dissociated neurons grown in cell cultures resemble the *in vivo* characteristics of these cells (Nishi and Willard, 1985). VIP, ACh, NO, calbindin, TK, ENK, calretinin, 5-HT, NPY, SOM, GABA and CGRP are major classes of neurotransmitters expressed by enteric neurons in the gut (Lomax *et al*, 2000; Costa *et al*, 1996; Furness, 2000; Krantis, 2000), and in ideal enteric cell culture model neurons expressing multiple neurotransmitters must be detectable.

In the present study, upon plating, uniform rounded cells devoid of processes were commonly observed suspended within the culture feeding medium, while small aggregates of viable cells were attached to the culture substrate. At this time-point, cell types were generally difficult to distinguish. However, some glial cells could be identified by their flattened appearance. Although the majority of cells continued to exhibit a uniform rounded appearance after 24 hrs *in vitro*, a small number of cells had extended short processes. By 48 hrs, cells were flattened upon the culture substrate and demonstrated cell-type characteristic morphologies. Neurons were generally clustered together, and commonly extended several branching processes which intersected with many other cell bodies and/or processes within the same cluster or in neighboring neuronal clusters. Neurons preferentially adhered to and overlaid sheets of glial cells, which exhibited characteristic flattened cell bodies with thin membranous expansions and a few processes. Cultured cells continued to extend branching processes to form complex networks of interconnecting ganglion-like structures by 7 days in culture. Pan-neuronal markers PGP9.5 and TUJ1 were used to identify myenteric neurons and their ganglionic

Figure 10. Activation and iNOS Upregulation in RAW264.7 Cells.

Morphological changes and iNOS upregulation were detectable 6 hrs after LPS or TNF- α + IFN- γ treatment. These changes lasted up to 24 hrs after the treatment. Cytokine treatment, in comparison to LPS, induced a less pronounced effect in terms of the number of iNOS immunoreactive cells.

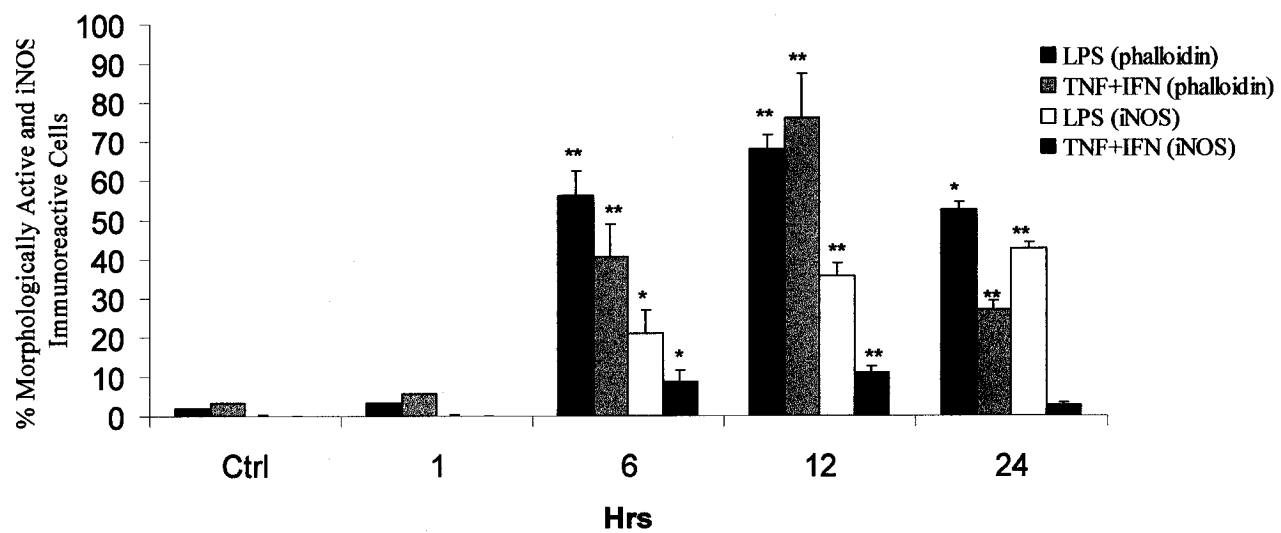
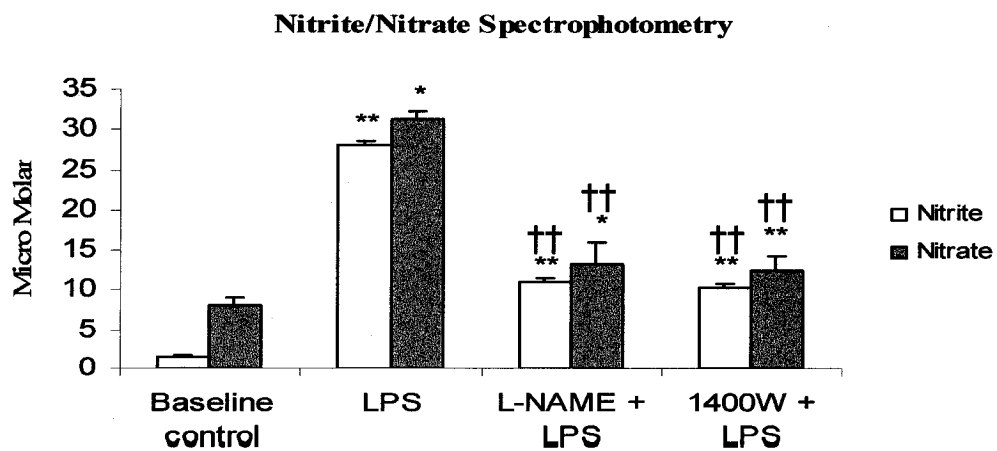


Figure 10

Figure 11. NO Synthesis in RAW264.7 Cell Cultures.

A. LPS treatment of RAW264.7 cells for 24 hrs resulted in approximately 6 fold increase in nitrogen metabolite synthesis. Pretreatment with iNOS inhibitors L-NAME hydrochloride or 1400W dihydrochloride blocked much of this nitrite / nitrate synthesis (significant with respect to the LPS treated group). **B.** Pro-inflammatory cytokines TNF- α or IFN- γ alone failed to induce a marked increase in nitrite / nitrate synthesis; however a combination of TNF- α and IFN- γ increased the production to a significant extend. Pretreatment of TNF- α + IFN- γ treated group with iNOS inhibitors prevented the synthesis. * $p < 0.05$, ++ or ** $p < 0.001$ (* shows the significance in comparison with baseline control, and + indicates the significance in comparison with LPS treated group in A).

A.



B.

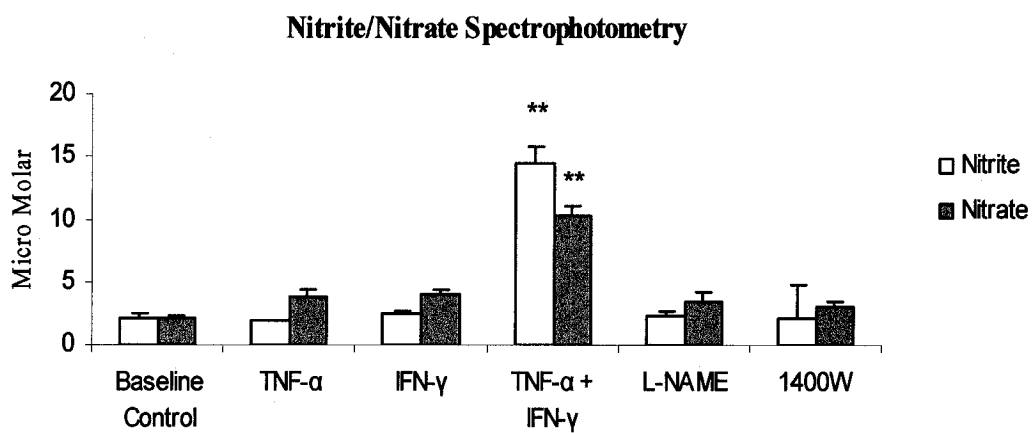


Figure 11

structure and network of axons and dendrites in cell cultures (Figures 12.A&E). Furthermore, by applying anti-pChAT and anti-NOS antibodies and NADPH-d assay the two major subsets of myenteric neurons, cholinergic and nitrergic (Furness, 2000), were detected in cell cultures (Figures 12.B-D). Applying GFAP antibody, glial cells were identified in the myenteric cell cultures, and myenteric neurons were located amongst these cells (Figure 12.E).

3.4. Neurodegeneration in Myenteric-Macrophage Cell Co-Culture Exposed to Bacterial Endotoxin

Neurons in co-cultures of myenteric-macrophage cells showed a normal appearance and high survival rate, i.e. $93.3 \pm 8.6\%$ compared with myenteric neurons in single cultures (Figure 13) ($n \geq 6$). In pure myenteric cell cultures, neuronal exposure to bacterial endotoxin LPS did not induce marked reduction in neuronal survival ($85.7 \pm 8.5\%$). However in co-cultures treated with LPS, activated macrophages (Figure 12.F) and apoptotic neurons were identified and survival rate of myenteric neurons was diminished to $41.2 \pm 1.2\%$ of basal controls (Figure 13).

PGP9.5 staining intensity was significantly reduced in LPS treated co-cultures (76.5 ± 13.1) as early as 6 hrs post exposure (control group: 222.3 ± 13.6) (Figure 14.A). The surviving neurons showed a gradual increase in PGP9.5 synthesis that reached to the similar level to that of controls 96 hrs after treatment (223.0 ± 8.1). Co-culture pretreatment with iNOS inhibitors L-NAME hydrochloride or 1400W dihydrochloride

Figure 12. Neurons in Myenteric Cell Cultures.

A. In a 7 day culture of myenteric cells the neuronal network and ganglionic structure were well established (PGP9.5 immunostaining) and different subtypes of neurons were present: cholinergic neurons were labeled with anti-pChAT antibody (**B**), and nitrenergic neurons were detected using nNOS immunostaining (**C**) and NADPH-d staining method (**D**). Glial cells were widespread, forming a substrate layer in the wells, and myenteric neurons were located amongst and upon them (**E**). Inflammatory neurodegeneration in co-culture of myenteric neurons and macrophages is seen 48 hrs after LPS treatment (**F**). Note the neuronal cell loss (only 4 neurons are seen in this field, compared to 20 neurons in E) and the activated macrophages in the vicinity of cytoskeletal remnants of myenteric neurons weakly stained with TUJ1 antibody. Bars represent 100 microns.

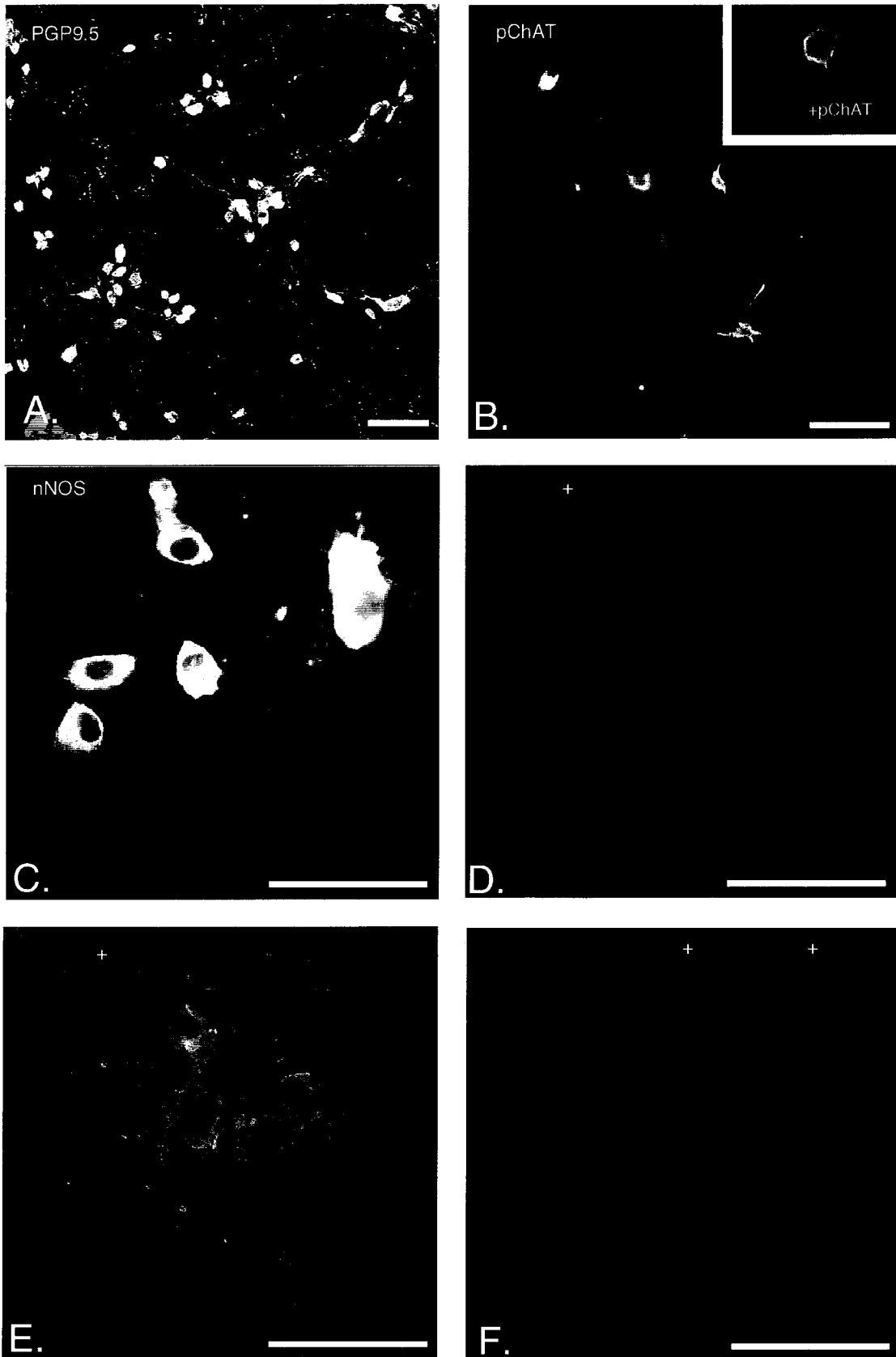


Figure 12

Figure 13. Neuronal Survival in Single Culture and Co-culture.

Although co-culturing the neurons with macrophage cells or treatment of single cultures with LPS did not affect their viability, exposure of myenteric - macrophage co-cultures to LPS induced 60% neuronal cell loss, based on cell count of PGP9.5 immunoreactive neurons. Co-culture exposure to single TNF- α or IFN- γ cytokine did not induce significant neurodegeneration. A combination of TNF- α and IFN- γ , however, had an effect similar to LPS on myenteric neurons in co-culture. * $p < 0.05$, ** $p < 0.001$.

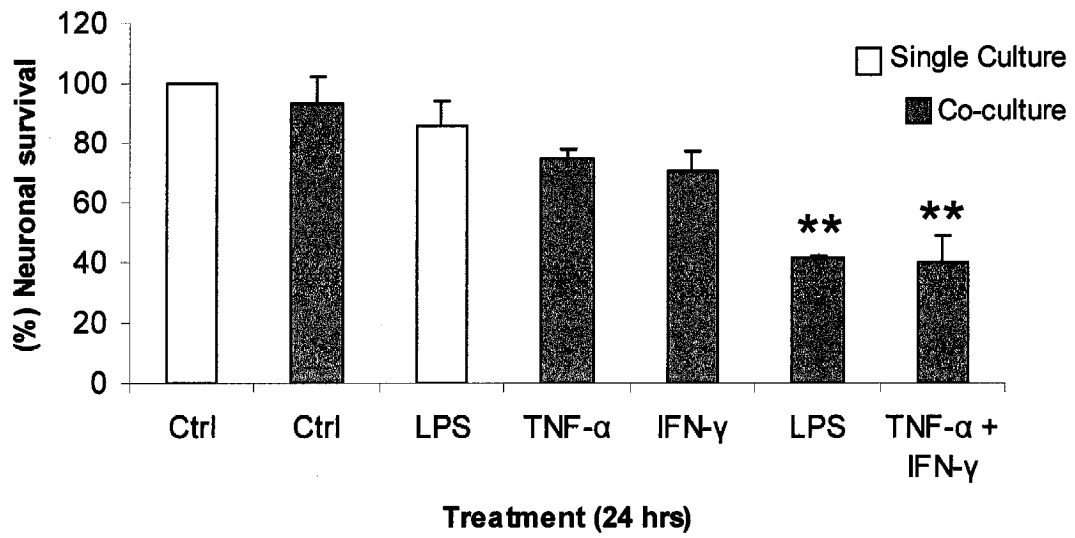


Figure 13

protected myenteric neurons against the neuroinflammatory effect of exposure to LPS, in terms of preservation of PGP9.5 synthesis (intensity in grey scale: 248.4 ± 6.6 and 225.5 ± 8.8 , in pretreated with L-NAME hydrochloride and 1400W dihydrochloride respectively, 6h post treatment) (Figure 14.A).

In co-cultures exposed to LPS, neuronal death rate increased in comparison to the control group: 24 hrs post treatment only $35.3 \pm 2.7\%$ of myenteric neurons survived and the cell loss was even greater 48 hrs after receiving the treatment (survival rate: $24.4 \pm 3.0\%$) (Figure 16.A). Pretreatment of co-cultures with iNOS inhibitors, L-NAME hydrochloride or 1400W dihydrochloride, reduced cell death, and the number of surviving neurons in pretreated wells was not significantly different than control group (survival rate $83.4 \pm 13.9\%$, and $89.8 \pm 12.9\%$ in L-NAME hydrochloride and 1400W dihydrochloride pretreated co-cultures respectively, 24 hrs post treatment). Broadening and multiplying the endpoints of the experiment from 6 to 96 hrs demonstrated that shielding effect of iNOS inhibitors remains for at least 96 hrs, and myenteric neurons did not undergo a delayed cell death (Figure 16.A) ($n \geq 5$).

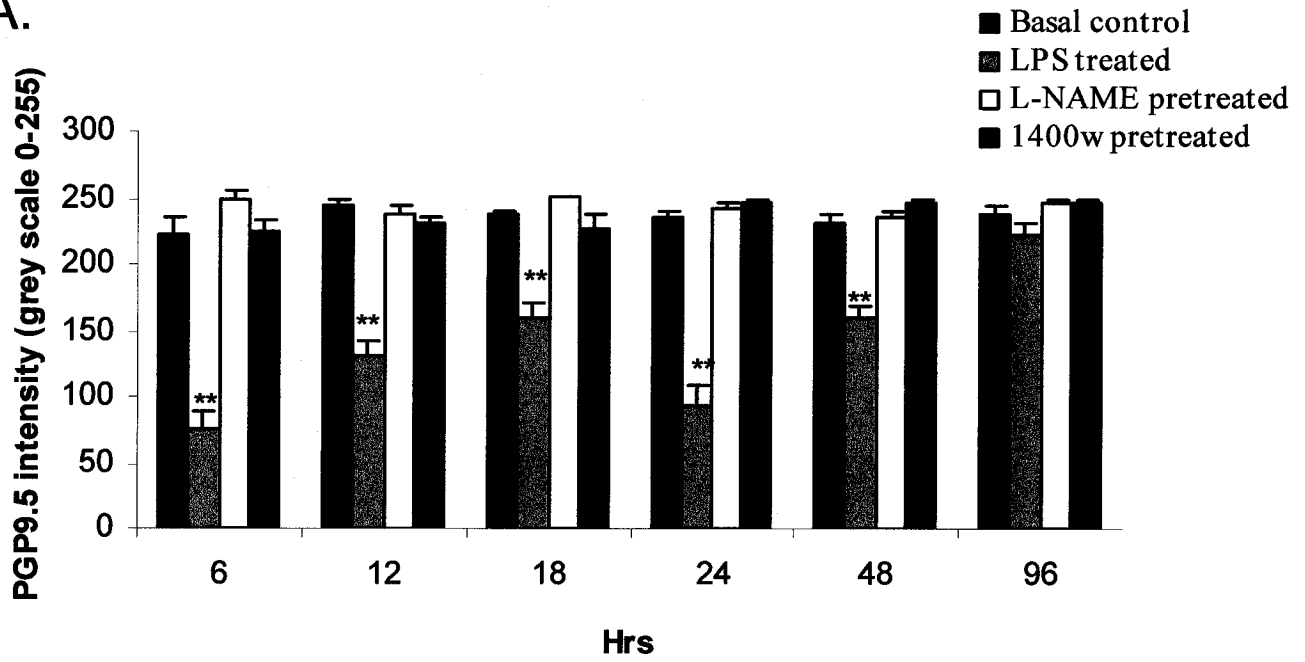
3.5. Neurodegeneration in Macrophage-Myenteric Cell Co-Cultures Treated with Pro-inflammatory Cytokines

Significant reduction in PGP9.5 staining intensity was observed as early as 6 hrs post treatment (121.6 ± 15.5 intensity in grey scale of 0-255; controls: 240.6 ± 4.2), which was prevented by L-NAME hydrochloride or 1400W dihydrochloride pretreatment (247.0 ± 2.6 and 243.4 ± 2.9 respectively) (Figure 14.B). Cytoskeletal protein TUJ1

Figure 14. PGP9.5 Staining Intensity in Myenteric Neurons.

PGP9.5 immunostaining is dramatically reduced in myenteric neurons as early as 6 hrs after LPS / cytokine treatment (A & B). iNOS inhibitors L-NAME hydrochloride or 1400W dihydrochloride protected myenteric neurons against this effect. Immunoreactivity was restored in surviving neurons and returned to a normal level after 96 hrs. * $p < 0.05$, ** $p < 0.001$.

A.



B.

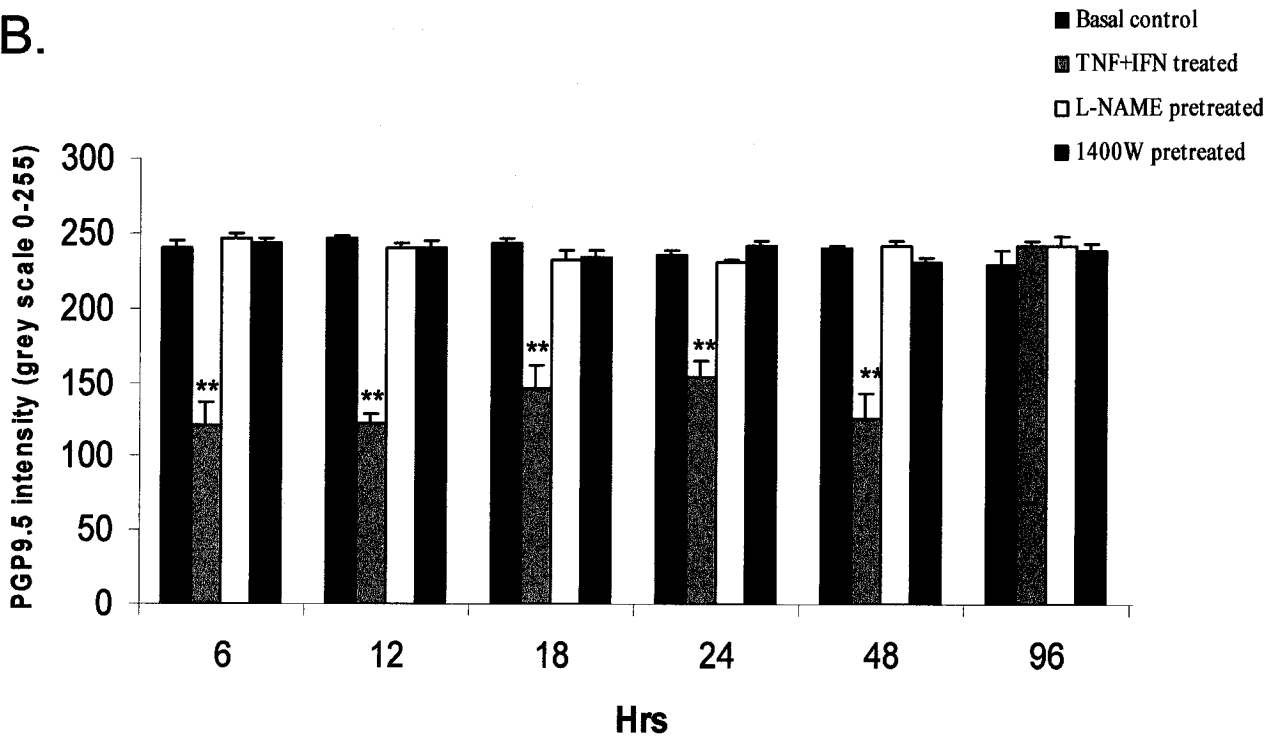


Figure 14

Figure 15. Viability of Myenteric Neurons in iNOS Inhibitor-pretreated Co-cultures.

Viabile neurites are characterized by the abundance of synapsin-1 labeled synapses (A). In a 1400W dihydrochloride pretreated culture, a similar distribution pattern of synapses is seen despite treatment with cytokines (24 hrs) (B). C. 1400W dihydrochloride protected myenteric neuronal cell bodies against cytokines (i.e. they showed both PGP9.5 and TUJ1 immunoreactivity) versus non pre-treated culture (D) in which TUJ1 immunostaining is combined with a weak PGP9.5 staining. Bars represent 100 microns.

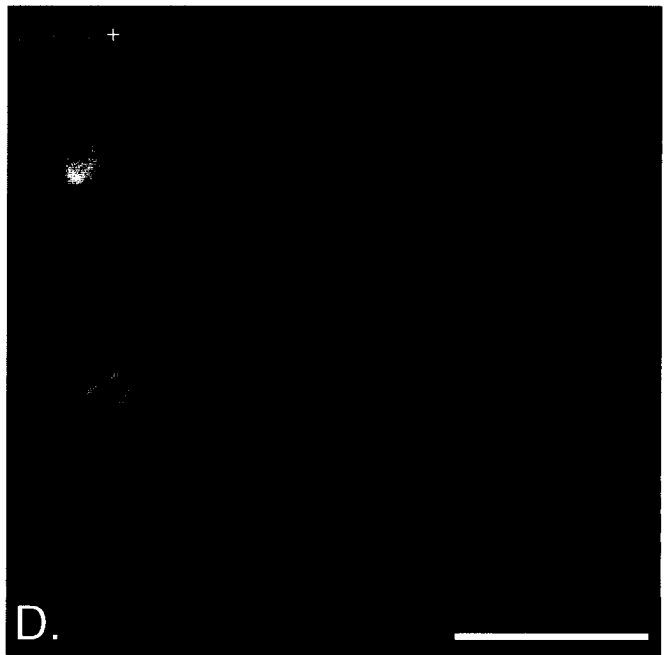
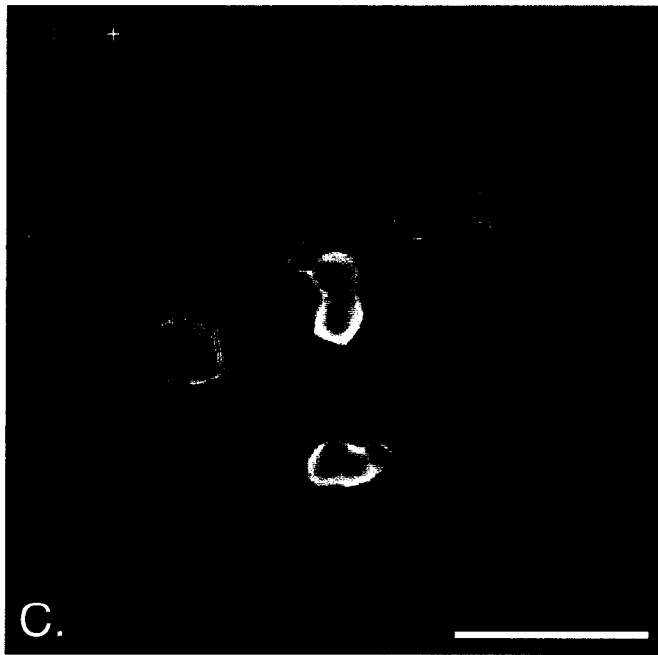
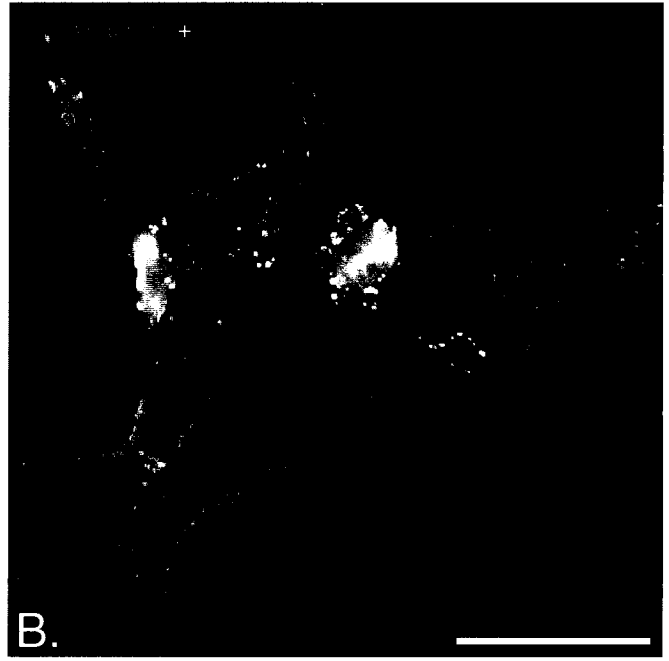
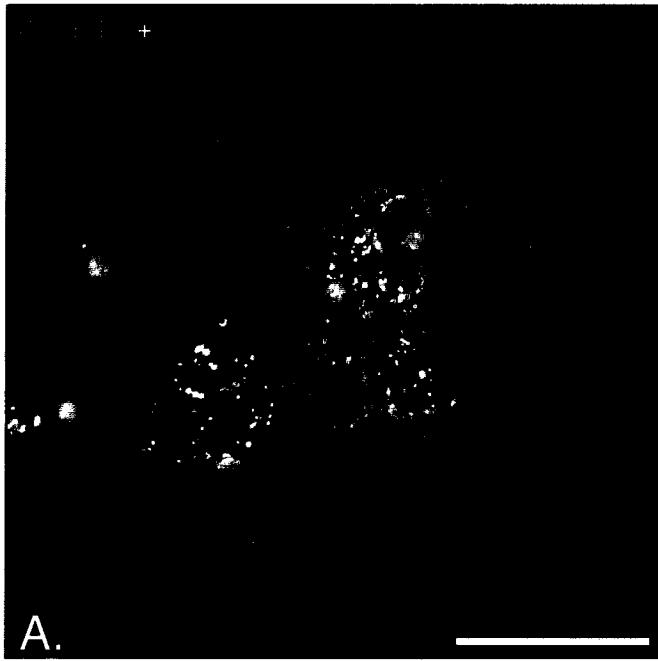


Figure 15

immunostaining does not change 24 hrs after cytokine challenge, whilst functional protein PGP9.5 immunoreactivity was diminished by that time (Figures 15.C-D).

The results of the synapsin-1 immunostaining confirmed the presence of synapses on axons, dendrites, and neuronal cell somas in pretreated co-cultures, resembling neurons in non-treated control group (Figures 15.A-B) (n = 5).

Myenteric neurons in co-cultures with RAW264.7 cells exposed to pro-inflammatory cytokines TNF- α or IFN- γ alone did not differ from controls in terms of neuronal survival; however applying a combination of the two cytokines induced an extensive neuronal death, i.e. only $39.8 \pm 8.8\%$ of myenteric neurons survived after 24 hrs exposure to insult (Figure 16.B). This effect was prevented by applying L-NAME hydrochloride or 1400W dihydrochloride prior to cytokine treatment (Figure 16.B) (n = 5).

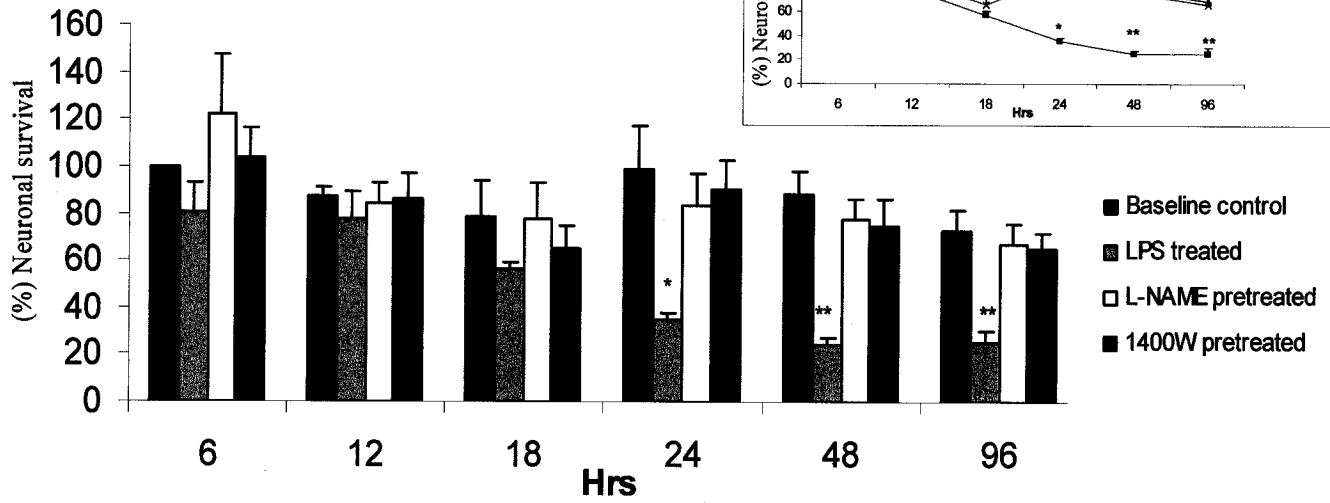
3.6. Neurodegeneration in Co-Cultures of Cortical Neurons and Macrophage Cells

In a macrophage-cortical neuronal cell co-culture, macrophage cells showed different phenotypes than controls 48 hrs after treatment with bacterial endotoxin or pro-inflammatory cytokines (Figures 17.A-B). Co-culturing cortical cells with RAW264.7 macrophage cells did not affect the viability of neurons ($102.2 \pm 16.2\%$ survival); however 48 hrs following the exposure of co-cultures to inflammatory components, only $23.6 \pm 6.9\%$ (LPS treatment) and $28.3 \pm 4.3\%$ (TNF- α + IFN- γ treatment) of cortical neurons survived (Figure 17.C). This effect was prevented by pre-treating co-cultures

Figure 16. Neurodegeneration in LPS / Cytokine Treated Co-cultures.

A. In time course experiments, significant myenteric neurodegeneration was observed after 24 hrs, and increased at 48 hrs post LPS treatment (based on neuronal cell counts of PGP9.5 immunoreactive cells). Pre-treatment of co-cultures with iNOS inhibitors L-NAME hydrochloride or 1400W dihydrochloride prevented neurodegeneration, allowing the cell survival rate to remain at control levels. Applying a combination of two pro-inflammatory cytokines had effects similar to LPS, and this degenerative effect was also blocked by pretreatment with iNOS inhibitors (**B**). * $p < 0.05$, ** $p < 0.001$.

A.



B.

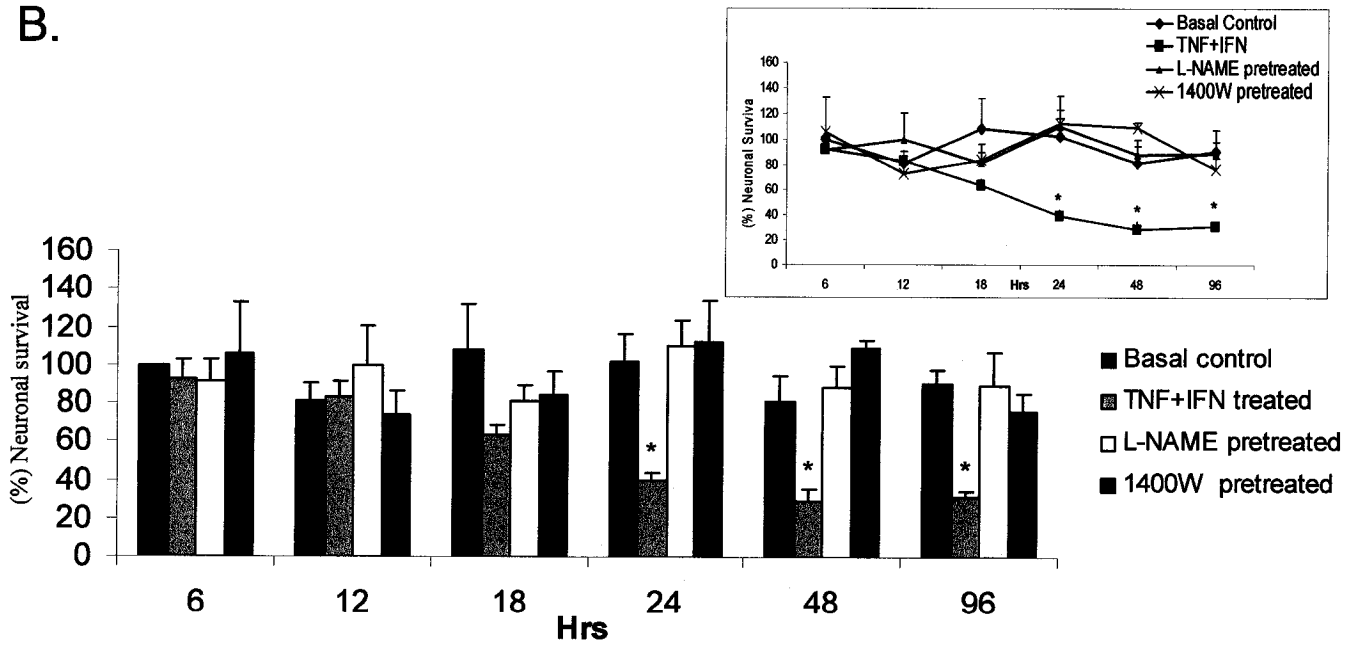


Figure 16

Figure 17. Neurodegeneration in Macrophage – Cortical Cell Co-culture.

Macrophage cells in control (A) and LPS treated (B) wells showed different phenotypes: Increased cell size in treated well demonstrating typical feature of activated macrophages. Concurrently there is a dramatic decrease in the number of neurons surviving in the treated co-cultures. C. LPS or cytokine treatment induced neurodegeneration; only 20% of neurons survived following endotoxin / cytokine challenge. This effect was prevented by applying iNOS inhibitors L-NAME hydrochloride or 1400W dihydrochloride. Bar represents 100 microns. * $p < 0.05$.

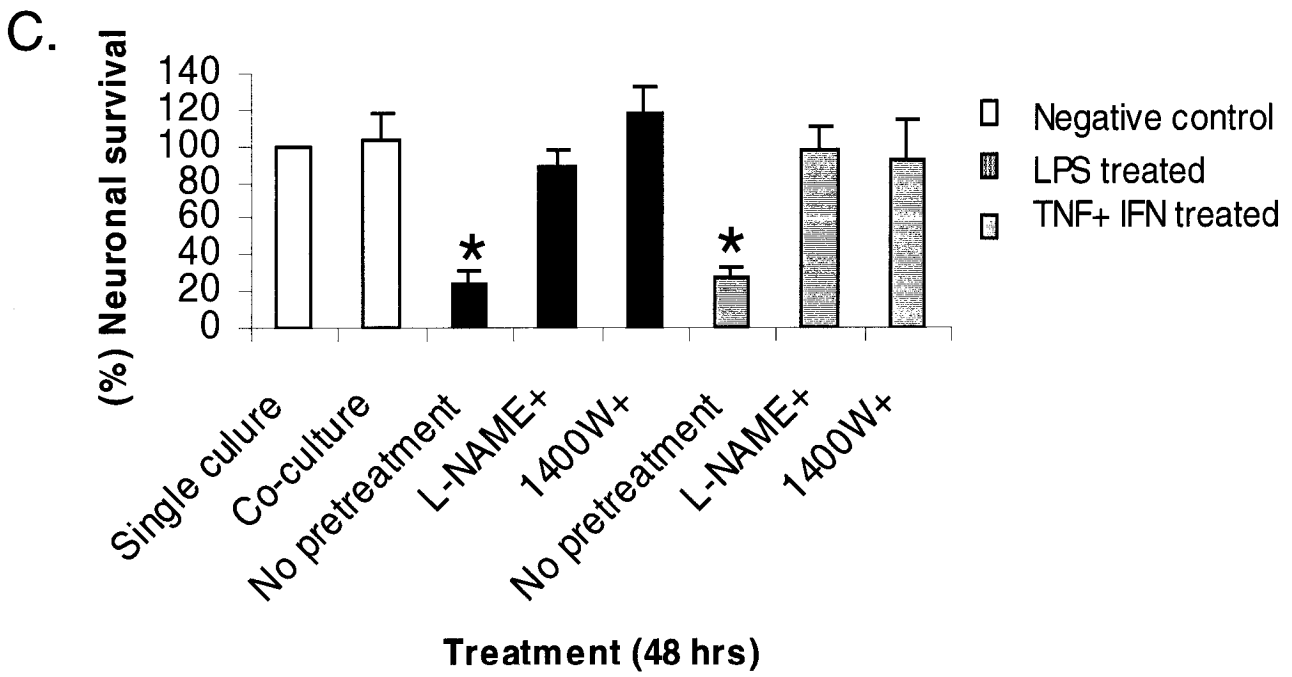
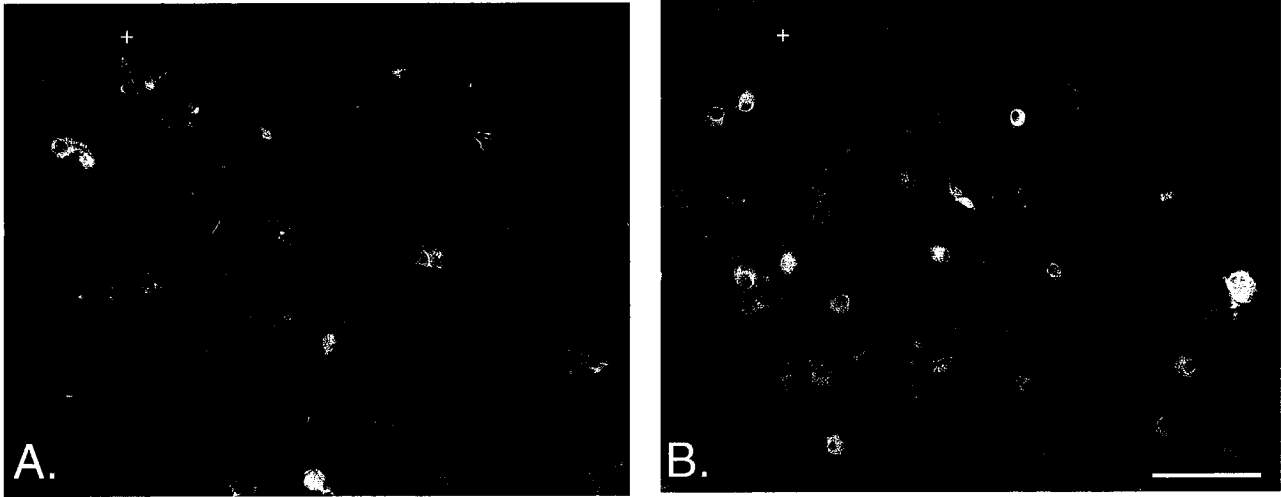


Figure 17

with iNOS inhibitors L-NAME hydrochloride ($88.9\pm 9.3\%$ and $97.9\pm 12.5\%$ survival in LPS and TNF- α + IFN- γ treated groups respectively) or 1400W dihydrochloride ($117.5\pm 15.6\%$ and $93.0\pm 20.7\%$ survival in LPS and TNF- α + IFN- γ treated groups respectively) ($n = 5$).

3.7. General Observations in Colitic Rats

DNBS treated rats displayed characteristics of acute colitis such as piloerection, unkempt appearance, diarrhea, and in some cases mild abdominal distension. Macroscopic signs of inflammation including hyperemia, edema, and ulceration were observed in longitudinally opened colon of rats (Figure 18.A). The inflammation showed signs of recovery 96 hrs post DNBS administration ($n \geq 5$).

DNBS treated animals lost weight, which reached a significant level 48 hrs after the treatment (change in weight after 48 hrs: -7.6 ± 1.5 g in DNBS treated rats, $+25.0\pm 8.2$ g in controls). Pretreatment with 1400W dihydrochloride did not have a remarkable influence on weight loss (weight change in 12 hrs: $+4.0\pm 2.5$ g, 24 hrs: $+1.0\pm 3.6$ g, 48 hrs: -5.0 ± 3.6 g, and 96 hrs: $+12.4\pm 4.6$ g) (Figure 19.A).

Increased weight of the colonic tissue, a consequence of edema and inflammation in the gut, was observed in animals receiving DNBS injection. Colonic tissues at 12, 24, 48 and 96 hrs post treatment time-points (2789 ± 342 mg, 3624 ± 759 mg, 3799 ± 87 mg, and 3085 ± 433 mg respectively) were dramatically heavier than controls (1860 ± 84 mg, 1691 ± 118 mg, 2029 ± 208 mg, and 1900 ± 120 mg, respectively; Figure 19.B).

Figure 18. Gross Morphologic Damage and Microscopic Changes in Colonic Tissue after DNBS Treatment.

A. Photographs taken from longitudinally opened large intestines: control animals showed no sign of inflammation in colon; however as early as 12 hrs after DNBS administration macroscopic signs of inflammation, hyperemia and edema, were observed. The recovery process started 96 hrs after injection.

Micrographs (B) confirm the results of macroscopic observations (dark field microscopy). In cross sections of gut, control animals showed a preserved architecture of villi, submucosa, and neuromuscular layers. This pattern was disrupted 12 hrs after applying DNBS. Signs of recovery were evident after 96 hrs (thickening of mucosa and recovery of its folia, as indicated by the arrows). Bar represents 100 microns.

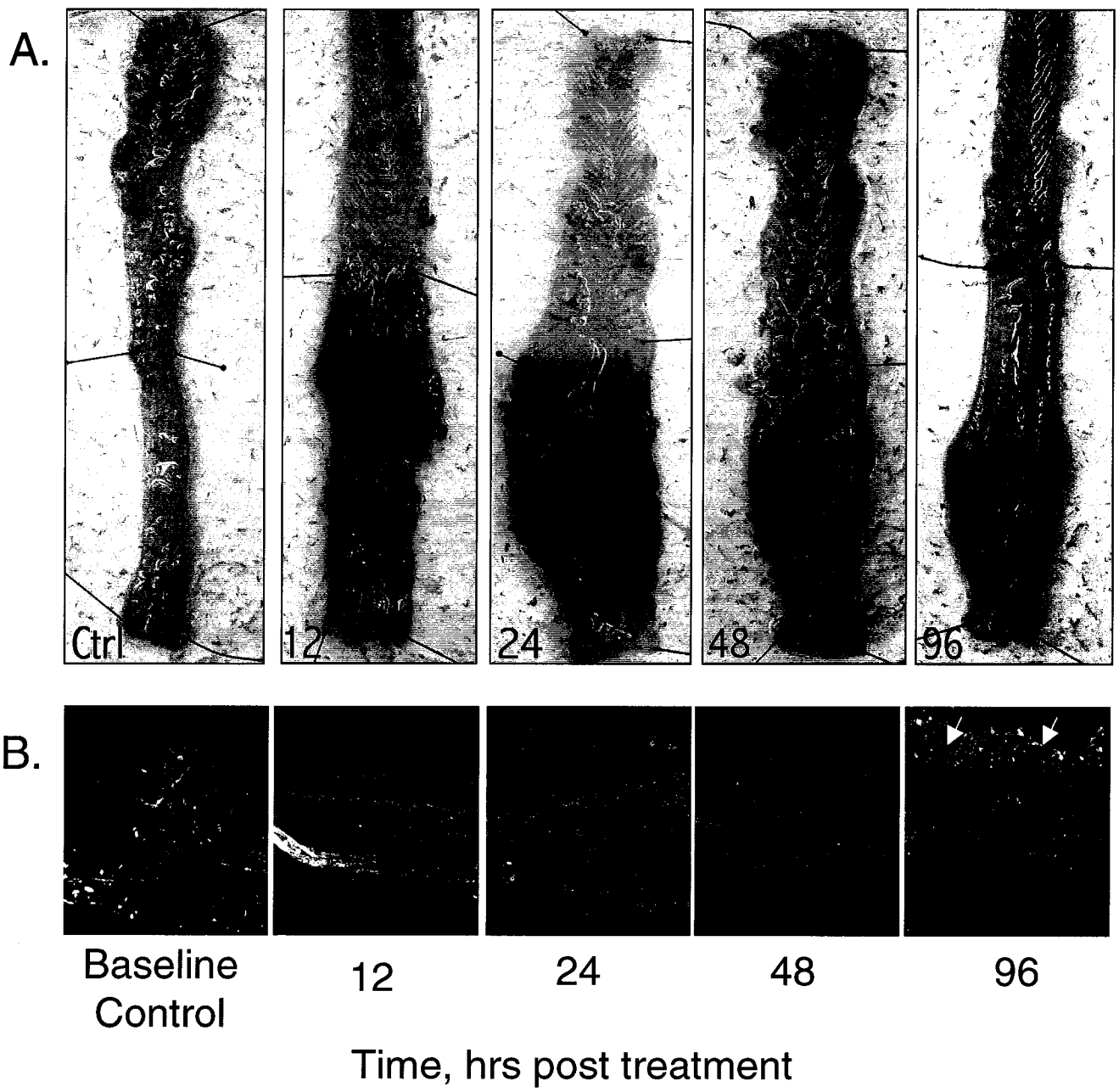
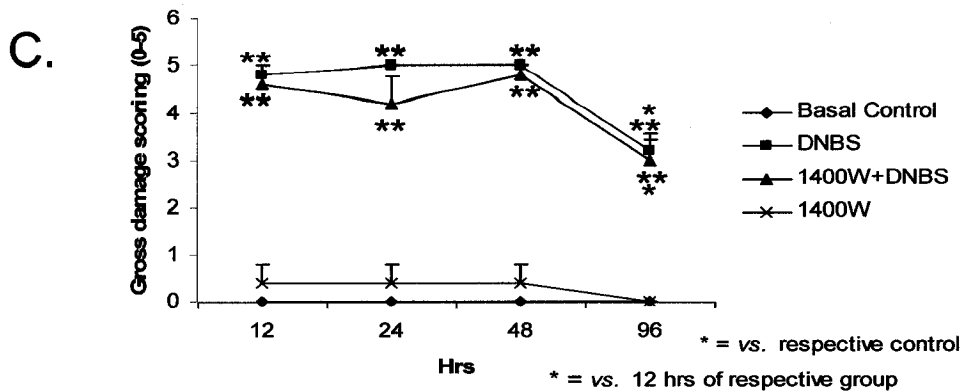
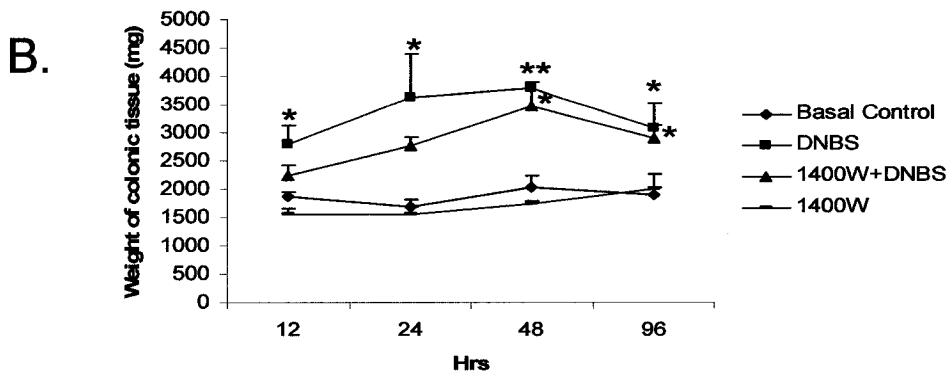
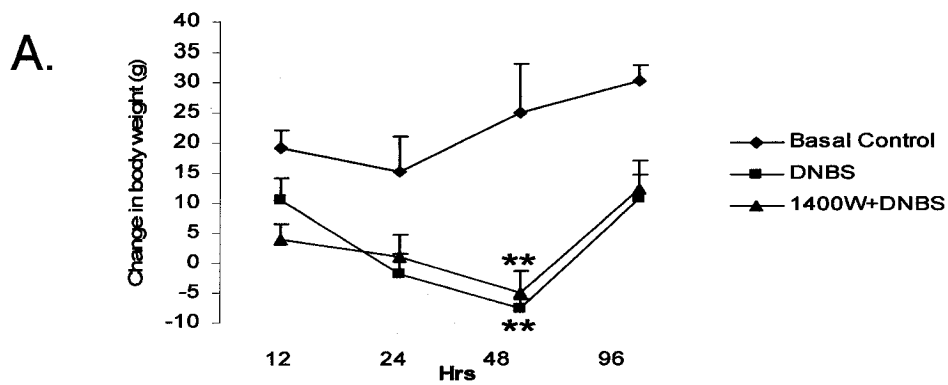


Figure 18

Figure 19. General Changes in DNBS Treated Rats.

A. Animals lost weight as early as 12 hrs after DNBS injection, reaching significance 48 hrs following treatment. DNBS treated rats gained weight and started to recover by 96 hrs post-treatment. 1400W dihydrochloride pretreated animals were slightly different than those in the single DNBS treated group (data is not available for the group receiving 1400W dihydrochloride alone). **B.** Contrary to the body weight changes in DNBS treated animals, they had a heavier wet colonic tissue in comparison to basal control and 1400W dihydrochloride treated animals. **C.** Criteria for gross morphology scoring were based on the absence, presence and severity of inflammation in the colonic tissue (Morris *et al*, 1989). Scoring the inflamed colon, DNBS treated rats (pretreated and non-pretreated with 1400W dihydrochloride) received scores significantly higher than controls and 1400W dihydrochloride treated animals at all time points. 96 hrs after DNBS administration a significant reduction in the severity of inflammation was observed (comparing to 12 hrs treated animals of respective group). * $p < 0.05$, ** $p < 0.001$.



Score	Gross Morphology
0	No damage
1	Localized hyperemia
2	Linear ulcers with no significant inflammation
3	Linear ulcer with inflammation at one site
4	Two or more sites of ulceration and / or inflammation
5	Two or more major sites of inflammation and ulceration or one major site of inflammation and ulceration extending >1cm along the length of the colon

Scoring chart adapted from Morris et al. (1989)

Figure 19

No inflamed area was observed in the intestine of control group animals. Micrographs taken from the gut sections corresponded with these results (Figure 18.B). Severe damage to the mucosa was observed 12 hrs after DNBS treatment (gross damage scoring 4.8 ± 0.2 , scale 0-5), and was detectable at later time-points up to 96 hrs (5.0 ± 0 , 24 hrs; 5.0 ± 0 , 48 hrs; 3.2 ± 0.4 , 96 hrs; Figure 19.C) ($n \geq 5$). In overall scoring the gross morphology of the gut based on severity of inflammation, DNBS injected rats received the highest scores, which were markedly different from control animals at all time-points (Figure 19.C). The length and severity of inflammation were slightly reduced in 1400W dihydrochloride pre-treated rats, i.e. 4.6 ± 0.4 , 4.2 ± 0.6 , 4.8 ± 0.2 , and 3.0 ± 0.4 for 12, 24, 48, and 96 hrs treated animals respectively. The scoring results of gross morphology damage: 0.4 ± 0.4 , 0.4 ± 0.4 , 0.4 ± 0.4 , and 0.0 ± 0.0 (12, 24, 48, and 96 hrs treated animals respectively) showed that animals received 1400W dihydrochloride injection alone did not differ from control animals (Figure 19.C).

3.8. Immunocyte Infiltration into the Site of inflammation in Colitic Rats

Myeloperoxidase (MPO) activity is a marker of the presence of neutrophil granulocytes in the tissue (Mullane *et al*, 1985). A MPO assay showed a significant increase in the activity of this enzyme in DNBS treated rats and reached its highest level 48 hrs after treatment; the calculated peroxidase activity was 6.7 ± 0.9 , 13.7 ± 4.2 , 5.7 ± 0.9 , and 5.7 ± 1.6 ng/g tissue compared to controls (0.6 ± 0.2 , 0.5 ± 0.1 , 0.6 ± 0.1 , and 0.7 ± 0.1 ng/g

Figure 20. Myeloperoxidase Activity in Colonic Tissue.

Myeloperoxidase activity, a marker of neutrophil infiltration into the inflamed site, was significantly increased in DNBS treated rats, and reached the maximum level 24 hrs after injection. In the animals pretreated with 1400W dihydrochloride, only a slight increase in MPO activity was detected (there are no data available for 1400W dihydrochloride treatment alone). * $p < 0.05$, ** $p < 0.001$.

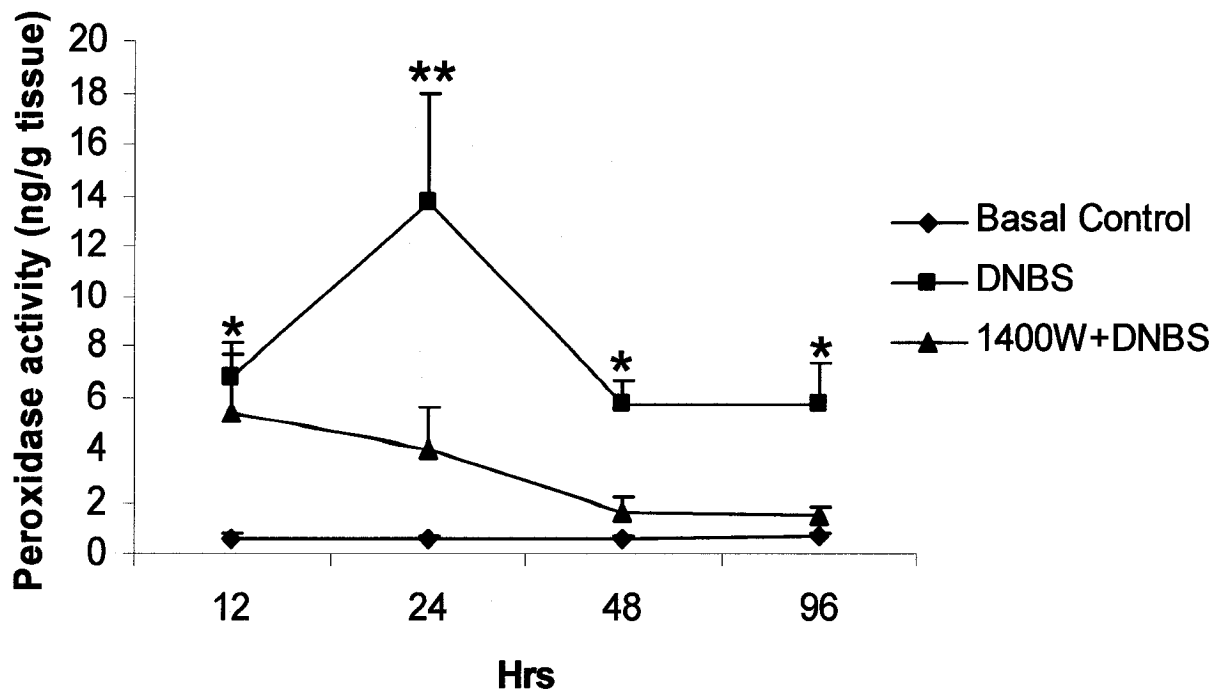


Figure 20

tissue) in 12, 24, 48, and 96 hrs treated animals respectively (Figure 20). 1400W dihydrochloride pretreated rats showed a reduced MPO activity in comparison with DNBS treated group. This activity, however, was slightly higher than respective control groups at all time-points as peroxidase activity in 1400W dihydrochloride pre-treated groups was recorded as 5.4 ± 2.6 , 4.0 ± 1.6 , 1.6 ± 0.6 , and 1.5 ± 0.3 ng/g tissue at 12, 24, 48, and 96 hrs time-points post treatment respectively (Figure 20) ($n \geq 5$).

As judged by immunohistochemistry, abundant macrophage and lymphocyte migration to the inflamed site of the gut was observed as early as 12 hrs after DNBS injection (Figure 21.A). These cells were found in mucosa, submucosa, and neuromuscular layers, and were detectable inside myenteric ganglia.

3.9. DNBS treated Rats and Intestinal iNOS Upregulation

iNOS mRNA expression was detectable 12 hr after DNBS treatment (Figure 21.B) ($n = 5$). This observation is in line with the results of the work of Kankuri *et al* (2001). Western blotting also showed the enzyme upregulation in DNBS treated rats ($n = 5$), and double immunostaining of ED-1 and iNOS antibodies labeled iNOS protein within macrophage cells (Figure 21.C).

Figure 21. Macrophage Infiltration and iNOS Upregulation in DNBS Treated Rats.

A. 24 hrs after DNBS injection, massive infiltration of macrophages was observed. These immunocytes were detectable adjacent to and inside myenteric ganglia. **B.** iNOS mRNA expression in inflamed colonic tissue was increased 12 hrs after DNBS injection; it was not detectable 24 hrs post treatment. **C.** iNOS immunostaining was colocalized with macrophage marker ED-1.

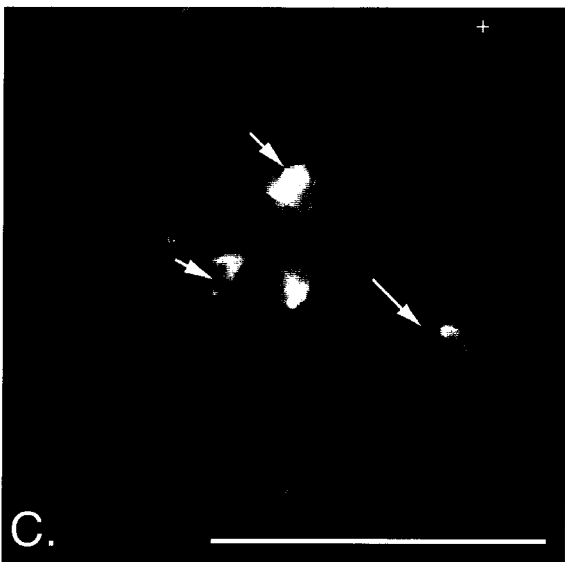
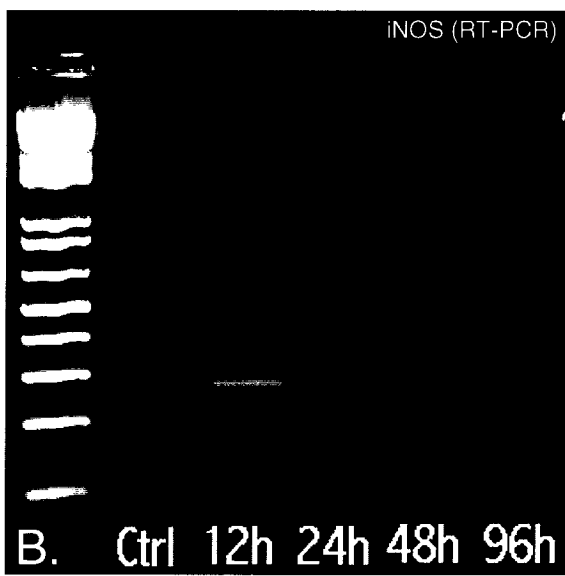
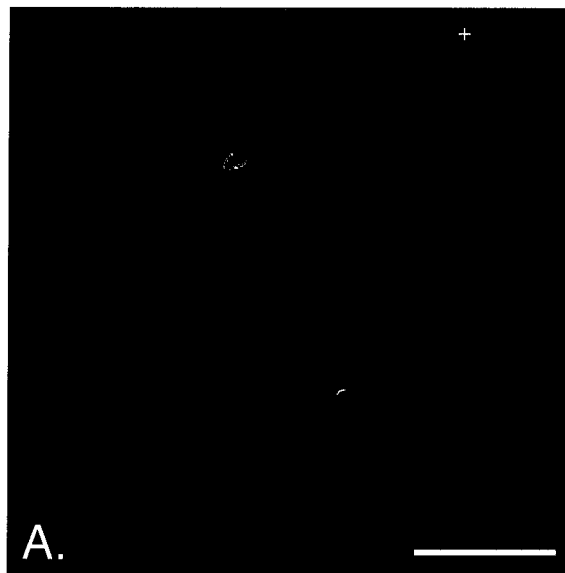


Figure 21

3.10. Experimental Colitis and Myenteric Neurodegeneration

Using the Fluoro-Jade assay to detect necrotic cells, no sign of necrosis was observed in either test groups; however cell degeneration ensued in DNBS treated animals, as cells underwent the process of apoptosis. Apoptotic cells were identified using three different approaches: casp-3 immunostaining for early phase, and TUNEL assay and Hoechst 33258 staining for late phase of apoptosis. Apoptotic cells were detectable in mucosal, submucosal, and myenteric neuronal layers (Figures 22.B&C).

Myenteric neurons in DNBS treated rats were affected by inflammation; the level of PGP9.5 synthesis was significantly reduced at early time-points of the experiments (89.9 ± 11.2 , 99.0 ± 7.6 , and 115.0 ± 12.1 ; 12, 24, 48 hrs post treatment respectively; in grey scale of 0-255) compared to controls (184.4 ± 7.401 , 187.0 ± 17.1 , and 212.5 ± 14.6 ; 12, 24, and 48 hrs post start-time; Figure 23.A). This reduction was iNOS dependent as pretreatment of the animals with iNOS inhibitor kept PGP9.5 immunointensity in myenteric neurons (176.5 ± 16.2 , 129.4 ± 24.3 , 178.9 ± 13.1 , and 188.5 ± 25.3 ; 12, 24, and 48 hrs post treatment respectively) to the level of control group. Enzyme immunoreactivity was partially restored in surviving neurons of DNBS treated group 96 hrs after receiving the treatment (138.4 ± 4.6) (Figure 23.A).

Although apoptotic neurons were detected in myenteric ganglia (Figure 22.B&C), the number of neurons per myenteric ganglion in DNBS treated group was not considerably different (6 ± 0.6 , 6.5 ± 0.6 , 6.4 ± 0.7 , 9.0 ± 1.2 ; 12, 24, 48, 96 hrs post treatment respectively) from controls (6.4 ± 0.6 , 8.2 ± 0.8 , 7.5 ± 1.0 , 6.9 ± 0.6 ; Figure 23.B) at any time-point (number of ganglia studied in each group = 20).

Figure 22. TNF- α Upregulation in DNBS Treated Rats and Apoptosis in Myenteric Ganglia.

Control animals showed no sign of TNF- α immunoreactivity (inset, A); however in gut mucosa and neuromuscular layer of DNBS treated rats (24 hrs after injection) it was noticeable. Panel (A) shows a myenteric ganglion positively stained with TNF- α antibody (arrow). B. In DNBS treated rats (24 hrs post injection) casp-3 positive cells were detectable in the myenteric ganglion (arrows). C. Chromatin condensation and nuclei fragmentation are features of the irreversible phase of apoptosis. In this panel a myenteric neuron undergoing apoptosis is shown (inset shows nuclear segmentation; Hoechst 33258 staining). Bars represent 100 microns.

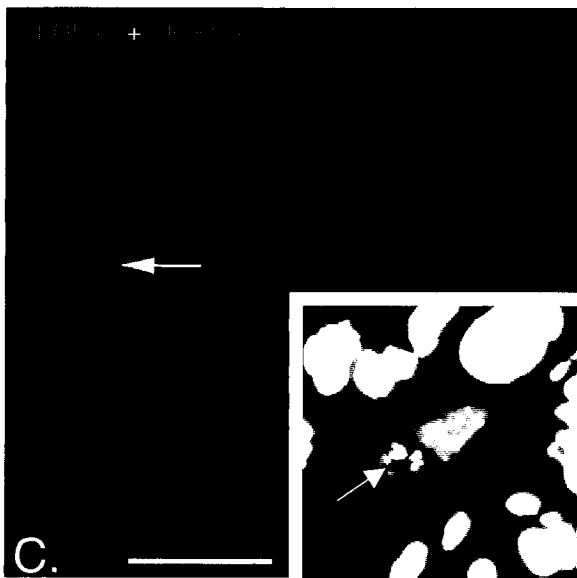
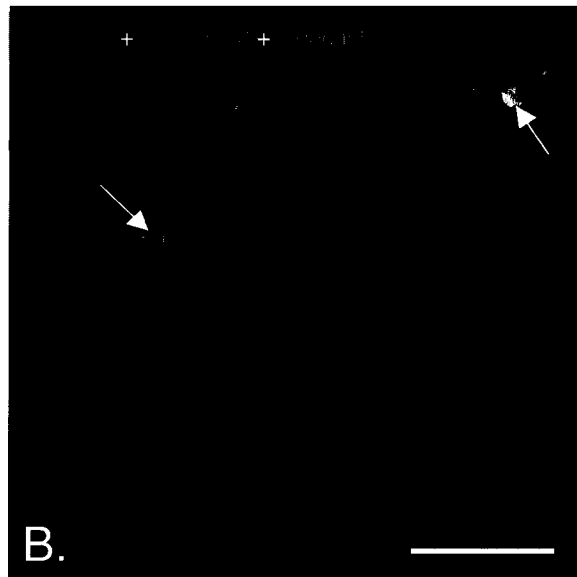
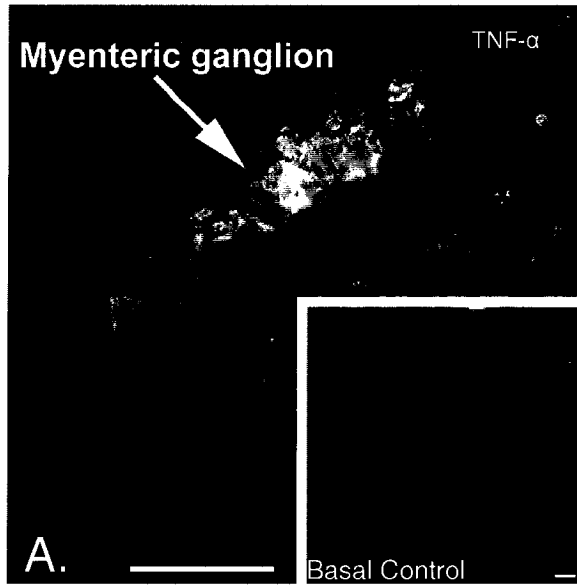


Figure 22

Figure 23. Neurons and Glia in Myenteric Ganglia of DNBS Treated / Non Treated Rats.

PGP9.5 staining intensity was significantly reduced in myenteric neurons of DNBS treated rats, an indicator of neuroinflammation. This effect was prevented by applying iNOS inhibitors, L-NAME hydrochloride or 1400W dihydrochloride (**A**); however no major change in the total number of neurons per ganglion was observed, in comparison with basal control group (**B**). Quantity measurement also showed no changes in glial cells in any of the treated groups comparing to basal control (**C**). * $p < 0.05$.

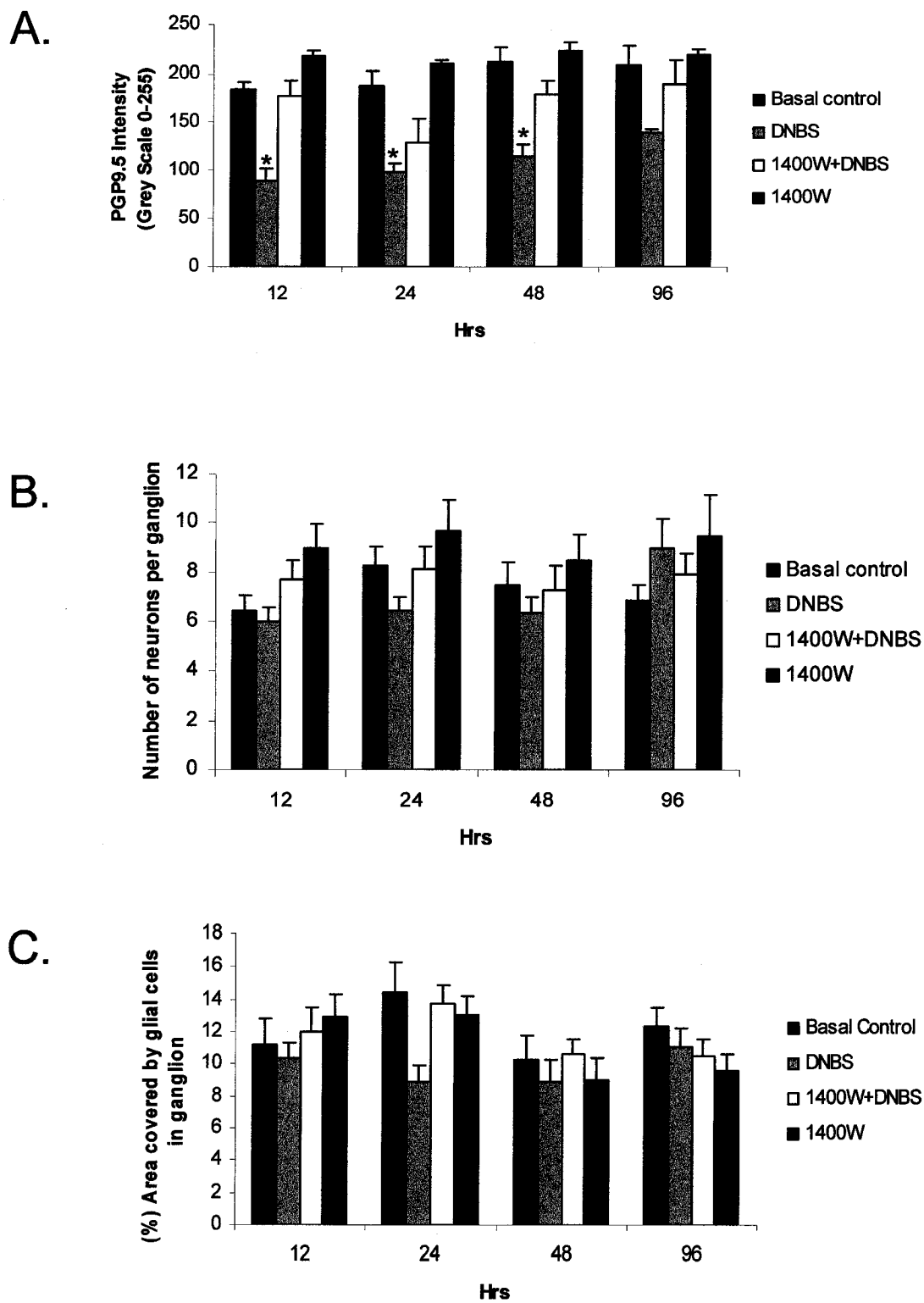


Figure 23

An upregulation in TNF- α expression was also observed in myenteric ganglia of DNBS treated rats, comparing with basal control groups (figure 22.A).

Glial cells have trophic and protective roles toward the enteric neurons, thus it is expected that glial cells proliferate and increase during the course of inflammation (Cabarrocas *et al*, 2003). However in our experiments quantitative measurement of glial cells in myenteric ganglia of DNBS treated and control groups did not show any significant difference from each other; i.e. area covered by GFAP positive cells in myenteric ganglia 12, 24, 48, and 96 hrs post treatment (11.2 \pm 1.6%, 14.4 \pm 1.8%, 10.3 \pm 1.5%, and 12.3 \pm 1.2%) was similar to that in controls (10.4 \pm 1.0%, 9.0 \pm 1.0%, 9.0 \pm 1.4%, and 11.1 \pm 1.2%; Figure 23.C) (number of ganglia studied per time-point \geq 10).

3.11. General Observations in DNBS and TNF- α Treated Mice

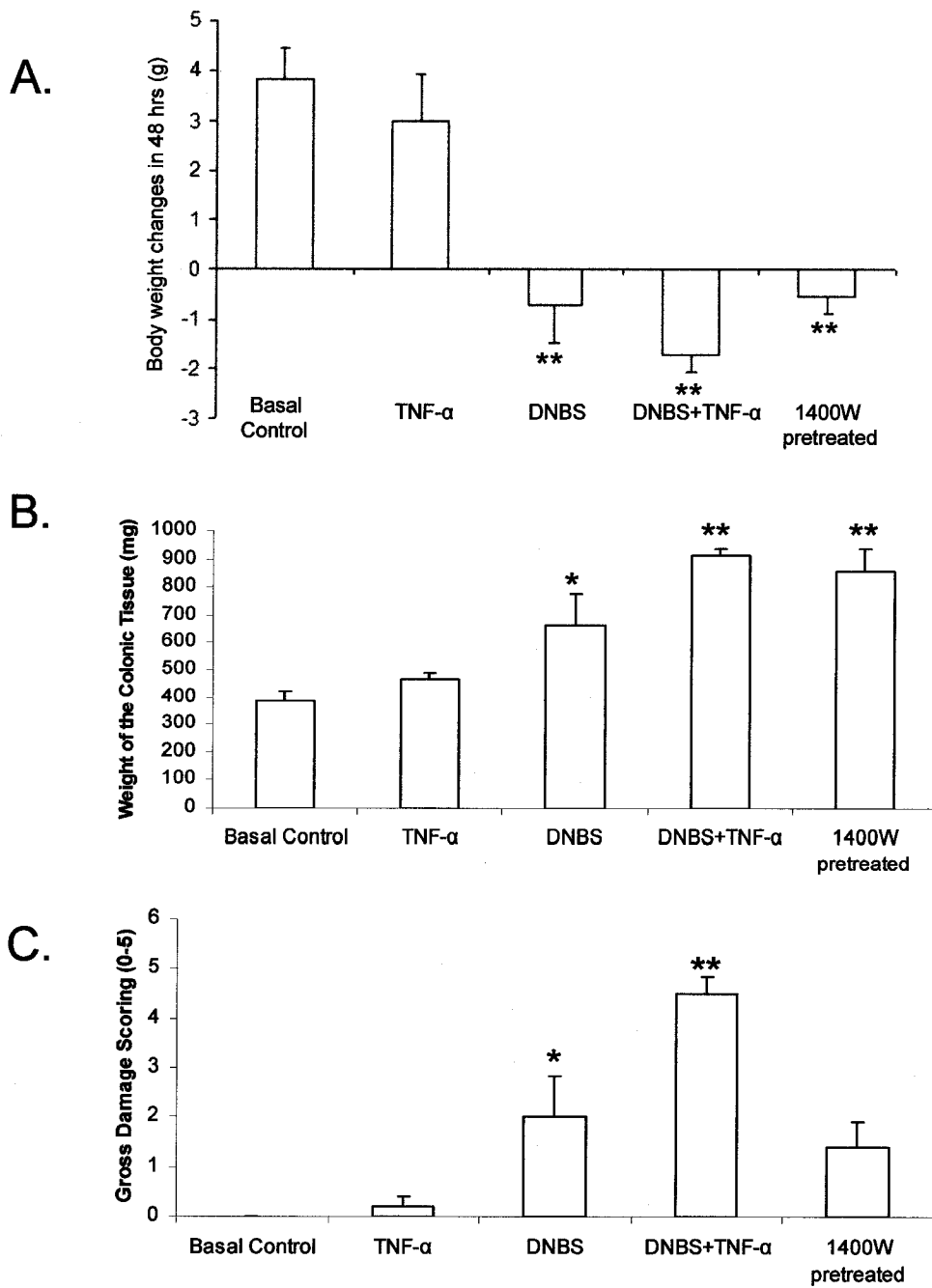
Diarrhea, piloerection, dehydration, mild abdominal distension, and unkempt appearance, classical indicators of experimental colitis in rodents (Morris *et al*, 1989) were observed in animals received DNBS alone or in combination with other treatments.

Control and TNF- α treated groups gained 3.8 \pm 1.5 g and 3.0 \pm 2.0 g weight during the course of experiment (48 hrs) respectively (n \geq 5), whilst mice received DNBS or DNBS+TNF- α injections lost weight (-0.7 \pm 1.8 g and -1.7 \pm 0.8 g respectively), and pretreatment with 1400W dihydrochloride did not significantly change it (-0.6 \pm 0.9 g) (Figure 24.A).

Because of cellular infiltration, mucosal inflammation and edema, wet colonic tissues in DNBS (666.8 \pm 112.6 mg) and DNBS + TNF- α (912.8 \pm 24.2 mg) treated mice

Figure 24. General Changes in DNBS and TNF- α Treated Mice.

Animals receiving only TNF- α did not differ from control group in terms of body weight, weight of the colonic tissue and gross damage scoring (**A**, **B**, & **C**). All of the animals receiving DNBS treatment, alone or in combination with other injections, showed significant weight loss and heaviness of the colonic tissue (**A** & **B**). Scoring the inflammation in the colonic tissue, DNBS and DNBS+TNF- α treated mice received scores significantly higher than control animals. 1400W dihydrochloride pretreatment prevented the inflammation (**C**). * $p < 0.05$, ** $p < 0.001$.



Score

Gross Morphology

0	No damage
1	Localized hyperemia
2	Linear ulcers with no significant inflammation
3	Linear ulcer with inflammation at one site
4	Two or more sites of ulceration and / or inflammation
5	Two or more major sites of inflammation and ulceration or one major site of inflammation and ulceration extending >1cm along the length of the colon

Adapted from Morris et al. (1989)

Figure 24

were heavier than control group (387.0 ± 32.5 mg). Pretreatment with 1400W dihydrochloride did not reduce the heaviness of colonic tissue (855.4 ± 83.1 mg; Figure 24.B). Weight of the colonic tissue of the animals receiving TNF- α alone were in the range comparable to control group (467.2 ± 21.4 mg) (n = 5).

Moreover, DNBS injection alone resulted in a high damage score (2.0 ± 0.8) (Figures 24.C & 25.B), and extensive gross damage was observed in animals receiving DNBS+TNF- α injections (4.5 ± 0.8). 1400W dihydrochloride pretreatment preserved the architecture of the colonic tissue and partially prevented the gross damage of the colon (1.4 ± 0.5 ; Figure 24.C). TNF- α alone did not induce a meaningful increase in damage scoring, comparing to basal control group (0.0 ± 0.0 , and 0.2 ± 0.2 ; control and TNF- α treated groups respectively).

3.12. Macrophage Infiltration and iNOS Upregulation in DNBS + TNF- α Treated Mice

Massive macrophage infiltration was detected in the vicinity and within myenteric ganglia, in all groups receiving DNBS injection (Figures 25.D&E). This feature was not observed in control group (Figure 25.C). Upregulation of iNOS enzyme expression was observed in DNBS treated mice, and co-localized with macrophage cells.

Figure 25. Inflammation in the Mucosa, Macrophage Infiltration, and Myenteric Ganglionic Loss in DNBS and TNF- α Treated Animals.

H&E staining reveals intact mucosa in basal control animals **(A)** and damaged mucosal tissue in DNBS treated mice **(B)**. Massive infiltration of macrophages to the site of inflammation **(D)** and adjacent to a myenteric ganglion **(E)** in DNBS-treated mice contrast with control **(C)**. A significant loss of myenteric ganglia was observed in DNBS+TNF- α injected mice **(G)** comparing to control **(F)**; however pretreatment with 1400W dihydrochloride prevented this effect **(H)**.

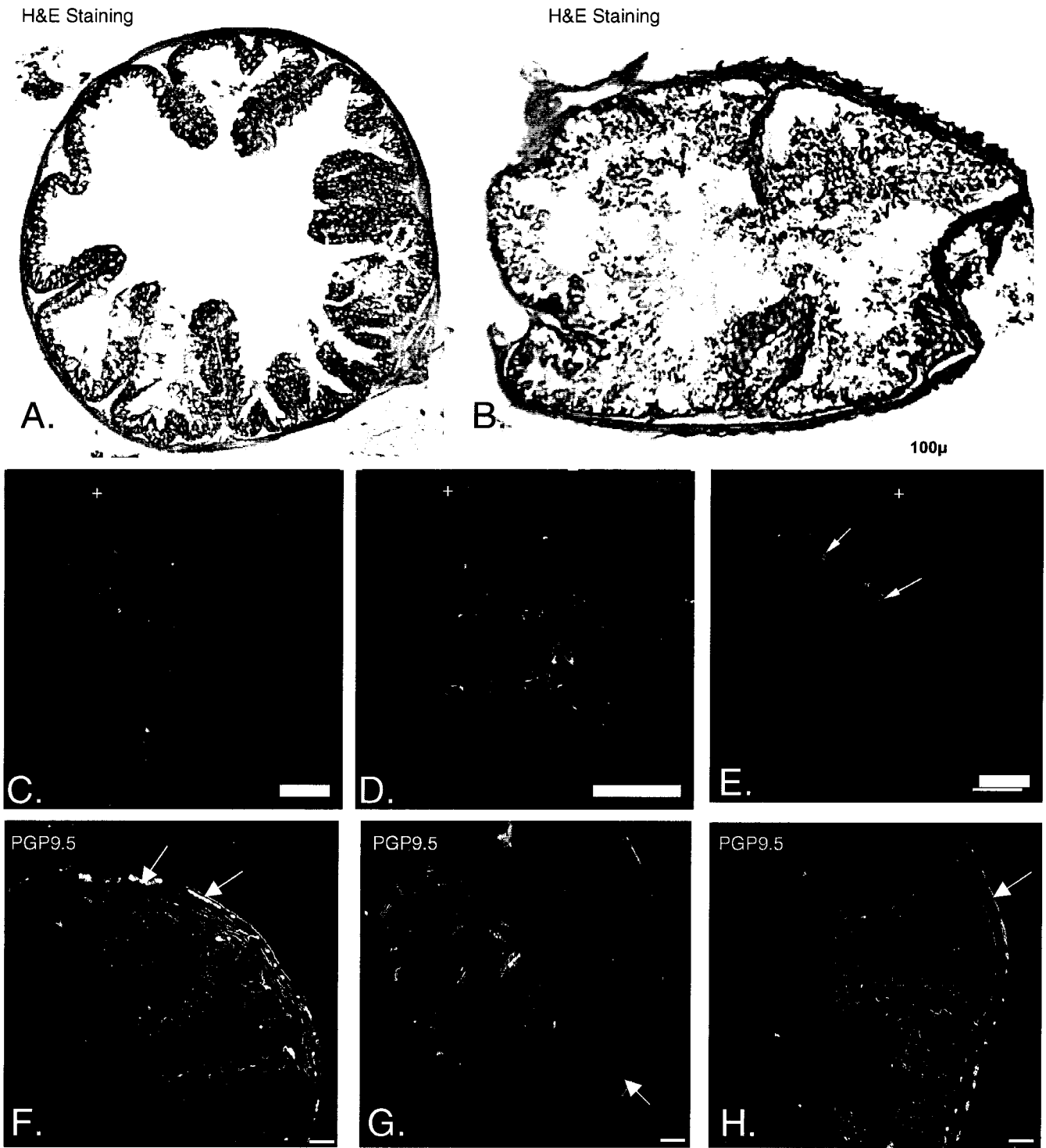


Figure 25

3.13. Effects of DNBS and TNF- α Injections on Myenteric Neuronal Survival

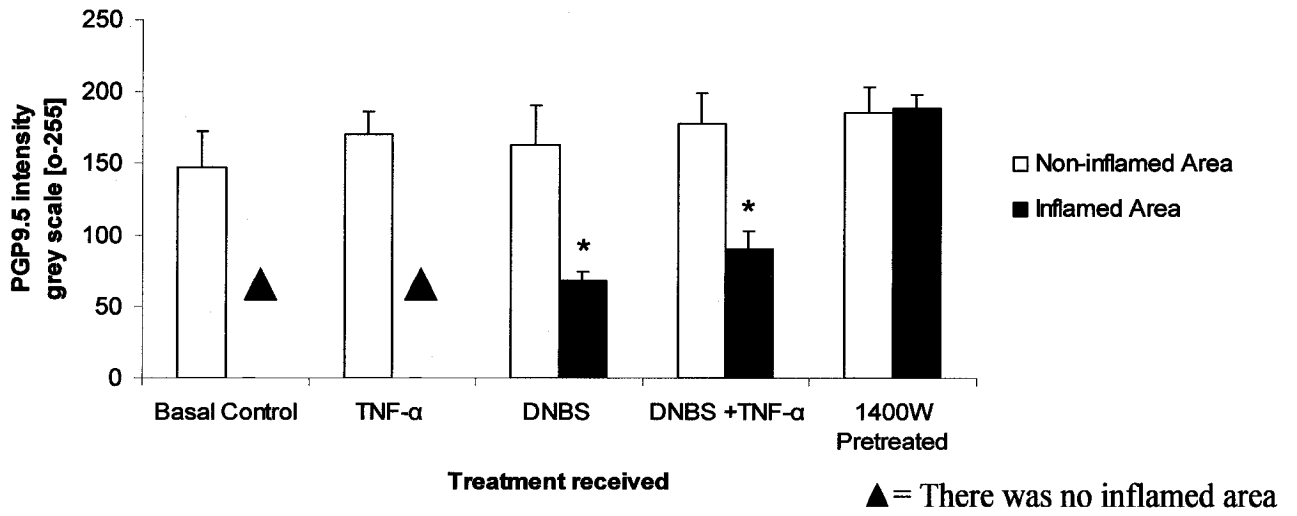
Major reduction in PGP9.5 synthesis was observed in the inflamed areas of the colon of DNBS and DNBS+TNF- α injected groups; the intensity was calculated as 68.0 ± 5.7 and 90.0 ± 12.9 respectively, in grey scale of 0-255 (basal control: 147.1 ± 25.4 ; n = 5). 1400W dihydrochloride pretreatment protected the neurons and PGP9.5 intensity in inflamed and non-inflamed areas of the gut in this group was similar (188.7 ± 9.5 and 185.2 ± 18.0 respectively) to that of control group (Figure 26.A).

Applying a TUNEL assay to gut sections, apoptotic cells were detectable in mucosa, muscular layer and myenteric plexuses of all groups receiving DNBS injection; however in single TNF- α (11.4 ± 0.6) or DNBS (non inflamed region: 11.1 ± 0.8 , inflamed region 9 ± 0.7) treated mice no major changes in the number of neurons per myenteric ganglion were observed, compared to the control group (10.5 ± 0.6 ; number of myenteric ganglia studied per group ≥ 19). On the other hand, DNBS+TNF- α injection induced significant neuronal death in myenteric plexuses in the inflamed regions of the gut (5.1 ± 0.2 surviving neurons per ganglion) (Figure 26.B), and quantitative measurements revealed a significant loss in the number of myenteric ganglia per length (1.7 ± 0.2 ganglia/mm) in this group, compared to controls (3.2 ± 0.3 ganglia/mm) (Figure 27.A). Pretreating with iNOS inhibitor prevented both these effects (10.5 ± 0.4 neurons/ganglion and 2.9 ± 0.3 ganglia/mm in the inflamed regions of the intestine) (Figures 26.B & 27.A).

Figure 26. Neuronal PGP9.5 Intensity and Neurodegeneration in DNBS + TNF- α Treated Mice.

A. PGP9.5 staining intensity was significantly reduced in DNBS and DNBS+TNF- α treated animals. 1400W dihydrochloride pretreatment of these animals prevented this effect. **B.** In the inflamed area of the colon of DNBS+TNF- α treated mice, the number of neurons per myenteric ganglion was significantly reduced, comparing to control group and non-inflamed areas of the colon of the same animals. This effect was also prevented by applying iNOS inhibitor, 1400W dihydrochloride. * $p < 0.05$, ** $p < 0.001$.

A.



B.

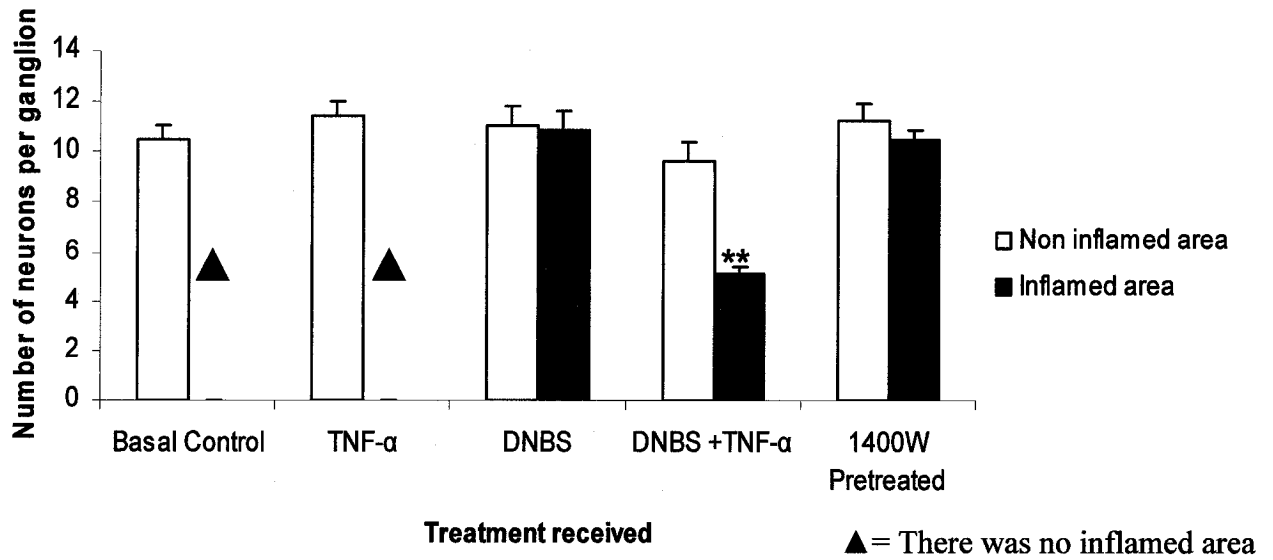


Figure 26

Figure 27. Effects of DNBS and TNF- α Administration on Myenteric Ganglia and Glial Cells.

A. The number of myenteric ganglia was significantly reduced in DNBS+TNF- α injected mice relative to all other experimental conditions. This loss was prevented by 1400W dihydrochloride. **B.** No signs of degeneration or regeneration were observed in glial cells in any of the treated group of animals. * $p < 0.05$.

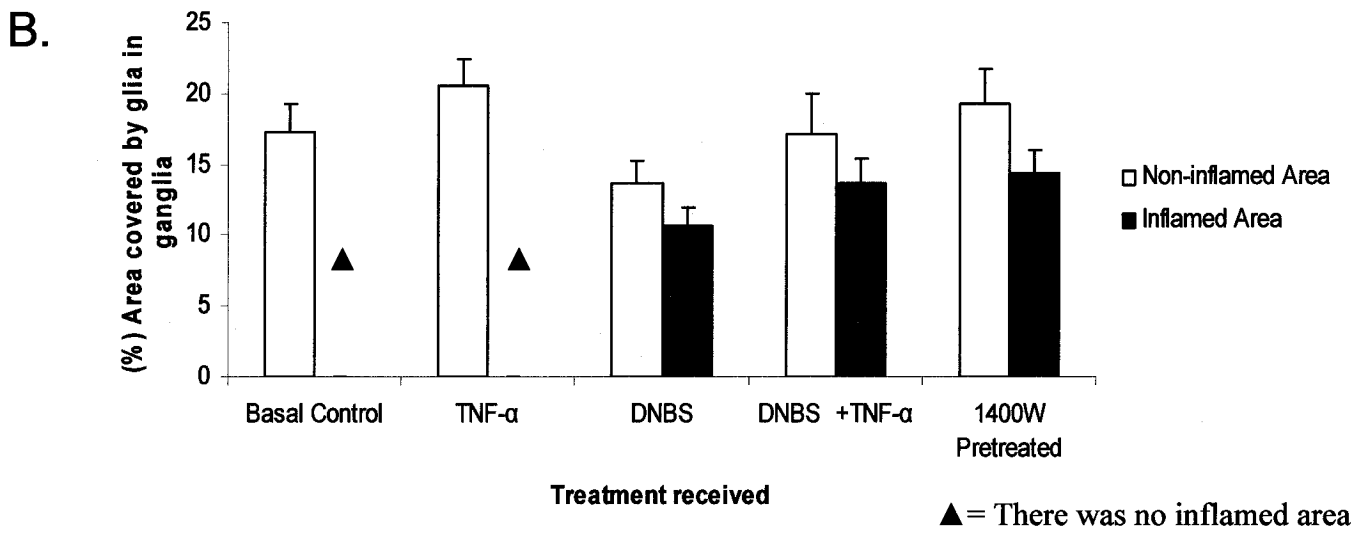
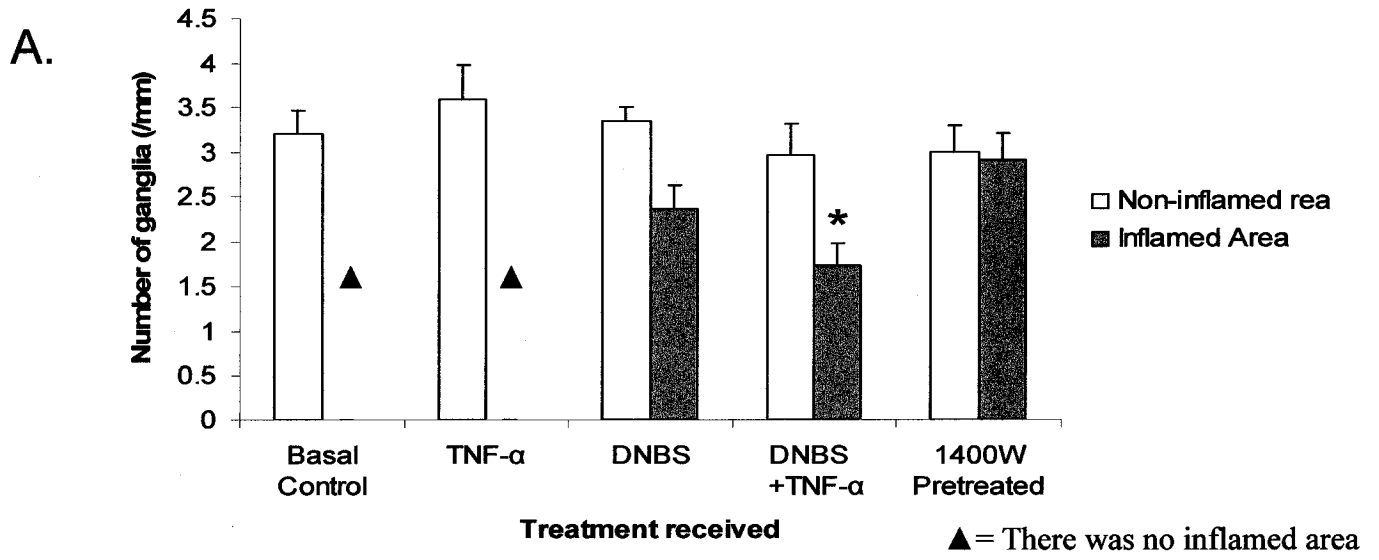


Figure 27

Quantitative measurement also showed that glial cells in all treated groups, including DNBS (inflamed region of the gut: $10.7 \pm 1.2\%$ area of ganglion covered by glial cells) and DNBS+TNF- α (inflamed region of the gut: $13.6 \pm 1.7\%$ area of ganglion covered by glial cells) treated groups were similar to control group ($17.3 \pm 1.2\%$ area of ganglion covered by glial cells) and no considerable reduction in glial cell survival was observed (Figure 27.B; number of ganglia studied per group ≥ 7).

CHAPTER 4:

DISCUSSION

4.1. Macrophage Cell Cultures

4.1.1. Macrophage Activation

Macrophages have been known to exist in a number of activity states since the turn of the twentieth century (Langermans *et al*, 1994). Activated macrophages are distinctive by virtue of their larger size and changes in morphology (Cohn, 1978; Edelson *et al*, 1975). At this stage, the macrophage undergoes changes in mRNA expression and protein synthesis; hence a common early approach to quantification of the phenomenon of activation was measurement of protein mass of the cell compared with that of naïve non-treated macrophages (Cohn, 1978; Edelson *et al*, 1975). Activation triggers an enhanced ability of the cell to adhere, infiltrate and migrate, due primarily to expression of different selective genes and changes in cytoskeletal structure (Nau *et al*, 2002; Jones, 2000). This process eventually leads to a cell with a distinctly different phenotype, usually much larger in size, stellate shape with pseudopodia, increased intracellular enzymes and lysosomal hydrolases, as well as increased surface receptors complement receptors and MHC class II (Adams and Hamilton, 1984).

In this study, we examined the changes in macrophage cells at the activation stage, in order to relate their capacities and functions at this stage with myenteric

neurodegeneration observed in co-culture and *in vivo* studies as related to inflammatory bowel diseases.

RAW264.7 cells generally showed remarkable changes in morphology after treatment with bacterial endotoxin or pro-inflammatory cytokines. In addition, these cells showed significant increase in iNOS protein expression and NO synthesis. These correspond with the known ability of endotoxins and pro-inflammatory cytokines to induce production of inflammatory mediators in macrophage cells (Boisse *et al*, 2003; Bradford *et al*, 2001; Drapier and Hibbs, 1988; Nau *et al*, 2002; Eskandari *et al*, 1999; Hori *et al*, 1987; Drapier *et al*, 1988).

These data also showed that even in a macrophage cell line culture (where all the cells have an identical origin), the responses to inflammatory stimuli range from non-responsiveness to a fully activated state. Interestingly, the percentage of morphologically 'activated' macrophages does not correspond to the percentage of iNOS immunoreactive cells. Therefore morpho-activation is not necessarily always accompanied, or followed by, increased iNOS expression and hence full activation. This phenomenon may be related in part to the fact that signal transduction pathways for the applied inflammatory mediators (LPS, IFN- γ , TNF- α) are quite different and mostly independent from each other (see sections 1.10.1 and 1.10.2). This fact could also explain the variety in the intensity of activation (i.e. percentage of activated cells) in different cultures. For example, macrophages showed neither morpho-activation nor iNOS upregulation after exposure of the culture to the pro-inflammatory cytokine TNF- α alone. This observation is in accordance with a study with macrophage cells by Drapier and Hibbs (1988), and the observation of Kinugawa *et al* (1997) in rat cardiac myocytes. We also found that

applying even very high doses of TNF- α did not raise an inflammatory response in RAW264.7 cells. This is similar to observations by others in this laboratory (unpublished data). By contrast, exposure to the pro-inflammatory cytokine IFN- γ induced morphological changes and re-arrangement in F-actin filaments in cytoskeleton; however it did not induce a distinct increase in iNOS expression nor nitrate and nitrite synthesis.

When a combination of the two pro-inflammatory cytokines was used for treating the culture, there were morphological changes, marked iNOS expression, and massive production of NO end-metabolites. This suggests that these two inflammatory mediators are acting synergistically to synthesize NO. The synergistic effects of TNF- α and IFN- γ have been previously described (Sartor, 1994; Hori *et al*, 1987; Drapier *et al*, 1988; Ding *et al*, 1988; Esparza *et al*, 1987); however the underlying mechanisms remain to be fully deciphered.

We found LPS at a concentration of 1 μ g/ml to be a vigorous inducer of morphological changes, iNOS upregulation, and NO synthesis in 264.7RAW macrophage cells in culture. These results are comparable with other studies, including Boisse *et al* (2003), Bradford *et al* (2001), Drapier and Hibbs (1988), Nau *et al* (2002), and Eskandari *et al* (1999).

4.1.2. Effects of iNOS Inhibitors and NO Blockade

LPS can stimulate RAW264.7 macrophage cells to express iNOS gene (Guha *et al*, 2001), and NO synthesis and iNOS upregulation have been observed in RAW264.7 cell cultures exposed to combined TNF- α and LPS (Bradford *et al*, 2001). TNF- α and IFN- γ can act synergistically to induce macrophage dependent killing of cells (Esparza *et al*,

1987) presumably by co-signaling induction of NO synthesis (Drapier *et al*, 1988, Ding *et al*, 1988).

In this study, pre-treatment of the 264.7RAW cells with specific iNOS inhibitors significantly reduced the level of NO synthesis in macrophage cell cultures, showing that NO is synthesized through an iNOS dependent mechanism and upregulation of iNOS has been the source of increased NO synthesis.

Interestingly, 1400W dihydrochloride was applied in 1,000 fold lower concentration than L-NAME hydrochloride, yet it reduced the NO production at a similar level to L-NAME hydrochloride, consistent with 1400W being a high affinity iNOS inhibitor. 1400W dihydrochloride has a higher affinity for iNOS enzyme ($IC_{50} = 0.23 \mu\text{M}$, in the presence of $30 \mu\text{M}$ L-Arginine; Alderton *et al*, 2001) in comparison to L-NAME hydrochloride ($IC_{50} = 6.6 \mu\text{M}$, in the presence of $30 \mu\text{M}$ L-Arginine; Alderton *et al*, 2001).

1400W dihydrochloride and L-NAME hydrochloride blocked NO production in cell cultures, regardless of the type of NO synthesis stimulus, bacterial endotoxin or pro-inflammatory cytokines. This observation confirms that the function of these inhibitors is based on the recognition of iNOS protein and blocking its binding site in competition with L-arginine.

4.2. Neurons and Glia in Myenteric Cell Cultures

Viability, morphology, neurochemistry and function of myenteric neurons and glia in culture have been extensively studied (Sandgren *et al*, 2003a, 2003b; Hanani *et al*,

1994; Jaeger, 1995; Blennerhassett and Lourenssen, 2000; Nishi and Willard, 1985; Willard and Nishi, 1985; Hanani, 1993). Glial cells in our myenteric cell-culturing system were typically small sized with fine short processes, and outnumbered the myenteric neurons. Myenteric neurons showed normal appearances comparable to their *in vivo* morphology. Myenteric neurons also expressed a variety of neurotransmitters, as detected by immunocytochemistry, which was similar to that shown by myenteric neurons in tissue sections of the gut, verifying the normal functional feature of these cells.

Plating neurons had spherical shapes lacking any processes, whilst in a matter of days they started growing long neurites and creating an interconnected ganglionic structure. This observation of growth and formation of neural fibers demonstrates the regenerative ability of the post-mitotic neurons; however to a limited extent.

4.3. Neuronal Survival, Inflammation and Degeneration in Macrophage-Neuronal Co-cultures

Co-culture of macrophages / microglial cells and neurons of the CNS has been the focus of several studies (Boje and Arora, 1992; Chao *et al*, 1992; McMillian *et al*, 1995; Adamson *et al*, 1996), and co-culture of macrophages and sympathetic neurons have been used to study neuroinflammation and neurodegeneration (Arantes *et al*, 2000). The present work, however, is the first (to my knowledge) report of employing macrophage-myenteric cell co-cultures.

4.3.1. Co-existence of Macrophages and Neurons

The fact that co-culturing of myenteric neurons with macrophages without any exposure to activation conditions, does not affect the survival of myenteric neurons, suggests that the presence and the products of non-activated macrophages are not damaging to nearby myenteric neurons. This observation is similar to what is observed in our co-culture of macrophage and cortical cells, where adding the macrophage cells to the cultures did not affect the survival rate of cortical neurons.

4.3.2. Inflammation and Degeneration

Exposure of myenteric-macrophage cell co-cultures to inflammatory initiators, i.e. bacterial endotoxin or pro-inflammatory cytokines, induced both neuroinflammation and neurodegeneration. After the inflammatory stimulation, large numbers of myenteric neurons underwent apoptosis and a majority of surviving neurons expressed diminished protein immunoreactivity. The possible mechanism underlying this phenomenon is: exposure of macrophage cells to inflammatory components increases iNOS protein expression and induces synthesis of massive amounts of NO. This gaseous molecule, after production and release from the cell, targets and affects the metabolism of myenteric neurons, mainly through oxidization of the cell membrane lipid structures, binding to and breaking DNA strands, and alteration in mitochondrial PTP, release of cytochrome c, and subsequent caspase cascade activation. In the presence of oxygen radicals, NO reacts with superoxide to generate peroxynitrite, an extremely reactive molecule with potent oxidant properties which readily diffuses across cell membranes to damage neighboring cells (Beckman *et al*, 1990).

A striking observation in these experiments was that pre-treatment of the cultures with two types of iNOS inhibitors protected the neurons against neuroinflammation and neurodegeneration. This strongly suggests that NO synthesized through iNOS activity is involved in the process of myenteric neurodegeneration *in vitro*.

4.3.3. Endurance of Protective Function of iNOS Inhibitors

The ability of L-NAME hydrochloride and 1400W dihydrochloride to protect against neurodegeneration was long lived, since 96 hrs after initial pre-treatment there was still no significant neuronal cell death, PGP9.5 protein synthesis remained at normal levels, the neurons showed normal morphology, and synapses were detectable on cell somas and neurites. Taken together, these data show that the protective effects of iNOS inhibitors were long lasting and not simply delaying neuronal cell death.

4.3.4. Comparison of Myenteric and Cortical Neuron Behavior in Co-culture with Macrophages

In order to place our results with myenteric neuronal-macrophage co-culture in context to the established central nervous system 'neuroinflammation' literature, we studied, in parallel, cortical neuronal survival in rat cortical neuronal-macrophage co-cultures. The results using cortical neuronal cells were similar to studies with myenteric neurons; however the ratio of macrophage cells to neurons necessary to induce neuroinflammation and neurodegeneration in cortical cells in culture was 2:5 macrophage-cortical neuronal cells comparing to 1:1 macrophage-myenteric neuronal cells. This provides evidence that cortical neurons are generally more susceptible to NO

mediated damage than myenteric neurons. The nature of this susceptibility is unclear. However, cortical neurons are highly protected in the brain through different mechanisms of defense and protection (Brightman and Reese, 1969), whilst myenteric neurons are highly prone to a variety of harmful mechanical, chemical and biological factors. We propose that through an evolutionary process the myenteric neurons have accomplished a higher stage of resistance and enhanced their own mechanisms of self protection which help them survive in a more aggressive environment, comparing to cortical neurons which normally reside in the highly regulated and immunologically privileged neurochemical milieu of the CNS. The advantageous characteristics of myenteric neurons may include changes in cytoskeletal structure, alteration in cell membrane and its permeability, and quantitative and qualitative changes in cell surface receptors.

4.4. The ENS, Inflammation, and *in vivo* Studies

4.4.1. Experimental Colitis in Rats

a. General Inflammation in the Gut:

The colitis induced in the present study met the classical criteria for experimental acute colitis established and used by numerous research groups (Morris *et al*, 1989; Sanovic *et al*, 1999; Colon *et al*, 2001; Poli *et al*, 2001; Yamada *et al*, 1992, 1993; Jacobson *et al*, 1995; Watanabe *et al*, 2001; Neurath *et al*, 1997; Kankuri *et al*, 1999; Zingarelli *et al*, 1999; Boisse *et al*, 2003; Bobin-Dubigeon *et al*, 2001). Diarrhea, unkempt appearance, gross morphological deformity, mild abdominal distension,

hyperemia, edema, ulceration, increased colonic weight, and increased colonic myeloperoxidase activity were the major standards taken into consideration in order to verify the occurrence of inflammation. The caveat with the current animal model is that we were able to produce the colitis in anaesthetized and analgesia treated rats, which represents a significantly easier and more humane way to create this disease model. The tendency of the inflammation to be resolved as early as 96 hrs after DNBS administration represents the acute nature of this model.

b. Inflammation in the ENS and Absence of Myenteric Neurodegeneration

In this rat model of colitis, the inflammation extended into the deep layers of the distal colon and infiltration of inflammatory cells induced irritation in the myenteric neurons of the ENS (indicated by the reduced synthesis of the specific neuronal protein PGP9.5). Treating with the highly selective iNOS inhibitor 1400W dihydrochloride prevented this alteration in PGP9.5 immunoreactivity and hence 'neuroinflammation', suggesting that neuroinflammation occurs in an iNOS dependent manner and represents the basis for involvement of NO in this disease related event.

We found iNOS expression and subsequent NO production have degenerative effects on non-neural layers of the proximal colon, and applying iNOS inhibitor 1400W dihydrochloride reduced this inflammation in the intestine. This observation is in accordance with the data presented by Kankuri *et al* (2001) showing that iNOS inhibitor pretreatment reduces the size of mucosal lesions, and suppresses inflammatory edema formation and neutrophil infiltration.

A surprising finding from our study was that we observed no signs of neurodegeneration in our colitis animals. Sanovic *et al* (1999) previously reported major enteric neuronal loss in inflamed region of the gut by 24 hrs after induction of colitis. This discrepancy may be explained by the fact that we employed a lower dose of DNBS. The applied DNBS dose was the highest possible one which was not accompanied with high rates of mortality in the tested groups. Our model also was different since in order to relieve the pain in the DNBS treated animals, buprenorphine hydrochloride, an analgesic drug, was continuously administered during the course of experiment. It is possible that cyclic treatment of the animals with analgesic have reduced the severity of inflammation and hence neurodegeneration. This is an intriguing finding and requires further study.

4.4.2. Experimental Colitis in Mice

TNF- α plays an important role in induction and maintenance of inflammation in the IBD (see introduction). It has been observed that treatment with TNF- α synthesis inhibitors has a beneficial role in recovery of TNBS-induced colitis in rats and dramatically reduces different features of inflammation. Moreover colitis could not be induced in mice in which TNF- α gene had been inactivated by homologous recombination (Bobin-Dubigeon *et al*, 2001; Neurath *et al*, 1997). Furthermore, it was also observed that striking colonic inflammation and lethal pan-colitis is induced in TNF- α -transgenic mice upon TNBS treatment (Neurath *et al*, 1997).

Previous studies in our lab have also shown that the intestine is one of the explicit targets of TNF- α injection, and in a study of a murine model of inflammation, single / double TNF- α administration was used as an initiator of inflammatory response in the

gut. However infiltration of macrophages or other immunocytes within the intestinal lumen was not detectable (unpublished data).

In order to boost the effects of DNBS-induced inflammation in a controlled way and study the phenomenon of inflammation and involvement of immune system, the combination of DNBS and TNF- α injection was chosen as the treatment regimen.

Myenteric Neuronal Inflammation and Degeneration: DNBS was injected at its critical dosage, i.e. any further increase in DNBS dose induced a high rate of mortality. This treatment induced the maximum non-lethal inflammation and immunocyte infiltration 24 hrs post treatment. The additional TNF- α injection induced an even greater effect in terms of synthesis of inflammatory mediators including NO. This could explain the occurrence of neurodegeneration whilst it was absent in the myenteric plexus of the animals treated with DNBS alone.

Blocking NO synthesis by applying iNOS inhibitor 1400W dihydrochloride reduced the rate of neurodegeneration significantly. This confirms the hypothesis that overproduction and synthesis of NO during the course of inflammation plays a vital role in induction of enteric neurodegeneration; therefore it must be considered a major, if not the only, contributing pathway to initiate inflammation and degeneration in the ENS.

Glial Cells and Inflammation: In addition to neurons, enteric plexuses contain glial cells. Glial cells represent an extensive cell population within the gut. They have trophic and protective functions toward enteric neurons, and integration and modulation of neuronal activities are dependent on the glial cells (Cabarrocas *et al*, 2003).

Survival, injury, degeneration and proliferation of enteric glial cells during gut inflammation are subjects of controversy. Bradley *et al* (1997) have observed increased

glial cell proliferation ensuing in rat myenteric plexus in inflammatory conditions; however they failed to show that glial cell proliferation leads to an increased number of glia, as it is possible that proliferation is just compensating a degenerative process in this class of cells. Conversely, Cornet *et al* (2001) reported a diminished enteric glial cell network in involved and non-involved parts of the intestine of CD patients, as compared with controls. They demonstrated that depletion of glial cells induced vasculitis and fulminant jejuno-ileo-colitis in mice, thus relating glial cell loss with induction and/or maintenance of inflammation in the bowel.

In the present research we did not observe a significant change in quantity of glial cells in either of the rodent models of colitis. This could be interpreted as evidence that there are no marked changes in number of glial cells and they are not affected by the inflammation. Alternatively, another explanation is that the rate of degeneration and proliferation of these cells are both increased in a similar way, therefore the final quantitative measurements do not reflect a remarkable change in numbers of glial cells in tested animals, compared to naïve specimens. This phenomenon requires further study.

In summary, our studies establish the phenomenon of myenteric neuronal susceptibility to NO mediated damage *in vivo* and in cell cultures. We have also shown the involvement of activated macrophages, and the significant role of iNOS inhibitors to protect cortical and myenteric neurons against inflammatory- and apoptosis-inducing modulators in neuronal cell cultures. The results of the *in vivo* experiments further suggest a significant role for TNF- α in induction and maintenance of inflammation and neurodegeneration in the inflamed intestine. The selective beneficial role of iNOS inhibitor 1400W dihydrochloride in attenuating the inflammation and preventing

neurodegeneration makes it an appropriate candidate for clinical trial and a potential therapeutic treatment for gastrointestinal diseases in which neuronal injury is a pathogenic feature.

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