

AMERICAN UNIVERSITY OF BEIRUT

EFFECT OF COLITIS ON CYTOKINES LEVELS
IN THE RAT INTESTINE

by
SARAH IBRAHIM SAWAH

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AMERICAN UNIVERSITY OF BEIRUT

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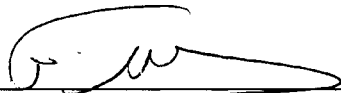
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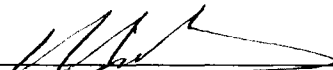
Dr. Kassem Barada, Associate Professor
Department of Internal Medicine

Advisor



Dr. Fadi Mourad, Associate Professor
Department of Internal Medicine

Co - Advisor



Dr. Nayef Saade', Professor
Departments of Human Morphology and Physiology

Member of Committee



Dr. Abdo Jurjus, Professor
Department of Human Morphology

Member of Committee

Date of Thesis Defense: October 5, 2004

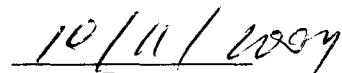
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DEDICATION

To

My Father and Mother for Their Continuous Prayers, Encouragement, Endless Love
and Care

My Brothers for Their Support, Encouragement and Patience

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AN ABSTRACT OF THE THESIS OF

Sarah Ibrahim Sawah for Master of Science
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Title: Effect of colitis on cytokines levels in the rat intestine

Colonic inflammation may be associated with functional derangements in non-inflamed segments of the gut. Small bowel dysfunction, nutrient malabsorption and altered motility occur in human and experimental colitis. Many cytokines have well known effects on the transport of nutrients and electrolytes in the small intestines of animals. This study aims to investigate the effect of colonic inflammation on the expression of cytokines in the small intestine. Colitis was induced in rats by rectal administration of 100 μ l of 6% Iodoacetamide (IA). Control rats were injected with the vehicle. Using the two-site ELISA technique, levels of TNF- α , IL-6, IL-1 β and NGF were measured in serum and in tissue homogenate sampled from the duodenum, jejunum, ileum and colon at zero time and at 6, 12, 24, 48, and 96 hours post colitis induction in 5-6 rats per each time interval.

No detectable levels of cytokines were present in serum of naïve and vehicle injected rats. These levels showed significant ($P < 0.01$) and early increase following the induction of colitis. Similarly, moderate concentrations of all cytokines were detected in full thickness strips and mucosal scrapings isolated from the duodenum, jejunum, ileum, and the colon of naïve and vehicle injected rats. These levels were increased by 10-20 folds for TNF- α ($P < 0.001$), 5-10 folds for IL-6 and IL-1 β ($P < 0.001$) and 2-5 folds for NGF, following the induction of colitis. This increase peaked at 12-24 hs after colitis. TNF- α showed an earlier and short-lived increase. Furthermore, the most important and sustained increase of cytokines was observed in the colon.

This study demonstrates significant increase in the expression of the proinflammatory cytokines in non-inflamed areas of the small intestine of rats with experimental colitis. The findings might explain, in part, the small bowel dysfunction observed in patients and in animals models for colitis.

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INTRODUCTION

A. Preamble

Inflammatory Bowel Disease (IBD) has been known for more than half a century, but the complete process of the disease remains unknown. Advances in research during the last decade unveiled the fact that there can be no single mechanism that can explain all aspects of IBD.

IBD is a general term that covers two disorders: Ulcerative Colitis (UC) and Crohn's Disease (CD). It is a chronic, relapsing and remitting inflammatory condition of the gastro intestinal tract. It results from interrelated genetic and environmental factors that may be channeled through an abnormality in the mucosal immune function, possibly due to dysregulated or excessive T helper cell responses. The major symptoms of IBD include diarrhea, rectal bleeding, the passage of mucus, and abdominal pain.

UC and CD are two distinct inflammatory disorders with some clinical and conceptual evidence that suggest that they may be part of a biological continuum. As this issue remains mystery, UC is mediated by a Th2 response, which involves the interleukins IL-4, IL-5, IL-6, and IL-10 that mostly affect the mucosal areas in the large intestine causing ulcerations. On the other hand, CD is mediate by a Th1 response, which involves the interleukin-2 and interferon-gamma that affect the entire gastrointestinal system and could extend into the deeper layers of the intestinal wall.

Although UC is confined to the large intestine, some studies have shown functional abnormalities in the small intestine of patients with UC. These abnormalities were manifested by a decrease in intestinal absorption of water (Binder and Ptak, 1970), aminoacids (Zetzel and Banks, 1942), D-xylose (Anderson et al., 1971) and fat (Chakravarti et al., 1973). Moreover, Barada et al., (2001) proved that UC impairs jejunal amino acid absorption and that this effect involves the vagal efferents as well as capsaicin sensitive primary afferents. Furthermore, another study done in our laboratories showed the inhibitory effect of chemical colitis on jejunal water absorption and that this effect involves both vasoactive intestinal peptide and nitric oxide (Mourad et al., 2001). In addition, Fries and his colleagues (1999) showed that experimental colitis increases tight junction permeability throughout the entire small intestine, and that the extent of alterations correlates with colonic damage.

The exact causes of small bowel dysfunction in colitis is not yet fully understood. It could be the result of a neuronal reflex, release of neurohormonal mediators or release of inflammatory mediators. Therefore, this work aims at studying the expression of serum and intestinal proinflammatory cytokines in experimental colitis, at different time intervals during the course of the disease starting from induction to recovery.

B. Functional Anatomy and Histology of the Small and Large Intestines

The small intestine is a convoluted tube, forming the longest section of the digestive tract. It is contained in the central and lower part of the abdominal cavity, and is surrounded above and at the sides by the large intestine. It forms a passage from the pylorus to the large intestine and is divided into three major segments: the duodenum,

the jejunum and the ileum. It is within the small intestine that the final stages of enzymatic digestion, nutrient and water absorption, as well as endocrine secretion occur.

Histologically, the wall of the small intestine is composed of four concentric layers known as the serosa, muscularis externa, submucosa, and mucosa. The serosa is a thin outer layer of loose connective tissue covered with mesothelium, extending from the mesentery. It is rich in blood and lymphatic vessels, as well as adipose tissue. The muscularis externa comprises two thick layers of smooth muscle. The outer shows longitudinal fibers and the inner layer is circular. Between these layers is the myenteric nerve plexus (Auerbach's plexus). The submucosa is a wide zone of fibro-elastic loose connective and supporting tissue containing small blood vessels, lymphatics and nerves of the submucosal plexus (Meissner's plexus), wandering leukocytes and variable quantities of fat. The mucosa consists of three layers: a surface epithelium composed of mucus-secreting goblet cells and many absorptive cells (enterocytes), a lamina propria of supporting tissue with abundant neurovascular supply and cells of the immune system and a thin double layer of smooth muscle, the muscularis mucosa. The villi are tall finger-type projections of the mucosa, between which tubular glands or crypts of Lieberkühn extend to or beyond the muscularis mucosa. From the bases of the crypts come the precursors of the villus epithelial cells and other crypt cells such as goblet cells, Paneth cells, and cells producing hormonal peptides that are active in the gastrointestinal tract. Enterocytes are characterized by thousands of surface microvilli known as brush border, which greatly increase the cell surface area, facilitating absorptive capacity of each columnar cell (Kerr, 1998).

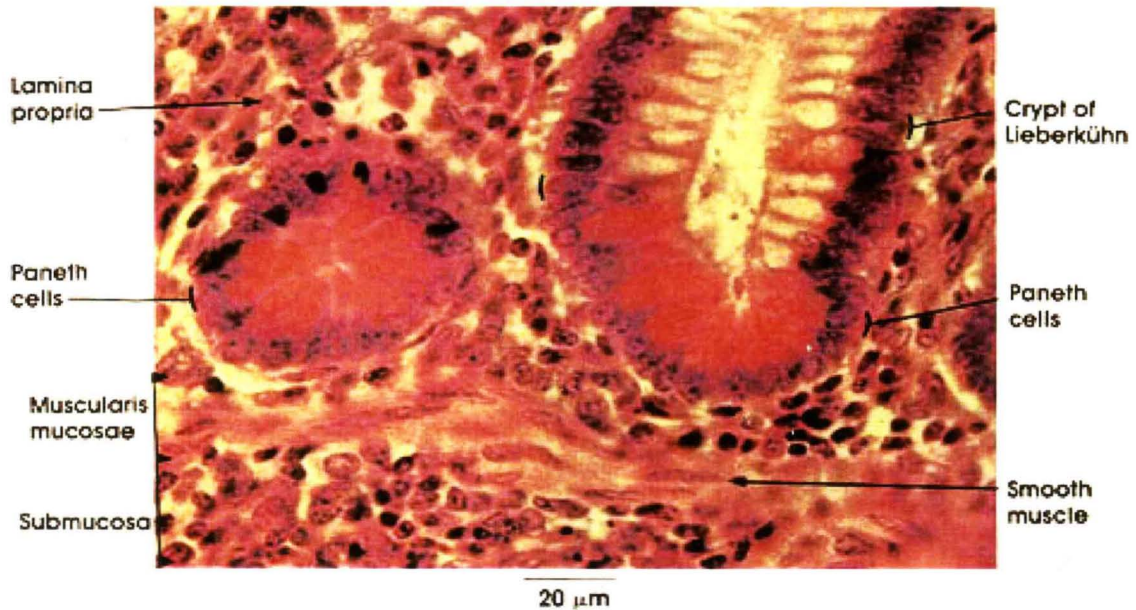


Figure 1.1 Jejunum (Bergman et al., 1999)

The large intestine is the part of the digestive tract between the terminal ileum and anus. It consists of three major segments: The cecum; the colon which is sub classified into ascending, transverse and descending segments; and the rectum which is the short, terminal segment of the digestive tube. Semisolid undigested food from the small intestine enters the large intestine through the ileocecal valve. The large intestine is the main site for fecal material formation and mucus production.

Histologically, colonic mucosa usually presents an undulating appearance with occasional infoldings. The characteristic feature is the presence of numerous crypts of Lieberkuhn, oriented as straight tubular glands extending down to the muscularis mucosae. Numerous lymphatic nodules are present and many goblet cells are noted, although they are outnumbered by the absorptive enterocytes. A unique feature of the colon is the transformation of the smooth muscle coat of the outer muscularis externa

into three distinct longitudinal strips, the tinae coli, which allow segments of the colon to contract independently (Kerr, 1998).

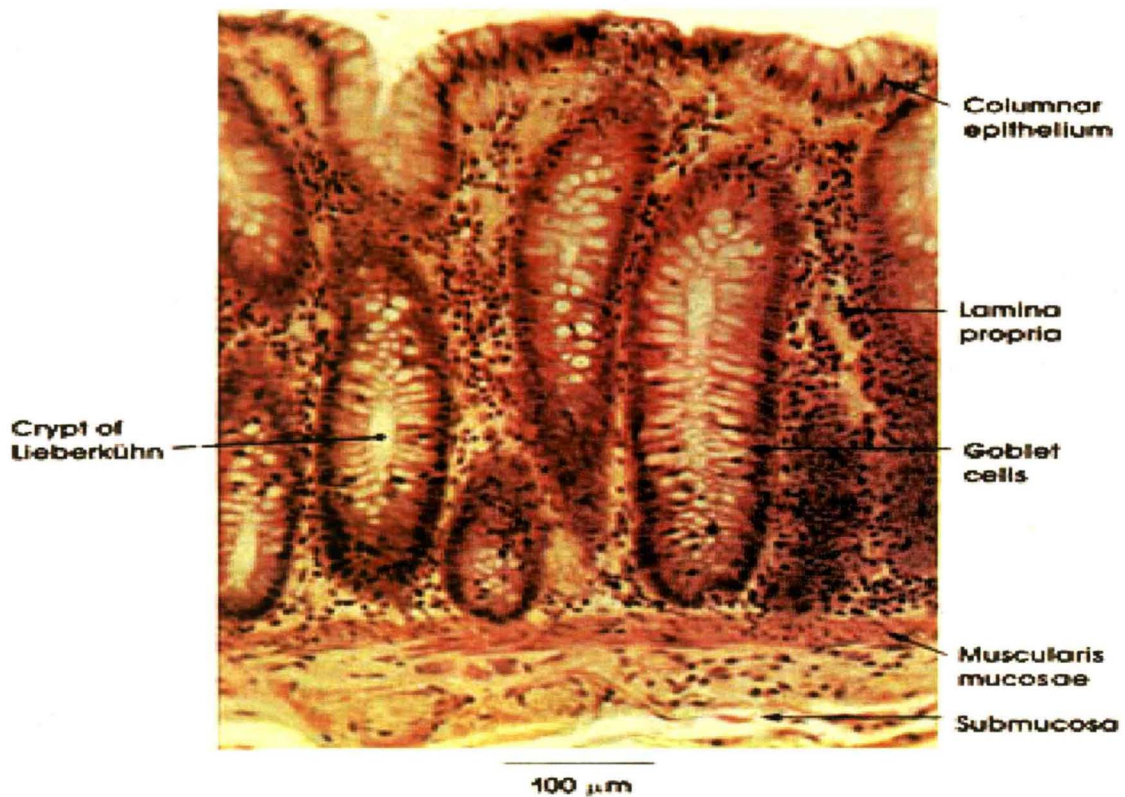


Figure 1.2 Colon (Bergman et al., 1999)

C. The Gastrointestinal Mucosal Immune System

The gastrointestinal mucosal immune system is a highly specialized system composed of organized lymphoid tissue and diffusely distributed lymphoid cells. This complicated immune system has evolved to provide the host with protective mechanisms against invasion by potential pathogens, such as bacteria, viruses, and protozoans, across mucosal surfaces. On the other hand, the gastrointestinal immune

system has evolved to permit immunological tolerance against the wide array of harmless products of normal digestion and the normal intestinal flora (Stephen, 1995).

1. General Defense Mechanisms

The non immunological defense mechanisms of the gastrointestinal tract, act in concert with immunological mechanisms to protect the host. They limit the growth of organisms and prevent the entry of pathogens across the mucosa. These mechanisms include gastric acid secreted by the stomach, normal motor propulsive activity of the gastrointestinal tract, mucus that can bind and trap bacteria, and Paneth cells that produce a class of defensive molecules named cryptdins which are antibacterial. Interestingly, the alteration or absence of one of these mechanisms, may not lead to any pathological consequences if other mechanisms remain intact (Stephen, 1995).

2. Specific Immune Defense Mechanisms

Lymphocytes associated with the gastrointestinal tract are located in 3 major compartments: the gut associated lymphoid tissue that is found in organized structures such as Peyer's Patches, tonsils, mesenteric lymph nodes, and appendix; the non organized lymphoid cells present diffusely throughout the lamina propria; and the lymphocytes present within the gastrointestinal epithelium (Stephen, 1995).

a. Organized Lymphoid Tissue: Peyer's Patches

Peyer's patches are located throughout the jejunum and ileum of humans, being most concentrated in the terminal ileum. Peyer's patches are macroscopic structures that

extend from the mucosa into the submucosa of the intestine. Lymphoid cells in Peyer's patches are non-mature cells that play an important role in the initiation of immune responses. They carry out functions of the "afferent" limb of the immune response by interacting with antigens. Following ingestion, antigens and micro-organisms are transported from the gut lumen via specialized M cells, which are scattered among conventional epithelial cells overlying the dome of Peyer's patch follicles. Compared with epithelial cells, with which they share a common progenitor cell, M cells have a pronounced capacity to transport a wide variety of substances and microbes to the subepithelial dome region (Stephen, 1995).

Peyer's patches have germinal centers that contain centrally activated B cells enriched in surface IgA-positive cells, antigen presenting cells and dendritic cells, with a surrounding dense layer of resting B cells. Adjacent to the germinal centers are interfollicular zones that contain predominantly CD4⁺ T cells. Antigen-stimulated T cells in this site carry out a range of regulatory functions, including cytokine production that is necessary for B cell class switching and maturation. In addition, cytolytic T cell precursors can be activated in this site (London et al., 1987). Functional antigen presenting cells are present directly beneath the epithelium of Peyer's patches (Lause et al., 1981). In addition to macrophages, dendritic cells are the main antigen-presenting cells that bind bacterial products with their Toll-like receptors. The latter are a part of the innate immune defense, recognizing conserved patterns on micro-organisms. Signals initiated by the interaction of Toll-like receptors with specific microbial patterns direct the subsequent inflammatory response (Sminia et al., 1982).

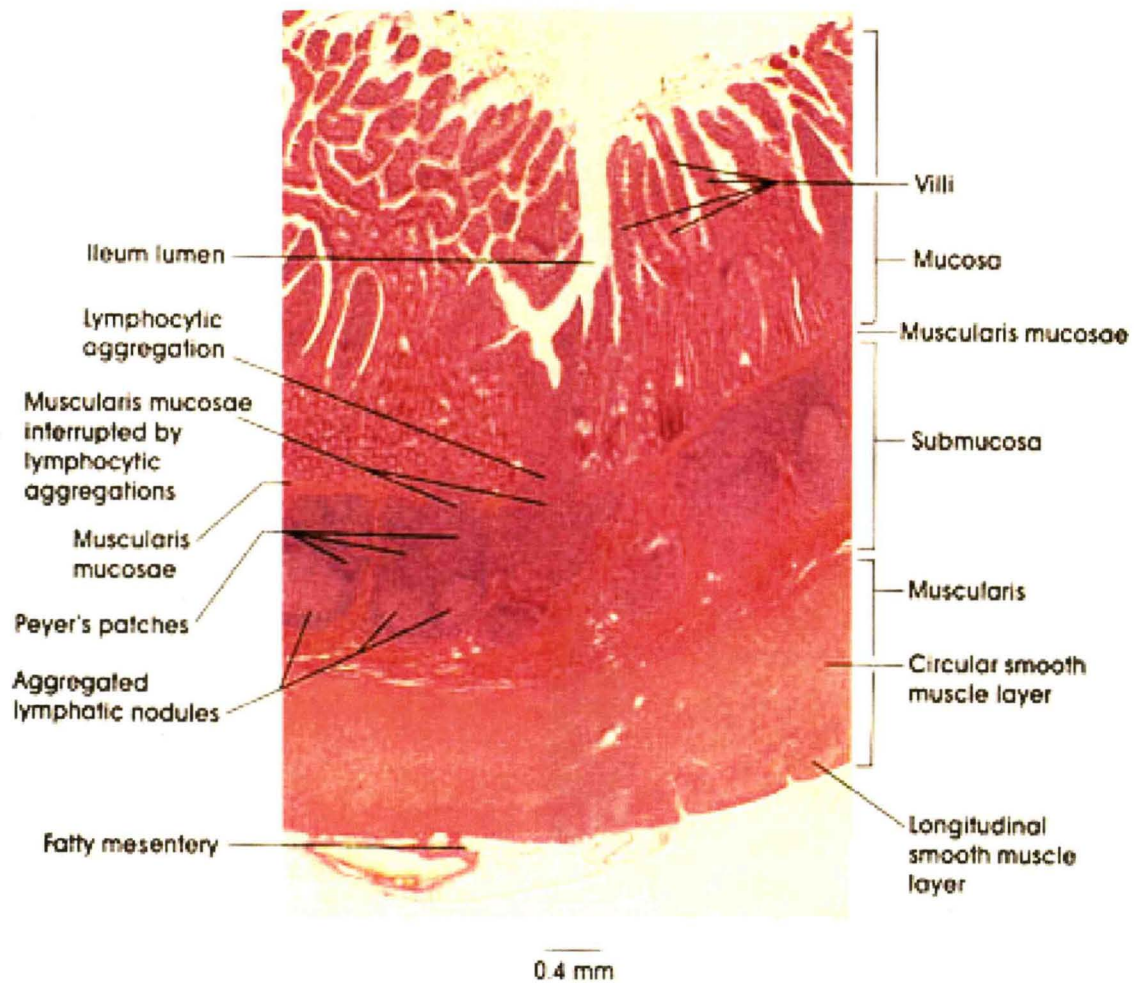


Figure 1.3 Ileum Peyer's patches (Bergman et al., 1999)

b. Diffusely Distributed Mucosal Lymphoid Cells

i. Intestinal Lamina Propria Lymphoid Cells

The non organized lymphoid cell compartment located diffusely within and beneath the gastrointestinal epithelium contains a large, complex mixture of cells that are part of the immune effector functions in the gastrointestinal tract. The lamina propria contains a large number of T cells, B cells, plasma cells, macrophages, and mast cells and smaller numbers of eosinophils, neutrophils, and dendritic cells. About 80% to 90% of the B cells and plasma cells in the normal gastrointestinal tract are IgA-positive. Most of the non-IgA cells are IgM-positive, with about 1% to 2% each of IgG and IgE

positive cells. About a third of the cells in the lamina propria are T cells. Because of the large size of the intestine, this is the largest T cell site in humans. Most T cells in the human lamina propria express either CD4+ or CD8+ and α , β T cell receptors, suggesting that they have arisen from the bone marrow and undergone thymic education. Moreover, the low proliferative rate of lamina propria lymphocytes under normal basal condition was confirmed by studies showing only rare Ki antigen-positive cells in the normal intestinal mucosa (this antigen is specifically expressed on dividing cells) (Stephen, 1995).

ii. Intraepithelial Lymphocytes

Intraepithelial lymphocytes (IEL) are a specialized subset of lymphocytes that are interspersed within the epithelial layer of the intestinal mucosa. The majorities of IEL's are CD8+, CD3+ and have a large granular lymphocyte morphology. In addition, although the majority of T cells in humans have α , β T cell receptors; there is an increased proportion of IEL's having γ , δ receptors, which are rare in the peripheral circulation (Ulrich et al., 1990). Thus it has been suggested that these T cells can recognize particular classes of highly conserved antigens, such as heat shock proteins. Furthermore, it is possible that they interact with classes of molecules expressed on epithelial cells besides typical human leukocyte antigen class I or II molecules, such as CD1 molecules. Finally, γ , δ IEL's can be found in germ-free mice, indicating that bacterial antigens and mitogens are not necessary for generation of these cells and suggesting that the localization and maturation of the IEL population do not require education in the thymus (Bandeira et al., 1990). Interestingly, in 1990 Ebert showed that

although IEL's proliferate poorly in response to mitogens, they do produce lymphokines, particularly IL-2 and IFN- γ .

D. Ulcerative Colitis

1. Definition

UC was first recognized as a distinct disease from bacillary dysentery by Samuel Wilks, a physician at Guy's Hospital in 1859. However, Sir Arthur Hurst was the first to complete the description of UC in relation to sigmoidoscopic appearances (Jewell, 2001). UC is an ulceroinflammatory disease affecting the colon but limited to the mucosa and submucosa except in more severe cases. UC begins in the rectum and extends proximally in a continuous fashion, sometimes involving the entire colon (Crawford and Kumar, 2003). It is divided into four categories, depending on the site of mucosal ulceration according to the Crohn's and Colitis Foundation of America:

- Proctitis: Inflammation involves only the rectum.
- Proctosigmoiditis: Inflammation of the rectum and the sigmoid.
- Colitis: Inflammation of the descending colon.
- Pancolitis: Inflammation of the entire colon, which is very uncommon.

UC rarely affects the small intestine except for the end section; the terminal ileum.

2. Epidemiology

UC is a worldwide disorder with a variable incidence. It is more common in industrialized countries. The Jewish population is more prone to colitis than non-Jewish

populations, but this also varies geographically (Farrokhyar et al., 2001). UC may be present at all ages, but primarily affects the young population (20 – 40 years of age) and tends to affect women more than men (Jewell, 2001).

3. Etiology:

Until now the etiology of UC remains unknown. Many studies suggest that UC results from environmental factors triggering a breakdown in the regulatory mechanism of mucosal immune responses to enteric bacteria in genetically susceptible individuals (Jewell, 2001). Thus, many hypotheses have been developed and many risk factors have been found to be associated with the prevalence of the disease.

a. Infections

An infectious etiology for UC, with a direct cause and effect relationship between a single microorganism and inflammation, has not been asserted (Jewell, 2001). Interestingly, genetically engineered animals developed colitis when exposed to non-pathogenic colonic bacteria, thus providing the most compelling evidence to prove the association between colonic micro flora and the pathogenesis of UC (Farrell et al., 2002). Further evidence was the presence of colonic mucus and mucosal barrier abnormalities in patients with UC (Jewell, 2001).

Furthermore, Bruke and Axon (1987) stated that *E. coli* isolated from patients with UC in complete remission phase, expressed more adhesions than those isolated from healthy individuals. They concluded that *E. coli* in UC patients has the potential of adhering to the epithelial cells of the colon thus causing damage. Moreover, some

clinical trials based on the above hypothesis, showed that tobramycin was significantly more effective than the placebo in treating UC (Bruke et al., 1990).

b. Environmental Risk Factors

Among the many puzzles of the pathogenesis of UC, the role of environmental risk factors is the most difficult to understand and study. The most associated environmental factors are smoking, oral contraception, appendectomy and diet.

The protective effect of cigarette smoking in UC remains the most puzzling, but the most consistent of all environmental factors. Studies consistently found that the concentration of proinflammatory cytokines IL-1 and IL-8 are significantly lower in smokers than in non-smokers with UC (Sher et al., 1999). Interestingly, another study showed that colonic levels of TNF-alpha, interleukin (IL)-1 beta, and IL-6 in colitis rats were alleviated by passive cigarette smoking. In contrast, the deprivation of colonic IL-10 during colitis was preserved in cigarette-smoking rats (Ko et al., 2001). Moreover, it was shown in an in vivo study that nicotine has an inhibitory effect on T-helper-2 cell, which predominates in UC (Madretsma et al., 1996).

In addition, Boyko et al., (1994) have shown that higher UC risk tends to occur among users of high estrogen dose oral contraceptives, while CD risk is similar regardless of estrogen potency. Moreover, Dijkstra et al., (1999) have confirmed that appendectomy protects against the chance of developing UC.

Several studies have examined the relation of many dietary items to the etiology of UC. One study found that the intake of refined sugar, starch and total proteins was significantly higher prior to the onset of the disease as compared to the

controls. In addition, another case-control study showed an association between high intake of Mono- and Polyunsaturated fats with increased risk of UC (Head and Jurenka, 2003). Moreover, one study analyzed the serum levels of antibodies to milk proteins in patients with UC and in controls. The antibody titers did not differ in comparing both groups suggesting that there is still no clear evidence that milk plays a role in the etiology of UC (Jewell, 2001).

c. Genetic Factors

Linkage studies and whole genome scans have suggested that there are susceptibility genes for UC on chromosomes 2,3,6,7, and 12. Nevertheless, other genes appear to influence the disease development independently of the susceptible genes. A strong association exists between genes of human leukocyte antigen region (HLA-DR2) (involved in the regulation of the immune response) and UC. HLA-DR2 has been one of the most reproducible genes in defined Japanese and Jewish populations with UC (Jewell, 2001). Another study in molecular genotyping has provided preliminary evidence that an inherited polymorphism of MUC3, a gene that encodes intestinal mucins, might also be associated with the pathogenesis of UC. It was proven that selective reduction of some mucin components may impair mucosal defense against luminal bacteria which in turn may stimulate the auto-immune response to epithelial antigens (Kyo et al., 1999).

It should not be forgotten however, that the concordance rates among monozygotic twins and dizygotic twins with UC are 13% and 2%, respectively. Thus, non-genetic factors are clearly indispensable to the development of UC through the interaction of multiple predisposing, but not sanctioning genes (Jewell, 2001).

4. Pathology of UC

a. Macroscopic Features

In UC patients with mild inflammation, there is destruction of the colonic mucosa with macroscopic hyperemia, edema, and granularity with friability and easy bleeding. As the disease becomes more severe, the mucosa becomes intensely hemorrhagic, and there is extensive and broad based ulceration of the mucosa which may extend deeply in the lamina propria. In long-standing disease the regenerating mucosa of the colon, bulges upward to create pseudopolyps. In rare cases, the muscularis propria is so compromised as to permit perforation and pericolic abscess formation. The exposure of the muscularis propria and neural plexus to fecal material may also lead to a complete shutdown of the neuromuscular function, thus causing colonic dilatation and gangrenes, a condition known as toxic megacolon.

In remission, the mucosa appears normal, but patients with recurrent attacks over several years, may develop atrophic and featureless mucosa. This is described by narrowing and shortening of the colon as a result of abnormalities in the muscular mucosal layer (Crawford and Kumar, 2003).

b. Microscopic Features

In mild UC, inflammation is predominantly confined to the mucosa. The lamina propria becomes edematous and the capillaries are dilated and congested, often with extravasations of red cells. Diffuse mononuclear inflammatory cells of neutrophils, lymphocytes, plasma cells, and macrophages infiltrate the lamina propria. Eosinophils

and mast cells are also present in increased numbers. The neutrophilic infiltration of the epithelial layer, usually in the crypts, give rise to cryptitis and eventually crypt abscesses. Interestingly, it was observed that all these changes can be nonspecific and may be confused with infective or self limiting colitis. However, features that suggest chronicity may help in the diagnosis of UC. These features include distorted crypt architecture, crypt atrophy, increased intercrypt spacing to fewer than 6 crypts per millimeter, an irregular mucosal surface, basal lymphoid aggregates, and chronic inflammatory infiltrates.

With increasing inflammation, the surface epithelial cells become flattened and eventually ulcerated. Some inflammation and vascular congestion may be seen in the submucosa. Furthermore, the ulceration and inflammatory infiltrates may extend to the muscularis propria, which may undergo ischemic necrosis.

In remission, the histological appearances may revert to normal, especially after mild attacks early in the course of the disease. After recurrent attacks, granulation tissues fill in the ulcer craters, followed by regeneration of the mucosal epithelium. Submucosal fibrosis and mucosal architectural disarray and atrophy persist, with the evidence of altered crypt architecture. Other chronic changes are sometimes seen including neural hypertrophy and fibromuscular hyperplasia of the muscularis mucosa (Crawford and Kumar, 2003).

E. Immunopathogenesis of Ulcerative Colitis

The immunologic changes found in the colonic mucosa of patients with UC involve both humoral and cellular responses. However, whether these mechanisms are

due to an antigenic challenge, a response to a specific etiology or indicate a defect in the mucosal immunoregulation, is still difficult to prove.

1. Humoral Responses

UC has been first described as an autoimmune disease in the 1950's, after Broberger and Perlmann (1959) have found antibodies in the serum of UC patients that cross reacted with human fetal colon cells in vitro. Furthermore, autoantibodies to epithelial cell – associated components (ECACs) have been described to a 40-kd epithelial antigen which has been isolated from inflamed colonic mucosa of patients with UC. This autoantibody response to the 40-kd autoantigen, which has been found on colonic epithelium, is proven unique in UC. Moreover, monoclonal antibodies against this 40-kd antigen identifies a shared epitope in human colon, skin, biliary epithelium as well as eyes and joints, locations compatible with the extraintestinal manifestations of UC (Das et al., 1990). Autoantibodies against members of topomyosin family of proteins have been found in all patients with UC (Das et al., 1993). Similarly the majority of patients with UC and a significant number of those with CD express antineutrophil cytoplasmic antibodies (pANCA) (Duerr et al., 1991). The antigen recognized by pANCA is myeloid specific and appears to be associated with neutrophil granules. Autoantibodies in IBD play a role in the cell-mediated immune destruction of intestinal epithelial cells. For example, NK cells could be armed with intraepithelial cell antibodies and engage in epithelial cell lysis.

Histologically, studies of the inflamed colon in active UC have indicated an increase in mucosal B cells and plasma cells. However, the production of immunoglobulins is not uniform between IgA, IgM, and IgG which increase by 2, 5,

and 30-folds, respectively. Also, much of the increase in the IgG synthesis results from an increase in the IgG1 and IgG3 subclasses, in contrast to CD, in which there is a marked increase in the IgG2 synthesis. This kind of response suggests that protein antigens may be the predominant triggers in UC (Kett et al., 1987).

2. Cellular Response

a. T cells

Perlamann and Broberger (1963) were also the first to show that immune cells, specifically lymphocytes, could recognize and destroy intestinal cells. They showed the cytotoxic action of circulating CD8+ lymphocytes against human fetal colon cells in vitro. Moreover, another study confirmed the action of circulating CD8+ lymphocytes against freshly isolated colon cells in vitro, suggesting that natural killer cells are responsible for this phenomenon (Shorter et al., 1969).

T cell function was also investigated in the lamina propria of patients with UC. CD8+ lymphocytes were reported to be cytotoxic to autologous colonic epithelial cells and the helper function of CD4+ T cells affected the production of immunoglobulins by B cells. Furthermore, many reports, but not all have found diminished activity of suppressor CD8+ T cells during active disease only (Jewell, 2001).

Regardless of their functional status, both peripheral and mucosal T cells (CD4+, CD8+) show evidence of activation in UC, and there is a direct connection between increased number of T cells and tissue destruction with mucosal damage in vitro (Raedler et al., 1985).

b. Intraepithelial Lymphocytes

Most intraepithelial lymphocytes (IEL's) in human intestine are T cells (CD3+) and of these, the majority are CD8+ cells. In the inflamed intestine of patients with UC, the absolute number of IEL's is normal or reduced and the CD4+ / CD8+ ratio is unchanged. Interestingly, the proportion of cells using the γ δ T cell receptor may increase by 30%-40% in patients with UC. However, the function and significance of these γ δ T cells are unknown (Trejdosiewicz et al., 1989).

c. Monocytes and Macrophages

The nonspecific cellular immunity in UC is evidenced by an increase production of circulating monocytes and mucosal macrophages. The mucosal macrophages in active UC exhibit a unique phenotype with cells having low-affinity Fc γ R (3G8+, CD14) and other cells that express RFD9, a marker for epitheloid cells (Mononuclear form of macrophages). The CD14+ macrophages are known to be sensitive to lipopolysaccharides which indicate its relevance for the pathogenesis of UC, but the RFD9+ cell function and origin are still unknown (Grimm et al., 1995) .

d. The epithelial cells

The colonic epithelial cells of patients with UC, exhibit an increased turnover rate in the active and remission phase of the disease (Allan et al., 1985). Moreover, these epithelial cells show a reduced metabolism of short fatty acids; and their membranes are abnormally permeable to labeled chromium (Gibson et al., 1988). In addition, the mucus production by these cells differs in patients and controls, suggesting

that a basic abnormality in the epithelial cells renders persons to be susceptible to UC (Podolsky and Isselbacher, 1984).

Furthermore, human enterocytes and colonocytes can express class II antigens and can function as antigen-presenting cells playing a role in inflammation (Mayer and Shlien, 1987). There is also evidence that epithelial cells are involved in the expression of functional cytokine receptors that respond to IL-15; secrete IL-7, and activate lamina propria mononuclear cells; produce chemokines, colony-stimulating factor, and TNF- α ; and express leukocyte adhesion molecule. It is also known that the colonic epithelial cells in patients with UC have a defective capacity in inducing suppressor T cells and they preferentially activate CD4+ helper T cells leading to an amplified local immune response (Fiocchi, 1998).

e. Immune Cells activation

The activation of immune cells results in the release of cytokines and inflammatory mediators that mediate tissue damage, amplify the immune response and promote further inflammation. Moreover, macrophages found in the inflamed colon of patients with UC, secrete IL-1 β , TNF- α , and IL-6, which stimulate the acute phase of the disease. Furthermore, epithelial cells in the inflamed mucosa present class II antigen to the CD4+ cells which may lead to the up regulation of the mucosal immune response (Selby et al., 1983).

In addition to cytokines release, activated mucosal cells release mediators that not only contribute to tissue damage and inflammation, but also affect epithelial cell permeability and ion transport. Some of these mediators are leukotrienes, thromboxane,

platelet-activating factor, nitric oxide, and reactive oxygen metabolites, predominantly produced by neutrophils and macrophages (Boughton-Smith and Pettipher, 1990).

f. Immunoregulatory Cytokines

Immunoregulatory cytokines are glycoproteins predominantly secreted by T lymphocytes. They direct the nature of the immune response and control the production of proinflammatory cytokines.

i. Interleukin-2 (IL-2)

The prototypic Immunoregulatory cytokine studied in UC is IL-2 which is mainly secreted by T helper cells. It acts as a growth factor/activator for T cells, NK cells and B cells and promotes the development of lymphokine-activated killer cells (Cohen and Cohen, 1996). Binding of IL-2 to the IL-2 receptor on T lymphocytes leads to cell proliferation, increased lymphokine secretion (IFN- γ , lymphotoxin, IL-4, IL-3, IL-5, GM-CSF), and enhanced expression of class II MHC molecules.

Studies have shown that circulating IL-2 in serum was not detected in health or in disease. However, decreased IL-2 bioactivity from isolated intestinal mucosal mononuclear cells was detected in patients with UC (Fiocchi et al., 1984). In another study, Murata et al., (1995) showed that IL-2 production was significantly decreased in active and inactive UC compared to controls, and the values in active UC were inversely correlated with the degree of inflammation. Another study using the reverse-transcription polymerase chain reaction, showed an increase in IL-2 mRNA in active CD but not in UC lesions. Furthermore, UC mucosal T cells are activated weakly by IL-

2, in contrast to CD mucosal T cells that show a hyper reactive response. Thus, suggesting the involvement of IL-2 in the pathogenesis of CD (Matsuura et al., 1993). Moreover, Van Damme et al., (2001) observed that IL-2 producing CD3+ cells are decreased in the lamina propria lymphocytes while no difference is noted in intraepithelial lymphocytes of patients with UC compared with controls.

ii. Interferon-gamma (IFN- γ)

Another Immunoregulatory cytokine investigated in UC was IFN- γ which is produced by activated T cells and NK cells. IFN- γ is known to enhance MHC class I and II expression on nucleated cells and to stimulate many of the effector functions of mononuclear phagocytes. Studies done by Lieberman et al., (1988) reported a decreased production of IFN- γ by intestinal mucosal mononuclear cells. Moreover, it was reported as well, that IFN- γ production in active UC is inversely correlated to the degree of inflammation. Other studies using ELISA technique, showed decreased levels of IFN- γ in the lamina propria of the colon of patients with UC compared with controls (MacDonald et al., 1990). Furthermore, another study showed a correlation between IFN- γ with the degree of inflammation in CD, by showing an increased IFN- γ mRNA expression by lamina propria mononuclear cells and the presence of IFN- γ secreting T cells in active UC. IFN- γ secreting T cells in activating the involvement of IFN- γ in the pathogenesis of CD, and strengthening the suggestion of the so called Th-1-like pattern in CD (Fais et al., 1991). Moreover, Van Damme et al., (2001) showed a proportional decrease of IFN- γ producing CD3+ and CD8+ cells in the colonic lamina propria of patients with UC.

g. Proinflammatory Cytokines

Proinflammatory cytokines such as IL-1, IL-6, TNF- α , are glycoproteins that tend to be consistently elevated in UC and are involved in most local and systemic components of acute and chronic inflammatory diseases.

i. Interleukin -1 (IL-1)

IL-1 consists of at least two types of polypeptides, IL-1 α and IL-1 β . Their main cellular sources are mononuclear phagocytes, fibroblasts, keratinocytes, and T and B lymphocytes. Both IL-1 α and IL-1 β can trigger fever by enhancing prostaglandin E2 synthesis by the vascular endothelium of the hypothalamus. Moreover, IL-1 elicits the release of histamine from the mast cells at the site of inflammation (Feghali et al., 1997). IL-1 increases the production of IL-6 and TNF alpha by monocytes/macrophages in both an autocrine and a paracrine fashion (Guimbaud et al., 1998). Furthermore, IL-1 induces the proliferation and differentiation of antigen-specific T cells, and B cells, and the release of granule contents from neutrophils (Ishiguro et al., 1999).

Studies have shown that IL-1 levels were only slightly elevated in the plasma samples of patients with IBD. While other studies of cultured mucosal specimens (Ligumsky et al., 1990; Stevens et al., 1992), cultured lamina propria mononuclear cells (Mahida et al., 1989), immunohistochemistry (Stevens et al., 1992), and the expression of IL-1 transcripts in LPMC (Youngman et al., 1993) have shown increased production of IL-1 in the inflamed mucosa of active UC. Moreover, Casini-Raggi et al., (1995) published results that supported the hypothesis that there is an imbalance between IL-1 and IL-1ra production in colonic mucosa of patients with UC. A markedly significant

decrease in the intestinal mucosal IL-1ra/IL-1 ratio was found in both CD and UC patients when compared with control subjects. Moreover, the IL-1ra/IL-1 ratio was correlated closely with the clinical severity of disease.

Interestingly, Mansfield et al., (1994) tried to determine whether UC was associated with a particular allele of IL-1 receptor antagonist. Allele 2 of interleukin-1 receptor antagonist was significantly over-represented in the UC patients versus that of the controls. This association with allele 2 of interleukin 1 receptor antagonist was greatest in patients with total colitis and was not seen in CD. There were no associations between UC and any of the other cytokine genes examined. Thus this observation provided an evidence that interleukin-1 receptor antagonist may have a role in determining the genetic susceptibility to and pathogenesis of UC.

ii. Tumor Necrosis Factor (TNF- α)

TNF- α is one of the products of activated macrophages/monocytes, fibroblasts, mast cells, and some T and natural killer cells. Like IL-1, TNF- α can induce fever, either directly via stimulation of PGE₂ synthesis by the vascular endothelium of the hypothalamus, or indirectly by inducing release of IL-1 (Warren, 1990). TNF- α shares an important inflammatory property with IL-6 that is the induction of acute phase reactant protein production by the liver. Furthermore, TNF- α and IL-1 exert secondary inflammatory effects by stimulating IL-6 synthesis in several cell types, thereby perpetuating the inflammatory response through a cascade of cytokines with overlapping properties.

Investigators have reported that the expression of TNF- α in UC mucosa tend to be elevated, though the serum, protein and mRNA content of this cytokine is variable. Murch et al., (1991) found that TNF- α was elevated in serum of children with active UC. This result was confirmed by the work of another British group, who detected elevated levels of TNF- α in stools of children with UC as compared to controls (Braegger et al., 1992). In another study however, Hymas et al., (1991) could not detect differences in TNF- α serum levels between children with and without UC. Moreover, Stevens et al., (1992) investigated the presence of TNF- α mRNA transcripts in inflammatory bowel disease (IBD), normal, and other inflammatory intestinal specimens utilizing the polymerase chain reaction (PCR). TNF- α mRNA levels did not vary between inflammatory bowel disease and control specimens. Yet other reports by Reinecker et al., (1993) showed that TNF- α production is greater in cultures of CD than UC mucosal mononuclear cells. However, direct evaluation of IBD tissue secretions by in situ hybridization showed elevated TNF- mRNA in macrophages (Cappello et al., 1992). In addition, the secretion patterns of TNF- α by lamina propria mononuclear cells (LPMNC) from patients with UC demonstrated a close correlation with the degree of tissue involvement and mucosal inflammation. Moreover, Guimbaud et al., (1998) characterized the network of colonic cytokines in vivo in patients with UC using the colonic perfusion method. TNF- α was found in high levels in the colonic perfusate of patient with UC and was not detected in controls. The high levels of TNF found suggested that this harmful cytokine could be a therapeutic target in UC, but the mechanism of anti- TNF- α is still unclear.

iii. Interleukin-6 (IL-6)

IL-6 also referred to as B-cell stimulatory factor-2 (BSF-2), is produced by a variety of cells including mononuclear phagocytes, T cells, and fibroblasts (Van Snick, 1990). In addition to the stimulation of acute phase protein synthesis by the liver, IL-6 acts as a growth factor for mature B cells and induces their final maturation into antibody-producing plasma cells. It is involved in T cell activation and differentiation, and participates in the induction of IL-2 and IL-2 receptor expression.

Mahida et al., (1991) observed that circulating IL-6 is particularly high in patients with active CD but not in patients with UC. In contrast, other studies reported that IL-6, primarily secreted by macrophages and epithelial tissues, was consistently elevated in colonic intestinal tissues of patients with UC (Kusugami et al., 1995). Furthermore, Hosokawa et al., (1999) showed that the levels of IL-6 and sIL-6R in colonic organ cultures were substantially elevated in patients with IBD, especially in those with histologically active inflammation. Also the levels of IL-6 and sIL-6R in colonic organ cultures, but not those in the serum, showed a significantly positive correlation with the degree of clinical disease activity in patients with UC. Moreover, the same group of scientists showed as well, that the expression of IL-6 and IL-6R mRNA in lamina propria mononuclear cells was in parallel with the results obtained in colonic organ cultures. In cell cultures, mucosal macrophages were the main cell type producing both IL-6 and sIL-6R on a per cell basis and other cell fractions including colonic epithelial cells and lymphocytes, produced substantially lower amounts of these molecules (Hosokawa et al., 1999). Interestingly, one study showed graded production of IL-6 in intestinal lamina propria and serum of newly diagnosed pediatric IBD patients confirming the presence of IL-6 in early IBD patients. In addition, it was

specified that serum IL-6 may be a good predictor of IBD in pediatric patients with suspected or newly diagnosed IBD (Brown et al., 2002).

iv. Effects of Proinflammatory cytokines on nerves and muscles

Intestinal inflammation causes marked changes in the architecture and function of the gut. It is not limited to the mucosa, and encompasses the smooth muscle layers and enteric plexuses. The proinflammatory cytokine, IL-1 β is known to induce its own gene expression in several cell types including vascular (Warner, 1987) and enteric smooth muscle cells (Khan et al., 1993). Thus, it is likely that the early mucosal production of IL-1 β is a stimulus for the induction of IL-1 β production in other tissue sites in the inflamed gut including the myenteric plexus (Khan and Collins, 1994). Moreover, IL-1 β and TNF- α influences the release of acetylcholine (Main et al., 1993) and noradrenaline (Hurst and Collins, 1993) from myenteric nerves, and that IL-6 potentiates the action of IL-1 β on nonadrenergic nerves (Ruehl et al., 1993). Furthermore, Kelles et al., (2000) showed that IL-1 β and IL-6 act as excitatory neuromodulators of gastrointestinal motility through direct excitatory actions on a subset of myenteric neurons and through presynaptic inhibition of acetylcholine release. Moreover, Lodato et al., (1999) showed that IL-1 β and TNF- α produce increased levels of the potent smooth muscle relaxant nitric oxide, thus impairing smooth muscle contractility. Interestingly, another study by Vrees and his colleagues (2002) demonstrated elevated levels of IL1 β in colonic circular muscle in patients with UC, which can explain the impaired contraction through production of hydrogen peroxide and depletion of releasable Ca²⁺ stores. A unique relationship exists between IL-1 β and substance P, which acts both as an excitatory neurotransmitter and a proinflammatory

mediator. Furthermore, Grider (2003) showed that IL-1 induces a selective increase in substance P synthesis and release from excitatory motor neurons, which is reflected by an increase in contractile response of longitudinal muscle and circular muscle during the ascending phase of the peristaltic reflex.

F. Involvement of the Small intestine in UC

UC is thought to be restricted to the colon. However, there is evidence that colitis in humans and experimental animals may be associated with small bowel dysfunction, including altered motility and malabsorption of fat, carbohydrates (Chakravarti et al., 1973), proteins (Zetzel and Banks, 1942), minerals, vitamins, water (Binder and Ptak, 1970) and D-xylose (Anderson et al., 1971). In addition previous data from our laboratory have shown that Iodoacetamide induced colitis is associated with decreased jejunal absorption of alanine, glucose and water (Mourad et al., 2001; Barada et al., 2001).

Furthermore, there are numerous and varied complications in the natural course of UC and the extracolonic manifestations of this disease have been described in many reviews and case reports. Well known bowel complications include toxic megacolon, colitis cystica profunda, backwash ileitis, and postcolectomy pouchitis. The extension of pathological changes of UC in a retrograde manner through the ileocecal valve into the ileum, responsible for back wash ileitis, only reaches a short distance into the ileum, and the upper small intestine, particularly the duodenum, is not affected. Despite this well-established and accepted dogma, rare cases of histologically proven diffuse duodenitis (DD) associated with UC appear in the literature.

Salem et al., (1964) described that the upper small intestine is frequently abnormal, with changes such as atrophy of the villi, in UC cases, speculating that this is due to the generalized disease because of the frequency with which organs other than the colon are affected, such as skin, eye, joints, mouth, and liver.

Based on a review of the literature from 1966 to 1995, only two cases with roentgenologic evidence of duodenitis and UC have previously been reported. However, Mitomi et al., (1997) reported the first surgical case of diffuse ulcerative duodenitis associated with UC without backwash ileitis. The radiographic, colonoscopic, and colonic histological findings pointed to typical UC, and no granulomas were found in either the duodenum or the colon. Moreover, evidence of other inflammatory conditions, including allergic disease, drug injury, and celiac disease was not found.

A retrospective review of five pediatric cases of UC with gastroduodenal inflammation was done by Kaufman et al., (1997). In these patients, upper GI tract and ileal inflammation led to the tentative diagnosis of CD, and after prolonged medical treatment, all were ultimately found to have refractory UC necessitating subtotal proctocolectomy. Thus, proving that the presence of inflammation in the duodenum does not exclude the diagnosis of UC, unless stereotypic CD findings such as aphthous ulceration and/or granulomas are present.

More recently, Terashima et al., (2001) reported on two patients with strongly suggested UC and extracolonic manifestations in whom diffuse duodenitis developed. Interestingly, duodenal lesions closely resembled the UC lesions in the large intestine, macroscopically and microscopically, and there were no granulomas in the biopsy specimens. Moreover, the radiographic, colonoscopic and colonic histological findings were more suggestive of UC with the absence of colonic granulomas as well.

Honma et al., (2001) analyzed gastroduodenal lesions in patients with UC. Endoscopic and histological changes (redness and deformity of villi) in the duodenum were more prominent in UC patients with pancolitis than those with left-sided/proctitis. Again, CD8+ cells infiltrating both the duodenum and stomach were increased in UC.

Most recently, Rubenstein et al., (2004) presented a case of a 38-year-old man with voluminous diarrhea following colectomy for well-documented UC; he was found to have ulcerative enteritis with histologic features identical to UC. Moreover, after an inclusive review of another 11 cases of UC associated enteritis reported in the literature, Rubenstein and his colleagues concluded that UC associated enteritis is distinct from CD, frequently present shortly after colectomy, and usually is responsive to traditional therapies for IBD.

G. Experimental Models of Inflammatory Bowel Disease

A steadily increasing number of experimental animal models with some pathological manifestations similar to those observed in inflammatory bowel disease have recently been developed and have contributed greatly to important advances in the current understanding of the immunological, pathological, and physiological features of chronic intestinal inflammation. Despite the varying nature of these models, the aspects which they have in common greatly support the concept that environmental factors affecting genetically susceptible hosts are responsible for the induction of mucosal inflammation. Interestingly, since the first description of the immune complex colitis in rabbits in 1961, overall 63 models have been described (Hibi et al., 2002). These IBD animal models can be divided into 5 main categories based on the methods of induction:

(1) gene knockout (2) transgenic (3) chemical (4) adoptive transfer, and (5) spontaneous (Jurjus et al., 2004).

In spite of the high overall number of models, none of them is the 'perfect' model and therefore numerous aspects need to be considered when choosing one model for a particular study. However, it is good to mention that as experimental mucosal inflammation can arise from various experimental models, a variety of defects can be the cause of IBD in humans, and the immunological defects seen in experimental colitis are due to immune dysregulation rather than immunodeficiency.

H. Purpose of the Study

Although UC is a disease confined to the colon, many studies have shown functional and structural abnormalities in the small intestine of UC patients and animal models with colitis. Such functional abnormalities were manifested by a decrease in intestinal glucose, amino acids, D-xylose, fat, water and sodium absorption. In addition, structural abnormalities were manifested by an increase in tight junction permeability throughout the entire small intestine. Nevertheless, some studies have described several cases of clinical UC associated enteritis and duodenitis. However, the effect of inflammation in the colon on the small intestine has not been fully elucidated. Thus, based on the IA induced model of colitis, the purpose of this study is to search for discrete factors that could explain altered small bowel function in UC without evident structural alteration and to investigate the expression of proinflammatory cytokines in serum and intestinal tissues in experimental colitis at different time intervals starting from induction to recovery.

MATERIALS AND METHODS

Adult female Sprague-Dawley rats weighing 200-300g were used in all experiments. They were kept under controlled environmental conditions for light, temperature and humidity and with free access to tap water and standard lab chow. The animals were fasted for 24hrs before the day of the experiment with free access to tap water.

A. Induction of Ulcerative Colitis

Based on a previously described experimental model (Sato et al., 1997), colitis was induced by the instillation of 100microliters of 6% Iodoacetamide (IA) [600mg of IA powder in 1% Methyl Cellulose (MC) solution-100mg of MC powder in 10ml of distilled water] 5cm distal to the anal verge using a Nelaton's catheter. Sham rats were treated with the vehicle (100microliter of 1% MC solution) and control rats were treated with saline.

B. Animal Grouping and Time Course of the Study

The study was divided into two sets of experiments. Each set contained eight groups (n=5-6) of rats. In the first set of experiments, whole tissue sections were taken from the large and small intestines. In the second set of experiments, mucosal scrappings were taken from the large and small intestines. Different time intervals were assigned for post-induction tissue removal. In the twelve experimental groups, tissues were removed 3, 6, 12,

24, 48, and 96 hours post induction. In the two saline groups and the two Methyl Cellulose (MC) groups, tissues were removed 24hs post induction.

C. Post-Induction Tissue Removal

On the day of surgery, rats were anesthetized according to each animal body weight with an intraperitoneal injection of sodium pentobarbital (50mg/Kg body weight). The abdominal cavity was opened by a midline incision, and the digestive system was exposed.

In the first set (set1) of experiments, the descending colon and the small intestines from the ligament of Treitz to the ileocecal junction were removed, flushed with ice-cold 0.9% NaCl, and cut along the mesenteric border. Whole intestinal tissue sections (1cm each) were taken from the mid-duodenum, mid-jejunum, mid-ileum and next to the site of injection in the descending colon. Tissue sections were placed on dry ice cakes, weighed and then stored at -80°C . Serum samples from the inferior vena cava were taken from all the animals in set 1 on the day of tissue removal for later cytokine determination.

In the second set (set 2) of experiments, the descending colon and the small intestines from the ligament of Treitz to the ileocecal junction were removed, flushed with ice-cold 0.9% NaCl, and cut along the mesenteric border. Mucosal scrappings were taken from the mid-duodenum, mid-jejunum, mid-ileum and next to the site of injection in the descending colon. Mucosal scrappings were placed on dry ice cakes, weighed and then stored at -80°C . The weight of the intestinal samples both tissue sections and mucosal scrappings ranged between 80mg-120mg.

D. Determination of Cytokine Level in Intestinal Samples

1. Tissue Homogenization

Tissue homogenization was done according to a protocol modified from Saade et al., (2002). Intestinal samples were homogenized for 1min using a polytron probe at a speed of 23000rpm, each in a 4 ml phosphate buffer solution [0.818g of NaCl, 0.02g of KCl, 0.02g of KH₂PO₄, and 0.115g of Na₂HPO₄ in 100ml DDW (pH= 7.2-7.4), modified by adding 2.3376g of NaCl, 0.5g of Bovine Serum Albumin (BSA), 50µl of Tween 20, and 2 tablets of protease inhibitors per 100 ml of PBS (pH= 7.2-7.5)]. The homogenates were then centrifuged for 1h at 4°C at a speed of 11000rpm. The supernatant for each intestinal sample was put in the deep freeze at -80°C until used.

2. Enzyme Linked Immunosorbant Assay (ELISA)

ELISA was done according to a protocol modified from Saade et al., (2002). The concentration of TNF- α , IL-1 β , and IL-6 in each intestinal sample supernatant was measured by a two-site ELISA.

The TNF- α , IL-1 β , and IL-6 assay used immunoaffinity purified polyclonal sheep anti-rat TNF- α , IL-1 β , and IL-6 serum to coat the high binding microtiter plates. Recombinant rat TNF- α , IL-1 β , and IL-6 were used as the standards and a biotinylated, immunoaffinity purified polyclonal sheep anti-rat TNF- α , IL-1 β , and IL-6 as recognition antibodies. For all assays, the color was developed for 15–20 min by using streptavidin horseradish peroxidase and 3, 3', 5, 5'-tetramethyl-benzidine in acetate buffer and 30%

H₂O₂. The enzymatic reaction was stopped by adding 100 µl of 2 M H₂SO₄ to each well and the optical density (OD) was measured at 450 nm. Results of the cytokine levels in the intestine were expressed as pg/mg of tissues.

3. Nerve Growth Factor Immunoassay System

The concentration of the nerve growth factor (NGF) in the supernatant of intestinal samples was determined by the NGF Immunoassay System designed for the detection of NGF in an antibody sandwich format.

4. Protein Quantification

The BioRad assay for protein quantification was used to determine the concentration of protein in each intestinal sample by measuring the protein's intrinsic UV absorbance generated by a protein-dependent color change. The cytokine concentration of each intestinal sample was expressed then in mg of protein.

E. Assessment of Colonic Inflammation in UC Rats

The descending colon 7 cm from the anus was removed, opened longitudinally, washed with saline and flattened on a filter paper. The area (mm²) and severity of

inflammation were assessed macroscopically using a scale of 0-3 described by sato and his colleagues (sato et al., 1997). Grade 0 was normal, 1 was mucosal erosion, 2 was a moderate lesion or ulcer and 3 was a deep lesion or ulcer.

F. Histology

In addition to the visual assessment of severity, segments of the colon, duodenum, jejunum, and ileum were studied histologically to determine if the change in tissue cytokine levels were accompanied by significant inflammation. Segments of tissues were removed, washed in ice cold physiologic saline and then fixed in 4% paraformaldehyde. Then they were cut into 5 μ m thick sections and stained with Hematoxylin and Eosin, Periodic Acid Schiff or silver for light microscopy.

G. Materials

Reagents used in this study were Iodoacetamide, methyl-cellulose, Tetra-Methyl Benzidine, glacial acetate, Na-Acetate (Sigma Chemicals, St. Louis, MO, USA), pentobarbital (AUB-MC pharmacy), bovine serum albumin, tween 20, HRPO (Amersham Pharmacia Biotech, England, UK), protease inhibitors (Roche Bioscience, Palo Alto, California, USA), Biotin Conjugated Antibody (Perotech Sciences, Ontario, CA), Normal Sheep Serum (Serotec, Oxfordshire, UK), hydrogen peroxide (Fisher Scientific Company, Houston, TX, USA), BioRad assay (Life Sciences Group, Hercules, CA), NGF ELISA kit (Promega Corporation, Madison, WI, USA), TNF- α , IL-1 β , and IL-6 ELISA kits (National Institute for Biological Standard and Control, England), microtiter plates (Nunc, USA).

H. Statistics

Data was expressed as the mean \pm SEM from a minimum of 5 rats per group for determining the cytokine level in intestinal samples for each time interval, and assessing the colitis ulcer score index. Statistical significance between the measurements in the sham, control, and colitic animals was determined by one-way ANOVA when multiple comparisons were made followed by Boferroni Mutiple comparison test (Graph Pad Instat; Graph Pad Software Inc., San Diego, CA, USA) taking $p < 0.05$ as the limit of significance.

RESULTS

A. General Observations

Rats with Iodoacetamide induced colitis showed signs and symptoms of UC, such as increased stool frequency with blood and mucus in the rectum. Moreover, these rats showed inhibition of body weight gain (Fig. 2.1), dilatation and adhesion of the colon and mucosal lesions. The intensity and severity of these symptoms in colitic rats increased in a time dependent manner starting from 3hs reaching a peak at 48hs then began to decline at 96hs.

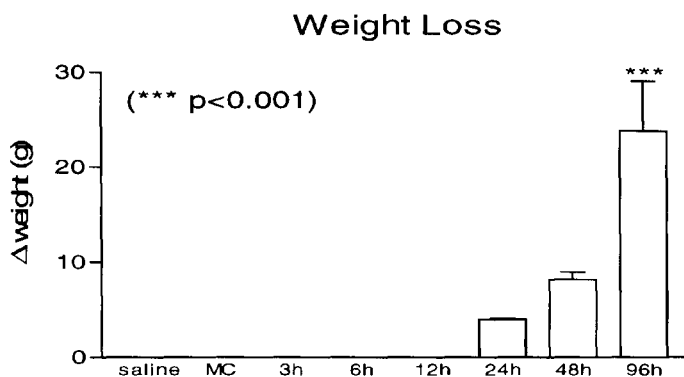


Figure 2.1 Gradual weight loss of rats at different time intervals following the induction of colitis. Results are represented as mean \pm SEM from different groups of 5 rats for each time interval. Weight loss is significant ($P<0.001$) at 24, 48 and 96 hours.

B. Ulcer Score Index

The mucosal colonic damage in colitic rats was recorded at different time intervals using an ulcer index score of 0-3 (0=normal, 1=mucosal erosion, 2=moderate lesion or ulcer, 3=deep lesion or ulcer). The mucosal colonic damage with a mean score of 1.8 ± 0.25 , 2.0 ± 0.26 , 2.08 ± 0.22 , 2.36 ± 0.2 , 2.84 ± 0.1 , 2.31 ± 0.36 ; ($p<0.001$) was

recorded in colitic rats at 3, 6, 12, 24, 48, 96 hours post ulcer induction with IA, respectively (Fig. 2.2).

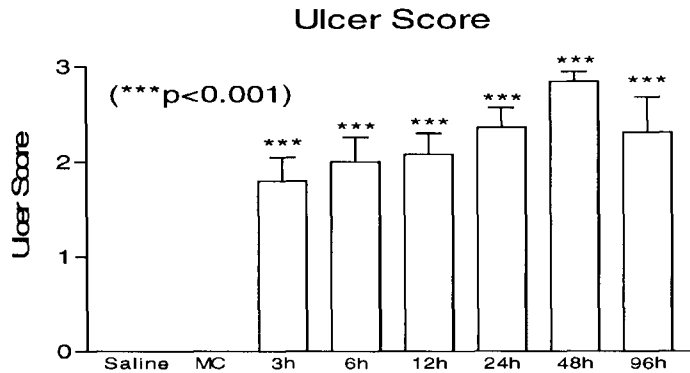


Figure 2.2 Ulcer Index Scores at different time intervals following the induction of colitis as compared to control rats (Saline and MC). Results are represented as mean \pm SEM from different groups of 5 rats for each time interval. $P < 0.001$ for all samples compared to the control (Saline & MC).

C. Detection and Temporal Evolution of Cytokines in Blood Serum

During the course of Iodoacetamide induced colitis, proinflammatory cytokine levels in the serum of rats were monitored at different time intervals following the induction of UC. In the serum of the saline and MC groups there were no detectable levels of TNF- α , IL-6 and IL-1 β . However, there was an early, moderate and short-lived increase in the TNF- α level which was only significant at 6hs (58.0 ± 26.0 pg/ml; $p < 0.05$) post instillation of IA followed by a decline and almost full recovery at 96hs (4.0 ± 4.0 pg/ml; $p > 0.05$). The IL-6 level showed a similar pattern of an early transient increase that started at 6hs (2275.0 ± 460.0 pg/ml; $p < 0.05$), peaked at 12hs (5000.0 ± 1000.0 pg/ml; $p < 0.001$), and then vanished thereafter. In contrast, the level of IL-1 β level showed an early increase at 6hs (315.0 ± 32.0 pg/ml; $p < 0.001$), which was maintained throughout the course of the disease (Fig. 2.3).

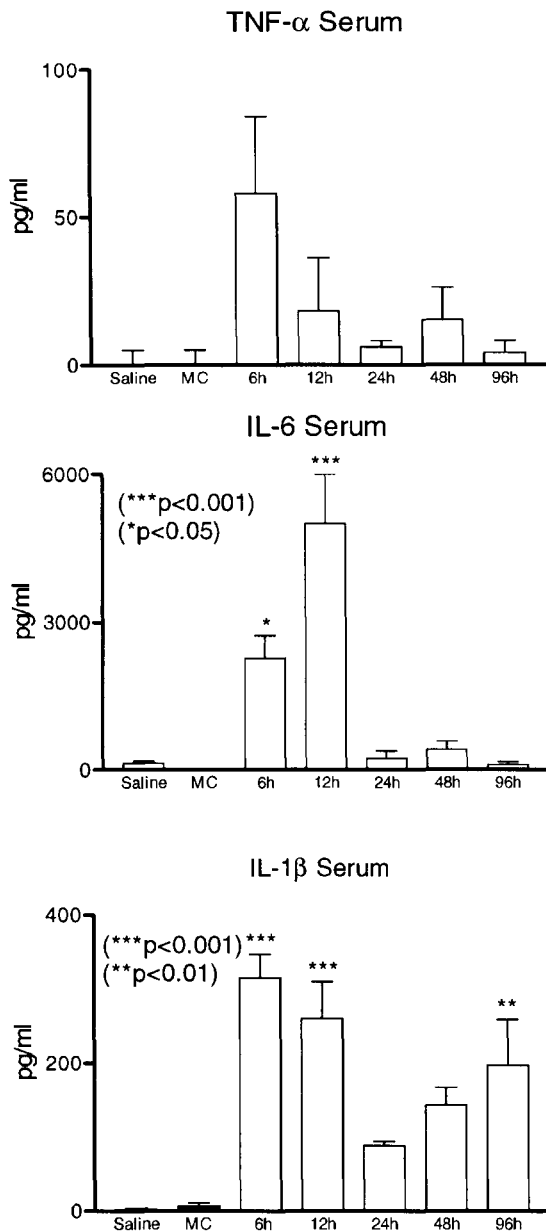


Figure 2. 3 Temporal evolution of TNF- α , IL-6 and IL-1 β levels in the serum of rats following the induction of colitis. Results are represented as mean \pm SEM from different groups of 5 rats for each time interval.

D. Detection and Temporal Evolution of Cytokine levels in whole Intestinal Tissues

I. TNF- α

There were no significant levels of TNF- α in the whole intestinal tissues of the saline and MC groups. There was an early significant rise of TNF- α level at 6hs

(5.4 \pm 0.65 pg/mg of protein; $p < 0.001$) post IA instillation, which was maintained till

24hs (7.7 ± 0.81 pg/mg of protein; $p < 0.001$) and then recovered at 96hs (0.96 ± 0.66 pg/mg of protein; $p > 0.05$). In the colon, TNF- α level was more significant reaching a maximal level at 48hs (18.0 ± 2.0 pg/mg of protein; $p < 0.001$) and was maintained throughout the observation period (Fig. 2.4).

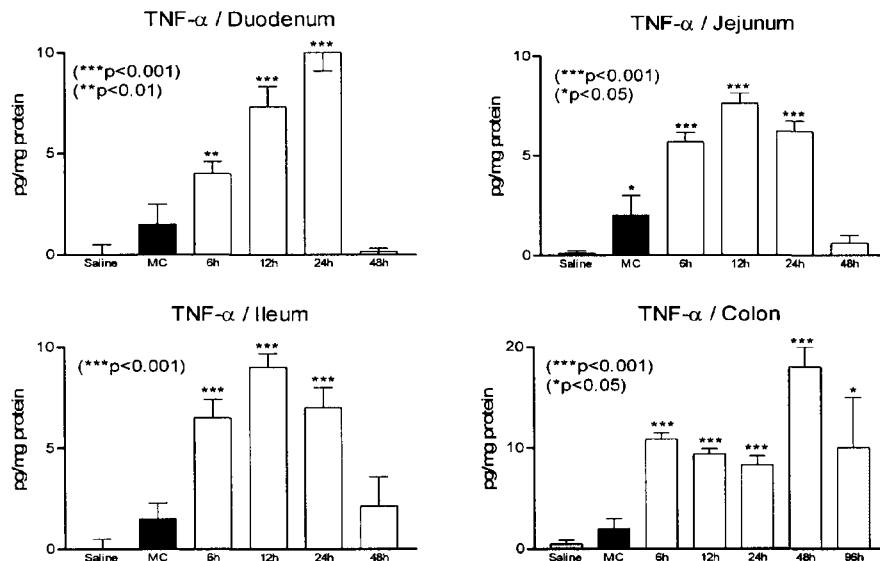


Figure 2.4 Temporal evolution of TNF- α in the whole intestinal tissues of rats following the induction of colitis. Results are represented as mean \pm SEM from different groups of 5 rats for each time interval.

2. IL-6

IL-6 was detectable in the small intestinal tissues of saline and MC groups. The increase of IL-6 level was significant starting 6hs (30.3 ± 5.3 pg/mg of protein; $p < 0.05$) post IA instillation reached a peak at 24hs (59.13 ± 4.82 pg/mg of protein; $p < 0.01$) and then recovered over the period from 48 to 96 hours (7.26 ± 0.63 pg/mg of protein; $p > 0.05$). This increase was more pronounced in the colon, peaked at 6hs (88.8 ± 6.4 pg/mg of protein; $p < 0.01$), and was maintained throughout the observation period till 96hs (7.7 ± 7.0 pg/mg of protein; $p > 0.05$) (Fig. 2.5).

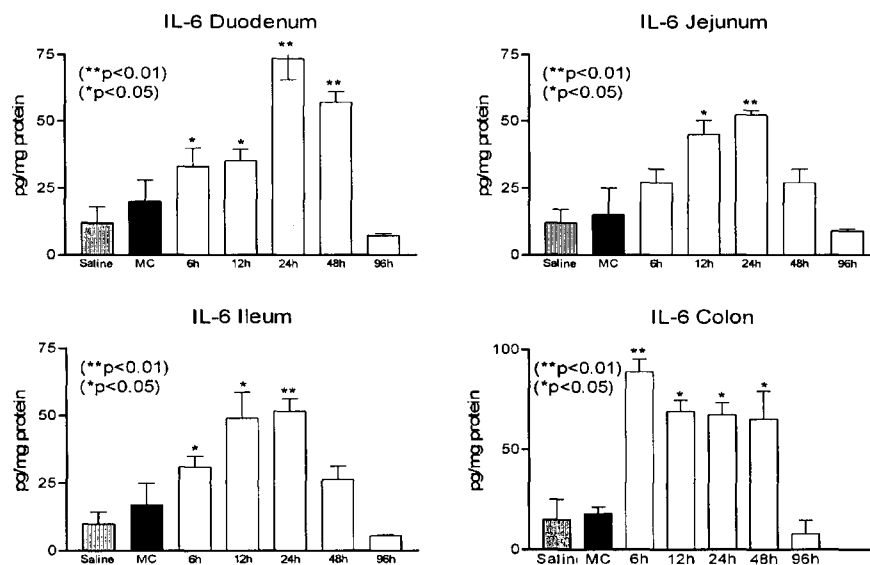


Figure 2. 5 Temporal evolution of IL-6 in the whole intestinal tissues of rats following the induction of colitis. Results are represented as mean \pm SEM from different groups of 5 rats for each time interval.

3. *IL-1 β*

Secretion of small amounts of IL-1 β was observed in the whole intestinal tissues of the saline and MC groups. A delayed but significant peak of IL-1 β level was evidenced at 12hs (624.0 ± 120.0 pg/mg of protein; $p < 0.001$) in the jejunum and ileum of rats with experimental colitis. A more delayed but also a significant peak of IL-1 β was detected at 24hs (719.25 ± 72.5 pg/mg of protein; $p < 0.001$) in the duodenum and colon of rats with experimental colitis. This increase of IL-1 β level was maintained throughout the observation period then recovered at 96hs (266.5 ± 85.75 pg/mg of protein; $p > 0.05$) in all the intestinal sections (Fig. 2.6).

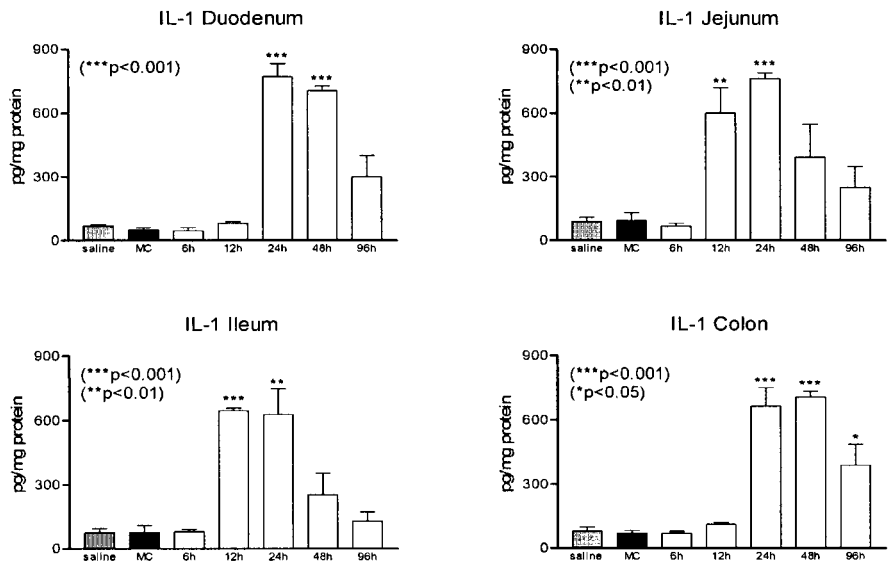


Figure 2.6 Temporal evolution of IL-1 β in the whole intestinal tissues of following the induction of colitis. Results are represented as mean \pm SEM from different groups of 5 rats for each time interval.

4. NGF

Low levels of NGF were detected in the whole intestinal tissues of the saline and MC groups. However, NGF level in the whole intestinal tissues increased significantly and progressively post induction of UC, peaked over the period between 24hs (2262.75 \pm 409.75 pg/mg of protein; p<0.05) and 48hs (2966.5 \pm 470.5 pg/mg of protein; p<0.001) and then almost recovered at 96hs (2005.5 \pm 577.75 pg/mg of protein; p>0.05) in all intestinal tissues (Fig. 2.7). Moreover, the level of NGF in the colonic tissues was 1.5 folds higher than that in the small intestinal tissues.

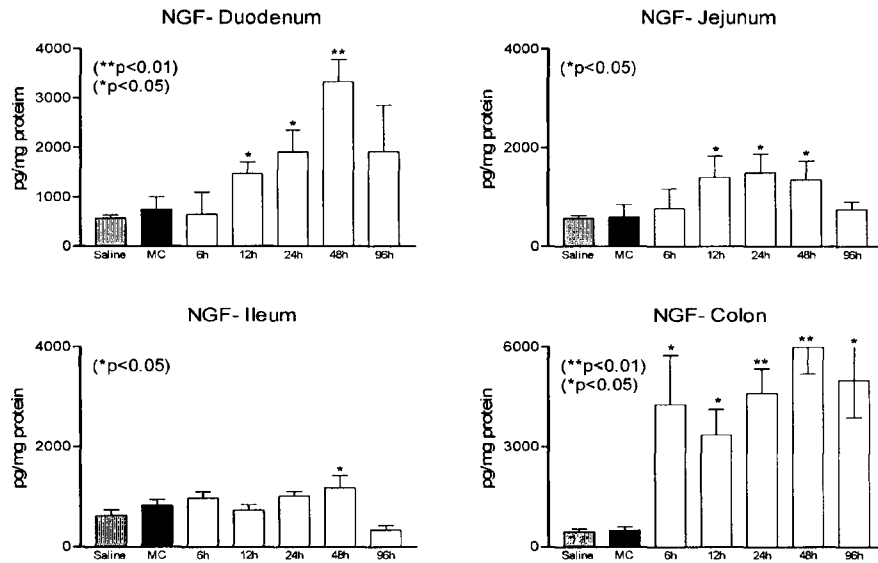


Figure 2.7 Temporal evolution of NGF in the whole intestinal tissues of rats following the induction of colitis. Results are represented as mean \pm SEM from different groups of 5 rats for each time interval.

E. Detection and Temporal Evolution of Cytokine levels in Mucosal Scrapings of Intestinal Tissues

1. *TNF- α*

There were no significant levels of TNF- α in the mucosal scrapings of the saline and MC groups. A significant level of TNF- α was detected very early in the small intestinal mucosa of rats with colitis at 3hs (28.5 ± 2.5 pg/mg of protein; $p < 0.001$), reached a peak at 6hs (34.0 ± 5.0 pg/mg of protein; $p < 0.001$) and then gradually recovered over the period of 24 to 96hs (10.0 ± 6.0 pg/mg of protein; $p > 0.05$) post IA instillation mainly in the duodenum and jejunum. In the ileum, there was a decline in the TNF- α level at 12hs (16.0 ± 4.3 pg/mg of protein; $p < 0.01$) then a significant relapse at 24hs (35.0 ± 2.9 pg/mg of protein; $p < 0.001$) which gradually recovered over the period of 48 to 96 hours (12.0 ± 6.0 pg/mg of protein; $p > 0.05$). Moreover, the TNF- α level in the Colonic mucosa of rats with colitis was significantly elevated at 3hs (79.0 ± 9.0

pg/mg of protein; $p < 0.001$) and was sustained throughout the observation period till 96hs (15.0 ± 5.0 pg/mg of protein; $p > 0.05$) (Fig. 2.8).

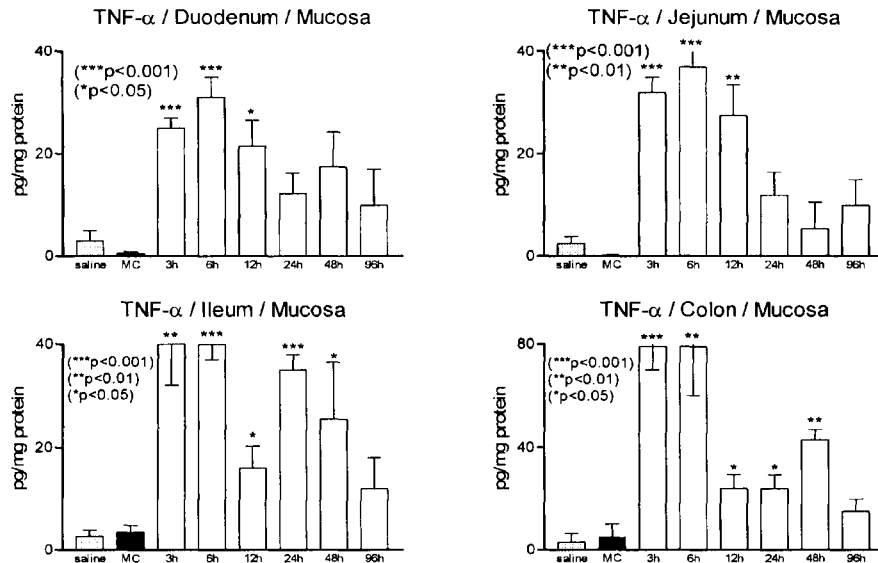


Figure 2.8 Temporal evolution of TNF-α in the mucosal scrapings of intestinal tissues of rats following the induction of colitis. Results are represented as mean \pm SEM from different groups of 5 rats for each time interval.

2. IL-6

IL-6 was detectable in small amounts in the mucosal scrapings of intestinal tissues in the saline and MC groups. The level of IL-6 showed a moderate but significant increase in the duodenal and jejunal mucosa of colitic rats, which peaked at 6hs (25.5 ± 6.0 pg/mg of protein; $p < 0.05$) then gradually declined over the period of 24 to 96 hours (13.5 ± 5.5 pg/mg of protein; $p > 0.05$) post IA instillation. Interestingly the pattern of IL-6 expression in the ileal and colonic mucosa was the same. IL-6 reached a significant peak at 24hrs (54.0 ± 4.85 pg/mg of protein; $p < 0.001$), and then gradually recovered over the period of 48 to 96 hours (22.5 ± 5.65 pg/mg of protein; $p > 0.05$) post IA instillation (Fig. 2.9).

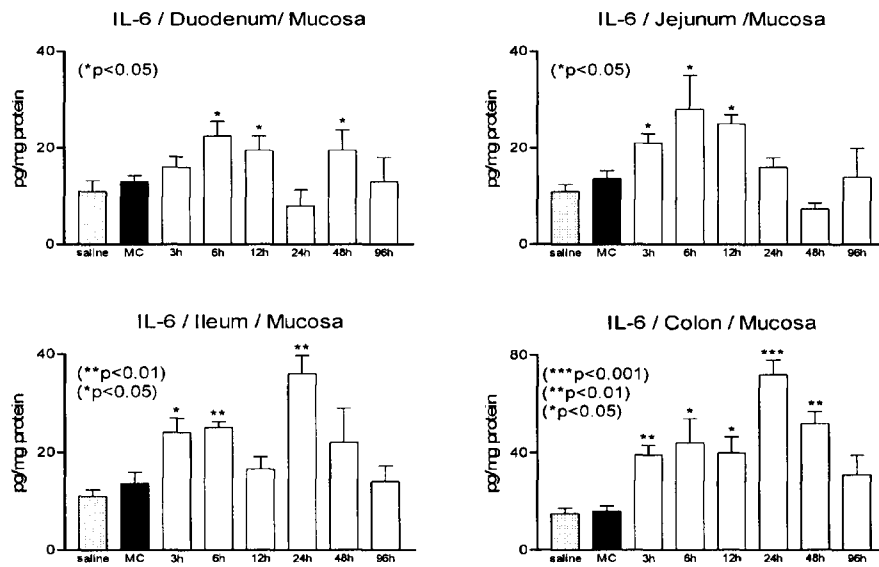


Figure 2.9 Temporal evolution of IL-6 in the mucosal scrapings of intestinal tissues of rats following the induction of colitis. Results are represented as mean \pm SEM from different groups of 5 rats for each time interval.

3. *IL-1 β*

Secretion of small amounts of IL-1 β was observed from mucosal scrapings of intestinal tissues in the saline and MC groups similar to the observation found in whole tissues. There was a delayed and moderate increase in IL-1 β production in the duodenal and jejunal mucosa of rats with colitis that peaked at 48hs (426.0 ± 83.5 pg/mg of proteins; $p < 0.01$) then directly dropped at 96hs (192.5 ± 98.0 pg/mg of protein; $p > 0.05$). In the ileal and colonic mucosa of rats with colitis, the level of IL-1 β was more pronounced, reached a peak at 24hs (865.5 ± 199.0 pg/mg of protein; $p < 0.001$) and then partially recovered at 96hs (222.0 ± 100.0 pg/mg of protein; $p > 0.05$) (Fig. 2.10).

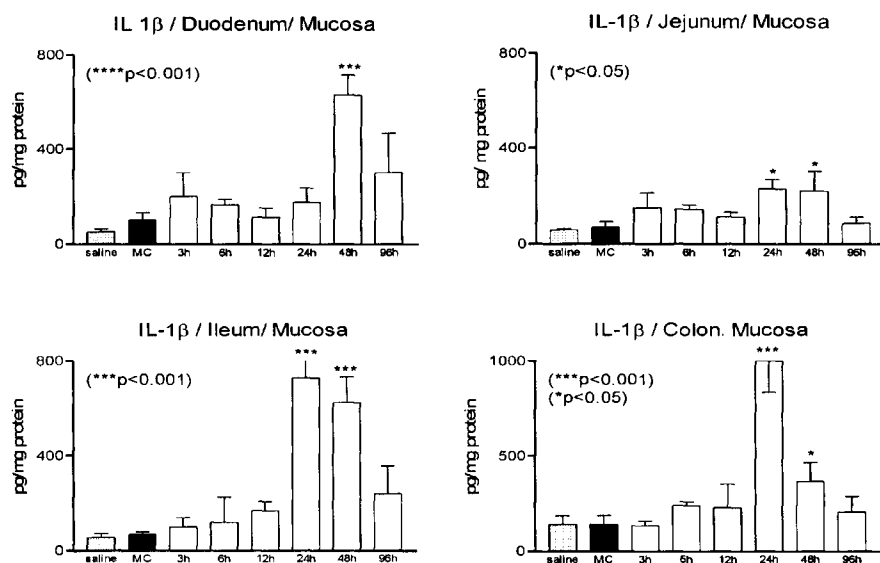


Figure 2.10 Temporal evolution of IL-1β in the mucosal scrapings of intestinal tissues of rats following the induction of colitis. Results are represented as mean ± SEM from different groups of 5 rats for each time interval.

4. NGF

NGF level was expressed in the mucosal scrapings of intestinal tissues in the saline and MC groups. NGF was detected early in the mucosal scrapings of all intestinal sections of rats with colitis. The level of NGF in the duodenal and ileal mucosa of rats with colitis was moderately increased starting 3hs (225.0 ± 56.5 pg/mg of protein; $p < 0.05$), then gradually decreased reaching a non significant level at 96hs (105.0 ± 53.5 pg/mg of protein; $p > 0.05$). Moreover, the level of mucosal NGF in the jejunum was more pronounced reached a peak at 3hs (286.0 ± 32.0 pg/mg of protein; $p < 0.001$) then gradually declined till recovery at 96hs (66.0 ± 25.0 pg/mg of protein; $p > 0.05$) post IA instillation. In the colon of rats with colitis, the expression of NGF was detected at 3hs (432.0 ± 75.0 pg/mg of protein; $p < 0.05$), reached a peak at 24hs (1297.0 ± 200.0 pg/mg of protein; $p < 0.001$) then showed a prolonged recovery with the NGF level still significant at 96hs (401.0 ± 21.0 pg/mg of protein; $p < 0.05$) (Fig. 2.11).

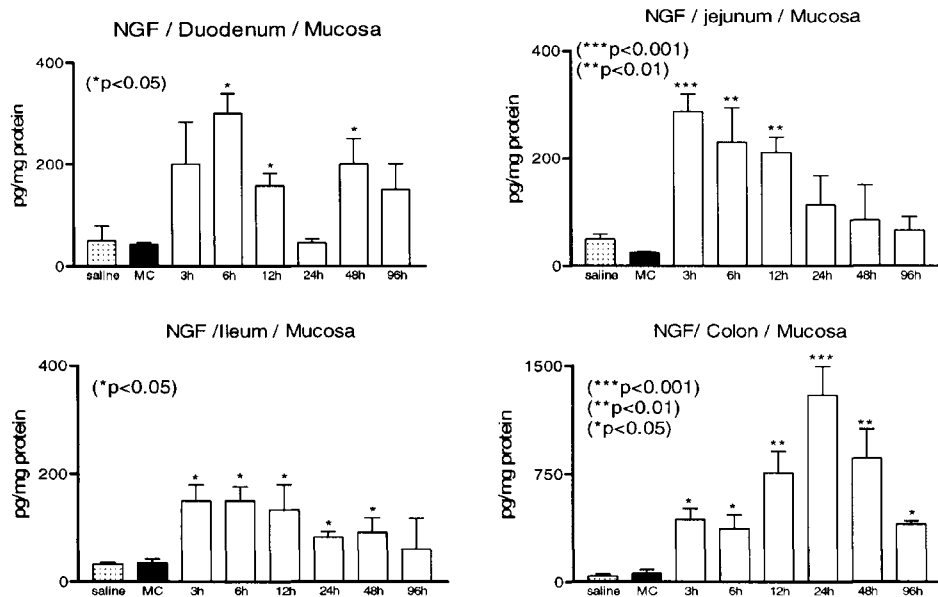


Figure 2.11 Temporal evolution of NGF in the mucosal scrapings of intestinal tissues of rats following the induction of colitis. Results are represented as mean \pm SEM from different groups of 5 rats for each time interval.

F. Histology

There was an evident inflammation in the colon of rats with experimental colitis. The main features were ulceration of the epithelium with complete loss of the crypts, heavy infiltration by inflammatory cells and tissue edema. Those changes peaked 2-3 days post induction of colitis and resolved by 8 days post induction.

However, the small intestine of rats with experimental colitis showed no evidence of alterations of villous architecture and no frank inflammation in the duodenum, jejunum or ileum.

Normal



Colitis

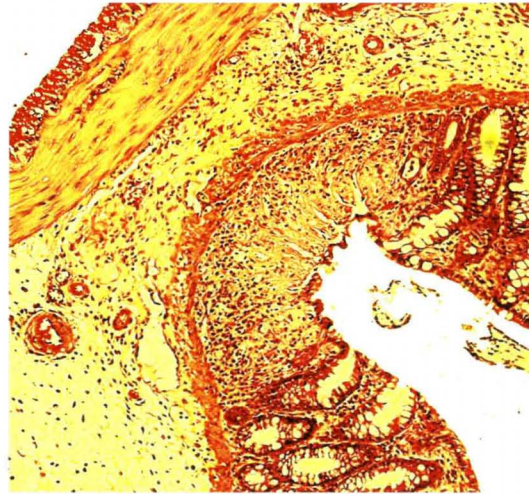


Figure 2.12 The histology of the colon 48hs following the induction of colitis.

Normal



Colitis

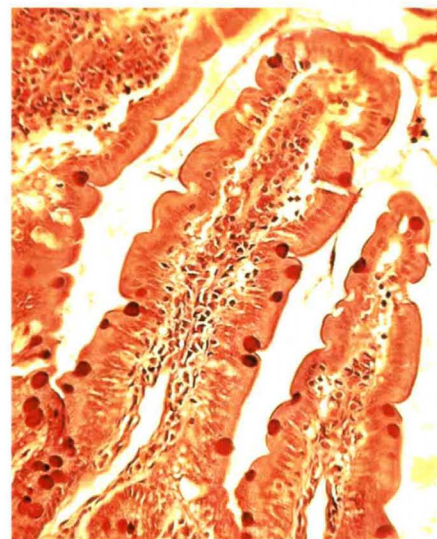


Figure 2.13 The histology of the jejunum 48hs following the induction of colitis.

DISCUSSION

Ulcerative colitis is a chronic inflammatory disease confined to the colon, whose etiology remains unknown. Nevertheless, some studies have shown structural and functional abnormalities in the small intestine of patients and animals models with UC. Interestingly, authors have described several cases of clinical UC associated enteritis (Rubenstein et al., 2004) and duodenitis (Honma et al., 2001). Moreover, human and experimental colitis have been associated with a decrease in intestinal water (Binder and Ptak, 1970; Mourad et al., 2001), aminoacids (Zetzel and Banks, 1942; Barada et al., 2001), D-xylose (Anderson et al., 1971), and fat absorption (Chakravarti et al., 1973). In addition, Fries et al., (1999) showed that experimental colitis increases tight junction permeability throughout the entire small intestine, and that the extent of alterations correlates with colonic damage. However, the impairment of the small intestine function in UC has not been fully elucidated. Thus, the aim of this study was to detect discrete factors that could explain altered small bowel function in ulcerative colitis.

In this study, Iodoacetamide (IA), a sulfhydryl group blocker, was intrarectally administered to induce ulcerative colitis in rats. This was based on the fact that endogenous sulfhydryl (SH) compounds such as glutathione play an important role in the protection of the gastric mucosa (Szabo et al., 1981, 1988). Thus, IA causes injury of the mucosa by decreasing the amount of defensive SH compounds. Moreover, Satoh et al., (1997) found that the instillation of IA in the colon caused UC similar to that observed in humans and characterized by mucosal damage with inflammation, diarrhea with bleeding, and decrease in body weight. In our study, similar observations were noticed in the rat model of UC.

Based on the above described experimental model, the results of this study appear to provide a molecular substrate for the different clinical observations seen in patients with UC. This substrate is based on the significant increase of proinflammatory cytokines in the serum, colon (the site of injury) and in intestinal compartments that did not show any alteration of villous architecture or frank inflammation.

Recently, Komatsu et al., (2001) was the first to report that the serum concentration of TNF- α in IBD patients was strikingly higher than in controls (\approx 390-fold higher) and that serum TNF- α could provide an important pathophysiologic marker for the presence and activity of IBD. Furthermore, studies have shown that a significant correlation exists between serum IL-6 levels and disease activity in patients with UC (Holtkamp et al., 1995) and that serum IL-6 may be a good predictor of UC in pediatric patients with suspected or newly diagnosed UC (Brown et al., 2002). Finally, Hymas et al., (1994) have shown that IL-1 β levels were only slightly elevated in the plasma samples of patients with IBD.

It is well established, that the increase in serum proinflammatory cytokines can alter the brain function and induce a spectrum of behavioural changes known as sickness behaviour. Sickness behaviour is characterized by changes such as fever, reduced activity, reduced appetite, and reduced social interaction (Konsman et al., 2002). Moreover, circulating cytokines may affect many functions of the central nervous system (CNS), such as impairments in learning and memory that are largely mediated by IL-1 (Banks et al., 2002). Interestingly, similar signs of sickness behaviour such as fever, reduced activity and reduced appetite were observed in the rats with experimental colitis. Furthermore, the observed changes in behaviour correlate with the severity of the disease.

Our study shows that there is a low level baseline production of TNF- α , IL-6, IL-1 β and NGF in sham and saline treated rats. This finding is supported by previous data reporting an increase in proinflammatory cytokines in the stools and biopsy specimens obtained from healthy controls (Nicholls et al., 1993). This observation can be attributed to the fact that there is a constant state of low-grade inflammation in the healthy gastrointestinal tract in response to foreign antigens in the lumen of the gut (Stephen, 1995).

Our results also show a significant increase in the level of cytokines in the whole intestinal tissues and absorptive mucosa of the small intestine in rats with experimental colitis. As an illustration, TNF- α level was very low in the full thickness strips and in the mucosa of control rats, but elicited an early up regulation in different parts of the small intestine as well as near the site of injury in the colon following the induction of colitis. This observation has suggested a key role of TNF- α in the cascade formation of proinflammatory cytokine that was at the origin of IL-6 and IL-1 β upregulation. Furthermore, this hypothesis was supported by the delayed upregulation of IL-1 β in the small intestinal sections at 24 hrs post IA instillation and this increase could be related to the jejunal absorptive deficit described in the same experimental model (Barada et al., 2001).

In addition, there was a correlation between the temporal evolution of TNF- α , IL-6 and IL-1 β levels and that of the ulcer scores. Ulceration in the colon was observed as early as 3 hours after Iodoacetamide administration and was associated with marked increase of TNF- α and IL-6 production. As the inflammation in the colon started to subside 96 hours after induction, the levels of TNF- α , IL-6 and IL-1 β returned to their control values. These results suggest a role of these proinflammatory

cytokines in the initiation, perpetuation and decline of the colonic inflammation in colitis.

Studies measuring TNF- α , IL-6 and IL-1 β levels in the inflamed and non-inflamed tissues of patients with IBD have yielded controversial results. However, most have found increased expression of these cytokines at the mRNA, and protein levels in the affected areas of the gut (Dieleman et al., 1996; Carty et al., 2000). Our animal model of colitis have provided an experimental confirmation of these results and expanded further the observation to other intestinal areas not directly involved in the inflammatory process. In addition, our results have presented a full account on the time course of the variations in the expression of these cytokines.

Relatively few studies have addressed cytokine effects on the intestinal epithelial function and several features contributed to the difficulties of interpretation of such studies. First, cytokines act in a coordinated interplay. Second, given the complex intercellular signalling that occurs within the gut, cytokine effects may involve other non-cytokine mediators. Finally, virtually every putative mediator of inflammation has been implicated in diseases of the intestine.

The enteric epithelium serves as a dynamic barrier which maintains regulated uptake of nutrients and water at the same time as excluding potential pathogens. It also regulates normal homeostasis, innate immunity and regulation of acquired immunity at both sensory and effector levels. Moreover, the epithelium have the capacity to produce bioactive cytokines and other immunoregulatory molecules, thus it is identified as both target and source of cytokines. Furthermore, epithelial derived cytokines have the potential to play an autocrine role or to influence adjacent non-epithelial cells.

Epithelial permeability, which reflects the integrity of the epithelial barrier, may be compromised in diseases. Luminal material crosses the epithelial sheet via the transcellular or paracellular pathways. Tight junctions form a circumferential seal at the luminal pole of adjacent epithelial cells and are the rate limiting factor regulating paracellular permeability. However, such junctions express a high degree of plasticity and may be greatly affected by proinflammatory cytokines, thus reducing barrier integrity (Madara et al., 1992).

Furthermore, transepithelial electrolyte movement creates driving forces for directed water and nutrient movement and results from coordinated activity of ion channels, transporters and pumps, which have polar distributions in the cell membrane. Electrophysiological studies using single epithelial cell types tend to show few rapid or direct effects of cytokines on electrogenic ion transport. IL-1 β (Chang et al., 1990) and TNF- α (Kandil et al., 1994) have been found to stimulate epithelial ion secretion. In each case, the effects of the cytokines were indirect with the change in ion transport being mediated by prostaglandins from subepithelial cells.

Interestingly, some very recent studies have shown that the injury, inflammation or manipulation of a part of the intestine can be associated a dysfunction in other part of the gut with or without any evident of inflammation, as in field effect. For example, the surgical manipulation of the jejunum may upregulate the expression of proinflammatory cytokines in the stomach and colon which might explain in part the post operative delay in gastric emptying and ileus (Schwarz et al., 2004). Moreover, it was reported that the function of the enteric nervous system is altered in the inflamed and non-inflamed areas of the gut in experimental colitis. Thus, explaining in part the altered gut motility reported in humans and animal models with UC (Jacobson et al, 1995).

Our study was the first to demonstrate increased expression of TNF- α , IL-6, IL-1 β and NGF in the colon as well as in the non-inflamed segments of the duodenum, jejunum and ileum. Our results have presented a full account on the time course of the variations in the expression of these cytokines. In our model, there was no evidence of major inflammation in the small intestine in rats with colitis. Thus, this was not a case of inflammation extending from the colon to the small intestine. The mechanisms and signals for the marked increase in proinflammatory cytokine expression in non-inflamed areas of the intestine are not clear.

In line with the above presented data, this study has opened the way to investigate the effect of each cytokine on intestinal absorption *in vivo* and *in vitro*. Preliminary results from our laboratories tend to confirm the hypothesis of alteration in intestinal absorption by proinflammatory cytokines such as IL-1 β and TNF- α .

In conclusion, proinflammatory cytokines are elevated in the inflamed colon as well as in the non-inflamed segments of the small intestine of rats with experimental colitis. These cytokines may serve as a substrate for the functional alterations in the small intestinal function that have been reported in patients and animals with UC. These include altered small bowel motility, reduced absorption of nutrient, water and electrolyte as well as changes in the enteric nervous system structure and function.

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