# Glucagon-like Peptide 2 and Inflammatory Bowel Disease

by

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## Abstract

Management of inflammatory bowel disease, including Crohn's disease, remains unsatisfactory despite currently available immunomodulatory and anti-inflammatory therapies. Regulation of the epithelial barrier of the gastrointestinal tract may provide a direct route for therapeutic intervention either preventing or ameliorating inflammation. The single cell thick epithelium lining the gastrointestinal tract provides a barrier to the entrance of non-desirable luminal contents such as bacteria, while allowing for the absorption of nutrients, ions and fluids. Disruption of this epithelial barrier occurs in inflammatory conditions such as parasitic or bacterial infections and in chronic inflammatory bowel disease.

The gastrointestinal epithelium is maintained through the actions of numerous mitogenic and cytokine peptides. Alterations in the mitotic index or in the rate of migration of cells along the crypt-villus axis may result from changes in the interstitial endocrine or cytokine milleu due to infection or inflammation. Glucagon-like peptide 2 (GLP-2) has been shown to be a significant and specific mitogen for the epithelia of the gastrointestinal tract. Recent development of a non-degradeable analog of GLP-2, ALX-0600 has precipitated investigation into the benefits of ALX-0600 therapy in models of gastrointestinal inflammation including radiation, chemotherapy and dextran sulfate induced enteritis. This thesis reports on the effects of ALX-0600 pretreatment on an acute model of ileitis in the guinea pig, and on experiments aimed at developing a reactivating model of ileitis. Further to these investigations, we report a seasonal variation in the development of TNBS ileitis. ALX-0600 pretreatment and therapy was also tested in an alternative model of inflammatory bowel disease, indomethacin-induced enteritis.

An acute ileitis was developed following the intraluminal injection of TNBS in ethanol; increased small intestinal permeability and severe macroscopic damage characterized disease. ALX-0600 pretreatment had no effect upon the development or progress of TNBS induced ileitis in the guinea pig, and did not appear to demonstrate its normal mitogentic effects upon the epithelium. ALX-0600 therapy in this model of ileitis does not suggest a use in the treatment or prevention of Crohn's disease. Reactivation of ileitis was not reproducible despite extensive investigation and therefore testing of the effects of ALX-0600 on reactivating ileitis was not carried out. Seasonal variations in the development of TNBS ileitis have not previously been reported. Our investigations indicate a distinct circannual rhythm whereby ileitis was not produced during the summer months but was clearly observable during other seasons. This distinct seasonal variation, in combination with the prolonged time course of each experiment may be cause for caution in employing this model in subsequent investigations.

Indomethacin-induced enteritis in the rat is another common model of Crohn's disease, which was employed in investigation of ALX-0600 as a treatment for inflammatory bowel disease. Pretreatment of rats with ALX-0600 for one week was protective against the damage induced through two daily injections of indomethacin, while administration of ALX-0600 beginning concurrently with the indomethacin was able to accelerate the healing of the enteritis. The overall increase in small intestinal permeability, a marker of damage, seen in the indomethacin controls, was not present in either treatment group. Granulocyte infiltration was reduced in the pretreated animals seven days following indomethacin. Fourteen days following induction of enteritis there was significantly less granulocyte infiltration in both the pretreatment and concurrent treatment

groups. Macroscopic damage was significantly reduced compared with the indomethacin controls in the pretreatment but not the concurrent treatment group; however, fourteen days following the induction of enteritis both the groups receiving ALX-0600 had significantly less macroscopic damage than did the indomethacin control group. Animals receiving only ALX-0600 and indomethacin vehicle demonstrated the previously documented effects of ALX-0600 including increased villus length. They also demonstrated a significant, and previously undocumented *in vivo* decrease in permeability compared with controls. ALX-0600 is an effective treatment in this model of inflammatory bowel disease and the decrease in permeability in the control group may indicate a method of preventing the development of IBD or relapse in patients with remissive IBD where increased permeability is supposed to be a predisposing factor.

The difference between these two models lies in both the system and the type of disease initiated. TNBS ileitis in the guinea pig is initiated through an immune reaction to a non-self epitope created when TNBS absorbs to lysine residues on the surface of interstitial cells. Indomethacin enteritis results from a direct cytotoxic effect upon the epithelial cells, particularly in the crypt. This initiates an inflammatory reaction due to tissue necrosis and the infiltration of luminal contents to the interstitial space. The inability of ALX-0600 to affect the course or development of TNBS ileitis may be due to the inability of the hormone analog to affect the anaphylaxis that initiates the disease. TNBS ileitis is a transient disease therefore increasing the healing rate of the epithelia by increasing the mitotic index may not have detectable effects. Indomethacin induced enteritis is the result of cytotoxicity and the inhibition of other mitogens such as $PGE_2$ and epidermal growth factor (EGF). The effects of ALX-0600 may have been the result of

replacing $PGE_2$ and epidermal growth factor and thereby increasing the healing rate of the enteritis. Treatment of human inflammatory bowel disease with ALX-0600 may be marginally beneficial as some of the damage is thought to result from disregulation of the gastrointestinal epithelia. Investigation of the effects of ALX-0600 on other models of inflammatory bowel disease and clarification of the underlying cause of IBD will aid in the development of this or other therapies.

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*To my family, with loving thanks for years of support and encouragement.*

*And for those special people who help to make it all worth while, you know who you are.*

Happiness lies in being privileged to work hard for long hours in doing whatever you think is worth doing.

<u>To Sail Beyond the Sunset</u>
Robert Heinlein

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| | |
|---|---|
| ALX-0600 | Human [$Gly^2$] Glucagon-like peptide 2 |
| BrDU | 5-bromodeoxyuridine |
| cm | centimetre |
| CTL | Cytotoxic lymphocyte |
| DABA | |
| EDTA | Disodium ethylenediamine tetraacetate |
| EGF | Epidermal growth factor |
| EGFR | Epidermal growth factor receptor |
| Fractional Excretion | [(volume recovered)*(concentration recovered)]/[(volume given)*(concentration given)] |
| g | gram |
| $G_0$ | Resting state of mitosis |
| $G_1$ | Beginning of mitosis |
| GLP-2 | Glucagon-like peptide 2 |
| HB-EGF | Heparin-binding epidermal growth factor-like growth factor |
| HPLC | High performance liquid chromatography |
| HTAB | hexadecyltrimethylammonium bromide |
| IBD | Inflammatory Bowel Disease |
| IGF | Insulin-like growth factor |
| IL-10 | Interleukin 10 |
| kg | Kilogram |
| L | litre |
| Lac/man | lactulose/mannitol excretion ratio |
| µl | microlitre |
| MHC | Major histocompatibility complex |
| mg | milligram |
| ml | millilitre |
| NSAID | Non-steroidal anti-inflammatory drug |
| PBS | Phosphate buffered saline |
| PC2 | Prohormone convertase 2 (Dhanvantari, 1996) |
| $PGE_2$ | Prostaglandin E 2 |
| TGF α | Transforming growth factor alpha |
| TGF β | Transforming growth factor beta |
| TNBS | Trinitrobenzene sulfonic acid |
| TNF α | Tumor necrosis factor alpha |

# Chapter 1 Introduction

## 1.1 Inflammatory Bowel Disease

Crohn's disease was described as early as 1612 by Hildenus and often described as an 'untreatable abdominal tumor'. Reports continue to describe details of pathological and histological features during the next 300 years although an increased number of cases were documented in the 1900s. It was one of the many conditions included in the general diagnosis of "bloody flux" and was characterized by diarrhea, fever and weight loss. Pathological involvement of the ileum or ileal-cecal valve was common (1). It was identified through the publication of a widely read article in the Journal of the American Medical Association in 1932 by Crohn, Ginzburg and Oppenheimer (2). Crohn's disease is one of two recognized members of the inflammatory bowel diseases, the other being ulcerative colitis. While Crohn's disease can affect any portion of the gastrointestinal tract from the mouth through to the anus, ulcerative colitis affects only the colon and rectum. The inflammation seen in Crohn's disease is transmural and patchy in appearance while ulcerative colitis affects the epithelial layer of the colonic mucosa in a continuos manner. (1) Today numerous factors are considered to contribute to the etiology of inflammatory bowel disease. Infectious agents, especially members of the *Mycobacterium* family, have been proposed to be the causative agent in Crohn' s disease; however, no bacterium has ever been consistently cultured from the luminal aspirates of patients with active disease. (3, 4) While the case of *H.pylori* cautions investigators against quick judgement in this matter, other factors present a more likely scenario (5). Genetic predisposition, in either an immunological or epithelial regulation factor, is strongly suggested to be a major

component in the development of the disease. Environmental factors such as dietary components, chemical contaminants, psychological stress and even hygiene have also been implicated in disease etiology (6). The luminal environment is a crucial component in disease as patients who undergo diverting ileostomy experience remission of the disease present distal to the diversion, but recurrence soon after the fecal stream is restored (7). Furthermore, animals such as the interleukin-10 knock-out mice that develop spontaneous colitis do not develop disease when raised in a germ-free environment (8). The most commonly held hypothesis remains that disease results from an abnormal immunological reaction to a normally present luminal antigen. (6) The mechanism of this reaction remains a source of controversy.

Crohn's disease is thought to be an abnormal immune response to a normally present luminal component that upon presentation to the mucosal immune system initiates an inflammation which becomes chronic due to disregulation of the immune response. Presentation of the luminal component, be it bacterial or virus derived or dietary, involves presentation of the antigen to the immune cells in the lamina propria. Normally, luminal antigens are taken up through the M cells in the Peyer's patches and presented to T cells in a manner similar to what is seen in the thymus during development, such that tolerance is initiated to the antigen, similar to the recognition of self versus non-self. There is also some evidence that luminal antigen may be taken up by epithelial cells and presented to CD8+ Tcells (suppressor T cells) or in the absence of co-stimulatory molecules to CD4+ Tcells, resulting in an anergic response, ie tolerance (9). In active inflammatory bowel disease, tolerance is abrogated to normally present luminal contents (10). This implies that antigen is being absorbed into the lamina propria through the paracellular space

between cells. This theory is supported by the study by Yacyshyn and Meddings who demonstrated, in unaffected relatives of Crohn's patients, that increased intestinal permeability is associated with increased levels of CD45RO, a marker of antigenic stimulation on B cells. (11) Paracellular permeability is a function of the tight junction, which exists between the epithelial cells, and is regulated by the actin-myosin ring that exists below the microvilli on the apical surface of the cells (12). Tight junctional morphology has been shown to be abnormal in non-inflamed areas of the gastrointestinal tract in patients with Crohn's where the tight junctional strands appeared fragmented and irregularly distributed (13, 14). The passage of molecules through the paracellullar space is referred to as permeability (15). *In vivo* permeability may be assessed using non-metabolized sugars such as mannitol, lactulose and sucralose (16) or molecules such as PEG 400 (17) or $Cr^{51}$-EDTA (18). Markers are excreted in the urine over periods of six to twenty-four hours and quantified to assess intestinal permeability (19). Patients with inflammatory bowel disease demonstrate increased permeability to most markers during active disease. Active inflammation can exacerbate permeability defects as the infiltration of granuloctyes destroys tight junctions (20) and the expression of tumor necrosis factor alpha (TNF-$\alpha$) and interferon gamma decrease the viability of colonic epithelial cells (21). Patients also demonstrate increasing permeability prior to relapse (22), and to proteins in non-inflammed areas of the ileum (23). A subset of patient family members, approximately 10%, demonstrate increased permeability in the absence of clinical disease, the same proportion as would be expected to develop the disease (24, 25). Use of non-steroidal anti-inflammatory drugs (NSAIDS) is known to cause an increase in intestinal permeability and patients with inflammatory bowel disease respond poorly to NSAIDS,

often experiencing relapse after use. Normal family members have been shown to respond with abnormal increases in permeability in response to NSAIDs. (26, 27) Abnormal permeability is considered by most experts to be one of the key factors in both the development and progress of Crohn's disease. There are no current methods of modifying permeability *in vivo*, therefore, avoidance of substances and circumstances known to exacerbate permeability is the current therapeutic advice to patients. (28)

Disease management currently consists of anti-inflammatory and anti-microbial therapies, which may induce remission for a period of time. Relapse is common and provides a continuous challenge to both patients and the medical care community. Factors which may precipitate relapse include psychological stress (29), use of non-steroidal anti-inflammatory drugs or infection in another region of the body, typically respiratory infections. A common therapeutic drug, misoprostol, attenuates an increase in permeability in an experimental model (30). While current therapies have focused upon relief of the patient's symptoms, and surgical removal of the affected portions of the gut in extreme cases, new immunomodulatory therapies are attempting to alter the course of the disease by treating one of the presumed underlying causes of the disease, immune deregulation. In this new treatment protocol, anti-tumor necrosis factor $\alpha$ is (TNF-$\alpha$) used to control inflammation by interrupting the recruitment of neutrophils and activation of inflammatory mediators, thereby lessening the immune reaction and down-regulating the inflammation (31). The development and testing of therapies for inflammatory bowel disease and investigation of the underlying defects in the development and progress of IBD has led to the development of many models of inflammatory bowel disease based upon immunological or cytotoxic mechanisms.

## 1.2 Models of Inflammatory Bowel Disease

Models of inflammatory bowel disease mimic human disease in that they develop inflammation in portions of the gastrointestinal tract in response to either directly toxic compounds or immune challenge or disregulation. Mice given dextran-sulfate orally develop a diffuse colitis which resemble inflammatory bowel disease (32). Administration of indomethacin results in severe small intestinal injury in rats through direct cytotoxic destruction of the crypt cells (33). Both result in erosion and ulceration and demonstrate increased intestinal permeability. Immune challenge through direct injection of bacterial derivatives peptidoglycan-polysaccharide to the lamina propria results in an inflammatory response which resembles Crohn's disease (34). Disruption of the barrier using a compound such as ethanol allows interstitial labeling of cell surfaces with haptens such as trinitrobenzene sulfonic acid which results in an immune response which also resembles inflammatory bowel disease, demonstrating increased permeability, alterations in mucosal morphology, ulceration and inflammatory cell influx (35). Molecular biology has allowed the development of models of inflammatory bowel disease, which result spontaneously from disregulation of the immune system. IL-10 knock-out mice develop a severe diffuse colitis within 10 weeks of birth. (8) There is only one spontaneous model of inflammatory bowel disease that does not require molecular alterations. Cotton-top tamarins kept in captivity develop a spontaneous form of intestinal enteritis (36). None of the models completely mimic human disease and all have the disadvantage of being primarily acute

models, which do not undergo spontaneous relapse. However, they do allow the investigation of either the underlying causes of inflammatory bowel disease, or preliminary assessment of therapeutic options.

## Chapter 2

### Regulation of Intestinal Epithelial Cell Proliferation and Integrity

The intestinal epithelial barrier is maintained through the actions of numerous growth factors including gastrin, growth hormone, and members of the epidermal growth factor superfamily. Because the development of Crohn's disease is proposed to include a disruption of the epithelial barrier, research into epithelial regulation in the small intestine has been intensifying in recent years. Also, the use of exogenous growth factors to attenuate the damage during active disease may provide a therapeutic intervention for patients who relapse. The life cycle of intestinal epithelial cells is brief. Cells are produced from progenitor cells in the crypt. They differentiate as they migrate along the crypt-villus axis, becoming specialized cells for the absorption of nutrients and are shed following apoptosis at the villus tip. This process, in humans, takes three days. While the production and migration of cells along the crypt-villus axis has been observed many times through BrDU labeling, the factors which influence the mitotic index for crypt progenitor cells is less well understood.

Members of the epidermal growth factor superfamily are important in the regulation of the gastrointestinal mucosa. Epidermal growth factor is secreted from many sources in the gastrointestinal tract including salivary and Brunner's glands. It acts through a g protein receptor to activate protein kinase C and elicits cellular responses, including proliferation. Administration of recombinant human EGF to rats for four weeks induces a marked increase in mucosal mass in the small intestine (37) and along with TGF-$\alpha$, which acts through the EGF receptor (EGFR) stimulates crypt cell proliferation (38). Disruption of the EGFR disrupts the morphology of the small intestine as the enterocyte

proliferative rate decreases resulting in fewer and shorter villi (39). Epidermal growth factor protects the gastric mucosa from damage due to numerous injurious substances (40, 41) and is integral to the maintenance of small intestinal mucosal integrity (42). Sialoadenectomy decreases luminal EGF and is associated with increased levels of intestinal permeability to $Cr^{51}$ - EDTA, an effect that was reversible by EGF supplementation. Lower levels of EGF in salivary secretions have been found in patients with Crohn'sdisease (43) suggesting a defect in either the regulation of mucosal integrity or a defect in mucosal repair. Treatment of intestinal inflammation with EGF has not been extensively investigated at this time, although there are some reports of therapy with EGF in children with necrotising enteritis (44) and congenital microvillus atrophy (45, 46). Procaccino did not find that EGF was useful in the therapy of colitis in rats, however, it has been shown to be trophic for parenteral nutrient induced atrophy when administered in combination with protease inhibitors (47). During wound healing and re-epithelization, the uptake of EGF is increased and is associated with increased proliferative rates in the associated tissues (48) and is elevated in the tissues surrounding healing gastric ulcers (40). There is some suggestion that EGF does not act alone in increasing the proliferative rate of enterocytes. Duncan *et al* demonstrated that EGF acts as a competence factor for insulin-like growth factor I (a member of the EGF superfamily) by promoting the transition from $G_0$ to $G_1$. The two growth factors acted synergystically to promote proliferation (49).

Insulin-like growth factor I (IGF-I) also mediates the effects of growth hormone on the gut (50, 51). Growth hormone stimulates growth of all soft organs and over-expression is clinically associated with acromegaly, however, in experimental situations

where growth hormone was associated with increased mucosal mass, or healing of enteritis, increases in IGF-I were demonstrated (52, 53). IGF-I transgene expression in mice increased both small bowel weights due to increased mucosal surface area and small bowel length (54); a similar effect was demonstrated through exogenous administration of IGF-I (55). Evaluation of IGF-I in mucosal injury has been limited, however, it has been suggested that it improves healing of the intestinal mucosa from radiation induced damage in rats (56). Members of the epidermal growth factor superfamily are clearly important in the maintenance of the intestinal barrier, being protective against the damaging effects of cytotoxic agents and augmenting the healing of wounds. Therapeutic use of these growth factors, EGF, TGF-α, or IGF-I, requires specificity for the crypt progenitor cells of the gastrointestinal tract. EGF and TGF-α and IGF- I are pluripotent mitogens, which limit their therapeutic potential in Crohn's disease.

Gastrin is a well-recognized hormone in the gastrointestinal tract. It acts primarily as a stimulant o gastric acid secretion but also functions as a mitogen for the oxyntic mucosa of the stomach (57) which is produced in response to feeding. It has also been shown to be trophic for the colon (58, 59) and the esophagus (60). There are no reports of trophic effects for gastrin in the small intestine however, the effects of gastrin may be mediated through a member of the epithelial growth factor superfamily member, heparin binding epidermal growth factor-like growth factor (HB-EGF), the expression of which is induced by gastrin. (61) The proliferative effects of gastrin on the stomach enterochromaffin cells can be completely blocked by blocking the epidermal growth factor receptor (EGFR). This suggests that gastrin is causing proliferation of cells indirectly through interaction with members of the epidermal growth factor superfamily. Therapeutic

use of gastrin for the treatment of IBD is not realistic given that it causes the secretion of gastric acid, however, interaction between the signaling mechanisms for other mitogens and the stimuli for gastrin make it a crucial component in monitoring the effects of growth hormone therapy in the gastrointestinal tract.

Keratinocyte growth factor is an epidermal growth factor expressed by mesenchymal cells, which plays a significant role in re-epithelization in the dermis. It is highly over-expressed in inflammatory bowel disease, particularly ulcerative colitis and around the base of mucosal crypts in Crohn's ileitis (62, 63). KGF is distributed similarly to the T lymphocytes in the tissue and the receptor is localized to the crypt epithelial cells (64). Both its distribution and overexpression during active inflammatory bowel disease implicate it in the regulation of the intestinal epithelial response to inflammation and therefore, it has been a subject of investigation for therapy in IBD. It is protective against both chemotherapy and radiation induced enteritis in mice (65) and TNBS induced colitis in rats (66) and dextran sulfate sodium induced colitis in mice (67, 68). However, it is not protective against indomethacin induced enteritis in rats (69). There is some preliminary evidence that KGF upregulates the expression of TGF-$\alpha$ during immune mediated cell proliferation (70) which may suggest interaction of KGF with members of the epithelial growth factor superfamily.

Inflammation in the gut is closely associated with proliferation of the crypt progenitor cells and is mediated through the mechanisms described above. Cytokines released during the inflammatory process are also influential in the regulation of the epithelial barrier. For example, TGF-$\beta$ is inhibitory to the proliferation of enterocytes and is upregulated during inflammation. Interleukins 11 and 3 stimulate crypt cell production

rate and IL-11 inhibits apoptosis in villus tip cells. (71,38). Interleukin 15 has also been shown to stimulate intestinal epithelial cells in culture (38). Clearly the normal regulation of the epithelia barrier is affected by the presence of wounds or inflammation and may be altered through endocrine, immune-mediated or neuroendocrine pathways. The specificity of the pathways remains the most significant problem in the therapeutic use of growth factors or cytokines in the management of inflammaoty bowel disease. With the possible exception of IGF-I, all the factors described above are pluripotent and systemic administration will have significant side effects.

## Chapter 3

### Glucagon-like Peptide 2

Enteroglucagon was identified in 1961 when antisera against pancreatic glucagon were shown to react against intestinal endocrine cells (72). Over time it was determined that although this peptide was chemically distinct from that produced in the pancreas, it resulted from the same gene through a process of differential processing (73). In the pancreatic A cell, glucagon is the major product after processing by PC2 whereas in the small intestine, glicentin, oxyntomodulin, glucagon-like peptide 1 (GLP-1) and glucagon-like peptide 2 (GLP-2) are produced in the L cell (74, 75, 76). GLP-1 was quickly determined to be glucoregulatory and to have a role in the ileal brake mechanism (77). A role for GLP-2 was not elucidated until 1996 when it was observed that nude mice with GLP-2 producing tumors demonstrated crypt cell proliferation resulting in villus growth and increased bowel weight (78).

GLP-2 was shown to increase villus height through stimulation of crypt progenitor cell proliferation and inhibition of apoptosis in the villus tip cells (79). Both neurotensin and GRP stimulate the release of proglucagon derived peptides, including GLP-2 and the beneficial effects of exogenous neurotensin for short bowel rats are likely mediated by GLP-2 (80, 81). GLP-2's effects are detectable within four days and are continue for the duration of GLP-2 administration; however, after discontinuation the villus morphology returns to normal (82). The small intestine functions normally following stimulation by GLP-2 however GLP-2 can upregulate both the expression of sodium dependent glucose transporter 1 (SGLT-1) (83) and basolateral glucose transporter 2 (GLUT2) activity (84).

JJ Holst's group elucidated a further role for GLP-2 in the ileal brake mechanism when they demonstrated that GLP-2 inhibits antral motility (85). Clearly GLP-2 was an important hormone in gastrointestinal physiology, both in terms of epithelial regulation but also for the normal regulation of nutrient absorption. Investigations of the use of GLP-2 in therapy for intestinal disorders began in 1997 when Chance *et al* (86) showed that GLP-2 was protective against parenteral nutrition-induced gut hypoplasia. Measurement of the circulating levels of GLP-2 led to the discovery that GLP-2 is rapidly broken down in the rat and human by the enzyme dipeptidyl peptidase IV (87). Development by Allelix Biopharmaceutical Inc., of Mississauga, of a non-degradable functional analog of GLP-2, ALX-0600, aided in the investigation of its function and possible uses in the treatment of intestinal disorders.

In 1999 Drucker's lab continued to demonstrate the usefulness of GLP-2 using the ALX-0600 analog to reduce the damage caused by dextran sulfate in the colon of the rat (88). Further investigation into the mechanisms of GLP-2 function and possible therapies demanded the identification of the GLP-2 receptor. Allelix Biopharmaceuticals Inc accomplished this in 1999 when they demonstrated that GLP-2 receptors are present in decreasing concentrations from the jejunum through colon and not in other tissues. They also demonstrate that the GLP-2 receptor (GLP-2R) is a member of the g protein superfamily that transduces the signal through adenylyl cyclase thereby increasing the intracellular cAMP levels (89). As previously mentioned, PGDP (proglucagon derived peptides) levels are increased by the hormones GRP and neurotensin. GLP-2 levels are also mediated through the ingestion of nutrients in humans, appropriately for their role in regulation of absorption. Consumption of carbohydrates and fat but not protein induced

an increase in circulating N-terminal immunoreactive GLP-2 in normal fasted humans (90). Identification of the cell types which have GLP-2 receptors will aid in elucidating the mechanisms of GLP-2 action upon the intestine, but identification of the receptor within the small intestine implies possible therapeutic use for intestinal inflammation. Crohn's disease, which primarily affects the ileum, may be a good candidate for GLP-2 therapy.

# Chapter Four

## Methods

### Animal Models

Male Hartley guinea pigs were obtained from Charles River Laboratories and group housed (3-4 per bin) and allowed a minimum of two weeks to acclimatize. They were fed standard rabbit chow, supplemented daily with fresh carrots and lettuce. They had free access to water which was supplemented daily with vitamin C. The guinea pigs were housed under standard 12-hour lighting intervals. During fasting they remained in their regular cages; however, for urine collection they were individually housed in metabolic caging where they had free access to water (with vitamin C supplements) but no access to food.

Male Wistar rats were obtained from Charles River Laboratories and individually housed in metabolic caging. They were allowed a minimum of two weeks to acclimatize. Rats had free access to standard rodent chow and water and were housed under 12-hour lighting intervals. During periods of fasting and urine collection they had free access to water. Prior to urine collection, the hoppers were changed to prevent any contamination with food particles.

All practices followed Canadian guidelines and were approved by the local animal care committee.

### Animal Model: TNBS

Male Hartley guinea pigs, 250-300g, were anaesthetized with halothane at 3.5 parts per litre, oxygen at 1 L/min. The animals were placed upon a heating pad set on low beneath

an absorbent bench pad to maintain body temperature. The abdomen was shaved, cleaned with isopropanol and swabbed with betadine. A midline incision was made using a number 10 scalpel blade and the ileal-cecal junction located and externalized. Ileum was externalized to a minimum of 15 cm proximal to the junction and 0.5 ml of TNBS in ethanol was injected with a tuberculin syringe (27 gauge needle) into the lumen. TNBS was administered in a dose of 20 mg per animal or at a dose of 60 mg/kg. Ethanol concentration was 15% in the initial experiment and was reduced to 10% for all subsequent experiments. All concentrations are indicated in each result. The intestine was returned to the perotineal cavity and moistened with an intraperotineal infusion of sterile saline (0.9 %). Garamycin (0.1 ml of a 1% solution) was administered intraperotineally. The muscle layer was closed with number 3 vicryl. The skin was closed with a number 3 silk. The abdomen was swabbed with soapy water and the guinea pig placed upon a heating pad set at low for recovery. Animals were returned to normal housing within four hours of surgery, during which time they were observed and had free access to water. The following day, the guinea pigs were checked for herniation or other surgery related problems, and corrective measures taken when necessary.

**Animal Model: Indomethacin**

Male Wistar rats, 200-225g, were given 7.5 mg/kg indomethacin in 5% sodium bicarbonate. This was administered by subcutaneous injection with a tuberculin syringe and was done twice at a twenty-four hour interval.

**GLP-2 Administration**

GLP-2 was administered in the form of the active, non-degradable analog $(GLU)_2$-GLP-2 otherwise and hereafter known as ALX-0600. A dose of 0.1μg/kg in a 50 μg/ml PBS solution was administered twice a day through subcutaneous injection with a tuberculin syringe with a 27 gauge needle. This dose was shown to be the most effective. (Allelix Biopharmaceuticals, unpublished data, 82)

**Permeability Probe**

Guinea pigs were administered 1000 mg sucrose, 120mg lactulose, 80mg mannitol and 60 mg sucralose dissolved in $ddH_20$. The solution was administered orally through an 18 gauge, three inch ball nosed needle on a 3 cc syringe. Rats were administered 500 mg sucrose, 120 mg lactulose, 80 mg mannitol, 60 mg sucralose and 50 mg D-xylose dissolved in $ddH_20$. Solution was administered by intragastric gavage using a 18 gauge, three inch ball nosed needle on a 3cc syringe.

**Permeability**

Permeability was assessed twice prior to the induction of inflammation (days -6 and -3), and every three days thereafter for fourteen days. Animals were fasted for four hours prior to the administration of the probe. Urine was collected for a period of 18 to 20 hours during which time the animals were fasted but had free access to water. Urine samples were frozen (-20 °C) until processing for HPLC. Samples for the quantification of sucrose, lactulose and mannitol were mixed with deionizing resin (1.5 of 400 : 1 of 120 Amberlite) and spun at 11,000 rmp for 10 minutes. Three millilitres of deionized urine

was the passed through a syringe filter (pore size 45 μm). The urine was diluted 1:2 , cellubiose added as an internal control and the sample passed over a Dionex MA-ion exchange column, eluting with sodium hydroxide at a flow rate of .4 ml/min. Peaks were detected using pulsed amperometric detection and analyzed for peak area. For the quantification of sucralose, the urine samples were filtered, diluted appropriately and phenyl-β-d-thiogalactoside added as an internal control. Samples were passed over a Dionex Inopac NS1 column, eluting with acetonitrile/water at 1 ml/min. with acetonitrile content increasing from 0% to 20% during the run. Peaks were determined and analyzed for peak area (16). D-xylose was quantified in the urine using a standard colourimetric assay with analysis by spectrophotometry. Fractional excretions of all sugars are calculated and small intestinal permeability assessed as the ratio of lactulose to mannitol excreted.

**Macroscopic Assessment of Damage**

Rats and guinea pigs were anaesthetized with halothane (5 parts per litre, 1 L/min oxygen) and sacrificed by cervical dislocation. The small intestine was removed from the ligament of Treitz to the ileal-cecal junction. The intestine was measured for length tension, opened along the anti-mesenteric border and washed with ice-cold saline to remove luminal contents. The width of the intestine and presence of ulcers and/or inflammatory changes (erosions, obvious edema) were assessed using the scale shown in Table 1. Planimetry was performed to assess the area of damage using a 1 mm grid.

Table 1 **Macroscopic Damage Score**

| Score | Damage |
|---|---|
| 0 | no apparent damage |
| 1 | slight thickening of intestinal wall, redness |
| 2 | thickening and adhesions, no apparent ulcers |
| 3 | adhesions and one or more pin-point ulcers |
| 4 | adhesions and pin-point ulcers with one or more linear ulcer < 1 cm in length |
| 5 | one or more linear ulcer > 1 cm in length |

**Myeloperoxidase Assay**

Myeloperoxidase assay was performed using standard techniques on one centimeter of proximal jejunum taken from within areas of inflammation but not ulceration when damage was apparent or 10 cm distal to the ligament of Treitz when damage was not observed. In brief, tissue was removed, weighed, and placed in eppendorf tubes on dry ice. Samples were stored at -80 ° C until assay was performed, a period not longer than one week. Samples were homogenized in 0.5 ml of HTAB. Protein content was assayed and the remaining homogenate diluted to 50 mg protein to 1 ml of HTAB. One ml of solution was centrifuged at 100,000 rpm for two minutes at 4 ° C. Seven µl of supernatant was pipteted into a 96 well microtitre plate, each sample was analyzed 5 times. 200 µl of o-dianisidine solution was added and the optical density of the solution assessed using a

plate reader. Optical density was converted to units of myeloperoxidase per mg tissue (91).

**Protein Assay**

The intestine was opened along the anti-mesenteric border, and flushed with ice cold saline. Glass slides were used to scrape tissue mucosa from 10 of proximal jejunum, weighed and suspended in ice-cold 25 mM EDTA in a ratio of 1 g tissue to 10 ml of EDTA. Tissue was homogenized and frozen at -80°C for a maximum of three months prior to assay. Samples were diluted 1/100 with 25 mM EDTA, and assayed using the commercially available BioRad Protein Assay kit. Samples were analyzed for optical density at 600 nm and protein content was determined.

**DNA Assay**

Tissue mucosa was scraped from 10 cm of proximal jejunum, weighed and suspended in 25 mM EDTA in a ratio of 1 g mucosa to 10 ml EDTA. Tissue was homogenized and 30 µl of homogenate dropped onto the bottom of a 13x 100 glass, siliconized test tube. Samples and standards (1mg/ml calf serum DNA), in duplicate were dried at 60°C overnight. DABA was added (0.1ml) and the tubes incubated at 37°C for 60 minutes, 3ml of 1N hydrochloric acid was added and the percent fluorescence measured using a fluorometer (92).

**Sucrase Assay**

Mucosa was scraped from 10 cm of proximal jejunum and suspended in 25mM ice cold EDTA in a ratio of 1 g tissue to 10 ml EDTA. Samples were homogenized and stored at -80°C for not longer than three months. Samples were defrosted and diluted 1/100 with

EDTA and 10 µl was placed in quadruplicate on the bottom of 16 x 100 siliconized glass test tubes. Blank tubes (duplicates) were placed in boiling water for two minutes. Three ml of a solution of TGO (.3 ml glucose oxidase, .5 mg peroxidase, .5 ml o-dianisidine, 1 ml Triton X-100 (10 ml in 40 ml 95% ethanol), Tris-HCl buffer (pH 7.0) to 100 ml) reagent was then added and the tubes were incubated at 37°C for 30 min. followed immediately by two min. in boiling water. Samples were analyzed by spectrophotometry at 420nm. The optical density was converted to units of sucrase activity per cm of tissue (93).

**Histological Examinations**

Tissue samples were taken from proximal jejunum in areas of apparent tissue damage but not ulceration, or 12 cm distal to the ligament of Treitz in the rats. In the guinea pigs, samples were taken from the distal ileum. Samples were fixed in 10% formalin for at least 48 hours, and mounted in paraffin blocks using standard techniques. The samples were cut to 7 µm thickness on a microtome, affixed to slides and stained with Hemotoxalin and Eosin using an autostainer and standard techniques. The muscle thickness, villus length, crypt depth, and villus width were assessed by a blinded technician under 40X magnification.

**Statistical Analysis**

Results are presented as means with standard errors. Analysis of Lac/Man curves for the assessment of small intestinal permeability, was done by ANOVA, with comparisons within each group to the baseline (days –6 and –3) and post tested with the Tukey test. In

histological and enzymatic assays, the results were analyzed by t test. Permeability in the indomethacin experiment is expressed as the sum of the area under the curve for each experimental group. These were analyzed by ANOVA with Tukey post-testing.

# Chapter 5

## Results: TNBS-induced Ileitis

### Experiment 1:Effects of ALX-0600 on Acute Ileitis

**Hypothesis:**

Pretreatment with ALX-0600 will prevent or ameliorate ileitis induced with TNBS and ethanol in the guinea pig.

### Experiment 1: Dose Finding Experiments

Methods:

Guinea pigs were randomized into weight matched groups of 10 and two baseline permeabilities were assessed. Ileitis was induced by intraluminal injection of the appropriate TNBS/ethanol solution, 15 cm proximal to the ileal-cecal junction. Group A received 20 mg TNBS in 15% ethanol, Group B received 20 mg TNBS in 10% ethanol and Group C received 15 mg TNBS in 10% ethanol. Permeabilities were assessed during following fourteen days. Histological and macroscopic examination of tissue from the distal ileum was performed to assess the damage induced in each group

**Results**

There was significant mortality (80%) within four days of administration of TNBS/ethanol to Group A. Sixty percent of the mortality was due to perforation or intestinal obstruction resulting from the TNBS-induced damage. The remaining 20% mortality was the result of investigator error during gavage which resulted in esophageal puncture. The surviving guinea pigs did not have increased gastric or colonic permeability. There was no change in small intestinal permeability (lac/man: $0.319 \pm 0.041$; $p>0.05$) four days following ileitis

induction. (Fig 1) Group C, which received the lowest dose of hapten (TNBS) and ethanol did not have any mortality. This group also did not demonstrate any increase in gastric or colonic permeability as the fractional excretions of sucrose and sucralose did not change. There was no indication of small intestinal disease in this group as the small intestinal permeability was not increased (lac/man: $0.354 \pm 0.053$; $p>0.05$). (Fig. 2) Macroscopic examination of the tissue did not reveal any ulceration or edema, which would indicate ileitis. Group B, which received 20 mg of TNBS in 10% ethanol had 30% mortality and demonstrated a significant increase in small intestinal permeability (lac/man: $0.206 \pm 0.029$; $p< 0.0002$) four days following the induction of ileitis. The control group for group B, which received ethanol intraluminally, did not have increased permeability (lac/man: $0.062 \pm 0.004$; $p>0.05$) four days following surgery, nor was there any mortality in this group. The small intestinal permeability resolved to baseline within 10 days, indicating rapid healing of the damage. (Fig. 3) Macroscopic comparison of Group B and their controls indicate that TNBS/ethanol caused significantly more damage to the terminal ileum ($3.67 \pm 1.33$; $p< 0.023$) than did the ethanol alone ($1.0 \pm 0.58$; $p> 0.05$). (Fig. 4) Histologically, there were significantly more granulocytes in the ileal wall of the TNBS group ($2.5 \pm 0.28$; $< 0.021$) than in the ethanol group. (Fig. 5)

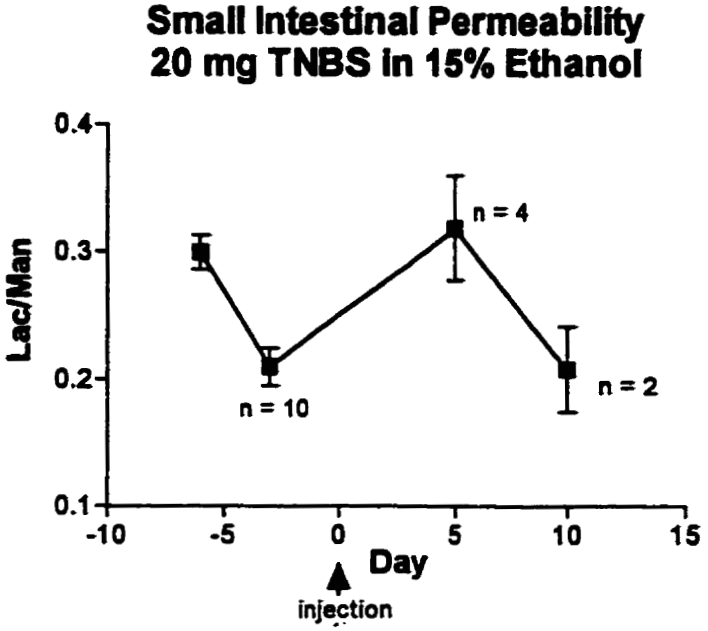


Fig. 1 Small intestinal permeability was not increased following administration of 20 mg TNBS in 0.5 ml of 15% ethanol.

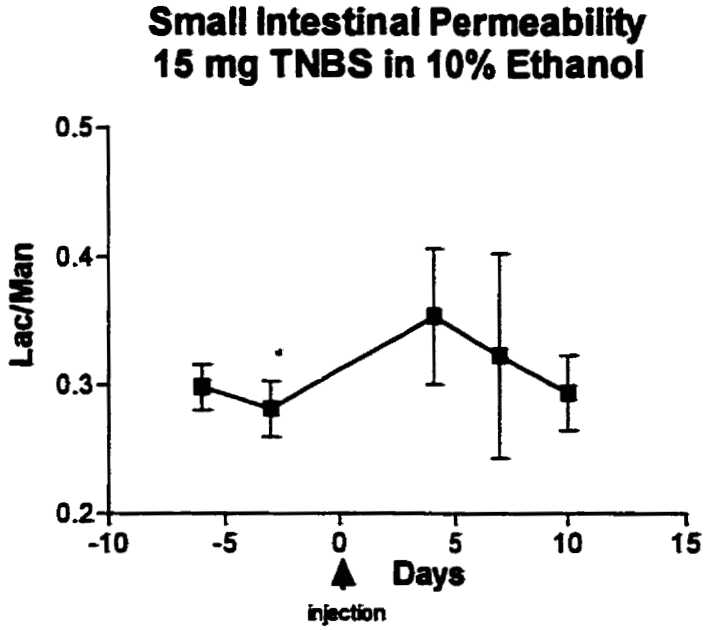


Fig. 2 Small intestinal permeability in the guinea pig (n = 10)was not altered by administration of 15 mg TNBS (■) in 0.5 ml of 10 % ethanol.

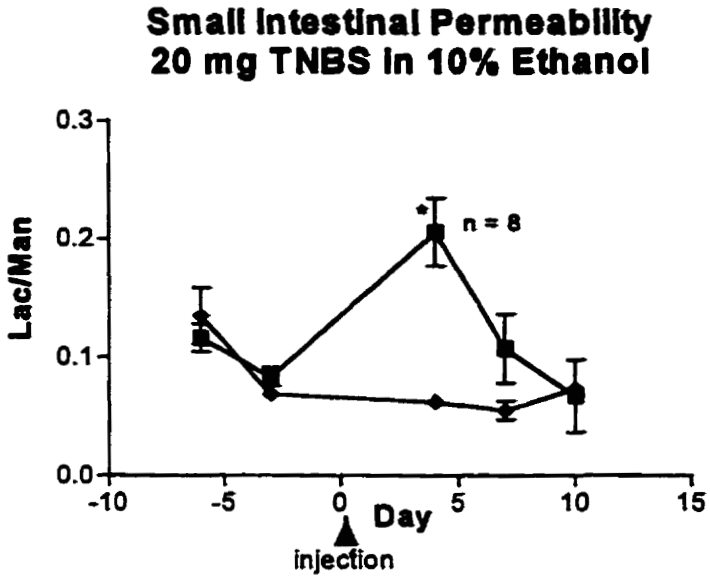


Fig. 3 Small intestinal permeability was significantly increased (* p< 0.0002) four days following induction of ileitis in the guinea pigs receiving TNBS/ethanol (■; Group B) but not in the ethanol controls (◆)

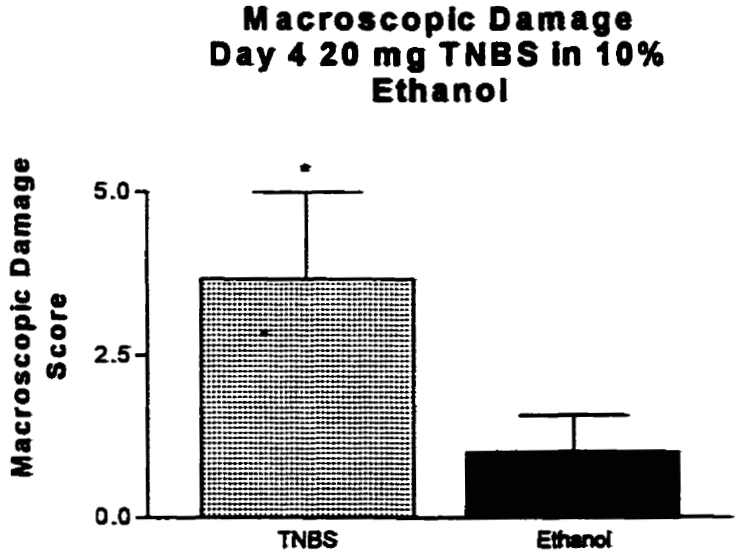


Fig. 4 Signficantly more damage was observed in the terminal ileum of guinea pigs receiving TNBS ( * p< 0.023; n ≥ 5) than in those receiving only ethanol four days after administration.

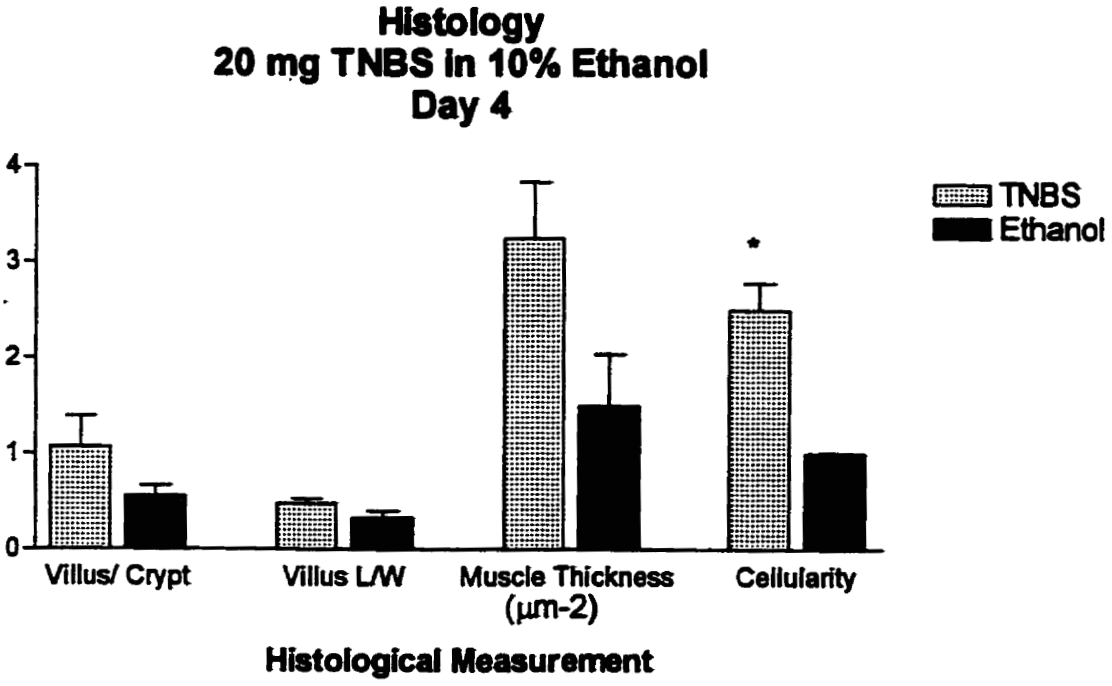


Fig. 5 There was significantly more (* $p < 0.021$) granulocyte infiltration in the TNBS group than in the Ethanol group. No other morphological features were different between the two groups.

**Experiment 1.2: Pretreatment of TNBS-ileitis with ALX-0600**

Methods

Guinea pigs were randomized into three groups, ALX-0600, ALX-0600 + TNBS and TNBS. ALX-0600 was administered at a dose of 0.1 μg/kg twice a day subcutaneously for once week prior to induction of ileitis, and for the entire period of the experiment. The TNBS group received volume equivalent doses of PBS (the ALX-0600 vehicle) for the entire period of the experiment. TNBS ileitis was induced through the intraluminal injection of 20 mg TNBS in 0.5 ml of 10% ethanol, 15 cm proximal to the ileal-cecal junction. Permeability was assessed, and five animals were sacrificed from each group four days following surgery for assessment of macroscopic and histological damage.

Results:

Permeability of the small intestine was significantly increased in the TNBS and ALX-0600 + TNBS groups four days after induction of ileitis (lac/man: $0.107 \pm 0.013$, $0.110 \pm 0.028$; $p<0.0316$). The ALX-0600 group which received ethanol intraluminally did not demonstrate altered permeability (lac/man $0.0889 \pm 0.013$; $p>0.5$). (Fig. 6) There was no difference in the macroscopic damage to the terminal ileum between the TNBS and the ALX-0600 + TNBS group. Both groups had significant ulceration, tissue necrosis and edema. Villus morphology was normal where there was no ulceration in both the TNBS and ALX-0600 + TNBS groups. The ALX-0600 group's villus morphology did not differ from either group receiving TNBS. Cellularity, assessed in non-ulcerated tissue was the same in all three groups. Muscle thickness was significantly higher in the TNBS group than in the ALX-0600 + TNBS or the ALX-0600 group ($3.25 \pm 0.595$; $p<0.0388$). (Fig. 7)

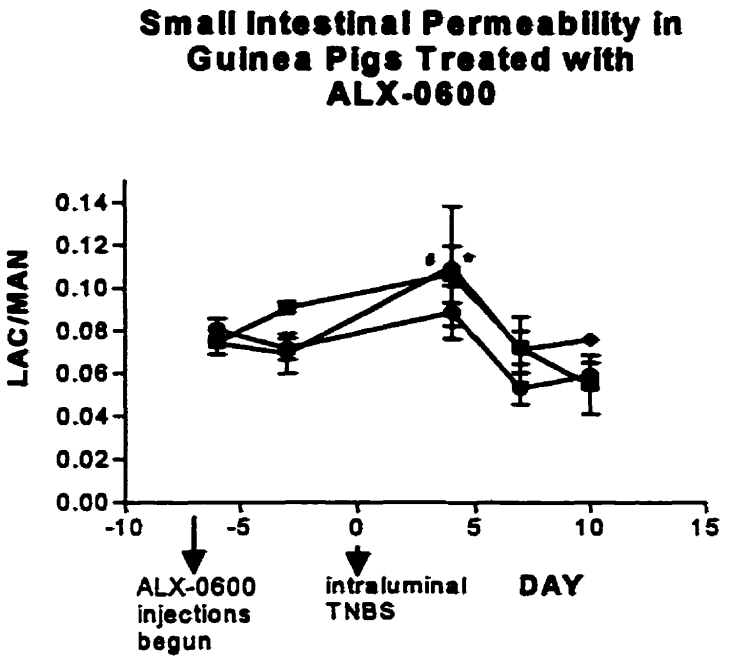


Fig.6 ALX-0600 pretreatment had no protective effect upon TNBS induced ileitis as both the TNBS (■; $p<0.0316$) and the ALX-0600 + TNBS group (◆ $p<0.0192$) had significantly increased permeability. ALX-0600 treatment had no effect upon permeability (●; $p>0.05$).

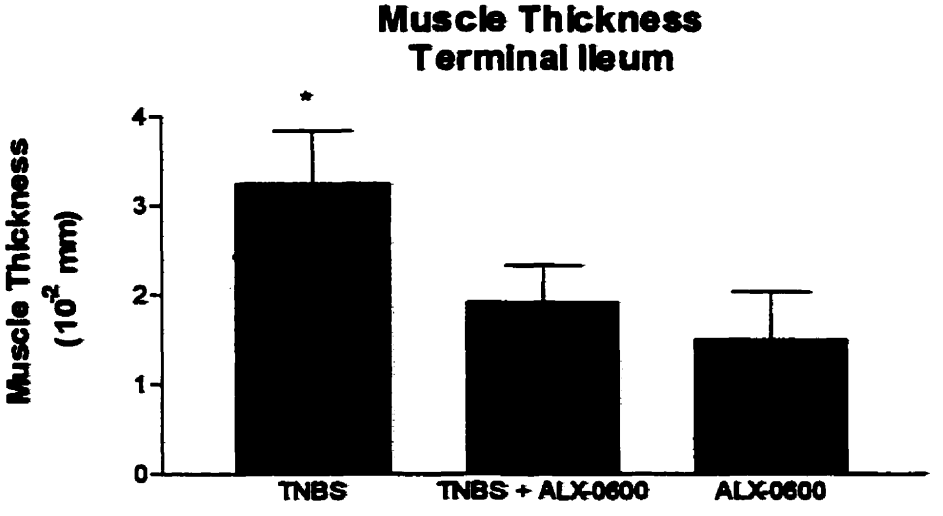


Fig. 7 ALX-0600 was protective against the increase in muscle thickness seen in the TNBS group (* $p< 0.0388$; $n\geq 5$). Neither the ALX-0600 nor the ALX-0600 + TNBS group demonstrated an increase in muscle thickness.

Discussion

Ileitis was induced by intraluminal injection of 20 mg TNBS in 10 % ethanol to the terminal ileum. This concentration of TNBS and of ethanol is much lower than that used by other investigators, including Miller (94); however higher doses of TNBS resulted in excessive mortality rates. The concentration of TNBS is sufficient to alter the cell-surface morphology of lamina propria cells through its interaction with the ε-amino groups of lysine residues, initiating an immune reaction by macrophages. Association of TNBS altered proteins with MHC molecules on the cell surface of macrophages and epithelial cells results in generation of a hapten specific CTL T cell population (95). These cumulative immune responses result in significant inflammation and ulceration detected as an increase in intestinal permeability. Generation of chemotactic cytokines by the macrophages contributes to the influx of granulocytes seen in the histological assessments of cellularity. The lack of damage observed in the TNBS groups in the histological assessment of epithelial morphology is the result of observing non-ulcerated areas. In areas of damage, the normal mucosal morphology was eradicated, therefore it was impossible to assess villus or crypt lengths. Muscle thickness may be an indicator of edema, fibrosis, hypoplasia or contraction. The increased muscle thickness observed in the TNBS groups is the result of inflammation; however the wet to dry ratios were not assessed and therefore edema cannot be determined to be the sole cause.

Administration of ethanol alone has no effect on permeability, indicating no significant effect of the barrier breaker on intestinal integrity in the absence of an immunological stimulant, such as the hapten, TNBS. This is confirmed by the observations of macroscopic damage and histology. While this result may seem

contradictory to the hypothesis that a normally present luminal antigen causes inflammation during times of compromised intestinal integrity, the administration of 0.5 ml of ethanol alters the contents of the intestine by flushing luminal contents into the cecum. Ethanol disrupts the actin-myosin filaments around the tight junctions resulting in increased permeability without cytotoxicity; damage that is transient, lasting a maximum of three hours (96). The relatively short abrogation of the barrier does not allow sufficient antigenic challenge to the mucosal immune system to initiate a significant inflammatory reaction. There is stimulation of histamine release from the mast cells; however, this apparently does not initiate a significant inflammation of the small intestine (96)

Normal fractional excretions of sucralose indicate that the colon is unaffected by the inflammatory reaction. Sucrose fractional excretion was not altered during the disease process that indicates the integrity of the gastric mucosa was not compromised. This may also indicate a lack of physiological stress in the guinea pigs. Stress is associated with the development of gastric ulcer (97). Gastric ulceration is preceded by an increase in gastric mucosal permeability (98); furthermore increased gastric permeability can be assessed using the sucrose fractional excretion (16). Therefore, the normal sucrose fractional excretion levels in the guinea pig may indicate a lack of stress.

ALX-0600 had no effect upon the induction of TNBS ileitis in the guinea pig. Small intestinal permeability was significantly increased in both groups receiving TNBS, there was no difference between them. This sensitive marker of small intestinal damage suggests that ALX-0600 pretreatment does not alter the immune response the hapten. This is supported by both the macroscopic damage scores, which were not different between the groups and the cellularity assessed during microscopic tissue examination.

The reduction in muscle thickness seen in the group receiving both TNBS and ALX-0600 may be the result of less fibrosis, or contraction. ALX-0600 has no documented effects beyond the epithelia; however it is possible that administration of this growth hormone altered the inflammatory response sufficiently to alter the muscle thickness.

The changes in villus-crypt morphology which are characteristic of ALX-0600 administration in mice (78) were not observed in the group receiving ALX-0600. It is possible that these effects were not discernable within twelve days of treatment; however, it is effective in both rats (99) and mice within seven days (81). ALX-0600 may not be effective in promoting growth of the small intestine of the guinea pig. However, GLP-2 has been shown to be highly conserved within all higher forms of life including mammals (100), fish (101), birds (101) and reptiles (102). It's structure is also highly conserved within mammalian species (38) therefore it is unlikely that ALX-0600 is non-functional in the guinea pig. The effects of ethanol on the ileum are transient and it is unlikely that they were sufficient to blunt the normal trophic response of the mucosal epithelium to ALX-0600. The lack of effect of ALX-0600 pretreatment upon sensitive markers of inflammation implies that this growth hormone is not effective in this model of inflammatory bowel disease.

**Experiment 2: Reactivation of TNBS – Induced Ileitis**

**Hypothesis:**

TNBS-induced ileitis will be reactivated through systemic but not oral administration of the hapten.

Methods:

TNBS ileitis was induced in 15 guinea pigs through intraluminal injection of 20 mg TNBS in 0.5 ml of 10% ethanol 15 cm proximal to the ileal-cecal junction. Control guinea pigs (10) received 0.5 ml of 10% ethanol, 15 cm proximal to the ileal-cecal junction Permeability was assessed by oral administration of the probe, and urinalysis by HPLC. One month following intraluminal administration of TNBS or vehicle, 4.45 mg/kg TNBS in PBS was administered subcutaneously twice a day for three days. Permeability was assessed on the third day of subcutaneous administration. Following systemic administration of the hapten TNBS and the control group were allowed one month to recover following reactivation. The guinea pigs were orally administered 4.45 mg/kg TNBS in the permeability probe twice a day for three days. Permeability was assessed on the fourth day after beginning administration of the hapten.

Results:

Four days after intraluminal administration of TNBS/ethanol there was a significant increase in small intestinal permeability (lac/man: $0.206 \pm 0.029$; $p<0.0002$) which was not observed in the ethanol controls (lac/man: $0.062 \pm 0.004$;$p>0.5$). (Fig 8) Excretion of the other sugars did not differ from baseline. Following subcutaneous administration of TNBS there was a significant increase in permeability in the group which had previously been inflamed (lac/man: $0.225 \pm 0.084$; $p<0.0217$) but not in the ethanol control group

(lac/man: 0.101 ± 0.006;p>0.5). There was a sub-population of guinea pigs who did not demonstrate reactivation (40%). There was no alteration in the other sugars excretion - levels. Mortality immediately following surgery was 50 %, in the TNBS group while there was no mortality in the control group. There was no mortality following systemic administration of TNBS.

Oral administration of TNBS following healing of the systemically induced reactivation did not result in re-inflammation of the ileum. Permeability did not change in either the sensitized or the control group (lac/man 0.123 ± 0.034, 0.079 ± 0.01 p>0.5). Excretion of sucrose and sucralose were not altered from baseline. There was no mortality following oral administration of TNBS.

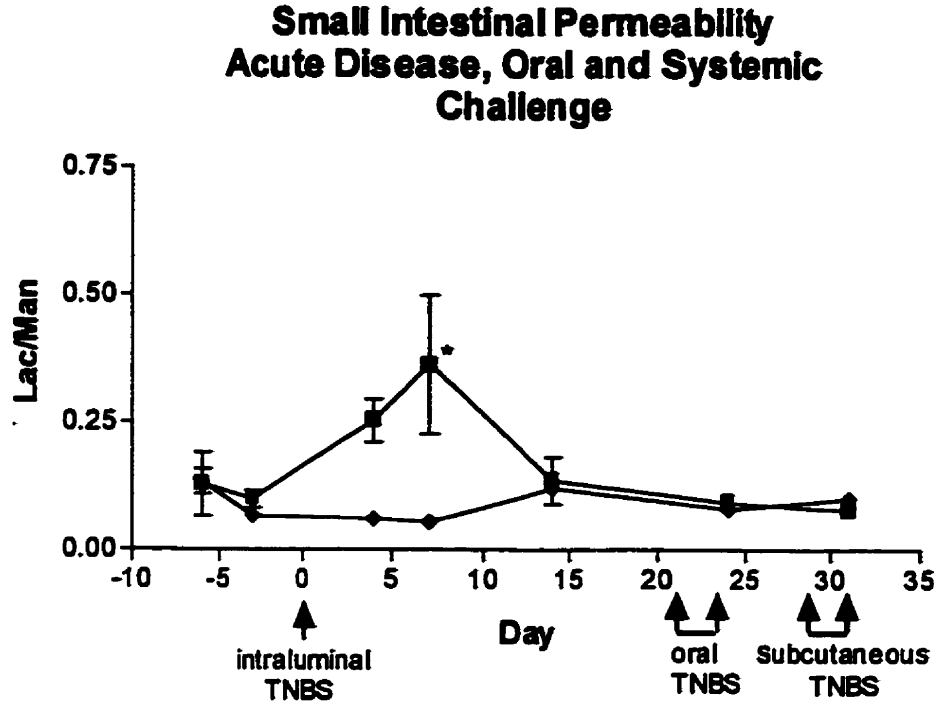


Fig. 8 Acute reaction in guinea pigs receiving TNBS/ethanol intraluminally was significant (■; * $p < 0.0001$, $n \geq 10$) however, there was no increase in permeability following either oral ( $p>0.05$) or systemic($p>0.05$) challenge. The ethanol control group (◆)did not demonstrate increased permeability at any time point.

Discussion:

Acute inflammation in the group receiving TNBS/ethanol was indicated by the increase in small intestinal permeability, which was not seen during the acute phase in the group receiving ethanol alone. The TNBS-induced increase in permeability resolved within one week. There was no indication of involvement of the colon, as the sucralose fractional excretions did not change. The animals did not experience any significant stress or gastric involvement that would be indicated by increased sucrose levels, as previously discussed. The mortality in the acute phase was limited to the TNBS-receiving group and resulted from intestinal obstruction that was secondary to inflammation.

Systemic administration of the hapten is a documented method of reactivating TNBS induced colitis (103), using a dose 1/15 that which is used during acute induction. Subcutaneous administration of 4.45 mg/kg TNBS resulted in a significant increase in the small intestinal permeability in the group which had recovered from ileitis but not in the ethanol control group or in a small sub-population of guinea pigs, referred to in the literature as non-reactors. (93) This group either does not becomes sensitized to the TNBS or possibly does not retain the small amounts of TNBS in the intestinal wall following acute phase inflammation. The initial reaction to TNBS modified cells in the intestine is due to the actions of macrophages and to CTL T cells following sensitization through the presentation of TNBS modified proteins on MHC cell surface moieties. (95) There is also generation of immunoglobulin G against the TNBS modified epitope (104). Systemic reactivation is most likely due to the proliferation of the hapten specific cytotoxic T cell population resulting in a delayed type hypersensitivity reaction. Systemic administration of the hapten did not alter the intestinal integrity when there was

no prior administration of the hapten to the intestine. If induction of inflammation in the intestine by a luminally present molecule requires increased permeability, then oral administration of the hapten to a sensitized animal with normal intestinal permeability should not cause reactivation because it would be excluded from the mucosal immune system. This has been demonstrated in the rat. Following TNBS-induced colitis intracolonic administration of TNBS does not reactivate the colitis (103). There was no reactivation of the disease in the animals that had been sensitized to TNBS through intraluminal administration in the presence of the barrier breaker ethanol, nor in those who were sensitized by systemic administration of the hapten. It is possible that TNBS is not fully excluded from the lamina propria but that tolerance has been induced. There is evidence that high doses of a compound delivered directly to the mucosal immune system will result in tolerance whereas low doses sensitize the mucosal T cells (105). Therefore it is possible that the combination of systemic and mucosal sensitization to TNBS has resulted in tolerance of the immune system to TNBS-labeled cells. Therefore, a subsequent experiment is necessary to demonstrate that TNBS is truly excluded from the lamina propria and is not merely tolerated by the immune system.

**Experiment 2.2: Oral TNBS will not reactivate Ileitis**

Hypothesis:

Following acute inflammation resulting from TNBS, oral administration of TNBS will not result in recurrent inflammation but subsequent systemic administration of TNBS will.

Method:

Acute inflammation was induced in the terminal ileum of the guinea pig through intraluminal injection of 0.5 ml of 40 mg/ml TNBS in 10% ethanol. Three weeks after actue phase induction, TNBS was administered orally at a dose of 4.45 mg/kg twice a day for three days. Four weeks following induction (therefore one week following oral administration) TNBS was administered systemically by subcutaneous injection of 4.45 mg/kg TNBS twice a day for three days. Permeability was assessed during the acute phase, and on the day following the last administration of TNBS both orally and subcutaneously.

Results:

Guinea pigs receiving TNBS/ethanol experienced an acute phase increase in permeability indicating inflammation which was significant and maximal at day 7 (lac/man: $0.254 \pm 0.042$; $p < 0.0001$) following surgery. The permeability had returned to baseline and was not significantly different from ethanol controls fourteen days following induction. Neither the sensitized nor the control group demonstrated a significant increase in permeability following oral administration of TNBS (lac/man: $0.0.093 \pm 0.003$, $0.08 \pm 0.007$; $p>0.5$). Systemic administration of TNBS did not result in increased permeability in either group (lac/man: $0.079 \pm 0.007$, $0.101 \pm 0.006$; $p>0.5$). (Fig. 9) There was no change in excretion of other sugars over the course of the experiment. Mortality was 75%

during the acute phase in the group receiving TNBS. There was no mortality in the control group during the acute phase or in any group during either reactivation phases.

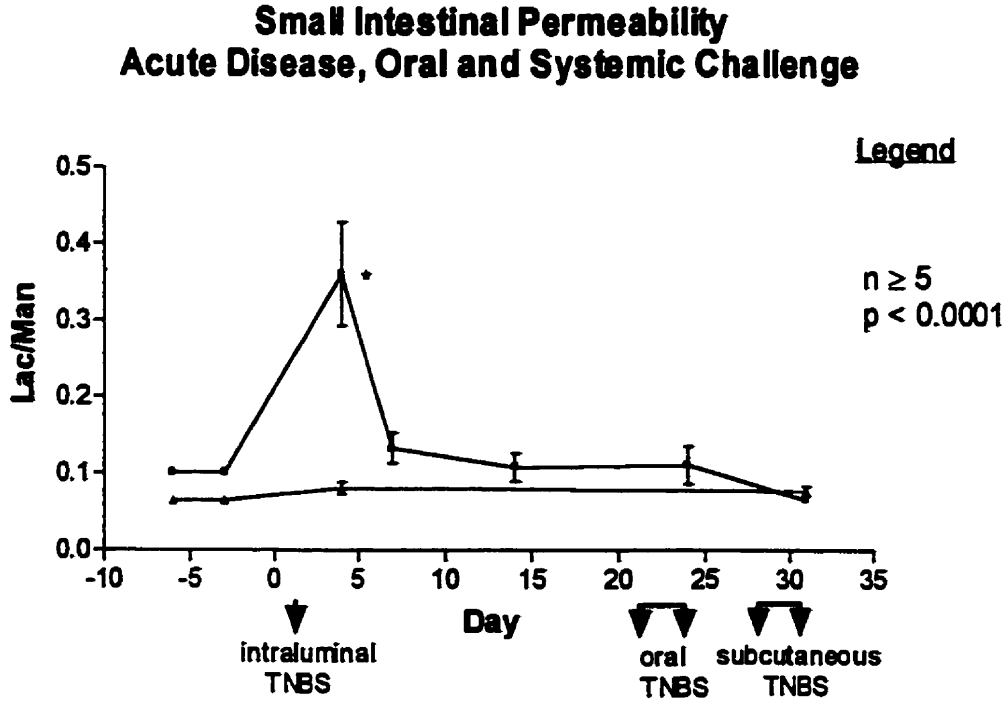


Fig.9 Small intestinal permeability is significantly increased in the group receiving intraluminal TNBS/ethanol at a dose of 60 mg/kg (* $p<0.0001$, $n \geq 5$). There is no increase in permeability following oral or systemic challenge. The ethanol control group demonstrated no increase in permeability at any time point.

Discussion:

Small intestinal permeability was significantly increased in the TNBS/ethanol group immediately following surgery, and was elevated for a longer duration that is normally observed in this model. It resolves to baseline and is not different from the ethanol control group within two weeks following the surgery. There is no change in permeability following either the oral or the subcutaneous administration of the TNBS. There was no alterations in the excretion of sucrose or sucralose in either group at any time point in the experiment, indicating the animals were not experiencing significant stress or colonic inflammation. There was a significant increase in the mortality rate of the guinea pigs during the acute phase of the reaction. This was likely a result of a decrease in the starting weights of the guinea pigs as the procedures have been progressing more rapidly. A retrospective analysis of the body weight per milligram of TNBS demonstrates that the amount of TNBS has been increasing gradually as the weights of the guinea pigs had been decreasing. The optimal dose appears to be 60 mg/kg; this was subsequently used as a standard dose, replacing the 20 mg dose of TNBS.

The lack of reactivation following systemic administration may be due to either tolerance induced by the oral administration of TNBS prior to systemic challenge or to elimination of the re-activating subpopulation in the survivors. In the previous experiment which demonstrated reactivation following systemic administration of TNBS, we demonstrated an approximately 60% reactivation rate, 40% of the guinea pigs did not reactivate. The hypothesis, which may explain the lack of reactivation following systemic administration of TNBS, is, that oral administration of the TNBS prior to the systemic administration may be inducing oral tolerance. If this is the case, small amounts of TNBS

or luminal proteins labeled with TNBS are being absorbed into the intestine in the absence of increased permeability and presented through normal immune systems to the T cells inducing tolerance. TNBS compares with mannitol in size and therefore may be absorbed passively through the paracellular spaces in a low enough dose to fail to initiate an immune response. However this may be overcome if the amounts of TNBS in the lumen reach a critical level and therefore the passive absorption of TNBS is sufficient to initiate an immune reaction. The following experiments will test these hypothesis.

**Experiment 2.3 : Oral Tolerance**

Hypothesis:

High doses of TNBS administered orally will result in sufficient passive absorption to initiate a reactivation of the TNBS-induced ileitis.

Methods:

TNBS-ileitis was induced with 60 mg/kg TNBS in 0.5 ml of 10% ethanol injected into the lumen 15 cm proximal to the ileal-cecal junction. Three weeks following induction, guinea pigs were administered 100 mg/kg, 1000 mg/kg or 2000 mg/kg TNBS over 6 doses in 3 days. Permeability was assessed during the acute phase and following the oral challenge.

Results:

There was significant small intestinal inflammation in the acute phase. There was no increase in permeability following oral challenge following 100 mg ($0.078 \pm 0.008$; $p>0.05$), 1000 mg ($0.149 \pm 0.067$; $p>0.05$) or 2000 mg ($0.015 \pm 0.017$; $p> 0.05$) doses of TNBS. (Fig. 11) There was no alteration in the excretion of other sugars. There was normal mortality in the acute phase ( 40%) and one mortality in the 2000 mg/kg group during the reactivation phase which was ascribed to colonic cancer (as diagnosed by a veterinarian during postmortem examination).

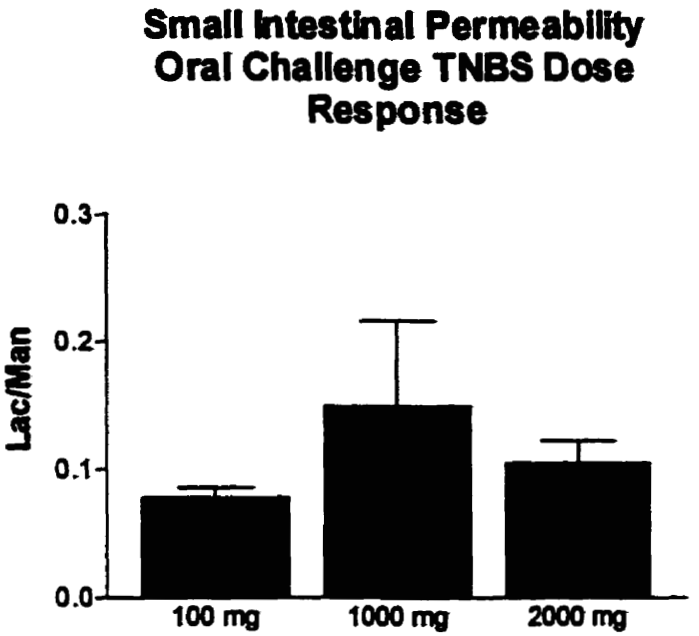


Fig. 10 Small intestinal permeability following high dose oral challenge in previously sensitized guinea pigs ($n \geq 4$). No significant increase in permeability was observed.

Discussion:

The ileitis was successfully induced however, exclusion of the TNBS from the interstitial compartment is sufficient to prevent passive absorption of high doses of TNBS from the lumen to the lamina propria and therefore to prevent induction of the immune response. If oral tolerance is induced through high doses of the hapten as has been that suggested then the amount of TNBS absorbed might have been sufficient to cause tolerance rather than initiate an immune response. However, if we assume that the TNBS is being absorbed paracellularilty in a mechanism similar to mannitol in the un-inflamed gut then there should have been some reaction of the macrophages to the presence of altered self cells even if there was no reaction of the cytotoxic T lymphocytes. Therefore, it is likely that TNBS was excluded from the interstitial compartment. To eliminate the possibility that oral administration of TNBS to sensitized guinea pigs is interfering with the systemic reactivation of ileitis, it is necessary to eliminate the oral administration.

**Experiment 2.4 Elimination of Oral Dosing**

Hypothesis:

Systemic challenge with TNBS to sensitized guinea pigs in the absence of an oral challenge will result in re-activation of ileitis.

Method:

Ileitis was induced as previously described using 60 mg/kg TNBS in 10% ethanol, and assessed by permeability. Four weeks following surgery, TNBS was administered at 4 mg/kg subcutaneously twice a day for three days and permeability assessed again.

Results:

There was a significant increase in small intestinal permeability during the acute phase reaction (lac/man: $0.181 \pm 0.028$; $p<0.001$) that resolved within three weeks. There was no increase in permeability following systemic administration of the hapten ($p>0.5$). (Fig. 11) There was no alteration in the excretion of either the gastrically absorbed sucrose, or the colonic sucralose. There was no mortality during any phase of the experiment.

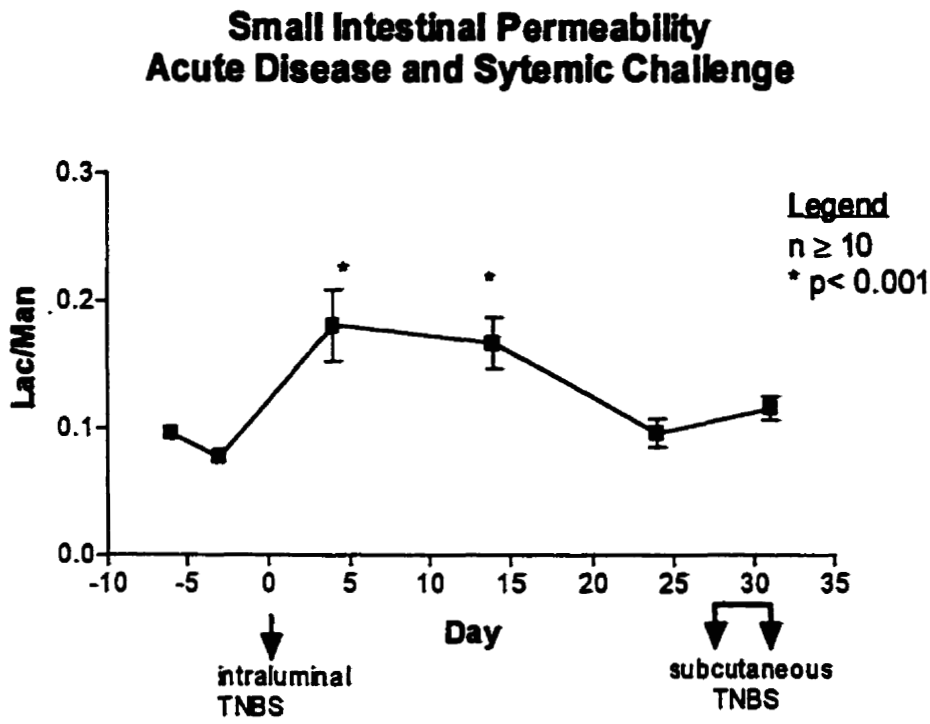


Fig. 11 There was a significant increase in small intestinal permeability immediately following surgery (0.181 ± 0.028; p< 0.001) which lasted for two weeks. There was no increase in permeability following systemic administration of TNBS.

Discussion:

Lack of reactivation following systemic administration may be due to elevated levels of TNBS absorption during the acute phase (100). The disease was active for longer periods of time than usual that may indicate a greater amount of cellular labeling. Intravenous administration of TNBS is sufficient to create T and B cell mediated tolerance to the hapten modified epitopes (101, 102) therefore it is possible that sufficient TNBS was absorbed into the systemic circulation during the acute phase that tolerance rather than sensitization was the result.

## Experiment 3: Seasonal Variation in TNBS-ileitis

### Background

During retrospective analysis of data, a pattern of circannual variation in the development of TNBS ileitis was suggested. Two studies conducted during the summer months were unsuccessful in inducing acute ileitis. This was initially explained to result from degradation of the TNBS; however, subsequent experiments proved the TNBS to be active.

### Hypothesis

Intraluminal injection of 60 mg/kg TNBS in 10% ethanol, a procedure that induces TNBS in winter months, will not induce ileitis during the summer.

### Method

Two groups of guinea pigs were administered 60 mg/kg TNBS in 0.5 ml of 10% ethanol, one group in July and one group in August. Ethanol controls were run as well in August. Intestinal permeability was assessed in July. Intestinal permeability, myeloperoxidase activity in the ileum, sucrase activity in the ileal mucosa were assessed and histological examination of the distal ileum was performed four days following induction of ileitis in August.

### Results

There was no acute increase in small intestinal permeability (lac/man: $0.102 \pm 0.014$; $p>0.05$, $n = 20$) was seen in July (Fig. 12). Neither the sucrose nor the sucralose fractional excretions changed following administration of TNBS in ethanol. Permeability was not altered in August following administration of TNBS in ethanol (data not presented). Macroscopic examination did not reveal any signs of inflammation, such as

edema or ulceration. Assessment of the myeloperoxidase activity in the distal ileum (Fig 13) and the sucrase activity in the mucosa of the distal ileum (Fig. 14) did not indicate any infiltration of granulocytes or decrease in digestive enzyme activity. Finally, histological examination of sections from the distal ileum did not reveal any changes in crypt/villus morphology or muscle thickness. Cellularity assessment confirmed the myeloperoxidase assay, as no significant increase in cellularity was found in the TNBS group.

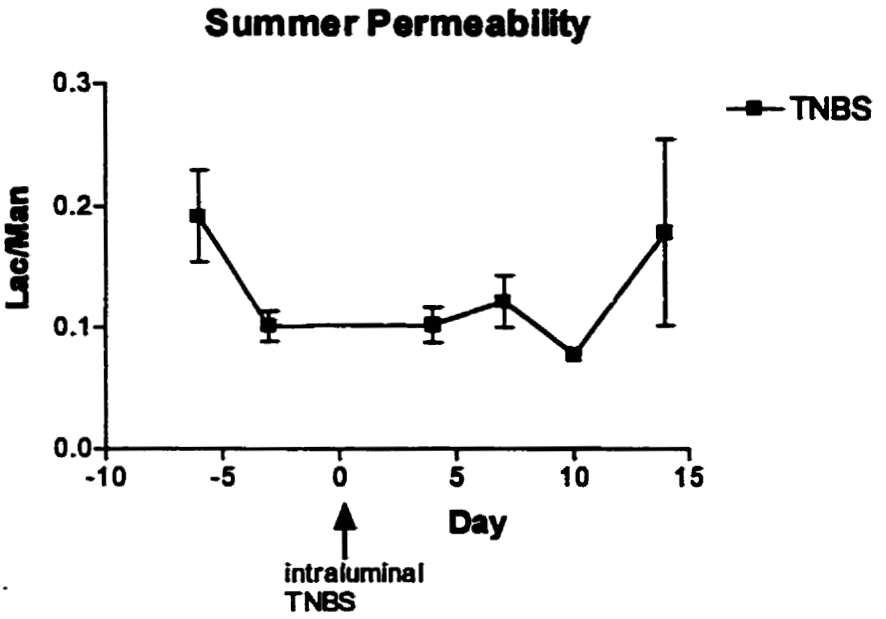


Fig. 13 Small intestinal permeability during a summer month. No effect upon permeability was elicited by intraluminal administration of TNBS/ ethanol.

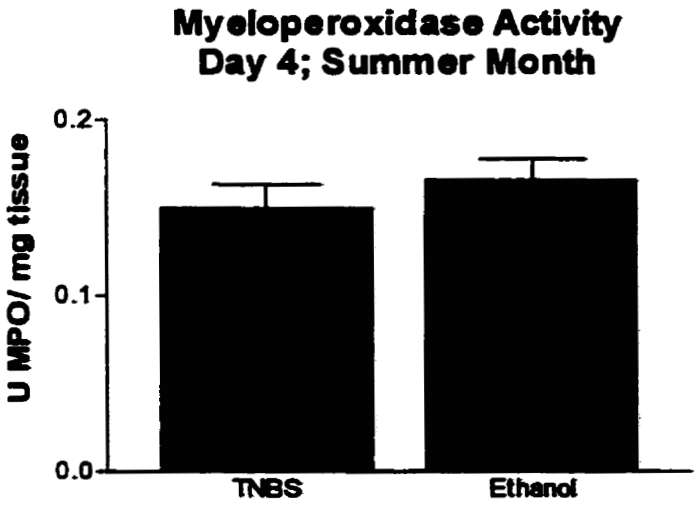


Fig. 13 Myeloperoxidase activity in the terminal ileum of guinea pigs four days following administration of 60 mg/kg TNBS/ ethanol or equivalent volumes of ethanol. No significant differences were observed.

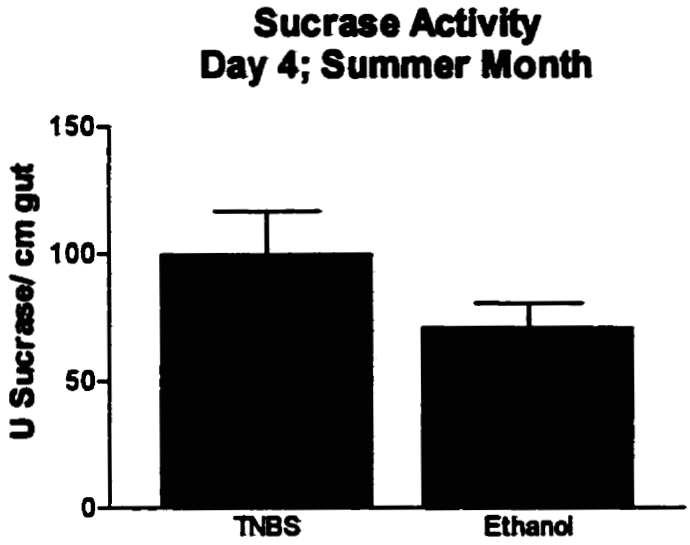


Fig. 14 No significant differences in the amount of sucrase activity in mucosal scrapings of the terminal ileum were observed following administration of TNBS/ethanol.

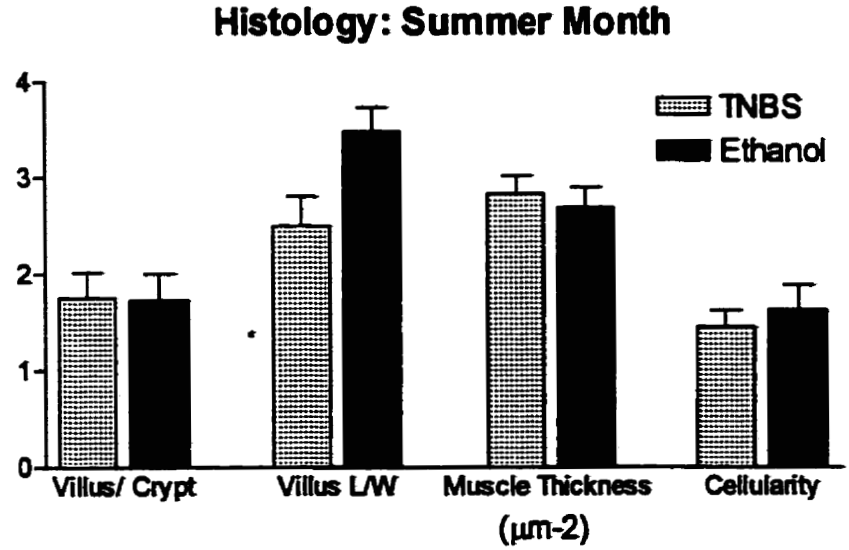


Fig. 15 No significant differences between the control and the TNBS recipients were found in terms of morphology of the gastrointestinal mucosa, the thickness of the muscle or the relative infiltration of granulocytes into the tissue.

## Discussion

There are no reports in the literature to support circannual rhythms in the development of TNBS ileitis. In humans, a retrospective analysis of ulceration and inflammation in the upper gastrointestinal tract found that there is a lower occurrence during the summer months (103). There is also some evidence in the literature to indicate the suppression of the immune system in response to heat stress (104). These animals were shipped during summer months and therefore it is possible that they underwent some heat stress that is subsequently suppressing the immune response to TNBS. The guinea pigs were acclimatized to the Animal Resource Centre climate for two weeks following shipping. The duration of the heat shock response was not reported; however, it is unlikely that the suppression of the immune response would be sufficent three weeks afterwards to prevent the TNBS-induced reaction. The circannual variation in the development of TNBS ileitis suggests caution in interpreting other studies of TNBS ileitis in guinea pigs. Additionally, investigation of the mechanism for non-reaction may provide insight into the regulation of the mucosal immune system that could provide alternatives in the management of inflammatory bowel disease.

# Chapter 6

## Results:Cytotoxicity model

## Indomethacin-induced enteritis in Rats

### Experiment 4

Hypothesis:

ALX-0600 will prevent or ameliorate the effects of indomethacin-induced enteritis in rats.

Methods:

Rats were divided into five weight matched groups, Controls, Indomethacin, Pretreatment, Concurrent and ALX-0600. Enteritis was induced through two daily subcutaneous injections of 7.5 mg/kg indomethacin in 5% bicarbonate in the Indomethacin, Pretreatment and Concurrent treatment groups. The Control and ALX-0600 group received equivalent volumes of 5% bicarbonate. ALX-0600 was administered at a dose of 0.1 μg/kg in PBS twice a day for seven days prior to indomethacin administration in both the ALX-0600 and Pretreatment groups; treatment continued for the entire study period. The Concurrent group received PBS subcutaneously (ALX-0600 vehicle) for the seven days prior to the administration of indomethacin. They began receiving ALX-0600 on the same day as they received the indomethacin, and ALX-0600 administration continued for the duration of the study period. The control group and the indomethacin group received equivalent volumes of PBS for the entire study period. (Fig. 16) Permeability was assessed using the method described in chapter 3. Briefly the rats were fasted for two hours, followed by administration of 500 mg of sucrose, 120 mg lactulose, 80 mg mannitol, 60 mg sucralose and 50 mg xylose by gavage. Urine was collected for 18 to 20 hours and excretion of the sugars was quantified by HPLC urinalysis. Rats were sacrificed seven and fourteen days

following indomethacin administration for macroscopic, histological and enzyme acivity assays.

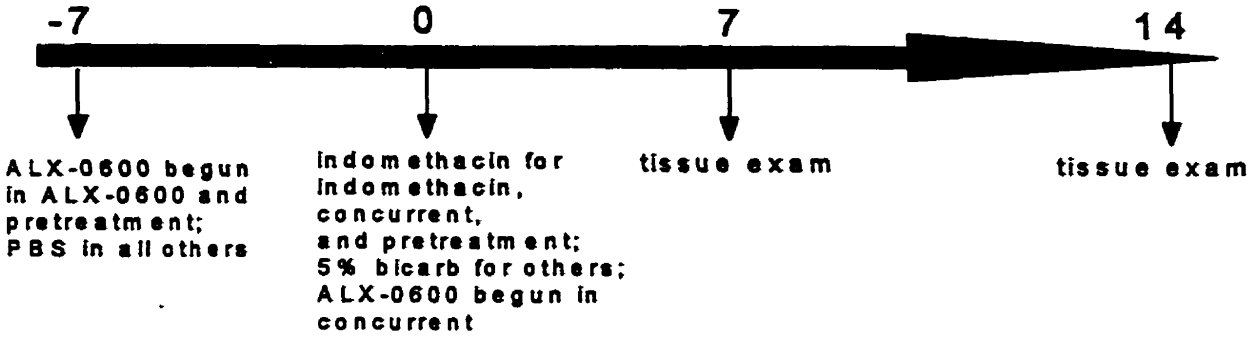


Fig. 16 Time scheme for groups in experiment 4

Results:

We assessed gastrointestinal permeability six times over the twenty-one day study period. (Fig 17) Ratios of the lactulose to mannitol fractional excretion were summed and the small intesinal permeability assessed as the area under the curve over the 21 day experiment. (Fig 18) Small intestinal permeability was increased ($1.68 \pm 0.07$ to $2.24 \pm .1$; $p < 0.001$) in the indomethacin group. Both the pretreatment and ($1.89 \pm 0.06$) and concurrent ($1.99 \pm 0.09$) groups had small intestinal permeability similar to the control group ($1.68 \pm 0.075$) and were also not significantly different from the ALX-0600 group ($1.38 \pm 0.09$; $p>.05$). Permeability in the ALX-0600 group was significantly lower than the control ($p< 0.01$). Neither gastric permeability, an assessment of psychological stress, nor colonic permeability, an indice of colitis, were altered in any group during the course of the experiment. Weight gain was depressed in all the groups receiving indomethacin and in the group receiving only ALX-0600. (Fig 19)

Visual examination of macroscopic damage in the small intestine revealed damage primarily in the jejunum, occasionally extending the entire length of the intestine. (Fig 20) Seven days after indomethacin administration, the macroscopic damage score for the indomethacin group ($3.9 \pm 0.31$; $p < 0.001$) was significantly higher, as was the concurrent group ($1.64 \pm 0.55$; $p < .01$). The pretreatment group was protected from macroscopic damage ($1.5 \pm 0.5$). Fourteen days following indomethacin exposure both the indomethacin group ($2.2 \pm 0.47$; $p < 0.01$) and the concurrent group ($0.64 \pm .054$; $p < .05$) had significant macroscopic damage. Total area of damage was assessed by planimetry to ascertain the extent of mucosal involvement in enteritis. (Fig 21) Indomethacin groups had significant areas of damage on both days seven and fourteen

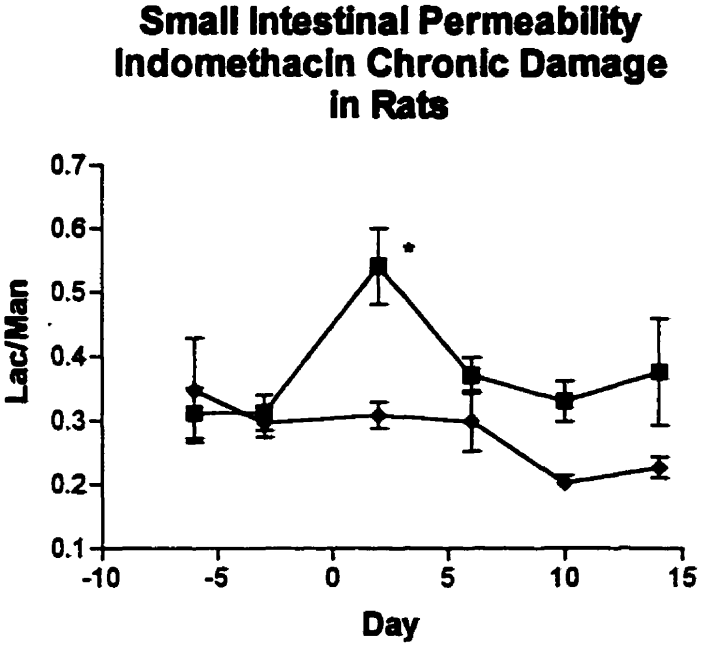


Fig. 17 Indomethacin significantly increases small intestinal permeability immediately following administration (■; * $p < 0.001$, $n \geq 20$). This resolves to baseline within fourteen days. There is no change in permeability following the administration of bicarbonate (◆).

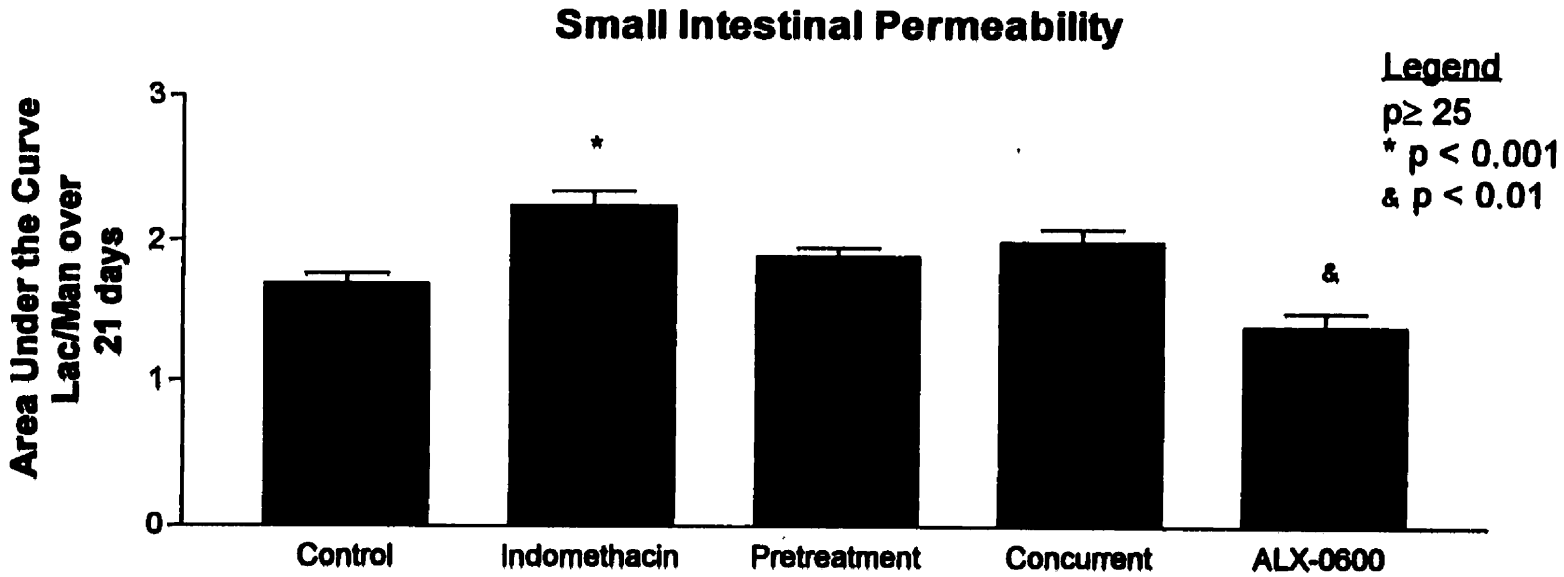


Fig. 21 Small intestinal permeability as a function of sugar excretion ratios over time. Rats receiving a seven day pretreatment or concurrent therapy with ALX-0600 did not demonstrate increased intestinal permeability compared with control as the indomethacin group did (* $p < 0.001$; $n \geq 15$). Animals receiving ALX-0600 for 21 days demonstrate a significant reduction in small intestinal permeability (& $p < 0.01$; $n \geq 15$) compared to control.

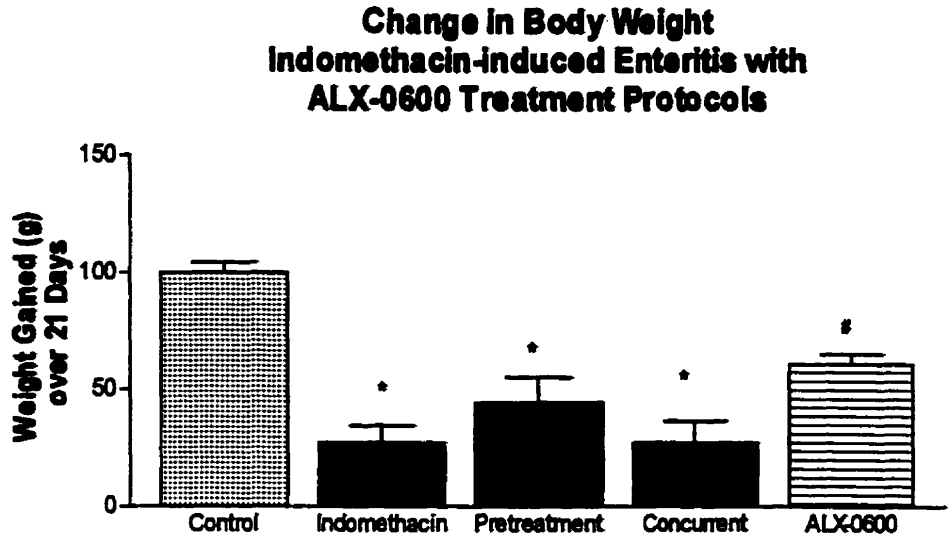


Fig. 19 ALX-0600 was not protective against the weight loss associated with indomethacin-induced enteritis (* $p < 0.001$; $n \geq 15$) in either the pretreatment ( * $p < 0.001$) or concurrent (* $p<0.001$) groups. ALX-0600 reduced overall weight gain significantly (# $p < 0.01$; $n \geq 15$) compared with controls.

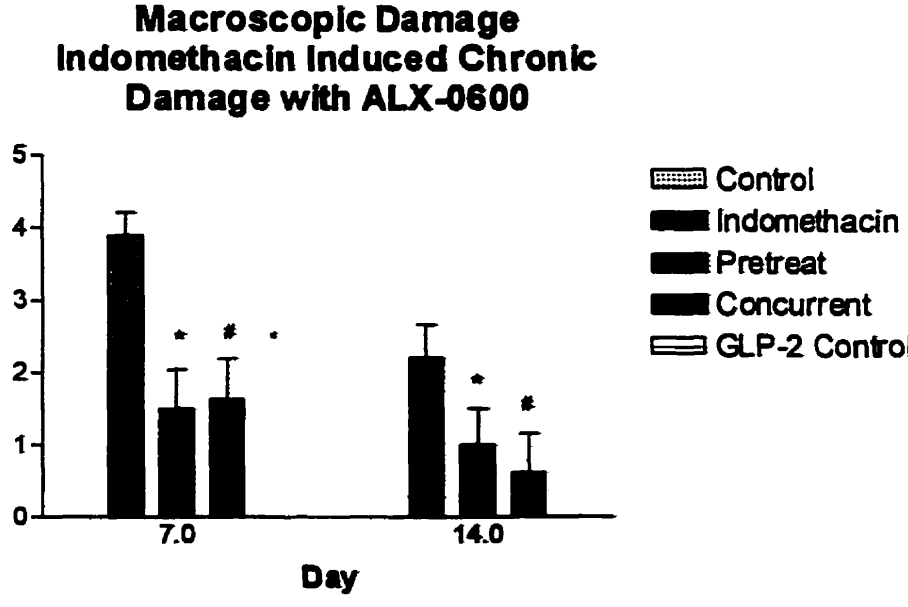


Fig. 20 ALX-0600 is protective for macroscopic damage caused by indomethacin when given as pretreatment (* $p < 0.001$) or concurrent (# $p < 0.01$) therapy.

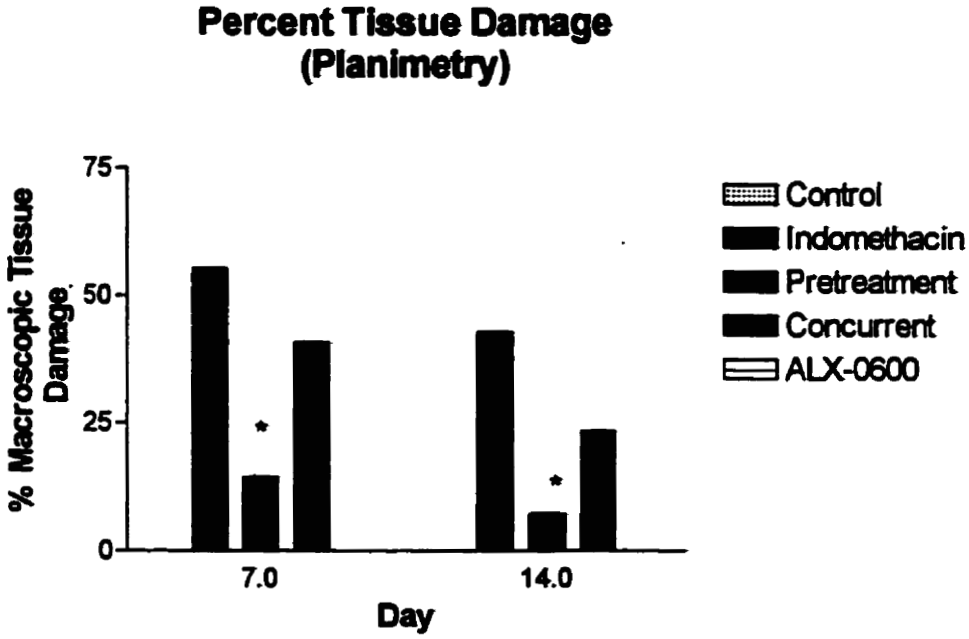


Figure 21. ALX-0600 pretreatment significantly (* $p < 0.001$, $n \geq 10$) reduces the macroscopically visible damage to the small intestine induced by indomethacin.

(55.27 % ± 0.09, 42.82 % ± 0.07; $p < 0.001$) while the concurrent group had significant area of damage only on day seven (40.7 % ± 0.12; $p < 0.01$). The pretreatment group did not exhibit significant area of damage (14.4 % ± 0.06; $p > 0.05$). Macroscopically, ALX-0600 pretreatment was highly effective in preventing significant small intestinal damage due to indomethacin exposure.

Inflammation is characterized by the infiltration of granulocytes, especially neutrophils, into the affected tissue. The extent of granulocyte infiltration was estimated through the quantification of the myeloperoxidase enzyme in segments of proximal jejunum showing non-ulcerative damage. (Fig 22) One unit of myeloperoxidase is taken to represent one granulocyte. Significant myeloperoxidase activity was assessed on day 7 in the indomethacin (0.44 ± 0.001; $p < 0.001$), pretreatment (0.42 ± 0.7; $p < .01$) and concurrent (0.65 ± 0.08; $p < .001$) groups. Inflammatory cell influx was reduced to normal levels in the pretreatment and concurrent group but remained significantly elevated in the indomethacin group (1.12 ± 0.2; $p < 0.01$) fourteen days following indomethacin exposure. Inflammation is also characterized by edema, which was assessed histologically through the measurement of muscle thickness. Indomethacin exposure resulted in significant edema in all exposed groups, ALX-0600 did not affect the extent of edema observed. (Fig 23) The protective effects of ALX-0600 demonstrated at the macroscopic level were not reflected at the microscopic level; however, ALX-0600 did accelerate the rate of healing in the pretreatment group.

To confirm the function of the peptide hormone analog ALX-0600, villus and crypt morphology was assessed in the proximal jejunum. (Fig 24) Villus length was significantly increased in the ALX-0600 group at both time points, resulting in a

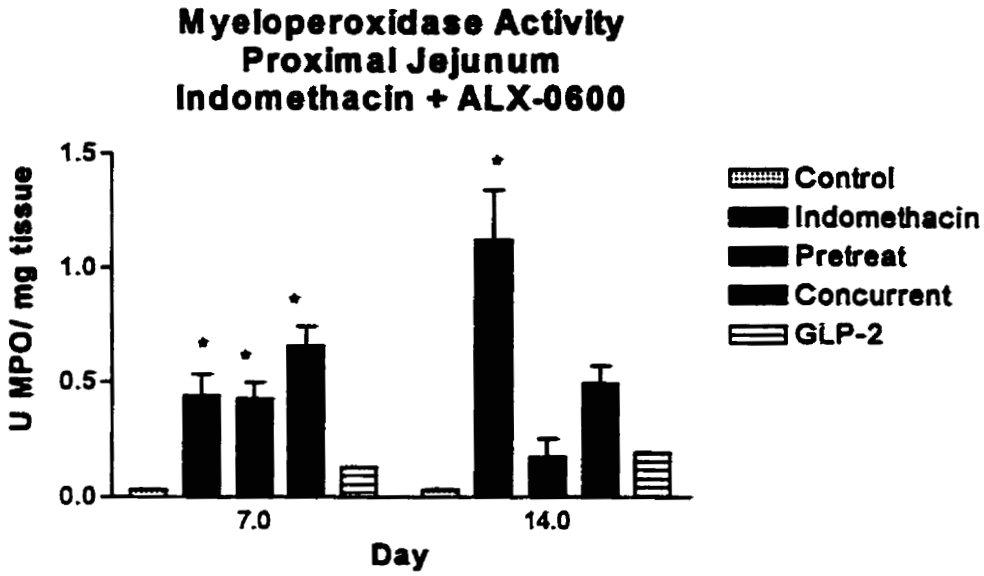


Figure 22 Myeloperoxidase activity is significantly (* $p < 0.001$, $n \geq 10$) increased on day 7 in all groups receiving indomethacin; by day 14 MPO remains elevated in the indomethacin control group only.

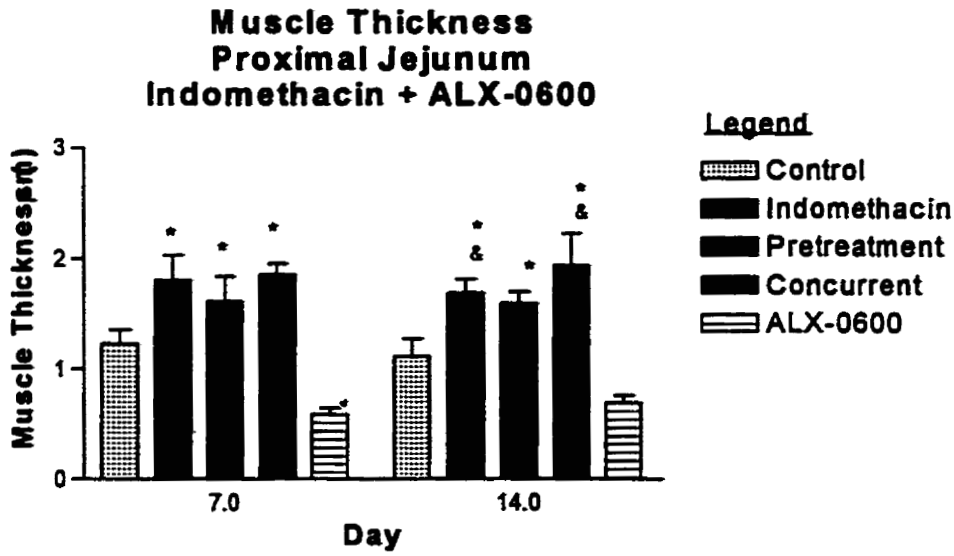


Fig. 23 Muscle thickness was significantly increased in the indomethacin, pretreatment and concurrent groups (* $p < 0.01$) at day 7 ($n \geq 10$). At day 14, ($n \geq 10$) the indomethacin, pretreatment and concurrent groups were all significantly thicker than controls ( * $p < 0.01$) and the indomethacin and concurrent groups were significantly thicker than the pretreatment group (& $p < 0.05$).

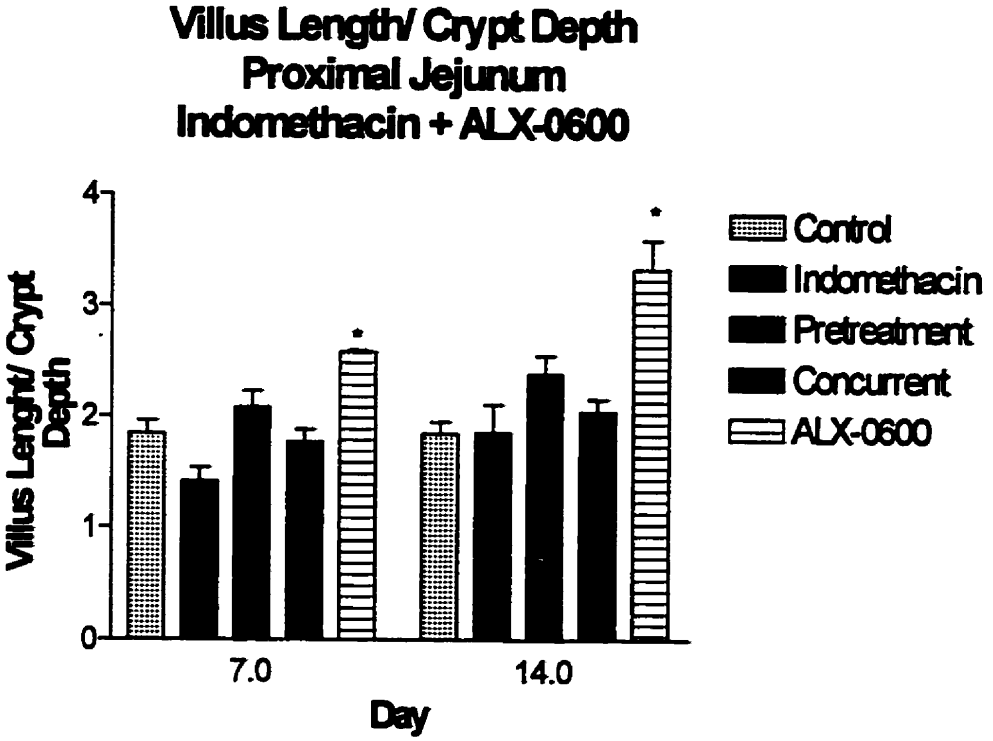


Fig. 24 Villus length was significantly increased in the ALX-0600 group (* $p < 0.001$, $n \geq 10$) on both days seven and fourteen resulting in an increased villus length/ crypt depth ratio.

significant increase in villus length to crypt depth ratio (2.58 ± 0.01; $p < 0.001$). Villus length to width ratio increased in the ALX-0600 group (4.5 ± 0.07; $p < .001$) on day 7, but not until day 14 in either the pretreatment (3.94 ± 0.29; $p < 0.001$) or concurrent (3.68 ± 0.029; $p < 0.01$). (Fig. 25) The ALX-0600 pretreatment group continued to demonstrate increased villus length to width ratios (3.5 ± 0.25; $p < 0.001$) while the indomethacin (2.75 ± 0.26) were not significantly different from controls (2.25 ± 0.13) The alterations in crypt villus morphology are consistent with previous descriptions of ALX-0600 and GLP-2 function and indicate a graded response to ALX-0600 dependent upon the severity of the inflammatory reaction to indomethacin.

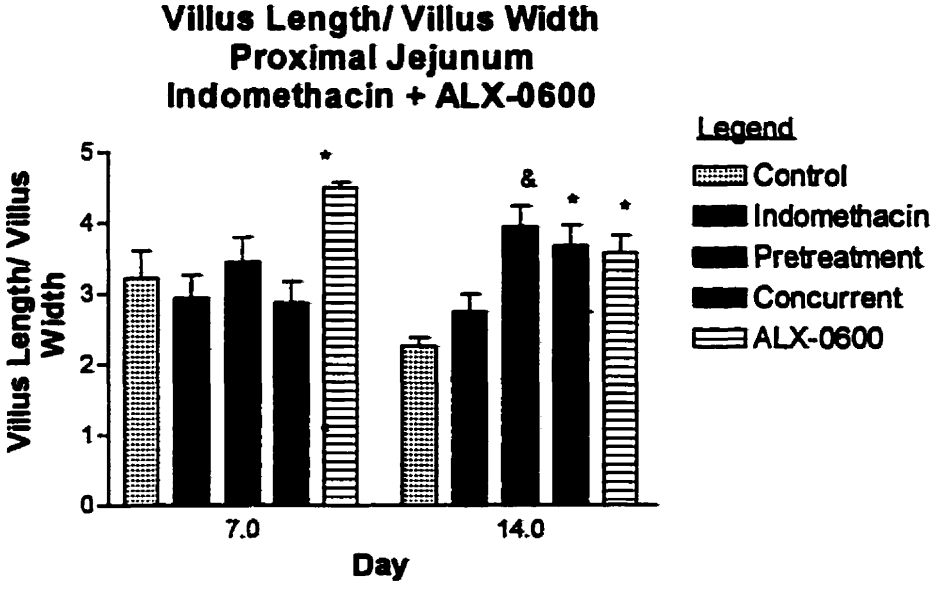


Fig. 25 Seven days following the onset of enteritis, only the ALX-0600 control group demonstrated significantly longer, narrower villi. (* $p < 0.001$, $n \geq 10$) Fourteen days following indomethacin the pretreatment (& $p < 0.01$, $n \geq 10$) and concurrent (* $p < 0.001$, $n \geq 10$) treatment groups also had increased villus length to width ratios.

**Discussion:**

NSAID enteritis induced either through chronic use in humans or acute doses in animal models such as utilized in this study, result in a characteristic increase in small intestinal permeability, inflammatory cell influx, ulceration and erosions (33). The similarities in damage to inflammatory bowel disease made this a good model of disease induced through direct cytotoxicity. Therapy of inflammatory bowel disease has focused upon relief of inflammation and secondary infection; however, acceleration of the healing process has become a subject of research in recent years. Glucagon-like peptide 2 has been shown to be a highly specific and potent mitogenic factor in the small intestine. It increases the mitotic index of crypt progenitor cells and decreases the apoptotic rate of villus tip cells (112).

We have shown that the GLP-2 analog ALX-0600 is effective in preventing indomethacin induced chronic enteritis in rats whether given in a pretreatment or concurrent treatment regime. GLP-2 significantly reduces the acute increase in small intestinal permeability. This correlates with a decrease in macroscopic damage scores, myeloperoxidase levels and histological changes seen in indomethacin control animals. While the concurrent treatment group did not perform as well as the pretreatment group, this is likely to be due to the different time period during which they received the hormone analog, as the effects of ALX-0600 and GLP-2 have been shown to accumulate over time. We observed the expected increase in villus length in the control animals receiving ALX-0600, confirming results from our (99) and other labs (81).

The first documented morphological changes following indomethacin treatment is the loss of crypt cells (113). This loss of crypts may result in the increase in permeability

and increased translocation of luminal antigen across the mucosal barrier. Treatment with ALX-0600 increases villi length but has no effect upon crypt morphology; however; crypt cells secrete a variety of compounds, including IgA that serves to protect the mucosa from luminal antigen (114). Antigen is found in a decreasing gradient from the villus tip to the crypt. Therefore, pretreatment with ALX-0600 may increase the distance which luminal antigen must cross to reach the breached barrier, resulting in less inflammation in the pretreated animals. The invasion of the mucosa by luminal contents has been shown to be essential to the development of indomethacin enteritis as germ free, bile-duct ligated or fasted rats are immune to the effects of indomethacin (115). Pretreatment with ALX-0600 allows a greater functional mucosa at the time of damage, preventing the functional malabsorption and diarrhea experienced by the concurrent and indomethacin control groups. Indomethacin undergoes enterohepatic circulation, which has been shown to be a primary factor in the pathogenesis of indomethacin-induced enteritis, and causes an increase in enteric bacterial numbers. The increased permeability resulting from continual exposure to a recirculating NSAID such as indomethacin or diclofenac would be the equivalent of multiple doses of a non-recirculating drug such as nitrofenac. The resulting continual increase in permeability coupled with the increase in bacterial colonization in the lumen, characteristic of NSAID induced damage, exacerbates the initial damage to the mucosa (116) Indomethacin also causes an accelerated turnover of cells in the small intestinal mucosa (117), the opposite effect of ALX-0600 therefore the effects of indomethacin may be blunted in the pretreated animals. Further investigations of luminal antigen translocation and nutritional status of these animals would be necessary to clarify

the mechanisms of the protective actions of ALX-0600 in NSAID induced chronic enteritis.

ALX-0600 given from the time of indomethacin aids in the healing of the direct cellular damage. One of the mechanisms postulated to contribute to NSAID enteritis is the inhibition of oxidative phosphorylation. This leads to a decrease in ATP production and calcium cytotoxicity (118). ALX-0600 acts through adenylyl cyclase to increase intracellular cAMP levels which may prevent the internal cytotoxic effects of indomethacin on mitochondria. Regulation of tight junctions is ATP dependent, therefore the increase in permeability associated with indomethacin treatment may be partially associated with lack of tight junctional control (119). ALX-0600 induced increases in the precursors of the TCA cycle may help to prevent disruption of normal cellular functions. Indomethacin is also a potent inhibitor of EGF release (120). Lack of EGF inhibits the healing of intestinal epithelia however ALX-0600 may substitute for the lack of EGF, stimulating healing through increased mitotic activity in the crypt. Prostaglandin $E_2$ ($PGE_2$) is inhibited through the actions of indomethacin on the enzyme cyclooxygenase. $PGE_2$ influences the migration rate and turnover time of epithelial cells (117), a function of ALX-0600 as well, therefore ALX-0600 may correct the deficit of this inflammatory mediator by increasing migration and delaying turnover without the other inflammatory side effects of $PGE_2$.

In summary, we have demonstrated that both pretreatment and concurrent therapy with ALX-0600, an analog to GLP-2, are effective in the treatment of NSAID induced chronic enteritis. Pretreatment is more effective during the acute phases of the disease,

however, both treatment groups demonstrated significant improvements in a wide variety of functional and morphological assessment in comparison to the control group.

# Chapter 7

## Conclusions

This thesis documents the characterization of an acute model of ileitis in the guinea pig induced by TNBS. TNBS ileitis was induced through the intraluminal injection of TNBS and ethanol initiating an inflammation that lasted for two weeks. Increased intestinal permeability, granulocyte infiltration and increased muscle thickness characterized the disease. ALX-0600 was used to test the hypothesis that pretreatment with this analog of GLP-2 would prevent or change the course of the disease, however there was no effect upon the disease. Attempts to develop a reactivating model of TNBS ileitis were not reproducible and therefore testing ALX-0600 on the reactivation phase of ileitis was not possible.

Indomethacin-induced enteritis was the other model of inflammatory bowel disease used to test the use of ALX-0600 in the treatment or prevention of IBD. ALX-0600 pretreatment was protective against the damage to the small intestine induced by indomethacin while concurrent therapy increased the rate of healing in the rats who received indomethacin. ALX-0600 in the absence of indomethacin induced the expected, previously documented changes in mucosal morphology and caused a previously undocumented decrease in small intestinal permeability. ALX-0600 is beneficial in this model of inflammatory bowel disease.

The difference between these two models lies in both the system and the type of disease initiated. TNBS ileitis in the guinea pig is initiated through an immune reaction to a non-self epitope created when TNBS absorbs to lysine residues on the surface of

interstitial cells. Indomethacin enteritis results from direct cytotoxicity of toward the epithelial cells, particularly in the crypt. This initiates an inflammatory reaction due to tissue necrosis and the infiltration of luminal contents to the interstitial space. The inability of ALX-0600 to prevent development of TNBS ileitis may be due to the inability of the hormone to affect the immunological response of macrophages and cytotoxic T cells to the presence of non-self epitopes. TNBS ileitis is a transient disease and increasing the healing rate of the epithelia by increasing the mitotic index may not have detectable effects on the course of the disease. Indomethacin induced enteritis is the result of cytotoxicity and the inhibition of mitogens such as $PGE_2$ and epidermal growth factor (EGF). The effects of ALX-0600 may have been the result of replacing $PGE_2$ and epidermal growth factor and thereby increasing the healing rate of the enteritis. Treatment of human inflammatory bowel disease with ALX-0600 may be marginally beneficial as some of the damage is thought to result from disregulation of the regulatory system of the gastrointestinal epithelia. Investigation of the effects of ALX-0600 on other models of inflammatory bowel disease and clarification of the underlying cause of IBD will aid in the development of this or other therapies

## Appendix 1 Materials

| Item | Source |
|---|---|
| ALX-0600 (GLP-2 analog) | Allelix Biopharmaceuticals Ltd, Mississauga, ON |
| Acetonitrile | VWR, Edmonton, AB |
| Amberlite 120 resin | BDH, Edmonton, AB |
| Amberlite 400 resin | BDH, Edmonton, AB |
| Americlear | Baxter, Deerfield, IL |
| Autotechnicon | Technicon Co., Chauncey, NY |
| Betadine | Perdue Frederick Inc.,Toronto, ON |
| BioRad Protein Assay Kit | BioRad, Richmond, CA |
| Cellubiose | Sigma, Oakville, ON |
| Centrifuge | Damon, IEC Division |
| Cover Slips, Glass 24x50 | VWR, Edmonton, AB |
| D-xylose | BDH, Edmonton, AB |
| Desk top eppendorf centrifuge | VWR, Edmonton, AB |
| Di-postassium hydrogen orthophosphate | BDH, Edmonton, AB |
| DABA | Kodak, Eastman Fine Chemicals, Rochester, NY |
| EDTA | Fisher Scientific |
| Entalin | BDH, Edmonton, AB |
| Eosin | VWR, Edmonton, AB |
| Ethanol | Commercial Alcohols Inc., Brampton, ON |
| Eppendorf tubes | VWR, Edmonton, AB |
| Formalin 10% | VWR, Edomonton, AB |
| Garamycin | Schering, Pointe Claire, QC |
| Glacial acetic acid | BDH, Edmonton, AB |
| Glucose oxidase | Sigma, Oakville, ON |
| HPLC - sucrose, lactulose, mannitol | Dionex, Oakville, ON |
| HPLC- sucralose | Hewlett Packard, Oakville, ON |
| Halothane | Benson Medical Industries, Markham, Ontario |
| Hemotoxalin | VWR, Edmonton, AB |
| HTAB | Sigma, Oakville, ON |
| Hisotomatic Slide Stainer | Fisher Scientific, Nepean, ON |
| Hydrochloric acid | BDH, Edmonton, AB |
| Indomethacin | Sigma, Oakville, ON |
| Ionpac NS1 column | Dionex, Oakville, ON |
| Isopropanol | BDH, Edmonton, AB |
| Lactulose | Solvay Pharma Inc., Scarbourgh, ON |
| MA-1 ion exchange column | Dionex, Oakville, ON |

| Item | Source |
|---|---|
| Male Hartley Guinea Pigs | Charles River Laboratories, St. Constant, QC |
| Male Wistar Rats | Charles River Laboratories, St. Constant, QC |
| Mannitol | BDH, Edmonton, AB |
| Microtome | Spencer |
| Mineral oil | BDH, Edmonton, AB |
| Mould Release | Fisher Scientific, Nepean, ON |
| o-diansidine | Sigma, Oakville, ON |
| Oxygen | PRAXAIR, Mississauga, Ontario |
| Parrafin | Baxter, Deerfield, IL |
| Phenyl-β-d-thiogalactoside | Sigma, Oakville, ON |
| Phloroglucinol | Sigma, Oakville, ON |
| Potassium phosphate monobasic | Fisher Scientific, Nepean, ON |
| Saline 0.9% | Baxter, Deerfield, IIL |
| Scalpel blade #10 | Fisher Scientific, Nepean, ON |
| Silk (3-0) with PS-2 cutting needle | Ethicon, Johnson & Johnson, Brampton, ON |
| Slides | VWR, Edmonton, AB |
| Sodium bicarbonate | BDH, Edmonton, AB |
| Sodium hydroxide | BDH, Edmonton, AB |
| Spectrophotometer | Hewlett-Packard, Oakville, ON |
| Sucralose | McNeil, Guelph, ON |
| Sucrose | Redpath Industries, Vancouver, BC |
| Syringe 3cc | Becton Dickson, Franklin Lakes, NJ |
| Syringe tuberculin | Becton Dickson, Franklin Lakes, NJ |
| Syringe filter (45µm) | Chromatographic Specialities Inc, Brockville, ON |
| Test tubes 13 x 100 glass siliconized | VWR, Edmonton, AB |
| Test tubes 16 x 100 glass siliconized | VWR, Edmonton, AB |
| Test tubes 10 x 75 glass siliconized | VWR, Edmonton, AB |
| Thymol | Sigma, Oakville, ON |
| Trinitrobenzene sulfonic acid (TNBS) | Fluka, Sigma, Oakville, ON |
| Triton-X100 | BioRad, Richmond, CA |
| Vicryl (3-0) with RB-1 tapered needle | Ethicon, Johnson & Johnson, Brampton, ON |

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