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Epithelial and Smooth Muscular Consequences of Gut Inflammation

by

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A THESIS

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You know a dream is like a river
Ever changin' as it flows
And the dreamer's just a vessel
That must follow where it goes
Trying to learn from what's behind you
And never knowing what's in store
Makes each day a constant battle
Just to stay between the shores...and

I will sail my vessel
'Til the river runs dry
Like a bird upon the wind
These waters are my sky
I'll never reach my destination
If I never try
So I will sail my vessel
'Til the river runs dry

Excerpt from "The River"
(Victoria Shaw, Garth Brooks)

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Epithelial and Smooth Muscular Consequences of Gut Inflammation" submitted by Bryn Alexander Watson in partial fulfilment of the requirements for the degree of Master of Science.

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Abstract

The gastrointestinal tract balances the roles of nutrient intake and host defense. This delicate balance can be subverted by noxious agents so that host defense becomes the overwhelming duty of the gut, and significant clinical diseases may arise. Alterations in motility and permeability may contribute to symptoms or the disease process. The aim of this study was to examine motility and permeability changes in two models of gut inflammation.

Motility alterations during *Yersinia enterocolitica* enteritis may originate due to aberrations at the longitudinal muscle plasma membrane. Purified smooth muscle plasma membranes had altered physical properties as well as muscarinic receptors with increased binding affinity. The plasma membrane may modulate the change in receptor function.

Inflammatory reactions in the intestine have demonstrated increased epithelial permeability using anesthesia and surgical techniques. An examination of conscious animals with extremely sensitive sugar probes revealed no increased permeability during food protein-induced anaphylaxis. Permeability variations may play little role in the pathophysiological consequences of food allergies.

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Dedication

This thesis is dedicated to my family:

To my dad, Bill, for teaching me the value of hard work and a sense of humour.

To my mom, Irene, for showing me the virtues of patience, and for putting up with me.

To my brother, John, for educating me on the value of experience in our many trips to the jungle, desert, coral reef and tennis net.

Table of Contents

List of Tables

List of Figures

List of Abbreviations and Symbols

^{3}H	tritium
5HT	5-hydroxytryptamine
^{51}Cr	isotope 51 of chromium
α	alpha
A2C	2-(2-methoxyethoxy)ethyl 8-(cis-2-n-octylcyclopropyl) octanoate
ACh	acetylcholine
ASA	acetylsalicylic acid
ATP	adenosine triphosphate
β	beta
B_{max}	total number of receptor sites
BSA	bovine serum albumin
Ca^{++}	calcium ion(s)
cAMP	cyclic adenosine monophosphate
CBB	coomassie brilliant blue
cc	cubic centimetre
CCh	carbachol
cm	centimetre(s)
CO_2	carbon dioxide
DAG	diacylglycerol
DPH	diphenylhexatriene
DTPA	diethylenetriaminopentaacetate
EA	egg albumin
$E_{D}50$	the dose of agonist at half maximal contraction
EDTA	ethylenediaminetetraacetic acid

ELISA	enzyme linked immunosorbent assay
γ	gamma
g	gram(s)
GI	gastrointestinal
GM-CSF	granulocyte-macrophage colony stimulating factor
HPLC	high performance liquid chromatography
ICAM	intercellular adhesion molecule
IgE	immunoglobulin E
Il-	interleukin
IP3	inositol-1,4,5-trisphosphate
K^+	potassium ion(s)
KCl	potassium chloride
K_d	receptor affinity
kPa	kilopascal(s)
lac:man	lactulose:mannitol ratio
Li	initial length
Lo	optimal length
LT	leukotriene
MBP	major basic protein
MDa	megaDalton(s)
µg	microgram(s)
mg	milligram
mg/kg	milligram(s) per kilogram of body mass
$MgCl_2$	magnesium chloride
MHC II	major histocompatibility complex type 2
µl	microlitre(s)

ml	millilitre(s)
ml/min	millilitre(s) per minute
mM	millimolar
mm	millimeter(s)
mm^2	square millmetre(s)
µmole	micromole(s)
n-AP	anthroyloxypalmitic acid
n-AS	anthroyloxystearic acid
Na^+	sodium ion(s)
NB	*Nippostrongylus brasiliensis*
nM	nanomolar
nm	nanometer(s)
NMS	[*N-methyl*-^{3}H]scopolamine
NSAID	non-steroidal antiinflammatory drug(s)
PAF	platelet activating factor
PCA	passive cutaneous anaphylaxis
PGD_2	prostaglandin D_2
PIP2	phosphatidylinositol-4,5-bisphosphate
PNS	post nuclear supernatant
RMCP II	rat mast cell protease type two
rpm	revolutions per minute
r_s	steady state anisotropy parameter
s.e.m.	standard error of the mean
U	units of activity
YE	*Yersinia enterocolitica*
°C	degrees Celsius

Chapter 1. Introduction

1.1 The Gastrointestinal (GI) Tract

The gastrointestinal tract is the first site of contact of nutrients and many potential antigenic substances. In a single lifetime, human beings ingest approximately 100 tons of food, which contains vital nutrients and countless other potentially hazardous substances. The role of the GI tract is one of selective nutrient and electrolyte absorption, with the exclusion of bacteria, viruses and toxic substances (Crowe and Perdue, 1992).

The epithelium is a continuous, single layer of cells lining the stomach, small intestine and colon. This layer is responsible for protecting the host against noxious substances in the gut lumen, and for selective uptake of required compounds. The single layer of cells is predominantly made up of enterocytes, whose roles are complex, but include secretion, digestion and nutrient absorption.

1.2 Composition of the Epithelium

A single layer of epithelial cells lines the stomach, intestine and colon. The composition and arrangement of the epithelium varies at different locations in the gut, reflecting different roles in GI tract function. In the stomach, gastric pits are found to contain mucous neck cells, chief cells and parietal cells. Chief cells secrete pepsinogen, and parietal cells secrete the hydrochloric acid that gives the stomach its low pH (Despopoulos and Silbernagl, 1991).

The small intestine is arranged into fingerlike projections out into the gut lumen called villi; indentations between the projections are the crypts. Intermixed with the enterocytes in

the villus epithelium are goblet cells, endocrine cells, intraepithelial leukocytes and M-cells. An additional cell type, Paneth cells, are found in the crypt.

The epithelium of the colon is arranged into pits, or crypts, which contain mucous secreting goblet cells (Despopoulos and Silbernagl, 1991).

1.3 Composition of the Gut

The stomach, small intestine and colon share a common structural arrangement. Epithelial cells are fastened together at their apical surface by tight junctions and are supported by a basement membrane. Together, the enterocytes and other epithelial cells secrete the basement membrane, a tough mat of type IV collagen, proteoglycans, laminin and entactin (Alberts *et al.*, 1989). Beneath the basement membrane is the lamina propria, which contains nerves, connective tissue and a variety of immune cells such as T- and B-lymphocytes, plasma cells, macrophages, mast cells, eosinophils and neutrophils (Sanford, 1992). These cells react to antigenic stimuli via receptors, by the ability to bind antibodies, or in a non-specific manner such as the response to bacterial endotoxin (Crowe and Perdue, 1992). Beyond the lamina propria is the muscularis mucosa, submucous plexus, longitudinal muscle layer, myenteric plexus and the circular muscle layer. The plexuses are part of the enteric nervous system, while the two muscle layers are involved in peristalsis, which propels luminal contents along the gastrointestinal tract. The final layer of the gut is the serosal layer, comprised of the visceral peritoneum merging with connective tissue (Sanford, 1992).

1.4 Smooth Muscle and Intestinal Motility

Encircling the gut is a bilayer of smooth muscle that handles the propulsive functions of the gastrointestinal tract. The external longitudinal muscle and inner circular muscle are named to reflect cellular and contractile fiber orientation. Contraction of the longitudinal muscle shortens segments of gut, while contraction of the circular muscle decreases the diameter of the gut lumen. These muscles contract in a phasic and tonic manner.

1.4.1 Phasic Contraction

Phasic contraction arises as a result of cyclic electrical activity in the plasma membranes of the muscle cells themselves. The oscillations in membrane potential are known as the slow wave. It is thought that continued leaking of ions results in the eventual depolarization of cell membranes, or that the membrane potential of the smooth muscle plasma membranes can be influenced by cells between the two muscle layers, the interstitial cells of Cahal (Sanford, 1992; Huizinga, 1991). When membrane depolarization occurs to a significant enough degree, voltage-gated Ca^{++} channels are opened and electrophysiological Ca^{++} "spiking" is observed. Spiking events do not occur with every oscillation of the slow wave, but their presence always results in contraction (Oigaard and Dorph, 1974). In the intestine, proximal segments have a higher slow wave frequency than distal segments, and therefore impose their frequency on more distal segments. Eventually, a segment will be reached where the smooth muscle cells are incapable of responding to the slow wave frequency, and the rate of the slow wave oscillations will fall (Sanford, 1992). If spiking is occurring in the traveling band of electrical activity, then propagating contractions will occur.

The electrical activity of the smooth muscle can be detected with the attachment of electrodes to the gut *in vivo*. Electrodes fastened in the smooth muscle layer of the GI tract detect electrical signals, such as the migrating myoelectric complex (MMC). The MMC is a band of electrical and contractile activity that propagates distally in the fasted state in all mammals, and in the fed state in herbivores (e.g. rabbits) (Szurszewski, 1969; Ruckebusch *et al.*, 1985). Its role in the fasted state is one of housekeeping, as lack of an MMC results in bacterial overgrowth (Granger *et al.*, 1985). The migrating myoelectric complex is comprised of three discrete regions known as Phases I, II and III (Code and Marlett, 1975).

Phase I is identified by a lack of spiking and contraction, which is followed by Phase II and its irregular spiking and contractions. Phase III is characterized by intense electrical spiking and contractile activity. The contractile activity in phase III effectively pushes luminal material aborally, preventing orad emigration of colonic bacteria (Granger *et al.*, 1985). Interruptions in the MMC may be associated with disease states such as *Yersinia enterocolitica* (YE) enteritis in the rabbit and food protein-induced intestinal anaphylaxis in the rat (Scott *et al.*, 1988a; Scott *et al.*, 1988b).

1.4.2 Tonic Contraction

The smooth muscle of the gastrointestinal tract also contracts in a continuous manner that is not associated with the rhythmic depolarization of cell membranes (Guyton, 1991). Tonic contraction of intestinal smooth muscle is mostly under the control of the myenteric plexus, a large and complex network of neurons between the two muscle layers. Neuronal interconnections with the submucous plexus are also involved in the modulation of muscle contraction, leading to a complex network of control. Furthermore, there may be some control exerted over intestinal motility by the central nervous system (Sanford, 1992). Many

compounds have been found to alter intestinal motility, including vasoactive intestinal peptide, somatostatin, substance P, opiod peptides, neurotensin, enteroglucagon and peptide YY. Many of these agents are thought to decrease motility. However, acetylcholine (ACh) released from cells of the myenteric plexus promotes motility by acting on muscarinic cholinergic receptors to initiate a signal cascade leading to contraction (Somlyo and Somlyo, 1994).

The signal cascade is initiated by ACh binding to muscarinic receptors of the M3 type (Jones, 1993). Upon binding to the receptor, a conformational change is induced in the receptor, the effect of which is transmitted across the bilayer to the inner hemi-leaflet of the plasma membrane. This change in conformation leads to a favorable association with a heterotrimeric G protein, Gq. The ensuing G protein conformational change leads to an exchange of bound GDP for GTP, and a final conformational change leads to the favorable dissociation of the G protein into two parts, α-GTP and a $\beta\gamma$ dimer. The α-GTP fragment activates a plasma membrane associated enzyme, phospholipase C. Phospholipase C hydrolyses the membrane phospholipid, phosphatidylinositol-4,5-bisphosphate, to release soluble inositol-1,4,5-trisphosphate (IP_3) and membrane associated diacylglycerol (DAG).

The target for intracellular IP_3 is a large membrane bound receptor found in the membrane of the sarcoplasmic reticulum, and a conformational change in the receptor allows the flow of Ca^{++} ions from the sarcoplasmic reticulum into the cytosol. The increase in $[Ca^{++}]_i$ can activate Ca^{++}-gated K^+ channels, and the subsequent depolarization may open plasma membrane bound voltage-gated Ca^{++} channels to further increase $[Ca^{++}]_i$. The IP_3 receptor is also found in the nuclear membrane (Malviya, 1994), and in specialized pockets of plasma membrane called caveolae (Fujimoto *et al.*, 1992). Although the presence of a nuclear IP_3 receptor probably has little, if any, role in muscle contraction,

the plasma membrane localized receptors may play a role in the influx of extracellular Ca^{++} that precedes contraction (Somlyo and Somlyo, 1994).

As the increase in $[Ca^{++}]_i$ continues, the intracellular protein calmodulin is activated. Calmodulin activates myosin light chain kinase, which phosphorylates the light chain of myosin. Once phosphorylated, myosin filaments interact with actin filaments in an ATP-dependent manner, and muscle contraction occurs (Somlyo and Somlyo, 1994).

This mechanism of smooth muscle contraction is not universal to all kinds of smooth muscle. Rather, the mechanisms of contractility may depend on the agonist and tissue site. Contraction of cat gallbladder strips induced by cholecystokinin-8 is dependent on IP_3 formation and intracellular calcium release (Lee *et al.*, 1989). However, in isolated cat esophageal smooth muscle cells, ACh induces contraction through M2 receptors coupled to G_{13} protein, which activate phospholipase D to liberate DAG from phosphatidylcholine (Sohn *et al.*, 1993). DAG activates protein kinase C (PKC), leading to muscle contraction; this contraction is independent of IP_3 formation and the release of $[Ca^{++}]_i$. Finally, the contributions of both M2 and M3 receptors and their associated phospholipase D and C pathways, respectively, leads to ACh-induced contraction of isolated cat gallbladder cells (Chen *et al.*, 1995).

Many mediators other than ACh are capable of inducing smooth muscle contraction, including histamine, PGD_2 and 5-hydroxytryptamine (5HT) (Scott and Maric, 1993) and several other products released in inflammatory events, such as leukotrienes (Freedman *et al.*, 1992; Scott *et al.*, 1990). These products may be in close proximity to the smooth muscle during the course of inflammatory events, and may act to influence the contractile behavior of the muscle.

1.4.3 Methods to Study Motility

1.4.3.1 *In vivo*

The electrical activity of the smooth muscle in its physiological state can be studied by the use of electrodes. Electrodes are attached to the gut wall, with their ends located in the vicinity of the smooth muscle layers, and are spaced apart by a known distance. The electrodes are also connected to a chart recorder for plotting electrical signals. As electrical signals are generated and travel from one region of the gut to another, the chart recorder plots the electrical activity. Calculations about the speed of propagation can be obtained by noting the time it takes for the signal to travel from one electrode to another, a known distance apart. The frequency and patterns of electrical activity can also be determined by this technique (Ruckebusch *et al.*, 1985).

The use of non-absorbable radio-labeled molecules such as $Na_2{}^{51}CrO_4$ is a rapid way of investigating intestinal transit (Maric *et al.*, 1989). Animals are given a known quantity of radioactivity, and are sacrificed after an appropriate time. The gut is divided into sections; for example, the stomach, equidistant segments of small intestine, cecum and colon. Samples of stool and blood should also be taken in order to account for all radioactivity. These experiments allow measurement of rates of transit of materials inside the lumen of the gut.

The use of an external gamma camera can also be employed to measure intestinal transit of radio-labeled compounds. This technique can be applied to humans as well as laboratory animals. Subjects ingest a known amount of a gamma emitting isotope (with a very short half-life) such as ^{153}samarium or ^{99m}technetium. A gamma camera is situated over the gastrointestinal tract to record gamma emissions. In this manner transit of compounds

through the gastrointestinal tract can be followed (Larouche *et al.*, 1995). A disadvantage to this technique is that over time, as the isotopes are diluted in transit, is becomes difficult to identify the area of interest in an accurate manner.

1.4.3.2 *In vitro*

Muscle strips as well as excised whole segments of gut can be utilized *in vitro*. The tissues are placed in tissue baths and immersed in an aerated, physiological buffer that strives to mimic the environment *in vivo*. Tissues can be oriented vertically to analyze the longitudinal muscle, or horizontally for the circular muscle, and are typically anchored to a force transducer to detect contractile activity. The contractile activity is amplified by a transducer amplifier, sent to a bioelectric amplifier and finally recorded by a multichannel chart recorder. With several organ baths, many gut segments can be analyzed in a single experiment. The chart recorder prints a continuous reading of the force generated by the muscle under experimental conditions. Typically, the muscle is allowed to attain a consistent pattern of phasic contraction-relaxation cycles, and is stretched to a point where a slight tension is placed on the muscle, called Li (initial length). The muscle is then induced to contract by standard agonists (e.g. carbachol - CCh), the agonists are washed out, and the muscle is stretched again. This process is repeated until a tension is attained where the maximum contractile response of the muscle is elicited in response to agonist; the position of the muscle is referred to as Lo (optimal length). After Lo is attained, chemicals (i.e. KCl), agonists (CCh), electric field or mechanical stimulation are applied to the tissue. The effects of various treatments on tension generation can be obtained upon analysis of the chart recorder data (e.g. Scott and Tan, 1992).

1.4.4 Diseases Affecting Motility

1.4.4.1 General

The appearance of symptoms such as cramping, pain and diarrhea in inflammatory bowel diseases lead to the idea that motility changes may be involved in these diseases. A number of animal models of human inflammatory bowel diseases demonstrate altered GI motility in inflammatory conditions: there is a decreased contractility in rabbit colitis (Xie *et al.*, 1990), YE enteritis (Scott and Tan, 1992), in jejunal circular muscle in inflammation by the nematode *Nippostrongylus brasiliensis* (NB) (Crosthwaite *et al.*, 1990), and in rabbit ileal muscle when directly exposed *in vitro* to *Clostridium difficile* toxin B (Gilbert *et al.*, 1989). In contrast, jejunal smooth muscle exhibits an increased tension in NB and *Trichinella spiralis* inflammation in the rat in response to CCh and 5HT (Farmer *et al.*, 1983; Vermillion and Collins, 1988). In all of these models, intestinal inflammation clearly alters the contractility of intestinal smooth muscle in a manner specific to the animal and inflammatory model.

1.4.4.2 YE enteritis

Yersinia enterocolitica (YE) enteritis is a recognized cause of bacterial enteritis in infants and adolescents (Maki *et al.*, 1980; Marks *et al.*, 1980). The spectrum of clinical illness encompasses acute gastroenteritis and chronic relapsing ileocolitis simulating Crohn's disease of the terminal ileum. Infants and young children commonly present with diarrhea, abdominal cramping, vomiting and fever (Carniel and Mollaret, 1990; Heesemann *et al.*, 1993). Older children and adolescents may experience fever, terminal ileitis and mesenteric lymphadenitis mimicking acute appendicitis. Additionally, patients may

experience secondary complications, including reactive arthritis of the knee, ankle or wrist, which can sometimes be associated with erythema nodosum (Heesemann *et al.,* 1993).

The cramping and diarrhea in infants and young children with YE infection may be contributed to by altered intestinal motility. The use of a rabbit model has allowed insight into some of the effects of YE enteritis on intestinal motility. Specifically, YE enteritis produces specific changes in the patterns of intestinal motor activity that are associated with an increase in aboral transit (Scott *et al.*, 1988a). The changes include a decrease in MMC cycle period and contractions per MMC, and an increase in phase III lifetime. The contractile properties of the longitudinal smooth muscle are also altered in YE enteritis.

Longitudinal muscle strips from the small intestine of a YE infected rabbit were found to have no difference in the ED_{50} concentration of CCh (a muscarinic cholinergic agonist) in the presence of tetrodotoxin, a nerve blocker (Scott and Tan, 1992). This indicated that the muscarinic receptor affinity between the control and infected groups was not different. Also, alterations in contractility were not mediated by changes in neuronal control.

In addition, the YE infected group's ability to generate tension in response to CCh was significantly decreased when compared to control animals, while the responsiveness of the pair-fed group was significantly increased when compared to the control group. This indicated that a decrease in nutrient intake, a hallmark of YE enteritis (Scott *et al.*, 1988a) was not sufficient to explain the impaired contractility in the YE infected group.

Finally, when the muscle strips were induced to contract with KCl, there was no difference between the YE infected and control groups, while the pair-fed group again exhibited a significantly increased tension compared to controls. The contraction of smooth

muscle strips by KCl is mediated through non-specific plasma membrane depolarization, which opens voltage-gated Ca^{++} channels in the plasma membrane, allowing extracellular Ca^{++} to enter the cell to induce contraction (De Ponti *et al.*, 1993). This step induces contraction independently of membrane bound receptors, and indicates that contractile proteins of the longitudinal smooth muscle are not impaired in YE enteritis.

Therefore, the decreased contractility of intestinal longitudinal smooth muscle of YE infected rabbits in response to CCh appears to be localized to a plasma membrane element of the signal cascade leading to contraction (Figure 1). The affected component(s) may be altered plasma membrane physical properties, ion channel or receptor abnormalities, altered G protein or phospholipase C expression/activity, or any combination thereof, that arise during inflammation.

1.5 Permeability

1.5.1 Regulation of Epithelial Permeability

The ability of the GI tract to act as a selective barrier lies with the epithelial cells lining the gut, and specifically, the tight junctions between the cells. The structure of human tight junctions is unknown, but recent findings have greatly advanced our understanding of these critical structures. The recent discovery of the chicken protein occludin (Furose *et al.*, 1993) as a potential candidate for the tight junctional extracellular protein was a quantum leap forward in understanding tight junctions. Occludin is a transmembrane protein that may strongly associate with occludin molecules on neighbouring cells to form the tight junction seal. Occludin is associated with the intracellular proteins (ZO-1 and ZO-2), which may interact with a contractile filament ring near the apical surface of epithelial cells. This

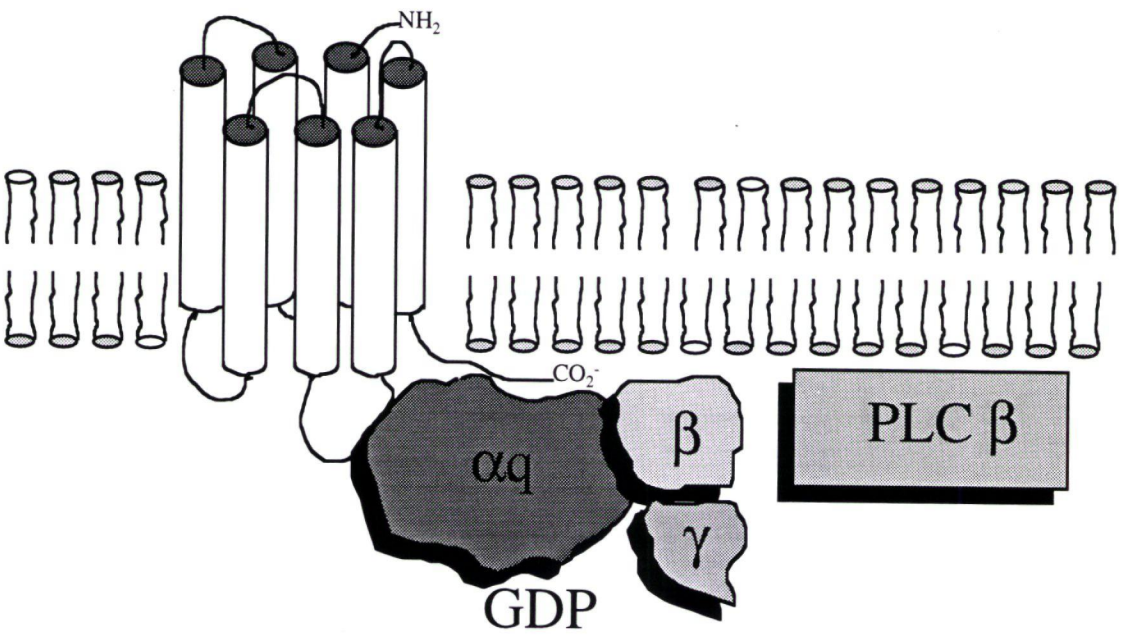

Figure 1. Schematic representation of plasma membrane associated molecules involved in signal transduction. The participants are: a muscarinic receptor with 7 transmembrane spanning domains, a heterotrimeric G protein with guanosine diphosphate (GDP) bound to the α subunit and phospholipase C beta (PLCβ).

association with the contractile ring may be a mechanism for regulation of the tight junctions (Anderson and Van Itallie, 1995).

1.5.2 Routes of Molecular Uptake

Despite the importance of the epithelium as a barrier to noxious substances, essential molecules must be taken up by the body to ensure survival. Two types of transport have been described, transcellular and paracellular.

1.5.2.1 Transcellular Transport

Transcellular transport refers to the uptake of molecules that pass through the plasma membrane of enterocytes. This transit is usually assisted by the involvement of protein carriers allowing for the uptake of specific molecules. However, some small water soluble and lipid soluble molecules are believed to passively diffuse through plasma membrane aqueous pores and directly through the membrane itself, respectively (Sanford, 1992; Figure 2). Transport of molecules across the plasma membrane occurs primarily by three processes: primary active transport, secondary active transport (facilitated diffusion) and simple diffusion (Sanford, 1992).

1.5.2.1.1 Primary Active Transport

Primary active transport is an energy dependent process whereby molecules are transported from an area of low concentration to an area of higher concentration. The energy to drive the transport comes from the large amount of free energy released when adenosine triphosphate (ATP) is hydrolyzed by the ATPase component of the transporter.

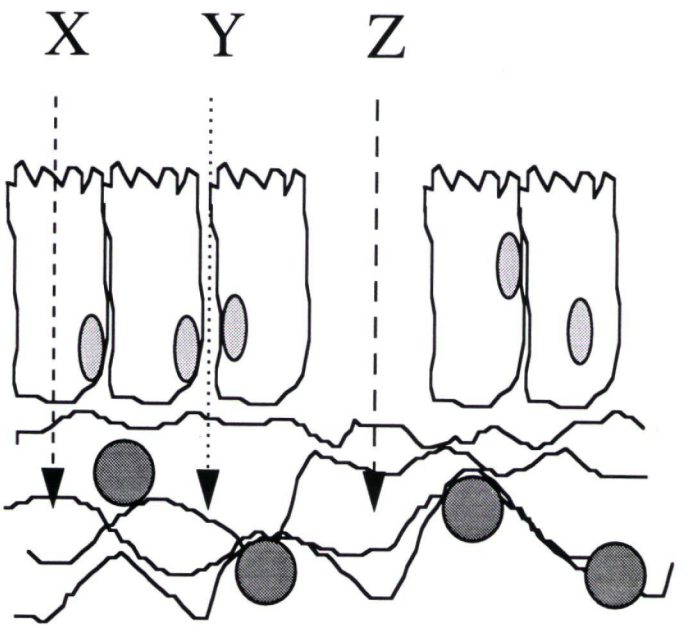

Figure 2. Uptake routes of luminal molecules. The top layer represents the enterocytes, sitting on a basement membrane of type IV collagen and connective tissue. X, transcellular transport; Y, paracellular transport through gap junctions; Z, paracellular transport through large gaps between enterocytes. Immune cells are underneath the epithelium.

An example of this type of transport is the Na^+/K^+ATPase in the plasma membrane (Guyton, 1991).

1.5.2.1.2 Facilitated Diffusion

With the continual pumping of Na^+ ions outside the cell by Na^+/K^+ATPase, the concentration of Na^+ outside of the cell is increased relative to the concentration inside the cell. Thus, a concentration gradient is made, which represents stored energy. Facilitated diffusion takes advantage of the stored energy in the gradient to literally pull other molecules across membranes in conjunction with Na^+ ions. The molecules are assisted in crossing plasma membranes by membrane-bound carrier proteins containing binding sites for Na^+ and other molecules. The direction of transport can either be in the same direction as Na^+ (co-transport) or in the opposite direction (counter-transport). An example of co-transport occurs in the well studied Na^+-glucose co-transporter, which carries glucose and Na^+ into the cell. Co-transport is also used to transport amino acids into the cell through five sets of amino acid transport proteins. An example of counter transport is the Na^+/Ca^{++} exchanger, whereby Ca^{++} is moved outside the cell in exchange for Na^+ ions. Again, the driving force of the transport is the large concentration gradient of sodium ions (Guyton, 1991).

1.5.2.1.3 Simple Diffusion

Simple diffusion is a process that requires no energy and occurs in the same direction as concentration or electrochemical gradients. Lipid solubility is an important factor in deciding if a substance can cross membranes; the higher the lipid solubility the greater the absorption. A second determinant of absorption by simple diffusion is the molecular size, as

there is an inverse relationship between molecular size and rate of uptake of small, lipid insoluble molecules (Sanford, 1992). An example of a molecule that is absorbed by simple diffusion is mannitol, which is thought to permeate cells through small water filled channels in the plasma membrane (Bjarnason, 1986a; Figure 2).

1.5.2.2 Paracellular Transport

While the mechanisms of uptake are relatively specific, the gut is inherently leaky, as trace amounts of intact macromolecules escape the GI tract in the absence of disease (Warshaw *et al.*, 1974). The escape of large molecules occurs via a paracellular route, through gap junctions or through large spaces between cells (Figure 2). It has been proposed that increases in paracellular transport may serve as a means to maximize nutrient intake.

In 1987, Madara and Pappenheimer demonstrated an increased paracellular permeability in rodent small intestine which was induced by Na^+-coupled transport across the apical membrane. In addition, they also observed peri-junctional actin aggregation, indicating that contraction of the ring of actin and myosin filament ring near the apical surface of epithelial cells may increase the width between cells and be a means for additional absorption of nutrients in a fed state. However, these results have yet to be reproduced in human studies.

1.5.3 Techniques to Study Gastrointestinal Permeability

Test molecules used in assessing GI permeability take advantage of paracellular and transcellular routes of uptake. The larger sugar probes, as well as ^{51}Cr-EDTA are all thought to permeate through paracellular routes to get into the bloodstream and urine.

Probes such as mannitol are thought to permeate the epithelium via aqueous pores in the plasma membrane. Finally, molecules of polyethylene glycol share both routes of transport; limited lipid solubility allows for some transcellular transport while the larger molecules permeate via a paracellular route (reviewed by Menzies, 1974).

The development of methods for assessing GI permeability was greatly advanced in the 1970s with the development of non-hydrolysable oligosaccharides as test molecules. The detection of permeability alterations with oligosaccharide probes was introduced by Menzies (1974), who also formulated the concept of differential excretion of orally administered substances.

Oral tests of permeability are safe, cheap, easy to perform and may one day replace the need for some of the more invasive techniques used to investigate disease such as endoscopy and biopsy. Measurements of permeability have an advantage over biopsy and endoscopy in that function of the entire epithelial surface area can be analyzed; biopsy may miss scattered disease and some areas of the gut are inaccessible to endoscopy (Lim *et al.*, 1993).

1.5.3.1 Stomach and Upper Duodenum

1.5.3.1.1 Sucrose

Recently, the use of sucrose as a probe of upper gastroduodenal damage has been introduced (Meddings *et al.*, 1993). Sucrose is an ideal molecule for measuring damage to the upper GI tract. The lifetime of sucrose in the intestine is limited to the proximal duodenum, as sucrose is readily hydrolyzed to fructose and glucose by the brush border

enzyme sucrase. The detection of elevated sucrose in the urine indicates a compromised epithelial barrier in the stomach and/or upper duodenum.

Patients ingest 100g of sucrose in 450ml of a commercially available drinking product (e.g. Kool Aid) after a 4 hour fast just before bedtime. The overnight urine is collected the next morning and analyzed by high performance liquid chromatography (HPLC).

The usefulness of sucrose as a permeability probe has yet to be fully realized, although it is useful in predicting disease observed by endoscopy and in following the course of gastric epithelial healing (Meddings *et al.*, 1995). Increased urinary sucrose has also been correlated with increasing severity of gastroduodenal disease (Meddings *et al.*, 1995) and has been demonstrated as a useful indicator of NSAID-induced damage (Meddings *et al.*, 1993). The sucrose assay is simple to perform, cheaper than endoscopy, and the sweet drink vehicle is readily accepted by patients (Steinhart *et al.*, 1996).

1.5.3.2 Small intestine

1.5.3.2.1 Polyethyleneglycol (PEG)

Pioneering work by Chadwick *et al.* (1977) lead to the widespread adoption of polyethyleneglycol as a probe of intestinal permeability. Ethylene glycol polymers have the general formula $H(OCH_2CH_2)_nOH$, with liquid PEGs having an n value of 4 to 150. Commercially available PEGs used in permeability studies (PEG 400, 600, 900, 1000, 3000 and 4000) are a mixture of different molecules (Bjarnason *et al.*, 1995).

PEG 400 is the molecule most often used for human studies. After an overnight fast, five to ten grams of PEG 400 are taken orally with water. Urine is collected for 5-6 hours and analyzed by gas chromatography or HPLC.

Although commonly used, currently PEG is not a suitable molecule for measuring intestinal permeability. The wide disparity in toxicity between species, interbatch variation, time-consuming analysis, unpleasant taste and variable urine excretion post-intravenous injection limits the usefulness as a probe and reliability and interpretation of the data (Bjarnason, 1986a).

1.5.3.2.2 Non-Hydrolysable Oligosaccharides

Many carbohydrate probes have been used as indicators of intestinal permeability including lactulose, melibiose, raffinose, stachyose, dextrans and cellobiose. The latter should be avoided as a probe because of small intestinal cellobiase activity (Dahlqvist, 1962).

The most common method for assessing intestinal permeability with non-hydrolysable oligosaccharides is the use of two probes simultaneously (such as lactulose and mannitol). This is the concept of differential excretion introduced by Menzies (1974). Excretion of a single test substance can be affected by factors other than permeation (such as intestinal motility and secretion). The concurrent use of a second probe that is subjected to the same factors allows each probe to act as an internal standard for the other. Any variation in pre- or post-mucosal determinants will affect both sugars equally, so the ratio of the sugars is independent of non-permeability factors (Bjarnason, 1994). The difference between the two sugars lies in the method of epithelial permeation, as mannitol permeates through aqueous

cellular pores and lactulose via a paracellular route. The urinary excretion ratio of lactulose:mannitol (lac:man) gives an indication of the relative function of these two permeation routes. Increases in the lac:man ratio are indicative of a compromised small intestinal epithelial barrier as both sugars are rapidly degraded by bacteria in the colon.

After an overnight fast, patients are given a mixture of hypertonic sugars and urine is collected for the next five hours. Subjects are allowed fluids only during the collection period, and the urine is analyzed by HPLC.

This technique is a relatively exclusive measure of intestinal permeability because both probes are rapidly digested by colonic microflora. However, lactulose and mannitol may be present in food, and small amounts of endogenous mannitol may underestimate intestinal permeability in some cases (Laker *et al.*, 1982).

1.5.3.2.3 ^{51}Cr-EDTA and ^{99m}Tc-DTPA

The use of 51Cr-EDTA as a marker of intestinal permeability was initiated in a study of celiac disease (Bjarnason *et al.*, 1983). A second commonly used probe is ^{99m}Tc-diethylenetriaminopentaacetate (DTPA; Bjarnason *et al.*, 1995). Both are approximately the same size as the oligosaccharide probes and permeate the gut by paracellular transport. Detection of these probes in the urine indicates a compromised small intestinal epithelial barrier. The choice of which one to use depends on the experimental protocol. ^{51}Cr-EDTA can be made in batches due to the 27 day half-life of ^{51}Cr, while the 6 hour half-life of ^{99m}Tc requires that doses be made up the same day and analyzed very soon after urine collection.

The radiolabeled chelates are ingested after an overnight fast in 200-300 ml water, subjects are fasted for an additional 2 hours before being allowed food and water, and urine is collected for 24 hours. Analysis of the urine is simple, requiring only a γ-counter.

The disadvantage of using these test molecules is the cost of the radioactive materials, and the radioactivity itself, as it may be difficult to convince human patients to ingest a radioactive substance. However, the radiation received following a 100 μCi dose of ^{51}Cr-EDTA is <0.12 milliSieverts, and even less following an equivalent amount of ^{99m}Tc-DTPA. This is considerably less than the radiation received by natural sources alone (approximately 2.0 milliSieverts/year) (Bjarnason *et al.*, 1986a).

1.4.4 Permeability and Disease

There have been exciting developments in the techniques of measuring permeability that may allow for the prediction of relapsing Crohns' disease (May *et al.*, 1993), the detection of gastroduodenal ulcers (Sutherland *et al.*, 1994) and possibly celiac disease (Turck *et al.*, 1987). Permeability tests are more commonly used to follow the response to treatment in many diseased states.

1.4.5 Intestinal anaphylaxis

Intestinal inflammation induced by reaction to antibody, or intestinal anaphylaxis, has been shown to increase permeability in the rat (Crowe and Perdue, 1992). Intestinal anaphylaxis is an immediate hypersensitivity type reaction, indicating a dependence on antigen exposure resulting in the production of antibodies that attach to high affinity receptors on mast cell surfaces. The antibodies are usually IgE, but can also be IgG4 in

humans (Halpern and Scott, 1986). Antigen re-exposure results in mast cell activation, and the release of stored and newly synthesized molecules. The released molecules recruit other immune cells (such as neutrophils, basophils, eosinophils and $CD4^+$T cells), which also contribute to allergic reactions. The recruitment of other immune cells (a late-phase reaction) begins 2-4 hours after immediate type hypersensitivity reactions start, and is maximal by about 24 hours. Late-phase reactions are commonly observed against helminth parasites and insect larval infestations (Gleich, 1982; Abbas *et al.*, 1994).

1.4.5.1 Role of the Stomach

The stomach is the first line of contact with immunogenic substances in food, and the stomach has been shown to react to immunogenic substances. Antigen challenge of the sensitized rat stomach results in increased acid secretion and retention time, a decrease in the antral motility index and a disruption of the duodenal migrating motor complex (Catto-Smith *et al.*, 1989; Catto-Smith *et al.*, 1994). This response was shown to be a mucosal mast cell IgE-mediated event, as challenge of sensitized animals resulted in a decreased staining of mucosal mast cells, and increased luminal histamine and serum rat mast cell protease II (RMCP II) concentrations. The increased RMCP II indicated that mucosal mast cells were degranulating in a manner specific to antigen challenge (Woodbury *et al.*, 1978).

1.4.5.2 Role of the Small Intestine

Intestinal anaphylaxis has been extensively studied in the intestine. Using isolated loops, investigators have found that food protein-induced anaphylaxis produced epithelial damage and edema, a decrease in mucosal histamine, granulated mucosal mast cells, ion and water transport, and an increased permeability, secretion, enterocytic markers in perfusate and

perfusate volume and systemic RMCP II (Perdue *et al*, 1984a; Perdue *et al.*, 1984b; Perdue and Gall, 1986; Patrick *et al.*, 1988). Food protein-induced anaphylaxis has also been associated with disruptions in myoelectric and motor activity, with an increased rate of aboral transit and diarrhea (Scott *et al.*, 1988b; Maric *et al.*, 1989)

1.4.5.3 Role of the Colon

The colon is also capable of being an active participant in an anaphylactic reaction. Studies with humans have shown that the colon reacts to foreign proteins in an IgE-dependent manner (Gray *et al.*, 1940), and it is well documented that the colon is a frequent site of damage in food protein intolerance in children under two (Stern, 1991). Antigen challenge of the colon *in vitro* and *in vivo* indicates that sensitized animals react with increased Cl^- secretion and impaired water and electrolyte transport in an IgE and mast cell-dependent manner (Forbes *et al.*, 1988). Additionally, work with isolated muscle strips of rat colon has demonstrated an antigen-specific contractile response in sensitized animals (Oliver *et al.*, 1995). The contraction is thought to be mediated by mast cell release of plasma membrane derived mediators such as LTD_4 and PAF.

1.4.5.4 Summary

The stomach, small intestine and colon are all capable of actively participating in intestinal anaphylaxis. There is clearly an increase in permeability observed during antigen challenge of the small intestine. The previous studies demonstrating an increased permeability during intestinal anaphylaxis all shared the common thread that animals were anaesthetized and surgical techniques were employed, or the animals were killed and isolated tissue strips were utilized *in vitro*.

1.5 Objectives

The overall goal of this study was to evaluate the effects of gastrointestinal inflammation on structure and function of the gut. Two distinct models of inflammation were utilized and two different endpoints of damage were chosen. The first goal was to examine alterations at the level of the smooth muscle plasma membrane during YE enteritis. The second goal was to determine the anatomic extent and severity of altered mucosal permeability after antigenic challenge of the stomach, jejunum and colon of conscious, sensitized rats using non-invasive techniques.

Chapter 2. The Smooth Muscle in YE Enteritis

2.1 Rationale

The decreased ability of the rabbit intestinal longitudinal muscle to generate tension during YE enteritis could be explained by a plasma membrane localized interruption in the signaling cascade that leads to contraction (Scott and Tan, 1992). Changes in receptor properties, plasma membrane physical properties, G protein expression/activity or second messenger generation (or any combinations of these) that prevent an efficient delivery of the contractile signal might explain the decreased contractile ability.

We hypothesized that YE enteritis affects plasma membrane physical characteristics, and/or muscarinic receptor availability or affinity. If this was found to be the case, we hypothesized that there might be a link between altered plasma membrane physical properties and muscarinic receptor function.

2.2 Methods

2.2.1 Animal Model

New Zealand white rabbits (900g) of either sex were studied. Animals were kept in a room with a 12 hour night-day cycle, and had access to standard chow and water *ad libitum.* Experimental procedures followed the guidelines of the Canadian Council on Animal Care.

The invasiveness of YE is jointly dependent on the presence of a 42 megaDalton (MDa) plasmid and chromosomal genes (Paerregaard, 1992). The YE strain MCH700S (serotype 0:3), which was originally isolated from a human patient and possesses the 42 MDa plasmid, was used. New Zealand white rabbits were infected with 10^{10} colony forming units of YE (grown overnight at room temperature on sheep blood agar plates) in 10% HCO_3 via

orogastric feeding tube on day 0 (O'Loughlin *et al.*, 1986). Controls received vehicle alone, and daily weights, food intake and stool pattern were recorded. Fecal excretion of YE was confirmed by daily rectal swabs onto *Salmonella-Shigella* agar, and the presence of diarrhea was confirmed by examining the perineum and hind legs. Acute YE enteritis in rabbits results in weight loss, diarrhea and a similar mucosal damage pattern as observed in humans (O'Loughlin *et al.*, 1986).

2.2.2 Smooth Muscle Plasma Membrane Preparation

Infected animals were sacrificed on day 6 when infection is maximal (O'Loughlin *et al.*, 1986) by an overdose of sodium pentobarbital (MTC Pharmaceuticals, Cambridge, ON) (approximately 1ml/kg) injected into the ear vein. The entire small intestine was immediately excised and flushed with normal saline. The intestine was split along the mesenteric border, the mucosa was scraped away with a glass slide and the muscle was immersed in ice-cold Buffer A (25mM TRIS pH 7.4/0.25M sucrose/10mM $MgCl_2$/0.6M KCl). Random one inch samples were kept for histological analysis. The remaining tissue was blotted dry, weighed and immersed in ice-cold (5ml/mg) tissue Buffer A plus 0.5mM phenylmethylsulfonylfluoride (a serine protease inhibitor; Sigma, MO). The tissue was finely minced with scissors and homogenized with a Polytron probe (setting 7) for 3-15 second pulses, with time allowed for cooling between pulses. All further membrane isolation steps were done at 4°C.

The homogenized muscle was subjected to a modified procedure of Kwan *et al.* (1986) for membrane isolation (Figure 3). This method has proven successful in separating smooth muscle plasma from neuronal plasma membranes, as well as from intracellular

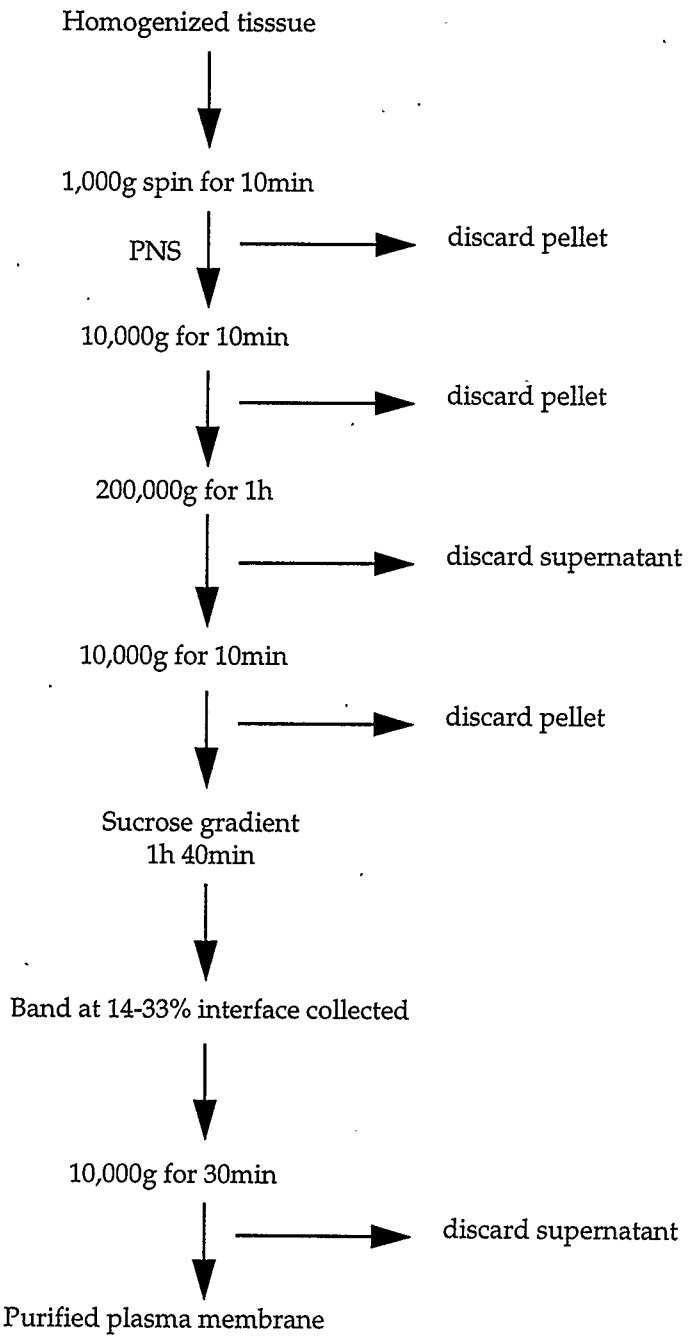

Figure 3. Flow diagram of smooth muscle plasma membrane purification procedure. This procedure is based on the method of Kwan (1986). PNS = post-nuclear supernatant.

membranes. Briefly, the homogenate was spun at 1000g for 10 minutes, and the supernatant (post nuclear supernatant - PNS) aspirated and spun at 10,000g for 10 minutes; the resulting supernatant was spun at 200,000g for one hour and the remaining pellet was resuspended in 8-10ml of Buffer B (25mM TRIS pH 7.4/0.25M sucrose/10mM $MgCl_2$); the suspension was spun at 10,000g for 10 minutes and the ensuing supernatant was loaded on top of a discontinuous sucrose gradient (14%-33% w/v) and spun for 100 minutes at 105,000g. Based on preliminary experiments, the band between 14% and 33% sucrose was collected and diluted approximately 10 times with Buffer Q (10mM TRIS-HCl pH 7.4/100mM NaCl/10mM $MgCl_2$), and spun at 10,000g for 30 minutes. The final pure plasma membrane pellet was resuspended in 1.0ml of Buffer Q and stored at -70°C.

2.2.3 Protein Quantitation

The concentration of protein in both the PNS and purified membrane was determined by the method of Bradford (1976), using bovine serum albumin as the standard. This procedure utilizes coomassie brilliant blue (CBB) added to plasma membrane; interactions between aromatic side chains of amino acids and aromatic regions of CBB alter the absorption spectra of CBB, and this is detected by a spectrophotometer.

2.2.4 Histology

Sections for histological examination were fixed in formaldehyde:50% ethanol overnight. The next day, tissue sections were immersed in increasing concentrations of ethanol, at increments of 5%, until the tissue was in 100% ethanol and was completely dehydrated. The following day, tissue sections were immobilized in paraffin, sectioned with

a microtome and stained with hematoxylin and eosin. Samples were viewed under a light microscope at 100X magnification.

2.2.5 Enzyme Assays

The oubain-sensitive Na^+/K^+ ATPase was used as a diagnostic marker of plasma membrane purity (Kelly *et al.*, 1972). Assays of the oubain-sensitive Na^+/K^+ ATPase were performed at 37 °C and expressed as units per milligram of protein, where one unit represents 1 mmol of molybdate reduced per hour. Contamination by intracellular membranes was measured by the diagnostic mitochondrial marker cytochrome c oxidase (1.9.3.1) according to the method of Wharton and Tzagoloff (1967). Results were expressed as U, where one U equals 1μmole of reduced cytochrome c converted to oxidized cytochrome c per minute. Membrane purity and contamination were calculated as a ratio of enzyme activity in the purified membrane:PNS.

2.2.5.1 Na^+/K^+ ATPase

Freshly prepared reaction mixture (0.05M Tris base/120mM NaCl/20mM KCl/2.5mM Na_2ATP/50mM $MgCl_2$) was heated to 37°C and the pH was adjusted to 7.2 with a mixture of 0.05M Tris base/120mM NaCl/20mM KCl pH 7.0. Oubain (Sigma, MO) was added to to approximately half of the reaction mixture to a final concentration of 0.2mM. One ml of reaction mixture was pipetted into 12x75 borosilicate glass tubes (total ATPase) and one ml of the oubain reaction mixture was pipeted into the oubain glass tubes (oubain). The tubes were places in ice-cold water, and 50μl of appropriately diluted PNS and purified plasma membrane were added to the tubes. All tubes were immersed in a 37°C water bath for 15 minutes, and the reactions were stopped by the addition of 0.5 ml ice cold 8% perchloric

acid to each tube. A standard curve was prepared by dissolving KH_2PO_4 in deionized water. Concentrations of free inorganic phosphate in one ml total volume ranged from 2-20µg. To each tube, 2.5ml of 0.5% (w/v) sodium molybdate/0.5M H_2SO_4 was added, followed by 1ml of 1% (w/v) p-methylaminophenol as reducing agent. Colored reactions were allowed to develop for 10 minutes, and read at 700nm in a spectrophotometer. The Na^+/K^+ ATPase activity in the oubain tubes was subtracted from total ATPase to yield plasma membrane specific Na^+/K^+ ATPase activity.

2.2.5.2 Cytochrome c Oxidase

A 1% (w/v) reduced cytochrome c solution was prepared by dissolving 100mg of cytochrome c (Signa, MO) in 8ml of reagent B (10mM $KHPO_4$ pH 7.0) in a dialysis bag. The dialysis bag was designed to hold back proteins larger than 10000 Daltons (molecular weight of cytochrome c is approximately 13000 Daltons). A small amount (3-5mg) of ascorbate was added to the bag to completely reduce the cytochrome c. Excess ascorbate was removed by dialyzing against 3 changes of reagent B over 24 hours, and the final volume of of the reduced cytochrome c was brought up to 10ml by addition of reagent B. The reduced cytochrome c was stored in the dark at 4°C.

The experiment was carried out at room temperature. Glass test tubes were labeled as blank and sample tubes, and 2.7ml of Reagent B was added to each tube. To each of these tubes, 0.2ml of reduced cytochrome c was added, followed by 0.1ml of 100mM sodium ferricyanide ($Na_4Fe(CN)_6 \cdot 10H_2O$) in 100mM $KHPO_4$ pH 7.0 to the blank tube only. The purpose of the sodium ferricyanide solution is to completely oxidize reduced cytochrome c. 100µl of membrane was added to each tube, the tubes were inverted, and the decrease in absorption at 550nm was recorded for 10 minutes at one minute intervals in a

spectrophotometer. The A_{550} of the blank tubes at 10 minutes incubation was subtracted from the sample A_{550} at each time point to yield the specific decrease in A_{550} (E). A plot of Ln(E) against time was constructed, and the slope of the curve was calculated using regression analysis. Determination of the slope allowed calculation of cytochrome c oxidase activity according to the method of Wharton and Tzagoloff (1967).

2.2.6 Muscarinic Receptor Binding Assay

Radioliogand binding studies allow determination of receptor number and affinity. Increasing concentrations (0-10nM) of [N-methyl-^{3}H]-scopolamine (NMS) (Amersham, Oakville, Ontario) were incubated in glass tubes with 50µg of membrane protein for 45 minutes at room temperature. The muscarinic antagonist NMS was used as a ligand because of its higher binding specificity and greater reversibility of binding than another commonly used muscarinic antagonist L-[3H] quinuclidinyl benzilate (Dehaye *et al.*, 1984). Non-specific binding was measured in the presence of a 500 fold excess of atropine, the definitive muscarinic antagonist. The total volume of the reaction mixture was 100µl. The binding reactions were stopped by the addition of 3ml ice-cold Buffer Q, and bound and free ligand were separated by vacuum filtration through a Whatman GF/B glass fiber filter on a 12 port Millipore filtration apparatus under a pressure of -60 kPa. The tubes and filters were washed four times with three millilitres of Buffer Q, and the filters were immersed in 10ml of liquid scintillation fluid (Ecolite, ICN) for one hour. Bound NMS was counted for five minutes in a beta counter (Beckman, LS 9800). In the time course assays, saturating amounts of ligand (10 nM) were added to 50µg membrane and the reactions were quenched at various time points. Total binding minus non-specific binding yielded specific binding. The receptor affinity (K_d) and density (B_{max}) were computed using non-linear regression analysis (Systat).

2.2.7 Plasma Membrane Physical Properties

To study the static component of membrane fluidity, we used the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH; Molecular Probes, Inc., Eugene, OR) excited at 360nm, with the emitted light measured at 450nm. The plasma membranes were labeled by adding 12µl of a freshly prepared 6.7mM stock solution of DPH (stored in darkness at -20°C) in tetrohydrofuran to 50µg of plasma membrane in 3ml of Buffer Q. The dynamic component of membrane fluidity was also examined using four isomers of n-(9-anthroyloxy) stearic acid (n-AS; Molecular Probes, Inc., Eugene, OR) and 16-(9-anthroyloxy) palmitic acid (16-AP; Molecular Probes, Inc., Eugene, OR) probes, at an excitation of 385nm and emission of 450nm. These probes are ideal for this purpose as the limiting anisotropy is very low, meaning the plasma membrane imposes very little rotational constraint upon the fluorescent moiety (Sawyer, 1988). The plasma membranes were labeled by adding 5µl of a 0.25mM stock solution in 95% ethanol to 50µg of plasma membrane in 3ml Buffer Q. The steady state anisotropy parameter (r_S) was measured for all probes in a SLM 4800C fluorometer (SLM-Aminco, Urbana, IL). When appropriate the fluidity of the membrane was manipulated by the use of 2-(2-methoxyethoxy) ethyl 8-(cis-2-n-octylcyclopropyl) octanoate (A2C) disolved in dimethylsulfoxide. The volume of A2C solution was always less than 5µl. This molecule decreases the packing of fatty acyl chains, which is equivalent to increasing the fluidity of the membrane. The membranes, probes and A2C were incubated in darkness and at room temperature for 30 minutes to allow for uniform incorporation.

When we compare r_S values obtained from different membranes, we assume that the lifetime of the excited state of the fluorescent probe is constant. However, it is possible that the different membranes we used offer environments that can alter fluorescent properties of the probe, such as fluorescent lifetime. If the fluorescent lifetimes are not consistent between

membranes, then r_S may be misinterpreted. In order to verify that the alterations in membrane fluidity that we observed were real changes and not artifacts, we calculated the total fluorescence of our probes by the following equation:

$$T_F = I_V + 2I_H$$

where T_F is the total fluorescence, and I_V and I_H are the intensities of the light in the vertical and horizontal orientations, respectively. Since total fluorescence is directly proportional to the fluorescent lifetime, increases or decreases in total fluorescence will reflect similar changes in fluorescent lifetime.

2.2.8 Statistics

The binding parameters B_{max} and Kd were acquired using non-linear regression by a statistical software program (Systat). The parameters were compared between pairs of assays by an F-test, and a Bonferroni correction was applied where appropriate. Comparisons between groups was determined using an unpaired Student t test with a second commercially available statistics program (Instat). A probability of less than 0.05 is considered significant.

2.3 Results

2.3.1 YE Enteritis

All infected animals were positive for YE cultures on Salmonella-Shigella plates by day 3. The YE infected rabbits exhibited significant weight loss and decreased food intake compared to controls by day 2 that were maintained for the duration of the experiment ($p<0.001$; Figure 4). All of the YE infected animals developed diarrhea, compared to none

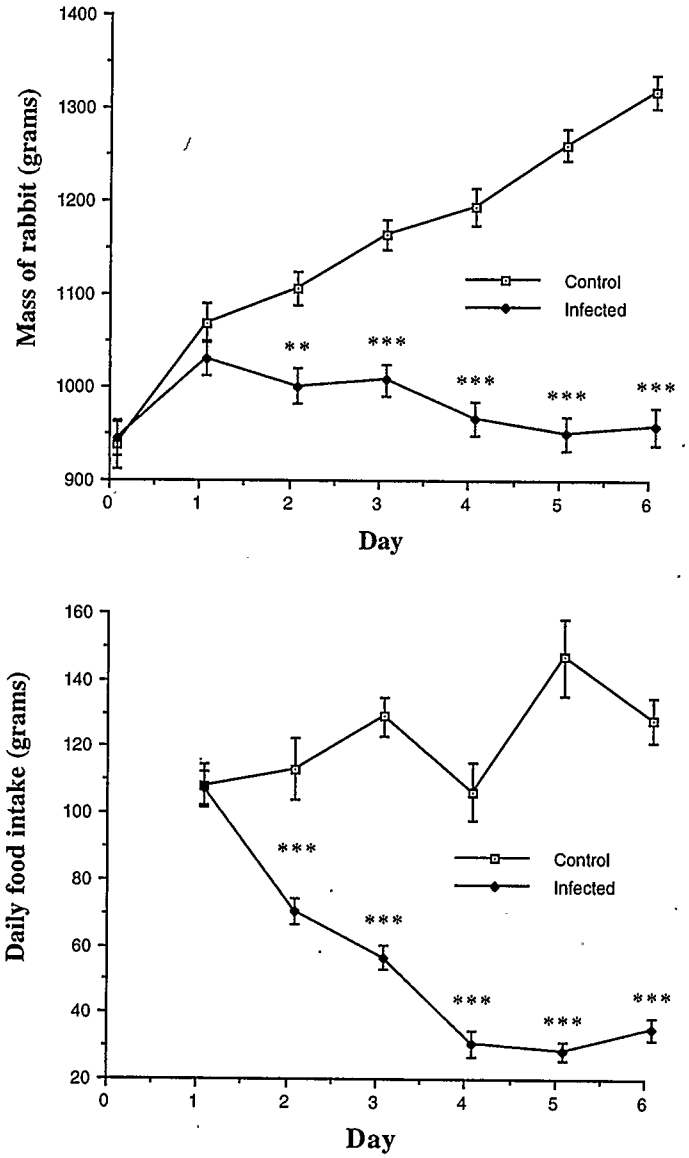

Figure 4. (Top panel) - Time course of weight gain in control and YE infected rabbits. YE infected rabbits demonstrated a significant decrease in weight gain starting at day 2. (n = 14 for control, and 32 for YE infected).**p<0.005, ***p<0.0001 vs control. (Bottom panel) - Time course of food intake in control and YE infected rabbits. YE rabbits demonstrated a significant decrease in food intake starting at day 2. (n = 13, 13, 12, 10, 5 and 11 for controls, and n = 33 for YE infected on days 1-6). ***p<0.0001 vs. control.

of the controls ($p<0.001$). The intestines of infected animals were scattered with abscesses that were most severe in the ileum.

Histological examination of scraped tissue samples revealed small amounts of remaining adherent mucosa, usually at the edges of the tissue (Figure 5). The small amount of adherent mucosa was a validation of the scraping technique. As the Na^+/K^+ATPase enzyme assay does not discriminate between cellular types, contaminating mucosal plasma membranes needed to be eliminated from the preparation.

2.3.2 Enzyme Activity of Membranes

Initial characterization of plasma membrane purity was assayed for by 5'-nucleotidase activity according to the method of Arkesteijn (1967). Preliminary work demonstrated that approximately 80% of the total 5'-nucleotidase activity was localized in the soluble fraction remaining after the 200,000g spin. Therefore, the purported smooth muscle plasma membrane marker 5'-nucleotidase (Ahmed *et al.*, 1987) was an inappropriate enzyme marker for plasma membrane purity in this model. A second biochemical marker of plasma membranes was chosen, the oubain-sensitive Na^+/K^+-ATPase enzyme. The oubain-sensitive Na^+/K^+-ATPase assay demonstrated that membranes from the control and infected animals were of comparable purity and the plasma membranes were not significantly contaminated with mitochondrial membranes (Table 1). This exclusion of mitochondrial membranes from the final preparation is expected as the procedure removes mitochondrial membranes at the second 10000g spin (Kwan, 1986).

Figure 5. Hematoxylin and eosin stained tissue sections of rabbit jejunum viewed at 100X magnification. Upper panel - cross section of unscraped intestine. Lower panel - cross section of scraped intestinal section. Note the general lack of adherent mucosa on top of the smooth muscle layers.

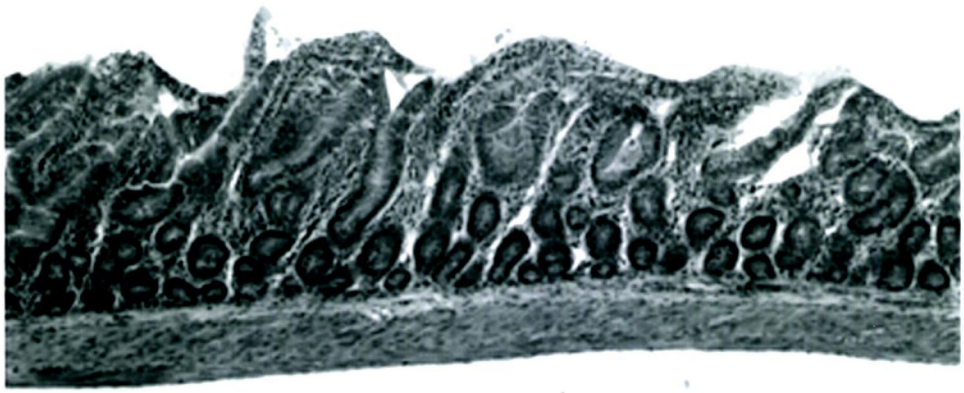

Table 1. Plasma membrane enrichment, contamination and DPH anisotropy in control and YE plasma membranes.

Membrane source	Enrichment	Contamination	DPH anisotropy
Controls	6.508 ± 0.641	0.76571 ± 0.083	0.240 ± 0.001
YE infected	7.413 ± 0.720	1.454 ± 0.474	0.245 ± 0.001[a]

n = 8, 3, and 3, respectively, for each of the two groups. Data are given as mean ± s.e.m.

[a] $p<0.001$ vs. controls

2.3.3 Radioligand Binding Studies

The ideal ligand for quantitation of receptor affinity and number is one that has rapid and reversible binding. A time course binding study showed that NMS rapidly bound membrane based receptors, with saturable and stable binding occurring approximately 20 minutes after incubation (Figure 6). We chose 45 minutes as the termination point of all future binding assays.

The reversibility and muscarinic nature of NMS binding was confirmed by the addition of 5μM atropine (the definitive muscarinic antagonist) to the assay. Excess atropine rapidly displaced NMS, with binding reaching minimal values 20 minutes after atropine addition (Figure 6).

A representative binding study is shown in Figure 7, along with non-specific binding in the presence of atropine. The maximal nonspecific binding never exceeded 12.5% of total binding, and was subtracted from total binding to attain specific binding at each time point. Three binding studies were performed (in triplicate) for each experiment, and the specific binding was calculated at each concentration of NMS.

Having demonstrated the time course and muscarinic nature of NMS binding, the next step was to determine if the functional characteristics of the muscarinic receptors were different between YE infected and control rabbits. While the absolute number of receptors was similar between the control and YE groups (3.202 ± 0.146 vs 2.48 ± 0.112; $p = 0.42351$; Figure 8), there was a significant decrease in the YE membrane receptor K_d group (1.5249 ± 0.22 vs 0.373 ± 0.08; $p<0.00001$; Figure 8), suggesting that YE enteritis results in increased muscarinic receptor affinity. The trend toward a decrease in the B_{max} in YE

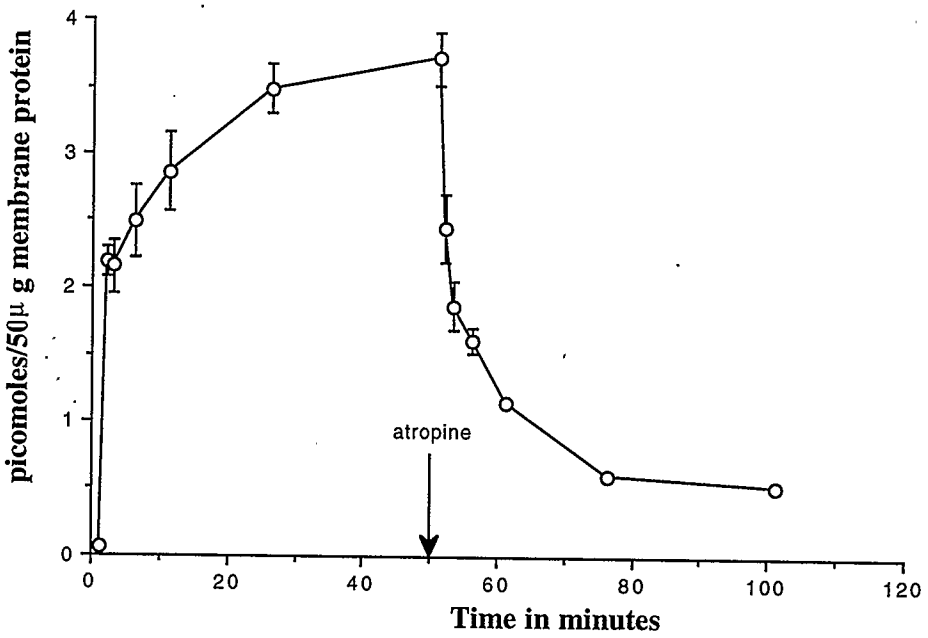

Figure 6. Time course binding-displacement curve of 10nM NMS added to 50µg of membrane protein. 5µM atropine was added at 50 minutes incubation to displace NMS (n = 3 for each point; mean ± s.e.m.). Some error bars are hidden behind the circles.

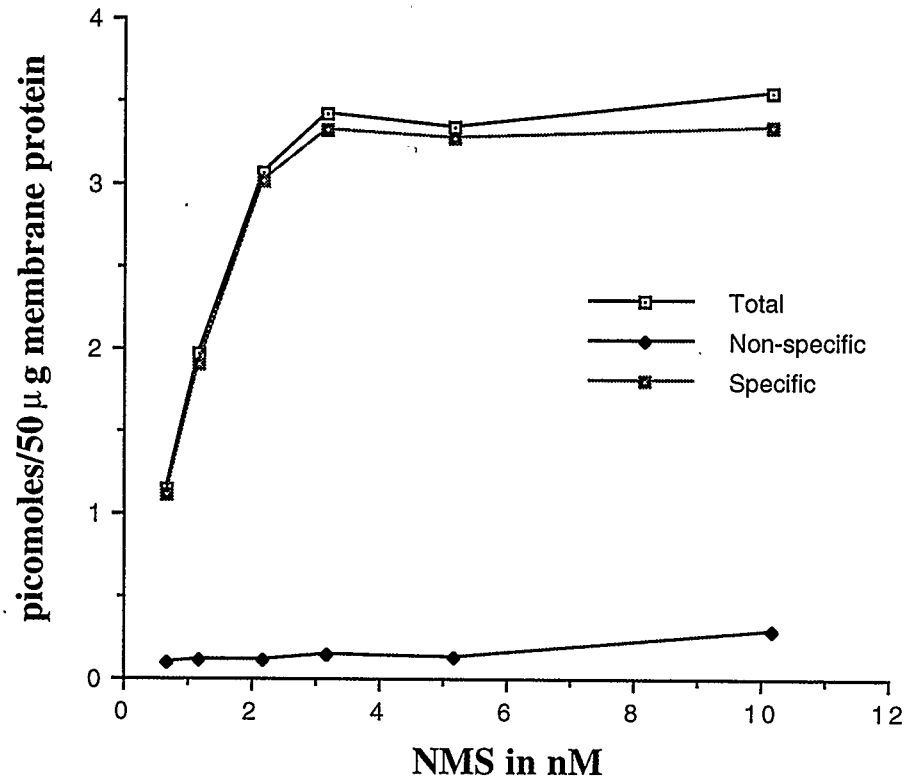

Figure 7. Representative binding curve demonstrating total, non-specific and specific binding of NMS to control purified plasma membranes. The binding reaction proceeded for 45 minutes at each concentration of NMS. Total binding minus non-specific binding yielded specific binding in each experiment.

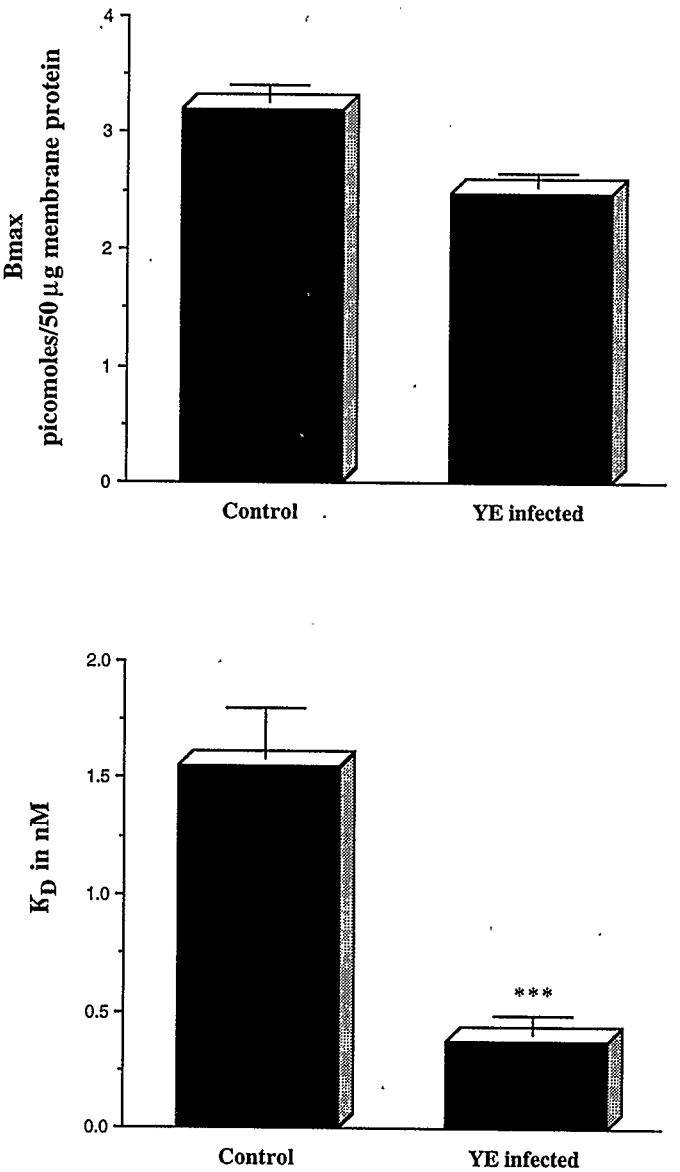

Figure 8. Apparent total number of muscarinic receptors (B_{max}) and receptor affinity (K_d) in control and YE infected purified smooth muscle plasma membranes. (n = 3 for each group; mean ± s.e.m.). * * * $p<0.00001$ vs. control.

compared to control membranes, and a small sample number (n = 3) might suggest a βII error in the statistical analysis, meaning the sample size was too small and therefore significance might have been overlooked.

2.3.4 Membrane Physical Properties

2.3.4.1 DPH

The probe DPH was used to determine the degree of acyl chain packing within the membrane. The r_S for DPH is determined mainly by its high hindered anisotropy (r_∞), a parameter that is directly related to the degree of membrane order (Sawyer, 1988). Larger values of r_S are indicative of a more rigid membrane. There was a significant increase in membrane rigidity associated with YE-induced intestinal inflammation when compared to controls (Table 1; $p<0.001$).

2.3.4.2 AS and AP Series

Our next step was to examine the physical properties of the plasma membranes throughout varying depths of the plasma membrane. The probes 2-, 6-, 9- and 12-AS, and 16-AP allowed us to investigate membrane physical properties from the surface of the membrane (2- and 6-AS) to the core (12-AS and 16-AP) (Figure 9). Using the probe 2-AS, a significant increase in the anisotropy parameter was identified at the surface of the YE membranes compared to control membranes ($p<0.0001$). There was no difference in the anisotropy parameter as we probed deeper into the membrane bilayer (AS-6 and -9). Deeper into the membrane bilayer, the anisotropy parameter with 12-AS and 16-AP probes was significantly decreased in membranes from infected compared to control animals ($p<0.0001$).

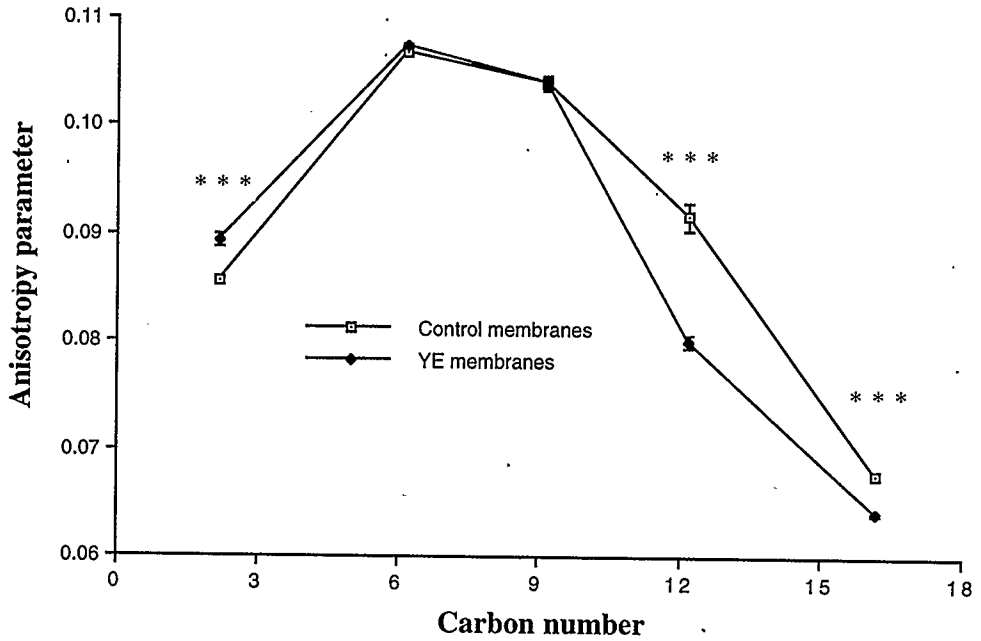

Figure 9. Steady state anisotropy parameter from control and YE membranes using 2, 6, 9 and 12-AS, and 16-AP fluorescent probes. The carbon number indicates the position of the anthroyloxy fluorophore on the fatty acid backbone (n = 3 for each point; mean ± s.e.m.). * * * $p<0.0001$ vs. control.

2.3.4.3 DPH and A2C

To determine if alterations in membrane physical properties of intestinal smooth muscle might alter the functional characteristics of muscarinic cholinergic receptors, radioligand binding assays were performed on membranes from control animals under conditions of increased membrane fluidity. The membrane mobility agent A2C fluidized the membrane to a stable anisotropy after 20 minutes incubation, which was maintained for the duration of the 45 minute experiments (Figure 10). Minor physiological increases in membrane fluidity (decreasing r_s) with increasing amounts of A2C were not associated with any change in B_{max} or K_d. However, a major increase in fluidity (r_s decreased to 0.201) was associated with a significant increase in the B_{max} [3.2024 $\pm$ 0.146 pmoles in controls to 3.6245 $\pm$ 0.034 pmoles in fluidized; $p<0.05$] in the absence of any change in K_d (Table 2).

2.4 Summary

YE enteritis decreases the contractility of the longitudinal smooth muscle of the rabbit intestine in a manner that is consistent with alterations at the level of the plasma membrane (Scott and Tan, 1992). We purified intestinal smooth muscle to permit examination of muscarinic receptor function and plasma membrane physical characteristics. YE enteritis resulted in a more rigid plasma membrane when examined by the probe DPH. Using the AS and AP series of probes, YE enteritis resulted in a significantly more rigid membrane at the surface, with a significant increase in fluidity at the core. Alterations in membrane fluidity were able to increase receptor density. The altered plasma membrane physical properties seen in YE enteritis were associated with an increased receptor avidity with no change in receptor density. Therefore, the defect in smooth muscle tension generation seen in YE enteritis cannot be explained by alterations in muscarinic receptor function.

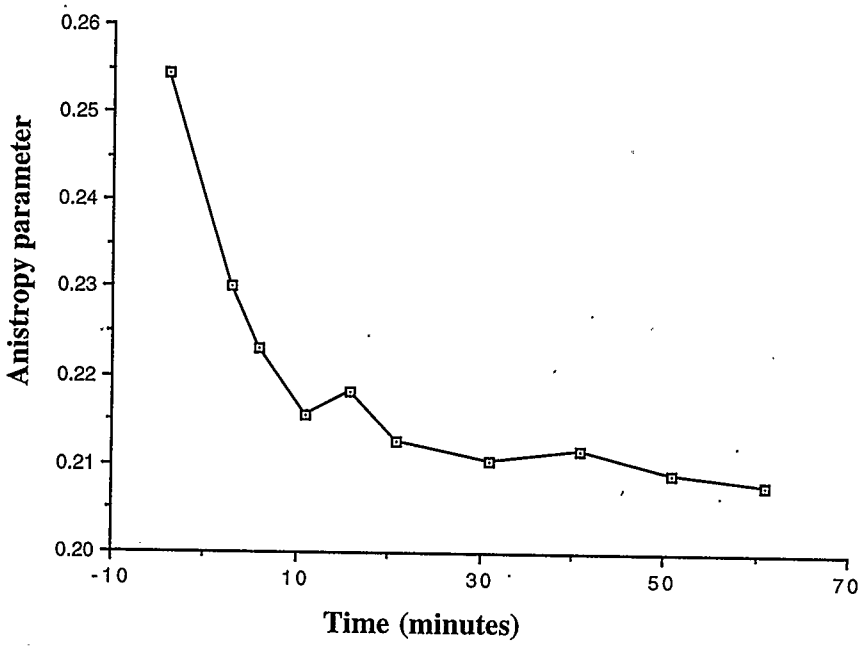

Figure 10. The decrease in DPH anisotropy, in the presence of A2C, with time. (n = 3 for each time point; mean ± s.e.m.). Error bars are hidden by the squares.

Table 2. B_{max} and K_d at different anisotropy parameters. Control membranes were fluidized by increasing amounts of A2C.

Anisotropy parameter	**B_{max} (picomoles)**	**Kd (nM)**
0.240 (more rigid)	3.202 ± 0.146	1.5948 ± 0.220
0.235	2.944 ± 0.159	1.1346 ± 0.206
0.218	2.774 ± 0.143	1.1916 ± 0.203
0.201 (more fluid)	3.625 ± 0.034 [a]	1.1409 ± 0.040

(n = 3 for each anisotropy). [a] $p<0.05$ vs 3.202 ± 0.146.

2.5 Discussion

The increased affinity for the antagonist NMS was a puzzling finding. YE enteritis results in a decreased ability of the longitudinal smooth muscle to generate tension when compared to controls. Therefore, one would expect a decreased affinity rather than the increase that we found ($p<0.00001$). To rationalize our findings, we need to consider receptor subtype, membrane composition, second messenger levels, and G protein and PLC expression/activity.

The muscarinic receptor family is divided into at least five classes of receptors, M1 to M5 (Jones, 1993). The M3 subtype is coupled to Gq and initiates the process of contraction when stimulated. Muscarinic M2 receptors act via Gi to inhibit adenylyl cyclase activity and thereby decrease inhibition of muscular contraction. In guinea pig ileal smooth muscle and in many other tissues, there is a preponderance of the M2 subtype (as high as 80%) with a minor M3 population (approximately 20%) (Eglen *et al.*, 1994). The disadvantage of the radioligand procedure we employed is that we get a gross indication of the number and affinity of all the muscarinic receptors present without any distinction of subclass. The different subtypes of muscarinic receptors have very different pharmacological binding profiles to antagonists (Eglen *et al.*, 1994). Therefore, the possibility of a sub-class shift from one to another during YE enteritis cannot be excluded, especially considering the increase in receptor affinity.

One of the major determinants of membrane rigidity is the cholesterol:phospholipid ratio; the higher the ratio the more rigid the membrane (Yeagle, 1985). Additionally, the composition of the phospholipids of the membranes might play a major role in the function of the muscarinic receptor, or in non voltage-gated Ca^{++} channels. Sunshine and

McNamee (1994) demonstrated that the function of the nicotinic ACh receptor in reconstituted lipid vesicles does not depend on membrane fluidity, but instead is affected by gross membrane composition. The nicotinic ACh receptor is a non-specific cation channel found in the plasma membrane of many tissues. We speculate that a change in membrane fatty acid composition is responsible for the alteration in membrane fluidity measured with DPH and the AS and AP probes, and may affect the function of non-voltage gated plasma membrane Ca^{++} channels, leading to less Ca^{++} entering the cell and a decreased contractility of the longitudinal smooth muscle. The voltage-gated calcium channels were previously demonstrated to be functionally intact by non-selective plasma membrane depolarization with KCl.

Additionally, while the apparent number of muscarinic receptors in the YE and control membranes were similar (although the possibility of a βII error cannot be excluded), membrane physical alterations in inflammation may affect the coupling of the receptor with the Gq protein, and/or the coupling of the Gq to PLC. Ma *et al.* (1994) demonstrated in the bile duct ligated model of cirrhotic cardiomyopathy that production of cAMP was impaired, and this impairment was associated with an increase in membrane rigidity. In this model the Gs protein is used to signal adenylyl cyclase to cleave ATP to cAMP. In our model, the Gq protein is utilized to signal phospholipase C to cleave PIP_2 to DAG and IP_3. However we can hypothesize that since the two G proteins share similar positioning to the intracellular hemileaflet a decrease in IP_3 generation in YE enteritis would be expected.

The decrease in contractility could also be explained by a decrease in the relative amount of G protein in the cell. Ma *et al.* (1996-in press) found that in cirrhotic cardiomyopathy there was a decrease in Gs protein levels. Since the β-adrenergic receptor uses Gs in its signaling cascade that leads to contraction, this was thought to be a mechanism for the

decreased heart muscle contractility. However, recent evidence suggests that G proteins are in great excess in signaling cascades and therefore it is unlikely that a decrease in protein at this level is sufficient to explain the effect seen with the longitudinal smooth muscle in YE enteritis (Post *et al.*, 1996). Additionally, the possibility of a decrease in PLC levels or activity cannot be ruled out and this possibility merits future study.

YE enteritis results in inflammation that is not usually continuous from the lumen of the intestine to the smooth muscle. While the inflammation is most concentrated in the ileum, contractility, motility and electrical alterations are observed throughout the small intestine (Scott and Tan, 1992; Scott *et al.*, 1988a), suggesting a systemic effect of YE enteritis with generalized effects throughout the small intestine. The release of oxidants by inflammatory cells adjacent to the smooth muscle may injure the plasma membrane of smooth muscle cells in close proximity to the oxidant source. If the systemic effect of YE enteritis induced oxidant release from immune cells throughout the length of the small intestine, it would be a mechanism whereby the contractile ability of the muscle may be impaired at sites distant to the concentrated inflammation.

Additionally, it has been shown that the myenteric plexus and smooth muscle cells are capable of participating in the immune response. It has been demonstrated that rabbit colonic smooth muscle can produce PGE_2 (Kao and Zipser, 1988), that rat muscularis externa and myenteric plexus are capable of producing cytokines (Khan and Collins, 1994), that cultured murine smooth muscle cells were capable of expressing MHC II complexes and ICAM (Hogaboam *et al.*, 1994), and that rat cultured smooth muscle cells are capable of producing Il-6 in response to Il-1β (Khan *et al.*, 1995). Taken together, these studies open up the possibility that the smooth muscle is not a bystander in the inflammatory process, but an active participant. Perhaps by being an active participant it recruits and

activates inflammatory cells to defend the body, and these cells damage the smooth muscle membrane unintentionally.

It is also possible that a substance is being released by the bacteria that is capable of affecting the smooth muscle directly. This substance could affect either the rigidity of the membrane, or the signal transduction pathway that leads to contraction. This is an unlikely hypothesis, as YE rarely penetrates beyond the muscularis mucosa, but the intriguing possibility exists nonetheless.

The increased membrane rigidity could come from peroxidation of plasma membranes (Jourd'heuil *et al.*, 1993). Oxidizing free radicals such as OH- and HOCl are used by cells of the immune system to combat infectious agents. These free radicals may stiffen the plasma membranes of cells by reducing double bonds, which increases the packing density of the phospholipids, thereby increasing membrane rigidity.

Ghosh *et al.* (1993) examined the effect of lipid peroxidation on the muscarinic receptor properties in rat frontal cortex. They found that peroxidation resulted in a more rigid membrane and a decreased muscarinic receptor number and affinity. We hypothesized that YE enteritis could result in a more rigid plasma membrane, and a decrease in receptor number and affinity to explain the decreased contractility observed during YE enteritis. The plasma membranes prepared from the YE infected animals were more rigid than those of control animals, having similar numbers of receptors with increased binding affinity. These results indicate that perhaps lipid peroxidation is not occurring at a significant level at the smooth muscle plasma membrane during YE enteritis, or that perhaps *in vitro* oxidation is more severe than the condition observed *in vivo*. Consider that 10 minutes of peroxidation in a test tube raised the anisotropy parameter from 0.241 to 0.268 while YE enteritis is

associated with a much smaller increase (0.2403 to 0.2447). Additionally, the greater *in vitro* oxidation of purified membranes may be damaging membrane protein, leading to fewer functional receptors. This might account for the decreased K_d and B_{max}.

There was a significant increase in DPH total fluorescence in smooth muscle plasma membranes of YE infected animals, but no difference in total fluorescence in the AS or AP series. Since total fluorescence is directly proportional to the fluorescent lifetime of a fluorescent probe, this indicates that the fluorescent lifetime of DPH in YE smooth muscle plasma membranes may have increased. An increase in fluorescent lifetime would allow the probe to move a greater distance in the bilayer and would therefore decrease the anisotropy parameter, suggesting a more fluid membrane. Therefore, if anything, we may have underestimated the rigidity of YE infected smooth muscle plasma membranes.

Chapter 3. Permeability Alterations in Food Protein-Induced Intestinal Anaphylaxis

3.1 Rationale

The stomach, small and large intestine are all capable of actively participating in intestinal anaphylaxis. There is clearly an increase in permeability observed during antigen challenge of the small intestine. Studies demonstrating an increased intestinal permeability during intestinal anaphylaxis shared the common methodological thread that the animals were anaesthetized and surgical techniques were employed, or the animals were killed and isolated tissue strips were utilized *in vitro*.

To date, few studies have examined the increase in permeability observed during food protein-induced intestinal anaphylaxis in conscious sensitized animals. Therefore, our aim was to determine the anatomic extent and severity of altered mucosal permeability after orogastric, jejunal or colonic antigen challenge of conscious, sensitized rats using non-invasive techniques.

3.2 Methods

3.2.1 Animal Model

Hooded-Lister rats of either sex (100-120g) were bred in the University of Calgary breeding colony, Calgary, Alberta. Animals were kept in a room with a 12 hour night-day cycle, allowed access to standard chow and water *ad libitum*. Experimental procedures followed the guidelines of the Canadian Council on Animal Care.

Hooded-Lister rats were sensitized by intraperitoneal injection of 10μg of chicken egg albumin (EA) (Sigma) in 0.5ml of 10% aluminum hydroxide as adjuvant in normal saline (154mM NaCl) (day 1). Sham animals were challenged with adjuvant and saline alone.

Seven days after sensitization and after an overnight fast with free access to water, animals for jejunal challenge were anesthetized with halothane. Using sterile technique, a plastic catheter was surgically inserted under the skin by making an incision in the abdomen and tunneling subcutaneously to an interscapular exit site. The catheter was brought into the peritoneal cavity by a stab incision and inserted into the proximal jejunum. The proximal jejunum was identified as a segment of intestine 10cm distal to the ligament of Treitz (which indicates an anatomical transition from duodenum to jejunum). Sutures on the tube and jejunum anchored the catheter in place, and a small piece of plasticene was inserted into the end of the catheter to maintain patency. Animals were maintained on water for 24 hours post-surgery, the liquid diet Isocal (MeadJohnson, Canada) for the next 24 hours to avoid irritating the intestinal injury and were then returned to a diet of standard rat chow.

On day 14, the animals were challenged with either EA, or bovine serum albumin (BSA) in the stomach, jejunum or colon. A stainless steel feeding needle (18 gauge, 6 cm long with a 2.25mm diameter round tip) (Fine Scientific Tools Inc., Canada) attached to a 5cc syringe was used for gastric dosing. Challenge of the jejunum was accomplished by injections through the jejunal catheter, and these animals received 20mg of BSA or EA in 0.5ml in distilled water via a 1cc syringe that attached to the surgically implanted catheter at the subscapular exit site at a rate of 0.5ml/min. Colonic challenge was performed by inserting a plastic feeding tube containing 50mg of BSA or EA in 0.5ml 8cm proximal to the anus. In all studies, the sugar probes were given by orogastric gavage. All animals received 2ml of a sugar solution containing 1g of sucrose, 0.08g of lactulose and 0.12g mannitol via an orogastric route. In the gastric challenged group, BSA or EA were given concurrently with the sugar probes. Preliminary experiments demonstrated that concurrent administration of sugars and EA were equally effective at evoking an immune response (measured as serum RMCP II) as when administered separately.

Rats were housed separately in metabolic cages with wire mesh flooring and urine was collected in polypropylene tubes, separate from stool, for 24 hours. Rats were allowed access to water three hours after dosing with sugar probes, but were fasted throughout the 24 hour urine collection period. The antibacterial agent 5% Thymol (100µl), dissolved in isopropyl alcohol, was added to each urine collection tube. After 24 hours, the urine was collected and the volumes recorded.

3.2.2 Urinalysis

The collected urine was then analyzed by HPLC for sugar content by a modified procedure of Meddings *et al.* (1993) using a Dionex DX500 HPLC (Dionex, Toronto, Canada) and an MA-1 analytical column. The mobile phase was 500mM NaOH with a flow rate of 0.4ml/min. Sugars were detected with an electrochemical detector using pulsed amperometric detection on a gold electrode.

3.2.3 Serum Markers

Blood was obtained by cardiac puncture 90 minutes post-challenge for determination of serum anti-EA IgE and RMCP II quantitation by passive cutaneous anaphylaxis (PCA) and enzyme-linked immunosorbent assay (ELISA), respectively. Animals were lightly anaesthetized with diethylether and approximately 500µl of blood was withdrawn using a 1cc syringe attached to a 25 gauge needle. The blood was allowed to settle for 10 minutes, and centrifuged at 4000rpm in a desktop centrifuge for 10 minutes. The serum was aspirated and immediately frozen at 20°C for future analysis.

3.2.3.1 Passive Cutaneous Anaphylaxis

For determination of specific anti-EA IgE titres, serial dilutions of serum (1:8 to 1:64) from sham and sensitized animals were injected intradermally in male Hooded-Lister or Sprague-Dawley rats. Seventy two hours later, 2.5mg EA and 0.5ml Evans blue in saline were given by intracardiac injection, and skin reactions were read after 60 minutes. The skin reactions were observed as rounded, blue discolorations of the skin. The principle of this assay is that IgE antibodies in sera, when injected under the skin, will encounter subcutaneous mast cells and bind to high affinity IgE receptors on the mast cell surfaces. Upon exposure to EA, the mast cells will be activated and degranulate. Degranulation products such as histamine increase vascular permeability and allow plasma proteins to leak out of the circulation (Alberts *et al.*, 1989). One of the plasma proteins that leaks out of the circulation is albumin. Since Evans blue is bound to albumin, it will cause the blue coloration wherever albumin is found. Titres were recorded as the greatest antibody dilution producing a colored reaction 5mm or more in diameter.

3.2.3.2 RMCP II

Mucosal mast cell granules contain RMCP II and the presence of RMCP II in tissue fluids and serum can be detected by ELISA. A sensitive sandwich ELISA allows for rapid and accurate quantitation; increased levels of serum RMCP II are indicative of mucosal mast cell degranulation. This enzyme has been reported to digest type IV collagen, which is found in basement membranes (Patrick *et al.*, 1988). It has been hypothesized that digestion of basement membranes may contribute to epithelial disruption observed in intestinal anaphylaxis.

Serum RMCP II was quantitated with a commercially available ELISA kit (Moredun Animal Health Limited, Scotland). Briefly, 96 well ELISA plates were coated overnight at 4°C with 50µl/well of an anti-RMCP II mouse monoclonal antibody diluted to 1mg/ml in 0.1M carbonate buffer pH 9.6 (0.1M Na_2CO_3/0.1M $NaHCO_3$). The plates were washed with an automatic plate washer 6 times with phosphate buffered saline (PBS) + 0.05% Tween 20 solution (PBS/Tween 20). Non-specific binding was blocked by the addition of 50µl of 4% BSA in PBS/Tween 20 for 30 minutes at room temperature. The plates were washed once with PBS/Tween 20, and 50µl of standards and samples (dilutions of 1:10, 1:100, 1:1000 and 1:10000), diluted in 4% BSA in PBS/Tween 20, were loaded into wells for 1.5 hours. Eight wells per plate were used to determine background optical density, where PBS/Tween 20 was used in place of samples or standards. Standards consisted of 0.5, 1, 2, 4, 6, 8, 10 and 12ng/ml RMCP II. The plates were washed 6 times with PBS/Tween 20, and 50µl of the secondary antibody (sheep anti-RMCP II-Horseradish peroxidase conjugate) was added to each well for 1 hour at room temperature. The plates were then washed 6 times with PBS/Tween 20, and the plates were incubated with 50µl of freshly prepared ortho phenylene diamine (OPD) solution (100ml 0.1M citric acid/0.2M Na_2HPO_4/40mg OPD and 40µl of 30% H_2O_2). Time for color development varied with each assay, but was usually sufficient within one hour. After adequate color development, the reaction was terminated with 25µl/well of 2.5M H_2SO_4. The plates were read at 492nm, and the average background optical density for each plate was subtracted from sample readings.

3.2.4 Statistics

To compare two means, a paired students' t-test was used. When comparing three or more means, ANOVA was used with a Tukey post test. All statistics were performed with a commercially available statistics program (Instat). A p value less than 0.05 was considered significant.

3.3 Results

3.3.1 Stress-Induced Damage

Sham-sensitized animals were given orogastric doses of sugars on days 1, 10 and 14. Urinary sucrose and lac:man ratios were significantly elevated on day 1 compared to day 10 (Figure 11), indicating damage in the absence of any experimentation. The day 10 level of urinary sugar output was maintained to day 14. Therefore, we decided to experiment only on animals that had been on site for at least 14 days.

3.3.2 Sensitization

Sham and sensitized rats were challenged 14 days after sensitization. There was no detectable anti-EA IgE in the sham sera, whereas all sensitized rats had IgE titres of >1:64.

Challenge of sham animals with BSA and EA, and sensitized animals with BSA did not result in any alteration in serum RMCP II, regardless of site of challenge. Therefore, these data have been pooled and are labeled as control in all graphs.

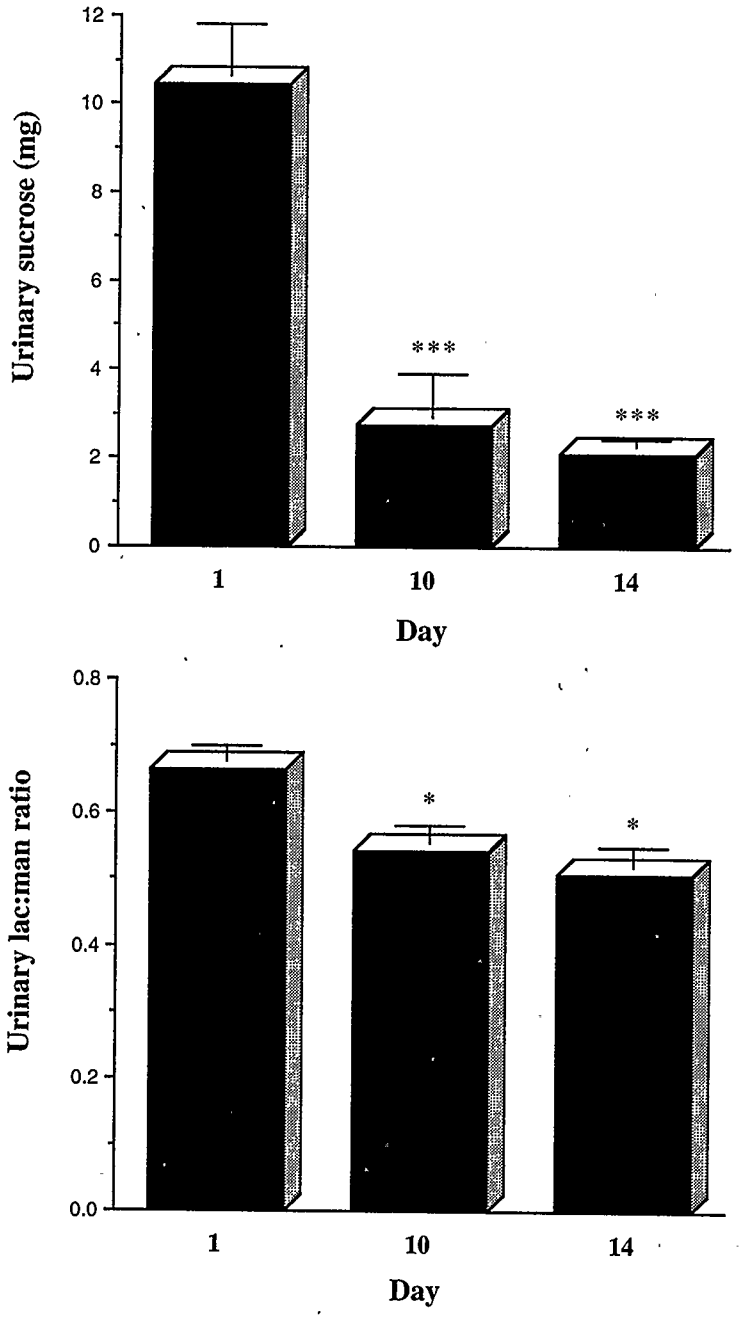

Figure 11. 24 hour urinary sucrose and lac:man ratio in control rats on days 1, 10 and 14. These rats were not experimented on for 14 days, and the increased urinary sugar output on day 1 was attributed to stress-induced increases in epithelial permeability (n = 6, 5 and 6, respectively; mean ± s.e.m.). * $p < 0.05$, * * * $p < 0.001$ vs. day 1.

3.3.3 Gastric Challenge

To examine the gastric response to antigen challenge, sensitized rats were challenged with 50mg EA via the steel feeding tube on day 14. When serum was analyzed by ELISA, there was a significant increase in RMCP II in sensitized rats compared to controls ($p<0.0001$; Figure 12). This indicated that mucosal mast cells, primarily those surrounding the stomach, were degranulating in a manner specific to sensitizing antigen.

When 24 hour urine was analyzed for non-hydrolyzable sugar probes, there was no detectable alteration in urinary sucrose or in the lac:man ratio in sensitized animals compared to controls (Figure 12). This lack of increased urinary sucrose indicated that gastric permeability was not increased in sensitized animals challenged with antigen. The lack of increased urinary lac:man means that antigen challenge of the stomach does not increase permeability in the small intestine in conscious, sensitized animals.

3.3.4 Jejunal Challenge

Sensitized rats with surgically implanted jejunal catheters were challenged with 20mg EA injected through a surgically implanted proximal-jejunal catheter on day 14. Challenge with EA on day 14 was associated with a significant increase in serum RMCP II compared to controls ($p<0.0001$; Figure 13). This indicated that mucosal mast cells of the proximal jejunum were degranulating in an antigen-specific manner.

Similar to what was observed in gastric challenged animals, there was no difference in urinary sucrose or lac:man ratio in EA challenged animals when compared to controls

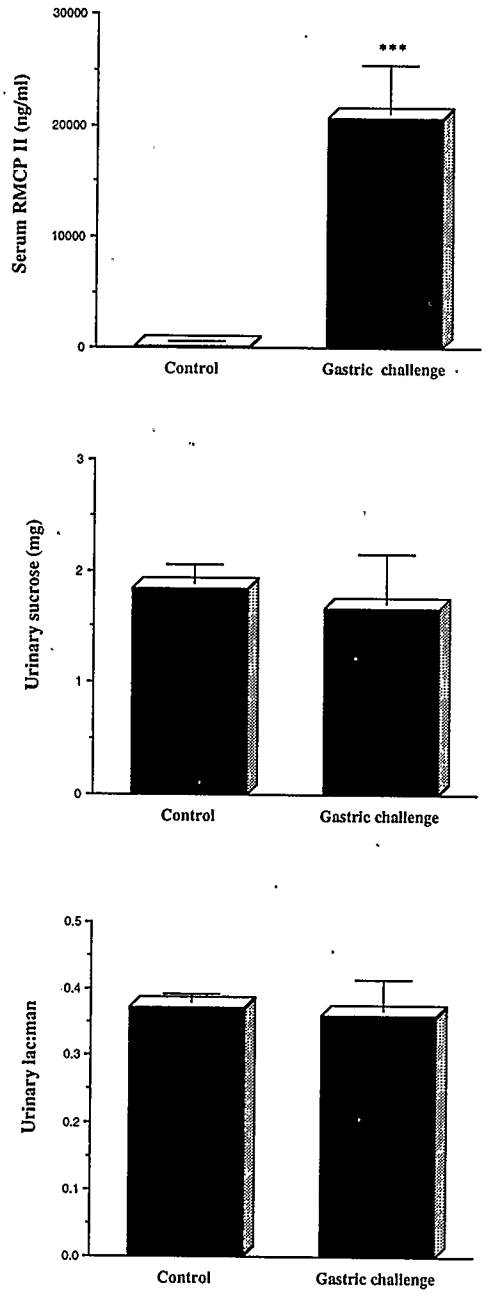

Figure 12. Serum RMCP II (upper panel), and urinary sucrose (mid panel) and lac:man (lower panel) in control and gastric challenged (50mg EA) sensitized rats. (n = 24, 23 and 23, respectively, for controls and n = 8, 7 and 7, respectively, for gastric challenge; mean ± s.e.m.). * * * $p<0.0001$ vs. control.

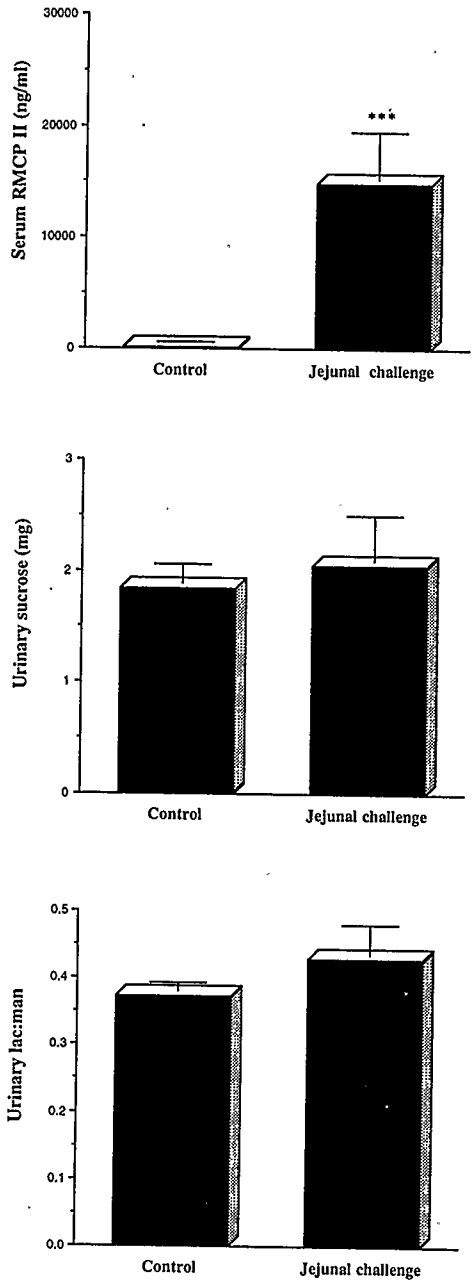

Figure 13. Serum RMCP II (upper panel), and urinary sucrose (mid panel) and lac:man (lower panel) in control and jejunum challenged (20mg EA) sensitized rats. Jejunal challenge was through a surgically implanted catheter. (n = 24, 23 and 23, respectively, for controls and n = 11, 10 and 10, respectively, for jejunal challenge; mean ± s.e.m.). * * * $p<0.0001$ vs. control.

(Figure 13). The lack of change in urinary sucrose indicates that antigen challenge of the proximal jejunum in sensitized rats does not lead to increased gastric permeability. The unaltered urinary lac:man ratio in sensitized rats suggests that in conscious, sensitized animals, there is no increase in small intestinal permeability post-antigen challenge.

3.3.5 Colonic Challenge

Colonic challenge of sensitized rats with 50mg EA on day 14 resulted in significantly elevated RMCP II levels compared to control animals ($p<0.0001$; Figure 14). This indicated that mucosal mast cells of the colon were degranulating in an antigen-specific manner.

Colonic challenge of sensitized animals with EA on day 14 resulted in unaltered levels of urinary sucrose and lac:man when compared to control animals (Figure 14). The lack of any change in urinary sucrose and lac:man would seem to indicate that challenge of the colon in conscious, sensitized animals does not result in permeability alterations in the stomach or small intestine.

We did not observe any significant increase in gastrointestinal permeability caused by food protein induced anaphylaxis. This was despite the release of an indicator of mucosal mast cell degranulation in three distinct sites.

The lack of any alteration in gastrointestinal permeability observed in intestinal anaphylaxis could be explained if the damage that has been shown be occur is transient, or perhaps our sugar probes are not sensitive indicators of GI damage. If the damage were transient, then our probes may not have reached the area of damage at a time where there

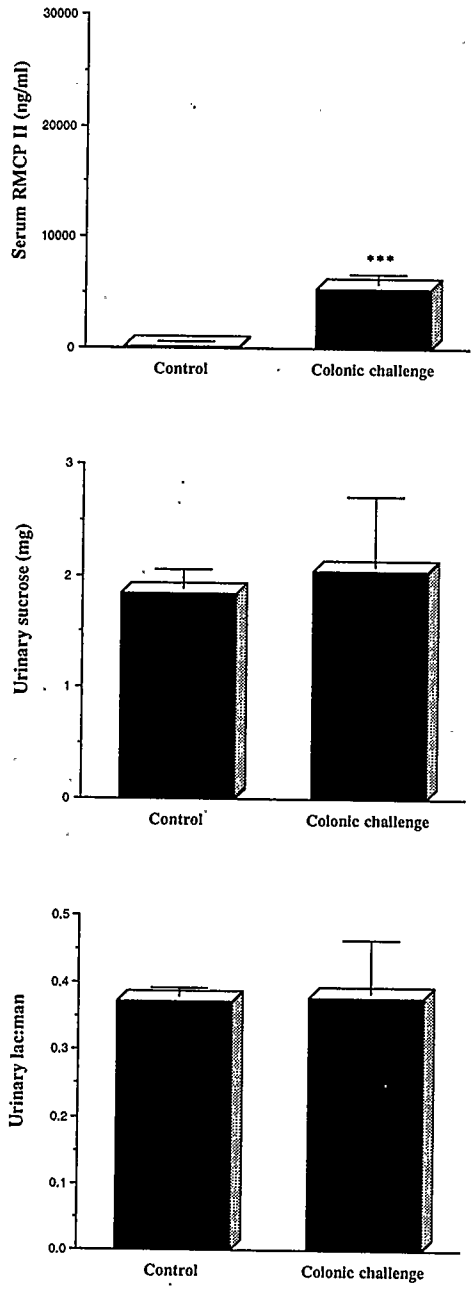

Figure 14. Serum RMCP II (upper panel), and urinary sucrose (mid panel) and lac:man (lower panel) in control and colonic challenged (50mg) sensitized rats. Colonic challenge was through a feeding tube, inserted 8cm proximal to the anus. (n = 24, 23 and 23, respectively for controls and n = 6 for all three colonic challenge groups; mean ± s.e.m.). * * * $p<0.0001$ vs. control.

was enough detectable damage that was within their detection range, and no damage would be detected.

Alternatively, the damage may be minimal, and below the detection limit of our probes. Therefore, to analyze the ability of our probes to detect quantifiable and reproducible damage, we decided to use a well established procedure for damaging gastric and small intestinal epithelia by the use of NSAIDs (Davies *et al.*, 1994; Davies *et al.*, 1995).

To damage the gastric epithelium we used acetylsalicylic acid (ASA, aspirin), which has been demonstrated to have little damaging effects on the epithelia beyond the stomach (Bjarnason *et al.*, 1986b). This may be due to the rapid absorption of aspirin in the stomach, and its rapid hydrolysis to salicylic acid (Rowland and Riegelman, 1968). This limits the direct exposure of the distal GI tract to aspirin as well as the systemic availability of functionally intact aspirin to the lower GI tract.

To damage the intestinal epithelium, we selected indomethacin. Indomethacin damages both the gastric and intestinal epithelia, but damage to the small intestine is augmented by the fact that glucuronated-indomethacin is recycled through the small intestine via the enterohepatic recirculation (Hucker *et al.*, 1966). The drug is then reactivated by β-glucuronidase activity of intestinal flora (Robert and Asano, 1977), and is capable of damaging the epithelium upon re-exposure.

3.4 Methods

3.4.1 ASA

Hooded-Lister rats (100-140g) were treated 14 days after arrival. For the ASA treatment, animals were fasted for 16 hours, with no water two hours before dosing. Rats were given ASA (50, 100 and 250mg/kg), suspended in a freshly prepared 0.5% carboxymethylcellulose (BDH) solution in deionized water into the stomach. One hour later, the rats were given 1g sucrose, 0.08g mannitol and 0.12g lactulose in 2.5ml sterile saline by feeding needle. Urine was collected for 24 hours and analyzed as before (section 3.2.2).

3.4.2 Indomethacin

Indomethacin treated rats were given orogastric indomethacin at doses of 5, 7.5 and 10.0 mg/kg, freshly prepared in a 5% Na_2CO_3 solution. Five hours later, food was withdrawn and the animals were fasted overnight. Water was withheld two hours before rats were given the same orogastric dose of sugars as used previously. The time between indomethacin and sugar probe administration was designed to allow significant small intestinal damage to occur before the sugars were given. This procedure allows 21 hours for damage to the small intestine and enterohepatic recirculation of indomethacin to occur. Urine was collected for 24 hours and analyzed as before (section 3.2.2).

3.4.3 Damage Assessment

3.4.3.1 Stomach

To assess damage caused by the ASA, animals were sacrificed by CO_2 inhalation one hour after ASA administration, and the stomachs and small intestines immediately were excised, opened along the mesenteric border and pinned flat onto a dissecting tray. Visual damage to the stomach appeared as blackened lines, which were measured by a ruler. Total damage was quantitated as the sum of damage surface area (mm^2).

3.4.3.2 Small Intestine

To assess damage caused by indomethacin, rats were sacrificed immediately after the 24 hour urine collection. The peritoneal cavity was exposed, and the ligament of Treitz (indicating an anatomical transition from duodenum to jejunum) was located. A 10cm portion of intestine 10cm distal to the ligament was excised, flushed with ice-cold normal saline, and opened along the mesenteric border. The tissue was laid flat along a plexiglass dissecting tray, and the mucosa was scraped off with a glass microscope slide. The mucosal scrapings were immediately stored in normal saline at -70°C for later sucrase enzyme activity.

The protein content of the mucosal homogenates was determined by the method of Bradford (1976), and all experiments were normalized to the protein content. Sucrase is an enzyme that is exclusively localized to the brush border membrane of the small intestine, primarily the jejunum. Sucrase activity was obtained by using a modified colorimetric assay of Dahlqvist (1964). Briefly, mucosal scrapings were diluted to 0.4mg/ml of protein content in 0.154M NaCl, and 100µl was pipetted into blank and reaction tubes. Sucrose substrate

(100µl of 0.056M sucrose/0.1M maleate buffer pH 6.4) was added to the reaction tubes, and the tubes were incubated for 30 minutes at 37°C in a water bath. At the end of the experiment, the reaction tubes were immersed in boiling water for 2 minutes. 100µl of substrate solution was added to the blank tubes and the reactions were immediately quenched by immersion in boiling water. A standard curve was constructed with glucose ranging from 0 to 50µg in 10µg increments, in a total solution volume 200µl. To each tube, 3ml of Tris-Glucose oxidase solution (0.25M Tris pH 7.0/0.3mg glucose oxidase reagent type V/0.5mg peroxidase reagent/1% (v/v) Triton X 100/5mg orthodianisidine). The colored reactions developed for one hour and were read at 420nm in a spectrophotometer. The results were expressed as Units/mg protein, where one Unit equals 1µM sucrose hydrolyzed per minute.

3.5 Results

3.5.1 Gastric Damage and Urinary Sucrose

Gastric damage was significant at a dose of 50mg/kg ASA and increased in a dose dependent fashion to 100 and 250mg/kg ASA (Figure 15); there was no visual damage to the small intestine.

As expected, there was a significant increase in urinary sucrose excretion when compared to controls at 50 and 100mg/kg ASA ($p<0.05$; Figure 15). The increase in urinary sucrose was even more pronounced at 250mg/kg ASA ($P<0.001$). This indicated that sucrose is a sensitive indicator of gastric damage induced by ASA.

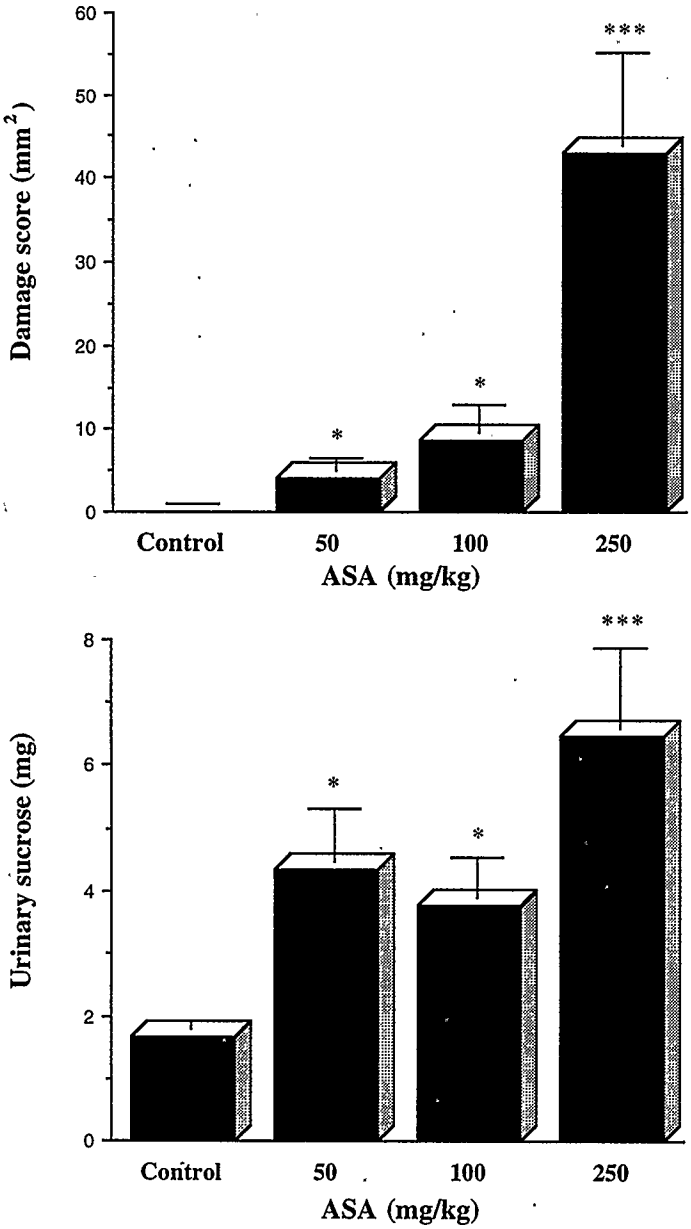

Figure 15. Visual gastric damage score (upper panel) and urinary sucrose (lower panel) in rats treated with increasing doses of ASA. (n = 6, 10, 11 and 9, respectively for damage scores and n = 23, 6, 16 and 14 for urinary sucrose, respectively; mean ± s.e.m.). * $p<0.05$ vs. control; * * * $p<0.001$ vs. 50 and 100.

3.5.2 Small Intestinal Damage and Urinary Lac:Man

Damage to the small intestine was quantitated by activity of the brush border enzyme sucrase in homogenates. A simple comparison of sucrase activity in control rats compared to indomethacin-treated rats allowed us to speculate about the functional state of the epithelium. There was a dose-dependent decrease in sucrase activity at 7.5 and 10mg/kg ($p<0.05$; Figure 16), indicating that indomethacin was damaging the small intestinal epithelium.

As a corollary to this damage score, there was a significant dose-dependent increase in the urinary lac:man ratio at 7.5 and 10mg/kg indomethacin ($p<0.001$; Figure 16). This indicated that the lac:man ratio was sensitive to small intestinal damage caused by indomethacin.

3.6 Summary

We have demonstrated that pharmacological doses of NSAIDs induce damage that can be visually and biochemically quantitated, and readily detected by urinary excretion of specific sugar probes. Urinary sucrose, a marker of gastric and upper duodenal permeability, was increased at therapeutic doses of ASA. The lac:man ratio, a specific marker of intestinal permeability, was increased at therapeutic doses of indomethacin. The results from NSAID administration demonstrate the sensitivity of our probes to pharmacologically relevant damage.

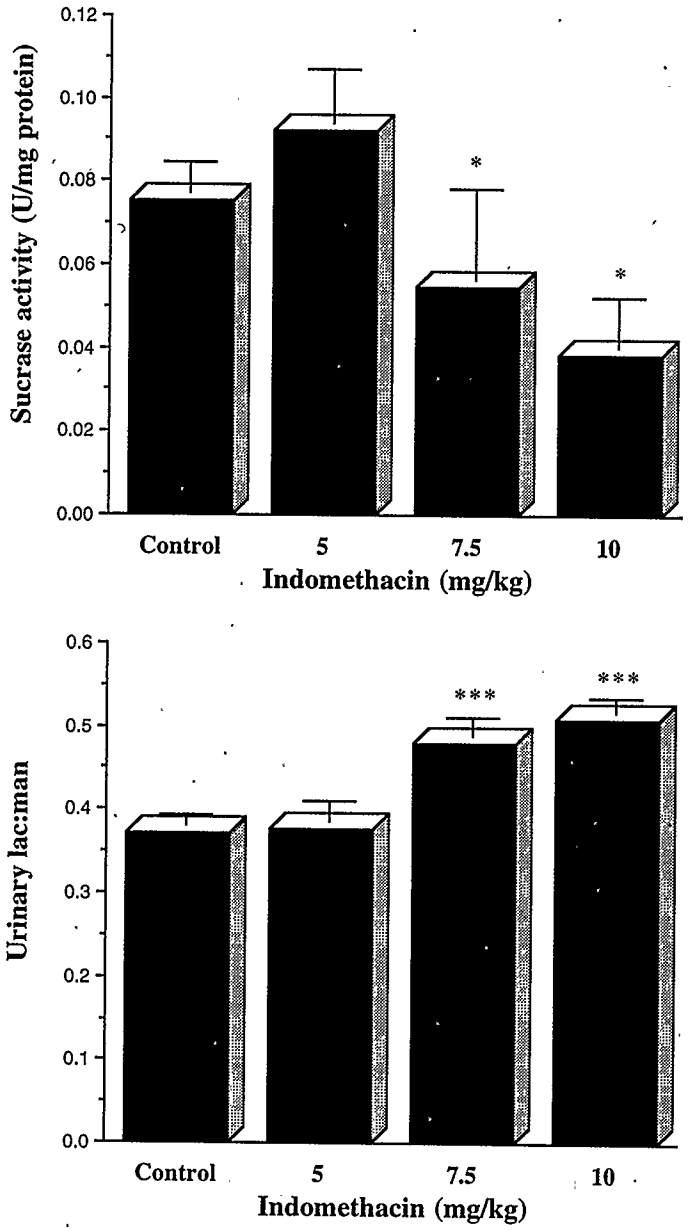

Figure 16. Sucrase activity in intestinal homogenates (upper panel) and urinary lac:man ratios (lower panel) in rats treated with increasing doses of indomethacin. (n = 7 for each sucrase group and n = 23, 7, 7 and 7, respectively, for urinary lac:man; mean ± s.e.m). * $p<0.05$, *** $p<0.001$ vs. control.

3.7 Discussion

We assumed the increased permeability observed on day 1 when compared to days 10 and 14 was due to stress. Acute stress has been shown to induce gastric lesions (Miyata *et al.*, 1995) which may explain the increased sucrose permeability. Additionally, acute stress has also been demonstrated to result in an increased permeability to ^{3}H-mannitol and ^{51}Cr-EDTA, and an increased tissue conductance *in vitro* (Saunders *et al.*, 1994). The stress might have been evoked by transportation from their birthplace to the Faculty of Medicine Animal Resources Centre, and the realities of a new environment. The behavior of the animals also indicated a reaction to early stress, as they were difficult to handle and were much more active on day 1 when compared to days 10 and 14.

The Hooded-Lister rat model is suitable for studying the effects of intestinal anaphylaxis on the function of the gut. When sensitized, this strain produces large quantities of IgE, much more than a "low responder" strain such as the Sprague-Dawley rat (Karlsson *et al.*, 1979); this phenomenon remains poorly understood. The titre of IgE has been shown to reach a maximum between days 13 and 15 after immunization (Sydbom and Karlsson, 1979). Therefore, we elected to experiment on animals on day 14, when both their stress and anti-EA IgE levels were appropriate.

There is a heterogeneous population of mast cells in the rat, consisting of the mucosal mast cells and the connective tissue mast cells. The two cell types are separated based on anatomical placement, and are also distinguished on the basis of morphology, granule constituents, IgE location, histochemistry, life span, proliferation, secretagogues and reactivity to mast cell constituents (Crowe and Perdue, 1992). The inherent leakage of intact

macromolecules across the epithelium activates mucosal mast cells via surface IgE to degranulate (Abbas *et al.*, 1994).

Sensitized rats with anti-EA IgE titres of >1:64 demonstrated specific mucosal mast cell degranulation and release of RMCP II in response to the challenging antigen at specific sites along the GI tract. Challenge of sensitized animals with EA at different sites (stomach, jejunum and colon) was not associated with increases in gastric or intestinal permeability as assessed using sugar probes. This may be because there is no damage or the probes are insensitive to small amounts of epithelial damage. To address the issue of probe sensitivity, two different NSAIDs were chosen to selectively damage gastric and small intestinal epithelia.

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most successful group of therapeutic drugs ever. They are clinically useful in the management of inflammatory diseases, fever and most recently cardiovascular disease (Somasundaram *et al.*, 1995). However, they are not without side effects such as damage to the gastrointestinal epithelium. The mechanisms of action occurring during absorption include the uncoupling of oxidative phosphorylation, resulting in the decrease in cellular ATP synthesis and mitochondrial Ca^{++} leakage. This defect in cellular energy function leads to the inability of the cells to maintain the integrity of the intracellular junctions, and an increase in gastrointestinal permeability ensues (Somasundaram *et al.*, 1995).

The second effect of NSAIDs is the inhibition of the enzyme cyclo-oxygenase, and a resultant drop in the levels of prostaglandins. Prostaglandins are 20 carbon molecules with a 5-carbon ring in the center that are essential for maintaining the integrity of the epithelium as well are essential for repair of the GI tract (Hudson *et al.*, 1992). Prostaglandins are

essential for the secretion of mucus and bicarbonate, mucosal blood flow and the flux of water from serosa to mucosa, repair and angiogenesis (Hudson, 1992). The inhibition of prostaglandin synthesis by NSAIDs allows for the changes in intestinal permeability to be prolonged.

The use of therapeutic single doses of NSAIDs to damage the gastric and small intestinal epithelia in a quantifiable manner was associated with a dose-dependent increase in urinary sucrose and lac:man, respectively. This demonstrated that the sugar probes are sensitive to small amounts of site-specific damage.

These results indicate the lack of any increases in GI tract permeability in this model of intestinal anaphylaxis. This is despite using sensitive probes, capable of detecting small amounts of epithelial damage. These results suggest that any damage to the epithelium in this model is either extremely minor, transient, or both.

It is possible that there is a significant amount of damage occurring, but that this damage is transient and therefore undetectable to our probes. We have no way of detecting whether this is the case. The possibility also exists that our probes are not as sensitive as ^{51}Cr-EDTA at measuring changes in intestinal permeability during food protein-induced anaphylaxis. Intriguing future studies could involve using ^{51}Cr-EDTA in our model, separately, or together with our sugar probes.

There was no increased permeability of sucrose in the stomach during intestinal anaphylaxis. Mast cells of the stomach are capable of degranulating in a specific response to ingested antigen. Increased retention time and acid secretion is observed when the sensitized stomach is challenged. This may be considered a defensive mechanism to

increase the chance that the protein antigen is denatured before contact with the intestine, thus lessening the exposure of the intestine to potentially harmful substances (Catto-Smith *et al.*, 1989). Intact antigen was able to cross the gastric epithelial barrier as there was an increase in serum RMCP II in EA-challenged, sensitized animals. It appears that mucosal mast cell degranulation, and the ensuing inflammatory reaction were not severe enough to damage the gastric epithelium to levels detectable by our sugar probes.

The factors that control the permeation of factors through the stomach are concentration gradient, surface area and retention time (Menzies, 1984). The stomach responds to antigen challenge with an increase in acid secretion, decreasing the concentration gradient by dilution; however, the retention time is increased. There is no known method of accurately assessing or controlling for the degree to which each factor is altered, and therefore changes to the concentration gradient and retention time remain two sources of error in this model.

There have been numerous studies demonstrating that intestinal anaphylaxis results in morphological alterations to the epithelium as well as increased permeability to test substances. Studies involving the use of the nematode *Nippostrongylus brasiliensis* have demonstrated that intestinal anaphylaxis results in increased permeability to ^{51}Cr-EDTA, ovalbumin, BSA and lactulose, a decreased permeability to mannitol, villus shortening and epithelial cell sloughing, decreased mucosal histamine and RMCP II, increased serum RMCP II, ion transport abnormalities and a decrease in brush border enzymes (Cobden *et al.*, 1979; Bloch *et al.*, 1979; Ramage *et al.*, 1988; D'Inca *et al.*, 1990). These alterations were also found to be independent of T cells, and were not inhibited by sodium cromoglycate, a stabilizing agent of connective tissue mast cells (D' Inca *et al.*, 1992a; D' Inca *et al.*, 1992b).

Despite the induction of an immune response and the use of sensitive probes, I was unable to find any increase in permeability. While the reaction in the NB model is immediate type hypersensitivity, the methods used as well as immune cells involved differ slightly. First, these studies were done with isolated intestinal loops in anaesthetized animals. While loops have the advantage of allowing for a control loop of intestine in the same animal, they have the disadvantage of inhibiting the increased motility observed during anaphylaxis. Also, the surgery and anesthesia are possible confounding factors. The biggest difference lies in the fact that a nematode parasite is used as the sensitizing agent.

Nematode parasites are ejected spontaneously within the second or third week post-infection (Ramage *et al.*, 1988). During the course of the build-up to the expulsion, there is an influx of neutrophils and eosinophils into the mucosa due to chemoattractive factors released by mast cells (LTB4 and PAF; Crowe and Perdue, 1992). Eosinophils are known to have low affinity IgE receptors and can therefore be activated by re-exposure to antigen. Upon re-exposure, it is possible that the eosinophils are activated and begin degranulating. One of the potent products released from eosinophil granules is major basic protein (MBP), which has been demonstrated to have a cytotoxic effect on mammalian cells in a dose-dependent manner, and can also activate neutrophils (Gleich *et al.*, 1979; Moy *et al.*, 1990). Neutrophils and eosinophils may be further activated by mast cell degranulation products such as GM-CSF (Gasson, 1991). It is possible that activation of the respiratory burst of neutrophils and eosinophils, along with the release of MBP is responsible for the consistently observed increase in permeability in the NB model of intestinal anaphylaxis.

In the food protein-induced model of intestinal anaphylaxis, there have been a number of studies indicating epithelial damage (Perdue *et al.*, 1984a; Perdue *et al.*, 1984b; Patrick *et*

al., 1988) and an increased intestinal permeability (Bloch and Walker, 1981; Turner *et al.*, 1988). These results differ from those presented here, and this needs to be examined.

In finding damage and edema, Perdue *et al.* (1984a and 1984b) and Patrick *et al.* (1988) used anesthesia and surgery to tie off two isolated loops of intestine, repeated injections of antigen and continuous perfusion. The damage was observed by light and electron microscopy, but was largely subjective and not subject to any quantitation. Additionally, the authors (Perdue *et al.*, 1984a) admit that sections of the same loop varied greatly, either having obvious edema, or having none at all. Finally, a possible effect of surgery and anesthesia on sensitized animals was not addressed.

The study by Bloch and Walker (1981) differs in two important ways. First, surgery that perforated the gastrointestinal tract was performed at the beginning of the experiment, and how much of the increased permeability could be attributed to this is unknown. Secondly, they used animals with PCA titres of 1:5 to 1:20, much less than the 1:64 that I used in my experiments. If anything, I would expect rats from this study to react more intensely to the antigen, and show an even greater increased permeability.

The study by Turner *et al.* (1988) utilized different methods again, including anesthesia, repeated cardiac punctures and gastrointestinal surgery the day of the experiment. Therefore, I am not sure what contributed to their observation of increased permeability. Also, they conclude that there is no simple correlation between gut permeation of low molecular weight sugars and macromolecules. However, they did not damage the gut in an immunological manner, preferring to use cetremide, a detergent. The effect of this detergent on the denaturation of intact protein was not analyzed, and the amount of damage induced by the detergent was not quantitated.

The colon is capable of being an active participant in GI anaphylaxis, as colonic mucosal mast cells were capable of degranulating in a manner specific to antigen challenge. There was no alteration in permeability in the stomach or small intestine, but what about the colon? The difficulties in measuring colonic permeability are the requirements that the probe(s) be resistant to bacterial degradation and avoid becoming extensively absorbed into feces. Exciting developments point to the possible use of a combination of ^{51}Cr-EDTA (which is not degraded in the colon) and lactulose to distinguish increases in small intestinal and colonic permeability (Jenkins *et al.*, 1992). Furthermore, the development of novel oligosaccharide derivatives resistant to bacterial degradation (i.e. sucralose) may allow for the determination of permeability throughout the entire GI tract (Meddings, personal communication).

In summary, it is not surprising that isolated loops of intestine and intestinal anaphylaxis lead to increases in permeability. Putting antigen into the lumen of a blocked small intestine prevents the defensive mechanisms of the gastrointestinal tract from being evoked. Upon antigen exposure, the stomach acts by increasing the secretion of acid and retention time, both presumably to denature the offending antigen thereby decreasing the detrimental effect on the intestine. The intestine reacts by increasing secretion and motility, presumable to decrease surface exposure time to antigen. Finally, the colon may be an active participant in response to antigen, further acting to propel the insult outside of the body. Bypassing these defensive mechanisms by using isolated loops of intestine is not a physiological condition and therefore may not be the most appropriate experimental technique for studying permeability alterations during intestinal anaphylaxis.

Chapter 4. General Conclusions

In summary, findings from this study demonstrate that there are alterations at the smooth muscle plasma membrane during the course of YE enteritis. Plasma membrane physical properties are altered and muscarinic receptors have an increased affinity. This may be an adaptive response of the muscle cell to its environment. Or, this may occur as a result of increasing oxidation of the plasma membrane, making it more rigid.

There was no increased permeability during intestinal anaphylaxis in the Hooded-Lister rat. This was in spite of inducing a specific gastrointestinal inflammatory response and the use of very sensitive probes. Food protein-induced permeability changes appear to less than that observed after a single therapeutic dose of NSAID. Permeability changes would seem to play little role in the pathophysiology of food protein-induced anaphylaxis.

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