Evaluation of a Bovine Colostral Preparation as Treatment for Gastrointestinal Injury

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

by

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October 2001

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For Smythe

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Evaluation of a Bovine Colostral Preparation

As Treatment for Gastrointestinal Injury

David N Floyd October 2001

Abstract

Growth factors play an important role in repair of the injured gut as well as maintaining gut homeostasis. De-fatted bovine colostrum is a health food supplement containing significant amounts of growth factors. Thus, derivatives from bovine colostrum might be an inexpensive treatment for gut injury [e.g. inflammatory bowel disease (IBD)], and as a prophylactic against injurious effects of drugs such as non-steroidal anti-inflammatory drugs (NSAIDs).

The studies presented in this thesis were devised to test the effects of a commercially-available bovine colostral preparation on wound healing and proliferation *in vitro*, and in an animal model of IBD. The effect of TGFβ2, a major colostral growth factor, on NSAID-induced murine small intestinal injury was also tested. Finally, the effects of bovine colostral preparation on intracellular signalling leading to stimulated DNA synthesis was investigated.

Bovine colostral preparation stimulated DNA synthesis and proliferation in cultured intestinal epithelial cells, and also stimulated epithelial migration following wounding. Motogenic effects were independent of proliferation. Colostral components greater than 30 kDa in weight were mostly responsible for these effects.

Motogenic, but not mitogenic, components were resistant to transient acidification, such as might be encountered in the stomach. Transient acidification liberated active TGFβ. TGFβ2, the major colostral TGFβ isoform, was prophylactic against murine small intestinal NSAID-induced damage.

Oral bovine colostral preparation stimulated healing of chemically-induced colitis in rats, but had no effect on the induction of colitis. Topical application of the colostral preparation by enema was ineffective.

Inhibition of growth factor receptors inhibited some, but not all, proliferative activity in colostral preparation. Some effects of colostral preparation were due to activation of the EGF receptor despite the absence of EGF in the preparation.

In conclusion, bovine colostral preparation might act as an orally-active prophylactic and healing agent for a variety of gastrointestinal injuries.

Acknowledgements

The studies presented in this thesis were performed under the initial direction of Professor R J Playford, currently at Imperial College Medical School. The analyses and writing-up were performed under the supervision of Dr Alan Bevington, Division of Renal Medicine, University of Leicester. Dr Bevington's unfailing kindness and wisdom have been invaluable.

The studies presented in this thesis were funded by the Medical Research Council, by SHS International Ltd, Wavertree, Liverpool, UK, and by Leicester General Hospital.

I received technical assistance from the following individuals whilst performing the experiments presented here: Mr Denis Calnan, currently at University College, London; Dr Robert Goodlad, Imperial Cancer Research Fund, London; Dr Mehmoona Khatri, University Hospitals of Leicester NHS Trust; Dr Zubair Khan, University Hospitals of Leicester NHS Trust; the staff of the biomedical services departments at the University of Leicester and at the Imperial Cancer Research Fund, Potter's Bar.

I gratefully acknowledge the constructive comments of the following during the preparation of this manuscript: Drs Martin Dickens, Lisa Jennings and Raj Patel, Biochemistry Department, University of Leicester; Dr Tom Robinson, Division of Medicine for the Elderly, Department of Medicine, University of Leicester.

The following people have kept me human: Denis and Anita Calnan; Carl Glennon, Kathryn Smith and Poppy – cheers!

Publications Relating to This Thesis

Floyd DN and Playford RJ. *In vitro* studies examining the potential value of a bovine colostrum preparation in the treatment of gut injury. Clin. Sci., 1999, 96, 4p.

Playford RJ, Floyd DN, MacDonald CE, Calnan DP, Adenekan RO, Johnson W, Goodlad RA and Marchbank T. Bovine colostrum is a health food supplement which prevents NSAID-induced gut damage. Gut, 1999, 44(5), 653-658.

Abbreviations.

Ac col	**transiently acidified colostral preparation**
ANOVA	**analysis of variance**
5-ASA	**5-aminosalicylic acid**
6MP	**6-mercaptopurine**
BCGF	**bovine colostral growth factor**
CBGF	**colostric basic growth factor**
CCK	**cholecystokinin**
CD	**Crohn's disease**
COX	**cyclo-oxygenase**
DMEM	**Dulbecco's modified Eagle medium**
DMSO	**dimethyl sulphoxide**
DNA	**deoxyribonucleic acid**
DSS	**dextran sodium sulphate**
EGF	**epidermal growth factor**
EC	**enterochromaffin cell**
ECL	**enterochromaffin-like cell**
ECM	**extracellular matrix**
FGF	**fibroblast growth factor**
GALT	**gut-associated lymphoid tissue**
GPCR	**G protein-coupled receptor**
HBSS	**Hank's balanced salt solution**
HMW	**high molecular weight**
HMWCP	**high molecular weight colostral preparation**
HMPC	**hydroxypropyl methylcellulose**
IBD	**inflammatory bowel disease**
ICC	**interstitial cell of Cajal**
IEL	**intra-epithelial lymphocyte**
IGF	**insulin-like growth factor**
Indo	**indomethacin**
ip	**intra-peritoneal**
JNK	**jun-N-terminal kinase**

kDa	**kilo Dalton**
KGF	**keratinocyte growth factor**
LAP	**latency-associated peptide**
M	**molar**
MAPK	**mitogen-activated protein kinase**
mg	**milligram**
μg	**microgram**
ml	**millilitre**
μl	**microlitre**
mM	**millimolar**
μM	**micromolar**
MPO	**myeloperoxidase**
mRNA	**messenger ribonucleic acid**
NEC	**necrotising enterocolitis**
ng	**nanogram**
nM	**nanomolar**
NSAID	**non-steroidal anti-inflammatory drug**
NSD	**not significantly different**
OD	**optical density**
PBS	**phosphate buffered saline**
PDGF	**platelet-derived growth factor**
PKC	**protein kinase C**
PSTI	**pancreatic secretory trypsin inhibitor**
PYY	**peptide YY**
RNA	**ribonucleic acid**
sc	**sub-cutaneous**
SCFA	**short-chain fatty acid**
SEM	**sub-epithelial myofibroblast**
TFF	**trefoil family factor**
TGFα	**transforming growth factor alpha**
TGFβ	**transforming growth factor beta**
TMB	**tetramethylbenzidine**
TNBS	**trinitrobenzene sulphonic acid**

TNFα	**tumour necrosis factor alpha**
UACL	**ulcer-associated cell lineage**
UC	**ulcerative colitis**
VIP	**vasoactive intestinal peptide**
v/v	**volume per volume**
w/v	**weight per volume**

Chapter 1.

General Introduction

"He [Dr Berlioz] cites with great deference the writings of Broussais, one of the foremost bleeders of the time, a systematist of the old school who ascribed all diseases to inflammation of the intestines."

David Cairns in *Berlioz: The Making of an Artist 1803-1832.*

Bleeder or not, Dr Broussais recognised early that the intestines play an important role in health and disease. Since he preceded the medical advances of the nineteenth and twentieth centuries we can forgive him for overlooking the equal importance of the stomach.

1.1 Growth Factors and the Damaged Gut

The gastrointestinal tract (or gut) is responsible for the digestion and absorption of nutrients, whilst preventing ingress of pathogens into the systemic circulation (Hogan et al, 1994; Mahida et al, 1997a). The gut mucosal layer is essential for these functions, and damage to this mucosa, such as mucosal denudation and increased epithelial permeability, results in dysfunction, infection and inflammation (Gibson, 1997; Hogan et al, 1994 Sigthorsson et al, 1998; Soderholm et al, 1999). Mucosal damage is known to occur in response to drugs, stress, or ingestion of alcohol (Allison et al, 1992; Fiocchi, 1998; Romano et al, 1992; Sanders, 1998; Wyeth and Pounder, 1994).

During the past twenty years a considerable amount of data have accrued demonstrating that growth factors have a central role in modulating gut mucosal function and integrity (table 1; Szabo et al, 1995b; Uribe and Barrett 1997; Wilson and Gibson, 1997a). Endogenous growth factors monitor mucosal integrity and stimulate the reparative process (restitution) by which breaches to the mucosa are healed (Alison et al, 1995; Goodlad and Wright, 1995; Playford, 1995). Exogenous growth factors protect the intact gut or stimulate repair of the damaged gut when administered orally or systemically (Lund and Zimmerman, 1996; Romano et al, 1992; Szabo and Sandor, 1996). Finally, growth factors stimulate migration *in vitro* and *ex vivo* of primary cells and cell lines derived from the gut (Dignass and Podolsky, 1993; Wilson and Gibson, 1997a).

Growth factors are important components of biological fluids including serum, milk and colostrum (Bastian et al, 2001; Lund and Zimmerman, 1996; Rogers et al, 1996; Srivastava et

Table 1.1 Growth Factors Involved in Maintaining Gut Epithelial Integrity.

EGF	Epidermal Growth Factor
TGFα	Transforming Growth Factor alpha
IGF-I & II	Insulin-Like Growth Factors I and II
TGFβ	Transforming Growth Factor beta
FGFs	Fibroblast Growth Factors
PDGF	Platelet-Derived Growth Factor

al, 1996; Szabo and Sandor, 1996; Xu, 1998). Colostrum is milk produced by the mother around parturition. It is a rich source of growth factors, protease inhibitors, antibiotics and antibodies (Kirihara et al, 1995a&b; Koldovsky et al, 1993; Korhonen et al, 2000; Kussendrager and Van Hooijdonk, 2000; Uruena et al, 1998; Van Hooijdonk et al, 2000). Ingestion of colostrum by the neonate stimulates growth and maturation of the gastrointestinal tract, increases body mass and confers passive immunity (Koldovsky, 1995; Odle et al, 1996; Xu, 1996). Products derived from ruminant (mostly bovine) colostra are marketed as health food supplements for adults, which might have beneficial side effects against a range of diseases, including arthritis, irritable bowel syndrome (IBS) and some cancers.[1] Bovine colostrum derivatives are also marketed as supplements to enhance athletic performance and anabolism during training (Antonio et al, 2001; Buckley et al, 2000). Many of these claims are based on known activities of isolated molecules found in colostrum, including anti-oxidants, casein-derived peptides, antibiotics, polyamines, cytokines, nucleosides and nucleotides, and growth factors (Gill and Cross, 2000; Lindmark-Mansson and Akesson, 2000; Koldovsky et al, 1993; Loser, 2000; Oguchi et al, 1997; Schlimme et al, 2000; Shah, 2000; Srivastava et al, 1996; van Hooijdonk et al, 2000; Xu, 1998).

Partially purified and concentrated colostral growth factors support the growth of a variety of cells in culture, demonstrating that the growth factors retain bioactivity during processing (Belford et al, 1997; Rogers et al, 1996; Shing and Klagsbrun, 1984). Derivatives of bovine colostrum are inexpensive when compared to recombinant growth factors, and might be cost-effective treatments for the damaged gut. The experimental data presented in this thesis were obtained from experiments designed to test the efficacy of a commercially available bovine colostral preparation as a treatment for the damaged gut using *in vitro* and *in vivo* methods.

This introductory chapter will provide overviews of the adult gastrointestinal mucosa, and of epithelial wound healing (restitution), before describing those situations where gut injuries are common. Finally, the nature of bovine colostral growth factors will be reviewed and discussed, with particular relevance to the normal and injured gut.

[1] Useful websites: www.bovinecolostrum.com; www.brightertomorrows.com; www.colostrum-health.com; www.healthwell.com.

1.2 The Gastrointestinal Tract.

Most of the basic information in this section is taken from *Wheater's Functional Histology: A Text And Colour Atlas.* Edited by Young and Heath. Fourth edition, 2000, Churchill Livingstone, London. All other sources are referenced appropriately.

1.2.1 Introduction. The length of the gastrointestinal tract is covered luminally by the mucosal/epithelial layer (Karam, 1999). This has evolved to protect against the extremely harsh environs of the gut lumen (acid, digestive enzymes, pathogens) and to enable rapid repair of breaches in the integrity of the epithelium (Sidebotham and Baron, 1994). The epithelium is responsible for chemical digestion and absorption of nutrients, prevention of translocation of pathogens into the local and systemic circulations, and immuno-modulation, including oral tolerance of ingested food antigens (Johnson, 2000b&c). This plethora of functions requires exquisite regulation, which is achieved locally and systemically (Johnson 2000a; Podolsky, 1993; Weisbrodt, 2000a). The mucosa adjoins the lamina propria, which contains many blood cells, sub-epithelial myofibroblasts and the gut microvasculature (Powell et al, 1999). Beneath the lamina propria lies the muscularis mucosa, made up of smooth muscle fibres. Their action aids secretory activity in the gut. The underlying submucosa makes up the rest of the mostly muscular gut wall (Powell et al, 1999).

The gastrointestinal tract comprises the oesophagus, stomach, small intestine (duodenum, jejunum and ileum) and large intestine, each with characteristic forms and functions (Karam, 1999). These shall be described first, with the exception of the oesophagus, followed by descriptions of individual cell types involved in the gastrointestinal tract.

1.2.2 The Gastrointestinal Mucosa

1.2.2.1 The Stomach. The stomach consists of a flat surface pitted with 'crypts', acid/protease/mucus-secreting oxyntic glands in the fundus (main part) and mucus-secreting glands in the distal pyloric antrum (Karam, 1999). The gastric lumen contains acidic juice and proteolytic enzymes, which degrade food and exert toxicity towards exogenous pathogens (Johnson, 2000b; Mahida et al, 1997a). Gastric epithelium is protected against the degradative capacity of gastric juice by a locally secreted layer of mucus and bicarbonate (Atuma et al, 2001; Hogan et al, 1994). This protective layer provides a neutral pH at the epithelial surface, resulting in a massive pH gradient over the layer of mucous (from a luminal pH as low as one unit, to pH7 at the mucus-epithelial barrier; Hogan et al, 1994).

Gastric function – secretion and peristalsis - is regulated by local and circulating peptide factors, such as growth factors and gut-regulatory peptides (Johnson, 2000a). Gut-regulatory peptides (e.g. gastrin, peptide YY) modulate secretion of acid and bicarbonate, gastric emptying and tissue growth (Carver et al, 1996; Matsuda et al, 1996; Johnson, 2000a). Release of these regulatory peptides is in turn regulated by ingestion of food, acid secretion and neurological activity (Johnson, 2000a; Weisbrodt, 2000a). The EGF-receptor ligands EGF and TGFα stimulate proliferation, modulate acid secretion and provide 'luminal surveillance', and maintain 'mucosal integrity' respectively (Goodlad and Wright, 1995; Playford, 1995; Szabo et al, 1995a&b; Uribe and Barrett, 1997). When breached, the gastric epithelium is also repaired by small peptides, including pancreatic secretory trypsin inhibitor (Marchbank et al, 1996) and trefoil peptides (Playford, 1993). This last group are referred to as 'rapid response' peptides, since they are quickly upregulated at sites of injury and stimulate restitution, as well as protecting the gut epithelium (Playford, 1993; Playford et al, 1996; Podolsky, 1999; Sands and Podolsky, 1996).

As a result of these processes, ingested food is chemically and physically semi-digested into *chyme*, a liquid substance which is forced by the pyloric sphincter into the duodenum of the small intestine (Weisbrodt, 2000b).

1.2.2.2 The Small Intestine. The small intestine is characterised by extensive folding of the epithelium to form invaginated crypts and larger, evaginated villi (Karam, 1999). This 'concertina-like' effect greatly increases the surface area of the small intestine, which increases its ability to digest further and absorb nutrients that enter from the stomach (Johnson, 2000c). The small intestine comprises the duodenum (proximal to the stomach), jejunum and ileum, the principal purpose of which is to digest fully, and absorb, nutrients from chyme (Johnson, 2000c). Duodenal bicarbonate secretion neutralises acid in gastric juice, and secreted mucus protects the length of the small intestine (Atuma et al, 2001; Hogan et al, 1994). Chyme is digested luminally by enzymes such as trypsin and elastase (protein digestion), amylase (carbohydrate digestion) and lipase (lipid digestion). Further digestion occurs by the action of enzymes bound to the surface of epithelial cells on the villi, prior to absorption (Johnson, 2000c). Absorption of digested lipids requires solubilisation, which

occurs by formation of aggregates of lipids and bile salts, which are derived from the liver and enter the duodenum via the gallbladder (Weisbrodt, 2000c). Intestinal functions (luminal enzyme secretion, bicarbonate secretion, mucosal growth and bile salt secretion) are regulated by local factors such as cholecystokinin (CCK), secretin and pancreatic polypeptide (PP) (Johnson 2000a&c). Release of intestinal regulatory peptides is controlled principally by the passage of acidic chyme from the stomach into the duodenum (Johnson 2000a). Intestinal motility (smooth muscle contraction) is controlled by regulatory peptides and by the autonomic nervous system (Johnson 2000a; Weisbrodt, 2000b)

Intestinal homeostasis is subject to nutritional regulation. For example, parenteral feeding reduces the wet weight of rat small intestines as well as reducing brush border enzyme activity, and these effects are reversed by growth factors, such as EGF, and nutrients, such as glutamine (Carver and Barness, 1996; Goodlad et al, 1991; Marchbank et al, 1995; Playford et al, 1993). Endogenous regulatory peptides, growth factors and trefoil peptides are important in stimulating growth and maintaining homeostasis of the small intestinal epithelium, in similar fashion to the stomach (see above; also Playford et al, 1996; Poulsen et al, 1999). In addition to epithelial cell proliferation, the intestinal epithelium grows by crypt fission, whereby whole crypts grow, then divide (StClair and Osborne, 1986). Crypt fission is stimulated by growth factors (Park et al, 1997).

Following passage though the small intestine chyme is depleted of nutrients, and passes into the large intestine, via the ileocaecal valve.

1.2.2.3 The Large Intestine. The epithelial surface of the large intestine, or colon, does not have villi, but is pitted with crypts, similar to those in the stomach, and is protected with a layer of mucous (Karam, 1999). Nutrient-depleted, liquid chyme enters the large intestine where it is increasingly desalted and dehydrated, to form solid, excretable faeces. Mucus is an important defence against the injurious effects of increasingly solid faeces; the rat mucus layer is at least twice as thick in the colon as in any other region of the gut (Atuma et al, 2001).

Short chain fatty acids (SCFAs) originate luminally by bacterial fermentation of faecal carbohydrates (Salminen et al, 1998). SCFAs (acetate, propionate and butyrate) are important regulators of the large intestine, rather than the regulatory peptides discussed above (Johnson,

2000a; Salminen et al, 1998). SCFAs are absorbed by the colonic mucosa, stimulate absorption of salt and water and induce epithelial proliferation (Salminen et al, 1998). Butyrate is the most potent trophic SCFA, which also modulates differentiation, inhibits growth of transformed colonocytes and stimulates restitution (Salminen et al, 1998; Wilson and Gibson, 1997b). However, Scheppach et al (1996) reported no effect of luminal butyrate on colonic restitution *ex vivo*.

Growth factors and other peptides modulate colonic epithelial integrity. EGF and TGFα are trophic for colonocytes *in vitro*, but only TGFα is expressed in the colonic epithelium *in vivo* (Barnard et al, 1995; Uribe and Barrett, 1997). Colonic crypt fission is stimulated by intravenous EGF, indicating that colonic growth is regulated by EGF-receptor ligands (Park et al, 1997). IGF also stimulates colonic growth and repair *in vitro* and *in vivo* (Lund and Zimmerman, 1996).

Pancreatic secretory trypsin inhibitor (PSTI) is found in the normal human colon, but is reduced in the colons of inflammatory bowel disease (IBD) patients (Playford et al, 1995). PSTI stimulates migration of colonocytes *in vitro* (Marchbank et al, 1996), in keeping with its designation as a mucosal integrity peptide (Playford, 1995). Trefoil peptides are upregulated at sites of colonic mucosal injury, as seen elsewhere in the gut (Alison et al, 1995).

The main features and functions of the stomach, small and large intestines, have been described, together with a simplified description of the sources of regulation of these tissues. Many functions of the gut arise from the several differentiated cell types that reside in the epithelium and sub-epithelium.

1.2.3 Cells of the Gastrointestinal Mucosa.

The gastrointestinal mucosa is made up of layers of mucus, on top of epithelial cells, which reside on a basement membrane of large matrix proteins, fibroblasts, cells of the immune system and neurones. The mucosa is exposed luminally to a variety of endogenous bacteria that affect the gut.

1.2.3.1 Epithelial Cells. The gastrointestinal tract is covered luminally with a single sheet of epithelial cells. Normal adult gut epithelial homeostasis is maintained by an exquisitely regulated balance of proliferation, differentiation and death (Hall et al, 1994; Podolsky, 1993). Stem cells in the proliferative zones of crypts and pits give rise to mature differentiated cells in the gut epithelium (Karam, 1999; Podolsky, 1993). Five distinct stages in the life of a gut epithelial cell have been identified:

(1) undifferentiated stem cells undergoing unbalanced proliferation, giving rise to stage 2 cells as well as maintaining the stem cell pool (Hall et al, 1994; Karam, 1999);
(2) uncommitted or committed precursors that undergo balanced proliferation;
(3) transit cells that translate and transcribe some of the genes associated with full differentiation;
(4) mature, fully differentiated cells;
(5) senescent cells cleared by phagocytosis, or by extrusion into the gut lumen.

The process of differentiation is accompanied by migration of the cell from the stem cell compartment to its final position in the crypt or pit, or on the villus (Karam, 1999).

Gastric oxyntic glands comprise a proliferating stem cell compartment from which increasingly differentiated cells migrate. Pit cells reside at the top of the oxyntic gland and are identified by dense, apical mucus granules; zymogenic, or chief, cells reside at the bottom of the gland, secreting pepsinogen; parietal cells are the acid-secreting cells in the stomach, and are found throughout the oxyntic gland (reviewed by Karam, 1999). The stomach also contains a number of endocrine cell types, including G cells (gastrin secretion), D cells (somatostatin secretion), enterochromaffin (EC) cells (serotonin secretion) and enterochromaffin-like (ECL) cells (histamine secretion) (Norlen et al, 2001).

The stem cell compartment in the small intestine is located towards the bottom of the crypt (Karam, 1999). Paneth cells migrate to the bottom of the crypt, where they secrete antimicrobial peptides (lysozyme, defensins) which are important in maintaining intestinal sterility (Ouellette, 1997). All other small intestinal cell types migrate upwards from the proliferative zone along the villus, from the tip of which senescent cells are sloughed into the lumen or engulfed by neighbouring cells (Hall et al, 1994; Karam, 1999). Goblet cells secrete mucus into the small intestine. However, the majority of small intestinal epithelial cells (more

than 80% of murine small intestinal epithelial cells) are absorptive, columnar cells, characterised by their prominent brush border (Karam, 1999). These cells express digestive enzymes in the surface membranes (Beaulieu, 1997). Endocrine cells are also found throughout the small intestine (Norlen et al, 2001), as are specialised antigen-sampling M cells which are found throughout the small intestine in regions called Peyer's patches (Gebert et al, 1996; Neutra, 1999; see section 1.2.3.2.1).

A highly specialised ulcer-associated cell line (UACL) is found at the edge of intestinal ulcers (Wright et al, 1990). The cells originate in the proliferative compartments of crypts adjoining the ulcer and secrete a range of peptides and growth factors that stimulate wound healing (Wright et al, 1990).

The large intestinal stem cell compartment resides in the colonic crypts, giving rise to vacuolated columnar cells and mucus-secreting goblet cells (Karam, 1999), in addition to the aforementioned endocrine cells (Norlen et al, 2001).

1.2.3.2 Cells of the Lamina Propria. Beneath the epithelial layer is the lamina propria, consisting of extracellular matrix and a variety of cell types associated with the gut epithelium. These include intraepithelial lymphocytes (IELs), neutrophils, macrophages and sub-epithelial myofibroblasts (SEMs) (MacDonald, 1999; Neutra, 1999; Powell et al, 1999). Beneath this layer of cells and matrix is the muscularis mucosa and the submucosa, comprising interstitial cells of Cajal (ICCs), another type of fibroblast, in association with the smooth muscle (Powell et al, 1999). The ICCs and associated musculature are mostly responsible for gut motility and peristalsis, and shall not be considered here.

The interaction of intestinal epithelial cells with the underlying lamina propria is essential for the maintenance of epithelial integrity and regulation of gut function (Beltinger et al, 1999; Strater et al, 1996). Immunological cells and sub-epithelial myofibroblasts contribute two essential aspects to mucosal function.

1.2.3.2.1 Gastrointestinal Immune Cells. The gut has a very large surface area, which is exposed to a variety of immunogens and pathogens, and is prone to physical and chemical injury (Hogan et al, 1994). Ingress of immunogens and pathogens is inevitable; indeed, the gut has been described as being in a state of 'physiological inflammation' (Hodgson, 1998).

The adult gastrointestinal tract has evolved its own local immune system to prevent local invasion leading to systemic disease, whilst allowing absorption of 'safe' food antigens (Hein, 1999; MacDonald, 1999). Thus, the gut immune system must be sampling constantly potential antigens as they pass through the epithelium. This is performed by specialised M-cells, in regions of 'gut-associated lymphoid tissue' (GALT), which are found throughout the small and large intestine (Gebert et al, 1996; Neutra, 1999). GALT regions in the small intestine are called Peyer's patches (Gebert et al, 1996). The epithelium of these GALT regions is called the 'dome epithelium', comprising both normal epithelial cells and M cells (Gebert et al, 1996). Underneath the dome is an array of immunological cells (T- and B-lymphocytes, macrophages) that regulate the response to antigens taken up by M cells (Mahida et al, 1997b). These cells act locally but also interact with the circulating immune system. Normal function is maintained by local secretion of cytokines (MacDonald, 1999).

1.2.3.3.2 Sub-epithelial myofibroblasts. SEMs play an important role in the form and function of the gut epithelium. SEMs form a syncytium underneath the epithelial layer, extending throughout the entire gut (Powell et al, 1999). Cells form homotypic-junction complexes, allowing intercellular communication; also, the SEM syncytium connects with the gut vasculature and with the nervous system (Powell et al, 1999). Thus, SEMs play a vital role in co-ordinating local signals with those from the nervous system and the circulation. In response to these signals, SEMs control local morphology, and secrete both soluble factors (growth factors and cytokines) and extracellular matrix (Beltinger et al, 1999; Mahida et al, 1997c; McKaig et al, 1999). As discussed below, all of these functions are central to the modulation of epithelial wound healing.

1.2.3.4 Gastrointestinal Bacteria. The gut lumen is populated by many species of beneficial bacteria. The level of 'flora' is comparatively low in the stomach, due to toxicity of gastric juice (Salminen et al, 1998; Mahida et al, 1997a). The small intestinal flora is limited also, because of short transit times and toxicity due to bile and secreted enzymes (Salminen et al, 1998). The intestinal flora has an important immunoregulatory function, contributing to oral tolerance and lymphocyte maturation (Salminen et al, 1998).

The colon has a large and varied flora, mostly non-aerobic, which is acquired shortly after birth (Salminen et al, 1998). Its principal function is to metabolise carbohydrates in faecal matter that were not digested and absorbed in the small intestine. Bacterial fermentation of

carbohydrates forms short-chain fatty acids (SCFAs), which are important regulators of colonocyte growth and differentiation (see section 1.2.2.3), and might stimulate repair of the damaged colonic epithelium (Wilson and Gibson, 1997b).

1.3 'Once more unto the breach, dear friends': Epithelial Restitution.

Restitution is a highly organised process whereby epithelial cells at the edge of a wound spread out and migrate to cover the denuded area without losing contact with neighbouring cells (Szabo et al, 1995a; Tarnawski and Halter, 1995; Wilson and Gibson, 1997a). This process restores epithelial integrity within hours of injury and proceeds independently of proliferation (Basson et al, 1992; Wilson and Gibson, 1997a). In situations where injury extends beyond the lamina propria additional remodelling of the tissue and replenishment of other cell types is necessary (Tarnawski and Halter, 1995; Wilson and Gibson, 1997a). This occurs following formation of a mucous 'cap', which is essential to the repair process (Wallace and Whittle, 1986).

1.3.1 Modulation of Restitution. Restitution is stimulated by a variety of molecules, including nucleotides, polyamines, nutrients, trefoil peptides, neuropeptides, cytokines and growth factors (reviewed by Wilson and Gibson, 1997a). These effects have been demonstrated *in vivo*, using animal models of gut injury (Adhip et al, 1996; Ameho et al, 1997; Barbara et al, 2001; Hirano et al, 1995; Playford et al, 1995; Playford et al, 1996; Romano et al, 1992; Szabo et al, 1995b; Wallace and Keenan, 1990); *ex vivo*, using explanted gut tissue (Blikslanger et al, 1997; Scheppach et al, 1996; Yanaka et al, 1996); and *in vitro*, using cultures of primary gastrointestinal cells or established cell lines (Andre et al, 1999; Basson et al, 1992; Dignass and Podolsky, 1993; Dignass et al, 1995; Playford et al, 1995; Szabo et al, 1995a&b). Each methodology has its drawbacks and its compensations. For example, *in vivo* studies show that pro-restitutive factors really do work in a biological system, but analysis of the molecular mechanisms by which the effect is made manifest is difficult, if not impossible. *Ex vivo* tissue culture (eg using Ussing chambers) allows more direct manipulation of the tissue, but is limited by tissue degradation over time and also makes single mechanisms difficult to evaluate. *In vitro* cell cultures provide a highly simplified system to measure the effects of soluble factors and extracellular matrix components on restitution, and to analyse further the intracellular signalling pathways and cytoskeletal changes that give rise to restitution. However, *in vitro* methods cannot account

for the complex interactions of local cell types, specialised basement membranes and local and systemic soluble factors mentioned in the preceding sections. Further, established cell lines might not always bear sufficiently close resemblance to the cells of origin, so results of mechanistic studies must be interpreted with caution. A mixture of the three approaches is most useful (Wilson and Gibson, 1997a).

1.3.2 The Epithelial Cell and Its Environs. Epithelial cells are polarised in both shape and function (Bredt, 1998; Devarajan and Morrow, 1996). A description of the features of a 'typical' epithelial cell is useful to understand the mechanisms by which restitution is modulated (figure 1.1).

The apical surface of gut epithelial cells faces the lumen and makes up the 'brush border', comprising microvilli (Heintzelman and Mooseker, 1992). Tight junctions form where apical surfaces of adjoining cells meet. Tight junctions separate the apical membrane from the baso-lateral membrane (Zahraoui et al, 2000). The basolateral membrane comprises the interface between adjoining cells (lateral region), and the interface between the cell and the underlying basement membrane (basal region). Intercellular junctions, called adherens junctions, enable communication between cells (Nagafuchi, 2001; Vasioukhin and Fuchs, 2001). Adherens junctions contain cadherins (transmembrane intercellular adhesion molecules), which connect intracellularly with catenins and thence to the actin cytoskeleton (Vasioukhin and Fuchs, 2001; Wheelock et al, 1996). The basal cell membrane connects to the extracellular matrix via transmembrane integrins, the cytoplasmic domains of which connect to intracellular signalling machinery via focal adhesions (Horwitz and Parsons, 1999). Thus, the epithelial cell is subject to regulation via three routes – the luminal surface, intercellular connections and interactions with the basement membrane. Stimulation of intracellular signalling machinery via these routes results in changes to the cytoskeleton, gene expression and proliferation (Anastasiadis and Reynolds, 2001; Basson et al, 1996; Cooper and Schafer, 2000; Downward, 2001).

1.3.3 Mechanisms of Restitution. Luminal exposure to a variety of molecules stimulates restitution both *in vitro* and *in vivo*. Conventional *in vitro* assays measure the effect of an exogenous compound at the 'apical' surface of a monolayer of confluent cells grown on a cell culture surface. Compounds known to stimulate restitution under these conditions include growth factors (EGF, TGFα, TGFβ, PDGF, FGF, IGF), cytokines, trefoil peptides,

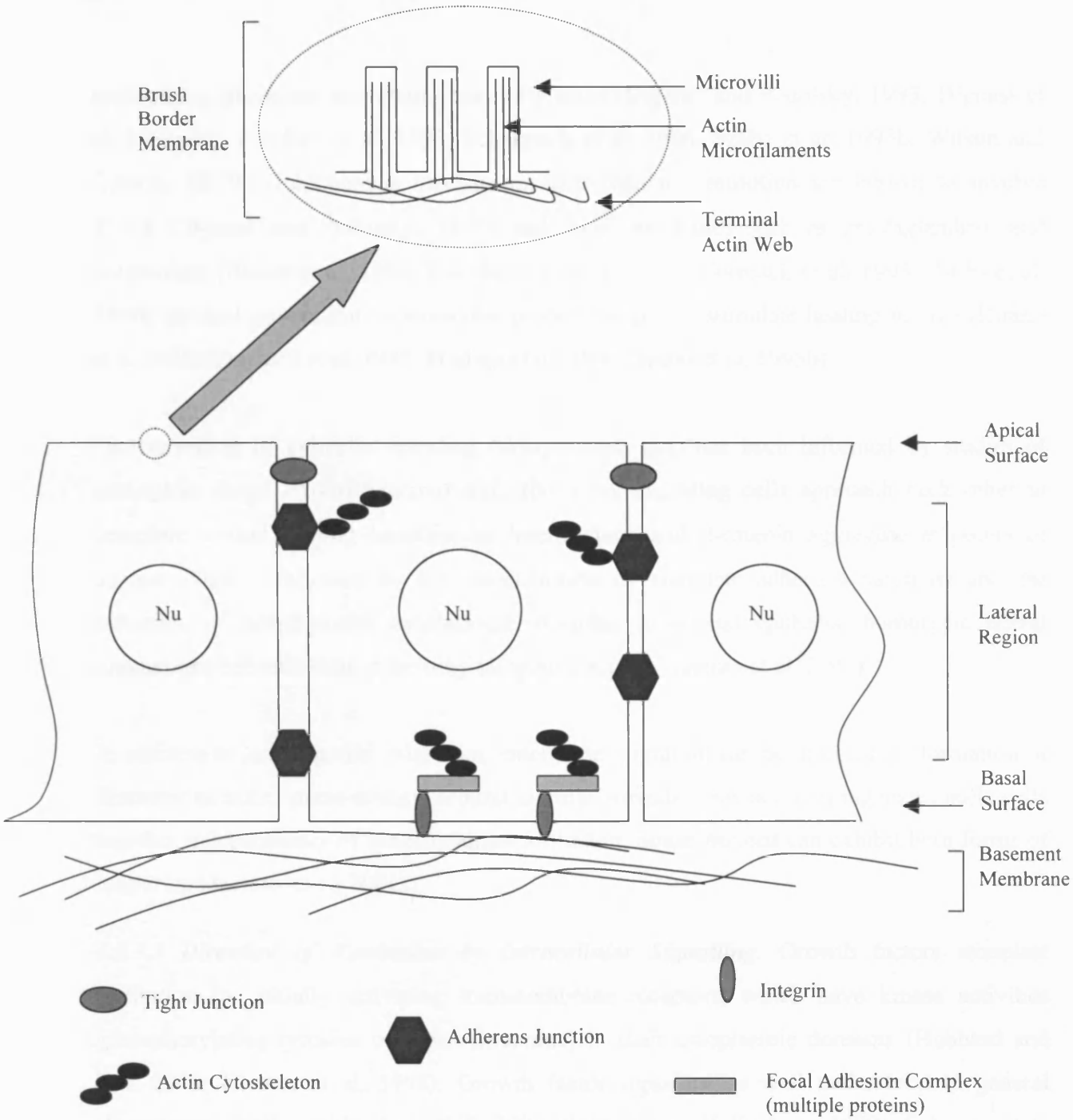


Figure 1.1. A Typical Epithelial Cell and its Environs.

The epithelial brush border membrane is shown in the expanded view. Actin microfilaments support the microvilli. These microfilaments connect with with cellular cytoskeleton via the actin terminal web. The cell is shown connecting with its neighbours via adherens junctions and with the basement membrane via integrins and focal adhesions. Tight junctions between cells separate the apical and lateral regions. Connections to the actin cytoskeleton are shown. See accompanying text for a full explanation (section 1.3.2).

nucleotides, glutamine and short chain fatty acids (Dignass and Podolsky, 1993; Dignass et al, 1994a&b; Playford et al, 1995; Scheppach et al, 1996; Szabo et al, 1995b; Wilson and Gibson, 1997b). Endogenous mechanisms that regulate restitution are known to involve TGFβ (Dignass and Podolsky, 1993), and small molecules such as prostaglandins, and polyamines (Banan et al, 1996; Blikslager et al, 1997; McCormack et al, 1993; Zushi et al, 1996). Several pro-restitutive molecules protect the gut, or stimulate healing *in vivo* (Hirano et al, 1995; Playford et al, 1995; Poulsen et al, 1999; Szabo et al, 1995b).

Understanding of epithelia resealing (skin, cornea, gut) has been informed by studies of drosophila dorsal closure (Jacinto etal, 2001). As migrating cells approach each other to complete wound healing lamellipodia interdigitate, and β-catenin aggregate at points of contact. This is followed by the establishment of complete adherens junctions and the retraction of interdigitated lamellipodia, resulting in normal epithelial homotypic lateral interactions between cells at the original wound margin (Jacinto et al, 2001).

In addition to lamellipodial migration, enterocyte migration can be directed by formation of filamentous actin 'purse-strings' around circular wounds. This actin arrangement pulls cells together independently of lamellipodium formation. Some wounds can exhibit both forms of migration (Jacinto et al, 2001).

1.3.3.1 Direction of Restitution by Intracellular Signalling. Growth factors stimulate restitution by initially activating transmembrane receptors, which have kinase activities (phosphorylating tyrosine or serine/threonine) in their cytoplasmic domains (Hubbard and Till, 2000; Klemm et al, 1998). Growth factor signalling is well understood in general (Downward, 2001; Hubbard and Till, 2000; Robbins and Hollenberg, 1999). Subsequent to receptor activation (i.e. dimerisation and trans-phosphorylation; Hunter, 1995; Klemm et al, 1998) signal transducing molecules are stimulated resulting in changes to morphology, migration, proliferation and/or differentiation (Downward, 2001; Hooshman-Rad et al, 2000; Zebda et al, 2000). The precise mechanisms by which *epithelial restitution* is stimulated following growth factor receptor activation are incompletely understood, with many extrapolations being made from data obtained using *drosophila* and cultured fibroblasts and keratinocytes (Jacinto et al, 2001).

Epithelial cells activate a number of intracellular signalling molecules (MAPK, ERK proteins, JNK) in response to wounding (Göke et al, 1998; Pai et al, 1998). In addition, proteins that regulate cytoskeletal change and lamellipodium formation are stimulated by exposure to growth factors and other pro-migratory molecules (Kaibuchi et al, 1999; Zebda et al, 2000). Growth factor receptor activation results in stimulation of downstream molecules such as G-protein-coupled receptors (GPCRs), protein kinase C (PKC) and the Rho family of small GTPases (Chiu and Rozengurt, 2001; Kaibuchi et al, 1999; Pitcher et al, 1998; Sturm et al, 1999).

Homotypic interactions between adjacent cells remain intact during restitution, so cells behind the wound edge must also migrate to maintain epithelial integrity (Basson et al, 1996; Walsh et al, 2001). Signalling through epithelial cells several rows away from the wound edge has been observed (Jacinto et al, 2001).

1.3.3.2.The Extracellular Matrix and Focal Adhesions. The extracellular matrix (ECM) plays an important role in restitution. Isolated ECM components, such as laminin, fibronectin and collagen, stimulate restitution *in vitro* (Basson et al, 1992), as well as modulating the activity of growth factors; for example, $CaCO_2$ cells migrate following exposure to EGF or TGFβ in the presence of laminin, but not collagen I (Basson et al, 1992). Local variations of matrix components *in vivo* suggest that they might contribute to specific control of physiological migration (i.e. during normal proliferation and differentiation) and of restitution (Beaulieu, 1997). ECM components interact with cells via transmembrane integrins (Mercurio et al, 2001; Hynes, 1992), which are affected by pro-restitutive growth factors (Basson et al, 1992; Basson, 1998). Data from studies of a variety of cell types, including epithelial cells and fibroblasts, show that 'outside-in' signalling via integrins stimulates changes in cell shape and migration via specialised structures called focal adhesions (Horwitz and Parsons, 1999; Huttenlocher et al, 1995). Focal adhesions are large complexes of structural and signalling molecules that modulate cell shape and migration in response to the ECM (Burridge and Chrzanowsky-Wodnika, 1996; Ezzell et al, 1997).

1.3.3.3 Sub-Epithelial Myofibroblasts. The role of SEMs in modulating restitution has already been mentioned (section 1.2.3.3.2). Molecules secreted by SEMs *in vitro* (TGFβ, HGF, collagen, laminin and fibronectin) stimulate epithelial restitution (McKaig et al, 1999;

Powell et al, 1999). However, SEMs play a more complex role than simply secreting useful proteins. Detergent-induced injury to guinea pig ileum *ex vivo* results in rapid reduction in villus height (Moore et al, 1989). Villus shortening results in increased cell densities. Electron microscopical examination reveals the appearance of electron-dense processes from SEMs in the lamina propria of the villi, due to accumulation of actin in an energy-dependent manner. 'Functional denervation' by exposure to tetrodotoxin shows that villus contraction by SEMs is partly modulated by neurotransmission. Thus, SEMs facilitate restitution by causing contraction of the injured villus, decreasing the area in need of immediate re-epithelialisation (Moore et al, 1989). Since villus shortening occurs in response to NSAIDs and chemotherapy drugs such as methotrexate (Playford et al, 1996; Howarth et al, 1996), the role of SEM-induced villus contraction deserves further study.

Clearly, epithelial restitution is a complex phenomenon that is regulated at many levels. Circulating soluble factors and matrix components stimulate migration, in addition to activation of endogenous intracellular molecules, whilst the denuded area is reduced by SEM-mediated villus contraction in the small intestine, resulting in rapid re-establishment of epithelial integrity following epithelial injury. Now it is appropriate to describe those situations where restitution is necessary.

1.4 Epithelial Injury in the Gut.

1.4.1 Introduction. Damage to the gastrointestinal epithelium results in denudation, loss of mucosal protection, exposure to acidic pH and digestive enzymes (in the stomach and duodenum), and in more severe cases damage to the lamina propria, smooth muscle and microvasculature, leading to necrosis and inflammation (Tarnawski and Halter, 1995). These types of damage can arise from several sources: treatment for unrelated diseases (for example, non-steroidal anti-inflammatory drugs [NSAIDs] or chemotherapy drugs), stress and from ingested substances (e.g. alcohol) (Tarnawski and Halter, 1995; Wilson and Gibson, 1997a). Infectious agents such as *Helicobacter pylori* are associated with gut injury, in this case peptic ulceration (Graham, 2000; Kokoska and Kauffman, 2001). Also, disease states manifest themselves as epithelial injury and inflammation, for example Crohn's disease and ulcerative colitis, collectively known as inflammatory bowel diseases (IBDs) (Fiocchi, 1998). Other forms of gut 'injury' include altered epithelial permeability to macromolecules, and altered tissue morphology, for example shrinkage of intestinal villi, due to parenteral feeding

and by enteral feeding with elemental diets (chemically defined, nutritionally minimal diets) (Sigthorrson et al, 1998; Söderholm et al, 1999).

1.4.2. Non-Steroidal Anti-inflammatory Drugs (NSAIDs). NSAIDs, such as aspirin and indomethacin, are commonly used to treat both short- and long-term inflammatory conditions (Wallace, 1997). Long term use of NSAIDs is associated with upper gastrointestinal tract bleeding and ulceration, which is fatal in some cases (Allison et al, 1992; Wallace, 1997). Even short-term, and/or low dose NSAIDs cause gut injury (Cryer and Feldman, 1999; Playford et al, 2001). This has important implications, since low dose, long term aspirin has been proven to reduce manifestations of cardiovascular disease (myocardial infarction or stroke) and might prevent gastrointestinal cancers (Langman and Boyle, 1998; Vanio and Morgan, 1998). Thus, gastrointestinal complications due to NSAID consumption can be expected to become even more widespread (Cryer and Feldman, 1999). Between 42 and 67% of NSAID users have gastrointestinal injury, depending upon the method of measurement (Leite et al, 2001).

The body does mount some defence against NSAID-induced injury, which results in *adaptation* (Doljanin et al, 1996). This is the process whereby repeated administration of the same injurious agent causes progressively less damage. Adaptation is characterised by increased gastric mucosal TGFα (Doljanin et al, 1996), and by increased salivary secretion and increased salivary EGF (Lipscomb et al, 1998).

The biochemical mechanisms by which NSAIDs cause epithelial injury, and by which the effects are manifest, are complex. Available data suggest more than one molecular mechanism. NSAIDs exert their anti-inflammatory effect by inhibiting cyclo-oxygenases (COXs) (Hawkey, 1999). COXs are key enzymes in prostaglandin (PG) synthesis. PGs are small molecules with a wide range of biological activities, including gut mucosal homeostasis, vasodilation and modulation of inflammation (Donnelly and Hawkey, 1997; Vane et al, 1998). Indiscriminate COX inhibition, for example by indomethacin, is anti-inflammatory, but also affects systems such as the gut mucosa that rely on prostaglandin synthesis (Donnelly and Hawkey, 1997). There are two main COXs – COX-1 is constitutively expressed, whereas COX-2 is upregulated during inflammation by the action of pro-inflammatory cytokines (Vane et al, 1998). Thus, COX-2 contributes to high levels of

prostaglandins local to sites of inflammation; COX-2 inhibitors should inhibit the pro-inflammatory effects due to COX-2 without affecting 'normal' prostaglandin synthesis (Wallace et al, 1998). However, effective doses of *selective* COX-2 inhibitors were found to inhibit COX-1 also (Hawkey, 1999; Wallace et al, 1998). Specific COX-2 inhibitors offer more hope for treatment with fewer side effects (Hawkey, 1999).

The mechanisms of NSAID-induced mucosal injury differ according to the region of the gut. Also, there appear to be multiple mechanisms of action (reviewed by Wallace, 1997). Gastric ulceration is partly a direct consequence of cyclo-oxygenase inhibition, which is also the mechanism by which NSAIDs exert their anti-inflammatory action (see previous paragraph). Indomethacin causes reduced blood flow in the stomach, which mediates injury and also exacerbates existing injury (Wallace, 1997).

Yamada et al (1993) studied the mechanisms of acute and chronic intestinal damage caused by indomethacin. Acute intestinal injury in rats was caused by a single injection of indomethacin (7.5 mg/kg). This damage was repaired endogenously within a week. Chronic damage, caused by 2 injections of indomethacin, persisted for two weeks. Epithelial injury was dependent upon enterohepatic circulation of indomethacin, since bile duct ligation abolished epithelial injury. Indomethacin and bile acid together resulted in cellular toxicity *in vitro* (Yamada et al, 1993). Mitochondrial damage and subsequent uncoupling of oxidative phosphorylation by indomethacin occurs in the small intestine. Decreased cellular ATP causes altered intercellular junctions i.e. increased permeability to macromolecules and bacteria; subsequent bacterial overgrowth exacerbates and maintains intestinal inflammation (Leite et al, 2001). The antibiotic metronidazole protects against the effects of indomethacin by killing bacteria and also by preventing uncoupling of oxidative phosphorylation (Leite et al, 2001).

NSAIDs are thought not to be injurious to the colon *per se*, but can aggravate existing inflammation due to imbalance between prostaglandins (inhibited by NSAIDs), and leukotrienes, which maintain inflammation and prevent healing (Wallace, 1997).

Options for NSAID users to reduce the possibility gut injury include cessation of NSAID therapy and prophylaxis. The former is not always practical, and predictions are that the latter will be expensive (Wyeth and Pounder, 1994; Wallace, 1997).

1.4.3 Peptic ulceration. Until the early 1980s, peptic ulceration (both gastric and duodenal ulcers) was treated as a disorder of acid secretion, and treated with proton pump inhibitors (PPIs), which inhibit acid secretion, and thus reduce chemical trauma to the denuded epithelium (Kokoska and Kauffman, 2001; Wyeth and Pounder, 1994). However, medical treatment of gastric ulceration has been revolutionised by the discovery of the aetiological role played by *Helicobacter pylori* (Marshall and Warren, 1984).

Infection with *H. pylori* is believed to occur in childhood, and infection is asymptomatic in up to 70% of infected individuals (Graham, 2000; Kokoska and Kauffman, 2001). *H. pylori* is a bacterium able to survive in the harsh environment of the stomach by burrowing into the epithelium and harnessing its host's anti-complement system (Kokoska and Kauffman, 2001; Rautemma et al 2001). *H. Pylori* causes ulceration directly, by damaging epithelial cells and degrading protective mucus, by stimulating release of cytokines that cause inflammation, and by stimulating acid secretion (Kokoska and Kauffman, 2001). Currently, up to eighty-five percent of peptic ulcer patients are treated successfully with combinations of antibiotics and PPIs (Graham, 2000; Kokoska and Kauffman, 2001) and recurrence rates are less than ten percent following eradication of H. pylori (Kokoska and Kauffman, 2001; Wolfe and Sachs, 2000).

Peptic ulceration not associated simply with *H. pylori* [for example due to NSAIDs (section 1.4.2) or stress (Mayer, 2000; Wyeth and Pounder, 1994; Wolfe and Sachs, 2000)] can require long-term maintenance therapy (Wolfe and Sachs, 2000). Experimental treatments for peptic ulcers which are not associated with *H. Pylori* include growth factors, such as FGF, PDGF, TGFβ and EGF, which modulate acid or bicarbonate secretion, angiogenesis and epithelial proliferation in addition to stimulating restitution (Alison et al, 1995; Szabo and Sandor, 1996; Szabo et al, 1995a&b; Tarnawski and Halter, 1995; Uribe and Barrett, 1997).

1.4.4.Inflammatory bowel diseases. Ulcerative colitis (UC) and Crohn's disease (CD) are complex, multifactorial inflammatory bowel diseases (IBDs), resulting in inflammation and ulceration of the colon (Fiocchi, 1998; Sands, 2000). Inflammation follows the expected pattern of ingress of antigens into the colonic mucosa, followed by an immunological response, including T-cell proliferation and differentiation, cytokine production and the

recruitment of circulating cytotoxic blood cells (Sands, 2000). However, there are marked differences between UC and CD, including:

(1) **morphology:** UC lesions are diffuse and shallow with clear delineation between normal and diseased tissue; CD lesions are patchy, and both shallow and deep ulcers are observed. A similar contrast of inflammation is observed.
(2) **submucosal fibrosis** and bowel wall thickening is associated with CD.
(3) **muscular and neuronal hypertrophy** is more common in CD than in UC.
(4) **immune cell functions;** CD is associated with cell mediated immunity (Th1 response – local production of IL-12 and interferon-γ stimulating mucosal macrophages to produce pro-inflammatory tumour necrosis factor-α), whereas UC has enhanced humoral immunity (Th2 response –peripheral white blood cells cytotoxic towards colonic cells).
(5) **ulcer-associated cell lineage** (UACL) is observed more often in CD than in UC.
(6) **autoantigens** against tropomyosin and against neutrophils are a feature of UC. (reviewed by Fiocchi, 1998; Sanders, 1998; Sands, 2000).

Both genetics and environment play a predisposing role to IBD, but their precise roles are not fully understood (Fiocchi, 1998). Colonic bacteria and their secreted peptides have an important role in UC, possibly due to the loss of tolerance towards normal intestinal flora (Campieri and Gionchetti, 2001; Fiocchi, 1998; Leiper et al, 2001). Endogenous alterations to epithelial function (permeability for example), to interactions between endothelial cells of the microvasculature and circulating blood cells, and to the mucosal immune system appear to result in an improperly balanced response to ingress of antigens (Fiocchi, 1998; Gibson, 1997; Soderholm et al, 1999). Subsequent inflammation arises from, and is maintained by, a complex mix of bacterial translocation, local B- and T-cell proliferation, cytokine production and action, recruitment of circulating mononuclear cells and vascular damage (Fiocchi et al, 1998; Pallone and Monteleone, 1998; Sands, 2000). In particular, tumour necrosis factor-α (TNF-α) has a central role in IBDs, modulating the balance between apoptosis and inflammation (Baud and Karin, 2001; Sands, 2000). Whilst the local immune system has an undoubted role in Crohn's disease and ulcerative colitis, the data reviewed by Fiocchi (1998) and Hodgson (1998) reveal discrepancies and contradictions suggesting that the Th1/Th2 dichotomy mentioned above is not so clear cut (Sands 2000).

Treatments for IBDs include aminosalicylates (inhibitors of cyclo-oxygenases and 5-lipoxygenase), corticosteroids and immune modulators (Jarnerot et al, 1998; Richter and Scheppach, 1997; Sands, 2000). Therapy against TNFα is promising, including thalidomide and recombinant anti-TNFα antibodies (Sands, 2000). Infliximab, an anti- TNFα antibody, has high success rates in reducing disease activity in both UC and CD (up to 80%), as well as maintaining remission in CD patients (Baert et al, 1999; Sands, 2000). However, this form of treatment is too new to allow assessment of its long-term effects and side effects (Sands, 2000). Other novel therapies include short-chain fatty acids (SCFAs), such as butyrate. Butyrate is a primary cellular fuel in the colon (Salminen et al, 1998), and improper metabolism of butyrate has been observed in ulcerative colitis, whereas irrigation of the colitic colon with short-chain fatty acids led to improvement in disease severity (Fiocchi, 1998). Clinical trials of SFCA enemas are promising (Richter and Scheppach, 1997). Probiotic therapy is another area of increasing interest. Probiotics are non-pathogenic bacteria that have positive health benefits (Salminen et al, 1998) and early data suggest that ingestion of probiotics might be beneficial (Campieri and Gionchetti, 2001; Sands, 2000).

In addition to localised colonic inflammation, IBDs are associated with anorexia and weight loss, particularly in childhood (Murch and Walker-Smith, 1998). These effects are most associated with CD, which can affect the small intestine as well, resulting in deranged nutrient absorption as well as affecting appetite and metabolism (Murch and Walker-Smith, 1998). Cytokines (TNFα and IL-1) and hormones (leptin) are elevated in IBDs and contribute to appetite suppression and anorexia (Murch and Walker-Smith, 1998; Sands, 2000). Nutritional therapy (such as elemental diets) and growth factors restore immunological function and stimulate growth and body mass, as well as reducing inflammation (Ameho et al, 1997; Chen et al, 1997; Murch and Walker-Smith, 1998). Growth factors have also shown anti-colitic activity *in vivo* using animal models of colitis (Giladi et al, 1995; Luck and Bass, 1993; Procaccino et al, 1994; Strober, 1998).

Corticosteroids, aminosalicylates and immunosuppressive agents are associated with a variety of side effects (summarised in table 1.2). Such complications can necessitate change or cessation of therapy (Connell and Taylor, 1997). Novel therapies, by their very definition, are not yet understood fully. Thus, whilst current therapies for inflammatory bowel disease can be effective, the range of associated side effects make desirable equally, or more, effective treatments, with fewer complications.

Table 1.2. Medications Commonly Used to Treat IBD, and Some of Their Adverse Events (Modified from Sands, 2000)

Agent	Adverse effects
5-ASA	
- Sulfasalazine	Anorexia, dyspepsia, nausea/vomiting; haemolysis, neutropenia, agranulocytosis, folate malabsorption, reversible male infertility, neuropathy; see also sulfa-free 5ASA's.
- Sulfa-free (mesalasine, olsalasine, balsalaside)	Headache; drug fever, rash, paradoxical exacerbation of colitis, pancreatitis; hepatitis, pericarditis, pneumonutus; nephritis; secretory diarrhoea (osalasine)
Antibiotics	
- Metronidazole	Anorexia, nausea/vomiting, peripheral neuropathy
- Ciprofloxacin	Nausea/vomiting; headache, restlessness; rash, pseudomembranous colitis, spontaneous tendon rupture
Corticosteroids	
- Classic	Sleep disturbance, mood disturbance, acne, striae, hirsutism, adrenal supression, proximal myopathy, glucose intolerance, hypertension, narrow angle glaucoma, cataracts, infection, oedema, impaired wound healing, growth retardation, osteoporosis, aseptic necrosis
Immunomodulators	
- 6MP/AZA	Nausea; drug fever, rash, arthralgia; thrombocytopenia, pancreatis; hepatitis; infection
- Methotrexate	Anorexia, nausea/vomiting; bone marrow suppression; megablastic anaemia, alopecia, hepatic fibrosis; neuropathy
- Cyclosporine	Reversible or irreversible decrease in renal function; hypertension; tremor, headache, paraesthesia, seizure, hepatotoxicity; infection; lymphoma.

1.4.5. Chemotherapy/Radiotherapy-Induced Mucositis. Chemotherapeutic drugs and radiotherapy usually act by killing the most rapidly dividing cells; thus it is no surprise that intestinal toxicity arises from these treatments, since there is a high level of cell turnover in the gut (Howarth et al, 1996). Mucositis is inflammation of the small intestine caused by chemotherapeutic drugs or by radiotherapy (Hirano et al, 1995; Howarth et al, 1997). Clinically successful treatments for mucositis are lacking; however, there is some interest in the role of growth factors in repair of mucositic damage (Hirano et al, 1995; Howarth et al, 1996&1997; Tanaka et al, 1998).

1.4.6 Necrotising Enterocolitis. Necrotising enterocolitis (NEC) is a severe, life-threatening bowel disorder in the neonate (reviewed by Neu and Weiss, 1999). Risk factors for NEC include prematurity and bacterial infection. Current treatment involves intensive care and ultimately surgery (Neu and Weiss, 1999). More successful treatments for NEC are required, and current possibilities include human milk, platelet activating factor acetyl hydrolase and recombinant EGF (Neu and Weiss, 1999; Sullivan et al, 1991).

1.4.7 Summary. Many treatments for gastrointestinal injury and disease are of limited success. New therapies are required that are effective but with minimal side effects. Other treatments are associated with serious side effects that require prophylaxis. In recent years the central role of growth factors in gut protection, and in healing of injury, have been described in many systems *in vitro* and *in vivo*. However, recombinant or purified growth factors are costly. Alternatively, bovine colostrum is a ready, inexpensive source of the growth factors that are known to be beneficial to the gut.

1.5 Bovine Colostrum.

The cow is of the bovine ilk;
One end is moo, the other, milk
(Ogden Nash, 1931)

1.5.1 General Considerations.

1.5.1.1 Overview. Colostrum, produced maternally around the time of parturition, is a nutritionally rich, complex mixture of fats, bulk proteins (casein, lactalbumin), growth factors, cytokines, antibodies, antibiotics, regulatory peptides, protease inhibitors and small

molecules (amino acids, nucleotides, sugars) (Koldovsky et al, 1993; Srivastava et al, 1996; Wu and Knabe, 1994; Xu, 1998). Ingested colostrum stimulates growth and maturation of the neonatal gastrointestinal tract, confers passive immunity and stimulates body growth (Guilloteau et al, 1993; Kelly et al, 1993; Reinhart et al, 1992; Xu, 1996). Colostra and milk from different species have similar constituents, although there are some important differences (Shing and Klagsbrun, 1984); for example, human colostrum is rich in EGF, whereas bovine colostrum contains large amounts of IGF (Odle et al, 1996; Xu, 1996; Zumkeller, 1992).

Colostrum has qualitative and quantitative differences from milk, such as amounts of growth factors, growth factor-binding proteins and hormones (Koldovsky, 1989; Zumkeller 1992). TGFβ (Pentilla et al, 1998), IGF-I and II (Skaar et al, 1991), and EGF (Koldovsky, 1989) increase around the time of parturition, but decrease in the two or three weeks post-partum. High molecular weight binding proteins (42-46 kDa) for IGF-II predominate around parturition in bovine colostrum, whereas smaller 30 kDa species increase post-partum (Skaar et al, 1991). Prolactin in human milk is maximal at parturition, but falls to one-seventh of its peak value within two weeks (Koldovsky, 1989).

1.5.1.2 Colostrum and the Neonate. Much is known about the role of colostrum, and the fate of colostral components, in the neonatal gastrointestinal tract in humans and animals (Koldovsky et al, 1993; Koldovsky, 1995; Odle et al, 1996; Oguchi et al, 1997).

Gastric juice in the newborn is less acidic, and contains less proteolytic activity than adult gastric juice, thus, colostral growth factors are less prone to degradation in the neonatal gut (Koldovsky et al, 1993; Oguchi et al, 1997).

Colostral growth factors act directly on the neonatal gut, stimulating proliferation (cell mass) and maturation (enzymatic activity and impermeability to macromolecules) (Guilloteau et al, 1993; Kelly et al, 1993; Oguchi et al, 1997; Reinhart et al, 1992; Xu, 1996). The gut is affected further by regulatory peptides in colostrum (see section 1.2.2). These include vasoactive intestinal peptide (VIP), gastrin, peptide YY (PYY), cholecystokinin (CCK) (Koldovsky, 1989; Koldovsky et al, 1993; Xu, 1996).

The neonatal gut has elevated permeability to macromolecules, which allows colostral components to enter the circulation and affect growth and maturation of the immune system and organs (Xu, 1996); antibiotic peptides and proteins, such as lactoferrin, prevent infection (van Hooijdonk et al, 2000). Thus, colostrum has evolved to perform a plethora of functions in the neonate, from maintenance to maturation.

1.5.2 Major Colostral Growth Factors

1.5.2.1. Introduction. Growth factors have been shown both to protect the gut and to stimulate repair of the damaged gut (see sections 1.2 and 1.3). Colostrum is a rich source of such growth factors. Thus, bovine colostrum can be considered as a natural source of growth factors for the treatment of gastrointestinal disease (Xu, 1996). Bovine colostral growth factors include transforming growth factor beta (TGFβ), insulin-like growth factors I and II (IGF I and II), fibroblast growth factor (FGF), betacellulin and bovine colostral growth factor (BCGF) (Belford et al, 1997; Koldovsky, 1995;Koldovsky et al, 1995; Rogers et al, 1996 Shing and Klagsbrun, 1984).

1.5.2.2.Transforming growth factor beta. The family of TGFβ proteins affect proliferation, migration and differentiation of many cell types, including epithelial cells, fibroblasts and cells of the immune system (Dignass and Podolsky, 1993; Lyons and Moses, 1990; Massague, 1998). There are three main TGFβ isoforms (1-3) which share significant sequence homology (Lyons and Moses, 1990).

TGFβ is secreted as an inactive dimer non-covalently associated with LAP, the latency-associated protein (Lyons and Moses, 1990; Massague, 1998). LAP is the N-terminal domain of the pro-TGFβ molecule which remains associated with, and masks bioactivity of, the mature TGFβ. TGFβ activity is further regulated by latent TGFβ-binding protein (LTBP), which associates covalently with LAP. LTBP interacts with extracellular matrix to cause pools of inactive TGFβ (Massague, 1998). TGFβ can be activated by changes in pH, heat, and exposure to proteases or glycosidases (Massague, 1998).

TGFβ binds to, and activates trans-membrane receptors (type I or II), which contain serine/threonine kinase activity in their cytosolic domain (Masague, 1992). Downstream signalling proceeds via SMAD proteins (Itoh et al, 2000; Massague, 1998).

TGFβ stimulates proliferation of fibroblasts and some blood cells, but inhibits epithelial cell proliferation (Lyons and Moses, 1990), and also stimulates cell migration (Ciacci et al, 1993; Yanaka et al, 1996). TGFβ stimulates production of extracellular matrix proteins, such as collagen, which can result in scarring at the site of wound healing (Shah et al, 1995). TGFβ1 and 2 isoforms are mostly responsible for scarring, whereas the β3 isoform stimulates 'better quality' wound healing, i.e. with reduced scarring (Shah et al, 1995).

The role of TGFβ in the immune system is complicated by 'context specific' effects, i.e. the effect of TGFβ depends on local production of cytokines and on membrane receptor expression by target cells, including T- and B-lymphocytes, macrophages and neutrophils (Letterio and Roberts, 1998). TGFβ has a central role in developing and maintaining immunologic tolerance, i.e. the ability of the immune system to differentiate between self and non-self antigens, failure of which results in autoimmune diseases (Letterio and Roberts, 1998). TGFβ is involved in restoration of immunologic tolerance in animal models of colitis (Neurath et al, 1996).

Bovine colostral TGFβ is mostly of the β2 isoform (80% of total colostral TGFβ; Jin et al, 1991; Rogers et al, 1996; Tokuyama and Tokuyama, 1993). Both colostrum and milk contain TGFβ in high molecular weight complexes (80 and 600 kDa) (Rogers et al, 1996). Transient acidification to pH 2 liberates low molecular weight, active TGFβ, which stimulates proliferation of fibroblasts and inhibition of epithelial cell proliferation (Rogers et al, 1996). A variety of milk-borne low molecular weight TGFβ species (7-15 kDa) have been reported (Tokuyama and Tokuyama, 1989), but the origin and purpose of these particular peptides remains to be determined.

TGFβ has an essential role in maintaining gut homeostasis and in stimulating repair of the damaged gut. TGFβ stimulates restitution; also, several growth factors have been shown to stimulate restitution via TGFβ (Basson et al, 1992, Ciacci et al, 1993; Dignass and Podolsky, 1993; Yanaka et al, 1996). Both endogeous and exogenous TGFβ are involved in gastric protection and stimulation of healing (Yanaka et al, 1996). Ablation of TGFβ signalling in the intestine, by generation of a TGFβ receptor knockout, renders mice more susceptible than

normal to induction of chemical colitis (Hahm et al, 2001), and TGFβ gene therapy stimulates healing of chemically induced colitis (Giladi et al, 1995).

1.5.2.3 Insulin-Like Growth Factors. The two insulin-like growth factors (IGF-I and –II, molecular weights approximately 7.5 kDa) regulate gut growth and maturation and also development of body mass and linear growth (Zumkeller, 1992). There is also an N-terminally truncated form of IGF-I, Des(1-3)IGF-I, which is more potent than the full length IGF-I (Ballard et al, 1996).

IGF stimulates cell signalling via transmembrane receptors containing a cytoplasmic tyrosine kinase domain (Rubin et al, 1993). Binding to and stimulation of these receptors triggers intracellular signalling cascades resulting in proliferation, differentiation, morphogenesis and/or migration (Hubbard and Till, 2000).

IGF-I and II are major growth factors in bovine milk and colostrum (Campbell and Baumrucker, 1989; Zumkeller, 1992). Truncated des(1-3)IGF-I is also found in bovine colostrum (Zumkeller, 1992). Milk and colostrum also contain IGF binding proteins; the profiles of expression, and the amounts, vary considerably with time (Campbell and Baumrucker, 1989; Skaar et al, 1991; Zumkeller, 1992). For example, IGF2-binding protein activities in bovine colostrum falls dramatically at birth, but increases shortly thereafter (Skaar et al, 1991).

Exogenous IGF stimulates growth and maturation of the neonatal gut (Carver and Barness, 1996), and maintains intestinal integrity during sepsis (Chen, 1995). IGF-I stimulates enterocyte proliferation and migration *in vitro* (Andre et al, 1999; Simmons et al, 1995), as well as having supra-additive effects with EGF on proliferation of cultured epithelial cells (Simmons et al, 1996). IGF stimulates cell migration and proliferation, as well as aiding repair of the injured gut (Howarth et al, 1997; Lund and Zimmerman, 1996; Sagiura et al, 1997).

1.5.2.3 Fibroblast Growth Factors. The FGF family includes FGF-I (also called acidic (a)FGF), FGF-2 (also called basic (b)FGF) and keratinocyte growth factor (KGF) (Jaye et al, 1992). These peptides are of low molecular weight (aproximately 18 kDa); however, high

molecular weight species of FGF-2 (HMW FGF-2; 22-34 kDa) have been detected, which arise from use of alternative start codons in FGF-2 mRNA (Delrieu, 2000). HMW-FGF-2 species play an important role in cellular transformation, proliferation, growth in culture medium containing low amounts of serum, and trans-differentiation (Belford et al, 1997; Delrieu, 2000).

FGFs are angiogenic and neurotrophic, in addition to stimulating proliferation and migration of fibroblasts and epithelial cells (Jaye et al, 1992). Extracellular FGFs exert their cellular effects via stimulation of transmembrane FGF receptors, which have cytoplasmic tyrosine kinase domains. Receptor/FGF complexes are internalised following ligand binding and receptor activation, and FGF remains active for several hours following internalisation (Jaye et al, 1992). HMW FGF-2 species are transported to the nucleus where they appear to bind directly to chromatin and modulate gene transcription (Delrieu, 2000).

FGF activity and localisation is modulated by FGF binding proteins, in a similar manner to IGFs (see above). FGFs bind to heparin-like molecules on the cell surface and in the extracellular matrix (Jaye et al, 1992). Cell membrane-bound heparins might act to regulate FGF activity, whereas matrix heparins might enable the establishment of pools of growth factors, to be released following disruption to the matrix, for example in response to injury (Delrieu, 1992; McCawley and Matrisian, 2001). Internalised FGF remains associated with heparan sulphate, which might protect FGF from proteolytic degradation, thus contributing to the intracellular activity of internalised FGF (Tumova et al, 1999).

Exogenous FGF-2 stimulates healing of gastrointestinal damage *in vivo*, both in animal models and in patients taking non-steroidal anti-inflammatory drugs (NSAIDs) (Paimela et al, 1993; Szabo et al, 1995a&b). FGF-2 stimulates both restitution and epithelial proliferation *in vitro* (Belford et al, 1997; Dignass et al 1994b). Also, patients taking NSAIDs and suffering from gastric ulceration were found to have significantly less gastric FGF-2 than those patients without ulceration (Hull, et al, 1998). Synthesis of nuclear HMW FGF-2 is increased, and 18 kDa FGF-2 is depleted, in the rat duodenum following cysteamine-induced ulceration (Szabo et al, 1995a).

FGF is found in human and bovine milk and colostra, albeit in quantities lower than those of TGFβ, IGFs and EGF (Belford et al, 1997). The quantity of FGF-2 in a bovine milk

preparation known to stimulate fibroblast proliferation (approximately 1 ng/ml; Belford et al, 1997) is greater than the binding constants for FGF receptors (Jaye et al, 1992). FGF is not considered a major colostral growth factor (Belford et al, 1997), thus the role of FGF in colostral bioactivity has not been considered.

1.5.2.5 Betacellulin. Betacellulin was originally identified as a secreted product of insulinoma cells, and probably modulates cellular differentiation in the pancreas (Dunbar and Goddard, 2000). Betacellulin is a member of the EGF receptor-ligand family (Kumar and Vadlamudi, 2000), which contains the typical EGF-like motif of six cysteine residues forming three disulphide bridges (Uribe and Barrett, 1997). However, betacellulin (21-22 kDa; Dunbar et al, 1999) is much larger than EGF (6 kDa; Uribe and Barrett, 1997).

Endogenous EGF-receptor ligands are important in maintaining gut homeostasis (Barnard et al, 1995; Goodlad and Wright, 1995; Uribe and Barrett, 1997). The central role of EGF and TGFα in proliferation, maintenance of homeostasis and stimulation of repair in the gastrointestinal tract has been discussed throughout this introductory chapter. Betacellulin has also been found thoughout the gut (Uribe and Barrett, 1997). Betacellulin is expected to have similar activities to EGF and TGFα (mitogenicity, wound healing), due to action via the same receptor (Dunbar and Goddard, 2000; Howarth et al, 2000; Tada et al, 2000). The EGF receptor is probably located basolaterally in the intact gastrointestinal mucosa, only becoming available luminally following epithelial injury (Bishop and Wen, 1994; Goodlad and Wright, 1995; Uribe and Barrett, 1997).

Betacellulin has been detected in bovine milk and colostrum (Bastian et al, 2001). The role of betacellulin in bovine colostrum has yet to be elucidated.

1.5.2.6 Bovine colostral growth factor. Bovine colostrum contains a 30 kDa growth factor with mitogenic activity for fibroblasts (Shing and Klagsbrun, 1987). This 'bovine colostrum growth factor' (BCGF) has chemical similarities to platelet-derived growth factor (PDGF); for example it has an alkaline isoelectric point, is resistant to acidic pH (pH 1) and is inactivated by dithiothreitol. (Shing and Klagsbrun, 1987). Exogenous PDGF stimulates repair of the damaged gastrointestinal mucosa *in vivo* (Arceiz et al, 1997; Szabo et al, 1995a&b).

A similar growth factor has been found in goat colostrum, called colostric basic growth factor (CBGF; Brown and Blakely, 1984) and in bovine milk (Shing and Klagsbrun, 1984). However, there are no sequence data to confirm their similarity to each other, and to PDGF. Available data are conflicting.

Bovine colostrum growth factor appears to bind to the PDGF receptor, since it competes with PDGF in radioreceptor assays (Shing and Klagsbrun, 1987). Goat CBGF inhibits binding of EGF to its receptor, however, CBGF does not bind to the EGF receptor itself (Brown and Blakely, 1984). Belford et al (1997) demonstrated immunoreactivity in bovine milk to an anti-PDGF-AB antibody, and competition for binding to the PDGF alpha-receptor. However, a neutralising antibody against PDGF was found not to inhibit the proliferative effects of a bovine milk growth factor preparation, despite using cells that responded to recombinant PDGF (Belford et al, 1997). Goat CBGF, like PDGF, acts as a 'competency factor', increasing bioactivity of both depleted foetal calf serum and platelet-poor serum *in vitro*, whilst having little effect itself (Brown and Blakely, 1984). No recombinant protein is available for further study of this protein(s) at this time. It is noteworthy that two groups have recently discovered and characterised PDGF-DD, a protease-activated ligand that forms homodimers of 30-40 kDa *in vitro* and *in vivo* (Bergsten et al, 2001; LaRochelle et al, 2001). PDGF-DD stimulated phosphorylation of PDGFR-β, and stimulated fibroblast proliferation (Bergsten et al, 2001). Colostral PDGF-like molecules may be related to this novel PDGF isoform.

1.5.3 Colostrum and Milk in vitro.

Colostrum and milk from several species, including human, cow, sheep and goat have been shown to support growth of cultured cells *in vitro* (Belford et al, 1997; Brown and Blakely, 1984; Ichibada et al, 1992; Shing and Klagsbrun, 1984). Derivatives of milk or colostrum modulate proliferation of fibroblasts and epithelial cells derived from several tissue types, and from several species (Belford et al, 1997; Brown and Blakely, 1984; Ichibada et al, 1992; Shing and Klagsbrun, 1984; Steimer et al, 1981).

Bovine milk supports growth of transformed fibroblasts independent of cell adhesion (Steimer and Klagsbrun, 1981). However, non-transformed fibroblasts require fibronectin, to facilitate attachment, before the growth supporting effects of milk become apparent. Milk and

fibronectin together result in transformed fibroblasts growing as an adherent monolayer (Steimer and Klagsbrun, 1981). Similar observations have been made using goat colostrum (Brown and Blakely, 1984). Thus, whilst milk and colostrum affect cell survival and proliferation, additional 'attachment' factors are required, such as fibronectin or serum components, for cultured cells to adhere to their culture surface.

Growth supporting and stimulating activity in colostrum and milk have been attributed to its constituent growth factors, including EGF, TGFβ, IGF, FGF, PDGF-like growth factors and casein-derived peptide (Belford et al, 1997; Brown and Blakely, 1984; Liu et al, 1996; Shing and Klagsbrun, 1984). However, whilst purified, concentrated peptides and growth factors stimulate potently the proliferation of cultured cells, these growth factors mixed together in quantities relevant to those in milk do not reproduce the effects of bovine milk on cell proliferation (Belford et al, 1997). Such data show clearly that biological effects of milk and colostrum are known, but poorly understood.

1.5.4 Colostrum and Milk in Experimental Gastroenterology.

Whilst many of the molecules in colostrum have been investigated for a role in treatment of gut injury, these have been considered in isolation (see discussion of colostral growth factors above), and the use of colostrum *per se*, has attracted little attention. Dai et al (1985) showed that a fraction of human milk, designated human milk growth factor III, prevented cysteamine-induced duodenal ulcer formation in mice. Human milk growth factor III has been shown to be EGF (Shing and Klagsbrun, 1984) which has proven action in preventing gastrointestinal damage using several model systems (discussed throughout this chapter).

Porter et al (1998) used bovine whey extract from milk in a rodent model of chemical colitis. Rats were given a diet supplemented with dextran sodium sulfate (DSS) to induce colitis. Disease severity over 10 weeks was measured by detecting ethane in rat breath. Ethane production results from reactive oxygen metabolites in the colitic colon. Also, disease severity was assessed by stool consistency and rectal bleeding. Bovine whey extract significantly reduced ethane production during weeks 3 and 6, but not during week 9 of the study. Stool consistency and rectal bleeding were unaffected by treatment with whey extract. Histological evaluation at the end of the study showed no significant effect of the whey extract; however, several criteria revealed little or no difference between normal and

untreated colitic animals, suggestive of almost complete endogenous healing. The protocol used by Porter et al did not allow direct evaluation of the effect of whey extract on the kinetics of healing. Porter et al also showed that body weight loss associated with colitis was less in animals treated with whey extract. Weight loss was unaffected by conventional treatments for colitis, such as sulfasalazine or prednisolone (Porter et al, 1998). Thus, whey might have a nutritional role in the treatment of IBD, in addition to effects due to the growth factor content.

Whey extract also had beneficial effects against mucositis caused by the anti-cancer drug methotrexate (Howarth et al, 1996). Methotrexate, administered sub-cutaneously to rats, reduced intestinal villus height, an effect which was prevented in the jejunum by oral administration of whey extract. Bacterial translocation was also reduced by whey extract.

Grazioso et al (1997) studied the effect of human milk on colitis induced in rats by adminstration of an acetic acid enema. This study used both aqueous and cream fractions of milk, and equated the mild anti-inflammatory effects of human milk with those of interleukin-1-receptor antagonist (IL-1-RA).

These *in vitro* and *in vivo* data indicate that derivatives from bovine colostrum or milk might be a source of treatment for the damaged gastrointestinal tract and/or provide prophylaxis against damage due to NSAIDs, for example. However, more complete and detailed study is required to evaluate the extent to which such preparations might be effective, and to elucidate the mechanisms by which their effects are made manifest.

1.6 An Outline of the Studies Presented in this Thesis.

The data presented in chapters 3 to 6 of this thesis are from studies designed to test the bioactivity of a commercial preparation from bovine colostrum (Bioenervi, Turku, Finland) in models of gastrointestinal injury *in vitro* and *in vivo*. The effect of Bioenervi on proliferation and restitution of cultured cells is tested, and the molecular weights of active components estimated (chapter 3). TGFβ is a major colostral growth factor and plays an important role in protecting the gut and repairing damage (section 1.5.2.2). Studies are presented analysing the role and stability of colostral TGFβ in Bioenervi bioactivity (chapter

4). The effects of Bioenervi on protection from, and healing of, chemically-induced colitis is tested (chapter 5). The use of chemical inhibitors of intracellular signalling is a novel approach to understanding the mechanisms by which bovine colostral preparation affects cells. Data from such studies are presented in chapter 6. The implications of these data for the clinical use of bovine colostral preparations, together with recommendations for further studies, are presented in chapter 7, the conclusion.

Chapter 2.

Materials and Methods

2.1 Materials and Suppliers.

All chemicals were purchased from Sigma-Aldrich, Poole, England, except where stated.

All cell culture media, animal sera and antibiotics were purchased from Life Technologies Inc., Paisley, Scotland. Falcon™ cell culture plastics were also purchased from Life Technologies Inc.

Defatted bovine colostrum (Bioenervi™, from Turku, Finland) was supplied by SHS International, Liverpool, England. The preparation contained IGF-I and –II (approximately 2 μg/ml) and TGFβ (25 ng/ml), with total protein of 4.3 mg/ml (data supplied by W Johnson, SHS International).

Recombinant epidermal growth factor was a gift from Dr J Berlanga, CIGB, Havana, Cuba.

Anti-TGFβ antibody, and recombinant TGFβ and PDGF-BB proteins were purchased from R&D Systems, Abingdon, England.

Trinitrobenzene sulfonic acid (TNBS) was purchased from Fluka, Poole, England.

Radiolabelled thymidine and mixed amino acids were purchased as aqeous solutions from Amersham Pharmacia Biotech UK Ltd., Little Chalfont, England.

2.2 Methods

2.2.1 Cell Culture

HT-29, RIE-1 and NIH-3T3 cells were a gift of Dr T Marchbank, Imperial Cancer Research Fund, London. HT-29 cells originated from a human colon adenocarcinoma (Fogh, 1975), and were used at passages (x + 5 to 84), where x denotes the passage at which they were obtained from Dr Marchbank. Their actual passage number at this stage could not be determined. RIE-1 is a non-transfomed epithelial cell line derived from the rat small intestine (Blay and Brown, 1984). RIE-1 cells were used between passages 38 and 120. NIH-3T3 fibroblastic cell line was derived from the NIH Swiss mouse embryo (Jainchill et al, 1969) and were used between passages (x + 5 to 112) (see above).

Complete medium was made up by supplementing DME medium with FCS (10% v/v), glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and fungizone (0.25 μg/ml). Serum free medium was made up similarly, but without the addition of FCS.

The cell lines were propagated by culture on plastic (25 or 80 cm^2 flasks) in complete medium. Flasks were housed in a humidified incubator supplied with 10% CO_2 at 37°C.

Cell lines were passaged at approximately 80% confluence. Cells were washed with HBSS (without Ca^{2+} and Mg^{2+}), and exposed to trypsin/EDTA (0.5 g/L and 0.2 g/L respectively) at 37°C for three to five minutes. Trypsin/EDTA was inactivated by 3 volumes complete medium, following disruption of the cell monolayer. After centrifugation (200*g*, 5 minutes) cells were resuspended in complete medium and diluted ten-fold for stock cultures. When cells were required for experiments, cell number and viability was determined using a Neubauer counting chamber. The number and percentage of cells impermeable to trypan blue was determined and the cell density adjusted accordingly by dilution in complete medium.

Stocks of frozen cells were made by mixing sub-confleunt cells (10^6 per ml) with FCS containing 10% v/v DMSO and transferring to Nunc cryotubes (Life Technologies Inc., Paisley, Scotland). After 16-24 hours at minus 80°C in a polystyrene container, vials were transferred to liquid nitrogen for long term storage.

2.2.2 DNA and Protein Synthesis Assays.

DNA synthesis was measured as described by Freshney (1987). Cell suspensions were obtained as described above (section 2.2.1) and seeded in 96-well tissue culture plates (10^4 cells per well) using a multi-channel pipette. Cells were cultured for 16-24 hours, to allow cells to adhere and spread out, then washed with HBSS and cultured for 24 hours in serum-free medium to halt proliferation. Cells were washed with HBSS then cultured for 24 hours in medium with serum and/or test substances are described for each experiment. During the last 6-8 hours of culture tritiated (^{3}H-methyl) thymidine (1 mCi/ml) was added (3 μl, 1μCi per well, diluted in HBSS). At termination of the experiment medium was removed and cells were washed four times with HBSS (200 μl per well). Plates were blotted dry and macromolecules were precipitated by exposure to ice-cold 30% w/v tri-chloroacetic acid

(TCA; 100 µl per well, one 10 minute exposure followed by two 5 minute exposures on ice). TCA was removed and wells washed briefly with 100 µl iso-propanol. Plates were allowed to dry at room temperature before insoluble material was dissolved in 0.3M NaOH containing 1% SDS (50 µl per well).

Protein synthesis assays were performed identically, except that ^{14}C-labelled mixed amino acids (50 µCi/ml) were added (3 µl, 100 nCi per well, diluted in HBSS) instead of tritiated thymidine.

Incorporation of radiolabel was quantified using liquid scintillation counting. Aliquots of the dissolved material (30 µl per sample) were tranferred to flexible 96-well plates (Perkin Elmer, England) containing 50 µl scintillation fluid (Optiphase Supermix™, Perkin Elmer, England). Plates were inserted into a Wallac MicroBeta Trilux computer controlled scintillation counter (Perkin Elmer, England) to perform scintillation counting.

2.2.3 Determination of Cell Number.

Cell suspensions were obtained as described in section 2.2.1 and seeded into 24-well plates at $2x10^5$ cells per well in 250 µl complete medium. After 16 to 24 hours in culture cells were washed in HBSS and medium serum-free medium containing test substances (500 µl per well) added as described in the text. Cells were cultured at 37°C in 5% CO_2 in an humidified incubator.

At the end of the experiments, as described in the relevant text, cells were washed in HBSS w/o Ca^{2+} and Mg^{2+} (1.5 ml per well), and trypsin/EDTA added (250 µl per well). Cells were incubated at 37°C until cells were dissociated, as judged by microscopical analysis. Vigorous pipetting was found to aid dissociation of cells.

Cells liberated into the trypsin/EDTA solution were transferred to microfuge tubes containing 1 ml complete medium. Cells were washed by centrifugation (200g, 5 mins) and resuspension in 100 µl HBSS containing trypan blue. The total number of cells, and the number of cells excluding trypan blue were counted using a Neubauer counting chamber, and visualised using a microscope fitted with a x10 objective lens. Three counts per sample were made, and a mean calculated for subsequent statistical analysis (see section 2.2.7).

2.2.4 Cell Migration Assays

HT-29 and RIE-1 cells were seeded into 6-well plates at $1x10^6$ cells per well, in 2 mls complete medium per well. Cells were grown to confluence, usually 7 to 10 days, with medium changes every 2 or 3 days. Cells were maintained at confluence for 2 or 3 days before performing migration assays.

Confluent cells were washed 3 times with HBSS (5 mls per well). HBSS (2 mls per well) was added and 'wounding' performed by scraping the cell monolayer with the end of a pipette tip (volume up to 200 µl). Cells were washed with HBSS 3 times to remove dead cells and pro-migratory factors released by wounding. Medium containing test factors and/or FCS was added (2 mls per well) as indicated for each experiment. Each experimental condition was performed in quadruplicate.

Before returning cultures to the incubator a mark was made using an indelible, fine-tipped pen on the external surface of the bottom of the plate, that was perpendicular to the wound. This mark was used as a reference for measuring wound widths. All photomicrographs of wounds were taken with the mark to the left of the field of view, using the ten times objective lens. Serial photomicrographs were taken of the wounds immediately after wounding, and subsequently after 3, 6, 9 and 12 hours (RIE-1 cells) or 12, 24, 36 and 48 hours (HT-29 cells).

Photomicrographs were processed and printed as 5"x7" pictures. They were calibrated using a photomicrograph of a section of a cell counting chamber (1 mm on the photograph corresponded to 4.45 µm *in vivo*). Wound widths were measured by placing a transparent grid over the picture, aligned to the mark, and wound widths measured in 5 places, each 5 cm apart. All data from replicates at each time point were pooled and the mean and SEM calculated.

2.2.5 Murine Small Intestinal Damage

The model used was that of Playford et al (1996). Small intestinal damage was induced in mice by a single sub-cutaneous injection of indomethacin in 5% bicarbonate (85 mg/kg). Twenty-four hours later, at autopsy, intestines were shorter and contained bloody chyme,

indicating intestinal injury and bleeding. Small intestinal tissue was subsequently processed and dissected in order to measure villus height. Reduced villus height is a marker of small intestinal damage in this model (Playford et al, 1996).

C57Bl mice were housed as groups of 10 animals. They were allowed free access to food and water during the experiment.

2.2.5.1 Protocol. Recombinant TGFβ2 was made up daily at 12.5 ng/ml in water containing 0.2% w/v BSA. All components were sterilised by passage though a 0.2 μm filter. This formed the test drinking water. In separate groups TGFβ2 (12.5 ng/animal/day) was administered via daily subcutaneous injection in a volume of approximately 100 μl. BSA (0.2% w/v) was included in the injection fluid. Control groups of mice received iso-proteinaceous, sterile water via the same two routes. Treatment with TGFβ/BSA continued daily for 6 days. On the sixth day half of the animals in each group received a single injection s.c. of indomethacin (85 mg/kg) in 5% bicarbonate solution. Control animals received just 5% bicarbonate solution. Two hours before sacrifice on the seventh day all animals received vincristine (1 mg/kg of 0.1 mg/ml solution in sterile water) via i.p. injection. At sacrifice, the gut of each animal was dissected, washed, blotted dry, weighed and measured. The gut was divided into the stomach, small intestine (5%, 30% and 75% of small intestinal length, 30 and 75% of colon length), and each sample fixed as described below. Sample containers were labelled according to the blinded code provided by the Biomedical technicians.

2.2.5.2 Tissue processing. Tissue fragments from the dissected guts were fixed in Carnoy's fluid (6:1:3 v/v ethanol:acetic acid:chloroform) in 2 ml cryotubes. After 3 hours the Carnoy's fluid was removed and 70% ethanol added for storage at room temperature.

2.2.5.3 Tissue Microdissection. Microdissection was performed as described by Goodlad (1994). Briefly, pieces of tissue fixed in Carnoy's fluid and stored in 70% v/v ethanol were transferred to 50% ethanol for 15 minutes, then to 25% ethanol for a further 15 minutes. Rehydrated tissue was hydrolysed at 60°C in 1M hydrochloric acid for 8 minutes before staining with Schiff's reagent for 45 minutes at room temperature, resulting in a deep magenta colour. A fragment of the stained tissue was cut off using a small scalpel blade, placed on a clean microscope slide and covered with a drop of 45% acetic acid. Tissue was

illuminated using a fibre-optic cold light source and viewed using a dissection microscope (Nikon, Japan). Groups of up to five villi were teased away from the tissue using a pair of dental probes and moved to a separate area of the slide. Enough tissue was microdissected to allow measurement of twenty villi and crypts per tissue sample.

The dissected tissue was wetted with 45% acetic acid, covered with a coverslip and transferred to the viewing stage of a microscope fitted with objective lenses (x4, x10, x25 and x40 magnification) and a drawing tube (Nikon, Japan). Villi were viewed using the x10 objective lens and crypts using the x25 objective lens. The images were projected via the drawing tube onto paper and the outline of 20 villi and of 20 crypts per tissue sample were traced. Gentle pressure to the coverslip was used to separate closely spaced crypts. Villus heights and crypt depths were measured from the traces on paper. Microdissection and drawing duties were shared between the author and Dr Khatri, Leicester General Hospital. Preliminary analysis of randomly selected tissue samples confirmed that there was no significant difference in measurements made by either observer.

Crypt metaphases were counted using the criteria of Goodlad (1994). Crypts were observed though the x40 objective, and the number of intensely stained metaphases, identified by loss of nuclear envelope and by chromatin condensation, were counted using a hand-held tally counter.

2.2.6 Rat Model of Colitis.

2.2.6.1 Animal Handling and Induction of Colitis. Severe, persistent colitis was induced in rats by the rectal administration of trinitrobenzene sulfonic acid (TNBS) in alcoholic solution. Alcohol disrupts the colonic mucosa allowing ingress of TNBS. This causes chemical modifications (haptenisation) to colonic proteins, resulting in an immunological response (inflammation). TNBS-induced colitis is characterised by severe ulceration and inflammation to the distal colon lasting for several weeks. Weight loss is common during the first few days after induction of colitis.

Male Sprague-Dawley rats (0.25-0.30 kg) were used for these studies. They were housed in groups of two or three. They were allowed free access to food and water except for the 24 hours preceding administration of TNBS. During this time they had free access to water supplemented with 20% glucose. Daily records were kept of food and water consumed per cage, and of each animal's body weight.

Rats were lightly anaesthetised with halothane before TNBS (0.25g per animal) was administered in a solution of 50% ethanol, via a lubricated paediatric feeding tube inserted 5 cm into the colon. Each enema was in a volume of 250 µl. The tube remained in place for 30 seconds after the enema was delivered. After the tube was removed animals were placed in fresh, wire-bottomed cages and allowed free access to food and water. Therapeutic interventions were performed as described in chapter 5.

Rats were killed by stunning and cervical dislocation. The distal 10 cm of the colon was removed and opened longitudinally using a scalpel blade. The tissue was pinned to a cork tile, with the luminal face uppermost, washed in sterile PBS and blotted dry. Macroscopic tissue damage was evaluated using the system given in tables 5.1 or 5.3. The weight of the tissue was recorded before being frozen in liquid nitrogen and subsequently stored at minus 70°C.

2.2.6.2 Tissue Analysis of Myeloperoxidase Activity. Colonic tissue was thawed at room temperature and stored on ice. Tissue was minced with scalpel blades and transferred to the barrel of a glass, hand-held homogeniser. Hexadecyltrimethylammonium-bromide (0.5% w/v) in phosphate buffer (2 mls) was added and homogenisation performed. The homogenate was transferred to plastic 7 ml 'Bijoux' containers and freeze-thawed three times. The resulting suspension was transferred into a microfuge tube (1.5 mls per sample) and centrifuged in a refrigerated microfuge for 15 minutes at full speed at 4°C. The supernatant was transferred to a fresh microfuge tube and stored at minus 70°C.

MPO activity was determined colorimetrically using tetramethylbenzidine (TMB) as a substrate for MPO (Suzuki et al, 1983). One TMB tablet was dissolved in 1 ml DMSO, then added to 9 ml phosphate buffer (50 mM, pH6). Hydrogen peroxide (30% v/v) was added to 0.0005% v/v. The resulting reaction buffer was added to the wells of a 96-well plate (100

µl/well). Thawed colonic tissue homogenate supernatant (7 µl) was added and the plate swirled to initiate the reaction. The reaction was incubated at room temperature for 120 seconds, then terminated by addition of 50 µl 2M H_2SO_4. Each sample was analysed in triplicate. MPO activity was determined by measuring the optical density at 492 nm using a Dynex plate reader (Prior Laboratory Supplies, England) controlled by Dynex Revelation 3.2 software. One unit was defined as a change of 1 OD unit in one minute (Sekizuka et al, 1988).

2.2.6.3 Tissue Extract Protein Content. Total protein content of separate aliquots of these samples was determined using a BioRad protein determination kit (Bio-Rad Laboratories, England), as per the manufacturer's instructions for the microassay. Samples were measured in triplicate using BSA as a standard.

2.2.7 Data analysis and statistics.

All statistical analyses and calculations were performed using SPSS software.

2.2.7.1 Data from cell culture assays. The following analysis of data was performed for each experiment: the mean and standard error of the mean (SEM) was calculated from the replicate raw data (n= 4 to 8) for each treatment. These data were used for graphical representations. All raw data from each experiment were used to perform an analysis of variance (ANOVA). Where statistical significance was detected ($F<0.05$; i.e. differences between mean values of groups of data are greater than differences between raw data within groups), a post-hoc modified t-test, based on the residual error, was performed on pairs of means. Graphs throughout the thesis represent this method of analysis of a single, typical experiment; they do not necessarily represent the best data. The author acknowledges that this method of analysis and presentation does not include the effects of inter-experimental variation. Thus, it is noted that each statistically significant effect was demonstrated in at least two out of three, or four out of five, separate experiments.

2.2.7.2 Data from small intestinal damage model. Mean values of villus height, crypt depth and mitoses per crypt were calculated for each tissue sample (i.e animal). These values were used to calculate mean +/- SEM values for each treatment group. Mean values per tissue

sample were used in ANOVAs to determine significance of treatments before post-hoc analysis, as described above.

2.2.7.3 Data from TNBS-induced colitis model. Means and standard errors of the means were calculated for graphical presentation. All raw data were analysed by ANOVA and post-hoc comparisons made where appropriate, except for macroscopic damage data. These data were not continuous, and were analysed non-parametrically using the Mann-Whitney U-test.

Chapter 3.

The Effects of Bovine Colostral Preparation on Wound Healing *In Vitro*.

3.1 Introduction.

Superficial damage to the gastrointestinal mucosa is repaired by *restitution*. Restitution is completed with just a few hours *in vivo* and followed by a period of increased epithelial cell proliferation to re-establish the original cell density (Wilson and Gibson, 1997a). Both phases of wound healing are modulated by many factors, including growth factors (Basson et al, 1992; Takeuchi et al, 1996), extra-cellular matrix (Basson et al, 1992) and tension of the underlying tissue (Moore et al, 1989; Osada et al, 1999). The mechanisms involved in restitution were discussed in chapter 1 (section 1.3). This plethora of wound healing stimuli operates in concert. Furthermore, the potency of any one component may be modulated by another. For example, the stimulatory effect of TGFβ on Caco-2 restitution requires laminin, but is inhibited by collagen (Basson et al, 1992). Such inter-dependence makes difficult the study of isolated components during wound healing *in vivo*.

The direct effects on restitution of a particular preparation can be studied using assays of proliferation and of migration by intestinal epithelial cells *in vitro*. These assays have been used widely (see references in preceding paragraph). There is a good correlation between activity in these assays and wound healing activity *in vivo* (Marchbank et al, 1998; McKenzie et al, 1997; Sturm et al, 1999).

3.2 Does Bovine Colostral Preparation Stimulate Intestinal Epithelial Cell Proliferation?

Studies were designed to observe the effect of bovine colostral preparation on the proliferation of gastrointestinal cell lines. Two cell lines were chosen - HT-29 cells, derived from human colonic carcinoma, and RIE-1, derived from rat small intestine. The first assays were designed to assess proliferation by measuring the incorporation of tritiated thymidine into DNA. This is commonly used to test the effects of compounds on cell proliferation, but measures DNA synthesis, not actual proliferation (Boulton and Hodgson, 1995). Subsequent studies were designed to measure directly the number of cells present in cultures exposed to colostral preparation. This latter method is more labour intensive but provides unequivocal information concerning the proliferation of cells.

The methodological details were presented in chapter 2 (sections 2.2.2 and 2.2.3). Briefly, sub-confluent cells were cultured in 96-well plates and serum starved before culture in serum-free medium supplemented with increasing amounts of colostral preparation for 24 hours. ^{3}H-methyl thymidine and ^{14}C-labelled amino acids were added during the last 6 to 8 hours. At termination of the experiment, acid-precipitable material was dissolved and the incorporated radiolabel measured by liquid scintillation counting. In separate experiments cells were seeded into 24-well plates and cultured in the presence of colostral preparation subsequent to adherence to the culture well. Cells were washed with HBSS (w/o Ca^{2+} and Mg^{2+}) and harvested using trypsin/EDTA. Cell number was determined using a counting chamber.

The data obtained from radioisotope incorporation assays for RIE-1 and HT-29 cells are shown in figures 3.1 and 3.2 respectively. DNA synthesis by RIE-1 cells was stimulated by colostral preparation (figure 3.1a). The effect of the bovine colostral preparation was dose dependent (maximum effect at 30% v/v, $p<0.001$). The stimulatory effect of bovine colostral preparation was less at the higher amounts tested, up to 60% v/v. However, none of the doses tested in these experiments caused DNA synthesis by RIE-1 cells to fall below the levels measured in control cells. Protein synthesis was measured by the incorporation of ^{14}C-labelled amino acids into acid precipitable material (figure 3.1b). Colostral preparation caused a significant increase protein synthesis by RIE-1 cells. The effect was dose dependent, with a maximum at 50% v/v ($p<0.01$).

Tritiated thymidine incorporation into HT-29 DNA was increased by colostral preparation up to 30 % v/v (figure 3.2a). Culture of HT-29 cells in medium containing higher amounts of colostral preparation revealed a progressive decrease in tritiated thymdine incorporation up to the highest amount tested (60% v/v). An identical pattern was observed when protein synthesis was assessed by the incorporation of ^{14}C-labelled amino acids (figure 3.2b).

These data showed stimulation of DNA synthesis by bovine colostral preparation, with a maximum effect by 30% v/v. Higher amounts of colostral preparation stimulated DNA synthesis by RIE-1 cells, albeit less potently. However, DNA synthesis by HT-29 cells was decreased below that in serum-free controls in the presence of bovine colostral preparation higher than 40% v/v. Protein synthesis assays were performed by measuring incorporation of

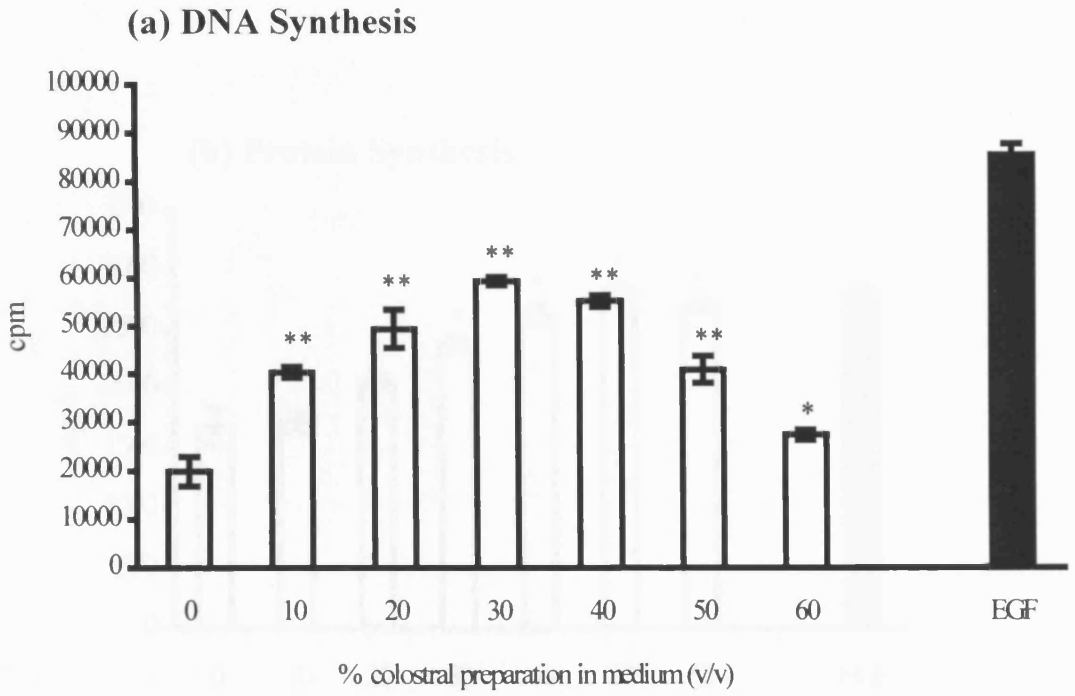


Figure 3.1 Bovine Colostral Preparation Stimulates DNA Synthesis and Protein Synthesis by Rat Intestinal Epithelial Cells.

RIE-1 cells were cultured in the presence of increasing amounts of colostral preparation. Data represent mean+/- SEM (n=4) from a typical experiment.
* $p<0.01$ ** $p<0.001$.

(a) DNA synthesis was measured by the incorporation of ^{3}H- thymidine into acid precipitable material. Colostral preparation stimulated significantly when present between 10 and 60% v/v.

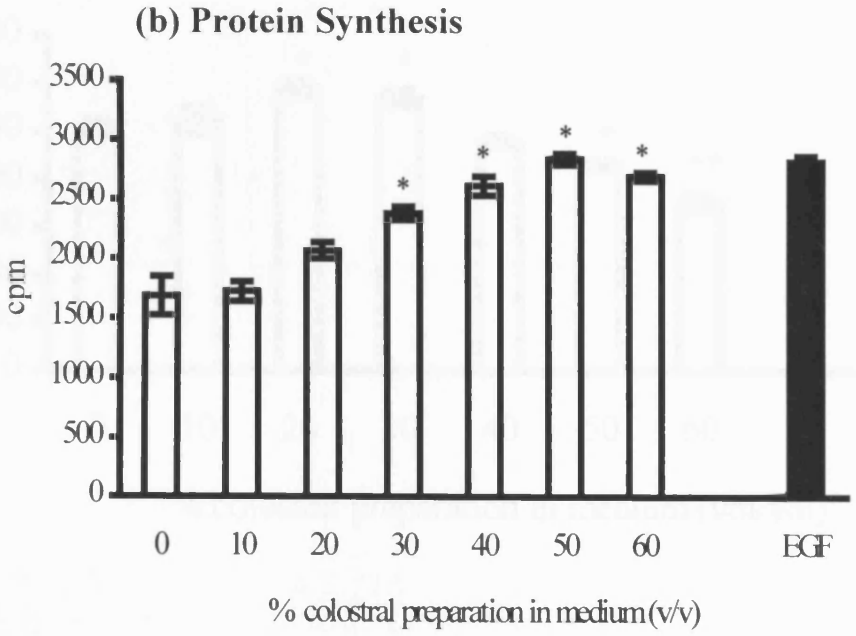


Figure 3.1 Cont'd.

(b) Protein synthesis was measured by incorporation of ^{14}C-labelled mixed amino acids into acid precipitable material. Colostral preparation stimulated significantly when present between 30 and 60% v/v.

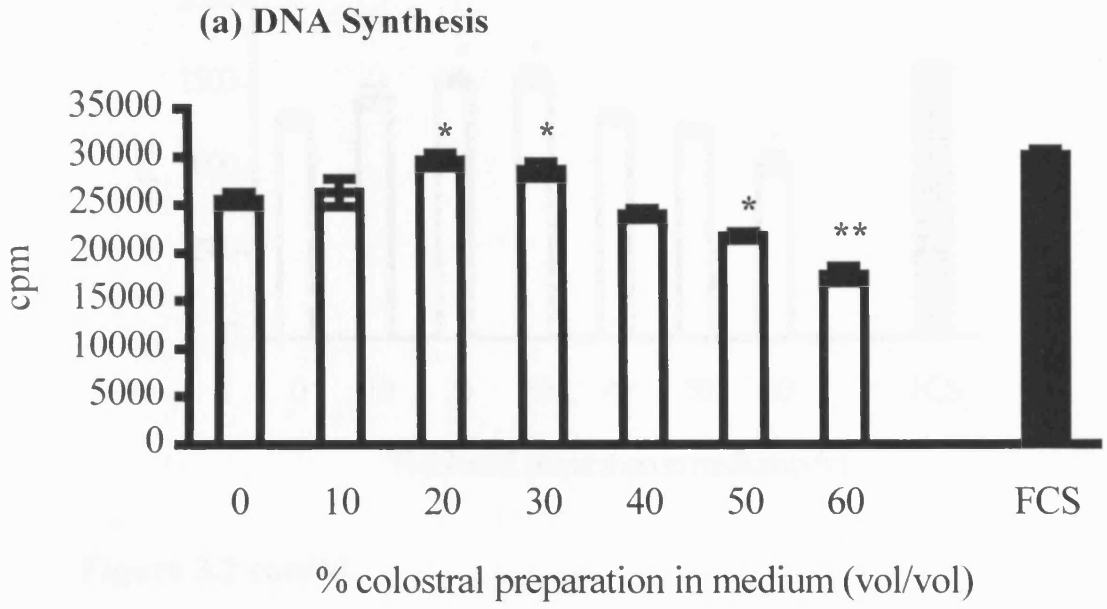


Figure 3.2 Bovine Colostral Preparation Stimulates or Inhibits DNA Synthesis and Protein Synthesis by Human Intestinal Epithelial Cells Depending on Amount of Colostral Preparation.

HT-29 cells were cultured in the presence of increasing amounts of colostral preparation.

(a) DNA synthesis was measured by the incorporation of ^{3}H- thymidine into acid precipitable material. Colostral preparation stimulated when present between 10 and 30% vol/vol, but inhibited in larger amounts. Data represent mean+/- SEM (n=4) from a typical experiment. * $p<0.01$ ** $p<0.001$.

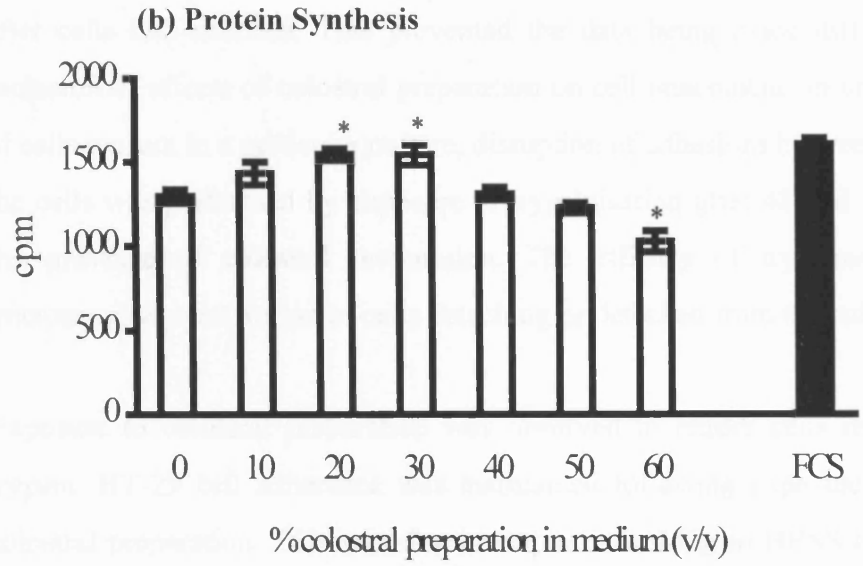


Figure 3.2 cont'd.

(b) Protein synthesis was measured by incorporation of ^{14}C-labelled mixed amino acids into acid precipitable material. Data are similar to those for DNA synthesis. Data represent mean+/- SEM (n=4) from a typical experiment. * $p<0.05$.

^{14}C-labelled amino acids. Similar patterns of stimulation and inhibition were observed, which might reflect direct effects on protein synthesis. Alternatively, protein synthesis may be altered due to changes in cell mass, resulting from the proliferative effects of bovine colostral preparation.

Cell counting assays were performed to determine if true cell proliferation was occurring in response to bovine colostral preparation. Cells were plated out in medium containing 10% (v/v) FCS, which was changed to medium supplemented with bovine colostral preparation after cells had attached. This prevented the data being made difficult to interpret by the inclusion of effects of colostral preparation on cell attachment. In order to count the number of cells present in a particular culture, disruption of adhesions between the culture surface and the cells was performed by exposure to trypsinisation after 48 and 72 hours after culture in the presence of colostral preparation. The efficacy of trypsinisation was checked by microscopical observation of cells detaching or detached from the culture surface.

Exposure to colostral preparation was observed to render cells resistant to the effects of trypsin. HT-29 cell adherence was maintained following exposure to as little as 10% v/v colostral preparation. This was despite copious washing in HBSS (w/o Ca^{2+} and Mg^{2+}) and exposure to trypsin of up to 30 minutes duration. Similar observations were made using RIE-1 cells. However, it was possible to recover all RIE-1 cells from the culture dishes following 48 hours' exposure to 10% v/v colostral preparation (figure 3.3). The absolute number of cells per well in the culture dish increased significantly over 48 hours ($p<0.01$). As shown in figure 3.1a, 10% v/v colostral preparation stimulated DNA synthesis significantly. Thus, bovine colostral preparation stimulates DNA synthesis, which results in increased cell proliferation. The increased cell mass is expected to contribute to the observed increase in protein synthesis.

3.2 Does Bovine Colostral Preparation Stimulate Intestinal Epithelial Cell Migration?

Restitution precedes proliferation in epithelial wound healing, and is independent of proliferation (Wilson and Gibson, 1997a). An *in vitro* assay of restitution was used to determine the effect of bovine colostral preparation on migration of the two cell lines – HT-

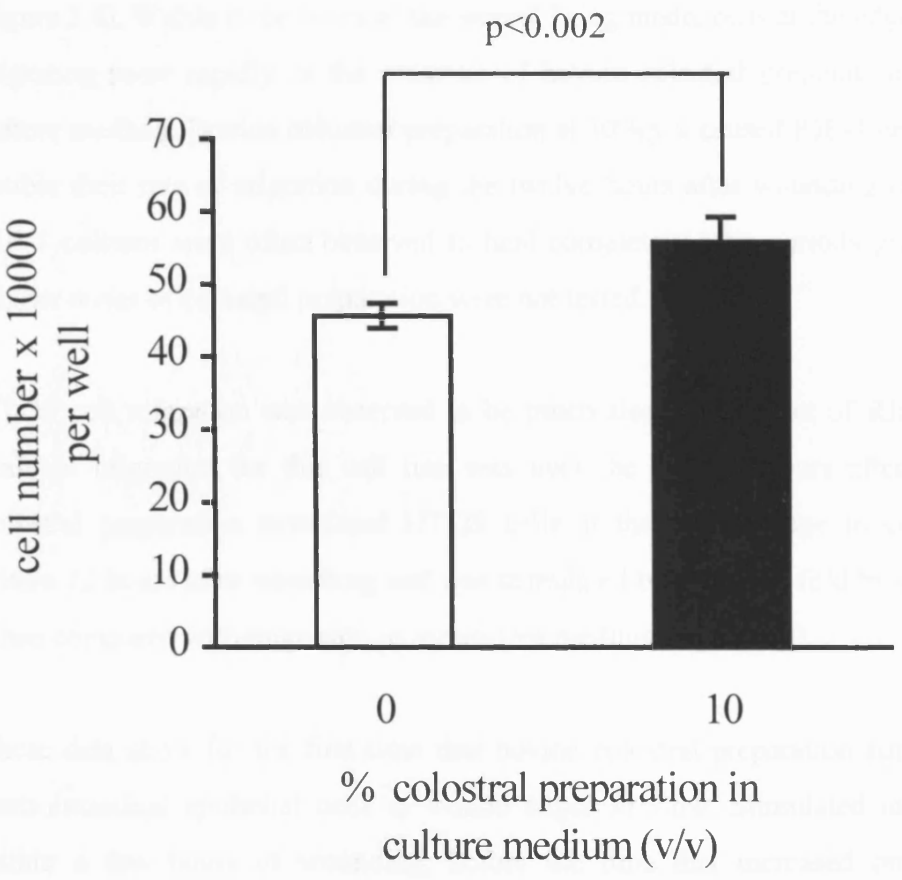


Figure 3.3 RIE-1 Cell Numbers Increase in Response to Colostral Preparation.

RIE-1 cells were plated in complete medium and allowed to attach to the culture surface before being washed and subsequently cultured in serum-free medium without or with colostral preparation. After 48 hours cells were harvested by trypsinisation and counted using a counting chamber.

Data represent mean+/- SEM (n=6) from a typical experiment.

29 and RIE-1 – at the edge of an artificially induced wound in a confluent monolayer. The rate of migration was determined from linear measurements of wound width taken from serial photomicrographs of the wound at various times after wounding (section 2.2.4).

Bovine colostral preparation stimulated migration by RIE-1 cells in a dose dependent manner (figure 3.4). Within three hours of the wound being made, cells at the edge of the wound were migrating more rapidly in the presence of bovine colostral preparation than in serum-free culture medium. Bovine colostral preparation at 30% v/v caused RIE-1 cells approximately to double their rate of migration during the twelve hours after wounding ($p<0.01$). Wounds to RIE-1 cultures were often observed to heal completely after periods greater than 12 hours. Higher doses of colostral preparation were not tested.

HT-29 cell migration was observed to be much slower than that of RIE-1 cells. The time-scale of migration for this cell line was over the first 30 hours after wounding. Bovine colostral preparation stimulated HT-29 cells at the wound edge to commence migration within 12 hours after wounding and was stimulated two- to four-fold by colostral preparation when compared with migration in serum-free medium (figure 3.5).

These data show for the first time that bovine colostral preparation stimulates migration of gastrointestinal epithelial cells at wound edges *in vitro*. Stimulated migration commences within a few hours of wounding, before the time that increased proliferation might be expected to contribute to epithelial cell density. Further studies were devised to elucidate whether the observed stimulation of migration was independent of cell proliferation.

3.4 Is Migration Stimulated by Colostral Preparation Dependent Upon Increased DNA synthesis?

In order to study the possible contribution of increased proliferation to the stimulated migration of intestinal epithelial cells in the presence of colostral preparation, DNA synthesis was inhibited before wounds were 'inflicted.' Mitomycin C has been used by others to inhibit DNA synthesis prior to analysis of restitution (Basson et al, 1992). Preliminary assays were performed on confluent cells to determine the optimal concentration of mitomycin C to use in

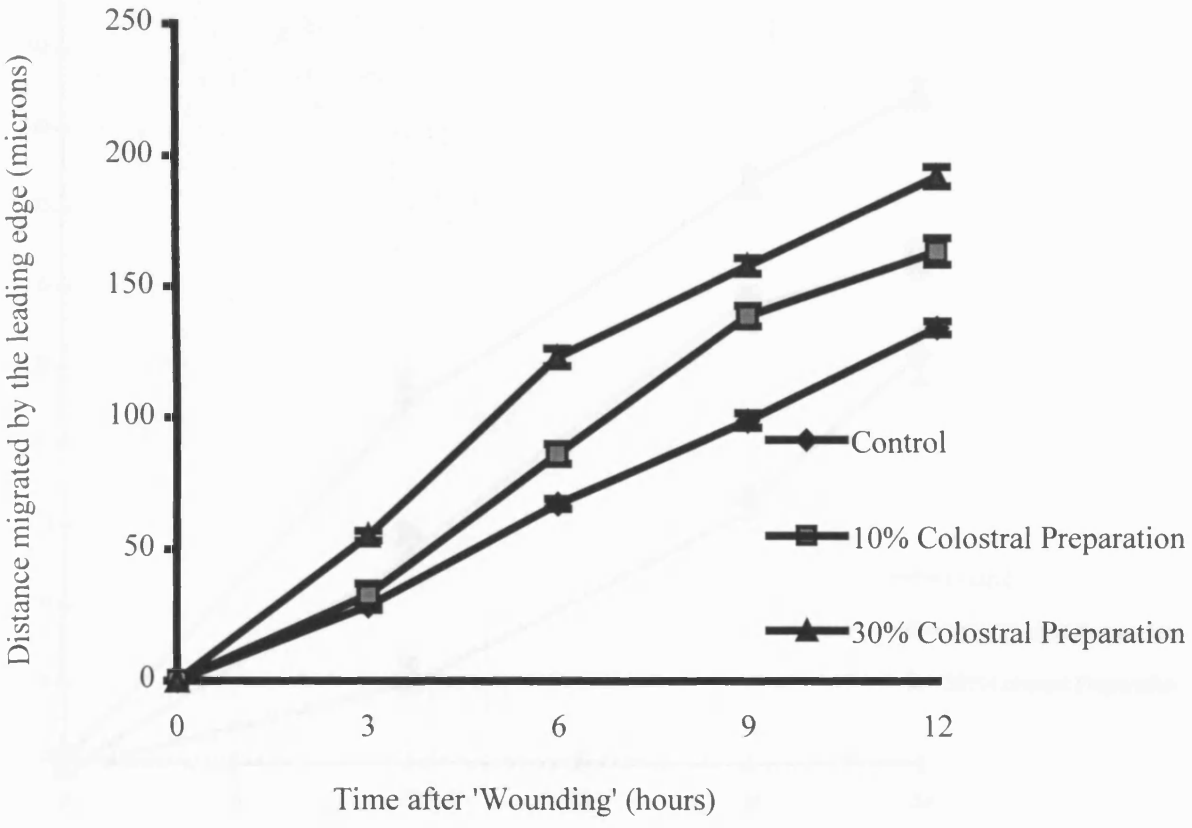


Figure 3.4 Bovine Colostral Preparation Stimulates Migration of Rat Intestinal Epithelial Cells .

Confluent RIE-1 cells were 'wounded' and cultured in the presence of colostral preparation at 10 and 30% vol/vol. Migration was measured as described in the text. Colostral preparation significantly stimulated RIE-1 migration compared with migration in serum-free medium at all time points ($p<0.01$).

Data show mean +/-SEM (n=4) from a typical experiment.

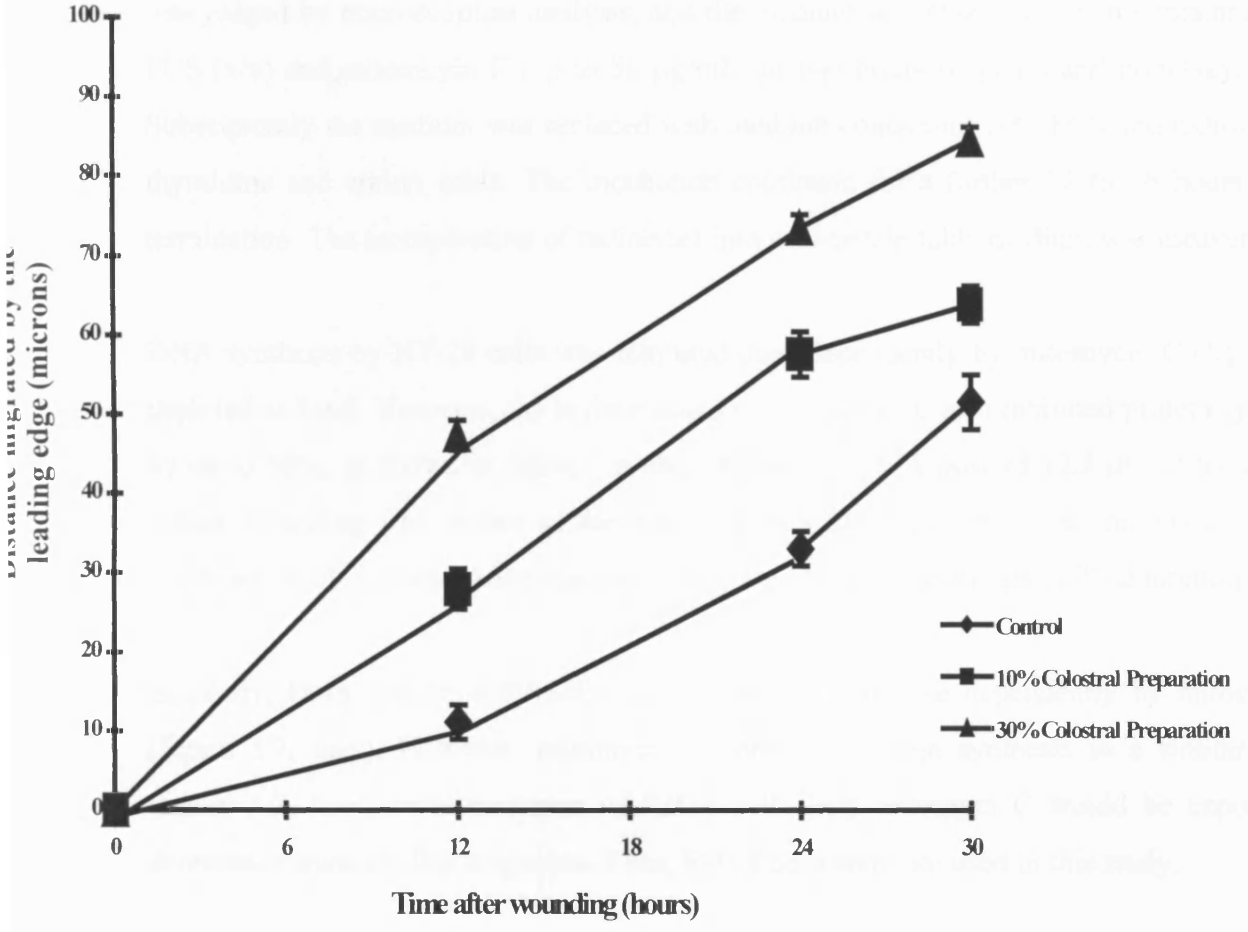


Figure 3.5 Bovine Colostral Preparation Stimulates Migration of Human Intestinal Epithelial Cells.

Confluent HT-29 cells were 'wounded' and cultured in the presence of colostral preparation at 10 and 30% vol/vol. Migration was measured as described in the text. Colostral preparation significantly stimulated HT-29 migration compared with serum-free medium at all time points ($p<0.01$). Data show mean +/-SEM (n=4) from a typical experiment.

restitution assays (Dignass and Podolsky, 1993). The optimal concentration is one that abolishes DNA synthesis without too large an effect on protein synthesis, which is necessary for restitution (Wilson and Gibson, 1997b).

DNA synthesis assays were performed as described (section 2.2.2). The assays were slightly different from those described earlier in this chapter. Cells were grown to confluence, which was judged by microscopical analysis, and the medium was changed to that containing 10% FCS (v/v) and mitomcyin C (up to 50 μg/ml) for two hours (Dignass and Podolsky, 1993). Subsequently the medium was replaced with medium containing 10% FCS and radiolabelled thymidine and amino acids. The incubation continued for a further 12 to 16 hours before termination. The incorporation of radiolabel into acid-precipitable medium was measured.

DNA synthesis by HT-29 cells was inhibited dose dependently by mitomycin C (figure 3.6, depicted as bars). However, the highest doses of mitomycin C also inhibited protein synthesis by up to 50%, as shown in figure 3.6 (depicted as a line). A dose of 12.5 μg/ml for 2 hours before wounding was chosen as the dose to give a balance of the most inhibition of DNA synthesis (80% inhibition) and the least inhibition of protein synthesis (15% inhibition).

Similarly, DNA synthesis by RIE-1 cells was inhibited dose dependently by mitocycin C (figure 3.7, bars). However, mitomycin C inhibited protein synthesis to a similar extent (figure 3.7, line). Prior treatment of RIE-1 cells with mitomyin C would be expected to decrease or even abolish migration. Thus, RIE-1 cells were not used in this study.

The migration of HT-29 cells at the wound edge was measured as described previously. Cells were incubated in medium containing FCS and mitomycin (12.5 μg/ml) for two hours before wounding. Subsequently, cells were cultured in serum-free medium with or without the optimal amount of bovine colostral preparation for 12 hours, typically the time point at which the difference between control and stimulated migration was greatest. When cells were cultured in serum-free medium pre-treatment with mitomycin C caused a small decrease (approximately 15%) in the rate of migration (figure 3.8). This was not statistically significant. Similarly, cells cultured in medium supplemented with colostral preparation

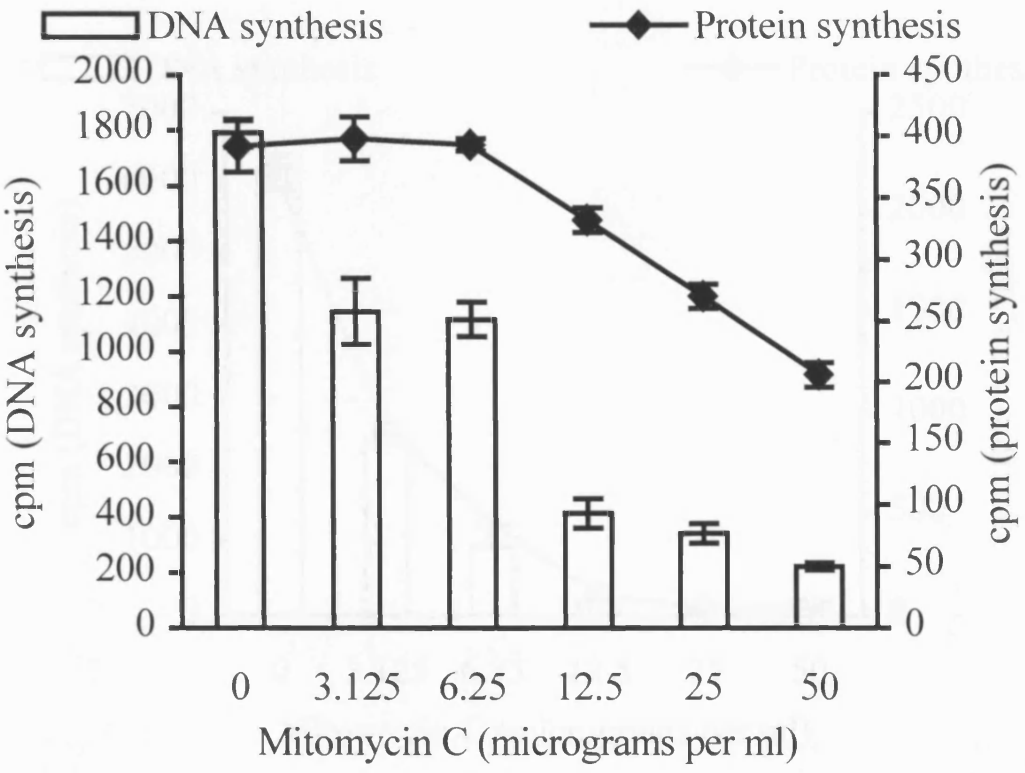


Figure 3.6 Mitomycin C dose-dependently Inhibits DNA and Protein Synthesis by HT-29.

HT-29 cells were cultured in complete medium containing for Mitomycin C for 2 hours, before determinations of DNA and protein synthesis were performed in complete medium. Data represent mean +/- SEM (n=4) of a typical experiment.

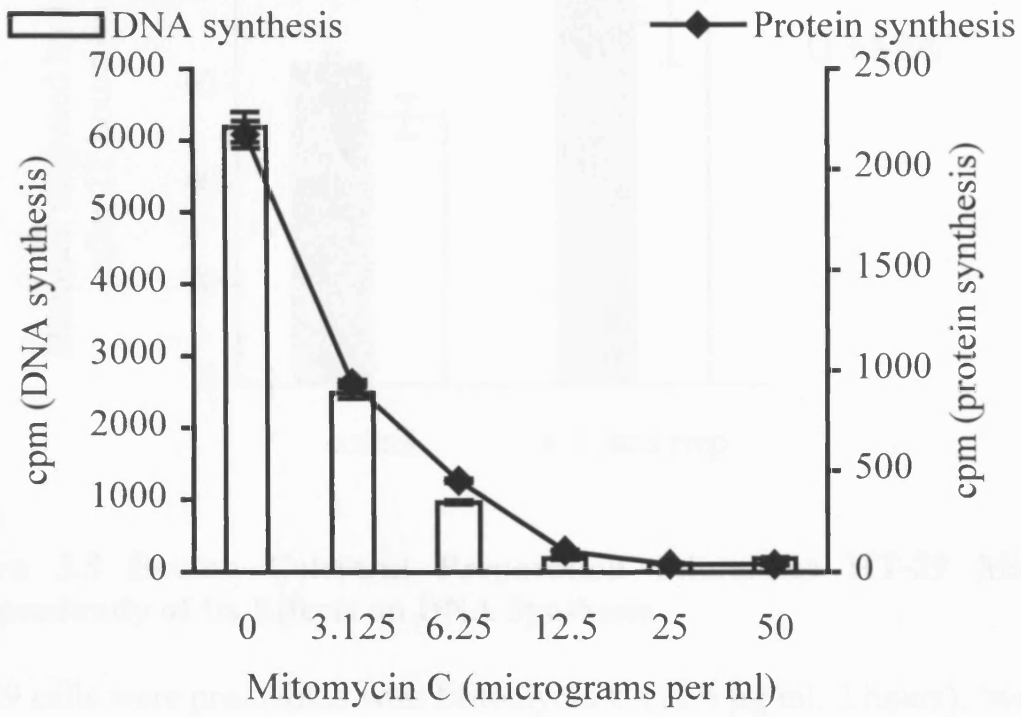


Figure 3.7 Mitomycin C dose-dependently Inhibits DNA and Protein Synthesis by RIE-1 Cells.

RIE-1 cells were cultured in complete medium containing for Mitomycin C for 2 hours, before determinations of DNA and protein synthesis were performed in complete medium. Data represent mean +/- SEM (n=4) of a typical experiment.

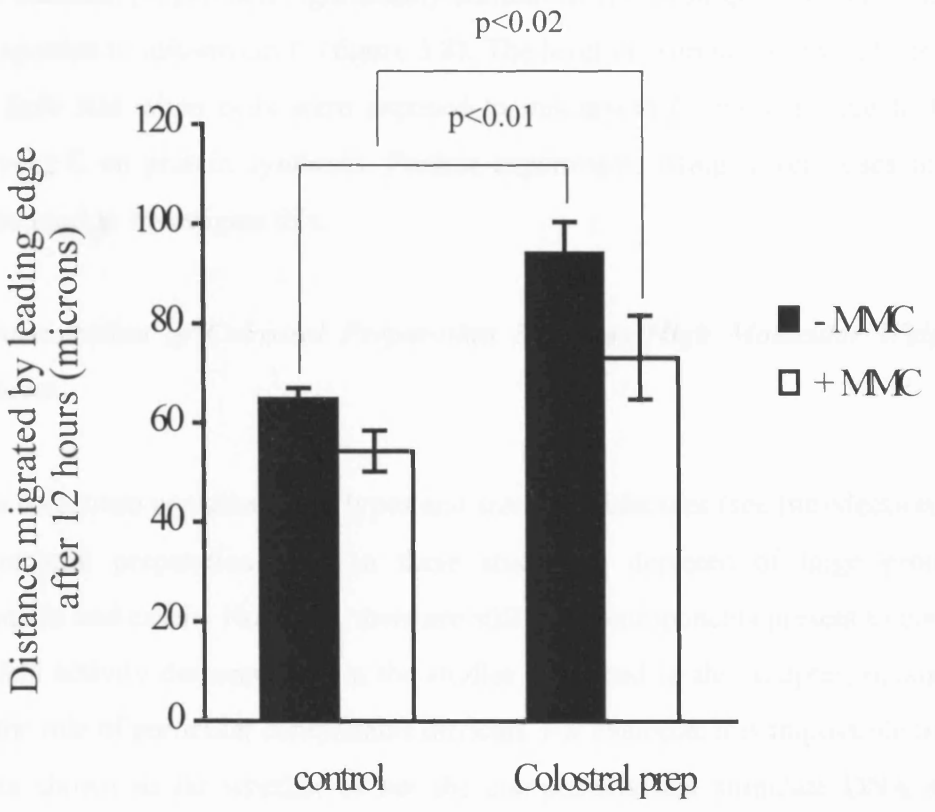


Figure 3.8 Bovine Colostral Preparation Stimulates HT-29 Migration Independently of Its Effects on DNA Synthesis.

HT-29 cells were pre-treated with Mitomycin C (12.5 μg/ml, 2 hours), 'wounded' then cultured in 30% vol/vol colostral preparation for 12 hours. Mitomycin C caused a small reduction in migration of samples cultured with and without colostral preparation. However, colostral preparation stimulated migration significantly with or without mitomycin C.

migrated more slowly when previously exposed to mitomycin C, probably due to inhibited protein synthesis.

Bovine colostral preparation significantly stimulated HT-29 migration both without and with prior exposure to mitomycin C (figure 3.8). The level of stimulation by colostral preparation was a little less when cells were exposed to mitomycin C, possible due to the effects of mitomycin C on protein synthesis. Further experiments using lower doses of mitomcin C could be used to investigate this.

3.5 Fractionation of Colostral Preparation Indicates High Molecular Weight Bioactive Complexes.

Bovine colostrum contains many types and sizes of molecules (see Introduction, section 1.5). The colostral preparation used in these studies is depleted of large proteins such as lactalbumin and casein. However, there are still many components present to contribute to the biological activity demonstrated in the studies described in this chapter, making deductions about the role of particular components difficult. For example, it is impossible to deduce from the data shown so far whether or not the components that stimulate DNA synthesis also stimulate migration.

There are various ways of fractionating further the colostral preparation, including fractionation by size and by electric charge (Brown and Blakely, 1984; Shing and Klagsbrun, 1984). In the experiments described here colostral preparation was separated using ultra-filtration into fractions with broad ranges of molecular weights. This method is simple, rapid and recovery of the colostral components at their original concentration is readily done. Colostral preparation was separated into broad ranges of molecular weights (less than 5 kDa; 5 to 10 kDa; 10 to 30 kDa; greater than 30 kDa) using centrifuge ultrafiltration units (Millipore, England) according to the manufacturer's instructions. The resulting concentrated fractions were restored to their original volume in sterile PBS.

These fractions were used subsequently to analyse the bioactivities in colostral preparation that stimulate DNA synthesis and/or cell migration. The cell line most sensitive to the

colostral preparation was chosen for each assay (i.e. DNA synthesis assays were performed using only RIE-1 cells and migration assays were performed using only HT-29 cells).

The effects of fractionated colostral preparation on DNA synthesis are shown in figure 3.9. DNA synthesis by RIE-1 cells was stimulated by whole colostrum (30% v/v). Fractions with molecular weights between 10 and 30 kDa, and greater than 30 kDa, stimulated DNA synthesis when present at 30% v/v. Components with molecular weights between 10 and 30 kDa had a small effect on DNA synthesis, much less than the effect of whole colostrum. Components greater than 30 kDa were observed to stimulate DNA synthesis more than unfractionated colostral preparation, suggesting that at least one component with a molecular weight below 30 kDa was inhibitory either to the action of a high molecular weight component or to DNA synthesis *per se*. Components with molecular weights below 10 kDa were observed to have no effect on RIE-1 DNA synthesis.

The effects of fractionated colostral preparation on cell migration are shown in figure 3.10. Fractions with molecular weights between 10 and 30 kDa and greater than 30 kDa stimulated migration of HT-29 cells. Most pro-migratory activity was found in the colostral fraction greater than 30 kDa. Pro-migratory activities in these fractions were less than the activity of whole colostral preparation, but when added together were similar in magnitude. Components less than10 kDa had no effect on cell migration.

These data show that bioactivity was found in high molecular weight components of bovine colostral preparation (i.e. greater than 30 kDa). It is noteworthy that fractionation of colostral preparation appears to increase the effects on DNA synthesis, but causes a decrease in the pro-migratory activities of the fractions considered here. These data suggest that there are components in the colostral preparation that stimulate either DNA synthesis or cell migration.

3.6 Discussion.

The data presented in this chapter demonstrate that bovine colostral preparation stimulated restitution and proliferation of gastrointestinal epithelial cells *in vitro*. Migration was stimulated by colostral preparation, independent of increased proliferation. Proliferation was

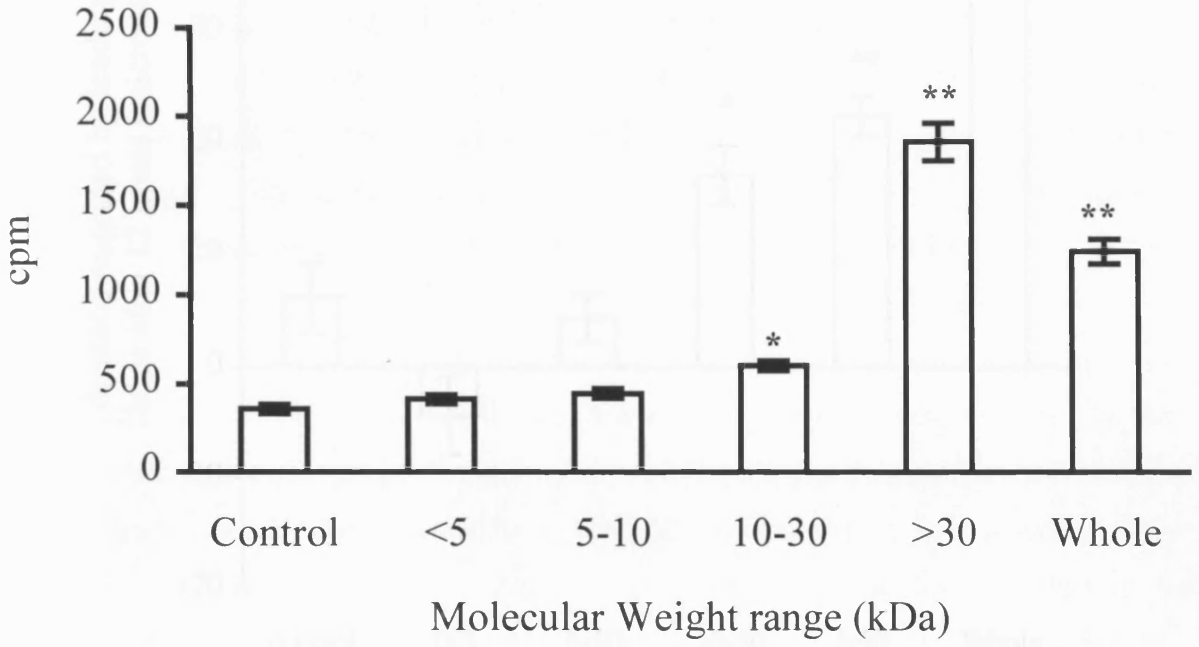


Figure 3.9 DNA Synthesis by RIE-1 Cells is Stimulated by High Molecular Weight Colostral Components.

Sub-confluent, serum-starved RIE-1 cells were cultured in colostral fraction at 30% v/v. Data show the incorporation of ^{3}H-thymidine into acid-precipitable material, representing mean +/- SEM (n=8) from a typical experiment.

* represents $p<0.05$ and ** represents $p<0.001$ compared with serum-free control.

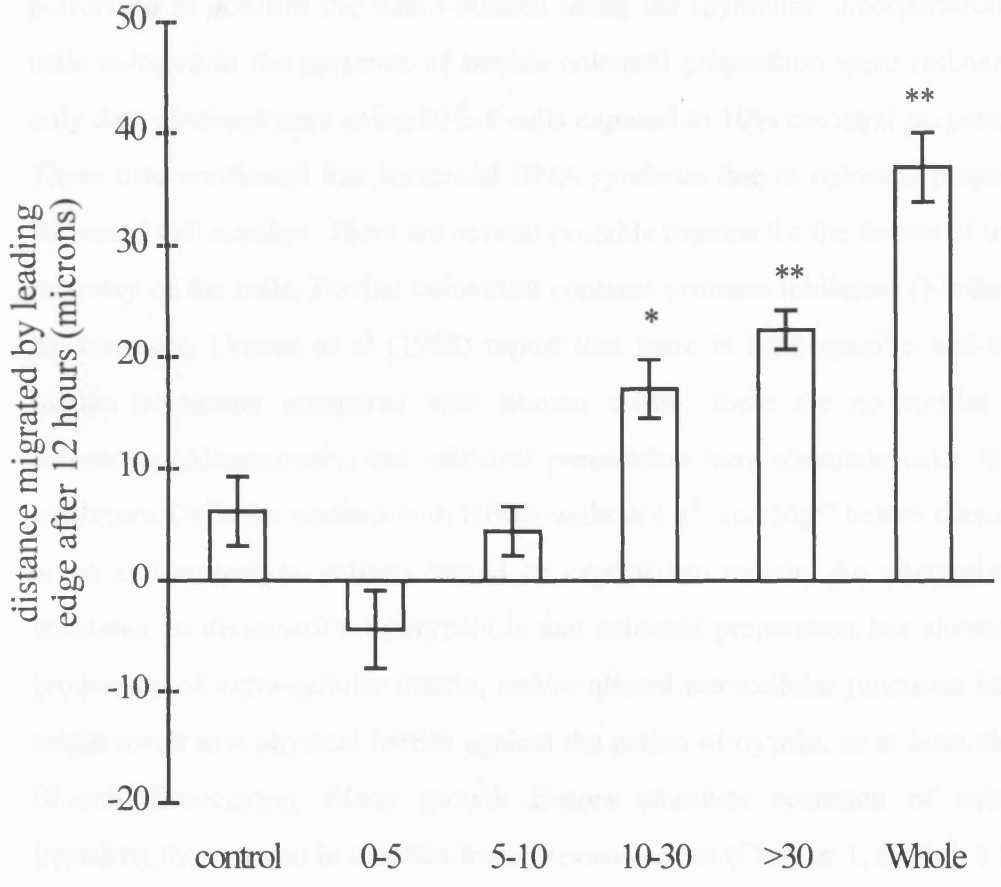


Figure 3.10 Pro-migratory Components in Bovine Colostral Preparation are Greater than 10 kDa.

HT-29 cells were 'wounded' then cultured in colostral fraction at 30% v/v. Data show the distance migrated by the wound edge 12 hours after wounding, representing mean +/- SEM (n=4). * represents $p<0.05$ and ** represents $p<0.01$ compared with serum-free controls.

stimulated by components of the colostral preparation with molecular weights greater than 30 kDa. Smaller components had no affect on proliferation. Pro-migratory activities were greater than 10 kDa in size, and mostly greater than 30 kDa.

Bovine colostral preparation stimulated the incorporation of tritiated thymidine into DNA (section 3.2). This is a commonly used, but indirect, method of measuring proliferation, for which the 'gold standard' is cell counting (Boulton and Hodgson, 1995). Cell counting was performed to confirm the data obtained using the thymidine incorporation assay; however, cells cultured in the presence of bovine colostral preparation were resistant to trypsin. The only data obtained were using RIE-1 cells exposed to 10% colostral preparation for 48 hours. These data confirmed that increased DNA synthesis due to colostral preparation resulted in increased cell number. There are several possible reasons for the failure of trypsin to facilitate recovery of the cells. Bovine colostrum contains protease inhibitors (Kirihara et al, 1995a & b); however, Uruena et al (1998) report that there is little specific anti-trypsin activity in human colostrum compared with human serum: there are no similar data for bovine colostrum. Alternatively, the colostral preparation may stimulate cells to secrete protease inhibitors. Cells are washed with HBSS without Ca^{2+} and Mg^{2+} before dissociation; thus little or no anti-proteolytic activity would be expected to remain. An alternative explanation for resistance to dissociation by trypsin is that colostral preparation has altered or increased the production of extra-cellular matrix, and/or altered intercellular junctions between cells. This might result in a physical barrier against the action of trypsin, or at least slow down the rate of cell dissociation. Many growth factors stimulate secretion of extracellular matrix, including those found in colostra from various species (Chapter 1, section 1.5).

Others have demonstrated increased cell proliferation and/or DNA synthesis in the presence of colostrum or milk. Direct comparisons of data are problematic due to the choices of milk or colostrum, the species from which milk or colostrum are derived, and the assay of proliferative activity used. Preparations from milk and colostrum support and stimulate growth of cells from different species (mouse, rat, human, canine, mink) and deriving from different tissues (skin, breast, lung, small intestine, kidney, muscle; Belford et al, 1997; Brown and Blakely, 1984; Ichibada et al, 1992; Liu et al, 1989; Shing and Klagsbrun, 1984). These studies contain discussion of the growth promoting activity of milk or colostral

preparations in terms of their growth factor content. The major growth factors in bovine colostrum - IGF-I and II, TGFβ, FGF - were discussed in detail in the introductory chapter (section 1.5.2).

Belford et al (1997) attempted to reconstruct the effect of bovine milk by mixing together relevant quantities of recombinant growth factors. However, this mixture provided just 50% of the growth stimulatory activity of bovine milk extract. Furthermore, the combination of growth factors was only twice as potent as any of the growth factors tested singly. These data are a powerful demonstration that a consideration of individual, known growth factors is insufficient. Non-growth factor components could also account for some proliferative activity. For example, Liu et al (1996) found that carboxy teminus fragments of casein from bovine milk stimulated proliferation of Rama 27 cells (rat mammary fibroblasts). Another protein found in bovine milk and colostrum - lactoferrin - stimulated DNA synthesis and proliferation by IEC-18 (rat intestinal epithelial) cells (Hagiwara et al, 1995). Supra-additive effects of lactoferrin and recombinant EGF were also demonstrated. Thus, bioactivity of colostral and milk preparations is probably due to many components, and due to interactions between some of them.

High doses of colostral preparation appeared to inhibit the proliferation of colonic HT-29 cells (figure 3.2). These data might be have been due either to inhibition, or to inherent toxicity of the colostral preparation. It is noteworthy that milk components have been observed to stimulate apoptosis in a number of cell types (Roy et al, 1999; Yoo et al, 1997). Pro-apoptotic activity in the colostral preparation under investigation might have predominated over proliferative activities at high percentages of the preparation. Also, milk-derived apoptotic factors have been shown to cause apoptosis of cancer cells and cell lines, but not of normal, mature cells (Hansson et al, 1995). These observations might account for the fact that data obtained using HT-29 cells were not comparable with those obtained using RIE-1 cells. The possible role of apoptosis in the effects of colostral preparation on HT-29 cells deserve further study.

Bovine colostral preparation stimulated migration of intestinal cells at the edge of wounds *in vitro* within hours of wounding (figures 3.4, 3.5 and 3.8). The pro-migratory effect of

colostral preparation was maximal at 30% v/v, similar to the most potent amount of colostral preparation for stimulated DNA synthesis. One possible interpretation of these data is that stimulated DNA synthesis was in some way connected to increased migration. The experiments described in section 3.4 were performed to test this possibility. DNA synthesis was inhibited by incubation with mitomycin C before wounding (Dignass and Podolsky, 1993). Mitomycin C decreased migration of HT-29 cells regardless of the presence of colostral preparation. These data may be explained by the small decrease in protein synthesis caused by mitomycin C, since protein synthesis is essential for restitution (Wilson and Gibson, 1997b). Alternatively, an endogenous mechanism by which cells migrate may be dependent upon proliferation (Nakajima and Kuwayama, 1995). Colostral preparation significantly simulated migration of HT-29 cells with or without prior treatment with mitomycin C, demonstrating that the majority of promigratory activity in bovine colostral preparation is independent of proliferative activity.

Bovine colostral preparation can be considered as a *nutriceutical*, i.e. a food product containing physiological quantities of bioactive molecules. It was not the purpose of this thesis to describe the isolation of a single, active component. However, it is possible that the bioactivity of the colostral preparation might be improved by some further purification if there are antagonistic components present. Thus, bovine colostral preparation was fractionated crudely into components with molecular weights up to 5 kDa, between 5 and 10 and between 10 and 30 kDa, and greater than 30kDa. Most growth stimulatory activity in bovine colostral preparation was due to components greater than 30kDa. This was surprising. As discussed in chapter 1, most of the growth factors in the colostral preparation have molecular weights below 30 kDa. However, the results of Belford et al (1997) indicate that components other than the major growth factors contribute to bioactivity (see above). These data indicate that there are growth stimulatory components in the colostral preparation under consideration here that cannot be readily assigned to known classes of mitogens based on their molecular weights. One growth factor specific to ruminant colostrum has been described with a molecular weight of approximately 30 kDa, referred to as bovine colostral growth factor (Brown and Blakely, 1984). This growth factor has partial similarity to PDGF. However, there are no data describing the complete characterisation of this growth factor,

including its precise mechanism(s) of action, nor has it been cloned. Such studies would be of great importance and interest in the light of the data presented in this chapter.

Pro-migratory activity of colostral preparation was observed in response to colostral components greater than 10 kDa. Unlike the effect of fractionation on proliferative components, fractionation reduced amount of pro-migratory activity per fraction. These data suggest that some pro-migratory activities were due to non-proliferative components, especially with molecular weights between 10 and 30 kDa, which had only a small effect on proliferation.

The data presented in this chapter show that bovine colostral preparation contains significant bioactivity *in vitro* and that this bioactivity is probably due to several components. Some components might regulate proliferation or migration separately, whereas others might regulate both. However, the bulk of the bioactivity described in this chapter cannot be accounted for by a simple consideration of the known colostral growth factors, based on their molecular weights.

Chapter 4.

The Effects Of Transient Acidification On Colostral Bioactivity *In Vitro*, And The Role Of TGFβ *In Vitro* and *In Vivo*

4.1 Introduction.

Colostrum and milk are rich in transforming growth factor beta (TGFβ) (Jin et al, 1991; Rogers et al, 1996; Tokuyama & Tokuyama, 1989). Bovine colostral TGFβ is predominantly of the β2 isoform (approximately 80%; Rogers et al, 1996).

High molecular weight (80 kDa), latent TGFβ is detectable immunologically in milk (Rogers et al, 1996). This form has no effect on growth of cultured cells; however, transient acidification (pH2 for 4 hours) causes the appearance of bioactivity (Rogers et al, 1996). Electrophoretic and gel filtration studies demonstrate that transient acidification liberates lower molecular weight forms of TGFβ (Rogers et al, 1996; Tokuyama & Tokuyama, 1989). However, the 24 kDa form, which is commonly observed in a number of systems, is absent from some post-acidification colostrum or milk preparations (Rogers et al, 1996; Tokuyama & Tokuyama, 1989) but not others (Jin et al, 1991). Thus, there is disparity between different sources of published data concerning the nature and effects of colostral TGFβ.

The acidification protocols used in the studies mentioned above (pH 2, 4 hours) are similar to those that would be experienced by ingested colostrum as it passes through the stomach (Hogan et al, 1994). Thus, ingestion of colostral preparation might liberate bioactive TGFβ, i.e. the acidic conditions in the stomach might actually make colostral preparation more bioactive. Active TGFβ would be expected to be protected from degradation by gastric proteases by the presence of other large proteins (Playford et al, 1993) found in the colostrum preparation (Belford et al, 1997). These facts suggest that TGFβ might not be an important component of colostral preparation *in vitro*, but may be important *in vivo,* after exposure to acidic conditions in the stomach.

The studies discussed above considered the effects of transient acidification on colostral bioactivity solely in terms of TGFβ. Exposure of ingested colostral preparation to transient acidification as it passes through the gut should affect other colostral components, although proteolysis is the major cause of growth factor degradation by gastric juice (Playford et al, 1993). It is desirable to know the effects of transient acidification on the bioactivities demonstrated in the previous chapter, to elucidate how colostral preparation exerts its effects *in vivo*.

The studies in this chapter were designed to test the effect of acidification on *in vitro* bioactivity of bovine colostral preparation. The role of colostral TGFβ in these effects was evaluated using anti-TGFβ neutralising antibodies. Also, the role of the major colostral isoform of TGFβ in NSAID-induced small intestinal injury was investigated. The dose of recombinant TGFβ2 chosen was equivalent to that in a volume of colostral preparation known to be effective in this assay (Playford et al, 1999).

4.2 High Molecular Weight Proliferative Activity In Bovine Colostral Preparation Is Sensitive To Transient Acidification.

This study was devised to measure the effect of transient acidification on the proliferative effect of colostral preparation. High molecular weight colostral components, greater than 30 kDa, contain the majority of bioactivity measured in the tritiated-thymidine incorporation assay for RIE-1 cells (chapter 3, figure 3.1). Thus, only high molecular weight components, referred to as HMW colostral preparation, were used in these studies, and were prepared as described in chapter 2. Tritiated thymidine incorporation assays were also performed using NIH-3T3 fibroblast cells. Fibroblast growth is stimulated by milk and colostral preparations, and by TGFβ (Brown & Blakely, 1984; Belford et al, 1997).

Acidification of HMW colostral preparation was performed as described by Rogers et al (1996). Initially, the pH of HMW colostral preparation was 5.6 to 6.0. The preparation was acidified by dropwise addition of 1M HCl at room temperature, to pH 2.0. The volume of acid required for this was not above 5% of the volume of the colostral preparation. Acidified samples were left to stand for 4 hours at room temperature before neutralisation to the original pH with 500 mM NaOH. Unacidified samples of HMW colostral preparation were left at room temperature for the same duration to control for potential degradation of bioactive components. Following neutralisation all samples were sterilised by passage through a 0.22 μm filter.

DNA synthesis assays were performed as described in chapter 2. Sub-confluent, serum-starved cells in 96-well plates were cultured in 30% colostral preparation (unacidified or acidified) for 24 hours. Tritiated thymidine was added for the last 6-8 hours. Incorporated radiolabel was measured in acid-precipitable material.

The effect of transient acidification on the proliferative activity of HMW colostral preparation on RIE-1 cells is shown in figure 4.1. Unacidified colostral preparation stimulated thymidine incorporation. However, following transient acidification proliferative bioactivity was abolished. Thymidine incorporation by RIE-1 cells exposed to transiently acidified colostral preparation was not significantly different from that measured by cells cultured in serum-free medium. This effect might be due to destruction of acid-sensitive, stimulatory components, or to liberation of inhibitory components – such as TGFβ - by transient acidification.

The effect of transient acidification on the proliferative activity of HMW colostral preparation on NIH-3T3 fibroblasts is shown in figure 4.2. HMW colostral preparation stimulated DNA synthesis. Following transient acidification the stimulatory effect of HMW colostral preparation was significantly reduced (approximately 25%), but not abolished. Indeed, substantial activity remained. Thus, transient acidification of the colostral preparation caused loss of some proliferative components, and/or the appearance of some inhibitory components.

These data show that a proportion of proliferative bioactivity for both cell lines is destroyed by transient acidification. The degree of loss of bioactivity is substantially different for each cell line, indicating that stimulatory components for each cell line may be partly different. Liberation of colostral TGFβ might account for some of these effects.

4.3 The Role of TGFβ in Proliferation Due to High Molecular Weight Colostral Preparation.

The data in section 4.2 show that transient acidification destroys some, but not all, components that stimulate DNA synthesis. However, liberation of active TGFβ by transient acidification may contribute to the observed effects. Thus, further studies were devised to analyse the role of colostral TGFβ in proliferative activity, using an antibody that abolishes TGFβ bioactivity.

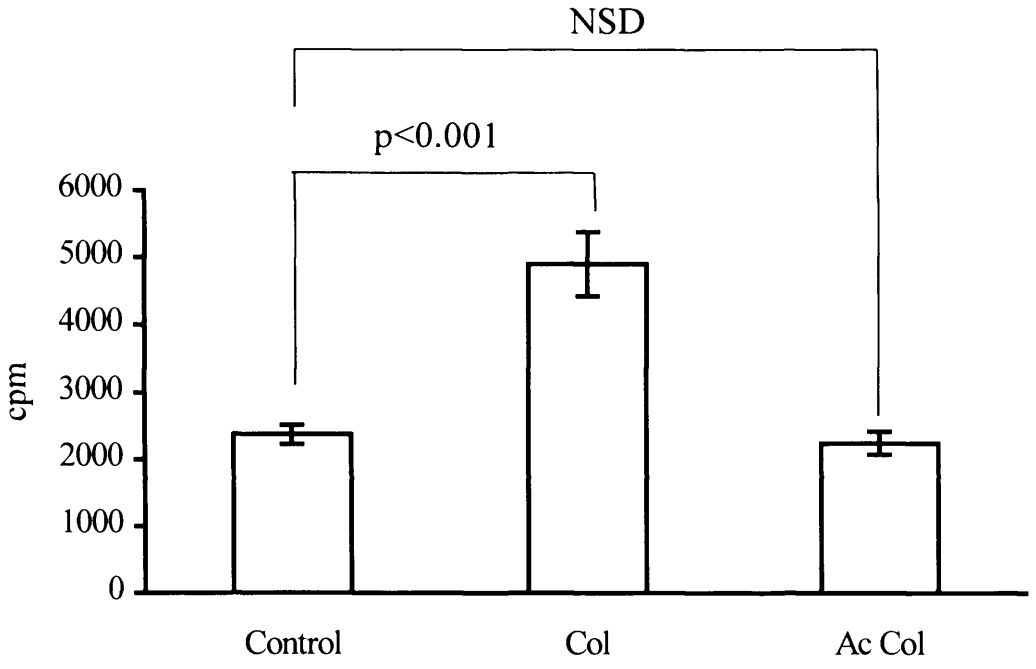


Figure 4.1 Transient Acidification Abolished Colostral Proliferative Activity In RIE-1 Intestinal Epithelial Cells.

Colostral preparation stimulated RIE-1 DNA synthesis (labelled Col, $p<0.001$ vs control). Transiently acidified colostral preparation (labelled Ac Col) had no effect on DNA synthesis by RIE-1 cells ($p>0.05$ vs control). Data represent mean +/- SEM ($n=4$) from a typical experiment. Three reproducible experiments were performed.

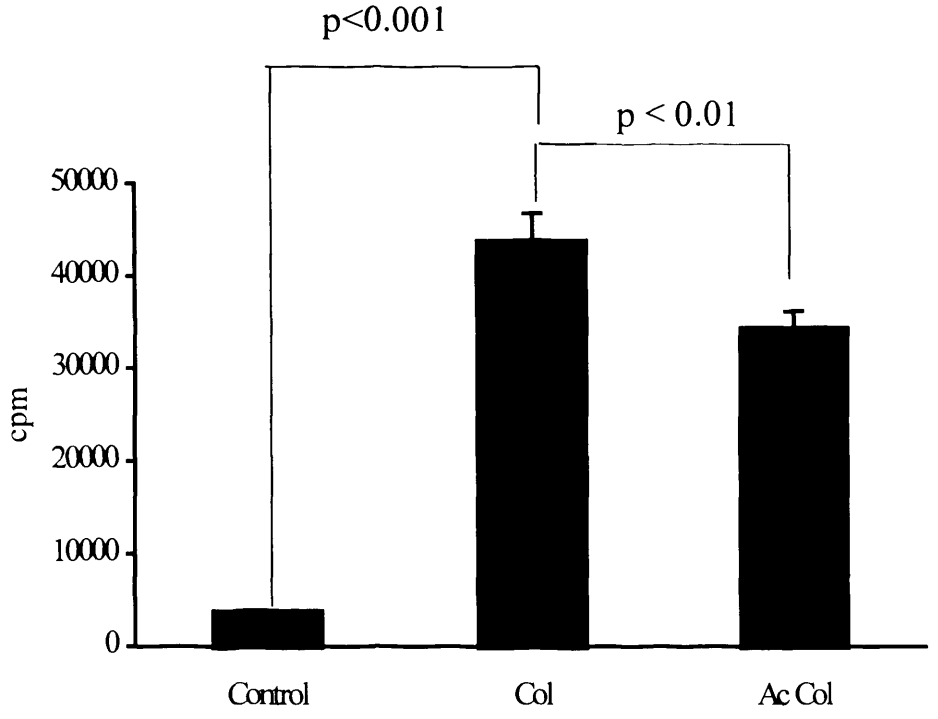


Figure 4.2 Transient Acidification Reduces, But Does Not Abolish, Fibroblast DNA Synthesis Stimulated By Colostral Preparation.

Colostral preparation stimulated DNA synthesis by NIH-3T3 fibroblasts (labelled Col, $p<0.001$ vs control). Transient acidification reduced the stimulatory effect of colostral preparation (labelled Ac Col, $p<0.01$). Data bars represent mean +/- SEM (n=4) from a typical experiment;. Three reproducible experiments were performed

First, the effect of recombinant TGFβ2 on DNA synthesis by the two cell lines was confirmed. Sub-confluent, serum starved cells were cultured in serum-free medium supplemented with rTGFβ2 (10 ng/ml). DNA synthesis was measured as previously described. Data from experiments using RIE-1 and NIH-3T3 cells are shown in figures 4.3a and b respectively. RIE-1 DNA synthesis was decreased by rTGFβ2, whereas NIH-3T3 DNA synthesis was stimulated, as expected.

Secondly, it was necessary to demonstrate that the chosen anti-TGFβ antibody could neutralise rTGFβ2 bioactivity. RIE-1 cells in serum-free medium containing anti-TGFβ antibody (10 μg/ml) synthesised more DNA than those cells cultured without antibody. These data could arise from the inhibition of the effects of endogenous TGFβ, which have been demonstrated for cultured cells (Belford et al, 1997). When rTGFβ2 was included in the culture medium DNA synthesis was inhibited. Inclusion of the antibody restored DNA synthesis to control levels when the effect of the antibody alone was taken into account (figure 4.3a).

Addition of the antibody (10 μg/ml) to NIH-3T3 cells exposed to TGFβ had no effect on stimulated DNA synthesis, since the antibody alone stimulated DNA synthesis (figure 4.3b).

DNA synthesis by RIE-1 was measured in the presence of HMW colostral preparation (unacidified and acidified) supplemented with anti-TGFβ antibody. (figure 4.4). Inclusion of the antibody in culture medium supplemented with unacidified preparation had little effect on DNA synthesis when effects of the antibody alone were taken into account. As demonstrated previously acidification of HMW colostral preparation resulted in loss of stimulated DNA synthesis. Inclusion of the anti-TGFβ antibody caused DNA synthesis to be slightly elevated (figure 4.4a). However, the difference between acidified colostral preparation with and without antibody was not significant. Thus, colostral components that modulated RIE-1 DNA

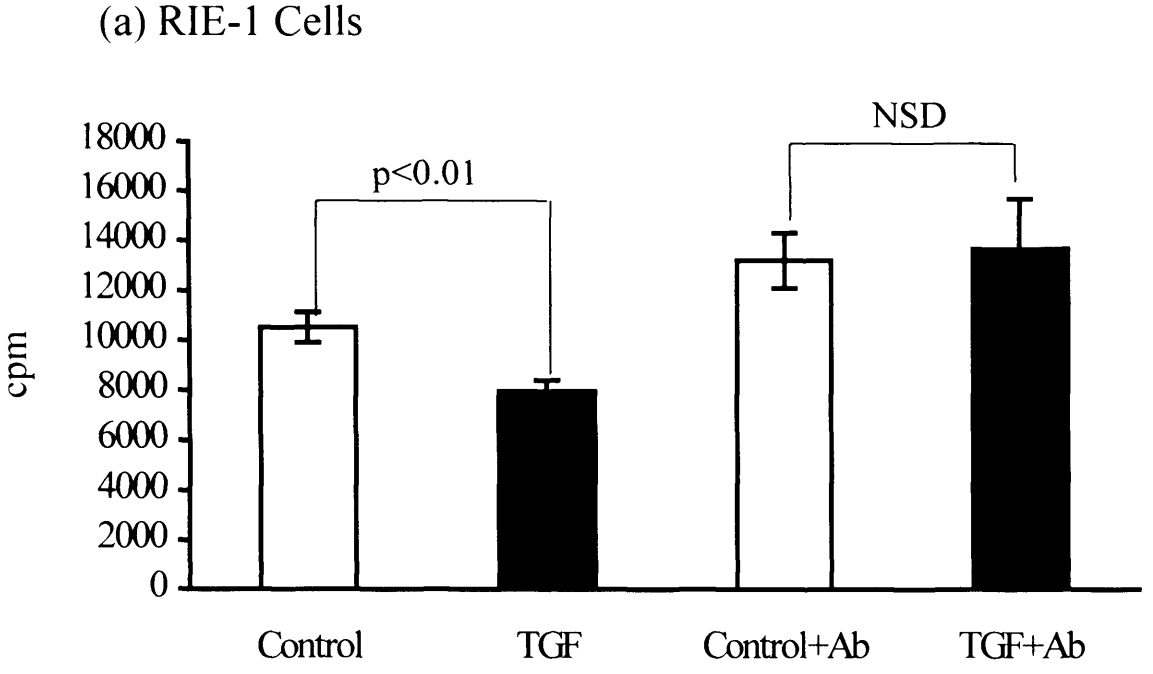


Figure 4.3 Demonstration of TGFβ Activity Using RIE-1 and NIH-3T3 Cells. Anti-TGFβ Neutralising Antibody Abrogated Effects of TGFβ.

(a) RIE-1 DNA synthesis was decreased in the presence of TGF (10ng/ml). In the presence of neutralising antibody (10 μg/ml) TGFβ has no effect on DNA synthesis compared with serum free conditions. Data represent the mean +/- SEM (n=4) of a typical experiment.

(b) NIH-3T3 Cells

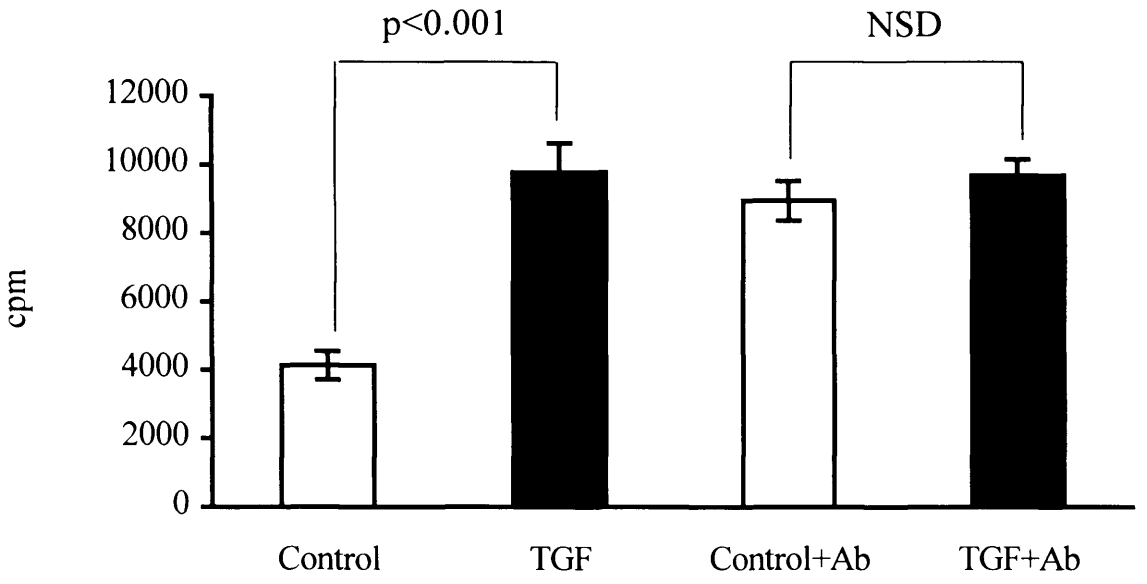


Figure 4.3 cont'd

(b) NIH-3T3 DNA synthesis was increased in the presence of TGFβ (10 ng/ml). Neutralising antibody (10 μg/ml) had no effect on has no effect on DNA synthesis stimulated by TGFβ compared with serum free conditions. Data represent the mean +/- SEM (n=4) of a typical experiment.

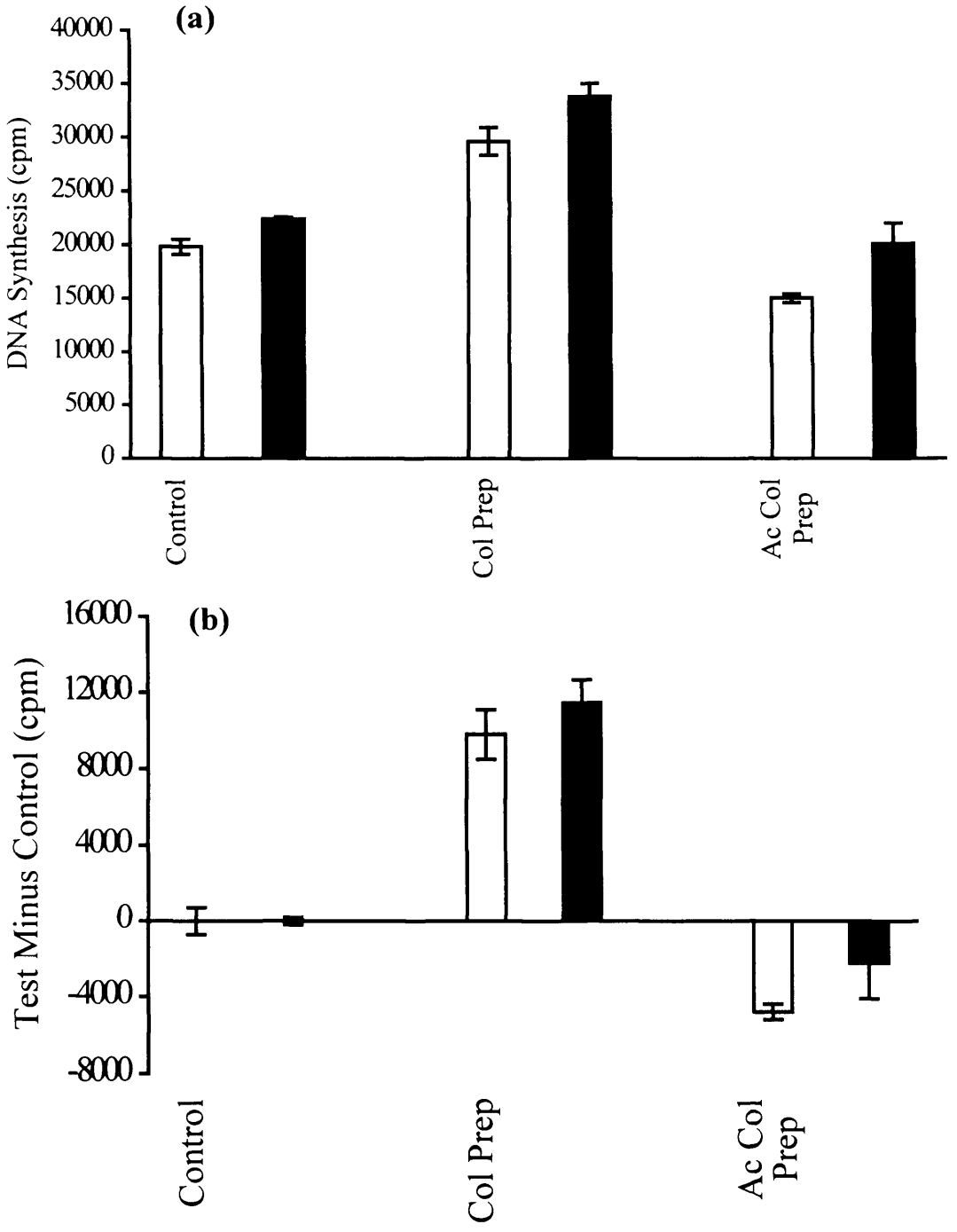


Figure 4.4 Effects of Bovine Colostral Preparation on RIE-1 DNA Synthesis Are Not Attributable To TGFβ.

RIE-1 DNA synthesis was measured in the presence of colostral preparation without (Col Prep) or with (Ac Col Prep) transient acidification (white bars). Identical cultures were performed incorporating 10 μg/ml anti-TGFβ neutralising antibody (black bars). (a) ^{3}H-thymidine incorporation is increased by antibody regardless of culture conditions (NSD). (b) When the effect of the antibody on control cells is taken into account there is no significant effect of neutralising antibody on colostral. Data represent mean+/-SEM (n=4). Three reproducible experiments were performed.

synthesis were mostly of the stimulatory, acid-sensitive kind, and acid-labile TGFβ was a minor component.

The data obtained using cultures of NIH-3T3 fibroblasts in the presence of HMW colostral preparation (unacidified and acidified), with or without the neutralising antibody, are shown in figure 4.5. Unacidified HMW colostral preparation stimulated NIH-3T3 DNA synthesis, and this stimulation was largely unaffected by inclusion of the neutralising antibody. However, the stimulatory effect of acidified HMW colostral preparation was reduced in the presence of neutralising antibody, and this was significant when the effect of the antibody alone was accounted for (figure 4.5b). Thus, unacidified HMW colostral preparation contained negligible active TGFβ, but transient acidification did liberate significant amounts of active TGFβ.

Overall, the data obtained in these studies show that there is TGFβ in the HMW colostral preparation being tested, which is enhanced significantly by transient acidification. The relative contribution of TGFβ and proliferative components to colostral bioactivity differed according to the model (i.e. cell line) being studied.

4.4 Transient Acidification Does Not Affect Colostral Pro-Migratory Activity, Which Is Independent Of TGFβ.

Most of the colostral activity measured using assays of DNA synthesis and cell migration was due to components of molecular weight greater than 30 kDa (chapter 3, figure 3.9 and 3.10). Some colostral components are capable of stimulating both DNA synthesis and migration, as demonstrated using some growth factors, including EGF (Basson et al, 1992) and PDGF (Szabo and Sandor, 1996). Thus, pro-migratory colostral components might also be destroyed by transient acidification. Studies were designed to analyse the pro-migratory bioactivity in HMW colostral preparation following transient acidification.

TGFβ plays a role in co-ordinating the cell signalling pathways that control cell migration in some models *in vitro* (Basson et al 1992; Dignass & Podolsky, 1993). However, there are several exceptions to these observations, including trefoil peptides, lysophosphatidic acid and short-chain fatty acids (Dignass et al, 1994a; Sturm et al 1999; Wilson & Gibson, 1997b).

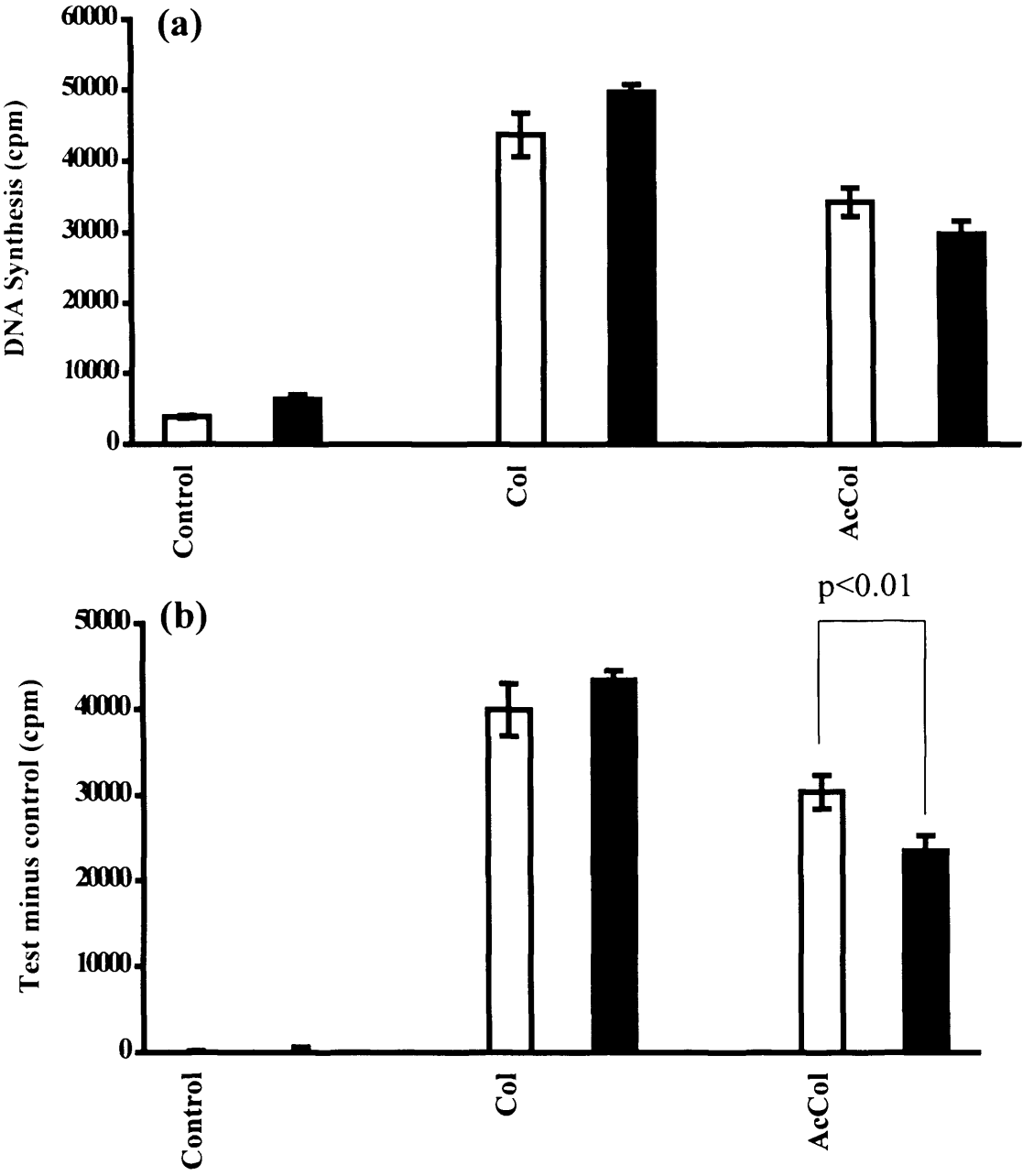


Figure 4.5. Colostral TGFβ Was Liberated by Transient Acidification.

NIH-3T3 fibroblasts were cultured in HMW colostral preparation without (Col) or with (Ac Col) prior transient acidification. Cultures were performed without (white bars) or with (black bars) anti-TGFβ antibody (10 μg/ml). (a) DNA synthesis was stimulated by colostral preparation with little effect due to the antibody. Antibody caused a small increase in DNA synthesis by control cells. (b) When the effect of antibody on control cells was taken into account, significant bioactivity in transiently acidified colostral preparation was abolished by the neutralising antibody ($p<0.01$ vs colostrum without antibody). Bars represent mean+/-SEM, n=4. Three reproducible experiments were performed.

The potential role of TGFβ in HMW colostral preparation was tested in a series of experiments using the HT-29 cell migration model. These migration assays were performed as described previously (chapter 2, section 2.2.4).

The data obtained using transiently acidified HMW colostral preparation are shown in figure 4.6. Colostral preparation stimulated migration of the leading edge within a few hours of wounding. Transient acidification of HMW colostral preparation did not reduce the potency of colostral preparation. Thus, pro-migratory colostral components were resistant to acidification. When these data are compared with those for RIE-1 DNA synthesis, it is clear that there is at least one colostral component that is resistant to severe, transient acidification, and that stimulates epithelial migration but not proliferation.

The HT-29 model of enterocyte migration was sensitive to exogenous TGFβ2, as demonstrated in figure 4.7. Migration was significantly enhanced by rTGFβ2 at 0.1 ng/ml. Incorporation of the neutralising antibody (10 μg/ml) caused increased migration by control cells, similar to the observations made using the thymidine incorporation assays. However, when antibody was incorporated into medium containing TGFβ2, migration was no different from control cells in the presence of the antibody. These data demonstrate that exogenous TGFβ2 stimulates migration, and that the neutralising antibody was effective in this assay.

The effect of the neutralising antibody on promigratory bioactivity in HMW colostral preparation was tested. Acidified colostral preparation was not tested, since transient acidification was shown not to have any effect (figure 4.6). The data from a typical experiment are shown in figure 4.8. HMW colostral preparation stimulated migration significantly. Neutralising antibody had no effect on the level of stimulation by colostral preparation. Thus, colostral TGFβ had no significant role in pro-migratory bioactivity in HMW colostral preparation, nor did any endogenous TGFβ that was stimulated by colostral components.

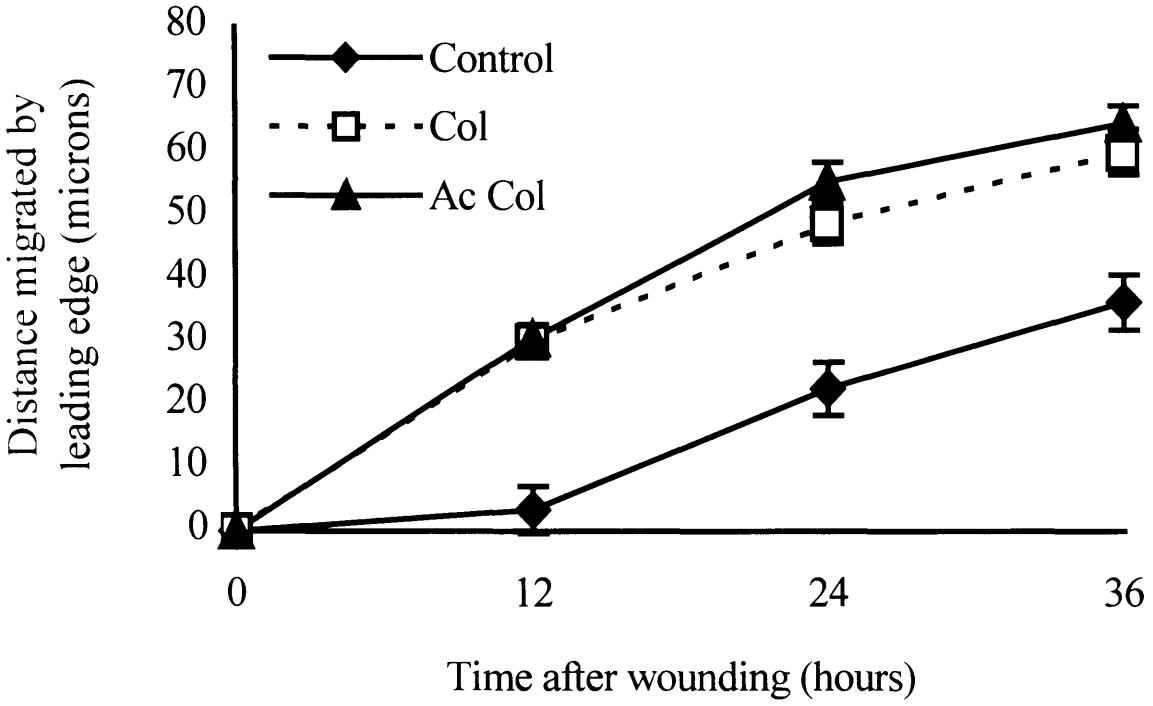


Figure 4.6 Transient Acidification of Colostral Preparation Had No Effect On Pro-Migratory Activity.

Wounded, confluent HT-29 cells were cultured in colostral preparation (30% v/v) in serum-free medium (white square), or in an equivalent solution of transiently acidified colostral preparation (triangles). Colostral preparation stimulated HT-29 migration at the wound edge ($p<0.01$ vs control at all time points). Transient acidification of colostral preparation had no effect on pro-migratory activity in colostral preparation ($p>0.05$ vs unacidified colostral preparation at all time points). Data represent the mean+/-SEM (n=4). Four reproducible experiments were performed.

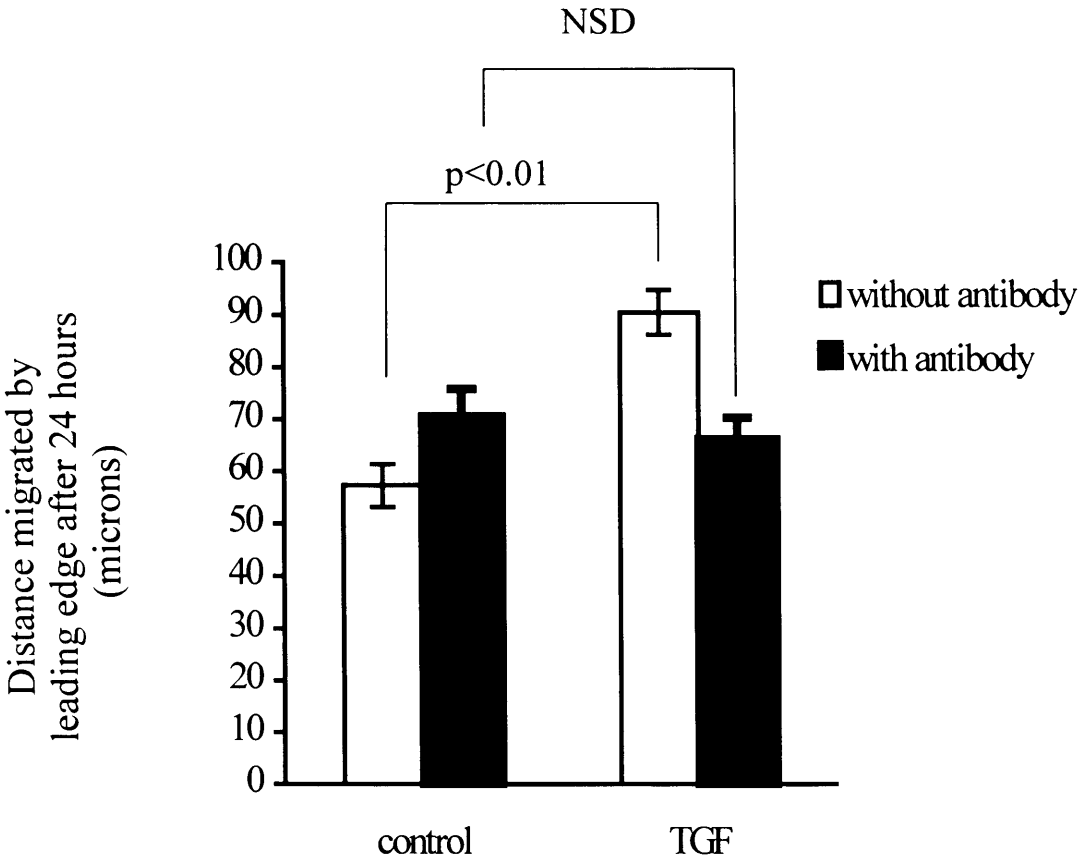


Figure 4.7 Exogenous TGFβ Stimulated HT-29 Migration.

Wounded, confluent HT-29 cells were cultured in the presence of recombinant TGFβ2 (0.1 ng/ml). TGFβ2 stimulated migration at the wound edge (white bars, $p<0.01$). The neutralising antibody (10 μg/ml) caused a small increase in the migration of control cells. Incorporation of the antibody abrogated the effects of *r*TGFβ2 (black bars). Results show the mean+/-SEM, n=4. Three reproducible experiments were performed.

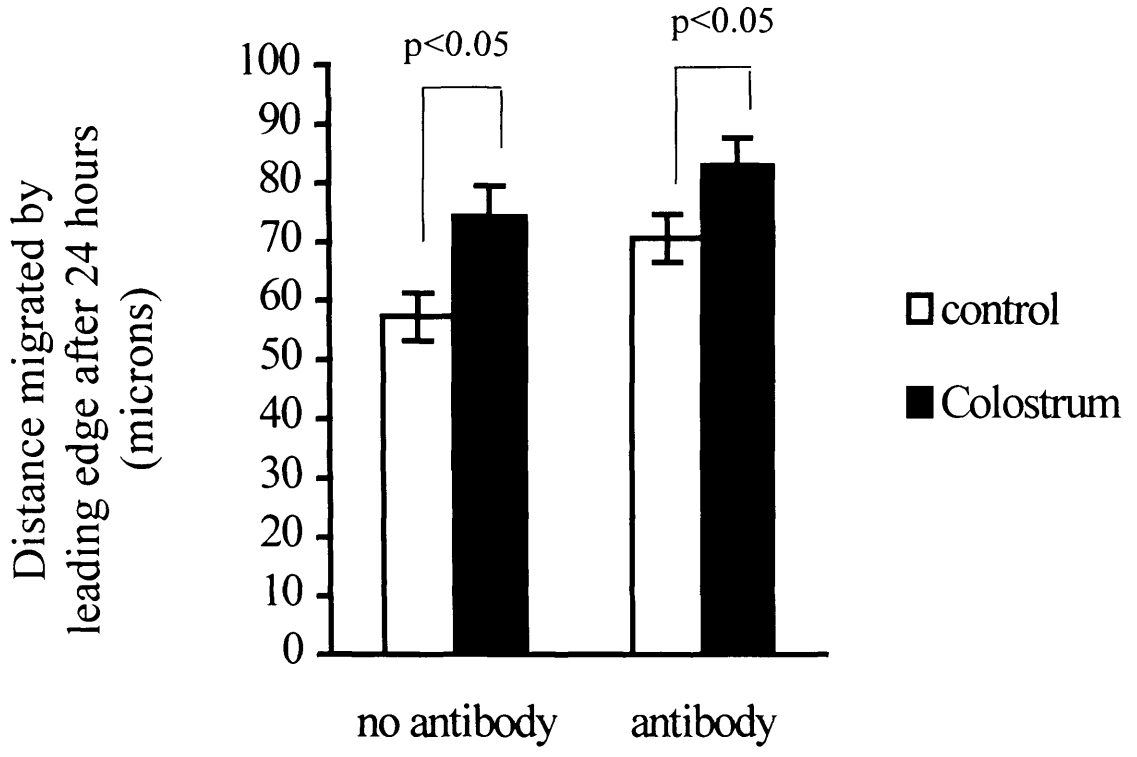


Figure 4.8 Pro-Migratory Activity In Bovine Colostral Preparation Was Not Due To TGFβ.

Wounded, confluent HT-29 cells were cultured in 30% v/v colostral preparation (grey bars) without or with anti-TGFβ neutralising antibody (10 μg/ml). Colostral preparation stimulated migration at the wound edge ($p<0.05$ vs appropriate control). The antibody had no effect on pro-migratory activity in colostral preparation. Data represent mean+/-SEM (n=4). Three reproducible experiments were performed.

4.5 Colostral-Equivalent Recombinant TGFβ2 Protects the Small Intestine Against NSAID-Induced Injury

TGFβ proteins are important modulators of wound healing *in vivo*, which can modulate cell proliferation and migration, stimulate production of extra-cellular matrix and affect the immune system (Dignass et al, 1993; Letterio and Roberts, 1998; Lyons and Moses, 1990; Yanaka et al, 1996). The mass of data published so far has concentrated on the TGFβ1 isoform, although increasing numbers of publications are presenting data obtained using TGFβ2, the major colostral isoform (Rogers et al, 1996).

The preceding studies in this chapter showed that TGFβ was present in HMW colostral preparation, which became active in response to transient acidification, whereas other components, such as those that stimulate epithelial DNA synthesis, were inactivated by transient acidification (figures 4.1-4.5). Thus, TGFβ might play a more significant role in the gut *in vivo* that *in vitro*, since other colostral components might not survive passage through the stomach.

Colostral preparation administered to mice via drinking water prevents NSAID-induced small intestinal damage, measured by the prevention of reduction in villus height, suggesting that colostral preparation might act as a prophylactic for patients receiving NSAID therapy (Playford et al, 1999). The volume of colostral preparation consumed per animal per day in the published study contains approximately 12.5 ng/ml TGFβ. The effect of this amount of recombinant TGFβ2 on NSAID-induced small intestinal injury was measured using the same model.

The details of the mouse small intestinal damage model have been described elsewhere (Playford et al, 1996; see chapter 2, section 2.2.5). C57Bl mice (20 per group) were used. Briefly, recombinant TGFβ2 was administered at 12.5 ng/ml in water containing 0.2% w/v BSA. Separate animal groups received subcutaneous TGFβ2 (12.5 ng/animal/day) in BSA (0.2% w/v). Control groups of mice received iso-proteinaceous, sterile water via the same two routes. Treatment continued daily for 6 days. On the sixth day half of the animals in each group received a single injection s.c. of indomethacin (85 mg/kg) in 5% bicarbonate solution.

Control animals received equivalent volumes of 5% bicarbonate solution by s.c. injection. Two hours before sacrifice on the seventh day all animals received vincristine (1 mg/kg of 0.1 mg/ml solution in sterile water) via i.p. injection. At sacrifice, the gut of each animal was dissected, washed, blotted dry, weighed and measured. The gut was sectioned, and each sample fixed in Carnoy's solution. Tissues were stained with Schiff's reagent and microdissected before analysis of villus height, crypt depth and the number of crypt cell mitoses (Goodlad, 1994).

At sacrifice, mice treated with indomethacin were observed to have darkened gut contents, indicative of bleeding into the lumen. The length of the small intestine of each animal was measured, and the mean length found to be reduced by indomethacin (figure 4.9). However, administration of rTGFβ2 did not modulate the effect of indomethacin.

Damage to the small intestine was measured by the reduction in the height of intestinal villi. The effect of indomethacin was greatest at the site around 75% of the small intestinal length, where villi were 22% shorter than those from control animals (figure 4.10). These data were similar to those of Playford et al (1996). Treatment with rTGFβ2 prevented villus shrinkage (figure 4.10). The villi of animals receiving rTGFβ2 orally or sub-cutaneously were not significantly different from those of control animals. TGFβ2, without subsequent indomethacin, had no effect on villus height when compared with control animals (figure 4.11). Thus, rTGFβ2, at an amount equivalent to that found in a volume of colostral preparation known to be effective in this model, protects the small intestine from NSAID-induced injury as measured by villus shrinkage.

Indomethacin did not affect intestinal crypt depths (figure 4.12a). However, treatment of animals with rTGFβ2, via either route, before injection with indomethacin resulted in significantly deeper crypts (figure 4.12a). Analysis of the intestinal crypts of animals who had received rTGFβ2 without indomethacin revealed that whilst oral administration had no effect there was a *significant reduction* in crypt depth caused by s.c. rTGFβ2 (figure 4.12b). These data suggest that the mechanisms by which s.c. rTGFβ2 protected the small intestine involved production of shallower crypts in the absence of injury, which subsequently became much deeper in response to exposure to indomethacin.

The transition from shallower to deeper crypts might have been due to a rapid increase in cell proliferation in response to indomethacin. The average number of mitoses per crypt following

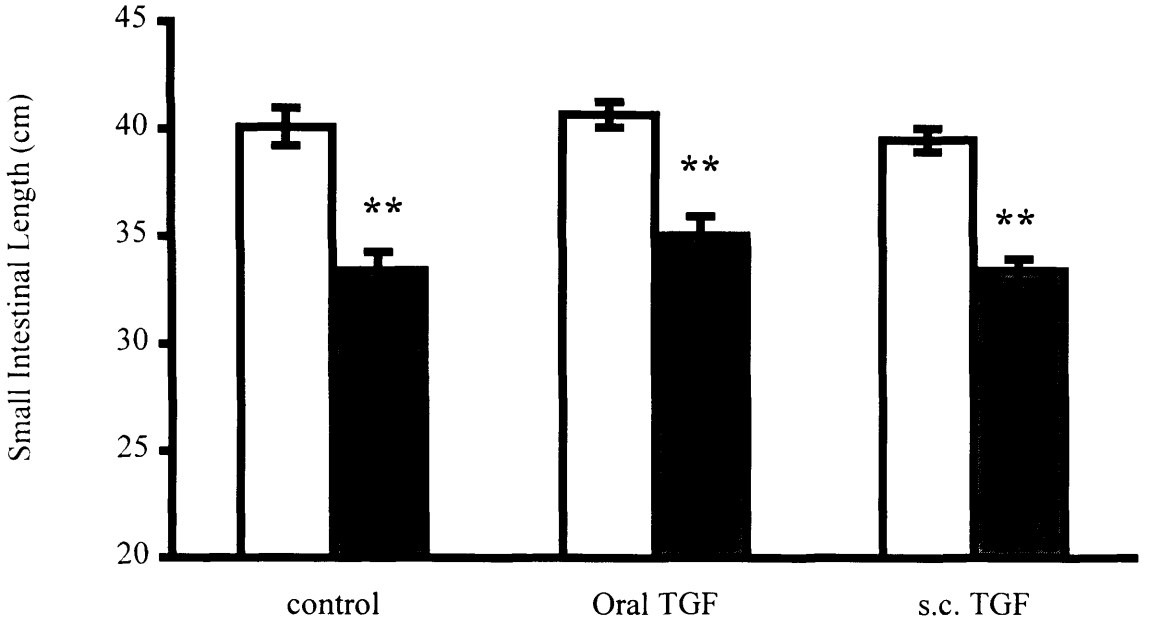


Figure 4.9 Small Intestinal Length Was Shorter Following Indomethacin.

Mice were injected s.c. with indomethacin in 5% bicarbonate (grey bars) or vehicle(white bars), which resulted in a significant decrease in small intestinal length, regardless of pre-treatment with *r*TGFβ2. ** $p<0.001$ vs controls. Data represent mean +/- SEM (n=10).

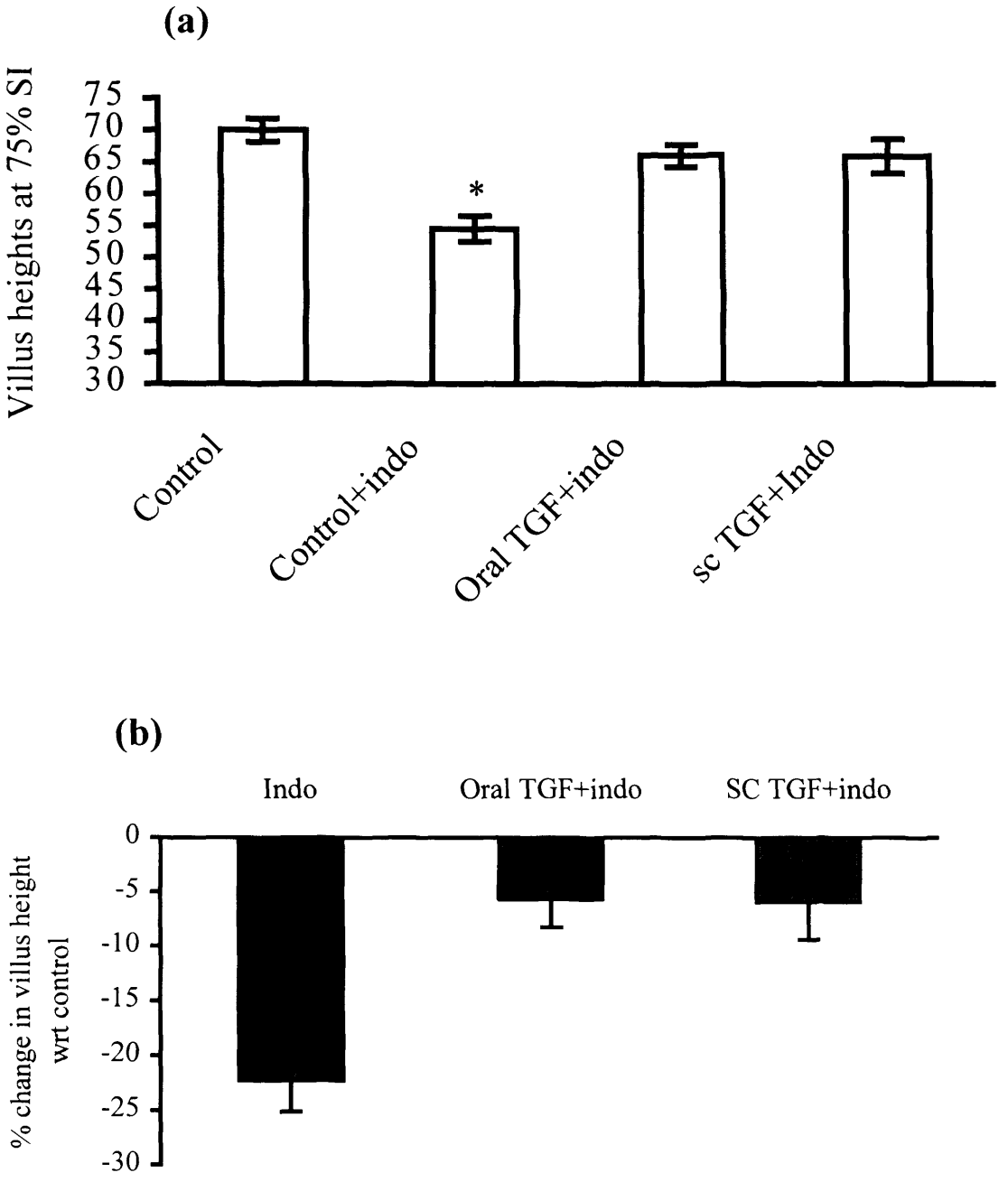


***Figure* 4.10 *r*TGFβ2 Prevented Villus Shrinkage Caused By Indomethacin.**

(a) Mean villus height was reduced at 75% of small intestinal length by indomethacin. *r*TGFβ2 adminstered orally or sub-cutaneously prevented indomethacin-induced villus shrinkage. (b) shows the percentage villus shrinkage compared with control. * $p<0.05$ vs control. Data represent mean+/-SEM (n=10).

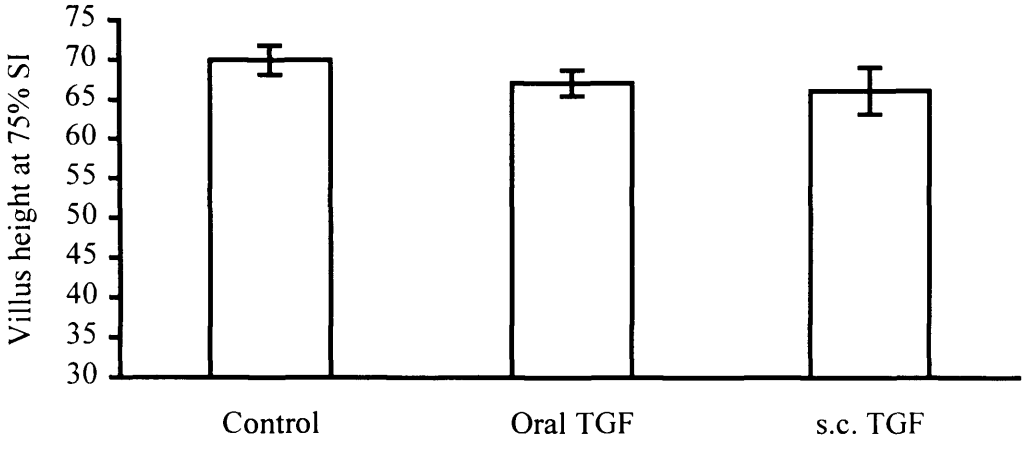


Figure 4.11 *r*TGFβ2 Had No Effect On Villus Height.

Mice were given oral or sub-cutaneous *r*TGFβ2 for 7 days. Small intestinal villus height wsa not significantly different from controls ($p>0.05$). Bars represent mean+/-SEM (n=10).

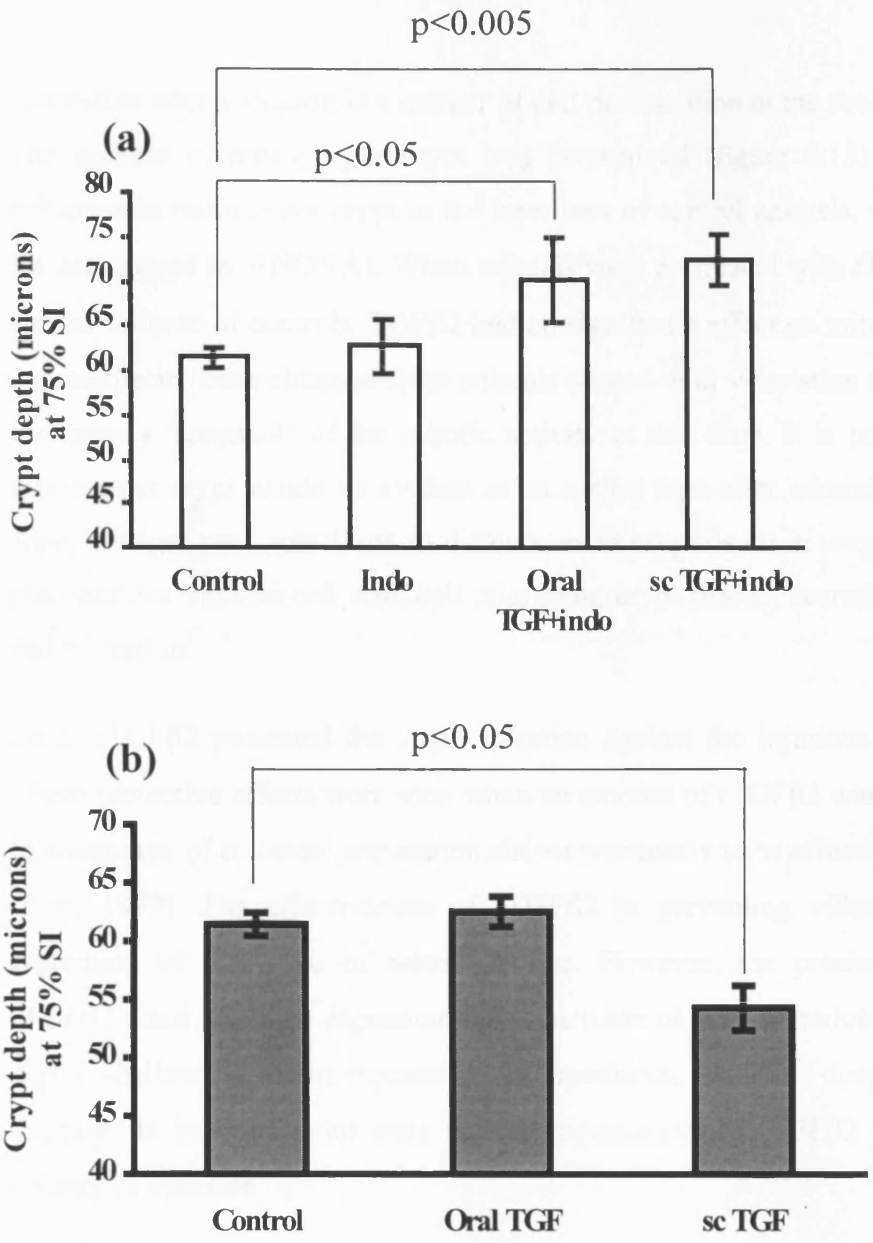


Figure 4.12 Small Intestinal Crypt Depth Was Greater Following Indomethacin and rTGFβ2.

(a) Indomethacin had no effect on the depth of intestinal crypts of control animals. Indomethacin caused a significant increase in crypt depth following rTGFβ2. (b) Administration of s.c. rTGFβ2 to control animals caused a significant reduction in crypt depth. Bars represent mean+/-SEM (n=10).

vincristine administration is a marker of cell proliferation at the time the animals were killed. The number of mitoses per crypt was determined (figure 4.13). Indomethacin caused a reduction in mitoses per crypt in the intestines of control animals, which was not significant (as determined by ANOVA). When animals were pretreated with rTGFβ2, crypt depths were similar to those of controls. TGFβ2 had no significant effect on mitoses per crypt without s.c. indomethacin. Data obtained from animals treated with vincristine two hours before sacrifice represent a 'snapshot' of the mitotic activity at that time. It is possible that differences in mitoses per crypt would be evident at an earlier time after administration of indomethacin. Also, changes may contribute to differences in crypt depth in ways that do not involve cell proliferation, such as cell size, cell migration, crypt fission, secretion of extracellular matrix and hydration.

Thus, rTGFβ2 protected the small intestine against the injurious effects of indomethacin. These protective effects were seen when an amount of rTGFβ2 was used that was equivalent to an amount of colostral preparation shown previously to be effective in this model (Playford et al, 1999). The effectiveness of rTGFβ2 in preventing villus shortening was similar regardless of the route of administration. However, the precise mechanisms by which rTGFβ2 acted might be dependent upon the route of administration, since its effects on crypt depth (shallower without exposure to indomethacin, becoming deeper than normal following exposure to indomethacin) were most pronounced when rTGFβ2 was administered by sub-cutaneous injection.

4.6 Discussion

The studies presented in this chapter were devised to test the effect of transient acidification, such as might be encountered during passage through the stomach, on HMW colostral preparation *in vitro*. Some of the colostral components that stimulated DNA synthesis were sensitive to the effects of transient acidification, whereas pro-migratory components were resistant to such effects. Acid lability is a demonstrated characteristic of colostral TGFβ (Rogers et al, 1996). The role of TGFβ in colostral preparation was tested with and without transient acidification. Intestinal epithelial cell DNA synthesis stimulated by colostral preparation was largely independent of TGFβ, whereas fibroblast DNA synthesis was stimulated partly by TGFβ in acidified colostral preparation. Acid-resistant, pro-migratory colostral bioactivity was not due to TGFβ, since a suitable neutralising antibody had no effect

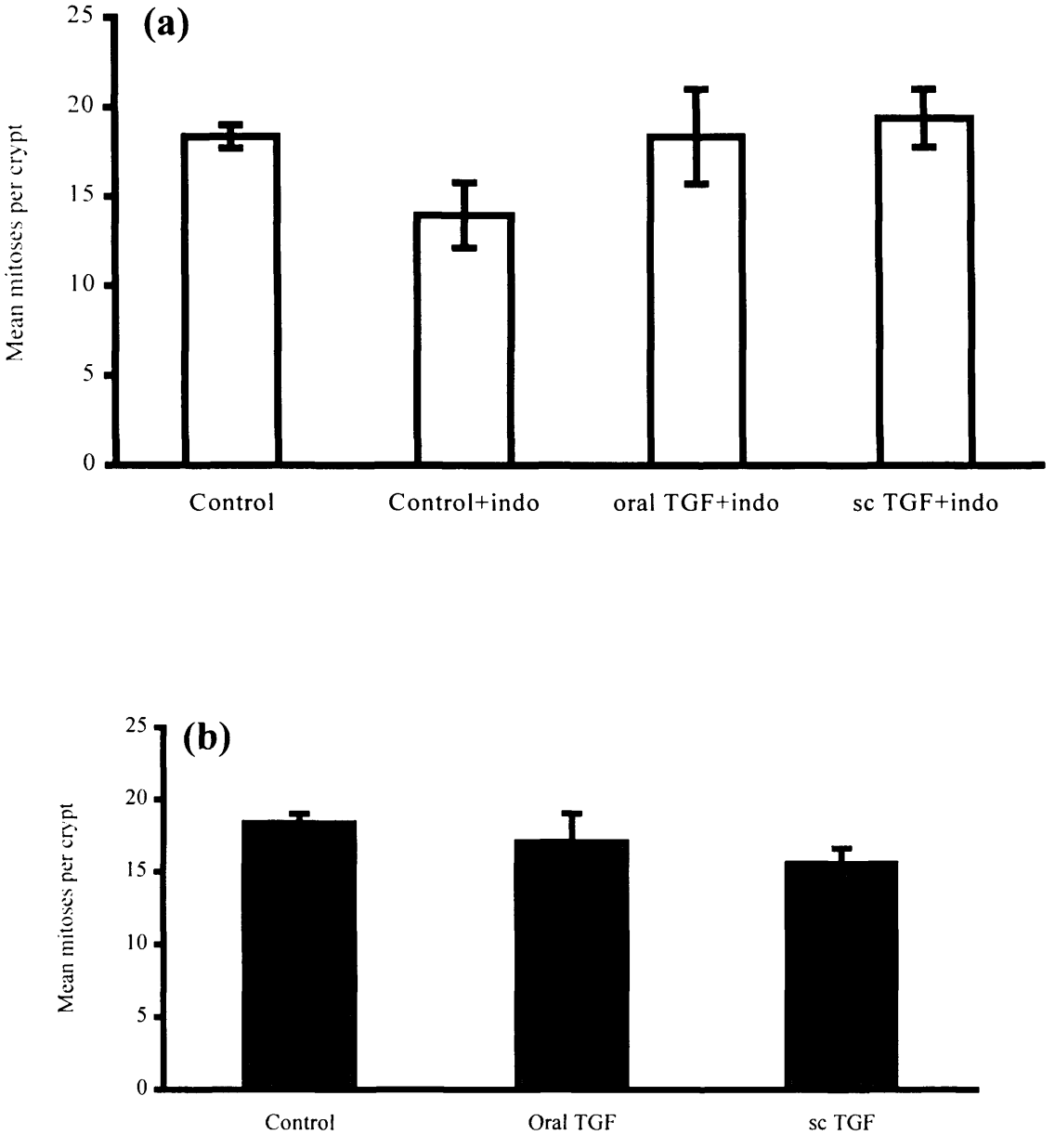


Figure 4.13 *r*TGFβ2 Prevents Indomethacin-Induced Reduction In Small Intestinal Crypt Mitoses.

(a) Indomethacin causes a small decrease in crypt mitoses, which is not evident when mice are pre-treated with *r*TGFβ2. (b) *r*TGFβ2 alone has no significant effect on crypt mitoses in control animals. Bars represent mean+/-SEM, n=10.

on the stimulatory effect of HMW colostral preparation. However, a subsequent study *in vivo,* utilising the injurious effects of indomethacin on the small intestine, demonstrated that TGFβ was present in colostral preparation in sufficient quantities to protect fully the small intestine against NSAID-induced injury.

The data obtained for enterocyte and fibroblast DNA synthesis showed that HMW colostral preparation must contain a variety of active molecules. Intestinal RIE-1 DNA synthesis was stimulated by acid-sensitive colostral components. Inclusion of an anti-TGFβ neutralising antibody showed that TGFβ had a negligible role in bioactivity on RIE-1 cells. However, NIH-3T3 fibroblast DNA synthesis stimulated by acidified colostral preparation was partly due to TGFβ, since the antibody reduced the level of stimulated DNA synthesis. These data might be due to incomplete titration of TGFβ by the neutralising antibody. However, the amount of TGFβ present in 100 μl of a 30% dilution (less than 10 ng/ml) was less than that used in preliminary assays (10 ng/ml), where the effects of TGFβ were completely abolished.

The proliferative effects of colostral preparation on gut epithelial cells might be severely decreased by the passage through the stomach. However, proliferation of other cell types, such as fibroblasts, is stimulated by transiently acidified colostral preparation (Rogers et al, 1996). Sub-epithelial myo-fibroblasts (SEMs) are important modulators of gut injury (see sections 1.2.3.2.2 and 1.3.3.3), which might contribute to the effects of colostral preparation *in vivo* (Playford et al, 1999). Studies of the effect of colostral preparation on SEMs, and on co-cultures of epithelial cells and SEMs are required for greater understanding of colostral bioactivity

High molecular weight (HMW) colostral preparation stimulated enterocyte migration independently of its effects on proliferation (chapter 3, figure 3.8). This might be accounted for by pluripotent colostral components, or by separate proliferative and pro-migratory components. Pro-migratory colostral components were resistant to acidification, unlike those responsible for epithelial DNA synthesis. Thus, there is at least one component in bovine colostrum that is greater than 30 kDa, resistant to transient acidification and stimulates enterocyte migration. Partially characterised PDGF-like colostral growth factors have been described that are suitable candidates in terms of their size and biochemistry (Brown and Blakely, 1984; Shing and Klagsbrun, 1984). However, their effects on cell migration are

untested. Ingested colostral preparation can be expected to retain its restitutive activity during passage through the gut, explaining partially its *in vivo* activity (Playford et al, 1999).

In addition to this interpretation, these data show that colostral growth factors and cytokines do not stimulate HT-29 migration via liberation of endogenous TGFβ, a phenomenon described by Dignass and Podolsky (1993). This difference might be due to the cell line used (Dignass and Podolsky used the non-transformed rat IEC-6 cell line) or to the non-TGFβ-dependent stimulation of HT-29 migration by colostral components.

The data shown in sections 4.2-4.4 in this chapter showed that the direct role of colostral TGFβ in epithelial wound healing *in vitro* was equivocal. However, the data obtained using fibroblasts (figure 4.5) suggested that colostral TGFβ might have indirect effects on epithelial injury, for example by its stimulating sub-epithelial myofibroblasts *in vivo*. Colostral preparation protected the murine small intestine from NSAID-induced injury (Playford et al, 1999). The effective dose of colostral preparation was equivalent to 12.5 ng TGFβ per animal per day. This amount of recombinant TGFβ2 also protected the murine small intestine from NSAID-induced injury as measured by prevention of villus shortening (section 4.5, figure 4.10). The growth factor was equally potent whether administered orally via drinking water, or via a single, daily s.c. injection. Oral administration of rTGFβ2 will deliver the growth factor to the luminal surface of the mucosa, whereas sub-cutaneous rTGFβ2 will act systemically. Thus, the preventative mechanisms of rTGFβ2 may be stimulated via different pathways depending upon the route of administration. It is clinically relevant that data obtained from oral administration demonstrated that ingested growth factor retained its bioactivity in the small intestine. Similar results were demonstrated using recombinant TGFβ1, the minor form of colostral TGFβ (Playford et al, 1999).

The combined effects of indomethacin and recombinant TGFβ2 on small intestinal crypt depths are particularly interesting, considering the protective effect of rTGFβ2. Sub-cutaneous TGFβ2, without subsequent indomethacin, resulted in shallower intestinal crypts than normal, whereas oral TGFβ2 had no effect. This may be due to inhibited proliferation, to altered cell size or to changes in secretion of extracellular matrix. When indomethacin was administered, crypt depths became greater rapidly, i.e. within 24 hours. This effect was seen regardless of the route of administration. However, increased crypt depth was greatest in animals receiving s.c. TGFβ. This might be accounted for by transient stimulation of

proliferation; however, there was no evidence of this when crypt mitoses were counted (figure 4.13). Any period of increased proliferation may have preceded the time of vincristine injection and sacrifice. Counting the number of cells per crypt would provide data to aid this interpretation.

Also, rapidly increased crypt depth might be due to increased cell size, or to changes in the basement membrane, which might facilitate restitution. These changes would require effects on the extracellular matrix and cytoskeletal changes in the sub-mucosal layers, which are known effects of TGFβ (Powell et al, 1999; Uribe and Barrett, 1997). These hypotheses require further study.

In conclusion, colostral preparation contains multiple components that affect cell growth and migration; some of these are sensitive to transient acidification, such as would be encountered in the stomach. Components that stimulate enterocyte migration *in vitro* are resistant to transient acidification. No large-scale appearance of acid-generated colostral TGFβ was found. However, there is enough TGFβ in colostral preparation to provide protection against NSAID-induced small intestinal injury, and is effective when administered orally. Thus, colostral TGFβ might have a more important role in colostral bioactivity *in vivo* than *in vitro*.

Chapter 5.

Evaluation of Bovine Colostral Preparation As Treatment For Inflammatory Bowel Disease Using An Experimental Model Of Colitis

5.1 Introduction

Inflammatory diseases of the large bowel – Crohn's disease (CD) and ulcerative colitis (UC) – are highly complex, involving genetic, environmental, immunological, vascular, epithelial and gut luminal components (see Chapter 1, section 1.4.4). Hodgson (1998) suggests that the diversity of mechanisms involved in Crohn's disease will limit the success of treatments targeting just one mechanism. Similarly, mechanisms of gut injury in ulcerative colitis are manifold (Fiocchi, 1998; Gibson, 1997), so that treatments for UC might similarly benefit from being multi-potent.

Corticosteroids and immunosuppressive agents are common treatments for inflammatory bowel diseases (Hanauer and Baert, 1995; Jarnerot et al, 1998). However, such treatments are associated with a wide variety of side effects, including osteoporosis, psychological disturbance, infection, obesity, hirsuitism, renal complications, growth retardation, gastrointestinal complications, malignancy, and anti- and peri-natal effects (table 1.2; reviewed by Connell and Taylor, 1997 and Sands, 2000). Such complications can necessitate change or cessation of therapy. Also, patients must be monitored extensively and thoroughly to avoid the appearance of these complications (Connell and Taylor, 1997). Thus, whilst current therapies for inflammatory bowel disease can be effective, the range of associated side effects make desirable equally or more effective treatments, with fewer complications.

A notional treatment for inflammatory bowel disease can be expected to be multi-faceted in its action, and preferably inexpensive. Bovine colostral preparation fits these criteria. The bioactivities in colostrum – growth factors, antibodies, antibiotic peptides – can be expected to contribute to a wide range of anti-inflammatory processes *in vivo* (Koldovsky et al, 1993; Korhonen et al, 2000; Srivastava et al, 1996; van Hooijdonk et al, 2000; Xu, 1996). Access to bovine colostral preparations is currently unregulated, suggesting that there are no serious side effects associated with consumption of this natural product. The studies presented in this chapter were devised to test the effect of bovine colostral preparation on bowel inflammation and ulceration caused by trinitrobenzene sulfonic acid (TNBS), an established model of inflammatory bowel disease (Morris et al, 1989; Yamada et al, 1992).

The model comprises two components, which are administered as an enema; ethanol breaks down the colonic epithelium, allowing ingress of TNBS, a hapten. The hapten reacts with

proteins to cause immunological responses (Morris et al, 1989). The TNBS model is generally accepted to be most like Crohn's disease, based on analysis of its immunopathology (Strober, 1998). However, it is noteworthy that one important 'Crohn's-like' characteristic – disregulated Th1-T cell response, governing local immune response – is one that may not be a real distinction of Crohn's disease from ulcerative colitis in humans (Hodgson, 1998; Sands, 2000). A variety of treatments have been found to modulate colonic injury resulting from ethanol/TNBS, including growth factors (Giladi et al, 1995; Luck and Bass, 1993; Procaccino et al, 1994), inhibitors of leukotriene synthesis (Wallace and Keenan, 1990), trefoil peptides (Tran et al, 1999), nutrients (Amenho et al, 1997), plant extracts (Zhou and Mineshita, 1999), inhibitors of nitric oxide synthase (Kiss et al, 1997) and induction of oral tolerance (Neurath et al, 1996).

5.2 Characterisitics of TNBS Model of Colitis in Rats.

TNBS-induced colitis is modulated by epidermal growth factor (EGF) (Procaccino et al, 1994). The conditions used by Procaccino et al were repeated to test the *in vivo* activity of a recombinant preparation of EGF (supplied by CIGB, Havana, Cuba). Data from this study are presented here to demonstrate the salient features of the model.

Briefly, male Sprague-Dawley rats (250-300g) received daily either rhEGF (600μg/kg, n=18) or BSA (0.1% w/v, n=24) intra-peritoneally. TNBS (30 mg in 50% ethanol, 0.25 mls) was administered by enema, one hour after the first EGF injection, to all animals in the EGF group and to 18 animals in the BSA (control group). The remaining 6 animals in the control group received an enema of PBS (0.25 mls). This group of six (PBS control) was sacrificed after 24 hours to provide normal data. Also, 6 animals in receipt of TNBS and BSA, and six in receipt of TNBS and rhEGF were sacrificed after 24 hours. Subsequently, colitic animals from the EGF and control groups (n=6) were sacrificed 48 hours and 7 days after induction of colitis. Colons were removed from freshly killed animals, then processed and analysed as described in chapter 2.

TNBS/ethanol caused a significant increase in colon weight compared with normal (figure 5.1). The mean weight of damaged colons continued to increase during the course of the

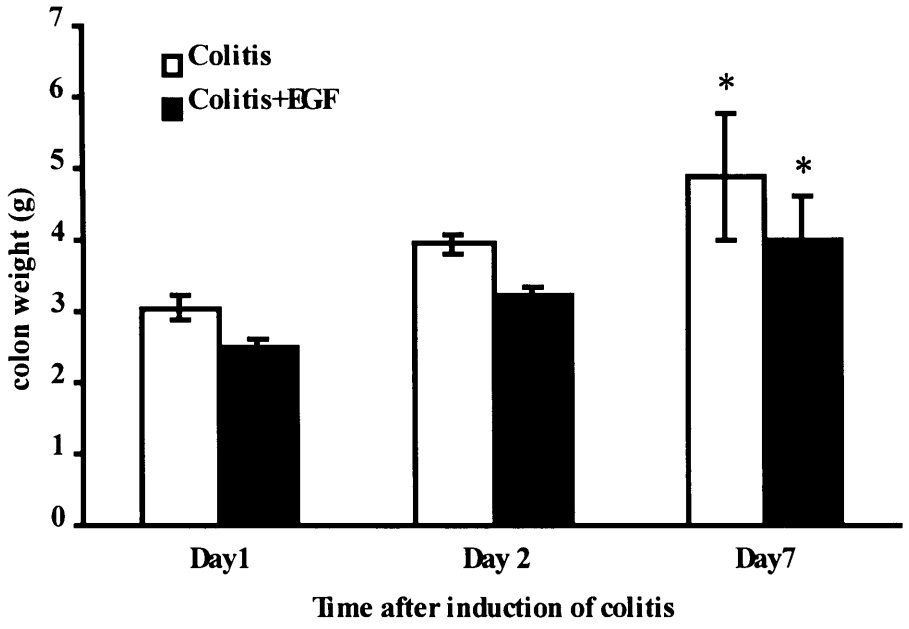


Figure 5.1 Colitis Caused Increased Colon Weight.

Rats were given TNBS enemas with or without i.p. rhEGF. Colons of colitic animals were heavier than normal (normal weights 1.53g, $p<0.01$). Colon weights continued to increase over time (heavier on day 7 than on day 1, $p<0.05$, signified by *). EGF caused a small reduction in colitis-associated colon weight (NSD). Bars represent mean+/-SEM, n=6.

experiment. Treatment with rhEGF caused a small reduction the mean colonic weight when compared to the weight of untreated, damaged colons.

Macroscopic damage to the mucosal surface of the excised colon was evaluated using the five-point scale of damage shown in table 5.1 (Morris et al, 1989). Colitic colons from all animals scored maximally after 24 and 48 hours after TNBS (figure 5.2). Recombinant hEGF had no effect on macroscopic damage at these times. Colitic animals not in receipt of rhEGF showed signs of healing after 7 days, signified by a small decrease in mean damage score (NSD). Animals treated with rhEGF showed increased healing after seven days. However, this effect was not statistically significant when compared with untreated colitic tissue ($p<0.02$).

Representative colons were photographed after longitudinal dissection and cleaning (figure 5.3). Untreated colitis resulted in extensive inflammation and ulceration along the length of the distal colon after 48 hours. Animals treated with rhEGF had visibly less damage, although still scored maximally according to the scale used. Untreated animals still exhibited severe colitis after 7 days, which was visibly reduced by treatment with rhEGF. The disparity between the damage scored according to table 5.1 and the visible damage showed this scale to be insufficient.

Bowel inflammation is characterised by infiltration of mononuclear cells from the circulation (Krawisz et al, 1984; Sekizuka et al, 1988; Smith et al, 1978). Myeloperoxidase (MPO) enzymatic activity is a marker of this infiltration, which can be modulated in response to some anti-colitic treatments (Procaccino et al, 1994; Wallace and Keenan, 1990), but not others (Giladi et al, 1995). The details of the colorimetric MPO assay are described in chapter 2 (section 2.2.6.2). TNBS increased MPO activity compared to normal values (figure 5.4). Treatment with rhEGF reduced MPO activity associated with colitis compared with untreated colitic tissue. This effect was not significant (determined by ANOVA, factor – treatment, $F<0.02$).

Colitic animals in the EGF study lost significant body weight (figure 5.5). A nadir was reached between 3 and 5 days, with signs of recovering body weight after 7 days. Treatment with rhEGF minimised weight loss during the first three days of colitis. After the fourth day,

Table 5.1 Scoring System For Assessment of Macroscopic Damage. (Taken from Morris et al, 1989)

Score	*Appearance*
0	No damage
1	Slight bowel wall thickening, no hyperaemia, no mucosal erosions
2	Hyperaemia and bowel wall thickening (inflammation), or severe inflammation without hyperaemia
3	Single erosion with hyperaemia and bowel wall thickening
4	Two or more erosions with hyperaemia and/or bowel wall thickening
5	Two or more major sites of damage or major damage extending more than 1 cm along the length of the colon

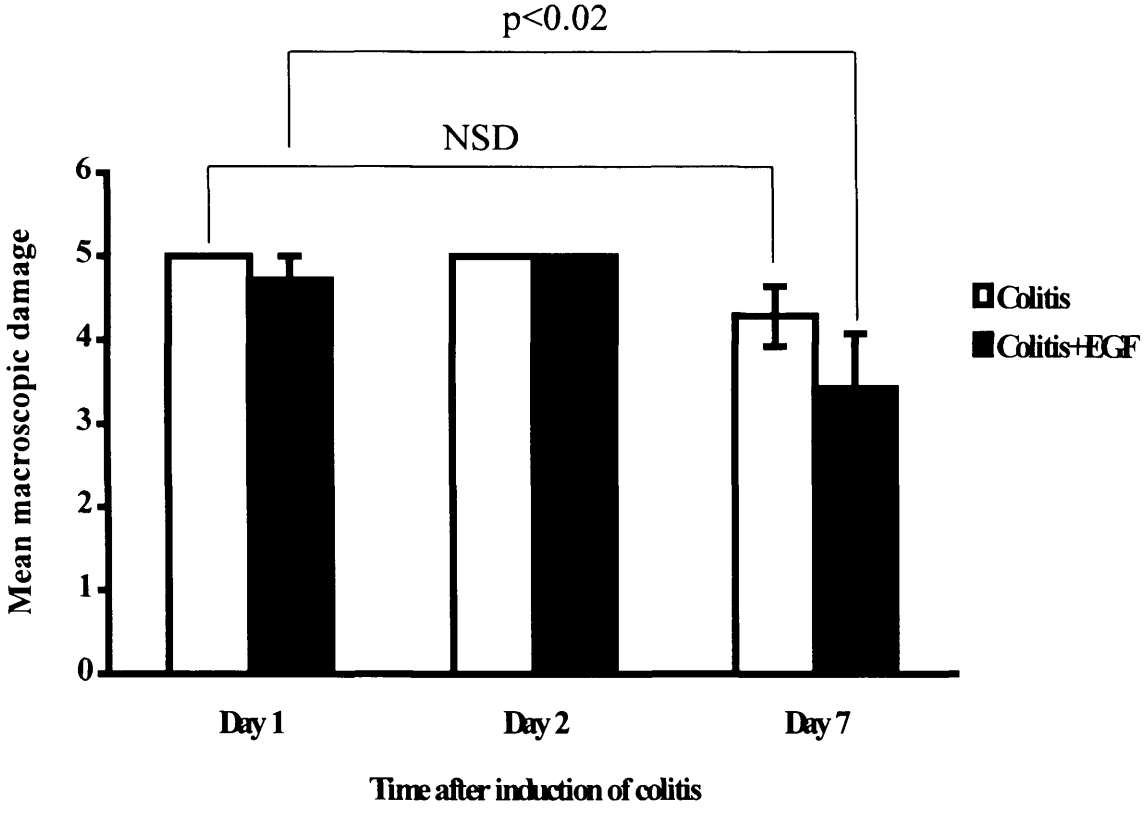


Figure 5.2 rhEGF Stimulated Healing of Macroscopic Colitic Damage.

Macroscopic damage to the colon was scored using the system described in table 5.1 (Morris et al, 1989). Damage was maximal after 24 and 48 hours post-TNBS enema (normal controls scored 0.42+/-0.2). rhEGF had no effect. Seven days after TNBS enema macroscopic damage was reduced in untreated animals (NSD vs Day 1) and more convincingly in EGF-treated animals ($p<0.02$ vs day 1). Bars represent mean +/- SEM, n=6.

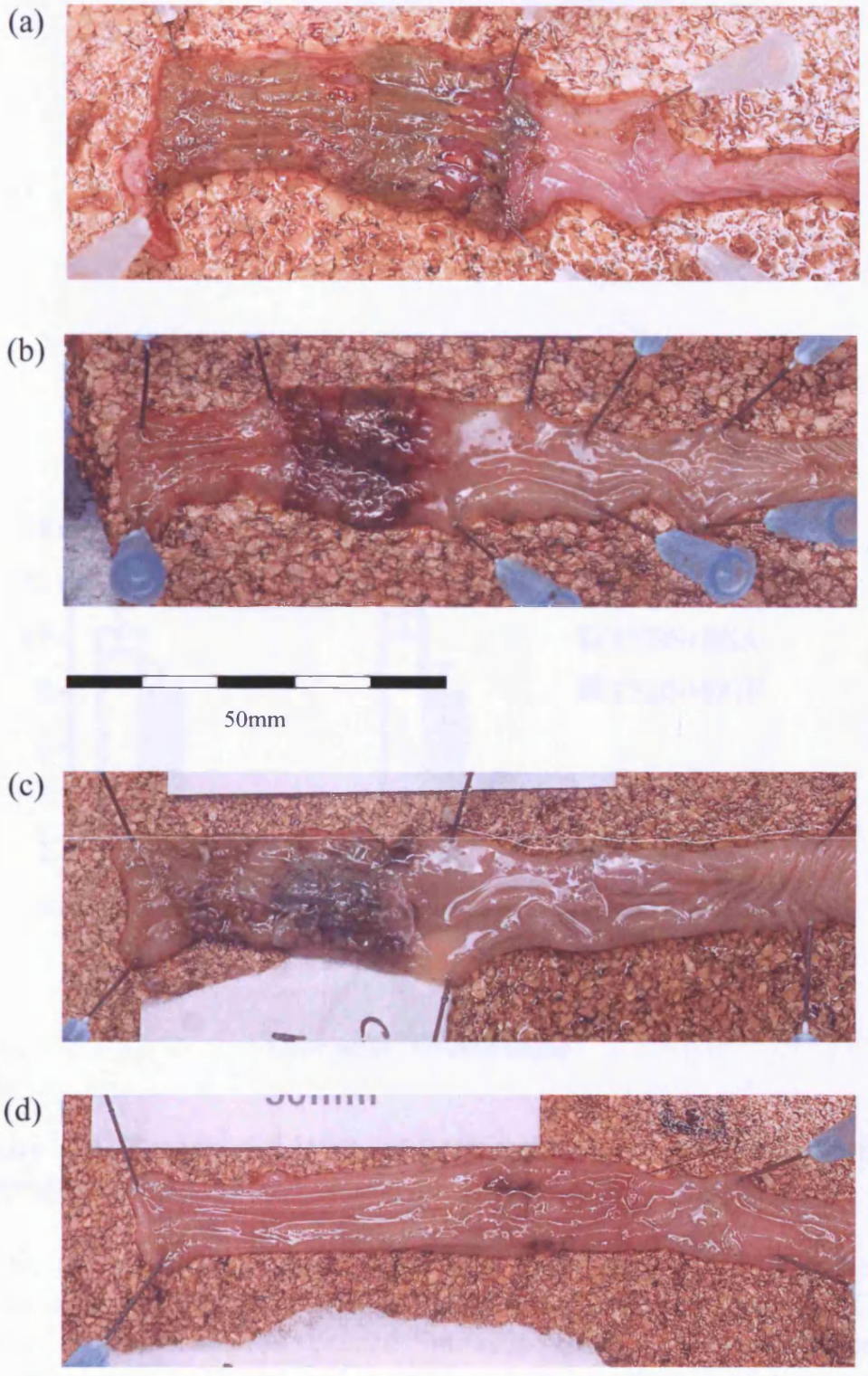


Figure 5.3 Treatment of TNBS-Induced Colitis with EGF.

(a) and (c), colitis induced by TNBS, sham treatment with i.p. BSA.
(b) and (d), colitis induce by TNBS, treated with i.p. EGF.

(a) and (b), colons from animals sacrificed 48 hours after induction of colitis.
(c) and (d), colons from animals sacrificed 7 days after induction of colitis.

See main text for discussion

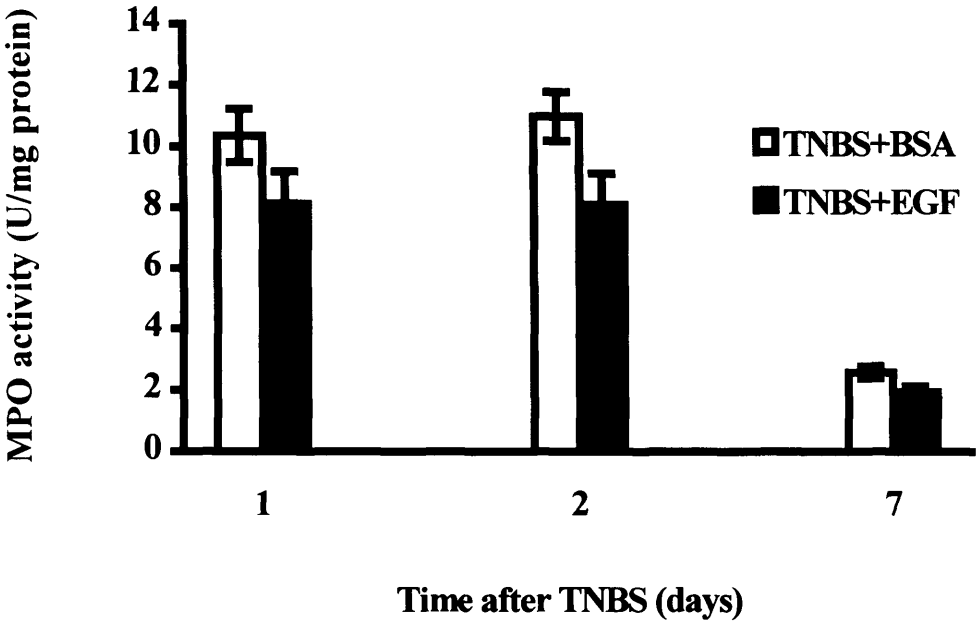


Figure 5.4 Treatment With rhEGF Caused A Small Reduction In MPO Activity Due To Colitis.

Colitic animals were treated with BSA or with EGF, as described in the text. Extracts of colonic tissue (0.5g) were made and tested for MPO activity and protein content. Colitis caused incresaed MPO (normal values 1.98+/-0.3). Treatment with EGF resulted in reduced MPO activity at all time points, although these differences were not statistically significant (ANOVA, factor: treatmetn, $F<0.2$). Bars represent mean+/-SEM, n=6-7.

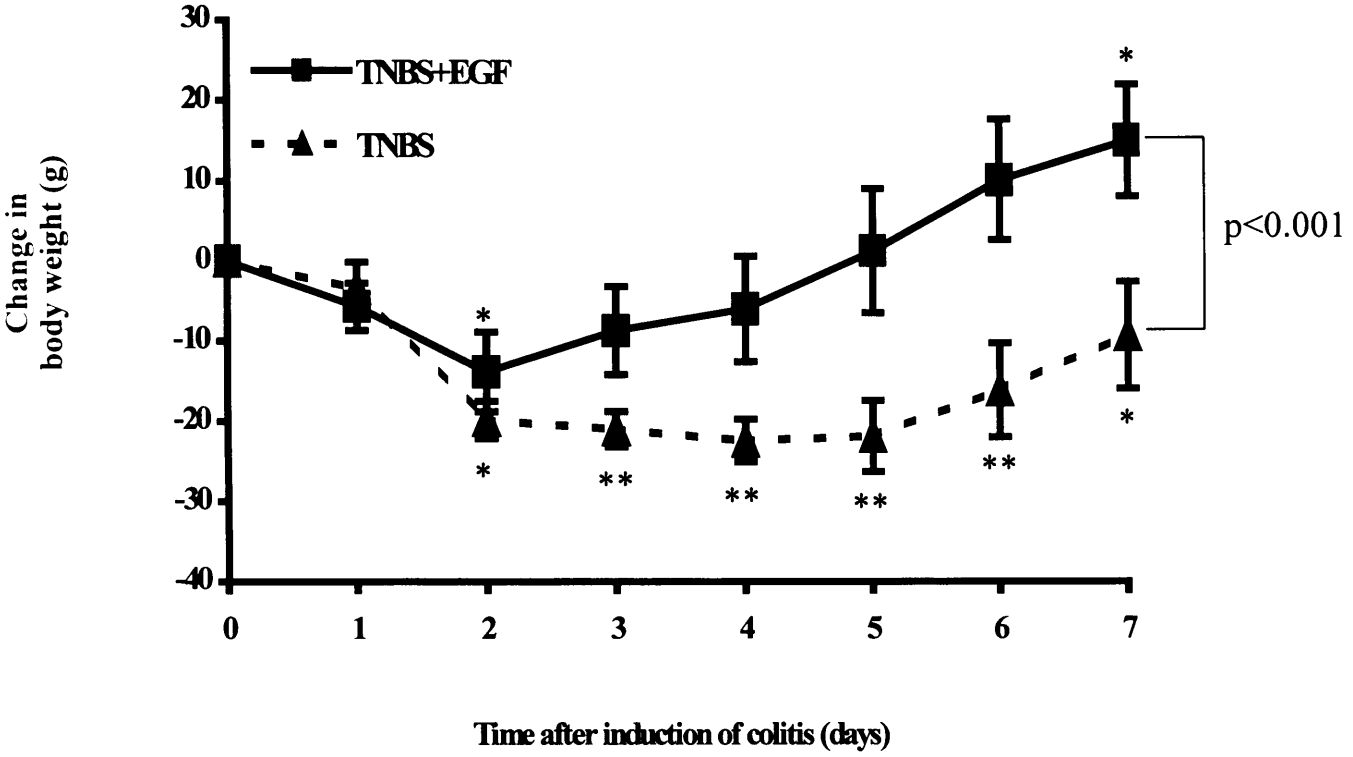


Figure 5.5 rhEGF Prevents Weight Loss Associated With TNBS-Indced Colitis.

TNBS caused significant weight loss from untreated colitic animals between 2 and 7 days after TNBS. Weight loss from animals treated with EGF was less than from untreated ($p<0.05$ vs untreated on days 3 and 4, $p<0.001$ on days 5, 6 and 7). Animals treated with rhEGF exhibited significant weight gain on day 7 compared with day 0. Data points represent mean+/-SEM, n=6. *$p<0.05$ vs day 0; **$p<0.01$ vs day 0.

body weights of animals treated with rhEGF were observed to increase above their initial weights.

This study has provided a demonstration of the essential features of the model, which are markers of injury, and of response to intervention (Table 5.2). There was disparity between the scored macroscopic damage and the observed damage, showing that the 'five point' scale (table 5.1) was insufficient to differentiate between levels of damage. This insufficiency might mask effects due to intervention with potential anti-colitic treatments. Thus, a modified scale was used subsequently that takes into account the length of the colon exhibiting inflammation and ulceration (table 5.3; Wallace and Keenan, 1990).

5.3. Evaluation of Bovine Colostral Preparation As Treatment for TNBS-Induced Colitis: Can Colostral Preparation Prevent Induction of Colitis?

Oral administration of the commercial bovine colostral preparation under investigation here has been shown to protect the stomach and small intestine against NSAID-induced injury (Playford et al, 1999). A study was devised to test the efficacy of bovine colostral preparation, when administered via drinking water, to protect the colon against TNBS-induced colitis. TNBS has been shown to produce colitis dose dependently between 10 and 30 mg per animal (Morris et al, 1989). TNBS was used at 10, 20 and 30 mg per animal in this experiment to evaluate whether or not bovine colostral preparation could modulate different amounts of colitis. It has been recommended that the optimum amounts of TNBS/ethanol be determined by the individual investigator (Morris et al, 1989).

Bovine colostral preparation (10% v/v) or isoproteinaceous BSA was administered via drinking water, starting 48 hours before induction of colitis by TNBS enema. Control animals received sterile saline (0.25 mls) by enema. All animals continued to receive glucose-supplemented drinking water until sacrifice 48 hours after the enema.

TNBS caused an increase in colon weight that was independent of the amount of TNBS (figure 5.6). Bovine colostral preparation had no effect on increased colon weight.

Macroscopic damage was scored according the scale described in table 5.3. TNBS induced damage dose-dependently (figure 5.7). Macroscopic damage was less in the colons of animals

Table 5.2. Features of TNBS-Induced Colitis Made Use of in These Studies.

Colon Weight	Increased following TNBS
Macroscopic Damage	Increased following TNBS
Colonic Myeloperoxidase	Increased following TNBS
Body Weight	Decreased following TNBS

Table 5.3 Modified Scoring System For Assessment of Macroscopic Damage. (Taken from Wallace and Keenan, 1990)

Score	*Appearance*
0	No damage
1	Slight bowel wall thickening, no hyperaemia, no mucosal erosions
2	Hyperaemia and bowel wall thickening (inflammation), or severe inflammation without hyperaemia
3	Single erosion with hyperaemia and bowel wall thickening
4	Two or more erosions with hyperaemia and/or bowel wall thickening
5	Two or more major sites of damage or major damage extending more than 1 cm along the length of the colon
6-10	Incremental increase in score for every centimetre length of damage above 1 cm

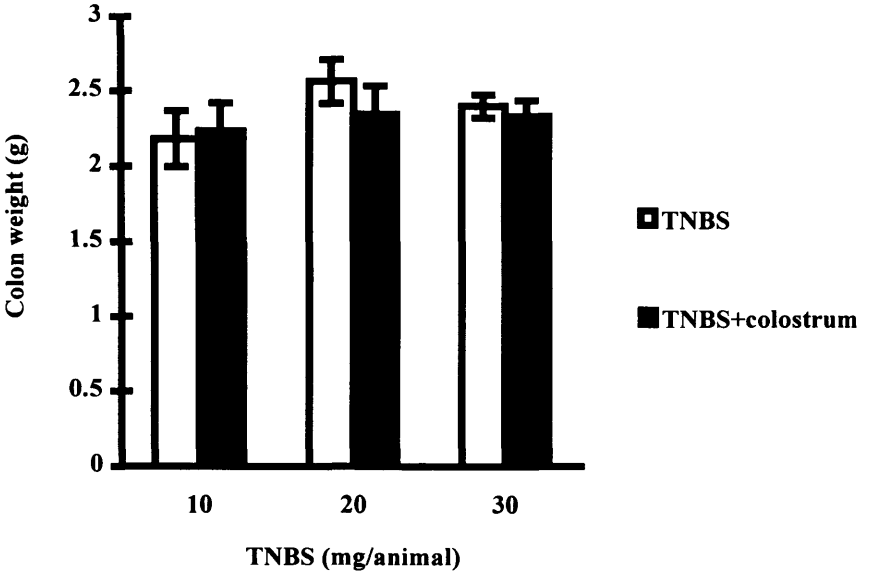


Figure 5.6. Colostral Preparation Did Not Prevent Increased Colon Weight Associated With Colitis.

TNBS-induced colitis caused colon weight to be significantly increased compared with control ($p<0.01$, mean colon weight of controls 0.62+/-0.02 g). Colostral preparation in drinking water had no effect on colon weights (NSD vs untreated colitic rats). Bars represent mean+/-SEM, n=6.

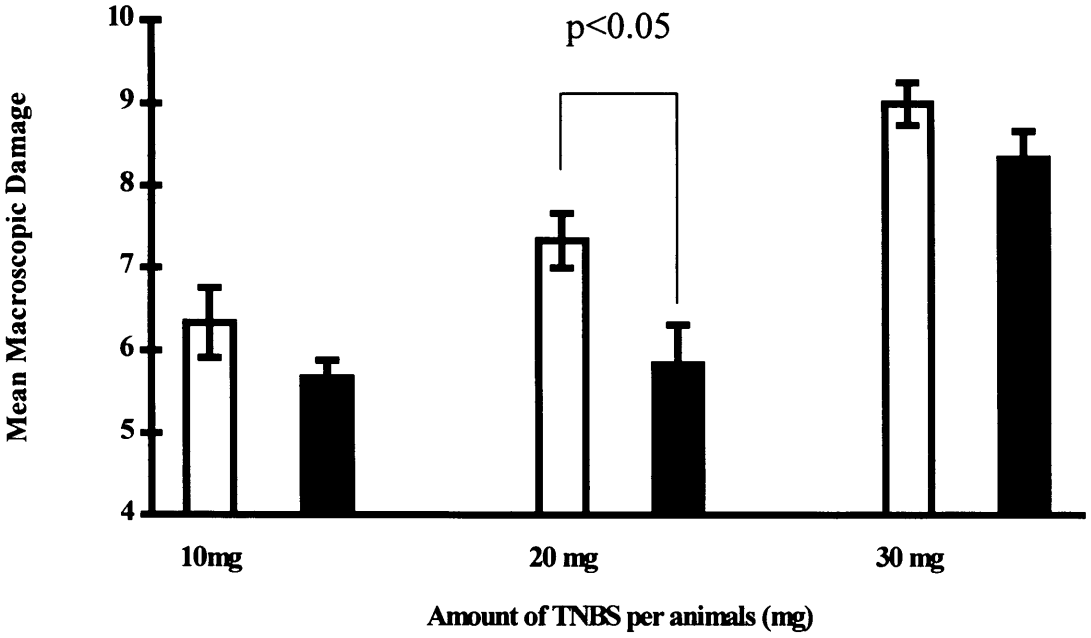


Figure 5.7 Bovine Colostral Preparation Reduced Colitic Damage Caused by TNBS.

Macroscopic damage was scored according the scheme in table 5.3. White bars represent animals with untreated colitis. Black bars represent animals treated with colostral preparation. Colitic animals had significantly more damage ($p<0.01$) than controls (control score 0.5+/-0.22). Colostral preparation caused a small reduction (8-20%) in damage. The reduction was significant for damage arising from 20 mg TNBS. Bars respresent mean+/-SEM, n=6.

receiving drinking water supplemented with bovine colostral preparation. The reduction in damage was significant for colitis induced by 20 mg TNBS ($p<0.05$).

TNBS caused increased MPO activity compared with controls. Bovine colostral preparation prevented the increase caused by 20 mg TNBS (figure 5.8). MPO activity resulting from administration of 10 mg and 30 mg was unaffected by colostral preparation.

TNBS-induced colitis caused a dose-dependent decrease in body weight (figure 5.9). Weight loss associated with lower amounts of TNBS (i.e. 10 and 20 mg) was unaffected by colostral preparation (figure 5.9a and b). However, the more severe weight loss arising from 30 mg TNBS was reduced by colostral preparation (figure 5.9c).

Table 5.4 shows the water consumed by the animals during this study. Three animals were housed in each cage; thus, there were two cages per group. The data reveal considerable differences between cages from the same treatment group. Thus, the amount of colostral preparation ingested by each animal would have been very different, and could have affected the results of this study. Administration of colostral preparation via drinking water is not suitable for delivering a controlled dose of colostral preparation.

Overall, bovine colostral preparation showed potential as an orally-administered treatment for colitis, but this potential might have been obscured by uncontrolled administration.

5.4. Evaluation of Bovine Colostral Preparation As Treatment for TNBS-Induced Colitis: Can Colostral Preparation Promote Healing of Colitis?

A follow-up study was devised to test the effect of bovine colostral preparation on the 'healing phase' of colitis by extending the period of treatment after TNBS enema. Treatment with bovine colostral preparation was performed by gavage. This route was chosen to eliminate the problems associated with consumption of drinking water experienced in the previous study. Data in table 5.4 showed that rats drank an average of 20 mls water per day. Supplementing drinking water with 10% v/v colostral preparation resulted in ingestion of approximately 2 mls colostral preparation per animal per day. This volume (2 mls) was chosen for gavage of animals with undiluted colostral preparation.

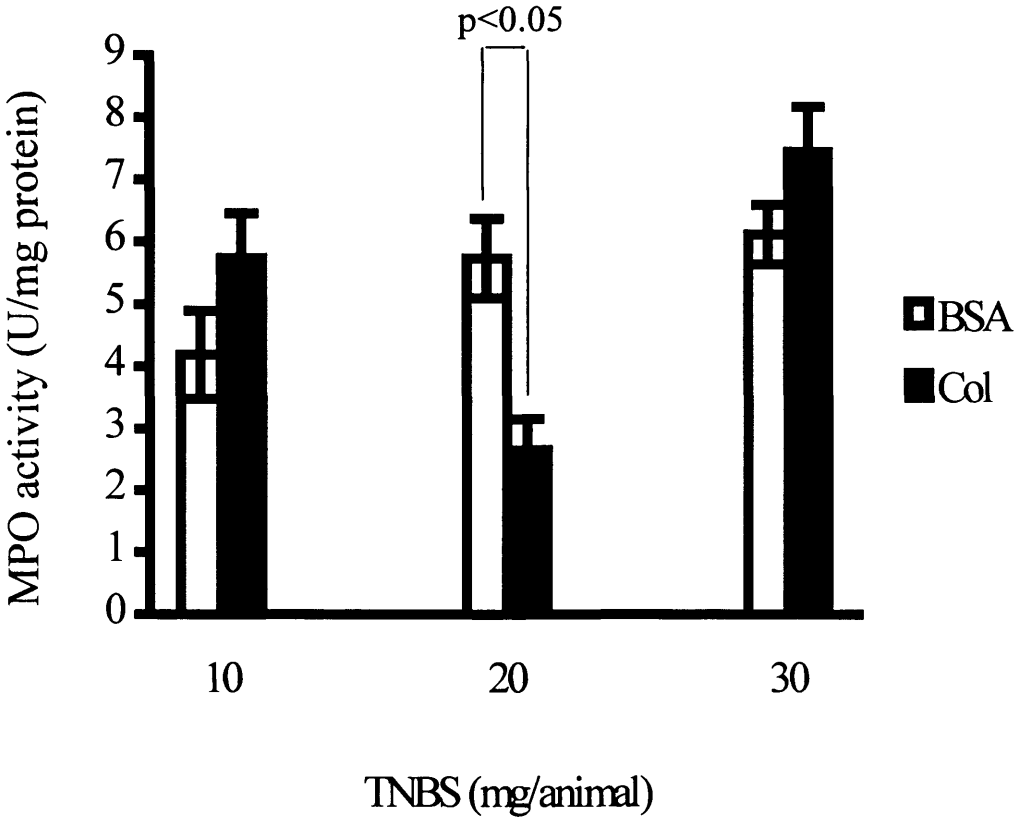


Figure 5.8 Oral Colostral Preparation Reduced Colonic MPO Activity.

Protein extracts of colonic tissue (0.5g/sample) were assayed for MPO activity and protein content. Colostral preparation administered via drinking water significantly reduced MPO activity associated with colitis caused by 20 mg TNBS ($p<0.05$ vs colitis without treatment with colostral preparation). There was no effect on MPO activity associated with 10 and 30 mg TNBS. Bars represent mean+/-SEM, n=6.

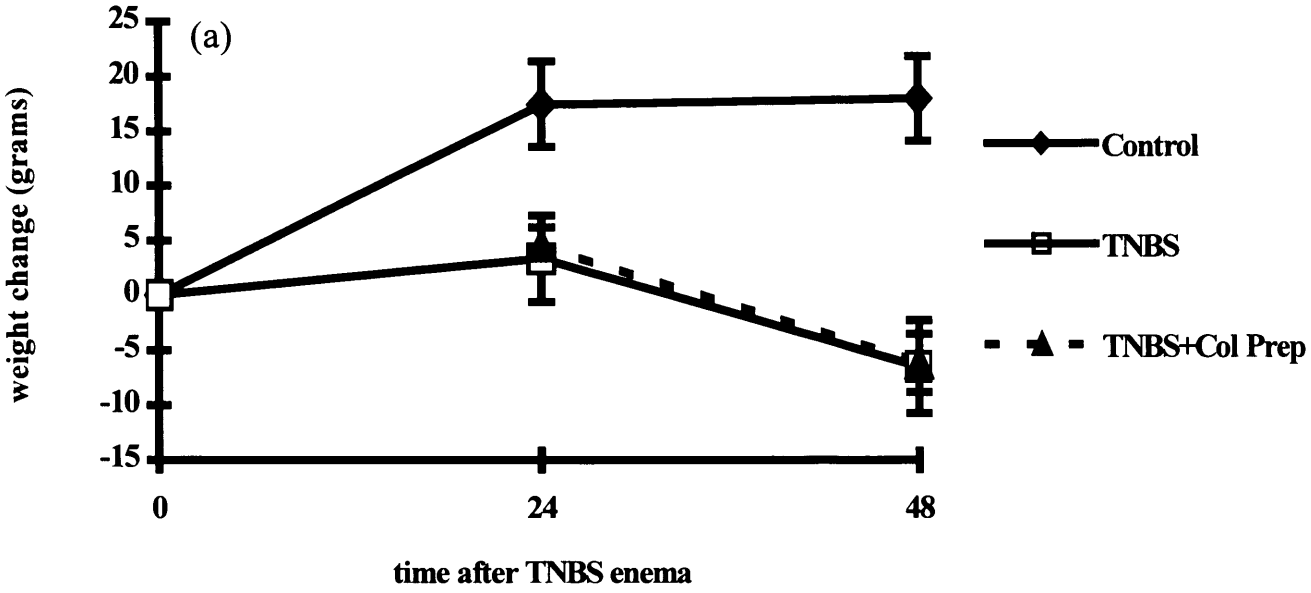
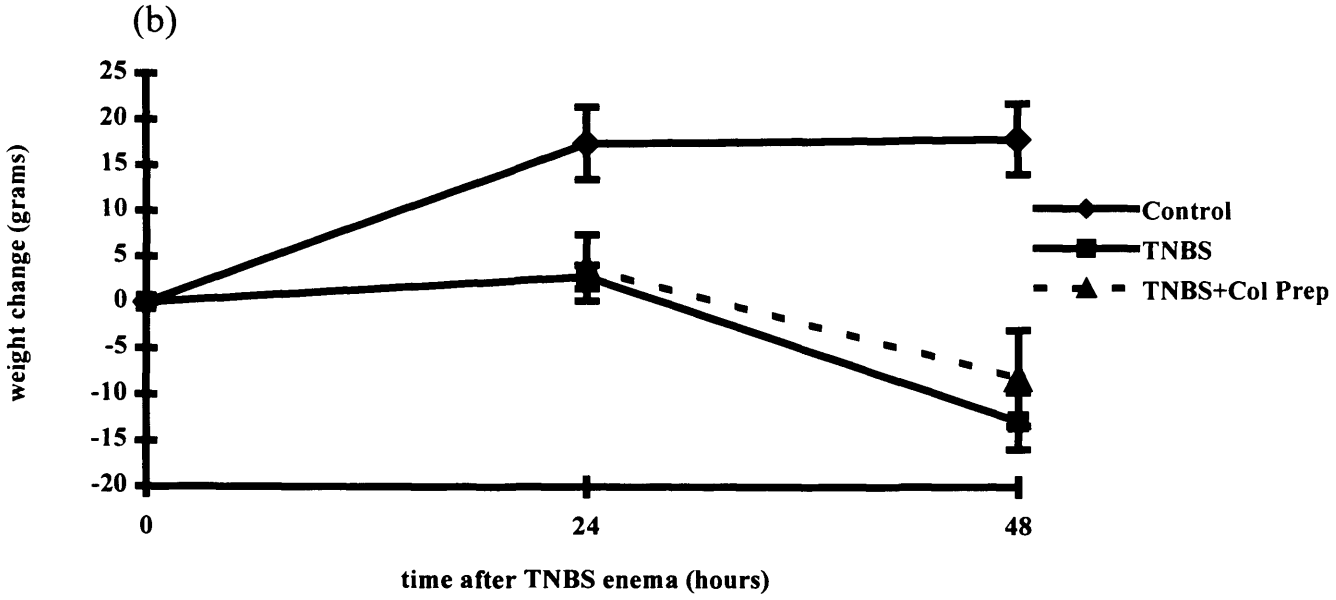


Figure 5.9 Weight Loss Associated With Severe TNBS Colitis Was Reduced By Oral Colostral Preparation.

TNBS caused significant weight loss ($p<0.05$) when administered as 10, 20 and 30 mg/animal (a, b and c respectively). (a) Colostral preparation had no effect on weight loss caused by 10 mg TNBS. (b) Colostral preparation had no effect on weight loss caused by 20 mg TNBS. (c) Forty eight hours after induction of colitis weight loss was significantly less from animals treated with colostral preparation ($p<0.05$). Data represent mean+/-SEM, n=6.

Figure 5.9 cont'd



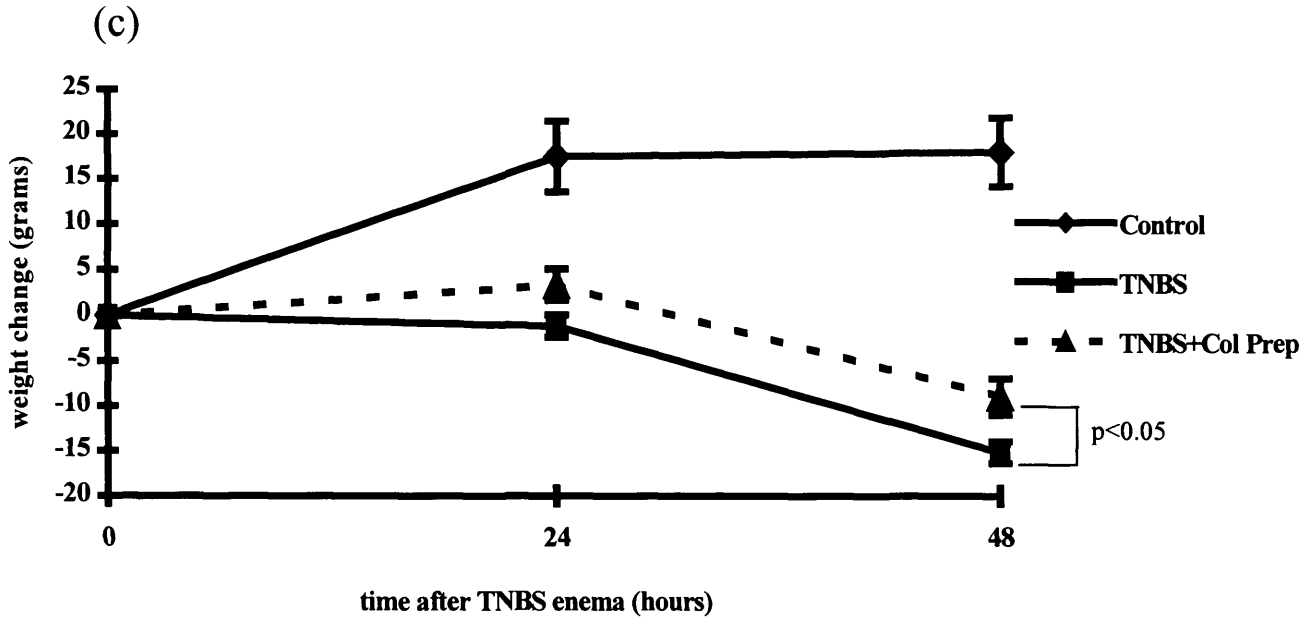


Table 5.4 Consumption of Drinking Water With or Without Colostral Preparation.

Animals were housed in cages, three per cage. The consumption of drinking water was monitored daily by weighing the water bottle (1 gram approximately equal to 1 cm^3 water). There was considerable variation in the volumes of water consumed per cage between treatment groups and also between cages within the same treatment groups.

			Volume of water/colostral preparation drunk per cage (mls)		
TNBS (mg per animal)	*Treatment*	*Cage*	*24 hours beforeTNBS*	*24 hours after TNBS*	*24-48 hours after TNBS*
0	Colostral prep	1	48	118	74
		2	72	135	75
10	BSA	1	64	105	86
		2	39	57	40
10	Colostral prep	1	98	100	58
		2	72	95	70
20	BSA	1	33	60	47
		2	48	58	50
20	Colostral prep	1	87	118	75
		2	40	64	5
30	BSA	1	22	43	15
		2	22	42	39
30	Colostral prep	1	57	80	56
		2	67	57	43

The design of the study was essentially as before – treatment with colostrum started 48 hours before induction of colitis. 20 mg TNBS in 50% ethanol was used, since colitis associated with this amount was found to be most sensitive to the effects of colostral preparation (figure 5.7). Colostral preparation (2 mls, thickened with 2% hydroxy-propyl methylcellulose, HPMC) was administered by daily gavage. Control animals received isoproteinaceous BSA/HPMC. Groups of animals were killed and their colons excised 48 hours and 7 days after induction of colitis.

TNBS colitis resulted in increased colon weight 48 hours after the TNBS enema. Gavage with colostral preparation did not modulate this increase (figure 5.10a; cf figure 5.6). Colon weights remained elevated seven days after induction of colitis. Treatment with oral colostral preparation caused a small reduction in the weights of colitic colons, which was not significant on statistical analysis (figure 5.10b). Oral colostral preparation caused a small increase in mean colon weight after nine days of administration (figure 5.10b). Thus, the use of colon weight as a marker of damage was compromised in this case.

Macroscopic damage to the colon was increased 48 hours after administration of the TNBS enema. Colostral preparation caused a small reduction (approximately 12%) in mean macroscopic damage (figure 5.11a), but was not statistically significant, in contrast to the previous study (figure 5.7; approximately 19% reduction, $p<0.05$). Seven days after the induction of colitis untreated animals still had significant colonic macroscopic damage (mean score approximately 5). Oral colostrum resulted in a significant 45% reduction ($p<0.01$) in macroscopic damage compared with untreated colitic animals (figure 5.11b). Photomicrographs of representative colons with macroscopic damage scores between 0 and 10 are shown in figure 5.12 (a-f). These pictures demonstrate the considerable differences between colons in terms of the amount of inflammation and the area of necrosis.

Data from the analysis of MPO activity are shown in figure 5.13. MPO activity was increased by TNBS, but was unaffected by colostral preparation 48 hours after the induction of colitis. MPO levels were reduced to near normal after 7 days (figure 5.13b; compare with figure 5.4). There was no significant difference in MPO activity between untreated and treated animals at this time.

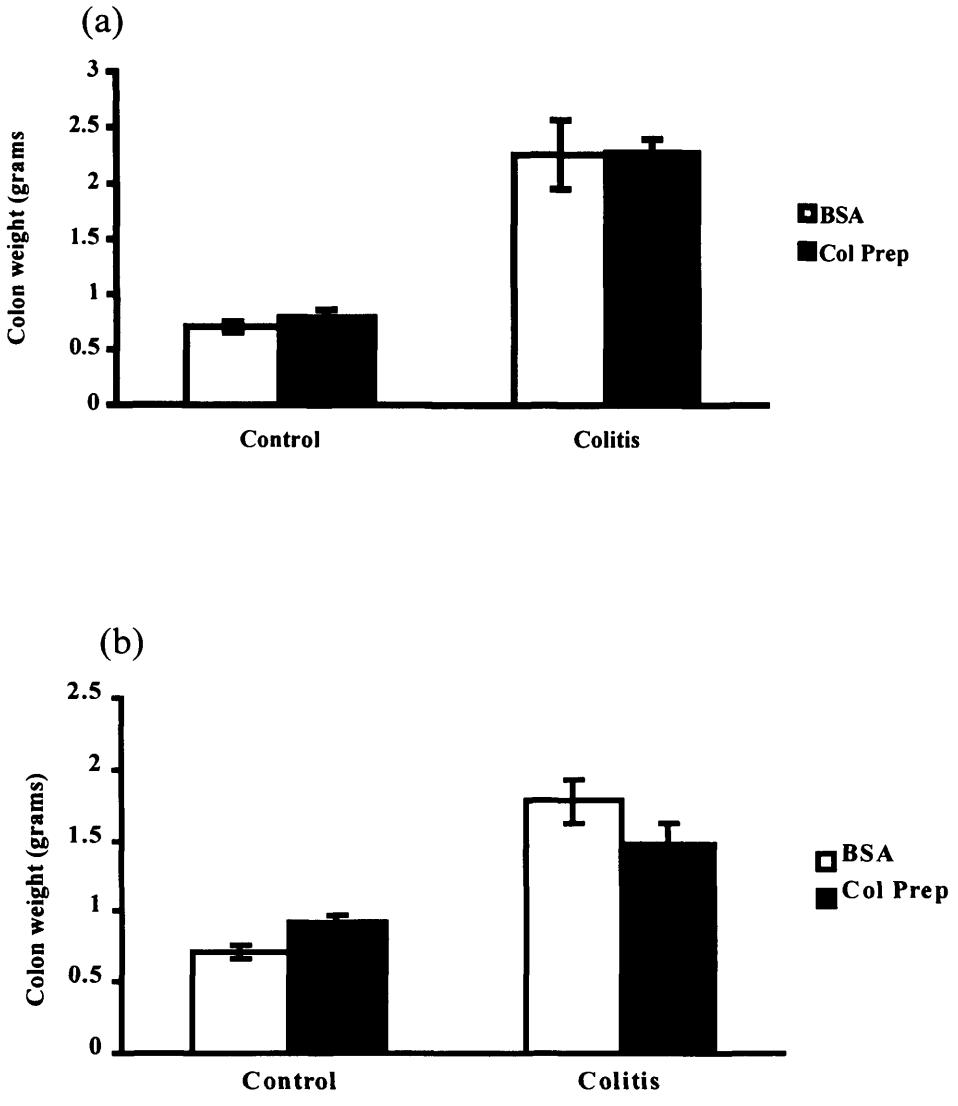


Figure 5.10 Effect Of Oral Bovine Colostral Preparation On Increases In Colon Weight Associated With Colitis.

The weights of the excised colons were measured at the time of dissection. (a) 48 hours after induction of colitis colon weights were greater than controls, but were unaffected by oral colostral preparation ($p>0.05$). (b) 7 days after induction of colitis colon weight was still greater than control. Colostral preparation caused a small decrease in colon weight (NSD). Bars represent mean+/-SEM, n=10-16.

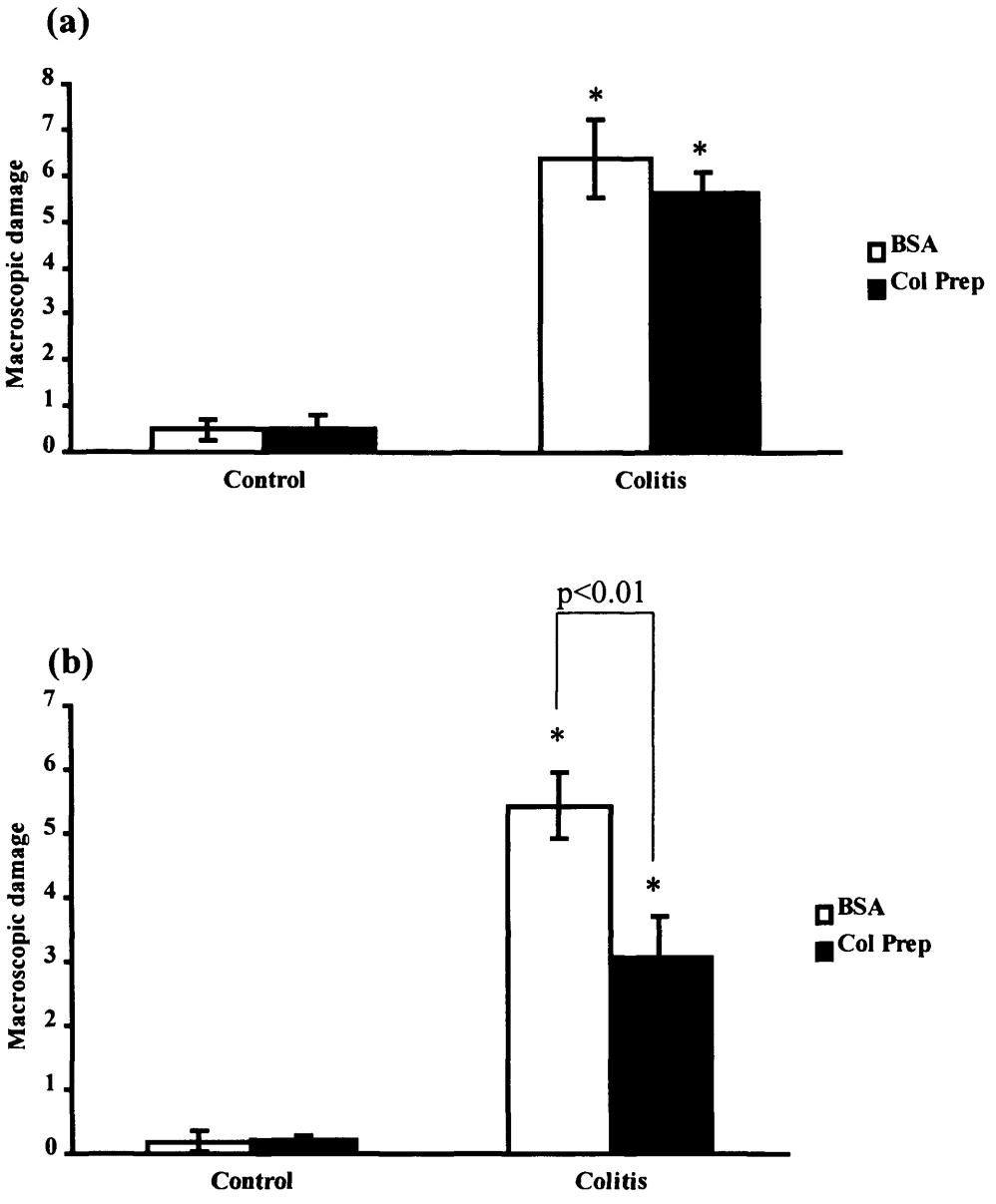


Figure 5.11 Oral Colostral Preparation Stimulate Healing Of Colitic Tissue Damage.

Macroscopic damage to the colon was measured according to a 10-point scale (table 5.2). (a) 48 hours after induction of colitis macroscopic damage was significantly increased ($p<0.01$ vs control). Colostral preparation caused a small decrease in damage score (NSD vs untreated colitis). (b) 7 days after induction of colitis macroscopic damage was still significantly increased ($p<0.01$ vs control). Colostral preparation caused a significant decrease in macroscopic damage ($p<0.01$ vs untreated colitis). Bars represent mean+/-SEM, n=10-16. *$p<0.01$ vs animals without colitis.

Figure 5.12. Colitic and Normal Colons *(over page).*

Colons were removed at sacrifice, 7 days after induction of colitis, opened longitudinally and macroscopic damage was scored according to the scale in table 5.3.

Photographs show colons with damage scores of 10 (a), 8 (b), 6 (c), 4 (d), 2 (e) and 0 (f).

See main text for discussion.

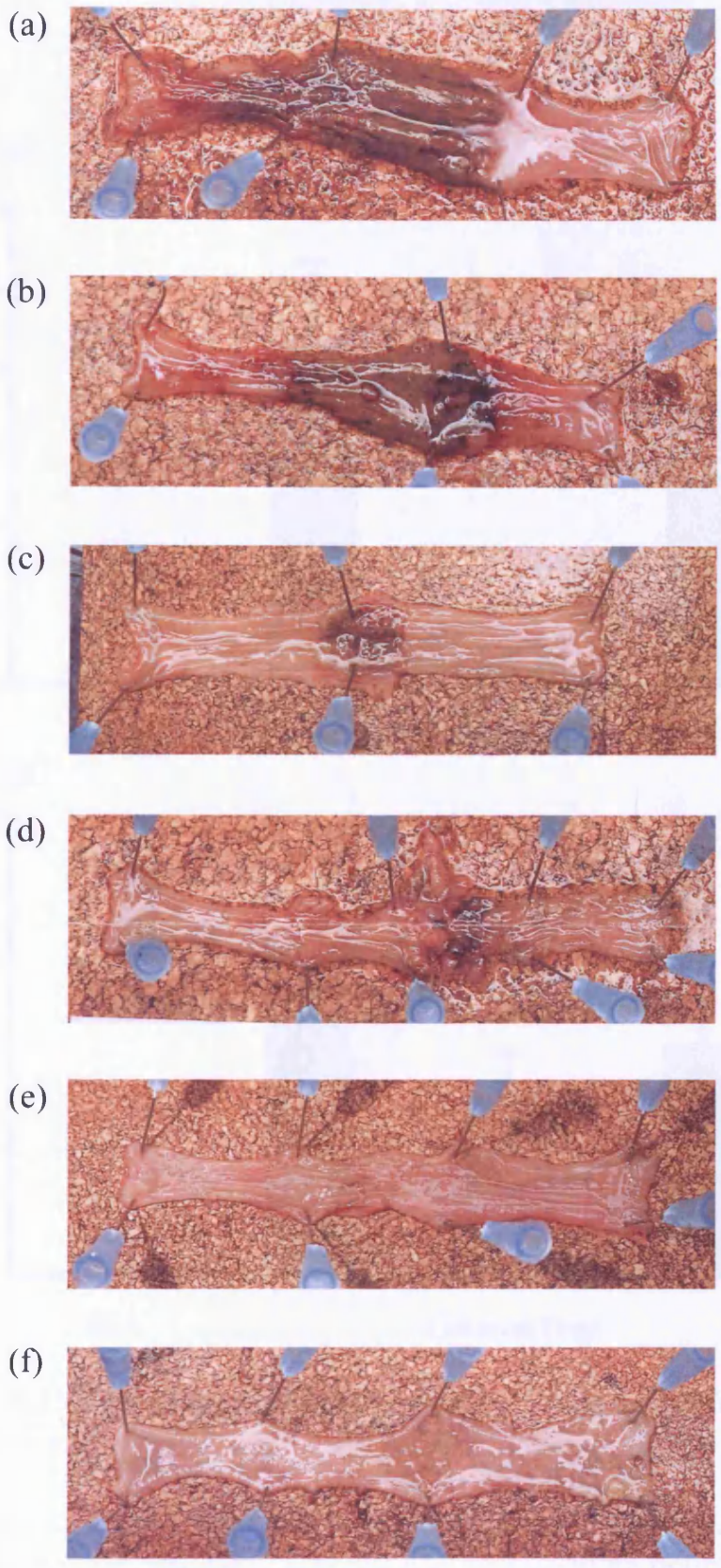


Figure 5.12 Colitic and Normal Colons.

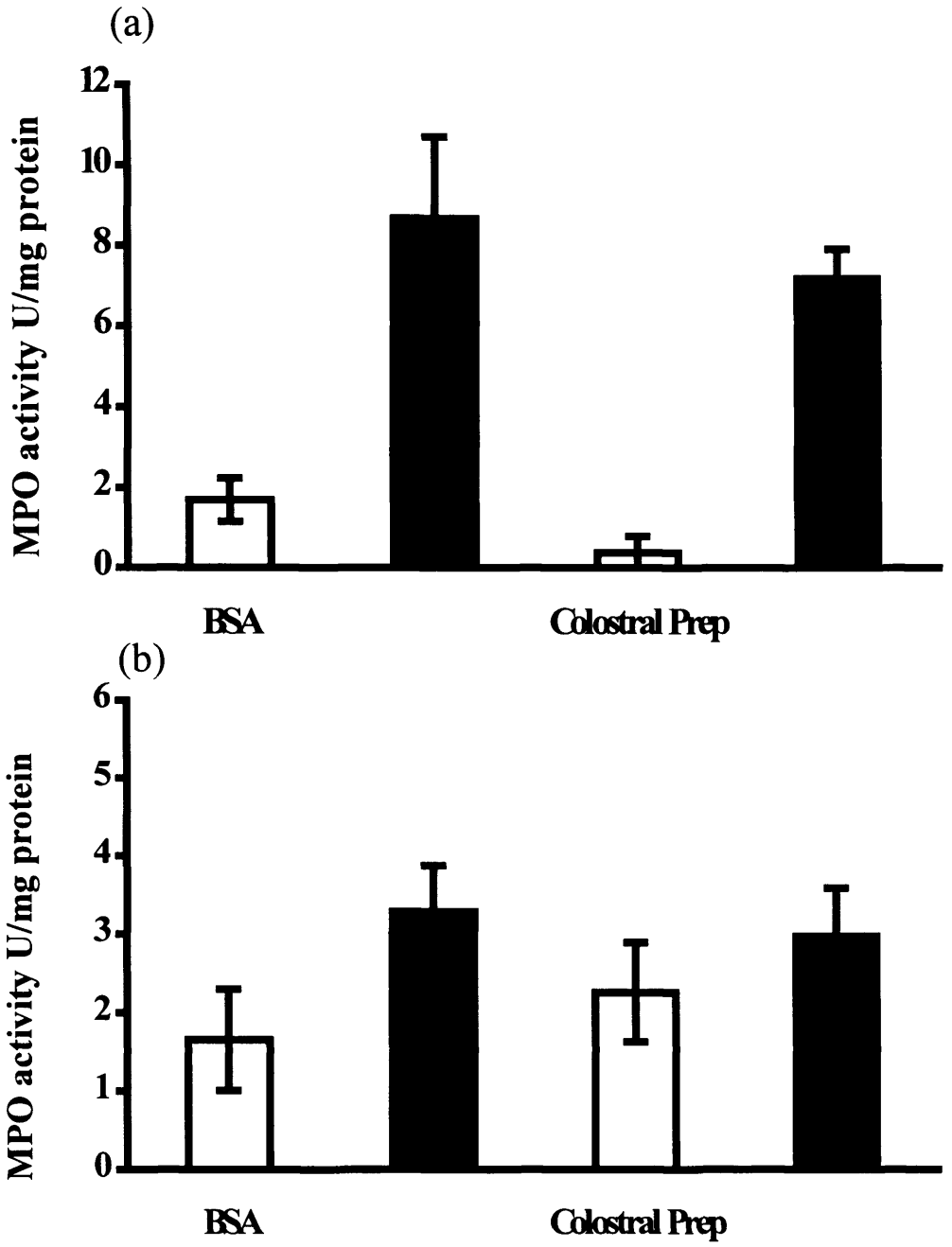


Figure 5.13 Myeloperoxidase Activity Was Unaffected By Colostral Preparation.

MPO was measured in colon extracts (0.5g per sample). White bars: control; black bars: colitic. (a) MPO activity was increased 48 hours after the induction of colitis. Bovine colostral preparation caused some diminution of this increase (NSD). (b) MPO activity had returned to levels comparable to controls after 7 days (note scale). There was no difference between MPO levels in untreated and treated colitic tissue. Bars represent mean +/- SEM, n=10-16.

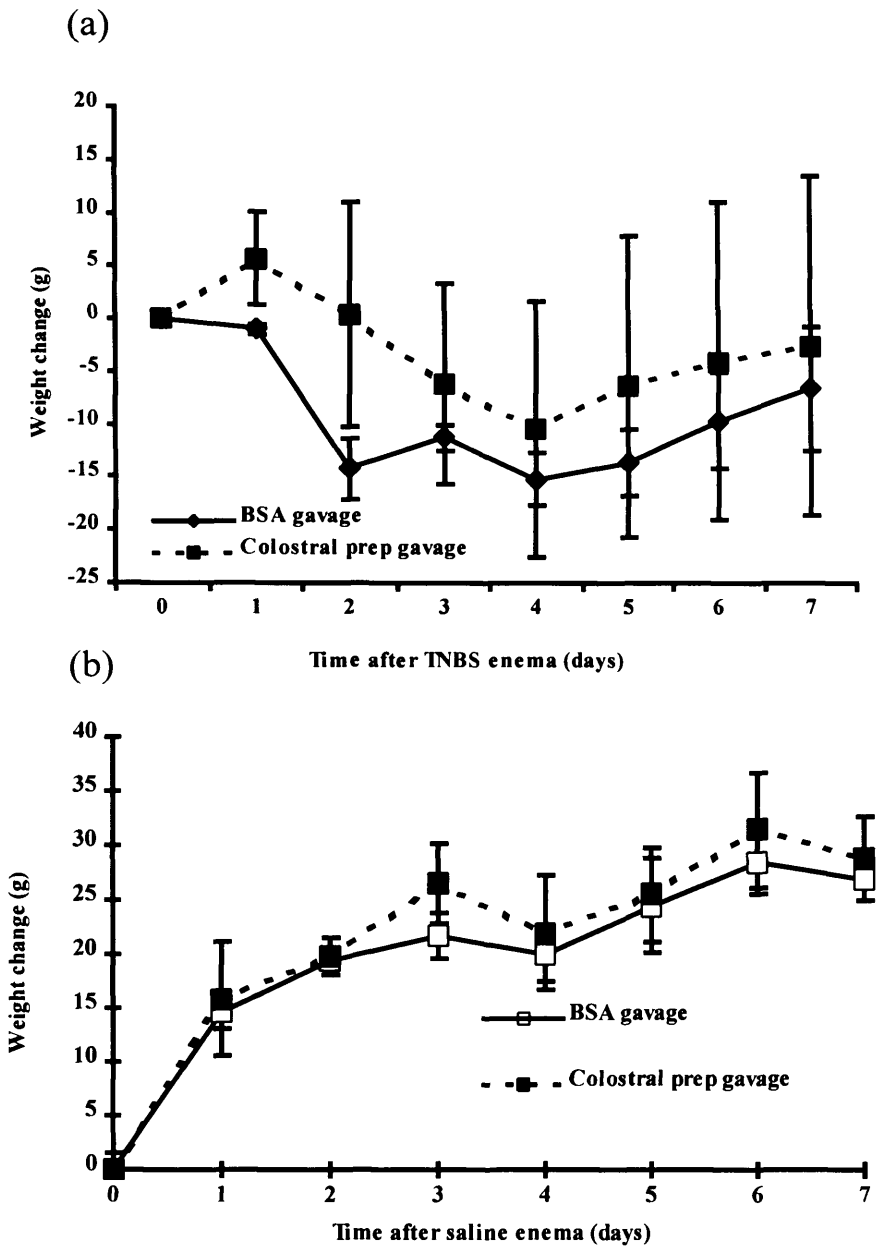


Figure 5.14 Effect of Oral Colostral Preparation on Body Weight in Normal and Colitic Rats.

Rats were administered oral colostral preparation or BSA (2 mls daily). (a) There was no effect of colostral preparation on body weight of healthy controls ($p>0.05$, $n=6$-12). (b) Colitic animals lost weight following TNBS enema. Weight loss was less in animals administered colostral preparation, but was not statistically significant ($p>0.05$, $n=10$-16).

The effect of colostral preparation on weight loss associated with colitis is shown in figure 5.14. TNBS caused weight loss throughout the course of the experiment (figure 5.14a). Colostral preparation diminished this weight loss, although the difference was not statistically significant. Colostral preparation had no effect on the body weights of normal animals (figure 5.14b).

Thus, orally-administered bovine colostral preparation did not protect against colitis, but did stimulate healing.

5.5. Evaluation of Bovine Colostral Preparation As Treatment for TNBS-Induced Colitis: What Is the Effect of Topical Application of Colostral Preparation?

Topical application of therapies for inflammatory bowel diseases might enable treatment whilst minimising the side effects due to systemic exposure (Richter and Scheppach, 1997). The data from chapters 3 and 4 of this thesis show that bovine colostral preparation has bioactivities *in vitro* that should benefit topical application. Considerable macroscopic damage to the mucosal surface is caused by TNBS. Daily administration of an enema might be problematical due to physical trauma caused by the route of delivery. Thus, a pilot study was performed, administering colostral preparation by enema to determine whether or not a full-scale test was warranted.

The design of the study was as before – treatment with colostrum started 48 hours before induction of colitis with 20 mg TNBS in 50% ethanol. Colostral preparation (2 mls, thickened with 2% hydroxy-propyl methylcellulose, HPMC) was administered by daily enema, except on the day of administration of the TNBS enema. Groups of animals were killed and their colons excised 48 hours and 7 days after induction of colitis.

Administration of colostral preparation was associated with methodological problems. Initially, before the induction of colitis, there were no problems with retention of the thickened colostral preparation. However, as the experiment progressed retention became a notable problem, with the enema occasionally leaking out whilst the delivery tube was in place. Also, the presence of substantial faecal matter was problematical. Retention of faeces was notable within three days after induction of colitis, and colostral preparation would have

been absorbed into the faeces. In some cases faecal matter prevented correct insertion of the catheter, so that the enema was not administered at the correct level in the colon.

TNBS caused a significant increase in colon weight 48 hours after administration. Colostral preparation administered by enema had no effect (figure 5.15). However, after seven days of colitis the colon weight of animals treated with colostral enemas was significantly increased. Similarly, macroscopic damage after 48 hours was unaffected by colostral enema (figure 5.16). Macroscopic damage after seven days was also unaffected by the colostral preparation.

MPO activity was increased by TNBS, and was unaffected by colostral enemas after 48 hours. MPO activity was increased by colostral preparation after 7 days, but the effect was not significant compared with untreated colitic tissue (figure 5.17).

Overall there was no effect of colostral enema on TNBS-induced weight loss (figure 5.18), although there was better maintenance of weight during the first 24 hours after induction of colitis.

The pilot study revealed methodological problems associated with delivery of treatment in this model. Colostral enemas did not affect macroscopic damage, and appeared to worsen colonic weight increases and MPO activity. These data did not warrant a properly controlled, large-scale study of colostral preparation administered as an enema using this model.

5.6 Discussion

The studies presented in this chapter were designed to test the efficacy of colostral preparation as a treatment for inflammatory bowel disease, and employed an established model of colitis – the TNBS/ethanol model (Morris et al, 1989). There are a number of markers of colitis in this model – macroscopic damage to the colonic mucosa, increased myeloperoxidase activity and colon weight, and overall weight loss (table 5.2). Previous studies showed improvements in at least some of these markers in response to treatment (Amenho et al, 1997; Giladi et al, 1995; Neurath et al, 1996; Procaccino et al, 1994; Wallace and Keenan, 1990). Colostral preparation administered orally caused a significant improvement in macroscopic damage, and caused signs of improvement in MPO activity. Also, weight loss associated with colitis was reduced by colostral preparation. Topical

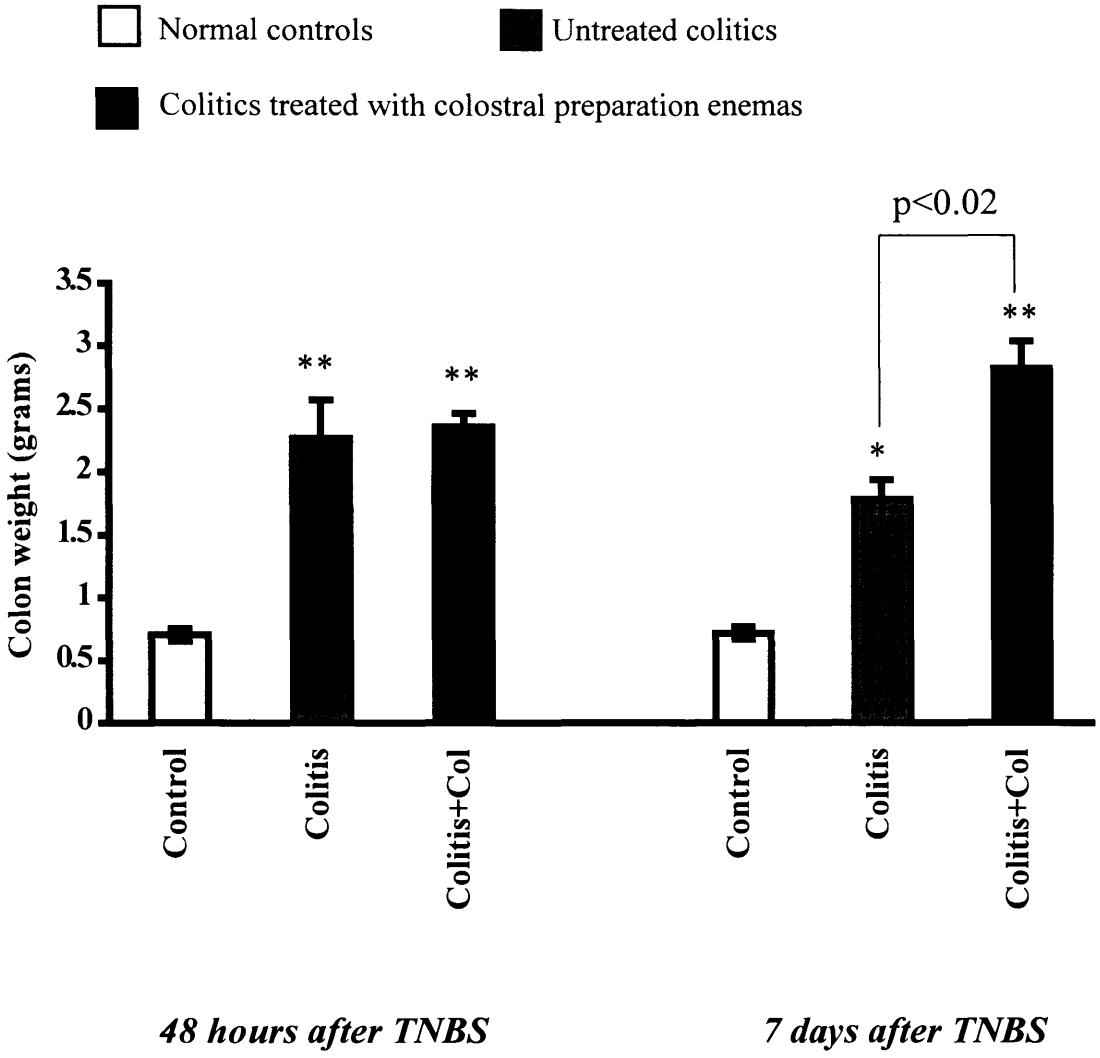


Figure 5.15 Administration Of Colostral Preparation By Enema Increased Colon Weight.

Colitic animals were treated with colostral preparation by daily enema. After 48 hours colitis caused a significant increase in colon weight that was unaffected by colostral preparation. After 7 days colon weights of animals with colitis were still greater than controls. Colostral preparation enemas resulted in a significant increase in colon weights ($p<0.02$ vs untreated colitic animals). Bars represent mean+/-SEM, n=10-16. *$p<0.02$ vs control; **$p<0.01$ vs control.

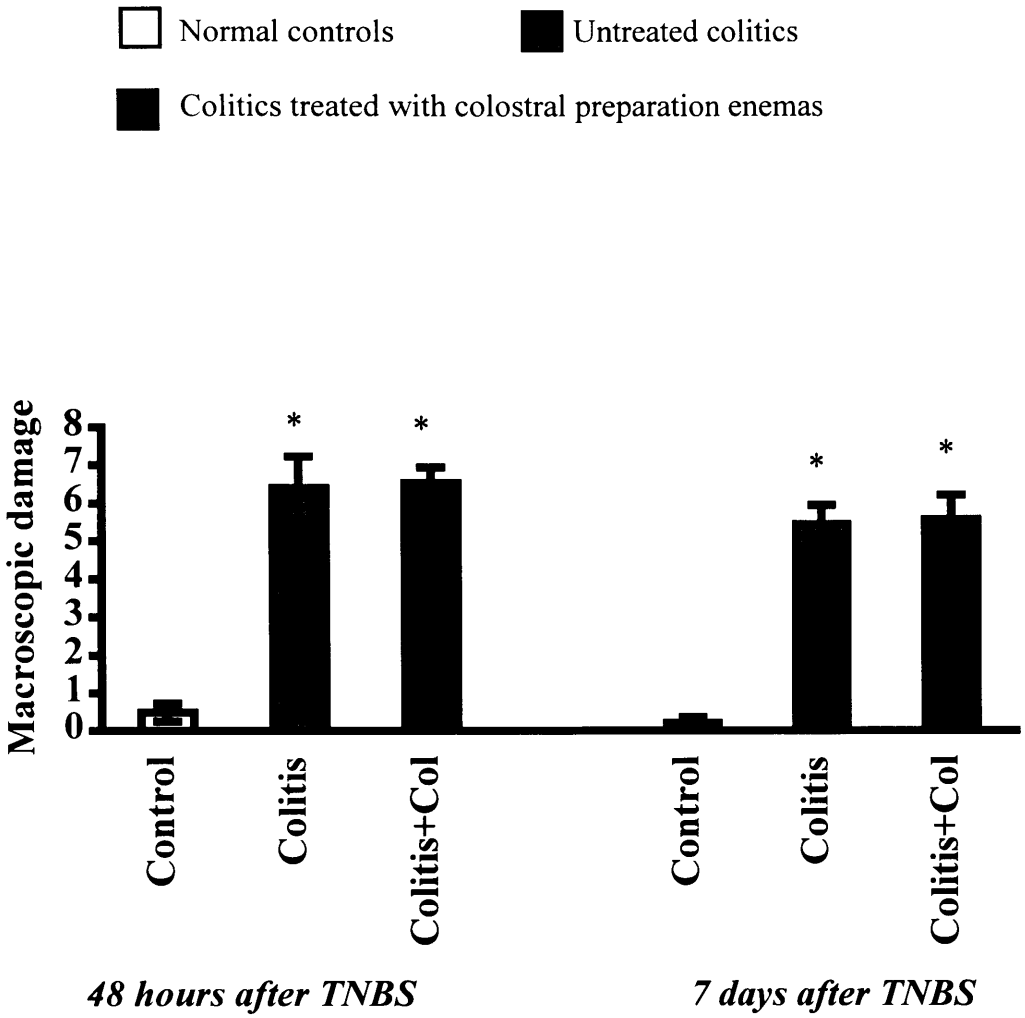


Figure 5.16 Administration Of Colostral Preparation By Enema Did Not Modulate Damage To The Colonic Mucosa.

Colitic animals were treated with colostral preparation by daily enema. After 48 hours, colitis caused a significant increase in macroscopic damage that was unaffected by colostral preparation. After 7 days, macroscopic damage remained. Colostral preparation enemas had no effect on macroscopic damage. Bars represent mean+/-SEM, n=10-16. *$p<0.01$ vs control.

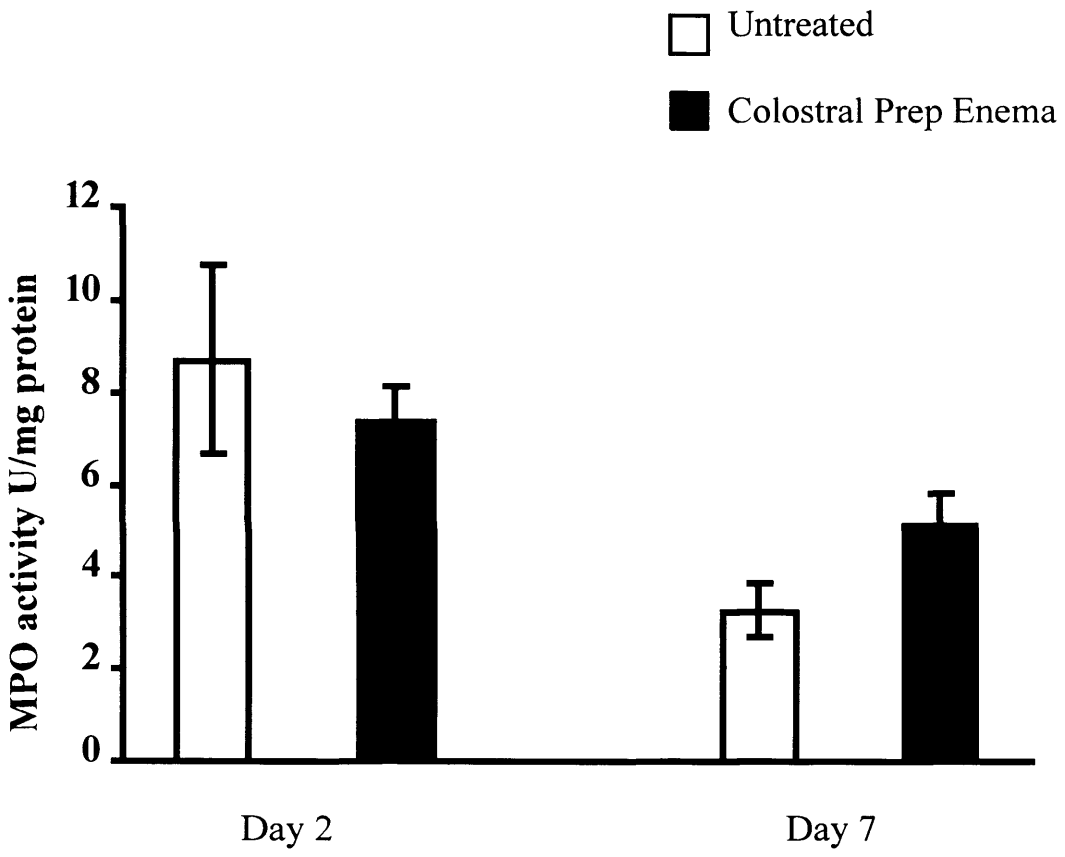


Figure 5.17 Administering Colostral Preparation By Enema Did Not Affect MPO Activity Associated With Colitis.

Colitic animals received colostral enema daily or were untreated. MPO activity was measured in extracts from 0.5g tissue samples. Bars represent mean+/-SEM, n=10-16.

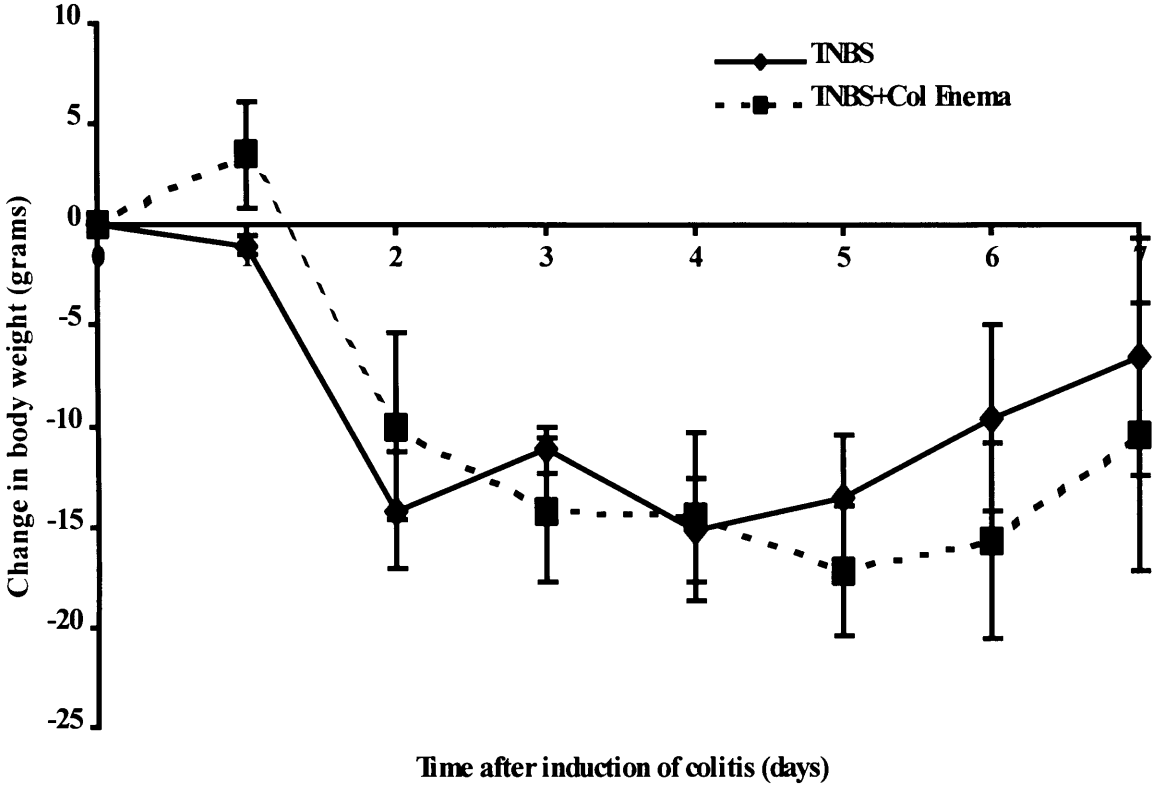


Figure 5.18 Colostral Preparation Administered By Enema Did Not Modulate Weight Loss Associated With Colitis.

TNBS caused weight loss during the first 3 days after administration. Colostral preparation enema did not modulate this loss. Data are represented by mean+/- SEM, n=10-16.

application of colostral preparation was unsuccessful, but this might have been due to methodological difficulties.

The combination of ethanol and TNBS produces a severe, extensive colitis that is evident within hours and lasts up to two months (Morris et al, 1989). The effectiveness of any treatment is ultimately the reduction in mucosal injury that is present in inflammatory bowel disease. Publications of data obtained using the TNBS model usually present data of macroscopic damage as an objective measure, either in accordance with a scale of injury (Morris et al, 1989) or as the measured area of necrosis (e.g. Procaccino et al, 1994). Pragmatically, the use of the scale of damage was most convenient. However, the scale described in table 5.1 (Morris et al, 1989) was observed not to reflect the amount of macroscopic damage to the colonic mucosa (revealed by comparison of figures 5.2 and 5.3). Thus, the effect of EGF on macroscopic damage was found to be less marked than that observed by Procaccino et al (1994), who measured the area of necrosis. A subsequent modification to the scale extended the upper values to account for differences between lengths of necrotic regions (Wallace and Keenan, 1990). This scale (table 5.3) was employed in subsequent studies and was found to reflect the observable damage more satisfactorily.

The severity of colitic damage due to 30mg TNBS was a cause of concern, and the recommendations of Morris et al (1989) were followed, using lower doses of TNBS. The first study was to test the prophylactic effects of colostral preparation on the initial damage due to TNBS. When TNBS was administered at 20 mg per animal, peak macroscopic damage (at 48 hours post-TNBS) was less than that caused by 30 mg TNBS. Colostral preparation administered via drinking water reduced the amount of damage due to 20 mg but not 30 mg TNBS. However, these effects were small, albeit statistically significant. In a subsequent study, a similar effect of colostral preparation was observed, but was not statistically significant (figure 5.11). Since considerable colitic damage was measurable, the difference between statistical and biological significance must be considered. For example, in studies where macroscopic damage was measured as the area of necrosis, successful treatments, such as NOS inhibitors (Kiss et al, 1997) and growth factors (Procaccino et al 1994) resulted in 30% of the colon being necrotic. This would be equivalent to a score of 5 on the 5-point scale of Morris et al (1989) and to 7 on the 10-point scale of Wallace and Keenan (1990). There is a need for clarification of what level of difference in macroscopic damage scores represents a real improvement in treatment during the initial phase of TNBS-induced colitis.

Myeloperoxidase (MPO) activity is a proven marker of gut damage (Grisham et al, 1986; Sekizuka et al, 1988; Smith and Castro, 1978) and assays of tissue MPO activity have been described (Krawisz et al, 1984; Suzuki et al, 1983). MPO generates a number of short-lived, highly reactive chemical species that are associated with cellular damage (McCafferty, 2000). Correlation between isolated neutrophils and MPO activity is good, but there is a relatively poor correlation between numbers of tissue neutrophils, evaluated histologically, and MPO activity (Krawisz et al, 1984). MPO activity during the initial phase of TNBS-induced colitis can be modulated (Kiss et al, 1997), although the effect is transitory, and MPO activity is the same for tissue from treated and untreated colitic animals at later time points. Other studies have shown no effect of anti-colitic treatment on MPO activity during the initial phase (Procaccino et al, 1994; Wallace and Keenan, 1990).

Whilst the initial phase of TNBS-induced colitis appears to be difficult to modulate, the healing phase has been demonstrated to be affected by a variety of treatments (e.g. Luck and Bass, 1993; Giladi et al, 1995; Wallace and Keenan, 1990). The study of the effect of colostral preparation on healing of colitic damage presented here was performed using gavage, rather than supplementation of drinking water, for the administration of colostral preparation. Discrepancies in the volumes of water/colostral preparation drunk per cage were discovered during analyses of the data from the previous study (table 5.4). Administration of colostral preparation by gavage overcame this problem. The different modes of administration might have contributed to the discrepancies between the data for macroscopic damage and MPO activity for the two experiments (figures 5.7 and 5.11, and 5.9 and 5.12 respectively). For example, administration of treatment by gavage involved handling of the animals in a way that caused stress (scruffing, gavage). Stress has been shown to contribute to severity of colitis (Million et al, 1999).

Bovine colostral preparation administered by gavage caused biologically significant healing of TNBS-induced macroscopic damage (figure 5.11b). The colons of animals treated with colostral preparation exhibited very little necrosis and inflammation, with most of the mucosal surface appearing normal, as shown photographically in figure 5.12. This level of healing is similar to that observed by others using different treatments (e.g. Giladi et al, 1995; Luck and Bass, 1993, Wallace and Keenan, 1990).

Many of the studies referred to above showed a decrease in MPO activity concomitant with decreased macroscopic damage. This was not demonstrated here. It is possible that the time course of healing was such that any differences would have appeared earlier. MPO activity in tissue from untreated colitic animals was greatly reduced on the seventh day of colitis (figure 5.13). Also, bovine colostrum contains cytokines that stimulate neutrophils, such as GM-CSF (Gilmore et al, 1994; Moore et al, 1992). However, bovine colostral preparation did not increase MPO activity in healthy animals (range 1-4 units/mg protein).

Porter et al (1998) investigated the effect of a powdered bovine milk (whey) preparation on colitis induced by dextran sulphate sodium (DSS). Control animals were fed a defined, casein-based diet. The difference between the administration of the powdered preparation used by Porter et al and the liquid preparation used here makes direct comparison difficult. Furthermore, investigation of the colonic mucosa was only performed after 10 weeks of colitis and treatment. At this time, there was no improvement due to the milk extract compared with untreated colitic animals. A non-invasive breath test for the production of ethane, resulting from peroxidation reactions in the damaged colon, showed reduced ethane production during the third week of the study but not at any other time. However, supplementation of the diet with milk preparation did help to maintain body weight. Overall, the study by Porter et al is disappointing, since no effect of the milk preparation on the injured colonic mucosa was demonstrated. This might be due to the time course of the experiment. The studies presented in this chapter do demonstrate an effect of the colostral preparation on healing, and are currently unique in this respect. Therefore, this system forms a more suitable basis for evaluating the ethane breath test.

Topical application of anti-colitic drugs is one method of improving the therapeutic ratio of treatment to side effects (Richter and Scheppach, 1997). Topical application is warranted where the treatment in question has been demonstrated to have local effects. Data presented in chapters 3 and 4 in this thesis show that bovine colostral preparation has considerable activity in assays of cell proliferation and migration *in vitro*. The nature of these *in vitro* assays – cells adherent to a surface, onto which medium/colostral preparation is added – can be viewed as equivalent to topical application, albeit in a much simplified system. Ingestion of colostral preparation clearly has therapeutic effects. However, passage of colostral preparation through the stomach and small intestine will result in the destruction of some colostral components (chapter 4). Thus, topical application (i.e. by enema) of colostral

preparation might be expected to be a favourable route of administration. Administration of 5-amino-salicylic acid (5-ASA) enemas reduced macroscopic damage due to TNBS, whereas EGF enemas did not (Luck and Bass, 1993).

A small pilot study was performed to assess the feasibility of administering colostral preparation by enema. This study showed that colostral preparation enemas did not affect healing of colitis. Indeed, colon weight and MPO activity was increased when compared with data from untreated colitic animals. Further, practical problems were encountered in administering the colostral enemas, including obstruction by faecal matter and poor retention. The effect of colostral enemas on colon weight and MPO activity are of concern. Daily administration of the enema under light anaesthetic might have interfered with the healing process. This would readily be evaluated by inclusion of an appropriate control in a larger study. Alternatively, the observed effects might have been due to the physical trauma of inserting the canula, irritating the mucosa and inhibiting 'spontaneous' healing. Another possibility is that the colostral enema has increased colonocyte proliferation, resulting in increased tissue mass, and stimulation neutrophils (chapter 3; Moore et al, 1992).

The role of TGFβ is a further consideration when interpreting these data. Activity of colostral TGFβ will increase by the action of gastric juice (chapter 4), therefore, colostral TGFβ will probably be inactive when colostral preparation is administered by enema. Therefore, further studies should include colostral preparation that has been transiently acidified.

These data show that colostral enemas administered according to this protocol do not have a role in the treatment of colitis using this model. It is not clear whether or not this is due to a failure of colostral bioactivity *per se*, or due to other, more pragmatic reasons. A recent paper by Tran et al (1999) administered enemas of TFF2 (formerly known as spasmolytic peptide) beginning five days *after* induction of colitis, to good effect. Experience of administering 'therapeutic' enemas suggests that the modified protocol might be employed for further studies.

The failure of topical application makes difficult a more informed discussion of the mechanisms by which colostral preparation affects healing of mucosal damage associated with TNBS-induced colitis. Little bioactivity will remain in the gut lumen after passage

through the stomach and the small intestine, due to degradation (chapter 4) and to absorption into the small intestinal mucosa. Indeed, small intestinal permeability to macromolecules is increased in colitis (Soderholm et al, 1999). Thus, the mechanisms by which colostral preparation exerted its effects in the colon are likely to be systemic. For example, Giladi et al (1995) used TGFβ gene therapy to treat successfully TNBS-induced colitis. However, they were unable to detect increased plasma TGFβ following intra-muscular injection of an expression vector containing TGFβ cDNA. The authors suggest that this might be due to problems quantifying small increases in circulating TGFβ. Whilst studies of the effects of oral colostral preparation on circulating growth factors are warranted, care must be taken in interpreting null results and in attributing increased levels to absorption rather than secretion stimulated by signalling through the gut mucosa.

Colitis is associated with dietary complications that result in anorexia and weight loss (Barbier et al, 1998; Murch and Walker-Smith, 1998). Two major components involved in weight loss and anorexia associated with IBDs are the growth hormone/IGF system (Ballinger et al, 2000; Chen et al, 1997) and the leptin system (Barbier et al, 1998). Murch and Walker-Smith (1998) recommend that treatment of patients with inflammatory bowel disease include consideration of nutrition to restore body weight and growth. Thus, therapies for IBD that also modulate weight homeostasis are likely to be valuable. TNBS caused dose-dependent weight loss during the first 48 hours after induction of colitis (figure 5.9). Colostral preparation reduced this effect even when there was no effect on colonic mucosal injury (compare figure 5.9c with figure 5.7). These data suggest that colostral preparation might be an important nutritional supplement that can minimise the 'indirect' effects of colitis, even in cases where the preparation has no effect against the colitis *per se*.

In conclusion, colostral preparation stimulated healing of the damaged colonic mucosa. Treatment with colostral preparation restored substantially the normal macroscopic appearance of the colon after 1 week of treatment. Ingestion of colostrum was more effective than topical application, suggesting a systemic action. A nutritional role of colostral preparation has been demonstrated, since weight loss associated with colitis was reduced by ingestion of colostral preparation.

Chapter 6.

The Effects of Inhibitors of Signal Transduction on DNA Synthesis Stimulated By High Molecular Weight Colostral Preparation: A Preliminary Study

6.1 Introduction.

Modulation of DNA synthesis by milk and colostral preparations *in vitro* has been attributed to colostral growth factors (Belford et al, 1997; Rogers et al, 1984; Shing and Klagsbrun, 1984). However, it has proved impossible to reconstruct total bioactivity by combining relevant quantities of the appropriate growth factors (Belford et al, 1997).

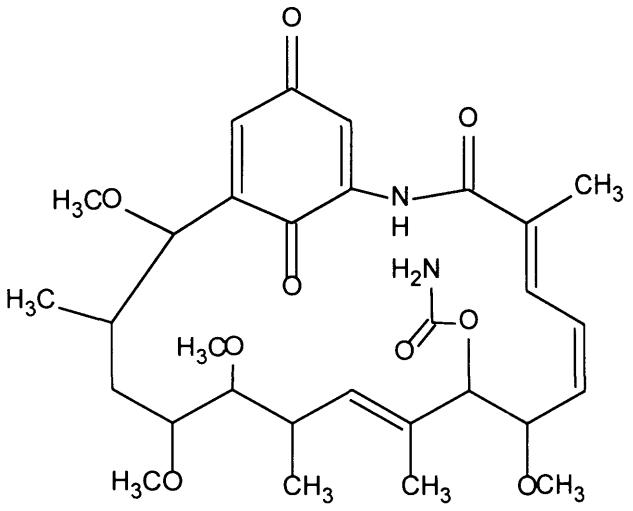
An alternative approach is to observe effects on intracellular signalling pathways; for example, inhibitors of signal transduction can be used in DNA synthesis assays to test which signalling pathways are activated (Levitzki 1992). Inhibition of pathways involved in DNA synthesis stimulated by colostral preparation will result in reduced efficacy of colostral preparation. Judicious choice of inhibitors should allow conclusions to be made regarding the pathways involved in the action of colostral preparation (Baghdiguian and Fantini, 1997; Kovalenko et al, 1994; Levitzki, 1992). These studies can be performed without working on individual growth factors, and without further fractionation of the colostral preparation, which might be important when considering the role of any PDGF-like molecule as a 'competency factor' that increases the activity of other growth factors (Brown and Blakely, 1984).

6.2 Small Molecule Signal Tranduction Inhibitors.

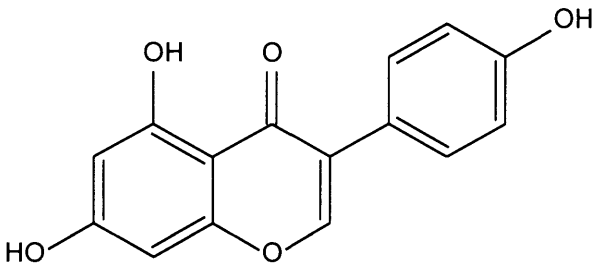
Several signal transduction inhibitors were chosen to study the effects of high molecular weight bovine colostral preparation on DNA synthesis. Their characteristics are described below and their chemical structures shown in figure 6.1.

Herbimycin A. Herbimycin A inhibits tyrosine kinases (Uehara et al, 1989). Several growth factor receptors have intrinsic tyrosine kinase activity, including those for IGF (Rubin et al, 1983), EGF (Ushiro and Cohen, 1980), PDGF (Ek, 1982; Nishimura et al, 1982) and FGF (Jaye et al, 1992). Herbimycin A was identified by its ability to reverse the transformed phenotype of cultured kidney cells infected with Rous sarcoma virus (Uehara et al, 1989). Also, herbimycin A interferes with growth factor receptor maturation (Sakagami et al, 1999).

Genistein. Genistein is another tyrosine kinase inhibitor (Akiyama et al, 1987). Micromolar amounts of genistein inhibit ribosomal S6 kinase (Linassier et al, 1990) and



HerbimycinA

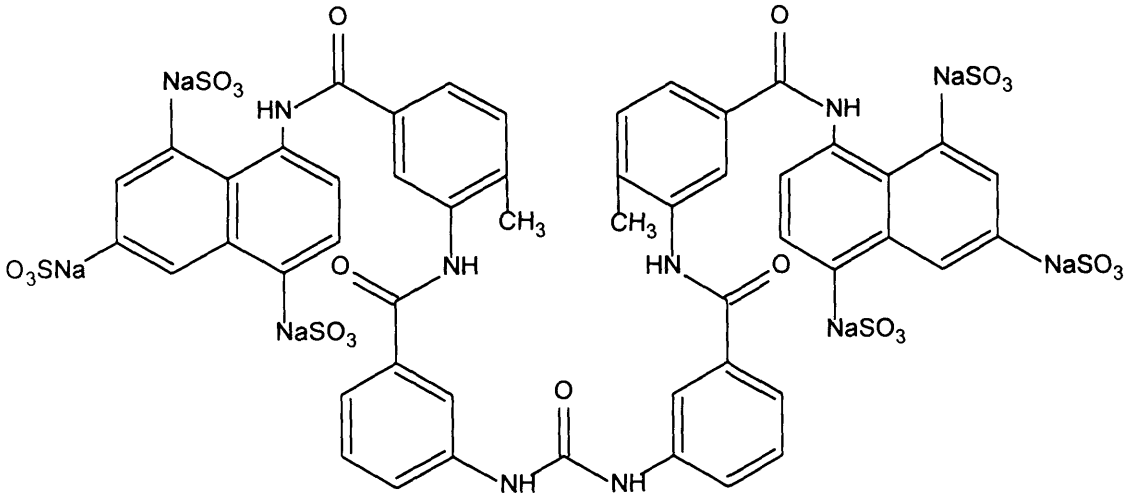


Genistein

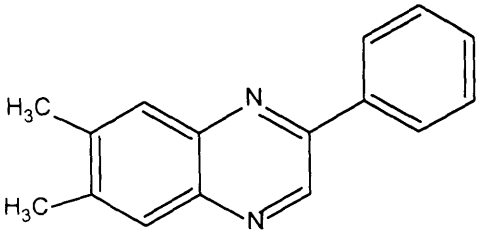
Figure 6.1

Chemical Structures of Signal Transduction Inhibitors

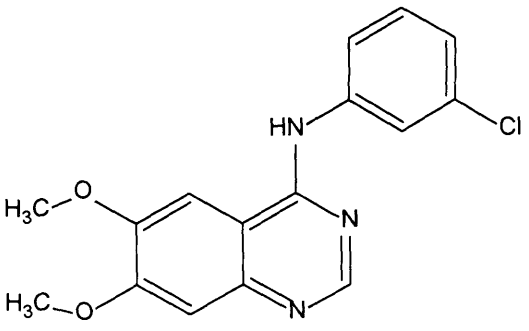
Used in This Chapter.



Suramin



Tyrphostin AG1295



Tyrphostin AG1478

autophosphorylation of the EGF receptor tyrosine kinase (Akiyama et al, 1987). Also, genistein induces differentiation of leukaemic and erythroleukaemic cells (Constantinou et al, 1990, Watanabe et al, 1991). The precise mechanism by which genistein inhibits tyrosine kinases remains unknown, although it has been postulated that genistein competes with ATP for its binding site at the kinase region (Akiyama et al, 1987).

Suramin. Suramin is a poly-ionic, poly-sulfonated naphthylurea. Originally it was used as an anti-trypanosomal treatment, for example for the treatment of sleeping sickness. In recent years suramin has undergone clinical trials, both as an anti-tumour agent and as a treatment for AIDS (reviewed by Baghdihuian and Fantini, 1997). Suramin molecules bear multiple ionic charges that result in the adsorption of growth factors, thereby inhibiting binding to their receptors (Middaugh et al, 1992). Suramin also inhibits intracellular signalling directly, by uncoupling G-proteins from receptors (Baghdihuian and Fantini, 1997; Pollack and Richard, 1990). Suramin inhibits purified DNA topoisomerase II, which might be a source of its anti-tumour activity (Bojanowski et al, 1992).

Tyrphostins. Erbstatin inhibits tyrosine kinases (Umezawa et al, 1986). A variety of synthetic analogues of erbstatin have been produced with either selectivity or specificity for tyrosine kinases associated with particular growth factor receptors. These are called tyrphostins (Gazit et al, 1989; Gazit et al, 1991). Tyrphostin AG1295 in micromolar quantities is specific for the PDGF receptor tyrosine kinase (Kovalenko et al, 1994). whilst similar amounts of tyrphostin AG1478 are specific for the EGF receptor tyrosine kinase (Oshirov and Levitzki, 1994).

In the experiments described in this chapter, DNA synthesis assays were performed as described in chapter 2. Sub-confluent, serum-starved cells were cultured in serum-free medium containing 10 or 30% HMWCP. Previously, 30% v/v colostral preparation was shown to have maximal effect on RIE-1 cells. The lower amount of HMWCP was also used in the assays described here, to reduce the chance of there being saturating amounts of competing bioactivities. Signal transduction inhibitors were added, at the amounts indicated in the text and figure legends, at the time of addition of HMWCP, except where stated. RIE-1 epithelial cells and NIH-3T3 cells have been used previously. HT-29 cells were also used in these studies since colostral preparation contained both stimulatory and inhibitory components for HT-29 DNA synthesis (chapter 3, figure 3.2). All data were analysed by ANOVA (using both the amount of colostrum and of inhibitor as the two fixed factors).

Where significance was reproducibly obtained, comparisons were performed using a modified t-test based on residual errors.

6.3. The Effect of Herbimycin A on DNA Synthesis Stimulated by HMWCP.

Herbimycin A was added to culture medium with or without colostral preparation (10 or 30% v/v). The quantities of herbimycin used were 0.1 and 1.0 μg/ml, equivalent to 0.17 and 1.7 μM, respectively. This dose range has been demonstrated to inhibit intracellular signalling via tyrosine kinases (Satoh et al, 1992).

Data from typical experiments are shown in figure 6.2 a, b and c, corresponding to RIE-1, HT-29 and 3T3 cells respectively. Basal DNA synthesis by RIE-1 cells was unaffected by herbimycin A. The response of RIE-1 cells to HMWCP, as measured by thymidine incorporation, was unaffected by 0.1 μg/ml herbimycin A. However, 1 μg/ml herbimycin A reduced significantly DNA synthesis stimulated by HMWCP ($p<0.01$ vs cultures with HMWCP and without genistein), although DNA synthesis was not reduced to basal levels.

HMWCP stimulated DNA synthesis by HT-29 cells, but there was no significant difference between stimulation by 10% and 30% HMWCP. Basal DNA synthesis by HT-29 cells was not affected significantly by herbimycin A (figure 6.2b). Similarly 0.1μg/ml herbimycin A had no effect on DNA synthesis stimulated by HMWCP. Herbimycin A at 1 μg/ml caused significant inhibition of DNA synthesis stimulated by 30% HMWCP. As with the RIE-1 cells, DNA synthesis due to HMWCP was diminished, but not abolished.

Fibroblast 3T3 cells were unaffected by herbimycin A (figure 6.2c).

These data showed that epithelial DNA synthesis was stimulated partly by colostral components acting on, or via, tyrosine kinases.

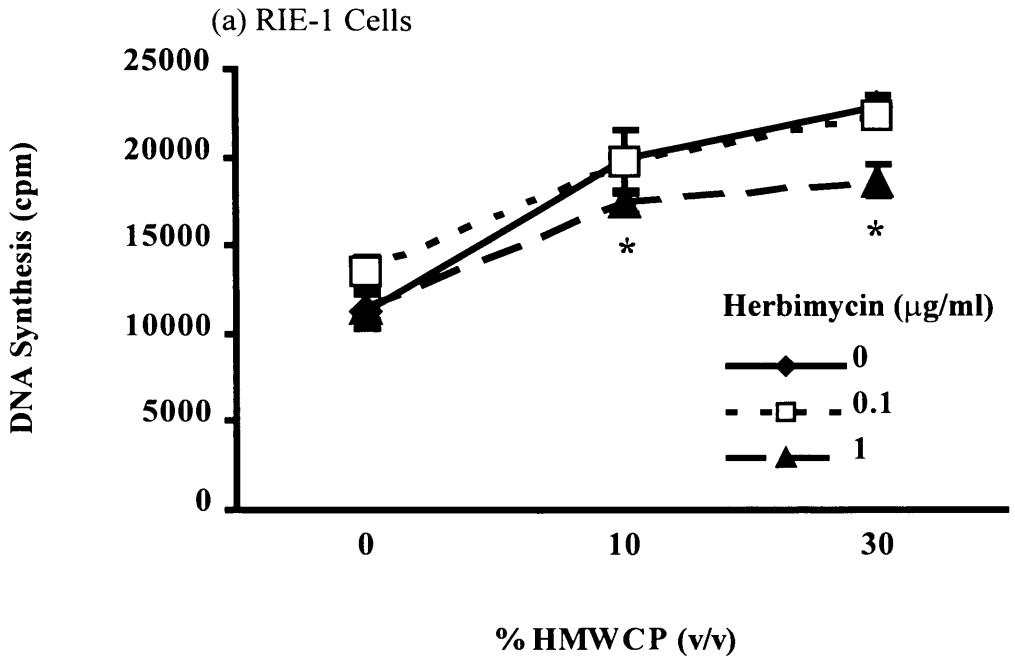
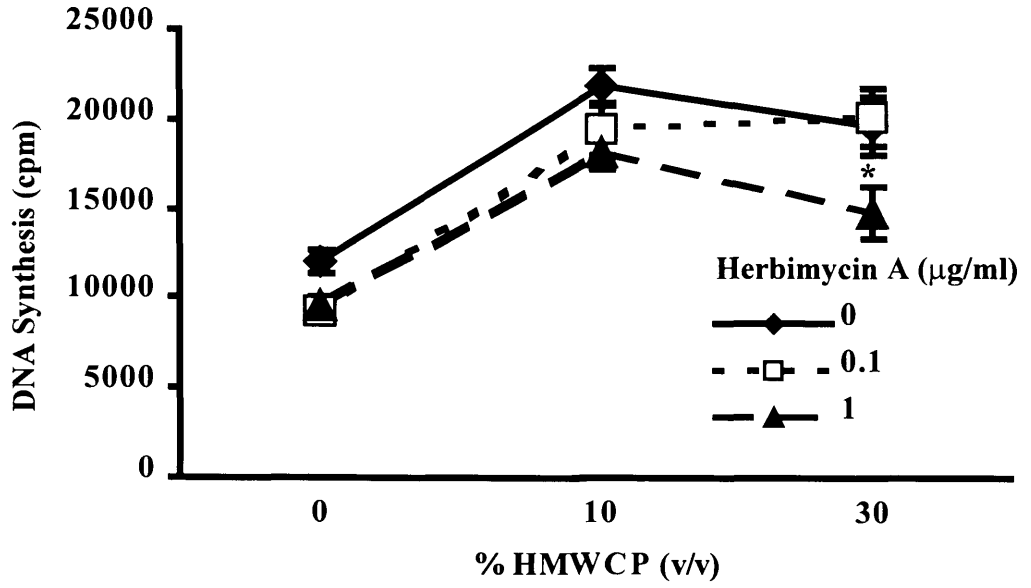


Figure 6.2 Herbimycin A Inhibited Partially the Effects of High Molecular Weight Colostral Preparation (HMWCP) on Epithelial Cells, But Not That On Fibroblasts.

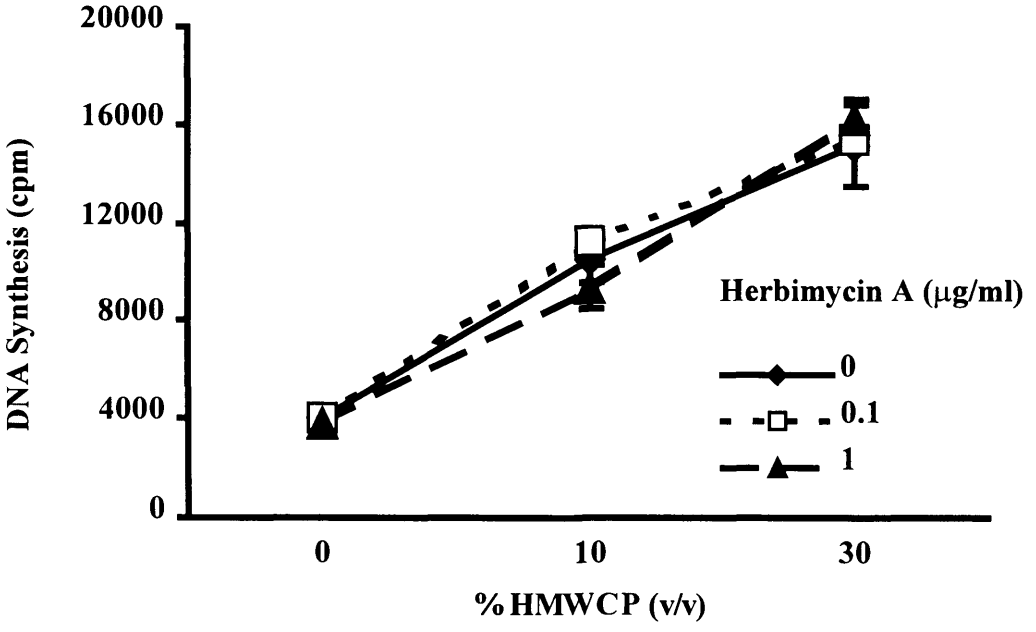
Cells were cultured in medium containing 10 or 30 % v/v HMWCP and herbimycin A as described in the text. (a) RIE-1 DNA synthesis stimulated by 10 and 30% HMWCP was reduced by 1 μg/ml herbimycin A ($p<0.01$ vs HMWCP without herbimycin A). (b) HT-29 DNA synthesis stimulated by 30% HMWCP was reduced by 1 μg/ml herbimycin A ($p<0.01$ vs HMWCP without herbimycin A). (c) NIH-3T3 DNA synthesis stimulated by HMWCP was unaffected by herbimycin A.

Data represent mean+/-SEM (n=4) of a typical experiment (n=3). *$p<0.01$ vs culture in HMWCP without herbimycin A.

(b) HT-29 Cells



(c) NIH-3T3 Cells



6.4. The Effect of Genistein on DNA Synthesis Stimulated by HMWCP.

Cell lines were cultured as described above, with the inclusion of genistein at 0.1, 1 or 10 μg/ml (0.4 to 40 μM). These doses have been demonstrated to inhibit signal transduction via tyrosine kinases (Akiyama et al, 1987; Linassier et al, 1990).

Data from typical experiments are shown in figure 6.3 (a-c). RIE-1 DNA synthesis stimulated by 10% HMWCP was inhibited partially by 0.1 and 1 μg/ml genistein. The stimulatory effect of 30% HMWCP was unaffected by these doses of genistein. Genistein at 10 μg/ml had a significant effect on both basal and stimulated DNA synthesis, making interpretation of these data difficult.

Basal HT-29 DNA synthesis was not affected significantly by genistein (figure 6.3b). DNA synthesis stimulated by 10% and 30% HMWCP was significantly decreased by 10 μg/ml genistein. The lower doses of genistein had no effect on stimulated DNA synthesis.

Low doses of genistein did not significantly affect basal DNA synthesis by 3T3 fibroblasts (figure 6.3c). DNA synthesis stimulated by 10% HMWCP was partially inhibited by genistein. However, genistein had no effect on DNA synthesis stimulated by 30% HMWCP. The highest dose of genistein significantly inhibited basal and stimulated DNA synthesis.

To summarise, HMWCP exerted its effects on the three cell lines through tyrosine kinase activities that were sensitive to genistein. However, these data did not account for all colostral bioactivity measured in these assays.

6.5. The Effect of Suramin on DNA Synthesis Stimulated by HMWCP.

Suramin was used in these studies both in the presence of HMWCP (figure 6.3), and also before cells were cultured in HMWCP (figure 6.4). This latter protocol was followed to determine the effects of suramin on cell signalling apparatus, without contributions from the effects of sequestration of exogous growth factors. The doses of suramin used in these studies – 10 and 100 μM – have previously been shown to be effective (Bojanowski et al, 1992; Fantini et al, 1989; Middaugh et al, 1992).

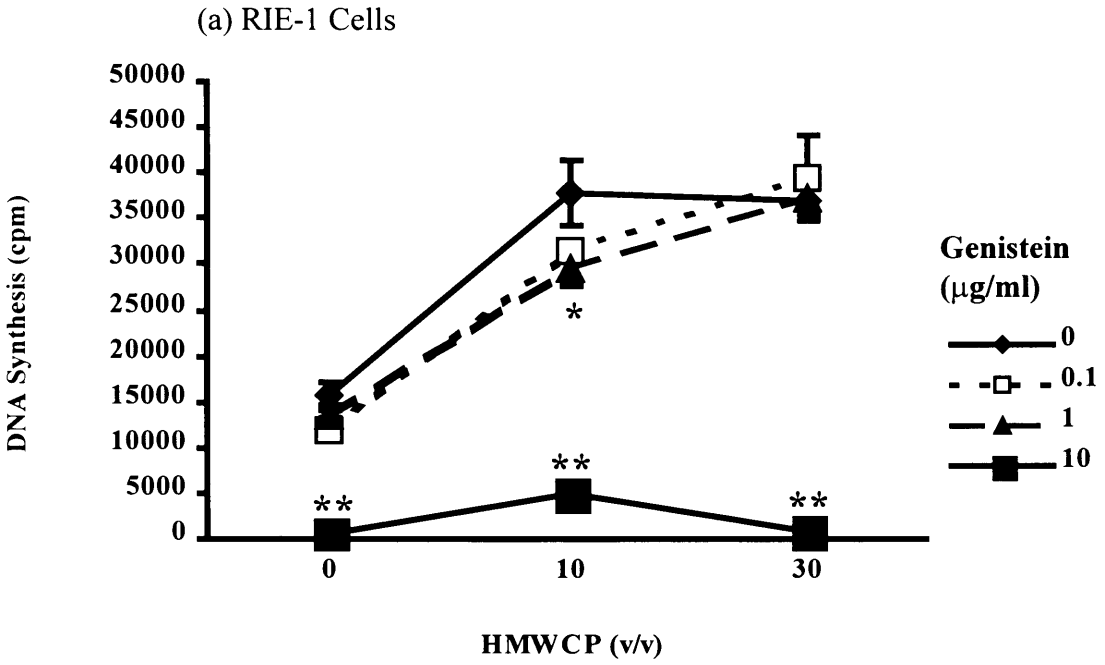
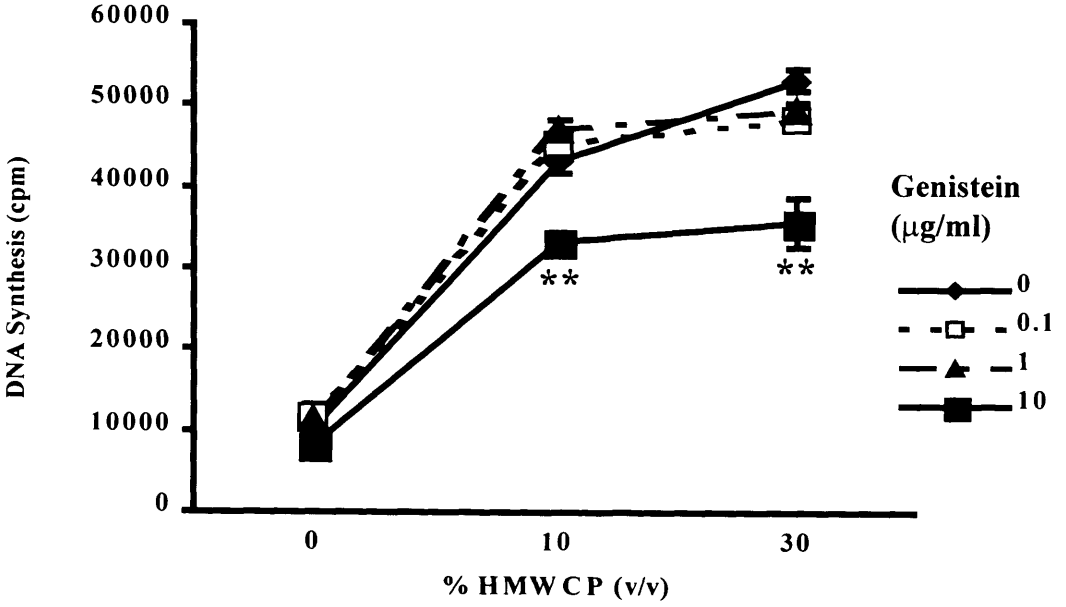


Figure 6.3 Genistein Inhibited Partially the Effects of High Molecular Weight Colostral Preparation (HMWCP) on Epithelial Cells and on Fibroblasts.

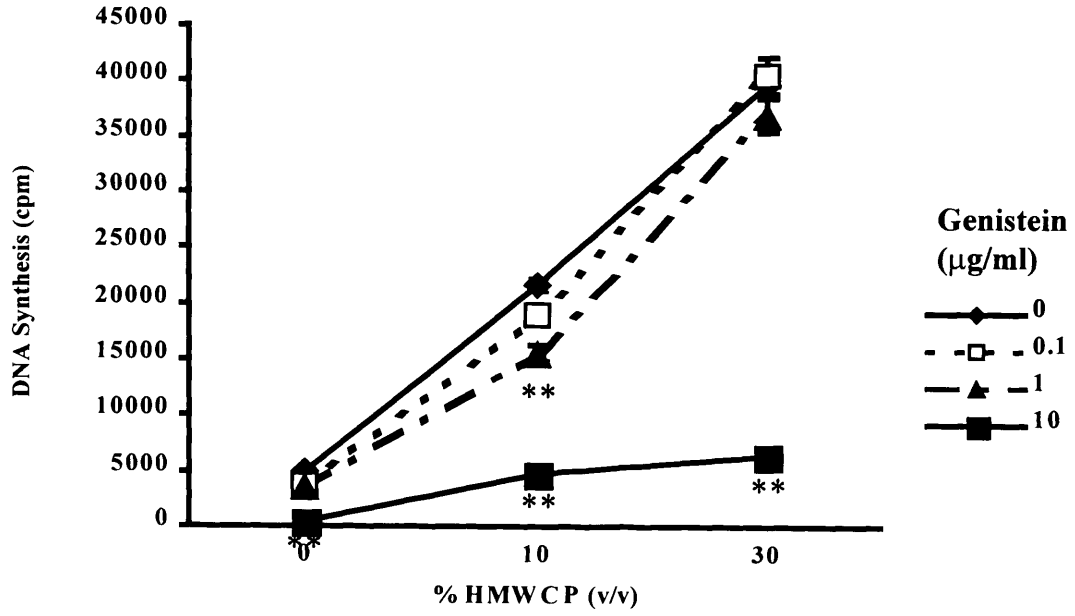
Cells were cultured in medium containing 10 or 30 % v/v HMWCP and genistein as described in the text. (a) RIE-1 DNA synthesis stimulated by 10% HMWCP, but not 30%, was reduced by 1 μg/ml genistein ($p<0.01$ vs HMWCP without genistein). (b) HT-29 DNA synthesis stimulated by 30% HMWCP was reduced by 10 μg/ml genistein ($p<0.01$ vs HMWCP without genistein). (c) NIH-3T3 DNA synthesis stimulated by HMWCP was unaffected by genistein.

Data represent mean+/-SEM (n=4) of a typical experiment (n=3). *$p<0.01$ vs culture in HMWCP without genistein. **$p<0.001$ vs culture in HMWCP without genistein.

(b) HT-29 Cells



(c) NIH-3T3 Cells



RIE-1 cells were exquisitely sensitive to suramin (figure 6.4a). It was not possible to make any conclusion regarding the effect of suramin on DNA synthesis in the presence of HMWCP because of the effects of suramin on basal DNA synthesis.

Suramin had no effect on basal DNA synthesis by HT-29 cells. DNA synthesis stimulated by HMWCP was inhibited by suramin (figure 6.4b). The effect was most pronounced at 100 μM suramin.

DNA synthesis by 3T3 fibroblasts was affected significantly by suramin. Suramin (10 μM) caused a reduction in DNA synthesis due to 10% HMWCP, and 100 μM suramin abolished the effect (figure 6.4c). However, suramin also significantly affected unstimulated NIH-3T3 cell. Similar data were obtained using 30% HMWCP.

These data did not reveal whether the effects of suramin were due to sequestration of growth factors, to effects within the cell, or to a mixture of the two. This problem was investigated by pre-treating the cells with suramin, and washing thoroughly before subsequent culture in colostral preparation.

Basal DNA synthesis by RIE-1 cells was unaffected by 10 μM suramin, but was inhibited significantly by 100 μM suramin. Similar data were obtained for DNA synthesis stimulated by HMWCP (figure 6.5a). The only reproducible effect was due to 100 μM suramin. These data were difficult to interpret due to the effect of suramin on unstimulated cells.

Similar data were obtained using HT-29 cells (figure 6.5b). However, there was a reduction in DNA synthesis stimulated by 10% HMWCP when cells were pre-treated with 10 μM suramin, suggesting that a small proportion of colostral bioactivity is due to pathways sensitive to suramin.

Basal 3T3 fibroblast DNA synthesis was unaffected by pre-treatment with suramin (figure 6.5c). However, DNA synthesis stimulated by HMWCP was significantly reduced by prior exposure of the cells to suramin.

(a) RIE-1 Cells

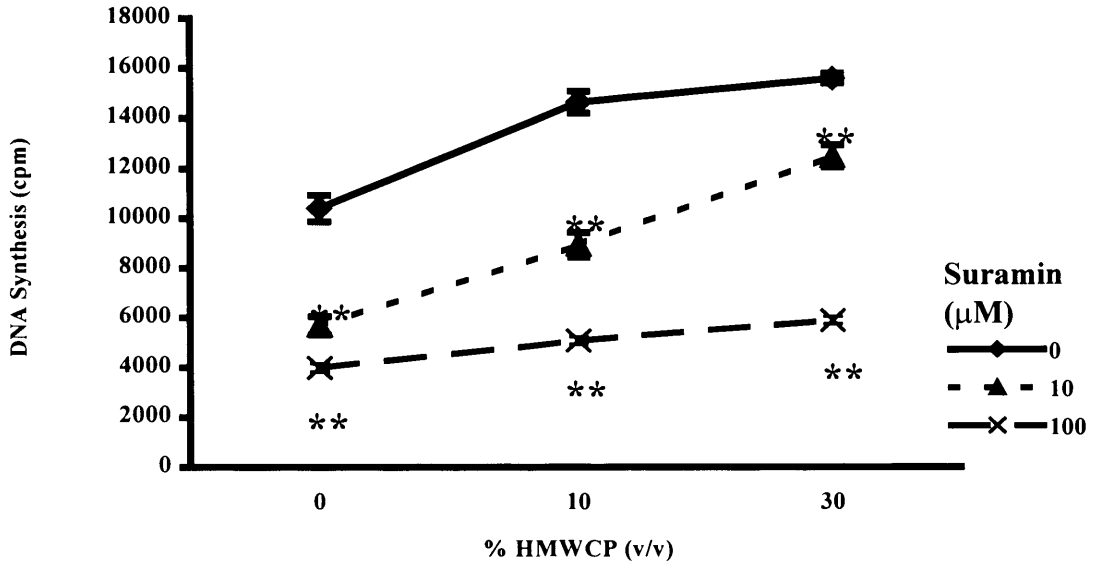
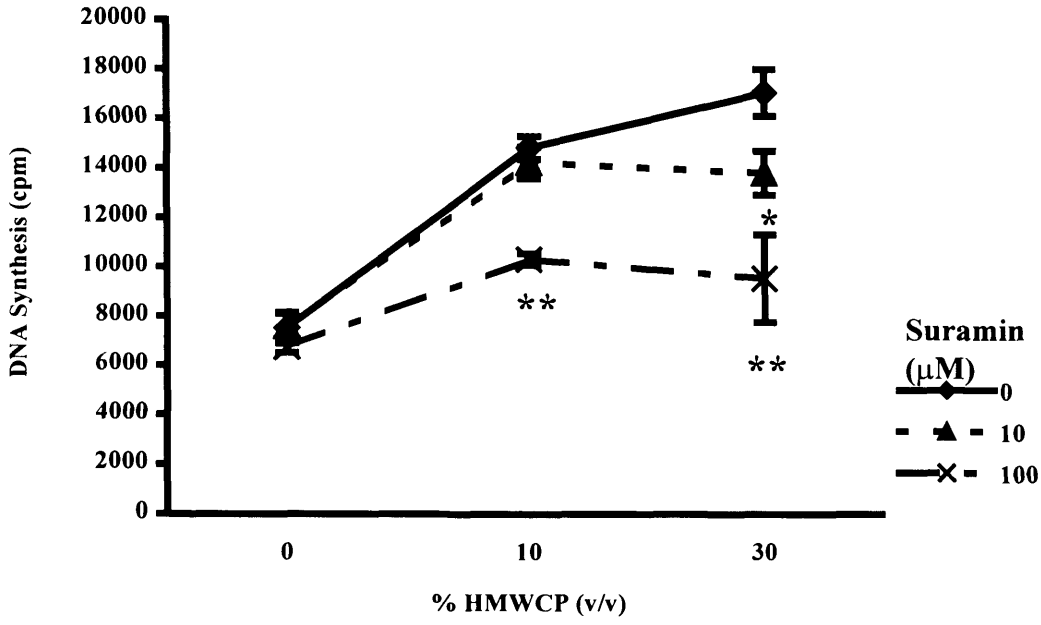


Figure 6.4 Suramin Affected HT-29 and NIH-3T3 DNA Synthesis Stimulated By High Molecular Weight Colostral Preparation (HMWCP).

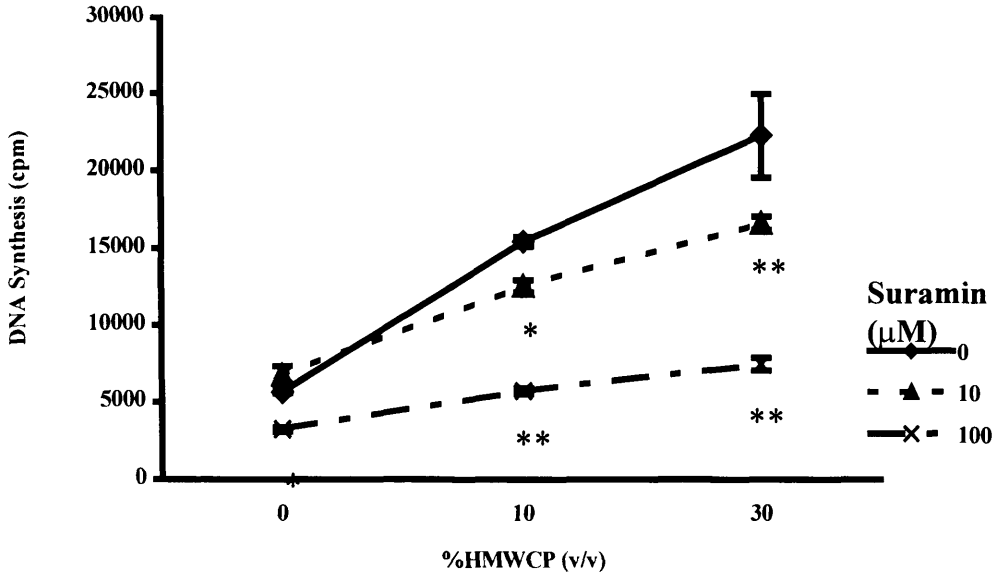
Cells were cultured in medium containing 10 or 30 % v/v HMWCP and suramin as described in the text. (a) RIE-1 basal DNA synthesis was inhibited by suramin dose dependently ($p<0.001$ vs control). (b) HT-29 DNA synthesis stimulated by HMWCP was reduced by suramin dose dependently ($p<0.05$ or $p<0.001$ as indicated). (c) NIH-3T3 DNA synthesis stimulated by HMWCP was inhibited by 10 μM suramin. 100μM suramin affected basal and stimulated DNA synthesis ($p<0.05$ and $p<0.001$ respectively vs appropriate conditions).

Data represent mean+/-SEM (n=4) of a typical experiment (n=3). *$p<0.05$ vs culture in HMWCP without suramin. **$p<0.001$ vs culture in HMWCP without suramin.

(b) HT-29 Cells



(c) NIH-3T3 Cells



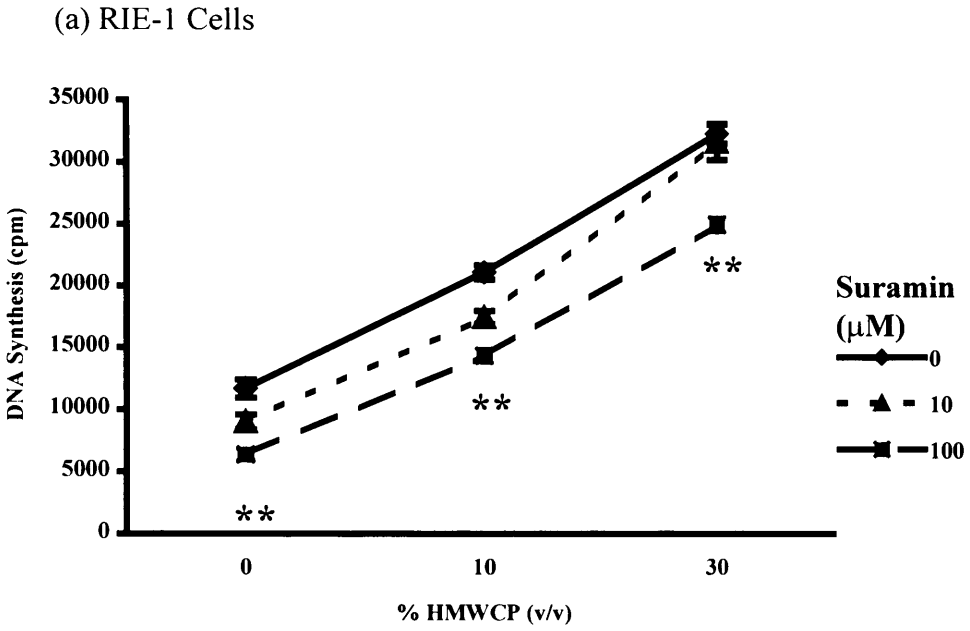
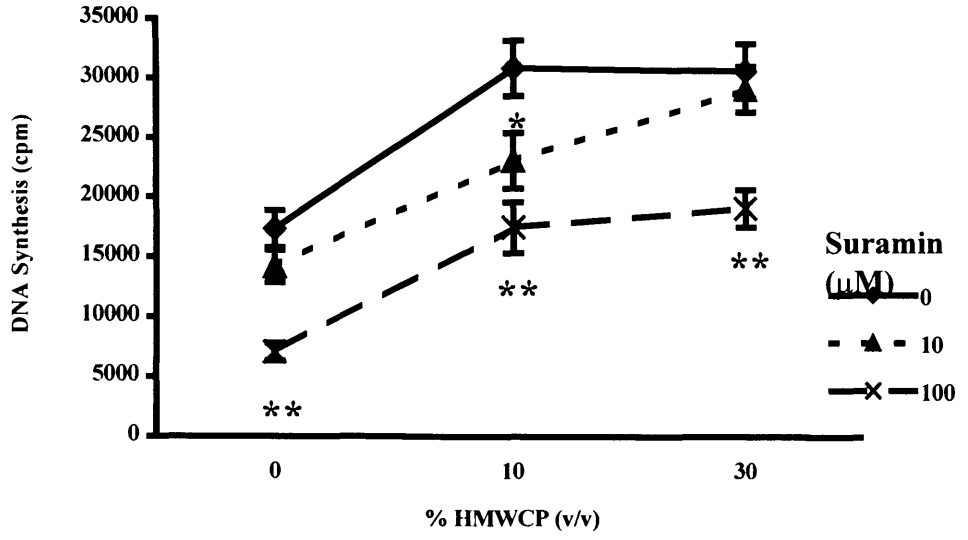


Figure 6.5 Pre-Treatment With Suramin Affected DNA Synthesis Stimulated By High Molecular Weight Colostral Preparation (HMWCP).

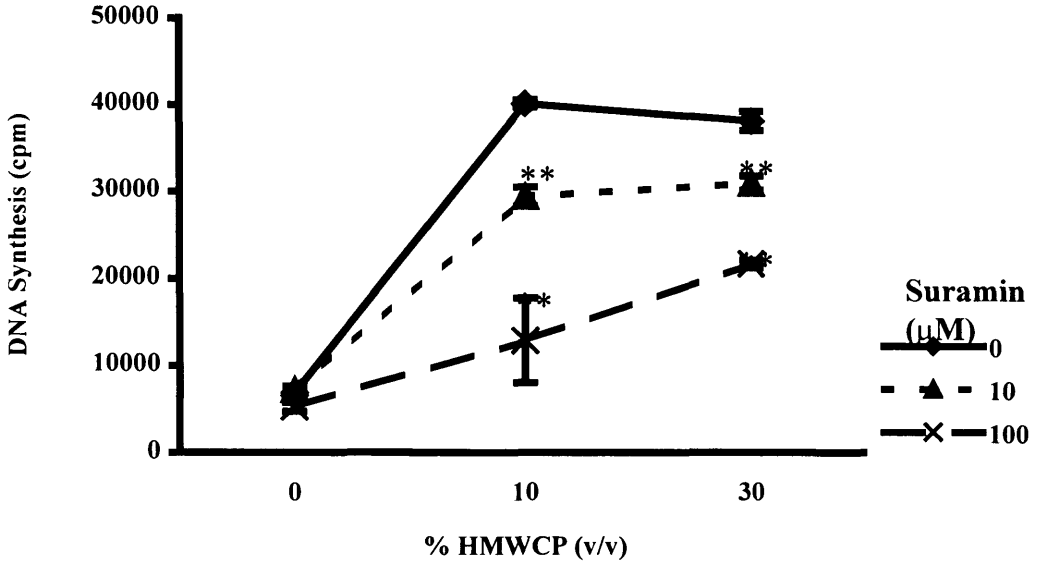
Cells were cultured in medium containing 10 or 30 % v/v HMWCP and suramin as described in the text. (a) RIE-1 basal DNA synthesis was inhibited by suramin dose dependently ($p<0.001$ 100 μM vs control). (b) HT-29 DNA synthesis stimulated by HMWCP was reduced by suramin dose dependently ($p<0.05$ or $p<0.001$ as indicated). (c) NIH-3T3 DNA synthesis stimulated by HMWCP was inhibited by 10 μM suramin. 100μM suramin affected basal and stimulated DNA synthesis ($p<0.05$ and $p<0.001$ respectively vs appropriate conditions).

Data represent mean+/-SEM (n=4) of a typical experiment (n=3). *$p<0.05$ vs culture in HMWCP without suramin. **$p<0.001$ vs culture in HMWCP without suramin.

(b) HT-29 Cells



(c) NIH-3T3 Cells



Data obtained using the three cell lines showed that there were differences between colostral bioactivity for each of the cell lines. Stimulated HT-29 and NIH-3T3 cells were sensitive to the presence of suramin, and prior exposure of NIH-3T3 cells to suramin prevented stimulation by HMWCP.

The data shown in figures 6.2 to 6.5 show that whilst the effects of colostral preparation in different models (such as cell lines) may be the same (increased DNA synthesis for example), the mechanisms by which the effects are produced are probably different.

6.6. The Role of the PDGF and EGF Receptor Tyrosine Kinases in Signal Transduction Stimulated by Bovine Colostral Preparation.

Bovine colostral growth factor (BCGF) has many characteristics of platelet-derived growth factor (PDGF; Brown and Blakely, 1984; Shing and Klagsbrun 1987). Receptors for EGF and PDGF appear to be involved in the action of this PDGF-like colostral growth factor (Brown and Blakely, 1984; Shing and Klagsbrun 1987). Experiments were devised to test the potential role of this colostral growth factor *in situ*. Signal transduction through the PDGF receptor was inhibited specifically with tyrphostin AG1295 (Kovalenko et al, 1994), and through the EGF receptor with tyrphostin AG1478 (Oshirov and Levitzki, 1994).

Preliminary experiments were performed to demonstrate the effect of the tyrphostins when the cells were exposed to the appropriate growth factor. PDGF (10 ng/ml) stimulated DNA synthesis by all three cell lines, and these effects were abolished by incorporation of 1 μg/ml tyrphostin AG1295 (figure 6.6 a-c). Tyrphostin AG1295 did not affect basal DNA synthesis in these cell lines.

EGF (10 ng/ml) stimulated DNA synthesis by RIE-1 and 3T3 cells (figure 6.7 a and c), but not by HT-29 cells (figure 6.7 b). The stimulatory effects of EGF were abolished by 1 μg/ml tyrphostin AG1478. Tyrphostin AG1478 had no effect on HT-29 DNA synthesis. Tyrphostin AG1478 significantly reduced basal DNA synthesis by RIE-1 cells.

Effect of tyrphostin AG1295 on Colostral Bioactivity. RIE-1 cells were unaffected by tyrphostin AG1295 (figure 6.8a). HT-29 DNA synthesis was stimulated by 10% HMWCP

(a) RIE-1 Cells

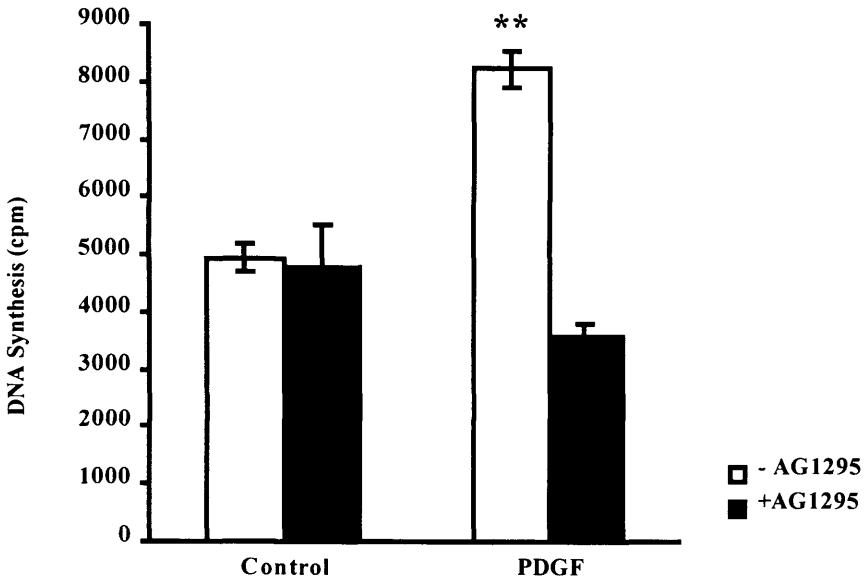
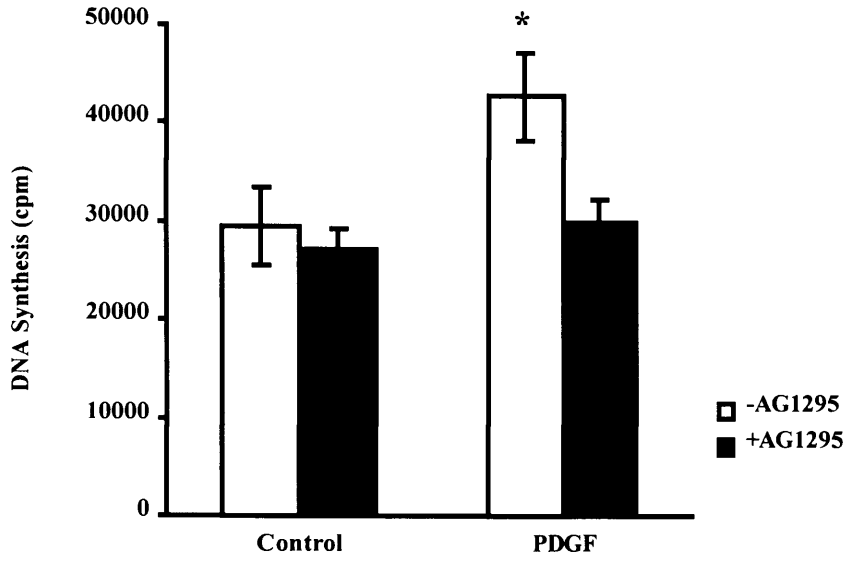


Figure 6.6 RIE-1, HT-29 and NIH-3T3 DNA Synthesis was Stimulated by PDGF-BB. Stimulated DNA Synthesis was Inhibited by 1 μg/ml Tyrphostin AG1295.

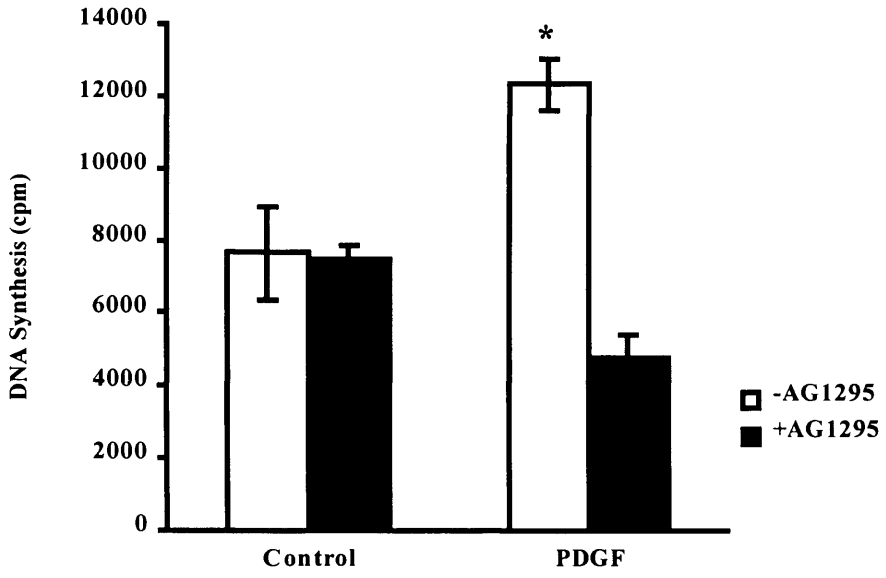
Cells were cultured in serum-free medium containing 10 ng/ml PDGF-BB with or without 1 μg/ml tyrphostin AG1295. This tyrphostin is specific for the PDGF receptor tyrosine kinase. DNA synthesis by RIE-1 (a), HT-29(b) and NIH-3T3 (c) were stimulated by PDGF ($p<0.05$ vs control cultures). Stimulated DNA synthesis was abrogated by tyrphostin AG1295. Tyrphostin AG1295 had no effect on basal DNA synthesis (NSD vs control).

Data represent mean+/-SEM (n=4) of a typical experiment (n=3). **$p<0.001$ vs control. *$p<0.05$ vs control

(b) HT-29 Cells



(c) NIH-3T3 Cells



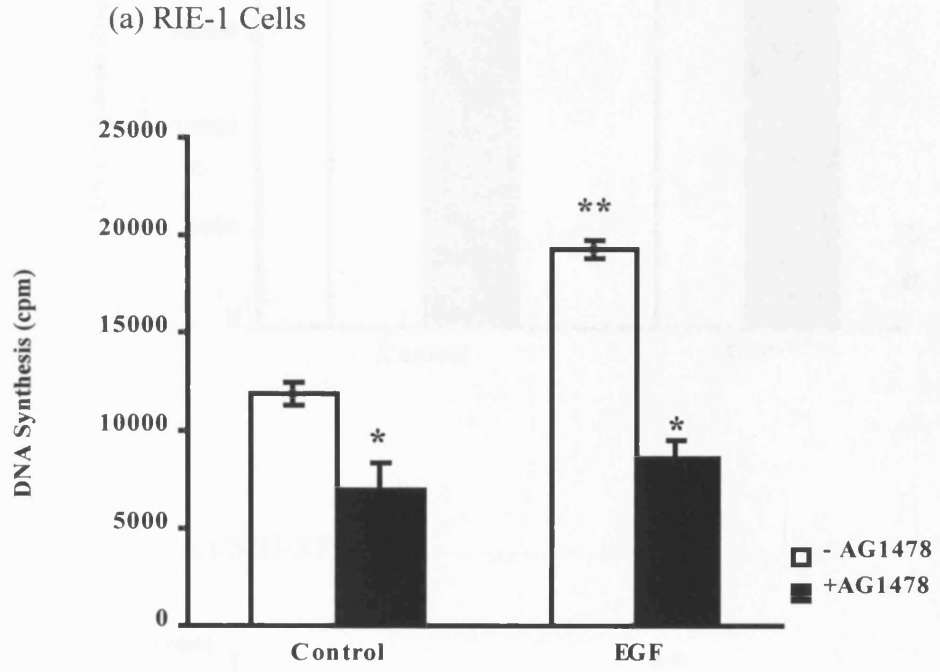
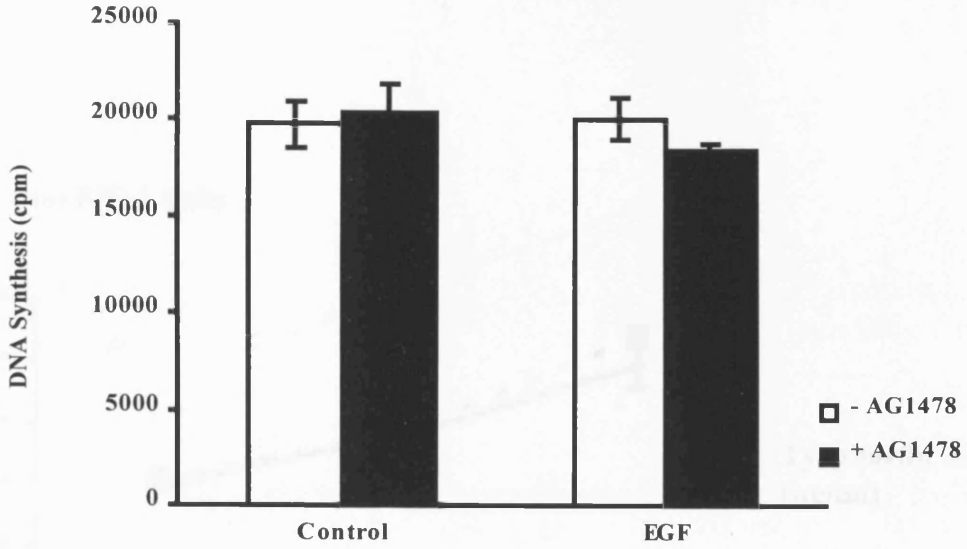


Figure 6.7 RIE-1 and NIH-3T3 DNA Synthesis is Stimulated by EGF. Stimulated DNA Synthesis is Inhibited by 1 μg/ml Tyrphostin AG1478.

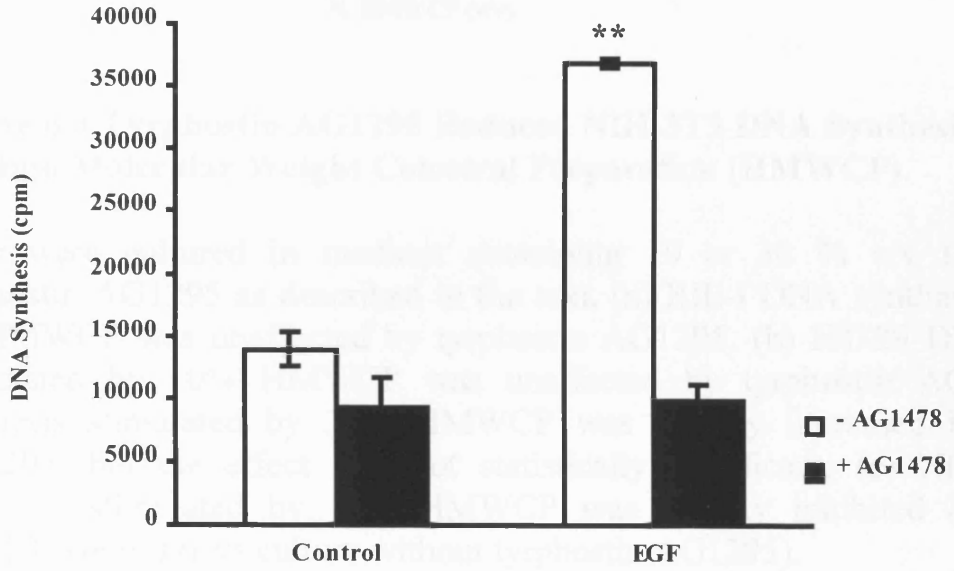
Cells were cultured in serum-free medium containing 10 ng/ml EGF with or without 1 μg/ml tyrphostin AG1478. This tyrphostin is specific for the EGF receptor tyrosine kinase. DNA synthesis by RIE-l (a) and NIH-3T3 (c), but not HT-29(b), was stimulated by EGF ($p<0.05$ vs control cultures). Stimulated DNA synthesis was abrogated by tyrphostin AG1478. Tyrphostin AG1478 reproducibly caused a significant decrease in basal DNA synthesis by RIE-1 cells ($p<0.05$ vs control).

Data represent mean+/-SEM (n=4) of a typical experiment (n=3). **$p<0.001$ vs control. *$p<0.05$ vs control

(b) HT-29 Cells



(c) NIH-3T3 Cells



(a) RIE-1 Cells

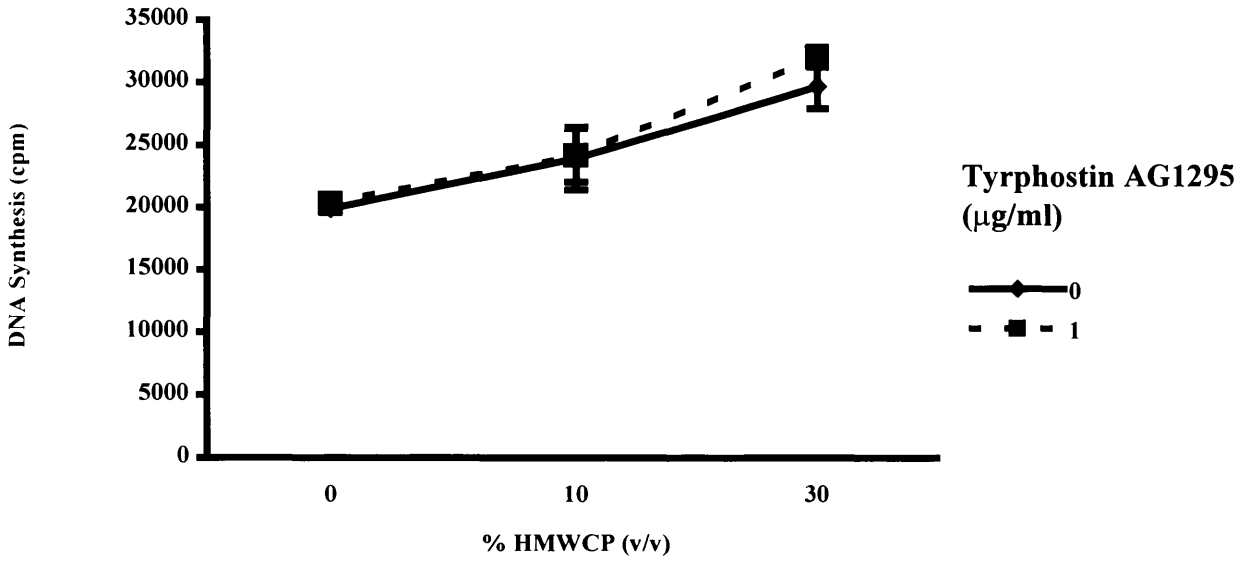
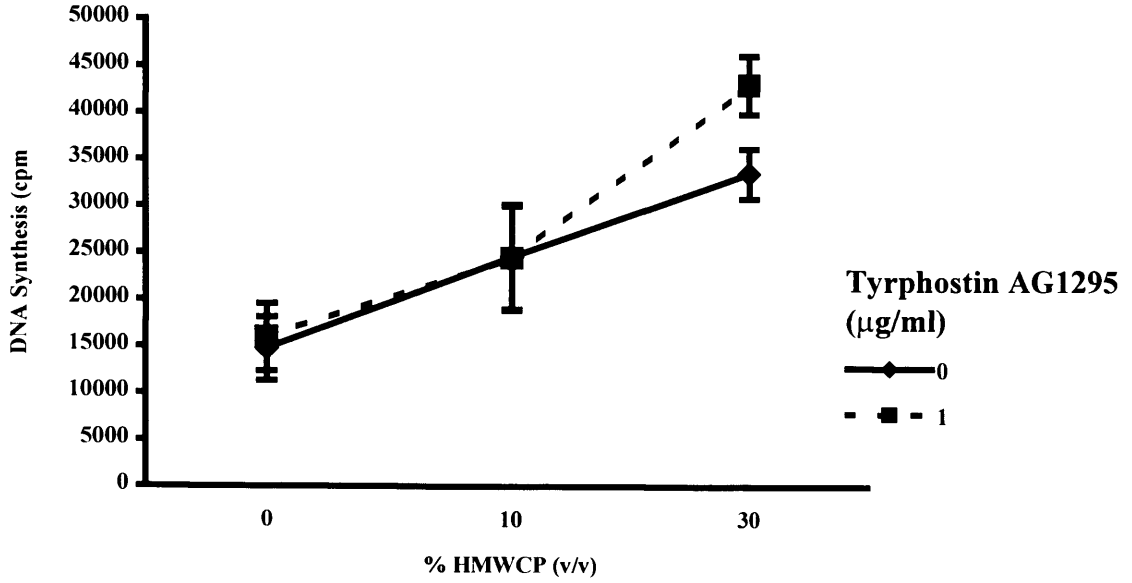


Figure 6.8 Tyrphostin AG1295 Reduced NIH-3T3 DNA Synthesis Stimulated By High Molecular Weight Colostral Preparation (HMWCP).

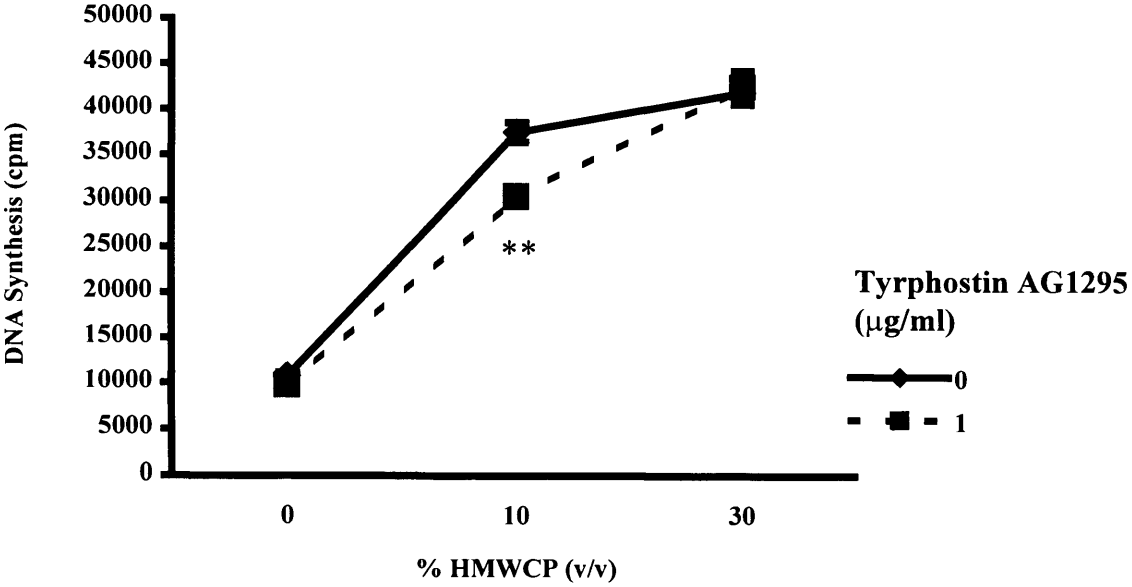
Cells were cultured in medium containing 10 or 30 % v/v HMWCP and tyrphostin AG1295 as described in the text. (a) RIE-1 DNA synthesis stimulated by HMWCP was unaffected by tyrphostin AG1295. (b) HT-29 DNA synthesis stimulated by 10% HMWCP was unaffected by tyrphostin AG1295. DNA synthesis stimulated by 30% HMWCP was slightly increased by tyrphostin AG1295, but the effect was not statistically significant. (c) NIH-3T3 DNA synthesis stimulated by 10% HMWCP was slightly inhibited by tyrphostin AG1295 ($p<0.001$ vs culture without tyrphostin AG1295).

Data represent mean+/-SEM (n=4) of a typical experiment (n=3). **$p<0.001$ vs culture in HMWCP without tyrphostin AG1295.

(b) HT-29 Cells



(c) NIH-3T3 Cells



without any effect of the inhibitor. However, DNA synthesis stimulated by 30% HMWCP was slightly *increased* in the presence of tyrphostin AG1295 (figure 6.8b). This effect was reproducible, but not significant when analysed by ANOVA (factors: presence of tyrphostin and amount of colostral preparation) and modified t-test. Fibroblast DNA synthesis stimulated by 10% HMWCP was slightly, but significantly, reduced by the inhibitor (figure 6.8c). No effect was found on DNA synthesis stimulated by 30% HMWCP.

Effect of tyrphostin AG1478 on Colostral Bioactivity. Unstimulated RIE-1 cells were very sensitive to the effects of tyrphostin AG1478 (figure 6.9a). The data obtained from cultures of RIE-1 cells exposed to HMWCP and tyrphostin AG1478 were thus difficult to interpret. However, tyrphostin AG1478 significantly reduced HT-29 DNA synthesis stimulated by 10 and 30% HMWCP (figure 6.9b). Basal 3T3 DNA synthesis was slightly, but not significantly, reduced by tyrphostin AG1478, whereas stimulated DNA synthesis was significantly reduced by tyrphostin AG1478 (figure 6.9c).

In summary, signal transduction via the PDGF receptor was not an important route by which colostral preparation exerted its effects on DNA synthesis. However, colostral bioactivity measured using HT-29 and NIH-3T3 cells was partly mediated via the EGF receptor.

6.7. Discussion.

DNA synthesis and cell proliferation stimulated by colostral preparations have been considered in terms of growth factors (Belford et al, 1997, Ichibada et al, 1992; Shing and Klagsbrun, 1984). However, the failure to reconstitute colostral, or milk, bioactivity using mixtures of recombinant growth factors required alternative approaches to understand the effects of bovine colostral preparation. The aim of the studies described in this chapter was to analyse the effects of colostral preparation without considering components in isolation. This was done using chemical inhibitors of signal transduction pathways, which required no assumption of the nature of colostral components. Instead, deductions were made based on the effects of colostral preparation on the cellular signalling machinery. These studies were performed using high molecular colostral fractions, in which most bioactivity resides (chapter 3, figure 3.9).

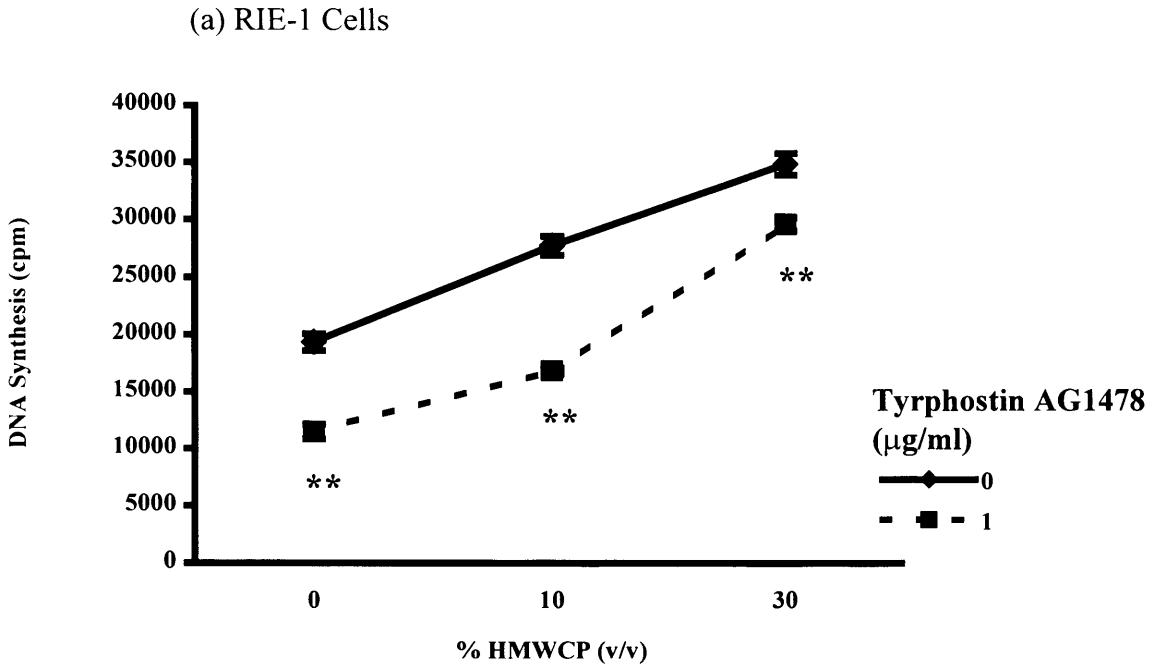
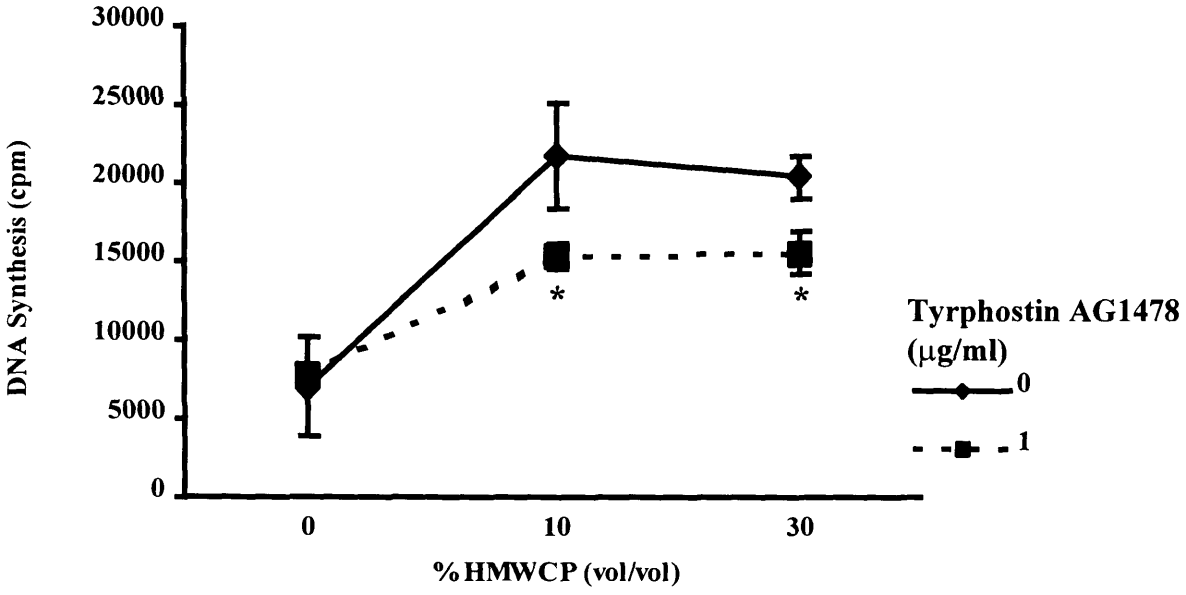


Figure 6.9 Tyrphostin AG1478 Reduced HT-29 and NIH-3T3 DNA Synthesis Stimulated By High Molecular Weight Colostral Preparation (HMWCP).

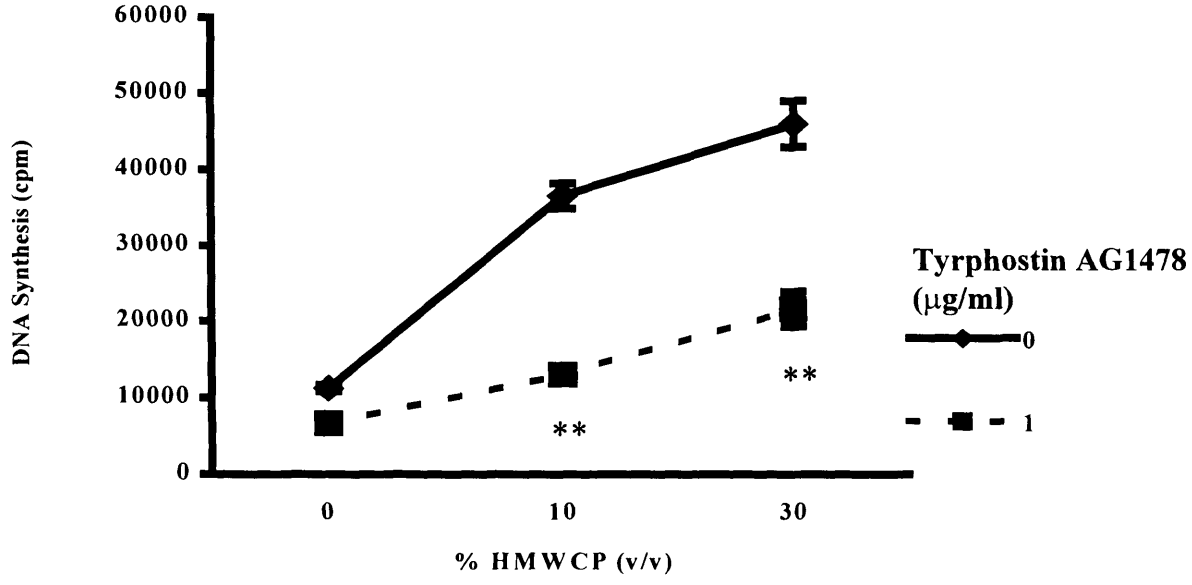
Cells were cultured in medium containing 10 or 30 % v/v HMWCP and tyrphostin AG1478 as described in the text. (a) RIE-1 basal and stimulated DNA synthesis was inhibited by tyrphostin AG1478. (b) HT-29 DNA synthesis stimulated by HMWCP was inhibited by tyrphostin AG1478 ($p<0.05$ vs cultures without tyrphostin AG1478). (c) NIH-3T3 DNA synthesis stimulated by HMWCP was inhibited by tyrphostin AG1478 ($p<0.001$ vs culture without tyrphostin AG1295). Tyrphostin AG1478 had a small, non-significant effect on basal DNA synthesis (NSD vs control).

Data represent mean+/-SEM (n=4) of a typical experiment (n=3). **$p<0.001$ vs culture in HMWCP without tyrphostin AG1478. *$p<0.05$ vs culture in HMWCP without tyrphostin AG1478.

(b) HT-29 Cells



(c) NIH-3T3 Cells



Data presented in figures 6.2-6.5 show that colostral bioactivity measured by stimulation of DNA synthesis was not due to dominant growth factors (IGF, TGFβ and FGF, Chapter 1, section 1.5.2), since the non-specific inhibitors herbimycin A, genistein and suramin reduced, but did not abolish, stimulated DNA synthesis, even at the highest doses used. These doses have been shown to be effective in abolishing signal transduction in other systems (Linassier et al, 1989; Miyata et al, 2000). The relative potency of active components in the high molecular weight colostral preparation differed according to the cell line being studied, a finding in agreement with that drawn from data presented in chapter 4. For example, epithelial cell lines were affected to a greater extent than were fibroblasts by tyrosine kinase inibitors (figures 6.2 and 6.3). Fibroblasts were sensitive to the effects of suramin on intracellular signalling pathways (figure 6.5), whereas epithelial cells were more sensitive to suramin in the presence of the colostral preparation, that is to growth factor sequestration and prevention of ligand-receptor binding (figure 6.4).

Tyrosine kinase activities play an important role in signal transduction (Pitcher et al, 1998). There are two types of tyrosine kinase: those intrinsic to growth factor receptors, and small, cytosolic proteins (reviewed by Robbins and Hollenberg, 1999). The role of these latter in signal transduction is much wider than first thought. Small, soluble tyrosine kinases are activated by G-protein coupled receptors (Luttrell et al, 1997). Receptors with tyrosine kinases can also co-ordinate multiple signalling pathways independently of their kinase activities (Daub et al, 1996; Yamauchi et al, 1997). The data presented in this chapter showed that inhibition by non-specific tyrosine kinase inhibitors did not result in total abrogation of the stimulatory effects of colostral preparation, suggesting that signalling pathways independent of tyrosine kinases were activated by colostral components. TGFβ acts via a serine/threonine receptor kinase (Massague, 1992 & 1998). However, previous studies showed that colostral TGFβ did not contribute significantly to the effects of colostral preparation on epithelial cells (chapter 4, figures 4.4), and only to the effects on fibroblasts following transient acidification of the colostral preparation (chapter 4, figure 4.5). Also, it is worth considering that inhibitors of tyrosine kinases might act generally, but not totally. Linassier et al (1990) found that genistein at less than 40 μM (10 μg/ml) inhibited NIH-3T3 proliferation stimulated by EGF or insulin, but did not inhibit other signal transduction pathways acting via soluble tyrosine kinases, such as induction of c-Myc.

Whilst the tyrosine kinase inhibitors have been used at doses shown previously to be effective, these earlier studies usually concentrated on single pathways (Linassier et al, 1990; Miyasa et al, 2000). Coffey et al (1987) showed an inhibitory effect of suramin (100 μM) on cells stimulated with FCS (5% vol/vol); however, the effect on 'unstimulated' cells was not documented. Higher doses of inhibitors might be required for analysis of a complex mixture such as colostral preparations, and whilst such studies are warranted, problems are expected due to the effects of the inhibitors on unstimulated cells (e.g. data shown in figures 6.3 a and c).

Suramin, when present *with* colostral preparation, had a significant inhibitory effect on DNA synthesis by HT-29 and NIH-3T3 cells. RIE-1 cells were exquisitely sensitive to suramin, showing significant inhibition of basal DNA synthesis. This might be due to toxicity in this particular cell line. However, the data in figure 6.5a show that cells exposed to high dose suramin can be subsequently stimulated by colostral preparation. Suramin had a significant inhibitory effect on fibroblast DNA synthesis stimulated by colostral preparation whether exposed prior to, or in concert with, colostral preparation. Thus, the epithelial cells are probably stimulated by proteins contained in colostrum that are adsorbed onto suramin by its multiple ionic charges (Middaugh et al, 1992), whereas fibroblast DNA synthesis is stimulated by colostral components that act via cellular pathways that are inhibited by suramin (Baghdiguian and Fantini, 1997). One potential signalling pathway affected by suramin involves G-protein coupled receptors (GPCRs; Baghdiguian and Fantini, 1997; Chahdi et al, 1998). A number of ligands operate via GPCRs, including lysophosphatidic acid (LPA), adrenaline, trypsin and thrombin (reviewed by Pitcher et al, 1998). Activation of GPCRs can result in stimulation of cell growth and adhesion (Miyata et al, 2000). Several minor colostral components are known to stimulate GPCRs (Xu, 1998), although some have been shown not to affect DNA synthesis and proliferation (Bjork et al, 1993).

The data discussed so far show clearly that a simple consideration of colostral growth factors is inadequate for a full description of colostral bioacitivity *in vitro.* The patterns of dose response in figures 6.2-6.5 also suggest that for a particular cell line there are components of differing potencies. For example, inhibition by genistein was observed when cells were stimulated by 10% HMWCP, but not by 30% HMWCP (6.3 a and c). These data suggest that there is a dose response to a colostral component(s) that is insensitive to genistein, and this

component(s) contributes progressively more to colostral bioactivity from 10% to 30% HMWCP.

Bovine milk and colostrum contain negligible amounts of EGF when compared with human milk and colostrum (Brown and Blakely, 1984). Instead, bovine milk and colostrum contains betacellulin, a 26 kDa EGF receptor ligand (see chapter 1, section 1.5.2.5). Ruminant colostra also contain a PDGF-like growth factor (Shing and Klagsbrun, 1984; Shing and Klagsbrun 1987). Partially pure bovine colostral growth factor (BCGF) is the bovine form of this PDGF-like growth factor, which stimulates fibroblast DNA synthesis and proliferation (Shing and Klagsbrun, 1984; Shing and Klagsbrun 1987). However, the role of these PDGF-like growth factors in modulating epithelial proliferation is equivocal (Corps and Brown, 1987). DNA synthesis by the cell lines used in this study was stimulated by recombinant PDGF. This effect was abrogated by tyrphostin AG1295, which specifically inhibits the intrinsic tyrosine kinase activity of the PDGF receptor. Tyrphostin AG1295 had no effect on RIE-1 DNA synthesis stimulated by colostral preparation. These data fit with those of Corps and Brown (1987), who showed that RIE-1 proliferation was stimulated by human and ruminant colostral preparations, but not by purified PDGF-like colostral growth factor.

The data in figure 6.8a show that any PDGF-like molecule in the colostral preparation does not act on RIE-1 cells via the PDGF receptor in the presence of other growth factors. Similar data were obtained using HT-29 cells, although there was a small increase in DNA synthesis stimulated by 30% colostral preparation when tyrphostin AG1295 was present. This increase was not statistically significant when analysed by ANOVA and modified t-test. Earlier studies showed that HT-29 DNA synthesis was stimulated by low amounts of unfractionated colostral preparation, but inhibited by higher amounts (chapter 3). Further studies of components acting on HT-29 cells via the PDGF receptor are required using higher concentrations of colostral preparation.

Proliferation of Swiss 3T3 fibroblasts is stimulated by PDGF-like colostral growth factor in the presence of serum (Brown and Blakely, 1984). Thus, the inhibitory effect of tyrphostin AG1295 on NIH-3T3 fibroblasts is to be expected. However, the absence of effect of the

tyrphostin on 30% colostral preparation (figure 6.8c) suggests that stimulatory activity acting via the PDGF receptor is a minor component of colostral preparation.

PDGF-like colostral growth factor inhibits binding of EGF to its receptor (Brown and Blakely, 1984). This effect is not due to binding of the PDGF-like colostral growth factor to the EGF receptor *per se* (Brown and Blakely, 1984). Clearly, the EGF receptor plays a central role in the modulation of basal RIE-1 DNA synthesis, since specific inhibition of the receptor tyrosine kinase with tyrphostin AG1478 significantly reduced basal DNA synthesis. This effect made difficult the interpretation of data obtained from cells stimulated by colostral preparation. The dose response curves of DNA synthesis to colostral preparation were approximately parallel (figure 6.9a), suggesting that colostral factors stimulating RIE-1 DNA synthesis do not act via the EGF receptor. This interpretation agrees with data presented by Corps and Brown (1987), showing that RIE-1 proliferation was stimulated by colostral preparations irrespective of the response of RIE-1 cells to exogenous EGF.

HT-29 and NIH-3T3 DNA synthesis stimulated by colostral preparation was inhibited by tyrphostin AG1478. Thus, EGF receptor tyrosine kinase activity plays an important role in relaying colostral stimulatory signals to the nucleus. This was particularly unexpected for HT-29 cells, which were insensitive to recombinant EGF (figure 6.7b). Colostral preparation contains betacellulin, a ligand for the EGF receptor. Inhibition of signalling stimulated by betacellulin might account for these data. However, tyrosine kinase activities in general were found to be relatively minor components in mitogenic signalling pathways stimulated by colostral preparation (figures 6.2. and 6.3). Alternatively, the EGF receptor might be acting as a 'scaffold' around which signalling molecules associate to modulate growth (Daud et al, 1996; Yamauchi et al, 1997). These complexes may play a central role in controlling enterocyte restitution and proliferation. This 'scaffold' function appears to be independent of intrinsic receptor tyrosine kinase activity (Yamauchi et al, 1997). However, tyrphostin AG1478 inhibits this 'scaffold function' (Daub et al, 1996). Further studies are warranted to analyse the role of the EGF receptor in cell signalling stimulated by colostral preparation.

In conclusion, growth factors played an important role in colostral bioactivity, but other molecules, as yet unidentified, also contributed. Major active components were different for different cell lines, probably due differences in receptor expression between cell lines. The role of the EGF receptor in signal transduction due to bovine colostral preparation was of

particular interest. Considerable further study is required to elucidate colostral components that contribute to bioactivity, and to determine the signalling pathways that mediate their effects.

Chapter 7.

Concluding Remarks and Suggestions for Further Study.

Bovine colostral preparations are commercially available health food supplements that contain active growth factors. Such preparations might be of use as prophylactics and healing agents for treatment of gastrointestinal injury. The studies presented in this thesis were designed to test and analyse bioactivity of one bovine colostral preparation in the context of the injured gut.

Bovine colostral preparation stimulated DNA synthesis by epithelial cells and fibroblasts, as shown by others (Belford et al, 1997; Shing and Klagsbrun, 1984), which resulted in cell proliferation. Data shown in chapter 3 demonstrated a motogenic effect on injured epithelial cells. This was a novel and important observation since gut epithelial integrity is essential for normal mucosal function, as well as preventing infection and inflammation (Hogan et al, 1994).

Preparations from bovine colostrum are taken orally, and the fate of bioactive components in the acidic/proteolytic milieu of the stomach must be considered. Motogenic colostral components were resistant to transient acidification (pH2 for two hours). Thus, motogenicity would be expected to survive in the gut and stimulate wound healing; however, proteolysis also occurs in gastric juice (Playford et al, 1993), and further experiments are required to test the effect of proteolytic enzymes on colostral bioactivity. Additional experiments mixing colostral preparation with gastric juice from healthy adults and patients suffering from gastrointestinal disorders would also be informative as to the *in vivo* stability of the preparation. Mitogenic colostral components were sensitive to transient acidification, and thus would be expected not to affect epithelial proliferation. Studies analysing proliferation *in vivo* of many cell types (for example, epithelial, fibroblastic, and blood cells) would provide information about potential mitogenic components that were not detected in the studies presented here. For example, passage through the stomach will increase amounts of active TGFβ2, which stimulates fibroblast proliferation. The importance of such studies cannot be underestimated when the long-term ingestion of colostral preparations is considered.

TGFβ2 has effects other than stimulation or inhibition of proliferation; these include stimulation of extracellular matrix secretion, and modulation of the immune system (summarised in chapter 1, section 1.5.2.2). These effects should be taken into account when

considering the data obtained from the study of prophylactic effects of TGFβ2 against NSAID-induced small intestinal injury (chapter 4). TGFβ2 was prophylactic when administered either orally or sub-cutaneously, although the mechanisms of prophylaxis might differ according the route of administration. The effect of TGFβ2 on intestinal crypt depth is interesting in this context. Detailed study of *how* TGFβ2 caused shallower crypts in normal animals, yet deeper crypts in animals with intestinal injury would be informative. Effects on crypt cell proliferation, on sub-epithelial myofibroblast growth and matrix secretion, and on physical tension in the basement membrane would need investigation. Additional studies analysing the effects of TGFβ2 on immune cell function and levels of circulating pro- and anti-inflammatory cytokines would also be informative. Data from such studies would add to current knowledge of how the gut is protected from injury, and how repair occurs. Subsequent studies using bovine colostral preparation would determine whether colostral TGFβ had similar effects in the presence of other colostral components.

Routes of administration and modes of action are also of interest when considering the healing effects of colostral preparation on colitis. Colostral preparation taken orally stimulated healing of colitis, but it is unlikely that much bioactivity would remain in the gut lumen after passage through the small intestine, given that intestinal permeability is elevated during colitis (Soderholm et al, 1999). The data presented in chapter 5 have important implications for the clinical use of bovine colostral preparations; however, mechanistic studies would provide invaluable data allowing understanding how the colostral preparation works. In the case of healing colitis, the most likely mode of action is through stimulation of circulating modulators both of gut function and of the immune system. Changes in circulating hormones, growth factors, and pro- and anti-inflammatory cytokines should be monitored, as well as analysis of gut mucosal levels of regulatory peptides, growth factors, and pro- and anti-inflammatory molecules and enzymes. Additional studies are warranted to determine the reasons why topical application of colostral preparation failed to stimulate healing of colitis. A beneficial effect was expected because of the direct effects of colostral preparation on epithelial restitution. Failure was most likely due to pragmatic problems with the rat model, especially obstructive faeces. The modified treatment protocol of Tran et al (1999), where administration of therapeutic enemas begins several days after induction of colitis, should be

employed to analyse the effects of colostral preparation on healing, and to analyse effects on colon weight and neutrophil content.

Novel methods are required for mechanistic studies of colostral bioactivity *in vitro* (chapter 6). Conventional studies employing physical or chemical fractionation, are useful; however, such methods study colostral components in more or less isolation from each other. Inhibition of the effects of one colostral component *in situ* may be more informative if there are supra-additive effects of two or more components. The data obtained from preliminary studies using inhibitors of signal transduction showed that the effects of bovine colostrum are partly, but not wholly, due to known growth factors. None of the inhibitors used were able to abolish DNA synthesis stimulated by bovine colostral preparation. The role of other cell signalling systems, including serine/theonine kinases and G-protein-coupled receptors should be investigated, as well as 'down stream' signalling molecules, such as mitogen-activated protein kinases (MAP kinases) and their substrates. These studies should be extended to include analysis of signalling pathways involved in stimulated cell migration, in particular, those acid-resistant components described in chapter 4.

One possible use of colostral preparation would be as part of a combination therapy, for example with proton pump inhibitors in peptic ulcer disease, or with 5-ASA in IBDs. It can readily be conceived that such combinations might allow the use of reduced doses of conventional treatments, thereby reducing side effects whilst providing a good level of treatment.

There are many sources and types of bovine colostral preparations available. The relative bioactivities of these different preparations is unknown, and must be evaluated if they are to be tested or used clinically, allowing doses to be adjusted accordingly.

Concern over the long-term effects of ingested colostral preparation has been mentioned. Any such concerns must include a consideration of any role in the induction or progression of cancers. For example, IGF-I is a potent mitogen for cancer cells *in vitro* (Baghdiguian et al, 1992; Pollak and Richard, 1990); however, these negative effects must be balanced against the anti-cancer properties of colostrum (Gill and Cross, 2000). Evaluation of the effects of

ingested colostral preparation on cancer induction and progression using animal models will be necessary to evaluate any long-term risks to patients.

In conclusion, bovine colostral preparation *is* a potential prophylactic and healing agent for gut injury, which requires further pre-clinical, as well as small-scale clinical, evaluation. Additional experimentation is necessary to understand fully the mechanisms by which colostral preparation exerts its effects.

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