

### III. THE INTESTINAL MUCUS BLANKET

#### 1. Effects of Specific Bacteria on Mucus Secretion

Mucus is secreted throughout the gastro-intestinal tract by a variety of specialised cells located in glands or in the surface epithelium. The prime function of the mucus layer is to protect the delicate underlying mucosa from damage by potentially harmful agents in the lumen (such as digestive enzymes and bacteria) and by the mechanical forces associated with digestion and the passage of solid material through the gut. Mucus exists in two distinct physical forms: 1. a thin layer of stable, water-insoluble gel firmly adhering to the mucosal surface and 2. a soluble layer mixed with luminal material that overlies the gel. The soluble mucus, although very viscous, can be removed from the mucosa by gentle washing. Both the adherent gel and the soluble mucus are composed of mucus glycoproteins (or mucins) which are responsible for the characteristic visco-elastic and gel-forming properties of the secretion. The relationship between the soluble and gel forms of mucus has not yet been clarified but, since proteolytic enzymes are capable of solubilising mucus gel and degrading mucus glycoproteins, it is presently believed that the sol layer is

likely derived from the gel by a combination of enzymatic and mechanical erosion (Allen and Carroll, 1985). Therefore, to maintain an intact mucus barrier, erosion from the luminal surface must be balanced by secretion.

In mucosal sections fixed and stained for light or electron microscopy, the adherent mucus layer on the epithelial surface is difficult to visualise and often appears discontinuous. This is because many of the fixatives used, such as ethanol and glutaraldehyde, dehydrate the mucus causing denaturation and shrinkage of the gel. However, gastric mucus can readily be observed in situ on unfixed mucosal sections mounted transversely under the light microscope. The gel appears as a thin but continuous translucent layer of varying thickness (50 to 450  $\mu\text{m}$  in man) between the surface epithelium and the luminal solution (Allen and Carroll, 1985). In the intestine and colon, a mucus 'blanket' (30 to 400  $\mu\text{m}$  thick, dependent on species and region of the gut) lines the crypts and covers the villi and surface mucosa (Sakata and Engelhardt, 1981; Rozee et al., 1982). Observation of the continuity of the mucus layer in the intestine and co-

**Table 2:** Methods used for measurement of mucin secretion

1. *In vitro* or *in vivo* incorporation of radiolabelled precursors (e.g.  $^{14}\text{C}/^3\text{H}$ -monosaccharides,  $^{35}\text{S}$ -sulphate) into tissue and release of high molecular weight labelled glycoproteins into secretions.
2. Colorimetric assays of glycoprotein or individual monosaccharide constituents (e.g. galactose, sialic acid) in secretions.
3. Determination of total carbohydrate content of secretions by gas liquid chromatography.
4. Ultrastructural morphologic assessment of mucus-producing tissue by light or electron microscopy, including autoradiography (presence/absence of full/empty goblet cells).
5. *In situ* determination of adherent mucus gel thickness over the surface mucosa.
6. Specific enzyme-linked or radio-immunoassays of mucin in secretions.

lon is particularly difficult but has been achieved at the electron microscopical level following stabilisation of the gel with anti-mucus antibody (Rozee et al., 1982). In these same studies, it was found that the surface mucus blanket contains a rich and varied population of indigenous bacteria and protozoa that are randomly distributed throughout the mucus layer but are physically separated from the underlying mucosa by the gel. Although most micro-organisms do not penetrate beyond the mucus barrier, some filamentous bacteria are anchored in the epithelium and are buried under or project into the mucus gel (Rozee et al., 1982).

Most of what is known about the effects of specific bacteria on intestinal mucus secretion is based on observations during particular infections. There are very few systems where goblet cells and mucin secretion have been studied in detail and any changes accurately quantitated. This is in part due to the multitude of difficulties associated with measuring mucin secretion. The gel layer adheres strongly to the mucosa and is therefore difficult to sample. Thorough washing of the tissue may not

remove all the adherent gel, particularly that trapped in the crypts, and scraping of the mucosa inevitably damages the underlying epithelium leading to contamination by intracellular mucin released from ruptured goblet cells. Measurement of soluble mucin in the lumen is not a satisfactory index of secretion since an increase in the amount present may reflect greater erosion of the insoluble gel layer rather than actual mucin release. Notwithstanding these problems, a variety of methods have been used to assess gastro-intestinal mucin secretion (Table 2). Many, however, are associated with pitfalls and require careful interpretation. The major drawback to most of the techniques is their lack of sensitivity and/or specificity. For example, precursor radiolabelling and colorimetric assays measure all glycoproteins present in secretions and not just mucus glycoproteins (Forstner, Maxwell and Romui, 1981; Specian and Neutra, 1982). Ultrastructural, morphologic assessment of mucus-producing tissue may be used to determine whether goblet cells have emptied (Specian and Neutra, 1982; Neutra, O'Malley and Specian, 1982) but has severe

**Table 3:** Effects of cholera toxin on rat intestinal mucin secretion

	Amount of toxin	Potency (‘blueing dose’)	µg mucin released mg tissue protein
Crude filtrate:	50 mg/ml	60	25
	25 mg/ml	30	22
Pure toxin:	50 µg/ml	950	15
	20 µg/ml	380	16
	10 µg/ml	190	17
Control:	no toxin	0	5

Rat intestinal tissue slices were incubated in vitro in modified Krebs-Ringer solution, pH 7.4, at 37°C, in a shaking metabolic incubator, in the presence of varying amounts of crude dialysed cholera filtrate or purified enterotoxin. The amount of mucin secreted into the incubation medium at the end of the 90 min incubation period was determined using a radio-immunoassay and mucin release was calculated relative to the protein content of the incubated tissue. Data taken from: Forstner et al., 1981.

quantitative limitations in terms of the amount of mucin released. Actual measurement of mucus gel thickness is possible in the stomach (Allen and Carroll, 1985) but cannot be reliably carried out in the intestine and colon due to the presence of crypts and the fragility of the tissue. Recently, however, the development of highly specific and sensitive immunoassays for intestinal mucins have allowed for accurate and reliable quantitation of secretion (Forstner, Maxwell and Roomi, 1981; Forstner et al., 1981; Roomi et al., 1984; Mantle et al., 1989).

There are two studies in which intestinal mucin secretion (as assessed by immunoassays) has been thoroughly investigated in response to specific bacteria, namely *Vibrio cholerae* (Forstner et al., 1981; Roomi et al., 1984) and *Yersinia enterocolitica* (Mantle et al., 1989). The first case represents an acute rapid response in mucin

secretion to a bacterial toxin, while the second study examines the more long-term effects of an entero-invasive organism.

Following exposure of the rat small intestine to cholera toxin, mucin secretion is rapidly increased within 30 minutes (Forstner et al., 1981). Since crude cholera filtrate stimulates mucin secretion to a greater extent than purified enterotoxin (Table 3), it appears that the crude extract contains another potent (but as yet unidentified) mucin secretagogue. Cholera toxin binds to GM1 ganglioside in the cell membrane and ultimately activates adenylate cyclase, leading to increased intracellular cAMP levels and elevated fluid and electrolyte secretion from the epithelium into the intestinal lumen. However, a variety of agents that are known to raise intracellular cAMP levels and/or promote intestinal fluid secretion have no effect on mucin secretion (Table 4),

**Table 4:** Effects of agents that stimulate fluid secretion and/or increase intracellular cAMP on mucin secretion in the rat small intestine

Agent	% change in mucin secretion
Control	100%
Crude cholera filtrate (125 mg)	1000%
Pure cholera enterotoxin (20 µg)	950%
Dibutyryl cAMP ( $10^{-2}$ M and $10^{-3}$ M)	120%
+theophylline ( $10^{-3}$ M)	120%
Theophylline ( $10^{-3}$ M)	100%
Isoproterenol ( $10^{-3}$ M)	85%
+theophylline ( $10^{-3}$ M)	85%
VIP ( $2 \cdot 10^{-7}$ M and $3 \cdot 10^{-8}$ M)	100%
+theophylline ( $10^{-3}$ M)	100%
Mannitol (450 mosmol/l)	170%

*In situ* loops were constructed in the small intestine of anaesthetised rats. Control or test solution was injected into the loop lumen, the abdomen was closed and the animal allowed to regain consciousness. After 4 hours, the rat was sacrificed, the loop was removed, opened and carefully washed to collect all secretions. Control secretion (0.8 - 1.2 µB mucin protein/mg tissue protein) was normalised to 100% and test solutions were calculated relative to this baseline. All the above agents (with the exception of mannitol) increase intracellular cAMP and promote intestinal fluid secretion. Theophylline is a phospho-di-esterase inhibitor. Isoproterenol is a β-adrenergic agonist. VIP, vaso-active intestinal peptide. Hyperosmotic mannitol stimulates fluid secretion but without increasing intracellular cAMP concentration. Data taken from: Roomi et al., 1984.

**Table 5:** Effect of *Yersinia enterocolitica* on rabbit intestinal and colonic mucin production

Mucin		Upper small intestine	Mid small intestine	Distal small intestine	Proximal colon
Tissue content ( $\mu\text{g}/\text{mg}$ )	INF	86 $\pm$ 12*	106 $\pm$ 24*	121 $\pm$ 13*	200 $\pm$ 19*
	PFC	41 $\pm$ 7	49 $\pm$ 13	64 $\pm$ 20	90 $\pm$ 20
	CON	30 $\pm$ 7	32 $\pm$ 8	43 $\pm$ 9	59 $\pm$ 15
Goblet cells per 100 enterocytes	INF	7.2 $\pm$ 0.2	8.7 $\pm$ 0.1*	11.1 $\pm$ 0.2*	ND
	PFC	7.5 $\pm$ 0.1	7.0 $\pm$ 0.2	5.5 $\pm$ 0.1	ND
	CON	6.0 $\pm$ 0.1	5.7 $\pm$ 0.1	7.0 $\pm$ 0.2	ND
Secretion ( $\mu\text{g}/\text{mg}$ )	INF	11 $\pm$ 2*	12 $\pm$ 2*	18 $\pm$ 2*	22 $\pm$ 3*
	PFC	6 $\pm$ 1	6 $\pm$ 1	7 $\pm$ 1	7 $\pm$ 3
	CON	5 $\pm$ 1	5 $\pm$ 1	6 $\pm$ 1	7 $\pm$ 2
Synthesis ( $^{14}\text{C}$ -dpm uptake/mg)	INF	37 $\pm$ 45*	547 $\pm$ 90*	900 $\pm$ 200*	2375 $\pm$ 375*
	PFC	128 $\pm$ 20	144 $\pm$ 19	158 $\pm$ 23	783 $\pm$ 178
	CON	165 $\pm$ 32	164 $\pm$ 39	159 $\pm$ 42	470 $\pm$ 106

Rabbits infected with *Yersinia enterocolitica* (INF) were compared on day 6 to pair-fed, malnourished (PFC) and unmanipulated, weight-matched control (CON) animals. Tissue samples from the upper, mid and distal small intestine and the proximal colon were homogenised and analysed for mucin content by an enzyme-linked immunoassay. Tissue mucin content (in  $\mu\text{g}$ ) is shown expressed relative to tissue protein content (in mg). Further tissue samples were fixed and stained with periodic acid-Schiff reagent and haematoxylin and eosin. Goblet cells were counted and are expressed as the number per 100 enterocytes in a crypt/villus unit. The remaining tissue was incubated in vitro in modified Krebs-Ringer solution, pH 7.4, containing 25  $\mu\text{Ci}$  ( $^{14}\text{C}$ )-glucosamine, at 37°C in a shaking metabolic bath. After 90 minutes, mucin in the tissue and medium was measured by the immunoassay and secretion into the medium (in  $\mu\text{g}$ ) was calculated relative to total (tissue + medium) protein content (in mg). Total glycoprotein synthesis was estimated from the amount of protein-bound radiolabel found in the tissue after 90 minutes of incubation and is calculated relative to total (tissue + medium) protein content (in mg).

ND: goblet cell and enterocyte numbers could not be determined in the proximal colon. No differences were detected between PFC and CON groups.

\*  $p < 0.05$  for INF compared to PFC and CON groups.

suggesting that cholera toxin-induced mucin secretion, unlike fluid and electrolyte secretion, is not controlled via the adenylate cyclase-cAMP system (Roomi et al., 1984). In subsequent studies, it was shown that cholera toxin-induced fluid/electrolyte and mucin secretion in the small intestine could actually be dissociated by a number of drugs. Cycloheximide (a protein synthesis inhibitor), cytochalasin B (a microfilament disrupter), colchicine (a microtubule disrupter), and verapamil (an inhibitor of  $\text{Ca}^{2+}$  transport) all abolished cholera toxin-induced mucin se-

cretion but had no effect on fluid/electrolyte secretion. Conversely, acetazolamide (a carbonic anhydrase inhibitor) decreased fluid/electrolyte secretion without inhibiting cholera toxin-induced mucin secretion (George and Leitch, 1983; Njoku and Leitch, 1983).

Thus, the secretory effects of cholera toxin are not all mediated by the same intracellular second messenger mechanism.

While similar rapid increases in fluid/electrolyte and mucin secretion are also observed following challenge with *Escherichia coli* enterotoxin (Moon,

Whipp and Baetz, 1971), not all enterotoxins behave in the same manner. For example, the toxins produced by various *Salmonella* and *Shigella* species promote small intestinal fluid secretion much more slowly (with a lag period of ~100 minutes) and do not apparently induce mucin release, as assessed by morphologic techniques showing no depletion of goblet cell mucin content (Steinberg et al., 1975; Formal, Hale and Sansonetti, 1983). In general, enterotoxin production causes fluid/electrolyte secretion in the small intestine without damaging the mucosa and it seems likely that this fluid secretion, either with or without concomitant mucin secretion, is a relatively early, non-specific mucosal response to bacterial toxins in an attempt to 'wash away' the organism and prevent colonisation of the gut.

*Yersinia enterocolitica* (YE) is an entero-invasive organism that causes severe gastro-enteritis. Rabbits infected with a human pathogenic strain of YE develop diarrhoea and weight loss by about day 3 (O'Loughlin et al., 1986). In the early stages of the disease, the organism penetrates the mucosa forming micro-abscesses throughout the small intestine and colon. By day 6 post-infection, mucosal abscesses become more localised to the ileo-caecal region, crypt hyperplasia occurs throughout the small intestine with villus atrophy in the ileum, fluid and electrolyte absorption are decreased, and the activities of brush border membrane di-saccharidases are markedly depressed. Thus, the diarrhoea developed by YE-infected animals likely results from maldigestion and malabsorption of nutrients, rather than active secretion (O'Loughlin et al., 1986; O'Loughlin, Pai and Gall, 1988).

Tissue mucin content is significantly elevated in the upper, mid and distal small intestine and in the proximal colon of infected animals compared to pair-

fed, malnourished and unmanipulated, weight-matched control animals (Table 5). These results suggest either goblet cell hypertrophy or hyperplasia. In fact, a significant increase in goblet cell numbers could only be demonstrated in the mid and distal small intestine (but not in the upper small intestine) and, even then, the increase was not in proportion to the rise in tissue mucin content, suggesting that goblet cell hypertrophy also occurs during the disease. Mucin secretion along with total glycoprotein synthesis is markedly elevated in infected animals compared to pair-fed and unmanipulated control animals (Table 5). Interestingly, a graded response is observed down the intestinal tract of infected animals with the greatest impact on tissue mucin content, mucin synthesis and secretion occurring in the distal small intestine and the proximal colon where mucosal injury is most severe (Mantle et al., 1989).

Goblet cell proliferation and increased mucin secretion in the small intestine and colon are not unique to YE infections, since a variety of other entero-invasive organisms have been observed to produce similar effects, including *Escherichia coli* (Khaviin et al., 1980), *Salmonella typhimurium* (Rout et al., 1974), *Shigella flexneri* (Rout et al., 1975), and *Treponema hyodysenteriae* (Hughes, Olander and Williams, 1975).

Since stimulation of an inflammatory response by intestinal invasion accompanies enhanced mucin production and secretion, it appears that there is close integration between the immune component and mucus secretion in the host's mucosal defence system. The reason for the enhanced mucin production is not yet clear but it likely represents an attempt by the mucosa to push away a damaging organism and clear it from the body, to prevent the organism from gaining access to and further dam-

aging the tissue, and/or to allow epithelial recovery under a thickened mucus blanket, protected from potentially injurious agents (such as digestive enzymes and bacteria) in the lumen.

Little is known about the effects of indigenous bacteria on mucus secretion. Since the gut flora is capable of degrading the mucus barrier by production of extracellular and membrane-bound proteases and glycosidases, a natural balance must exist *in vivo* between bacterial erosion and mucin secretion. Indigenous microflora may also contribute to mucosal protection by a. modifying potential receptors in mucus for pathogenic organisms, and/or b. competitively inhibiting the attachment of pathogens to mucus and their subsequent multiplication in or penetration of the mucus layer. If the normal situation is compromised, for example, by mal-

nutrition or a change in the gut flora by antibiotic therapy, tissue mucin content and mucin secretion decreases (Sherman et al., 1985). This can result in reduced mucosal protection in the host and therefore enhanced susceptibility to epithelial damage by indigenous bacteria or increased vulnerability to enteric pathogens.

During bacterial overgrowth, mucin secretion is increased (Sherman et al., 1987), indicating that the normal gut flora can affect mucin production in particular circumstances. Whether interaction of these bacteria and the immune system is required for the stimulation of mucin secretion is not known at present.

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## 2. Mucus Structure and Physiology

The mucus gel creates a stable unstirred microenvironment at the mucosal surface which is - when measured under stirred condition - about 70 to 80 microns thick in the rat and about 180 microns in man. In the rat stomach topical administration of prostaglandin increases mucus secretion. Therefore, it is thought that increased mucus secretion is often mediated by prostaglandins.

### *Macroscopic and Microscopic Localisation of Mucus Production*

Mucus is produced throughout the length of the gastrointestinal tract by a variety of cell types: oesophagus mucous glands, cardiac, fundic and pyloric glands of the stomach, Brunner's glands of the duodenum, goblet cells of the small and large intestines and superficial mucous cells found all through the tract (Filipe, 1979). The control of mucus production by these cells can occur

at two levels:

- a. secretion of preformed mucus, and
- b. mucus biosynthesis.

In a normal mucus-secreting cell, one might expect these two processes to be interdependent, since an increase in secretion alone would soon exhaust the cell of mucus unless there was also an increase in its biosynthesis. However, stimulation of mucus secretion by excessive mechanical or chemical irritation might exceed the rate of biosynthesis and exhaust the mucus within the cells. This ultimately would cause a breakdown of the mucosal barrier. Scanning electron microscopy studies of anaesthetised dog gastric mucosa by Zalewsky and Moody (1979) have shown three mechanisms by which mucus is released:

- a. a continuous exocytosis of a few granules at a time,
- b. an explosive release of mucus by

- apical expulsion of the older cells in the interfoveolar area and
- c. the relatively rare event of cell exfoliation.

The explosive release of mucus from mucosal cells in contact with irritants is in keeping with apical expulsion (Kelly et al., 1979; Zalewsky and Moody, 1979), which is maximal on gastric mucosa away from the foveolae; this is the site of maximal drainage in gastric injury.

#### *Stimulation of Mucus Secretion and Release*

There is a large body of evidence that the output of mucus can be increased in the luminal juice by neural, hormonal or chemical (irritants) stimulation. The production of a thick mucous secretion in the stomach in response to acid was first observed by Ivy and Oyama (1921) and has been observed with a number of other chemical irritants, e.g., mustard oil and ethanol (Dinosa, Ming and Meniff, 1976; Forstner, 1978).

Salivary mucus is stimulated by either sympathetic or parasympathetic stimulation. Two different glycoproteins or groups of related glycoproteins are formed, depending on which nerve is stimulated to evoke submaxillary secretion. Thus the ratio of sialic acid to fucose or hexosamines was higher and the size of the salivary glycoproteins smaller in secretion evoked by sympathetic than parasympathetic stimulation (Dische et al., 1970).

Stimulation of the splanchnic or vagus nerves or topical application of acetylcholine produces a copious gastric mucous gel in dogs (Horowitz and Hollander, 1961). Acetylcholine stimulated radioactive mucus release from rabbit and human colonic biopsies *in vitro* but did not affect the rate of glycoproteins biosynthesis (MacDermott, Donaldson and Trier, 1974).

The two best-documented hormonal

stimulants of gastric mucus production are secretin and prostaglandins. Secretin increases the sugars of mucous glycoproteins in gastric juice from humans (Andre, Lambert and Descons, 1972), cats (Vagne and Perret, 1976) and dogs (Kowalewski et al., 1979). Prostaglandins, which, as judged visually, cause an increased production of viscous mucus (Domschke et al., 1978), produce a rise in the glycoprotein-bound sialic acid content of gastric washouts in man when given with pentagastrin (Domschke et al., 1978). Prostaglandins also increase the soluble mucus in rat gastric juice, although no change in the glycoprotein content of the surface mucous gel was observed (Bolton, Palmer and Cohen, 1978). Finally, various other gastrointestinal hormones, including gastrin, CCK-PZ, and histamine have been shown to increase the fucose and galactose content of gastric juice from cats prepared with fistulas or pouches (Vagne and Perret, 1976), although there was no information on viscosity or on whether the glycoprotein was degraded and therefore the result of peptic erosion. Both 5-HT and carbachol increased mucus production by the rat colon, as measured by increased hexose content of the perfused lumen *in vivo* (Black, Bradbury and Wyllie, 1979). Atropine abolished the action of carbachol and application of either histamine or isoprenaline alone had no effect on the hexose content.

#### *Chemical and Physical Characteristics of Mucus*

Mucus is a gel like agar. If one cuts agar it remains cut, but if one cuts mucus it reforms. Its protective function may rely on this ability to reform and to flow slowly along the epithelial lining. These properties enable mucus to make a continuous cover over the mucosal surface. The mucus gel is made up of complicated molecules, which are re-

sistant to pH 1 to 8 and are not dissolved by bile. Mucus is, however, affected by proteolysins - in particular pepsin and bacterial proteolysins - by reduction of disulphide bridges. By breaking these bridges, one dissolves the mucus. The quantification of mucus gel secretion poses special problems since it is necessary to distinguish qualitatively the adherent layer from both soluble mucus in the lumen and from the intracellular reserves of unsecreted, preformed mucus. To measure the mucus gel one of the most successful techniques is called mechanical spectroscopy (*Keress, Allen and Garner, 1982*). In this technique, the mucus gel is put between two plates of a rheogoniometer. The top plate is oscillated under controlled computerised conditions. Very small oscillations do not break down the gel but disturb its structure. These oscillations are picked up by the plate below. If there is a solid substance between both plates, the two plates will move at the same frequency and phase. Frequency and phase of the bottom plate change when the substance between both plates becomes viscous to liquid. Purified glycoprotein of intestinal mucus has the same curve as the native gel. Treatment with pepsine changes the structure and therewith the curve registered by the bottom plate substantially. Studies with this apparatus have revealed that the gel glycoprotein consists of approximately 20% protein and some 80% carbohydrate chains. The latter chains are up to 20 chivers long surrounding the central protein core so protecting it from proteolysis.

The molecular weight of undegraded glycoprotein from pig gastric mucus, which is  $2 \times 10^6$  daltons, is markedly de-

creased in size to  $5 \times 10^5$  daltons following proteolysis by pepsin and other proteases (*Allen and Snary, 1972*).

Furthermore, the undegraded glycoprotein is dissociated on reductive cleavage of the disulphide bridges with mercapto-ethanol into four equal sized subunits of MW  $5 \times 10^5$  daltons (*Snary, Allen and Pain, 1970*), the same size as those obtained by proteolysis. However, there is also a bit of the protein core that has no carbohydrate around it. It has been shown that a separate protein of 70,000 MW joined to the glycoprotein subunits by disulphide bridges is the main component of the naked protein of each glycoprotein molecule (*Pearson and Allen, 1980*). The importance of naked protein and disulphide bridges to the gel-forming structures of glycoproteins from a wide variety of mucous secretions is clear from the ready solubilisation of these secretions by proteolytic enzymes and thiol reagents.

The structure of the carbohydrate chain consists of five negatively charged sugars in defined order. With this structure of carbohydrate chains of up to 12 sugars in size, it may become understood that mucus gels can in fact do differ between animal species and even between different sites in the digestive tract. These differences in carbohydrate chain cause a great antigenic variation. The terminal sugars of human mucus are alike glycoproteins of the blood group (ABH) system on erythrocytes and other cells.

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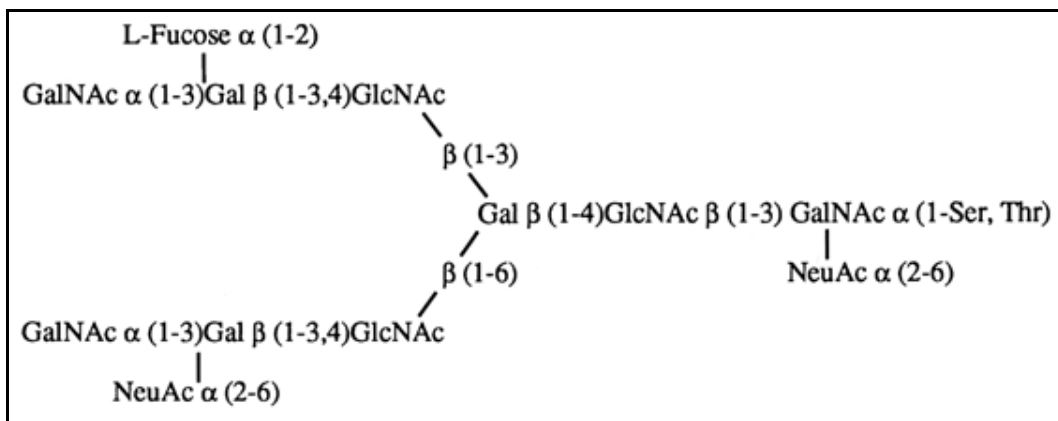
### 3. Gut Mucus as a Nutrient Milieu for Enteric Bacteria

#### *Degradation of the Oligosaccharide Chains of Mucin Glycoproteins in the Colon*

The gastrointestinal mucus layer's principal constituents are mucus glycoproteins (Allen and Hoskins, 1988). Each of these macromolecules is comprised of numerous oligosaccharide chains linked at their reducing end to a common polypeptide core, and each chain is comprised of from 2 to 18 glycosidically linked monosaccharides. Since the oligosaccharide side chains account for 70% or more of the dry weight of mucus glycoproteins the monosaccharides comprising the side chains would constitute an excellent endogenous carbon and energy source for enteric bacteria providing that their glycoside linkages could be cleaved. The longer oligosaccharide chains of mucin glycoproteins have a backbone of

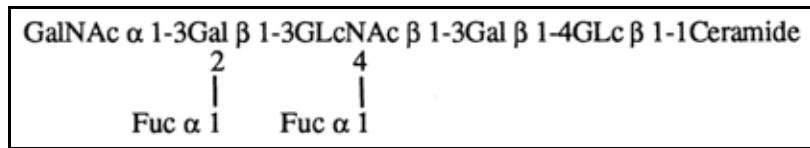
branched and unbranched sequences of alternating  $\beta$ -galactose and  $\beta$ -N-acetylglucosamine residues. Each chain is linked at the reducing end to the core polypeptide by a glycoside bond between a terminal  $\alpha$ -N-acetylgalactosamine residue and the hydroxyl group of serine or threonine (Figure 1). In glycosphingolipids of gastrointestinal epithelium similar chains are linked to the ceramide lipid moiety via a "lactosyl" (galactosyl- $\beta$ -(1-4)-glucosyl-) sequence (Figure 2) (Gustafsson et al., 1986; Bjork et al., 1987).

The outer, non-reducing end of fully completed chains in mucin glycoproteins terminate in  $\alpha$ -linked monosaccharides that include sialic acid and saccharides conferring ABH and Lewis blood group antigenic specificities. Similar  $\alpha$ -linked glycosides are present on the non-reducing terminus of many of the oligosaccharide chains of gut mucosal



**Figure 1:** Structure of a typical branched oligosaccharide chain of mucin glycoproteins from rat (Slomiany et al., 1980) and human (Podolsky, 1985) colonic mucus showing the core N-acetylgalactosamine in an  $\alpha$ -linkage with a serine or threonine residue in the polypeptide core on the right, a branched backbone of  $\beta$ -linked saccharides, and  $\alpha$ -linked saccharides at the outer, non-reducing end of the chain that include the blood group A and H antigenic determinant N-acetyl-galactosaminoyl- $\alpha$ (1-3)- and L-fucosyl- $\alpha$ (1-2)- moieties.

Abbreviations: GalNAc = N(acetyl)galactosamine, GLCNAc = N(Acetyl)glucosamine, Gal = galactose, NeuAc = N(acetyl)neuraminic acid, Ser,Thr = serine, threonine.



**Figure 2:** "A-7", a representative neutral glycosphingolipid isolated from human meconium and rat intestinal mucosal epithelium showing the galactosyl-β(14)glucosyl-core sequence linked to a ceramide, and blood group A, H and Lewis-b a-linked saccharides at the non-reducing end. Abbreviations as in Figure 1.

glycolipids. Catalytic hydrolysis of each glycoside linkage requires a glycosidase, each of which exhibits a high degree of specificity for its particular substrate glycosidic linkage. This specificity is determined by the structure of the monosaccharide, the anomeric configuration of the linkage, the location of the carbon on the penultimate monosaccharide to which it is bonded, and, frequently, the structure of the penultimate monosaccharide itself. Thus, chain degradation requires the simultaneous presence of several glycosidases, each having the requisite specificity to degrade a specific glycoside linkage as it is exposed during sequential degradation of the chain. In the absence of a particular glycosidase further chain degradation will cease at the glycoside linkage it would have otherwise cleaved.

It is now clearly established that the enteric microbiota degrade the complex oligosaccharide side chains of secreted mucin glycoproteins and cell membrane glycolipids that are normally sloughed into the gut lumen. Studies have shown that large amounts of undegraded mucin glycoproteins are excreted in faeces of germfree animals but not in faeces of conventional animals nor in faeces of germfree animals monocontaminated with certain strains of enteric bacteria (Lindstedt, Lindstedt and Gustafsson, 1965; Hoskins and Zamcheck, 1968; Gustafsson and Carlstedt-Duke, 1984). Analysis of mucus constituents in gastrointestinal contents of healthy humans autopsied shortly after sudden death re-

vealed high molecular weight glycoconjugates in ileal contents where indigenous bacteria are sparse and loss of these large molecules in the colon where bacterial populations are large (Vercellotti et al., 1977). Mucin glycoproteins added to sterile, anaerobic, bacterial culture media are extensively degraded during growth of bacteria following inoculation with faeces (Hoskins and Zamcheck, 1968; Variyam and Hoskins, 1981), and the cell-free supernates of these cultures as well as cell free faecal extracts will also degrade mucin glycoproteins (Variyam and Hoskins, 1981).

Degradation does not occur with initial colonisation in new-borns but is gradually established until 20 months of age when all faecal samples exhibit mucin degradation (Norin et al., 1985). A similar pattern also occurs with faecal glycosphingolipid degradation; it most likely occurs around weaning time (Larson et al., 1987).

Degradation of mucin glycoproteins by cell free faecal extracts and cell free supernates of anaerobic faecal cultures is due to their containing a multiplicity of highly active bacterial glycosidases required for degradation of the oligosaccharide chains of mucin glycoproteins (Prizant and Koningsberg, 1981; Hoskins and Boulding, 1976a; Hoskins and Boulding, 1981). Although endoglycosidases may be present and may play a role in chain degradation, evidence to date indicates that the major degradation is accomplished by sugar-

specific exoglycosidases acting sequentially to hydrolyse one glycoside linkage at a time beginning with the terminal sugar at the outer, non-reducing end of each chain. Yet, the ability to produce all the glycosidases required for chain degradation is a property that is restricted to a small number of normal commensal bacteria. In man the largest populations of these bacteria comprise a subset that averages 1% of cultivatable faecal bacteria (Miller and Hoskins, 1981). This was first clearly shown by Salyers and associates (Salyers et al., 1977a, 1977b) who made a systematic study of the ability to ferment hog gastric mucin by strains of bacteria isolated from human faeces. Out of a total of 342 tested, representing 8 genera and 32 species, only 8 strains fermented the mucin. These were 2 of 5 strains of *Bifidobacterium bifidum* and 6 of 9 strains of *Ruminococcus torques* (Salyers et al., 1977a). My colleagues and I obtained identical results using a different approach (Hoskins et al., 1985).

In our studies we tracked down and isolated the strains in fresh faecal samples that were responsible for producing the  $\alpha$ -glycosidases which cleave the A, B or H blood group antigenic determinant glycosides from their terminal positions on the oligosaccharide chains of mucin glycoproteins. We found that production of these enzymes was strongly associated with the ability to degrade hog gastric mucin, which has blood group A, and H antigenic determinants. The 5 isolates we obtained were *Ruminococcus torques* strains (2), *Ruminococcus* AB (1), and *Bifidobacterium* species (2) (Hoskins et al., 1985). These are Gram-positive, non-sporulating, obligate anaerobes, which are normal inhabitants of the colon in healthy humans (Moore and Holdeman, 1974; Holdeman, Good and Moore, 1976) (Figure 3). The salient feature of these strains is their production of a va-

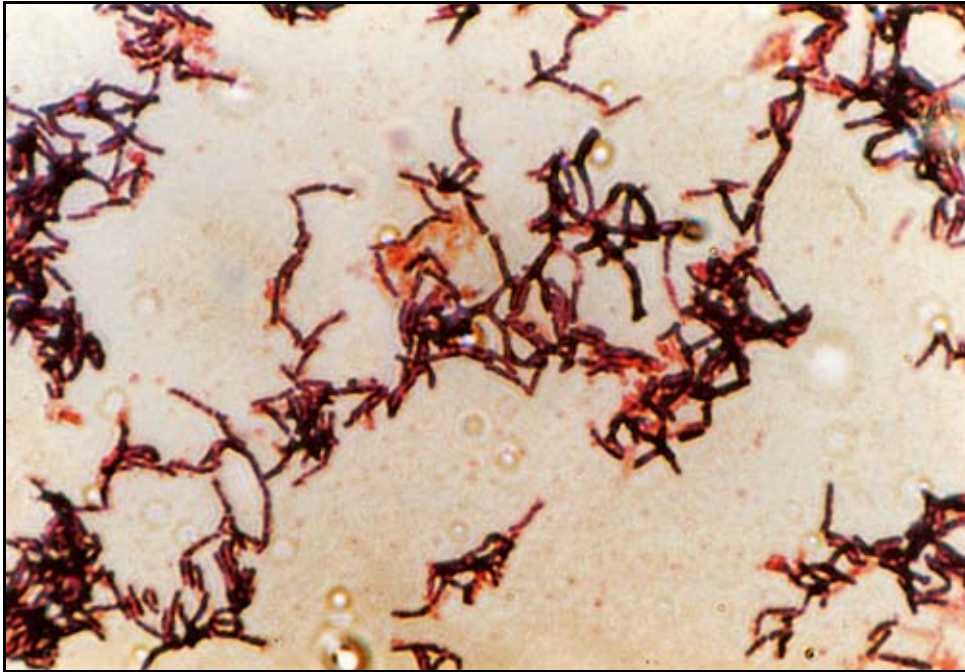
riety of glycosidases that include  $\beta$ -galactosidases and  $\beta$ -N-acetylhexosaminidases that degrade the backbone of each oligosaccharide chain, sialidases, and one or more  $\alpha$ -glycosidases that cleave the ABH and Lewis blood group sugars from the non-reducing end (Table 6). The strains differed from one another in their ability to produce the blood group-degrading  $\alpha$ -glycosidases. Thus, all strains produced  $\alpha$ -L-fucosidases that cleave the H and Lewis  $\alpha$ -L-fucosyl moieties from the chains, but only the 2 *R. torques* strains produced a blood group A-degrading  $\alpha$ -N-acetylgalactosaminidase, and only the *Ruminococcus* AB strain produced a blood group B-degrading  $\alpha$ -galactosidase. When inoculated in culture medium containing B salivary glycoproteins from a blood group B secretor only the *Ruminococcus* AB strain was able to cleave the B antigenic determinants from the outer end of the chain. But, alone among these isolates, the *Ruminococcus* AB strain did not produce  $\beta$ -N-acetylhexosaminidases so that its ability to further degrade the chains was limited. However, when this strain was co-cultured in medium containing B-salivary glycoprotein with one of the other strains that produced  $\beta$ -N-acetylhexosaminidases, a symbiotic association occurred wherein each supplied a glycosidase the other lacked, resulting in extensive degradation of the blood group B-salivary glycoprotein and greater growth of each than either had achieved when grown alone in the same medium (Hoskins et al., 1985). Degradation of mucin glycoproteins by these strains is likely to be confined to the oligosaccharide moieties and not to the polypeptide core. Using azo-albumin as a general substrate we were unable to demonstrate any protease activity at pH 8.0 in culture supernates and cell sonicates of any of the 5 strains nor at pH 6.5 in one of these.

**Table 6:** Extracellular glycosidases produced by human faecal mucin-degrading bacteria (adapted from *Hoskins et al., 1985*)\*

Strain	BGD activity**			Glycosidase activity			
	A	vs. B	H	Sialidase	$\beta$ -galacto- Sidase	$\beta$ -N-acetyl- glucosaminidase	$\beta$ -N-acetyl galactosaminidase
Ruminococcus torques IX-70	4+	0	2+	3+	4+	4+	2+
Ruminococcus torques VIII-239	4+	0	2+	3+	4+	4+	2+
Ruminococcus AB VI-268	0	4+	2+	2+	1+	0	0
Bifidobacterium bifidum VIII-210	0	0	3+	3+	4+	4+	2+
Bifidobacterium infantis VIII-24-	0	0	3+	2+	4+	4+	1+

\* Enzyme activities, originally expressed as units per mg bacterial protein, are expressed here on a scale of 0, 1+ to 4+ for simplicity.

\*\* BGD = Blood group degrading activity.



**Figure 3:** Gram-stained preparations of two mucin-degrading strains of bacteria isolated from human faeces.  
Top: *Bifidobacterium bifidus* strain VIII-210. Bottom: *Ruminococcus* AB strain VI-268.  
Magnification: 500x.

A second feature of glycosidase production by these strains is that it is constitutive and not inducible (Hoskins et al., 1985). Competition studies in chemostat cultures (Dykhuizen and Hart, 1982) suggest that, under conditions where the concentration of nutrient substrate is low, strains that constitutively produce enzymes required for utilising that substrate have a competitive growth advantage over strains whose synthesis of these enzymes is substrate-induced. Such conditions probably apply to the caecum and colon; here the total amount of mucus secreted per 24 hrs is not known, but measurements of ileostomy secretions suggest that not more than 2-3 g of combined mucus secretions from stomach and small intestine enter the caecum every 24 hrs (Englyst and Cummings, 1986).

A third important feature of glycosidase production by these strains is that the glycosidases are released into the environment as extracellular enzymes (Hoskins and Boulding, 1981; Hoskins et al., 1985). Therefore they can act on a variety of complex glycoconjugates which may not be in the immediate vicinity of the bacterial cell that produces them.

These properties of glycosidase production by mucin-degrading bacteria distinguish them from most other enteric bacterial populations. Thus, some strains of *Bacteroides* and *Bifidobacterium* outnumber mucin-degraders by about 100:1 and dominate the enteric microbial ecosystem (Moore and Holdeman, 1974; Holdeman, Good and Moore, 1976). When we tested them we found that these strains possessed cell-bound sialidase,  $\beta$ -galactosidase and  $\beta$ -N-acetylglucosaminidase activities, but they lacked the  $\alpha$ -glycosidases required to cleave the terminal ABH and Lewis blood group glycosides on the outer, non-reducing end of the mucin oligosaccharide chains and therefore

could not make the initial cleavage to degrade such chains (Hoskins et al., 1985). These strains had a limited ability to degrade gut mucin glycoproteins and display little or no growth in anaerobic culture media containing intact mucin glycoprotein as the sole carbohydrate source (Harouny and Hoskins, 1983). It is presently unclear whether their lack of producing the  $\alpha$ -glycosidases required for initial cleavage, or the cell-bound nature of their other glycosidases, or both factors, is the more important limiting factor in their ability to degrade mucins. We are currently performing experiments to clarify this point.

It is important to emphasise that the subset of mucus-degrading bacteria described above may not be the only bacteria capable of degrading mucus in human colon contents. Our quantitative studies suggest, however, that they constitute the largest populations of mucus-degrading bacteria in human faeces which produce the requisite glycosidases as extracellular enzymes (Hoskins and Boulding, 1981; Hoskins et al., 1972). Other incompletely characterised strains including *Bacteroides* sp. have been reported to ferment mucin (Bayliss and Houston, 1986) while some strains of *Clostridium perfringens*, which are recovered in low and variable numbers from human faeces, produce the requisite glycosidases for oligosaccharide chain degradation (McGuire, Chipowsky and Roseman, 1972; Aminoff, 1972). Furthermore, host species differences may influence the type of enteric bacteria degrading mucus glycoproteins. Thus, a mucin-degrading *Peptostreptococcus* was recently isolated from rat faeces (Carlstedt-Duke et al., 1986) while some *Bacteroides* strains isolated from swine colons have mucin-degrading properties (Stanley et al., 1986) (Table 7).

**Table 7:** Faecal bacteria that degrade mucin oligosaccharides

Source	Species	Reference
I. Human	Ruminococcus torques strains	Saylers et al., 1977a; Hoskins et al., 1985; Bayliss and Houston, 1986
	Ruminococcus AB strain	
	Bifidobacterium strains	Bayliss and Houston, 1986
	"Unidentified Gram-pos. rods"	
II. Pig	Clostridium perfringens A strains Bacteroides strains	Stanley et al., 1986
III. Rat	Peptostreptococcus strain	Carlstedt-Duke et al., 1986

*Nutritional Consequences of Mucus Degradation for Enteric Bacteria*

With few exceptions the great majority of enteric bacteria are saccharolytic i.e. utilise monosaccharides as carbon and energy sources. Monosaccharides of the types comprising the oligosaccharides of mucin glycoproteins and glycolipids are readily utilised by enteric bacteria *in vivo* and *in vitro*. Thus, only small amounts of these monosaccharides were recovered in the dialysable constituents of faeces from conventional rats in whom bacterial degradation of gut mucus is extensive (Hoskins and Zamcheck, 1968), and utilisation of galactose and other monosaccharides by several numerically dominant enteric species has been demonstrated during growth in pure cultures (Hoskins and Boulding, 1976a; Miller and Hoskins, 1981).

A study performed by Victor Harouny in the laboratory of Hoskins demonstrated how degradation of mucin glycoproteins by the subset of mucin degrader strains could have a nutritional support role for other enteric bacteria *in vivo* (Harouny and Hoskins, 1983). Fresh faeces from 5 healthy subjects were serially diluted and  $10^{-10}$  g were inoculated into

- a. anaerobic medium without any carbohydrate,
- b. same as a. but containing intact hog gastric mucin as the sole carbohydrate source,

- c. same as a. but containing a mixture of galactose, fucose and N-acetylhexosamine monosaccharides in the same amount as were present in the chains of intact mucin, and
- d. same as b. but with the mucin pre-incubated for 24 hrs with the sterile culture supernate of the mucin-degrader strain R. torques IX-70.

This supernate contained the glycosidases that extensively degraded the oligosaccharide chains of the mucin prior to introducing the faecal inoculum. At  $10^{-10}$  g faecal inoculum the mucin degrader strains were diluted out and only the larger dominant strains of enteric bacteria remained in the inoculum. Bacterial growth failed to occur in the anaerobic medium lacking any carbohydrate and was slight in the medium containing intact mucin. By contrast, there was marked and comparable growth in the media containing either the monosaccharide constituents of the mucin or mucin pre-degraded with the glycosidases produced by the mucin-degrader strain IX-70. *In vivo*, the extracellular glycosidases produced by this much smaller mucin-degrader population presumably would perform a similar function. Larger populations of enteric bacteria could be sustained by these means under conditions such as host fasting when fermentable dietary carbohydrates are not entering the

caecum. It has been suggested that about 70 g of fermentable carbohydrate are required daily by enteric bacteria in order to sustain their colonic biomass (Smith and Bryant, 1979). This is far in excess of the 1-2 g mucus recovered daily in ileostomy effluent (Englyst and Cummings, 1986). Therefore, it is very doubtful that the nutritional needs of enteric bacteria could be met by gut mucus secretions alone. Nevertheless, the continuous secretion of mucin into the mucus layer overlying the mucosa probably sustain focal bacterial populations in the micro-environment of the mucus layer with the help of glycosidases from mucin degrader strains.

The amount of carbohydrate in epithelial cell glycosphingolipids shed daily into the gut lumen is negligible compared to the normal carbohydrate consumption of enteric bacteria. For example, only 1-2 mg is excreted per day in faeces of germfree rats (Gustafsson et al., 1986). In man, one can estimate the amount of mucosal glycolipids entering the lumen daily from measurements of epithelial shedding rates. About 6-12 g of small intestinal epithelial protein is lost by epithelial cell shedding each day (Croft and Cotton, 1973). With the glycosphingolipid concentration of intestinal epithelium at 8-10 mg per gram protein (Bjork et al., 1987), this is equivalent to a daily shedding of 50 to 120 mg of glycosphingolipids.

Another feature of oligosaccharide chain degradation pertinent to microbial nutrition is that it enhances the susceptibility of the polypeptide core of mucus glycoproteins to proteolytic degradation. Colonic contents exhibit strong protease activity (Hoskins and Boulding, 1976a; Variyam and Hoskins, 1983). While the intact multimeric mucin glycoprotein molecule has unglycosylated regions of the polypeptide core that are susceptible to cleavage by proteases, the intervening

highly glycosylated subunits resist proteolysis, probably because of steric hindrance by the numerous oligosaccharide chains linked to the core polypeptide in these subunits. We found that if the oligosaccharide chains were first degraded from these subunits by incubation with the glycosidases in cell free culture supernates of mucin degraders, the underlying polypeptide core became highly susceptible to pancreatic proteases (Variyam and Hoskins, 1983). Although ammonia is readily utilised as a nitrogen source by many enteric bacterial strains (Bryant, 1974), others appear to require amino acids, and some seem to utilise peptides better for growth than mixtures of amino acids (Bryant, 1974; Russell, 1983). The gut mucin polypeptide core is highly enriched in serine, threonine, and proline (Allen and Hoskins, 1988). Hence, it would be of ecological interest to determine whether enteric bacteria might obtain special growth advantages from utilising fragments of this mucin core polypeptide obtained by proteolytic degradation of the deglycosylated subunits.

#### *Other Actions of Glycosidases from Mucin Degradors in the Gut Lumen*

The extracellular glycosidases produced by mucin degrader strains of enteric bacteria act not only on the oligosaccharide chains of mucin glycoproteins but also on related oligosaccharides on the surface of enteric bacteria. Thus, faecal extracts and the cell free supernates of anaerobic faecal cultures will degrade the blood group B-like antigen from the surface of *E. coli* 086, releasing the antigenic determinant  $\alpha$ -D-galactose from the surface structures (Cromwell and Hoskins, 1977). Such actions *in vivo* may markedly alter the spectrum of bacterial surface antigens presented to the host's mucosal barrier from that found in pure cultures of the



same bacteria. Immune responses of the host to enteric bacteria which lead to autoimmune tissue damage have been implicated in the pathogenesis of idiopathic ulcerative colitis, some forms of arthritis, and more recently Graves' disease (*Fiocchi and Farmer, 1987; Inman, 1987; Heyma, Harrison and Robins-Browne, 1986*). Degradation of bacterial antigens in the gut lumen by bacterial glycosidases from mucin degraders and by intraluminal proteases may ordinarily protect against such immune responses.

Extracellular glycosidases from mucin degrading bacteria also act on glycosphingolipids extracted from the gut mucosa. Recently, *Larson, Falk and Hoskins (1988)* showed that glycosidases from cell free culture supernates of mucin degrader strains rapidly degraded the oligosaccharide of chains of GM3 and the blood group active fucoglycolipids extracted from human meconium and rat intestinal mucosa. While the amount of mucosal glycosphingolipids entering the colonic contents from sloughed epithelium is too small to be a major source of nutrient carbohydrate for enteric bacteria, the significance of their degradation may be on its effects on the functions of glycosphin-

golipids on the luminal membranes of colonic mucosal cells. There is considerable evidence to indicate that the oligosaccharide moieties of cell surface glycolipids have an important biological role as receptors for macromolecules like cholera toxin as well as binding sites for bacterial adherence (*Curatolo, 1987; Holgersson et al., 1985; Bock et al., 1985*). One of the latter, lactosylceramide, preferentially binds strains of several genera of enteric bacteria when these are overlain onto developed thin layer chromatograms of mucosal glycolipids (*Holgersson et al., 1985; Bock et al., 1985; Hansson et al., 1983*). We found that lactosylceramide was the principal product accumulating during degradation of more complex glycosphingolipids by glycosidases from 4 of the 5 mucin degrader strains (*Larson, Falk and Hoskins, 1988*). Thus, glycosidases from mucin degrader strains may have another important ecological role in the gut lumen: they may promote adherence of normal commensal bacterial populations by degrading mucosal glycolipids to simpler structures like lactosylceramide.

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#### **4. The Role of Lectins in the Organisation of the Mucus Blanket**

As mentioned in previous paragraphs, the intestine is covered by a very thick mucus blanket of up to 200 microns. This makes it very difficult to process for electron microscopy. However, by stabilising the mucus layer using antibodies developed against the mucus of the host species concerned (*Costerton, Rozee and Cheng, 1983*), it is possible to hold the mucus blanket on the tissues during careful processing (Figure 4).

In the mucus layer covering the mucosa of adult host individuals, a large

bacterial population can be seen (Figure 5). In addition, in some, protozoan species have been observed (Figure 6). In the bottom layer of the mucus very few types of bacteria appear to be attached. In fact, often as few as only two types of organisms may be found in large numbers associated with the intestinal surface. The majority of the bacterial population of the mucus is not actually in contact with the mucosal surface but inhabits the overlying mucus (Figure 7).

To obtain better insight in the role of the mucus in determining the colonisa-

tion pattern of the gut, new-born animals have been studied where the mucus structure is not fully set up. In four-hour-old calves electron-microscopic sections revealed villi with goblet cells starting to produce fairly large amounts of mucus (Figure 8) and large amounts of bacteria associated with these pockets of mucus. The general, yet uncovered, microvilli were not associated with large numbers of organisms. In case of neonatal diarrhoea in the calve, like in *E. coli* K99 diarrhoea, this leaves a 'window' of uncovered tissue. The intestine is relatively bare of mucus at this stage so that the *E. coli* K99 can adhere to the surface using their pili, as was seen when monoclonal antibodies were used specific for these pili (Figure 9). This experiment of nature shows that when there is not a full and complete mucus blanket over the tissue, the intestine is very vulnerable to infection.

In calves of six days, the surface of the microvilli is completely covered (Figure 10). At that time, the mucus is nicely continuous and a large 'natural bacterial population' in this mucus surface renders these animals not longer susceptible to neonatal diarrhoea. It could therefore be interesting to the agricultural science to put in a large number of 'natural' (bovine) bacteria immediately after birth in order to initiate rapid colonisation as soon as mucus secretion starts. By inoculating calves orally with large numbers of organisms of 29 different species isolated from the cow, a better weight gain has been obtained in an experimental station (Table 8). In addition, it was found absolutely impossible to implant the enterotoxin producing *E. coli* K99.

Other elucidating studies have been done in the rumen of calves. For colonisation of the roots of plants, lectins - proteins with affinity for specific carbohydrates - are extremely important. This effect of lectins is not confined to

plants. In the rumen, the cow makes a lectin with affinity for certain organisms, the great majority of which is Gram-positive (Figure 11). The presence of relevant lectins is important for proper colonisation of the rumen. This may explain why natural colonisation in the rumen of the calf is fairly complete at four days after birth and absolutely complete at ten days (*Cheng, Irvin and Costerton, 1981*). This ruminal population stays with the animal throughout its life. Obviously, serious physiologic disturbance may occur when this bacterial population is affected by antibiotic therapy.

Investigations in the neonatal gut of the rat have been performed to study the hypothesis that endogenous lectins call in and mediate with a specific beneficial population of bacteria. The approach was indirect by adding exogenous lectin - phytohaemagglutinin (PHA) - to the system (*Banwell et al., 1985*). This had a specific effect as instead of the 'clean intestinal surface' with a very few adherent bacteria of the normal untreated rat, overgrowth was observed by cocci as well as rods (Figure 12). These organisms colonised down the microvillous surface, which affected the tissue very profoundly. Influx of leukocytes (lymphocytes) was found associated with a great thinning of the mucus. Scanning electron microscopy provided the same picture: Large numbers of cocci and rods at the surface of the villi and far less mucus than there would normally be present (Figure 13).

The explanation for the dramatic effect of PHA on the type of mucosal colonisation could be as follows: when the mucus is excreted by goblet cells, it shows an affinity for neighbouring tissue. It would roll over in a highly structured way (Figure 14). In this manner the thick layer of complex structured mucus, as is seen in older animals, is built up; a process in which

**Table 8:** Effect of inoculation on weight gain

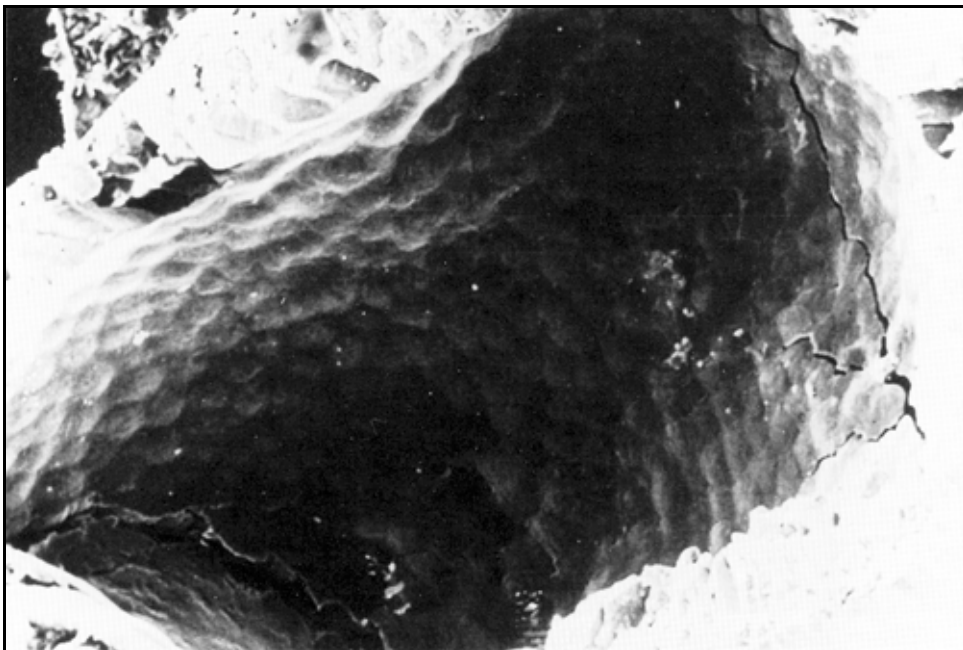
Animals	Weight (kg) at slaughter	% Increase over control	Digestive tract (kg)	% Increase over control
Control	26.5	-	2.1	-
Treated	32.4	23	2.6	24

endogenous lectins play an important role. There are two possible ways in which PHA could have deranged the system. The first possible explanation is that PHA could have affinity for the bacteria and take them to the surface of the microvilli. The second possible explanation to be considered is that PHA attaches to the villi and thus prevents normal spread and rolling over of endogenous lectins so that bacteria can attach directly to the microvillous surface (Figure 15).

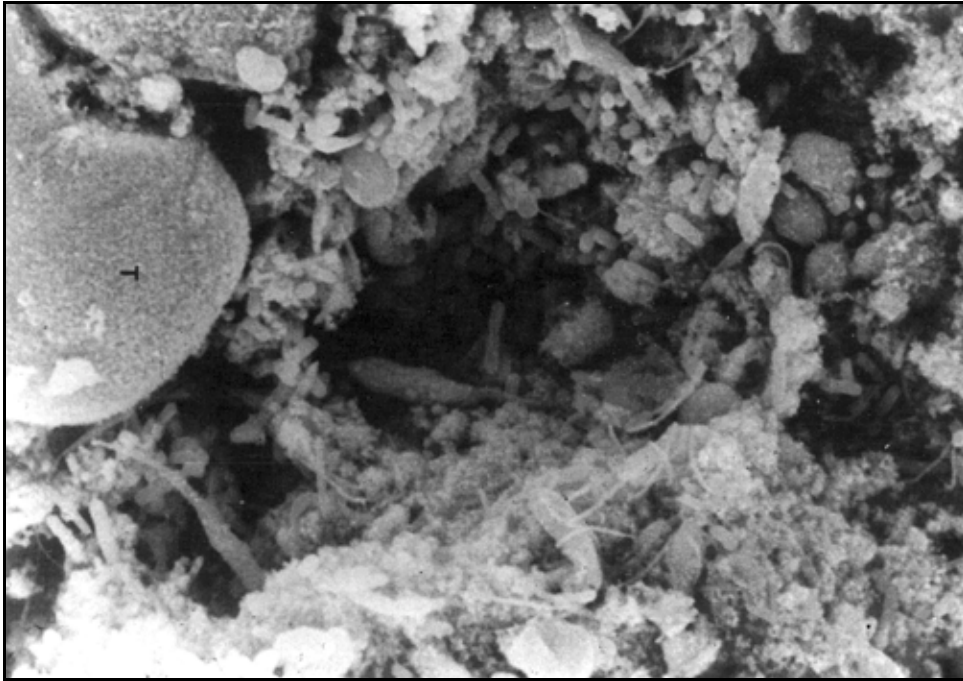
Bacteria, isolated from the overgrowing population in PHA treated newborn rats, did not show affinity to PHA *in vitro*. Therefore, the second hypothe-

sis is the most likely, e.g. if PHA is an antagonist of endogenous lectins and thus interferes with the function of the endogenous lectin, then, rather than having a well structured mucus over the surface of the microvilli, there may be an aperture where the tissue is exposed to luminal bacteria (Figure 15). Then, bacteria that would normally not or only sparsely grow in the mucus blanket would be able to express their very high affinity for tissue and colonise it to cause the damage reported above.

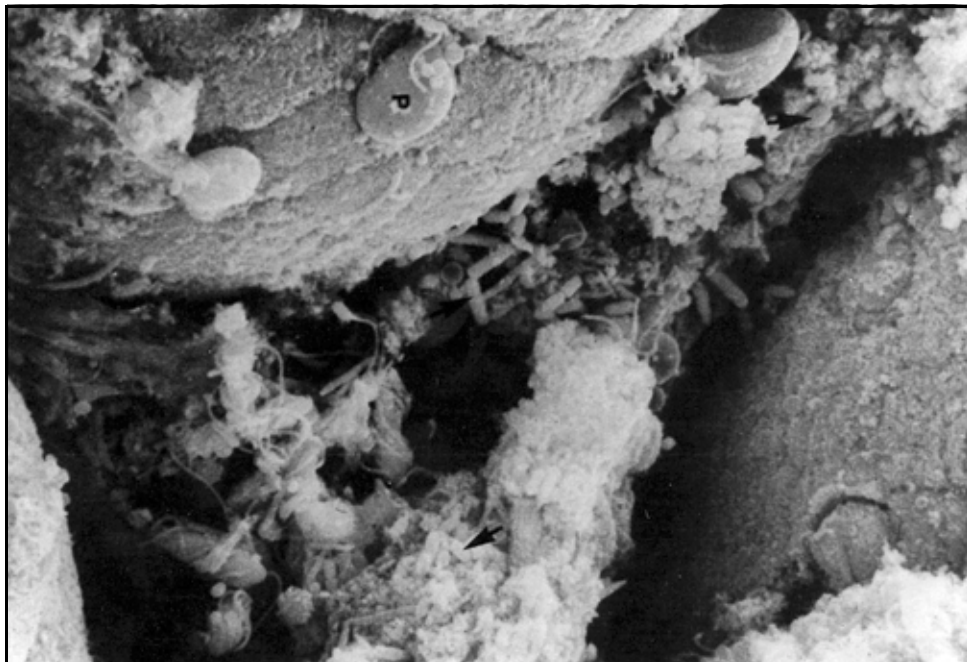
*J. William Costerton, Department of Biology, University of Calgary, Calgary, Alberta, Canada T2N 1N4.*



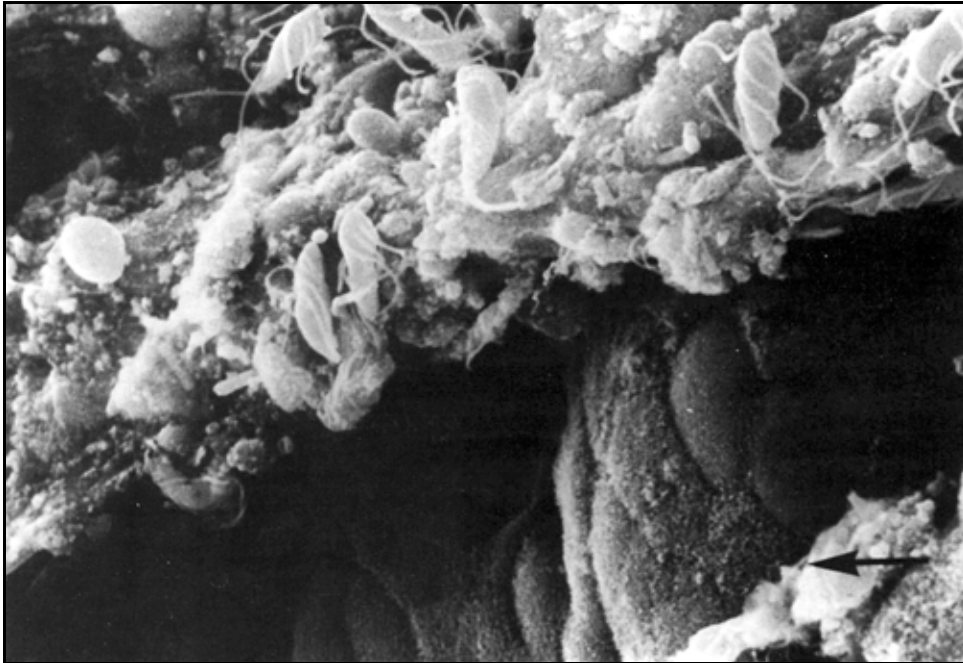
**Figure 4:** SEM of the surface of rat intestine showing the complete retention of the thick mucus layer.



**Figure 5:** Higher magnification of the same SEM showing large bacterial populations within the mucus blanket.



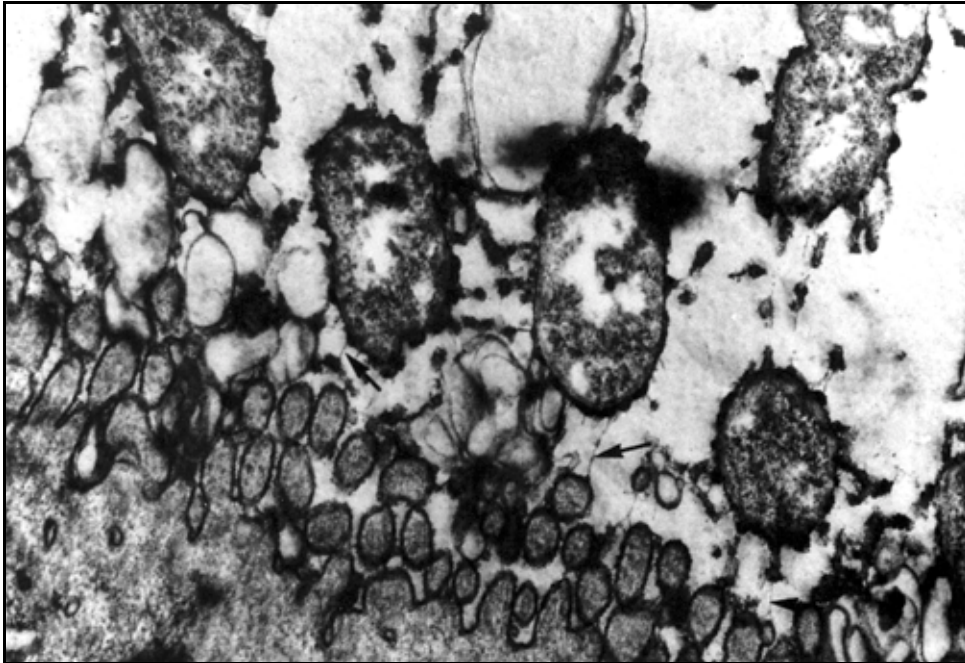
**Figure 6:** High magnification SEM of the same tissue surface showing adherent protozoa (*Giardia*) in the mucus blanket of the normal intestine.



**Figure 7:** SEM of the intestinal surface of a different rat showing the association of bacteria and protozoa with the mucus layer and their absence from the microvillar surface.



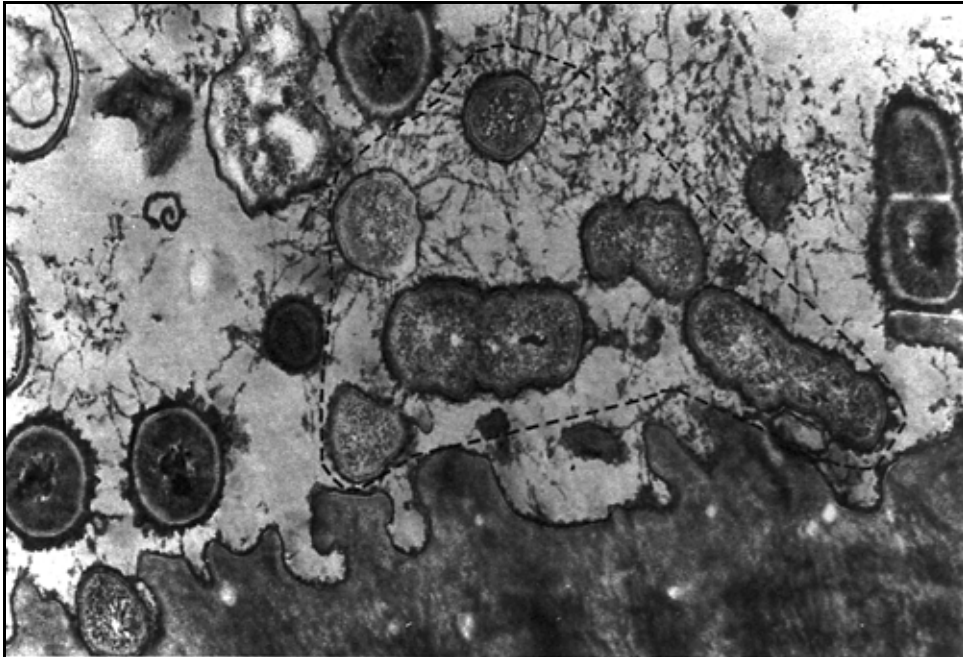
**Figure 8:** SEM of the intestinal surface of a four-hour-old calf showing the production of mucus masses by individual goblet cells.



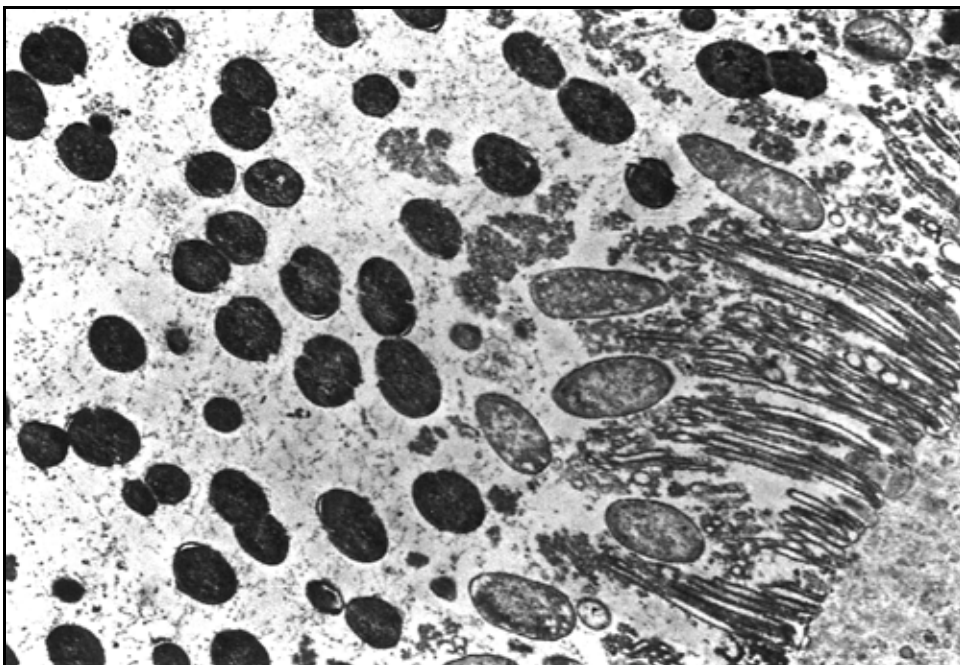
**Figure 9:** TEM of the surface of the intestine of a new-born calf infected by a K99 strain of *E. coli* and treated with specific anti-K99 pilus antibody in order to visualise these pilus structures (arrows).



**Figure 10:** SEM of the intestinal surface of a six-day-old calf showing the complete occlusion of the tissue surface by a mucus blanket colonised by bacteria.



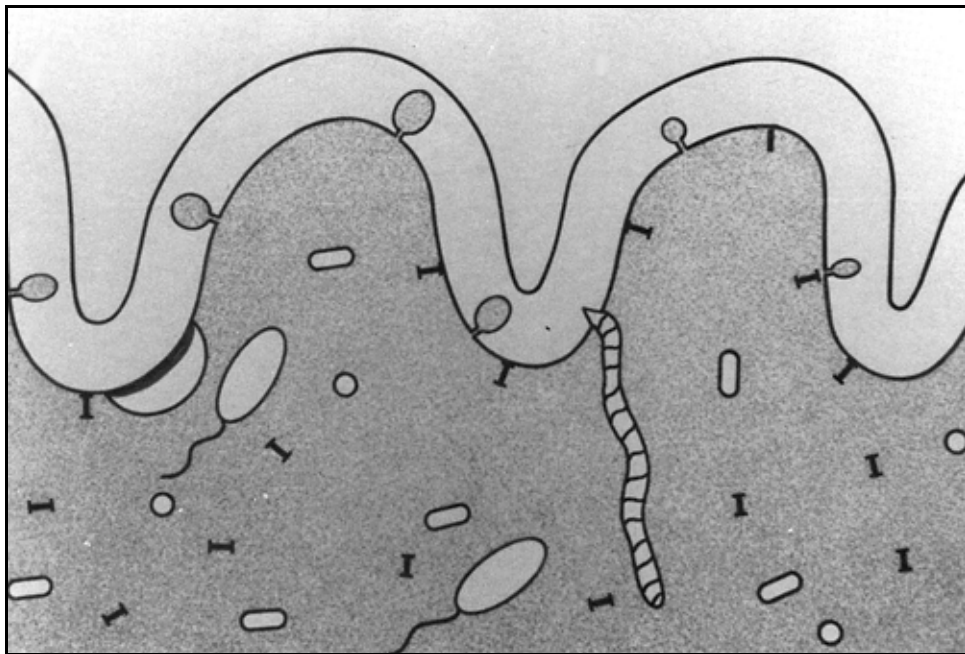
**Figure 11:** TEM of the surface of the bovine rumen showing a preponderance of Gram-positive bacteria next to the tissue in a complex layer of adherent bacteria.



**Figure 12:** TEM of the surface of the intestine of a PHA-fed rat in which the microvillar surface was heavily colonised by Gram-negative rods and Gram-positive cocci.

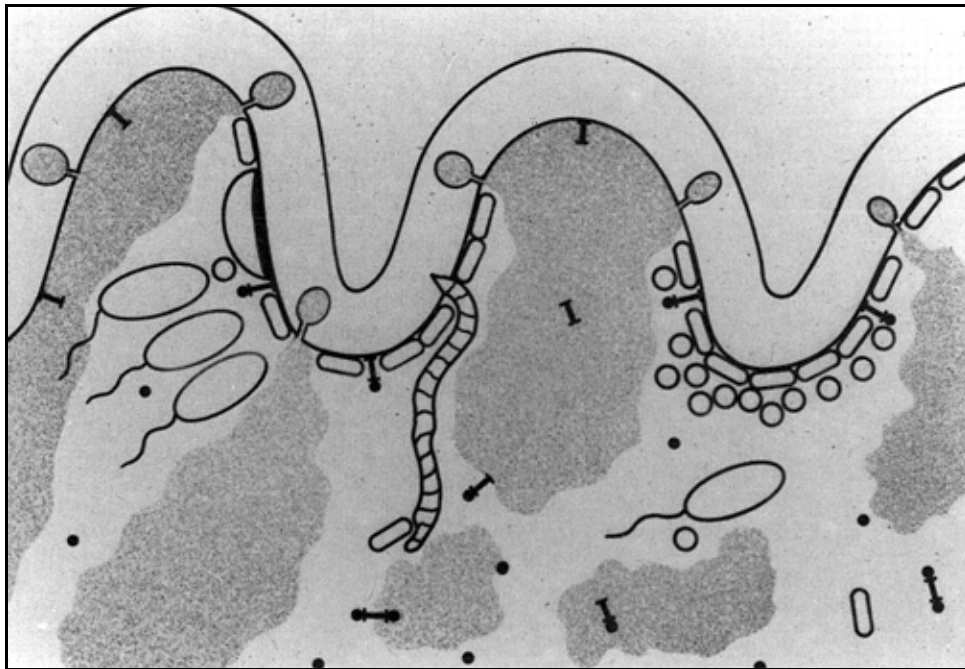


**Figure 13:** SEM of the intestinal surface of the same animal seen in Figure 9 showing both rods and cocci embedded in the microvillar layer of the colonised intestine.



**Figure 14:** Diagrammatic representation of mucus secretion in the normal intestine in which mucus is produced in goblet cells and forms an even layer because of the affinity of natural lectins (I) from both the tissue surface and the mucus.





**Figure 15:** Diagrammatic representation of mucus secretion in the intestine of a PHA-treated animal showing the disruption of mucus structure caused by the association of PHA (●) with the natural lectin (≡) and the consequent colonisation of the unprotected gut surface by bacteria and protozoa.

## 5. The Mucus Blanket as a Protective and Chemotactic Factor at the Epithelial Surface

The consequences of microbial interactions in the intestine are a titration of various host factors against virulence characteristics of the organism. These consequences have profound medical implications in both the developed and the Third World. In addition to relieving human suffering, an understanding of and ability to control disturbances of intestinal microecology will have tremendous economic, social and political impact. An indication of the variety of processes responsible for the microecology of the intestine which must be understood is provided in Tables 9 and 10.

Walker and colleagues have studied some factors which contribute to microbial partition in the gut. Specifically,

they examined host-microbial events responsible for infection with *Campylobacter jejuni*, a major enteric pathogen and also those associated with the development of opportunistic infections in immunocompromised rodents.

### *Enteric Disease Studies*

Infections similar to clinical *Campylobacter* diarrhoea can be induced in rabbits with the Removable Intestinal Tie Adult Rabbit Diarrhoea (RITARD) model (Spira et al., 1981; Caldwell et al., 1983; Caldwell et al., 1986). This procedure ensures an experimental ileus as it inhibits normal peristalsis for several hours so that the bacterial inoculum can establish sufficiently to cause a mucoid diarrhoea associated with bacter-

**Table 9:** Factors responsible for determining intestinal microecology

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**Indigenous population stabilisers**

1. Gel embedding (entrapment)
2. Gel attachment (chemical bond)
3. Epithelial attachment sites
4. Unique niche fitting (*C. pylori*)
5. Favourable nutrient environment
6. Multiplication rate to match population size vs. environment
7. Immunologic tolerance to indigenous flora

**Exogenous population inhibitors**

1. Immobilisers - antibody - mucus glycoprotein
  2. Multiplication reduction
    - colonisation resistance factors (largely unknown)
  3. Physical barriers/removers
    - viscosity of mucus
    - peristalsis
    - mucus secretion
    - fluid secretion
    - cell sloughing
- 

aemia after a few days. Many animals die as a consequence of the infection. If the rabbit is fed the organism without the RITARD procedure, colonisation for two to three weeks takes place, but no diarrhoea occurs. Rechallenge of these transiently colonised animals shows immunity with no disease, no bacteraemia, and rapid gut clearance of the organism, usually in less than 24 hours (Burr et al., 1988).

*In vitro* studies were conducted to better understand control mechanisms involved in *Campylobacter* infection and immunity in the rabbit. These studies used adherence assays not only to epithelial cells (INT 407), but also to rabbit intestinal mucus (McSweegan and Walker, 1986; McSweegan, Burr and Walker, 1987) as the organism must interact first with the latter substrate. Strains of *C. jejuni* used varied greatly in their ability to adhere to either the cell or mucus substrate (McSweegan and Walker, 1986). Lipopolysaccharide was an adhesin which bound both substrates, but flagella, another adhesin,

bound only to cells. Binding to epithelial cells *in vivo* may not occur, but *in vitro* mucus as well as other defences are removed. Lee et al. (1986) found that *C. jejuni* colonises crypts, but does not seem to attach to or penetrate epithelial cells. The spiral morphology, motility, mucinase production and chemotaxis to mucin are all bacterial characteristics, which would enhance this process.

A more realistic *in vitro* system for *C. jejuni* was created by overlaying INT 407 cells with mucus. Bacteria were placed on top of the mucus and penetration and subsequent attachment to epithelium were quantitated. Mucus reduces adherence to cells by about 50%. If the rabbits were immune to *C. jejuni*, their mucus totally blocked adherence to epithelial cells in this assay (McSweegan, Burr and Walker, 1987).

The immune mucus phenomenon described above could be associated with titres of SIgA in the mucus. Whole eventually provide secretory IgA to mucosal surfaces (Figure 16). It is of

**Table 10:** Factors responsible for a disturbed ecosystem

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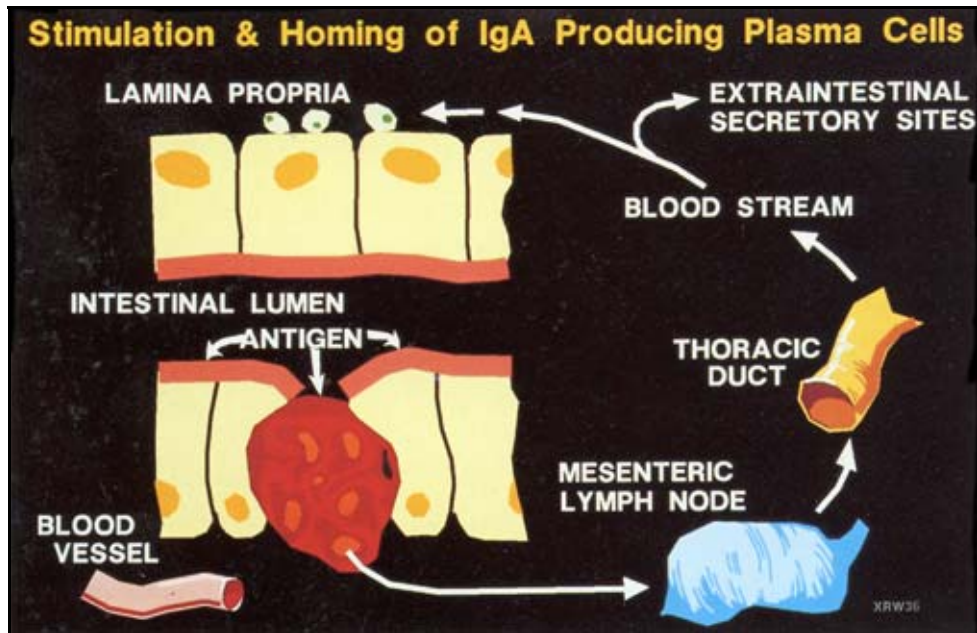
<b>Exogenous population colonisation mechanism</b>
1. Motility
2. Mucinase (protease)
3. Morphology
4. Invasive properties
5. Numbers
6. Metabolic ability to exploit altered environment
<b>Compromised states</b>
1. Traumatic injury/radiation
- peristalsis impaired
- mucus blanket disruption
- loss of cell surface fibronectin
- crypt cell damage
- immunosuppression
2. Viral enteritis
- virus destroys enterocytes
- bacteria adhere to lesion created by virus
3. <i>Clostridium difficile</i>
- antibiotic impairment of colonisation resistance
- age associated immunity reduction
4. Autoimmune dysfunction (Ulcerative colitis; Ankylosing spondylitis)
- genetic material from bacteria transferred to host cell
- viral antigen attached to self-antigen
5. Carcinogenesis
- substrates available for bacterial carcinogen production
6. Blind loop syndrome
- intestinal stasis
7. Hepatic encephalopathy
- metabolic derangement
8. Abnormal drug metabolism due to unique flora

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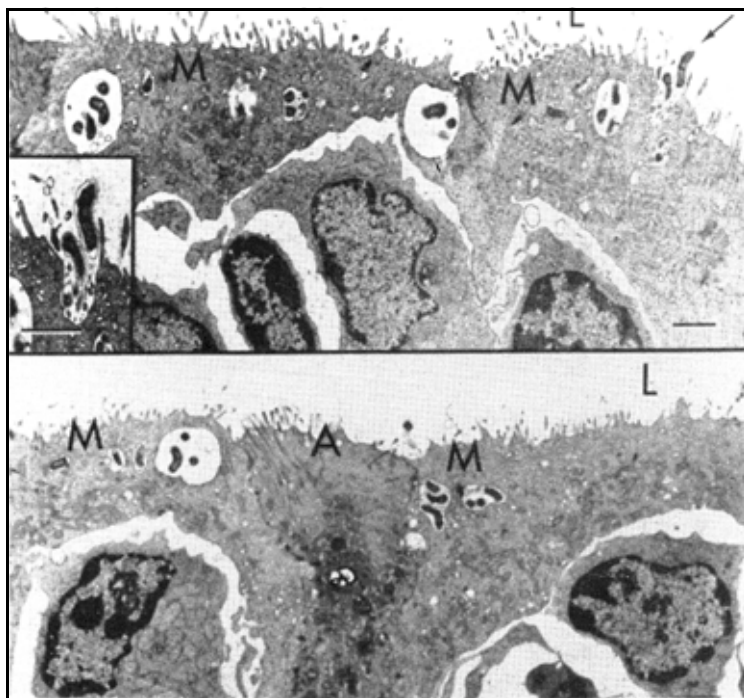
interest that these antigen-sampling organs are relatively free of mucus due to bacterial cells but not flagella or *E. coli* could absorb the SIgA from the mucus and loss of SIgA titre eliminated the immune effect. SIgA alone, collected by lavage (Burr et al., 1987), did not inhibit adherence to cells. When added to non-immune mucus, however, lavage fluid from immune animals caused a total reduction in bacterial attachment to INT 407 cells. This suggests that the antibody and mucus must work together to effect inhibition of bacterial passage.

Mucus and its flow due to secretion by goblet cells and peristalsis is, thus, a major component of host defence which

can be augmented significantly by specific antibody. This interaction of immune and non-immune components suggested that the intestinal lymphoid tissue response to *Campylobacter* should also be examined. This system is scattered throughout the gut, but can be found concentrated in Peyer's patches where specialised epithelial cells, called M cells, cover follicles containing lymphocytes and macrophages (Owen, 1982; Owen et al., 1986; Sneller and Strober, 1986). M cells take up antigen and pass it apparently unchanged into the follicle where it is processed to scarcity of goblet cells in the adjacent area.



**Figure 16:** Antigen in the intestinal lumen interacts with lymphoid tissue sites to produce IgA producing plasma cells. These cells move systemically via the lymphatic and blood circulatory systems to extra-intestinal mucosal surfaces.



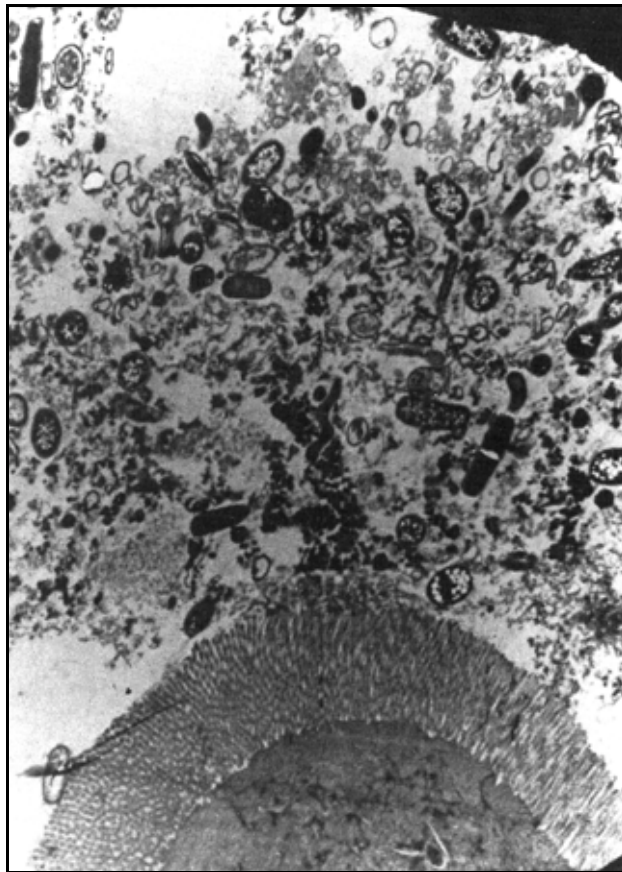
**Figure 17:** Endocytosis of *C. jejuni* by rabbit M cells (M). Bacteria are seen in vacuoles within the M cells. Inset in top photograph shows bacteria being endocytosed from intestinal lumen (arrow). Relationship between M cells and absorptive epithelial cells (A) lining the lumen (L) is shown in bottom photo. Space bar represents 2 micrometers.

Walker et al. (unpublished data) found that *Campylobacter* preferentially binds to and is transported through M cells (Figure 17), but not to other intestinal epithelial cells (enterocytes). This may be a portal for translocation of the campylobacters which could contribute to the disease process (Walker et al., 1986) as well as cause immunity. The fact that bacteria could be found free in the M cell follicle, suggests that this could also be a route for translocation

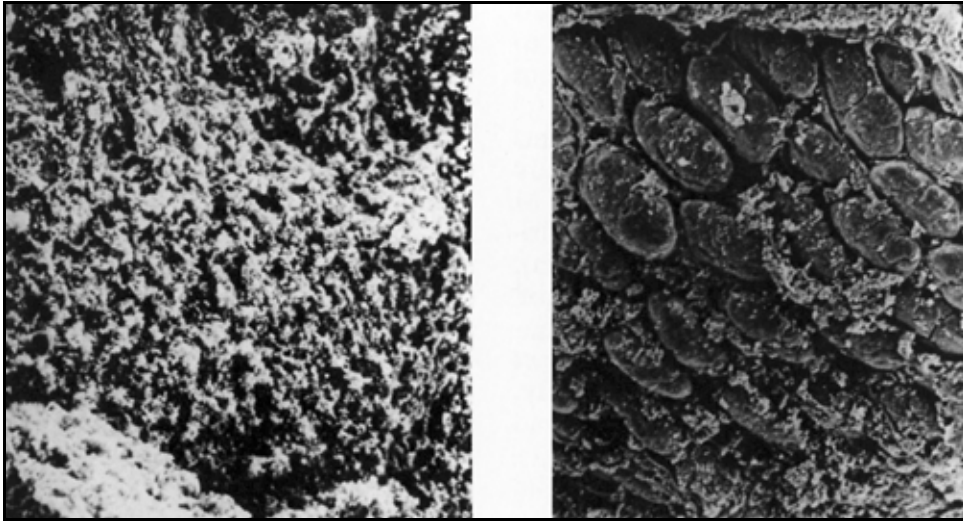
by opportunistic pathogens in immunocompromised individuals.

#### *Opportunistic Infection Studies*

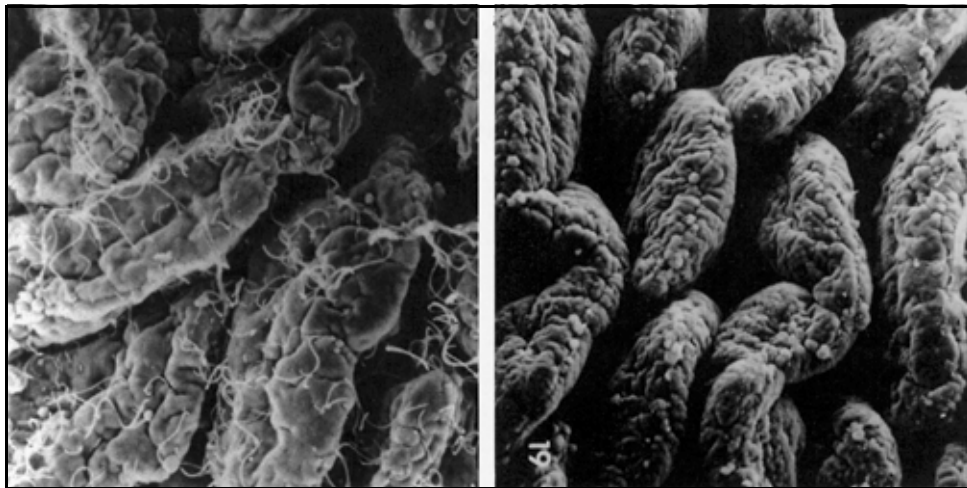
Although the M cell still needs to be studied as a means for translocation of microorganisms across the mucosa in immunocompromised subjects, other studies already completed provide some insight into non-specific factors affecting microecology of the gut in the immunocompromised host.



**Figure 18:** The mucus gel in a rabbit ileum was stabilised with specific antiserum to show relation of gel to the brush border of an epithelial cell. Note the numerous microorganisms seen in the gel layer.



**Figure 19:** Scanning electron micrograph of surface of mouse ileum in which mucus gel has been stabilised with anti-mucus antibody. The mucus material in the sample from a normal mouse is seen to virtually occlude the mucosal surface (left hand side of figure). The blanket is reduced to patchy accretions and the villi are clearly seen through this discontinuous layer 3 days after exposure of mice to 10 Gy of gamma radiation (right hand panel). Space bars represent 50 micrometers.

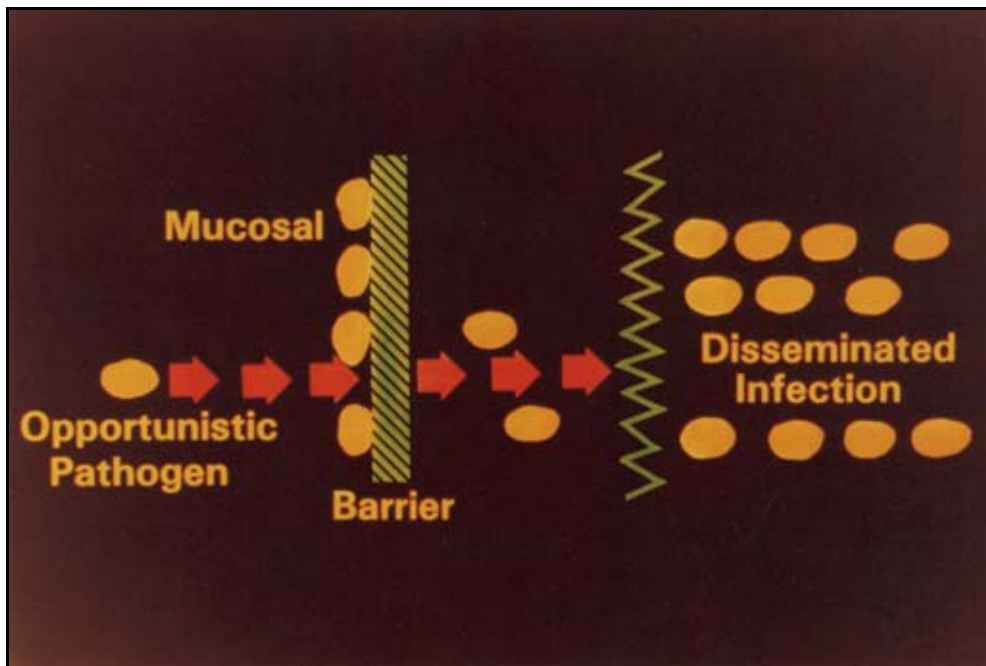


**Figure 20:** The normal rat ileum is healthy colonised with segmented filamentous microflora (SFM) which are inserted at one end into the intestinal epithelial cell (left hand panel). After sub-lethal (5 Gy) or lethal (10 Gy) gamma radiation the SFM are significantly reduced in number and, in contrast, to rats given sublethal radiation, they never returned in the lethally irradiated animals (right hand figure).

Disruption of tight junctions between goblet cells and adjacent absorptive epithelial cells following wounds and/or irradiation not only provide a route for escape of endotoxin from the gut, but also indicates activity by the goblet cells (*Walker and Porvaznik, 1983*). Also, intestinal stasis may permit host proteases to accumulate in the intestine. These events suggest that the mucus blanket could be changed following injury. Since most microorganisms are found in the mucus (Figure 18), this could be very significant.

*Walker* and colleagues (1985) have looked at the normal mucus blanket and at the mucus blanket on days 1 to 3 after lethal irradiation of mice (Figure 19). They saw progressive disruption of blanket integrity so that exposed villi were visible.

Segmented filamentous microflora (SFM) were examined in rats by scanning electron microscopy. These organisms were used as an indicator of disruption of the gut ecosystem and possible loss of colonisation resistance (*Walker and Porvaznik, 1983*). Following sublethal radiation SFM decreased but returned to normal levels by day 3. After lethal radiation they never returned (Figure 20). When rats were exposed to sublethal radiation facultative flora decreased and then returned to normal levels after the SFM returned. This was in contrast to a more rapid return and overgrowth of facultative flora in lethally exposed animals. This event is associated with translocation and lethal systemic infections (*Walker and Porvaznik, 1983*). The importance of colonisation resistant flora is shown by



**Figure 21:** Opportunistic pathogens in the lumen of the intestine must overcome a series of defence systems to colonise the epithelial surface of the mucosal barrier. If sufficient organisms colonise this surface, some may translocate into normally sterile tissues. If normal systemic defences (jagged line) are lost due to radiation or other immunocompromising injuries, disseminated infection can occur.

experiments with metronidazole. If mice are treated with systemic metronidazole, the decrease in anaerobes is accelerated and lethal opportunistic infections occur earlier (*Brook, MacVittie and Walker, 1988*).

Figure 21 illustrates diagrammatically how breakdown of various mucosal defences such as described above could permit colonisation of the mucosal surface by opportunistic pathogens. If

sufficient numbers of these organisms translocate through the mucosal barrier, they can cause disseminated infections if normal defences such as granulocytes (illustrated by jagged line) are not present.

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